

N-170 Microbial Diversity-Based Novel Crop Protection Products

Ronald Flannagan¹, Rafael Herrmann², James Presnail², Tamas Torok³, and Nasser Yalpani¹

¹Pioneer Hi-Bred International Inc., Johnston, IA 50131; ²DuPont Experimental Station, Wilmington, DE 19880; ³Lawrence Berkeley National Laboratory, Berkeley, CA 94720

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 patented, 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

Extremophilic 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 from 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 dosed military bases in Alameda and Fort Ord, California, in the Sierra foothills, in and around Lake Baikal and the Kronotsky 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 soft 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 under went 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).

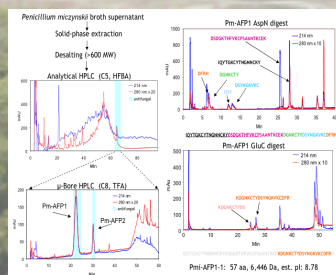


Figure 1. Example antifungal peptide (AFP) isolation and identification.

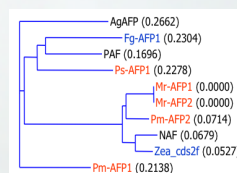


Figure 2. Phylogenetic analysis of AFPs (neighbor joining method of Saitou and Nei with a utility in Vector NTI-AlignX)

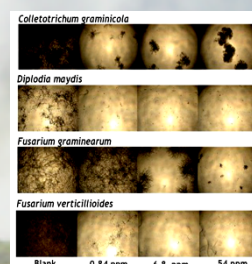


Figure 3. *In vitro* antifungal activity assay (Ps-AFP1; 48 h)

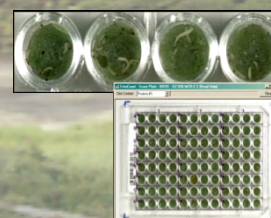


Figure 4. *In vitro* insecticidal activity assay

Acknowledgements

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

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<i>Aspergillus terreus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus oryzae</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus carbonarius</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus clavatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumigatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus terreus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus oryzae</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus carbonarius</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus clavatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumigatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus terreus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus oryzae</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus carbonarius</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus clavatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumigatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus terreus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus oryzae</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus carbonarius</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus clavatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumigatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
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<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
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<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumigatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
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<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus oryzae</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus carbonarius</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus clavatus</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus nidulans</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus niger</i>	Antifungal activity	Antifungal activity	Antifungal activity
<i>Aspergillus fumig</i>			

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