Investigating the antimicrobial potential of cultivable high Arctic bacteria

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April 2023

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

"Look deep into nature, and then you will understand everything better."
Albert Einstein

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Acknowledgements

I would like to express my gratitude to Dr. Lyle Whyte for his guidance and for fostering my scientific creativity. Thank you for believing in me.

I am also very appreciative of the tremendous support offered by my collaborators: Dr. Samantha Gruenheid, Dr. Dao Nguyen, Dr. Roger Levesque, Dr. Jennifer Ronholm, Dr. Francois Alain Le Mauff, Evelyne Côté, and the 2017 MIMM-212 cohort.

I must also acknowledge the outstanding mentorship and collaboration involving Dr. Donald Smith and Dr. Rachel Backer. I would like to send a heartfelt *thank you* to both of you.

I am particularly grateful for my supportive lab mates and dear friends: Dr. Jacqueline Goordial, Dr. Isabelle Raymond-Bouchard, Dr. Ianina Altshuler, Dr. Mira Okshevsky, Dr. Miguel Angel Fernandez Martinez, Dr. Ya-Jou Chen, Catherine Maggiori, Brady O'Connor, Olivia Blenner-Hassett, Esteban Gongora, Elisse Magnuson, David Touchette, and Adam Classen. Thank you all for your support and for our stimulating scientific discussions.

To Tina - my partner and the pillar of strength in my life - thank you for inspiring me and for encouraging me to actualize my ambitions. Lastly, to my little Jason, despite being too young to understand the gratitude that I feel towards you: thank you for bestowing on me a beautiful new perspective on life that was unfathomable before your arrival to the world.

Abstract

Antimicrobial resistance (AMR) is one the major healthcare and environmental crises that we face in the 21st century. The slow discovery rate of new antimicrobials over the last four decades has led to an exponential increase in bacterial and fungal pathogens resistant to our available arsenal of antimicrobial agents. Consequently, the negative impacts of resistant pathogens are currently affecting several major pillars of human civilization. Within the context of healthcare, it is projected that deaths attributed to AMR will surpass deaths due to diabetes and cancer, combined, by 2050. Additionally, within agricultural contexts, AMR is causing losses in livestock and food loss resulting in major global economic losses. Bioprospecting for novel antimicrobial natural products derived from microorganisms living within extreme environments represents a promising strategy in identifying new antimicrobial agents. Specifically, high Arctic bacteria represent an underexplored source of antimicrobial metabolites due to the logistical challenges in accessing their remote geographical habitats and their fastidious growth requirements in laboratory settings. Within this framework, the overarching goal of my doctoral project was to isolate, screen, characterize, and apply cultivable high Arctic bacteria exhibiting antimicrobial properties.

The first part of this project involved employing two cultivation approaches to first isolate and subsequently screen bacterial isolates derived from Canadian high Arctic habitats for antibacterial activities. These approaches included employing the cryo-iplate, an *in situ* cultivation device that I designed, and crowdsourcing standard bulk soil plating. The combination of both cultivation methods resulted in the isolation and identification of Arctic *Pseudomonas* and *Paenibacillus* spp. that exhibited antibacterial activity against clinically relevant pathogens including methicillin resistant and susceptible *Staphylococcus aureus* (MRSA

and MSSA), *Listeria monocytogenes, Salmonella enterica Escherichia coli* O157:H7, *Acinetobacter baumanii, Enterococcus faecium*, and *Enterococcus faecalis*. Genomic mining of these isolates for antimicrobial biosynthetic gene clusters (BGCs) led to the detection of undiscovered biosynthetic pathways clusters, sharing low homology (<50% coverage, <30% identity, and e-values >0) with known BGCs, that could potentially lead to novel antimicrobial molecules.

Since resistant fungal pathogens are increasingly playing a role in the global AMR crisis, the focus of my second doctoral chapter was to characterize the antifungal properties of a putatively novel Arctic species*, Pseudomonas* sp. B1.1W. This isolate was found to inhibit the growth of the second most prevalent phytopathogenic mold species, Botrytis cinerea, responsible for global crop losses. By mining its genome for antimicrobial BGCs and by biochemically analyzing organic extracts derived from liquid culture supernatants, B1.1W was found to produce an antifungal cyclic lipopeptide part of the viscosin group.

The final component of my doctoral project evaluated B1.1W as a potential biocontrol agent against *B. cinerea* in *Cannabis sativa*. Cellular inoculants and bioactive extracts prepared from liquid culture supernatants of B1.1W were tested as foliar biocontrol treatments on *C. sativa* challenged with *B. cinerea*. Infected cannabis leaves that were treated with cellular inoculants and organic extracts of B1.1W featured 43.79% and 89.83%, respectively, less total mold cell counts. Furthermore, challenges cannabis plants that received B1.1W treatments displayed fewer counts of symptomatic foliage throughout the course of a 14-day observation period compared to untreated controls. Targeted 16S amplicon sequencing of *C. sativa* leaves treated with B1.1W cells revealed that B1.1W dominated the foliar bacterial community, suggesting that it colonized

leaf tissue. By evaluating its antifungal effect *in planta* and its potential to colonize leaf tissue, I found that B1.1W has biocontrol potential against *B.cinerea* in cannabis.

The studies comprising this thesis ultimately demonstrate how high Arctic bacteria represent a promising source of potentially novel antimicrobial specialized metabolites with biotechnological applicability.

Résumé

La résistance aux antimicrobiens (RAM) est l'une des principales crises sanitaires et environnementales auxquelles nous sommes confrontés au XXIe siècle. La lenteur de la découverte de nouveaux antimicrobiens au cours des quatre dernières décennies a entraîné une augmentation exponentielle des agents pathogènes bactériens et fongiques résistants à notre arsenal d'agents antimicrobiens. Par conséquent, les effets négatifs des agents pathogènes résistants affectent actuellement plusieurs piliers majeurs de la civilisation humaine. Dans le contexte des soins de santé, on prévoit que les décès attribués à la résistance aux antimicrobiens dépasseront les décès dus au diabète et au cancer, combinés, d'ici 2050. En outre, dans le domaine de l'agriculture, la RAM entraîne des pertes de bétail et des pertes alimentaires qui se traduisent par des pertes économiques mondiales considérables. La bioprospection de nouveaux produits naturels antimicrobiens dérivés de micro-organismes vivant dans des environnements extrêmes représente une stratégie prometteuse pour l'identification de nouveaux agents antimicrobiens. En particulier, les bactéries du Haut-Arctique représentent une source sous-explorée de métabolites antimicrobiens en raison des défis logistiques liés à l'accès à leurs habitats géographiques éloignés et à leurs exigences de croissance fastidieuses en laboratoire. Dans cette optique, l'objectif principal de mon projet de doctorat était d'isoler, de cribler, de caractériser et d'appliquer des bactéries cultivables du Haut-Arctique présentant des propriétés antimicrobiennes.

La première partie de ce projet consistait à utiliser deux méthodes de culture pour isoler puis cribler des isolats bactériens provenant d'habitats de Haut-Arctique canadien pour leurs activités antibactériennes. Ces approches comprenaient l'utilisation de la cryo-iplate, un dispositif de culture in situ que j'ai conçu, et le crowdsourcing d'un ensemencement standard de sol en vrac.

La combinaison de ces deux méthodes de culture a permis d'isoler et d'identifier des espèces de *Pseudomonas* et de *Paenibacillus* arctiques qui présentent une activité antibactérienne contre des agents pathogènes cliniquement pertinents, notamment *Staphylococcus aureus* sensible et résistant à la méthicilline (MRSA et MSSA), *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli* O157:H7, *Acinetobacter baumanii*, *Enterococcus faecium*, et *Enterococcus faecalis*. L'exploration génomique de ces isolats à la recherche de groupes de gènes de biosynthèse antimicrobienne (BGC) a permis de détecter des groupes de voies de biosynthèse non découverts, partageant une faible homologie (<50% de couverture, <30% d'identité et des valeurs e >0) avec des BGC connus, qui pourraient potentiellement conduire à de nouvelles molécules antimicrobiennes.

Étant donné que les pathogènes fongiques résistants jouent un rôle de plus en plus important dans la crise mondiale de la résistance aux antimicrobiens, l'objectif de mon deuxième chapitre de doctorat était de caractériser les propriétés antifongiques d'une nouvelle espèce arctique présumée, *Pseudomonas* sp. B1.1W (Mota Fernandes et al., 2021). Cet isolat s'est révélé capable d'inhiber la croissance de la deuxième espèce de moisissure phytopathogène la plus répandue, *Botrytis cinerea*, responsable de pertes de récoltes à l'échelle mondiale. En recherchant dans son génome des BGC antimicrobiens et en analysant biochimiquement des extraits organiques dérivés des surnageants de culture liquide, on a découvert que B1.1W produisait un lipopeptide cyclique antifongique appartenant au groupe des viscosines.

Le dernier volet de mon projet de doctorat a évalué B1.1W en tant qu'agent de biocontrôle potentiel contre *B. cinerea* dans le *Cannabis sativa*. Des inoculants cellulaires et des extraits bioactifs préparés à partir de surnageants de culture liquide de B1.1W ont été testés en tant que traitements de biocontrôle foliaire sur *C. sativa* attaqué par *B. cinerea*. Les feuilles de cannabis

infectées qui ont été traitées avec des inoculants cellulaires et des extraits organiques de B1.1W présentaient respectivement 43,79% et 89,83% de moins de cellules de moisissures totales. En outre, les plantes de cannabis contestées ayant reçu des traitements au B1.1W présentaient moins de cas de feuillage symptomatique au cours d'une période d'observation de 14 jours que les témoins non traités. Le séquençage ciblé de l'amplicon 16S des feuilles de *C. sativa* traitées avec les cellules B1.1W a révélé que B1.1W dominait la communauté bactérienne foliaire, ce qui suggère qu'il a colonisé le tissu foliaire. En évaluant son effet antifongique *in planta* et son potentiel de colonisation des tissus foliaires, j'ai trouvé que B1.1W a un potentiel de biocontrôle contre *B.cinerea* dans le cannabis.

Les études réalisées dans le cadre de cette thèse démontrent en fin de compte que les bactéries du Haut-Arctique représentent une source prometteuse de métabolites antimicrobiens spécialisés potentiellement nouveaux et applicables sur le plan biotechnologique.

Contributions to Knowledge

1. By sequencing and mining the genomes of Arctic isolates, I identified biosynthetic gene clusters (BGCs) sharing low homology with publicly available clusters suggesting that high Arctic bacteria have the potential to produce novel antimicrobial metabolites. In so doing, this thesis contributes to the limited existing descriptions of functional antimicrobial traits exhibited by bacterial strains from the Canadian high Arctic against bacterial and fungal pathogens.
2. Using an innovative cultivation device that I designed, and 3D printed (the *cryo-iplate*), I isolated a putatively novel bacterial species, *Pseudomonas* sp. B1.1W, from active layer permafrost. I demonstrated that this isolate has antifungal properties against the phytopathogenic mold, *Botrytis cinerea*. By bioinformatically mining this isolate's genome, and by biochemically analyzing its organic extract, B1.1W was found to produce a potentially novel antifungal cyclic lipopeptide within the viscosin group. To my knowledge, this represents the first description of a high Arctic bacterium capable of biosynthesizing a viscosin-like compound with antifungal activity against *B. cinerea*.
3. One of the most prevalent fungal phytopathogens responsible for cannabis crop losses is *Botrytis cinerea*, the causal organism of gray mold disease. To my knowledge, this thesis includes one of the few descriptions of how an Arctic strain of bacteria could be applied as a biocontrol agent against *Botrytis cinerea* in *Cannabis sativa*. Overall, this work illustrates how active layer permafrost of the Canadian high Arctic harbors underexploited bacteria with agricultural biocontrol applicability.

Contribution of Authors

Chapter 3: Marcolefas E. carried out field work, lab work, analysis, and writing. Okshevsky M.carried out analysis, participated in experimental design, and contributed significantly to writing. Leung T. and McKay G. conducted the dereplication assay and screening against clinical pathogens. Hignett E. conducted the dereplication assay and participated in isolating and screening cryo-iplate strains. Hamel J. and Boyle B. conducted whole genome sequencing and assembly. Aguirre G. and Blenner-Hassett O. assisted in isolating and screening cryo-iplate strains. Whyte L., Levesque R.C. and Nguyen D. advised in experimental design and writing. Gruenheid S. coordinated the MIMM212 undergraduate crowdsourcing laboratory course, advised, and contributed significantly to writing and analysis. Whyte L. supervised the project and significantly contributed to project design, writing and analysis.

Chapter 4: Marcolefas E. carried out field work, lab work, analysis, and writing. Levesque R.C. and Gauthier J. conducted whole genome sequencing and assembly. Côté E. and Le Mauff F.A. conducted biochemical analyses on the crude extracts prepared by Marcolefas E. Levesque R.C, Gruenheid S., Nguyen D. and Sheppard D. advised in experimental design and writing. Whyte L. supervised the project and significantly contributed to project design, writing and analysis. Lastly, Liu L., part of the Bayen S, group, was contracted to perform LC-MS and process the data output.

Chapter 5: Marcolefas E. carried out field work, all lab work, analysis, and writing. Backer R. assisted in writing, propagating and growing cannabis plants, and with assisted in applying B1.1W treatments. Schwinghammer T. performed statistical analyses on the whole plant lesion count data and assisted in manuscript editing. Smith D. and Whyte L. supervised the project and significantly contributed to project design, writing and analyses.

List of Abbreviations

ACP: acyl carrier protein
AMR: antimicrobial resistance
ANI: average nucleotide identity
ARP: Antibiotic Resistance Platform
ASV: amplicon sequence variant
AT: acyl transferase
BGC: biosynthetic gene cluster
CBD: cannabidiol
CDC: Centers for Disease Control & Prevention
CLP: cyclic lipopeptide
DH: dehydratase
DLA: detached leaf assay
ER: enoyl reductase
ESKAPE: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterococcus
HPLC: High Performance Liquid Chromatography
IPC: inositol phosphorylceramide
IPC: inositol phosphorylceramide
ISR: induced systemic resistance
LARGT: lateral antimicrobial resistance genetic transfer
LB: lysogeny broth
LC-MS: liquid chromatography mass spectrometry
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MARS: McGill Arctic Research Station
MGI: mycelial growth inhibition
MRSA: methicillin resistant Staphylococcus aureus
MS-MS: Tandem mass spectrometry
MSSA: methicillin susceptible Staphylococcus
NRP: non-ribosomal peptide
NRPS: nonribosomal peptide synthetase
PABA: p-amino benzoic acid
PBP: penicillin binding proteins
PCA: principal component analysis
PCP: peptidyl carrier protein
PDA: potato dextrose agar
PKS: polyketide synthetase
RAST: Rapid Annotation using Subsystem Technology
SARS-CoV-2: severe acute respiratory syndrome coronavirus 2
TE: Thioesterase
THC: tetrahydrocannabinol
TSA: tryptic soy agar
3-HDA:3-hydroxy decanoic acid

Chapter 1. Introduction: Exploring the polar microbiome as a potential sources of novel antimicrobial natural products

Within the context of current bioprospecting efforts focused on mining underexplored microbial communities for novel antimicrobial natural products, high Arctic habitats represent an understudied reservoirs (Hagestad, 2021; Liu et al., 2013). An example illustrating how Polar bacteria have the potential to biosynthesize novel antimicrobial molecules includes a new angucyclinone called frigocyclinone produced by *Streptomyces griseus (*Bruntner et al., 2005*).* This organism was isolated from Antarctic soil and inhibits Gram-positive bacteria. Another example is the antimycobacterial pigment violacein expressed by *Janthinobacterium* sp. Ant5-2 (J-PVP), initially isolated from land-locked freshwater lakes in East Antarctica (Bruntner et al., 2005; Mojib et al., 2010). Despite the potential for novel antimicrobial biochemistry, the biosynthetic potential of microorganisms from the Canadian high Arctic currently represents a knowledge gap in natural product research. The main impediments in studying high Arctic microorganisms include their geographically remote habitats and their fastidious cultivation requirements within laboratory settings (Hagestad, 2021; Liu et al., 2013; Vester et al., 2015).

1.1 Doctoral research objectives and hypothesis

The overarching objective of my doctoral thesis was to bioprospect various Canadian high Arctic habitats for novel antimicrobial specialized metabolites; this work is detailed in Chapters 2, 3 and 4 of this thesis. Specifically, environmental samples and microbial strains originating from various habitats within the vicinity of the McGill Arctic Research Station (MARS), Expedition Fiord, Axel Heiberg Island, Nunavut, Canada (79^{o}26'N, 90^{o}46'W), were screened for novel antimicrobial metabolites. Given that cold-adapted microorganisms from the Canadian high

Arctic possess unique molecular adaptations evolved to survive under extreme physicochemical conditions, it was hypothesized that potentially novel antimicrobial specialized metabolites can be identified.

In Chapter 3, I identified high Arctic bacterial isolates exhibiting antibacterial activity against clinically relevant pathogens. To achieve this, two cultivation approaches were used: one involving the deployment of an *in situ* cultivation employing the cryo-iplate device that I designed, and the other involved crowdsourcing bulk soil plating to a cohort of undergraduate researchers. Once bacteria from high Arctic habitats were isolated, they were then screened against foodborne and nosocomial bacterial pathogens using dual-culture antagonistic assays. To gain insight into their biosynthetic potential, the genomes of Arctic isolates displaying broad spectra of antibacterial activity were mined for antimicrobial BGCs.

In Chapter 4, I characterized the putatively novel species *Pseudomonas* sp. B1.1W that I isolated using the *in situ* cryo-iplate device from the previous chapter that displayed antifungal activity against *B. cinerea*. This was achieved by taxonomically identifying this isolate through average nucleotide identity (ANI) comparisons of its genome with phylogenetic relatives. To characterize B1.1W's biosynthetic potential, I bioinformatically mined its genomic sequence for BGCs using antiSMASH and compared the detected clusters with publicly available BGC sequences that I detected within the genomes of closely related strains. To identify the antifungal causative agent produced by this strain, MALDI-TOF MS-MS and LC-MS was performed on antifungal organic extracts derived from liquid cultures of B1.1W.

Lastly in Chapter 5, I tested the biocontrol potential of *Pseudomonas* sp. B1.1W *on Cannabis sativa* challenged with *B. cinerea*. This was achieved by applying cellular inoculants and fermentation extracts of B1.1W on cannabis infected with *B. cinerea*. To further evaluate this

isolate's biocontrol potential, the strain's capacity to colonize foliar tissue was assessed using 16S community profiling on leaves treated with B1.1W inoculants and the B1.1W genome was mined for genes families known to be implicated in foliar colonization. Lastly, Chapter 6 synthesizes the findings of this doctoral project highlighting how high Arctic bacteria represent a potential reservoir of novel antimicrobial natural products with biotechnological applicability.

Chapter 2. Literature Review: The Need for Novel Antimicrobial Agents

Portions of this literature were adapted from:
Marcolefas, Evangelos, Ianina Altshuler, and Lyle G. Whyte. "1. Advanced microbial cultivation methodologies and their applicability in cryoenvironments." *Advanced Techniques for Studying Microorganisms in Extreme Environments*. De Gruyter, 2019. 1-22.

Contributions of authors: E.M. developed and wrote this review. I.A and L.G.W provided editing support.

1.1 Combatting pathogens with antimicrobials

Viruses, bacteria, fungi, and microscopic unicellular/multicellular eukaryotes form the ecological foundation of the planet's biosphere (Michael et al., 2014). They ubiquitously inhabit most environmental niches including intra-and-extracellular environments of larger organisms spanning the entire tree of life (Thakur et al., 2019; Whitman et al., 1998). Most microorganisms associated with other organisms typically form benign or beneficial symbiotic relationships with their hosts (Miller et al., 2018). However, a small portion of host-associated microbes are predatory (Miller et al., 2018). These microbial predators, categorized as pathogens, infect their hosts causing morbidity, and in certain cases, death (Van der Putten et al., 2007; Vonaesch et al., 2018). Despite the fact that they represent a small subset of the planet's total microbial diversity, pathogens are phylogenetically diverse and have evolved sophisticated mechanisms to infect their hosts and subsequently mediate disease for their survival (Martínez, 2018; Van der Putten et al., 2007).

To combat pathogenic microorganisms, chemical agents known as antimicrobials are used to prevent, kill or limit their propagation (Moellering Jr, 2011; Serwecińska, 2020). Most antimicrobials are either naturally produced by plants and microorganisms or are anthropogenically designed (Miethke et al., 2021; Saleem et al., 2010). Such agents include

antivirals, antibacterials, antifungals, antiparasitics, and antiseptics which encompass a diverse plethora of chemical structures (Gallagher & Baker, 2020; Moore & Payne, 2004). The following chapters will focus on antibacterial and antifungal specialized metabolites also referred to as antimicrobial natural products.

1.2 Different classes of antibacterial and antifungal agents

1.2.1 Antibacterials

Antibacterials are agents that kill or inhibit the growth of bacteria and are classified based on their mechanism of action (Terreni et al., 2021; Yoneyama & Katsumata, 2006). The main antibacterial mechanisms of action include inhibiting the biosynthesis of: (i) bacterial cell walls and membranes, (ii) nucleic acids, (iii) proteins, and (iv) folic acid metabolism (Hutchings et al., 2019; Yoneyama & Katsumata, 2006). These agents could either inhibit bacterial growth (bacteriostatic effect) or kill bacterial cells (bactericidal effect). Certain antibacterials exhibit different inhibitory profiles against Gram-positive and negative bacteria depending on the biomolecular targets to which they bind (Kapoor et al., 2017).

Inhibitors of bacterial cell wall and cell membrane biosynthesis

Beta-lactam and glycopeptide antibiotics inhibit cell wall synthesis. By targeting penicillin binding proteins (PBPs), β-lactam agents (such as penicillin) disrupt cross-linkages between D-alanyl-alanine and glycine portions of peptide side chains that form peptidoglycan polymers (Kahne et al., 2005; Kapoor et al., 2017). Glycopeptides, such as vancomycin, bind to the D-alanyl-alanine subunits of peptide side chains blocking the binding of PBPs that perform transglycosylation to form peptidoglycan (Stogios & Savchenko, 2020). Through these mechanisms, β-lactams and glycopeptides compromise the structural integrity of peptidoglycan polymers that form bacterial cell walls (Marschall et al., 2019; Reynolds, 1989). This disruption

of peptidoglycan biosynthesis ultimately leads to bactericidal lysis of cells (Naclerio & Sintim, 2020; Nakae, 1995; Reynolds, 1989). Antibacterial agents that inhibit cell walls synthesis are more effective at killing Gram positive bacteria since they do not feature outer cell membranes (Impey et al., 2020). Other groups of antibacterial that target cell membranes are more effective at killing both Gram negative and positive bacteria (Impey et al., 2020; Naclerio & Sintim, 2020; Yoneyama & Katsumata, 2006). These include cationic peptides such as polymyxins that interact with the cell membrane lipids disrupting membrane integrity (Landman et al., 2008). The affected membranes then become hyperpermeable and leads to bacterial death (Kuhn, 2019; Landman et al., 2008; Moffatt et al., 2019).

Inhibition of protein biosynthesis

Another large class of antibacterials includes those that inhibit protein synthesis by targeting either the 30S or 50S subunit of the bacterial ribosomes (Baquero & Levin, 2021). Aminoglycosides bind to the A site of the ribosomal 30S subunit causing misreading and termination of mRNA translation (Böttger & Crich, 2019). Tetracyclines prevent tRNA binding to the A site by binding to conserved sequences of 16S rRNA of the 30S ribosomal subunit (Gasparrini et al., 2020; Vannuffel & Cocito, 1996). Chloramphenicol, macrolides, lincosamides, streptogramins B, and oxazolidinones bind to the 23S rRNA of the 50S ribosomal subunit blocking tRNA from binding to the A site (Champney, 2020). In addition to binding to 23S rRNA, oxazolidinones also interact with peptidyl tRNA and consequently suppress 70S ribosomal subunits (Wilson et al., 2020). These agents are commonly bacteriostatic whereby bacterial growth and reproduction are inhibited (Wilson et al., 2020).

Inhibitors of nucleic acid biosynthesis

Other antibacterial agents include inhibitors of bacterial DNA replication. Quinolones, such as fluoroquinolones, bind to bacterial DNA gyrases responsible for nicking negative supercoils in

double-stranded DNA required for replication and transcription (Yilancioglu, 2019). Quinolones bind to the A subunit of gyrases inhibiting the enzymes' DNA nicking activity (Bush et al., 2020). Unable to replicate and transcribe essential genes, quinolones are often bactericidal agents that exhibit higher potency against Gram positive bacteria (Bush et al., 2020). This discrepancy in effectiveness is due to these agents' greater affinity for topoisomerase IV enzymes of Gram-positive bacteria that are responsible for nicking and separating daughter strands of DNA after replication (Higgins et al., 2003; Kokot et al., 2022).

Inhibitors of folic acid metabolism

Lastly, sulfonamides (sulfa-drugs) and trimethoprim inhibit bacterial folic acid metabolism. Sulfonamides competitively inhibit dihydropteroate synthase given their higher affinity for the enzyme compared to its native p-amino benzoic acid (PABA) substrate (Connor, 1998; Fernández-Villa et al., 2019). Trimethoprim inhibits the dihydrofolate reductase enzymes required at a later step of folic acid biosynthesis (Manna et al., 2021). Since folic acid is an essential nutrient required for protein and nucleic acid synthesis, these agents inhibit bacterial growth (Fernández-Villa et al., 2019). When used individually, these agents are bacteriostatic but confer bactericidal effects when used in combination (Manna et al., 2021).

1.2.2 Antifungals

Antifungal agents are also classified based on their mechanisms of action. Some of the main mechanisms include inhibiting the biosynthesis of: (i) ergosterol , (ii) cell walls , (iii) cell membranes (iv) nucleic acids, or (v) proteins (Campoy & Adrio, 2017). These agents could either exhibit fungistatic or fungicidal effects depending on the cellular processes that they inhibit (Perfect, 2016; Van Daele et al., 2019). Unlike the diverse set of antibacterial drug classes, there are only five classes of antifungal drugs: azoles, echinocandins, polyenes, pyrimidine analogs, and allylamines (Castelli et al., 2014; Van Daele et al., 2019).

The lack of development in antifungal treatments is partially due to the challenge in identifying drug candidates with minimal host cell toxicity (Gintjee et al., 2020). Since fungal drug targets share common eukaryotic biomolecular evolution with mammalian host cells, many candidate antifungal compounds have low therapeutic potential because of their toxic effects to human cells (Gintjee et al., 2020). Most approved antifungal drugs have either been developed through synthetic chemistry or discovered from natural sources. The only recently developed new class of synthesized antifungals are the orotomides (Kirchhoff et al., 2020). Olorofim is the only representative agent within this new class which was developed by F2G Limited and is currently in phase III clinical trials for its activity against *Aspergillus fumigatus (*Kirchhoff et al., 2020*)*. The main antifungal classes will be discussed in further detail below.

Inhibitors of ergosterol biosynthesis

Of the antifungal agents that inhibit the synthesis of ergosterol, azoles, allylamines and morpholines are most commonly used as human therapeutics (Campoy & Adrio, 2017). Azoles are cyclic compounds that inhibit the cytochrome P450-dependent enzyme 14α-lanosterol demethylase (CYP51) responsible for converting lanosterol to ergosterol in fungal cell membranes (Nicola et al., 2019; Sheehan et al., 1999). This causes inhibition of fungal growth and replication (Wall & Lopez-Ribot, 2020). Allylamines are synthetic compounds that bind and inhibit squalene epoxidase (ERG1) which converts squalene into 2,3-squalene epoxide (Sant et al., 2016). Consequently, this leads to an accumulation of squalene in fungal cells causing increased permeability and cellular disorganization (Sant et al., 2016). Morpholines inhibit both Δ^7–Δ^8 isomerase (ERG2) and the Δ^{14}-reductase (ERG24) enzymes involved in ergosterol synthesis (Denning & Hope, 2010; Sant et al., 2016). These agents could have fungistatic or

fungicidal effects depending on which fungal pathogen that they are used against (Denning & Hope, 2010; Wall & Lopez-Ribot, 2020).

Disruptors of fungal cell membranes

The first group of antifungal molecules used in clinical settings were polyenes (Caffrey et al., 2022; Hossain & Ghannoum, 2000). These macrocyclic fungal membrane disruptors bind to the lipid bilayer of fungal cells and form complexes that produce pores (Hossain & Ghannoum, 2000, 2001). These pores disrupt the structural integrity of cell membranes leading to leakage of intracellular contents resulting in fungal death (Hossain & Ghannoum, 2001). Such fungicidal molecules have broad spectra of activity against a diverse set of fungal pathogens including but not exclusive to *Cryptococcus, Candida, Aspergillus* and *Fusarium* spp. (Shahandashti & Lass-Flörl, 2019; Zhong et al., 2000). Notable polyene drug examples include amphotericin B and nystatin (Lalitha et al., 2008). Other antifungal agents that disrupt fungal cell membrane integrity act by inhibiting sphingolipid synthesis (Zhong et al., 2000). An example of such agents includes aureobasidin A, a cyclic depsipeptide that inhibits inositol phosphorylceramide (IPC) synthase (Teymuri et al., 2021). This inhibitor halts sphingolipid synthesis disrupting the structural integrity of fungal cell membranes(Teymuri et al., 2021).

Inhibitors of cell wall biosynthesis

Antifungal agents that disrupt cell wall synthesis typically inhibit β-glucan or chitin biosynthesis. Echinocandins are lipopeptides that noncompetitively inhibit β-(1,3)-d-glucan synthase enzyme complex (Lorand & Kocsis, 2007; Pristov & Ghannoum, 2019). This results in fungicidal osmotic instability (Pristov & Ghannoum, 2019). The most studied inhibitors of chitin biosynthesis include nikkomycins and polyoxins (Akins, 2005; Larwood, 2020; Osada, 2019). Peptidyl nucleosides are analogs of UDP-N-acetylglucosamine and competitively bind chitin synthases (Akins, 2005; Larwood, 2020; Osada, 2019). By inhibiting chitin synthesis, the

structural integrity of fungal cell walls become osmotically unstable (Sucher et al., 2009). Owing to their fungal target specificity, echinocandins have favorable safety profiles compared to other antifungal classes but have limited activity against pathogens that feature lower concentrations of β-(1,3)-d-glucan in their cell walls (Arendrup & Perlin, 2014).

Inhibitors of nucleic acid biosynthesis

First developed in the 1950s, fluorinated pyrimidine analogs such as flucytosine (5-FC; 5-fluorocytosine) disrupt pyrimidine metabolism and the synthesis of RNA/DNA (Liu et al., 2016). 5-fluorocytosine is consumed by fungal cells and converted to 5-fluorodeoxyuridine monophosphate (5-FdUMP) which inhibits thymidylate synthase. In the case of 5-FC, the drug preferentially binds to fungal cytosine permeases found in cell membranes and is then deaminated to form 5-fluorouracil (5-FU) which is incorporated into DNA and RNA (Billmyre et al., 2020). This disrupts DNA synthesis and arrests nuclear division (Onishi et al., 2000). However, the binding specificity of 5-FC to fungal cytosine permeases is promiscuous as the drug could also bind to commensal bacteria within mammalian hosts capable of performing the deamination step to form 5-FU (Arendrup & Perlin, 2014; Vermes et al., 2000). The accumulation of 5-FU within the mammalian hosts often leads to hepatotoxic, hematopoietic, and gastrointestinal side effects (Vermes et al., 2000).

Inhibitors of protein biosynthesis

Examples of antifungals that inhibit protein biosynthesis include tavaborole, sordarin, and cispentacin (Liang, 2008). Tovaborole binds to leucyl-tRNA synthetase blocking amino acids from transferring to the ribosome (Rock et al., 2007). Sordarin inhibits translation elongation factor 2 (EF2) while Cispentacin, a β-amino acid, inhibits isoleucyl tRNA synthetase (Liang, 2008). The targets of these agents are conserved amongst eukaryotic cells however they bind to fungal targets with higher affinity (Liang, 2008). Another recent example includes MGCD290 by

Mirati Therapeutics which is a Hos2 fungal histone deacetylase (HDAC) inhibitor that also targets non-histone proteins such as Hsp90 (Gintjee et al., 2020). By targeting these fungal proteins, cellular stress responses could be impaired leading to fungicidal effects. Unfortunately, MGCD290 only exhibited antifungal effects against *Candida* spp. in vitro and failed phase II trials evaluating it as an adjunct therapeutic to fluconazole in vaginal candidiasis (Gintjee et al., 2020). However, fungal HDACs and Hsp90 enzymes remain to be the focus of potential fungal targets for future antifungal drug design given their critical roles in gene regulation and control of essential fungal cell functions (Gintjee et al., 2020; Zhen et al., 2022).

1.3 The Antimicrobial Resistance (AMR) Crisis

Inherently, pathogens exposed to antimicrobials are put under selective evolutionary pressure to develop mechanisms of resistance. Antimicrobial resistance (AMR) arises when microbial pathogens including bacteria, fungi and viruses develop resistance mechanisms that render antimicrobial agents ineffective (Venkatasubramanian et al., 2020). Such mechanisms include: 1) developing enzymatic machinery that digest or inactivate antimicrobial molecules, 2) altering receptor binding sites of antimicrobial molecules 3) expelling molecules from the intracellular environment *via* active efflux, or 4) modifying the microbial cell wall to render them impermeable to antimicrobial molecules (Munita & Arias, 2016). These mechanisms could be spread amongst microbial communities through lateral antimicrobial resistance genetic transfer (LARGT) pathways by mobile elements including plasmids, integrons and transposons (Munita & Arias, 2016). AMR is innate to the microbial world and evolved before the anthropogenic use of antimicrobials (D'Costa et al., 2011). However, the widespread overuse of antimicrobial agents by humans during the last century has intensified the selective pressures favoring AMR (D'Costa et al., 2011; Sykes, 2010).

The increased global emergence of resistant pathogens over the course of the last two decades that negatively impact human society is reaching a dire point (Kadri, 2020). This phenomenon has been termed the AMR Crisis (Gross, 2013). It has almost been a decade since the Centers for Disease Control & Prevention (CDC) in the USA declared that human civilization has entered a "post-antimicrobial era" (Kwon & Powderly, 2021). This period is marked by AMR rendering our current arsenal of antimicrobial agents ineffective in treating infectious diseases (Kwon & Powderly, 2021). Untreatable infections would set modern medicine back by at least a century (Michael et al., 2014). Medical interventions addressing infectious disease would resort to antiquated techniques such as debridement, disinfection, amputation, and isolation; such approaches are invasive and drastically less effective at resolving infections (Michael et al., 2014). Without the development of new therapeutics, infectious diseases could rapidly become the leading cause of death worldwide (Prestinaci et al., 2015).

Exacerbating this crisis, it is predicted that the overuse of antibiotics, biocides and disinfectants during the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic will further drive the emergence of AMR (Rezasoltani et al., 2020). Two healthcare trends are causing an increase in global antibiotic administration: 1) treating patients co-infected with SARS-CoV-2 and bacterial pathogens and 2) inappropriate antibiotic stewardship towards patients presenting with respiratory symptoms in medical settings lacking access to laboratory diagnostics to determine disease etiology (Huang et al., 2020; Lansbury et al., 2020). In addition to antibiotic overuse, the uncontrolled use of biocides and disinfectants released at unprecedented levels into the environment to mitigate the spread of SARS-CoV-2 will also intensify selective pressures on resistant pathogens (Hsu, 2020; Murray, 2020).

1.3.1 Resistant bacterial and fungal pathogens of concern

In terms of resistant bacterial pathogens, the Infectious Disease Society of America has identified *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* species (referred to as the ESKAPE pathogens) that pose the most significant threat to public health (De Oliveira et al., 2020). Notably, nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium*, and fluoroquinolone-resistant *P. aeruginosa* cause more deaths annually in U.S. hospitals than HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008; Klevens et al., 2006; Mulani et al., 2019).

