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Ice Nucleation Induced by *Pseudomonas syringae*[1]

LEROY R. MAKI, ELIZABETH L. GALYAN, MEI-MON CHANG-CHIEN, AND DANIEL R. CALDWELL
Division of Microbiology and Veterinary Medicine, University of Wyoming, Laramie, Wyoming 82071

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Broth cultures of suspensions of *Pseudomonas syringae* isolated from decaying alder leaves (*Alnus tenuifolia*) were found to freeze at very warm (−1.8 to −3.8 C) temperatures. The initiation of freezing appears associated with the intact cell and not with extracellular material. Chemical treatments and physical destruction of the cell destroy activity. Bacteria must be in concentrations of approximately 10^6/ml before freezing at warm temperatures occurs.

Weather modification by seeding clouds with particles which serve as ice formation nuclei is a widely used procedure to alleviate the effects of severe weather conditions and to increase precipitation in arid regions (1). Most current seeding procedures use inorganic chemical particles (including AgI) (2) or artificially produced organic particles (3) as ice formation nuclei, but the use of these agents is both economically and environmentally undesirable compared with the use of naturally occurring, biologically generated particles. The occurrence of biologically formed ice nucleation particles in the atmosphere can be readily demonstrated (5, 6), and the work of Schnell and Vali (5) suggests that decaying tree leaves are an important source of these particles. R. Fresh (unpublished data) isolated a bacterium from decaying alder leaves (*Alnus tenuifolia*) which was highly active in initiating ice nucleation at relatively warm temperatures (−2.5 to −5.0 C). The isolation of a bacterium active in ice nucleation from decaying tree leaves suggests the possibility that the production of ice-nucleating particles during tree leaf decay is a microbially mediated process and that study of Fresh's isolate (designated as strain C-9) may lead to increased understanding of biologically mediated ice nucleation. Using the scheme described by Lelliott et al. (4), we have determined that strain C-9 is apparently a strain of *Pseudomonas syringae*. This report concerns the conditions influencing the production and stability of ice nucleating activity by strain C-9.

MATERIALS AND METHODS

Culture sources, media, and growth conditions. Cultures of *P. syringae* strain C-9 were obtained from the Department of Botany, University of Wyoming, and a culture of known *P. syringae* was obtained from D. J. Hagedorn, Department of Plant Pathology, University of Wisconsin, Madison. All other cultures used were obtained from the Division's stock collection. Cultures were maintained on Trypticase soy agar (TSA; BBL) slants at room temperature. The basal medium used for all tests of ice nuclei production was Koser broth (Difco) sterilized in Erlenmeyer flasks. Cultures were aerated by bubbling air through a glass tube which was passed through a cotton stopper into the medium. Cultures were incubated at room temperature (20 to 25 C).

Determination of cell numbers, growth curve, and nucleating activity. The growth curve and associated activity were determined by inoculating three 1-liter flasks containing 250 ml of Koser citrate broth at 12-h intervals, using 1.0 ml of a 10^{-3} refrigerated dilution (5.6×10^3 cells/ml) of a Koser citrate broth-grown culture. Sampling of all three flasks was begun at the time the third flask was inoculated. Sampling was repeated at 4- or 6-h intervals over a 24-h period, the nucleation activity was determined, and viable colony counts were made by plating dilutions of the cultures on Trypticase soy agar.

To determine the relation between bacterial numbers and activity, twofold dilutions of active cultures were prepared, and plate counts were made to determine viable cell numbers. Nucleating activities of successive dilutions were also determined.

Determination of freezing nucleus content. The freezing nucleus content of the bacterial cultures was measured by the nucleus spectrometer and procedures described by Vali (7). Thirty 0.01-ml drops of test material were placed on a controlled temperature surface and the temperature was slowly lowered from ambient temperature to −25 C. The temperatures at which 1, 10, 50, 90, and 100% of the drops froze were recorded. The temperature required to freeze either 50% (T_{50}) or 90% (T_{90}) was used as an end point.

Effect of physical and chemical agents on nucleating activity. The effect of disruption of active cell preparations on nucleating activity was determined by breaking concentrated cell suspensions either by sonic disruption (Sonifer cell disrupter, model W185, Heat Systems-Ultrasonics, Inc.) or with a Sorvall RM cell fractionator equipped with a Ribi

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valve. The latter was operated at 0 C at 30,000 lb/in².

