VOL. 54 NO. 1, JAN. 2001 THE JOURNAL OF ANTIBIOTICS pp. 56 — 65

Extremophilic Organisms as an Unexplored Source of Antifungal Compounds

CHARLES H. PHOEBE, Jr.[a], JOAN COMBIE[b], FRED G. ALBERT[b], KIM VAN TRAN[a], JESSICA CABRERA[c], HEIDI J. CORREIRA[c], YUEHUA GUO[c], JOHANNA LINDERMUTH[c], NICOLE RAUERT[d], WILLIAM GALBRAITH[c] and CLAUDE P. SELITRENNIKOFF[d,*]

[a] Waters Corporation, Mail Stop CRD, 24 Maple Street, Milford, MA 01757-3696, USA
[b] Montana Biotech Corporation, 1740 East Baseline Road, Belgrade, MT 59714, USA
[c] Alpha-Beta Technology, Inc., One Innovation Drive, Worcester, MA 01569, USA
[d] MycoLogics, Inc., 4200 East Ninth Avenue, Box B-111, Denver, CO 80262, USA

(Received for publication September 8, 2000)

Extracts of the biomasses and fermentation broths of 217 extremophilic microorganisms isolated from a number of locales were screened for antifungal activity using whole-cell and mechanism-based *in vitro* assays. Importantly, eleven broth extracts had activity against several *Candida* species and *Aspergillus fumigatus* in whole-cell *in vitro* assays. One broth specifically inhibited (1,3)β-glucan synthase activity and four specifically inhibited ketol-isomerase activity, suggesting a mode of action of the antifungal compound(s) present in these extracts. The extract from one thermophile, a novel species of *Pseudomonas*, was fractionated, an active compound purified and its structure determined. The compound was identified as pyochelin, a previously identified iron-binding compound with heretofore undescribed antifungal activity. To our knowledge, this is the first report demonstrating that extremophiles synthesize compounds that have antifungal activity.

During the last three decades there has been a dramatic increase in the frequency of fungal infections in immunocompromised patients[1~3]. Deep-seated mycoses are increasingly observed in patients undergoing organ transplants and in patients receiving aggressive cancer chemotherapy[4]. For example, the incidence of fungal infections following solid organ transplantation ranges from 5% in kidney recipients, between 15 and 35% in lung and heart recipients, and up to 40% in liver recipients[4]. The most common pathogens associated with invasive fungal infections are the opportunistic yeast, *Candida albicans*, and the filamentous fungus, *Aspergillus fumigatus*[5,6]. Also adding to the increase in the numbers of fungal infections is the emergence of Acquired Immunodeficiency Syndrome where virtually all patients become affected with some form of mycosis[4,7]. The most common organisms encountered in these patients are *Cryptococcus neoformans*, *Pneumocystis carinii*, and *C. albicans*[7,8]. Current treatments for fungal infections are limited to three therapeutic classes: Amphotericin B (a macrolide polyene), which interacts with membrane sterols, flucytosine (a fluoropyrimidine), which interferes with protein and DNA biosynthesis and a variety of azoles (*e.g.*, ketoconazole, itraconazole and fluconazole) that inhibit membrane-sterol biosynthesis[4,9]. Even though amphotericin B has a broad range of activity and is viewed as the "gold standard" of antifungal therapy, its use is limited due to infusion-related reactions and nephrotoxicity[4,6]. Flucytosine use is also limited due to the development of resistance and its narrow spectrum of activity while the wide-spread use of azoles is causing the emergence of clinically-resistant strains of *Candida* spp[4,10]. Although advances in the formulation of

* Corresponding: claude.selitrennikoff@uchsc.edu

amphotericin B have decreased its nephrotoxicity[11] and new classes of antifungal agents such as the lipopeptides[12] and sordarins[13] are in various stages of clinical development, the impact of fungal infections in the clinical management of infected patients underscores the clear need for new antifungals.

The identification of new chemical entities is becoming more difficult. Historically, the screening of soil microorganisms and extracts obtained from terrestrial plants and animals has yielded novel natural products which themselves, or through chemical modification and synthesis, have been a rich source of drugs for the treatment of human disease[14,15]. A review of the 520 newly-approved drugs reported between 1983 and 1994 indicates that 157 (30%) are derived from unmodified natural products or semi-synthetic analogs[16]. Unfortunately, many potential sources have been screened numerous times, decreasing the probability of finding additional, useful lead compounds. One way to avoid this stumbling block is to screen organisms from unusual and previously ignored ecosystems.

Some of the oldest life-forms on Earth that withstand and even thrive in unusual ecosystems are known as "extremophiles". The unique genomes of these organisms are believed to have been adapted initially to suit the extreme environments that the earliest organisms on this planet faced. Today these organisms live in habitats that will support no other form of life. Most thermophilic extremophiles are moderate and grow at temperatures above 45°C[17,18]; they are still able to grow but slowly between 25°C and 40°C[18]. Research on thermophiles has been on-going for several decades with the most noted being the discovery of heat-stable DNA polymerase used in the polymerase chain reaction[19]. More recent work has been focused on other unusual ecosystems where extremophiles have been isolated from waters above 100°C in terrestrial hot springs and deep-sea thermal vents, at temperatures below 0°C in arctic waters, at immense pressure under several miles of water on the ocean floor, in the saturated salt environment of the Great Salt Lake, at very high osmolarities (*e.g.*, high sugar concentrations), at pH values less than pH 2 in drainage in certain mines and geothermal sulfur-rich springs, at pH values greater than pH 11, and in media containing toxic compounds[20]. The research thus far has focused on the isolation of "extremozymes" that might provide novel opportunities for biocatalysis in research and in industrial chemical processing[20,21].

