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Distribution of Ice Nucleation-Active (INA) Bacteria from Rain-water and Air

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Certain bacteria that induce biological ice nucleation are suspected to play an important role in condensation and ice nuclei formation in clouds. Those bacteria can produce biological ice nucleator[1], which is a protein and usually found on leaf surface and air. Most studies on INA bacteria were conducted in subtropical areas. In this study, INA bacteria were isolated from rain-water and air between March to May 2008 from Jakarta, Bogor, Bekasi, Tangerang, and Depok. The percentage of INA bacteria from rainfall for those area are 19.4, 18.7, 5.3, 2.2, and 6.4% respectively, while percentage for air are 9.5, 6.5, 0, 2.7, and 1.8%. The highest incidence of INA bacteria were from rain-water and air found in sample from Jakarta and then followed by the samples from Bogor. It was shown that the percentage of INA bacteria from rain-water was higher than air for all of the samples from different areas. The isolate from Jakarta (isolate A_{32}) which had the highest activity for ice nucleation, with the temperature classification at -2.7 °C, revealed 100% similarity with *Pantoea* sp. The presence of INA bacteria in rain-water and air might play an important role in nucleation process which is required for rainfall induction.

Key words: INA bacteria, rain-water, air

INTRODUCTION

Although ice melts at 0 °C, water does not necessarily freeze at this temperature. Instead water can be supercooled to several degrees below the melting point (0 °C) of its solid state, ice. This is because initiation of ice crystallization depends on the presence of ice nuclei, particles of a critical size and shape that allow formation of the ice lattice around them (Pooley & Brown 1990). In liquid state, because of the presence of suitable catalysts (ice nucleation-active substances), the liquid-to-solid phase transition or freezing will occur at temperatures only slightly below 0 °C. The initiation of the transition from the liquid state to ice is called nucleation. Substances that facilitate this transition so that it takes place at a relatively high sub-zero temperature are called ice nucleators. Such catalysts may be ice crystals themselves or a variety of organic or inorganic heterogeneous ice nucleation agents (Orser *et al.* 1985). Pure water freezes at -40 °C, many chemicals and contaminants, such as dust particles, will act as ice nuclei at temperatures below -5 °C, though few are as effective as the ice nucleating bacteria, which are very active at -2 to -3 °C. So be sure to stress that the freezing point of pure water is -40 °C and that less pure water is unlikely to supercool below -10 °C (Handelsman *et al.* 2002). Several genera of Gram-negative bacteria are able to nucleate the crystallization of ice in supercooled water (Warren *et al.* 1986).

In the presence of these ice nucleation active (INA) bacteria, plant tissues cannot reach their normal supercooling point and thus cannot avoid freezing injury. Supercooling is predicted to be a frost protection mechanism. Damage to 'frost-sensitive' plants under natural conditions usually occurs between -2 and -5 °C. At these temperatures, ice forms from supercooled water in such plants, propagates throughout the plants (inter- and intracellularly), and frost damage occurs. In the absence of sites capable of ice nucleation, the water in plant tissues can supercool; freezing will not occur until the temperature becomes low enough that the most active ice nucleus associated with the plant is able to catalyze crystallization of supercooled water. Supercooling may protect blossoms in orchads against spring frost injury (Lindow *et al.* 1982). Because these INA bacteria species are common plant epiphytes, they are important incitants of frost injury in a variety of agricultural crops at relatively warm subzero temperatures (0 to -6 °C) (Lindow *et al.* 1978).

Numerous studies have shown that the ability of bacteria such as *Pseudomonas syringae*, *P. fluorescens*, and *Erwinia herbicola* to nucleate ice formation in supercooled H_2O was due to the products of specific genes called *inaZ* for *P. syringae*, *inaW* for *P. fluorescens*, and *iceE* for *E. herbicola*. All of these related bacterial genes produce membrane-bound proteins with marked similarities to each other; all containing an essential and unique repetitive primary sequence (Turner *et al.* 1991).

