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Association of an ice-nucleating pseudomonad with cultures of the marine dinoflagellate, *Heterocapsa niei*

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ABSTRACT

The observations that terrestrial ice nuclei can have a biogenic origin and that certain bacteria can initiate freezing at exceptionally warm temperatures (−1.5°C) are now well documented. Less well understood are active ice nuclei (−2 to −5°C) found in sea water, marine fog and the marine atmosphere in general. Recently, the authors have isolated an ice nucleation-active (INA) bacterial strain (FB 1032) from cultures of the marine dinoflagellate, *Heterocapsa niei.* FB 1032 is halotolerant and phenotypically similar to *Pseudomonas fluorescens* biotype G, although it differs from biotype G strains in its bacteriophage sensitivity and expression of the INA phenotype. A search for the INA pseudomonad in sea water at La Jolla, California was unsuccessful, but several INA *Erwinia* sp. were isolated. The possible terrestrial origin of these INA bacteria is suggested.

1. Introduction

The observations that ice nuclei can have a biogenic origin and, more particularly, that certain bacteria can initiate freezing of water at exceptionally warm temperatures are relatively recent discoveries. Studies by Maki and coworkers (see Maki and Willoughby, 1978) led to the identification of two closely related bacteria, namely *Pseudomonas syringae* and *P. fluorescens* biotype G strains, as major sources of active ice nuclei. Lindow *et al.* (1978) showed that isolates identified as *Erwinia herbicola* were also active in forming ice nuclei. These ice nucleation active (INA) bacteria have been found on plants, in water, and in the atmosphere in North America, Europe, Asia and the Arctic (Lindow *et al.,* 1978; Yankofsky *et al.,* 1981; Lindemann *et al.,* 1982; Jayaweera and Flanagan, 1982).

Less well understood are ice nuclei formed in sea water, marine fog, and the marine atmosphere in general. That some of these nuclei are biogenic in origin was first suggested by Schnell and Vali (1975). They asked the question whether sources of ice nuclei might exist in oceans. Surface waters from the Atlantic, Caribbean and Pacific oceans were tested for ice nuclei by a drop freezing technique, and samples from the Atlantic ocean exhibited activity associated with a bloom of phytoplankton. Isolated

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phytoplankton, dispensed in distilled water, showed ice nucleation activity at temperatures as warm as $-2.5°C$ and contained 10^7 nuclei g^{-1} active at $-10°C$. These ocean-derived nuclei were <1 μm in diameter, heat labile, and easily aerosolized, properties similar to the nuclei of terrestrial INA bacteria.

Bigg (1973) reported an analysis of airborne ice nuclei over southern seas and found bands of nuclei along latitudes 40 to 55S. Schnell (1975) noted that Bigg's ice nuclei zones correspond to regions of heavy marine phytoplankton productivity. This zone of high primary productivity occurs in a region of upwelling, persistent high winds, and violent, wave-tossed seas dubbed the "Roaring Forties" by early whalers. It is interesting to note that the transfer of materials from oceans to the atmosphere has been well documented, particularly whenever breaking waves are present. For organic material in particular, Stevenson and Collier (1962) have shown that air above the oceans contains many marine microorganisms, and Blanchard reported in 1964 that bubbles bursting on the surface of the sea could inject high concentrations of organic matter into the atmosphere. Blanchard (1970) further showed that the bursting bubble phenomenon was particularly effective at concentrating and ejecting high concentrations of bacteria from marine surfaces. Thus, the presence of elevated concentrations of active ice nuclei in the atmosphere along 40–50S latitude is consistent with both a preferred source region and an active sea-to-air transfer mechanism. The above observations prompted further research into identifying specific active marine ice nucleants as described below.

