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Isolation, Characterization, and Genetic Diversity of Ice Nucleation Active Bacteria on Various Plants

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Ice nucleation active (INA) bacteria is a group of bacteria with the ability to catalyze the ice formation at temperature above -10 °C and causing frost injury in plants. Since, most of the literature on INA bacteria were from subtropical area, studies of INA bacteria from tropical area are needed. We sampled eight fruits and 36 leaves of 21 plant species, and then identified through biochemical and genetic analysis. INA bacteria were characterized for INA protein classification, pH stability, and optimization of heat endurance. We discovered 15 INA bacteria from seven plants species. Most of bacteria are oxidase and H_2S negative, catalase and citrate positive, gram negative, and cocoid formed. These INA bacteria were classified in to three classes based on their freezing temperature. Most of the isolates were active in heat and pH stability assay. Some isolates were analysed for 16S rRNA gene. We observed that isolates from *Morinda citrifolia* shared 97% similiarity with *Pseudomonas* sp. Isolate from *Piper betle* shared 93% similarity with *P. pseudoalcaligenes*. Isolate from *Carica papaya* shared 94% similarity with *Pseudomonas* sp. While isolate from *Fragaria vesca* shared 90% similarity with *Sphingomonas* sp.

Key words: INA bacteria, isolation, characterization, genetic diversity, plants

INTRODUCTION

The melting temperature of ice is 0 °C, but water does not necessarily freeze at that temperature. In fact, liquid water has the ability to supercool to temperatures well below zero before the phase transition to ice occurs. Small volumes of pure water will not spontaneously freeze until the temperature reaches -38 °C, the homogeneous nucleation temperature (Th) (Edwards *et al.* 1994).

Ice nucleation at temperatures greater than Th is induced by heterogeneous ice nucleating agents. The agents are believed to act by mimicking the structure of polar groups that exist on the surface of ice, inducing epitaxial growth of ice at the water INA interface. Several organic and inorganic materials have been shown to have heterogeneous ice-nucleating ability (Lindow 1983). Ice nucleating agents has also been identified from biological sources. The most thoroughly characterized biological heteronucleator is found in ice nucleation active (INA^+) bacteria. The INA^+ phenotype has been found in species of three gram-negative genera: *Erwinia*, *Pseudomonas*, and *Xanthomonas.* All INA + bacteria are epiphytic and often ubiquitous on agronomically important crops.

These bacteria, as epiphytes, are a primary cause of frost damage to plants. Ice nucleating bacteria have also evolved as symbionts that facilitate early freezing as part of cold adaptation processes in some plants and animals. Ice nucleating bacteria are present in large numbers throughout the biosphere on the surfaces of nonconiferous plants, and are likely widely dispersed in the earth's atmosphere (Wowk & Fahy 2002).

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The presence of INA bacteria on leaves and other plant parts prevents water on and within frost-sensitive plants from supercooling, thus leading to internal ice formation and frost damage. It has been hypothesized that by virtue of their ice-nucleation capability, these bacteria may play a role in global climatology, serving as atmospheric freezing nuclei important in precipitation processes.

Physiological and biochemical investigations have shown that the ice-nucleation site of INA bacteria is proteinaceous and preferentially localized in the outer membrane. Lipids and other membrane components, while not sufficient to serve as a nucleating site, may be act to stabilize the active catalytic site (Arepura *et al*. 1988).

The genetic basis for ice nucleation in bacteria is known to be a single chromosomal gene, *ina*, which is both necessary and sufficient for the INA^+ phenotype. To date, *ina* genes (alternatively called *ice* in one species) have been cloned and sequenced from five species: *inaZ* from *Pseudomonas syringae*, *inaW* from *Pseudomonas fluorescens*, *iceE* from *Erwinia herbicola*, *inaA* from *Erwinia ananas*, and *inaX* from *Xanthomonas campestris* pathovar translucens (Edwards *et al.* 1994).

