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Identification of a Novel Ice-Nucleating Bacterium of Antarctic Origin and its Ice Nucleation Properties

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A novel ice-nucleating bacterium (INB) was isolated from Ross Island, Antarctica. INBs could be isolated more frequently than was generally thought. INB strain IN-74 was found in the white colony group. Strain IN-74 was identified from its taxonomic characteristics as a novel INB, *Pseudomonas antarctica* IN-74. When strain IN-74 was cultured aerobically in a medium consisting of the ice-nucleating broth (pH 7.0) for 6 days at 4°C, the ice-nucleating activity of strain IN-74 cells was obtained. Strain IN-74 cells produced ice nuclei only at extremely low growth temperatures. The nuclei appeared to be less thermolabile than those of INB *Pseudomonas fluorescens* KUIN-1. The freezing difference spectra in D_2O and H_2O at ice-nucleating temperature for strain IN-74 cells and conventional INBs (*Pseudomonas fluorescens* KUIN-1, *Pseudomonas viridiflava* KUIN-2, and *Pseudomonas syringae* C-9) exhibited different curves.

Key Words: ice-nucleating activity; ice-nucleating bacterium; ice-nucleating temperature; *Pseudomonas antarctica* IN-74.

Ice-nucleating bacteria (INBs) are defined as those bacteria that can initiate ice nucleation in water at temperatures above −10°C (17). At least six species of INBs have been found: *Pseudomonas syringae* (16), *Pseudomonas fluorescens* (16), *Pseudomonas viridiflava* (20), *Erwinia herbicola* (13), *Erwinia ananas* (5), and *Xanthomonas campestris* (5). Some strains of *Fusarium* (22) and related genera of fungi are also active in ice nucleation.

Bacterial nucleation is attributed to the presence of a protein, consisting of highly repetitive sequences of amino acids, in the bacterial outer membrane that serves as a template for ice crystallization (7). Although the broad distribution of INBs in soils, in leaf mulch, and on plants as well as their significance in promoting frost damage in crops is well documented, the number of known INB species is relatively small (6, 14, 18).

A new process for freeze texturing of food materials using INB (*Erwinia ananas* IN-10) was proposed by Arai and Watanabe (2, 3). Recently, the study of INBs has developed rapidly and achievements within the field have generated attention.

In a previous paper (21), we identified an INB, KUIN-3, isolated from strawberry leaves, as a strain of *Erwinia uredovora.* The presence of a novel INB of Antarctic origin has not been previously reported. This paper describes the identification of a new INB, strain IN-74, and its ice-nucleating properties. A detailed study of this strain should contribute to a better understanding of the physiology of ice-nucleating bacteria in general.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Strain IN-74 was isolated from Ross Island, Antarctica. The ice-nucleating medium was composed of DL-Serine (2.0*g*), DL-Alanine (2.0*g*), K_2SO_4 (8.6*g*), KCl (1.4*g*), $MgSO_4 \cdot 7H_2O$ (1.4*g*), sucrose (10*g*), and yeast extract (30*g*) in 1 liter of distilled water. The medium was adjusted to pH 7.0 with 0.1 N NaOH before autoclaving. One loopful of cells grown on a

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trypticase soy agar-slant for 24 h was inoculated into 50 ml of the above medium. Cells were grown in shaking flasks (50 ml) at 4°C. Growth was monitored by measuring the optical density (OD) at 660 nm. Cells were harvested from the culture broth and washed with 50 mM phosphate buffer (pH 7.0). All samples were transferred to 50 mM phosphate buffer (pH 7.0) for testing ice-nucleating activity.

Identification of Strain IN-74 of Antarctic Origin

Identification of the microorganism was carried out using previously described methods (4, 12). The physiological and biochemical characteristics of the microorganism were studied by the methods of Stanier *et al.* (23). DNA was isolated by Marmur's method (19). The DNA base composition was calculated from the thermal denaturation temperature (T_m) by following the procedure of Marmur and Doty (18).

