tivity of the assay was found to be independent of antiserum concentration used.

The competitive inhibition ELISA developed showed a sensitivity limit as low as 10 fg. This can be further adopted as a routine test for the detection of chlorpyrifos as a rapid and cost-effective tool for onsite mass screening of a large number of samples simultaneously.

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P. GOEL
J. KAPUR-GHAI*

Department of Zoology,
Punjab Agricultural University,
Ludhiana 141 004, India
**For correspondence.*
e-mail: kapurghaij@yahoo.com

Ice nucleating bacteria in hailstones during a hailstorm over Karad, India

Hailstones are a form of solid water formed within thunderstorms produced by cumulonimbus clouds. They are formed when warm air with high moisture rises up rapidly and meets the cold front of clouds in the atmosphere. Spontaneous crystallization of supercooled water is known to occur only at –40°C, the homogenous nucleation temperature. For crystallization to occur at higher temperatures, other heterogenous ice nucleating agents are required. Bacteria are known to produce special membrane-associated proteins called 'ice nucleation proteins' (INP) that are known to crystallize water at –2 to –14°C and serve as templates for ice crystals[1]. Bacteria are also known to form ice on leaf surfaces and have been discovered in clouds as well[2–5], but their role in the formation of hailstones is little known. Michaud *et al.*[6] reported the occurrence of bacteria in hailstones in 2011 and postulated on their possible role in the hailstone formation as well as in bioprecipitation. Here we report two bacterial species obtained from hailstones that show a marked ability to catalyse crystallization of supercooled water.

Four hailstones around 3 cm in diameter were collected during a thunderstorm in March 2011 on the campus of Yashwantrao Chavan College of Science, Karad, Maharashtra, India, in a sterile polypropylene beaker of 500 ml capacity. The largest hailstone having three layers was allowed to melt layer by layer in a sterile watch glass under aseptic conditions in a horizontal Laminar Air Flow Workbench (Yorco Sales Pvt Ltd, Model No. HL-42) at ambient temperature. The melted water of each layer was transferred to sterile tubes by means of sterile pipettes and streaked onto sterile nutrient agar plates that were incubated at 28°C till bacterial colonies appeared (around 48–72 h). The isolates were analysed for their phenotypic characters and their partial 16s rRNA sequences were determined using universal Eubacteria-specific primers[7]. They were analysed for sequence matching using the web-based SeqMatch of RDP-II and submitted to the database of DDBJ (available in DDBJ/EMBL/GenBank under accession numbers AB716761 and AB716762). Their evolutionary history was inferred by the neighbour joining method using MEGA 4 (refs 8 and 9).

The ice nucleation (IN) potential was studied by the 'tube nucleation test' method described by Hirano and Baker[10]. Glass test tubes of 18 × 150 mm size

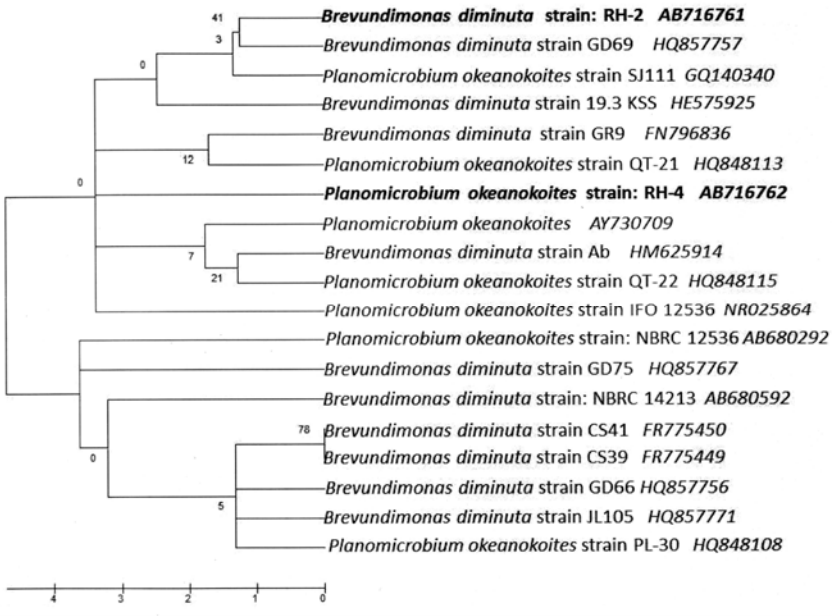

Figure 1. Neighbour joining tree of isolates RH-2 and RH-4 using bootstrap method.

