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Antimicrobial properties and the influence of temperature on secondary metabolite production in cold environment soil fungi

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Abstract:

The Arctic and Antarctic share environmental extremes. To survive in such environments, microbes such as soil fungi need to compete with or protect themselves effectively from other soil microbiota and to obtain the often scarce nutrients available, and many use secondary metabolites to facilitate this. We therefore (i) screened for antimicrobial properties of cold-environment Arctic and Antarctic soil fungi, and (ii) identified changes in the secreted secondary metabolite profiles of a subset of these strains in response to temperature variation. A total of 40 polar soil fungal strains from King George Island, maritime Antarctic and Hornsund, Svalbard, High Arctic, were obtained from the Malaysian National Antarctic Research Centre culture collections. The plug assay technique was used to screen for antimicrobial potential against Gram-positive and Gram-negative human pathogenic bacteria (*Bacillus subtilis*, *B. cereus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*). About 45% of the tested fungal strains showed antimicrobial activity against at least one tested microorganism. Three fungal isolates showed good bioactivity and were subjected to secondary metabolite profiling at different temperatures (4, 10, 15 and 28°C). We observed a range of responses in fungal metabolite production when incubated at varying temperatures, confirming an influence of environmental conditions such as temperature on the production of secondary metabolites.

Keywords: Fungal metabolites, *Penicillium flavigenum*, *Pseudogymnoascus* sp., temperature influence

1. Introduction

Fungi are remarkable microorganisms known to produce a diverse range of compounds extracellularly, usually of low molecular weight, known as secondary metabolites (Keller et al., 2005). Often these secondary metabolites are unique to particular microbial species (Larsen et al., 2005). There is a general consensus that secondary metabolites are not essential for growth, development or reproduction, even though they are produced by many fungi (Madigan et al., 1997). Produced generally near the end of the active growth phase, these compounds are synthesized from compounds that are themselves derived from primary metabolic pathways (Davies, 1985). Although their ecological role often remains unclear, many exhibit antifungal or antimicrobial activity (Peláez, 2006) and are therefore likely to provide the producing microorganism with a competitive advantage facilitating survival in their natural environment.

Although the systematic study of fungal secondary metabolites began in 1922 (Raistrick, 1985), it was not until the discovery of penicillin by Alexander Fleming in 1928 (Alharbi et al., 2014; Ligon, 2004a,b) that the exploration of secondary metabolites started to intensify. Over recent decades considerable efforts have been devoted to the study of these compounds. This is, in part, driven by the rapidly increasing levels of resistance towards many of the currently available antibiotics (Cooper and Shales, 2011).

It is widely accepted that differences in evolutionary pressures have led to some level of specificity associated with ecological niches (Schutz, 2001). To date, the search for pharmaceutically-active fungal strains has largely been focussed in the temperate and tropical regions, and there have been relatively few studies in the cold and stressful environments of the Arctic and Antarctic. Cold-adapted fungi may provide a valuable and currently untapped source of novel metabolites. Their survival in the extreme polar environment requires high

adaptability and resistance against many stresses including low nutrient availability, high UV radiation and prolonged exposure to both chronic cold and freezing temperatures and short term thermal maxima during summer (Montiel, 2000; Madronich et al., 1998, Nishiyama, 1977; Des Marais, 1995). Tolerance of these stresses leads to the expectation of evolutionary adaptations being developed in polar microbes enabling them to remain viable and function at low temperatures.

Research on extreme environments started to gain momentum the middle of the 20th Century. Margesin et al. (2007) reported that, by 2007, there were 30,000 studies published on extremophiles, but also that two-thirds of these studies focused on thermophiles and studies from cold areas remained rare. Recently, there has been greater emphasis on studies of cold environment microbes. The production of extracellular cold-tolerant bioactive compounds and enzymes by polar microbes has been a subject of increasing research interest, particularly in the context of their potential for biotechnological and pharmaceutical application (Groudieva et al., 2004; Priscu et al., 1999).

A number of secondary metabolites have been described from Arctic and Antarctic fungal isolates that exhibit antimicrobial and antifungal activities (e.g. O'Brien et al., 2004). Li et al. (2008) reported five novel bioactive asterric acid derivatives from an Antarctic ascomycete (*Pseudogymnoascus* sp.). Some of these compounds displayed antifungal activity against *Aspergillus fumigatus*, and some showed antimicrobial activity against Gram-positive and Gram-negative bacteria. *Penicillium griseofulvum*, collected in Greenland, produced a range of secondary metabolites including griseofulvin, fulvic acid, mycelianamide, roquefortine C, roquefortine D, chanoclavine I and elymoclavine, all of which contributed to antimicrobial activity (Frisvard et al., 2004). Niu (2014) reported isolation of new natural products with unique structural features in *Spriromastix* sp. from deep sea sediments of the South Atlantic Ocean.

Abiotic environmental stresses are particularly important in the terrestrial ecosystems of the polar regions (Hogg et al., 2006; Tufto, 2000; Convey et al., 2014). Recent rapid changes in global and regional climates have prompted a range of studies on factors that may influence microbial interactions in these regions. Chwedorzewska (2010) stated that climate change may affect polar terrestrial biota via three fundamental factors - temperature, water and solar irradiance - and small shifts in these factors may result in greater biological impacts. The recent Climate Change 2014 synthesis report by the Intergovernmental Panel on Climate Change has predicted an increase of global average temperature likely to be in the range of 0.3°C to 0.7°C for the period 2016 to 2035 and projection for the end of the 21st Century (2081-2100) likely to exceed 1.5°C, while the polar regions will continue to warm more rapidly than the global mean (IPCC, 2014). However, the effects of temperature variation on Antarctic organisms, as elsewhere, have largely not been studied in detail (Convey et al., 2014). A number of temperature manipulation studies, particularly using methodologies such as Open Top Chambers (OTCs), carried out in recent years have shed some light on the response of native Antarctic soil arthropod, lichens and plant communities towards elements of climatic change (Bokhorst et al., 2008, 2011, 2013). Although studies on temperature-driven biodiversity shifts in polar microbial communities are available (Benhua et al., 2014; Dennis et al., 2012; Newsham et al., 2015), limited data currently exist on the effects of warming on secondary metabolism in soil microfungal communities.

