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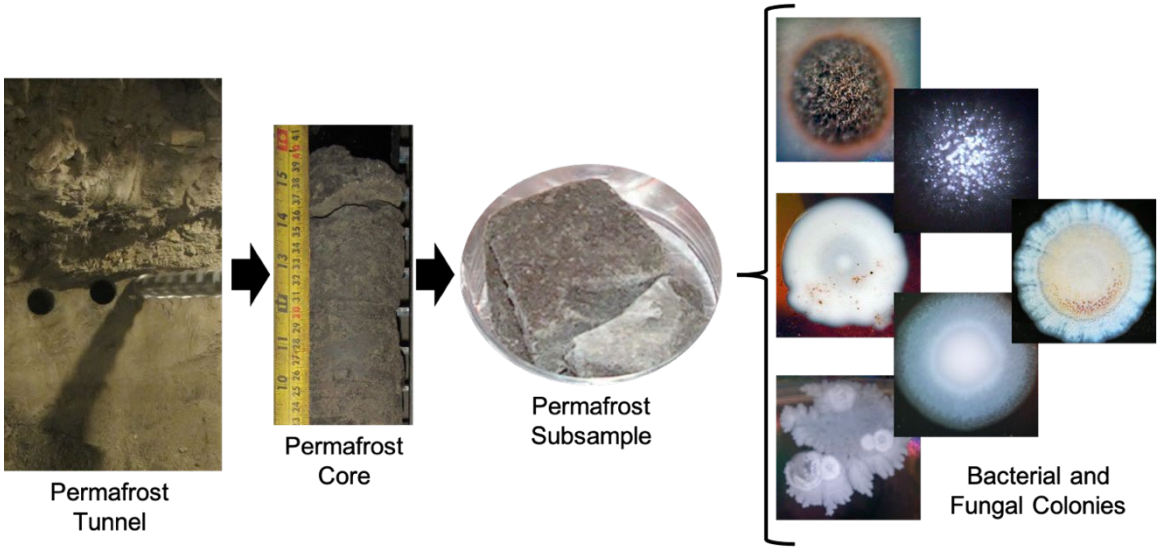
Isolation and Characterization of Bacterial Isolates from Alaskan Permafrost for Synthetic Biology Applications

Alison K. Thurston, Logan M. Gonzalez, Flora Laurent, Elizabeth J. Corriveau, and Robyn A. Barbato

September 2023



Cold Regions Research and Engineering Laboratory

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Abstract

Operations in the Artic and other cold regions require technologies that can perform reliably under extreme cold conditions. Permafrost and frozen soils harbor a wide range of microorganisms that have adapted to extremely low temperatures and have unique metabolic capabilities relevant to military operations and that could be exploited to develop biotechnologies optimized for cold environments. Cold-tolerant bacteria (psychrophiles and psychrotrophs) are critical to the development of synthetic biology technologies meant to work in cold environments like the Arctic.

Using bacteria isolated from Alaskan permafrost, we applied an experimental pipeline to test the best candidates for use as biological platforms, or chassis, for low-temperature synthetic biology. Since synthetic biology constructs will perform only as well as their chassis, it is critical that circuits expected to perform under extreme cold conditions are housed in chassis that are adapted to those conditions. We identified one permafrost isolate, PTI8, related to *Rhodococcus fascians*, that is capable of growing from −1°C to at least 25°C and which we experimentally confirmed to uptake and express the broad host range plasmid pBTK519, suggesting PTI8 is a candidate for use as a novel cold-adapted chassis for synthetic biology.

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Preface

This study was conducted for Headquarters, US Army Corps of Engineers, under PE 0601102A, Project T14. The technical monitor was Dr. Elizabeth Ferguson, US Army Engineer Research and Development Center (ERDC), Environmental Laboratory.

The work was performed by the Biogeochemical Sciences Branch of the Research and Engineering Division, ERDC Cold Regions Research and Engineering Laboratory (CRREL). At the time of publication, Mr. Nathan Lamie was branch chief; and Dr. John Weatherly was acting division chief. The acting deputy director of ERDC-CRREL was Dr. Ivan P. Beckman, and the director was Dr. Joseph L. Corriveau.

COL Christian Patterson was commander of ERDC, and Dr. David W. Pittman was the director.

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1 Introduction

1.1 Background

1.1.1 Permafrost Microbiome

Permafrost, which affects 25% of the northern hemisphere, is ground that has been frozen for at least two consecutive years (T. Zhang et al. 2008; Brown and Haggerty 1998). Seasonally thawed active-layer soils and permafrost harbor a variety of microorganisms, including bacteria, archaea, and fungi (Steven et al. 2008; Wilhelm et al. 2012). These organisms sustain life at subzero temperatures between −10°C and −20°C in Arctic regions, often surviving within the high solute regions of soil pores (Rivkina et al. 2000).* Many studies have characterized the biodiversity in permafrost (Steven et al. 2008; Mackelprang et al. 2011; Bellemain et al. 2013; Hultman et al. 2015; R. A. Barbato et al. 2022), determined metabolic processes in permafrost (Messan et al. 2020; Wu et al. 2022), and identified the limits of growth at subzero temperatures as low as −35°C (Panikov and Sizova 2007; Wilhelm et al. 2012; Heinz et al. 2018). Extremophiles that are adapted to low-temperature environments are known as psychrophiles (cold loving) and psychrotrophs (cold tolerant) (Morita 1975). An example of a psychrotroph is *Planococcus halocryophilus*, a gram-positive bacterium that was isolated from permafrost active-layer soil in the Canadian high Arctic. It is capable of growing under a range of temperatures from 25°C to as low as −15°C (Mykytczuk et al. 2013). Additionally, a gram-negative bacterium capable of growing at as low as −10°C was discovered in 43,000-year-old Siberian permafrost (Bakermans et al. 2003). These bacteria are able to survive at very low temperatures, making them attractive candidates for further investigation and low-temperature synthetic biology applications.

* For a full list of the spelled-out forms of the units of measure used in this document, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 248–52, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

1.1.2 Cold-Adaptive Properties

Low temperatures result in multiple challenges for biology. For instance, decreased membrane fluidity impacts nutrient and waste transport; decreased enzymatic reaction rates slow cellular processes like transcription, translation, and cell division; improper protein folding or cold denaturation interfere with protein function; and intracellular ice crystallization can cause physical cellular damage (reviewed in D'Amico et al. 2006). As a result, organisms growing in cold regions have evolved a diverse set of adaptations, both genetic and physiological, many of which are still far from understood. One common property of cold-adapted organisms is a change in cellular membrane structure. Because reduced temperatures restrict the kinetic motion of membranes, psychrophiles and psychrotrophs often have a greater proportion of unsaturated fatty acids in their bilipid membrane to counter this effect (Collins and Margesin 2019; D'Amico et al. 2006). Another common adaptation is the production of extracellular polymeric substances (EPS), which are produced by microbes (Schiraldi and De Rosa 2016) for a variety of reasons, including biofilm formation (Deming and Young 2017). Their ability to form a hydrated gel matrix in the extracellular region, in large part due to EPS production, is believed to serve as cryoprotection by acting as a physical barrier to ice crystal formation and the entry of solutes (Caruso et al. 2018). Many proteins found in cold-adapted organisms are known to be specially adapted to fold and function properly at low temperatures. Folding dynamics can be influenced by temperature-dependent changes in the strength of molecular forces, including hydrophobic interactions (Guo et al. 2012; Baldwin 1986). Reaction rates also decrease at low temperatures due to reduced molecular movement (Laidler 1984). To overcome this issue, many proteins found in cold-adapted organisms have evolved to increase flexibility, either globally or near the active site. This is commonly achieved through amino acid substitutions that decrease intramolecular bonding and torsional restricted loops (Feller 2013).

1.1.3 Cold-Adapted Organisms in Synthetic Biology and Biotechnology

The term synthetic biology was first used to describe genetically engineering an organism using recombinant DNA technology (Hobom 1980) and has evolved to encompass a wide field of techniques and approaches, including cell-free systems, minimal systems, creating unnatural organisms, or expressing unnatural biomolecules within a living system (Benner and

Sismour 2005). In short, it is engineering biology for an application. Domesticated *Escherichia coli* (*E. coli*; a bacterium) and *Saccharomyces cerevisiae* (*S. cerevisiae*; a yeast) are the workhorses for microbial laboratory-based synthetic biology (de Lorenzo et al. 2021). Use of these mesophile synthetic biology chassis* microorganisms comes with the advantage of thousands of available strains, such as in the yeast deletion collection, consisting of the deletion of approximately 6000 open reading frames (Giaever and Nislow 2014), and commercially available molecular tools with interchangeable genetic parts, such as Golden Gate assembly (Engler et al. 2008) and Gibson assembly (Gibson et al. 2009), allowing genetically modified strains to be rapidly designed, built, and tested. However, mesophiles typically grow between 20°C and 45°C, with an optimum growth temperature range of 30°C to 39°C (Schiraldi and De Rosa 2016), limiting their usefulness in low-temperature research and as cold-studies chassis. Additionally, because these laboratory organisms lack fitness in the natural environment (Tang et al. 2018), they are not reliable as fieldable technologies (Brophy et al. 2018). Using environmental organisms as chassis is advantageous, as they have already adapted to the dynamic natural environment (Robyn A. Barbato 2021).

One of the most popular uses of cold-active enzymes, including lipases (Li et al. 2014), proteases, and amylases (Hmidet et al. 2009), are as additives in commercially available cleaning products (Sarmiento et al. 2015). In laundry detergents, these enzymes offer similar efficiency but at lower temperatures, thereby reducing the need for heating, which can reduce energy costs. Certain enzymes from cold-adapted organisms have also found use in a laboratory setting. For example, heat labile recombinant alkaline phosphatases, derived from an Antarctic bacterium, can be used in restriction cloning to prevent the recircularization of an empty vector (Rina et al. 2000). The advantage of using a heat labile alkaline phosphatase during the cloning process is the ability to irreversible heat inactivate the enzyme. This eliminates the need for harsh chemicals, which are otherwise necessary to inactivate non–heat labile alkaline phosphatases (Sarmiento et al. 2015). Additionally, ice-binding proteins from cold-adapted organ-

* For our study, we define a chassis as an organism used to host genetic components, providing both a physical structure (cell) and the resources (basic cellular machinery) for function (Adams 2016). De Lorenzo et al. (2021) provides a review of the evolution and suggested standard definition of the term chassis.

isms have been considered as promising candidates for use as cryoprotectants in food science, agriculture, and biomedicine due to their ability to control ice formation (Voets 2017).