Measurement of Ice-Nucleating Activity

For precise measurement of ice-nucleating activity, each cell suspension was initially diluted with 50 mM phosphate buffer (pH 7.0) to 1.0×10^7 cell/ml ($OD_{660} = 0.1$) and was further diluted by serial 10-fold dilutions. The ice-nucleating temperature was measured using a freezing nucleus spectrometer (thermoelectric plate Mitsuwa Model K-1), as described by Vali (26). That is, 30 drops, 10-μl each, were placed on a controlled-temperature surface and the temperature was slowly lowered from the ambient temperature to -20°C at a rate of 1°C/min. The temperatures required to freeze 10% (T_{10}), 50% (T_{50}), and 90% (T_{90}) of the drops were used in the analyses. The ice nucleation spectra of bacterial suspensions were obtained by the droplet-freezing method similar to that of Vali (26), as modified by Lindow *et al.* (15). Namely, the concentration of ice nuclei was calculated from the formula of Lindow *et al.* (15):

$$N(T) = (-\ln f)/V$$

where $N(T)$ is the nucleation frequency at temperature T, f is the proportion of droplets unfrozen, and V is the volume of individual droplets. The number of nuclei per cell was calculated by dividing the concentration of nuclei/ml by the density of the cell suspension.

Freezing Difference Spectra in D_2O and H_2O at Ice-Nucleating Temperature

Cells were centrifuged at 10,000*g* for 15 min at 4°C, and the cell pellets were suspended in the same volume of either ordinary 50 mM phosphate buffer (pH 7.0) in H_2O or 50 mM phosphate buffer (pH 7.0) in 90% (v/v) D_2O. The 90% (v/v) D_2O-phosphate was used instead of 100% D_2O because the cell pellets already contained water which could not be removed. The freezing difference spectra in D_2O versus H_2O were measured on a cold plate by the method described by Turner *et al.* (24).

Transmission Electron Microscopy

Specimens for electron microscopy were prepared by placing a drop of the bacterial culture (liquid) on a Formvar-coated grid. Cells were fixed for 10 min in 10% glutaraldehyde. After fixation, cells were stained with 2% phosphotungstate for 10 min. The stained specimen was observed using a JEM-1210 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Isolation and Identification of a Novel Strain IN-74 of Antarctic Origin

Bacteria previously isolated from Antarctica were used throughout this study. The samples were collected by Dr. G. Matumoto (Ohtsuma Women's University, Tokyo). A new ice-nucleating bacterium, IN-74, was isolated from sand of Ross Island in the McMurdo Dry Valleys region. We were able to find ice-nucleating bacteria of 11 strains from 135 strains by the droplet-freezing method (26). Ice-nucleating bacteria were isolated more frequently than is generally thought. Strain IN-74 grew at 0–30°C with an optimum temperature of about 10°C.

TABLE 1
Comparison of Conventional Ice-Nucleating Bacteria and Strain IN-74 of Antarctic Origin

Strain[a]	Incubation		Growth (OD_{660})	pH	Ice-nucleating temperature (°C)[b]		
	Temperature (°C)	Time (day)			T_{10}	T_{50}	T_{90}
P. antarctica IN-74	18	2	18.9	8.3	−15.7	−17.4	−22.7
P. antarctica IN-74	4	6	4.6	8.2	−4.4	−4.4	−4.6
P. antarctica IN-74[c]	0	15	7.5	8.0	−3.7	−3.7	−3.8
P. fluorescens KUIN-1	18	2	17.0	8.3	−2.5	−2.5	−2.6
P. viridiflava KUIN-2	18	2	10.7	8.8	−4.6	−5.9	−8.4
P. syringae IFO-3310[d]	18	2	11.7	8.0	−2.6	−2.7	−2.8
Distilled water	—	—	—	—	−18.7	−21.6	−24.1

[a] Cells were grown for 2 days at 18°C with shaking in the ice nucleation medium.
[b] Temperatures required to freeze 10% (T_{10}), 50% (T_{50}) and 90% (T_{90}) of cells.
[c] Cells were grown for 15 days at 0°C with shaking in the ice nucleation medium.
[d] IFO, Institute for Fermentation, Osaka.