Clearly one of the primary ecological roles of secondary metabolites is to help the producing organisms function efficiently in their natural environment (Kliebenstein, 2004) and, often, their production is influenced by environmental factors (Akula and Ravishankar 2011; Alvarez et al., 2000). Various studies have examined the effects of external factors such as temperature, UV-B radiation, soil composition, CO_2 and humidity on the production of secondary metabolites (Abreau and Mazzafera, 2005; Eichholz et al., 2011; Rosa et al., 2001;

Szakiel and Henry, 2011). Here, we report an initial screening of the antimicrobial properties of 40 polar soil fungal strains, and examine in detail the influence of temperature on the overall secondary metabolite production profiles of three of the most active of these strains.

2. Methodology

2.1. Fungal cultivation and maintenance

A total of 40 polar fungal isolates were obtained from the National Antarctic Research Centre Fungal Collection, held at the University of Malaya, Kuala Lumpur. These fungal strains were originally isolated from soils collected at different locations in Hornsund, Svalbard (High Arctic) and King George Island, South Shetland Islands (maritime Antarctic) during the boreal summer of 2010 and austral summer of 2007/08, respectively. These fungal isolates had previously been identified using BLAST searches (where sequence data existed) and/or phylogenetic approaches, including the sequencing of type or voucher specimens. The available Genbank accession numbers for the identified isolates are provided in supplementary Table 1.

The 40 fungal strains were cultivated by transferring actively growing mycelia onto potato dextrose agar plates (PDA; Difco Laboratories, Detroit, MI, USA), with incubation at 4°C. PDA was prepared by suspending 19.5 g of potato dextrose agar powder in 500 ml of distilled water and heating with frequent agitation for 1 min to dissolve the powder before autoclaving at 121°C for 15 min. The autoclaved media was then poured into petri dishes and allowed to cool. The prepared PDA had a pH of 5.6 ± 0.2. Sub-samples of each strain were preserved in individual universal bottles containing slants of PDA.

2.2. Bioactivity screening

2.2.2. Preliminary screening for biological activity

148 The antimicrobial activities of all 40 polar fungal isolates were tested in triplicate using the
149 plug assay technique (Hoskisson at al., 2001; Ezra et al., 2004) against five Gram-positive
150 and Gram-negative human pathogenic bacteria. The Gram-positive bacteria used were
151 *Bacillus subtilis* (Ehrenberg) Cohn. (ATCC 6051), *Enterococcus facaellis* (Andrewes and
152 Horder) Schleifer and Kilpper-Balz (ATCC 29212) and *B. cereus* Frankland and Frankland
153 (ATCC 11778). The Gram-negative bacteria were *Pseudomonas aeruginosa* (Schroeter)
154 Migula (ATCC 27853) and *Escherichia coli* (Migula) Castellani & Chalmers MTCC 443
155 (ATCC 25922). All the ATCC standard test human pathogenic bacteria were supplied by the
156 Microbiology Department, University of Malaya and were maintained on Luria Broth (LB)
157 agar at 4°C.

158 Fungal isolates were cultivated on sterile PDA for 14 d prior to carrying out the bioassay, and
159 bacterial test microorganisms were prepared in liquid suspension to provide a concentration
160 equivalent of 0.5 McFarland units. Plugs of fungal mycelium growing tangentially to the
161 edge of the colony were then cut and transferred using a 5 mm cork borer into bacterial-
162 pathogen-seeded Muller Hinton Agar (MHA) plates. Muller Hinton Agar was prepared by
163 suspending 19g of MHA powder (Difco^{TM}) in 500 ml of purified water, and the mixture was
164 then heated with frequent agitation. Next the mixture was autoclaved for 15 min at 122°C.
165 Once cool enough to handle (45-50°C), it was poured into sterile petri dishes and left to cool
166 to room temperature. The plates (n = 3 replicates per pathogen) were then incubated at 37°C
167 for 24 h. After the incubation period inhibition zones were observed and measured. The
168 degree of toxicity of the fungi on the test microorganisms was determined by the diameter of
169 the inhibition zone in millimetres (mm). Antimicrobial activities were classified based on the
170 diameter of the zone of inhibition, with < 7mm considered as no activity, 7-9 mm considered as
171 weak activity, 9-11 mm as moderate activity and > 11 mm as good activity. Only isolates
172 with good bioactivity were considered for further analysis.