As far as we are aware, there are no reports of screening extremophiles for antifungal activity. The Montana Biotech Corporation (MBC) in-house collection includes isolates from 1400 ecosystems from around the world. Many of the sampling sites were the thermal waters of Yellowstone National Park, which has the world's most diverse array of readily accessible, extreme microbial habitats.

In this manuscript, we report the screening of biomass and fermentation broth extracts of extremophiles for antifungal activity using whole-cell and *in vitro* enzyme assays. Importantly, we have identified fermentation broths of extremophiles and thermophiles that have potent activity against several human fungal pathogens.

Materials and Methods

Chemicals

UDP-glucose [glucose-^{14}C] and UDP-*N*-acetyl-glucosamine [glucosamine-^{14}C] were obtained from New England Nuclear (Boston, MA). Yeast extract, casamino acids, tryptone and Bacto-Peptone were obtained from Difco (Detroit, MI). Beef extract, proteose peptone and malt extract were from Accumedia. SytoStain 13 was purchased from Molecular Probes (Eugene, OR). All other reagents and enzymes were purchased from Sigma (St. Louis, MO).

Microorganisms

The database covering the in-house collection of Montana Biotech Corporation was used to select organisms from a diversity of locations. Microorganisms were chosen for testing based on locality data, *e.g.*, waters of pH between pH 3 to pH 9, temperatures from 20°C to 70°C and of various chemistries. Organisms that were either isolated in waters >45°C, could grow under laboratory conditions at >45°C, or were able to grow on exotic carbon or nitrogen sources or on toxic compounds were tested for antifungal activity (COMBIE, unpublished). Organisms were isolated and grown on media previously described[23~26]. *Zalerion arboricola* (ATCC 20868), *Emericella rugulosa* (ATCC 58398), *Actinomadura hibisca* (ATCC 53557), *Streptomyces nodosus* (ATCC 14899), *Aspergillus nidulans* var. *echinulatus* (ATCC 16825), *A. fumigatus* (ATCC 16424), *Candida albicans* wild-type strain (ATCC 20402), *C. glabrata* (ATCC 48435), and polyene-resistant *C. albicans* (ATCC 38247) were obtained from the American Type Culture Collection and were reconstituted according to the directions provided. Each of the first five ATCC organisms is known to produce antifungal compounds (*e.g.*, echinocandins) and was grown in 125 ml of the medium recommended by ATCC in 500-ml shaker flasks at 30°C for

72 hours at 180 rpm. Fermentation broths and biomasses were separated and processed as described below. Cultures of *Candida* spp. cells were grown and harvested as described[26].

Each extremophile was grown to stationary phase in 500 ml medium in one liter mini-fermentors with individually controlled air flow using a two port system (air inlet and outlet) at air flows of 250 to 1000 ml air/minute. In general, microorganisms were incubated in the medium on which an organism was originally isolated. Media were adjusted to the pH approximating that of the original habitat. A loopful of cells from a storage slant was used to inoculate each mini-fermentor. Incubations were performed at the temperature of the original habitat. In general, organisms grown at 45°C or above were incubated for three days, while those at less than 45°C were incubated for four to seven days. Organism 8(C)X was isolated from Beach Springs Lake, Wyoming; the pH of the lake was pH 6.7 while the ambient water temperature was ~20°C.

Analysis of 16S rDNA

Organism 8(C)X was harvested and genomic DNA isolated. The 16S rRNA encoding gene was PCR-amplified from genomic DNA. Primers used for the amplification corresponded to *E. coli* positions 005 and 1540. The sequence of the amplification products was determined on an ABI Prism 377 DNA Sequencer and analyzed using PE/Applied Biosystems DNA editing and assembly software[28] by Dr. J. BARTELL of MIDI Labs (Newark, DE).

Extract Preparation

Ethyl Acetate Extract: After incubation, each sample was centrifuged at 1900 *g* for 30 minutes to separate the supernatant (broth) and cells (biomass). Each broth sample was passed through a 0.22 μm filter and extracted with an equal volume of ethyl acetate. The extracted broth sample (aqueous phase) was discarded, the ethyl acetate extract was reduced to 5 ml under a stream of dry air at 27°C, transferred to pre-weighed screw-top glass tubes (16×100 mm) and taken to dryness *in vacuo* at ambient temperature in a SpeedVac concentrator (Savant Instruments, Inc., Holbrook, NY). The weight of each ethyl acetate extract was determined, DMSO was added to yield 100 mg extract/ml, solutions were transferred to individual wells of deep 96-well plates and stored frozen at −20°C. Uninoculated media were processed in the same manner.

