Ice nucleation proteins - a synthetic pathway to alleviate ice loss

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The Arctic sea ice moderates the global climate by reflecting approximately 90% of solar radiation into space, preventing the ocean and land from absorbing and releasing heat into the atmosphere. However, global warming is melting the Arctic sea ice, which raises the atmospheric temperature. This temperature increase accelerates the rate of ice melt, creating a feedback loop of deteriorating ice cover. Melted ice raises sea levels significantly, and humanity will eventually find most coastal cities submerged if no remedial measures are taken. Our team proposes a method to slow the melting process by increasing the Arctic ice size. The eventual objective is to insert *Pseudomonas syringae's inaZ*, a gene encoding an ice nucleation protein (Xin et al., 2018), into *Marinomonas primoryensis*, a gram-negative bacterium that naturally exists and binds Arctic ice (Delesky et al., 2021). However, since minimal research has been done on *M. primoryensis*, we will conduct a prototypical design with the use of *Pseudoalteromonas haloplanktis*, a well-researched Arctic bacterium, as our target bacterium for transformation. Upon inserting the *inaZ* gene into *P. haloplanktis*, the ice nucleation proteins can increase ice crystallization temperature, in turn alleviating the melting through the formation of new ice. Our design utilizes the pFF plasmid, a revised chassis of pUC18, into which we will clone the *inaZ* gene with the PhaspC promoter. Once transformed into *P. haloplanktis*, the plasmid will increase ice crystallization within the bacteria when added to existing ice. This new strain of *P. haloplanktis* will allow new sheets of ice to form. A successful completion of the project would alleviate the loss of and perhaps restore Arctic ice.

Keywords: Ice nucleation, *Pseudoalteromonas haloplanktis*, global climate change, psychrophiles, lnaZ

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Watch a video introduction by the authors at https://youtu.be/wO7BNuvTpIY

Background

The Arctic sea ice moderates the global climate by reflecting the sunlight back into space. However, the increased greenhouse gas emission has caused rapid loss of Arctic ice, in turn resulting in more heat being absorbed by the ocean surface. Starting from 1980, the Arctic ice sheets have been retreating at a rate of 2.6 percent per decade (National Snow & Ice Data Center, 2021), and more rapid ice loss is projected to be underway (NASA's Earth Science News Team, 2020). As temperatures rise, ice sheets will melt faster than they can form new ones, resulting in a rise in global sea levels. In order to slow the loss of Arctic ice, our team proposes a design that utilizes Pseudomonas syringae and *Marinomonas primoryensis*.

P. syringae is a bacterium that induces frost damage in plants. The gene INA, or *inaZ*, codes for the ice nucleation proteins, which *P. syringae* uses to cause frost injuries to plants and gain entry into the plant tissue (Xin et al., 2018). The gene *inaZ* includes a transport sequence that anchors the ice nucleation proteins on the surface of *P. syringae* (Kassmannhuber et al., 2020), which prompts the crystallization of ice when in contact with water molecules. Ice nucleation proteins induce the formation of ice with a planar arrangement of hydrogen binding groups that complements the arrangement of an ice crystal surface. When linked together, the hydrogen binding groups can induce massive amounts of ice formation (Sherman & Lindow, 1993).

We plan to transform *M. primoryensis* with the gene *inaZ*. *M. primoryensis* was first isolated from coastal sea-ice (Romanenko et al., 2003) and can be cultivated on Marine Agar at up to 20°C (Vance et al., 2018). Being a psychrophile, it is capable of surviving in extremely low temperatures. The organism also has a tendency to bind to ice, which will allow the recombinant strain to create ice crystals around existing sheets of ice. However, there have been no documented transformations of *M. primoryensis*, and the feasibility of various transformation methods remain unknown. Hence, we plan to transform *Pseudoalteromonas haloplanktis* as the prototype of our design since it has been successfully transformed to secrete α-amylase through interspecies conjugation (Tutino et al., 2002). *P. haloplanktis* is a psychrophilic bacterium typically cultured in YTP media at 4°C (Parrilli, 2008).

The final rendition of our project will create a recombinant *M. primoryensis* strain transformed with the *inaZ* gene. As it naturally binds to ice, the ice nucleation proteins coded by the *inaZ* gene allows the transformed *M. primoryensis* to promote ice crystallization around existing ice sheets.

Systems Level

Our initial design is to engineer the gene *inaZ* into *M. primoryensis*, an existing bacterium in the Arctic ice (see Figure 1). However, since there have been no documented successful transformations of *M. primoryensis*, we plan to implement the design, instead, by inserting *inaZ* into *P. haloplanktis* TAC125, one of the most well-researched psychrophiles with previous cases of successful transformation (Duilio et al., 2004), through interspecific conjugation. *P. haloplanktis* presents a high level of similarities with the originally targeted organism, *M. primoryensis*, as both are gram-negative bacteria isolated from Antarctic coastal seawater.

