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COMPARATIVE STUDY OF POTENTIAL VIRULENCE FACTORS IN HUMAN PATHOGENIC AND SAPROPHYTIC *TRICHODERMA LONGIBRACHIATUM* STRAINS*

ZSUZSANNA ANTAL[1], L. KREDICS[1], J. PAKARINEN[2], ILONA DÓCZI[3], MARIA ANDERSSON[2], MIRJA SALKINOJA-SALONEN[2], L. MANCZINGER[4], A. SZEKERES[4], L. HATVANI[4], CS. VÁGVÖLGYI[4] AND ELISABETH NAGY[1,3]

[1]Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701, Szeged, Hungary; [2]Department of Applied Chemistry and Microbiology, University of Helsinki, P.O. Box 56, FIN-00014 Helsinki, Finland; [3]Department of Clinical Microbiology, University of Szeged, Somogyi Béla tér 1, H-6725, Szeged, Hungary; [4]Department of Microbiology, University of Szeged, P.O. Box 533, H-6701, Szeged, Hungary

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* Dedicated to the 80th anniversary of the birth of Professor Ilona Béládi.

Abstract

Potential virulence factors of 9 saprophytic and 12 clinical *Trichoderma longibrachiatum* strains were examined in the present study to compare their capacity to cause human infection. All of the strains were able to grow at temperatures up to 40 °C and at pH values ranging from 2.0 to 9.0. In the carbon and nitrogen source utilization experiments it was revealed that all of the strains were able to utilize a series of basic amino acids both as sole carbon and nitrogen sources. The MIC value of the antifungal drugs were found to be between 0.016 and 8 μg/ml for amphotericin B, 64 and 256 μg/ml for fluconazole, 0.5 and 32 μg/ml for itraconazole, 0.008 and 1 μg/ml for ketoconazole in the case of *Trichoderma* isolates. Metabolites of the strains inhibited the growth of different bacteria, furthermore, in the case of three clinical *Trichoderma* strains they reduced motility of boar spermatozoa, indicating their toxicity to mammalian cells as well. On the whole, there were no significant differences in the examined features between strains derived from clinical or soil samples. The question, however, whether all environmental *Trichoderma longibrachiatum* strains have the capacity to cause infections or not, remains unanswered.

Introduction

More and more common air-borne and soil-borne organisms are being added to the list of emerging fungal pathogens of humans. Infection may occur when normal barriers are broken (traumatic inoculation, surgery), host defenses are weakened by medical conditions or treatments, or in patients with chronic sinusitis. Emerging of the incidence as well as the seriousness of the outcome is primarily the result of the increasing number of immunocompromised patiens.

Strains of the *Trichoderma* genus are widespread in the environment, mainly as soil habitant filamentous fungi. Several isolates have outstanding abilities to produce extracellular degrading enzymes. This property is related to their ecological role as decomposers of plant litter, and makes them economically important as producers of industrial enzymes [1, 2]. Many *Trichoderma* strains are potential biocontrol agents of plant pathogens which is based on their antagonistic abilities including the mechanisms of stimulation plant defense responses, competition for space and nutritive elements, antibiosis by the production antifungal metabolites and mycoparasitism [3-7]. Recently, as emerging fungal pathogens, *Trichoderma* strains were detected in humans on skin and lung infections or as the causative agents of peritonitis in peritoneal dialysis patients, and of infections disseminated in the liver, brain, heart or stomach of immunocompromised patients [8-16].

To get more information about this uncommon opportunistic pathogen, this study was designed to examine *Trichoderma longibrachiatum* strains as emerging human pathogens, and to investigate and compare potential virulence factors in strains derived from clinical and soil samples.

Materials and methods

Isolates

Trichoderma longibrachiatum strains isolated from soil or clinical samples and identified on the basis of ITS sequences were used in the experiments (Table I). Bacterial strains of *Bacillus subtilis, Escherichia coli, Micrococcus luteus, Serratia marcescens* and *Staphylococcus aureus* derived from the SZMC (Szeged Microbiological Collection).

Effect of pH and temperature on the colony growth rate

To study mycelial growth at different temperatures, the examined strains were inoculated onto Petri plates containing minimal (0.5 % glucose, 0.1 % KH_2PO_4, 0.1 % $(NH_4)_2SO_4$, 0.1 % $MgSO_4$ x $7H_2O$, 2 % agar in tap water) or yeast extract (0.5 % glucose, 0.2 % yeast extract, 1 % KH_2PO_4, 2 % agar in distilled water) agar medium. The influence of pH on mycelial growth was studied on both minimal and yeast extract agar medium adjusted with McIlvain buffer to pH values from 2 to 9. Mycelial discs (3 mm diameter) cut from the growing front of young colonies, were used for inoculations. After incubation at 25 or 37 °C, mycelial growth was determined by colony diameter measurements.