Comparison of conventional ice-nucleating bacteria and strain IN-74 of Antarctic origin is shown in Table 1. Cells were grown on ice-nucleating broth for 15 days at 0°C, for 15 days at 4°C, or for 2 days at 18°C. The various ice-nucleating bacteria, other than strain IN-74, were found to be active in ice nucleation from −2.5°C (T_{50}) to −5.9°C (T_{50}) for 2 days at 18°C. However, when strain IN-74 was cultured aerobically in a medium consisting of ice-nucleating broth for 15 days at 0°C or for 6 days at 4°C, the ice-nucleating activity of strain IN-74 cells was obtained. But, the ice-nucleating activity of strain IN-74 cells was not obtained for 2 days at 18°C. Ice-nucleating temperature (T_{50}) was detected at −3.7°C for cell suspensions (1.0×10^7 cells/ml) of strain IN-74 incubated at −4°C and at −17.4°C (T_{50}) for cells incubated at 18°C. Ice-nucleating bacteria (*P. fluorescens* KUIN-1, *P. viridiflava* KUIN-2, and *P. syringae* IFO-3310) were not grown on ice-nucleating broth for 15 days at 0°C (data not shown).

Figure 1 shows a transmission electron micrograph of cells of strain IN-74 of Antarctic origin. The bacterium was a Gram-negative rod (0.6 to 0.7 by 1.1 to 2.2 μm), motile by polar flagella, strictly aerobic, catalase-positive, oxidase-positive, and fluorescent pigments-positive. The temperature range for growth was from 0 to 30°C. The mol % G + C of the DNA was 58.6 in strain IN-74. Morphological, physiological, and biochemical characteristics of strain IN-74 are summarized in Table 2. These characteristics indicate that strain IN-74 belongs to the genus *Pseudomonas*. Use of carbon source by strain IN-74 is shown in Table 3. The key properties of strain IN-74 were fluorescence pigment—positive, levan formation from sucrose—positive, Arginine dihydrolase—positive, Lipase (Tween 80 hydrolysis)—positive, denitrification—negative, Voges–Proskauer test—negative, trehalose—use, and L-valine—use. From these results, strain IN-74 was identified as *P. antarctica* IN-74 (12, 18, 19, 23).

Ice Nucleation Spectra for Strain IN-74 Cells Grown at Different Temperatures

The ice nucleation spectra for strain IN-74 cells grown at different temperatures is shown in Fig. 2. The ice-nucleating activity of strain IN-74 was greatly inhibited by the growth temperatures. High ice nucleation activity was observed in the range of 0 to 4°C, but the ice nucleation activities of strain IN-74 grown at 10, 18, and 30°C were much lower. Between