Understanding the variety, habitat, biology characteristic, and genetic diversity of the INA bacteria is required to control the amount of these bacteria in nature, or even to use these bacteria in several needs. Data of the presence of INA bacteria on the leaf surfaces are commonly from subtropical areas. 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 (Morris *et al.* 2004). Christner *et al.* (2008) found INA bacteria are abundant in fresh snow samples. Riupassa *et al.* (2005) studied the Indonesian edible plants and the

abundant of their phyllosphere bacteria. However, the role of these bacteria on tropical leaves need to be scrutinized more. Therefore, we studied INA bacteria from tropical area, especially Indonesia. We have isolated and identified INA bacteria from Indonesian edible leafy plant Poh-pohan (*Pilea glaberina*) (Waturangi *et al.* 2008).

In this study we explored INA bacteria from other plants, focused on fruits, vegetables, medicinal plants, and decorational plants.

Here, we reported the isolation, identification, and characterization of the INA bacterias and their ice nucleation proteins from several plants.

MATERIALS AND METHODS

Sampling Procedure and Enumeration of Surface Bacterial Population. Fruits and leaves samples were collected from several plants species (Table 1). The samples (0.5-5 g weight) and vigorously washed for 2 hours in sterile phosphate buffer (10 mM, pH 7.2). Washing solution was decanted and centrifuged at 10,000 x g for 20 min, and the pellet was suspended in 5 ml sterile phosphate buffer. After appropriate five-fold dilutions in phosphate buffer, samples were plated in King'S B agar. Plates were incubated at 28 ^{o}C for 1-2 days, and then total bacteria were counted. All single colonies were purified onto new King'S B medium agar. The representative colonies was suspended in 0.5 ml of phosphate buffer (6×10^4 CFU/ml) and tested for ice nucleation activity after equilibration to -5 or -10 ^{o}C or both in a circulating alcohol bath.

Determination of Ice Nucleation Frequencies. A cell suspension was prepared in 0.5 ml of sterile phosphate buffer. Ten-fold dilution series in phosphate buffer were prepared from the suspension and 20 x 50 ìl droplets of each dilution were placed on paraffin/margarine-coated aluminum foil 'boats' floating on a circulating alcohol bath with temperature maintained constant at -5 ^{o}C (Lindow *et al.* 1978). In this study, frozen droplets intensity test was performed to determine the intensity of ice nucleation protein (INP) from ice nucleation activity (INA). Every frozen droplets suspension bacteria were counted according to Vali (1971). The nucleation frequency for each strain was determined by the ice nucleus concentration and cell density of the suspension as determined by dilution plating.

Protein Classification Based on Freezing Temperature. Positive colonies was suspended in 0.5 ml of phosphate buffer (6×10^4 CFU/ml) and 0.1 ml of suspension was added to 0.9 ml of phosphate buffer and tested for ice nucleation activity at -2 to -10 ^{o}C in a circulating alcohol bath.

pH Stability Assay. All of the positive colonies were suspended in 0.5 ml of phosphate buffer (6×10^4 CFU/ml) and 0.1 ml of suspension was added to 0.9 ml of phosphate buffer pH 3-10 in each reaction tube and tested for ice nucleation activity at -10 ^{o}C in a circulating alcohol bath. Positive ice nucleation activity was indicated by the formation of ice after 5 minutes incubation (Kieft & Ruscetti 1990).

Optimization of Heat Endurance. The colonies were suspended in 0.5 ml of phosphate buffer (6×10^4 CFU/ml) and 0.1 ml was added to 0.9 ml of phosphate buffer in reaction tube. The suspensions were heated at 30 to 90 ^{o}C and tested for ice nucleation activity in a circulating alcohol bath (Kieft & Ruscetti 1990).

Biochemical Assays. Any positive colonies of INA bacteria were assayed for Gram staining, indole, citrate, H_2S, catalase, and oxidase assays.

PCR Amplification of 16S rRNA Gene. First step of this analysis was DNA extraction with CTAB method (Doyle & Doyle 1987). Then the 16S rRNA genes were amplified using Polymerase Chain Reaction (Perkin Elmer geneAmp PCR System 2400). PCR reactions were performed in a 25 ìl volume containing 12.5 ìl GoTaq (buffer, dNTP mix, and DNA polymerase) (Promega, USA), 1 ìl of 63 forward primer (New England BioLabs, USA) (25 pmol) (63f: 5'-CAGGCCTAACAC ATGCAAGTC-3'), 1 ìl of 1387 reverse primer (New England BioLabs, USA) (25 pmol) (1387r: 5'-GGGCGGAWGTGT ACAAGGC-3 '), 1 ìl of DNA template, and 9.5 ìl ddH_2O. The cycles used were as follow: pre-denaturation at 94 ^{o}C for 2 min, 25 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 55 ^{o}C for 30 sec, elongation at 72 ^{o}C for 60 sec, followed by a prolonged extension at 72 ^{o}C for 20 min (Marchesi *et al*. 1998).