An analysis for ice nuclei in 23 marine phytoplankton cultures at Scripps Institution of Oceanography (Schnell, 1975) revealed that one was especially active. This culture of the dinoflagellate *Heterocapsa niei*[3] contained ice nuclei active at $-3°C$. Since these algae cultures were (and are) mixed cultures containing many different marine bacteria it seemed possible that the bacteria were the source of the ice nuclei, although this was not established. In subsequent studies bacteria were isolated from surface seawater and marine fog off the coast of Nova Scotia (Schnell, 1977), and both salt-requiring and nonrequiring strains active as ice nuclei were isolated. These strains were not identified or maintained.

The above observations, taken together, suggest that marine bacteria contain ice nucleants analogous to those known to be present on three species of terrestrial bacteria mentioned above. It was the goal of this study to initiate the isolation, identification and characterization of INA bacteria from marine sources. The work described herein focuses on INA bacteria isolated at SIO.[4]

2. Materials and methods

a. Marine algae. Cultures of marine algae were obtained from the sources listed in Table 1. They were transferred to sterile tubes and maintained in suitable sea water

3. This dinoflagellate, previously referred to as *Cachonina niei*, has been placed in the genus *Heterocapsa* (Morrill and Loeblich, 1981).
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Table 1. Screening for ice nucleation activity in marine phytoplankton cultures (January 1983)*.

Algal division† and species	Growth‡ medium	Ice nucleation§ t_{50}(°C)
Chlorophycophyta		
Chlorella salina UTEX 1809	BSW	−20.8
Platymonas sp.	GPM	−22.1
Ulva lactuca UTEX 1857	GPM	−22.0
Ulva taeniata UTEX 829	GPM	−21.7
7-75 green, motile	GPM	−20.9
Chrysophycophyta		
Chatonella kagosima	GPM	−21.2
Closterium sp.	GPM	−21.4
Isochrysis sp.	GPM	−22.9
Phaeodactylum (Guam)	BSW	−20.2
P. tricornutum UTEX 642	BSW	−19.8
S16 (Mono Lake)	GPM	−21.0
Pyrrhophycophyta		
Amphidium carterae PY-4	GPM	−19.5
Cryptothecodinium cohnii	MLH	−19.7
Gonyaulax polyhedra PY-11	GPM	−20.3
Gymnodinium splendens	GPM	−22.1
Gyrodinium resplendens	GPM	−20.7
Heterocapsa niei PY-5	GPM	−11.5(−4.8)∞
Prorocentrum micans	GPM	−21.8
Protoceratium (Salton sea)	GPM	−21.0
Cryptophycophyta		
Chroomonas salina	GPM	−20.7
Cryptomonas sp. (La Jolla)	GPM	−19.5

*Cultures were obtained from F. Haxo, R. Lewin and W. Thomas, Scripps Institution of Oceanography (La Jolla, California), and W. Barclay (Boulder, Colorado) and from the Culture Collection of Algae (UTEX). Each was screened by the drop freezing technique. In each case 10 to 30 ten microliter drops of the cultures plus a similar number of drops of the uninoculated growth media were analyzed for ice nucleating activity over the temperature range, 0°C to −24°C.

†Using the taxonomic divisions of Bold and Wynne (1978).

‡All the growth media are standard marine media, described in the text.

§t_{50} represents the temperature at which 50% of the drops were frozen.

∞The temperature in parentheses is the t_{50} for a cell pellet of *H. niei* resuspended in one-tenth its original volume with distilled water, as in Schnell (1975).

media. The media used were: GPM (Loeblich, 1975) for most of the strains; BSW (Brune *et al.*, 1981) for *Chlorella salina* and *Phaeodactylum* isolates; and MLH containing 0.2% glucose (Tuttle and Loeblich, 1975) for *Cryptothecodinium cohnii.* All strains were maintained under fluorescent lights at 20°C except for *C. cohnii* which was maintained in the dark at 20°C.