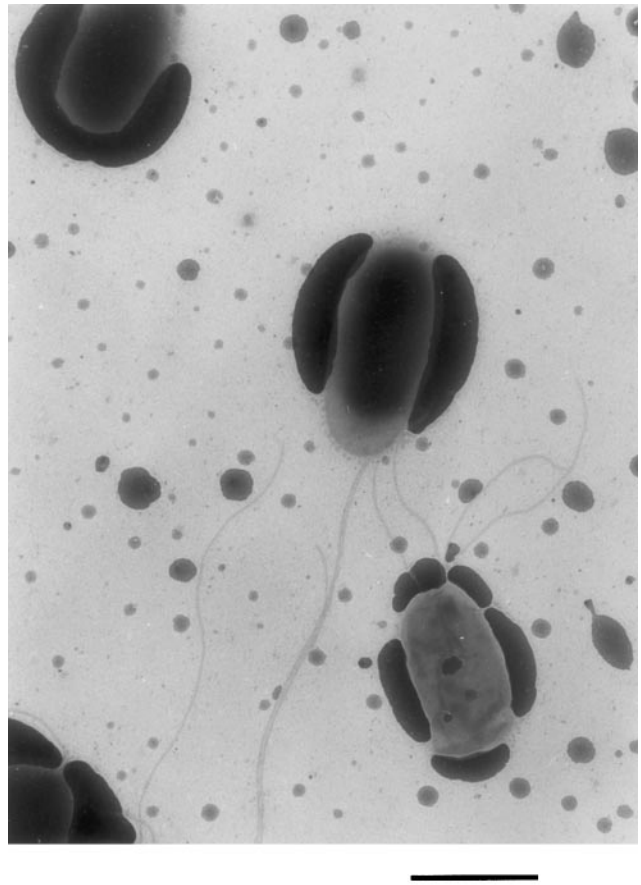

FIG. 1. Transmission electron micrograph of cells of a novel ice-nucleating bacterium, strain IN-74. Bar denotes 1 μm.

-4.0 and -8.5°C, the nucleation frequency of the intact cells (1.0×10^7 cells/ml) grown at 0°C increased from about 1.0×10^{-7} to about 5.0×10^{-3}. On the other hand, in strain IN-74 cells grown at 10°C, between -8.5 and -10°C the nucleation frequency of the cells increased from 1.0×10^{-7} to 1.0×10^{-5}.

Growth Characteristics of Strain IN-74

The relationship between the growth curve and the ice-nucleating temperature of strain IN-74 is shown in Fig. 3. Experiments were conducted over 20 days with the incubation period at 0°C. Strain IN-74 has a generation time of 14.4 h at 0°C in aerated culture. After incubating for 15 days, the organism reached a stationary growth phase. The ice-nucleating activity of the organism remained unchanged regardless of its growth phase.

Cell suspensions were incubated with 50 mM phosphate buffer (pH 7.0) at various temperatures for 30 min, and the ice-nucleating temperature was measured (data not shown). The ice-nucleating temperatures, T_{50} (°C), of cells were -3.7°C at 0°C, -8.6°C at 20°C, and -13.5°C at 90°C. This stability was not equal to that of the cells in other INBs (*P. fluorescens* KUIN-1, *P. viridiflava* KUIN-2, and *E. herbicola* IFO-12686). Strain IN-74 cells were sensitive to temperature (at

TABLE 2
Morphological, Physiological, and Biochemical Characteristics of Strain IN-74

Size (μm)	0.6 to 0.7 by 1.1 to 2.2	Levan formation from sucrose	Positive
Shape	Rods	Nitrate reduction	Negative
Gram stain	Negative	Denitrification	Negative
Motility	Positive	Gelatin hydrolysis	Negative
Growth in air	Positive	Lipase (Tween 80 hydrolysis)	Positive
Growth anaerobically	Positive	Voges–Proskauer test	Negative
Catalase	Positive	Methyl red test	Negative
Oxidase	Positive	Lecithinase (egg yolk)	Positive
Glucose (acid)	Positive	Indole production	Negative
Carbohydrates [F/O/−]	Oxidation	Starch hydrolysis	Positive
Fluorescens pigments	Positive	H_2S production	Positive
Arginine dihydrolase	Positive	Urease	Negative
Lysine decarboxylase	Negative	Protocatechuate, ortho cleavage	Positive
Ornithine decarboxylase	Negative	Mol% G + C of DNA	63.2

25°C or above). Furthermore, the pH stability held between pH 4.0 and pH 9.0. This result was nearly equal to the stability in other properties of the extracellular ice-nucleating substance and resembled those of ice nuclei from other INBs (*P. fluorescens* KUIN-1 and *Erwinia uredovora* KUIN-3) (8).