Genetic diversity was conducted by using amplified ribosomal DNA restriction analysis (ARDRA) for all positive INA bacteria. Three ìl of DNA was mixed with 2 ìl of 10X buffer and 2 U/ml of restriction enzyme *Hae*III (New England BioLabs) in a sterile microtube. The volume was adjusted to 20 ìl with ddH_2O. The tube was incubated at 37 ^{o}C for overnight. Profiles of digested 16S rRNA fragments were separated by gel electrophoresis using 2.5% agarose in 1X TAE buffer for 2.5 h at 45 V. Restriction pattern were analyzed using Treecon software to construct the phylogenetic tree.

DNA Sequencing Analysis. Some of the PCR products of 16S rRNA gene were purified using PCR DNA Fragments Extraction (Geneaid, Taiwan) Kit and the products were cloned to *Escherichia coli* DH5á with pGEM®-T (Promega, USA) easy vector systems. Plasmid from transformant cells were isolated using HiYield™ Plasmid Mini (RBC, China) Kit. Plasmid DNA were then sequenced by using ABI PRISM 310 Genetic Analyzer. Finally, the bacterial sequences were subjected to bioinformatics analysis (Basic Local Alignment Search Tool /BLASTN).

RESULTS

INA bacteria were isolated and calculated their number compared to the total of bacterial population on every samples. The samples for this study were collected from 4 species of fruits and 17 species of leaves. From those samples, we found 5 INA bacteria only from *Fragaria vesca* and 10 INA bacteria from 7 leaves species (Table 1).

All of INA bacteria were tested for the ice nucleation frequencies using drop test method and classified into three classes of INA bacteria. All isolates from *F.vesca* St1, St2, St3, St4, St5, and isolates Sl1, Sl2, Md1, and Py1 were classified to class A based on their freezing temperature. Isolates Sl3, Md2, and Db1 were classified to class B. Isolates Sr1 and So1 were

Table 1. Total and INA bacterial populations in sample washings from various plant species

Plant specimen	log [(cells/g)/total bacteria/ total INA bacteria]
Fruits	
Psidium guajava	5.8/ND
Eugenia aquea	5.3/ND
Solanum lycopersicum	6.7/ND
Fragaria vesca	6.4/0.5
Leaves	
Solanum lycopersicum	6.8/ND
Fragaria vesca	5.9/ND
Manihot esculenta	6.7/ND
Dahlia sp.	6.2/ND
Ipomoea aquatica	5.9/ND
Lilioasphodelus flava	6.3/ND
Capsicum sp.	5.1/ND
Morinda citrifolia	7.4/0.9
Daucus carota	5.6/ND
Alstroemeria L.	5.9/ND
Piper betle	7.2/0.9
Brassica rapa	6.4/ND
Arachis hypogaea	6,1/ND
Carica papaya L.	7.3/1.2
Allium fistulosum	6.9/1.2
Lactuca sativa	6.8/0.7
Ficus thunbergi	7.6/1.2

ND: INA bacteria were not detected.

classified to class C. Isolates St2 and St5 showed the highest nucleation activity and the lowest are So1 and Md1 (Table 2).

Most of the isolates are cocoid and Gram negative, except Sl3 with coccus formed, isolate Sl2 and Py1 with bacil formed, and isolate Sr1 and Db1 are cocoid, gram positive. Biochemical tests revealed that five isolates from *F. vesca* had identical biochemical properties (Table 3).

Some of the isolates (Md1, St2, Py1, and Sr1) were picked out based on their phylogenetic variety and subjected to DNA sequencing. The bacterial DNA sequences were subjected to analysis and submitted to Genbank for the accession numbers (Table 4).