b. Sample collection. Seawater samples were collected from the flume at the base of SIO pier using sterile flasks; samples were immediately transported to the laboratory and filtered (sterilely) using a Millipore filter apparatus and 0.2 micron filters (Nucleopore). Such filters retained >95% of bacterial cells present in sea water, as measured by viable cell counts (below), and may retain ice nuclei associated with other biological materials or insoluble organic or inorganic residues. Volumes ranging from 100 to 700 ml were filtered, and the filters were either (a) plated directly on marine agar (Baumann and Baumann, 1981) or (b) vortexed in sterile artificial sea water (MacLeod, 1968) with aliquots plated on marine agar or taken for drop freezing analysis. In some experiments laboratory water, air and flowing sea water at SIO was filtered by similar standard procedures.

c. Bacteriological methods. Terrestrial bacterial strains were maintained by subculture on TYG medium (0.5% Bacto-tryptone, 0.2% yeast extract, 2% glycerol) solidified with agar. Marine strains were subcultured on marine agar (Difco Marine broth 2216 solidified with agar).

Authentic bacterial strains were obtained from the American Type Culture Collection, including (strain numbers in parentheses) *P. fluorescens* biotypes A(17575), B(17467), C(17574), F(17513), and G(17386, 17518, 17573). An INA^+ culture of *P. fluorescens* F-12 (Maki and Willoughby, 1978) was obtained from L.R. Maki, University of Wyoming.

Bacterial colonies detected after plating algal cultures or filtered sea water fractions on marine agar were quantified to give a viable cell count and for comparison to the total number of ice nuclei retained on filters. Identification of INA isolates was carried out by transferring colonies with sterile toothpicks to 200 microliters of high purity water (HPLC grade, Fisher Chemicals), and determining the freezing profile of ten microliter drops of each bacterial suspension. Identification of strain FB 1032 was carried out using published procedures (Baumann and Baumann, 1981; Stolp and Gadkari, 1981). Identification of some INA isolates as *Erwinia* sp. was carried out as described in Bergey's Manual (Buchanan and Gibson, 1974).

d. Phage isolation and host range determination. Phages were isolated from a variety of vegetation sources collected in California, Colorado and Florida using the general procedures outlined by Crosse and Garrett (1963). The routine plating medium was TYG agar (1.5%) overlaid with TYG agar (0.5%). Plates were scored for plaque formation after 24 to 48 hours at 20°C. Single plaques were picked with sterile toothpicks, suspended in phage buffer (0.01M potassium phosphate, 0.01M $MgSO_4$, pH 7.0), and purified by the double agar overlay method. High liter phage stocks ($>10^{10}$ pfu/ml) were prepared and stored over chloroform at 4°C. Tests for phage host range were carried out by diluting phage to 10^6 pfu/ml and spotting 10 microliter drops on lawns of the various host cultures. The properties of the phages used here, including pf1B, pf1Cm, pf1E, pf1G and pf1H, will be described elsewhere.

e. Freezing tests. Detection of ice nuclei was carried out by the drop freezing technique described in detail by Schnell (1975, 1977). Drops of algae cultures were tested directly, or where indicated the culture was centrifuged at 1,000 g for 10 min and the cell pellet was taken up in one-tenth volume of distilled water before drops were tested. Routinely, the temperature at which 50% of the drops froze (t_{50}) was recorded. In some experiments the total number of ice nuclei (at a given temperature) was quantitated as described by Vali (1971). The ice nucleation frequency of bacterial suspensions was determined as described by Lindow *et al.* (1982).

3. Results and discussion

In previous work ice nucleation activity was found to be associated with a culture of *H. niei* maintained at SIO (Schnell, 1975). Recently, we carried out a similar screening of marine phytoplankton, again searching for ice nucleation activity. The results of this screening are shown in Table 1. Of twenty-one different marine algae cultures, only one exhibited ice nucleation activity at temperatures warmer than −12°C. This culture, *H. niei* PY-5, is the same as that described as INA by Schnell (1975); it has been maintained continuously since that time in the laboratory of F. Haxo at SIO. When a sample of the culture was centrifuged 10 minutes at 10,000 g, and the resulting cell pellet resuspended in distilled water, a t_{50} of −4.8°C was obtained (Table 1).

