Some Basic Characteristics of Bacterial Freezing Nuclei

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ABSTRACT

Freezing spectra of INA bacteria from different parts of the world were compared. A slight increase in efficiency of freezing nuclei produced by strains from warmer climates was observed. Whole cells of the most efficient strain produced nuclei active at temperatures ranging from −2 to −10°C, whereas fragments from these cells exhibited activity only at −8 to −10°C. In all cases, the frequency of active cells in a population proved low. Thus, activity at −8 to −10°C was evidenced by 1 cell in about 300, the corresponding ratio being 1 in 10^4 at −2 to −4°C. It was shown in several ways that the variety of "freezer" individuals was not due to a need for multicell aggregation or any other cooperative process. Also, the time at which a given individual in a cell population expressed its latent freezing potential was shown to vary with time and cell physiological state.

1. Introduction

The freezing nucleus activity of most microorganisms is limited to temperatures colder than −20°C (Soulage, 1957). Bacteria active as freezing nuclei at −2 to −5°C, and probably responsible for the susceptibility of certain plants to frost damage at relatively warm temperatures, however, recently have been recovered from plant surfaces (Lindow *et al.*, 1978a). Two types of such "ice nucleation-active" or "INA" (Lindow *et al.*, 1978b) bacteria have been described: 1) producers of water-soluble fluorescent pigments; and 2) producers of water-insoluble yellow pigments. The former types, strains of *Pseudomonas syringae* and *P. fluorescens* (Maki *et al.*, 1974; Maki and Willoughby, 1978) come from various North American habitats, while the latter are known to occur both in North America (Lindow *et al.*, 1978a) and the Middle East (Yankofsky *et al.*, 1981; Levin *et al.*, 1980). Yellow INA bacteria from these different geographical regions can be placed in a single group on the basis of their common sensitivity to certain bacterial viruses (Yankofsky *et al.*, 1981). Detailed studies on their freezing nucleus behavior, however, have not yet been reported.

Other than ice itself, INA bacteria happen to be among the most efficient freezing nuclei yet found in natural environments (Vali *et al.*, 1976). Some also show activity as ice nuclei in cloud chambers (Maki and Willoughby, 1978). Therefore, depending on their relative abundance in the atmosphere at different times and places, INA bacteria may be of direct meteorological, as well as of agricultural and ecological significance. The possibility of employing them as efficient ice nuclei for cloud seeding also arises. For example, efforts to enhance rainfall over Israel in winter involve seeding with aerosols of silver iodide. The highest temperatures at which these inorganic nuclei act are around −8 to −12°C, as compared to −2 to −4°C for freezing nucleus activity by yellow INA bacteria indigenous to the same region (Yankofsky *et al.*, 1981; and Levin *et al.*, 1980). Finally, insight into the highly efficient bacterial nucleation process may eventually permit construction of artificial ice nuclei which are better, cheaper and ecologically safer than the ones presently used. However, only one or two basic studies on ice nucleation in cloud chambers by bacteria have yet been published (Schnell, 1976; Maki and Willoughby, 1978). The present paper primarily describes basic aspects of the freezing nucleus process in a yellow INA bacterium.

2. Methods

a. Cultivation and titration of bacterial organisms

Routine culture of all bacteria at 30–33°C in liquid medium under aerobic conditions was as described elsewhere (Yankofsky *et al.*, 1981). Culture development was monitored by turbidity increase and cell number densities determined by counting colonies arising from cells deposited on nutrient agar surfaces. It is assumed that the colony-forming unit (cfu) in each case is a single cell. Therefore, despite the fact that the assumption does not strictly hold, we shall henceforth use the terms cell and cfu interchangeably.