The INA bacteria have protein that acts as a core for the nucleation processes. This result showed the stability of INP

Table 4. BLAST result of 16S rRNA INA bacterium genes and Genbank Accession num

Isolate	Alignment		Genbank
	Organism	Identity (%)	Accession No.
Md2	*Pseudomonas* sp. NF1a	97	EU913770
Sr1	*P. pseudoalcaligenes*.	93	EU913771
Py1	*Pseudomonas* sp. clone DS109	94	EU913772
St2	*Sphingomonas* sp.	90	EU913773

Table 2. Ice nucleation frequencies of bacterial strains

Isolate	Number of freezing drop	Total drop	Ratio (freezing drop/total drop)	Drop volume	Ice nucleation activity
Fragaria vesca 1 (St1)	17	20	0.85	50	4.89
F. vesca 2 (St2)	18	20	0.90	50	5.30
F. vesca 3 (St3)	13	20	0.65	50	4.05
F. vesca 4 (St4)	11	20	0.55	50	3.80
F. vesca 5 (St5)	18	20	0.90	50	5.30
L. sativa 1 (Sl1)	16	20	0.80	50	4.61
L. sativa 2 (Sl2)	16	20	0.80	50	4.62
L. sativa 3 (Sl3)	14	20	0.70	50	4.20
Piper betle 1 (Sr1)	10	20	0.50	50	3.70
P. betle 2 (Sr2)	16	20	0.80	50	4.61
Ficus thunbergi 1 (So1)	1	20	0.05	50	3.05
Morinda citrifolia 1 (Md1)	1	20	0.05	50	3.05
M. citrifolia 2 (Md2)	5	20	0.25	50	3.28
Allium fistulosum 1 (Db1)	2	20	0.10	50	3.10
Carica papaya 1 (Py1)	4	20	0.20	50	3.21

Table 3. Biochemical tests for INA bacteria

Isolate	Bacterial cell		Bacterial test of				
	Gram	Shape	Catalase	Oxydase	Citrate	H_2S	Indole
Fragaria vesca 1 (St1)	-	Cocoid	-	+	+	-	M +
F. vesca 2 (St2)	-	Cocoid	-	+	+	-	M +
F. vesca 3 (St3)	-	Cocoid	-	+	+	-	M +
F. vesca 4 (St4)	-	Cocoid	-	+	+	-	M +
F. vesca 5 (St5)	-	Cocoid	-	+	+	-	M +
L. sativa 1 (Sl1)	-	Bacil	+	-	+	-	M -
L. sativa 2 (Sl2)	-	Cocoid	+	-	+	-	M -
L. sativa 3 (Sl3)	-	Coccus	+	-	+	-	M -
Piper betle 1 (Sr1)	+	Cocoid	+	-	+	-	M -
P. betle 2 (Sr2)	-	Cocoid	+	-	-	-	nM -
Ficus thunbergi 1 (So1)	-	Cocoid	+	-	+	-	M -
Morinda citrifolia 1 (Md1)	-	Cocoid	+	+	+	-	M -
M. citrifolia 2 (Md2)	-	Cocoid	-	-	+	-	M -
Allium fistulosum 1 (Db1)	+	Cocoid	+	+	+	-	M -
Carica papaya 1 (Py1)	-	Bacil	+	-	-	-	M -

at a wide range of pH, from pH 3 to 10 (Figure 1). The INP was also tested for the stability of heat endurance. Commonly, many proteins will be denaturated or inactive after heat treatment. In Figure 2, we found that the INP also has the same characteristics as other proteins.

Some isolates were tested for genetic diversity by using ARDRA method. *HaeIII* was used for the restriction process. This restriction enzyme will cut the 16S rRNA gene sequence at the position of 5' GG*CC 3' as the restriction site. From this method we obtained several digestion profiles that were showed in Figure 3. The phylogenetic tree was constructed from the binary data of ARDRA result. From the phylogenetic tree, the INA bacteria were classified into 3 clusters (A, B, and C) (Figure 4).