In many mesophilic hosts, some proteins tend to fold improperly at the organisms' required growth temperatures, leading to the formation of insoluble protein aggregates, or inclusion bodies, reducing the proportion of active-form proteins (Mitraki et al. 1991). This effect is known to be temperature dependent, and studies have shown that it is somewhat alleviated at low temperatures, leading to a greater production of active-form proteins (Vera et al. 2007). Therefore, cold-adapted organisms have been utilized to overcome barriers for recombinant protein expression. *E. coli* was engineered to express cold-adapted chaperonins from *Oleispira antarctica*, a psychrophilic organism (Ferrer et al. 2003) allowing recombinant protein folding from 4°C to 12°C (Strocchi et al. 2006). Additionally, *Pseudoalteromonas haloplanktis* TAC125, a marine bacterium and the most commonly studied cold-adapted bacteria, has been used to produce recombinant proteins down to –2.5°C (Sannino et al. 2017), serving as the first known case of subzero recombinant protein expression. Not only does this organism exhibit relatively fast doubling times at low temperatures (approximately 4 h at 4°C [Margesin and Feller 2010; Sannino et al. 2017; Piette et al. 2011]) but also it has been successfully used to heterologously produce proteins in at least 12 different studies, including difficult-to-express proteins like human nerve growth factor hβ-NGF (Vigentini et al. 2006; Parrilli and Tutino 2017).

Cold-adapted organisms exhibit high diversity, abundance, and representation spanning all branches of the tree of life (De Maayer et al. 2014; Margesin and Miteva 2011). Our research attempts to outline the necessary steps to isolate and characterize cold-adapted bacteria for biotechnology applications. Indeed, with the growing market for cold-active detergents, an increasing interest in environmental biotechnology, a greater amount of genomic data, and the availability of genome-scale metabolic models for cold-adapted organisms, biotechnological interest in cold-adapted organisms is likely to continue expanding well into the future.

1.2 Objective

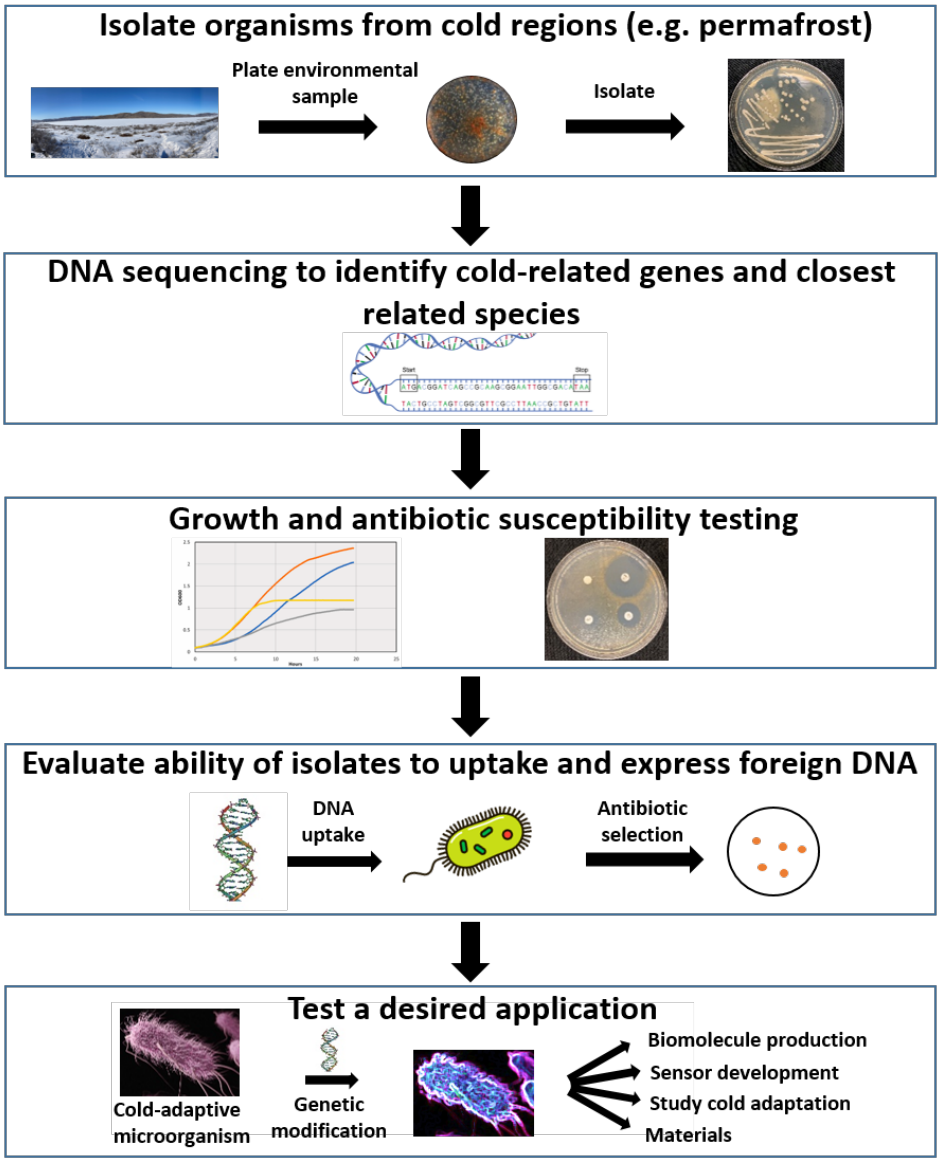
The goal of this project was to evaluate microorganisms isolated from permafrost as candidates for chassis in synthetic biology applications. Specific aims included the following:

1. Evaluate the ability of a genetically engineered *Escherichia coli* strain to grow at 4°C.
2. Characterize four environmental isolates from permafrost.
3. Test an environmental isolate's ability to uptake exogenous DNA by electroporation transformation.

1.3 Approach

Twenty-three bacteria isolated from permafrost are preserved in the Innovative, Collaborative, Exploratory, Cold Regions Organism Library for Discovery (ICE COLD) at the US Army Engineer Research and Development Center's Cold Regions Research and Engineering Laboratory (ERDC-CRREL). Of those 23, our team selected 4 isolates to characterize through our synthetic biology pipeline (Figure 1). To obtain some initial information on the isolates, we interrogated the last common taxon by sequencing the 16S rRNA gene. We grew the isolates at –1°C, 4°C, 10°C, and room temperature to examine growth at various temperatures and to measure growth rates. We tested the susceptibility of isolates to antibiotics commonly used in molecular biology through disk diffusion and minimum inhibitory concentration (MIC) assays. Finally, we introduced exogenous plasmid DNA into our best chassis candidate using electroporation transformation.

Figure 1. Pipeline for evaluating cold-adaptive organisms for potential use as a synthetic biology chassis.



2 Methods

2.1 Isolate Preparation

Four isolates, PEI1, PEI4, PTI8, and PTI10, from ICE COLD, housed at CRREL, were processed through the synthetic biology pipeline (Figure 1). Culturing conditions of the isolates are described in each method section. Unless otherwise noted, isolates were revived from −80°C storage, streaked for single colonies on tryptic soy agar (TSA) or Reasoner's 2A agar (R2A) plates, and grown at room temperature prior to experimentation.

2.2 *Escherichia coli* (*E. coli*) Strains

This study used two *Escherichia coli* (*E. coli*) strains: *E. coli* C-3000, a bacteriophage host derived from *E. coli* K-12 (ATCC 15597) and *E. coli* DH5α (F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ^- *thi*$^-$1 *gyr*A96 *rel*A1) (Invitrogen, 18258012). *E. coli* C-3000 was provided by the University of New Hampshire. *E. coli* C-3000 was used for a long-term cold phenotype assay. *E. coli* DH5α was utilized in the cold growth phenotype studies, disk diffusion assays, minimal inhibitory concentration assays, and electroporation studies. DH5α was revived from −80°C storage on Luria-Bertani (LB) plates and grown at room temperature for 2–3 days unless otherwise stated.

2.3 Long-Term Cold Phenotype

A starter culture of *E. coli* C-3000 was grown overnight in tryptic soy broth (TSB) media. The culture was diluted to an OD_{600}* of approximately 0.5 in TSB media. Then, 1 mL of the diluted culture was centrifuged at 3000 rpm for 3 min (Eppendorf Centrifuge 5430 R). The supernatant was removed. The cell pellet was resuspended with 1 mL of sterile 1× phosphate-buffered saline (PBS) at a pH of 7.4. Tenfold serial dilutions were carried out, and 5 µL of each dilution was spotted in duplicate onto two sets of agar plates. A set of agar plates consisted of LB, R2A, TSA, and 1% $NaCl^+$ in TSA . Plates were left at room temperature until the spots had

* Optical density.

† For a full list of the spelled-out forms of the chemical elements used in this document, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 265, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

completely dried. Once dry, one plate set was placed in a room temperature incubator (Amerex Instruments Gyromax 737), and the other plate set was placed in a 4°C incubator (Yamato IN602CSW). Both plate sets were imaged after 20 h of growth in the dark using an iPhone (SE, iOS 15.5). The 4°C plate set remained in the 4°C incubator for 148 days.

2.4 Gram Stain

We followed the Gram staining procedure published by Smith and Hussey (2005). A single colony from an agar plate was mixed with 5 µL of sterile PBS on a microscope slide. The cells were heat fixed onto glass slides using an alcohol burner. The fixed cells were flooded with crystal violet solution (2 g crystal violet dissolved in 20 mL 95% ethanol and 0.8 g ammonium oxalate dissolved in 80 mL of distilled water mixed and filtered to remove solids) for 1 min, followed by a brief deionized water rinse. Then, the cells were flooded with Gram's iodine solution (1 g iodine, 2 g potassium iodide in 300 mL distilled water) for 1 min and followed by a brief water rinse. Next, the slides were washed with decolorizer (equal parts acetone and 95% ethanol) to remove excess stain. Slides were flooded with the counter stain safranin solution (2.5 g safranin O dissolved in 100 mL 95% ethanol, diluted tenfold in distilled water) for 30 seconds and rinsed with water. The slides were allowed to air dry. Cells were examined at a 1000× magnification with an Olympus BX40 microscope using immersion oil.

2.5 DNA Extraction and Nearest Relative

To determine the last common taxonomic classification of each isolate, genomic DNA was extracted. Cell cultures of each isolate were inoculated and allowed to grow to stationary phase. Cell pellets were collected by centrifugation, followed by removal of the media. Cell pellets were kept frozen until DNA extractions were carried out. Genomic DNA was extracted using the Qiagen DNeasy UltraClean Microbial Kit (Qiagen, 12224-250) following the standard kit procedure. DNA concentrations were measured on the Qubit 4 Fluorimeter (Thermo Fisher Scientific, Q33226) using the broad range assay kit (Thermo Fisher Scientific, Q32850). DNA was sent to Genewiz (South Plainfield, New Jersey) for identification; genomic DNA was PCR amplified, and the 16S rRNA gene was sequenced using Sanger sequencing. The DNA sequences were compared against the National Center for Biotechnology Information (NCBI) database to determine the closest nucleotide match by using the basic local alignment search tool (BLAST) blastn algorithm; the top candidate for each isolate is reported in the results.

2.6 Temperature Phenotypic Assay

To assess growth phenotypes at –1°C, 4°C, 10°C, and room temperature, single colonies of PEI1, PEI4, PTI8, PTI10, and DH5α were streaked using autoclaved toothpicks onto R2A plates. A single colony was streaked repeatedly on the same plate in slash pattern in an attempt to mimic a serial dilution. The plates were placed in incubators (Yamato IN602CSW, Amerex Instruments Gyromax 737, and Thermo Scientific Precision) at the desired temperatures and imaged periodically using an iPhone (SE, iOS 15.5) to capture growth differences between the isolates and DH5α (Thermo Fisher Scientific, 18258012).

