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Inhibition of bacterial ice nucleation by polyglycerol polymers☆

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Abstract

The simple linear polymer polyglycerol (PGL) was found to apparently bind and inhibit the ice nucleating activity of proteins from the ice nucleating bacterium *Pseudomonas syringae*. PGL of molecular mass 750 Da was added to a solution consisting of 1 ppm freeze-dried *P. syringae 31A* in water. Differential ice nucleator spectra were determined by measuring the distribution of freezing temperatures in a population of 98 drops of 1 μL volume. The mean freezing temperature was lowered from −6.8 °C (control) to −8.0, −9.4, −12.5, and −13.4 °C for 0.001, 0.01, 0.1, and 1% w/w PGL concentrations, respectively (SE < 0.2 °C). PGL was found to be an ineffective inhibitor of seven defined organic ice nucleating agents, whereas the general ice nucleation inhibitor polyvinyl alcohol (PVA) was found to be effective against five of the seven. The activity of PGL therefore seems to be specific against bacterial ice nucleating protein. PGL alone was an ineffective inhibitor of ice nucleation in small volumes of environmental or laboratory water samples, suggesting that the numerical majority of ice nucleating contaminants in nature may be of non-bacterial origin. However, PGL was more effective than PVA at suppressing initial ice nucleation events in large volumes, suggesting a ubiquitous sparse background of bacterial ice nucleating proteins with high nucleation efficiency. The combination of PGL and PVA was particularly effective for reducing ice formation in solutions used for cryopreservation by vitrification.

Keywords: Polyglycerol; Polyvinyl alcohol (PVA); Bacterial ice nucleation; Antinucleation; Ice blocker; Vitrification; Supercooling

Ice growth is thermodynamically favored in water at temperatures below 0 °C. However, the initial formation (nucleation) of ice is not kinetically favored in pure water at temperatures above approximately −40 °C, the homogeneous nucleation temperature (T_h). Ice nucleation at temperatures greater than T_h is induced by heterogeneous ice nucleating agents (INAs). INAs are ubiquitous in the environment and are known to be of both mineral and biological origin [39]. INAs are believed to act by mimicking the structure of polar groups that exist on the surface of ice, inducing epitaxial growth of ice at the water–INA interface.

Among the largest and most efficient INAs in nature are assemblies of ice nucleating proteins found on the surface of certain bacteria, such as *Pseudomonas syringae*, *Erwinia herbicola*, and *Pseudomonas fluorescens* [14]. By presenting

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protein aggregates of large spatial extent [4,10, 15,29], these ice nucleating bacteria are able to initiate ice growth at temperatures as warm as −1 °C. These bacteria, as epiphytes, are a primary cause of frost damage to plants [1,21–23]. Ice nucleating bacteria have also evolved as symbiotes that facilitate early freezing as part of cold adaptation processes in some plants and animals [18,47].

Ice nucleating bacteria are present in large numbers throughout the biosphere on the surfaces of nonconiferous plants [23], and are likely widely dispersed in the earth's atmosphere. Ice nucleating bacteria and their proteins may therefore be suspected as a common cause of high temperature nucleation in both natural and laboratory environments. In natural settings, inhibition of bacterial INA is of considerable interest for environmental ice control, particularly prevention of frost damage to crops. In the laboratory, bacterial INA contamination may contribute to heterogeneous nucleation events that foil attempts to cryopreserve large systems by vitrification [6–8,46], and especially attempts to supercool solutions without cryoprotectant [3,25,35]. Clearly there is a need for methods to inhibit the activity of ice nucleating bacteria and proteins.

Bacterial ice nucleation can be inhibited by agents that kill whole cells such as bactericides [16,17,27,33], antibiotics [13,20,22], copper salts [13,20,22,26], and agents that alter protein–membrane anchoring such as surfactants [12], organic solvents [19,37], and phospholipases [11,38]. Nucleation by isolated INA proteins can be further inhibited by cationic surfactants [30,42], proteases [24,33], and compounds that chemically alter proteins such as sulfhydryl reagents and *N*-bromosuccinimide [17,31,36].

Bacterial INA can also be inhibited by binding interactions with *cis*-hydroxyl binding borates [17,38], certain lectins [17], antifreeze proteins [5], antifreeze glycoproteins [32,43], and antibodies raised against INA protein [34]. For sensitive applications such as cryopreservation, inhibition by selective binding is obviously preferable to harsher chemical treatments. However, simple nontoxic compounds that selectively bind and inhibit bacterial INA are not known.