Utilization of carbon and nitrogen sources

Amino acids, each as sole carbon source, were tested at concentrations of 0.2 %, dissolved in liquid minimal medium (1 % KH_2PO_4, 0.5 % $(NH_4)_2SO_4$, 0.1 % $MgSO_4$ x $7H_2O$, in tap water). Two hundred µl volumes from each liquid medium were added into the wells of a microtiter plate, and inoculation was carried out by adding 20 µl from the conidial suspensions of the strains. The mycelial growth was determined after incubation for 5 days at 30 °C.

The abilities of the strains to utilize amino acids as sole nitrogen source were tested by inoculating small drops (10 µl) of conidial suspensions onto the surface of minimal media (0.2 % glucose, 1 % KH_2PO_4, 0.1 % $MgSO_4$ x $7H_2O$, 1.5 % agar in distilled water) containing one of the nitrogen sources at a concentration of 0.1 %. Incubation was performed at 30 °C and

growth characteristics were checked 4 and 10 days after inoculation, comparing with a control (minimal medium without a nitrogen source).

Etest for antifungal susceptibility testing

The *Trichoderma* isolates were tested to determine their susceptibilities to amphotericin B, fluconazole, itraconazole and ketoconazole by the Etest method, according to the guidelines of the manufacturer (AB Biodisk; Solna, Sweden) using either Casitonine medium or RPMI 1640 medium supplemented with 0.03 % L-glutamine, and buffered to pH 6.5 with 0.2 M phosphate buffer. The plates were inoculated with $5x10^4$ CFU/plate and dried at room temperature before the application of the Etest stripes. The MIC values were read after incubation at 30 °C for 24 or 48 hours depending on the growth conditions of the isolates.

Toxicity studies on bacterial stains

To investigate the toxicity of the *T. longibrachiatum* isolates, the strains were inoculated into 20 ml liquid medium containing 5 ml human plasma as inducer and 0.2 % glucose, buffered to pH 7 by Sorensen phosphate buffer. After incubation by agitation at 30 °C for 6 days, the ferment broths were collected, filtrated on cotton bed and lyophilised. The precipitates were dissolved in 1 ml methanol and the extracts used in the toxicity assay. Petri plates containing yeast extract agar medium (0.2 % glucose, 0.2 % yeast extract, 2 % agar in distilled water) were massive inoculated by bacterial strains (*Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Serratia marcescens*) and dried on air. Paper discs (5 mm in diameter) were placed on the surface of the medium, and 10 µl methanol

extract from the culture filtrate of each strain was dropped onto them. The inhibitions were read after incubation at 30 °C for 24 or 48 hours depending on the growth conditions of the bacteria.

Toxicity studies on mammalian cells

The soil derived CECT 2412, CECT 2606, CECT 2937, CECT 20105, and the clinical *Trichoderma* strains (except CM-1798, CM-2171 and CM-2277) were cultivated on YEGK media at room temperature for 4 days, approximately 20 mg of biomass of each strain was harvested, and methanol extracts were prepared by suspending the harvested biomass in 400 µl of 100 % methanol in a 4 ml glass bottle. The suspensions were incubated at 100 °C for 15 minutes.

A semiquantitative sperm toxicity bioassay was performed as described by Andersson *et al*. [17] by exposing 200 ml of boar sperm suspension to 1 µl, 2 µl and 5 µl of *Trichoderma*-methanol extracts. The sperm suspensions were incubated for 15 min at room temperature and 5 min at 37 °C, than the motility of the spermatozoa was estimated by phase-contrast microscopy**.** More than 90% of the spermatozoa were motile in a non-exposed control sample and in a sample exposed to 5 µl of methanol. EC_{50} values were determined by the treatment of boar sperm suspension with different concentrations (25, 50 and 100 µg biomass per ml) of methanol soluble substances.

Mitochondrial membrane damage in spermatozoa was studied by epiflurescence microscopy using the JC-1/propidium iodide staining [18, 19].

