

THE SEARCH FOR “GREEN INHIBITORS:” PERTURBING HYDRATE GROWTH WITH BUGS

Emily I. Huva¹
Raimond V. Gordienko¹
John A. Ripmeester^{1,3}
Huang Zeng^{1,3}
Virginia K. Walker^{1,2*}

¹Department of Biology & ²Department of Microbiology & Immunology, Queen’s University
Kingston, Ontario, K7L 3N6, CANADA;

³The Steacie Institute for Molecular Sciences, National Research Council of Canada
100 Sussex Drive, Rm 111, Ottawa, Ontario, K1A 0R6, CANADA

ABSTRACT

Certain organisms, including some bugs (both insects and microbes) are able to survive low temperatures by the production of either ice nucleating proteins (INPs) or antifreeze proteins (AFPs). INPs direct crystal growth by inducing rapid ice formation whereas AFPs adsorb to ice embryos and decrease the temperature at which the ice grows. We have also shown that certain AFPs can inhibit the crystallization of clathrate hydrates and eliminate more rapid recrystallization or “memory effect”. Here we examine several bacterial species with ice-associating properties for their effect on tetrahydrofuran (THF) hydrate crystallization. The bacteria *Chryseobacterium* sp. C14, which shares the ice recrystallization inhibition ability of AFPs, increased induction time to THF hydrate crystallization in isothermal experiments. In an effort to understand the association between AFPs and THF hydrate we have produced bacterially-expressed AFPs as probes for hydrate binding. Although the structure of hydrates is clearly distinct from ice, the apparent potential for these products to perturb clathrate hydrate growth compels us to explore new techniques to uncover “green inhibitors” for hydrate binding.

Keywords: gas hydrate, tetrahydrofuran, kinetic inhibitors, antifreeze protein, ice nucleating protein, memory effect

NOMENCLATURE

AFP antifreeze protein
CFU colony forming units per mL
CHAP clathrate-hydrate affinity purification
GFP green fluorescent protein
INP ice-nucleating protein
 n_c fraction of samples crystallized
 t time [h]
THF tetrahydrofuran

INTRODUCTION

Gas hydrates are formed when gas molecules are encaged by water molecules under conditions of

low (but not subzero) temperatures and modest pressures. Although deposits of natural gas hydrates are regarded as a very promising potential energy resource [1], unscheduled hydrate formation during hydrocarbon recovery and transport can be costly, dangerous, and harmful to the environment. [2,3].

Alternatives to thermodynamic inhibitors like methanol, which are required in large amounts to be effective, are the low-dosage hydrate inhibitors, either anti-agglomerants or kinetic inhibitors. Recently, a third group of low-dosage hydrate inhibitors have been reported. Antifreeze proteins

* Corresponding author; Phone: 613-533-6123; Fax: 613-533-6617; Email: walkervk@queensu.ca

(AFPs), which confer cold tolerance to a variety of organisms by adsorbing to ice crystals and inhibiting their growth, can also slow the growth of hydrate crystals [3,4]. These studies additionally present evidence that some AFPs can eliminate the faster recrystallization of hydrate after a brief melt, the so called, “memory effect.” Experiments designed to explore this phenomenon are presented elsewhere in this volume [5].

To date, only AFPs from animals have been reported with hydrate inhibition activity. However, they are costly to harvest from natural sources and it is difficult to obtain high yields of active protein when expressed by recombinant *Escherichia coli*. Our hypothesis was that bacterial AFPs would be more practical candidates as low dosage hydrate inhibitors for large-scale production. We further believe that industry would be open to this novel approach to hydrate inhibition because of the precedent set by the uses of bacterial proteins associated with hydrate storage and transport. Commercial biosurfactants from *Bacillus subtilis*, *Pseudomonas aeruginosa*, *P. syringae*, and *Acinetobacter calcoaceticus* have been used to accelerate the formation of CO₂ and natural gas hydrates [6,7,8].