Ethanol extract: The biomass from each fermentation was transferred to a 50-ml disposable centrifuge tube and brought to a final volume of 40 ml with absolute ethanol. Tubes were shaken for 10 minutes and then centrifuged (1700 *g*) for 30 minutes. The ethanolic supernatants were decanted into 40-ml glass vials, the extracted biomasses discarded, and each solution reduced to ~2 ml under a stream of dry air at 27°C. The concentrated samples were transferred to pre-weighed screw top glass tubes (16×100 mm) and taken to dryness *in vacuo* at ambient temperature in a SpeedVac concentrator. The weight of each ethanolic extract was determined, the extracts dissolved in one ml 50% (v/v) aqueous methanol and 500 μl of each sample dispensed into individual wells of deep 96-well plates. The plates were placed into a SpeedVac concentrator and the samples reduced to dryness. Fifty μl DMSO was added to each well to dissolve each extract and the plates stored frozen at −20°C.

In vitro Antifungal Whole-cell Assays

C. albicans Protoplast Regeneration Assay

Inhibition of *C. albicans* (ATCC 20402) protoplast cell-wall generation and growth was measured using an agar overlay method. Protoplasts were released from mid-log cells, grown in 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose (YPD), using lyticase (0.38 mg/ml in 0.6 M KCl, 50 mM $NaHPO_4$, 5 mM tetrasodium EDTA, and 0.34% [v/v] 2-mercaptoethanol, 37°C) to digest the cell wall. Protoplasts were washed by centrifugation and resuspended in 0.6 M KCl. Test samples or vehicle (2.5 μl) were placed in 96-well microtiter plate wells and molten agar (75 μl; 1.5 % [w/v] agar in YPD containing 0.6 M KCl) was added and allowed to cool. Protoplasts were added in a molten agar layer (30 μl containing 2×10^5 cells/ml in 0.5% [w/v] agar in YPD with 0.6 M KCl). The microtiter plates were incubated at 30°C and microscopically scored for protoplast regeneration and growth after 24 and 48 hours of incubation.

C. albicans Whole-cell Agar Diffusion Assay

Inhibition of *C. albicans* (ATCC 20402) cell growth was measured using an agar overlay method. Test samples, standard inhibitors, or controls (2.5 μl) were placed in 96-well microtiter plate wells and molten agar (75 μl; 1.5% [w/v] agar in YPD) was added and allowed to cool. *C. albicans* cells, obtained as described above, were added in an agar layer (30 μl containing 3×10^5 cells/ml in 0.5% [w/v] agar in YPD) and allowed to cool. The microtiter plates were incubated at 30°C and evaluated visually at 40~48 hours using an inverted light microscope.

C. albicans Broth Assays

All *Candida* spp. broth assays were performed in RPMI

1640 medium with glutamine, without bicarbonate, buffered with MOPS to pH 7.0. Inhibition of *Candida* spp. by biomass and broth extracts was determined as follows: *C. albicans* wild-type (ATCC 20402), *C. glabrata* (ATCC 48435), and polyene-resistant *C. albicans* (ATCC 38247) cells were grown to mid-log phase, washed by centrifugation, resuspended in medium, and added to microtiter plate wells at 1×10^3 cells per well (~110 μl). Extracts or vehicle were added (2 μl), plates were incubated at 30°C with shaking for 15~16 hours and washed. Cells were resuspended to the original volume with RPMI containing 0.165 M MOPS and SytoStain 13 added (10 μl of 10 μM in RPMI-0.165 M MOPS). Fluorescence was measured using a 7620 Microplate Fluorometer (Cambridge Technology, Inc., Cambridge, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

A. fumigatus Broth Assay

The microtiter assay procedure was a modification of the NCCLS protocol M-27T. Liquid (200 μl) RPMI 1640 medium with glutamine and phenol red, without bicarbonate, buffered with MOPS to pH 7.0, in 96-well microtiter plates was inoculated with 2×10^3 *A. fumigatus* (ATCC 16424) conidia per well (conidia were obtained from −80°C glycerol stocks). Samples (2 μl prepared in DMSO) were added to each well and plates incubated at 37°C for 48 hours. The amount of growth was determined using a microtiter optical plate reader at 680 nm or, alternatively, by scoring each well by eye with the aid of a light microscope.

Enzyme Assays

(1,3)β-glucan synthase activity (EC.2.4.1.34; UDP-glucose; 1,3-β-D-glucan 3-β-glucosyl transferase) was assayed as described by WOOD *et al.*[27]. Chitin synthase activity (EC.2.4.1.16; chitin : UDP-acetylamino-deoxy-glucosyl transferase) from *C. albicans* was assayed essentially as described in YARDEN and YANOFSKY[29]. Ketol-isomerase activity (EC. 5.3.1.19: 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase [amino transferring]) was measured using lysates from *A. fumigatus* as enzyme sources as described by SELITRENNIKOFF and OSTROFF[30]. The inhibitor, 6-diazo-5-oxo-norleucine, was used as a control for enzyme inhibition and had an IC_{50} of 17 ng/ml (~0.1 μM). The amounts of protein in cell extracts were determined using the Bio-Rad kit following the manufacturer's directions.