While drug-resistant bacterial pathogens are often the focus of discussions surrounding AMR, the prevalence of resistant fungal pathogens is also increasing worldwide (Mota Fernandes et al., 2021). Globally, fungal infections affect approximately 1.7 billion individuals, resulting in ~1.7 million deaths annually (Denning & Bromley, 2015). The severity of fungal disease is primarily determined by underlying health conditions, where immunocompromised individuals with autoimmune diseases, HIV/AIDS, and persons undergoing organ transplantation or chemotherapy are at higher risk of developing severe morbidity (Hasim & Coleman, 2019; Mota Fernandes et al., 2021; Roemer & Krysan, 2014). Amid the SARS-CoV-2 pandemic, the global incidence of fungal infections has risen amongst the expanding number of immunocompromised individuals weakened by the virus (Gangneux et al., 2020; Song et al., 2020).

In terms of mycopathogens, there are 300 fungal species known to cause disease in humans (Mota Fernandes et al., 2021; Schmiedel & Zimmerli, 2016). Some of the most prevalent pathogens include: *Candida albicans*, *Candida auris*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis, Malassezia furfur*, *Blastomyces*

dermatitidis, *Sporothrix* spp., *Fusarium* spp., *Rhizopus* spp. and *Scedosporium* spp. (K Mazu et al., 2016; Perfect, 2017; Schmiedel & Zimmerli, 2016) . Over the course of the last decade, the prevalence of fungal resistance against antifungal therapies is proliferating because of the selection pressures imparted by their overuse and their lengthy treatment regimens (Mota Fernandes et al., 2021). Fungal mechanisms of drug resistance include cellular modifications that prevent drugs from penetrating cells, altering molecular drug targets, or expelling drugs from the intracellular environment *via* efflux transporters (Berman & Krysan, 2020; Cowen, 2008; Lee et al., 2020). Additionally, since drug targets within fungal pathogens often feature eukaryotic evolutionary conservation with mammalian cells, many antifungal agents are toxic to humans (Chau et al., 2014; Parente-Rocha et al., 2017). This often causes adverse physiological events with limited therapeutic indices that challenge their clinical applicability (Chau et al., 2014; Mota Fernandes et al., 2021; Parente-Rocha et al., 2017).

Beyond the scope of human healthcare, AMR also has significantly negative impacts on agriculture. Mass application of antimicrobial agents in agricultural settings used for optimizing yield has led to selective pressure on resistant veterinary and plant pathogens (Wright, 2010). Large-scale application of antimicrobials to improve fitness of livestock and crops has been justified by commercial benefits and to improve food supplies to large portions of human populations experiencing food shortages (Michael et al., 2014; Wright, 2010). However, emergent agricultural pathogens that are uncontrollable by current antimicrobial agents are causing stock and crop losses worldwide (Fisher et al., 2012). Resistant phytopathogenic fungi, in particular, cause major pre- and post-harvest losses of valuable crops (Lucas et al., 2015). Fungal phytopathogens are responsible for the destruction of a third of all food crops leading to major economic losses and decrease global food supplies (Lucas et al., 2015). The fungal

phytopathogens that cause the greatest economic losses worldwide include *Magnaporthe oryzae*, *Puccinia* spp., and *Botrytis cinerea*. These plant pathogens have a wide host ranges spanning many valuable crops including wheat, rice, lettuce, bananas, and ornamental plants causing billions of dollars in global economic loss annually (Lucas et al., 2015).

1.4 Novel antimicrobial natural products: part of the solution to the AMR Crisis

Discovering novel natural products (NPs) of microbial origin represent a promising strategy in addressing the AMR crisis (Udwary et al., 2021). Other recent strategies such as phage therapeutics, photodynamic light therapy, and inorganic nanoparticle antimicrobials also represent promising methods but are currently often coupled with conventional NP agents to synergistically increase their effectiveness (Mulani et al., 2019; Wang et al., 2020; Westwater et al., 2003).

Microbial NPs encompass a vast chemical diversity that has been naturally selected to bind molecular targets with high affinity and to efficiently permeate into microbial cells (Roemer et al., 2011). Most currently used antimicrobial agents are NPs comprised of antimicrobial specialized metabolites originating from environmental microorganisms (Abdel-Razek et al., 2020; Scheffler et al., 2013). Antimicrobial specialized metabolites are biomolecules produced by microorganisms or plants that are not essential for their growth, development or reproduction (Tyc et al., 2017). These metabolites typically mediate ecological interactions such as competition and predation that increase the survival of the producing organism (Tyc et al., 2017).

Bacterial specialized metabolites are a set of distinct biomolecular classes featuring high degrees of structural variation with diverse physicochemical and bioactivity profiles (Tyc et al., 2017). Antimicrobial specialized metabolites of bacterial origin are produced by biosynthetic pathways that are encoded within compact and modular gene clusters (Grubbs et al., 2017). These biosynthetic gene clusters (BGCs) encode the enzymatic machinery that synthesize the molecular

building blocks and tailoring steps involved in producing antimicrobial metabolites (Albarano et al., 2020; Blin et al., 2013; Grubbs et al., 2017). Bacterial genera known to produce antimicrobial specialized metabolites include: *Streptomyces*, *Pseudomonas*, *Bacillus*, *Burkholderia*, and *Myxococcus* (Chen et al., 2006; El-Sayed et al., 2003; Stinear et al., 2004; Tyc et al., 2017). Antimicrobial-producing bacteria inhabit most ecological niches (Udwary et al., 2021). Some of the most studied niches include rhizospheric soils and benthic sediments (Milshteyn et al., 2014). Several main antimicrobial specialized metabolite classes include ribosomally synthesized and post-translationally modified peptides (RiPPs), nonribosomal peptides (NRPs), polyketides (PKs), and terpenes (Tyc et al., 2017). Each of these will be explored in more detail.

RiPPs

RiPPs are ribosomally-produced peptides initially synthesized as longer precursor peptides (Arnison et al., 2013; Rowe & Spring, 2021). This class of specialized metabolites includes bacteriocins, lanthipeptides, linaridins, proteusins, linear azol(in)e-containing peptides (LAPs), cyanobactins, and thiopeptides (Arnison et al., 2013). It is estimated that more than 99% of all bacterial species have the ability to biosynthesize at least one type of RiPP (Arnison et al., 2013; Riley & Wertz, 2002). In particular, rhizospheric soil-dwelling bacteria and members of mammalian gut microbiotas are well studied producers of such antimicrobial peptides (Hammami et al., 2009; Kommineni et al., 2015).

Nonribosomal peptides

Nonribosomal peptides are synthesized by large multi-modular nonribosomal peptide synthetases (NRPSs)(Khabthani et al., 2021). Each module of NRPSs typically extend the peptide by one amino acid in an iterative assembly-line fashion (Figure 1). Each module contains an adenylation (A) domain which selects and loads a single amino acid, a condensation (C) domain responsible for catalyzing the formation of a peptide bond and a phosphopantetheinylated peptidyl carrier

protein (PCP) that binds that extending peptide chain (Niquille et al., 2021; Tyc et al., 2017). Thioesterase (TE) domains are optional additional NRPS domains that release or cyclize the nascent peptide chain (Tyc et al., 2017). Important examples of antimicrobial NRPS include β-lactam antibiotics and lipopeptides (Raaijmakers et al., 2010; Tahlan & Jensen, 2013).

Figure 1. Non-ribosomal peptide biosynthesis. Large multi-modular nonribosomal peptide synthetases (NRPSs) comprising initiation, elongation and termination modules extend the peptide by one amino acid. Each module contains an adenylation (A) domain, a condensation (C) domain, and a phosphopantetheinylated peptidyl carrier protein (PCP) that binds that extending peptide chain. Thioesterase (TE) domains in termination modules release or cyclize the final peptide chain. Created using BioRender.com.

Polyketides

Polyketides represent another large class of specialized metabolites that are synthesized by multidomain polyketide synthases (Almalki, 2020). Their biosynthesis is analogous to Claisen condensation of fatty acid production involving acyl transferase (AT) domains that select the elongation units and transfer them to an acyl carrier protein (ACP) that carries the growing acyl chain (Staunton & Weissman, 2001). Ketosynthases (KSs) then condense the next monomer unit extension while optional dehydratase (DH), and enoyl reductase (ER) domains perform reductive modifications (Staunton & Weissman, 2001). Lastly, the terminal TE domain releases the final

product forming either an acid or a lactone (Shen, 2003). Notable examples of antimicrobial compounds pertaining to the PKs include tetracyclines and macrolides (Hertweck et al., 2007).

Terpenes

Terpenes are built from dimethylallyl pyrophosphate and isopentenyl pyrophosphate substrates originating from mevalonate or deoxy xylulose phosphate pathways (Avalos et al., 2022; Dickschat, 2011). Soil-dwelling *Streptomyces* are well studied producers of terpenes (e.g. geosmin) (Cane et al., 2006; Gürtler et al., 1994). Another notable bacterial terpene with antimicrobial bioactivity includes the antimalarial compound artemisinin (Helfrich et al., 2019).

1.4.1 Historical discoveries and applications of antimicrobial natural products

Since antiquity, antimicrobial natural products from microbial sources have been used to combat pathogens that negatively affect human civilization (Simon et al., 2009). For example, the antibiotic properties of moldy bread were used by ancient Egyptians to heal wounds (Simon et al., 2009). Despite being used for millennia, the microbial origin and the molecular understanding of antimicrobial specialized metabolites remained unidentified until the last century: The serendipitous discovery of penicillin by Sir Alexander Fleming in 1928 catalyzed the strides accomplished during the golden era of antibiotic discovery (1950s-1970s). During this era, most of the antimicrobials that are still used today (e.g. streptomycin, neomycin, vancomycin, bacitracin) were largely discovered from environmental microbial strains (Lewis, 2012). Following this era, the high re-discovery rates of known compounds and the high costs of screening made antimicrobial natural product research and development less attractive to industry. This has caused a large discovery void in the last three decades (Zarins-Tutt et al., 2016). While recent efforts to discover novel antibacterials have successfully identified of over a dozen structural classes, antifungal discovery efforts have been fewer and not as rewarding (Roemer et al., 2011).

Partly because of lacking research incentives during the past, only polyenes and echinocandins have been discovered from natural sources (Ostrosky-Zeichner et al., 2010; Udwary et al., 2021). However, in the face of increasing AMR, the current need for new compounds is driving efforts to explore understudied natural reservoirs, including extreme ecosystems (Chávez et al., 2015; Jiang et al., 2014; Lopes et al., 2016; Sayed et al., 2020)

While the pharmaceutical sector's interest in antimicrobial natural products as a source of novel therapeutics declined over the last four decades, the agricultural sector increasingly pursued developing antimicrobial natural products as biological control agents (Raaijmakers et al., 2002). Public ecological concerns over the use of synthetic pesticides to combat phytopathogens continues to drive research and development of sustainable biological control agents (Raaijmakers et al., 2002). Bacterial genera capable of synthesizing specialized metabolites such as *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, and *Streptomyces*, have been found to control phytopathogens when applied to crop soil, or in certain, cases directly onto phyllospheres (Cesa-Luna et al., 2020; Weller, 1988; Whipps, 2001). Biocontrol agents typically involve treating crop soil or plant tissues with purified metabolites or live cell applications of beneficial bacteria (de Almeida Lopes et al., 2018). Such biocontrol treatments either directly inhibit the growth of phytopathogens and/or stimulate endogenous plant defense system, induced systemic resistance (ISR) (Berendsen et al., 2015; de Almeida Lopes et al., 2018; Morales-Cedeno et al., 2021; Weller, 2007).

1.4.2 Approaches to discover novel antimicrobial natural products

Equipped with novel culturing methods and next generation sequencing tools of the 21st century, a resurgence of antimicrobial natural product research is emerging (Zarins-Tutt et al., 2016). In the case of antimicrobial specialized metabolites, either culture-dependent or culture-independent

approaches may be used. With the advent of low-cost genomic sequencing in the 21st century, culture-independent techniques now represent keystone approaches in the search for novel antimicrobial specialized metabolites (Hover et al., 2018a; Katz et al., 2016). These approaches either involve sequence-based screening or functional metagenomic screening. Sequence-based (or homology-based) screening involves mining genomic or metagenomic DNA sequences *in silico* for specialized metabolite clusters BGCs homologous to sequences of previously characterized gene clusters encoding antibiotics (Malit et al., 2022). On the other hand, functional metagenomic approaches focus on capturing metagenomes in clone libraries which are then functionally screened for antimicrobial activity (Alam et al., 2021; Vester et al., 2015). The expectation of this approach involves capturing BGCs from metagenomes and expressing the specialized metabolite recombinantly in a suitable host (Banik & Brady, 2010; Ferrer et al., 2016; Katz et al., 2016; Vester et al., 2015). A successful application of heterologous BGC expression includes the discovery of the calcium dependent antibiotics, malacidins, that were found to exhibit inhibitory activity against MRSA and did not select for resistance under *in vitro* conditions (Hover et al., 2018a). The malacidins comprise a distinct class of antibiotics that require calcium for antibacterial activity (Kovalenko et al., 2021). They are commonly encoded in soil microbiomes but have never been successfully isolated using culture-based screening approaches. By cloning environmental DNA and expressing large BGC fragments in *E. coli*, bioactive malacidins were successfully isolated from clone libraries, illustrating the potential of capturing BGCs encoded within the genomes of fastidious organisms that cannot be cultured and screened using plate-based assays(Bauman et al., 2021; Hover et al., 2018a).

In the past, classical culture-dependent approaches involved screening organic extracts of cultured environmental strains for antibiosis (Lewis, 2012). In addition to high re-discovery

rates, another limitation of this approach includes the fact that BGCs are often not expressed in monoculture conditions - referred to as silent or cryptic BGCs (Thaker et al., 2013). Therefore, the majority of potentially novel specialized metabolites are undetected using the bioassays used in the previous century (Thaker et al., 2013). Despite these hurdles, recent novel culturing methods, such as the isolation chip (ichip), discussed further below, have improved the isolation rates of environmental microorganisms. This has once again brought attention to culture-dependent screening approaches for novel antibiotic discovery (Nichols et al., 2010).

1.4.3 Importance of Microbial Cultivation in Natural Product Discovery

Identifying and isolating novel antimicrobial compounds heavily depends on *in vivo* screening of cultivable strains (Vester et al., 2015). Yet, despite the significant strides made in modern science, the "great plate anomaly", a fundamental limitation in microbiology first observed over 119 years ago, remains largely unresolved (Winterberg, 1898). The term "great plate anomaly" was coined by Staley and Konopka in 1985 and refers to the discrepancy between total bacterial cells in a given environment compared to how many could be cultured on artificial media (Staley & Konopka, 1985). It has been estimated that a mere 0.1 to 1% of the total microbial species in the biosphere can be isolated using classical cultivation techniques (Staley & Konopka, 1985). This limitation in microbial cultivation arises from a number of contributing factors such as the: lack of specific nutrients, inappropriate incubation temperatures, unsuitable pH or osmotic conditions, incorrect oxygen levels, missing growth factors secreted by syntrophic organisms - or any combination thereof (X. Liu et al., 2021; Vartoukian et al., 2010).

The development of 16S rRNA next generation sequencing led to the realization that the portion of cultivable microorganisms does not accurately represent the community of the native

environment (Abellan-Schneyder et al., 2021; Olsen et al., 1986). This holds true both in terms of diversity and abundance of species (Dojka et al., 2000; Park et al., 2021). Instead, microorganisms capable of growing on artificial media represent the phylotypes that are best adapted to laboratory conditions (Prakash et al., 2021; Stewart, 2012). Although culture-independent molecular approaches have significantly improved our knowledge of microbial diversity and ecosystem composition, the need to culture microbes prevails (Prakash et al., 2021). Efforts to address the issue of the uncultivability of environmental microorganisms have either centered around developing novel culturing methods that better mimic the environment or have attempted to culture microbes in their natural environments *in situ (*Lewis et al., 2021*)*. Improved cultivation techniques are expanding the diversity of cultivable isolates that could be phenotypically screened for antimicrobial phenotypes (de Pascale et al., 2012; Lewis et al., 2021; Vester et al., 2015).

Isolation chip (ichip)

The ichip was initially developed in 2010 by Nichols *et al*. This device offers a high throughput platform for parallel cultivation of environmental microbes (Nichols et al., 2010). This multichannel assembly allows for simultaneous isolation and culturing of environmental microbial strains (Figure 2) (Nichols et al., 2010). It consists of three of hydrophobic plastic polyoxymethylene (DuPont, Delrin) plates (Nichols et al., 2010). The middle plate contains solid growth media where each well is inoculated with approximately one microbial cell from a diluted environmental sample (Nichols et al., 2010). The top and bottom layers are overlaid with 0.03 μm-pore-size, 47-mm polycarbonate membranes which cover the inoculated wells of the central plate (Nichols et al., 2010). Screws hold the three layers together and seal the assembly. Following inoculation, the ichip is placed in an environmental substrate for incubation. Only nutrients, growth factors from the native environment can diffuse through the assembly while

propagating microbes cannot exit (Goh et al., 2022; Nichols et al., 2010). Since its initial description, the core design ichip has been adapted to better suit a variety of applications that require microbial cultivation (Goh et al., 2022). For instance, a miniaturized version of the ichip made of surgical steel has been used to culture the human oral microbiota whereas NovoBiotic Pharmaceuticals uses a proprietary industrial-scale adaptation of the ichip as the principal cultivation tool to isolate and screen microorganisms for novel antimicrobials (Berdy et al., 2017; Sizova et al., 2012).

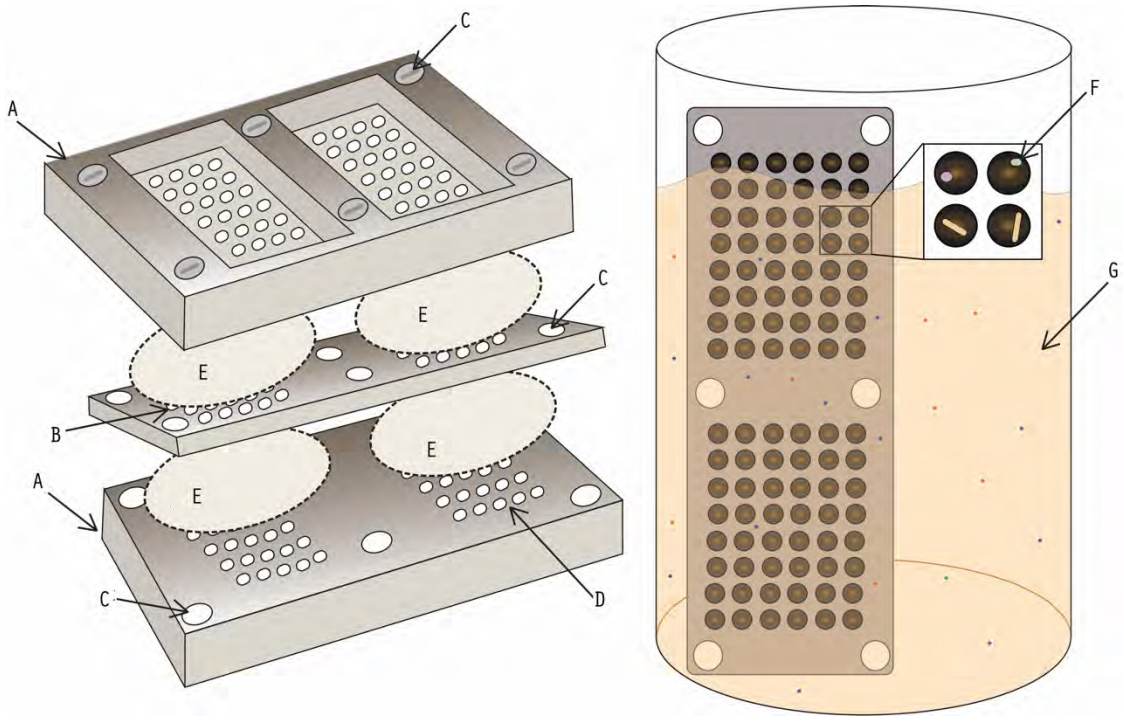

Figure 2. Isolate chip (ichip). A) Hydrophobic plastic polyoxymethylene plate (Du- Pont) (72 by 19 by 1 mm). B) Central plate where environmental cells suspended in agar contained in through holes. C) Screws and screw holes. D) Through holes where nutrients can diffuse through the semipermeable membranes and reach propagating cells in the central plate. E) Semi-permeable hydrophilic polycarbonate membranes (with 0.03 μm pores). F) Propagating environmental microorganisms G) Substrate (either environmental or simulated). Illustration adapted from Nichols et al. 2010.

In an initial study, Ling *et al.* 2015 used the ichip method to discover Teixobactin - a new class of antibiotic effective against drug-resistant Gram-positive bacteria. This unusual depsipeptide,

inhibits cell wall synthesis by binding to precursors of peptidoglycan and cell wall teichoic acid (lipid II and III respectively) (Ling et al., 2015a). In this study, grassland soil was used to inoculate ichips. Chemical extracts from 10,000 isolates reared in ichips were screened for antimicrobial activity against *Staphylococcus aureus.* The extract from a novel species of Betaproteobacteria, *Eleftheria terrae,* a class of bacteria not previously known to express antimicrobials, was found to express this new antibiotic molecule (Ling et al., 2015a). The discovery of this new antibiotic compound is a testament of how the discovery rate of new natural products will predictably increase as the capacity of novel culturing methods to isolate a larger portion microbial diversity broadens.

The first application of an ichip in a cryoenvironment was conducted by Goordial *et al.* 2017. In this study, a larger field prototype of the ichip (dubbed cryo-iplate) was used to cultivate microbes *in situ* within polygonal active layer permafrost soils (J. Goordial, 2017). This soil overlays ice wedges in polygonal permafrost. The cryo-iplate prototype design was inspired by the initial ichip by Nichols *et al.* 2010. Instead of using three hydrophobic plastic polyoxymethylene plates, this study used disassembled 200 µl-pipette-tip boxes to create the device assembly (J. Goordial, 2017). Using this technique, 39 microbial isolates were isolated including several that were identified as putatively novel through 16S rRNA gene sequencing (J. Goordial, 2017). One such example included a novel *Pedobacter* sp. displaying 98% closest similarity in GenBank) was isolated (J. Goordial, 2017). This study demonstrates the versatility of the ichip-like devices in cryoenvironments and the potential of recovering new microbial isolates in Arctic soils.

Connecting Text: Chapter 3

The aim of Chapter 3 was to isolate, screen and genomically mine bacterial isolates from various high Arctic habitats for antibacterial natural products. The Canadian high Arctic remains an understudied and underexploited frontier for natural product discovery. By using two cultivation approaches, followed by dual-culture screening and genomic mining, this chapter demonstrates how certain cultivable Arctic bacteria have the functional and biosynthetic capacity to produce potentially novel antibacterial metabolites. The cultivation approaches involved deploying an *in situ* cultivation device that I designed and engineered and an innovative crowdsourcing initiative where 120 undergraduate researchers partook in isolating bacterial strains from environmental Arctic samples that I collected. These antibacterial products inhibited clinically relevant bacterial pathogens highlighting their potential applicability as candidate therapeutic agents.

This chapter appears in:

Marcolefas, E., Leung, T., Okshevsky, M., McKay, G., Hignett, E., Hamel, J., Aguirre, G., Blenner-Hassett, O., Boyle, B., Lévesque, R.C. and Nguyen, D., 2019. Culture-dependent bioprospecting of bacterial isolates from the Canadian high Arctic displaying antibacterial activity. Frontiers in microbiology, 10, p.1836. (In press)

Chapter 3. Culture-dependent bioprospecting of antibiotic producing bacteria isolated from the Canadian high Arctic.

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Abstract

Background & Objective

The goal of this study was to isolate, screen, and characterize Arctic microbial isolates from Expedition Fjord, Axel Heiberg Island, Nunavut, Canada capable of inhibiting the growth of foodborne and clinically relevant pathogens.

Methods

Arctic bacteria were isolated from twelve different high Arctic habitats pertaining to active layer permafrost soil, saline spring sediments, lake sediments, and endoliths. This was achieved using (1) the cryo-iPlate, an innovative *in situ* cultivation device within active layer permafrost soil and (2) bulk plating of Arctic samples by undergraduate students that applied standard culturing. To mitigate the possibility of identifying isolates with already-known antibacterial activities, a cell-based dereplication platform was used.

Results & Conclusion

Ten out of the twelve Arctic habitats tested were found to yield cold-adapted isolates with antibacterial activity. Eight cold-adapted Arctic isolates were identified with the ability to inhibit the entire dereplication platform, suggesting the possibility of new mechanisms of action. Two promising isolates, initially cultured from perennial saline spring sediments and from active layer permafrost soil (*Paenibacillus sp.* GHS.8.NWYW.5 and *Pseudomonas sp.* AALPS.10.MNAAK.13 respectively), displayed antibacterial activity against foodborne and clinically relevant pathogens. *Paenibacillus sp.* GHS.8.NWYW.5 was capable of inhibiting methicillin resistant and susceptible *Staphylococcus aureus* (MRSA and MSSA), *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli* O157:H7. *Pseudomonas sp.*

AALPS.10.MNAAK.13 was observed to have antagonistic activity against MRSA, MSSA, *Acinetobacter baumanii*, *Enterococcus faecium*, and *Enterococcus faecalis*. After whole genome sequencing and mining, the genome of *Paenibacillus sp.* GHS.8.NWYW.5 was found to contain seven putative secondary metabolite biosynthetic gene clusters that displayed low homology (<50% coverage, <30% identity, and e-values >0) to clusters identified within the genome of the type strain pertaining to the same species. These findings suggest that cold-adapted Arctic microbes may be a promising source of novel secondary metabolites for potential use in both industrial and medical settings.

Introduction

The rise of antibiotic resistance is one of the most urgent challenges the world currently faces. Antimicrobial resistance has steadily increased in clinical settings (Bitnun & Yeh, 2018).We are on the cusp of returning to a pre-antibiotic world in which common infections and minor injuries will once again become deadly (Ferri et al., 2017; Laxminarayan et al., 2013; V Pawar et al., 2017). Simultaneously, natural product discovery efforts on the part of the pharmaceutical industry have largely dwindled since the end of the 20th century (Baker et al., 2007). Given that the current arsenal of effective antibiotics is decreasing, innovative discovery workflows for the identification of novel antibiotics are needed. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* species (ESKAPE) are recognized by the Infectious Disease Society of America as the bacteria posing the most significant risk to public health in the United States (Boucher et al., 2009). The ESKAPE pathogens are responsible for the majority of nosocomial infections in the United States, with an estimated 722,000 infections acquired in 2011 (Magill et al., 2014). Of particular concern are the increasing levels of antibiotic resistance occurring in these organisms, especially

methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium*, and fluoroquinolone-resistant *P. aeruginosa* (System, 2004). MRSA infections are now responsible for more deaths annually in U.S. hospitals than HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008; Klevens et al., 2006).

Many of the antibiotics currently in use are secondary metabolite natural products from soil bacteria, particularly from the *Streptomyces* genus. Since the pioneering experiments of Selman Waksman in the 1940's, this resource has been extensively studied (Schatz et al., 1944; Waksman & Woodruff, 1940). New approaches or modification to existing methods may be necessary to increase the probability of finding novel compounds. Once an isolate with antibiotic activity is identified, a "dereplication" procedure is required to avoid the re-discovery of already-known antibiotics. Compound extraction, purification and biochemical analyses are costly and technically challenging. Instead, we have employed a cell-based dereplication platform (Cox et al., 2017), in which isolates are tested for inhibitory activity against a panel of *E. coli* strains expressing specific antibiotic resistance genes. The presence of inhibitory activity against all dereplication strains that comprise the Antibiotic Resistance Platform (ARP) suggests that the isolate produces antimicrobial secondary metabolite(s) with a potentially novel mechanism of action. As an additional dereplication measure, genomic sequencing and in *silico* genome mining could be used to prioritize isolates for downstream testing. Microbial genomic sequences could be mined *in silico* to detect secondary metabolite biosynthetic gene clusters (BGCs) using open source web-based pipelines such as the antibiotics and secondary metabolite analysis shell (antiSMASH) (Blin et al., 2019). Once microbial genomes have been mined, isolates can be prioritized on the basis of low BGC sequence homology to known clusters (Medema et al., 2015).

In the search for new antibiotics, interest has been growing in underexplored environments such as marine systems (Jang et al., 2013; Machado et al., 2015) and the deep biosphere (Orsi et al., 2013). The Canadian high Arctic is characterized by extreme environmental conditions such as high salt and prolonged sub-zero temperatures. The unique ecological niches (i.e. hypersaline springs, permafrost and endoliths) of the high Arctic harbour diverse microbial communities which remain largely unexplored (Lay et al., 2013; Perreault et al., 2007; Perreault et al., 2008; Steven et al., 2007). Recent macro- and microdiversity studies have revealed that Arctic microbiomes do not always follow the latitudinal diversity paradigm (Gregory et al., 2019). Arctic environments have been observed to be a cradle for microbial diversity with biodiversity patterns that are in certain cases analogous to ones from macro-organisms (Gregory et al., 2019). Due to the rich microbial diversity and unique selective pressures experienced by the Arctic microbiome, we hypothesize that this extreme environment has potential for harbouring novel antibacterial secondary metabolites. Previous bioprospecting studies conducted in polar cryohabitats have successfully identified microbial isolates expressing new natural products and to encode unknown secondary metabolite BGCs (Dhaneesha et al., 2017; Mitova et al., 2005; Tedesco et al., 2016). One such example includes the discovery of *Streptomyces artemisiae* MCCB 248 isolated from sediments in the Arctic fjord Kongsfjorden. This isolate was shown to produce secondary metabolites with anticancer properties and was found to encode unique polyketide synthetase (PKS) and non-ribosomal peptide (NRP) genes (Dhaneesha et al., 2017). Isolating secondary-metabolite-producing microbes from Arctic environments has the additional benefit of yielding strains with inhibitory activity at cold temperatures. Cold-active antimicrobial enzymes - such as cold-active alkaline phosphatases with antibiofilm activity - represent

appealing biopreservatives for food processing industries since they can extend the shelf life of refrigerated consumables (Balabanova et al., 2017).

Identifying and isolating new antibacterial secondary metabolites often depends on cell-based screening of cultivable strains. Unfortunately, only a small percentage of all microbial species can be isolated using classic cultivation techniques (Ward et al., 1990; Winterberg, 1898). Co-culture assays are known to activate silent BGCs in antibiotic-producing strains that would not be expressed in pure culture (Zarins-Tutt et al., 2016). Innovative cultivation techniques are also expanding the portion of cultivable isolates that could be phenotypically screened using cell-based assays (Vartoukian et al., 2010). The efficacy of such *in situ* cultivation techniques for drug discovery is illustrated by the recent discovery of the antibiotic Teixobactin, derived from an ichip isolate (Ling et al., 2015b). Based on the initial design of the ichip, the cryo-iPlate prototype designed and implemented by Goordial *et al.* (Goordial et al., 2017) was the first *in situ* cultivation device deployed in the Arctic. Here, a re-designed and updated version of the cryo-iPlate is described for the first time and is used to isolate Arctic microorganisms for antibacterial screening. In addition to sampling unique ecological habitats and implementing the new cryo-iPlate cultivation device, the bioprospecting workflow described here uses a crowd-sourced screening approach first developed by Jo Handelsman at Yale University in 2012, which harnesses the manpower of undergraduate microbiology teaching labs to screen bacterial isolates for antibiotic activity (Davis et al., 2017).

Here, we employed two cultivation methods to isolate and then screen bacteria derived from Canadian high Arctic habitats for antibacterial activities (Figure 3). Arctic isolates were screened for antibiotic activity against a collection of both foodborne and clinical pathogens. Cold-adapted antibiotic-producing bacteria were derived from Arctic permafrost, saline spring sediments, and

cryptoendoliths suggesting that high Arctic environments could be a potentially untapped source of novel antibacterial secondary metabolites.

Materials and methods

Sampling Arctic microbiomes

The various soil and rock samples used in this study were collected within the vicinity of the McGill Arctic Research Station (MARS) on Axel Heiberg Island, Nunavut, in the Canadian high Arctic. The sediment, soil and rock samples were collected for crowdsourced bulk plating whereas the *in situ* cryo-iPlate cultivation approach was only applied to active layer permafrost soil (described below). Sediments from spring outflow channels and soil in this study were collected with an ethanol-sterilized spatula used to exhume the first 15 cm of soil/sediment horizons. Rocks with visible lithic microbial biomass were sampled by breaking large rocks into smaller pieces using an ethanol-sterilized hammer and chisel. All rock and soil samples were collected in sterile WhirlPak bags and stored in coolers kept at 5°C until returned to McGill University. Upon return to the laboratory, all samples were stored at 5°C.

Cryo-iPlate procedures

The cryo-iPlate design was based on the Nichol *et. al* 2015 ichip and the Goordial *et al.* 2017 cryo-iPlate prototype (Goordial et al., 2017; Nichols et al., 2010). It was designed using the Rhino 6 software and 3D-printed at Fablab Inc., Montreal, using durable PC-ISO polycarbonate plastic. Previous prototypes of the cryo-iPlate such as the one described in Goordial *et al.* 2017, consisted of empty pipette boxes that were not as durable in the field. The dimensions of the improved version of the cryo-iPlate described herein are 15 cm by 12 cm. It features 160 wells (as opposed to 96 in previous prototypes) that are 0.5 cm wide and deep. The wells of the central plate were filled with 2% w/v gellan gum (Alfa Aesar) prior to being deployed in the field. Based

on previous studies of total microbial counts in active layer permafrost at the MARS study site, dilutions of the soil were made in the field using sterile water (Wilhelm et al., 2011). This device was only applied to active layer permafrost. Each well of the central plate was inoculated in the field with 10 µl of diluted soil containing ~1-10 microbial cells. The top and bottom layers were overlaid with sterile semi-permeable 0.03 µm-pore-size polycarbonate membranes (WhatmanTM, GE healthcare Life Sciences) and were glued using a silicon adhesive (DuPont). The assembly of three plates was then screwed together using eight stainless steel 10-24 screws and left to incubate *in situ*. After incubating in the field for 10 days, the cryo-iPlate was collected in a large sterile Whirl-Pak sampling bag along with the soil in which it was incubating. The assembly was stored in a cooler at 5°C during transport back to McGill University where it was subsequently incubated *ex situ* at 5°C for three months before being disassembled for subculturing. After disassembling the cryo-iPlates, well contents from the central plate were pushed into individual Eppendorf tubes containing 0.1% w/v pyrophosphate using sterile 1000 µl pipette tips (Diamed Canada). Eppendorf tubes containing well contents from the cryo-iPlate in buffer solution were vortexed for 30 seconds and 100 µl of the solution was used to inoculate plates of ½Reasoner's 2A (R2A) broth media solidified with 2% gellan gum (Alfa Aesar). The plates were incubated at room temperature for two weeks. Morphologically distinct colonies (selected based on distinct colony form, elevation, margin, size, texture and colour) that grew on the ½ R2A plates were subcultured three times to attain clonal populations before being screened against the ESKAPE relative strains.

Crowdsourced screening of isolates against ESKAPE pathogen relatives

The twelve sample types described in Table 1 were processed by students in the Introduction to Microbiology Laboratory Course at McGill University in the following manner: One gram of

sample was serially diluted in sterile water to 10^{-5}, and 100 μL of each dilution were spread onto lysogeny broth (LB) plates solidified with gellan, tryptic soy agar (10% or 0.1%), potato dextrose agar or sheep blood agar. Each student pair participating in the course was provided with one of the Arctic samples and was allowed to select one of the culturing media to increase the diversity of isolates obtained. Plates were incubated for 1 week at room temperature and morphologically distinct colonies were picked (selected based on colony form, elevation, margin, size, texture and colour) and re-streaked to produce libraries of clonal populations. Isolates were screened against ESKAPE pathogen relatives (Table S1) using the spread-patch technique (Figure 4.A). Since antibiotic activity screening is performed in teaching labs, isolates were tested against non-pathogenic relatives of high priority threat pathogens. A diluted suspension of tester bacteria was evenly spread across on agar plate, and the Arctic isolates spotted on top. Plates were incubated for one week at room temperature and observed for a zone of pathogen inhibition surrounding isolates. All isolates that produced a zone of inhibition were given a name and stored as glycerol freezer stocks and selected for dereplication. If isolates were surrounded by a visually discernible zone of clearance devoid of any tester strain growth, then they were deemed to have positive antibacterial activity (Figure 4.A).

