Testing the nematophagous biological control strain
*Paecilomyces lilacinus* 251 for paecilotoxin production

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Abstract

*Paecilomyces lilacinus* is a nematophagous fungus currently developed as a biological control agent. In order to evaluate potential toxin production, culture extract and concentrated culture supernatant of *P. lilacinus* strain 251 were tested against Gram-negative and Gram-positive bacteria. High-performance liquid chromatography analysis was carried out to compare the chromatograms of *P. lilacinus* strain 251 with the chromatogram of known paecilotoxin. It was found that the 251 strain of *P. lilacinus* did not produce detectable levels of paecilotoxin or other toxins with antimicrobial activity.

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1. Introduction

The use of fungi in biological control of pests has been adopted recently in agriculture. *Paecilomyces lilacinus*, a common soil hyphomycete is well known as an egg parasite of plant parasitic nematodes [1–3] and is currently developed as biocontrol agent [4]. In addition to infecting plant parasitic nematodes, *P. lilacinus* has been reported to infect humans [5,6] and animals [7]. Therefore, potential production of toxins such as paecilotoxin by isolates applied for biological control presents a safety risk and should be assessed carefully. Even though not compulsory for product registration, testing for toxin production is recommended by regulatory authorities in some countries such as Australia.

Toxins produced by microorganisms are typically secondary metabolites featuring peptides, polypeptides and non-peptide antibiotics [8–10]. Mycotoxin production and toxic effects vary according to the fungal strain, culture medium and target organism. For example, culture filtrates of *P. lilacinus* grown on a medium containing malt, tested against 17 species of nematodes, were shown to be toxic only against *Meloidogyne* and *Heterodera* spp. The toxic effect of the unknown toxic metabolite in nematodes was neurotropic [11]; however, it is not known whether the culture filtrates had any antibacterial activity.

Several *P. lilacinus* isolates from Japan, some of which are of clinical origin, have been shown to produce paecilotoxins, known as leucinostatins [12]. These paecilotoxins are neutral straight peptides that contain an unsaturated fatty acid and an amine residue in their N-terminus and C-terminus, respectively. They exhibited uncoupling activity against rat liver mitochondria [13] and antimicrobial activity against Gram-positive bacteria [12]. It was also demonstrated that the paecilotoxins caused oral toxicity and formation of mycoses in humans [13] and in the infection of insects and nematodes [12]. Since *P. lilacinus* kills nematodes by invading nematode eggs, paecilotoxins may not be involved in the infection process and therefore their absence is not likely to affect the overall ability of the fungus to control nematodes.

*Paecilomyces marquandii* (Massee) has been reported to produce different forms of paecilotoxins [14], which have been found to be identical with paecilotoxins produced by...
2. Materials and methods

2.1. Fungal strain and cultivation conditions

Two liquid culture media were used to induce toxin production by \textit{P. lilacinus} strain 251 (deposited at Australian Government Analytical Laboratory, accession number 89/030550). The first medium (CM1) contained sucrose 5\% (w/v), bactopeptone (Oxoid) 0.5\% (w/v), yeast extract (Difco) 0.5\% (w/v), \(\text{Na}_2\text{CO}_3\) 1\% (w/v), \(\text{K}_2\text{HPO}_4\) 0.1\% (w/v), and \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) 0.02\% (w/v) at pH 8.5 [12]. The second medium (CM2) was a standard potato dextrose broth (Difco). Spores of \textit{P. lilacinus} were harvested in sterile water (about 10 ml per plate) from a potato dextrose agar (PDA) plate grown for 10 days and 150 ml of medium in 500 ml Erlenmeyer flasks was inoculated with the spores at 2.75\(\times\)10\(^6\) ml\(^{-1}\). The flasks were incubated at 27°C for 2 weeks on an orbital shaker at 125 rpm.