Fractionation of Organism 8(C)X

To identify the active compound present in the extract from 8(C)X, the organism was grown at 25°C in 80 500-ml fermentors as described above and the medium was extracted three times with EtOAc (~40 liters, total). The combined organic extracts were evaporated *in vacuo* to dryness (~1.5 g). The extract was separated using automated preparative reversed-phase HPLC and solid phase extraction (described in the next section) and 40-ml fractions collected. One ml aliquots of each fraction were taken to dryness *in vacuo*, re-suspended in DMSO (10 μl) and tested for antifungal activity using the *C. albicans* whole cell assay. Two active fractions were further separated by HPLC and three resulting active fractions analyzed by NMR.

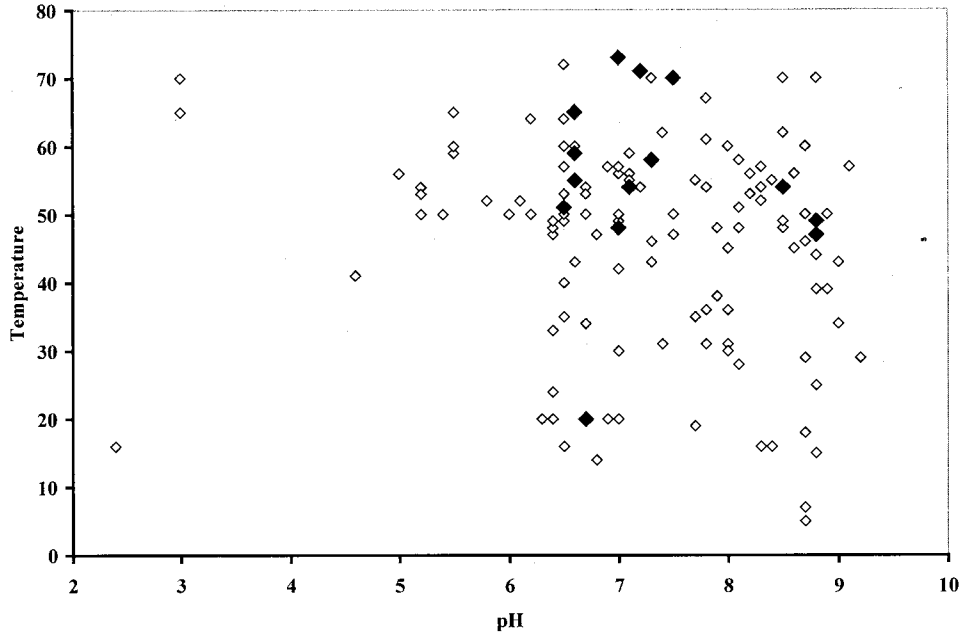
Analytical Methods

^1H and ^{13}C NMR spectra were obtained on either a Bruker DRX 500 or Bruker 400 spectrometer, with $CDCl_3$ as a solvent. Negative-ion and positive-ion ESI mass spectra were recorded on a Perkin-Elmer Sciex API 165, single quadrupole instrument. Analytical reversed-phase HPLC-ELSD-ESIMS of the active fractions was performed on a LiChrosphere 60, RP Select B column (4×250 mm, 5 μm particle size, Merck, employing a 30 minute linear gradient elution from (eluent A) 5 mM ammonium formate in water to (eluent B) acetonitrile/methanol (1 : 1) (A/B=85/15 to 0/100). The flow rate was 0.9 ml/minute. Automated preparative reversed-phase chromatography/solid phase extraction trapping was performed on a SEPBOX (AnalytiCon). The initial preparative fractionation was performed on a C-4 reversed-phase column (50×250 mm) employing a 30 minute linear gradient elution from (eluent A) 5 mM ammonium formate in water to (eluent B) methanol (A/B=0/100 to 100/0, hold for 20 minutes). The flow rate was 109 ml/minute and eighteen fractions were collected. Subsequent subfractionation was performed on a C-18 reversed-phase column (25×250 mm) employing various linear gradients of 5 mM ammonium formate in water and methanol (Figure 3A and 3B). The identification of the active compound in fractions K7, K8 and L7 by MS and NMR analyses was performed by AnalytiCon AG (Potsdam, Germany).

Results

Extremophiles include groups of organisms that have not been screened as sources of antifungal agents, thus making them potential sources of novel compounds. We selected

Fig. 1. Diversity of microbial habitats screened.



Original habitat pH *vs.* habitat temperature for organisms in this study. Open symbols represent all 217 organisms. Closed symbols represent organisms with anti-fungal activity. In some instances, several organisms are represented by one symbol at the same pH and temperature.

217 microorganisms for screening for antifungal compounds from the Montana Biotech collection based on the properties described in the Materials and Methods. A plot of the pH of the habitat and the temperature of the thermal feature of each of the 217 organisms is shown in Figure 1. The organisms that had antifungal activity, as described below, are indicated by closed symbols.

Each microorganism was grown and the fermentation broths and biomasses processed as detailed in the Materials and Methods. Extracts from each of five organisms known to produce antifungal compounds were similarly prepared to serve as controls. A total of 217 fermentation broths and biomass extracts and the 5 antifungal-producing organisms were screened for antifungal activity. Twenty-nine broth extracts (13% of total) and each of the antifungal-producing control organisms had activity against *C. albicans* (results not shown). To confirm these observations, a second fermentation of these 29 microorganisms and the five antifungal-producing organisms was prepared and an ethyl acetate extract made from the spent broth as well as an ethanolic extract of each biomass. As expected, each extract of the fermentation broth and/or biomass of the five antifungal-producing organisms had antifungal activity in both the whole-cell and the protoplast-regeneration assays (Table 1). Of the 29 microorganisms re-tested, the ethyl acetate extracts of only 15 were active against *C. albicans* (Table 1). The remaining 14 microorganisms failed to reproduce the original activity for reason(s) not understood (results not shown). None of the biomass extracts was active in the *C. albicans* whole-cell agar diffusion assay while seven were active against protoplasts (Table 1).