The preparation of test pathogens involved streaking each test culture onto Trypticase Soy Agar (TSA) and incubation overnight at 37°C. Trypticase Soy Agar was prepared by suspending 20g of TSA powder (Difco™) in 500 ml of purified water, then heating with frequent agitation, and autoclaving for 15 min at 122°C. Once the mixture was cool enough to handle (45-50°C), it was poured into sterile petri dishes and left to cool to room temperature. The test-pathogen-streaked TSA plates were incubated overnight at 37°C. After 24 h incubation, 4-5 single colonies were inoculated using a sterile loop into 15 ml universal bottles containing sterile Mueller Hinton Broth (MHB) and shaken thoroughly. MHB was prepared by suspending 21g of MHB powder (Difco™) in 1L of purified water, and again heating with frequent agitation, followed by autoclaving for 15 min at 121°C. Once cool enough to handle (45-50°C), the mixture was poured into 28 ml Universal Bottles and left to cool to room temperature. The densities of the test pathogens in MHB were matched with 0.5 McFarland standards that were prepared by adding 0.5 ml of 0.048 M $BaCl_2.2H_2O$ (1.17 % wt/vol) into 99.5 ml of 0.18 M H_2SO_4 (1 % v/v). This standard is considered to equate to 1.5 x 10^8 cfu/ml (Hendrickson and Krenz, 1991).

2.3. Disk diffusion assay

Inhibition of test bacterial growth by the polar fungal strains at different temperatures (4, 10, 15 and 28°C) was measured by the disk diffusion method. All experiments were repeated three times. Prior to each experiment, two-week-old fungal plugs were transferred into 500 ml conical flasks containing 250 ml PDB and incubated at 4, 10, 15 or 28°C for 21 d. The cultures were then centrifuged, filtered and extracted with equal volumes of ethyl acetate (EtOAc). The organic layer was collected and dried using a rotary evaporator and the crude extract was stored at 4°C until further use.

The test pathogens were prepared as described above and swabbed onto the surface of MHA plates. A sterile 6 mm filter disk was loaded with 10 µl of 1mg/mL crude extract and placed onto the test microorganism seeded plates. As a positive control gentamycin was used and, as a negative control, the solvent used to dissolve the extracts was included. The plates were then incubated for 24 h at 37°C. The diameters (mm) of inhibition zones were then measured and compared across the different incubation temperatures.

2.4. Temperature manipulation studies

Temperature manipulation studies of fungal strains with good bioactivity were carried out following the in-house protocol MECSUS (Microtiter plate, Elicitors, Combination, Solid phase extraction, UHPLC, Statistical analysis) (Rasha, 2013) with modifications. MECSUS is a protocol for microbial metabolite studies that involves miniaturized parallel fermentations in 96 well plates, parallel extraction and data analysis.

Three fungal strains that showed good bioactivity were selected for micro-scale cultivation at four different temperatures, 4, 10, 15 or 28°C. Prior to Micro Titre Plate inoculation, fungal strains were grown in 15 ml Falcon tubes containing sterile 70% Potato Dextrose Broth (PDB). The growth medium for inoculation was prepared by adding 6 ml of 70% PDB into a 15 ml Falcon tube together with five small steel balls. The Falcon tubes were then covered with cotton plugs and aluminium foil before being autoclaved at 121°C for 15 min.

Three 5 mm plugs of fungal mycelium were cut out from two-week-old fungal cultures using a 5 mm cork borer and transferred into separate 15 ml Falcon tubes containing 70% PDB. Inoculated tubes were then incubated at 4, 10, 15 or 28°C for 14 d.

At the end of 14 d incubation, the tubes containing fungal isolates were transferred into a biosafety cabinet where the cotton wool packing and aluminium foil cover were replaced

with sterile screw caps. The closed tubes were then shaken in a Genogrinder® at 1500 rpm for 30 min in order to disrupt the fungal mycelia before cultivation on MT plates.

Cultivation in 2 mL square deep 96-well MTPs involved adding 900 µl of 70% PDB into each MTP well. The plates were then each covered with a silicon mat, sealed with a custom-made clamp board, and autoclaved. Once the materials were cool enough to be handled, 100 µl of disrupted mycelial solution of each of three fungal strains was pipetted into the MTP wells. Each fungal strain was assigned individual rows of 12 wells. The MTPs were then covered with sandwich covers (System Duetz®, Germany) and a clamp board before being incubated at 4, 10, 15 or 28°C for 21 d.

After incubation, the MTP plates were uncovered in a biosafety cabinet, sterile silica SPEX beads added, and the cultures were then homogenised using a Genogrinder® SPEX® (SamplePrep, New Jersey) at 1500 rpm for 20 min. This was followed by centrifuging the plates at 4000 rpm for 30 min to allow the cell debris to settle at the bottom of the wells. After this, 500 µl of supernatant from the fungal culture from each well of a row (corresponding to one fungal isolate) was collected in single a sterile glass tube, giving a total volume of 6 ml (12 wells x 500 µl) per fungal strain.

The collected supernatants were then subjected to extraction with an equal volume of EtOAc. After shaking, the upper organic layer was transferred into a new and pre-weighed 30 ml vial. This step was repeated three times and the organic layers were combined. The EtOAc was evaporated to dryness using a rotary evaporator. The mass of each extract was recorded and the extracts were then stored at 4°C prior to HPLC analysis.

2.5. HPLC analysis

Extracts were chromatographically analysed to identify changes in the overall secondary metabolite profiles obtained at the different culture temperatures. Analytical HPLC was performed on an Agilent 1260 system (Agilent Technologies, United States) equipped with a column Synergy 4 µm Hydro-RP 80 Å 150 × 4.6 mm, 4 µm particle size (Phenomenex®, USA). The HPLC system (Agilent Technologies, United States) consists of the following components: quaternary pump G1311A, vacuum degasser pump G1322A, auto sampler G1316A and diode detector (DAD) G1315B. As an internal standard (IS), 1 mL of 1 mg/mL solution of 4-bromobiphenyl was added to the samples. Samples were prepared by diluting extracted crude sample of unknown amount into 1 mL of HPLC grade methanol (MeOH). The IS was then added to the sample. The mobile phase (H_2O/water) was prepared by filtering acetonitrile (ACN) and deionized water on 0.45 µm nylon membrane filters. The elution gradient was set as follows (solvent B, ACN): 10% at 0 min, 46% at 10 min, 70% at 15 min, 100% at 20 min, 100% at 30 min, 10% at 31 min, equilibration 10 min. The flow rate was set to 1 ml/min. The injection volume was set at 10 µL. UV-vis data were recorded from 190 to 600 nm and displayed at 220 nm. A Chemstation® chromatography data system (Agilent Technologies, United States), was used to control the chromatographic system and process the chromatograms. Chromatographic peaks with intensity > 50 mAU appearing between retention times (R_t) of 3 and 26 min were considered. The variations in size of major peaks were computed with reference to the IS.