From the previous preliminary studies, there are three chemically distinct classes of INA bacteria, A, B, and C. It appears that the most active ice nucleation structure (class A) contains the ice nucleation protein linked to phosphatidylinositol and mannose. The class B structure has been found to contain protein presumably linked to the mannan and glucosamine moieties but definitely not to the phosphatidylinositol. The class C structure, which

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has the poorest ice nucleation activity, appears to be the ice nucleation activity linked to a few mannose residues and to be partially imbedded in the outer cell membrane (Turner *et al.* 1991).

The application of the INA cells also caused freezing of certain model solutions at -6 °C, such as sucrose solution (10%), which did not freeze at the same conditions without INA bacterial cells. Additions of INA cells also shortened the total freezing time of the model systems by between 20 and 38%. These results suggest that with the application of bacterial ice nucleation, some current food freezing processes may be modified to operate at higher subzero temperatures to provide guaranteed freezing, energy savings and improvement of efficiency and product quality (Li *et al.* 1997).

Some steps are needed to incite the rainfall process. Condensation of water on earth because of sun heating and evaporation of water vapour into clouds are needed. Some INA bacteria are also predicted to play an important role in condensation and ice nuclei formation in clouds. Ice formation in tropospheric clouds is required for snow and most rainfall. Studying the ubiquity of biological ice nucleators in snowfall has been done. Biological ice nucleators at warm temperature are abundant in fresh snow samples (Christner *et al.* 2008). Most of the studies on INA bacteria are conducted in subtropical areas. Little is known about the presence and distribution of INA bacteria from tropical area. Therefore, studies on INA bacteria from tropical area, especially Indonesia, need to be conducted. It also reported by Morris *et al.* (2004) that these bacteria also play a role in atmospheric processes leading to rain, given that they are readily disseminated into the atmosphere and have been found in clouds at altitudes of several kilo meters. That they participate in a sort of biological cycle of precipitation - whereby they are transported into clouds from plant canopies and incite rain thereby causing favourable conditions for their growth on plant surfaces - was proposed about 20 years ago. Today, sufficient evidence and meteorological tools have emerged to re-ignite interest in bioprecipitation and in the ways in which plants play a role as cloud seeders.

The objectives of these studies are to isolate the INA bacteria from rain-water and air, and also to get data of the distribution of these bacteria, in these two habitats.

MATERIALS AND METHODS

Sampling Procedure and Enumeration of Bacterial Population from Rain-Water. Rain-water samples were collected from several locations (Jakarta, Bogor, Bekasi, Tangerang, and Depok) between March to May 2008. Some rain-water samples were diluted 2 to 4-fold in sterile phosphate buffer, and others were not before spread onto King'S B medium agar.

Plates were incubated at 30 °C for 1-2 days, and then total bacteria were counted. Single colony was purified onto fresh King'S B medium agar. One loop of representative colonies (~ 6 x 10^4 CFU/ml, measured at A_{600}) was suspended in 400 µl of phosphate buffer and tested for ice nucleation activity after equilibration to -10 °C in a circulating alcohol bath (the tube nucleation test) (Lindow *et al.* 1978).

Sampling Procedure and Enumeration of Bacterial Population from Air. Luria Agar medium were opened in several locations (Jakarta, Bogor, Bekasi, Tangerang, and Depok) for 0.5-1 minute at 2-3 different days, between March to May 2008. Plates were incubated at 30 °C for 1-2 days, and then total bacteria were counted. Single colony was purified onto fresh LA medium. One loop of representative colonies (~ 6 x 10^{-4} CFU/ml, measured at A_{600}) was suspended in 400 µl of phosphate buffer and tested for ice nucleation activity after equilibration to -10 °C in a circulating alcohol bath (the tube nucleation test).

Ice Nucleation Protein Classification Based on Freezing Temperature. One loop of positive colonies (~ 6 x 10^4 CFU/ml, measured at A_{600}) was suspended in 400 µl of phosphate buffer and tested for ice nucleation activity at -2 to -10 °C in a circulating alcohol bath. Tube with positive result will be shown like Figure 1. The phosphate buffer will be frozen because of the presence of INA bacteria (Lindow *et al.* 1978).

One of the isolates, which is has the highest ice nucleation activity, was continue for PCR amplification of 16s rRNA gene and DNA sequencing analysis.