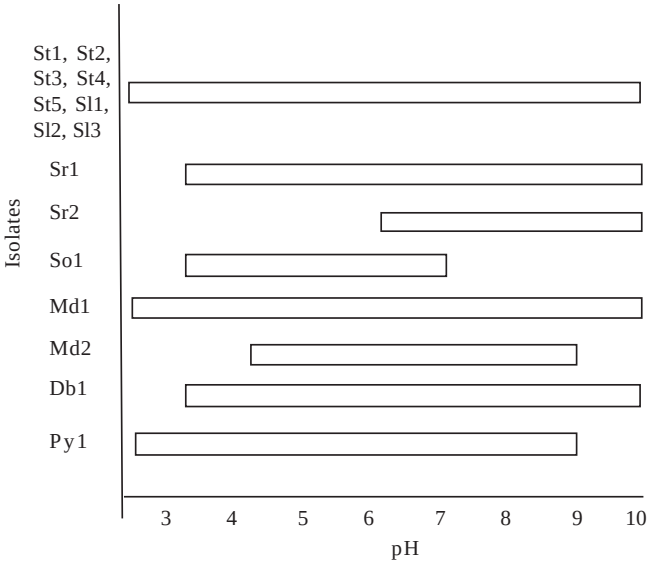

Figure 1. pH stability.

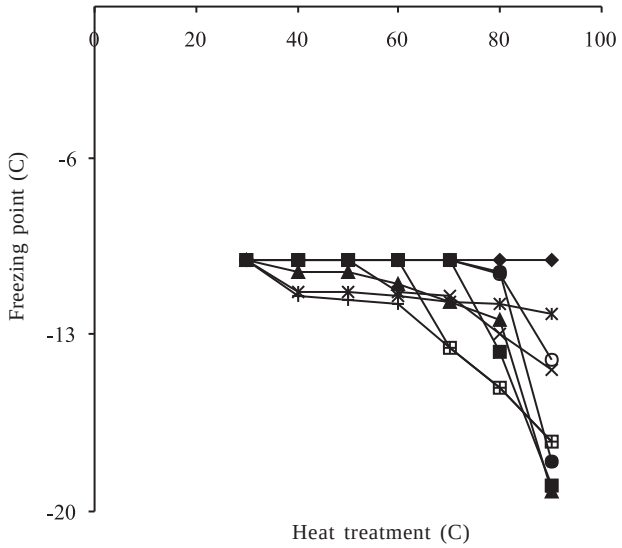

Figure 2. Optimization of heat endurance. ♦: St1, St2, St3, St4, St5, Sl1, Sl2; ■: Sl3; ▲: Sr1; ×: Sr2; ✱: So1; ●: Md1; l: Md2; □: Db1; ○: Py1.

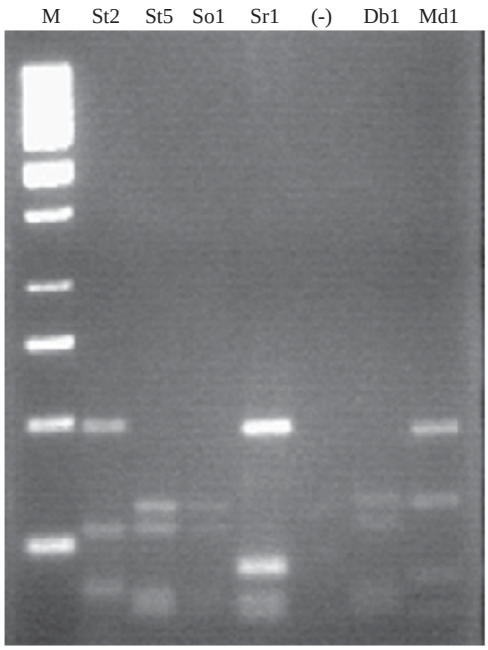

Figure 3. Profile of 16S rRNA gene digestion with *Hae*III Marker (M): 1 kb-ë ladder DNA (Fermentas), (-): negative control.

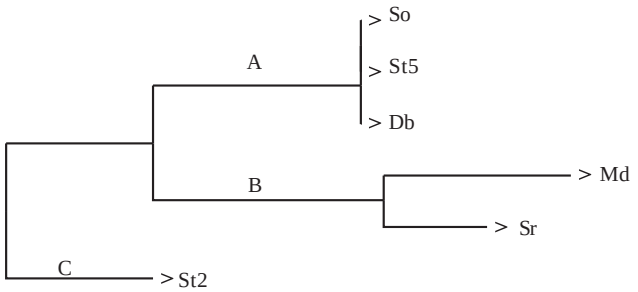

Figure 4. Phylogenethics tree of INA bacterium based on ARDRA result.