Ice Nucleation Spectra of Strain IN-74 Treated with D_2O

The ice-nucleation spectra of strain IN-74 treated with D_2O are shown in Fig. 4. The ice nucleation spectra of strain IN-74 and conventional INBs exhibited different curves. The cells exhibited typical spectra in which nucleation began at about −3.7°C for H_2O and 0.2°C for D_2O. It can be seen in Fig. 4, however, that the ice nucleation spectra in H_2O and D_2O for cells do not show curves with fixed differences.

After examining ice nucleation spectra, several laboratories (25) have proposed that, for convenience, three types (classes A, B, and C) of ice-nucleating activity could be inferred to occur on the bacterial surfaces.

Since these structures cannot be readily isolated and analyzed, their components have been

TABLE 3
Use of Carbon Source by Strain IN-74

Carbon sources	Use	Carbon sources	Use	Carbon sources	Use
Glucose	+	Geraniol	+	Citrate	+
Trehalose	+	Gluconate	+	Erythritol	−
2-Ketogluconate	+	Glycerol	+	Ethanol	+
meso-Inositol	+	Itaconate	−	D-Fructose	+
Geraniol	−	Lactose	−	D-Galactose	+
L-Valine	+	Maltose	−	D(−)-Tartrate	−
β-Alanine	+	Mannitol	+	L(+)-Tartrate	−
DL-Arginine	+	D-Mannose	+	L-Rhammose	+
Adonitol	−	Methanol	−	D-Xylose	+
L-Arabinose	+	Salicin	−	Caproate	−
n-Butanol	+	D-Sorbitol	+	Sucrose	+
Butyrate	−	Starch	−		

Note. Symbols: +, use; −, no use.

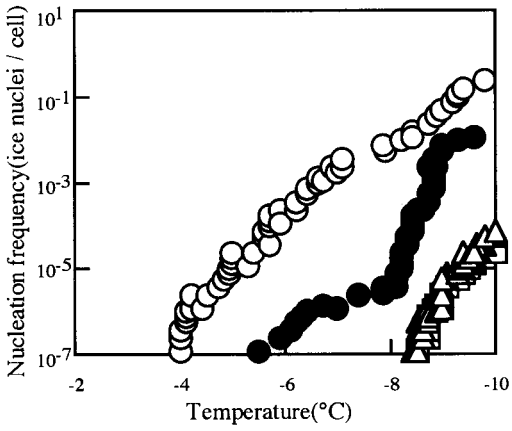

FIG. 2. Ice nucleation spectra for strain IN-74 cells grown at different temperatures. Ice nucleation spectra for strain IN-74 cells grown on ice nucleation medium at 0°C (○), 4°C (●), 10°C (▲), 18°C (△), and 30°C (□). Cells were harvested for 2 days at 18°C and then shifted to various temperatures.

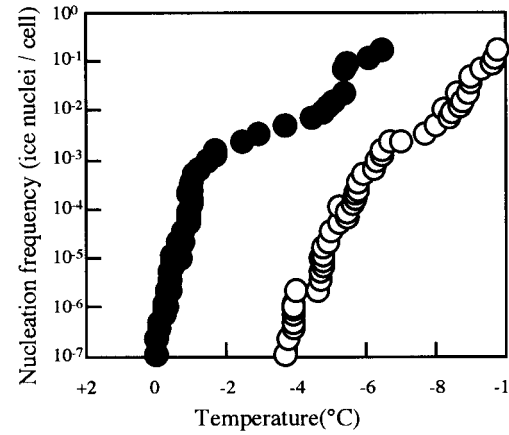

FIG. 4. Ice nucleation spectra of cells of strain IN-74 treated with D_2O. Cells (OD_{660} = 0.1) were treated with H_2O and D_2O in 50 mM phosphate buffer (pH 7.0). Cell suspensions were left for 24 h at 4°C. ●, 90% (v/v) D_2O; ○, H_2O.