2.2. Extraction of secreted metabolites

Potential toxins were extracted using the method described earlier [12] with minor modifications. Culture supernatants (100 ml) were adjusted to pH 3.0 with 1 N HCl and extracted with the same volume of ethyl acetate. The extracts were washed with 5\% (w/v) \(\text{NaHCO}_3\) and vacuum-concentrated. Crude metabolite fraction prepared in this way was dissolved in a small amount (300-400 \mu l) of methanol. Each sample was passed through a 0.2-\mu m pore size teflon filter (Advantec MFS, CA, USA). The resulting extracted potential toxin (EPT) was analyzed by high-performance liquid chromatography (HPLC) and assayed against Gram-positive and -negative bacteria to reveal possible antibacterial activities. Concentrated culture supernatant (CCS) was also tested in the assay.

2.3. Detection of antimicrobial activity against bacteria

Two Gram-positive bacteria, \textit{Bacillus subtilis} (strain CL062) and \textit{Micrococcus luteus} [15], and the Gram-negative \textit{Escherichia coli} (strain JM109) were prepared as follows. Standard medium (SM) plates (1.2\% (w/v) agar, 1\% (w/v) bactopeptone, 1\% (w/v) glucose, 0.1\% (w/v) yeast extract, 0.1\% (w/v) \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\), 0.22\% (w/v) \(\text{K}_2\text{HPO}_4\)) were inoculated with 100 \mu l of a culture previously grown overnight in axenic broth [16] at 37°C.

Antibacterial activity was tested with the following methods. Method 1: a 3-mm hole was cut aseptically in the center of each 24-h-old culture plate of \textit{B. subtilis} and \textit{E. coli} and 10 \mu l of either EPT or CCS was pipetted into each well with four replications. Plates were then incubated at either at 33 or 37°C up to 48 h. Method 2: 7.5\mu l of an overnight culture of \textit{E. coli}, \textit{B. subtilis} or \textit{M. luteus} was spread on SM plates with three replications. The bacteria were allowed to dry on the plate, after which 100 \mu l of either EPT or CCS was added on top and the plates were incubated as above. Antibacterial activity was assessed by counting the number of colony forming units (cfu) and observing the diameter of bacterial colonies by eye. Method 3: 6-h-old liquid cultures of \textit{E. coli}, \textit{B. subtilis} and \textit{M. luteus} were mixed with CCS from fungal cultivation (bacterial culture:CCS ratio was 5:2) for 18 h at 37°C. After incubation, 100 \mu l aliquots of the mixtures were plated on SM plates. Bacterial growth inhibition was assessed by counting the number of cfu and comparing the numbers to those from the treatment without CCS.

2.4. Comparison of antimicrobial activity of EPT with a crystallized paecilotoxin

This method involved a culture of \textit{M. luteus} and paper disks. The crystallized toxin from \textit{P. lilacinus} Odashima strain [12] was kindly donated by Dr. Y. Mikami, Research Center for Pathogenic Fungi and Microbial Toxicoines, Chiba University, Japan. Sterile paper disks (7 mm) were soaked either in EPT (T1) crystallized toxin in methanol (T2), chloramphenicol (5 \mu g ml\(^{-1}\)) in 70\% (v/v) methanol (T3), and methanol only (T4). The disks were then air-dried and placed on a 1-day-old bacterial lawn on SM. The plates containing paper disks with three replications were incubated at 26°C for 7 days. Toxic effect was shown by formation of clearing zones around the disks on the bacterial lawn.

2.5. HPLC analysis

HPLC was employed to compare the chromatograms of EPT from \textit{P. lilacinus} strain 251 and the crystallized paecilotoxin from Odashima strain [12]. All chromatograms were performed using a C8 column (Pharmacia, Sweden). The solvent used was methanol:2-propanol:water:acetonitrile:diethylamine in a ratio 40:30:20:10:0.1. The flow rate was 1 ml min\(^{-1}\) and total running time was 20 min for each run.
3. Results and discussion

3.1. Effects of EPT and CCS on plate assays

The EPT and CCS of \textit{P. lilacinus} 251 applied in a hole of a \textit{B. subtilis} plate (method 1) showed almost no effect on the growth of bacteria (Table 1). A very small effect on the growth was observed by the application of CCS at 33°C, showing a 1-mm clearing zone around the hole. However, the clearing zone disappeared under bacterial growth when the plates were checked 48 h after incubation. There was no effect against \textit{E. coli}.