The effect of chemical agents was determined by adding known concentrations of the agents to bacterial cultures showing good nucleating activity, incubating them for 4 h at room temperature, and subsequently measuring the activity.

The association of the activity with the bacterial cell was determined by using a membrane filter (0.45-μm pore size, HA; Millipore, Corp.) for filtration experiments or centrifuging for 20 min at 30,000 × *g* in a Sorvall RC-2 centrifuge.

RESULTS

Relationship between growth curve, cell concentration, and ice-nucleating activity. The relationship between the growth curve and ice-nucleating activity (T_{50}) is shown in Fig. 1. Isolate C-9 has a generation time of 2.5 to 3.0 h at 20 C in aerated culture. The ice-nucleating activity did not appear until the bacterial cell concentration reached 10^7 to 10^8/ml. The activity then rapidly reached a maximum at approximately −2 C.

When high activity was obtained, the culture could be diluted to 10^6 bacteria per ml before activity rapidly decreased (Fig. 2). It appeared that active cells must be in a concentration of 10^6/ml (10^4 per 0.01 ml drop tested) before one ice-nucleating event took place at warm temperatures. Further dilution rapidly decreased the temperature of ice nucleation by the suspension, and dilutions containing less than or equal to 10^4 cells/ml approximated the activity of the uninoculated medium.

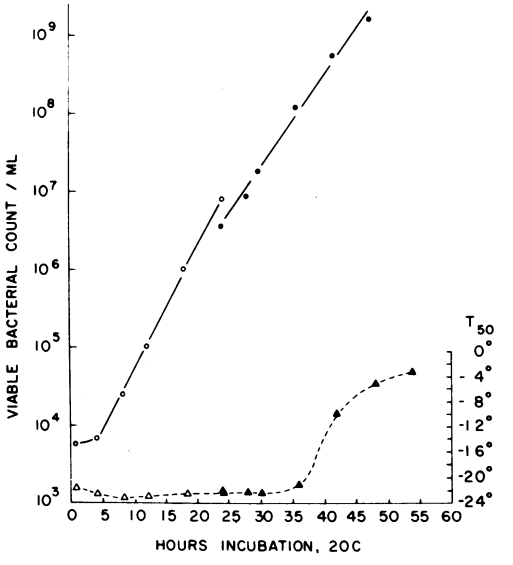

FIG. 1. *Relationship between viable cell numbers per milliliter and the production of ice nuclei by P. syringae strain C-9 as measured by the temperature required to freeze 50% (T_{50}) of the test samples. Symbols: ○, 0- to 24-h cultures; ●, 24- to 54-h cultures; △, freezing point (T_{50}) 0- to 24-h culture; ▲, freezing point (T_{50}) 24- to 54-h culture.*

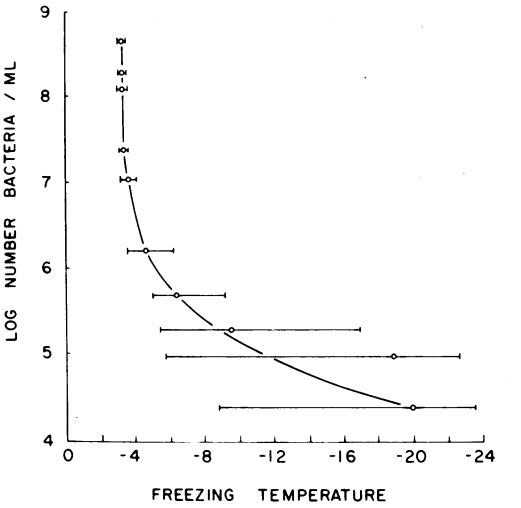

FIG. 2. *Effect of cell concentration on the nucleating activity of P. syringae strain C-9. The 1 to 90% (T_1, T_{90}) freezing range is given with the circles representing the point at which 50% (T_{50}) of the drops were frozen.*

Relationships between activity and whole cells. The ice-nucleating activity of the culture appeared to be associated with the intact cell. Heating a suspension to 65 C for 5 min, or disrupting the cells, apparently destroyed or inactivated the freezing nuclei which were active in the −2 C range (Table 1). The freezing nuclei which remained were those active at cooler (−8 to −12 C) temperatures. Further evidence of cell-associated activity was the observation that centrifugation for 20 min at 30,000 × *g* or filtration of active cultures through a 0.45-μm membrane filter removed the activity from the supernatant fluid or filtrate, but activity remained associated with the cells (Table 1).