PCR Amplification of 16S rRNA Gene. The genomic DNA was extracted with CTAB method (Doyle & Doyle 1987). Then the 16S rRNA genes were amplified using Polymerase Chain Reaction (Perkin Elmer geneAmp PCR System 2400). The PCR reaction were performed in a 25 µl volume containing 12.5 µl GoTaq (buffer, dNTP mix, and DNA polymerase) (Promega), 1 µl of 63 forward primer (New England BioLabs) (25pmol) (63f: 5'-CAGGCCTAACACAT-GCAAGTC-3'), 1 µl of 1387 reverse primer (New England BioLabs) (25 pmol) (1387r : 5'-GGGCGGAWGTGTACAAGGC-3'), 1 µl of DNA template, and 9.5 µl ddH_2O. The cycles used were as follow 94 °C for 2 min then 25 cycles: 94 °C for 30 sec denaturation, 55 °C for 30 sec annealing, 72 °C for 1 min elongation, 72 °C for 20 min after 25 cycle, and was held at 4 °C until further use and then checked by electrophoresis.

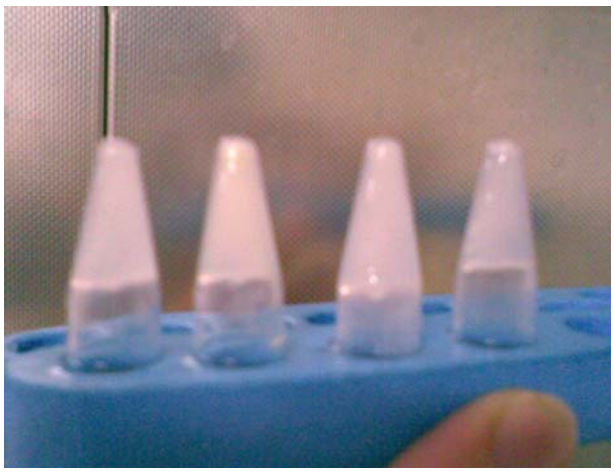

Figure 1. Positive results of tube nucleation test.

DNA Sequencing Analysis. The PCR product of 16S rRNA gene was purified using PCR DNA Fragments Extraction (Geneaid) Kit and continued by measurement of DNA concentration using Gene Quant (Amersham Biosciences). The DNA was then sequenced by using ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City, Calif.). DNA sequence was compared with data base sequences using ORF Finder and BLAST Nucleotide tools.

RESULTS

Jakarta had the highest average number of INA bacteria percentage among 5 different locations in Indonesia, and then followed by Bogor (Table 1). The percentage of INA bacteria from rain-water for Jakarta, Bogor, Bekasi, Tangerang, and Depok are 19.4, 18.7, 5.3, 2.2, and 6.4% respectively while percentage from air are 9.5, 6.5, 0, 2.7, and 1.8%.

The quantity of the bacteria was counted to know both of the total population and total INA bacteria on each sample. We discovered that the total population of the bacteria from rain-water variated from 10^1 to 10^3 cells/ml (Table 2), while the total population of bacteria from air variated from 10^1 to 10^2 cells/min (Table 3).

There are 2 isolates (A32 and A35 isolates) from Jakarta that able to freeze water at -2.7 °C. Just one isolate (A32) was continued for DNA sequencing analysis. It was showed that A32 isolate had 100% similarity with *Pantoea* sp. From rain water samples in Bogor, one positive colony was identified as *Pseudomonas putida* (99% similarity) and from air samples in Bogor we obtained another positive one and identified as *Acinetobacter baumannii* which was showed 98% similarity (Table 4).

DISCUSSION

From Table 1, we found that the presence of INA bacteria from rain water majority higher than the air from same location of sampling. From those data we hypothesized that INA bacteria might be play an important role in nucleation process which is required for rainfall. This result support research conducted by Christner *et al.* (2008), who found high frequency of INA bacteria from snowfall and they suggest the role of these bacteria in snowfall.

INA bacteria has been detected from rain-water samples and air with a various number. King's B Agar was use as selective growth medium for bacteria from rain-water because this medium expected have suitable nutrition for INA bacteria growth, while LA medium, which is more nutritious, is preferable for bacteria from air. From our preliminary study, there is no growth of bacteria when we used the King's B Agar medium for isolation of bacteria from air.