We now report the observation that the simple synthetic polymer polyglycerol (PGL) ($H[OCH_2CHOHCH_2-]_nOH$) was found to apparently bind and inhibit INA from the ice nucleating bacterium *P. syringae*. In vitrification solutions the polymer was found to complement the ice inhibiting effects of the general nucleation inhibitor, polyvinyl alcohol PVA [46]. These results have been previously presented in preliminary form [45].

Polyglycerol is a fully water-soluble polymer that contains both pendant hydroxyls and ether linkages. It is in some respects a hybrid between polyvinyl alcohol and polyethylene glycol (PEG). Polyglycerol is a metabolite of polyglycerol esters, which are widely used as emulsifiers and fat substitutes in the food industry [2,28,44]. The apparently harmless presence of polyglycerol in both the digestive tract and bloodstream [28] after ingestion of polyglycerol esters is illustrative of the low biological toxicity of the polymer. This makes polyglycerol promising for use in applications such as crop protection and tissue cryopreservation.

Materials and methods

Materials

All solutions were prepared gravimetrically (w/w) using ultrapure lab water (Milli-Q Biocel, 18 MΩ), except for the solution of Fig. 5a, which was prepared using bottled distilled water (Hinckley & Schmitt, Orange, CA, USA). Bacterial INA solutions were prepared from freeze-dried *P. syringae 31A* (Snomax, York International, Victor, NY, USA). INA concentrations are stated by weight because the cell density of this product is not known. Reagent-grade ethylene glycol was obtained from Spectrum Quality Products (Gardena, CA, USA). PGL was used as formulated in the product "Supercool Z-1000" (21st Century Medicine, Rancho Cucamonga, CA, USA). This polymer was produced by a proprietary source and certified by the source to be polyglycerol of mean molecular mass 750 Da. Polyvinyl alcohol (PVA) was used as formulated in the product "Supercool X-1000" (21st Century Medicine), a PVA copolymer of mean molecular mass 2000 Da in which 20% of the hydroxyls are replaced by acetate groups [46].

The environmental water samples tested in this study consisted of water drawn from the surface of Lake Elsinore, California (in the month of August) and rain water collected in Rancho Cucamonga, California (November).

Ice nucleation spectra

Differential nucleator spectra were determined by measuring the distribution of freezing temperatures in a population of 1-μL drops according to

the drop freezing assay of Vali [39,40]. Solutions were dispensed as seven 1-μL drops by a Hamilton microliter syringe into an aluminum sample pan (Perkin–Elmer 0219-0041) and covered with a drop of mineral oil to reduce evaporation. The open sample pan was placed inside the oven of a Perkin–Elmer DSC 7 differential scanning calorimeter, and the sample was cooled at 2 °C/min until all drops were frozen. The freezing temperature of each drop was recorded from its peak on the thermogram. Freezing data for a population of 98 drops (14 sample pans) were obtained to determine each spectrum. Spectra were computed by the formula [39,40]

$$k(\theta) = -(1/V\Delta\theta)\ln[1 - \Delta N/N(\theta)],$$

where V is the drop volume (1 μL), $N(\theta)$ is the number of unfrozen drops at temperature θ, and ΔN is the number of drops observed to freeze in the temperature interval $\Delta\theta$. Nucleation by causes extrinsic to the solutions tested was ruled out by observing that samples of ultrapure lab water did not freeze at temperatures above −20 °C.

Vial vitrification experiments

The ability of concentrated cryoprotectant solutions to vitrify (cool to below the glass transition temperature without ice formation) was tested by preparing either 10- or 15-g solution samples in 20-mL glass scintillation vials. The vials were then suspended in cold (−160 °C) nitrogen vapor 2 cm above the surface of liquid nitrogen in a TA-60 dewar (MVE, Burnsville, MN, USA), resulting in a mean cooling rate of approximately 7 °C/min [46]. Upon reaching a temperature of approximately −130 °C, the vials were removed from the dewar, briefly dipped in methanol to prevent exterior frost formation, and photographed.

Flask vitrification experiments

The ability of large solution volumes to vitrify was tested by preparing 500-g solution samples in 500-mL Erlenmeyer flasks and placing the flasks in a −130 °C freezer. The resulting passive convection cooling resulted in solution core temperatures of −41, −69, −85, −98, −106, and −112 °C after 1, 2, 3, 4, 5, and 6 h, respectively. A final temperature of −128 °C was reached after approximately 15 h. After at least 24 h, the flasks were removed from the freezer, wiped with methanol, and photographed.