Fractionation of the cell culture by membrane filtration revealed the following: the majority of the ice nuclei active at −5°C passed through 10 micron and 3 micron filters, but were retained by a 0.2 micron filter. This suggested that the ice nuclei were associated with bacteria present in the *H. niei* culture, or with algal cell fragments. Alternatively, these nuclei could be insoluble organic or inorganic particles analogous to those seen in marine aerosols (Nagamoto *et al.*, 1984).

Bacteria in *H. niei* cultures were isolated by plating dilutions on marine agar, a nonselective general medium for marine bacteria (ZoBell, 1941; Baumann and Baumann, 1981). Five major bacterial types were observed, none of which exhibited the INA^+ phenotype when tested by the drop freezing method. Of 142 colonies tested at random, only 3 were INA^+. The INA^+ isolates formed small pale yellow colonies on marine agar. One of these, strain FB 1032, was identified as outlined in Table 2. The identification scheme followed that described by Baumann and Baumann (1981) for marine bacteria. When it was discovered that strain FB 1032 does not require sodium ion for growth, it was plated on various media used for terrestrial bacteria. This led to the discovery that the strain produces fluorescent pigment on King B agar, and its identification as *Pseudomonas fluorescens* biotype G was completed following the procedures outlined by Stolp and Gadkari (1981).

The relatedness of isolate FB 1032 to other *P. fluorescens* biotype G strains was tested. These strains have been isolated from a variety of sources, including fresh water, soil, and polluted sea water (Stolp and Gadkari, 1981). Each strain was tested

Table 2. Key characteristics for the identification of strain FB 1032 as a *Pseudomonas fluorescens* biotype G*.

Characteristic	
Colonies on marine agar	+
Ice nucleation activity†	+
Gram stain	gram negative
Fermentation of glucose	–
Growth factors required	–
PHB accumulation	–
Sodium requirement	–
Fluorescent pigment	+
Arginine dihydrolase	+
Oxidase	+
Gelatin hydrolysis	+
Denitrification	–
Levan from sucrose	–
Non-fluorescent pigments	–

*Details of these key characteristics, except for ice nucleation activity, are given in Baumann and Baumann (1981) and Stolp and Gadkari (1981).

†Ice nuclei active at -2.8 to -3.2°C were routinely detected.

for the INA phenotype as well as susceptibility to phages selected for virulence towards FB 1032. The results are shown in Table 3. From these results, FB 1032 is very similar to the INA strain FY-12 isolated by Maki and Willoughby (1978), but different than the other 3 biotype G strains tested. FB 1032 was more halotolerant than any of the other strains shown in Table 3, possibly a reflection of its ability to survive in marine growth medium.

That the association of FB 1032 with *H. niei* can be a long lasting one is suggested by the following results. We detected the INA pseudomonad on three separate occasions in *H. niei* cultures at SI0 between January 1983 and February 1984. Addition of the bacterium to a culture of *H. niei* UTEX 1564[5] maintained by subculture in GPM medium led to its establishment as a permanent member of the bacterial microflora. Maximum cell density of FB 1032 was $<10^3$/ml, representing only a minor constituent of the microflora associated with *H. niei*.

The source of FB 1032 at SIO was investigated. Sea water, and laboratory water and air samples were filtered with 0.2 micron filters. The filters were plated on King B agar; no fluorescent INA colonies were seen in any of the samples. In similarly filtered sea water, ice nuclei active at temperatures warmer than -7°C were detected on several occasions (Table 4). Total bacteria, INA^+ bacteria, and total ice nuclei on the filters were also determined (Table 4). Although ice nuclei active at -7°C could be detected at concentrations of 2×10^3 to 2×10^4 per l of sea water, very few INA

5. *H. niei* UTEX 1564 is a culture of the PY-5 strain maintained at the Culture Collection of Algae, El Paso, Texas. As received, the culture contains a variety of bacteria, but no ice nucleation activity at temperatures warmer than -15°C.