In this paper we present the results of initial tests concerning the THF hydrate affinity of several ice-associating bacteria. *Chryseobacterium* sp. C14 was initially isolated in our lab as a highly freeze-thaw resistant microbe and was shown to exhibit ice recrystallization inhibition [9]. *Pseudomonas putida* has AFP activity and ice nucleating activity [10; B.R. Glick, personal communication; our unpublished observations]. *P. borealis* was isolated in our lab by ice affinity and shows ice nucleation activity [11] as do strains of *P. syringae*. We also examined the potential of ice affinity selection, developed for microbial isolation [11], to be adapted for hydrate affinity selection.

METHODS

Hydrate formation and reformation in the presence of ice-associating bacteria

Bacteria were cultured in 10% Bacto™ Tryptic Soy Broth (Becton, Dickinson and Co., Franklin Lakes, NJ), inoculated with a single colony and grown to stationary phase (~10⁹ CFUs/mL) over 48 h at room temperature. Cultures (40- 100 mL) were then transferred to 3°C for 48-62 h. These cultures were mixed with OmniSolv® tetrahydrofuran (>99.9% purity, unstabilized; EMD Chemicals Inc., Gibbstown, NJ) at a 3.34:1 culture:THF

volume ratio (15:1 molar ratio). Aliquots (3 mL) were dispensed into a series of 16 mm x 125 mm Pyrex® culture tubes. Samples were immediately immersed in a 0.0 ± 0.2°C bath and stirred individually at 300 rpm while temperature was monitored using thermocouples, as previously described [4]. The temperature was chosen so that ice could not form [12]. For each solution tested, 3-4 independent experiments (a total of 33-56 vials) were conducted. All vials, thermocouples and stir bars were well washed between experiments: first with Sparkleen™ 1 (Fisher Scientific Co., Pittsburg, PA), then with tap water, 75% acetone, and finally Milli-Q®-filtered H₂O.

The temperature-time output of the thermocouples was used to measure the induction time, *t*, between the time of sample equilibrium at 0°C and the onset of crystallization, indicated by a sudden increase in temperature. Note that the induction time to crystallization is distinct from the “time to nucleation,” as nucleation events did not necessarily result in bulk crystallization recorded by the thermocouples (see Results).

To examine THF hydrate recrystallization, the same procedure was used, except that freshly prepared samples were first frozen on dry ice (10 min), left at room temperature for 5 min and then thawed for 1 hr in a 6.4°C circulating bath (Model 9712; Polyscience, Niles, IL).

Data from all experiments were pooled to examine trends graphically, while means and standard deviations were calculated across experiments.

The development of a clathrate hydrate affinity purification (CHAP) technique

Initially cyclopentane and Milli-Q® water were mixed at volume ratios of 1:3.34 or 1:6.68. Sufficient bromophenol blue (Fisher Scientific) was added so as to be visible. THF and Milli-Q® water were similarly mixed. Samples were held at 3°C for days, both stirred and unstirred, after being nucleated by a cooled copper wire. The solid and liquid fractions were then separated and the solid fractions were rinsed with cold water before being visually compared.

Next, THF hydrate affinity purification was explored using several different recombinant proteins marked with jelly fish green fluorescent protein (GFP). *E. coli* strains were transformed with pET24a(+) plasmids encoding a His-tagged Type III AFP from the fish *Macrozoarces americanus*, and a His-tagged AFP-GFP from the

plant *Lolium perenne*. Controls were produced from *E. coli* transformed with a pET20b(+) plasmid bearing a His-tagged GFP.

After induction of the recombinant bacteria and collection of supernatants from cell lysates, the proteins were purified [13] by immobilized metal affinity chromatography with a cobalt-based resin (Clontech, Mountain View, CA). Purified proteins were dialyzed overnight against a 0.1 M Tris HCl buffer (pH = 8) at 4°C. Protein concentration was determined using a dye-binding assay [14].