The ethyl acetate extracts of the fermentation broths from the 15 active microorganisms were tested against a polyene-resistant strain of *C. albicans* as described in the Materials and Methods. The broth extracts of four organisms were not active (not shown); none of these four organisms was considered further. Extracts of the fermentation broths of the eleven remaining microorganisms (and the five control antifungal-producing organisms) were tested further to determine their breadth of activity and to elucidate possible mechanisms of antifungal activity. These results are presented in Table 2. Again as expected, each of the five control organisms was active. Note that extracts of four microorganisms were also active against *C. glabrata*, showing that these extracts had broad anti-candidal activity. Importantly, the ethyl acetate extracts

Table 1. Activity of extracts in *C. albicans* protoplast regeneration and whole-cell assays*.

Microorganism	Broth Extract		Biomass Extract	
	Protoplast	Whole-cell	Protoplast	Whole-cell
253	WALL	RCS	N	N
8 (C) X	WALL	KILL	WALL	N
24 (B) F	WALL	RCN	RCN	N
30 (A) X	WALL	RCN	WALL	N
236	WALL	KILL	N	N
237	KILL	RCN	N	N
244	KILL	RCN	RCN	N
245 (B)	KILL	RCN	N	N
248	WALL	RCN	WALL	N
252 (A)	KILL	RCN	N	N
430 PER	WALL	KILL	N	N
23 X	WALL	RCN	RCN	N
56 (B) X	RCN	RCS	N	N
197 PER	WALL	KILL	WALL	N
88 X	KILL	KILL	N	N
Zalerion arboricola	KILL	KILL	KILL	KILL
Emericella rugulosa	KILL	KILL	RCN	KILL
Streptomyces nodosus	N	N	KILL	KILL
A. nidulans	N	N	WALL	KILL
Actinomadura hibisca	KILL	N	N	N

* The indicated microorganisms were grown, the broths and biomasses isolated and extracted as described Materials and Methods. Each extract was tested in the *C. albicans* protoplast and whole-cell assays as described in the Methods. Each assay was scored as follows: N= no effect; RCS= reduced colony size; RCN= reduced colony number; WALL= individual protoplasts present but unable to regenerate a cell wall and grow; or KILL= no protoplasts or colonies present.

of broths from 8(C)X, 430 PER and 197 PER were active against *A. fumigatus*, *C. glabrata* and wild-type *C. albicans*.

In an effort to ascribe a mechanism of action to the antifungal compounds in each extract, we tested broth extracts from each of the eleven active microorganisms for inhibition of chitin synthase, (1,3)β-glucan synthase and ketol-isomerase activities (each activity is essential for fungal cell-wall formation) as described in the Materials and Methods. These results are summarized in Table 3. None of the extracts inhibited chitin synthase activity (results not shown). The extract from 430 PER inhibited (1,3)β-glucan synthase activity and the extracts from broths of 8(C)X, 248, 237, and 88X specifically inhibited ketol-isomerase activity. The extracts from broths of 236, 252(A), and 197 PER inhibited both (1,3)β-glucan synthase and ketol-isomerase activities. The remaining three extracts did not inhibit (1,3)β-glucan synthase or ketol-isomerase activities (not shown) and their mode of action is (are) unknown at this time.

Based on the observation that the extract from organism 8(C)X had broad antifungal activity against *C. albicans*, polyene-resistant *C. albicans*, *C. glabrata*, and *A. fumigatus*, we decided to identify the active component(s) present in this extract. Previously, we had determined that organism 8(C)X grew between pH 4 and pH 9, and had a T_{max} of ~50°C (unpublished results). Importantly, the 16s rDNA nucleotide sequence from organism 8(C)X was similar but not identical to *Pseudomonas veronii* (not shown), indicating that organism 8(C)X is a novel species of *Pseudomonas*. Details of the species identification of this and other antifungal producing microorganisms will be presented elsewhere (SELITRENNIKOFF, *et al.*, in preparation).

Second, we pooled the broths from 80 500-ml

Table 2. Effect of ethyl acetate broth extracts on the growth of *C. albicans*, *C. glabrata* and *A. fumigatus**.

Microorganism	*C. albicans*	*C. glabrata*	*A. fumigatus*
253	KILL	N	N
8 (C) X	KILL	KILL	KILL
24 (B) F	KILL	N	N
236	KILL	N	N
237	KILL	N	N
244	KILL	RCN	N
248	KILL	N	N
252 (A)	KILL	N	N
430 PER	KILL	KILL	KILL
197 PER	KILL	KILL	KILL
88 X	RCN	N	N
Zalerion arboricola	KILL	KILL	KILL
Emericella rugulosa	KILL	KILL	KILL
Streptomyces nodosus†	KILL	KILL	KILL
Aspergillus nidulans†	KILL	KILL	N
Actinomadura hibisca	N	KILL	N

* The indicated organisms were grown, the broths extracted with ethyl acetate and the extracts tested for inhibition of growth of the above fungi as described in Materials and Methods. The effects of the extracts were evaluated as follows: N= no effect; RCS= reduced colony size; RCN= reduced colony number; or KILL= no visible growth.