Taxonomic identification of isolates

Isolates from the cryo-iPlate and from the crowdsourced bulk soil plating that displayed zones of inhibition against at least one of the ESKAPE relatives were selected for 16S rRNA gene sequencing. Colony PCR was first conducted to amplify the 16S rRNA gene using the 27F (forward) bacterial primer: 5'-AGAGTTACCTTGTTACGACTT-3' and the 1492R (reverse) bacterial primer: 5'-GGTTACCTTGTTACGACTT-3'. The 16S rDNA amplification PCR reaction cycling program consisted of 1) 95 ºC for 7 minutes, 2) 94 ºC for 45 seconds, 3) 55ºC

for 45 seconds, 4) 72 ºC for 1 minute, (where steps 2 to 4 were repeated 30 times), 5) 72 ºC for 10 minutes. PCR products from the cryo-iPlate and from the bulk soil plating were sent for Sanger sequencing at the Plateforme d'Analyses Genomiques sequencing center at Laval University and the McGill University and at the Génome Québec Innovation Centre (respectively). All generated sequences were manually curated using 4Peaks software (http://nucleobytes.com/4peaks/) where sequence segments with an average Q>40 were used as queries against the EzBioCloud database for taxonomical identification (Yoon et al., 2017).

Dereplication platform

Isolates that demonstrated antibacterial activity against at least one ESKAPE relative were tested against a dereplication platform described in Table S2, and provided by the Wright lab at McMaster University (Cox et al., 2017). This dereplication platform consists of two parental strains: Wild type *E. coli* BW25113, and *E. coli* Δ*bamB* Δ*tolC* BW25113 harbouring mutations to increase outer membrane permeability and decrease efflux pump activity, thereby increasing its susceptibility to antibiotics. In all cases except the *fluB* mutant, the Wright lab transformed these parent strains with plasmids harbouring antibiotic resistance genes. Isolates were screened against the dereplication strains using the wagon-wheel assay, in which dereplication strains were streaked in a circle to form the "wheel" on LB plates, and Arctic isolates were streaked across the wheel to form "spokes" (Figure 4.B). Plates were incubated for one week at room temperature and it was subsequently observed in which cases the isolates were able to inhibit the growth of the dereplication strain. Isolates able to inhibit the growth of all dereplication strains were considered to have "passed."

Screening Arctic isolates that passed the ARP against ESKAPE pathogens

Arctic isolates that passed the ARP were screened for inhibitory activities against a panel of ESKAPE pathogens, namely *Enterobacter cloacae* (strain CO794 provided by the G. Wright laboratory), methicillin-resistant *S. aureus* (MRSA) (ATCC 43300), methicillin-susceptible *S. aureus* (MSSA) (ATCC 29213), *Klebsiella pneumoniae* (clinical strain HO132323 provided by the G. Wright laboratory), *Acinetobacter baumanii* AB5075, *P. aeruginosa* WT PAO1, *Enterococcus faecium* (ATCC 700221) and *Enterococcus faecalis* (ATCC 29212), using the wagon-wheel co-culture technique as described above (Figure 4.B). Frozen glycerol stock cultures of the Arctic isolates and of the tester pathogens were streaked on LB plates. The pathogen tester strains were incubated at 37°C for 18 hours whereas Arctic isolates were incubated at room temperature for 48 hrs. Once sufficient growth appeared on plates, liquid cultures of the Arctic isolates and the pathogenic tester strains were made using LB media. Liquid cultures of the Arctic isolates were incubated with agitation at 250 rpm for 48 hrs. at room temperature whereas pathogenic tester strains were incubated with agitation at 250 rpm for 24 hrs at 37°C. Loopfuls of the pathogenic strains in liquid culture were streaked in a circular line on a LB plate, while the Arctic isolates were streaked outwards starting from the middle of the plate (Figure 4.B). Triplicates of these co-culture plates were incubated at room temperature, and observed after 24, 48, and 72 hours of incubation. Regions of contact between the Arctic isolate and pathogenic tester strain in which the tester strain did not produce visible growth were recorded as inhibition.

Assessing growth of Arctic isolates that passed the ARP at various temperatures

Liquid TSB cultures of Arctic isolates were incubated with agitation at 250 rpm for 48 hrs at room temperature and then streaked on tryptic soy agar (TSA) plates. Inoculated plates were then

incubated at 37°C, 25°C, 10°C, 5°C, 0°C, -5°C, -10°C. TSA media intended for sub-zero incubation was amended with 3% v/v glycerol to avoid complete freezing of the media. Growth was visually assessed after 48 hrs of incubation at 37°C and 25°C. The remaining incubations were assessed for growth after 20 days.

Screening Arctic isolates that passed the ARP against foodborne pathogens

In addition to screening against clinical pathogens, all Arctic isolates that passed the dereplication assay were screened against foodborne pathogens using an overlay co-culture assay (Figure 4.C). Liquid TSB cultures of Arctic isolates were incubated with agitation at 250 rpm for two days at room temperature and then streaked in a line in the center of the TSA plates. The TSA plates were then left to incubate at room temperature for seven days. Following incubation, each Arctic isolate was overlaid with 7 ml of molten agar media inoculated with 100 µl of overnight liquid cultures of Staphylococcus *aureus* WT, *Listeria monocytogenes* HPB# 1870 serotype 1/2c, *Salmonella enterica* serovar Heidelberg, and *E. coli* 0157:H7. To assess whether any of the secreted secondary metabolites produced by the Arctic isolates retained inhibitory activity at human body and standard refrigeration temperatures, the overlay plates were incubated at 37°C and 5°C respectively. Zones of inhibition were observed after 24 hrs of incubation for plates incubated at 37°C and after 20 days for those incubated at 5°C. This assay inherently relies on the diffusion of secreted molecules within the cultivation media's semisolid agar matrix. Specifically, to observe zones of clearance, diffusible biomolecules are required to reach the top agar layer to inhibit the tester strains. This co-culture assay was conducted in triplicate and an established antibiotic-producing strain, *Paenibacillus terrae* NRRL B-30644, was used a positive control. This environmental strain has been previously reported to produce zones of inhibition in co-culture assays against foodborne pathogens (van Belkum et al., 2015).

The zones of inhibition produced by *P. terrae* NRRL B-30644 served as a visual reference for positive inhibitory activity for all the co-culture assays conducted in this study (Figure 4.C).

DNA sequencing, genome assembly, and analyses of isolates passing the ARP

Bacterial isolates were inoculated from single isolated colonies into R2A broth and grown at room temperature for periods ranging from 12 to 72 hours. The genomic DNA was extracted using the E-Z 96 Tissue DNA Kit (Omega Bio-tek, Norcross GA, USA). Between 250 and 700 ng of DNA were fragmented via sonication using a Covaris M220 (Covaris, Woburn MA, USA) for 40 seconds at 18-22°C. The peak power was set at 50.0, duty factor at 20.0 and the number of bursts at 200. The libraries were prepared using the NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs, Ipswich MA, USA) following the manufacturer's instructions. The libraries were barcoded with TruSeq HT adapters (Illumina, San Diego CA, USA) and sequenced using an Illumina MiSeq 300 bp paired-end run at the Plateforme d'Analyses Génomiques at the Institut de Biologie Intégrative et des Systèmes (Université Laval, Québec, Canada). The raw reads were assembled using the A5 pipeline (Tritt et al., 2012). Assemblies were validated using the metagenomic identification software Centrifuge and contig uniformity was verified to confirm that each assembly originated from the same source DNA and from not contaminants (Kim et al., 2016). To taxonomically identify the isolates that inhibited the ARP, their assembled genomes were first annotated using the Rapid Annotation using Subsystem Technology (RAST) version 2.0 using standard parameters (Aziz et al., 2008; Brettin et al., 2015b; Overbeek et al., 2013). Following annotation, full 16S rRNA gene(s) were identified within each genome and subsequently used as a query sequences in the EzBioCloud database for corroborating taxonomical identification (Yoon et al., 2017). To predict the secondary metabolite biosynthetic gene clusters within the genomes of the eight isolates of interest, all genomes were

mined using the antiSMASH database v.4 using default search parameter settings (Blin et al., 2017; Medema et al., 2011)The BGCs of the isolates showing the broadest activity spectra were used as query sequence in a blastn alignment against genomes of type strains of the same species: *Pseudomonas prosekii* LMG 26867 (genome accession GCA_900105155.1), and *Paenibacillus terrae* NRRL B-30644 (accession GCA_000943545.1). The assembled genomes of these two promising isolates were deposited at GenBank under the BioSample accessions (SAMN12211638 and SAMN12211639).

Organic extraction using liquid culture supernatants from two promising isolates

To confirm whether the antagonistic activities of promising isolates were caused by secreted secondary metabolites of bacterial origin, organic extracts using liquid culture supernatants were prepared. Isolates were incubated overnight in TSB media at room temperature with agitation at 250 rpm. Following overnight incubation, 14 mL of the cultures were mixed with 7.5 mL of ethyl acetate in a 50 mL Falcon tube (giving a final volume ratio of 2:1 culture supernatant to ethyl acetate). Solutions were vortexed on the highest setting until solutions were homogenized and then centrifuged at 7830 rpm for 15 minutes at room temperature. Following centrifugation, the organic layers were removed from both solutions and were collected in separate pre-weighed 7.5 mL borosilicate glass culture tubes using a Pasteur pipette. The ethyl acetate was then left to evaporate for 20 min by direct air filtered with a 0.3 μm HEPA filter (Whatman #0974479). After the solvent fully evaporated, both extracts were resuspended in 200 μL of methanol. To assess whether the crude organic extracts retained antibacterial activity, 30 μL of the extracts were spotted on agar plates. Once the methanol fully evaporated from the agar, 7 ml of molten agar containing the tester strains (*E. coli* ATCC 11775 and *E. coli* Δ*bam*Δ*tolC* BW25113) were poured over the dried extract spots. Control spots consisting only of methanol were plated to

confirm that the observed zones of clearance did not arise from the solvent used to resuspend the extract. The plates were then incubated at room temperature for 24 hrs before being observed for zones of clearance.

Results

Antibiotic activity of Arctic isolates against ESKAPE pathogen relatives

Twelve environmental samples were collected in the regions of Expedition Fiord, Gypsum Hill, Lost Hammer, Color Lake, and White Glacier on Axel Heiberg Island in the Canadian high Arctic. The sampled habitats included active layer permafrost, sediments, and lithic communities. Active layer permafrost samples included trough soil from polygonal tundra terrain, surface soil from polygonal tundra terrain, arid soil, and hummock soil. Sediment samples included perennial hypersaline spring outflow channel sediment, acidic freshwater lake sediment, acidic freshwater lake sediment covered with microbial mats, perennial saline spring sediment, and sediment from glacial terminus moraines. Lithic communities included gypsum cryptoendoliths and unknown rock cryptoendoliths (Table 1). All these samples were distributed to the undergraduate students for traditional plate cultivation, and 3120 distinct colonies were subcultured and further tested. After co-culture screening, 54 isolates (1.7%) were identified as having antibacterial activity against at least one ESKAPE pathogen relative. Of these, three isolates originated from acidic freshwater lake sediment covered with microbial mat, four from a gypsum cryptoendolith, seven from Gypsum Hill hummock active layer permafrost, three from Gypsum Hill perennial saline spring sediment, one from a cryptoendolith collected at Lost Hammer, 25 from active layer tundra permafrost, seven from arid active layer permafrost, two from polygonal terrain surface soil, one from polygonal terrain trough soil, and one from glacial terminus moraine sediment (Table 2). The genera represented by these 54 isolates were

Pseudomonas (27 isolates), *Bacillus* (5), *Nocardia* (6), *Janthinobacterium* (3), *Streptomyces* (3), *Paenibacillus* (2), *Mycetocola* (1), *Flavobacterium* (1), *Micrococcus* (1), *Curtobacterium* (1), *Arthrobacter* (1), *Pseudoarthrobacter* (1), *Rahnella* (1), and *Siccibacter* (1).

Microbial cultivation using the cryo-iPlate incubated in active layer permafrost soil within the vicinity of the MARS lead to the isolation of ~300 morphologically distinct colonies. A total of 16 isolates (~5%) exhibited antibacterial activity against at least one ESKAPE pathogen relative. All cryo-iPlate strains with antibacterial activity only displayed zones of inhibition against *P. putida* except for *Pedobacter* isolate B7.1 that inhibited *S. epidermidis,* and *Flavobacterium* strain C9.2 that inhibited the growth of *E. coli* and *P. putida* (Table 3). Genera represented by these 16 isolates were *Pseudomonas* (4 isolates), *Pedobacter* (3), *Flavobacterium* (3), *Janthinobacterium* (2), *Agreia* (1), *Pararhizobium* (1), *Sphingomonas* (1), and *Stenotrophomonas* (1) (Table 3). The genera isolated by both the classical soil plating approach and the cryo-iPlate were *Pseudomonas, Flavobacterium* and *Janthinobacterium*. Among both bulk plated and cryo-iPlate isolates, *Pseudomonas* represented the genus with the highest proportion of total isolated strains (50% of total bulk plated isolates and 25% of total cryo-iPlate isolates).

Antibacterial activity of Arctic isolates that passed the ARP against clinical pathogens

Eight (8) out of the total seventy (70) antibiotic-producing isolates (11%) were found to inhibit the growth of all strains comprising the ARP. These strains were prioritized for further investigation. To assess whether these isolates could inhibit the growth of clinically significant pathogens, the isolates were screened against ESKAPE organisms *P. aeruginosa*, MRSA, *A. baumanii,* MSSA, *E. cloacae*, *K. pneumoniae*, *E. faecium*, and *E. faecalis*. *Pseudomonas sp.* AALPS.10.MNAAK.13 was observed to have the broadest antibacterial activity spectrum. It

inhibited the growth of MRSA, *A. baumanii*, MSSA, *E faecium*, and *E. faecalis* (Table 5). *Flavobacterium sp.* GHHS.3.LBZX.4 and *Pseudomonas sp.* MAL.4.ABES.21 were not observed to inhibited any of the pathogenic tester strains. *Pseudomonas sp.* MAL.10.WYTK.25, *Pseudomonas sp.* AALPS.4.MSMB.5 and *Bacillus sp.* C11E23 inhibited the growth of MRSA, MSSA, and *E. faecium*. *Paenibacillus sp.* GHS.8.NWYW.5 was capable of inhibiting MRSA and MSSA. Pseudomonas sp. GHCE.5JVZL.12 inhibited the growth of MRSA, *A. baumanii*, MSSA, and *K. pneumoniae*.

Growth of Arctic isolates that passed the ARP at various temperatures

After screening against clinically significant pathogens, we assessed whether the eight prioritized isolates were capable of growing at various temperatures (37, 25, 10, 5, 0, -5 °C). All eight isolates were capable of growth at 5°C. Only two isolates (*Paenibacillus sp.* GHS.8.NWYW.5 and *Pseudomonas sp.* GHCE.5.JVZL.12) could grow at 37°C after 48 hrs of incubation. All isolates except *Pseudomonas sp.* GHCE.5.JVZL.12 grew at 0°C after 20 days of incubation. Apart from, *Flavobacterium sp.* GHHS.3.LBZX.4 and *Pseudomonas sp.* GHCE.5.JVZL.12, all other Arctic isolates that passed the ARP were capable of growing at -5°C after 20 days of incubation (Table S3).

Antibacterial activity of prioritized isolates against foodborne pathogens

The eight prioritized isolates were tested for antibacterial activity against four foodborne pathogens (*S. aureus*, *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7 at two different incubation temperatures (37 and 5°C). *Paenibacillus sp.* GHS.8.NWYW.5 was observed to inhibit the growth of *S. aureus*, *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7 at 37°C and 5°C. *Pseudomonas sp.* AALPS.10.MNAAK.13 was observed to inhibit *S. aureus* at 37°C after 24 hrs of incubation. *Pseudomonas. sp.* MAL.10.WYTK.25 was observed to inhibit the growth

of S. *enterica at* 37°C after 24 hrs. *Bacillus sp.* C11E23 inhibited *L. monocytogenes* at 37°C and 5°C (Table 6). The zones of inhibition produced by these Arctic isolates were consistent with the known antibiotic-producing strain *P. terrae* NRRL B-30644 which displayed inhibitory activity against all foodborne pathogens used in this assay.

Genomic analysis of bacterial isolates capable of inhibiting the entire ARP

After the Illumina MiSeq 300 bp paired-end run, the range of number of raw reads was 665502-2929247 and the fragment size range was 400-2000 bp (Supplementary Table S4, Figure S1). The genomes of the eight prioritized isolates were then mined using the antiSMASH database to identify secondary metabolite gene clusters that they encode (Table 4). These genomes contained ≥ 6 putative BGCs that were homologous to NRP, PK, bacteriocin/ ribosomally synthesized and post-translationally modified peptide (RiPP), and trans-polyketide synthetase (trans-PKS). All clusters labelled as "other" corresponded to either aryl polyene, terpene, betalactone, siderophore, N-acetylglutaminylglutamine (NAGGN) amide or phosphonate clusters (not shown in table). The genome of *Paenibacillus sp.* GHS.8.NWYW.5 featured the greatest number of BGCs compared to all other isolates with 23 secondary metabolite clusters. These clusters included 17 NRP clusters, three bacteriocin/ RiPP clusters, one trans-PKS cluster, and two other clusters (one siderophore and one phosphonate cluster). The isolate featuring the least number of BGCs within its genome was Flavobacterium *sp*. GHHS.3.LBZX.4 with a total of six secondary metabolite clusters. These included one type-3 PK, one NRP and four other clusters (one aryl polyene cluster, two terpene clusters, and one betalactone cluster). *Pseudomonas sp.* AALPS.10.MNAAK.13 and *Paenibacillus sp.* GHS.8.NWYW.5 represent the two isolates that displayed the broadest spectra of antibacterial activities against foodborne and clinical pathogens. Eight BGCs were identified within the genome of *Pseudomonas sp.*

AALPS.10.MNAAK.13 using antiSMASH (Table 7). After conducting a blastn alignment between the isolate's clusters with the *Pseudomonas prosekii* LMG 26867 type strain genome, none of the detected clusters displayed low homology within the type strain genome. The genome of the *Paenibacillus sp.* GHS.8.NWYW.5 featured 23 BGCs (Table 7). The clusters with low homology (<50% coverage, <30% identity, and e-values >0) to the type strain genome of *Paenibacillus terrae* NRRL B-30644 included: one NRP cluster displayed 11% coverage and 98% identity, one NRP cluster matching to pelgipeptin displayed 45% coverage and 97% identity, one NRP cluster displayed 41% coverage and 75% identity, and two unknown NRP clusters that did not have any significant homology with the type strain genome.

Organic extraction using liquid culture supernatants

Organic extracts using liquid culture supernatants from isolates *Paenibacillus sp*. GHS.8.NWYW.5**,** *Pseudomonas sp*. AALPS.10.MNAAK.13, *Pseudomonas sp*. AALPS.10.EMMH.23, *Pseudomonas sp*. AALPS.10.JKNJ.7, *Pseudomonas sp*. AALPS.4.MSMB.5, *Pseudomonas sp*. GHCE.5.JVZL.12, *Pseudomonas sp*. MAL.10.WYTK.25 (same isolate as 7.EKIG.16 in) were prepared using ethyl acetate. The crude organic extracts were then tested for retained antibacterial activity using a plate-based spot assay against the parental strains of the ARP (*E. coli* Δ*bam*Δ*tolC* BW25113 and *E. coli* BW25113). After 24 hrs. of incubation at room temperature, the extracts of *Pseudomonas sp*. AALPS.10.MNAAK.13, *Pseudomonas sp*. AALPS.10.EMMH.23 and *Paenibacillus sp*. GHS.8.NWYW.5 exhibited zones of inhibition against *E. coli* Δ*bam*Δ*tolC* BW25113 (.B).

Discussion

Arctic bacteria demonstrate antibiotic activity against ESKAPE pathogen relatives

Bacteria capable of inhibiting ESKAPE pathogen relatives were isolated from all sampled Arctic environments, apart from perennial hypersaline spring sediment sample from Lost Hammer. A total of 70 antibiotic producing isolates were identified, with 54 coming from a classical bulk soil plating approach, and 16 from cryo-iPlate cultivation. More isolates were obtained from the bulk plating approach aided by the greater throughput of approximately 130 undergraduate students processing samples in parallel, while the cryo-iPlate samples were processed by only two graduate students. Additionally, the volume of Arctic samples used for classical plating to isolate microorganisms was ~60 times greater than that used to inoculate the cryo-iPlate. None of the isolates with antibacterial activity were obtained from perennial hypersaline spring sediment from Lost Hammer, which may be because the salt content of the cultivation media was not adjusted to select for halophilic strains. It is known that the perennial hypersaline spring sediment at Lost Hammer hosts an active microbial community including members of the *Loktanella*, *Gillisia*, *Halomonas* and *Marinobacter* genera (Niederberger et al., 2010), and lack of isolates therefore cannot be attributed to lack of viable bacteria in the source material.

The sample type that yielded the greatest number of isolates with antibiotic activity was active layer soils overlaying permafrost, which consists of a low carbon mineral cryosol. It is ~60 cm deep during the Arctic summer and completely freezes during winter and spring (Doran, 1993; Goordial et al., 2017; Lau et al., 2015). Previously cultured phylotypes from this habitat predominantly included Actinobacteria (Niederberger et al., 2010), and indeed 26% of isolates identified by the bulk soil plating approach belonged to the Actinobacteria. This phylum includes the genus *Streptomyces* which are known to be prolific producers of antibiotics (Kiesser et al.,

2000; Manivasagan et al., 2014). A previous study using functional metagenomics showed that antibiotic resistance genes are present in similar permafrost soils at nearby Eureka, Ellesmere Island (Perron et al., 2015). The presence of antibiotics will create selective pressure for the development of antibiotic resistance. The presence of both antibiotic activity and antibiotic resistance therefore suggests interactions between these functions in the microbial permafrost community.

All cryo-iPlate strains were derived from active layer permafrost soil, because this was the only sample type in which the cryo-iPlate was incubated. The cryo-iPlate yielded genera (*Pararhizobium*, *Sphingomonas*, *Stenotrophomonas*, *Agreia*, *Pedobacter*) that were not observed in the strains isolated by the classical method from the same environment. In the initial implementation of the cryo-iPlate prototype, many of the same genera were isolated, including a putatively novel *Pedobacter* strain (Goordial et al., 2017). Similarly, *Pedobacter* strain F9.3 derived from the cryo-iPlate in this study was found to have 95.6% 16S rRNA gene sequence identity to its closest match *Pedobacter terrae* (Table 3). While none of the cryo-iPlate derived strains inhibited the entire ARP, they were able to inhibit the growth of both Gram-positive and Gram-negative relatives of the ESKAPE pathogens. Given that these strains were isolated from cryohabitats, their antibacterial activity has potential applications in food safety, where inhibitory activity is required at refrigeration temperatures.

Eight Arctic bacterial isolates inhibited all strains comprising the ARP

Here we have identified eight isolates able to inhibit the ARP which corresponded to either Firmicutes, Bacteroidetes or γ-Proteobacteria. It is worth noting that the dereplication method employed in this study is not a comprehensive approach in identifying isolates expressing novel antibiotic compounds. For example, if a given antibiotic-producing isolate expresses more than

one antimicrobial secondary metabolite, it would be capable of inhibiting the growth of all strains comprising the ARP regardless of whether the mechanisms of action of its secondary metabolites are novel. Given this inherent limitation of the ARP, the benefit of using this platform is an initial quick and cost-effective method of screening isolates with inhibitory activities for potentially novel activities. Further identification and characterization of the causative agents responsible for the inhibitory activities are required to confirm the novelty of a natural product.

The phyla of the prioritized isolates are known to make up a significant proportion of the active layer permafrost microbial community and are established secondary metabolite producers (Pathma et al., 2011; Wilhelm et al., 2011). Five out of the eight isolates that passed dereplication were *Pseudomonas spp.* This observation is not unexpected, as these Gram-negative heterotrophic γ-Proteobacteria are routinely isolated from soil samples using classical cultivation techniques such as the ones employed here (Sbrana et al., 2002). Additionally, the microbial community living in active layer permafrost within the vicinity of the MARS has been found to contain 19.4% γ-Proteobacteria (Wilhelm et al., 2011).

The only isolate that passed dereplication pertaining to the phylum Bacteroidetes was *Flavobacterium sp.* GHHS.3.LBZX.4. Two Firmicutes (one *Bacilllus sp*. and one *Paenibacillus sp.*) were also observed to inhibit all strains comprising the ARP. These two genera are well-documented secondary metabolite producers known to produce a plethora of antimicrobial compounds (Caulier et al., 2019; Pathma et al., 2011).

Interestingly, *Paenibacillus sp.* GHS.8.NWYW.5 and *Pseudomonas sp.* GHCE.5.JVZL.12 were the only isolates passing dereplication that were not derived from active layer permafrost. *Paenibacillus sp.* GHS.8.NWYW.5 was isolated from sediments of the Gypsum Hill saline

spring (~8% salts) and *Pseudomonas sp.* GHCE.5.JVZL.12 was isolated from a gypsum cryptoendolith. Our current knowledge of high Arctic saline spring sediments and cryptoendoliths as potential reservoirs of secondary metabolites is very limited and thus this finding serves as an interesting starting point for future studies.

Two promising isolates inhibit the growth of foodborne and clinically relevant pathogens. After the antibiotic activity of all eight isolates that passed the dereplication assay was tested against foodborne and clinically relevant pathogens, *Pseudomonas sp.* AALPS.10.MNAAK.13 and *Paenibacillus sp.* GHS.8.NWYW.5 were identified to have a broad spectrum of antibacterial activity. *Pseudomonas sp.* AALPS.10.MNAAK.13 was found to have the broadest activity against clinically relevant pathogens; inhibiting the growth of both Gram-positive and Gram-negative pathogens including MRSA, *A. baumanii*, MSSA, *E. faecium*, and *E. faecalis.* *Paenibacillus sp.* GHS.8.NWYW.5 was capable of inhibiting all four foodborne pathogens tested in this study. It displayed inhibitory effects at 37°C and at 5°C against *S. aureus*, *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 (Tables 5 and 6). The zones of inhibition produced by these Arctic isolates were visually similar to ones produced by the environmental reference strain *P. terrae* NRRL B-30644 which was initially isolated from a Russian poultry production environment and documented to have inhibitory activity against the foodborne pathogen *Campylobacter jejuni* (Lohans et al., 2014). To confirm that the antagonistic activities observed during the initial co-culture assays were due to secreted bacterial biomolecules, promising isolates were selected for organic extraction and tested against the parental strains of the ARP. The organic extract of isolates *Pseudomonas sp.* AALPS.10.MNAAK.13, and *Paenibacillus sp.* GHS.8.NWYW.5 exhibited zones of clearance against *E. coli* Δ*bam*Δ*tolC* BW25113. These observations coupled with our detection of secondary metabolite gene clusters

within Arctic isolates' genomes (Table 7), suggest that the inhibitory activities observed in this study arose from secreted antibacterial secondary metabolites. Ongoing testing is taking place to identify the causative agent(s) within the crude extracts and to determine their potency and spectrum of activity.

Interestingly, both Arctic isolates displayed antibacterial activity against MRSA and MSSA. MRSA's prevalence and the number of deaths it causes yearly qualify this pathogen as a major global public health concern (Martin et al., 2019), and antibacterial activity against MRSA is therefore of particular interest. The recent discovery of malacidin (effective against MRSA and other Gram-positive pathogens) illustrates how natural products from soil-dwelling bacteria continue to represent promising drug leads for endemic antibiotic resistant pathogens of the 21st century (Hover et al., 2018b). The secondary metabolites expressed by the Arctic isolates surveyed herein will be further characterized in future studies to determine if they have any chemotherapeutic potential.

The genome of *Paenibacillus sp.* GHS.8.NWYW.5 was found to 7 BGCs displaying low homology with clusters of its respective type strains of the same species. Conversely, all clusters detected within the genome of *Pseudomonas sp.* AALPS.10.MNAAK.13 displayed high homology when aligned with the type strain genome sequence. The genome of *Paenibacillus sp.* GHS.8.NWYW.5 featured the greatest number of total BGCs with five NRP clusters displaying low homology to its corresponding type strain genome. The type strain of this isolate, *P. terrae* NRRL B-30644 has been shown to express paenicidins and tridecaptin A_1 (Lohans et al., 2014). Clusters encoding both paenicidin and tridecaptin were identified within the genome of *Paenibacillus sp.* GHS.8.NWYW.5, however, it was also found to encode five NRP clusters with low homology to the type strain genome. Follow-up experiments such as genome-wide

transposon mutagenesis or targeted cloning and exogenous expression of specific clusters are required to determine which cluster(s) are responsible for the observed antibiotic activity exhibited by these isolates.

Arctic isolates that passed the ARP are capable of growth at refrigeration temperatures

All isolates that passed the dereplication assay (except for *Pseudomonas sp.* GHCE.5.JVZL.12) were capable of growing at temperatures ≤ 0°C within 20 days of incubation and grew more quickly at room temperature. Only two isolates (*Paenibacillus sp.* GHS.8.NWYW.5 and *Pseudomonas sp.* GHCE.5.JVZL.12) were capable of growing at 37°C. This observation suggests that these isolates are eurypsychrophiles which are typically characterized by their tolerance to sub-zero temperatures and display optimal growth at ~20 °C (Raymond-Bouchard, Tremblay, et al., 2018). Previous studies conducted with similar soil samples from the MARS site have led to the isolation of eurypsychrophillic strains including *Planococcus halocryophilus* Or1 that is capable of growth at -15 °C (Mykytczuk et al., 2016). The initial incubation of the bulk soil plates was performed at room temperature precluded the isolation of stenopsychrophiles that would have required lower cultivation temperatures (Raymond-Bouchard, Tremblay, et al., 2018).

The antibacterial activity of *Paenibacillus sp.* GHS.8.NWYW.5 against foodborne pathogens at common household refrigeration temperatures makes it a promising candidate for biotechnological applications in food-safety. The use of cold-adapted environmental strains as biopreservatives intended to inhibit the growth of foodborne pathogens within refrigerated commodities have been previously developed (Rovira & Melero, 2018). For example, certain strains of bacteriocin-producing *Carnobacteria spp*. isolated from natural environments have been used to limit the growth of pathogens within preserved seafood products (Wiernasz et al.,

2017). The isolates identified in this study that were capable of sub-zero growth and displayed antibacterial activity at refrigeration temperatures could serve as interesting starting points for future food-safety applications.

Conclusions

The results of our bioprospecting demonstrate how crowdsourced classical microbial cultivation and cryo-iPlate cultivation contribute to a culture-dependent workflow that can lead to the identification of Arctic bacteria capable of antibacterial activity. First results suggest that the habitats within the vicinity MARS on Axel Heiberg Island harbor promising cold-adapted bacteria with antibacterial activities against both foodborne and clinically significant pathogens. Further investigation will be required to identify the causative agents responsible for the observed antibacterial activities and to explore potential clinical and food-safety applications.

Acknowledgments

We acknowledge the hard work of the students and teaching assistants that participated in the MIMM212 Laboratory in Microbiology in 2016 and 2017. We thank Gerry Wright for generously providing the ARP strains. We thank Adam Classen, Maria Lee Ai Lan, Dr. Sudhakar G. Bhandare from the laboratories of Dr. Jennifer Ronholm and Dr. Lawrence Goodridge for providing the foodborne pathogens. We also thank Samy Coulombe for assisting in designing the cryo-iPlate and for subculturing Arctic isolates.

Chapter 3. Tables and Figures

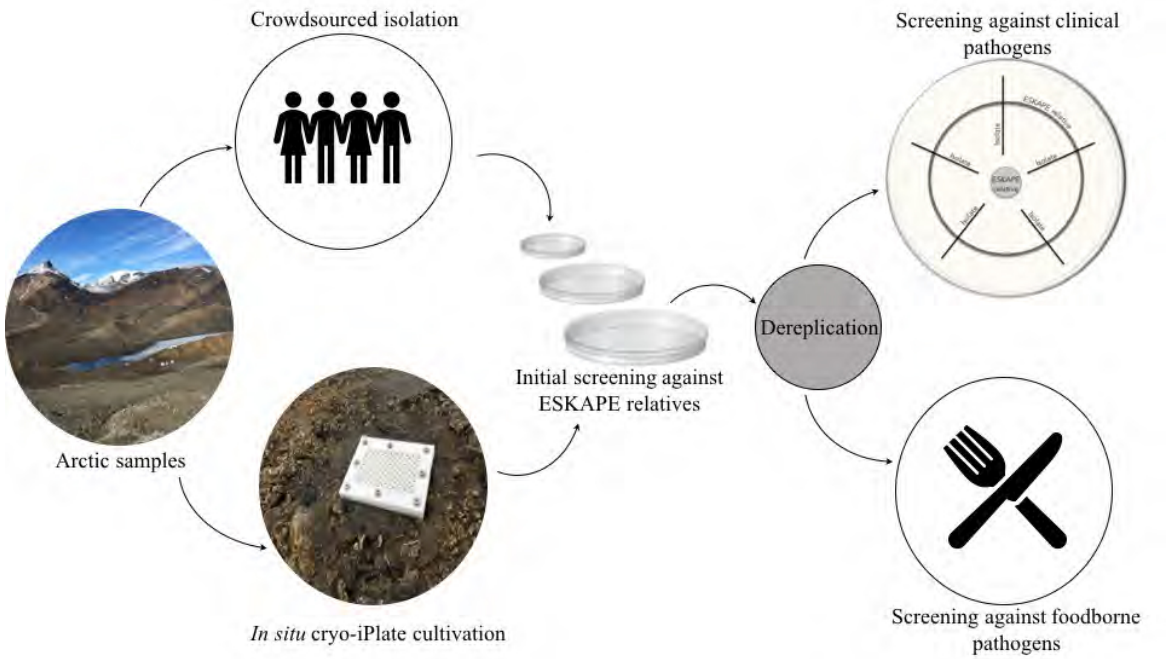

Figure 3. Overview of the Arctic bioprospecting workflow.

Figure 4. Three different co-culture techniques used to screen Arctic isolates for antibacterial activity. **A)** Spread-patch assay applied by the undergraduate crowdsourcing initiative to screen Arctic isolates against ESKAPE relatives. The image above features an Arctic bacterial isolate from the undergraduate crowdsourcing initiative exhibiting a zone of clearance against a lawn of an ESKAPE relative tester strain. **B**) Wagon wheel assay used to dereplicate and screen Arctic isolates against clinically relevant pathogens. The image above displays five Arctic isolates: GHCE.5.JVZL.12, AALPS.10.MNAAK.13, AALPS.10.EMMH.23, AALPS.10.JKNJ.7, MAL.10.WYTK.25 (in clockwise order) tested against the tester pathogen A. baumanii. **C**) Overlay assay used to screen Arctic isolates against foodborne pathogens. The image above displays the positive control strain Paenibacillus terrae NRRL B-30644 inhibiting the growth of L. monocytogenes at 5°C

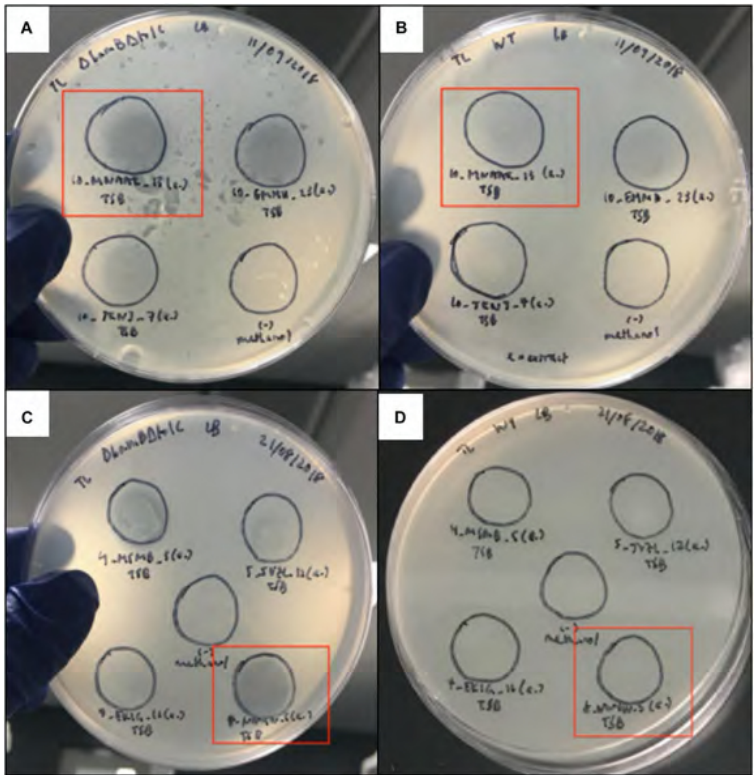

Figure 5 Spot assay used to assess antibacterial activity of organic extracts derived from Arctic isolate supernatants. (**A,B**) Organic extracts of isolates *Pseudomonas* sp.AALPS.10.MNAAK.13, *Pseudomonas* sp. AALPS.10.EMMH.23 and *Pseudomonas* sp. AALPS.10.JKNJ.7 tested against both parental strains of the dereplication platform (*E. coli* ΔbamΔtolC BW25113 and *E. coli* BW25113, respectively). Promising isolate *Pseudomonas* sp. AALPS.10.MNAAK.13 that displayed the broadest antibacterial activity in co-culture assays is boxed in red. Negative control spots solely consisting of the methanol are displayed at the bottom right in panels (A,B). (**C,D**) Organic extracts of isolates *Paenibacillus* sp. GHS.8.NWYW.5, *Pseudomonas* sp.AALPS.4.MSMB.5, *Pseudomonas* sp. GHCE.5.JVZL.12, *Pseudomonas* sp.MAL.10.WYTK.25 (same isolate as 7.EKIG.16 in figure) tested against both parental strains of the dereplication platform (*E. coli* ΔbamΔtolC BW25113 and *E. coli* BW25113, respectively). Promising isolate *Paenibacillus* sp. GHS.8.NWYW.5 that displayed the broadest antibacterial activity in co-culture assays is boxed in red. Negative control spots solely consisting of methanol are displayed in the center of panels (C,D).