THF-hydrate crystals were grown using the ice finger apparatus [15] with the following modifications. The crystal was seeded at a bath temperature of 2.3°C and later lowered into a pre-chilled 100 mL beaker containing one of the various proteins at 2 μ M. The bath temperature was dropped approximately 1°C/h until the crystal diameter approached half the original volume of the beaker. The hydrate crystal was then removed from the copper finger and washed with 30 mL distilled water (<4°C) and observed under UV light of wavelength 302 nm (Chromato-Vue transilluminator TM-36, UVP Incorp., San Gabriel, CA)

RESULTS & DISCUSSION

THF hydrate formation and reformation

The presence of microbial cells in THF induction experiments varied depending upon the type of bacteria examined. *P. borealis* and *P. syringae* appear to act as hydrate nucleators; supercooling of THF solutions in the presence of these cells was reduced by $\sim 2^\circ\text{C}$ compared to controls (unpublished observations). However, the addition of *P. syringae* cultures to THF did not result in a significantly higher fraction of crystallized samples in our experiments (Figure 1A; Table 1). Similarly, *E. coli* cells did not decrease THF hydrate induction time compared to H₂O-THF controls (Figure 1A; Table 1). Crystallization of both *P. putida* and *P. borealis* solutions was reduced by up to 10% initially, but after 5 h was indistinguishable from *E. coli*. It should be noted that THF is highly toxic to bacteria. Cells treated with THF at the concentrations used here or even at 70% of this concentration (1:5.2 vol/vol) were unculturable, forming no CFU (unpublished observations). Therefore, it is possible that any putative hydrate-inhibiting molecules associated with the cells could have been denatured or disaggregated by the THF and therefore have been ineffective in this assay.