Results and discussion

Effect of temperature and pH on the linear mycelial growth rate

Linear growth rates of the *Trichoderma* strains indicated that all investigated strains had optimum growth at 30 °C on both minimal and yeast extract agar media, but all of the strains were able to grow at 40 °C as well. As growth at elevated temperatures is one of the virulence factors of fungi, and the ability to grow at higher temperatures is caracteristic to *T. longibrachiatum* species it is not surprising that most of the strains in case reports about *Trichoderma* infections are belonging to this species.

The pH ranging from 2 to 9 supported the growth of most of the examined strains at 25 °C, with an optimum at pH 4. At elevated temperature (37 °C) the range was narrower, but all of the strains were able to grow at pH 7, which is a prerequisit of growth within the human host.

Carbon and nitrogen source utilization

To investigate the utilization ability of the strains, amino acids were tested as sole carbon, and as sole nitrogen sources. L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid and L-ornithine were utilized by all of the strains as both carbon and nitrogen source. The parallel utilization of these amino acids, which are basic components of the human body seems to be frequent among *Trichoderma* strains, and this ability can promote their growth as facultative human pathogens.

Antifungal susceptibilities

The *in vitro* antifungal susceptibilities of the isolates were tested to four antifungal drugs by the Etest method (Table II). All the strains were resistant for fluconazole (MIC value ≥ 64 μg/ml) irrespectively of their origin. In the case of itraconazole, most of the clinical isolates proved to be resistant (MIC value ≥ 1 μg/ml) and just one of them was susceptible-dose-dependent (MIC value between 0.25 and 0.5 μg/ml). The soil isolates were all resistant for itraconazole. The amphotericin B MIC values of the strains were lower and variable, as four of the soil isolates were susceptible (MIC value < 1 μg/ml) and five of them were resistant (MIC value > 2 μg/ml), while among clinical isolates was one susceptible and 11 resistant strains. In the case of ketoconazole, both the soil derived and the clinical strains were susceptible (MIC value < 8 μg/ml). In conclusion, although some variability was detected in the case of susceptibility for amphotericin B, there were no significant differences between strains derived from clinical and soil samples, and all the investigated *Trichoderma longibrachiatum* strains proved to be resistant for fluconazole and with one exception for itraconazole, and all the isolates were susceptible for ketoconazole.

Investigation of toxicity to bacteria and mammalian cells

The methanol extracts of all strains inhibited the growth of *S. marcescens* at a low level. Three soil-derived strains (CECT 2412, CECT 2606 and TB 19) and two clinical isolates (CM-2277 and UAMH 7955) were the most effective, while in the case of *E. coli,* three soil isolates (TB 12, TB 19 and TB 33) caused the highest inhibition and only UAMH 7955 had no effect on this bacterium at all. One soil (TB 33) and six clinical (ATCC 208859, CBS 446.95, CM-382, CM-2277, IP 2110.92 and UAMH 9515) strains caused reduced growth of *S. aureus*, while *M. luteus* was inhibited by two soil (TB 12, TB 19) and two

clinical (CM-2277 and UAMH 7955) isolates. The least susceptible to the toxic metabolites produced by the *Trichoderma* strains proved to be *B. subtilis*, as only the saprophytic TB 12 and TB 19, and the clinical CM-2277 had an inhibition effect on it. Altogether, strain CM-2277 had the strongest inhibition effect on all the five tested bacteria, but in the case of the other *T. longibrachiatum* strains there were no significant differences between isolates from soil and clinical samples.

In the case of the mammalian cells, more than 90 % of the spermatozoa were immotile in samples exposed to *T. longibrachiatum* UAMH 9515, ATCC 208859 and CM-382 in a concentration of 1.25 mg biomass ml^{-1} (5 µl of methanol extract), indicating that these strains produce heat-stable substances toxic to sperm cells. The EC_{50} value for ATCC 208859 and CM-382 was between 50 and 25 µg methanol soluble substances per ml, while in the case of UAMH 9515 it proved to be 100 µg. The other studied strains had no toxic effect on spermatozoa at the highest tested concentration, which was 100 µg per ml. Exposure to the fungal extracts of strains ATCC 208859, CM-382 and UAMH 9515 resulted in quenching of the yellow fluorescence of the midpiece of boar spermatozoa stained with JC-1, indicating that the dissipation of the mitochondrial membrane potential may be involved in the toxic effect.