Table 1. Arctic samples collected from Expedition Fiord, Axel Heiberg Island, Nunavut, used as source material for bioprospecting of antibiotic-producing strains.

Sample type	Source location	Geographic coordinates	Habitat
Active Layer Permafrost	MARS station Expedition Fiord	(79.412872, – 90.740343)	Trough soil from polygonal tundra terrain
			Surface soil from polygonal tundra terrain
			Arid soil
			Active layer tundra permafrost
	Gypsum Hill, Expedition Fiord	(79.403906, – 90.732321)	Hummock soil
Sediments	Junction Diapir (Lost Hammer)	(79.076755, –90.195299)	Perennial hypersaline (22–23% salt) spring outflow channel sediment (Pollard et al., 2009)
	Color Lake, Expedition Fiord	(79.416300, – 90.764081)	Acidic (pH ~3.6) freshwater lake sediment (Johannesson and Lyons, 1995)
			Acidic (pH ~3.6) freshwater lake sediment covered with microbial mat (Johannesson and Lyons, 1995)
	Gypsum Hill, Expedition Fiord	(79.403906, – 90.732321)	Perennial saline (7.5–15.8% salt) spring sediment (Perreault et al., 2007)
	White Glacier, Expedition Fiord	(79.431663, – 90.647073)	Sediment from glacial terminus moraines
Lithic Communities	Gypsum Hill, Expedition Fiord	(79.412151, – 90.740644)	Gypsum cryptoendolith
	Junction Diapir (Lost Hammer)	(79.076755, – 90.195299)	Unknown rock cryptoendolith

Table 2. Antibiotic activity of bulk soil isolates against ESKAPE pathogen relatives.

Source location	Habitat	Isolate name	Closest matching taxon	% identity	% coverage of 16S gene	*S. epidermidis*	*E. raffinosus*	*E. coli*	*P. putida*	*A. baylyi*	*E. aerogenes*
Color Lake, Expedition Fiord	Acidic freshwater lake sediment covered with microbial mat	CLMS.5.EMGM.4	*Janthinobacterium lividum*	99.87	54.6						
		CLMS.5.EMGM.9	*Pseudomonas yamanorum*	100	54.9						
		CLMS.6.VMCS.4	*Janthinobacterium lividum*	99.87	29.6						
Gypsum Hill, Expedition Fiord	Gypsum cryptoendolith	GHCE.C1.AWCA.27	*Streptomyces avidinii*	100	48.2						
		GHCE.C1.AWCA.31	*Mycetocola miduiensis*	99.75	56.3						
		GHCE.5.JVZL.12	*Pseudomonas fulva*	100	100						
		GHCE.5.JVZL.15	*Streptomyces avidinii*	100	46.4						
	Hummock soil	GHHS.3.LBZX.4	*Flavobacterium panaciterrae*	99	100						
		GHHS.3.LBZX.18	*Janthinobacterium svalbardensis*	100	59.5						
		GHHS.LB.ZXLB.24	*Pseudomonas helmanticensis*	99.75	54.8						
		GHHS.LB.ZXLB.15	*Pseudomonas helmanticensis*	100	54						
		GHHS.LB.ZXLB.15	*Pseudomonas arsenicoxydans*	100	54.8						
		GHHS.LB.ZXLB.9	*Pseudomonas arsenicoxydans*	100	46.2						
		GHHS.13.PTLY.2	*Pseudomonas arsenicoxydans*	100	53.9						
	Perennial saline spring sediment	GHS.C1.RZCG.3	*Bacillus nakamurai*	100	45.9						
		GHS.C1.RZCG.16	*Micrococcus aloeverae*	99.85	46.8						
		GHS.8.NWYW.5	*Paenibacillus terrae*	99.23	100						
Junction Diapir (Lost Hammer)	Unknown rock cryptoendolith	LCE.5.TZJR.21	*Pseudomonas lurida*	99.87	54.7						
MARS station Expedition Fiord	Active layer tundra permafrost	MAL.2.HSSH.5	*Pseudomonas kilonensis*	99.86	48.5						
		MAL.4.ABES.12	*Pseudomonas arsenicoxydans*	99.86	49.6						
		MAL.4.ABES.21	*Pseudomonas frederiksbergensis*	100	100						
		MAL.11.AHCHQX.12	*Pseudomonas arsenicoxydans*	100	100						
		MAL.10.WYTK.25	*Pseudomonas extremaustralis*	99.73	100						
		C2C25	*Curtobacterium pusillum*	99.54	100						
		C5C25	*Pseudomonas migulae*	99.6	100						
		HTAG2	*Pseudomonas jessenii*	99.34	100						
		C6A2	*Nocardia coeliaca*	99.62	100						
		C6A4	*Streptomyces netropsis*	100	100						
		C7A1	*Nocardia coeliaca*	99.61	100						
		C4F16	*Arthrobacter oryzae*	99.46	100						
		C6D6	*Nocardia globerula*	99.34	100						
		C6D9	*Pseudoarthrobacter phenanthrenivorans*	99.09	100						
		C7B19	*Nocardia coeliaca*	99.61	100						
		C7B21	*Bacillus mycoide*	99.69	100						
		C7D7	*Paenibacillus xylanexedens*	100	100						
		C8B11	*Rahnella inusitata*	98.59	100						
		C8C11.16	*Bacillus subtilis*	99.74	100						
		C10F22	*Nocardia coeliaca*	99.59	100						
		C10F26	*Pseudomonas fluorescens*	99.28	100						
		C10G6	*Nocardia globerula*	99.34	100						
		C11E10	*Bacillus subtilis*	100	100						
		C11E23	*Bacillus tequilensis*	99.87	100						
		MAL.11.SPBF.1	*Pseudomonas arsenicoxydans*	99.75	55.9						
	Arid soil	AALPS.3.MFAL.10	*Pseudomonas lutea*	100	36						
		AALPS.2.EKRD.20	*Pseudomonas mandelii*	99.73	100						
		AALPS.4.MSMB.5	*Pseudomonas mandelii*	99.66	100						
		AALPS.10.JKNJ.7	*Pseudomonas prosekii*	100	100						
		AALPS.10.EMMH.23	*Pseudomonas yamanorum*	98.68	100						
		AALPS.10.MNAAK.13	*Pseudomonas prosekii*	99.93	100						
		AALPS.13.YAYA.22	*Pseudomonas prosekii*	100	21						
	Surface soil from polygonal tundra terrain	PSALP.2.BBCP.18	*Pseudomonas lurida*	99.84	42.6						
		PSALP.2.JQGR.13	*Pseudomonas kilonensis*	99.87	53.8						
	Trough soil from polygonal tundra terrain	PWALP.2.KADK.9	*Pseudomonas fluorescens*	100	46.9						
White Glacier, Expedition Fiord	Sediment from glacial terminus moraines	GS.C1.SPSB.3	*Siccibacter turicensis*	100	53.9						

Red boxes: inhibitory activity. Black boxes: no inhibitory activity.

Table 3. Antibiotic activity of cryo-iPlate isolates against ESKAPE pathogen relatives.

Isolate name	Closest match	% identity	S. *epidermidis*	E. *raffinosus*	E. *coli*	P. *putida*	A. *baylyi*	E. *aerogenes*
A4.3Y	*Janthinobacterium lividum*	99.31						
B1.1W	*Pseudomonas trivialis*	100						
B4.6	*Pseudomonas migulae*	100						
B7.1	*Pedobacter humicola*	99.01						
C3.3	*Pedobacter alluvionis*	99.09						
C6.2	*Pseudomonas koreensis*	100						
C7.3	*Agrela pratensis*	100						
D4.1	*Pseudomonas arsenicoxydans*	100						
G2.2	*Flavobacterium oncorhynchi*	100						
F9.3	*Pedobacter terrae*	95.59						
D1.3	*Janthinobacterium lividum*	99.25						
C12.2A	*Stenotrophomonas rhizophila*	99.49						
C12.1B	*Flavobacterium frigidimaris*	99.2						
C11.2	*Pararhizobium herbae*	100						
C11.3	*Sphingomonas aerolata*	99.68						
C9.2	*Flavobacterium hydatis*	100						

Red boxes: positive inhibitory activity. Black boxes: negative inhibitory activity.

Table 4. Identification of secondary metabolite biosynthetic gene clusters within genomes of isolates inhibiting the ARP.

Isolate	Genome size (Mb)	Isolate Identification	%16S gene ID	AntiSMASH (total clusters)	NRP[1]	PK[2]	Bacterion/ RiPP[3]	Trans PKS[4]	Other
GHHS.3.LBZX.4	6.1	*Flavobacterium panaciterrae*	87.54	6	1	1	0	0	4
GHS.8.NWYW.5	5.7	*Paenibacillus terrae*	99.23	23	17	0	3	1	2
MAL.10.WYTK.25	6.8	*Pseudomonas extremaustralis*	99.73	9	4	0	2	0	3
GHCE.5.JVZL.12	5	*Pseudomonas fulva*	100	11	9	0	0	0	2
AALPS.4.MSMB.5	6.3	*Pseudomonas mandelii*	99.66	10	3	0	3	1	3
AALPS.10.MNAAK.13	6.1	*Pseudomonas prosekii*	99.93	8	2	0	1	0	5
MAL.4.ABES.21	7.1	*Pseudomonas frederiksbergensis*	99.86	7	3	0	1	0	3
C11E23	4.3	*Bacillus tequilensis*	100	12	4	1	3	1	3

[1] *Number of non-ribosomal peptide gene clusters identified using AntiSMASH.* [2] *Number of polyketide gene clusters identified using AntiSMASH.* [3] *Number of bacteriocin and ribosomally synthesized and post-translationally modified peptides (RiPP) identified using AntiSMASH.* [4] *Number of trans-polyketide gene clusters identified using AntiSMASH.*

Table 5. Screening Arctic isolates that inhibited the ARP against clinical pathogens.

Isolate name	Closest matching species	Closest matching strain	*P. aeruginosa*	*MRSA*	*A. baumanii*	*MSSA*	*E. cloacae*	*K. pneumoniae*	*E. faecium*	*E. faecalis*
GHHS.3.LBZX.4	*Flavobacterium panaciterrae*	DCY69(T)								
GHS.8.NWYW.5	*Paenibacillus terrae*	AM141(T)		+		+				
AALPS.10.MNAAK.13	*Pseudomonas prosekii*	LMG 26867		+	+	+			+	+
MAL.10.WYTK.25	*Pseudomonas extremaustralis*	14-3(T)		+		+			+	
GHCE.5.JVZL.12	*Pseudomonas fluorescens*	DSM 50090(T)		+	+	+		+		
AALPS.4.MSMB.5	*Pseudomonas mandelii*	NBRC 103147(T)		+		+			+	
MAL.4.ABES.21	*Pseudomonas frederikbergensis*	JAJ28(T)								
C11E23	*Bacillus tequilensis*	KCTC 13622(T)		+		+			+	

(+) Positive inhibitory activity.

Table 6. Screening Arctic isolates that inhibited the ARP against foodborne pathogens.

			Staphylococcus aureus		*Listeria monocytogenes*		*Salmonella enterica*		*Escherichia coli O157:H7*	
Isolate name	**Closest matching species**	**Closest matching strain**	**37°C**	**5°C**	**37°C**	**5°C**	**37°C**	**5°C**	**37°C**	**5°C**
GHHS.3.LBZX.4	*Flavobacterium panaciterrae*	DCY69(T)								
GHS.8.NWYW.5	*Paenibacillus terrae*	AM141(T)	+	+	+	+	+	+	+	+
AALPS.10.MNAAK.13	*Pseudomonas prosekii*	LMG 26867	+							
MAL.10.WYTK.25	*Pseudomonas extremaustralis*	14-3(T)					+			
GHCE.5.JVZL.12	*Pseudomonas fluorescens*	DSM 50090(T)								
AALPS.4.MSMB.5	*Pseudomonas mandelii*	NBRC 103147(T)								
MAL.4.ABES.21	*Pseudomonas frederikbergensis*	JAJ28(T)								
C11E23	*Bacillus tequilensis*	KCTC 13622(T)			+	+				

(+) Positive inhibitory activity.

Table 7. Comparing specialized metabolite gene clusters identified within genomes of promising isolates with type strains of the same species.

Supplementary Figures and Tables

Table S1. ESKAPE relatives used to screen for antibacterial activity

ESKAPE pathogen	Safe ESKAPE relative	ATCC
Enterococcus faecium	*Enterococcus raffinosus*	49464
Staphylococcus aureus	*Staphylococcus epidermidis*	14990
Klebsiella pneumoniae	*Escherichia coli*	11775
Acinetobacter baumannii	*Acinetobacter baylyi*	33305
Pseudomonas aeruginosa	*Pseudomonas putida*	12633
Enterobacter spp.	*Enterobacter aerogenes*	51697

Table S2. Antibiotic resistant strains comprising the dereplication platform used in this study (Cox et al., 2017).

Antibiotic class	Antibiotic	Resistance Gene	Plasmid	Promoter	Parent *E. coli* strain
Aminoglycosides	Streptomycin	*aph(3')-Ia*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
	2-Deoxystreptamine	*rmtB*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
	Apramycin	*apmA*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
	Spectinomycin	*Aph(9)-Ia*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
B-lactams	Penicillin	*NDM-1*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
	Cephalosporin	*NDM-1*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
	Carbapenam	*NDM-1*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
Lincosamides	Lincosamides	*ermC*	pGDP4	P_{lac}	Δ*bam*Δ*tolC* BW25113
Macrolides	Macrolides	*ermC*	pGDP4	P_{lac}	Δ*bam*Δ*tolC* BW25113
Type B Streptogramins	Type B Streptogramins	*ermC*	pGDP4	P_{lac}	Δ*bam*Δ*tolC* BW25113
Type A Streptogramins	Type A Streptogramins	*vatD*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
Streptothricin	Streptothricin	*STAT*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
Tetracyclines	Tetracycline	*tet(A)*	pGDP4	P_{lac}	Δ*bam*Δ*tolC* BW25113
Chloramphenicols	Chloramphenicols	*CAT*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
Fosfomycins	Fosfomycins	*fosA*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
Rifamycins	Rifamycins	*arr*	pGDP3	P_{bla}	Δ*bam*Δ*tolC* BW25113
Polymyxins	Polymyxins	*MCR-1*	pGDP1	P_{bla}	Wild-type BW25113
Echinomycins	Echinomycins	*uvrA*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113
Sideromycins	Albomycin	*fhuB mutant*	-	-	Δ*bam*Δ*tolC* BW25113
Tuberactinomycins	Viomycin	*vph*	pGDP1	P_{bla}	Δ*bam*Δ*tolC* BW25113

Table S3. Assessing growth of Arctic isolates that inhibited the ARP at various temperatures.

Isolate name	**Closest matching taxon**	**Closest matching strain**	**37°C t= 48hrs**	**25°C t= 48hrs**	**10°C t= 20 days**	**5°C t= 20 days**	**0°C t= 20 days**	**-5°C t= 20 days**
GHHS.3.LBZX.4	*Flavobacterium panaciterrae*	DCY69(T)		+	+	+	+	
GHS.8.NWYW.5	*Paenibacillus terrae*	AM141(T)	+	+	+	+	+	+
AALPS.10.MNAAK.13	*Pseudomonas prosekii*	LMG 26867		+	+	+	+	+
MAL.10.WYTK.25	*Pseudomonas extremaustralis*	14-3(T)		+	+	+	+	+
GHCE.5.JVZL.12	*Pseudomonas fluorescens*	DSM 50090(T)	+	+	+	+		
AALPS.4.MSMB.5	*Pseudomonas mandelii*	NBRC 103147(T)		+	+	+	+	+
MAL.4.ABES.21	*Pseudomonas frederikbergensis*	JAJ28(T)		+	+	+	+	
C11E23	*Bacillus tequilensis*	KCTC 13622(T)		+	+	+	+	+

(+) Visible growth

Table S4. Genomic sequencing statistics

Isolate	Contigs	Scaffolds	Genome Size	Longest Scaffold	N50	Raw reads	Error-corrected Reads	% reads passing Error-correction	Raw nucleotides	Error-corrected nucleotides	% nt passing Error-correction	Raw coverage	Median coverage	10th percentile coverage	Bases >= Q40
GHHS.3.LBZX.4	69	53	6116726	806308	501802	1145402	1129059	98.57	320233758	267677262	83.59	43.76	51	39	6112985
GHS.8.NWYW.5	73	55	5662149	609831	274348	3038748	2928247	96.36	889616513	647752279	72.81	114.4	149	126	5660236
AALPS.10.MNAAK.13	36	32	6090438	798849	523870	1272094	1192269	93.72	338765113	248848688	73.46	40.86	50	38	6084513
MAL.10.WYTK.25	62	60	6826261	442973	256343	676206	665502	98.42	179401430	138717033	77.32	20.32	25	16	6807762
GHCE.5.JVZL.12	48	44	4977715	625630	423958	1259932	1207488	95.84	332870865	244986609	73.6	49.22	62	48	4974582
AALPS.4.MSMB.5	79	60	6323405	523195	223859	1837634	1800987	98.01	506142769	380213292	75.12	60.13	76	60	6321054
MAL.4.ABES.21	23	21	6812670	1022152	638679	972954	945125	97.14	268813615	194890214	72.5	28.61	37	27	6809466
C11E23	25	18	4271209	2223793	2223793	1675208	1637939	97.78	485678175	416677039	85.79	97.55	110	90	4265909

Figure S1. High sensitivity DNA electropherogram of the pooled Illumina MiSeq library. The electropherogram was obtained from an Agilent High Sensitivity DNA Chip on an Agilent 2100 Bioanalyzer. The x-axis represents DNA fragment size, the y-axis represents fluorescence units.

Connecting Text: Chapter 4

The goal of chapter 4 was to characterize *Pseudomonas sp*. B1.1W, a putatively novel species that was isolated from active layer permafrost exhibiting antifungal activity against the phytopathogenic mold, *Botrytis cinerea*. By coupling MALDI-TOF MS-MS performed on liquid culture organic extracts of B1.1W and genomic mining, we found that this isolate biosynthesized a 1148 Da viscosin-like cyclic lipopeptide (CLP) with antifungal activity. This study represents one of the scarce reports of high Arctic bacteria capable of producing a viscosin-like CLP with antifungal activity against a phytopathogenic mold.

Chapter 4: *Pseudomonas* sp. B1.1W, a putatively novel cyclic lipopeptide producing high Arctic species exhibiting inhibitory activity against *Botrytis cinerea.*

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Abstract

Rhizospheric *Pseudomonas* species are prolific producers of antimicrobial specialized metabolites. Culturable representative strains from high Arctic soils, however, remain to be explored for their biosynthetic potential. The objective of this study was to characterize the antifungal properties of *Pseudomonas* sp. B1.1W, a putatively novel species isolated from active layer permafrost at Expedition Fjord on Axel Heiberg Island in Nunavut, Canada. Through dual-culture antagonistic assays, B1.1W was observed to inhibit the growth of *Botrytis cinerea*. After performing LC-MS and MALDI-TOF MS-MS on bioactive fractions of liquid culture supernatant extracts of B1.1W in co-culture with *B. cinerea,* viscosin-like signatures with molecular masses of 1148 and 1126 Da were detected. Genomic mining of B1.1W revealed a viscosin-like biosynthetic gene cluster (BGC) that included putative nonribosomal synthetase modules featuring amino acid specificities consistent with the oligopeptide moiety composition of the 1148 Da compound characterized through MALDI-TOF MS-MS. The B1.1W viscosin-like gene cluster shared sequence homology with the viscosin cluster initially characterized in *Pseudomonas fluorescens* SBW25 and with nonribosomal peptide clusters that we detected within publicly available genomes of soil-dwelling *Pseudomonas* spp. from Antarctica. Additionally, we identified 17 putative genes predicted to be part of B1.1W's viscosin-like cluster that are not present in the SBW25 viscosin cluster and whose biosynthetic roles remain unmapped. To our knowledge, this is the first report of a high Arctic *Pseudomonas* sp. with the biosynthetic potential to produce a viscosin-like cyclic lipopeptide with antifungal activity against *B. cinerea*.

Introduction

Soil-dwelling *Pseudomonas* species associated with plants commonly feature diverse sets of functional traits spanning plant growth promotion, degradation of xenobiotics, inhibition of phytopathogens and elicitation of endogenous plant defense systems (Coggan & Wolfgang, 2012; Raaijmakers et al., 2006). Their capacity to biosynthesize multiple specialized metabolites with biotechnological applicability encompassing bacteriocins, antibiotics, toxins and antifungals, has placed *Pseudomonas* spp. at the focus of numerous natural product studies (Gross & Loper, 2009). One large class of specialized metabolites that plant-associated *Pseudomonas* spp. produce are cyclic lipopeptides (CLPs) (Raaijmakers et al., 2010). Species of soil-inhabiting *Pseudomonas* capable of biosynthesizing CLPs include *P. syringae*, *P. tolaasii*, *P. fuscovaginae*, *P. corrugata*, *P. putida* and *P. fluorescens* (Nielsen & Sørensen, 2003; Nybroe & Sørensen, 2004). These molecules are composed of a fatty acid tail linked to a cyclic oligopeptide moiety (Martin H et al., 2019; Nielsen et al., 1999) .

CLPs are a versatile and structurally diverse class of molecules, including surfactin, amphisin, tolaasin, syringomycin, and massetolide compounds such as viscosin (Nielsen & Sørensen, 2003). Their bioactivity profiles include antibacterial, antifungal, antitumor, plant-growth promotion and surfactant properties (Bender et al., 1999; Loper et al., 2012). This functional diversity originates from structural variations in the composition and chain length of CLP fatty acid tails and differences in number, chirality and configuration of the amino acids comprising the oligopeptide moiety (De Bruijn et al., 2007). Additional structural variability arises from functional groups attached to the fatty acid tail and oligopeptide portions. CLPs are biosynthesized by large, multidomain nonribosomal synthetases (NRPSs) whose expression is

often regulated by two-component systems and quorum sensing (Bender et al., 1999; Grewal et al., 1995; Raaijmakers et al., 2010).

CLPS that are part of the viscosin group have been isolated from diverse habitats including plant rhizospheres, marine organisms, and frog skin (Gerard et al., 1997; Martin H et al., 2019). They typically exhibit antifungal activity against phytopathogens including *Pythium* spp. and *Rhizopus solani* (Martin H et al., 2019). The viscosin oligopeptide moiety consists of nine amino acids (Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile) synthesized by three NRPSs encoded by the genes *viscA, viscB* and *viscC* that were first mapped in *P. fluorescens* SBW25 (Grewal et al., 1995; Oni et al., 2020; Zhao et al., 2019). While *viscB* and *viscC* are usually encoded within a single biosynthetic gene cluster (BGC) and include a LysR regulator and efflux protein encoding genes downstream in the same reading frame, the *viscA* locus is typically ~1Mbp upstream and has LysR regulator and efflux protein encoding genes upstream in antisense configuration (Loper et al., 2012). Each NRPS contains elongation modules consisting of an adenylation (A) domain which selects an amino acid, a thiolation (T) domain responsible for the thioesterification of the activated amino acid, and a condensation (C) domain that catalyzes peptide bond formation that elongates the peptide chain (Loper et al., 2012; Raaijmakers et al., 2006). To release the cyclic peptide moiety, two thioesterase domains (TE) cyclize the linear peptide chain at the end of the biosynthetic assembly line in module 9 of the NRPs encoded by *viscC* (De Bruijn et al., 2007; Loper et al., 2012; Zhao et al., 2019).The oligopeptide moiety is linked at the N-terminus to 3-hydroxy decanoic acid (3-HDA) which is hypothesized to derive from primary metabolism (De Bruijn et al., 2007; Loper et al., 2012; Zhao et al., 2019).

In this study, we identified a 1148 Da CLP exhibiting inhibitory activity against *Botrytis cinerea* that was produced by *Pseudomonas* sp. B1.1W, a putatively novel species isolated from high

Arctic initially cultured from active layer permafrost using an *in situ* cultivation device called the cryo-iplate. Considered the second most destructive necrotrophic fungal phytopathogen after *Magnaporthe oryzae, B. cinerea* can infect a broad host range of plants causing major annual crop losses worldwide (Hua et al., 2018). Given the emerging prevalence of *B. cinerea* pathovars resistant to current fungicides and the magnitude of economic loss that they cause, novel antifungal natural products are needed to control this pathogen (Abbey et al., 2019; Cosseboom et al., 2020; S. Liu et al., 2021). By coupling genomic mining for antimicrobial BGCs with fractionation and structural analysis of organic extracts derived from B1.1W liquid cultures, we demonstrate how this isolate could potentially biosynthesize an antifungal compound part of the viscosin group capable of inhibiting *B. cinerea*.

Methods

Isolation of Arctic isolate *Pseudomonas* sp. B1.1W

B1.1W was initially cultured using the cryo-iplate, an *in situ* microbial cultivation device that is described in detail within (Marcolefas et al., 2019). This device was incubated within the first 15 cm of the active layer permafrost horizon of Gypsum Hill located at Expedition Fjord, Axel Heiberg Island, Nunavut, Canada (79.412872, -90.740343). This soil horizon featured rhizospheres of tundra plants including *Cassiope tetragona* (Figure S2). To attain pure culture after *in situ* incubation, *Pseudomonas* sp. B1.1W was subcultured three times on tryptic soy agar (TSA) (Difco™ Tryptic Soy Agar, BD) and incubated at 25ºC. The growth curve of B1.1W was determined by measuring liquid pure culture optical density at 600 nm ($OD600_{nm}$) in triplicate every hour over the course of 24 hrs at 25ºC in tryptic soy broth (TSB) (Difco™ Tryptic Soy Broth, BD).

Dual-culture antagonistic assay

Given that B1.1W originated from rhizospheric soil and displayed a limited antibacterial activity profile in Marcolefas *et al.,* 2019 , we explored the strain's antifungal potential by screening it in dual culture against phytopathogenic fungi (*Botrytis cinerea* WT*, Penicillium olsonii* WT*, Fusarium oxysporum* WT and *Pythium myriotylum* WT*).* The bacterial inoculant, *Pseudomonas* sp. B1.1W, was prepared from a stock culture in 15 mL of tryptic soy broth (TSB) incubated for 20 h at 25ºC with 250 rpm agitation. The tester fungal pathovars were grown from stock cultures on potato dextrose media (Difco™ Potato Dextrose Media, BD) at 28ºC for 10 d in the dark. Dual-culture assay plates were prepared by introducing 50 µL of the bacterial inoculant (1.0×10^{10} CFU/mL) into a 5 mm well at the center of the Petri dish and by placing a 5 mm agar disc of the fungal tester (containing 1.0×10^4 conidia after counting on hemocytometer) 3, 5, 7 cm adjacently from the bacterial inoculum. After 10 days of incubation at 25ºC, zones of inhibition areas were measured using imaging software Assess 2.0.

Whole Genome Sequencing

DNA extraction

An isolated colony of B1.1W was inoculated in Brain Heart Infusion (BHI) Broth and grown at 35°C without agitation. Cells were harvested by centrifugation for 10 minutes at 5000 x g to obtain approximately $2x10^9$ cells per pellet. Pelleted cells were frozen at -80°C until DNA extraction. The DNeasy Blood and Tissue kit (Qiagen) was used to extract DNA, following the manufacturer's protocol for Gram negative bacteria with some modifications. First, to avoid DNA shearing, all vortexing steps were replaced by tube inversions. After the bacterial lysis step, RNA was digested by adding 10 µl of RNase Cocktail (Invitrogen) to the lysate and incubating at room temperature for 5 minutes. A second wash with 500 µl of AW2 buffer was performed instead of one. Finally,

DNA was eluted twice with 50 µl of 10 mM Tris-HCl pH 8. Extracted DNA was quantified by the Qubit dsDNA BR method (Invitrogen).

Sequencing

Long read sequencing libraries were prepared from genomic DNA with Oxford Nanopore's SQK-LSK109 sequencing kit. A total of 600 ng was loaded on a MinION FLO-MIN106 flow cell (version R9.4). Sequencing was performed with Oxford Nanopore's proprietary software MinKNOW version 8.3.1 with run length set at 72 hours and time between mux scans set at 3 hours (Van der Verren et al., 2020). The raw sequencing data (in fast5 format) was basecalled and demultiplexed post-sequencing using Guppy v.4.2.2-GPU (Wick et al., 2019). Nanopore reads that were longer than 4000 bp with a Phred quality score over 12 were selected with NanoFilt v2.3.0 (Wick et al., 2019). Adapter sequences were trimmed afterwards with poreChop v0.2.4.

Short paired-end reads (2 x 300 bp) were obtained for each sequenced strain by Illumina MiSeq sequencing as previously described (Freschi et al., 2015; Freschi et al., 2019). Trimmomatic v0.36 was used to trim adapter sequences and filter short reads (Phred score >30, minimum length of 200 bp) (Bolger et al., 2014).

Hybrid genome assembly

The Unicycler assembly pipeline v0.4.8 was used to construct a hybrid genome assembly (Wick et al., 2017). Briefly, Illumina short reads were assembled into contigs and then bridged by MinION long read contigs. Then, contigs are extended and gap-closed with long reads and, if circular, rotated to the nearest replication/mobilization gene. Contigs are then polished with short reads to improve consensus quality and break long read misassemblies.

Assembly statistics (number of contigs, total bases, longest contig, N50, etc.) were calculated and compiled using assembly-stats v1.0.1 (https://github.com/sanger-pathogens/assembly-stats) and in-house Unix shell scripts.

Genomic analysis and Taxonomic Identification

To construct a circular genome map, the B1.1W genomic assembly was annotated and mapped using the Prokka pipeline within the CGView Server v. BETA (Grant & Stothard, 2008; Seemann, 2014; Stothard et al., 2019). To determine B1.1W closest phylogenetic relatives, the NCBI Prok tool within MiCrobial Genomes Atlas (MiGA) Online that queries complete genomic sequences against the NCBI Genome database using Average Nucleotide Identity (ANI) (Rodriguez-R et al., 2018). The closest relatives were then downloaded within the PATRIC RASTtk-enabled Genome Annotation Service to construct a codon tree using single-copy PATRIC PGFams that employs the RAxML program (Brettin et al., 2015a; Davis et al., 2020; Stamatakis, 2014).

Small scale organic extract preparation

A liquid-liquid organic extract, using the cell-free supernatant of a dual-culture fermentation (between B1.1W and *B. cinerea* WT), was prepared with ethyl acetate. This was achieved by placing a 5 mm agar disc from a ten-day old culture of *B. cinerea* on PDA (containing ~1.0 x 10^4 conidia) into 15 mL TSB within a 125 mL Erlenmeyer flask. The agar discs were incubated in TSB for 48 hrs at 25ºC with 250 rpm agitation before introducing 100 µL of B1.1W overnight liquid culture (1.0 x 10^{11} CFU/mL) to the growth culture. The dual-culture fermentation was incubated at 25ºC for 7 days with continuous agitation at 250 rpm. Cells were removed from the dual-culture through two rounds of centrifugation at 8000 rpm at 4 ºC for 15 minutes followed by filter filtration using a 0.22 µm polyethersulfone filter (Corning). The cell-free supernatant was mixed with 15 mL of ethyl acetate (≥99.8%, HiPerSolv CHROMANORM® for HPLC,

VWR Chemicals BDH®) and vortexed on high for 20 min. The organic layer was harvested by centrifugation at 8000 rpm at 4 ºC for 20 minutes, placed in a sterile 40 ml amber borosilicate (VWR®, TraceClean®) vial and the solvent was removed by filtered air drying for 1 hr. The dry weights of the crude extracts were weighed before being resuspended in methanol to confirm bioactivity.

Larger scale organic extract preparation

Three 5 mm agar discs from a ten-day old culture of *B. cinerea* on PDA (each disc containing ~1.0 x 10^4 conidia) into 500 mL TSB within a 2 L Erlenmeyer flask. The agar discs were incubated in TSB for 48 hrs. at 25ºC with 250 rpm agitation before introducing 5 mL of B1.1W overnight liquid culture (1.0 x 10^{11} CFU/mL) to the fermentation. The dual-culture fermentation was incubated at 25ºC for 7 days with continuous agitation at 250 rpm. Microbial biomass was removed from the dual-culture by two rounds of centrifugation at 10000 rpm at 4 ºC for 20 minutes followed by vacuum filtration using a 0.22 µm polyether sulfone filter (Corning). The cell-free supernatant was mixed with 1 L of ethyl acetate (≥99.8%, HiPerSolv CHROMANORM® for HPLC, VWR Chemicals BDH®) and mixed by shaking at 300 rpm for 1 hr. The organic layer was harvested using a separatory funnel and the solvent was removed by rotary evaporation at 40 ºC with 100 rpm rotation (Yamato RE500, with BM100 water bath).

Mycelial Growth Inhibition Assay

To confirm the antifungal activity of the organic extracts, a mycelial growth inhibition (MGI) assay was performed according to Sayago et al. (Sayago et al., 2012). This was achieved by impregnating Petri dishes containing 10 mL of TSA with 0, 100, 200 and 500 µL of crude dual-culture extract reconstituted in methanol (37.5 mg/mL); the experiment was conducted in triplicate for each test volume. Once the methanol evaporated, 5 mm agar discs containing *B.*

cinerea (1.0×10^4 conidia) were placed at the center of each plate. MGI assay plates were incubated at 25ºC for ten days until colonies on negative control plates extended to the edge of the plates. MGI was calculated by MGI (%) = $[(D_c - D_t)/D_c] \times 100$ where D_c corresponds to colony diameter on untreated control plates and where D_t corresponds to colony diameter on plates impregnated with crude extract.

Crude extract fractionation

A C18 Sep-pak column (Resprep) was primed with 2mL of ethanol and 2mL of Milli-Q water. 60µL of the B1.1W large scale extract was added to the column and eluted stepwise with acetonitrile (ACN) starting from 0% up to 100% with 5% increments. All fractions were dried and reconstituted in 60 µL of Milli-Q water. To test the fractions for antifungal activity, 15 µL of the ACN fractions reconstituted in Milli-Q water were added in triplicate to wells of a flat-bottom microplate containing 10^4 conidia/mL of *B. cinerea* WT in 85 µL potato dextrose broth (PDB) (Difco™ Potato Dextrose Media, BD). After 72hrs of incubation at room temperature in the dark, the antifungal activity of the tested fractions was determined by optical density using a spectrophotometer (SpectraMAx M2, Molecular Devices) set at 625 nm and experimental triplicates were compared to blank control wells that did not receive fractionated extract and positive control wells containing amphotericin B at 8 µg/mL.