identified by the use of specific enzymes or chemical probes and by stimulation of the formation of specific classes of freezing structures (28). From these preliminary studies, it appears that the most active ice-nucleation structure (class A) contains the ice-nucleation protein linked to phosphatidylinositol and mannose, and possibly glucosamine. 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 has a very weak ice nucleation activity, appears to be the ice-nucleation protein linked to a few mannose residues and to be partially embedded in the outer cell membrane (11, 25). It is suggested that strain IN-74 cells have the nucleation structures of the three classes (A, B, and C). However, the ice nucleation spectra of strain IN-74 cells and conventional INBs exhibited different curves (class B) (19).

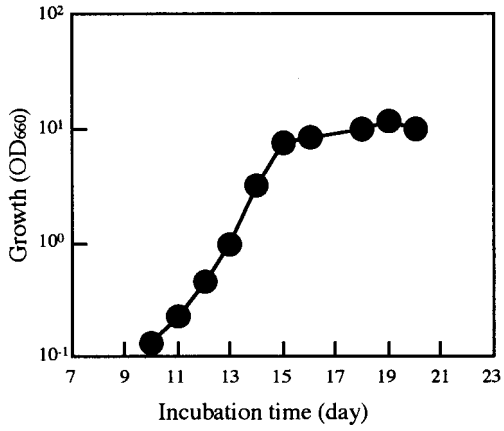

FIG. 3. Growth curve of strain IN-74. Cells were grown on ice nucleation medium (pH 7.0) at 0°C with shaking.

The freezing spectra of *P. fluorescens* KUIN-1 cells (19) and *P. syringae* C-9 cells (24) exhibited similar curves. Since D_2O is known to react more strongly than does H_2O with the hydrophobic domains of proteins (1), it seemed possible that class A and C structures were much more hydrophobic than were class B structures.

Freezing Difference Spectra in D_2O versus H_2O

Figure 5 shows the freezing difference spectra in D_2O and H_2O at ice-nucleating temperature for strain IN-74. A freezing difference spectrum (24) was calculated by subtracting the

freezing temperature for cells in H_2O from the freezing temperature of cells in D_2O at the same number of freezing nucleus units per cell. The freezing difference spectra in D_2O versus H_2O at the same number of ice nuclei/ml for three different bacteria (strain IN-74, *P. fluorescens* KUIN-1, and *P. viridiflava* KUIN-2) are shown in Fig. 5. The freezing difference spectrum of strain IN-74 and convenient INBs (*P. fluorescens* KUIN-1, and *P. viridiflava* KUIN-2) showed different curves. Cell activities at −3.7 to −4.6, −5.1 to −6.5, or −7.5 to −9.2°C were arbitrarily assigned as class A, class B, or class C, respectively. Since these structures cannot be readily isolated and analyzed, their components have been identified by the use of specific enzymes or chemical probes and by stimulation of the formation of specific classes of freezing structures (28). The freezing difference spectrum of strain IN-74 is distinct from the curve obtained for the ice nucleation-active *E. herbicola* and *P. syringae* C-9 (28). We postulate that the difference spectra indicate that there are three classes (A, B, and C) of nucleating structures on strain IN-74 or conventional INBs. However, it is suggested that the class B structure of strain IN-74 is distinct from the class B of conventional INBs. The freezing difference spectra in D_2O and H_2O at ice-nucleating temperature for strain IN-74 is analogous to the freezing difference spectra in D_2O and H_2O at the ice-nucleating temperature for the spermidine added extracellular ice-nucleating matter from *Erwinia uredovora* KUIN-3 (9).