Growth at elevated temperatures and tolerance of neutral pH are prerequisites of an infection in the case of *T. longibrachiatum*. The ability of the examined strains to utilize amino acids both as sole carbon and nitrogen source may also represent potential virulence factors, moreover, the low susceptibility levels to antifungal drugs are important features which may cause difficulties in the medical treatment of infected patients. On the basis of the collected data, most of the examined *T. longibrachiatum* strains have these properties. Although just three of the investigated clinical strains proved to produce substances toxic to

mammalian cells, the relevance of this result have to be analysed. As there were no other significant differences between strains derived from clinical and soil samples the question, whether all environmental *Trichoderma longibrachiatum* strain have the capacity to cause infections, remains unanswered. Further studies including toxicological, enzymological and molecular investigations are being carried out in order to determine, whether there are any special properties that make certain strains capable of causing opportunistic infections in humans.

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Addresses of the authors:

Corresponding author: Dr. Zsuzsanna Antal, Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701 Szeged, Hungary, Fax: 06 62 544 823, E-mail: zs.antal@bio.u-szeged.hu

Dr. László Kredics: same address as above, kredics@sol.cc.u-szeged.hu

Dr. J. Pakarinen, prof. Maria Andersson, prof. Mirja Salkinoja-Salonen: Department of Applied Chemistry and Microbiology, University of Helsinki, P.O. Box 56, FIN-00014 Helsinki, Finland; maria.a.andersson@helsinki.fi, salkinoj@millipede.helsinki.fi

Dr. László Manczinger, András Szekeres, Lóránt Hatvani, Dr. Csaba Vágvölgyi: Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary, Fax: 06 62 544 823, manczing@bio.u-szeged.hu, szandras@bio.u-szeged.hu, lori@szegedkendo.hu, csaba@bio.u-szeged.hu

Prof. Dr. Elisabeth Nagy, Ilona Dóczi: Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, Somogyi Béla tér 1, H-6725 Szeged, Hungary, nagye@mlab.szote.u-szeged.hu

Table I

Origin of *T. longibrachiatum* isolates

Isolate	**Origin**
CECT 20105	Soil; Egypt
CECT 2412*	Mushroom compost; UK
CECT 2606	Soil; Sierra Leone
CECT 2937*	Soil; Antarctica
TB 12 (SZMC)	Soil; Hungary
TB 19 (SZMC)	Soil; Hungary
TB 21 (SZMC)	Soil; Hungary
TB 33 (SZMC)	Soil; Hungary
TB 35 (SZMC)	Soil; Hungary
ATCC 208859	AIDS patient; USA
ATCC 201044	Skin biopsy; USA
CBS 446.95	Lung; Austria
CM-382*	Peritoneal fluid; Spain
CM-2277*	Sputum; Spain
CM-2171	Cutaneous lesion; Spain
CM-1798*	Blood; Spain
IP 2110.92*	Lung, brain, heart, stomach; France
UAMH 7955	Sinus of a transplanted patient; USA
UAMH 7956	Feaces, liver, lung; USA
UAMH 9515	Peritoneal fluid; Canada
UAMH 9573*	Peritoneal catheter; Canada

Collections: ATCC (American Type Culture Collection), CBS (Centraalbureau voor Schimmelcultures), CECT (Spanish Type Culture Collection), CM (Mycelial Collection of the Spanish National Centre for Microbiology), IP (Institut Pasteur), SZMC (Szeged Microbiological Collection), UAMH (University of Alberta Microfungus Collection & Herbarium). *: reidentified on the basis of the ITS sequences.

Table II

MIC-values (μg/ml) of the antifungal drugs in the case of *Trichoderma* species, obtained by the Etest method

Isolate	Fluconazole	Itraconazole	Ketoconazole	Amphotericin B
CECT 20105	≥256	4	0.5	2
CECT 2412	≥256	>32	0.064	1
CECT 2606	≥256	2	0.25	2
CECT 2937	≥256	2	0.25	0.064

TB 12 (SZMC)	≥256	32	1	4
TB 19 (SZMC)	≥256	16	1	1
TB 21 (SZMC)	≥256	16	0.25	0.032
TB 33 (SZMC)	≥256	>32	1	0.125
TB 35 (SZMC)	≥256	8	1	0.25
ATCC 208859	≥256	8	0.25	2
ATCC 201044	64	0.5	0.008	2
CBS 446.95	≥256	2	0.5	2
CM-382	≥256	2	0.25	8
CM-1798	≥256	1	0.125	0.016
CM-2171	≥256	16	0.5	2
CM-2277	≥256	32	0.25	2
IP 2110.92	≥256	16	1	2
UAMH 7955	≥256	32	0.125	1
UAMH 7956	64	16	0.25	2
UAMH 9515	≥256	16	0.5	2
UAMH 9573	64	32	0.25	2