2.7 Growth Curves

Growth curves were collected to determine the growth rate and doubling times of the bacteria during log phase. Starter cultures were incubated at room temperature or at 4°C until reaching stationary phase. Starter cultures were used to inoculate new cultures at a starting OD_{600} of approximately 0.05 for the growth curve. The cultures were grown at 4°C or at room temperature on shaker tables set at 140 rpm or 220 rpm, respectively. At 4°C, the cultures were not attached to the shaker table, and so a lower speed was selected. OD_{600} readings were taken repeatedly to capture all stages of the growth curve, and the absorbance was measured using a Genesys 30 spectrophotometer (Thermo Scientific, 14-380-442). Growth curves were run in duplicate (pPEI1 at 4°C and room temperature, PTI8 at 4°C) or triplicate (PEI4 at room temperature, PTI8 at room temperature, PTI10 at room temperature), depending on available space on the shakers.

2.8 Disk Diffusion Assay

Starter cultures were incubated at room temperature, shaking at 220 rpm until reaching stationary phase. Cultures were diluted in media to a starting OD_{600} of 0.05. The new cultures were allowed to incubate until reaching an OD_{600} of 0.1, after which 100 µL of culture was spread onto LB agar (20 mL agar media at a depth of 4 mm) using presterilized cotton swabs (Puritan, 806-WC) to ensure a homogenous dispersal of cells. Once the liquid culture was fully absorbed into plates, antimicrobial susceptibility disks (Table 1) were placed onto agar plates by using flame-sterilized tweezers. Plates were incubated in the dark at room temperature until a lawn of growth was observed on each plate (about 1–3 days, isolate dependent). Zones of inhibition (ZOI) around each antibiotic disk were measured with

a ruler, and diameters were compared to common ranges of ZOI by using the M100-21 from the Clinical and Laboratory Standards Institute (CLSI 2011) for each antibiotic to determine if each organism was resistant to each antibiotic. DH5α was included with each assay as a positive control to ensure the antibiotics in the disks had not degraded.

Table 1. Antibiotic disks tested on permafrost isolates.

Antibiotic	Abbreviation (Disk ID)	Mass on Disk (µg)	Oxoid Product Number
Amoxicillin	amc (AMC30)	30	CT0223B
Ampicillin	amp (AMP10)	10	CT0003B
Azithromycin	azm (AZM15)	15	CT0906B
Cefotaxime	ctx (CTX30)	30	CT0166B
Chloramphenicol	chl (C30)	30	CT0013B
Ciprofloxacin	cip (CIP5)	5	CT0425B
Gentamicin	gen (CN10)	10	CT0024B
Kanamycin	kan (K30)	30	CT0026B
Penicillin G	pen (P10)	10	CT0043B
Tetracycline	tet (TE30)	30	CT0054B

2.9 Minimum Inhibitory Concentration Assay

MIC assays were carried out to determine the appropriate antibiotic concentration for plasmid selection. We performed MIC tests using four antibiotics: ampicillin (Corning, 45000-612), chloramphenicol (MP Biomedicals, 190321), kanamycin (MP Biomedicals, 150029), and tetracycline (Fisher Scientific, BP912100) in LB media. Cells were grown to stationary phase in sterile culture tubes containing 5 mL LB, after which they were diluted to a starting OD_{600} of 0.05 using a Genesys 30 spectrophotometer (Thermo Scientific, 14-380-442). PEI4 displayed flocculent growth in LB media and was instead grown in Reasoner's 2A broth (R2B). The appropriate amount of antibiotic for a specific dilution (ampicillin, chloramphenicol, kanamycin, or tetracycline) and 200 µL of the diluted culture were added to each well in a 96-well plate (BrandTech, 781663). Concentrations were antibiotic specific: ampicillin (0, 50, 100, 150, and 300 µg/mL), chloramphenicol (0, 12.5, 25, 50, 100 µg/mL), kanamycin (0, 1, 2, 8, 12.5, 25, 50, 100, 200 µg/mL) and tetracycline (0, 1, 2, 4, 8, 12.5, 25, 50, 100 µg/mL). DH5α, known to be susceptible to all four antibiotics, was used as a control. The 96-well plates were incubated in the dark in a SpectraMax iD3 microplate reader (Molecular Devices, ID3-STD) for a total of 72 h at 25°C. Plate reader settings were as follows:

- Mode: ABS (absorbance)
- Read type: kinetic
- Plate: 96-well standard, clrbtm (clear bottom), lidded
- Wavelength: 600 nm
- Detection method: precise
- Shaking intensity: high
- Kinetic measurement interval: 5:56 min
- Shaking interval: 5:00 min

2.10 Electrocompetent Cells

To make electrocompetent cell stocks of PTI8 And DH5α, we adapted the Krantz Laboratory protocol (Krantz Lab, n.d.). Starter cultures were prepared by growing each organism at room temperature in LB media overnight. Cultures were diluted to a starting OD_{600} of 0.05 in LB media for DH5α and either LB or LB media containing 1.5% glycine for PTI8. Glycine was added to electrocompetent stock media to improve electrocompetence in *Kocuria sp*. (Matsumura et al. 2011). Freshly inoculated cultures were allowed to grow until reaching an OD_{600} of approximately 0.3–0.5 using a Genesys 30 spectrophotometer (Thermo Scientific, 14-380-442), after which the cultures were chilled on ice for 20–30 min. Cultures were transferred to autoclaved 250 mL centrifuge bottles and centrifuged at 2400 rpm for 20 min at 4°C using a Sorvall RC 6 centrifuge (Sorvall, 74800). The supernatant was carefully removed, and the cell pellets were washed twice with chilled sterile Milli-Q water followed by centrifugation at 2400 rpm for 20 min at 4°C. A final wash was done in 10% glycerol followed by centrifugation at 2100 rpm for 20 min at 4°C. The cell pellet was resuspended in 1 mL of chilled sterile 10% glycerol. We determined the OD_{600} of the concentrated culture. The concentrated PTI8 culture was diluted with 10% glycerol solution as necessary to reach a target OD_{600} of about 20. Aliquots (100 µL) were placed into sterile 1.5 mL microcentrifuge tubes and stored at −80°C until use.

2.11 Candidate Plasmids

Fourteen commercially available plasmids (Table 2) were used in the electroporation trials. To increase our odds of success, we compiled a set of plasmids containing different combinations of broad or narrow host range origins of replication (ORIs) and antibiotic selection. The plasmids con-

tained antibiotic resistance genes for ampicillin, chloramphenicol, kanamycin, or tetracycline, as these are commonly used antibiotics in molecular biology.

Table 2. Plasmid candidates for genetic modifications in cold-adapted microorganisms.

Plasmid Name	Origin of Replication	Antibiotic Markers	Size (base pair)	Copy	Reference
pAM5406	RSF1010 *oriV*; RK2-*bom* + pUC	amp	8107	low copy; high copy	(Bishé et al. 2019)
pBAV1K-T5-gfp	pWV01	kan	3653	high copy	(Bryksin and Matsumura 2010)
pJet1.2 cat	pUC	amp	4010	high copy	(Diebold-Durand et al. 2019)
pJet1.2 kanR	pUC	amp	4440	high copy	(Diebold-Durand, et al. 2019)
pBTK501	RSF1010 *oriV*	amp	8801	low copy	(Leonard et al. 2018)
pBTK519	RSF1010 *oriV*	kan	8118	low copy	(Leonard et al. 2018)
pBTK001	p15A	chl	2481	low copy	(Leonard et al. 2018)
pBTK102	ColE1	chl	1710	low copy	(Leonard et al. 2018)
pMM656	R6K *y ori*	amp	5647	low copy	(Mimee et al. 2015)
pSVJ21	p54; pUC	amp (*E.coli*), chl (*E.coli* and gram negative)	6002	low copy; high copy	(Miteva et al. 2008)
pUC19	pMB1 (derivative)	amp	2686	high copy	(Norrander et al. 1983)
pMRE135	pBBR1 *oriV*	kan, chl	7600	high copy	(Schlechter et al. 2018)
pMRE155	pBBR1 *oriV*	kan, chl	8454	high copy	(Schlechter et al. 2018)
pCAP05	pRK442(H)	tet	12730	low copy	(J. J. Zhang et al. 2017)

2.12 Electroporation

Plasmid DNA was transformed into PTI8 and DH5α cells using a Gene Pulser Xcell Microbial System (Bio-Rad, 1652662). Initial electroporation transformations with PTI8 were done with the plasmids pUC19 and

pSVJ21. Electroporation parameters were varied and are reported in Table A-1. A standard procedure was adapted from Desomer et al. (1990). Briefly, 24 µL of thawed electrocompetent cell stocks were mixed with 1 µL of plasmid DNA (1 ng/µL) and transferred to electroporation cuvettes prechilled at 4°C. Electroporation was performed using an exponential pulse, and parameters for PTI8 and DH5α are listed in Table 3. Immediately after electroporation, 975 µL of super optimal broth with catabolite repression (SOC) recovery media was added to cuvettes to resuspend cells, and the suspensions were transferred to 1.5 mL microcentrifuge tubes. Cells were allowed to recover in an Eppendorf thermomixer (Eppendorf, 5350) for 4 h (for PTI8) or 30 min (for DH5α). After the recovery period, 100 µL of the cell suspension was plated onto antibiotic-containing LB agar plates and incubated at room temperature for up to 2 weeks (for PTI8) or 2 days (for DH5α). Plates were checked daily for colony formation. To confirm antibiotic resistance of positive PTI8 transformants, multiple colonies were streaked twice consecutively onto antibiotic-selective LB plates. After the second plating, cells were cultured in antibiotic-selective media for plasmid recovery and for long-term cold storage. Colonies that were detected on antibiotic-containing agar plates were considered to be successful transformations, and they were counted to estimate transformation efficiency.

Table 3. Electroporation parameters.