Effect of dyes, antibiotics, and chemical agents. Further support that the activity is cell associated and may be associated with the cell wall was the observation that cetyl-pyridinium chloride, dyes, and physical disruption destroyed the activity (Table 2). Congo red, which does not combine with the cell wall, did not affect the activity. Certain antibiotics, notably streptomycin and polymyxin B, killed the cells without destroying activity (Table 2).

The activity was enhanced by aerobic culture and was quickly reduced when the culture was incubated without access to air. Activity could

TABLE 1. *Effect of cell disruption, centrifugation, and filtration on ice nucleation activity by P. syringae strain C-9*

Sample treatment	Ice-nucleating temperatures[a]	
	T_1	T_{90}
Cell concentrate control	−2.8	−4.1
Cells after Ribi-cell disruption[b]	−7.5	−8.8
Cell concentrate control	−1.8	−2.3
Cells after sonic disruption[c]	−8.0	−8.4
Control (not centrifuged)	−3.2	−7.5
Centrifuged, sediment[d]	−2.0	−2.3
Centrifuged, supernatant fluid	−7.4	−19.6
Control, not filtered[e]	−2.7	−3.3
Filtrate	−11.4	−23.9
Resuspended cells from membrane	−3.0	−3.4

[a] Temperatures required to freeze 1% (T_1) and 90% (T_{90}) of the test samples.
[b] Sorvall RM cell fractionator.
[c] Sonifer cell disruptor, model W185, Heat Systems-Ultrasonics, Inc.
[d] 30,000 × *g*, 20 min.
[e] Type HA, 0.45 μm (Millipore Corp.).

be restored by vigorous aeration.

Survey of other bacterial cultures for ice-nucleating activity. Initial observations that the activity appeared associated with the cell led us to examine other flagellated and non-flagellated bacteria to see whether ice-nucleating activity was a widespread phenomenon. All of the bacterial isolates examined except for the C-9 culture and the known *P. syringae* culture were inactive (Table 3).

DISCUSSION

P. syringae may play a role in the production of ice nuclei found in nature. It has been shown that the numbers of ice nuclei are most abundant in the temperate zones of the world (7). Recent research has shown that decomposing litters of plant leaves contain large numbers of freezing nuclei (10^9/g of decaying leaves active at −10 C) (5). The *Pseudomonas* C-9 isolate studied here is a plant-associated bacterium which, when added in pure culture to sterile leaves, has the potential of producing ice nuclei (R. Fresh, unpublished data).

The identification of the *Pseudomonas* C-9 isolate as *P. syringae* may limit the usefulness of this organism as a potential cloud-seeding agent. Because *P. syringae* is phytopathogenic, the culture must be killed or the freezing nuclei active at warm temperatures (−2 C) must be freed from the viable bacteria.

The effect of a number of chemical and physical agents was tested in an attempt to retain the nuclei active at warm temperatures while killing the bacteria or to separate the active nuclei from the cell. Treatment with

TABLE 2. *Effect of dyes, antibiotics, and chemical agents on nucleating activity*

Sample treatment	Ice-nucleating temperatures[a]		Viable cells/ml
	T_1	T_{90}	
Control (no treatment)	−2.8	−3.3	10^5
Methylene blue (0.001 M)	−12.5	−19.9	0
Crystal violet (0.001 M)	−17.4	−20.0	0
Safranin (0.001 M)	−17.2	−20.0	0
Control (no treatment)	−2.3	−2.7	10^6
Cetyl-pyridinium chloride (0.01 M)	−10.1	−20.0	0
Congo red (0.07 M)	−2.2	−2.8	10^6
Cetyl-pyridinium chloride (0.01 M) plus congo red (0.07 M)	−13.4	−20.0	0
Control (no treatment)	−2.6	−3.6	2×10^7
Penicillin (1,000 U/ml)	−2.5	−3.6	10^7
Streptomycin sulfate (100 μg/ml)	−2.7	−3.4	10^5
Polymyxin B (100 μg/ml)	−2.8	−4.7	10
Tetracycline (100 μg/ml)	−2.6	−4.0	10^6
Control (no treatment)	−1.8	−2.3	[b]
Mercuric chloride (2 mg/ml)	−6.4	−6.9	
Heat, 65 C for 5 min	−8.5	−12.0	

[a] Temperatures required to freeze 1% (T_1) and 90% (T_{90}) of the test samples.
[b] Not done.