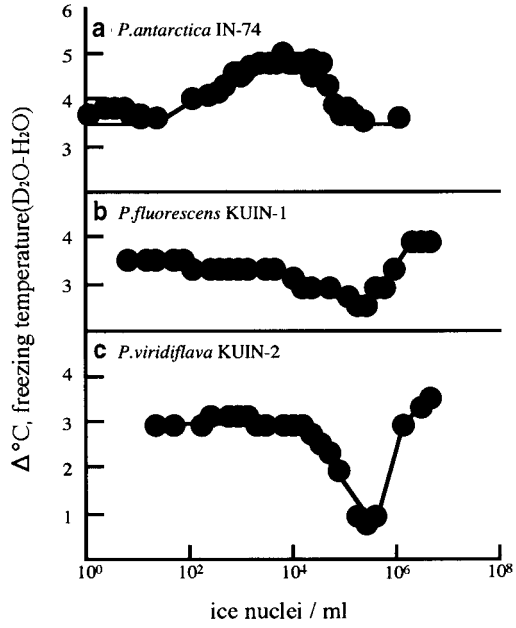

FIG. 5. Freezing difference spectra in D_2O and H_2O at ice-nucleating temperature. The freezing difference spectra was calculated from ice nucleation spectra shown in Fig. 4.

TABLE 4
Effects of Denaturants and Protein-Modifying Reagents on the Ice-Nucleating Temperature of Strain IN-74

Reagent[a]	Ice-nucleating temperature (°C)[b]		
	T_{10}	T_{50}	T_{90}
Cells	−3.7	−3.8	−3.9
+10% (v/v) Ethanol	−6.2	−6.5	−6.7
+3 mM Urea	−14.7	−14.9	−15.0
+3 mM *N*-Bromosuccinimide	−17.2	−20.0	−22.3
+9 mM Succinic anhydride (pH 4.0)	−6.4	−6.7	−7.0
+9 mM Iodo acetic acid (pH 4.0)	−6.7	−7.5	−7.8
+9 mM 2-Hydroxy-5-nitrobenzyl bromide (pH 4.0)	−4.8	−5.4	−5.8
+9 mM 4-(Hydroxymercuri) benzoic acid, sodium salt	−5.4	−5.7	−6.0
+3 mM *N*-Acetylimidazole (pH 4.0)	−4.9	−5.9	−6.5

[a] Cells (1.0×10^7 cells/ml) were treated with denaturants or protein-modifying reagents (pH 7.0) for 30 min at 0°C with shaking.

[b] After physical removal of reagent, for precise measurement of the ice-nucleating temperature, the cell suspension was diluted with a 50 mM phosphate buffer (pH 7.0) solution to 1.0×10^7 cells/ml (OD_{660} = 0.1). Temperature required to freeze 10% (T_{10}), 50% (T_{50}) and 90% (T_{90}) of test samples.

Effects of Denaturants and Protein-Modifying Reagents on the Ice-Nucleating Activity of Strain IN-74

Effects of denaturants and protein-modifying reagents on the ice-nucleating temperature of strain IN-74 are shown in Table 4. The ice-

nucleating activity of strain IN-74 was strongly inhibited by the addition of urea or *N*-bromosuccinimide. Further, ethanol, succinic anhydride, iodo acetic acid, *N*-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide, 4-(hydroxymercuri) benzoic acid, and sodium salt had an effect on the ice-nucleating activity of strain IN-74. Kozoloff (10) and Obata (19) have shown that the ice-nucleating activity in intact *P. syringae, P. fluorescens,* and *E. herbicola* strains is sensitive to protein-modifying reagents, suggestive of a role for protein in the ice-nucleating activity site. Therefore, the sensitivity of the ice-nucleating activity to protein sulfhydryl reagents and denaturants suggests the presence of a protein at the ice-nucleating site. Ice-nucleating *P. syringae* S 203 promotes the formation of ice in supercooled water by ice nucleators that contain a unique protein associated with the cell membrane (27). Green and Warren (7) sequenced an ice-nucleation gene from *P. syringae* S 203. We are attempting to characterize the molecular components of these bacterial ice nuclei. Details of an ice-nucleating substance from strain IN-74 will be reported at a date.

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