3. Results and Discussion

3.1. Antimicrobial activity of polar fungi

Forty cold environment soil fungal isolates originating from various habitats were screened for their antimicrobial properties using the plug assay method against five human pathogenic bacteria, with their antimicrobial activity being classified according to the extent of the

inhibition zone produced. Forty-five percent of tested fungal isolates showed antimicrobial activity against at least one of the five test pathogens. A majority of the fungi tested showed high inhibition activity against the Gram-positive bacteria, *B. subtilis* and *B. cereus* (Table 1), but only two isolates showed positive activity against the Gram-negative *E. coli* and *P. aeruginosa*. None of the isolates showed activity against *E. faecalis*. Two fungal strains, *Pseudogymnoascus* sp (AK 102 R1-4 sp 5) isolated from King George Island and *Penicillium flavigenum* (HND 11 R8-1) isolated from Hornsund, exhibited broad spectrum activity. Of the five *Pseudogymnoascus* sp. strains tested, three exhibited good activity. Of four *Penicillium* sp. strains tested, three exhibited inhibition.

Most of the fungi tested showed some inhibition activity against Gram-positive bacteria, comparable to the findings of Nedialkova and Naidenova (2005), who tested antimicrobial activities in actinomycetes isolated from Antarctica. Previous studies have also indicated that antibiotic agents of fungal origin most strongly inhibit Gram-positive rather than Gram-negative bacteria (Kumar el al., 2005). The reason for the disparity in the inhibitory activity between Gram-positive and Gram-negative bacteria relates to differences between the two groups' cell wall structures, as Gram-negative bacteria are equipped with a cell wall barrier that prevents the passage of large molecules and fat-soluble molecules, thus making them less susceptible to the action of antibiotics in comparison with Gram-positive bacteria (Tortora et al., 2001).

Two strains in this study displayed activity against both Gram-positive and Gram-negative bacteria, *Pseudogymnoascus* sp (AK 102 R1-4 sp 5) and *Penicillium flavigenum*. (HND 11 R8-1). *Pseudogymnoascus* sp. showed broad activity against three test pathogens. This fungal species is commonly encountered in cold environments around the world (e.g. Adams et al., 2006; Singh, 2011), and various *Pseudogymnoascus* strains have been reported to be endemic or indigenous to Antarctic and sub-Antarctic regions (Arenz et al., 2006, 2011; Hughes et al.,

2007). This genus belongs to the order Helotiales, which includes typically saprophytic fungi playing an active role in nutrient recycling. Members of the genus *Pseudogymnoascus* have been reported to produce a range of extracellular enzymes including amylase and cellulase (Krishnan et al., 2014), keratinases (Mercantini et al., 1989), lipase, chinase and urease (Finotti et al., 1993, 1996). Li et al. (2008) reported that an Antarctic *Pseudogymnoascus*strain exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, consistent with the current study. A study on Antarctic marine fungi (Henriquez et al., 2014) also confirmed the antimicrobial and antitumoral potential of *Pseudogymnoascus* sp.

A second fungal strain identified in the current study that exhibited broad spectrum bioactivity was the "classic" antimicrobial compound producer *Penicillium flavigenum* (HND11 R8-1), isolated from High Arctic Svalbard. Representatives of this genus are globally distributed and clearly capable of thriving in various ecosystems. Sonjak et al. (2006) reported that *Penicillium* species sourced from various habitats showed no or very few differences in their secondary metabolite profiles. In the current study, isolate HND11 R8-1 exhibited good inhibition activity against both Gram-positive and Gram-negative bacteria, unlike several other *Penicillium* strains tested here whose activity was limited to only one pathogenic bacterium, *Bacillus subtilis*.

The bryophyte-associated fungus *Atradidymella* sp. inhibited the growth of Gram-positive bacteria. This species is also known to be able to infect mosses, and produces soluble polyphenolic oxidases and cellulases (Davey et al., 2009), but no previous studies have reported antimicrobial activity.

The three fungal strains mentioned above (*Pseudogymnoascus*sp., *Penicillium flavigenum* and *Atradidymella* sp. were selected for further temperature manipulation studies to examine

(a) their overall secondary metabolite production profiles at different temperatures, and (b) their antimicrobial activity at different temperatures assessed via a disk diffusion assay.

3.2. Temperature influence on secondary metabolite production profile

Inspection of chromatograms and determination of the relative concentrations of major compounds with reference to an internal standard revealed an overall increase in compound concentrations for the major peaks at 4°C for *Atradidymella* sp. The other two isolates, *Pseudogymnoascus* sp. and *Penicillium flavigenum*, differed with higher concentrations obtained at 15°C. An overall reduction of concentrations produced by all strains was observed at 10°C (Figure 1 a, b, c). These data confirm that culture temperature influences the production of secondary metabolites in all three tested fungal strains, although not in a simple fashion.