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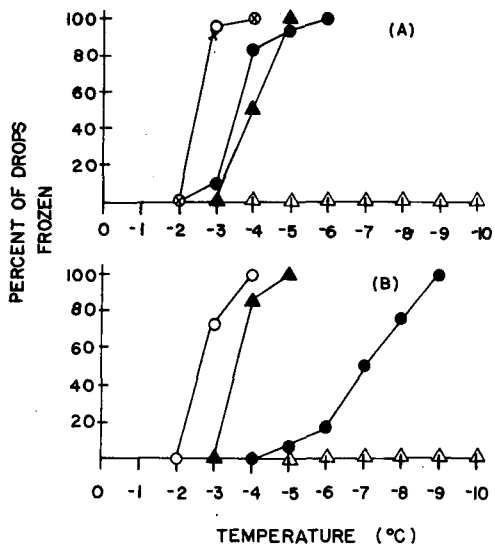

FIG. 1. Freezing spectra of drops from glucose-containing nutrient broth cultures of various INA bacteria. Cultures were allowed to develop at 32°C with shaking to (A) exponential and (B) stationary phase of growth. Each spectrum represents a total of 30 to 100 drops of 5 μL size. (○) bacterium M1; (×) bacterium M4; (△) bacterium L2; (▲) *E. herbicola*; (●) *P. syringae*.

b. Determination of freezing nucleus activity

Freezing temperature spectra of uniform drops (56 μL) from suspensions of bacterial freezing nuclei were determined in the drop freezing spectrometer (Vali and Stansburg, 1966) as detailed elsewhere (Yankofsky *et al.*, 1981). The freezing nucleus content of larger samples was determined at fixed temperatures below 0°C in a Colora KT 40 K constant temperature bath filled with methanol, or in a Tenney Jr environmental chamber. Samples of 0.4–4.0 mL volume were contained in 13 mm test tubes sealed with parafilm. The term freezing nucleus unit (fnu) derived from the above operations represents the smallest unit capable of initiating ice formation in a given volume at a given temperature over a defined period of time.

c. Production of M1 cell fragments

Cells suspended in saline were broken by exposure to ultrasound pulses of 20 s duration at 100 W in a Braun Labsonic sonifier. The standard procedure followed was three such pulses at 1 min intervals for heat dispersion. Sonicates were then filtered through 0.45 μm pores in order to remove any residual particles of cell size or larger.

3. Results

a. Freezing spectra of different INA bacteria

The five bacteria whose freezing spectra are given in Fig. 1 include three yellow-pigmented isolates (strains M1, M4 and L2) from Israel (Yankofsky *et al.*, 1981), one yellow INA bacterium (*Erwinia herbicola*) from the United States (Lindow *et al.*, 1978a) and one fluorescent INA bacterium (*Pseudomonas syringae*) from the United States (Maki *et al.*, 1974). Among the yellow bacteria mentioned above, strains M1, M4 and *E. herbicola* can be related by their common sensitivity to certain bacterial viruses (Yankofsky *et al.*, 1981). Although morphologically indistinguishable from bacterium M1, or M4, the yellow strain designated L2 is insensitive to viruses which attack the others and also lacks the INA trait. Its inclusion here serves as a methodology control and as a means of emphasizing the inability of most bacteria from natural environments to produce efficient freezing nuclei at any stage of growth.

The spectra in Fig. 1 represent freezing point distributions of uniform drops from dividing (Fig. 1a) and stationary (Fig. 1b) cell cultures. Dividing cultures were allowed to exponentially grow out to about 2×10^8 cfu mL^{-1} before being tested for their freezing response. Corresponding analysis of stationary cultures was performed several hours after they reached maximal cell number densities of $1-4 \times 10^9$ cfu mL^{-1} and stopped multiplying because of nutrient exhaustion or some other environmental limitation. Aside from strain L2, it is clear that cultures of all the microorganisms examined contained efficient freezing nuclei active at −10°C or warmer both during and after the multiplication phase of their growth cycles. However, spectra of exponentially-dividing M1 and M4 culture were displaced to the warmer side of those obtained with *P. syringae* or *E. herbicola* by 1–1.5°C. Since equal numbers of cells grown under identical conditions were compared, the observed differences are probably significant. Accordingly, the efficiency of the freezing nuclei produced by a given INA bacterium appears at least partially influenced by its geoclimatic origin. Stability of cell-derived freezing nuclei seems, on the one hand, entirely due to phylogenetic factors. Thus, growth cessation had no apparent affect on