TABLE 3. *Ice-nucleating activity of selected bacterial cultures*

Culture	Ice-nucleating temperatures[a] (C)	
	T_1	T_{90}
Pseudomonas syringae C-9	−2.9	−3.5
Known *P. syringae*[b]	−3.2	−3.9
P. aeruginosa	−7.5	−17.8
Staphylococcus epidermidis	−6.9	−19.5
Escherichia coli	−8.3	−17.1
Enterobacter aerogenes	−9.6	−17.0
Proteus mirabilis	−8.0	−19.4
P. vulgaris	−7.8	−17.0
Bacillus subtilis	−10.6	−18.0
B. cereus	−6.9	−17.0
Uninoculated medium	−9.2	−17.0

[a] Temperatures required to freeze 1% (T_1) and 90% (T_{90}) of the test samples.
[b] Obtained from Department of Plant Pathology, University of Wisconsin, Madison.

antibiotics such as polymyxin B offers the best means of killing the culture while retaining the nuclei active at warm temperatures (Table 2).

The ice-nucleating activity, which is predominant at warm temperatures (−2 C), appears associated with the intact cells since disruption of the cell is accompanied by a decrease in activity. Only freezing nuclei active at colder temperatures then remain. Attempts to remove the freezing nuclei from the intact cell have been unsuccessful.

After centrifugation the freezing nuclei active at warm temperatures are always associated with the intact cells. The activity of freezing nuclei remaining in the supernatant fluid approximates that found in the uninoculated medium.

The activity appears associated with the cell, and suspensions of cells grown on agar or in broth retained the activity. The identity of the nucleation catalyst is unknown.

The production of freezing nuclei by *Pseudomonas* C-9 during the period of logarithmic growth appears only after the cell concentration reaches 10^7 to 10^8/ml. Once this concentration of cells has been reached and the nuclei active at warm temperatures have been produced, the culture can be diluted to 10^5/ml before the freezing temperature is apparently lowered. It seems that a critical concentration of bacteria (10^7 to 10^8/ml) is required in a growing culture before freezing nuclei active at warm temperatures are produced. Once formed, the culture may then be diluted 100- to 1,000-fold before activity decreases and the nuclei active at cooler temperatures become apparent.

It is generally believed that a single event will initiate the freezing of each drop and that each ice nucleus will initiate freezing at a specific temperature. There is evidence that the initiation of freezing by *P. syringae* C-9 is a dynamic event since thawing and refreezing of large numbers of test drops often result in different freezing sequences, and the freezing temperatures of individual, repeatedly frozen drops may differ as much as 5 C.

In addition, it appears that one freezing event at −2 C occurs only when bacteria have reached concentrations of 10^4/0.01 ml, suggesting that only one bacterium in 10,000 is active at this temperature.

If the activity could be stabilized and isolated from dead cells, the active material might be compared with material isolated from leaf litter to determine whether ice-nucleating agents occurring in nature are derived from leaves or bacteria. It might also be possible to use this biodegradable material as an ice-nucleating agent in weather modification studies.

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LITERATURE CITED

1. Battan, L. J. 1969. Harvesting the clouds. Advances in weather modification. Doubleday & Co., Inc., Garden City, New York.
2. Cadle, R. D. 1966. Particles in the atmosphere and space. Reinhold Publishing Corp., New York.
3. Head, R. B. 1961. Steroids as ice nucleators. Nature (London) **191**:1058–1059.
4. Lelliott, R. A., E. Billing, and A. C. Hayward. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. **29**:470–489.
5. Schnell, R. C., and G. Vali. 1972. Atmospheric ice nuclei from decomposing vegetation. Nature (London) **236**:163–165.
6. Schnell, R. C., and G. Vali. 1973. World-wide source of leaf-derived freezing nuclei. Nature (London) **246**:212–213.
7. Vali, G. 1971. Quantitative evaluation of experimental results on the heterogenous freezing nucleation of supercooled liquids. J. Atmos. Sci. **28**:402–409.