Table 3. Ice nucleation activity, halotolerance and bacteriophage sensitivity of strain FB 1032 and other *P. fluorescens* biotype G isolates.

Strain	Ice nucleation activity* t_{50}	(nuclei/cell)	Halotolerance†	Susceptibility to phage‡ pf1B	pf1C	pf1E	pf1G	pf1H
FB 1032	−3.7	0.05	520	+	+	+	+	+
17518	−17.5	0	250	−	−	−	−	−
17573	−18.2	0	220	−	+	−	−	−
17586	−17.9	0	270	−	−	−	−	−
F-12	−4.2	0.02	240	+	−	+	+	+

*The number of ice nuclei active at −5°C in serial dilutions of cells in distilled water. Cells were grown at 20°C in TYG broth to cell densities of 3–5 × 10^9 cfu/ml. The t_{50} value shown is for suspensions of 10^6 cells/ml.

†Concentration of NaCl (mM) that results in a 50% decrease in growth rate, when cells were grown in a sodium-free basal medium containing 0.1% glycerol and 0.1% potassium acetate (Baumann and Baumann, 1981) supplemented with increasing levels of NaCl.

‡Five different phages, based on plaque morphology, were used.

bacterial colonies were recovered (7 INA out of 1950 tested). This leads to the conclusion that there are ice nuclei in sea water that may be associated (a) with bacteria that are not able to grow on marine agar, or (b) with nonviable bacteria. Alternatively, these nuclei may be associated with other particles of biogenic or nonbiogenic origin (Nagamoto *et al.*, 1984).

Analysis of the 7 INA^+ isolates revealed the following. All had the same phenotype: gram negative rods; yellow pigment; motile; capable of anaerobic growth; fermentation of glucose; oxidase negative; no arginine dihydrolase; and growth in the presence of 5% NaCl. Based on these criteria, these INA bacteria are tentatively identified as *Erwinia*

Table 4. Detection of ice nuclei, total bacteria, and INA^+ bacteria in sea water*.

Date/Sample	Total ice nuclei per l (active at −7°C)	Total bacteria per l (average)	Bacterial colonies tested for INA %INA^+	(INA^+/INA^-)
Jan 83	21,200	67,300	0.7	3/447
May 83	1,900	263,700	0	0/300
Jul 83	2,700	197,400	0	0/750
Feb 84	11,700	94,900	0.9	4/446

*All samples were collected at SIO pier, La Jolla, California, and filtered (in triplicate) onto 0.2 micron filters. Material retained on the filters was suspended in sterile artificial sea water (MacLeod, 1968), and aliquots were analyzed for ice nuclei active at −7°C by drop freezing or were plated on marine agar for total bacterial counts. After growth for 72 h at 20°C plates were analyzed for INA^+ and INA^- colonies.

sp. (Starr, 1981). Each isolate grew on nonmarine growth media and showed no dependence on sodium ion.

A key point to be established is whether the ice nucleating bacteria described here are true *terrestrial* or *marine* species. The distinction between terrestrial and marine bacteria is reviewed in some detail by Baumann and Baumann (1981), and relies primarily on whether strains exhibit a dependence on sodium ion for growth; most marine strains show such a dependency. Another criterion that can be applied is whether the isolated bacterium will (a) grow in sea water (preferably from the isolation site) supplemented with traces of carbon and nitrogen sources, or (b) just survive but not grow under these conditions. We are working to test these possibilities with the INA *Erwinia* isolates. It also remains to be determined whether unidentified INA bacteria isolated from marine sources previously (Schnell, 1977) are true marine or terrestrial species.

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