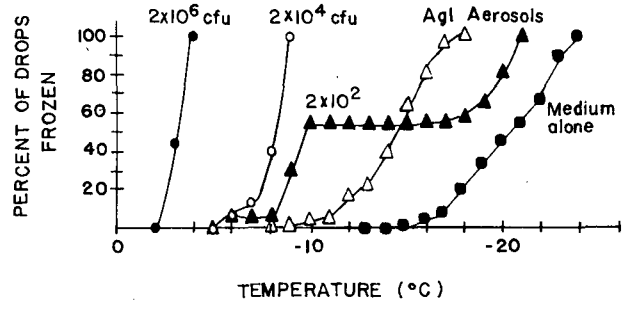

FIG. 2. Freezing spectra of drops from a nutrient broth of bacterium M1 at late-exponential phase. Curves represent successive 1:100 dilutions to give the indicated cell number per 5 μL drop.

the freezing nucleus distribution in cultures of yellow INA bacteria, but caused a sharp temperature downshift in the spectrum of *P. syringae* (cf. Fig. 1a and 1b). Instability of efficient freezing nuclei in cultures of fluorescent INA bacteria also has been observed by others (Maki *et al.*, 1974; Vali *et al.*, 1976; Maki and Willoughby, 1978).

b. *Influence of cell number on freezing temperature of drops from cultures of bacterium M1*

The way in which bacterium M1 was cultivated had no marked effect on its subsequent freezing response; drops from undiluted cultures consistently froze at temperatures between −2 and −4°C as depicted in Fig. 1. Nevertheless, such "Group I" nuclei were not the only ones produced by bacterium M1, and their concentration relative to that of cells was always low. Illustrative of these latter findings are the freezing spectra in Fig. 2 each representing a successive 1:100 dilution of the same late-exponential culture. Thus, while essentially every drop containing 2×10^6 cfu also exhibited activity at −2 to −4°C, fewer than 1 in 100 of the drops diluted to contain only 2×10^4 cfu did so. In other words, approximately 10^4 cells were required to produce a single Group I nucleus. Nevertheless, Groups II and III nuclei, particles respectively active at −5 to −7°C and −8 to −10°C were still present in drops from which the more efficient Group I activity had been removed by dilution. Dilution steps smaller than those shown in Fig. 2 indicated that particles active at temperatures between Groups I and II were not represented in the culture, and that Group II particles did not respond linearly to dilution. The reason for the latter finding is not clear. On the other hand, since particles active at temperatures warmer than −10°C were absent from the growth medium itself, it is clear that all three of the above particle types must be of cellular origin. Also, note that even the least efficient (and most abundant) Group III nuclei initiated freezing under the conditions of the test at significantly higher temperatures than nonbiogenic freezing nuclei associated with silver iodide aerosols dispersed in water. Finally, almost half of the drops in a set averaging 2×10^2 cfu each lacked even Group III nuclei (Fig. 2). Therefore, even at −10°C, freezing nuclei produced by cells were 200 to 300 times less frequent than the cells which produced them. Similar freezing nucleus to cell ratios have been reported for fluorescent INA bacteria (Maki *et al.*, 1974; Maki and Willoughby, 1978), and suggested for *E. herbicola* strains (Lindow *et al.*, 1978a).

c. *Physical characterization of freezing nuclei produced by bacterium M1*

It has been convincingly demonstrated that the freezing nuclei of fluorescent INA bacteria are located on cells, and probably on cell surfaces (Maki *et al.*, 1974; Maki and Willoughby, 1978). Since centrifugation of M1 cultures simultaneously precipitated out cells and efficient freezing nuclei, the same probably holds for our yellow INA bacterium. Additionally, the data in Table 1 show that membrane filters with diameters of 0.45 μm or less removed both colony-forming ability and freezing activity at −7°C from M1 cultures. Since electron microscopy indicates 0.4 μm to be the diameter of the smallest M1 cells in a population (Yankofsky *et al.*, 1981), the above finding suggest that a Group II freezing nucleus unit (fnu) can be a single cell, or a post-filtration aggregate of individual cells. Needless to say, filtration trials at −4 and −10°C, respectively, suggested groups I and III nuclei to have single cell (or smaller) dimensions, also. Additional evidence for the unit cell size of freezing nuclei will be presented in another section of this paper.

TABLE 1. Filterability of freezing ability from whole cells.

Filter pore size (μm)	Filtrate or culture[1] (cfu mL^{-1})	Least volume of culture or filtrate[2] still exhibiting Group II freezing nucleus activity (mL)
None	1.1×10^9	5×10^{-6}
1.2	3.4×10^8	5×10^{-6}
0.8	1.2×10^8	5×10^{-4}
0.6	1.5×10^6	0.5
0.45	<10	>0.5
0.3	<10	>0.5

[1] Determined by spreading 0.1 mL aliquots on nutrient agar surfaces after appropriate dilution and counting the colonies which arose after incubation at 33°C for 48 h.