P. haloplanktis will be transformed through interspecies conjugation, which requires transformed Escherichia coli cells that express the *inaZ* gene. Similar to *P. syringae*, the recombinant *P. haloplanktis* TAC125 should display ice nucleation proteins on its outer membrane (Kassmannhuber et al., 2020). Upon confirming the efficacy of our design, we will begin experimentation with *M. primoryensis*. While attaching itself to ice, the ice nucleation proteins on the outer membrane of recombinant *M. primoryensis* will promote ice nucleation at a higher temperature. Both the recombinant *P. haloplanktis* and *M. primoryensis* will be tested for efficacy with a droplet-freezing assay of ultra-pure water (Kassmannhuber et al., 2017). An aliquot of the transformed bacterial culture, mixed with ultra-pure water, will be cooled in a partially submerged well plate with an ethanol bath. Light is transmitted through the aliquot and its intensity, which changes upon freezing, is monitored by a camera. This method allows for the detection of freezing temperatures (David et al. 2019).

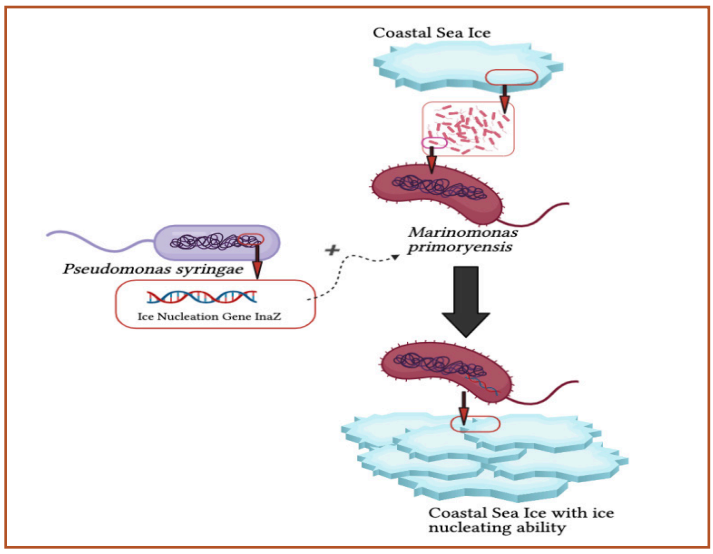

Figure 1. *The experimental process. The inaZ gene coding for the ice nucleation proteins will be transformed into M. primoryensis, which naturally binds to ice. The recombinant M. primoryensis, still capable of binding to ice, will produce ice nucleation proteins that facilitate the formation of ice.*

et al., 2002). The *inaZ* gene will be cloned into pFF, a plasmid capable of replication in both E. coli and psychrophilic bacteria. The vector will be first inserted into *P. haloplanktis* TAC125 via interspecies conjugation with transformed *E. coli* (Tutino et al., 2002). If the ice nucleation protein is successfully expressed in *P. haloplanktis*, *M. primoryensis* will be transformed with the same plasmid and procedures (Figure 2).

Parts Level

Constructed from the pUCL vector (modified from pUC18 by removing the restriction site NdeI, a promoter, and the lacI gene), the pFF contains OriR (cold-adapted origin of replication) and OriT (origin of conjugative transfer), enabling it to function within *E. coli* and psychrophiles through conjugation (Tutino et al., 2002). The pFF vector ☐Figure 3) also possesses the PhaspC promoter and the Tasp terminator, both of which have been proven to function within psychrophiles such as *P. haloplanktis* in low temperatures. The *inaZ* gene will be synthesized along with a restriction enzyme and cloned into the pFF

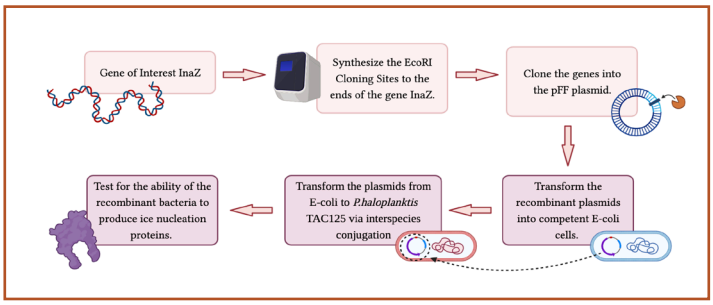

Figure 2. *Procedure of our experimentation. The inaZ will be synthesized along with our selected restriction enzymes and cloned into the pFF plasmid, which will be transformed first into E. coli cells in preparation for the transformation of our chassis. The transformed E. coli will then engage in interspecies conjugation with P. haloplanktis and M. primoryensis.*

plasmid utilizing the restriction site EcoRI (see Figure 4). The EcoRI restriction site is found between the Pasp promoter and the Tasp terminator in the pFF plasmid and produces an overhang, ensuring that the *inaZ* gene will be ligated in the correct orientation. Since the sequence of the pFF plasmid is currently unknown, a restriction digest will be performed with EcoRI to ensure only one EcoRI restriction site is present within the plasmid. The pFF plasmid is chosen despite its unclear sequence because it has been shown to function effectively within psychrophiles such as *P. haloplanktis*, and is compatible with interspecies conjugation (Tutino et al., 2002).