DISCUSSION

INA bacteria were found from various plant's leaves and fruits. We used King's B agar as a selective medium which was expected to have nutrition similar to those in the leaves. From the isolation results, we obtained many colony variation in quantity, morphology, and even colour. After counting total number of the bacteria, we discovered that total population of the bacteria varied from 10^5-10^7 cells/g (Table 1).

All of the bacterial colonies with different morphological characteristics were purified before the ice nucleation assay. This assay revealed that there were 15 isolates of INA bacteria. Five of them were recovered from *F. vesca* fruit, 3 isolates from *L. sativa*, 2 isolates from *P. betle*, 2 isolates from *M. citrifolia*, and 1 isolate each from *F. thunbergi*, *A. fistulosum*, and *C. papaya* leaves.

INA bacteria were divided into 3 classes, class A, B, and C, depending on their ice forming temperature. Belonging to class A can freeze water at -2 to -5 °C. While the freezing point for bacteria from class B and C are -5 to -8 °C and below -8 °C, respectively. In this study, most isolated INA bacteria belonged to class A (Table 2).

pH stability and optimization of heat endurance assays were conducted to understand the ice nucleation protein (INP) characteristics. Most of the isolates that contained INP were active and stable in pH and heating treatment. INP from Class A bacterium was more stable than other class in reference to pH and temperature stability (Figure 1 & 2).

Genetic diversity by using ARDRA showed most of the isolates revealed same digestion profile, such as isolate St5, So1, and Db1. Meanwhile, isolates St2, Sr1, and Md1 each have unique digestion profiles (Figure 3). The phylogenetic tree was constructed by Treecon using binary data from the whole digestion profile of 16S rRNA gene by *Hae*III. There were 3 different clusters based on the differences in the branch. Cluster 1 consisted 3 isolates with similar conformation restriction (So1, St5, and Db1). Cluster 2 consisted of 2 parts which were Md1 isolate and Sr1 isolate. St2 isolate was the third cluster (Figure 4).

Four isolates (Md1, St2, Py1, and Sr1) were picked out based on their phylogenetic variety and subjected to DNA sequencing. Using ABI PRISM 310 Genetic Analyzer, isolates from *M. citrifolia* (Md1) shared 97% similiarity with *Pseudomonas* sp. NF1a. Isolate from *P. betle* (Sr1) shared 93% similarity with *P. pseudoalcaligenes*. Isolate from *C. papaya* (Py1) shared 94% similarity with *Pseudomonas* sp. Meanwhile, isolate from *F. vesca* (St2) revealed 90% similarity with *Sphingomonas* sp. (Table 4). From these result, we found three isolates (Isolates Py1, Sr1, and St2) performed low level of similarity with the references. These isolates might be novel species of INA bacteria, since all of the references isolates collected in Genbank are from subtropical areas.

The phylogenetic tree analysis showed isolates Md1 and Py1 were classified as different cluster. The DNA sequencing result showed that these two isolates were similar to *Pseudomonas* sp., But actually the level of similarity of these two isolates with *Pseudomonas* is different. Isolate Md2 showed 97% similarity while isolate Py1 shared only 94% similarity. When we compared the biochemical test of INA bacteria (Table 3). According to Holt *et al.* (1994) the genus *Pseudomonas* are Gram negative, bacillus formed, and oxidase negative. *Pseudomonas* is one of the most popular INA bacteria genus. Soil, water, and air are the habitats of this bacteria. Every cubic of air contains approximately 40 *Pseudomonas* sp. cells. Some species of this bacteria are plant pathogenic bacteria that are found on the surface of the leaves. This bacteria may come from the air surrounding the plants and may contribute as a source of athmospheric ice nuclei. Isolates Sr, Md, and Py1 were similiar with bacteria that classified to genus *Pseudomonas* sp. and refer to biochemical result, these isolates were Gram and oxidase negative, but the shaped of the bacteria were different. Therefore, if we continue the study to analyzed the whole genome of the bacteria, we might get more discriminative results.

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