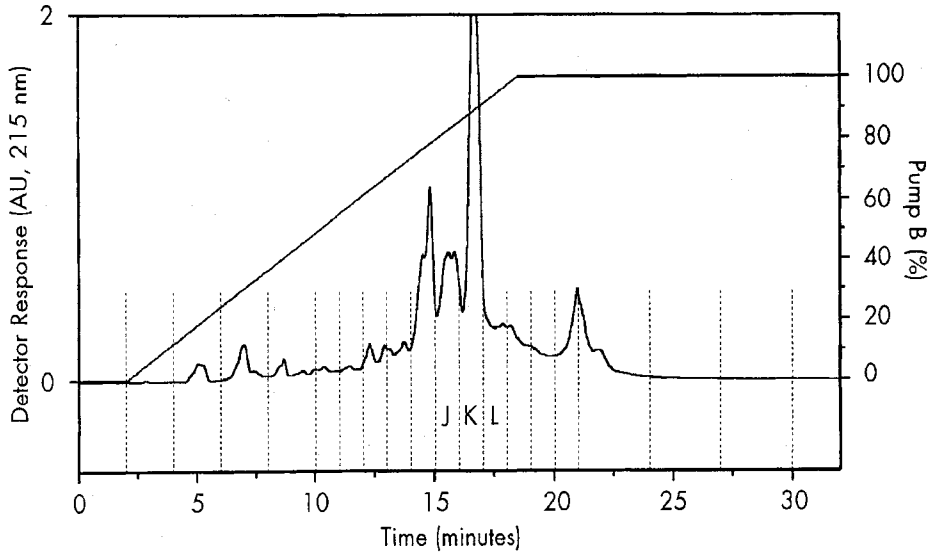
†Results are for the ethanolic extracts of the biomass.

Table 3. The effect of broth extracts on (1,3)β-glucan synthase and ketol-isomerase activities*.

Microorganism	(1-3)β-glucan synthase activity IC_{50} (mg/mL)	Ketol-isomerase activity IC_{50} (μg/mL)
430 PER	1.3	N
8 (C) X	N	40
248	N	155
237	N	75
88 X	N	160
197 PER	2.1	130
252 (A)	1.5	100
236	2.6	110

* The indicated extremophiles were grown, the biomasses and broths separated and extracted as described in Materials and Methods. Each broth extract was tested for inhibition of (1,3)β-glucan synthase and ketol-isomerase activities as described. N= no effect.

Fig. 2. Fractionation of *Pseudomonas akbaalia* extract.



The fermentation broths of 80 500-ml cultures of *P. akbaalia* were grown, harvested and extracted with ethyl acetate as described in the Materials and Methods. The extract was separated by gradient elution (gradient shown in right-hand Y-axis) and 18 fractions (each shown by dashed vertical lines) collected using the columns and conditions described in the Methods. The left-hand Y axis is the absorbance at 215 nm.