MALDI-TOF MS-MS

Each collected fraction was spotted on the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) steel plate in a ratio of 1:1 (v:v) with 5mg/ml of alpha-Cyano-4-hydroxycinnamic acid resuspended in 70% acetonitrile. Spectra were recorded using Bruker Daltonics UltrafleXtreme MALDI-TOF in the positive reflector mode and an accumulation of 5000 shots. Spectra were analyzed for unique ions founds within the elution fractions exhibiting

antifungal activity. Further structural information of molecules of interest were obtained by fragmentation using MALDI-TOF MS-MS technology as well as ESI-Orbitrap-MS-MS Bruker AmaZon SL technology.

LC-MS

Triplicate small scale ethyl acetate extracts, prepared using the method described above, consisting of: 1) B1.1W in pure culture, 2) B1.1W in co-culture with *B. cinerea*, 3) *B. cinerea* in pure culture and 4) procedural and procedural blank TSB medium (total of twelve independent ethyl acetate extractions) were subjected to LC-MS and their features were compared. The samples were first fractionated using a reverse phase C18 column and the mobile phases consisted of water and acetonitrile containing 5 mM NH4Ac and 0.1% formic acid. The liquid chromatography parameters consisted of a10 µL injection with a flow rate of 0.25 mL/min involving an increasing concentration gradient of ACN at 0-1 min until 20%, then increased to 100% from 1-32 min, and then decreased to 20% from 32.1-35 min. The mass spectroscopy parameters consisted of a 100-1700 m/z range, capillary voltage set to 3500 v (-) or 4000 v (+), gas temperature of 250 °C, drying gas flow 10 L/min, nebulizer 35 psi, fragmentor 175 v, skimmer 65 v, all ion MS-MS: collision energy = 0, 10, 20, 40 v with the data acquisition rate set to 2 scans/s.

LC-MS data analysis

The output was then analyzed using the MassHunter Profiling software series. For both positive and negative ion modes, raw data were first aligned using Agilent MassHunter Profinder (B.10.0) with Batch recursive feature extraction mode set to mass window: +/- 20 ppm, retention time window: +/- 0.15 min and peak filter: >/= 300 counts. The statistical comparison of the chemical profiles between pairwise extract types (1 to 4 listed above) was achieved using MassHunter Profiler Professional (MPP, version B15.0). MPP screening parameters consisted of a 75% shift in normalization algorithm, with a median of all samples setting for the baseline. Lastly, a fold change analysis within

the MPP software was used to identify chemical features that has at least a 2-fold higher intensity among the pairwise extract types.

Genome mining

The whole genome sequence of B1.1W was mined for antimicrobial biosynthetic gene clusters (BGCs) using antiSMASH v.6.0.1 (Blin et al., 2021). The translated genomic DNA sequences of genes comprising a viscosin-like BGC detected within the B1.1W genome were queried in the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database and BLASTp (Kautsar et al., 2020). The genomes of B1.1Ws closets relatives were also mined using antiSMASH v.6.0.1. To build a NRP cluster similarity network and BGC phylogenetic tree between B1.1W and its closest relatives, BiG-SCAPE 1.0.1 was used (Navarro-Muñoz et al., 2020). The NRP cluster network was constructed with Cytoscape v.3.8.0 employing a perfuse force open network type constructed using DSS anchored domain sequence homology between BGCs using 0.3 DSS cutoff.

Results

***Pseudomonas* sp. B1.1W ANI comparisons with relatives are below species-level cutoff.**

The genome of *Pseudomonas* sp. B1.1W was 6.674233 Mbp in length featuring 60.09% GC content (Figure 6). After querying the B1.1W genome using the NCBI Prok tool within the MiGA database, its closest relatives based on ANI percentage were: *Pseudomonas* sp. R76 (92.28%), *Pseudomonas poae* strain PMA22 (92.19%), *Pseudomonas* sp. IB20 (91.36%), *Pseudomonas antarctica* strain DSM 15318T (91.27%), *Pseudomonas antarctica* strain BS2772 (91.14%), *Pseudomonas antarctica* strain CMS 35 (91.1 %), *Pseudomonas* sp. S35 (87.41%), *Pseudomonas cremoris* WS5106 (87.26%) , *Pseudomonas fluorescens* strain W6 (87.13%),

Pseudomonas yamanorum strain GZD14026 (85.04%), and *Pseudomonas* sp. ADAK18 (84.9%) (Figure 8, Table S5).

Pseudomonas sp. B1.1W cells and organic extract inhibited growth of _B. cinerea_ WT.

The Arctic isolate *Pseudomonas* sp. B1.1W was screened for antifungal activity using a dual-culture antagonistic assay against four phytopathogenic fungi (*Botrytis cinerea* WT*, Penicillium olsonii* WT*, Fusarium oxysporum* WT and *Pythium myriotylum* WT*).* The isolate only displayed zones of fungal growth inhibition against *B. cinerea* WT. After incubating B1.1W cellular suspension adjacent to an agar disc containing *B. cinerea* for five days, B1.1W produced zone of inhibition against *B. cinerea* WT (Figure S4). The organic extract prepared from a dual-culture fermentation between B1.1W and *B. cinerea* exhibited dose-dependent inhibition of *B. cinerea* as shown in the MGI assay (Figure 9). The mean colony radius of *B. cinerea* that grew on plates with 100, 200 and 500 uL of a 37.5 mg/mL crude extract was 3.5, 2.67, and 1.17 cm; corresponding to 30, 46.67 and 76.67 % MGI, respectively (Figure 9). Extracts prepared from liquid culture supernatants of B1.1W in pure culture did not exhibit antifungal activity against *B. cinerea*.

A fraction of the crude B1.1W containing a 1148 Da compound displayed antifungal activity against _B. cinerea_ WT

After C18 Sep-pak column fractionation of the B1.1W large scale extract, a bioactive sub-fraction was found to be eluted at 40% CAN. This fraction displayed antifungal activity against *B. cinerea* when tested in a microplate assay (Figure 10). By subjecting the fraction to MALDI-TOF MS-MS, a1148.656 Da compound was detected featuring peaks that corresponded to a peptide sequence consisting of Val – Ile/Leu – Ser - Ile/Leu – Ser – Ile/Leu (Figure 11). The MS-MS spectrum also featured a strong 1035 Da fragment ion. The LC-MS profile on B1.1W ethyl

acetate extracts contained a 1126 Da compound. LC-MS detected a compound with chemical formula $C_{54}H_{95}N_9O_{16}$ that was eluted after ~26 min in ethyl acetate extracts derived from B1.1W in pure culture and B1.1W in co-culture with *B. cinerea* (Figure S5, Table S6). Extracts derived from *B. cinerea* in pure culture and procedural blanks did not contain this chemical signature.

A viscosin-like BGC was detected within the B1.1W genome and within genomes of Antarctic relatives

After mining the genome of Pseudomonas B1.1W, 12 BGCs were detected: one arylpolyene cluster, one betalactone, five NRPS, three RiPP-like, one RRE-containing cluster, and one thiopeptide (Table 8). The NRP cluster network between B1.1W and its closest relatives constructed with a threshold of 0.3, showed that three of the five NRP clusters detected within the genome of B1.1W shared NRPS domain sequence similarity with the NRP clusters detected in the genomes of its relative strains (Figure 14). The viscosin-like gene cluster in region 7 of the B1.1W genome shared the highest degree of sequence homology with viscosin-like clusters detected within the genomes of *Pseudomonas antarctica* strains BS2772 (82% ID and 100% coverage) and CMS35 (74% ID 90% coverage) (Figure 12, 13) . Both BS2772 and CMS35 NRP clusters differed by 0.1075 domain sequence similarity (DSS) with the B1.1W viscosin-like cluster (**Figure 12**). Both BS2772 and CMS35 clusters featured putative NRPS genes containing elongation modules identical to the ones detected within the region 7 cluster of the B1.1W genome (Figure 14).

The viscosin-like cluster contains putative NRPS genes with the potential to produce the viscosin peptide moiety.

The viscosin-like gene cluster in region 7 of the B1.1W genome was predicted to be 61275 bp in length and 62% of its genes shared sequence homology with the publicly available viscosin

cluster initially characterized in *P. fluorescens* SBW25 (MIBiG cluster BGC0001312) (Figure 12, Table S7). The B1.1W viscosin-like cluster contained two NRPS genes with locus tag designations ctg1_3707 and ctg1_3708 in the same predicted reading frame. The putative gene ctg1_3707 shared 88% nucleotide sequence identity and 100% coverage with the *viscC* gene encoding the NRPS in the known viscosin BGC (MIBiG cluster BGC0001312) (Figure 12.B). This gene featured four putative modules with adenylation domains specific for Thr-Val-Leu-Ser (Figure 13). The putative gene ctg1_3708 shared 89% ID and 100% coverage with the *viscB* gene and contained three modules with putative adenylation domains specific for Leu-Ser-Ile in addition to two thioesterase domains on the Ile-specific module (Figure 13). Downstream of ctg1_3707 and ctg1_3708, the putative gene ctg1_3706 was predicted to encode a drug efflux protein that shares 96% ID and 100% coverage with the efflux protein CAY48790.1. Predicted gene ctg1_3706 shared 97% ID and 100% coverage with the macrolide-specific ABC-type efflux carrier protein CAY48791.1. In the antisense reading frame relative to genes ctg1_3705 through ctg1_3708, the locus ctg1_3704 featured 88% ID and 97.3% coverage with the gene encoding a LuxR-family regulatory protein CAY48792.1. On a separate putative NRP gene cluster within region 9 of the B1.1W genome, 437124 bp upstream from to ctg1_3708, a putative NRPS gene ctg1_4021 shared 84.25% ID and 100% coverage with the viscosin NRPS encoded by *visc*A CAY50416.1. Directly upstream in the opposite reading frame, ctg1_4022 was predicted to encode LuxR family DNA-binding response regulator (65% ID and 100% coverage with banamide cluster BGC0001346, protein AOA33120.1). Ctg1_4023 shared 73% ID and 100% coverage a multidrug/solvent efflux pump outer membrane protein MepC (CCJ67633.1) encoded within the tolaasin cluster BGC0000447. It was predicted to contain two modules with putative adenylation domains specific for Leu-Asp/Glu (Figure 13).

Discussion

Soil-inhabiting *Pseudomonas* spp. represent a rich source of antimicrobial natural products. In this study, we isolated a putatively novel species with antifungal properties from active layer permafrost using the *in situ* cryo-iplate device. After sequencing the genome of *Pseudomonas* sp. B1.1W and conducting genomic ANI comparisons with its closest relatives, the B1.1W genome was found to share <95% ANI with the genomes of *Pseudomonas* sp. R76, *P. poea*, and *P. antarctica* that pertain to sub-lineages of the *P. fluorescens* group. Given that ANI comparisons between B1.1W and its closest relatives are below the species-level ANI cutoff (<95%), this isolate putatively represents a novel *Pseudomonas* species with p<0.011confidence. During dual-culture challenges, B1.1W only exhibited antifungal activity against *B. cinerea* WT and not against *Penicillium olsonii* WT*, Fusarium oxysporum* WT and *Pythium myriotylum* WT. Ethyl acetate extracts prepared from liquid co-culture supernatants between B1.1W and *B. cinerea* also exhibited antifungal activity against *B. cinerea* WT and not the other tester pathogens at the concentrations tested herein. Extracts prepared from pure cultures of B1.1W did not display mycelial inhibition of *B.cinerea* on MGI assays at the tested concentrations. Given that Lux-R proteins were detected in BGCs of the B1.1W genome, a possible explanation for this observation could be that the expression of the antifungal causative agent(s) is upregulated using Lux-R systems mediated by inter-kingdom transcriptional regulators present under co-culture conditions leading to higher yields of the agent(s) in organic extracts (De Bruijn & Raaijmakers, 2009; Gao et al., 2018; Hoshino et al., 2019; Zhang & Elliot, 2019).

After performing C18 Sep-pak column fractionation and MALDI-TOF MS-MS on the large scale B1.1W extract, a 1148 Da compound containing six discernable amino acids matching the amino acids comprising the viscosin peptide moiety was identified. This fraction displayed

inhibitory activity against *Botrytis cinerea.* The mass of this detected compound is consistent with a protonated ion of viscosin containing a sodium adduct (Laycock et al., 1991; Martin H et al., 2019). Supporting these findings, the LC-MS analysis performed on ethyl acetate extracts derived from liquid culture supernatants of B1.1W, in pure culture and in co-culture with *B. cinerea*, contained signals of a compound with the identical chemical formula and consistent mass with viscosin ($C_{54}H_{95}N_9O_{16}$ and 1126 Da, respectively) (De Bruijn & Raaijmakers, 2009; Laycock et al., 1991; Martin H et al., 2019). These signals from the B1.1W extracts were detected at retentions times of 26 minutes that are consistent with previous LC-MS profiles of viscosin that were observed under similar analytical conditions (Laycock et al., 1991; Martin H et al., 2019). However, a strong 1035 Da fragment ion was detected in the MALDI-TOF MS-MS spectrum of the active B1.1W fraction which is not typically derived from a viscosin parent ion. Additionally, the MS-MS spectrum of this fraction only contained two out of the five typical strong peak signals (with weights of 714 and 843 Da) that correspond to fragments regularly derived from a viscosin parent ion (Laycock et al., 1991; Renard et al., 2019). The corresponding peaks detected in the active B1.1W fraction were consistent with 714 and 843 Da fragments containing protonated sodium adducts; with respective observed weights of 736, and 865 Da. The possibility of whether B1.1W can produce viscosin-like derivatives in addition to viscosin, which may lead to the typical and atypical ion fragment signals that we detected, requires confirmation through further structural characterization.

Genomic mining of B1.1W revealed a viscosin-like BGC containing three putative NRPS-encoding genes homologous to *visc*A *visc*B and *visc*C initially found in *P. fluorescens* SBW25 that lead to the biosynthesis of the viscosin nonapeptide moiety (Braun et al., 2001). Similar to the NRPS-encoding genes in SBW25, the three NRPS genes in the B1.1W genome contain nine

modules that include A domains with predicted amino acid specificities consistent with the viscosin nonapeptide moiety composition (Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile) (Braun et al., 2001). The predicted amino acid specificities of these NRPS A domains were also consistent with the amino acid composition of the 1148 Da molecule detected by MALDI-TOF MS-MS which displayed inhibitory activity against *B. cinerea*. The first two NRPS genes were located within one genomic locus and shared 88% and 89% predicted amino acid sequence identities with the NRPSs encoded by *visc*B and *visc*C (respectively) (Braun et al., 2001). The modules of these two putative NRPSs were predicted to have A domain specificities for Thr-Val-Leu-Ser-Leu-Ser-Ile consistent with the substrate specificities of the A domains encoded within *visc*B and *visc*C (Braun et al., 2001). Additionally, a thioesterase domain was predicted within the terminal Ile-specific module as found in the NRPS encoded by *visc*C (Braun et al., 2001). These two NRPS genes were found to be conserved in B1.1W's relatives; strains *Pseudomonas antarctica* strains BS2772 and CMS35 isolated from plant rhizospheres within Antarctic soil (Reddy et al., 2004).

The third putative NRPS gene in the B1.1W genome was found 437124 bp upstream from the first two and featured modules containing A domains with predicted substrate specificities for Leu-Glu/Asp. This third NRPS gene shared 84.25% amino acid sequence homology with the NRPS encoded by *viscA*. The genomic organization of the three NRPS encoding genes in B1.1W is consistent with the viscosin gene cluster organization in SBW25 (Braun et al., 2001). The *visc*A gene is genomically disconnected from *viscB* and *viscC* by 1.62Mbp whereas the third NRPS-encoding gene in B1.1W was approximately 437124 bp upstream from the other two putative NRPS genes (Braun et al., 2001). Physical separation between one NRPS gene and the other two is typical in *Pseudomonas* spp. capable of producing viscosin-type CLPs (Braun et al.,

2001; Loper et al., 2012) (De Bruijn et al., 2007). The third disconnected NRPS gene in B1.1W was also detected in the genomes of relative *Pseudomonas* sp. R76 and *P. poae* strain PMA22 but not in the genomes of its Antarctic relatives. Additionally, the B1.1W viscosin-like gene cluster organization was consistent with the viscosin cluster of SBW25. Specifically, the B1.1W cluster featured Lux-R and efflux protein encoding genes directly downstream of the NRPS genes in similar synteny organization and reading frame as the SBW25 viscosin cluster (Loper et al., 2012).

Despite the high degree of homology and similar gene cluster organization between the predicted NRPSs of B1.1W and those found in SBW25, the B1.1W NRPSs' substrate specificities remain to be confirmed empirically. We cannot exclude the possibility that the observed differences in protein sequences between the predicted B1.1W NRPSs and those of SBW25 could lead to new biosynthetic capabilities. For example, it has been reported that relaxed substrate selectivity originating from mutations in NRPS A domain sequences could lead production of viscosin isoforms with different amino acid configurations (De Bruijn et al., 2007). Additionally, there are 17 genes predicted to be part of the viscosin-like cluster in the B1.1W genome that are not found in the SBW25 cluster whose biosynthetic roles, if any, remain unmapped. Should these putative genes that include acyltransferases and methyltransferases lead to functional biosynthetic enzymes, they may potentially impart structural modifications to the fatty acid tail or to the peptide moieties leading to the formation of viscosin derivatives. Since the amino acid composition and configuration of the peptide moiety along with the composition of the fatty acid tail dictate the antimicrobial properties of CLPs, the biosynthetic potential of this strain to produce viscosin group CLP(s) merits further characterization (De Vleeschouwer et al., 2020). To our knowledge, this study is one of the few reports describing a soil-dwelling high Arctic

Pseudomonas containing the biosynthetic genes that could lead to the production of a viscosin-like CLP with inhibitory activity against *Botrytis cinerea*.

Chapter 4. Tables and Figures

A

B

Genome Length	6.674233 Mbp
GC Content	60.09%
CDS	6809
tRNA	65
rRNA	16
Hypothetical proteins	1259
Proteins with GO assignments	6123
Proteins with Pathway assignments	6240

Figure 6. Hybrid assembly of the *Pseudomonas* sp. B1.1W genome combining Illumina short reads bridged with MinION long read contigs. **A)** Circular genome map of hybrid assembly annotated using Prokka pipeline within the CGView Server v. BETA. **B**) Genomic annotation analysis using PATRIC RASTtk-enabled Genome Annotation Service.

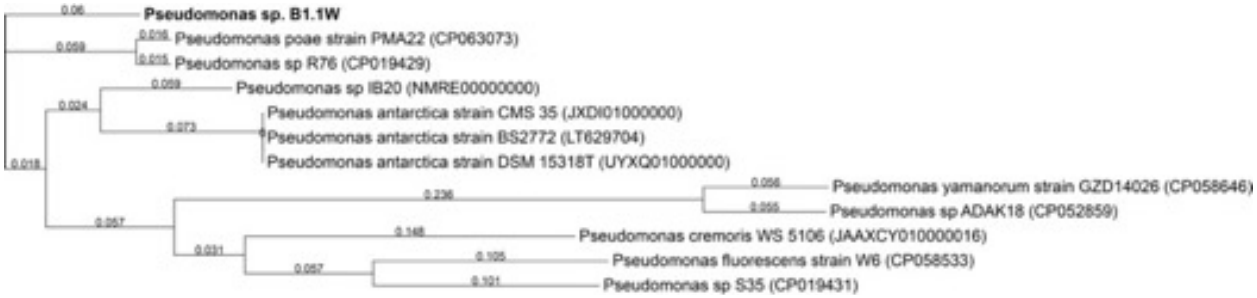


Figure 7. Phylogenetic codon tree including *Pseudomonas* sp. B1.1W and its closest relatives within the *P. fluorescens* group. Codon tree constructed using the PATRIC RASTtk-enabled Genome Annotation Service with single-copy PATRIC PGFams that employs the RAxML Bootstrapping method.

Figure 8. Average nucleotide identity (ANI) comparisons between the genome of *Pseudomonas* sp. B1.1W and its closest relatives. NCBI Prok tool within MiCrobial Genomes Atlas (MiGA) Online was used to query complete genomic sequences against the NCBI Genome database using Average Nucleotide Identity (ANI).

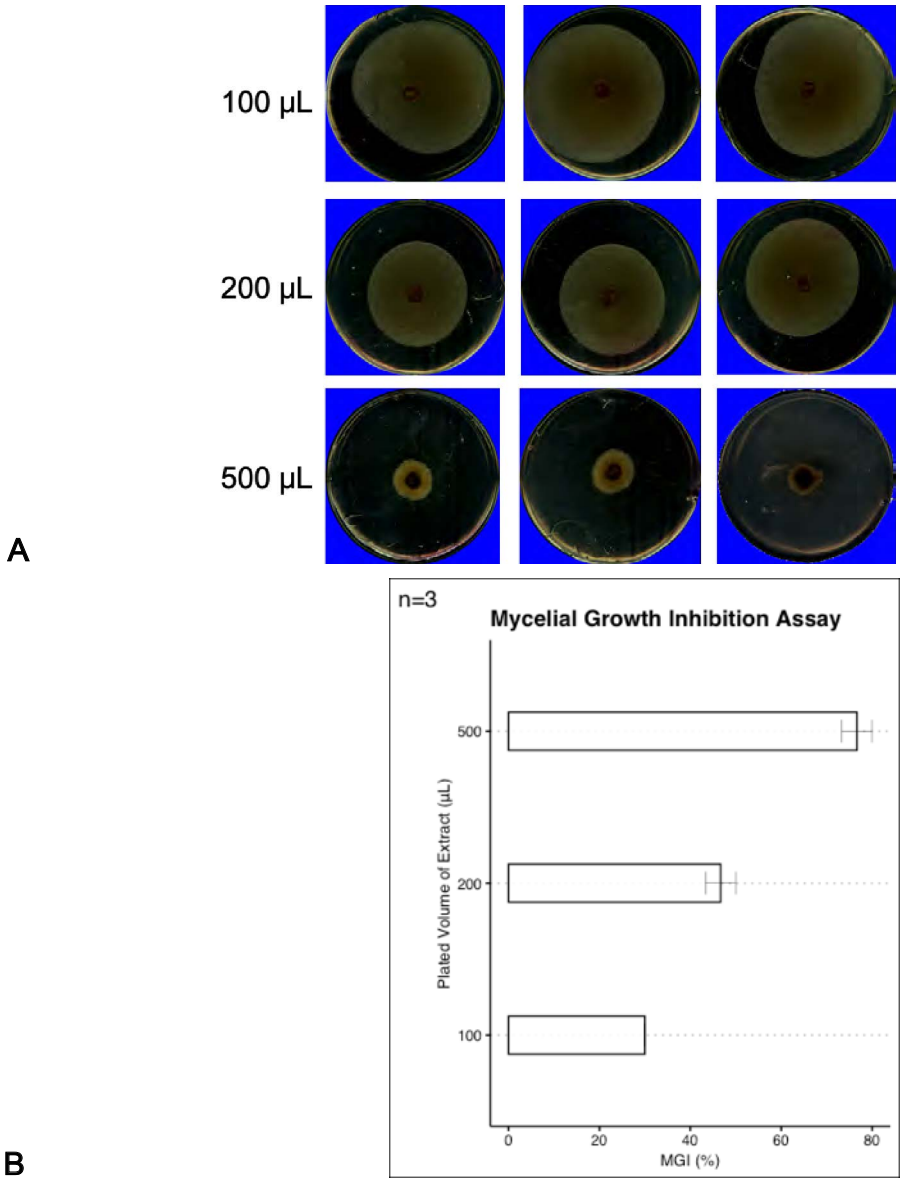


Figure 9. Antifungal activity of *Pseudomonas* sp. B1.1W and the organic extract. **A)** MGI assay where Petri dishes containing 10 mL of TSA were impregnated with 100, 200, and 500 µL of crude extract prepared using cell-free extracts of Pseudomonas sp. B1.1W and overlaid with 1×10^4 conidia of B. cinerea WT. **B)** MGI (%) plot showcasing dose-dependent mycelial inhibition.

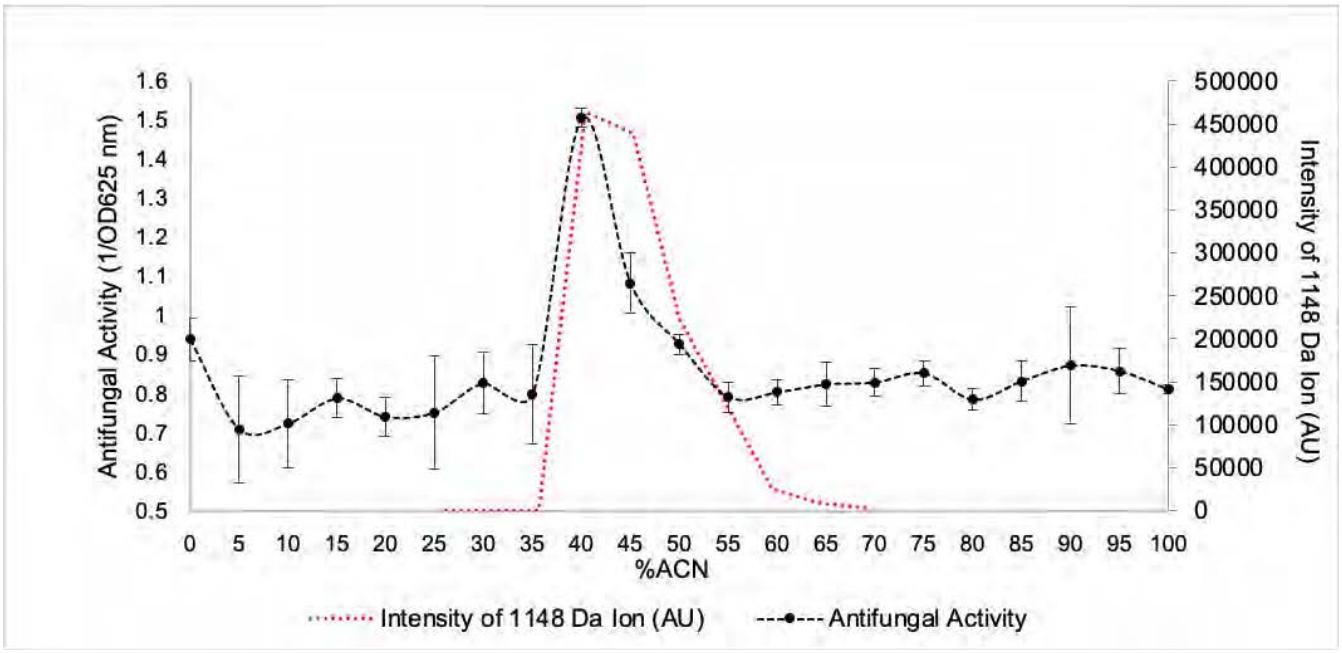


Figure 10. C18 Sep-pak column fractionation of the B1.1W large-scale extract eluted stepwise with acetonitrile (ACN) starting from 0% up to 100% with 5% increments. The 40% acetonitrile elution exhibited antifungal against *B. cinerea* and corresponds to a 1148 Da compound. The inverse absorbance at 625 nm peaked at the corresponding fraction intensity of the 1148 Da compound.

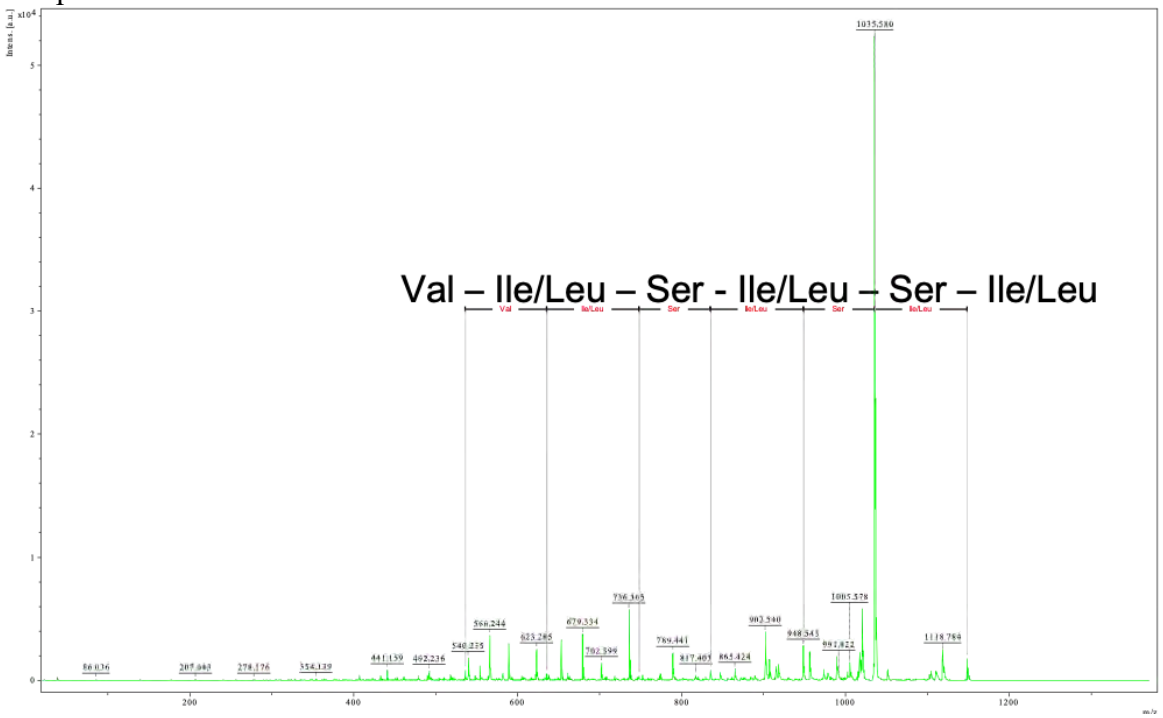
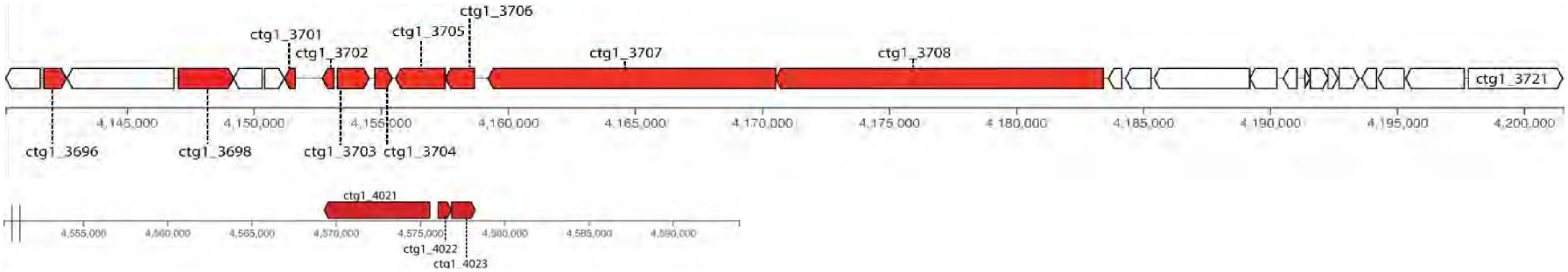


Figure 11. MALDI-TOF MS-MS chromatograph of the fraction from B1.1W fermentation extract containing the1148 Da ion that is active against *B. cinerea*. Spectrum was recorded using Bruker Daltonics UltrafleXtreme MALDI-TOF in the positive reflector mode Amino acid sequence of peptide moiety displayed above corresponding peaks.

A

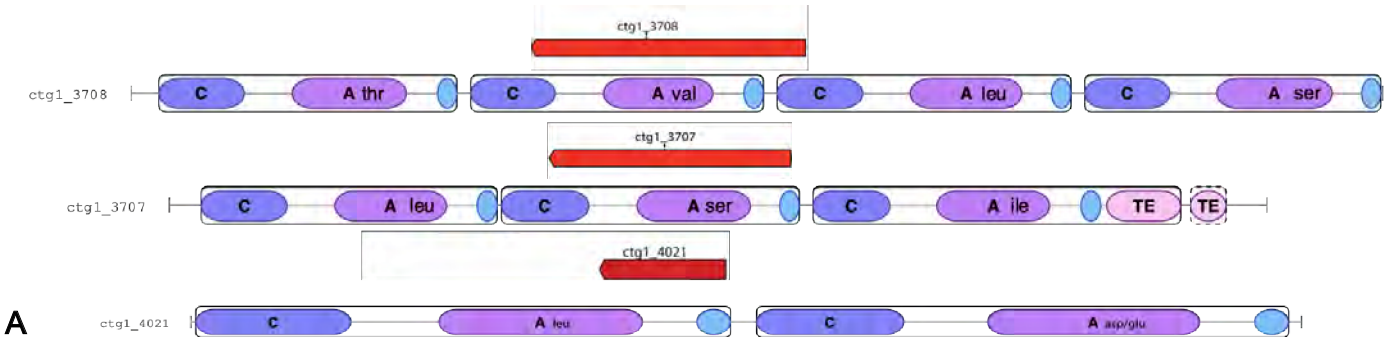


B

Locus Tag	MIBiG Protein	Description	MIBiG Cluster	MiBiG Product	% ID	% Coverage	E-value
ctg1_3696	CAY48797.1	Putative arac-type regulatory protein	BGC0001312	viscosin	89	99.3	3.00E-152
ctg1_3698	CAY48796.1	Putative ferric siderophore receptor	BGC0001312	viscosin	90	97.1	0
ctg1_3701	CAY48795.1	Conserved hypothetical protein	BGC0001312	viscosin	97	100	3.90E-76
ctg1_3702	CAY48794.1	AsnC family regulatory protein	BGC0001312	viscosin	97	100	1.50E-81
ctg1_3703	CAY48793.1	Methionine gamma-lyase	BGC0001312	viscosin	93	98.6	3.10E-219
ctg1_3704	CAY48792.1	Putative LuxR-family regulatory protein	BGC0001312	viscosin	88	97.3	3.60E-105
ctg1_3705	CAY48791.1	Macrolide-specific ABC-type efflux carrier	BGC0001312	viscosin	97	100	0
ctg1_3706	CAY48790.1	Putative drug-efflux protein	BGC0001312	viscosin	96	100.3	1.80E-197
ctg1_3707	CAY48789.1	Putative non-ribosomal peptide synthetase encoded by viscC	BGC0001312	viscosin	88	100.1	0
ctg1_3708	CAY48788.1	Putative non-ribosomal peptide synthetase encoded by viscB	BGC0001312	viscosin	84..25	100	0
ctg1_4021	CAY50416.1	Non-ribosomal peptide synthetase encoded by viscA	BGC0001312	viscosin	65	101.1	0
ctg1_4022	AOA33120.1	LuxR family dna-binding response regulator	BGC0001346	bananamide	65	100	3.60E-88
ctg1_4023	CCJ67633.1	Multidrug/solvent efflux pump outer membrane protein MepC	BGC0000447	tolaasin	73	100.2	3.20E-188

Figure 12. Putative viscosin-like gene cluster detected within B1.1W genome using antiSMASH. **A)** Locus tags corresponding to putative ORFs highlighted in red are homologous to known viscosin cluster BGC000132. Uncoloured ORFs represent genes that are not present in the *P. fluorescens* SBW25 viscosin cluster. **B**) Description table of locus tags represented in gene cluster in **A**).