From the isolation results, we obtained many colony variations, mostly in colour. Colony of bacteria that produced a shiny green pigment on King's B medium, which resembled *P. syringae*, were found repeatedly with a vast number from Bogor rain-water samples. This

Table 2. Total and INA bacterial populations in sample from rain-water collected from five different locations

Locations	{log[cells/ml(spread sample)]/[total/INA]}*
Jakarta 1	3.2/1.0
Jakarta 2	3.2/0.5
Depok 1	0.3/1.0
Depok 2	3.7/1.4
Bekasi 1	3.3/1.1
Bekasi 2	0.9/1.5
Tangerang 1	3.6/1.7
Tangerang 2	3.8/1.6
Bogor 1	3.9/1.2
Bogor 2	0.3/ND**
Bogor 3	NV***/ND

*First figure of each pair is log of the total bacterial population; the second is the log of the INA population, **ND, INA bacteria were not detected, ***NV, total bacterial were not valid.

Table 3. Total and INA bacterial populations from air at five different locations

Locations	{log[cells/minute(open time)]/[total/INA]}*
Jakarta 1	1.4/1.4
Jakarta 2	1.3/1.3
Jakarta 3	0.8/0.8
Jakarta 4	1.0/1.0
Depok 1	NV**/ND***
Depok 2	1.1/ND
Depok 3	0.8/ND
Depok 4	NV/ND
Bekasi 1	NV/ND
Bekasi 2	0.8/ND
Bekasi 3	1.8/1.5
Bekasi 4	2.0/1.4
Tangerang 1	2.0/ND
Tangerang 2	2.4/1.6
Tangerang 3	1.6/ND
Tangerang 4	2.5/1.9
Tangerang 5	1.8/1.5
Tangerang 6	2.2/1.0
Bogor 1	1.8/1.5
Bogor 2	1.4/ND
Bogor 3	1.7/0.7
Bogor 4	1.8/1.5
Bogor 5	2.0/1.2
Bogor 6	1.3/1.0

*First figure of each pair is log of the total bacterial population; the second is the log of the INA population, **NV, total bacterial were not valid, ***ND, INA bacteria were not detected.

Table 1. Data of positive and total colonies obtained along with the percentage from five different areas

Area	Rain-water			Air		
	Positive colonies	Total colonies	Percentage (%)	Positive colonies	Total colonies	Percentage (%)
Jakarta	143	735	19.40	2	21	9.50
Bogor	142	761	18.70	5	80	6.50
Bekasi	5	98	5.30	0	95	0
Tangerang	6	259	2.20	16	598	2.70
Depok	29	450	6.40	1	56	1.80

Table 4. Classification data and alignment result of isolates with highest nucleating activity

Isolate number	Source	Classification	Temperature of nucleating activity (°C)	Alignment result
A32	Jakarta rain water	A	-2.7	100% similarity with *Pantoea* sp.
B11	Bogor rain water	B	-5.0	99% similarity with *Pseudomonas putida*
BkA7	Bekasi air	B	-7.0	98% similarity with *Acinetobacter baumannii*

morphology was also found in leaf samples from California that were studied by Lindow *et al.* (1978). *P. syringae* first known as a pathogen on tomato plants and able to produce a pigment, known as *siderophore pyoverdin*. It is a Gram-negative bacteria, rods, not producing spore, arginine dihydrolase and oxidase negative. Classification of this bacteria is: subdivision Gammaproteobacteria, order Pseudomonadales, family Pseudomonada-ceae, and genus *Pseudomonas*. *P. syringae* is a nutritionally versatile organism (Lindoe *et al*. 1982).

Meanwhile, isolates from other locations were mostly yellowish and resembled *E.herbicola* in appearance. *Erwinia* is a Gram-negative bacteria, able to use citrate, H_2S production, motil, and have 50-58% of G+C. Member of the genus *Erwinia* are major pathogens of crop plants and cause blights, wilts, and several other plant diseases. Classification of this bacteria is: domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae, genus *Erwinia*. Some bacteria from Jakarta rain-water also produce a pink pigment, which is resembled to *Pseudomonas mesophilica*.