Organism	Cuvette Gap Size (mm)	Voltage (kV)	Capacitance (µF)	Resistance (Ω)	Recovery Media	Recovery Time
PTI8	2	2.5	25	400	SOC	4 h @ 25°C
DH5α	2	2.5	25	400	SOC	30 min @ 37°C

2.13 Confirmation of Plasmid Expression of pBTK519

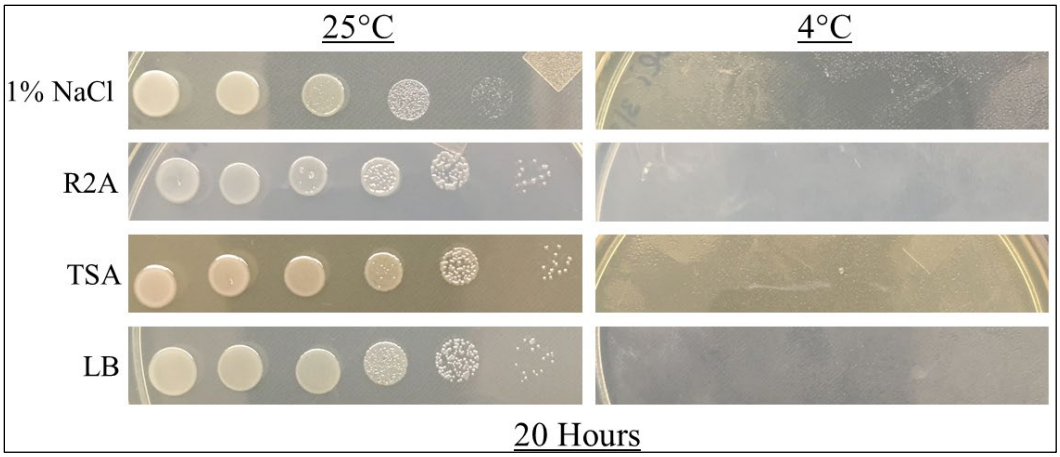
Once colony resistance was confirmed, the transformed pBTK519 plasmid was recovered for verification. To recover the plasmid, a 5 mL culture of the transformed PTI8 was grown in the LB + 50 µg/mL kanamycin media and incubated at room temperature on a shaker set to 220 until the culture reached stationary phase. The plasmid was purified from the cell cultures by using a Qiagen miniprep kit (Qiagen, 27106X4). The recovered plasmid concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, ND-2000). The recovered plasmids were used to transform DH5α cells by electroporation following the protocol in Section 2.10.

3 Results

3.1 Testing *E. coli* C-3000 Growth at 4 °C

Escherichia coli (*E. coli*) is a model organism used for laboratory studies, with a wide range of genetic tools specific to this organism available for synthetic biology. However, the optimal growth temperature is 37°C (body temperature), and thus *E. coli* and the tools are not conducive for cold-based studies. To demonstrate *E. coli's* sensitivity to low temperatures, we grew *E. coli* C-3000 at 4°C on four types of media (Figure 2). After 20 h, *E. coli* growth was observed on all media types at 25°C; however, no growth was observed on plates incubated at 4°C (Figure 2). The plates were left incubating at 4°C for 148 d with no *E. coli* growth observed.

Figure 2. *E. coli* C-3000 grown on 1% NaCl in tryptic soy agar (TSA), Reasoner's 2A agar (R2A), TSA, and Luria-Bertani (LB) agar plates at 25 °C and 4 °C.



3.2 ICE COLD Isolates

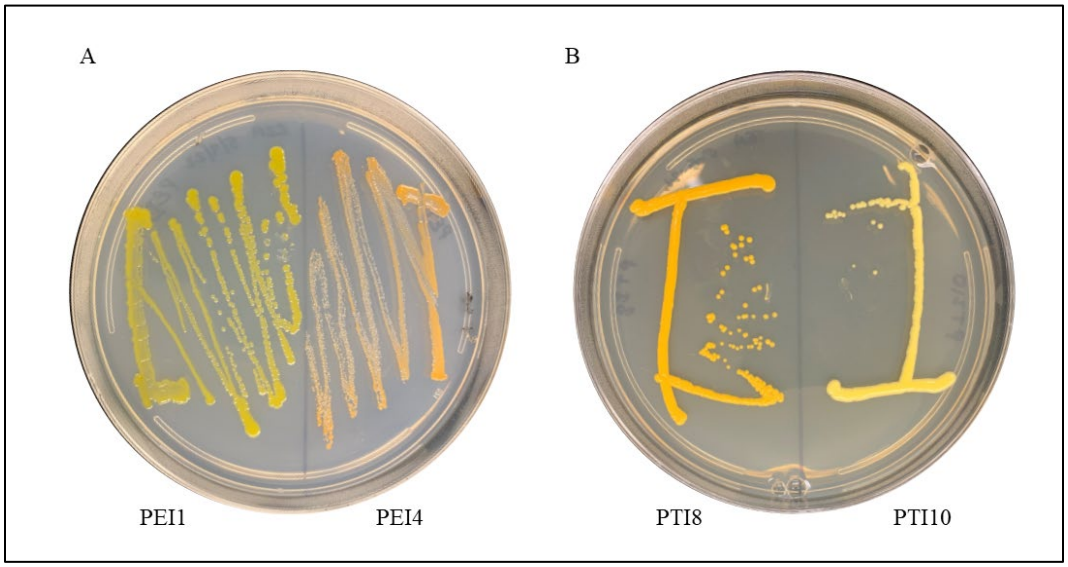
Four isolates, PEI1, PEI4, PTI8, and PTI10, were selected from ICE COLD to carry through our synthetic biology pipeline (Table 4 and Figure 1). These bacteria were isolated from a permafrost core collected in 2017 at the CRREL Permafrost Tunnel Research Facility (PTRF) in Fox, Alaska. The permafrost core was collected 45 m from the entrance of the northern tunnel (R. A. Barbato et al. 2022). PEI1 and PEI4 are gram-negative bacteria; PTI8 and PTI10 are gram-positive bacteria (Table 4). PEI1 and PTI10 colonies appear yellow in color, while PEI4 and PTI8 colonies appear orange in color (Table 4 and Figure 3). Sequencing of the 16S rRNA gene revealed the last common taxon of each isolate in the NCBI database. PEI1 and PEI4 are most closely related to *Massilia aurea* and *Sphingomonas*

faeni, respectively. PTI8 and PTI10 are most closely related to *Rhodococcus (Corynebacterium) fascians* and *Kocuria rhizophila*, respectively. Interestingly, closest relatives either at the species or genus level for each of these 4 organisms have previously been isolated from cold regions (Busse et al. 2003; Reddy et al. 2003; Gesheva et al. 2010; Shen et al. 2015)

Table 4. Description of ICE COLD isolates.

Isolate Name	Sample Source	Gram Stain (+/−)	Cell Morphology	Color	Closest Match	Base Pair Coverage (% Identity)
PEI1	CRREL PTRF	−	rods	Yellow	*Massilia aurea*	1436/1442 (99.6%)
PEI4	CRREL PTRF	−	rods	Dark Orange	*Sphingomonas faeni*	1385/1409 (98.3%)
PTI8	CRREL PTRF	+	cocci	Orange	*Rhodococcus fascians*	1403/1406 (99.8%)
PTI10	CRREL PTRF	+	cocci	Bright Yellow	*Kocuria rhizophila*	1402/1403 (99.9%)

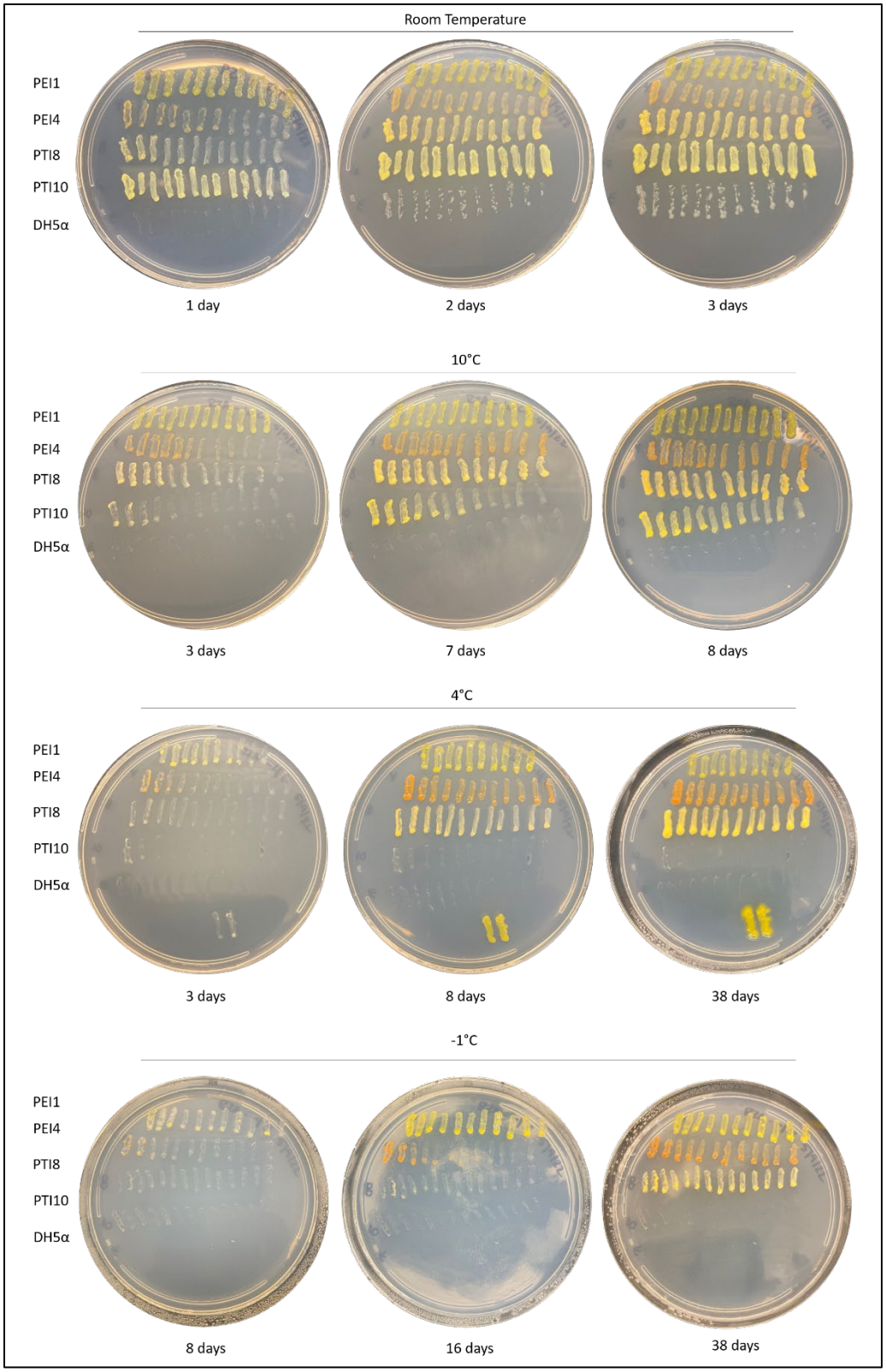
Figure 3. ICE COLD isolates PEI1 and PEI4 (*A*) and PTI8 and PTI10 (*B*) growing on R2A and TSA plates, respectively.



3.3 Isolate Growth at Various Temperatures

Isolates PEI1, PEI4, PTI8, and PTI10 were incubated on R2A plates at −1°C, 4°C, 10°C, and room temperature to approximate minimal growth temperatures. At room temperature, PEI1 and PTI10 had full growth after 1 day; PEI4 and PTI8 were fully grown after 2 days. DH5α did not grow well on R2A; even after 3 days, colony formation was limited (Figure 4).

Figure 4. R2A agar plates showing growth of PEI1, PEI4, PTI8, PTI10, and DH5α grown at room temperature, 10°C, 4°C, and −1°C.