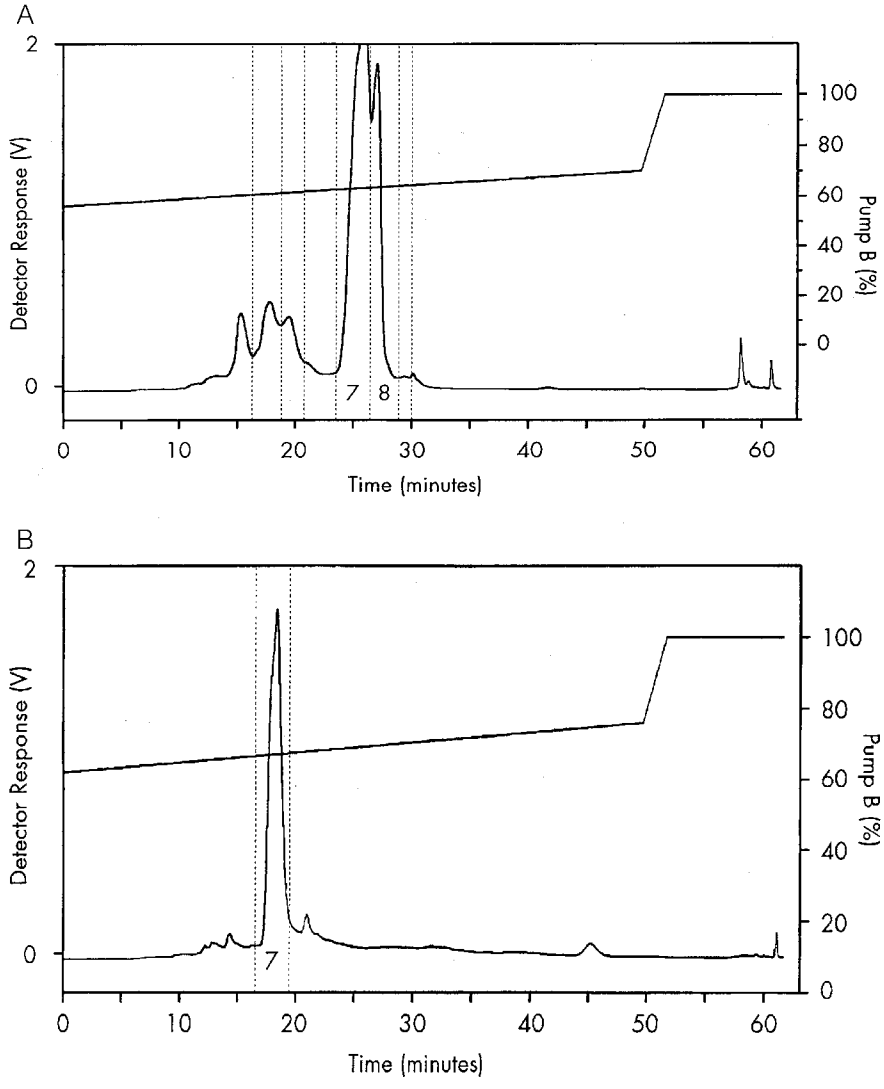
minifermentors, extracted the ~40 liters with ethyl acetate and separated the resulting ethyl acetate extract by HPLC as described in Materials and Methods. The elution results are shown in Figure 2. Each fraction of Figure 2 was assayed for antifungal activity using the *C. albicans* whole-cell assay described in the Methods. Fractions K and L were the only fractions with antifungal activity (results not shown). Fractions K and L were further separated as described in the Methods and these results are shown in Figure 3A for Fraction K and Figure 3B for Fraction L. Each fraction was tested for antifungal activity using the *C. albicans* whole cell assay and fractions K7, K8, and L7 were the only fractions with activity (not shown). Each of these fractions (*i.e.*, K7, K8 and L7) was subjected to MS and NMR analysis as described in the Methods in order to determine the structure of the active compound. In each case, the MS and NMR patterns were consistent to that of pyochelin and pyochelin derivatives (not shown). The structure of pyochelin is shown in Figure 4. To confirm that the antifungal activity was indeed due to pyochelin, we obtained *bona fide* pyochelin from Dr. C. COX (University of Iowa) and determined that pyochelin had antifungal activity against *C. albicans* (SCHIMOLER, unpublished results).

Discussion

To our knowledge, we have discovered for the first time, antifungal activity from extracts of fermentation broths of extremophilic and thermophilic bacteria. From screening 217 organisms, we have identified 11 organisms that produced antifungal compounds. Of these, three organisms (430 PER, 197 PER and 8(C)X) were fungicidal against *C. albicans*, *C. glabrata* and *A. fumigatus*. None of the eleven organisms was active against chitin synthase activity, one organism (430 PER) extract inhibited (1,3)β-glucan synthase activity and four organism extracts inhibited ketol-isomerase activity. The remaining 6 organism extracts inhibited either both (1,3)β-glucan synthase and ketol-isomerase or neither enzyme-their mode(s) of action remain undetermined. The minimum inhibitory concentration values of 430 PER crude extracts against *C. albicans* and *A. fumigatus* ($<14\,\mu$g/ml and $31\,\mu$g/ml, respectively [not shown]) clearly demonstrates the production of highly potent fungicidal compounds.

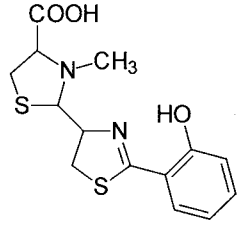
We chose to examine one organism in detail and determined that organism 8(C)X is a novel species of *Pseudomonas* (based on 16S rDNA sequence comparisons). We have named this species *Pseudomonas akbaalia*.

Fig. 3. Subfractionation of active fractions.



Fractions K and L of Figure 2 were subfractionated by HPLC as described in the Methods. A: Fraction K; B: Fraction L. Axes are as in Figure 2.

Fig. 4. Structure of pyochelin.



Importantly, we have identified an active component of the extract of the fermentation broth of *Pseudomonas akbaalia* as pyochelin, an iron-binding compound. Authentic pyochelin was also antifungal, confirming that pyochelin is in part responsible for the antifungal activity observed in the extract of the fermentation broth. Whether other active compounds are also present is not known at this time.

Taken together, our results indicate that at least 11 microorganisms warrant further investigation in order to identify the biologically active compounds present in the extracts. Importantly, the work presented in this manuscript points to extremophiles as an unexplored source of novel

antifungal compounds. We are currently, under a Phase II SBIR project, screening an additional 400 organisms and preliminary results show that we have identified ~36 organisms with activity against *C. albicans* and *A. fumigatus* (WILSON *et al.*, in preparation). These extracts are currently being fractionated to isolate and identify the active compound(s). These results will be the subject of future manuscripts elucidating the biological activities and structures of the active compounds.

Acknowledgments

This work was supported by a Phase I SBIR award to C. P. SELITRENNIKOFF of MycoLogics, Inc., (NIAID AI 40813). We would like to thank Ms. R. SCHIMOLER for performing the antifungal assays and to Dr. C. COX (University of Iowa, Iowa City) for providing us with a sample of authentic pyochelin.