Table 1. Crystallization of supercooled buffer by wet and dry biomass of the isolates

	RH-2		RH-4		*Escherichia coli*		*Pseudomonas aeruginosa*	
Time (h)	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
0	+	+	–	–	–	–	–	–
1			–	–	–	–	–	–
2			–	–	–	–	–	–
3			–	–	–	–	–	–
4			–	–	–	–	–	–
5			–	–	–	–	–	–
6			+	–	–	–	–	–
7				–	–	–	–	–
8				–	–	–	–	–
9				–	–	–	–	–
10				–	–	–	–	–
11				–	–	–	–	–
12				+	–	–	–	–
13					–	–	–	–
14					–	–	–	–
15					–	–	–	–
16					–	–	–	–
17					–	–	–	–
18					–	–	–	–

+, Crystallization of supercooled water; –, No crystallization of supercooled water.

were filled with 10 ml K-phosphate buffer (10 mmol, pH 7.0), plugged with cotton and sterilized by autoclaving at 121°C/15′. After cooling to room temperature, they were supercooled to –10°C by refrigerating in a 23% NaCl water bath overnight. The next morning, they were shaken vigorously and the tubes in which liquid was frozen or froze during shaking were discarded. The ones with liquid were used further. These tubes were equilibrated to ambient temperature (28°C in a water bath) and inoculated with 1 ml of cell suspension previously prepared in phosphate buffer to a density of 10^8 cells/ml of the isolates cultivated on nutrient agar. The tubes were mixed for cell dispersion, placed at –10°C and observed for freezing of the buffer at 30 min intervals.

In a separate experiment, the cell mass was concentrated to a pellet by centrifugation at 5000 rpm/15′ and added directly into the supercooled tubes by means of a spatula and monitored every hour for crystallization. A similar lot of bacterial cell mass was dried at 37°C to constant weight and also tested for IN potential. One tube without any bacterial inoculation and two tubes, one inoculated with *Escherichia coli* and the other with *Pseudomonas aeruginosa* were also observed in the same manner for controls.

Five bacterial strains were isolated from the three layers of hailstone. Two were from the outermost, one from the middle and two from the innermost core or embryo layer. All were studied for their IN properties; only two from the innermost layer showed this property. From phenotypic (data not shown) and genotypic (Figure 1) studies, these two isolates, RH-2 and RH-4 were identified as *Brevundimonas diminuta*[11] and *Planomicrobium okeanokoites*[12] respectively. When tested for their IN potential, both showed this activity prominently, with RH-2 (*B. diminuta*) being the most efficient among them. It crystallized the supercooled buffer within the first 30 min, whereas RH-4 (*P. okeanokoites*) froze the buffer after 5 h. When the concentrated cell mass was used, *B. diminuta* froze the buffer instantly (supplementary video, available online) with both wet and dry cell mass, whereas *P. okeanokoites* could achieve the freezing only after 5 h with wet and 12 h with dry cell mass respectively. Biomass of *E. coli* and *P. aeruginosa* used as control was unable to crystallize supercooled buffer even after 18 h (Table 1).

The fact that moist cell mass is more efficient than dry cell mass in the nucleation activity indicates that a certain amount of moisture is required by the bacteria for their IN activity. In the case of RH-2, however, it does not seem to make any difference. In light of the work of Michaud *et al.*, this study holds significance for the role of ice nucleating bacteria in the formation of hailstones. Further studies on isolation and purification of IN proteins involved in the nucleation are in progress.

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AVINASH A. RAUT*
SUMIT S. LOKARE
SHYAM S. BAJEKAL

Department of Microbiology,
Yashwantrao Chavan College of Science,
Karad,
Vidyanagar,
Karad 415 124, India
**For correspondence.*
e-mail: avinashraut@aol.in