At 10°C, PEI1 had full growth by 3 days, PEI4 and PTI8 after 7 days, and PTI10 after 8 days. We observed minimal DH5α growth after 16 days. At 4°C, we observed full growth of PEI1 after 6 days, PEI4 after 7 days, and PTI8 after 8 days. PTI10 colonies were not detected on agar plates incubating at 4°C after 38 days of incubation; however, growth was observed in TSA liquid media at 4°C, confirming its ability to grow at lower temperatures when in a liquid media. At –1°C, by day 16, PEI1 had full growth. PEI4 and PTI8 had some growth on day 16 and full growth by day 38. No growth was observed for PTI10 (Figure 4).

3.4 Determining Growth Rates and Doubling Times

To further characterize the growth of the isolated bacteria, growth curves were performed at room temperature and at 4°C in R2B and TSB. Flocculent growth while performing the growth curves made it difficult to quantify growth rates. PEI1 and PEI4 displayed flocculent growth when grown in TSB at both room temperature and 4°C. In addition, PEI4 had flocculent growth when grown in R2A at 4°C. PTI10, while able to grow at 4°C in liquid media, was flocculent when grown at 4°C, independent of the media choice. The flocculent growth prevented accurate spectrophotometer readings, so doubling times and growth rates were not calculated for these specific cases, denoted as ND (not determined) in Table 5.

Table 5. Growth rates and doubling times of isolates. (ND = not determined due to flocculent growth.)

Isolate	Growth Media	Room Temperature		4°C	
		Growth Rate ($\times 10^{-1}$ h^{-1})	Doubling Time (h)	Growth Rate ($\times 10^{-2}$ h^{-1})	Doubling Time (h)
PEI1	R2B	2.8	2.5	8.8	7.9
	TSB	ND	ND	ND	ND
PEI4	R2B	1.5	4.6	ND	ND
	TSB	ND	ND	ND	ND
PTI8	R2B	2.1	3.3	3.7	19
	TSB	2.6	2.7	4.3	16
PTI10	R2B	2.6	2.7	ND	ND
	TSB	3.0	2.3	ND	ND

At room temperature in R2B media, PEI1 grew the fastest with a doubling time of 2.5 h, while PEI4 grew the slowest at 4.6 h. PTI8 and PTI10 had doubling times of 3.3 h and 2.7 h, respectively (Table 5 and Figure A-2). PEI1 had a doubling time of 7.9 h in R2B at 4°C, twice as fast as PTI8,

which had doubling times of 19 and 16 h in R2B and TSB, respectively (Table 5 and Figure A-2). For reference, *Pseudoalteromonas haloplanktis*, a well-studied Antarctic psychrophile, has a doubling time of approximately 4 h at 4°C in marine broth (Piette et al. 2011).

3.5 Disk Diffusion Antibiotic Testing

Antibiotic resistance or susceptibility was tested on the four permafrost isolates by measuring the zones of inhibition using the disk diffusion susceptibility assay (Hudzicki 2009). The ten commercially available selected antibiotics inhibit bacterial growth by different mechanisms of action. Amoxicillin, penicillin G, ampicillin, and cefotaxime inhibit cell-wall biosynthesis, which ultimately leads to cell lysis. Gentamycin, kanamycin, and tetracycline inhibit protein synthesis by binding to the 30S ribosome, while chloramphenicol and azithromycin bind the 50S large ribosomal subunit, inhibiting protein synthesis. Ciprofloxacin inhibits cell division by causing DNA replication fork arrest (Werth 2022).

The disk diffusion susceptibility assay is commonly used to screen potential antibiotic sensitivities but cannot determine the minimal inhibitory concentration of the antibiotic. The ZOI is dependent on the interrogated organism and how the antibiotic molecules diffuse through the media. ZOIs can be clear or cloudy, and the boundaries can be discrete or diffuse. ZOIs may contain satellite colonies due to the growth of dormant microbes that grow once the antibiotic concentration decreases due to degradation (Table 6).

Of the ten antibiotics tested, we focused on four antibiotics commonly used in molecular biology, which include ampicillin, chloramphenicol, kanamycin, and tetracycline (*bolded*, Table 6). Selecting organisms susceptible to these conventionally used antibiotics allows for the use of commercially available plasmids. The commercially available plasmids contain genes of interest, including antibiotic resistance genes. When the antibiotic resistance genes are expressed, it indicates that the plasmid has been successfully transferred to the recipient microorganism. PEI1, PTI8, and PTI10 were sensitive to ampicillin, chloramphenicol, kanamycin, and tetracycline (Table 6 and Figure A-1). PEI4, was sensitive to kanamycin and tetracycline and resistant to ampicillin and chloramphenicol (Table 6 and Figure A-1). Susceptibility results from disk diffusion assays were used in deciding which combination of isolates and antibiotics to use in the MIC assay.

Table 6. Antibiotic zones of inhibition (ZOIs), measured in millimeters, for PEI1 (72 h of growth), PEI4 (96 h of growth), PTI8 (40 h of growth), and PTI10 (48 h of growth) on commercial antibiotic disks. *Symbols* denote a clear ZOI (*), cloudy ZOI (**), discrete boundary (#), diffuse boundary (##), and satellite colonies (~). *Bold* denotes antibiotics used in MIC testing.

Antibiotic	PEI1	PEI4	PTI8	PTI10	E. coli DH5α	*E. coli* ATCC 25922 (from CLSI)[a]
Gentamycin (CN10)	17*#	19*#	16*#	30*##~	23*##	19–26
Amoxycillin (AMC30)	30*#	31*#	34*##	80*##~	32*#	19–25
Chloramphenicol (C30)	40*#	11**##	34*##	55*##	33*##	21–27
Kanamycin (K30)	18*#	35*#	30*#	30*#~	30*##	17–25
Ciprofloxacin (CIP5)	44*##	49*#	42*##	30*##	52*##	30–40
Azithromycin (AZM15)	31*##	35*#	22*#	9*#	14*##	0
Ampicillin (AMP10)	17*##	0	24*##	>90	**26*##**	16–22
Cefotaxime (CTX30)	27*#	13**##	30*##	74*##~	48*##	29–35
Penicillin (P10)	17*#	0	32*##	>90	0	0
Tetracycline (TE30)	44*#	49*##	34*##	48*##	32*#	18–25

[a] Clinical and Laboratory Standards Institute

If a ZOI was not detected for a particular antibiotic, or the ZOI was smaller than the susceptibility threshold for known susceptible organisms, the bacterium was deemed resistant, and MIC testing was not performed with the antibiotic.

3.6 Minimum Inhibitory Concentrations

MIC assays were performed on the isolates using ampicillin, chloramphenicol, kanamycin, and tetracycline to inform the appropriate antibiotic concentration to use in agar plates for plasmid selection and retention after transformation. Although PEI1 appeared to be a good candidate, showing some sensitivity to all of the antibiotics in the disk diffusion assay (Table 6), it was resistant to ampicillin and chloramphenicol at the tested concentrations for the MIC (Table 7, denoted as ND, and Figure 5). When grown with ampicillin, there was an initial increase in the OD prior to inhibition. PEI1 appears able to overcome inhibition of chloramphenicol over time, even when grown in the presence of 100 µg/mL. PEI1 growth was suppressed in 8 µg/mL (lowest tested) kanamycin and tetracycline (Table 7 and Figure 5). PEI4 was fully inhibited for all concentrations when grown in kanamycin and tetracycline, including the lowest concentration tested, 2 µg/mL (Table 7 and Figure 5). PEI4 was resistant to both ampicillin and chloramphenicol in the disk diffusion assay (Table 6), so a MIC assay was not carried out in these antibiotics (Table 7, denoted as NT). PTI8 was tested against all four antibiotics; we observed delayed growth (approximately 48 h) at 50 µg/mL ampicillin, indicating that a higher antibiotic concentration was necessary; full inhibition was observed at 100 µg/mL and higher. In chloramphenicol, kanamycin, and tetracycline, growth was inhibited at 25 µg/mL, 8 µg/mL, and 1 µg/mL, respectively (Table 7 and Figure 6). For PTI10, growth was inhibited at 50 µg/mL, 12.5 µg/mL, 8 µg/mL, and 2 µg/mL, for ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively (Table 7 and Figure 6).

Table 7. Minimal inhibitory concentrations (µg/mL) of PEI1, PEI4, PTI8, and PTI10 in ampicillin, chloramphenicol, kanamycin, and tetracycline.

Isolate	Ampicillin	Chloramphenicol	Kanamycin	Tetracycline
PEI1	ND	ND	8	8
PEI4	NT	NT	2	2
PTI8	100	25	8	1
PTI10	50	12.5	8	2

NT = Antibiotic was not tested because isolate was not sensitive in disk diffusion assay.

ND = Value was not determined because growth was not suppressed by tested antibiotic concentrations.

Figure 5. MIC curves from PEI1 and PEI4 grown for 72 h in LB media and increasing concentrations of ampicillin, chloramphenicol, kanamycin, and tetracycline. Data is the average of two replicates (PEI4 and PEI1 in kanamycin and tetracycline) and three replicates (PEI1 in ampicillin and chloramphenicol).