Water supercooling experiments

The effect of additives on the ability of water to supercool was measured by preparing 22-mL water samples in glass scintillation vials. For each additive, 12 identical vials were prepared and instrumented with thermocouple probes. The vials were then immersed in a 0 °C ethylene glycol bath in a −20 °C freezer, resulting in a cooling rate of approximately 1 °C/h. The temperature readings of all thermocouples were electronically recorded at 1-min intervals, and the freezing temperature (last temperature before freezing exotherm) was documented.

Results

Bacterial INA spectra

The control curve of Fig. 1 is the cumulative freezing spectrum obtained for a 1 ppm (by mass) solution of *P. syringae* bacterial INA. PGL was added to the solution in concentrations up to 1% w/w, and the resulting freezing spectra are also shown in Fig. 1. The corresponding differential ice nucleator spectra are computed in Fig. 2. The mean freezing temperature was lowered from −6.8 °C (control) to −8.0, −9.4, −12.5, and −13.4 °C for 0.001, 0.01, 0.1, and 1% PGL concentrations, respectively (SE < 0.2 °C). PVA was also tested at

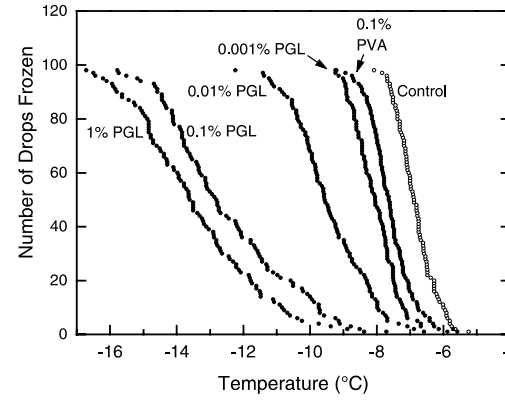

Fig. 1. Number of drops frozen (of 98) as a function of temperature for various concentrations of PGL and PVA in water containing 1 ppm *P. syringae* ice nucleating bacteria. Small concentrations of PGL were highly effective at lowering the temperature at which water freezes in the presence of the *P. syringae* INA. PVA was only slightly effective by comparison.

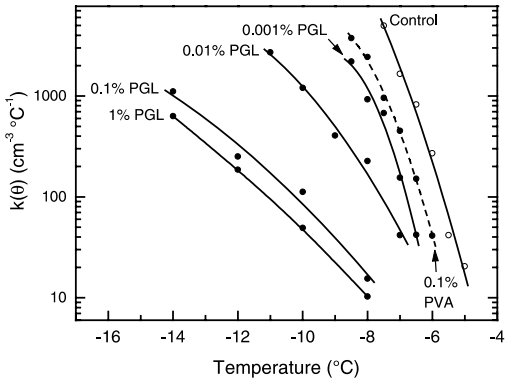

Fig. 2. Differential ice nucleator spectra computed from the data of Fig. 1. The spectra are defined such that $k(q)\mathrm{d}q$ gives the concentration of ice nucleating particles that initiate freezing within the interval dq at temperature q.

0.1% concentration and found to exhibit significant ($P \ll 0.001$) nucleation inhibition (Figs. 1 and 2). However, PVA was approximately two orders of magnitude less effective than PGL. The water-soluble polymers polyvinyl pyrrolidone (M_w 5000), polyethylene glycol (M_w 1000), polyacrylamide (M_w 1500), and dextran (M_w 40,000) were also tested at 0.1% concentration and found to have no effect on the nucleator spectrum.

A 1 ppb solution of *P. syringae* bacterial INA was also studied. The mean freezing temperature of 15 1-μL drops was found to be −16 °C (SE 1 °C). Adding 1, 0.1, or 0.01% PGL reduced the mean freezing temperature to $-20, -20$, and −19 °C, respectively. Adding 0.5% PGL and 0.5% PVA reduced the mean freezing temperature to −22 °C, which was identical to the mean freezing temperature of laboratory water without any added INA.

Crystalline organic ice nucleators

A large number of organic compounds are known to nucleate ice while present as crystals in aqueous solution [9]. Seven diverse compounds were selected based on their purported resemblance to different crystal faces of ice [9]. Each compound was mixed into a 55% ethylene glycol solution, and 10-g solution samples were cooled in a vial vitrification assay.