[2] Determined by incubating 0.5 mL of the appropriate dilution in a tenfold step series at −6.5 ± 0.5°C for 2 h in an environmental chamber.

Maki and Willoughby (1978) have shown that sonic rupture of *P. fluorescens* cells destroys cell-derived freezing activity at temperatures above, but not below −8°C. Moreover, residual activity at −8°C or colder by fragments of the above fluorescent INA bacterium was not appreciably less than that of comparable cell suspensions. The Group II freezing activity of a sonically produced fragment preparation of bacterium M1 is given in Table 2. To a first approximation, M1-derived fragments which were clearly smaller than cell size initiated freezing at −10°C about as well as the corresponding cell suspension from which they arose. Also, as indicated by the freezing spectra in Fig. 3, physical disruption of cell organization led to complete loss of Group I activity. We have already shown corresponding disappearance of Group I nuclei from undiluted cultures of M1 after treatment with protein synthesis inhibitors such as streptomycin (Yankofsky *et al.*, 1981). Consequently, it seems reasonable to conclude that the most efficient (i.e., Group I) freezing nuclei

TABLE 2. Activity of M1 cell fragments as freezing nuclei at −10°C.

Dilution factor	Intact cell suspension*	Fragment size** 0.6 μm	0.45 μm	0.3 μm	0.1 μm	Saline control†
10^3	+‡	+	+	+	+	−
10^4	+	+	+	+	−	−
10^5	+	+	+	+	−	−
10^6	+	+	+	+	−	−
10^7	−	−	−	−	−	−

* Exponential-phase M1 cells were collected by centrifugation and resuspended in 1/10 volume of saline at around 2×10^9 cfu/mL^{-1}.

** Above cell suspension was exposed to three ultrasound pulses as detailed in Section 2 and aliquots of the sonicate filtered through membranes of the indicated pore sizes under sterile conditions.

† One volume of saline was sonicated and diluted in parallel to the cell suspension. This was to ensure that observed nuclei did not originate from the walls of the plastic container.

‡ Positive responses represent freezing of 0.5 mL sample within 60 min of exposure to −10°C.

produced by all INA bacteria require a physically intact and physiologically normal cell for their expression, while those active at lesser temperatures do not. The physical-chemical significance of these observations remains to be seen. Also unclear at the moment is the relation of intermediate Group II nuclei to particles of either the Groups I or III class.

d. Expression of freezing nucleus capability by single cells as a function of time

The notion of time has not been stressed in any of the freezing results previously mentioned in this paper. However, experiments in the freezing nucleus spectrometer, or with larger sample volumes exposed to fixed temperatures for extended time periods, soon made it clear that time was an important factor in the nucleation process initiated by M1 cells. Take, for example, the data presented in Table 3. In this experiment, cells from a stationary culture were distributed to test tubes so that each tube in the set received the same number of cells in a volume of 0.4 mL. Twenty of the tubes so prepared were then sealed with parafilm, another 20 were brought to a final volume of 2.0 mL with cell-free medium before sealing, and the volume in still another subset of 20 adjusted to 4.0 mL prior to closure. All 60 samples were then placed at -10 ± 0.4°C in a temperature-controlled bath and the number of frozen sample tubes in each subset then recorded at 5 min intervals out to 165 min. It might be explicitly noted that the number of M1 cells per sample tube was deliberately made too low for every sample to have received an active "freezer" bacterium. As can be seen, the rate at which samples froze (number of tubes per min) for the first 5 min of the experiment was clearly more rapid in comparison to the second rate observed at later times. Nevertheless, however slow, this final rate of sample freezing was real. The time it took a given sample to equilibrate to the temperature of the bath was under 2 min. Also, the measured temperature fluctuation cycle of the bath (0.4°C in either direction) was less than 2 min long. Therefore, samples which froze at the slow rate after 5 min were surely not affected by such fluctuations. It is also possible to conclude that, within experimental error, there was no apparent dependence of sample freezing rate from 0 to 5 or 5 to 165 min on cell concentration. Taken above, this latter finding almost certainly rules out any necessity for cell-to-cell cooperation in the freezing process and proves that single cells (or fragments thereof) do act as freezing nuclei.