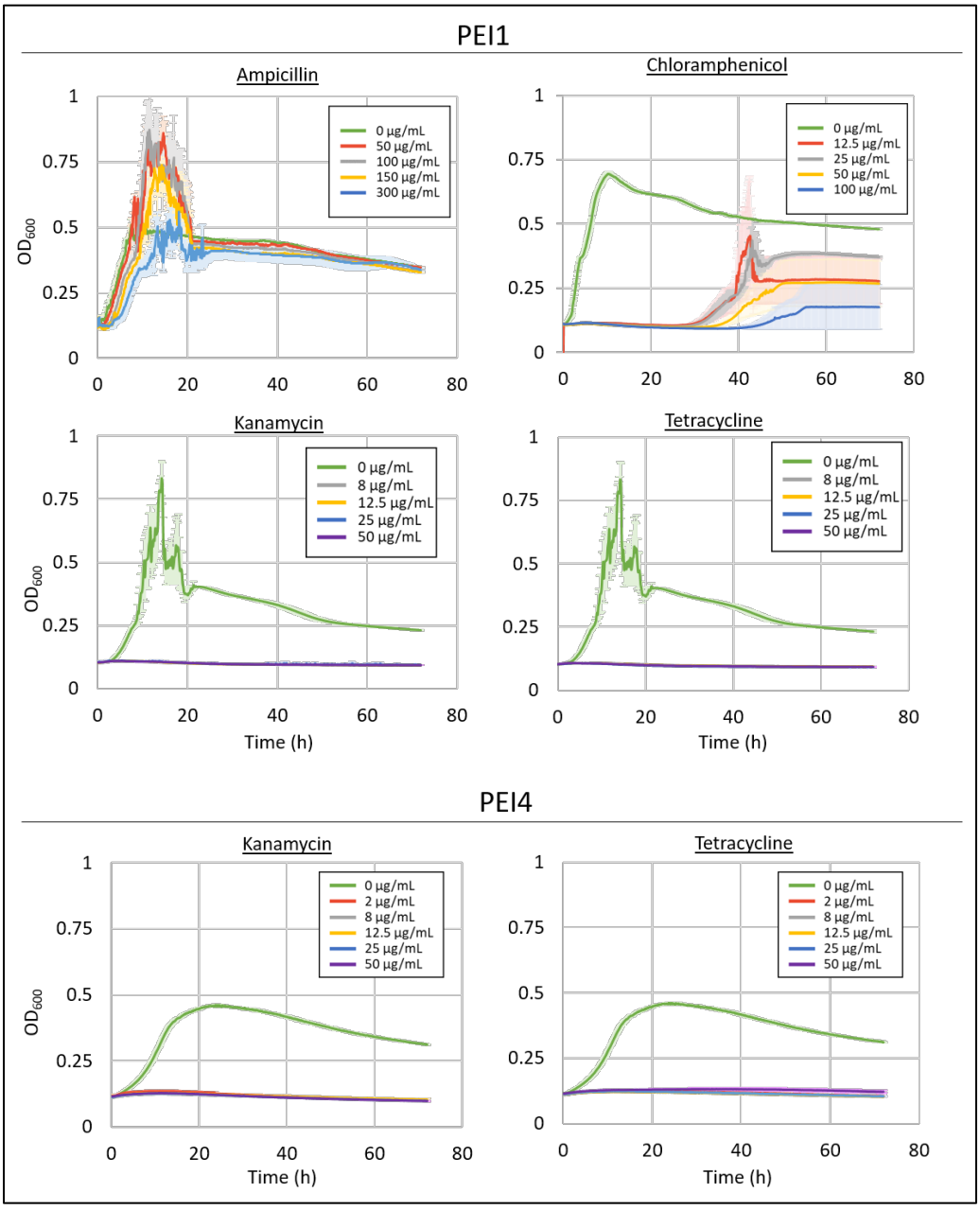
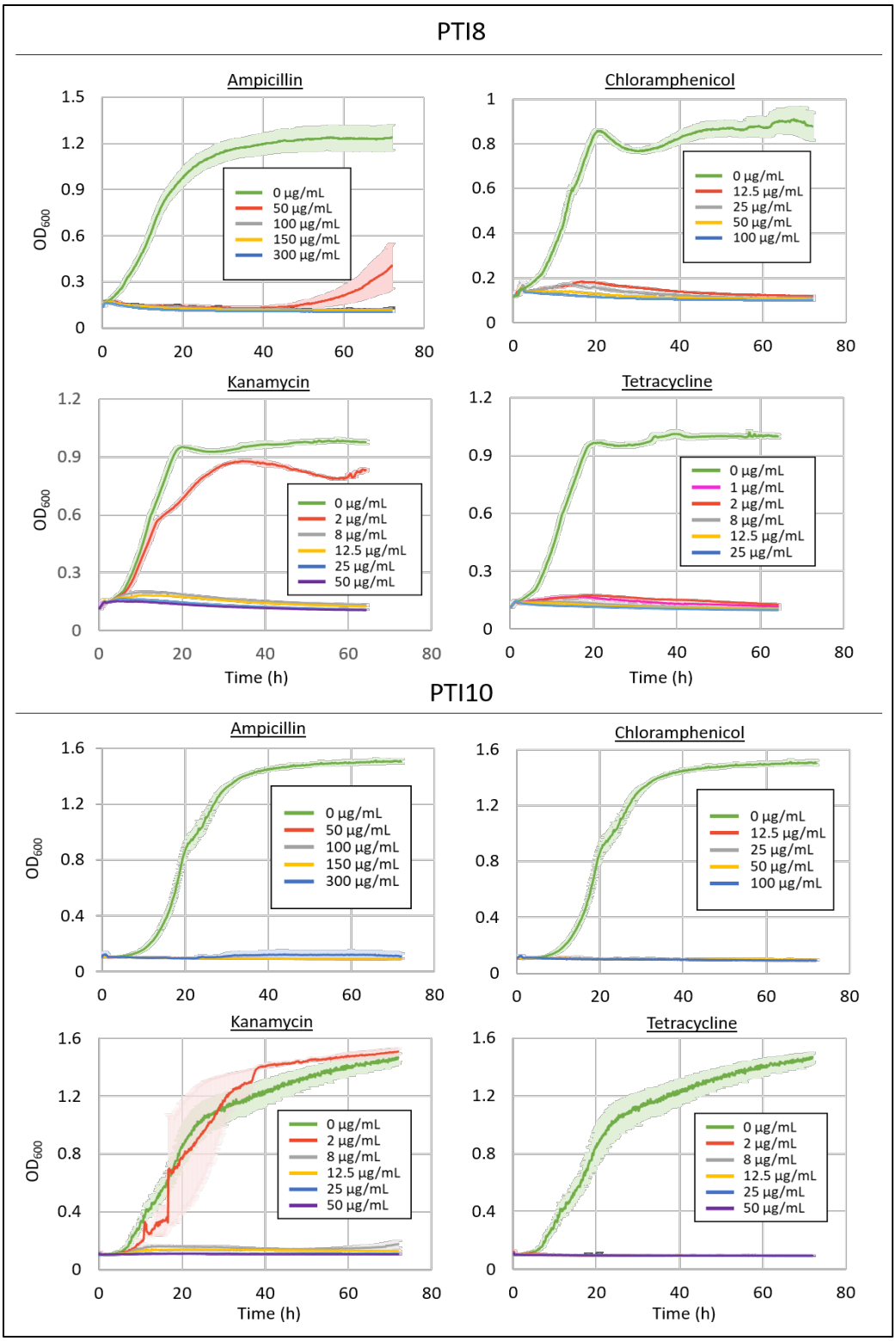


Figure 6. MIC curves from PTI8 and PTI10 grown for 72 h in LB media and increasing concentrations of ampicillin, chloramphenicol, kanamycin, and tetracycline. Data is the average of two replicates (PTI8 in kanamycin and tetracycline and PTI10 in all antibiotics) and three replicates (PTI8 in ampicillin and chloramphenicol).



3.7 Transformation of Exogenous DNA into PTI8

PTI8 was deemed the best candidate for transformation experiments because it met the following criteria: PTI8 was isolated from permafrost, grew at all test temperatures, and was sensitive to the four antibiotics tested. The sensitivity allowed us to perform electroporation transformations using all available plasmids, increasing the likelihood of getting a positive transformation event. Two plasmids, pUC19 and pSVJ21, were tested initially. pUC19 is a commonly used cloning vector for *E. coli* (Norrander et al. 1983), and pSVJ21 was reported as a psychrophilic bacterium shuttle vector (Miteva et al. 2008). The parameters for voltage, resistance, capacitance, plasmid concentration, and recovery time were varied (Table A-1). No positive transformants were obtained from the initial transformation studies. To increase the likelihood of a transformation event, we tested a larger selection of plasmids. The parameters (cuvette gap size, voltage capacitance, resistance, and recovery media) found in Desomer et al. (1990), reported in Table 3, were selected for the transformations with the additional 12 plasmids (Table 2). Desomer et al. (1990) reported successful electroporation transformations in *Rhodococcus fascians,* which is the nearest neighbor of our transformation candidate, PTI8.

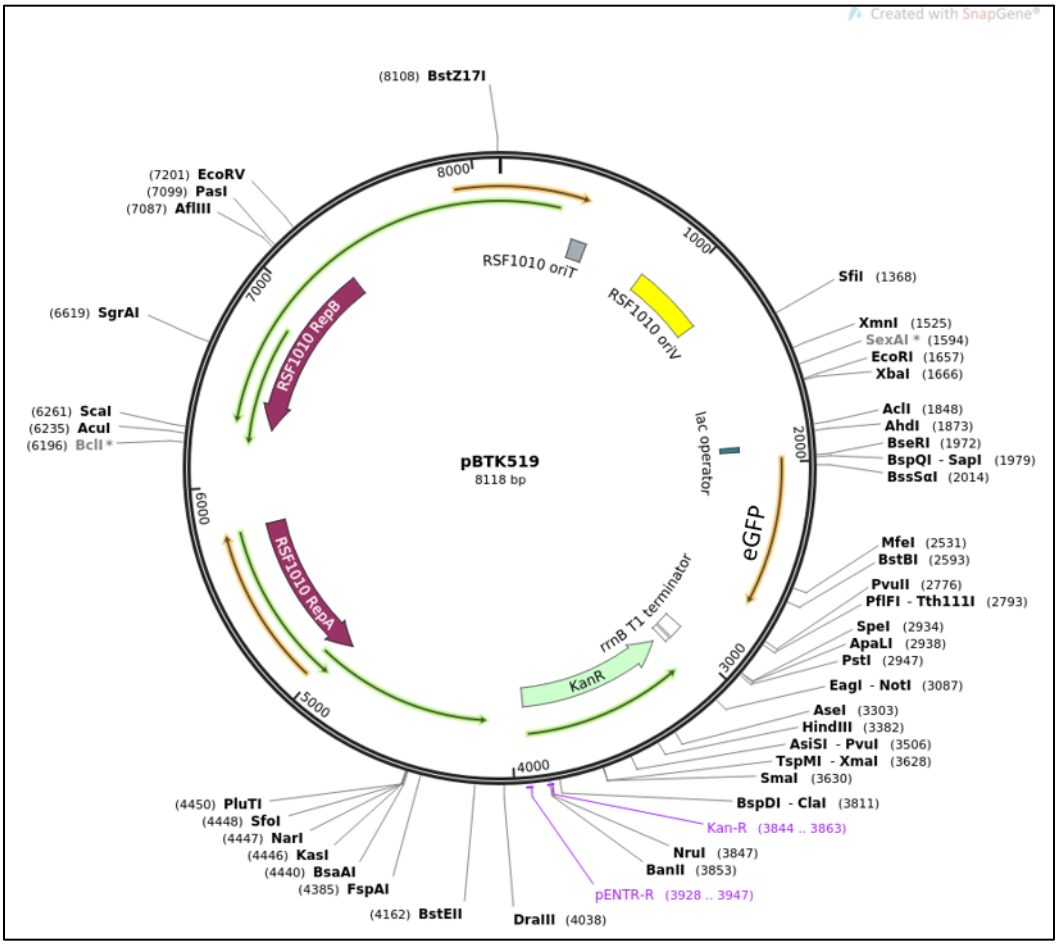
Electroporation of PTI8 with the plasmid pBTK519 resulted in positive colony growth. Colony growth appeared after a week of incubation on LB media containing 50 μg/mL kanamycin incubating at room temperature in the dark (Figure 8*A*). The positive transformants remained resistant to kanamycin following two rounds of restreaking to confirm resistance.