As summarized in Table 1, some of the compounds apparently dissolved completely (“soluble”), dissolved very slowly (“partly soluble”), or not at all (“insoluble”). The “partly soluble” compounds were tested in the partly dissolved state. If they were stirred long enough to dissolve completely, no ice nucleation occurred. The compounds listed as “soluble” nucleated ice even in the absence of any visible undissolved crystals. However, it is likely that these solutions contained an equilibrium amount of microscopic crystals because the individual molecules would presumably be too small to act as nucleators.

As Table 1 shows, the visual estimates of the number of ice crystals formed was unchanged in the presence of PGL for any of the compounds. However, all of the “soluble” and “insoluble” compounds were inhibited by PVA. Only the “partly soluble” compounds were unaffected by either polymer.

Environmental water samples

Ice nucleation spectra were obtained from a sample of natural lake water (Fig. 3). The INAs present in this sample were inhibited by PVA, but were comparatively unaffected by PGL.

The inhibition sensitivity of nucleators in rain water was also studied by a simple vitrification

Table 1
Approximate number of ice crystals formed by organic nucleators in 55% ethylene glycol solution in the presence of PGL and PVA polymers

Compound	Conc. (%)	Solubility	Control	0.1% PGL	0.1% PVA
Phloroglucinol	0.5	Soluble	10^4	10^4	10^2
3-Aminophenol	0.1	Soluble	10^3	10^3	10^1
Phenazine	0.1	Insoluble	10^3	10^3	10^2
Metaldehyde	0.1	Insoluble	10^2	10^2	10^1
Pthalic Anhydride	0.2	Insoluble	10^2	10^2	10^1
Acetoacetanilide	0.1	Partly soluble	10^3	10^3	10^3
2-Nitrodiphenylamine	0.1	Partly soluble	10^2	10^2	10^2

Note. Concentrations are by weight; 10-g solution samples cooled to −130 °C.

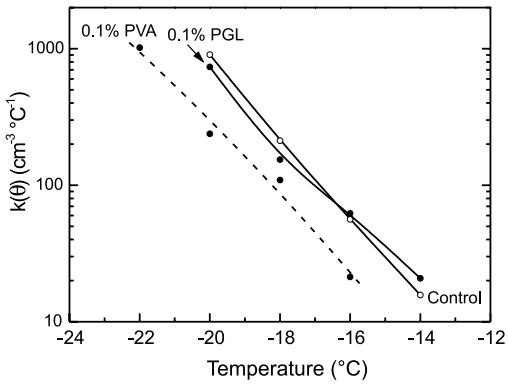

Fig. 3. Differential ice nucleator spectra for a sample of water drawn from the surface of Lake Elsinore, California, in the presence and absence of 0.1% PVA or PGL. The nucleators in this water were inhibited much more strongly by PVA than by PGL.

assay. Ten-gram samples of a 50% ethylene glycol solution in rain water were placed in three vials. One vial contained added 0.1% PVA, and another 0.1% PGL. The vials were then cooled for 7 min as described under Materials and methods until they reached −80 °C. Both the control vial and the PGL vial were observed to contain approximately 100 ice nucleation events (discrete ice growth sites), while the PVA vial contained only approximately 10 ice nucleation events. The appearance of the PVA vial was similar to that of a 50% ethylene glycol solution prepared in ultrapure water, indicating that the PVA had effectively neutralized the additional nucleators present in rain water compared to ultrapure water.

Complementary nucleation inhibition

The effects of PGL and PVA singly and in combination against *P. syringae* INA were tested in a vial vitrification assay. 1 ppm (by mass) *P. syringae* INA was added to 55% ethylene glycol in water, with an additional 1% of either PEG (M_w 1000), PVA, PGL, or a mixture of PVA and PGL.

