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N-170 Microbial Diversity-Based Novel Crop Protection Products

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Abstract

Extremophilic microorganisms are adapted to survive in ecological niches with high temperatures, extremes of pH, high salt concentrations, high pressure, radiation, *etc.* Extremophiles produce unique biocatalysts and natural products that function under extreme conditions comparable to those prevailing in various industrial processes. Therefore, there is burgeoning interest in bioprospecting for extremophiles with potential immediate use in agriculture, the food, chemical, and pharmaceutical industries, and environmental biotechnology.

Over the years, several thousand extremophilic bacteria, archaea, and filamentous fungi were collected at extreme environmental sites in the USA, the Chernobyl Exclusion Zone surrounding the failed nuclear power plant in Ukraine, in and around Lake Baikal in Siberia, and at geothermal sites on the Kamchatka peninsula in Russia. These organisms were cultured under proprietary conditions, and the cell-free supernatants were screened for biological activities against plant pathogenic fungi and major crop damaging insects. Promising peptide lead molecules were isolated, characterized, and sequenced.

Relatively high hit rates characterized the tested fermentation broths. Of the 26,000 samples screened, over thousand contained biological activity of interest. A fair number of microorganisms expressed broad-spectrum antifungal or insecticidal activity. Two-dozen broadly antifungal peptides (AFPs) are already patent protected, and many more tens are under further investigation.

Tapping the gene pool of extremophilic microorganisms to provide novel ways of crop protection proved a successful strategy.

Introduction

Extremophillic microorganisms have become a valuable bioprospecting target, as they often possess unusual physiological properties and the ability to produce unique secondary metabolites with potential commercial value. These natural products may be advantageous to the microorganisms in their respective environment and defend against predators or competitors.

Detailed characterization of microorganisms contributes to a greater understanding of their diversity, unique metabolism, and ecological function. Over a 20-year period, large numbers of microorganisms were isolated at contaminated sites and closed military bases, in deserts and forests in the USA, in Lake Baikal sediments in Siberia, and at geothermal and hydrothermal sites on the Kamchatka peninsula in Russia, as well as in and around the failed nuclear power plant, and the surrounding 30-km "Exclusion Zone" in Chernobyl, Ukraine. These environmental field sites were considered extreme in terms of their water activity, xenobiotic contamination, temperature, or biogeochemistry. Published or novel isolation techniques and growth media were adapted or modified and applied to simulate the eco-physiological conditions at the respective environmental niche. Pure cultures of the microorganisms were preserved and stored at ultra-low temperature for long-term maintenance.

In a collaborative effort between Pioneer Hi-Bred International, DuPont Experimental Station, and Lawrence Berkeley National Laboratory, we screened some 26,000 samples for novel crop protection products of microbial origin, and detected many biological activities of mutual interest. Here we focus on novel antifungal peptides as an example of the rich potential the gene pool of extremophilic microorganisms represents for science and industry.

Materials and Methods

Microorganisms. Field samples were collected at the closed military bases in Alameda and Fort Ord, California, in the Sierra foothills, in and around Lake Baikal and the Kronotzky National Park on the Kamchatka peninsula in Russia, and in and around the failed nuclear power plant and the surrounding 30-km "exclusion zone" in Chernobyl (ChEZ), Ukraine.

Isolation techniques and conditions targeted for their novel biotechnology potential endospore-forming bacteria, actinobacteria, and filamentous fungi. In short, sub-samples of the environmental materials were air-dried for 10 days at room temperature in a biosafety cabinet under aseptic conditions. The dry samples were homogenized with a sterile pestle and stamped with sterile foam plugs onto a set of ten media routinely used in our laboratory for microbial isolation. The isolation plates were incubated at room temperature for up to 2 months. The plates were examined for colony formation at weekly intervals. Pure cultures were preserved and transferred to a -84°C ultralow-temperature freezer for long-term maintenance.

Activity detection and identification. Isolated microorganisms were grown under proprietary conditions. The cell-free supernatants underwent primary screening. Samples with confirmed insecticidal or antifungal activities were subjected to HPLC purification. Molecular mass of the peptides of interest was determined by LC-ESI-TOF-mass spectrometry (LCMS). Ultimately, peptides were sequenced (**Figure 1**). BLAST analysis revealed novel peptides. Phylogeny of mature amino acid sequences was calculated using the neighbor joining method (**Figure 2**). As an example, **Table 1** lists the fungal species that expressed biological activities of interest.