To confirm that pBTK519 was transformed into the PTI8 isolate, we extracted the pBTK519 plasmid from transformed PTI8 cells. We transformed the recovered pBTK519 plasmid from the PTI8 cells back into untransformed DH5α cells, which pBTK519 contains a sequence encoding for enhanced green fluorescent protein (eGFP; Figure 7). When pBTK519 is expressed in DH5α, the normally shiny off-white colonies have a visually observable green tint. For a control, we also included the pBTK519 plasmid recovered from previously transformed DH5α cells. Positive green-tinted colonies were observed on the antibiotic-selective media plates, indicating expression of eGFP and providing evidence of successful transformation of pBTK519 into PTI8 cells (Figure 8). A color change was not observed in transformed PTI8 cells; however, it does not necessarily indicate an unsuccessful transformation. Possible reasons for a successful

transformation that was not observed visually include that pBTK519 is a low-copy-number plasmid, so the eGFP signal may be too low to detect, or PTI8's pigmentation might obscure the eGFP signal.

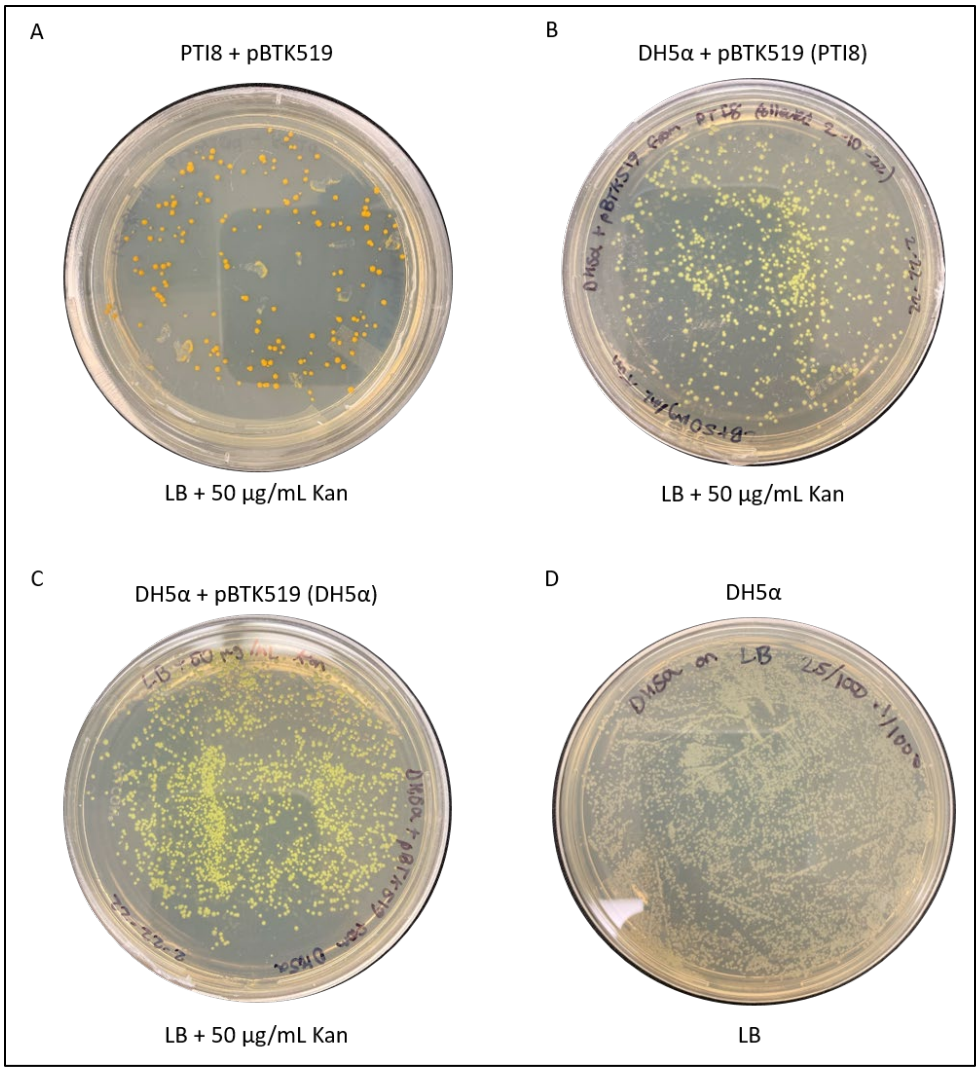
Transformation efficiencies for PTI8 transformed with pBTK519 ranged from 3.4×10^5 to 1.72×10^6 CFU*/µg DNA. These efficiencies fall within the range previously measured for *R. fascians* electroporation; Desomer et al. (1990) reported transformation efficiencies of 10^5 to 10^7 CFU/µg DNA for *R. fascians* when using a cloning vector constructed from an indigenous plasmid and *E. coli*. For reference, commercially engineered strains, such as max efficiency DH5α competent cells (Invitrogen, 18258012), have transformation efficiencies up to 10^9.

Figure 7. Plasmid map of pBTK519. (Images generated by SnapGene software from GSL Biotech; available at snapgene.com).



* Colony-forming unit.

Figure 8. (*A*) PTI8 transformed with pBTK519 growing on LB + 50 µg/mL kanamycin. (*B*) *E. coli* DH5α transformed with pBTK519 isolated from PTI8 growing on LB + 50 µg/mL kanamycin. (*C*) E. coli DH5α transformed with pBTK519 isolated from *E. coli* DH5α growing on LB + 50 µg/mL kanamycin. (*D*) Colonies of untransformed *E. coli* DH5α grown on LB.



4 Conclusions and Recommendations

In this study, we evaluated the potential of four environmental microorganisms isolated from Alaskan permafrost to be genetically engineered chassis for cold studies. We found that these organisms are capable of growing in the temperature range of 4°C to room temperature; and PEI1, PEI4, and PTI8 are capable of growing at –1°C. Isolates capable of growing relatively fast at 4°C may be very useful for low-temperature applications. We found that the isolates were sensitive to commonly used antibiotics. This is advantageous for two reasons: First, the antibiotics are easily purchased, well studied, and characterized. Second, we were able to take advantage of the Addgene repository to buy a variety of plasmids that already contain the antibiotic gene of interest to test with our isolates. We were also able to successfully demonstrate how our synthetic biology pipeline can yield positive results by transforming a commercially bought plasmid pBTK519 into an environmental isolate PTI8. Further testing is necessary to fully understand the genetic manipulability of these psychrotrophs. For future investigations, we recommend incorporating additional plasmids, examining plasmid retention, and assessing the microorganism's performance at low temperatures.

In subsequent studies, we would like to expand testing to other microorganisms in ICE COLD that have unique functions or adaptations for specific applications. For example, there has been interest in using cold-adapted microorganisms in cold regions for cleaning up hazardous waste such as oil spills, which have experienced higher frequency in recent years due to the increased human traffic in regions like the arctic (Giudice et al. 2010). Psychrotrophs and psychrophiles have been found to be able to degrade a variety of environmentally harmful hydrocarbons including naphthalene and biphenyl (Schultz and Rosado 2020). Low-temperature hydrocarbon degradation has also been found in members of *Rhodococcus* spp., located in hydrocarbon-contaminated alpine soils (Margesin et al. 2005), as well as in the cold-adapted bacterium *Pseudoalteromonas haloplanktis*, which was engineered to express tolueneoxylene monooxygenase for efficient aromatic hydrocarbon degradation at low temperatures (Papa et al. 2009). Psychrophilic and psychrotrophic organisms are also being explored for use in water treatment plants. A strain of the Arthrobacter genus was found to be able to induce clarification of a turbid synthetic wastewater at 10°C, suggestive of its use to treat wastewater (Gratia et al. 2009).

Our body of work provides fundamental knowledge necessary to expand biotechnology and synthetic biology approaches to cold environments. Cold-tolerant platforms will support the development of future technologies to enable soldiers to operate in changing environments and will enhance Army operations by allowing the development of cold-specific technologies.

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Appendix: Supplemental Figures

Figure A-1. Disk diffusion assay of (*A*) PEI1, (*B*) PEI4, (*C*) PTI8, and (*D*) PTI10.