The results are shown in Fig. 4. Adding bacterial INA to these solutions caused a high ice nucleation density that was unaffected by the presence of PEG. (It should be noted that all these solutions would vitrify without visible ice if nucleator were not added.) In contrast, both PVA and PGL inhibited ice nucleation, but in different ways. PVA reduced the total amount of ice formed, but left thousands of very small crystals that it either failed to inhibit or perhaps caused to nucleate at a lower temperature than they would otherwise. PGL completely inhibited most nucleation events, but allowed a small number of ice

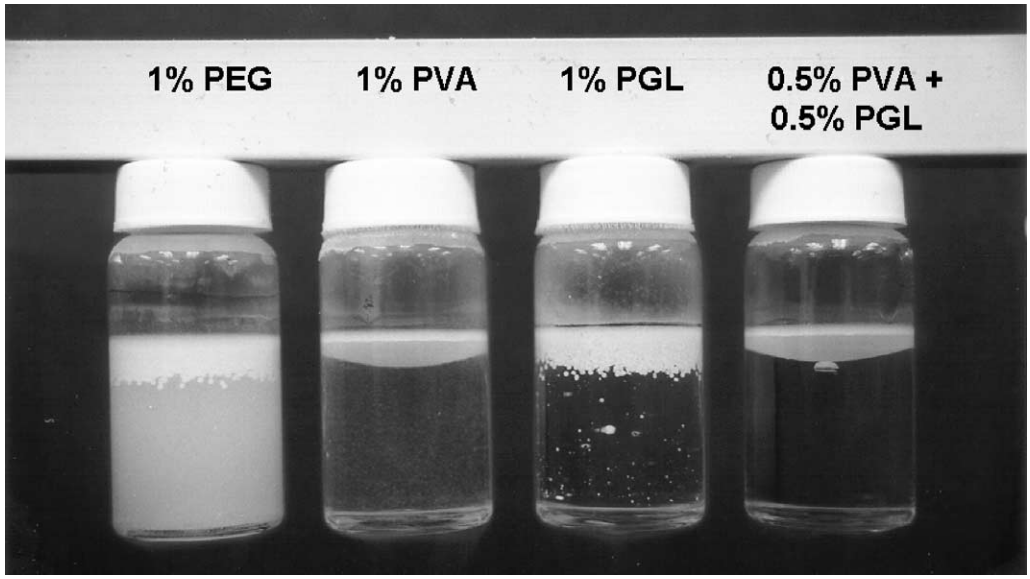

Fig. 4. Vials containing 15 g of 55% ethylene glycol and 1 ppm freeze-dried *P. syringae* in water cooled to −130 °C. From left to right, the vials contain the additives 1% polyethylene glycol 1000 (control), 1% PVA, 1% PGL, and 0.5% PVA + 0.5% PGL. The opacity of the first vial is caused by millions of tiny ice crystals, each initiated by a discrete nucleation event. Visible ice formation was completely inhibited in only the last vial, which contains both PVA and PGL.

nuclei to escape inhibition and grow to large size. The combination of the two agents caused complete inhibition of all visible nucleation events. These results suggest that PGL and PVA may act in a complementary fashion on different subpopulations of the nucleators present. Without PGL or PVA, approximately 62% ethylene glycol was required to achieve comparable inhibition.

Bulk solution vitrification

To test the effectiveness of PGL and PVA against background nucleators present in the laboratory, the effects of the polymers were observed during vitrification of large solution volumes without added nucleator. The results are shown in Fig. 5.

Figs. 5a and b show that background nucleator concentrations can depend strongly on water purity, as has been observed previously [46]. The solution prepared in distilled water is completely opaque with ice, while the solution prepared in ultrapure water contains discernible vitreous spaces between ice balls.

Fig. 5c shows that adding PGL alone does not seem to reduce the net amount of ice formed. Large ice balls (caused by early high-temperature nucleation events) are suppressed, but at the expense of an increase in very small ice balls caused by low-temperature nucleation events. This may happen because suppression of early bacterially mediated nucleation delays freeze-concentration of the cryoprotectant, thereby enhancing susceptibility of the solution to freezing caused by