In vitro testing. Purified and quantified peptides were *in vitro* tested against target fungal pathogens and insect pests of major crop plants. If the cell-free supernatants yielded insufficient amount of peptides, the appropriate *E. coli* codon-optimized genes were expressed in a proprietary strain of *E. coli*. Proprietary *in vitro* assays scored the inhibition of fungal spore/conidium germination and hyphal growth (**Figure 3**) or insect larval growth (**Figure 4**).

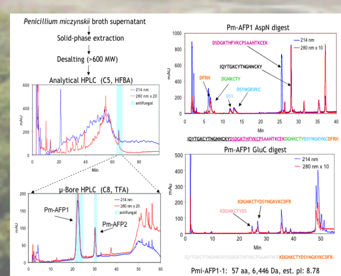

Figure 1. Example antifungal peptide (AFP) isolation and identification

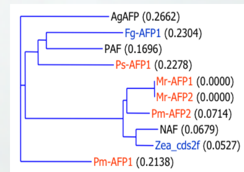

Figure 2. Phylogenetic analysis of AFPs (neighbor joining method of Saitou and Nei with a utility in Vector NTi –AlignX)

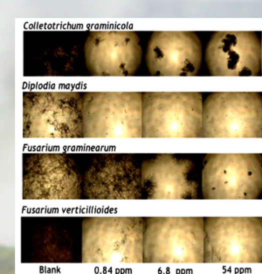

Figure 3. *In vitro* antifungal activity assay (Ps-AFP1; 48 h)

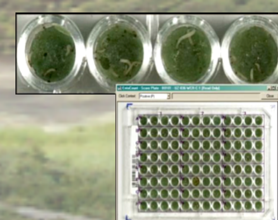

Figure 4. *In vitro* insecticidal activity assay

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Table 1. List of fungal species that expressed biological activities of interest