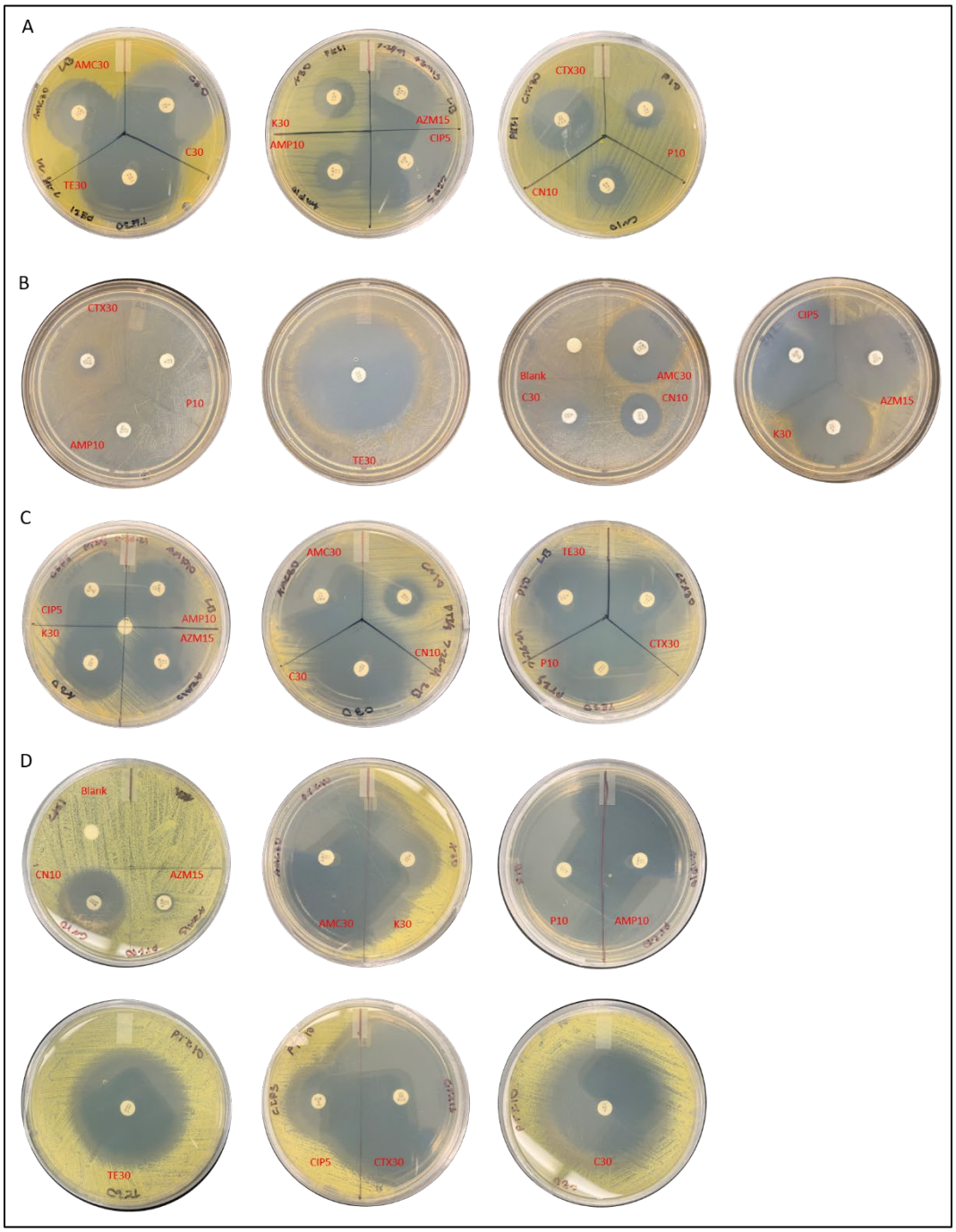
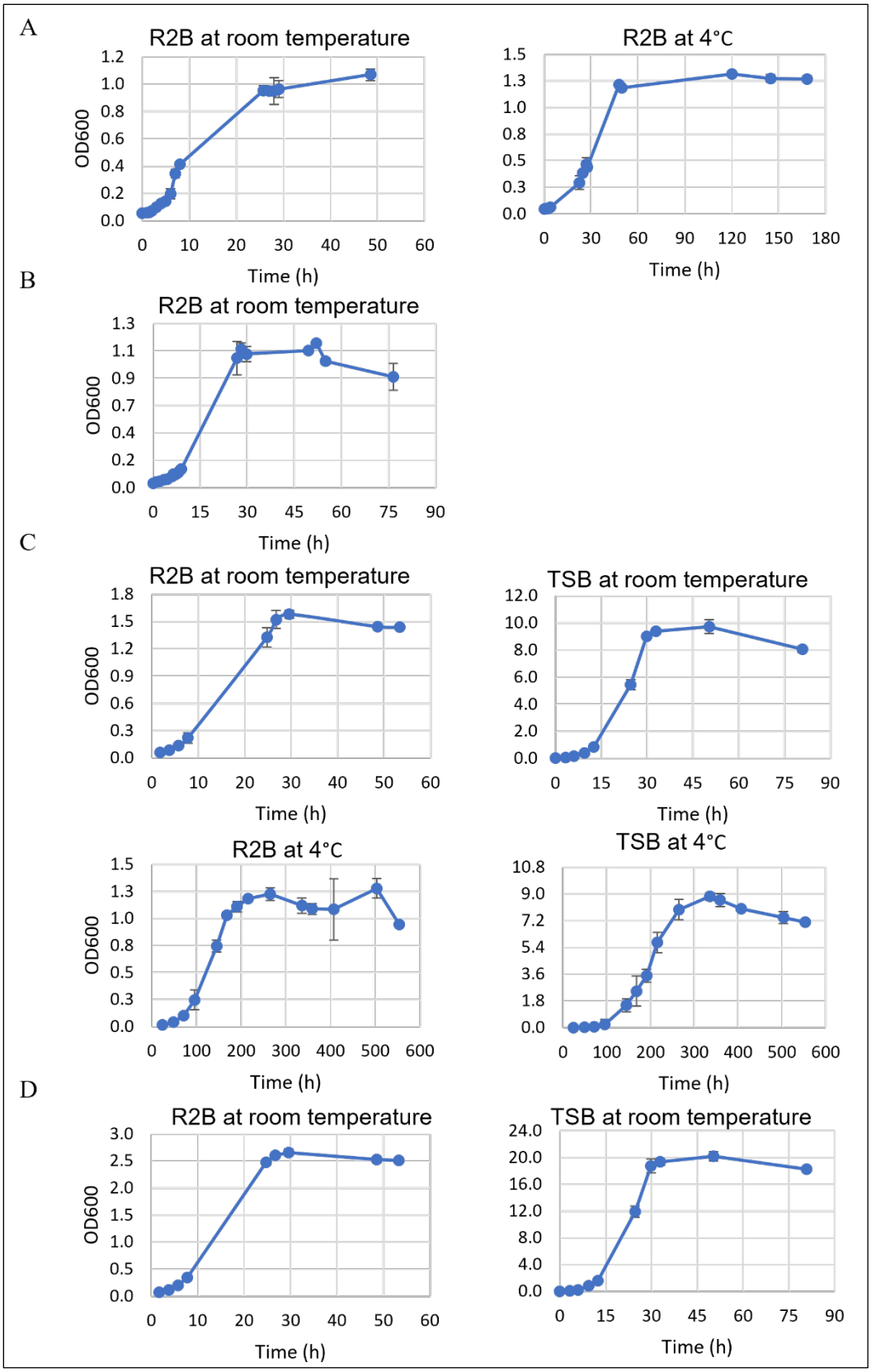


Table A-1. Electroporation parameters for transforming PTI8 with pSVJ21 and pUC19.

Isolate	Plasmid	Selection	Voltage (kV)	Capacitance (µF)	Resistance (ohms)	Cell Stock	Cells (µL)	Plasmid Volume (µL)	Plasmid Concentration	Recovery Time (h)	Recovery Media	Recovery Temperature (°C)	Cuvette Size (mm)
PTI8	pSVJ21	100 µg/mL amp	1.25	25	200	gly +	24	1	50 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	400	gly +	24	1	50 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	800	gly +	24	1	50 ng/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	200	gly +	24	1	10 pg/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	400	gly +	24	1	10 pg/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	800	gly +	24	1	10 pg/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	200	gly −	24	1	50 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	400	gly −	24	1	50 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	800	gly −	24	1	50 ng/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	200	gly −	24	1	10 pg/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	400	gly −	24	1	10 pg/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	800	gly −	24	1	10 pg/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.25	25	200	gly −	24	1	96.8 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	1.8	25	200	gly −	24	1	96.8 ng/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.25	25	200	gly −	24	1	48.9 ng/µL	1	SOC	25	1
PTI8	pUC19	100 µg/mL amp	1.8	25	200	gly −	24	1	48.9 ng/µL	1	SOC	25	1
PTI8	pSVJ21	100 µg/mL amp	2.5	25	400	gly −	24	1	96.8 ng/µL	1	SOC	25	2
PTI8	pUC19	100 µg/mL amp	2.5	25	400	gly −	24	1	48.9 ng/µL	1	SOC	25	2
PTI8	pSVJ21	50 µg/mL Chl	2.5	25	400	gly −	24	1	96.8 ng/µL	1	SOC	25	2
PTI8	pSVJ21	50 µg/mL Chl	2.5	25	400	gly −	24	1	96.8 ng/µL	5	SOC	25	2

Figure A-2. Growth curves in indicated media and temperatures for (*A*) PEI1, (*B*) PEI4, (*C*) PTI8, and (*D*) PTI10.



The following bulleted list provides information about the plasmid source and order information for reference and procurement.

- pAM5406 was a gift from Susan Golden (Addgene plasmid #132661 ; http://n2t.net/addgene:132661 ; RRID:Addgene_132661)
- pBAV1K-T5-gfp was a gift from Ichiro Matsumura (Addgene plasmid #26702 ; http://n2t.net/addgene:26702 ; RRID:Addgene_26702)
- pJet1.2 cat cassette was a gift from Stephan Gruber (Addgene plasmid #117119 ; http://n2t.net/addgene:117119 ; RRID:Addgene_117119)
- pJet1.2 kanR cassette was a gift from Stephan Gruber (Addgene plasmid #117122 ; http://n2t.net/addgene:117122 ; RRID:Addgene_117122)
- pBTK501 was a gift from Jeffrey Barrick (Addgene plasmid #110602 ; http://n2t.net/addgene:110602 ; RRID:Addgene_110602)
- pBTK519 was a gift from Jeffrey Barrick (Addgene plasmid #110603 ; http://n2t.net/addgene:110603 ; RRID:Addgene_110603)
- pBTK001 was a gift from Jeffrey Barrick (Addgene plasmid #110573 ; http://n2t.net/addgene:110573 ; RRID:Addgene_110573)
- pBTK102 was a gift from Jeffrey Barrick (Addgene plasmid #110574 ; http://n2t.net/addgene:110574 ; RRID:Addgene_110574)
- pMM656 was a gift from Timothy Lu & Christopher Voigt (Addgene plasmid #68887 ; http://n2t.net/addgene:68887 ; RRID:Addgene_68887)
- pSVJ21 was obtained from the laboratory of Jean Brenchley, PhD, Penn State University. (Kerafast plasmid #EQ2001 ; https://www.kerafast.com/item/773/psvj21-psychrophilic-bacterium-shuttle-vector)
- pUC19 was obtained from Thermo Fisher Scientific. (pUC19 DNA, Product #SD0061 ; https://www.thermofisher.com/order/catalog/product/SD0061?SID=srch-srp-SD0061)
- pMRE135 was a gift from Mitja Remus-Emsermann (Addgene plasmid #118489 ; http://n2t.net/addgene:118489 ; RRID:Addgene_118489)
- pMRE155 was a gift from Mitja Remus-Emsermann (Addgene plasmid #118505 ; http://n2t.net/addgene:118505 ; RRID:Addgene_118505)
- pCAP05 was a gift from Bradley Moore (Addgene plasmid #102920 ; http://n2t.net/addgene:102920 ; RRID:Addgene_102920)

Abbreviations

ABS	Absorbance
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
CRREL	Cold Regions Research and Engineering Laboratory
eGFP	Enhanced green fluorescent protein
EPS	Extracellular polymeric substances
ERDC	Engineer Research and Development Center
ICE COLD	Innovative, Collaborative, Exploratory, Cold Regions, Organism Library for Discovery in Biotechnology
LB	Luria-Bertani
MIC	Minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
ND	Not determined
NT	Not tested
OD	Optical density
ORI	Origin of replication
PBS	Phosphate-buffered saline
PTRF	Permafrost Tunnel Research Facility
R2A	Reasoner's 2A agar
R2B	Reasoner's 2A broth
SOC	Super optimal broth with catabolite repression
TSA	Tryptic soy agar
TSB	Tryptic soy broth
ZOI	Zone of inhibition

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14. ABSTRACT

Operations in the Artic and other cold regions require technologies that can perform reliably under extreme cold conditions. Permafrost and frozen soils harbor a wide range of microorganisms that have adapted to extremely low temperatures and have unique metabolic capabilities relevant to military operations and that could be exploited to develop biotechnologies optimized for cold environments. Cold-tolerant bacteria (psychrophiles and psychrotrophs) are critical to the development of synthetic biology technologies meant to work in cold environments like the Arctic.

Using bacteria isolated from Alaskan permafrost, we applied an experimental pipeline to test the best candidates for use as biological platforms, or chassis, for low-temperature synthetic biology. Since synthetic biology constructs will perform only as well as their chassis, it is critical that circuits expected to perform under extreme cold conditions are housed in chassis that are adapted to those conditions. We identified one permafrost isolate, PTI8, related to *Rhodococcus fascians*, that is capable of growing from −1°C to at least 25°C and which we experimentally confirmed to uptake and express the broad host range plasmid pBTK519, suggesting PTI8 is a candidate for use as a novel cold-adapted chassis for synthetic biology.

15. SUBJECT TERMS

Antibiotics, Bacterial growth, Biotechnology, Cold-adapted bacteria, Microbial chassis, Permafrost, Permafrost--Microbiology, Psychrophiles and psychrotrophs, Synthetic biology

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