Note

Interaction of an Antinucleating Chemical and an Ice Nucleation Active Bacterium: a Case Study with an *n*-Octylbenzyltrimethylammonium Salt and *Erwinia ananas*

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Frost injury to plants is known to be caused by some strains of *Pseudomonas* and *Erwinia* species.¹⁻⁵ At a subzero temperature in the range of 0 to -3°C , they act as heterogeneous ice nuclei to freeze dewdrops on the surface of leaves and stems. We have already reported that a series of tetra-substituted ammonium salts inhibited the ice nucleation activity of *Erwinia ananas* cells and their outer membranes.⁶⁾

This paper reports the interaction of antinucleating ammonium salts with cells and their parts by the use of ESR techniques.

E. ananas IN-10 cells and their outer membrane fraction were obtained according to the method described in the previous paper.⁶⁾ The cells were sufficiently rinsed with physiological saline, before the membrane was dispersed in a sufficient volume of water and then ultracentrifuged at

$150,000 \times g$ for 1 hr. An ice nucleation active (INA) fraction was obtained from the outer membrane fraction by ion-exchange chromatography under the following conditions: Column, DEAE-Sephadex A-50 (1.6×34 cm); mobile phase, 25 mM phosphate (pH 7.0) with NaCl linear-gradient elution from 0 to 1 M (400 ml each); elution rate, 57 ml/hr; and temperature, 4°C . The INA fraction, which was eluted at an NaCl concentration of 0.2 M, was dialyzed against water and obtained as a precipitate by ultracentrifugation at $150,000 \times g$ for 1 hr. *n*-Octylbenzyltrimethylammonium iodide (OBDA) was synthesized as reported in the previous paper.⁶⁾

A water suspension (1.8 ml) of cells (1×10^8 cells), a water dispersion (1.8 ml) of the membrane with an optical density at 280 nm of 0.011, and the INA fraction with an optical density at 280 nm of 0.56 were each mixed with a water solution (0.2 ml) of OBDA at final concentrations of 0.1, 1, 10 and 100 ppm. Each mixture was stored at 5 or 15°C for 1 hr prior to measuring its degree of supercooling (DS).⁷⁾ DS is expressed as $T_m - T_i$, where T_m is the melting point and T_i is the temperature at which the bulk water of the mixture began to freeze. Table I shows the result, indicating that the ammonium salt was more effective in increasing the DS when this was used at 15°C than at 5°C . The result also shows that the INA fraction responded most sensitively to the ammonium salt.

To quantify the sensitivities of the cells, the membrane and the INA fraction to the ammonium salt, we used a spin probe of a structural analogue to the ammonium salt. The spin probe was synthesized from 5-DOXYL stearic acid (Aldrich, 10 mg) in dimethylformamide (0.2 ml), which was condensed with 1.5 molar equivalents of choline chloride (Tokyo Chemical Industry) with the aid of 1.5 molar equivalents of dicyclohexylcarbodiimide in the presence of 1.5 molar equivalents of pyridine as a catalyst at 4°C for 3 days. The reaction mixture was supplemented with 0.01 N HCl (5 ml) and then filtered to remove dicyclohexylurea. The filtrate, after being washed twice with

TABLE I. ANTINUCLEATING ABILITY OF THE *n*-OCTYLBENZYLTRIMETHYLAMMONIUM SALT (OBDA) AT 5°C AND 15°C

Nucleus ^a	Temperature ($^{\circ}\text{C}$)	Concentration of OBDA (ppm)			
		100	10	1	0.1
Cell	15	$>5^b$	4.1 ± 0.6^b	0.1 ± 0.1^b	0.1 ± 0.1^b
	5	4.4 ± 0.5	0.7 ± 0.5	0.2 ± 0.1	0.2 ± 0.2
Membrane	15	>5	>5	>5	3.0 ± 0.6
	5	>5	>5	3.5 ± 0.7	1.0 ± 0.6
INA fraction	15	>5	>5	>5	>5
	5	>5	>5	>5	4.3 ± 0.5

^a Treated at 5°C or 15°C for 1 hr.

^b Each datum shown is an average value of DS with a standard error ($n=5$). >5 indicates that at least one sample froze at a temperature lower than DS 5.

TABLE II. INCORPORATION OF THE SPIN PROBE INTO THE CELLS, MEMBRANE AND INA FRACTION AT 5°C AND 15°C

Temperature (°C)	Duration (hr)	Incorporated spin probe ($\times 10^{-8}$ mol)		
		Cell (/100 mg)	Membrane (/1 mg)	INA fraction ^a (/1 ml)
5	3	1.0 ± 0.3	1.2 ± 0.2	0.5 ± 0.3
5	24	7.7 ± 1.3	11.3 ± 0.7	0.7 ± 0.3
15	3	25.5 ± 1.0	28.9 ± 1.5	1.3 ± 0.4
15	24	40.3 ± 3.2	34.0 ± 3.1	2.6 ± 0.7

^a OD₂₈₀: 0.5.

FIG. 1. ESR Spectra of the Ammonium Type of Spin Probe Incorporated into the INA Fraction and the Membrane at 5, 15 and 25°C.

sufficient diethyl ether, was chromatographed on thin-layer silica gel (Merck) with chloroform-methanol-water (65:25:4) as a solvent. Since the reaction product, 5-DOXYL stearyl choline, was detected at an *R_f* value of 0.31, it was developed on preparative thin-layer silica gel, and a zone of *R_f* 0.28~0.35 was eluted with methanol to obtain the ammonium spin probe in powder. When the spin probe was used at a concentration of 1 ppm, it was effective in increasing both the DS of the membrane dispersion and that of the INA fraction dispersion.

To understand the effectiveness, the following experiment was undertaken. The cells (100 mg), membrane (1 mg) or INA fraction (0.5 as a final optical density at 280 nm) were each suspended (or dispersed) in 0.1 M phosphate at pH 6.0 (0.9 ml) and then mixed with the ammonium spin probe (1 mg) dissolved in the same buffer (0.1 ml). The mixtures were stored at 5°C for 3 and 24 hr, and at 15°C for the same periods, and then supplemented with a sufficient volume of cold water. The resulting precipitate was collected by centrifugation at $8000 \times g$ for 20 min in the case of the cell suspension and by ultracentrifugation at $150,000 \times g$ for 1 hr in the case of the membrane and INA fraction dispersions. Each of the precipitates was freeze-dried, suspended in chloroform (0.5 ml) and then submitted to a spin probe assay by ESR

(JEOL JES-3000 Radic Sensor). A standard curve was calibrated using $1 \sim 50 \times 10^{-8}$ mol of the ammonium spin probe in chloroform (0.5 ml). Table II shows that the spin probe was incorporated into the cells, the membrane and the INA fraction; in each case the rate of incorporation was higher at 15°C than at 5°C. This result indicates that the temperature effect on the antinucleation activity of OBDA (Table I) may have been due to its different incorporation rate.

The wet states of the membrane and the INA fraction, after incorporation of the ammonium spin probe at 5°C, were also submitted to an assay at 5, 15 and 25°C with an ESR spectrometer (JEOL JES-RE3X) equipped with a temperature regulator (DVT-2), each spectrum being recorded at 9.21 GHz. Figure 1 shows that the structures around the spin probe were rigid at 5°C and that they become loose with temperature. The loosening of the structure is considered to have resulted in the increasing rate of incorporation of the antinucleating chemical to decrease the bacterial ice nucleation.

It is thus suggested that the ammonium type of antinucleating chemicals be sprayed in the daytime for effective antifrost results, although further details await field testing.

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