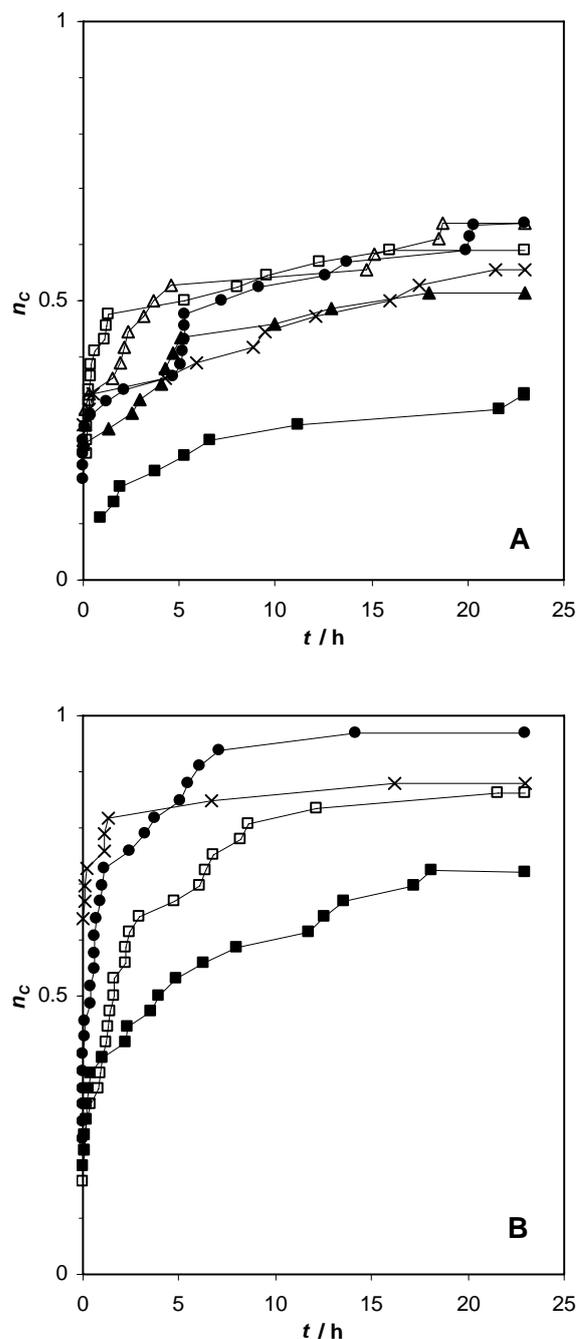


Figure 1. Crystallized fraction, n_c , of (A) freshly made and (B) pre-frozen samples that had formed THF hydrate after time t at 0°C. Solutions consisted of THF in a 1:3.34 (vol) ratio with: H₂O (×), *E. coli* (□), *Chryseobacterium* (■), *P. putida* (●), *P. borealis* (▲), and *P. syringae* (Δ). Cultures were approximately 10^9 CFU. Pre-frozen samples had been melted for 1h at 6.4°C prior to the experiment. Data points at $t = 0$ are not displayed.

Table 1. Comparison of isothermal THF hydrate formation at 0°C for various bacterial cultures.

Culture in solution with THF	Known activity ¹ :		Mean % Samples Crystallized at:					No. of:	
	AF	IN	<i>t</i> = 5 h		<i>t</i> = 23 h			Exps.	Vials
None (H ₂ O-THF)			36	± 13	56	± 13	3	36	
<i>E. coli</i>			46	± 22	58	± 14	4	44	
<i>Chryseobacterium</i>	•		19	± 13	33	± 14	3	36	
<i>P. putida</i>	•	•	36	± 7	64	± 13	4	44	
<i>P. borealis</i>	•	•	40	± 22	51	± 15	3	36	
<i>P. syringae</i>		•	54	± 25	64	± 16	3	37	
- Recrystallization ² Experiments -									
None (H ₂ O-THF)			82	^A ± 9	88	± 5	3	33	
<i>E. coli</i>			67	^{AB} ± 8	86	± 5	3	36	
<i>Chryseobacterium</i>			53	^B ± 10	75	± 22	3	36	
<i>P. putida</i>			82	^A ± 9	97	± 5	3	33	

¹ observed antifreeze (AF) or ice nucleating (IN) activity in ice, published and unpublished solutions were previously frozen and then melted (1 h at 6.4°C)

² groupings of samples that do not differ significantly (Tukey-Kramer HSD; q*=3.28 fresh samples, 3.20 pre-frozen; α=0.05).

Despite this challenge, it is all the more remarkable that samples with the ice-associating bacteria *Chryseobacterium* C14 took consistently longer to crystallize (Figure 1A). At the conclusion of the experiment only 33% of the *Chryseobacterium*-containing samples had crystallized, compared to 58% of the *E. coli* controls (Table 1).

Curiously, some *Chryseobacterium* and *P. putida* samples formed hydrate slurries instead of the solid hydrate blocks normally seen after crystallization. As many as 20% of these vials contained suspensions of small macroscopic crystals, identified as millimeter-wide octahedra in later morphology experiments (not shown). Such freezing was not seen on the thermocouple readout, indicating that the multiple nucleation and growth events in these vials were spread out over time; slurries were only recorded at the conclusion of the experiments (*t* = 23 h).

Thus, the presence of *Chryseobacterium* sp. C14 appears to inhibit THF hydrate, at least partially, by keeping crystals small for a certain time, superficially like the anti-agglomerant low dosage hydrate inhibitors. Although THF is toxic to the cells, it is possible then that at least some of the bacteria's ice-associating molecules survived and were responsible for mediating this effect. The proportion of *Chryseobacterium* vials containing slurries was almost equivalent to that bacteria's n_c reduction compared to controls. This suggests that the lower fraction of crystallized vials was due to growth inhibition and not to nucleation inhibition.

Nucleation inhibition did not occur, and indeed for *P. putida*, inclusion of an estimated slurry fraction of 0.18 would raise n_c to well above that of the control by 23 h. It may not be a coincidence that this increased THF hydrate nucleation combined with the observed inhibited growth (Figure 1) mirrors *P. putida*'s known dual ice nucleating and antifreeze activities.