Environmental stressors such as temperature, ultra-violet radiation, water availability, salinity, pH, micronutrient and water availability, and CO_2 concentration are important factors that affect the physiology and metabolic pathways of fungi (Magan, 2007). These factors play crucial roles in determining microbial community composition in soils (Castro et al., 2010) and small shifts in these abiotic factors could have significant effects on the growth and productivity of key microorganisms (Bell, 2013). Although the above-mentioned environmental factors often do not act independently in polar soil ecosystems (Peck, 2006), temperature is a critical environmental variable that affects soil ecology in the polar regions (Convey et al., 2014), and seasonally changing temperatures influence the relative abundance of microorganisms (Bell, 2013). Temperature is an important environmental factor that influences physiological function of all organisms. This is particularly the case for microorganisms as they are poikilotherms whose temperature follows that of their external environment. Temperature variation in any given habitat is a natural phenomenon, taking

place over various timescales from the immediate and dynamic, through diurnal to seasonal and longer, and these variations can have important impacts on the survival and evolution of microorganisms (Convey et al., 2014; Davey et al., 1992; Peck et al., 2006). In polar soils, microbial activities have been demonstrated at temperatures as low as -15°C (Steven, 2007). Significant effects of microbial competition on biogeochemical flux were observed over the summer where warmer temperatures led to an increase in overall activity (Bell, 2013). In the current study, we have demonstrated that culture temperatures clearly affect the production of secondary metabolites in the three polar soil fungal strains examined in more detail, but clear and consistent patterns did not emerge and the fungal responses were complex, as also noted by Shohael (2006).

3.3 Effects of temperature on antimicrobial activity

Microorganisms, particularly soil-dwelling fungi, are known to produce a number of secondary metabolites with antimicrobial properties as a defence mechanism, thought to aid in competition for resources (Gallo et al., 2004), and temperature has also been reported to exert a profound effect on the antimicrobial production and activity of fungi (Himabindu and Jetty, 2006). Castro (2010) demonstrated a substantial increase in antagonistic activity between many Arctic soil bacterial isolates with increased temperature, proposing two possible explanations: 1) increased production of antimicrobials or, 2) shifts in relative growth rates. In the current study the antimicrobial activities of the selected *Pseudogymnoascus* sp. strain tested varied at different temperatures (Table 2). After crude extraction for the disk diffusion assay, the extracts obtained from incubations at 4°C or 15°C inhibited four of the five tested human pathogens (*E. coli, B. subtilis, S. aureus* and *C. albicans*). The widest diameters of the zones of inhibition, inferred to indicate the greatest activity, were observed for extracts from cultures incubated at 15°C (Table 2). Only two test organisms (*B. subtilis* and *C. albicans*) were inhibited at 10°C. All extracts were able to

inhibit the yeast *C. albicans*, although the diameter of the zone of inhibition reduced with increasing temperature.

Three of the five test microorganisms were inhibited by *Penicillium flavigenum* crude extracts obtained at 10 and 15°C. At 4°C two test microorganisms were inhibited and at 28°C only *C. albicans* was inhibited. *S. aureus* was only inhibited by the crude extracts produced at 10 and 15°C. *P. aeruginosa* and *E. coli* were not inhibited by any of the crude extracts. Although the ability to produce compounds that inhibited the test organisms appeared to be retained over a wide range of culture temperatures, when exposed to the highest experimental temperature the inhibitory ability reduced dramatically.

All crude extracts obtained from cultures at 10°C showed moderate biological activity (Table 2). Good inhibitory activity was visible at 4 and 28°C for *Pseudogymnoascus* sp. and *Atradidymella* sp. The crude extract of *P. flavigenum* exhibited moderate activity across all culture temperatures. This suggests that the responses of the fungi tested here to temperature were species-specific. More detailed studies of the optimal growth conditions and production of secondary metabolites, along with formal identification of specific metabolites, of each of these strains are required to further understand the relationship between growth dynamics and secondary metabolite production at different temperatures.

4. Conclusions

This study investigated the antimicrobial properties of 40 cold-adapted soil fungi from the Arctic and Antarctic. The data obtained showed that 45% of tested fungal cultures expressed at least some antimicrobial activity towards at least one of the five human pathogenic bacteria tested. *Pseudogymnoascus sp*., *P. flavigenum* and *Atradidymella* sp. showed the strongest antimicrobial activity. These taxa are found abundantly in polar soils and have also been reported to exhibit antifungal activities and produce cold-adapted enzymes, consistent with

active roles in decomposition and nutrient recycling in the soil ecosystem. Our study provides further confirmation that these species are rich producers of extracellular substances. Culture temperature clearly influenced the production of secondary metabolites, including compounds with antibacterial activity, in *Pseudogymnoascus* sp., *Penicillium flavigenum* and *Atradidymella* sp. Further genomic and functional analyses of interspecific competition and synergistic interactions in polar soil microbial communities are required to help identify the effects of climate variability and warming on soil ecosystems.

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Table 1. Bioactivity of fungal isolates against five test microorganisms.