It has already been demonstrated that most cells in a population do not possess freezing nucleus capability at −10°C or warmer at any given moment. Not surprisingly then, samples diluted to contain around 10 cells in a volume of 0.1 mL failed to freeze after incubation at either −4 or −10°C for periods exceeding 24 h. However, when such supercooled samples were spread on semi-solid medium at the end of the cold-incubation period, and surviving cells allowed to form colonies, all resulting cell lines, representing millions of clonal descendants of a single cell in most cases, contained active "freezer" individuals in normal frequency (see Fig. 4). Therefore, genes for the INA trait are present in all normal M1 cells within a population; the rarity of "freezer" individuals must be sought in some sort of control mechanism which prevents expression of such genes most of the time in the majority of cells. If so, it becomes of interest to determine how time and circumstances govern the expression of cell nucleation potential. A start in this direction is given by the studies described below.

All of the experiments summarized in Fig. 4 in-

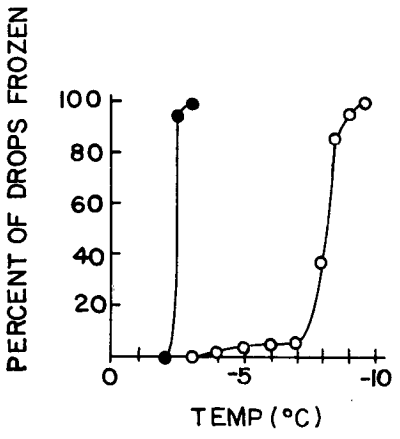

FIG. 3. Freezing spectra of intact versus sonically fragmented M1 cells: (●) intact cells from a stationary-phase M1 culture (see legend to Fig. 1 for details of culture); (○) fragments of 0.45 μm diameter or less obtained by sonication as detailed in Section 2. Each 5 μL drop tested contained either 2×10^6 cfu or an amount of fragment material equivalent to this number of intact cells.

TABLE 3. The effect of cell concentration on the rate of freezing at −10°C of samples containing equal numbers of Group III nuclei from bacterium M1.

Sample volume (mL)	Measured cell number per sample	Sample fraction frozen after 5 min	Sample fraction frozen after 165 min	Apparent freeze rate (min^{-1})	
				0–5 min	5–165 min
0.4	1100	4/20	7/20	0.8	0.04
2.0	1100	2/20	8/20	0.4	0.05
4.0	1100	4/20	8/20	0.8	0.05

volved determination of sample freezing times in replicate cell sample sets of a result of exposure to −4 ± 0.4°C (Fig. 4a) and −10 ± 0.4°C (Fig. 4b). The plots represent apparent ratio of freezer to total cells per sample ($n^{-1} \ln N_u/N$) as a function of time. When N_u is number of unfrozen samples, N is total sample number and n is the measured cell count at the start of the experiment. The distribution of freezer cells in a normal population was assumed to follow Poisson probability ($N_u/N = P_{(0)} = e^{-m}$, where m is the mean number of freezer cells per sample). It is strikingly evident that the time response of M1 freezer cells was very different during exponential growth as compared to stationary phase. First consider exponential cells. At −10°C, all samples which were to freeze over the entire 2 h period of the experiment did so within 2 min. It is, therefore, simple to conclude that the population examined contained one freezing nucleus unit (fnu) in 625 cfu. A comparable picture arises with exponential cells at −4°C, albeit that the time to saturation is nearer 5 min and the plateau level implies only 1 fnu in 10^4 cfu. By contrast, in the case of stationary cell populations there is no true saturation within 2 h. Consequently, the fnu frequency appears to change with time. However, it is still true that the initial −10°C response is faster than at −4°C, and that fnu frequencies at both temperatures exceed those observed with exponentially dividing populations by six or seven times.

The results examplified here, although influenced to some degree by growth conditions, are quite reproducible. For example, in contrast to what has been reported for *P. syringae* (Maki and Willoughby, 1978), the influence of growth temperature on the subsequent freezing nucleus activity of bacterium M1 is essentially zero. It is therefore suggested that the latent capacity of bacterial nucleants is not expressed in constant fashion. Whether or not a given particle of biological origin will initiate freezing at any given moment very much depends on its identity, its history prior to the moment it was placed at the temperature of interest, the number of particles per sample, and the length of the test.