All cultures recrystallized more rapidly after melting for 1 h at 6.4°C, demonstrating that the presence of cells did not interfere with memory effect. Despite some variability at 5 h, after 23 h n_c was between 0.2 and 0.4 higher for every sample tested (Table 1, Figure 1B). This overall increase in proportion of samples frozen is consistent with previous experiments on memory effect that show characteristic increases in freezing probability [16,17]. Significantly, no slurries were seen with *P. putida* or *Chryseobacterium*, or in any of the other samples, suggesting that for these microbes the formation of nuclei inevitably resulted in solid freezing by the end of the experiment.

Vials containing *Chryseobacterium*, again showed the lowest fraction frozen, ~0.12 below *E. coli*, over most of the experiment (Figure 1B). This was significantly slower than the THF-water controls and for *P. putida* (Table 1). Certain AFPs eliminate the memory effect [3,5], but in this case since recrystallization in the presence of *Chryseobacterium* was faster than the initial crystallization, there was no effect on memory.

Developing clathrate hydrate affinity purification

When ice or hydrates grow they exclude solutes, hence our initial experiments to develop CHAP used a dye so that the exclusion could be easily visualized. Unstirred cyclopentane solutions formed globular masses of hydrate that incorporated the bromophenol blue dye (Figure 2A). Solutions that were stirred formed slurries of small crystals that made the assessment of dye incorporation difficult. However, unstirred experiments using THF were more promising: THF hydrate excluded bromophenol blue (Figure 2B).

This successful demonstration with polycrystalline hydrate encouraged us to examine hydrate affinity for AFP. For ease of purification, the proteins were marked with a poly(His) tag, and in order to visualize them in the hydrate a GFP tag was also incorporated into the design of the plasmid constructs. When GFP alone was added to the THF-water solution, there was only minimal incorporation into the polycrystalline solid that was formed (Figure 3). In contrast, both AFP-GFP constructs, one with a fish AFP and the other with a plant AFP, appeared to be uniformly incorporated into the THF hydrate. These results show much promise for the use of CHAP, or a “hydrate finger,” for the isolation of novel hydrate-associating molecules and proteins.

CONCLUSIONS

THF hydrate formation was inhibited in the presence of cultures containing the ice-associating bacterium *Chryseobacterium* C14. There was a 40% reduction in crystallization, showing potential as a commercial “green inhibitor” for hydrates. There was no elimination of memory effect, however, and little compelling evidence was seen for nucleation inhibition by any of the bacteria. Although cyclopentane hydrates proved to be impractical, while THF’s toxicity to bacteria may be limiting, a novel method for isolating hydrate-associating molecules, with the potential for “green inhibition,” shows much promise.

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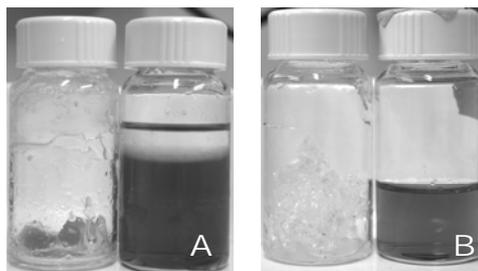


Figure 2. Hydrate (left) and aqueous (right) fractions of dilute (A) cyclopentane and (B) THF solutions containing bromophenol blue, after being nucleated and left unstirred at 3°C for several days.

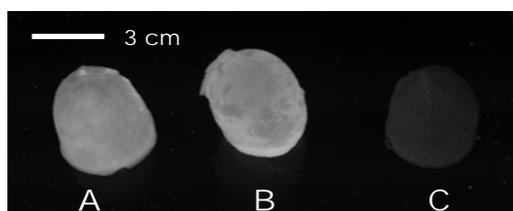


Figure 3. Incorporation into THF hydrate of (A) fish and (B) plant antifreeze proteins tagged with green-fluorescent protein (GFP), as visualized under UV light. Image (C) is a control with GFP alone. The experiment was repeated three times.

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