Strain code	Test microorganism				
	Bacillus subtilis	*Escherichia coli*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia faecalis*
AK07KGI102 R2-3	-	-	+++	-	-
AK07KGI 301 R2-2 ASCO 6	++	-	-	-	-
AK07KGI 102 R2-3 SP 1	-	-	+++	-	-
AK07KGI 105 R3-2 SP 17	-	-	-	-	-
AK07KGI 1001 R1-1 SP 2	-	-	-	-	-
HND 10 R2-4	+	-	-	-	-
HND 9 R1-1 SP 34	+	-	-	-	-
HND 11 R2-2 SP 36	+	-	-	-	-
HND 10R1-3	+	-	-	-	-
HND 5 R5-3	-	-	-	-	-
HND 2 R8-2	-	-	-	-	-
HND 1 R7-2	-	-	-	-	-
HND 4 R7-3	++	-	-	-	-
AK07KGI 102 R1-4 SP 5	+++	-	++	+++	-
AK07KGI 102 R3-3 SP 30	-	-	+++	-	-
AK07KGI 102 R3-1 SP 16	-	-	-	-	-
AK07KGI 1001 R3-2 SP 1	++	-	-	-	-
AK07KGI 105 R3-1	-	-	-	-	-
HND 12 R8-1 SP 5	-	-	-	-	-
HND 2 R5-3	-	-	-	-	-
HND 1 R7-3	-	-	-	-	-

AK07KGI 102 R2-1 SP 18	++	-	-	-	-
HND 7 R1-1	-	-	-	-	-
HND 5 R6-2	-	-	-	-	-
AK07KGI 103 R2-1	+	-	-	-	-
HND 7 R6-3C	-	-	-	-	-
AK07KGI 102 R1-3 ASCO 2	++	-	-	-	-
AK07KGI 102 R3-2 ASCO 4	-	-	-	-	-
HND 11 R8-1	+++	+++	++	++	-
HND 2 R5-4	+++	-	-	-	-
HND 4 R5-1	-	-	-	-	-
AK07KGI 101 R2-3SP 1	-	-	-	-	-
AK07KGI 101 R3-1 SP 2	-	-	-	-	-
AK07KGI 1001 R2-1	-	-	-	-	-
AK07KGI 102 R3-1 SP 2	-	-	-	-	-
HND 12 R2-1	-	-	-	-	-
AK07KGI 1001 R1-1 SP 4	++	-	-	-	-
HND 12 R8-2(2)	-	-	-	-	-
AK07KGI 102 R1-4 SP 16	-	-	-	-	-
AK07KGI 2001 R2-1 SP 1	-	+	-	-	-

* Activities are classified as: - = no activity, weak activity = (+), moderate activity = (++), high activity = (+++).

Table 2. Antimicrobial activity of *Atradidymella* sp., *Pseudogymnoascus* sp., and *Penicillium flavigenum* crude extracts measured at different temperatures.

Isolates	Test organisms	mean ± standard deviation diameter of inhibition zone (mm) at different culture temperatures			
		4°C	10°C	15°C	28°C
HND 10 (*Atradidymella* sp.)	*E. coli*	0.0	7.0 ± 0.5	7.0 ± 1.0	0.0
	B. subtilis	0.0	0.0	6.0	7.0 ± 0.5
	S. aureus	15.0 ± 0.5	9.0 ± 0.5	7.0 ± 1.0	9.0 ± 1.73
	P. aeruginosa	0.0	0.0	0.0	0.0
	C. albicans	11 .0	13.0 ± 0.5	15.0	15.0
AK 102 (*Pseudogymnoascus* sp.*)*	*E. coli*	23.0 ± 1.7	0.0	24.0 ± 1.5	0.0
	B. subtilis	16.0 ± 1.0	6.0 ± 0.5	17.0 ± 1.7	7.0 ± 0.5
	S. aureus	13.0 ± 1.7	0.0	18.0 ± 1.3	16 .0± 1.7
	P. aeruginosa	0.0	0.0	0.0	0.0
	C. albicans	18.0 ± 0.5	10.0 ± 0.5	18.0	8.0
HND 11 (*Penicillium flavigenum*)	*E. coli*	0.0	0.0	0.0	0.0
	B. subtilis	7.0	7.0 ± 1.5	7.0	0.0
	S. aureus	7.0	8.0 ± 1.0	7.0 ± 1.5	0.0
	P. aeruginosa	0.0	0.0	0.0	0.0
	C. albicans	8.0	7.0 ± 1.6	7.0	7.0

Strain ID	Collection Location	Thermal class	(Genebank Accession no)	Identity
AK07KGI 102 R2-1 SP18	King George Island	Psychrophillic	KY646431	*Pseudorotium*.sp
AK07KGI 2001 R2-1 SP1	King George Island	Psychrophillic	KY646433	*Pseudogymnoascus*.sp
AK07KGI 102 R1-4 SP5	King George Island	Psychrotolerant	KY623481	*Pseudogymnoascus*.sp
AK07KGI 1001 R3-2 SP 1	King George Island	Psychrophillic	KY646435	*Pseudogymnoascus*.sp
AK07KGI 105 R3-2 SP17	King George Island	Psychrophillic	n/a	n/a
AK07KGI 103 R2-1	King George Island	Psychrophillic	n/a	n/a
AK07KGI 102 R2-3 ASCO 2	King George Island	Psychrophillic	KY646438	*Penicillium commune*
AK07KGI 101 R2-3SP 1	King George Island	Psychrophillic	n/a	n/a
AK07KGI 102 R3-3 SP 30	King George Island	psychrotolerant	n/a	n/a
AK07KGI 301 R3-3 SP2	King George Island	psychrotolerant	KY646432	*Pseudogymnoascus*.sp
AK07KGI 102 R1-4 SP16	King George Island	Psychrotolerant	KY646441	*Tausonia pullulans*
AK07KGI 301 R2-2 ASCO 6	King George Island	Psychrophillic	n/a	n/a
AK07KGI 101 R3-1 SP 2	King George Island	Psychrophillic	KY623479	*Leuconeurospora*.sp
AK07KGI 102 R3-1 SP 2	King George Island	Psychrophillic	n/a	n/a
AK07KGI 102 R3-2 ASCO 4	King George Island	Psychrophillic	n/a	*n/a*
AK07KGI 1001 R1-1 SP4	King George Island	Psychrophillic	n/a	n/a
AK07KGI 102 R2-3 SP1	King George Island	Psychrotolerant	KY623480	*Penicillium commune*
AK07KGI 1001 R1-1 SP2	King George Island	Psychrophillic	KY646437	*Pseudgymnoascus*.sp
AK07KGI 1001 R2-1	King George Island	Psychrophillic	KY646434	*Pseudogymnoascus*.sp
AK07KGI 105 R3-1	King George Island	Psychrophillic	KY646436	*Pseudogymnoascus*.sp
AK07KGI 102 R3-1 SP 16	King George Island	Psychrotolerant	KY646440	*Tausonia pullulans*
HND11 R8-1	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrophillic	KY623482	*Penicillium flavigenum*
HND10 R2-4	Hyrneodden peninsula,	Psychrotolerant	KY934270	*Atradidymella* sp