It should be evident from the above discussion that data obtained with bacterial freezing nuclei will be difficult to interpret in terms of nucleation theory. Just how difficult can be inferred from Fig. 5. In this figure the fractional rate of change ($N_u^{-1} = dN_u/dt$) is plotted against time. Except for the curve representing cell fragments, all data comes from the same source as in Fig. 4. In one case (exponential cells, −10°C) the curve falls sharply to zero after an initial rise, suggesting that the singular

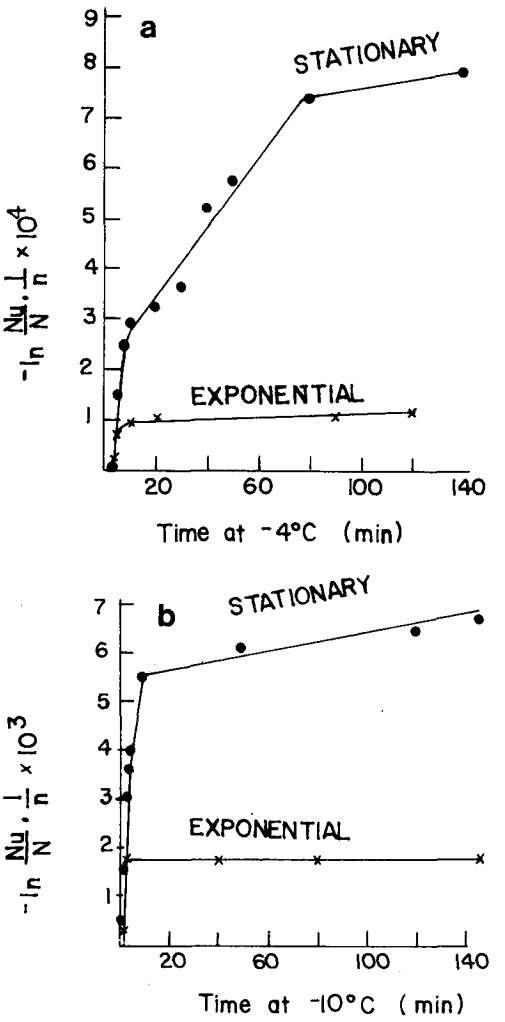

FIG. 4. Apparent active (freezer) to total cell ratio as a function of time at constant supercooling: (A) −4 ± 0.4°C; (B) −10 ± 0.4°C.