References

1) HERBRECHT, R.: The changing epidemology of fungal infections: Are the lipid-based forms of amphotericin B an advance? European Journal of Haematology 56: 12~17, 1996
2) COX, G. & J. PERFECT: Fungal infections. Current Opinion in Infectious Diseases 6: 422~426, 1993
3) FOX, J. L.: Fungal infection rates are increasing. ASM News 59: 515~518, 1993
4) ALEXANDER, B. D. & J. R. PERFECT: Antifungal resistance trends towards the year 2000-Implications for therapy and new approaches. Drugs 54: 657~678, 1997
5) BOW, E. J.: Invasive fungal infections in patients receiving intensive cytotoxic therapy for cancer. British Journal of Haematology 101: 1~4, 1998
6) WARNOCK, D. W.: Fungal infections in neutropenia: current problems and chemotherapeutic control. Journal of Antimicrobial Chemotherapy 41: 95~105, 1998
7) HOOD, S. & D. W. DENNING: Treatment of fungal infection in AIDS. Journal of Antimicrobial Chemotherapy 37: 71~85, 1996
8) POLIS, M. A. & J. A. KOVACS: Fungal infections in patients with the acquired immunodeficiency syndrome. In AIDS: Biology, Diagnosis, Treatment and Prevention, fourth edition. *Eds.*, DEVITA, Jr., V. T., HELLMAN, S. and ROSENBERG, S. A., pp. 231~244, Philadelphia: Lippincott-Raven, 1997
9) CLARK, A. M.: The need for new antifungal drugs. In Approaches for Antifungal Drugs. *Ed.*, FERNANDES, P. B. pp. 1~19, Boston, New Birkhauser, 1992
10) STEVENS, D. A. & K. HOLMBERG: Resistance to antifungal drugs: Current status and clinical implications. Curr. Opin. Anti-infective Invest. Drugs 1: 306~317, 1999
11) GRAYBILL, J. R.: The future of antifungal therapy. Clinical Infectious Diseases 22 (Suppl. 2): S166~S178, 1996
12) DENNING, D. W.: Echinocandins and Pneumocandins–a new antifungal class with a novel mode of action. J. Antimicrob. Chemother. 40: 611~614, 1997
13) ANON.: GM-237354. Drugs Future 22: 1221~1225, 1997
14) GULLO, V. P.: The discovery of natural products with therapeutic potential. Boston, Butterworth-Heinemann, 1994
15) SHU, Y.-Z.: Recent Natural Products Based Drug Development: A Pharmaceutical Industry Prospective. 61: 1053~1071, 1998
16) CRAGG, G. M.; D. J. NEWMAN & K. M. SNADER: Natural products in drug discovery and development. J. Nat. Prod. 60: 52~60, 1997
17) HORIKOSHI, K. & W. GRANT: Superbugs p. 4, Japan Scientific Societies Press, Tokyo, 1991
18) STETTER, K: Hyperthermophiles: Isolation, Classification and Properties. Extremophiles: Microbiol Life in Extreme Environments. pp. 1~24, Wiley-Liss, Inc, 1998.
19) BROCK, T. D.: Early Days in Yellowstone Microbiology. ASM News 64: 137~140, 1998
20) ADAMS, M. W. W. & R. M. KELLY: Enzymes from microorganisms in extreme environments. Chem. Engineer. News 73: 32~42, 1995
21) PERSIDIS, A: Extremophiles. Nature Biotechnol. 16: 593~594, 1998
22) ANKENBAUER, R. G.; T. TOYOKUNI, A. STALEY, K. RINEHART & C. COX: Synthesis and biological activity of pyochelin, a siderophore of *Pseudomonas aeruginosa*. J. Bacteriol. 170(11): 5344~5351, 1988
23) COMBIE, J.; K. RUNNION & M. WILLIAMSON: Acquisition of heat stable enzymes from thermophilic microorganisms: peroxidases, ureases and glucose oxidase. CRDEC-CR-152 , 1992
24) RUNNION, K. & J. COMBIE: Organic sulfur removal from coal by microorganisms from extreme environments. FEMS Microbial Review 11: 139~144, 1993
25) RUNNION, K. & J. COMBIE: Microorganisms from extreme environments as source of thermally stable enzymes for removal of polyurethane aircraft coatings (Phase II), report prepared for Naval Surface Warfare Center, Silver Spring, MD, NSWCCD/TR-95/229, 1996
26) COMBIE, J. & K. RUNNION: Looking for diversity of Yellowstone extremophiles. J. Indust. Microbiol. 17: 214~218, 1996
27) WOOD, R. L.; A. WRIGHT, P. MCCARTHY, T. MILLER, C. TAFT, S. POMPONI & C. P. SELITRENNIKOFF: Characterization and optimization of *in vitro* assay conditions for (1,3)β-glucan synthase activity from *Aspergillus fumigatus* and *Candida albicans* for enzyme inhibition screening. J. Antibiotics 51: 665~675, 1998
28) STACKEBRANDT, E. & B. GOEBEL: Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846~849, 1994
29) YARDEN, O. & C. YANOFSKY: Chitin synthase I plays a major role in cell wall biogenesis in *Neurospora crassa*. Genes Development 5: 2420~2430, 1991
30) SELITRENNIKOFF, C. P. & G. OSTROFF: Emerging therapeutic cell-wall targets in fungal infections. Emerging Therapeutic Targets 3: 53~72, 1999