	Mariesletta, Hornsund			
HND2 R5-4	Hyettevika,Hornsund	Psychrophillic	n/a	n/a
HND5 R6-2	Ralstranda, Hornsund	Psychrophillic	KY646429	*Isafaria farinosa*
HND5 R5-3	Ralstranda, Hornsund	Psychrophillic	KY623484	*Isafaria farinosa*
HND2 R8-2	Hyettevika,Hornsund	Psychrophillic	KY646428	*Isafaria farinosa*
HND2 R5-3	Hyettevika,Hornsund	Psychrophillic	KY646439	*Penicillium flavigenum*
HND1 R7-2	North-western slopes of Kvartsittknattane, Hornsund	Psychrophillic	KY646430	*Phialocephala* sp.
HND12 R8-1 SP 5	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrophillic	KY646427	*Isaria farinosa*
HND7 R6-3	North-western slopes of KvartsittknattaneHornsund	Psychrophillic	n/a	n/a
HND4 R5-1	Revdalen, Hornsund	Psychrophillic	KY623483	*Tausonia pullulans*
HND4 R7-3	Revdalen, Hornsund	Psychrophillic	n/a	n/a
HND11 R2-2 SP 36	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrotolerant	n/a	n/a
HND9 R1-1 SP 34	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrotolerant	n/a	n/a
HND1 R7-3	North-western slopes of KvartsittknattaneHornsund	Psychrophillic	n/a	n/a
HND12 R2-1	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrotolerant	n/a	n/a
HND12 R8-2(2)	Hyrneodden peninsula, Mariesletta, Hornsund	Psychrophillic	n/a	n/a
HND10 R1-3	Hyrneodden peninsula,	Psychrotolerant	n/a	n/a

	Mariesletta, Hornsund			
HND7 R1-1	Rotjesfjellet, Hornsund	Psychrophillic	n/a	n/a

Figure 1 a. Changes in relative concentrations of major secondary metabolite peaks produced by *Atradidymella* sp. at 4, 10, 15 and 28°C.