A



B

Locus Tag	Putative AMP-binding Domain Location	NRPSPredictor2	Large clusters prediction	Stachelhaus sequence	Nearest Stachelhaus code	Stachelhaus code match
ctg1_3708	552-946	thr	ser, thr, dhpg, hpg	DFWNIGMVHK	thr	100% (strong)
ctg1_3708	1622-1999	val	gly, ala, val, leu, ile, abu, iva	DALWIGGTFK	val	100% (strong)
ctg1_3708	2678-3063	leu	gly, ala, val, leu, ile, abu, iva	DAWFLGNVVK	leu	100% (strong)
ctg1_3708	3733-4128	ser	ser, thr, dhpg, hpg	DVWHMSLVDK	ser	100% (strong)
ctg1_3707	568-953	leu	gly, ala, val, leu, ile, abu, iva	DAWFLGNVVK	leu	100% (strong)
ctg1_3707	1606-1997	ser	ser, thr, dhpg, hpg	DVWHMSLVDK	ser	100% (strong)
ctg1_3707	2636-3027	ile	gly, ala, val, leu, ile, abu, iva	DAMFLGCTYK	ile	100% (strong)
ctg1_4021	466-849	leu	gly, ala, val, leu, ile, abu, iva	DAWFLGNVVK	leu	100% (strong)
ctg1_4021	1498-1898	asp	asp, asn, glu, gln, aad	DgWKLGVVDK	asp	90% (moderate)

C

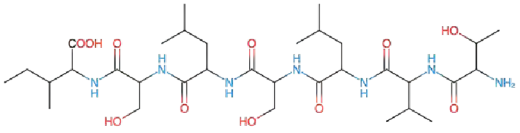


Figure 13. Putative domain predictions of nonribosomal synthetase genes detected within genome of B1.1W responsible for the production of the peptide moiety of the viscosin-like lipopeptide. **A)** Adenylation (A) with amino acid specificity, condensation (C), and peptide carrier domains within nonribosomal synthetase genes encoded within ORFs ctg1-3707,3708, 4021. **B)** Description table of putative adenylation domains with predicted amino acid specificity detected within ORFs ctg1-3707,3708, 4021. **C)** Predicted peptide moiety produced by putative NRPSs encoded within ORFs ctg1-3707,3708, 402.1.

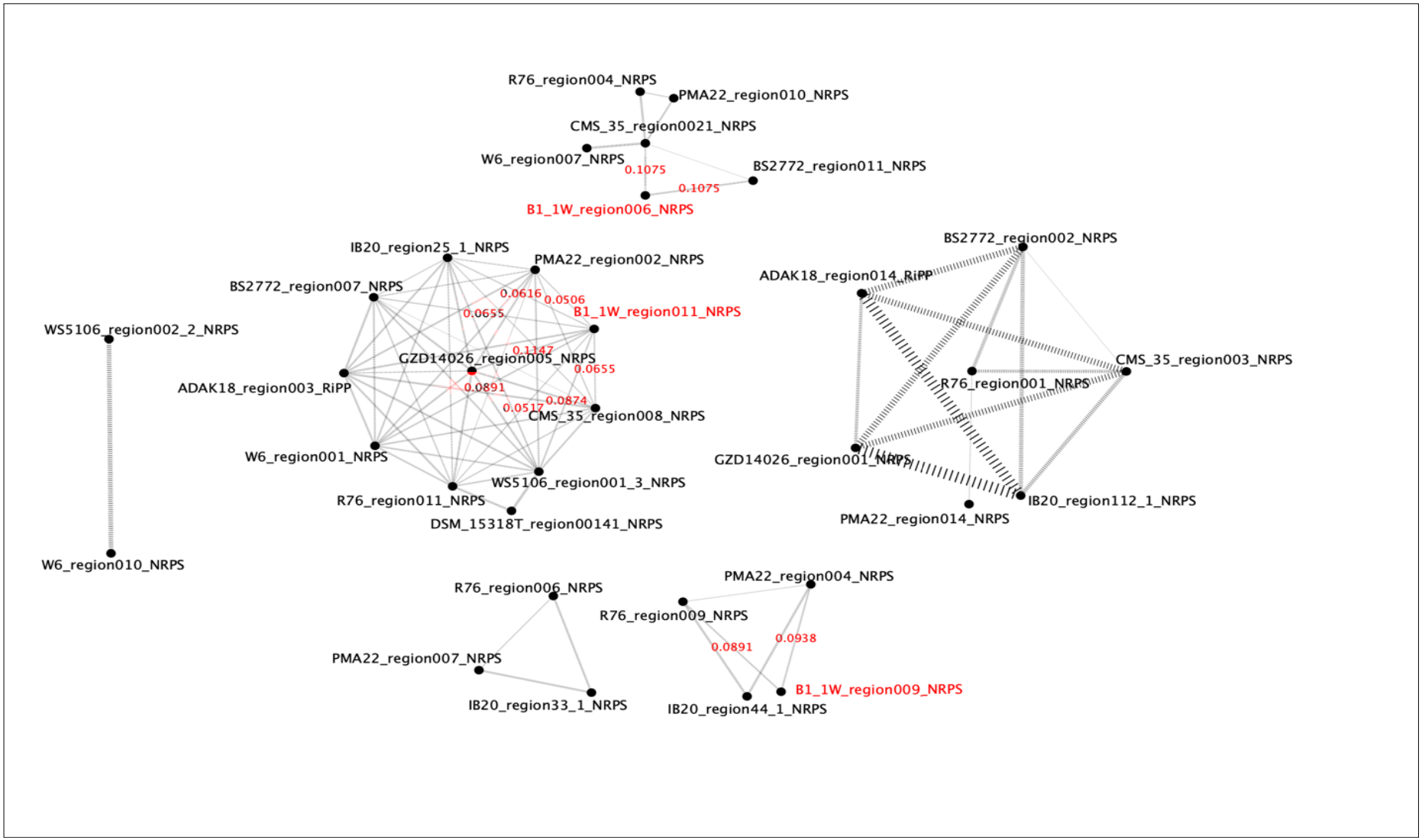


Figure 14. BGC sequence homology network of NRPs detected within B1.1W and *Pseudomonas* relatives. Nodes represent nonribosomal gene clusters detected within genomes related to B1.1W (red labelled nodes). Perfuse force open network constructed using DSS anchored domain sequence homology between BGCs using 0.3 DSS cutoff.

Table 8. BGCs detected within *Pseudomonas* sp. B1.1W genome using antiSMASH.

Region	Type	Length	Most similar known cluster	Similarity
Region 1	RiPP	9,364	None	0%
Region 2	arylpolyene	43,575	APE Vf	40%
Region 3	RiPP	9,216	None	0%
Region 4	NRPS	39,745	None	0%
Region 5	NRPS	64,123	pyoverdin	11%
Region 6	thiopeptide	29,089	lipopolysaccharide	5%
Region 7	NRPS	61,275	viscosin	62%
Region 8	RiPP	10,824	None	0%
Region 9	betalactone	28,475	fengycin	13%
Region 10	NRPS	43,610	anikasin	44%
Region 11	NRPS	52,900	pyoverdin	10%
Region 12	RRE-containing	20,275	lankacidin C	13%

Supplementary Materials

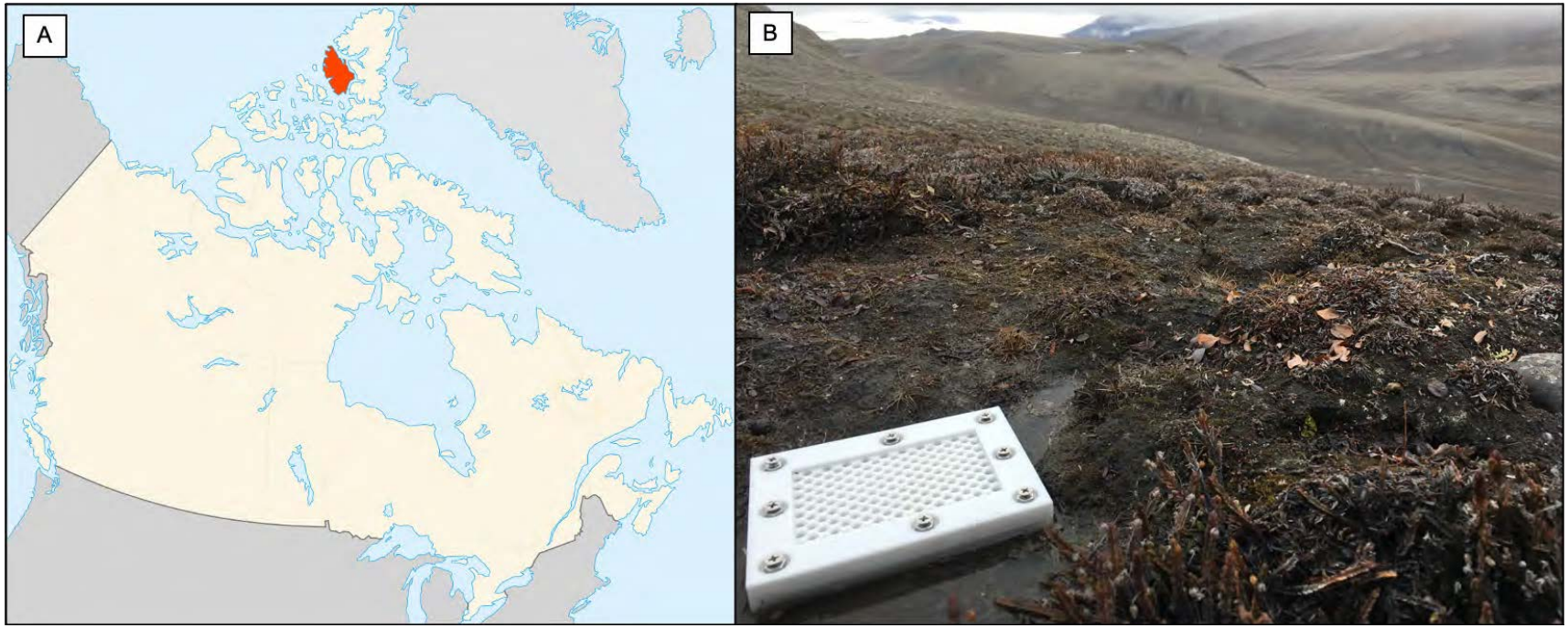


Figure S2. Sampling and microbial cultivation technique employed in this study**. A)** Geographic location of sampling location: Expedition Fjord, Axel Heiberg Island, Nunavut, Canada 79.412872, -90.740343. **B)** *In situ* microbial cultivation using the cryo-iplate device incubated within the active layer permafrost horizon which includes rhizospheres of *Cassiope tetragona*.

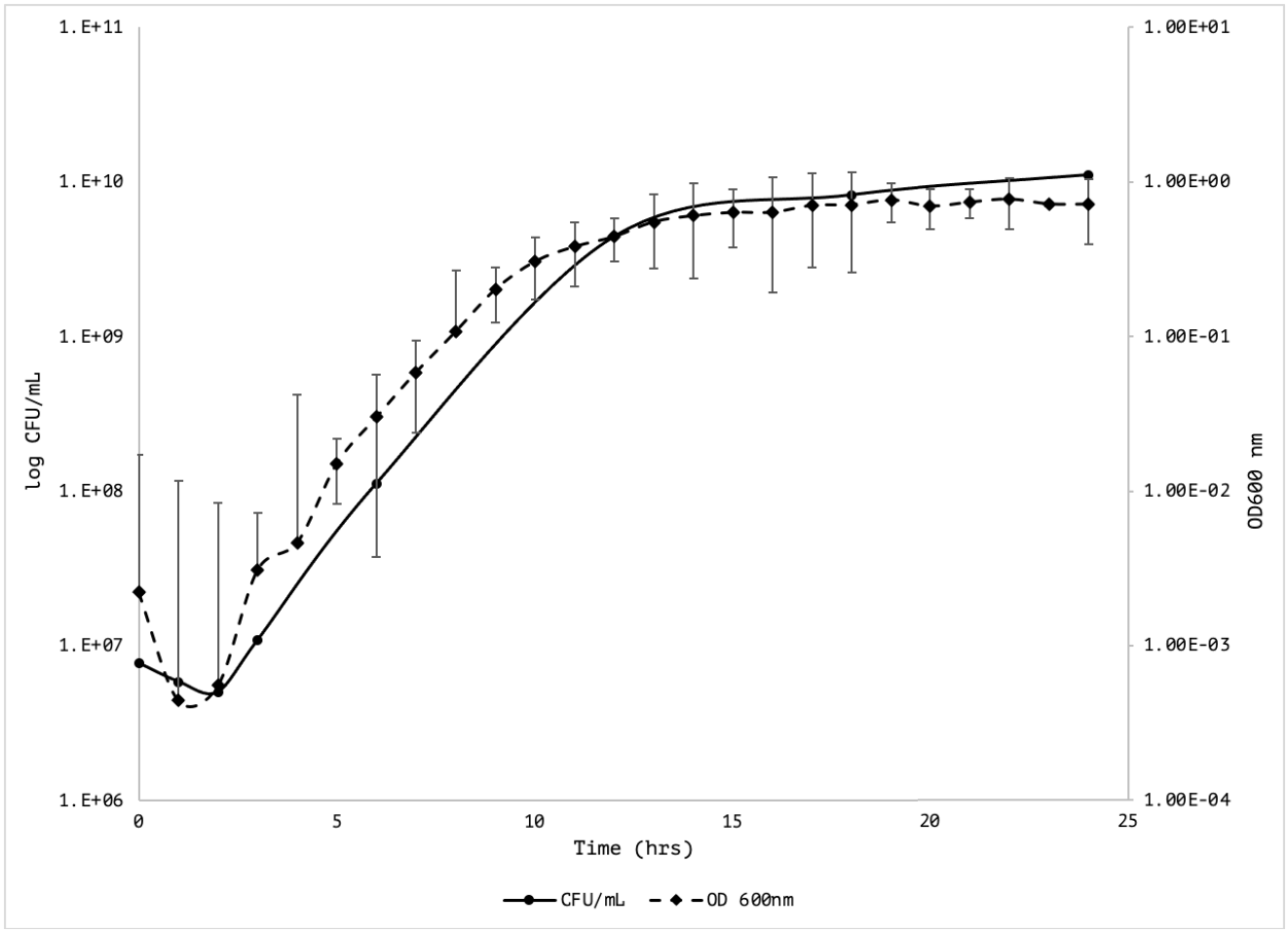


Figure S3. Growth curve of Arctic isolate Pseudomonas sp. B1.1W incubated at 25 ºC within tryptic soy broth for 24 hrs.

Figure S4. Co-culture method used to screen *Pseudomonas* sp. B1.1W against *Botrytis cinerea*.
A) 50 µL of 24 hr liquid cultures containing approximately 1.0 x 1011 CFU/mL of Arctic bacteria were inoculated in 5 mm wells at the base of plates containing 10 mL of TSA. **B**) 5 mm agar plugs of fresh *B. cinerea* culture were placed at the top of the TSA plate. Zones of inhibition were observed after incubating at room temperature for 10 days.

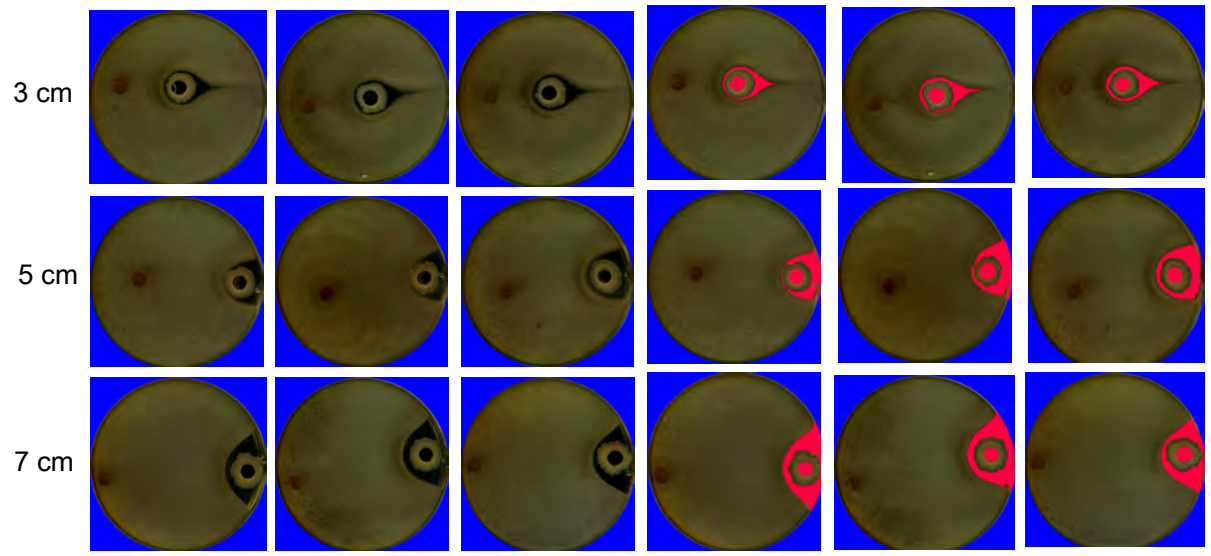


Figure S5. Co-culture challenges between B1.1W and B. cinerea. Inocula spaced 3, 5, 7 cm apart on plates containing 10 mL TSA. After 10 days of incubation, zone of inhibition areas were measured using imaging software Assess 2.0.

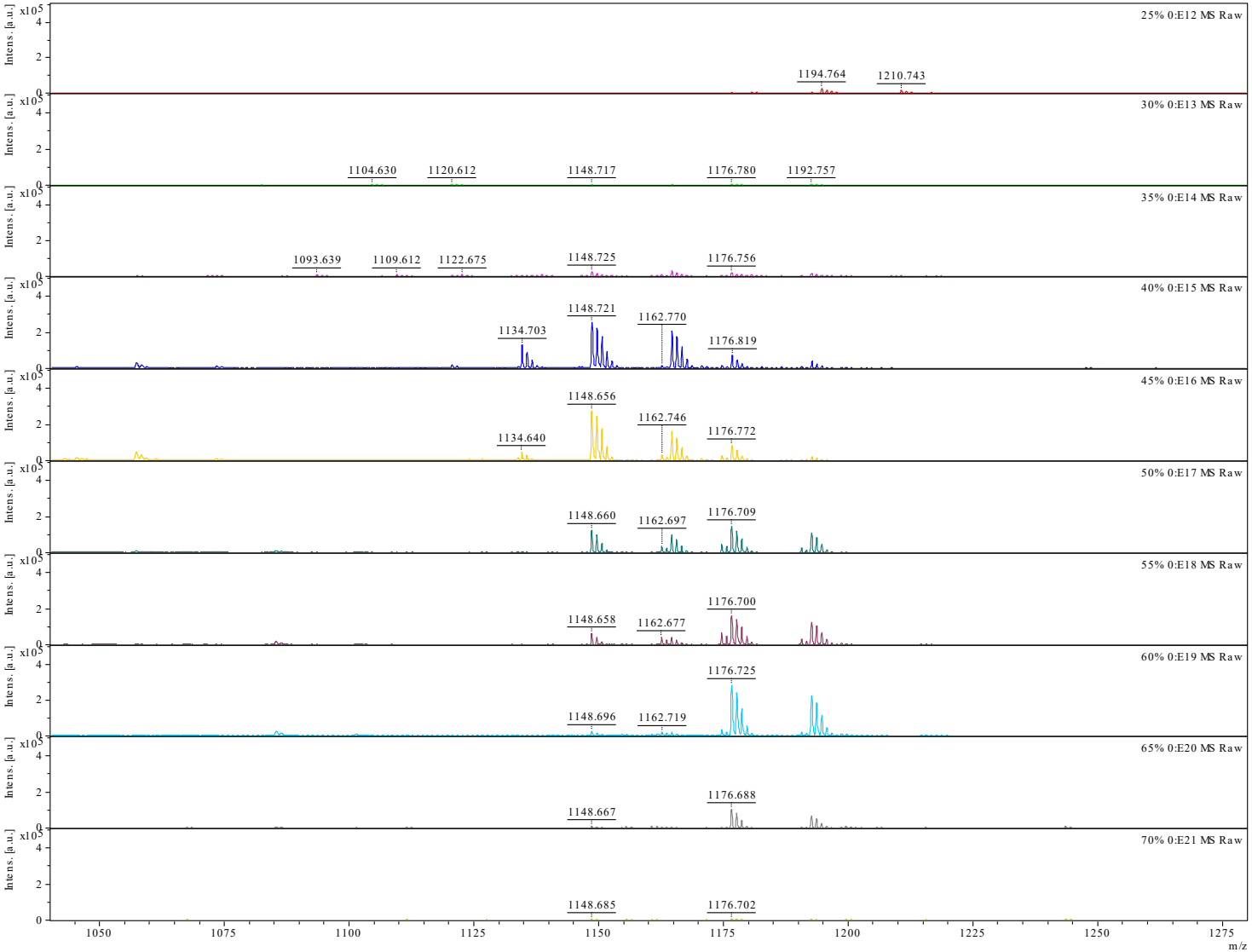


Figure S6. Fractionation of B1.1W crude extract. The B1.1W large scale extract was added to a C18 Sep-pak column (Resprep) and eluted stepwise with acetonitrile (ACN) starting from 0% up to 100% with 5% increments. The peak pertaining to 1148 Da molecule displayed antifungal activity against *B. cinerea*.

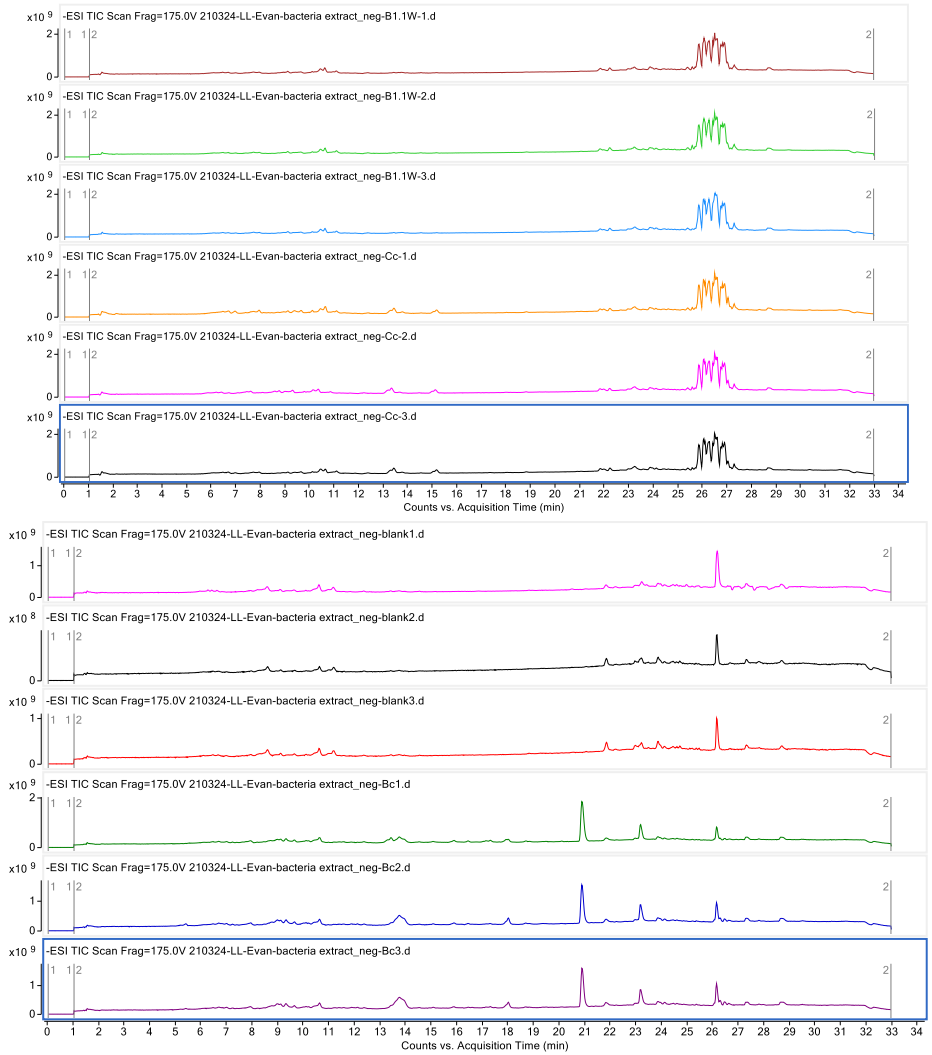


Figure S7. LC-MS performed in negative ion mode on small scale ethyl acetate extracts of liquid cultures containing: 1) B1.1W in pure culture (panels B1.1W-1-3.d) 2) B1.1W in co-culture with *B. cinerea* (panels Cc-1-3.d) 3) *B. cinerea* in pure (panels Bc-1-3.d) and 4) procedural and procedural blank TSB medium (panels blank-1-3.d)**.** Compound with chemical formula C54H95N9O16 identical to viscosin eluted at ~26 min in extract groups 1 & 2.

Table S5. Taxonomic novelty of *Pseudomonas* sp. B1.1W based on genomic ANI comparisons.

Taxonomic Rank	Taxonomic classification	p-value
Phylum	*Proteobacteria*	0.999
Class	*Gammaproteobacteria*	0.998
Order	*Pseudomonales*	0.991
Family	*Pseudomonadacae*	0.98
Genus	*Pseudomonas*	0.946
Species	*Pseudomonas sp. R76*	0.011

p-value indicates the probability of any two genomes not being classified in the same taxon at the specified taxonomic level.

Table S6. Chemical feature consistent with viscosin detected in B1.1W extracts using LC-MS in negative ion mode.

Compound	Formula	Mass (Da)	Retention Time (min)	Height	Score (MFG)
B1.1W extract	$C_{54}H_{95}N_9O_{16}$	1125.69	26.208	1511814	99.75
Viscosin*	$C_{54}H_{95}N_9O_{16}$	1126	26	-	-

*LC-MS profile of viscosin molecules described in (Laycock et al., 1991; Martin H et al., 2019)

Table S7. BLASTp of translated gene sequences comprising the viscosin-like gene cluster in *Pseudomonas* sp. B1.1W.

Gene Locus Tag[a]	Genomic Location[b]	Gene Length (nt)[c]	Protein Product Length (aa)[d]	BlastP Closest Matching Protein[e]	Organism of Closest Match[f]	Query Cover (%)	ID (%)[h]	E-value	Accession
ctg1_3695	4,140,243 - 4,141,601	1359	452	Glutamine synthetase family protein	Unclassified *Pseudomonas* sp.	100	99.56	0.00	WP_159957331.1
ctg1_3696	4,141,740 - 4,142,633	894	297	Helix-turn-helix domain-containing protein	*Pseudomonas* sp. R76	99	98.31	0.00	WP_159957332.1
ctg1_3697	4,142,659 - 4,146,867	4209	1402	Leucine-rich repeat domain-containing protein	*Pseudomonas fluorescens*	99	72.02	0.00	WP_191950129.1
ctg1_3698	4,147,028 - 4,149,214	2187	728	TonB-dependent siderophore receptor	*Pseudomonas* sp. MF6747	95	94.65	0.00	WP_200625519.1
ctg1_3699	4,149,204 - 4,150,334	1131	376	MFS transporter	Unclassified *Pseudomonas* sp.	100	93.88	2.00E-167	WP_094952729.1
ctg1_3700	4,150,433 - 4,151,203	771	256	Helix-turn-helix transcriptional regulator	*Pseudomonas* sp. ArH3a	99	95.69	9.00E-180	WP_242155738.1
ctg1_3701	4,151,208 - 4,151,645	438	145	Carboxymuconolactone decarboxylase family protein	Unclassified *Pseudomonas* sp.	100	99.31	7e-89	WP_049708741.1
ctg1_3702	4,152,704 - 4,153,174	471	156	Lrp/AsnC family transcriptional regulator	Unclassified *Pseudomonas* sp.	100	98.72	6.00E-109	WP_106580315.1
ctg1_3703	4,153,298 - 4,154,542	1245	414	Methionine gamma-lyase	*Pseudomonas* sp. ArH3a	100	96.14	0.00	WP_242155736.1
ctg1_3704	4,154,774 - 4,155,451	678	225	Helix-turn-helix transcriptional regulator	Unclassified Pseudomonas sp.	100	92	3.00E-140	WP_242168309.1
ctg1_3705	4,155,588 - 4,157,546	1959	652	MacB family efflux pump subunit	Pseudomonas sp. R76	100	98.62	0	WP_177015725.1
ctg1_3706	4,157,550 - 4,158,698	1149	382	Macrolide transporter subunit MacA	Pseudomonas sp. R76	100	97.65	0	WP_159955854.1
ctg1_3707	4,159,222 - 4,170,546	11325	3774	Non-ribosomal peptide synthetase	Pseudomonas poae	100	93.77	0	WP_197628781.1
ctg1_3708	4,170,543 - 4,183,448	12906	4301	Non-ribosomal peptide synthetase	Pseudomonas poae	100	94.14	0	WP_197628784.1
ctg1_3709	4,183,646 - 4,184,161	516	171	VWA domain-containing protein	Pseudomonas sp. D2002	97	97.01	2.00E-96	WP_218175106.1
ctg1_3710	4,184,317 - 4,185,315	999	332	AAA family ATPase	Unclassified Pseudomonas sp.	100	94.88	2.00E-178	WP_065906454.1
ctg1_3711	4,185,441 - 4,189,202	3762	1253	Cobaltochelatase subunit CobN	Pseudomonas sp. R76	100	97.77	0	WP_159955846.1
ctg1_3712	4,189,206 - 4,190,273	1068	355	Cobalamin biosynthesis protein CobW	Pseudomonas sp. RGB	100	98.03	0	WP_145164671.1
ctg1_3713	4,190,523 - 4,191,062	540	179	Hypothetical protein SAMN05216237_1687	Pseudomonas yamanorum	41	89.33 %	2.00E-37	SDU04421.1
ctg1_3714	4,191,379 - 4,191,567	189	62	CbtB-domain containing protein	Pseudomonas sp. EYE_354	70	100	7.00E-23	WP_248082527.1
ctg1_3715	4,191,579 - 4,192,286	708	235	CbtA family protein	Unclassified Pseudomonas sp.	100	96.17	4.00E-116	MCJ7959835.1
ctg1_3716	4,192,283 - 4,192,675	393	130	Cobalamin biosynthesis protein	Pseudomonas sp. R76	100	93.08	7.00E-59	WP_159955838.1
ctg1_3717	4,192,715 - 4,193,491	777	258	Precorrin-4 C(11)-methyltransferase	Unclassified Pseudomonas sp.	96	97.58	5.00E-161	WP_106578733.1
ctg1_3718	4,193,612 - 4,194,196	585	194	Fe-S biogenesis protein NfuA	Unclassified Pseudomonas sp.	100	100	1.00E-140	WP_017136914.1
ctg1_3719	4,194,256 - 4,195,278,	1023	340	Acyltransferase	Unclassified Pseudomonas sp.	100	94.41	0	WP_106578732.1
ctg1_3720	4,195,342 - 4,197,648	2307	768	Fatty acid cis/trans isomerase	Pseudomonas sp. R76	95	96.19	0	WP_159955832.1
ctg1_3721	4,197,815 - 4,201,525	3711	1236	Methionine synthase	Pseudomonas sp. R76	100	98.79	0	WP_201123554.1

Chapter 5: Connecting Text

The objective of Chapter 5 was to test the biocontrol potential of *Pseudomonas* sp. B1.1W described in Chapter 4 against *Botrytis cinerea* WT in *Cannabis sativa*. As the causative agent for grey mold disease in cannabis*, B. cinerea* is one of the most prevalent phytopathogens affecting pre- and post-harvest cannabis crops. Whole cell and cell-free liquid culture supernatant extracts of B1.1W were applied onto foliar tissues of *C. sativa* that were challenged with *B. cinerea*. We found that whole cell and extract treatments of B1.1W reduced total mold counts and stunted *B. cinerea* disease progression when applied to the plants. We observed a greater reduction of total mold cell counts on detached leaves treated with cell-free liquid culture supernatant extracts of B1.1W compared to the commercial biocontrol product Actinovate designed to mitigate *B. cinerea* growth on cannabis. Additionally, we found that B1.1W has the potential of colonizing leaf tissues through 16S community profiling of cannabis leaves treated with B1.1W cells. To our knowledge, this is the first report of high Arctic bacteria being applied as a foliar biocontrol agent on *C. sativa*.

Chapter 5. A high Arctic isolate, *Pseudomonas* sp. B1.1W provides biocontrol against *Botrytis cinerea* in *Cannabis sativa* L.

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Abstract

As the legalization of cannabis cultivation continues to expand globally, multiple strategies will be required to combat crop-losses attributed to fungal diseases. *Botrytis cinerea*, the causative agent of bud rot, is one of the most prevalent fungal phytopathogens responsible for pre- and post-harvest losses of cannabis. In this context, the goal of this study was to evaluate the biocontrol potential of *Pseudomonas* sp. B1.1W, a putatively novel species isolated from active layer permafrost in the Canadian high Arctic capable of inhibiting *B. cinerea*. Cellular inoculants of B1.1W and bioactive organic extracts, derived from cell-free liquid culture supernatants of B1.1W, were evaluated as foliar biocontrol agents on *C. sativa* strain CDB Kush (CBK) using detached leaf assays and whole plant evaluations. In detached leaf assays where *C. sativa* leaves were soaked in cellular inoculants and organic extracts of B1.1W, total mold counts were reduced by 43.79% and 89.83% (respectively) relative to untreated samples when challenged with 10^4 *conidia* of *B. cinerea*. Whole plants sprayed with B1.1W cellular inoculants and organic extracts displayed lower counts of symptomatic leaves exhibiting chlorotic and sporulating lesions throughout the course of a 14-day observation period post-challenge with *B. cinerea* compared to the commercial biocontrol strain *Streptomyces lydicus* strain WYEC 108. Targeted 16S amplicon sequencing of *C. sativa* leaves treated with cellular inoculants of B1.1W revealed that this strain dominates the foliar bacterial community, suggesting that it colonized leaf tissue. Supporting these findings, putative genes known to be implicated in foliar colonization pertaining to amino acid, polysaccharide, and type III secretion system biosynthesis, were detected within the genome of B1.1W. This study shows that B1.1W cellular inoculants and extracts offer *in planta* biocontrol protection against *B. cinerea* when applied as foliar treatments on *C. sativa.*

Introduction

Cannabis is an annual herbaceous plant where female inflorescences are studded with glandular trichomes laden with specialized metabolites including phytocannabinoids (Sirikantaramas et al., 2005; Tanney et al., 2021). The cannabinoids with medicinal and recreational relevance include tetrahydrocannabinol (THC), the main psychotropic constituent and cannabidiol (CBD), a non-psychotropic cannabinoid (Abrams, 2018; Sirikantaramas et al., 2005). Until recently, the cultivation of psychoactive cultivars of cannabis was prohibited in several jurisdictions internationally (Backer et al., 2019). As the cultivation of cannabis is increasing globally, so is the demand for high quality cannabis products. In Canada, the Cannabis Act was passed in 2018 which legalized cannabis cultivation for both commercial and scientific research purposes as well as recreational consumption (Cox, 2018).

One of the most prevalent fungal phytopathogens responsible for cannabis crop losses is *Botrytis cinerea*, the causal organism of gray mold disease, commonly referred to as bud rot and globally classified as the second most destructive fungal phytopathogen for all crops after *Magnaporthe oryzae* (Lucas et al., 2015). Within cannabis hosts, this necrotrophic fungus can infect seeds and tissues that comprise the phyllosphere (van Kan, 2006). Pathogenesis of *B. cinerea* is mediated by the secretion of lytic enzymes and phytotoxins breach plant tissues. These secreted biomolecules then trigger programmed cell death within the host, providing the pathogen with access to nutrients required for its growth from necrotized tissues (van Kan, 2006).

Common approaches for controlling *B. cinerea* combine synthetic fungicide usage with cultivation practices such as humidity control and heating within greenhouses. Outdoor cultivation measures include crop rotation, decreasing plant density, and increasing fertilization frequency (Elad, 2016). Unfortunately, indoor and field cultivation practices are ineffective in

mitigating the spread of *B. cinerea* given its robust infectivity. Thus, synthetic fungicides (such as Potassium bicarbonate (MilStop®) and hydrogen peroxide (ZeroTol®) remain the most popular methods for controlling the pathogen in Canada (Scott & Punja, 2021). However, the use of these compounds leads to several issues. One of these issues is the imparting of selective pressure on fungal pathogens to develop resistance against the finite arsenal of fungicides (Garrido et al., 2017). Further issues include environmental pollution and risks to human health when these synthetic fungicides remain on products destined for human consumption (Seltenrich, 2019). These concerns have led to the stringent regulation of fungicide usage in jurisdictions where cannabis cultivation is legally practiced (Seltenrich, 2019). As such, biocontrol agents comprised of environmental microorganisms with antimicrobial properties represent sustainable alternatives in controlling fungal diseases that commonly compromise cannabis production (Vujanovic et al., 2020).