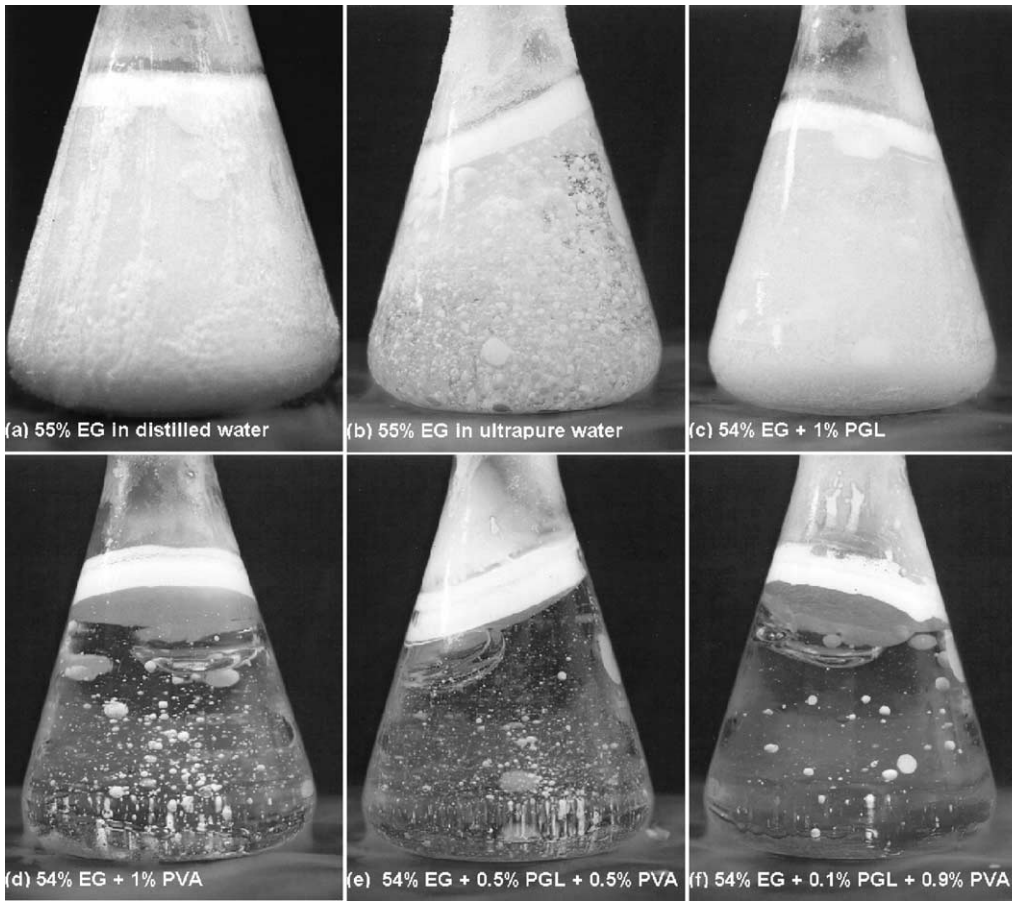

Fig. 5. Five-hundred-gram ethylene glycol (EG) solutions cooled to −128 °C. The difference between (a) and (b) demonstrates the effect of water purity on ice crystal nucleation. The remaining solutions were prepared using ultrapure water with ice blocking polymers added as indicated. In solutions containing both polymers, the number of small vs large ice balls can be shifted by changing the relative balance of each polymer.

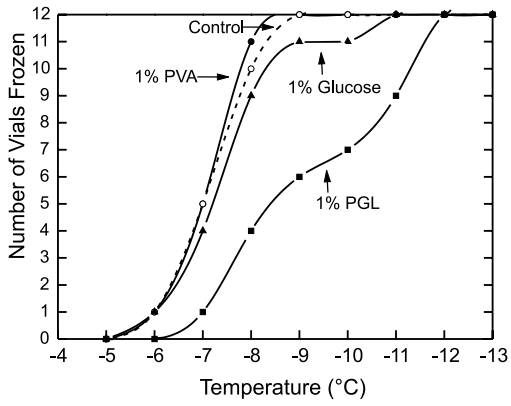

Fig. 6. Number of vials frozen (of 12) as temperature was decreased. Each vial contained 22 mL water with 1% of the stated additive. The frozen vial count is plotted at integer temperature increments. PGL was the only additive that significantly promoted supercooling compared to the control vials.

low-temperature nucleators that PGL cannot inhibit.

Solutions containing PVA achieve the most dramatic suppression of ice nucleation (Figs. 5d–f). The combination of PVA and PGL achieves better suppression of large ice balls, supporting the hypothesis that early high-temperature nucleation events in these solutions are caused by bacterial INA contamination. The lowest number of visible ice nucleation events is achieved with 0.1% PGL plus 0.9% PVA, although this reduction of PGL in favor of PVA brings an increase in the number of very large ice balls.

Water supercooling experiments

Fig. 6 shows the cumulative number of vials frozen as a function of temperature for laboratory water and laboratory water with three different additives. Only the vials containing PGL supercooled to a markedly greater extent than pure water ($P < 0.005$). PGL is therefore a better inhibitor of initial ice nucleation in these macroscopic volumes than is PVA.