EPT and CCS applied on the dried \textit{E. coli}, \textit{B. subtilis} and \textit{M. luteus} growth on SM plates (method 2) had little or no effect on the growth of bacteria (data not shown). A minor effect by CCS was observed 24 h after incubation on \textit{B. subtilis} and \textit{M. luteus} but this effect was minimal after 48 h. When CCS of \textit{P. lilacinus} was mixed with 6-h-old liquid cultures of \textit{E. coli}, \textit{B. subtilis} and \textit{M. luteus} and the mixture incubated for 18 h prior to plating on SM plates (method 3), no significant inhibition of bacterial growth was observed based on the cfu numbers (data not shown).

The negative results obtained suggest that \textit{P. lilacinus} strain 251 does not produce detectable levels of antibacterial toxins, paecilotoxin included, or other metabolites that may be toxic to Gram-positive and Gram-negative bacteria. Using a similar test with \textit{B. subtilis}, it was found that 19 of the 20 \textit{P. lilacinus} isolates obtained mainly from humans but also including isolates from nematodes, insects and soil, produced toxins [12]. The toxic effect was observed only in Gram-positive bacteria.

Production of toxins seems to depend on a particular fungal isolate. This has shown to be true with a range of fungal species, for example, \textit{Aspergillus flavus} in relation to production of aflatoxins, cyclopiazonic acid and aflatrem [17]. Also, it has been demonstrated that 28 of 42 isolates of \textit{Fusarium graminearum} produced deoxynivalenol (DON) [18]. DON is one of the most common contaminating mycotoxins in food.

3.2. Production of potential paecilotoxin

In order to verify the production or non-production of paecilotoxin by the strain \textit{P. lilacinus} 251, paper disks soaked with EPT (T1), crystallized paecilotoxin (T2) obtained from Mikami and an antibiotic chloramphenicol were placed on 1-day-old culture plates of \textit{M. luteus}. A clearing zone around the bacterial culture indicating inhibition was observed only when using crystallized paecilotoxin (T2) and chloramphenicol (T3). No inhibition of \textit{M. luteus} was observed by the EPT of \textit{P. lilacinus} strain 251 (T1) or methanol only (T4) after incubation of the plates for 7 days (Fig. 1). In the case of T2 and T3, the clearing zone was significantly visible from the second day of incubation onwards. Inhibition of bacterial growth was not observed either using EPT (T1 in Fig. 1) or CSS of \textit{P. lilacinus} in the same conditions (data not shown). Again, the results indicate that \textit{P. lilacinus} strain 251 does not produce metabolites that are toxic to Gram-positive bacteria. Mikami et al. [12] observed toxic activity against another Gram-positive bacterium, \textit{B. subtilis}, with the same crystallized paecilotoxin used in this experiment.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Extract</th>
<th>24 h after incubation</th>
<th>48 h after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>EPT</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>33</td>
<td>CCS</td>
<td>1 mm clearing around hole</td>
<td>no effect</td>
</tr>
<tr>
<td>37</td>
<td>EPT</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>37</td>
<td>CCS</td>
<td>no effect</td>
<td>no effect</td>
</tr>
</tbody>
</table>

EPT or CCS were applied on a central hole cut in agar.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Effect of EPT of \textit{Paecilomyces lilacinus} 251 (T1), crystallized toxin from Odashima strain by Mikami (T2), chloramphenicol (T3) and methanol (T4) on growth of \textit{M. luteus}. Paper disks containing a particular compound are seen as white in the middle of the photograph. Plates were incubated at 26°C for 7 days. Clearing zone, a dark circle around the paper disk is seen only in T2 and T3. Shadow of the paper disk appears on T1 and T4.}
\end{figure}
3.3. HPLC analysis for evidence of toxin produced by P. lilacinus

HPLC chromatograms of the EPT of P. lilacinus strain 251 were compared with the chromatogram published for the crystallized paecilotoxin from the Odashima strain [12]. The chromatograms presented in Fig. 2B,C were obtained from the EPT of P. lilacinus strain 251 cultured under conditions promoting paecilotoxin synthesis; C: EPT of P. lilacinus strain 251 cultured in PD broth. Paecilotoxin peaks (a, b and c in panel A) are not produced by P. lilacinus strain 251 under the culture conditions applied.

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References