In the search for novel biocontrol agents, recent studies have identified bacterial strains isolated from polar habitats capable of inhibiting phytopathogens (Torracchi C et al., 2020; Yarzábal, 2020; Zhuang et al., 2020). In this study, we evaluate the biocontrol effect of *Pseudomonas* sp. B1.1W, a putatively novel species originating from Arctic permafrost with inhibitory activity against *Botrytis cinerea* through a viscosin-like cyclic lipopeptide. Cellular inoculants and organic extracts prepared from supernatants of liquid cultures of B1.1W were tested as foliar biocontrol treatments on *C. sativa* challenged with *B. cinerea*. 16S targeted amplicon sequencing of cannabis leaves treated with B1.1W cells and extract was conducted to elucidate its biocontrol effects on the foliar bacterial community.

Methods

Detached Leaf Assay

To test the biocontrol potential of B1.1W and the organic extract, a detached leaf assay (DLA) was performed using fully developed *C. sativa* leaves excised from a mother plant in the vegetative growth stage. The assay employed a whole-cell suspension of B1.1W (1.0×10^{10} CFU/mL), an active organic extract of B1.1W (37.5 mg/mL in dH_2O) following the large scale extraction method described in chapter 4, Actinovate, and 10 mM $MgSO_4$ (negative control). To prepare the whole-cell treatment, liquid cultures of B1.1W, incubated in 15 mL TSB for 24 h at 25ºC with continual agitation at 250 rpm, were centrifuged twice at 10,000 rpm at 4ºC for 20 min to harvest cells. Cellular pellets were rinsed twice with cold sterile dH_2O and then suspended in 15 mL 10 mM $MgSO_4$.

For the DLA, *C. sativa* leaves were surfaced sterilized with 70% v/v ethanol, rinsed with sterile dH_2O, aseptically blot dried and then soaked for 30 min in 15 mL of the appropriate biocontrol treatment described above. Each treatment was conducted in quadruplicate. After treatment, *C. sativa* leaves were air dried and transferred to a Petri dish containing two pieces of sterile qualitative filter paper and a sterile cotton ball moistened with 5 mL of sterile dH_2O; the stem was inserted into the cotton ball to maintain leaf turgor. Each leaf was inoculated by placing a 5 mm agar disc from a ten-day old culture of *B. cinerea* on PDA (each disc containing ~1.0×10^4 conidia) on the mid-rib of the central leaflet. Petri dishes were sealed using adhesive gauze tape and then placed in a growth chamber and incubated at 30ºC under LED lights (Fluence Bioengineering) at approximately 100 µmol $m^{-2}s^{-1}$ for five days. At the end of the experiment, the extent of fungal biocontrol imparted by B1.1W and the organic extract was quantified using

the SimPlate for Yeast and Mold Color Indicator (Y&M-CI) (Millipore Co., Bedford, MA, USA) as per the protocol of the manufacturer.

Genomic analysis

To gain insight into the potential for B1.1W to colonize plant tissue, an annotation search of the B1.1W genome was conducted for the occurrence of 78 different protein families that are associated with epiphytic and apoplastic fitness originally found in *Pseudomonas syringae* strain B728a according to Helmann et al. 2019 (Helmann et al., 2019). A list of proteins within functional categories associated with amino acid and polysaccharide biosynthesis (with fitness scores of t>-3) along with type III secretion system was used to screen the annotated B1.1W genome. This was achieved using the protein family analysis tool within the PATRIC RASTtk-enabled Genome Annotation Service. Using the PATRIC genus-specific families (Plfams) filter, the occurrence of predicted proteins leading to epiphytic and apoplastic fitness found within the genome of *P. syringae* strain B728a were compared to occurrences within the genome of B1.1W and eleven closely related *Pseudomonas* strains as determined by the PATRIC Similar Genome Finder tool (Brettin et al., 2015a; Davis et al., 2020; Rodriguez-R et al., 2018). A heatmap of the protein family occurrences within these eight genomes was generated using PATRIC protein family analysis tool.

16S Community Profiling On Treated Detached Leaves

DNA Extraction and sequencing

Genomic DNA (gDNA) was extracted from biological triplicates for leaves treated 10 mM $MgSO_4$ inoculation (negative control), Actinovate (positive control), B1.1W whole cells (1.0×10^{10} CFU/mL) suspended in 10 mM $MgSO_4$, an active extract of B1.1W, 37.5 mg/mL in dH_2O, and untreated leaves aseptically collected from source plant. For each replicate, gDNA was

extracted from 100 mg of central leaflet tissue using the Dneasy Plant Pro DNA Isolation Kit (Qiagen) according to the protocol of the manufacturer. DNA concentrations were determined by Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). Extracted gDNA was then purified using Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA) according to the protocol of manufacturer. PCR amplification of the 16S rRNA gene was conducted using primers featuring Illumina overhang adapters 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016). PCR reactions were performed in triplicate on each biological replicate. Each 25 µL PCR reaction consisted of: 1X HotStarTaq Master Mix (Qiagen, Hilden, Germany), 0.6 µM of each primer, and 1 µL of DNA. The following conditions were used for the PCR amplification: 95°C for 15 min, 25 cycles of 94°C for 1 min, 50°C for 45 s, 72°C for 1 min, and 72°C for 10 min. Amplicons were then purified using Agencourt AMPure XP magnetic beads using a 0.8 bead-to-PCR volume ratio (Beckman Coulter, USA) before being indexed using the Nextera XT index kit (Illumina, San Diego, CA) following the protocol of the manufacturer. The Indexed library was then purified with AMPure XP beads using a 1.12 bead-to-sample volume ratio and quantified using the Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, USA). The indexed library was pooled in equimolar ratios to 4nM, and the quality was verified with an Agilent DNA 1000 kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The pooled denatured library was diluted to 4pM and sequenced on the Illumina MiSeq platform with the MiSeq Reagent Kit v3, 600 cycles.

Sequence data processing

Sequence processing of raw amplicon data was performed using R (v.4.1.1, R Core Team, 2021) (Team, 2013). To group microbial reads into amplicon sequencing variants (ASVs), the DADA2

package pipeline (Callahan *et al.,* 2016) was used (Callahan et al., 2016). To remove low-quality base calls, reads with expected errors ('maxEE' parameter) values higher than 3 and 4 were trimmed from the forward and reverse reads, respectively. Primer sequences were trimmed from both forward and reverse reads based on nucleotide lengths of each respective primer. To exclude contaminant sequences, the decontam package (Davis *et al.* 2018) was used with a prevalence-based identification method with a set threshold of 0.5 followed by manual removal of spurious ASVs with a threshold relative abundance of >0.25% per sample (n = 75) (Reitmeier, Sandra, et al. 2021, (Davis et al., 2018; Reitmeier et al., 2021). Taxonomic classification was performed using the SSU Ref NR 99% Silva database v. 1.9.5 (Quast et al., 2012). In addition to the decontam package, host DNA pertaining to *C. Sativa* was manually excluded after taxonomic classification by removing ASVs with chloroplast designations (n = 52). Detected contaminant sequences were omitted from the dataset before further analysis. Bacterial and fungal and ASV richness (*S*) and Shannon diversity index (*H'*) were calculated per treatment type using the vegan package v2.0-0 in R for community ecology (Oksanen et al., 2013). To detect broad trends in similarities and differences in bacterial families according to treatment types, a principal component analysis (PCA) was performed based on the UniFrac metric measure (Lozupone & Knight, 2005).

Cannabis plant experiments

Plant growth conditions

Cannabis plants were cultivated at the Macdonald Campus of McGill University under a Health Canada research license (LIC-5AZZW7S4GM-2021). CBD Kush starting material was obtained from a licensed producer (Markham, Ontario). Plants were propagated from cuttings, taken from a mother plant, and rooted in rockwool under LED lights (Koda™, LM030015F-1, 120 Vac, 60

Hz, 48 W, 4600 Lumens) for 14 days. Rooted cuttings were transplanted into 15.24 cm diameter pots filled with Promix (PRO-MIX All Purpose Mix, Plant Products, Laval, Quebec). Potted plants were grown under Fluence LED lights (Fluence Bioengineering, VYPR 2P, Austin Texas) under an 18/6 h day/night period at approximately 25°C and 65% relative humidity for 3 weeks for the vegetative growth period. Plants were watered on alternate days with 200 mL water and Remo Nutrients (Remo Nutrients Grow, Remo Brands Inc., Maple Ridge, BC) for the duration of the experiment according to the fertigation schedule of the manufacturer.

Experimental design

At the time of transplant, plants were sorted into experimental blocks according to height and leaf number per plant; within blocks plants were randomly assigned to treatment groups. The biocontrol treatment groups consisted of: 10 mM $MgSO_4$ inoculation (negative control), Actinovate (positive control), B1.1W whole cells (1.0×10^{10} CFU/mL) suspended in 10 mM $MgSO_4$, and an active extract of B1.1W, 37.5 mg/mL in dH_2O (as described in Detached Leaf section above). There were four plants per treatment for a total of 16 experimental plants. Plants were inoculated with the biocontrol treatment after seven days of growth in pots: each plant was sprayed with 10 mL of the treatment solution/suspension using a spray bottle (Adjustable Spray Bottle 23609-182 VWR International). Two days after receiving the biocontrol treatments, plants in the *B. cinerea* treatment group were challenged by spraying with 10 mL of *B. cinerea* spores suspended in water (4×10^4 conidia/mL, 0.05% v/v Tween20); plants were covered with plastic bags for 48 h after spraying with *B. cinerea* to maintain high humidity and promote disease progression.

Data collection and analysis

Since plants grown from cuttings are less uniform than plants grown from seeds, data was collected prior to the application of experimental treatments to record a baseline height, leaf number, node number, and SPAD readings for each plant to assign plants to treatment blocks and use as covariates in statistical analysis. Subsequently, the number of leaves displaying lesions attributed to *B. cinerea* infection were recorded every 48 hrs. after treatment and grouped based on disease severity: yellow chlorotic lesions, necrotic lesions, or sporulating lesions (Balthazar et al., 2020).

Generalized linear mixed modeling was performed using SAS PROC GLIMMIX (SAS version 9.4, SAS Inc., Cary, NC). The Gaussian/Normal distribution was not assumed and therefore the models were "generalized". The distributions that modeled the responses were selected based on the model fit statistics, that is, the Bayesian information criterion (BIC). The models included fixed factors and the random factor, *Pot*, and therefore the models were "mixed". The homogeneity of response variance was not assumed and models that included separate terms for treatment variances were selected for yellowing lesions and sporulating lesions, based on the BICs. In most cases, the variance components option (TYPE = VC), that is, a different variance component for each random effect, was selected as the covariance structure.

Results

***Pseudomonas* sp. B1.1W extract led to the greatest reduction in total fungal cell counts on detached leaves**

After quantifying total yeast and mold counts on leaves challenged with *B. cinerea* WT using the SimPlate method, we observed that the B1.1W crude extract treatment led to the greatest reduction in total fungal cell counts. The average total yeast and mold cells per gram amongst

triplicate leaves treated with the B1.1W crude extract were 6,333 cells/g, while counts for the B1.1W cell treatment, Actinovate, and $MgSO_4$ controls were 35,000, 27,333, and 62,266 cells/g, respectively (Figure 15).

Foliar bacterial community and diversity shifted according to biocontrol treatments

Phylloplanes treated with *Pseudomonas* sp. B1.1W cells were dominated by *Pseudomonales* accounting for 98.45% relative abundance whereas phylloplanes treated with B1.1W culture supernatant extract were dominated by *Rhizobiales* (83.16% relative abundance) and *Rickettsiales* (10.99%) (Figure 16). The Actinovate control phylloplanes were dominated by *Burkholderiales* (73.2%) and *Rhizobiales* (24.32%). Phylloplanes treated with $MgSO_4$ (negative control treatment) were dominated by *Burkholderiales* (59.15%) and *Pseudomonales* (34.78%). Blank phylloplanes (i.e., untreated leaves) predominantly featured *Rickettsiales* (70.8%) and *Burkholderiales* (21.71%). The foliar bacterial community composition of detached *C. sativa* leaves varied depending on the biocontrol treatment received (Figure 16, 18). In terms of ASV richness, the bacterial community of leaves treated with B1.1W cells had the lowest community richness ($S = 10$) whereas the Actinovate treatment featured the greatest richness ($S = 24$) (Figure 17). Leaves treated with B1.1W cells featured the lowest Shannon diversity values ($H' = 0.18$) whereas leaves treated with the B1.1W organic extract featured the highest diversity ($H' = 2.21$) (Figure 17).

The B1.1W genome contains predicted proteins involved in leaf colonization

Using the PATRIC RASTtk-enabled Genome Annotation Service, the presence or absence of 65 protein genes found within the genome of *P. syringae* strain B728a that have been linked to epiphytic and apoplastic fitness, were compared amongst B1.1W and eleven of its close relative

strains (Figure 19). Compared to its relatives, the genome of B1.1W and B728a featured the highest proportion of shared genes that contribute to leaf colonization (44 of the 68 genes used for the comparison). When compared to the genome of *P. syringae* strain B728a, the genome of B1.1W did not feature six genes pertaining to glycosyl transferases, 14 pertaining type III secretion proteins, and one gene pertaining to glutamine synthase. Specifically, the B1.1W genome did not feature certain glucosyl transferase proteins pertaining to group 1 and 2 families and type III secretion proteins HrpJ, HrpP, HrcQa, HrcQb, HrpT, HrpG, YscL, HrpD, Hrp, and HrpA pilin. The genomes of closely related *Pseudomonas antarctica* strains (BS2772, CMS 35, DSM 15318T) shared 39 of these genes (Figure 19).

B1.1W foliar treatments reduced *B. cinerea* disease symptoms *in planta.*

During a 14-day observation period post *B. cinerea* challenge, *C. sativa* plants treated with B1.1W cellular inoculant and extract displayed fewer leaves featuring yellow chlorotic and sporulating lesions when compared to the $MgSO_4$ (negative) and Actinovate (positive) controls (Figure 20). The number of leaves with chlorotic yellow lesions peaked on day eight for control groups whereas B1.1W cell and extract treatment groups peaked on day ten. The number of leaves displaying sporulating lesions started increasing on day five for plants that received B1.1W treatments, but the positive and negative control groups started increasing on day ten. After ten days, plants that received the B1.1W cellular inoculant displayed fewer leaves with chlorotic lesions than the extract treatment. By the end of the fourteen-day period, plants that received the B1.1W extract displayed fewer leaves with sporulating lesions (Figure 20).

Discussion

Cellular inoculants of B1.1W and the crude extract were tested as biocontrol treatments against *B. cinerea* on detached leaves and whole plants of *C. sativa*. Leaves soaked in cellular

suspensions of B1.1W and its extract led to 43.79% and 89.83% reductions in total mold counts relative to blank control treatments after being challenged with *B. cinerea* conidia. Given that B1.1W was previously shown to secrete a cyclic lipopeptide part of the viscosin family, a group of nonribosomal peptides with established biocontrol properties against phytopathogenic fungi, our observed reductions in DLA mold counts could be at least partially attributable to this agent (Marcolefas et al. 2022). Our observations support previous findings from culture-based studies illustrating the biocontrol potential of Polar *Pseudomonas* spp. against phytopathogens. *Pseudomonas* strains isolated from Antarctic marine sediments, and Arctic soils have been documented to inhibit the growth of various phytopathogens including members of phyla *Ascomycota* and *Oomycota* (de MELO et al., 2014; Gonçalves et al., 2015; Gorantla et al., 2014; Hennessy et al., 2015; Liu et al., 2019; Michelsen, Watrous, et al., 2015; Yarzábal et al., 2018). Previous studies characterized the biocontrol activity of polar strains of *Pseudomonas* but did not explore the applicability on cannabis. Whether B1.1W could inhibit the growth of other pathovars of *B.cinerea* affecting cannabis remains to be assessed.

When applied as foliar sprays onto *C. sativa* plants, cellular inoculant, and organic extract treatments of B1.1W mitigated *B. cinerea* disease symptoms. Compared to control treatment groups, plants sprayed with B1.1W cellular inoculant and organic extract displayed lower counts of leaves with *B. cinerea* lesions during a fourteen-day observation period. This was observed for both yellow chlorotic and sporulating lesion symptoms associated with *B. cinerea* infection in cannabis. The counts of leaves displaying chlorotic *B. cinerea* lesions on plants that received the B1.1W cellular inoculant and extract peaked at approximately two and five days, respectively, later than the control treatment groups. Therefore, B1.1W treatments can delay the onset *B. cinerea* infection *in planta*. Fourteen days post-challenge with the pathogen, plants treated with

the cellular inoculant of B1.1W exhibited fewer leaves with chlorotic lesions compared to the extract treatment. A possible explanation for this discrepancy could be that the viscosin-type CLP antifungal agent within the B1.1W extract may have photodegraded. Alternatively, it could have been potentially rendered inactive by oxidation during the course of the observation period given the inherent structural instability of this molecular group (Abellán et al., 2009; Wang & Zhuan, 2020). In contrast, the cells in the inoculant treatment could have colonized the plant tissues and secreted the bioactive agent(s), thus potentially offering a prolonged biocontrol effect when compared to the extract. Previous reports have demonstrated that the production of viscosin by *Pseudomonas fluorescens* SBW25 is implicated in plant tissue colonization by increasing the efficiency of surface spreading due to its surfactant properties (Alsohim et al., 2014). Although the disease prevention efficacy of B1.1W as a soil amendment was not tested in our study, the ability of B1.1W to control the disease when applied as a foliar spray treatment permits mitigation of the disease directly on infected tissues. Unlike seed and root dip biocontrol treatments, foliar sprays could be used repeatedly throughout the growing season and treatment frequency could be increased based on disease severity (Preininger et al., 2018). The effects of B1.1W cellular and extract treatments were effective during a two week period consistent with other commercially-available biocontrol treatments that follow a reapplication regimen of 14 days (Preininger et al., 2018).

Since plant tissue colonization is an important function for effective biocontrol strains, 16S targeted amplicon sequencing was conducted on detached leaves five days after being soaked in the biocontrol treatments (Ali et al., 2014; Helmann et al., 2019; Maldonado-González et al., 2015). Overall, the *C. sativa* foliar bacterial community composition shifted depending on the treatment type. Leaves treated with the B1.1W cell inoculant were heavily dominated by

Pseudomonales with low community diversity and richness indices, suggestive of B1.1W colonization. Putative proteins that contribute to epiphytic and apoplastic fitness in *P. syringae* strain B728a were detected in the genome of B1.1W. Compared to its relatives, the B1.1W and B728a genomes shared the greatest proportion of putative proteins known to be implicated in foliar colonization. These proteins pertained to amino acid, polysaccharide, and type III secretion system biosynthesis, suggesting that B1.1W has certain genetic resources that could potentially lead to epiphytic and/or apoplastic fitness. It is worth noting that genes encoding the pilin needle protein of the type III secretion system was not detected in the B1.1W genome. Whether B1.1W could produce a fully functional type III secretion system and whether it is implicated in foliar colonization requires further study. However, when considering the detected genomic features of B1.1W and the dominant *Pseudomonales* foliar community relative abundance, our findings suggest that B1.1W can colonize *C. sativa* leaf tissue (Cole et al., 2017; de Souza et al., 2019; Meneses et al., 2011; Preston et al., 2001; Yaron & Römling, 2014).

The B1.1W crude extract contributed to increased relative abundances of *Rhizobiales* while the community richness and diversity levels were not statistically significantly different to the untreated controls, which indicated that the bacterial community was not affected as much as on the leaves treated with the B1.1W cellular inoculant. The positive control Actinovate treatment, which consists of a cellular inoculant formulation of *Streptomyces lydicus* strain WYEC 108, was not detected in our 16S community analysis. None of the detected ASVs corresponded to *Streptomycetales* that would have pertained to the control inoculant WYEC 108. Therefore, we hypothesize that WYEC 108 remained in spore-form after the five-day incubation period for the treated detached leaves (Yuan & Crawford, 1995), which inhibited DNA extraction and targeted amplification of 16S gene(s) (Dineen et al., 2010). WYEC 108 has been demonstrated to

colonize plant roots and seeds while its capacity to colonize *C. sativa* leaves has not been evaluated (Dineen et al., 2010). Regardless of whether the Actinovate strain was detected using targeted amplicon sequencing, biocontrol activity on detached *C. sativa* leaves led to lower total mold counts five days after *B. cinerea* challenge compared to B1.1W cellular treatment. WYEC 108 produces antifungal specialized metabolites and chitinases that hydrolyze fungal cell walls (Dineen et al., 2010; Yuan & Crawford, 1995). The B1.1W crude extract, however, reduced total mold counts on detached *C. sativa* leaves and decreased *B. cinerea* symptoms on whole plants more effectively compared to the Actinovate control.

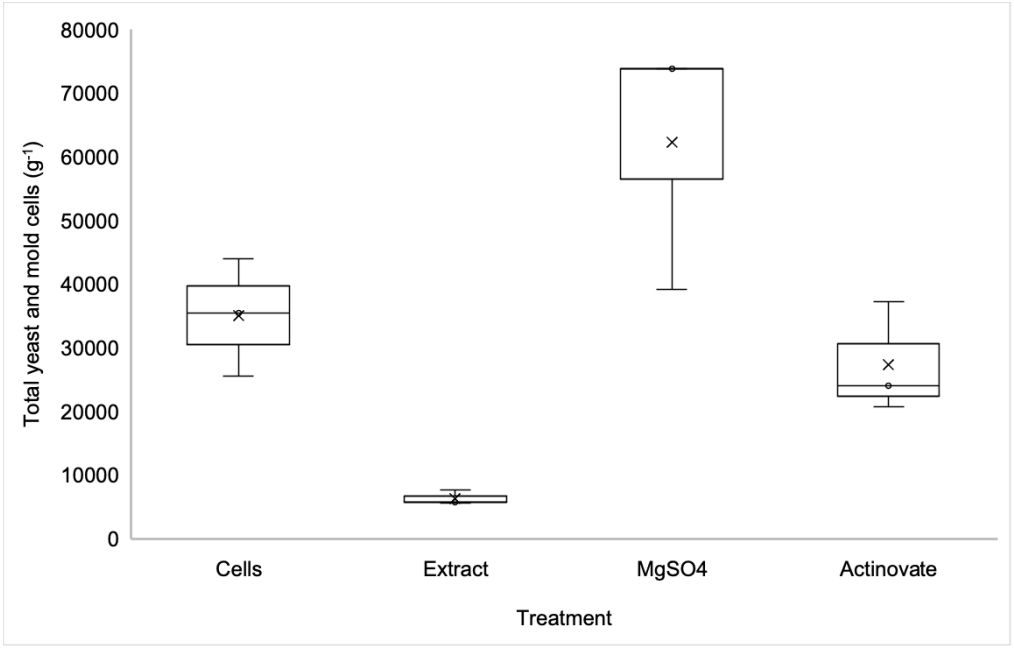
We thus conclude that B1.1W cellular inoculants and extract displayed biocontrol protection against *B. cinerea* on detached leaves and on whole plants of *C. sativa*.

Bacterial community analysis and genomic analysis indicated that B1.1W cells can colonize leaf tissue and therefore can persist with biocontrol effects, compared to the experimental extract. Although the mechanism of biocontrol action remains to be characterized, the biocontrol effects compare favourably with the commercially available biocontrol product, *Streptomyces lydicus* strain WYEC 108.

Future studies on *Pseudomonas* sp. B1.1W will identify the antifungal agents synthesized by B1.1W and explore whether this strain can stimulate endogenous plant defense systems and explore whether they are safe for human consumption when applied to cannabis plants. Additionally, *Pseudomonas* sp. B1.1W could be tested experimentally as a biocontrol treatment for other valuable plant hosts that are affected by *B. cinerea*. Overall, our findings contribute to the growing field of research on biocontrol agents isolated from biospheres that are ecologically distinct from agricultural habitats.

Chapter 5. Tables and Figures

A.



B.

Figure 15. Detached leaf assay. **A**) Total yeast and mold counts (cells • g^{-1}) on DLA leaves using SimPlate method. **B)** Fungal lesions on detached leaves treated with crude ethyl acetate extract from *Pseudomonas* sp. B1.1W (right) and untreated negative control using $MgSO_4$.

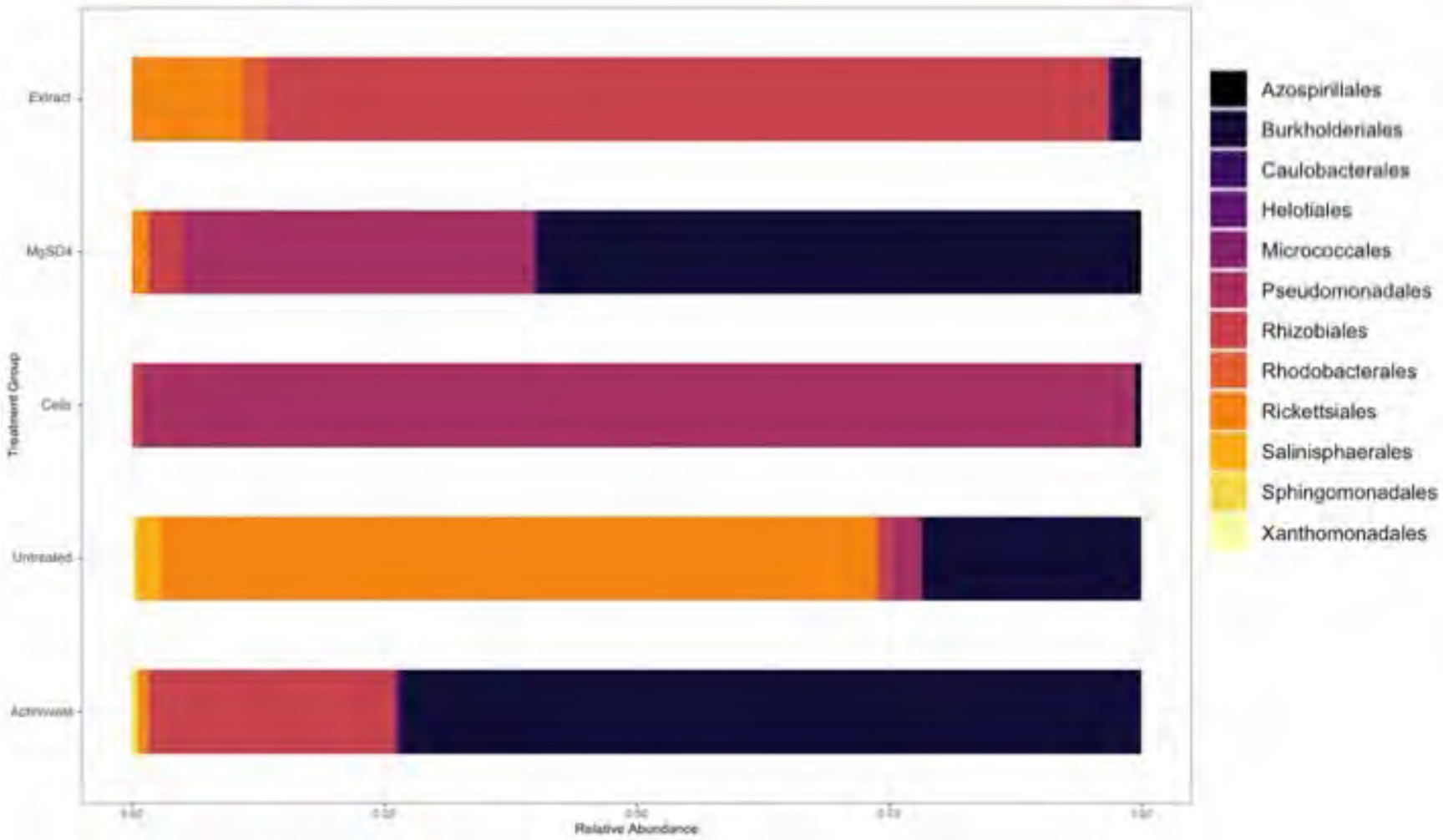
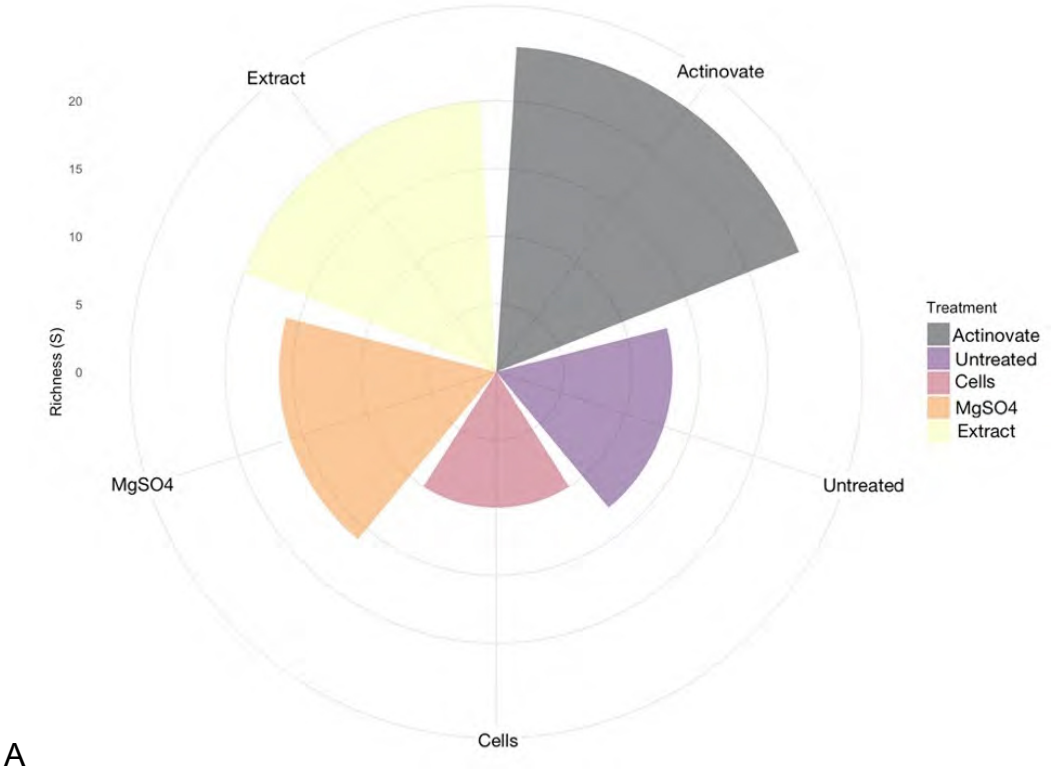
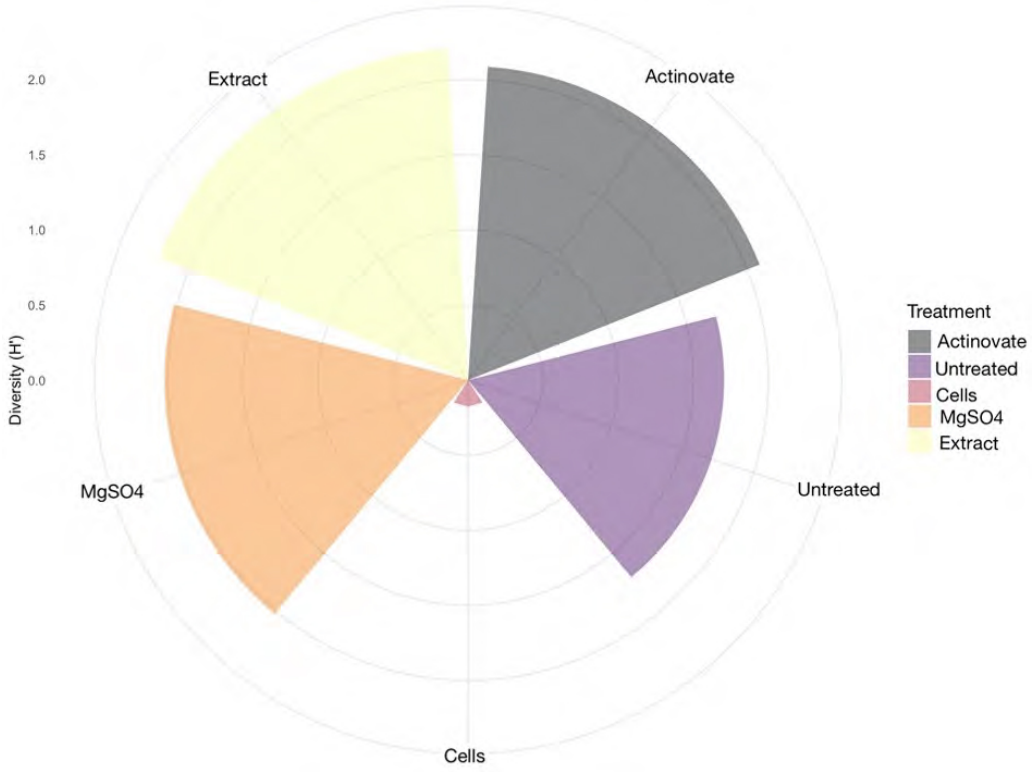


Figure 16. Taxonomic classification at the Order level of the ASVs detected from detached *C. sativa* leaves treated with different biocontrol treatments. Dominance of *Pseudomonales* among leaves treated with Arctic isolate *Pseudomonas* sp. B1.1W



A



B

Figure 17. A). Richness (S) and **B**) Shannon diversity (H') indices for ASVs detected on *C. sativa* leaves treated with different biocontrol treatments showing lower richness and diversity for leaves treated with *Pseudomonas* sp. B1.1W cells.

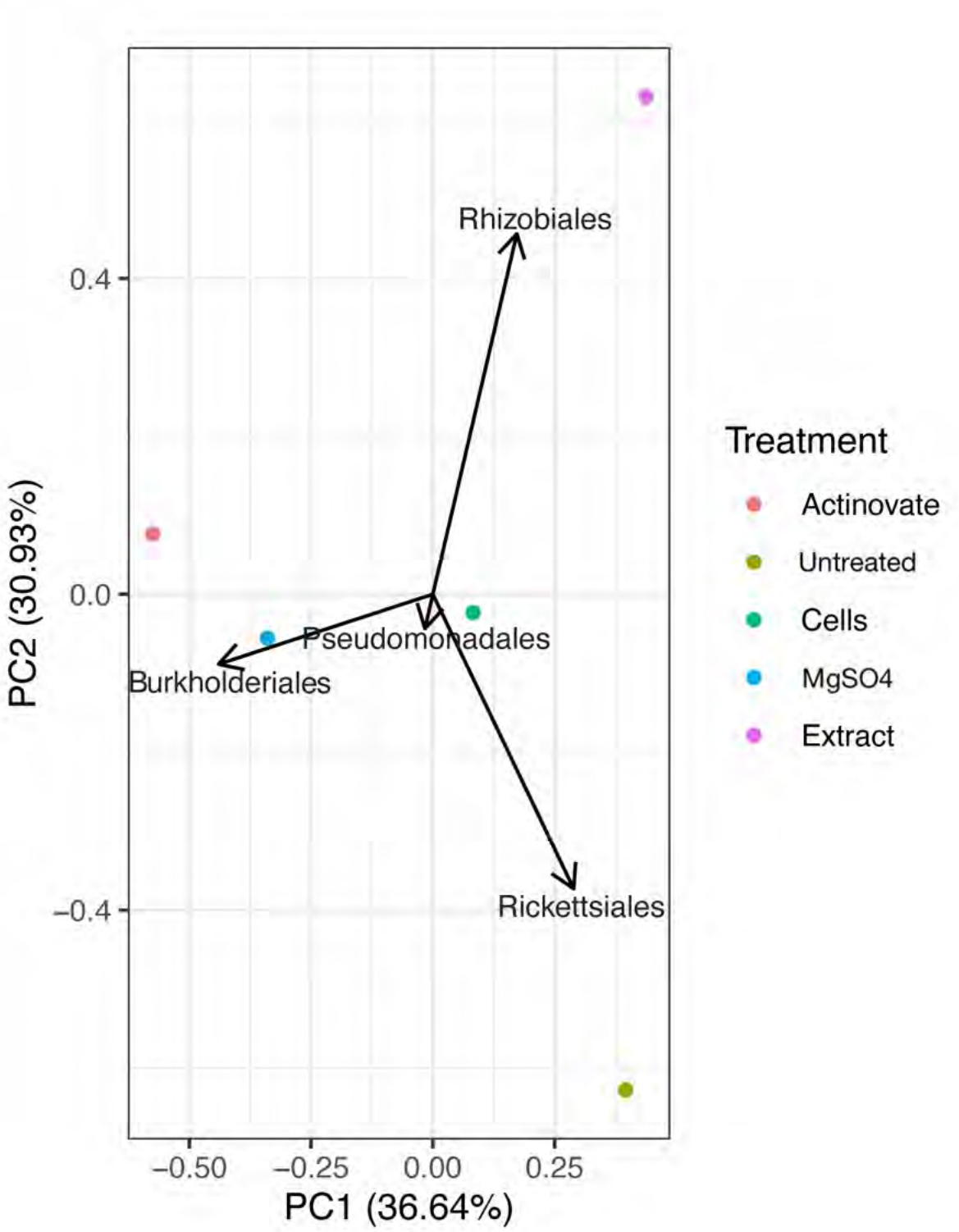


Figure 18. Principal component analysis (PCA) of ASVs detected on *C. sativa* leaves treated with different biocontrol treatments performed with ASV counts for each treatment group. Eigenvectors of most abundant ASVs indicate direction of the effect.