Safety

Both *M. primoryensis* and *P. haloplanktis* are bacteria native to Arctic regions. Since the only change in the recombinant *M. primoryensis* is the addition of the ice nucleation protein, we anticipate no negative effect on the Arctic ecosystem. Future experimentations with recombinant strains will be carried out to ensure the insertion of *inaZ* does not interfere with metabolic pathways of the bacteria. *P. haloplanktis* produces Volatile Organic Compounds that suppress the growth

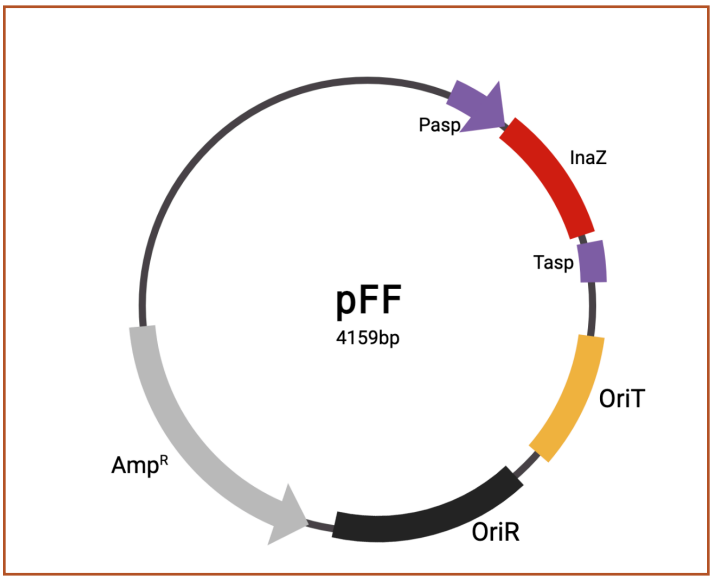

Figure 3. *The plasmid structure. The pFF plasmid is compatible with interspecies conjugation.*

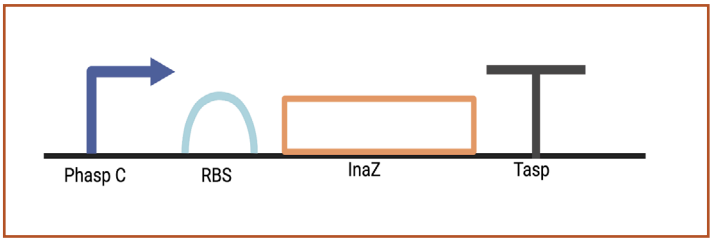

Figure 4. *Diagram of biological parts. The inaZ gene, coding for the production of the ice nucleation proteins, will be controlled by the PhaspC promoter (also notated as Pasp) and the Tasp terminator once cloned into the pFF vector.*

of *Burkholderia cepacia* complex strains (Sannino et al., 2016), pathogenic strains of bacillus present within the water, soil, and animal surfaces (Zhang & Xie, 2007). The recombinant *P. haloplanktis* will be deployed into seawater and should have no effect on the native Arctic *Burkholderia* genera, as they are commonly found within heathland (Hill et al., 2015) and tundra soils (Tao et al., 2020). We realize that the organism will need to be fully vetted before it is released into the environment and will conduct experiments and simulations to determine its exact effects over the environment.

M. primoryensis and *P. haloplanktis* have been cultivated under lab conditions (Vance et al., 2018). They are not pathogens and are safe to grow.

Discussions

We chose *M. primoryensis* as our chassis because of its ability to bind to ice. Ideally, the recombinant *M. primoryensis* would allow the formation of ice on existing ice sheets. However, *M. primoryensis* has not been well-researched, hence the results of the transformation are relatively unpredictable. The presence of ice-binding proteins within the organism might also affect the efficacy of our design, as they have been shown to demonstrate ice interactivity, influencing ice crystal growth and shaping the ice morphology (Delesky 2021). Ice binding proteins are typically present within the organism to prevent freezing of their body fluids (Dolev et al., 2016), but we are currently unsure of its influence over our design. The recombinant *M. primoryensis* will be tested by a droplet-freezing assay. If the recombinant organism does not show significant ice nucleating abilities due to the interference of the ice-binding proteins, *P. haloplanktis* TAC125 will be employed as the chassis for the final design.

Next Steps

Our design currently remains in the theoretical phase. For further development of the design, the gene *inaZ* will be cloned into the pFF vector, which will be used for the subsequent transformation of *E. coli. P. haloplanktis* and *M. primoryensis* will then be transformed with recombinant E. coli through interspecies conjugation.

Author Contributions

The prototype of the design was proposed by A.T. and improved upon by Y.L., A.T. and S.Y. Y.L. produced all graphics in this article; A.T. produced the video.

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