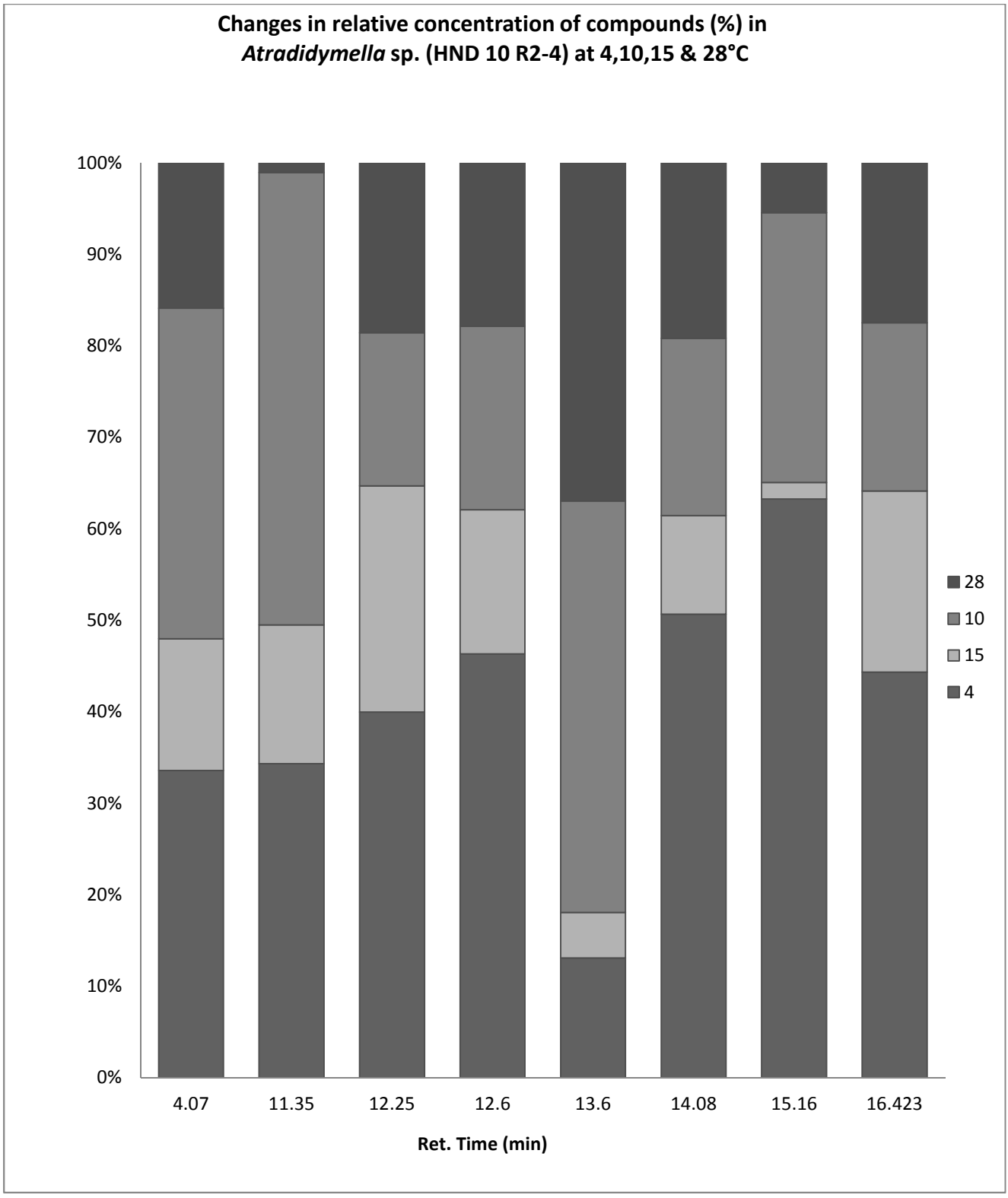

Figure 1 b. Changes in relative concentrations of major secondary metabolite peaks produced by *Pseudogymnoascus* sp. at 4, 10, 15 and 28°C.

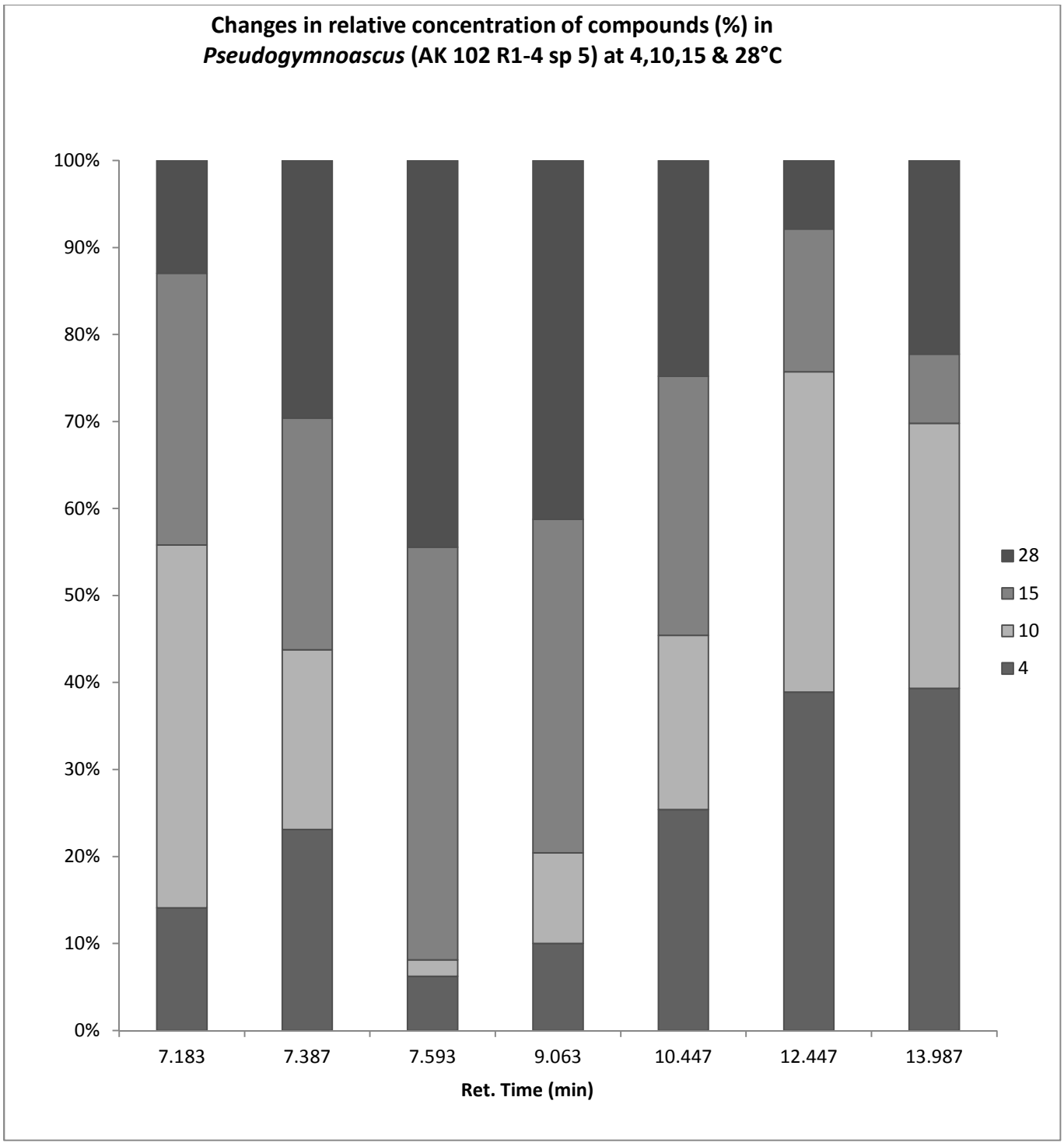

Figure 1 c. Changes in relative concentrations of major secondary metabolite peaks produced by *Penicillium flavigenum* at 4, 10, 15 and 28°C.