Heterogeneous ice nuclei are necessary, and the common epiphytic ice nucleation active (INA) bacteria *P. syringae* and *E. herbicola* are sufficient to incite frost injury to sensitive plants at -5 °C. The ice nucleation activity of the bacteria occurs at the same temperatures at which frost injury to sensitive plants occurs in nature. Bacterial ice nucleation on leaves can be detected at about -2 °C, whereas the leaves themselves, i.e. without INA bacteria, contain nuclei active only at much lower temperatures. The temperature at which injury to plants occurs is predictable on the basis of the ice nucleation activity of leaf discs, which in turn depends on the number and ice nucleation activity of their resident bacteria. INA bacteria incited frost injury to all of the species of sensitive plants tested (Lindow *et al.* 1982).

It was reported by Hirano *et al.* (1985) that the bacterial ice nucleation frequency (NF = ratio of number of ice nuclei to number of bacterial cells) was dependent on the conditions under which the cells were grown, including composition of the medium and temperature during growth and on the assay temperature itself. Lindow *et al.* (1982) also reported that not every cell of a given bacterial isolate that has ice-nucleating properties can serve as an ice nucleus at any given time and temperature. The ratio between the number of ice nuclei and number of bacterial cells in a culture (i.e. nucleation frequency) was found to vary with incubation temperature, growth medium composition, culture age, and genotype.

Some of the INA bacteria isolates were classified based on freezing temperature, class A, B, and C. Bacteria that belonged to class A can freeze the water at -2 to -5 °C. While the freezing point for bacterias from class B and C are -5 to -8 °C and below -8 °C, respectively. Bacteria samples are mostly belong to class C. There are 2 isolates (A_{32} and A_{35}) from Jakarta that able to freeze water at -2.7 °C. Just one isolate (A_{32}) was continued for DNA sequencing analysis. It is showed that isolate A_{32} has 100% similarity with *Pantoea* sp. From Bogor rain sample, one positive colony identified as *P. putida* (shows 99% similarity) and from Bekasi air we obtained another positive one and identified as *Acinetobacter baumannii* which is show 98% similarity.

Acinetobacter baumannii is a species of pathogenic bacteria called aerobic gram-negative bacillus and is naturally sensitive to relatively few antibiotics. *A.baumannii* forms opportunistic infections. *Acinetobacter* have no flagellum and cause nosomical infections. Classification of this bacteria is: domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Moraxellaceae, genus *Acinetobacter*.

Pantoea is a genus of Gram-negative bacteria of the family Enterobacteriaceae. It is facultative anaerobic, rods, catalase-positive and oxidase-negative. This bacteria found in soil, water and plants, and also in animals ranging from insects to humans (Paradis *et al.* 2005). *Pantoea ananatis*, which was first reported as a pathogen of pineapple fruit causing brown rot, is also classified as an INA bacteria and has *inaA* gene (Watanabe & Sato 1998).

In this study Jakarta showed higher frequency of INA bacteria than Bogor. In facts, Bogor has higher rain intensity than Jakarta (Table 1 and 2). One explanation might be because Bogor is located in higher place, which have lower temperature. Low temperature is one of the important factors for the expression of ice nucleation protein because the INA bacteria need the supercooling condition to be induced. That mean even though the number of INA bacteria found from Bogor on that period of time was lower, but the INA protein from those bacteria were expressed, since the expression were induced by lower temperature.

It was shown that the distribution of INA bacteria were different for each location (Table 2 and 3). It was also reported by Hirano *et al.* (1985) that population of bacteria on leaf surface, which also related with the INA bacteria population in aerosols, influenced by temperature, relative humidity, radiation, wind speed, and other weather parameters that changed diurnally. Rain or irrigation may change the environment of the leaf surface suddenly and drastically. Reported by Lindow *et al.* (1978), the overall abundance of INA bacteria on plan surface, taken together with the extent of terrestrial plant cover, may provide an

excellent source of atmospheric INA at relatively warm temperatures. Study further need to be conducted by increasing the number of samples from rain-water and air.

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