Discussion

PGL was found to be an effective inhibitor of ice nucleation by *P. syringae* INA, even at concentrations as low as 10 ppm. Since PGL is a biochemically inert polyhydroxy compound, such strong inhibition is presumably due to a binding interaction with INA proteins. Effectiveness against INAs from other species of ice nucleating bacteria remains to be studied. However, it would be remarkable if the effect were specific to *P. syringae* given the strong homology between INA proteins of different ice nucleating bacterial species [41].

PVA was found to be partially effective against almost all ice nucleating agents tested, including *P. syringae* INA. In contrast, *P. syringae* INA was the only defined nucleator that PGL was effective against. This suggests that the inhibitory activity of PVA is very general, perhaps involving direct interaction with ice embryos, whereas the more potent inhibitory activity of PGL is specific to *P. syringae*-like INA. This difference is remarkable considering the structural similarities between the two molecules.

PGL's failure to inhibit unidentified INAs found in rain water and microliter samples of lake water suggests that the numerical majority of atmospheric ice nucleators do not resemble *P. syringae*. This raises the question of whether environmental nucleators are of bacterial origin. Further study is required to better characterize atmospheric ice nucleators given the pivotal role that they play in precipitation and other meteorological phenomena.

The ineffectiveness of PGL alone for reducing the number of ice nucleation events in large solution volumes (Fig. 5) suggests that the numerical majority of ice nucleating contaminants in the laboratory also do not resemble *P. syringae*. The observed results are consistent with the presence of a sparse amount of bacterial INA (causing rare high-temperature nucleation events) combined with a much more numerous background of inefficient (low-temperature nucleating) INA of nonbacterial (or at least non-*P. syringae*) origin.

It may seem contradictory that in Fig. 4 PGL inhibits most nucleation events, leaving a small number of large ice crystals, whereas in Fig. 5d it is PVA that creates this appearance. Actually this is to be expected if the majority of nucleators in Fig. 4 are bacterial (having been added to the solution) and if in Fig. 5 the majority of nucleators are background nucleators of nonbacterial origin. In each case, the polymer that most effectively reduces the number of nucleation events is the polymer that is most effective against the majority nucleator present. The few ice nuclei that

escape inhibition are presumably either statistical outliers or minority-type nucleators that are inhibited poorly or not at all by the polymer.

The view of *P. syringae*-like bacterial INA forming a minority population of nucleators that are especially active at high temperature is further supported by the results of the water supercooling experiments. When water with little or no cryoprotectant is supercooled, the ice created by the first nucleation event will rapidly grow through the supercooled volume until the solution warms to 0 °C. The freezing properties of pure water are thus entirely determined by the most efficient (highest-temperature) nucleating particle in the sample. Ice nucleation spectra experiments with microliter samples without added INA were unaffected by PGL, suggesting that bacterial INA are not naturally present on a microliter scale. However, the supercooling of 22-mL water samples was profoundly affected by PGL, suggesting that the most efficient nucleator in these volumes was of bacterial origin.

Thus the relative importance of PGL vs PVA for ice suppression clearly depends on the solution volume and cryoprotectant concentration. In concentrated cryoprotectant solutions, such as those used for vitrification, ice grows very slowly so that all nuclei present contribute to the total amount of ice formed in the solution. In this context PVA plays a larger role than PGL in reducing ice formation. However, in solutions of substantial volume ($\gg$1 μL), the first nucleation event will probably be caused by bacterial INA and thus be susceptible to suppression by PGL. In dilute solutions, the first nucleation event will also be the last nucleation event, so PGL is more powerful than PVA for facilitating the supercooling of macroscopic water volumes with little or no cryoprotectant.

In all cases, the combination of PGL and PVA was more effective than either agent alone. This was especially true when *P. syringae* was deliberately added to samples, such as a 1-ppb solution in pure water or the ethylene glycol solution of Fig. 4. In these experiments the combination of both polymers was able to completely neutralize the effect of the INA, restoring the behavior of the solution to the same as that of the controls.

In conclusion, polyglycerol was found to be a potent and specific inhibitor of ice nucleation caused by the ice nucleating bacterium *P. syringae*. Low-molecular-weight polyvinyl alcohol was found to be a less powerful, but more broadly active antinucleation agent that complements the ice inhibiting activity of polyglycerol. The combination of the two ice blocking polymers is particularly effective, with the ideal balance depending on the application.

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