Fungal species			
Absidia spinosa	*Doratomyces microsporus*	Mycelia sterilia (white)	*Peyronella* sp.
Acremonium rutilum	*Embellisia helianthi*	*Myrothecium cinctum*	*Phoma fimeti*
Acremorium flavum	*Emericella nidulans*	*Myrothecium roridum*	*Phoma glomerata*
Acremorium strictum	*Emericella variecolor*	*Myrothecium* sp.	*Phoma* sp.
Alternaria tenuissima	*Emericella violacea*	*Myrothecium verrucaria*	*Sclerotinia ricini*
Apiospora montagnei	*Epicoccum purpurascens*	*Nectria inventa*	*Scolecobasidium tshawytschae*
Arthrinium phaeospermum	*Eupenicillium alutaceum*	*Nigrospora sphaerica*	*Scopulariopsis brevicaulis*
arthrinium state of *Apiospora montagnei*	*Eupenicillium erubescens*	*Paecilomyces lilacinus*	*Scopulariopsis candida*
Aspergillus alliaceus	*Eupenicillium javanicum*	*Paecilomyces marquandii*	*Scopulariopsis koningii*
Aspergillus deflectus	*Eupenicillium lapidosum*	*Penicillium citrinum*	*Stachybotrys bisbyi*
Aspergillus flavipes	*Eurotium halophilicum*	*Penicillium* sp.	*Thermoascus aurantiacus*
Aspergillus flavus	*Eurotium halophilicum*	*Penicillium canescens*	*Tolypocladium inflatum*
Aspergillus fresenii	*Exophiala* sp.	*Penicillium chrysogenum*	*Trichoderma album*
Aspergillus ochraceus	*Fusarium acuminatum*	*Penicillium citreonigrum*	*Trichoderma hamatum*
Aspergillus rhizopodus	*Fusarium avenaceum*	*Penicillium claviforme*	*Trichoderma harzianum*
Aspergillus sclerotiorum	*Fusarium bullatum*	*Penicillium clavigerum*	*Trichoderma koningii*
Aspergillus sp.	*Fusarium culmorum*	*Penicillium dierckxii*	*Trichoderma polysporum*
Aspergillus sydowii	*Fusarium culmorum*	*Penicillium expansum*	*Trichoderma pseudokoningi*
Aspergillus terreus	*Fusarium equiseti*	*Penicillium frequentans*	*Trichoderma* sp.
Aspergillus ustus	*Fusarium equiseti*	*Penicillium funiculosum*	*Trichoderma virens*
Aspergillus versicolor	*Fusarium heterosporum*	*Penicillium glandicola*	*Trichotecium roseum*
Aureobasidium pullulans	*Fusarium javanicum*	*Penicillium griseolum*	*Trimmatostroma* sp.
Bartalinia sp.	*Fusarium oxysporum*	*Penicillium griseum*	*Ulocladium botrytis*
Bipolaris australiensis	*Fusarium poae*	*Penicillium herquei*	*Ulocladium consortiale*
Bipolaris sorokiniana	*Fusarium sambucinum*	*Penicillium hirsutum*	Undetermined agonomycete
Chaetomium aureum	*Fusarium sambucinum* var. *ossicolum*	*Penicillium islandicum*	Undetermined agonomycete
Chaetomium cochliodes	*Fusarium semitectum*	*Penicillium janczewskii*	Undetermined ascomycete
Chaetomium dolichotrichum	*Fusarium solani*	*Penicillium janthinellum*	Undetermined ascomycete
Chaetomium globosum	*Fusarium tricinctum*	*Penicillium lanosocoeruleum*	Undetermined ascomycete
Chaetomium homopilatum	*Fusarium tumidum*	*Penicillium lanosum*	Undetermined ascomycete
Chaetomium nigricolor	*Gliocladium solani*	*Penicillium luteum*	Undetermined ascomycete
Chaetomium subspirale	*Gliocladium varians*	*Penicillium miczinskii*	Undetermined ascomycete
Chrysosporium pannorum	*Gliocladium viride*	*Penicillium oxalicum*	Undetermined ascomycete
Chrysosporium sp.	*Helicorhoidion pulchrum*	*Penicillium purpurogenum*	Undetermined ascomycete
Chrysosporium sp.	*Humicola grisea*	*Penicillium restrictum*	Undetermined ascomycete
Cladosporium cladosporoides	*Monascus ruber*	*Penicillium rugulosum*	Undetermined ascomycete
Coniothyrium fuckelii	*Mortierella vinacea*	*Penicillium simplicissimum*	Undetermined ascomycete
Corynascus sepedonium	*Mucor hiemalis*	*Penicillium* sp.	Undetermined ascomycete
Cunninghamella echinulata	*Mucor plumbeus*	*Penicillium variabile*	Undetermined basidiomycete
Cunninghamella elegans	*Mucor racemosus*	*Penicillium varians*	Undetermined coelomycete
Curvularia inaequalis	Mycelia sterila	*Penicillium vermiculatum*	*Wardomyces anomalus*
Dichobotrys sp.	Mycelia sterilia (orange)	*Penicillium verrucosum* var. *cyc*	*Wardomyces columbinus*
		Penicillium waksmani	*Zygorhynchus moelleri*

Table 2. Spectrum characteristics of microbial natural products in primary screens

Activity	Strains with 4 targets	Strains with 3 targets	Strains with 2 targets	Strains with 1 target
Antifungal	48	73	151	524
Insecticidal*	21	30	200	961

*includes non-protein hits

Results and Discussion

Over 26,000 primary samples were screened over a three-year period and some 1,000 showed insecticidal or antimicrobial activities. These samples were produced under proprietary cultivation conditions by over 74 bacterial and 86 fungal species (**Table 1**). Many microorganisms expressed broad-spectrum antifungal or insecticidal activities (**Table 2**). As an example, we show that activity-guided purification led to the identification of novel antifungal peptides (AFP) from several filamentous fungi **Figure 1**). These peptides were characterized, sequenced, phylogenetically analyzed (**Figure 2**), and their antifungal activity validated. They were shown to be inhibitory to fungal pathogens of maize that cause stalk rot and ear mold. These cysteine rich and basic peptides have 55 to 61 residues respectively, and can be aligned as one protein family together with some previously characterized AFPs. DNA sequence of several of these indicates that they are synthesized as preproproteins from which the mature AFP is cleaved. Promising AFPs were filed for patent protection in the USA and abroad.

Bioprospecting has elevated the search for microorganisms with novel capabilities to a safe, ethical, and meaningful conduct that equally benefits science and industry. Extremophilic microorganisms represent an immensely rich and mostly untapped genetic pool of novel bioactive capabilities. Microbial diversity-based new crop protection products and technologies are replacing traditionally used synthetic chemicals and agricultural practices. Transgenic plants expressing heterologous genes of extremophilic microorganisms may provide a secure solution to agricultural production of major crop plants and industrial feedstocks.

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