Note

Characteristics of Ice-nucleation Activity in *Fusarium avenaceum* IFO 7158

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The extracellular ice nuclei of *Fusarium avenaceum* IFO 7158 (FEIN) were stable at pH levels from 2 to 12 and tolerated temperatures up to 40°C. In an activity of the FEIN, proteins were important, however, saccharide and lipid which were proved to be a part of components of bacterial ice nuclei did not participate.

In the presence of suitable catalysis such as organic or inorganic heterogenous ice nucleating agents, the liquid-to-solid phase transition of water occurs at temperatures slightly below 0°C. Some bacteria strains belonging to the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia* can nucleate the formation of ice in supercooled water. The characterization and genetic analysis of the ice-nucleation properties produced by these bacteria were made considerably easier by the demonstration that a small region of the chromosome, cloned from *P. syringae*, *P. fluorescens*, or *E. herbicola* could confer the ice-nucleation activity (INA) on *E. coli.*

However, very few studies have been done to find INA in fungi. It has been reported that two *Fusarium* species, *F. avenaceum* and *F. acuminatum*, could produce ice nuclei.

In this report, we describe the characteristics of INA on *F. avenaceum* IFO 7158 and the complementarity of the *ina* gene between *F. avenaceum* and ice-nucleating bacteria.

*F. avenaceum* IFO 7158 was obtained from the Institute for Fermentation Osaka, Japan. INA of *F. avenaceum* IFO 7158 was measured by the droplet freezing method. The concentration of nuclei per milliliter (*N*) active at a particular temperature (*T*) of extracellular ice-nucleating matter was calculated from the equation devised by Vally: 

\[ N = \frac{-\ln(1-f)}{V \times D}, \]

where *f* is the proportion of drops frozen, *V* is the volume of each drop, and *D* is the dilution factor. To investigate the complementarity of the *ina* gene between *F. avenaceum* and ice-nucleating bacteria, we synthesized a oligonucleotide probe having the sequence 5'-GCGGTTATGGCAGCAGCTGACC-3', which encodes a main repetition motif of a protein produced by the *inaZ* gene (*Pseudomonas syringae*). A positive signal hybridizing with the probe was detected by a non-radioactive DNA labeling and detection system (New DNA Chemiprobe; Takara Shuzo Co., Ltd.).

A mycelium mat from a culture (3 days to 7 days) of *F. avenaceum* IFO 7158 grown on a potato dextrose agar plate was suspended in 10 ml of sterile distilled water. INA of *F. avenaceum* IFO 7158 was estimated before and after filtration through a 0.20-μm-pore size filter (cellulose nitrate, Advantec Co.). Both samples showed nearly the same INA, and the ice-nucleation temperatures, (*T*30), were -4.2 to -5.1°C. This observation seems to mean that *F. avenaceum* IFO 7158 secretes the ice nuclei into the extracellular milieu and the ice nuclei has a class A (-5°C) structure. From the freezing spectra of extracts passed through 0.20-μm-pore size filter, the extracts samples showed nucleus densities of at least 1 x 10³ nuclei/ml of extracts at -5°C.

To characterize the fungal extracellular ice nuclei of *F. avenaceum* IFO 7158 (FEIN), the effects of pH and heat treatments of FEIN, and the participation of protein, saccharide, and lipid, were tested. The FEIN was stable at pH level from 2 to 12 similarly to that of ice nuclei of *F. avenaceum* described by Pouleur et al.* Fig. 1. Relating to thermal stability, the FEIN began to be reduced from 40°C and became constant from -12.5°C to over 70°C without decreasing in two steps, (about 30°C and above 80°C), described for bacterial INA. However, the FEIN was not as stable against heat as ice nuclei of lichen or *F. avenaceum*.

It has been reported that bacterial ice nuclei are classified into class A, B, and C structures; the class C structure as a core protein, the class B structure as a glycoprotein, and the class A structure as a lipoglycoprotein. On the other hand, lichen ice nuclei did not were saccharide and lipid which were proved to be components of bacterial ice nuclei. So, we investigated on the participation of protein, saccharide, or lipid of the FEIN. The ice-nucleating temperature of the FEIN was significantly decreased by the addition of protease K (20U/ml; 60 min at 30°C), and then the

![Fig. 1](image-url) **Fig. 1.** Effects of pH on Ice-nucleating Temperature of FEIN. FEIN was treated at various pHs for 60 min.

![Fig. 2](image-url) **Fig. 2.** Effects of Phenylboric Acid on Ice-nucleating Activity of FEIN. FEIN was treated with 50 mM phenylboric acid (final concentration) for 90 min at 20°C. •, with phenylboric acid; ○, without phenylboric acid.
temperature, \( T_{s0} \), was above \(-12^\circ\)C. This suggests that proteins are important in an activity of the FEIN.

To test for the participation of saccharide, the FEIN was treated with phenylboric acid (50 mM) for 90 min at 20°C and dialyzed for 24 h at 4°C. As shown in Fig. 2, it is suggested that the FEIN is not a glycoprotein because the FEIN is not affected by phenylboric acid treatment. In addition, it was found that lipids did not participate in the FEIN from the result of chloroform treatment for 24 h at 20°C (Fig. 3). These results suggest that the FEIN is more similar to lichen ice nuclei than to bacterial ones.

However, a main constituent of these biological ice nuclei are essential proteins that nucleate crystallization in supercooled water. Therefore, the complementarity of ice nuclei between the bacterial one and FEIN was investigated at DNA level. \( F. avenaeceum \) IFO 7158 DNA was prepared by a modification of the method of Heregord.\(^{13}\) By Southern blotting, it was found that \( F. avenaeceum \) IFO 7158 DNA did not hybridize with the bacterial \( ina \) gene that was cloned from \( Pseudomonas viridiflava \) KUIN-2\(^{14}\) or the 24-mer oligonucleotide probe having a sequence that encoded a main repetition motif of a protein produced by the bacterial \( ina \) gene (Fig. 4). This suggests that \( F. avenaeceum \) IFO 7158 DNA has not the consensus repeat sequence for bacterial \( ina \) gene homology. However, it is possible that this result may be due to differences in codon usage between the bacterial \( ina \) gene and the \( F. avenaeceum \) IFO 7158 FEIN gene.

The complementarity of ice nuclei between the bacterial one and FEIN at protein level, and the cloning of FEIN genes from \( F. avenaeceum \) IFO 7158 DNA is now in progress and will be reported elsewhere.

References