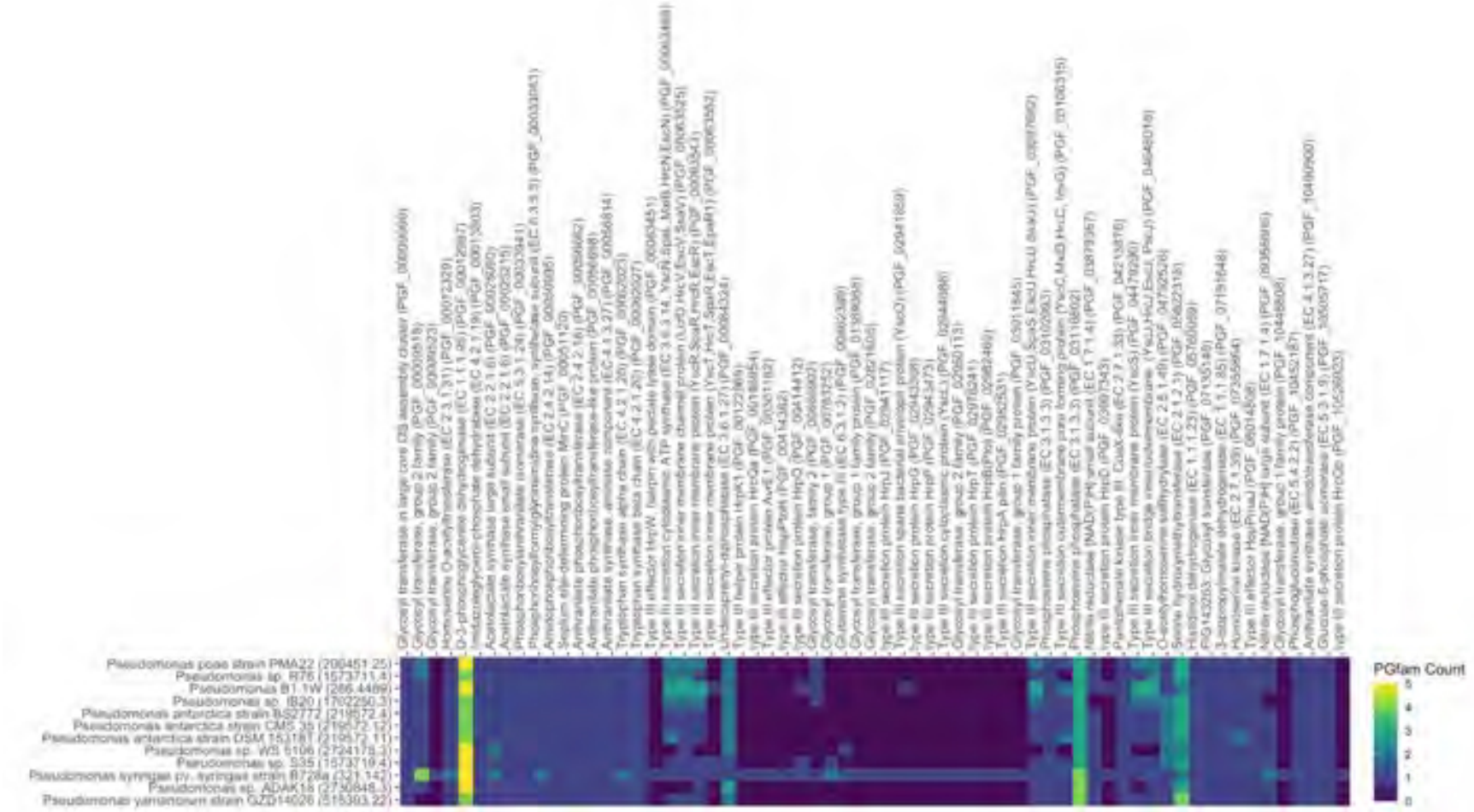


Figure 19. Heatmap of gene counts encoding proteins associated with plant colonization (biofilm, flagellar and Type III secretion systems) detected withing the genome of *Pseudomonas* sp. B1.1W and its closest phylogenetic relatives using PATRIC RASTtk-enabled Genome Annotation Service.

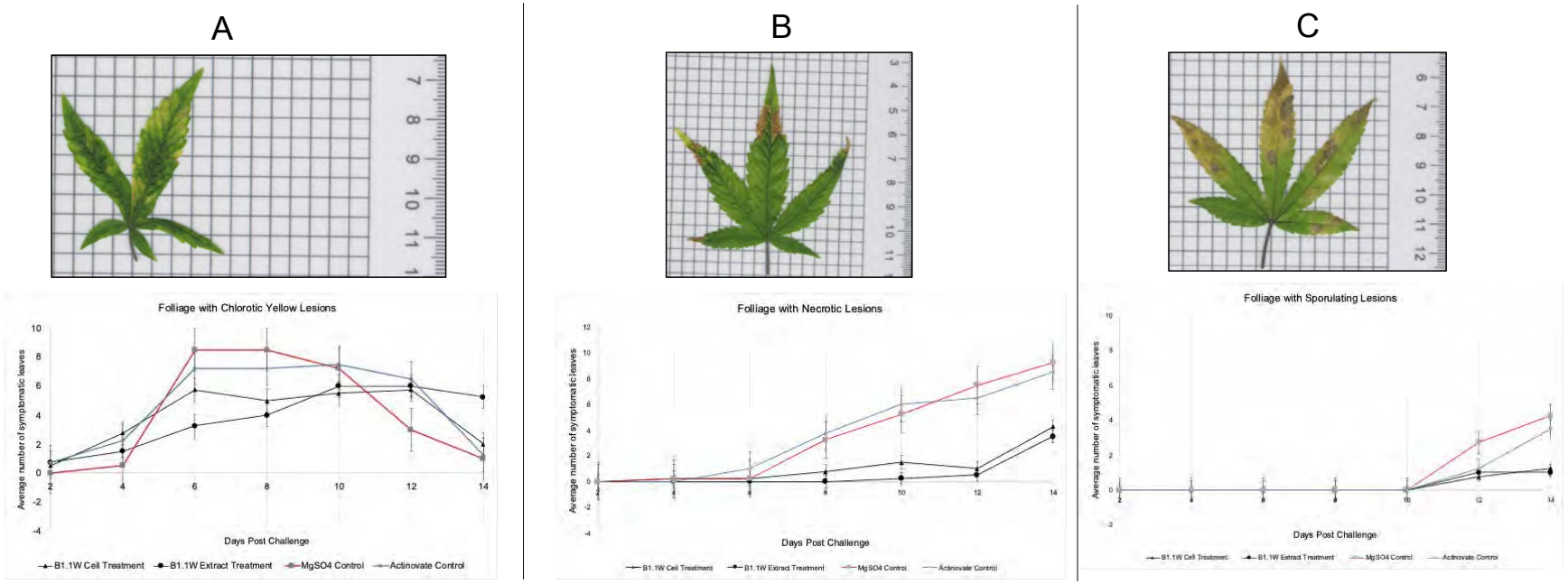


Figure 20. Whole plant assay testing B1.1W extract and cellular inoculants. Each plant was sprayed with 10 mL of the treatment solution/suspension (10^{11} CFU/mL Pseudomonas sp. B1.1W cells, 37.5 mg/mL Pseudomonas sp. B1.1W extract, 10mM MgSo4, 0.4g/L Actinovate. Two days after receiving the biocontrol treatments, plants were challenged with 10 mL of *B. cinerea* spores suspended in water (4 x 10^4 conidia/mL, 0.05% v/v tween). Disease progression was observed every 48 h by counting number of diseased leaves categorized by lesion type **A**. chlorotic yellow **B.** necrotic **C.** sporulating.

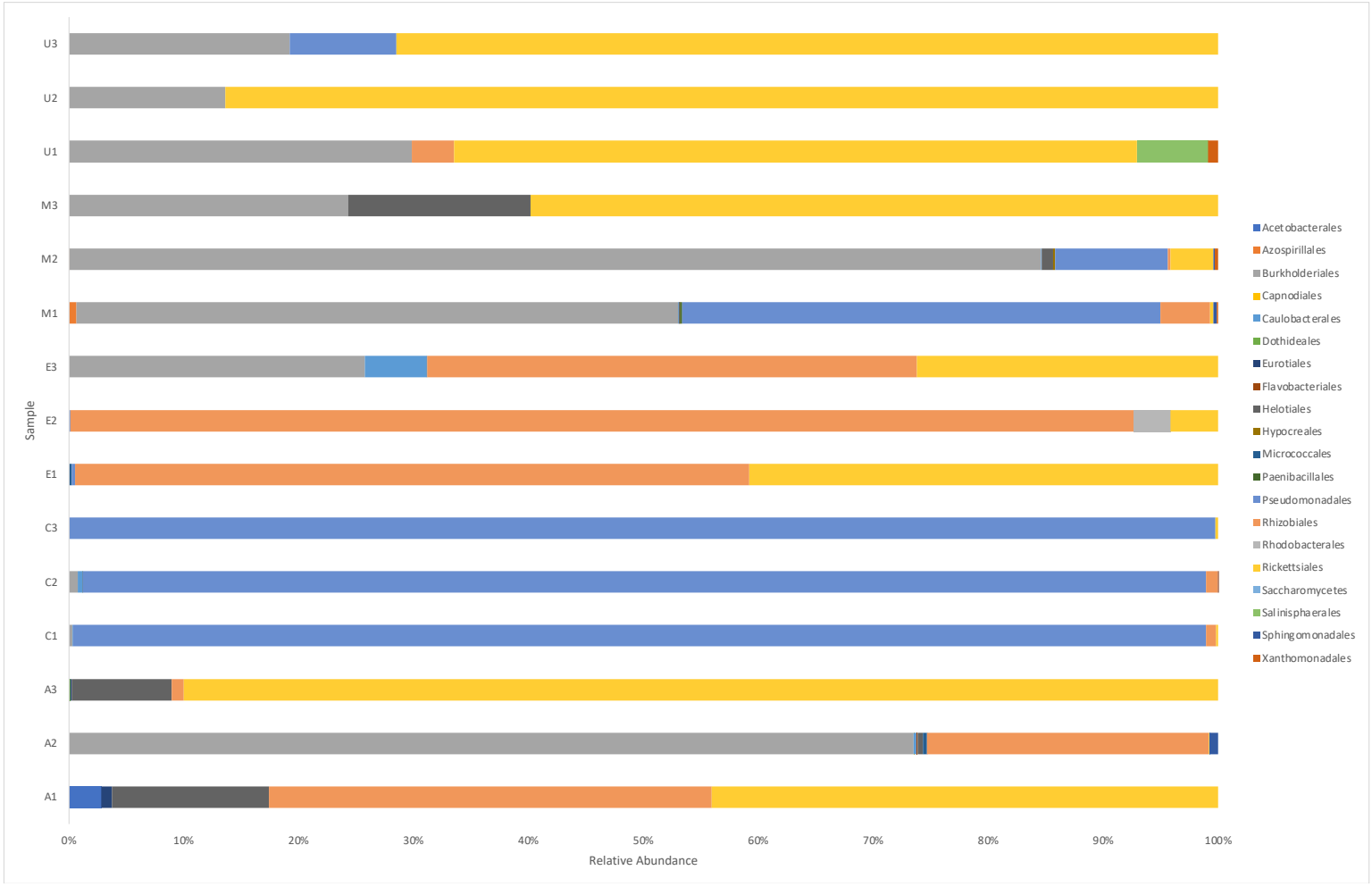


Figure S8. Taxonomic classification at the Order level of the ASVs detected from detached *C. sativa* leaves treated with different biocontrol treatments samples. Samples A1-A3 represent triplicates of leaves treated with the commercial product Actinovate that served as the positive control group, C1-C3 represent leaf triplicates treated with B1.1W cells, E1-E3 represent triplicate samples treated with B1.1W extract, M1-M3 represent leaf triplicates treated with MgSO4, and U1-U3 represent untreated control samples.

Figure S9. Principal component analysis (PCA) of ASVs detected on *C. sativa* leaves treated with different biocontrol treatments performed with ASV counts for each treatment group. Eigenvectors of most abundant ASVs indicate direction of the effect. Samples A1-A3 represent triplicates of leaves treated with the commercial product Actinovate that served as the positive control group, C1-C3 represent leaf triplicates treated with B1.1W cells, E1-E3 represent triplicate samples treated with B1.1W extract, M1-M3 represent leaf triplicates treated with MgSO4, and U1-U3 represent untreated control samples.

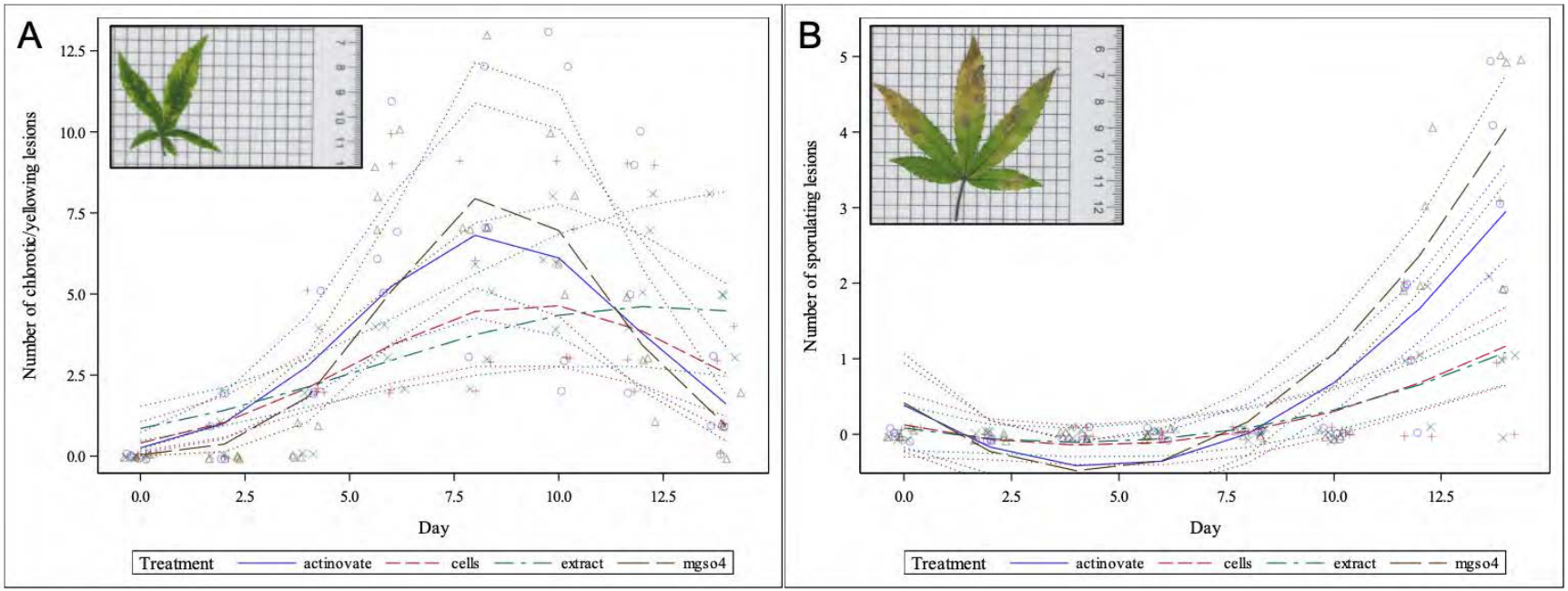


Figure S10. Generalized linear mixed modeling of leaf counts displaying **A)** chlorotic yellowing and **B)** sporulating lesions. Circles, crosses, triangles, and cross-mark symbols represent jitter points. Solid and dashed lines represent the predicted values. Dotted lines border the 95% confidence bands.

Chapter 6. Discussion

The Limitations of Addressing AMR with Natural Products

Owing to their intrinsic efficiency to penetrate microbial cells and the high affinity with which they bind to their molecular targets, antimicrobial natural products continue to represent promising solutions to address this crisis (Liang, 2008). Despite their potent activity profiles and versatile biomedical applicability, antimicrobial natural products feature several limitations. For one, microbial pathogens regularly evolve mechanisms of resistance against antimicrobial biomolecules of microbial origin. This phenomenon was famously first described by Alexander Fleming when he reported his discovery of Penicillin (Fleming, 1941; Howard et al., 2013). Another major limitation includes their prevalently broad antimicrobial spectra, often indiscriminately killing bacterial or fungal cells (Hagan et al., 2019). With our increasing appreciation of the beneficial functions that resident microbiotas impart within their host organisms, the need for targeted antimicrobials used to selectively inhibit pathogens of interest is increasingly apparent (Langdon et al., 2016).

These limitations have led to development of alternative antimicrobial methods and agents such as bacteriophage therapy, nanoparticles, antimicrobial adjuvants, and photodynamic antimicrobials (Liu et al., 2013; Spirescu et al., 2021; Westwater et al., 2003). These methods and agents are still under initial stages of development or regulatory approval and so they are often experimentally used in synergy with conventional antimicrobial agents - including natural product therapeutics (Mulani et al., 2019). For these reasons, the discovery of novel natural product antimicrobial agents remains a relevant endeavor in addressing the AMR crisis.

As we continue exhausting our existing arsenal of antimicrobials - most of which consist of natural products initially discovered from environmental microorganisms - innovative strategies are needed to identify new molecules given the significant risks of rediscovering already known agents using classical screening methods. This risk of natural product rediscovery is shifting the focus of antimicrobial research towards underexplored microbial communities living within extreme environments as a source for novel compounds (Orsi et al., 2013).

Cultivable Arctic bacteria: a source of novel antibacterial natural products.

Several high Arctic habitats within the vicinity of the MARS on Axel Heiberg Island served as the study sites for this doctoral work. These habitats include permafrost soil and sediments of perennially active saline springs which feature microbial communities with high proportions of Phyla known to produce antimicrobial specialized metabolites, including *Proteobacteria* and *Actinobacteria* (Lay et al., 2013; Perreault et al., 2007; Perreault et al., 2008; Steven et al., 2007).Importantly, these diverse microbial communities remain largely unmined for novel antimicrobial natural products. Some of the molecular adaptations that cold-adapted Arctic bacteria have evolved to survive in their ecological niches include producing higher levels of unsaturated methyl-branched fatty acids and short acyl-chain fatty acids to maintain membrane fluidity at lower temperatures (D'Amico et al., 2006). Since most liquid water within Arctic habitats at sub-zero temperatures is saline, cold-adapted microorganisms biosynthesize compatible solutes to maintain cellular osmotic balance with their extracellular environment (Raymond-Bouchard, 2018). To inhibit intracellular ice crystal nucleation, cold-adapted microbes produce proteins with higher proportions of hydrophobic residues and express cold-shock proteins that ensure proper protein folding (Phadtare, 2004; Raymond-Bouchard, 2018). The fact that Arctic microorganisms have evolved such specific molecular machinery to survive

within their harsh environment drove the overarching hypothesis of this doctoral project that they also might be capable of biosynthesizing unknown antimicrobial specialized metabolites.

To this end, we identified two promising isolates in Chapter 3, *Pseudomonas sp.* AALPS.10.MNAAK.13 from active layer permafrost and *Paenibacillus sp.* GHS.8.NWYW.5 from perennial hypersaline springs. Both isolates exhibited broad spectrum of antibacterial activity against clinically relevant pathogens which was determined by functionally screening through dual-culture antagonistic screening and through genomic mining for antimicrobial BGCs. Through this screening process, AALPS.10.MNAAK.13 was observed to inhibit the growth of MRSA, *A. baumanii*, MSSA, *E. faecium*, and *E. faecalis*. Whereas *Paenibacillus sp.* GHS.8.NWYW.5 inhibited *S. aureus*, *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 at 37°C and at 5°C. The genomes of both isolates featured at least one putative BGC sharing low nucleotide sequence homology with clusters of their respective type strains of the same species. GHS.8.NWYW.5 featured five NRP clusters displaying low homology to its corresponding type strain genome *Paenibacillus terrae* NRRL B-30644. The NRRL B-30644 strain is known to biosynthesize paenicidins and tridecaptin A_1 that inhibit *Campylobacter jejuni* (Lohans et al., 2014). Putative BGCs encoding both paenicidin and tridecaptin were detected within the genome of GHS.8.NWYW.5 along with five other NRP clusters sharing low sequence homology with NRRL B-30644 BGCs.

These results suggest that certain culturable representatives within the microbial communities found in habitats surrounding the MARS on Axel Heiberg Island can inhibit clinical pathogens. Whether the antimicrobial specialized metabolites produced by the isolates identified in this study also function as cold adaption traits remains unknown and could serve as a point of future

investigation. However, the findings of Chapter 3 suggest that the Canadian high Arctic could be an underexploited reservoir of potentially novel antibacterial natural products.

Arctic *Pseudomonas* as reservoirs for novel antifungal molecules

Since most of the agents currently in use consist of specialized metabolite from environmental sources, soil-dwelling bacteria continue to represent a focal point in the search for novel antifungal natural products (Chávez et al., 2015; Jiang et al., 2014; Lopes et al., 2016; Sayed et al., 2020). The genus *Pseudomonas* features species that hold biosynthetic potential to produce antimicrobial metabolites and represents a promising source for new antifungal biomolecules (Coggan & Wolfgang, 2012) (Raaijmakers et al., 2006).

Compared to the dozen or so classes of antibacterial NPs discovered over the course of the last five decades, the discovery rate of antifungal NP has been relatively slower (Roemer et al., 2011). Partially due to lacking research incentives from the private sector, antifungal natural product discovery efforts have only revealed polyenes and echinocandins (Ostrosky-Zeichner et al., 2010). As the incidence rates of fungal diseases continue to increase worldwide, the current need for new antifungal drugs is reinvigorating antifungal research (Chávez et al., 2015; Jiang et al., 2014; Lopes et al., 2016; Sayed et al., 2020). Several strategies could be used to develop new antifungal agents. These involve screening libraries of natural products (NPs), designing semi/fully synthetic derivatives of natural scaffolds, or repurposing drugs intended for other indications (Calderone et al., 2014). Unfortunately, synthetic drug developments have been largely unsuccessful because of their poor penetration into fungal cells (Calderone et al., 2014).

One of the bacterial isolates I cultured using the *in situ* cryo-iplate device from Chapter 3, *Pseudomonas* sp. B1.1W, was found to display antifungal activity against *Botrytis cinerea*

during whole cell dual-culture assays. Following HPLC fractionation, MALDI-TOF MS-MS, on the crude extract derived from B1.1W in co-culture with *B. cinerea*, a 1148 Da molecule active against *B. cinerea* featuring an amino acid peptide fragment consisting of Val – Ile/Leu – Ser - Ile/Leu – Ser – Ile/Leu. The amino acid sequence of this molecule's peptide moiety was found to be consistent with the conserved amino acid sequence of the viscosin molecule produced by *P. fluorescens* SBW25 (Laycock et al., 1991). Given that a viscosin-like BGC was detected within the genome of B1.1W, we suggest that a CLP part of the viscosin group was produced by B1.1W. A scarce number of previous studies have isolated and characterized bacteria from polar regions capable of producing CLPs with similar chemical structure and bioactivity profiles as viscosin (Ding et al., 2018; Janek et al., 2012). One example includes, *Pseudomonas fluorescens* BD5, a bacterium within the same phylogenetic lineage as B1.1W that was isolated from the Archipelago of Svalbard that produces a CLP named pseudofactin with antimicrobial activity against *E. coli*, *E. faecalis, Enterococcus hirae*, *S. epidermidis*, *Proteus mirabilis* and *Candida albicans (*Janek et al., 2012*).*

Additionally, I found that there were 17 genes in the B1.1W viscosin-like cluster that did not share nucleotide sequence homology with any genes comprising the SBW25 viscosin cluster. Since these genes encode biosynthetic enzymes including acyltransferases and methyltransferases, it is possible that these putative enzymes could impart biosynthetic alterations to the CLP produced by B1.1W, potentially leading to viscosin derivatives. However, the biosynthetic roles of these genes require empirical characterization. The MS-MS spectrum of the active B1.1W extract fraction containing a 1148 Da molecule featured both typical and atypical fragment signal peaks; that would be expected from a viscosin parent ion. As such, it is

possible that the B1.1W produces both viscosin and viscosin-like derivatives. This theory would need to be confirmed, however, by further fractionating the B1.1W extract and additional structural characterization through NMR.

The viscosin-like BGC within the B1.1W genome was homologous to NRP BGCs found in *P. antarctica* genomes. The nonribosomal synthetase genes were conserved among strains *Pseudomonas antarctica* strains BS2772 (82% ID and 100% coverage) and BS2772 (74% ID 90% coverage). Strains BS2772 and BS2772 were isolated form Antarctic permafrost (Reddy et al., 2004). Other previous studies have suggested that CLPs produced by cold-adapted bacteria may have unique \functional properties that aid with survival in cold environments (Perfumo et al., 2018; Saritha et al., 2021). For example, the biosurfactant properties of CLPs may enhance the solubility and bioavailability of hydrophobic carbon sources within nutrient-limited polar habitats (Perfumo et al., 2018). Whether the viscosin-like molecule(s) produced by B1.1W impart functions related to cold-adaptation merits further investigation.

This study represents one of the few investigations of a CLP-producing Arctic *Pseudomonas* with antifungal activity against a fungal phytopathogen. Although it is conceivable that the antifungal activity of this isolate may arise from the synergistic effects of multiple specialized metabolites or enzymes, we identified at least one of the candidate antifungal agents produced by this putatively novel species. This study serves as a proof of principle that Arctic bacteria harbour uncharacterized BGCs that could lead to the biosynthesis of novel antimicrobial natural products.

Applying Cultivable Arctic as Biocontrol Agents

Typical biocontrol strains include soil-dwelling rhizobacteria that have intricate co-evolutionary relationships with plants. These rhizobacteria can inhibit phytopathogens by secreting

antimicrobial specialized metabolites and/or by eliciting the endogenous plant defense system, induced systemic resistance (ISR) (Berendsen et al., 2015). Effective biocontrol also involves the colonization of the biocontrol strain within plant tissue (Mercado-Blanco & Bakker, 2007). Several studies have identified bacterial genomic and proteomic features implicated in plant colonization, where genes pertaining to amino acid, polysaccharide, and type III secretion system biosynthesis were determined to increase epiphytic and apoplastic fitness in the model organism *Pseudomonas syringae* (Ali et al., 2014; Helmann et al., 2019; Maldonado-González et al., 2015).There are several bacterial genera with such biocontrol properties, for example, *Streptomyces*, *Bacillus*, and *Pseudomonas* spp. (Abo-Zaid et al., 2020; Fischer et al., 2013; Jiménez-Gómez et al., 2017; Ly et al., 2015; Samaras et al., 2021; Sujarit et al., 2020). In the search to develop novel biocontrol strains, interest has grown in studying bacteria residing in extreme habitats.

Within this framework, certain eurypsychrophillic polar microbes (that can tolerate warmer temperatures up to 30°C, with optimal growth temperatures ranging between 20-25°C) could be applied in agricultural settings (Raymond-Bouchard, Goordial, et al., 2018; Raymond-Bouchard et al., 2017). Certain species of polar Pseudomonads feature highly adaptable metabolisms allowing them to: digest various of carbon sources, grow at different temperatures, colonize various substrates, and synthesize specialized metabolites. For example, *Pseudomonas fluorescens* In5 that was isolated from Greenlandic soil was found to antagonize phytopathogens *Pythium aphanidermatum, Fusarium* sp., and *Rhizoctonia solani* by producing two antifungal non-ribosomal peptides: nunapeptin and nunamycin (Christiansen et al., 2020; Hennessy et al., 2017; Michelsen, Jensen, et al., 2015; Michelsen, Watrous, et al., 2015). Another example includes the cold-adapted *Pseudomonas* strain GWSMS-1, isolated from Antarctic marine

sediments that displayed antifungal activity against *Verticillium dahlia* CICC 2534 and *Fusarium oxysporum* f. sp. cucumerinum CICC 2532, by secreting chitinases capable of lysing fungal cell walls (Liu et al., 2019). The findings of these studies illustrate how bacteria living in cryoenvironments, which are geographically separated and ecologically distinct from agricultural habitats, can be used as biocontrol agents. Such bacteria could be exploited as biocontrol agents against fungal pathogens infecting cannabis and be used as alternatives to synthetic fungicides. The final goal of this doctoral research (Chapter 5) was to evaluate *Pseudomonas* sp. B1.1W as a foliar biocontrol agent against *B. cinerea* on *Cannabis sativa* L. (cannabis). The global legal landscape of cannabi*s* is rapidly changing as legalization of medical and recreational use continues to expand (Backer et al., 2019). In North America, cannabis is one of the fastest growing industries representing a multi-billion-dollar market (Lancione et al., 2020).Given the expanding global prevalence of cannabis cultivation, the demand for natural alternatives to mitigate crop-losses attributed to fungal diseases is increasing.

Cannabis is susceptible to fungal diseases caused by over ninety different species of phytopathogenic fungi (McPartland, 1996; McPartland et al., 2000). Fungal diseases targeting cannabis plants can inhibit product quality control, and contribute to pre- and post-harvest loss (Punja et al., 2019). One of the most prevalent fungal phytopathogens responsible for cannabis crop losses is *Botrytis cinerea*, the causal organism of gray mold disease, commonly referred to as bud rot (Scott & Punja, 2021). Owing to the broad host range of *B. cinerea*, it is globally classified as the second most destructive fungal phytopathogen after *Magnaporthe oryzae* (Lucas et al., 2015). It infects over two hundred different host species of plants spanning vegetables, fruits, legumes, and ornamental plants causing $10-100 billion worth of crop losses annually. Given that synthetic fungicidal products are strictly regulated in cannabis cultivation, natural

alternatives such as beneficial environmental bacterial strains applied as biocontrol agents represent promising alternatives to mitigate fungal disease (Seltenrich, 2019; Vujanovic et al., 2020).

In chapter 5, Cannabis leaves soaked in cellular inoculants and organic extracts of B1.1W featured reductions in total mold cell counts 43.79% and 89.83%, respectively, compared to untreated samples after being challenged with *B. cinerea* conidia. This reduction in mold count could be partially attributed to the viscosin-like CLP secreted by B1.1W, although we have not determined whether other specialized metabolites could be acting synergistically in the strain's antifungal effect. Additionally, whole plants sprayed with B1.1W cellular inoculants and organic extracts displayed fewer counts of symptomatic foliage during a 14-day observation period post pathogen challenge compared to the commercially available biocontrol agent *Streptomyces lydicus* strain WYEC 108. By mining the genomic sequence of B1.1W and by 16S community profiling C. *sativa* leaves treated with cellular inoculants of B1.1W, we found that this strain has putative genes associated with foliar colonization and can dominate the foliar bacterial community. The viscosin-like CLP might also be implicated in B1.1W's foliar colonization since previous studies have demonstrated that viscosin produced by *Pseudomonas fluorescens* SBW25 increases the efficiency of surface spreading over plant tissue (Alsohim et al., 2014). These findings suggest that B1.1W has the capacity to colonize cannabis leaf tissue. Viscosin-like CLPs have also been reported to impart plant growth promotion depending on the length of their fatty acid tails (Raaijmakers et al., 2006). Whether the CLP produced by B1.1W has such effects on plant hosts remains to be determined. To our knowledge, this study represents the first description of an Arctic bacterial strain to be applied as a foliar biocontrol agent on cannabis effective against *B. cinerea*.

Future Directions

The studies presented in this thesis demonstrate the biosynthetic potential of high Arctic bacteria to produce antimicrobial specialized metabolites. The antibacterial metabolites expressed by the Arctic isolates described in Chapter 3 require biochemical isolation and structural characterization. Genome-wide transposon mutagenesis or targeted cloning involving exogenous expression of specific clusters would be needed to determine which specific BGCs are responsible for the antibacterial phenotypes observed in this initial study. Subsequent animal model studies could be conducted to determine if they hold chemotherapeutic potential.

For the antifungal viscosin-like CLP produced by the putatively novel species *Pseudomonas* sp. B1.1W described in Chapter 4, the complete chemical structure of the candidate agent and the biosynthetic role of the ancillary genes that comprise its BGC require identification and characterization. The purified compound could then be tested for activity against other agriculturally and medically relevant fungal pathogens. Although this doctoral project focused on the antifungal properties of B1.1W's viscosin-like molecule(s), further studies could explore whether these molecule(s) have different bioactivities relevant to biotechnology since molecules part of this family have been previously shown to display antitumor and biosurfactant properties. Furthermore, the ecological role of the viscosin-like CLP produced by B1.1W could be investigated as to whether it is implicated in cold-adaption. Lastly, the biocontrol potential of B1.1W reported in Chapter 5 could be evaluated in other plant hosts and determine whether this strain can induce endogenous plant immune responses (Berendsen et al., 2015). Additionally, the role of B1.1W's viscosin-like CLP in foliar colonization and whether it promotes plant growth remains to be determined.

Chapter 7. Conclusion

The main goal of this doctoral thesis was to determine the biosynthetic potential of microorganisms living in Canadian high Arctic habitats to produce antimicrobial specialized metabolites. This was accomplished by screening microbial strains isolated from various habitats within the vicinity of MARS, Expedition Fiord, Axel Heiberg Island, Nunavut, Canada (79°26'N, 90°46'W). Through this work, high Arctic bacterial isolates exhibiting antibacterial activity against clinically relevant pathogens were identified (Chapter 3). This work combined two separate cultivation methods. One method involved the engineering, and subsequent application, of the cryo-iplate, an *in situ* cultivation device that we designed. The second method involved crowdsourcing bulk soil plating to a cohort of undergraduate researchers using Arctic samples. The resulting Arctic isolates were then screened against foodborne and nosocomial bacterial pathogens. The genomic sequences of strains displaying broad spectra of antibacterial activity were mined for BGCs encoding antimicrobial metabolites.

Furthermore, this doctoral project aimed to characterize *Pseudomonas* sp. B1.1W, a putatively novel species isolated using the *in situ* cryo-iplate device *cinerea* (Chapter 4) which exhibited antifungal activity against *B. cinerea*. The biosynthetic potential of B1.1W was determined by mining its genomic sequence for BGCs using antiSMASH. The antifungal causative agent produced by this strain was subsequently identified by employing MALDI-TOF MS-MS and LC-MS on bioactive organic extracts derived from liquid culture supernatants of B1.1W in co-culture with *B. cinerea*.

Lastly, the biocontrol potential of *Pseudomonas* sp. B1.1W on *Cannabis sativa* challenged with *B. cinerea* was evaluated (Chapter 5). This was achieved by applying cellular inoculants and liquid culture supernatant extracts of B1.1W on cannabis challenged with *B. cinerea*.

Additionally, the capacity of B1.1W to colonize foliar tissue was assessed by mining its genome for genes families known to be implicated in foliar colonization and through 16S community profiling cannabis leaves treated with B1.1W.

Overall, this doctoral work highlights how high Arctic bacteria from permafrost soils could represent understudied reservoirs of antimicrobial natural products with biotechnological applicability.

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Last Printed On: 4/11/23 7:51:00 AM
As of Last Complete Printing
Number of Pages: 177
Number of Words: 105,224 (approx.)
Number of Characters: 628,194 (approx.)