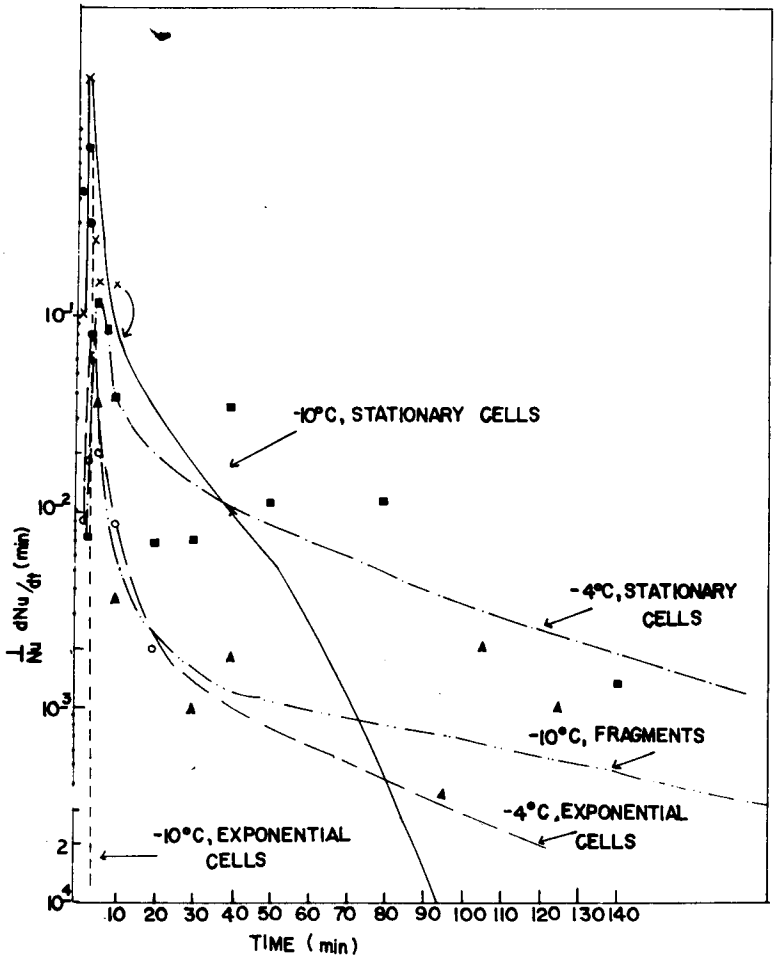

FIG. 5. Fractional rate of change of unfrozen samples in replicate sets as a function of time at constant supercoolings.

model of freezing might hold (e.g., Langham and Mason, 1958). However, in all other cases, including that of a cell fragment preparation, the curves taper off slowly to zero or some constant value. These latter instances, which incidently do not differ all that much from results obtained by Vali and Stansbury (1966) with undefined nuclei in distilled water at −20°C, are consistent with some sort of a combination between singular and stochastic (e.g., Bigg, 1953; Carte, 1956) freezing. In short, much work remains to be done before the respective mechanisms of bacterial and non-bacterial freezing nucleus action becomes clear.

4. Discussion

The data presented here establish that all INA bacteria so far isolated around the world are basically similar to one another in terms of their nucleus behavior. Such differences as exist are related to the highest temperature at which a given species can act, and to the stability of its nuclei. On the other hand, all strains produce cell-associated freezing sites active at −2 to −4°C, (Group I), at −5 to −7°C (Group II) and at −8 to −10°C (Group III). It is also evident that Group I sites of INA bacteria, the least abundant ones in any cell population, require metabolically normal and physically intact cells for their expression. The less efficient and most abundant Group III sites, on the other hand, are also present in cell-free fragment preparations of both yellow and fluorescent INA bacteria. Nevertheless, while it is convenient for us to functionally separate bacterial freezing nuclei into three classes on the basis of temperature, we are by no means certain as to the physical significance of such classification. For example, it may hold that a given cell capable of initiating freezing at −10°C will also do so at −4°C if given enough time. Alternatively, cell state rather than time may decide whether, and with what efficiency, a given individual can act. Evidence for the contribution of both time and growth phase to freezing nucleus expression has been obtained in these studies (see Figs. 4 and 5). In any case, frac-

tional rates of sample freezing observed here raise serious questions about the applicability of existing stochastic or singular freezing theories to the action of biogenic freezing nuclei. The data of Vali and Stansbury (1966) with undefined freezing nuclei further suggest that these models, in fact, may have no general validity.

The possibility of employing INA bacteria or their fragments as cloud seeding agents is obvious and has been suggested (Maki and Willoughby, 1978). However, the fact that the number frequency of active freezer bacteria in a population is always low raises a practical obstacle to attempts to use the organisms in actual cloud seeding trials. For example, in typical seeding operations one would like to release 10–30 artificial nuclei per liter of cloud. For a seeding volume of 3 km^3 bounded by -3 and -8°C isotherms, one requires 6×10^3 active particles to satisfy a demand for 10 nuclei per liter. At -4°C the average abundance of Group I particles is ~ 1 in 10^4 cells. Therefore, 3×10^{17} cells are required per experiment. Standard cell yields are 5×10^{12}/L of culture. Accordingly, as things now stand, we would require a 100 000 L fermentation to produce the necessary particles. This is unfortunately not a competitive situation. However, if every cell in the population were active, only 10 L would suffice. Lindow has reported the existence of a strain of *P. syringae* in which the freezing nucleus to cell ratio approaches unity (Lindow, 1977). Recent experiments in our laboratory have shown that it is also possible to induce each cell of bacterium M1 to become active at -4 to -5°C by treatment with certain chemical agents. These latter findings, as well as those relating to genetically altered strains of bacterium M1, will be reported elsewhere.

Finally, in those situations where cloud temperatures of -8 to -10°C are reached, it also becomes possible to contemplate the use of cell-derived fragments as seeding agents in place of intact cells.

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