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Original article

The antimicrobial potential of algicolous marine fungi for counteracting multidrug-resistant bacteria: phylogenetic diversity and chemical profiling

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Abstract

Marine fungi represent an important but still largely unexplored source of novel and potentially bioactive secondary metabolites. The antimicrobial activity of nine sterile mycelia isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea was tested on four antibiotic-resistant bacterial strains using extracellular and intracellular extracts obtained from each fungal strain. The isolated fungi were identified at the molecular level and assigned to one of the Dothideomycetes, Sordariomycetes or Eurotiomycetes classes. Following assessment of inhibition of bacterial growth (IC_{50}), all crude extracts were subjected to preliminary ^{1}H NMR and TLC analysis. According to preliminary pharmacologic and spectroscopic/chromatographic results, extracts of fungal strains MUT 4865, classified as *Beauveria bassiana*, and MUT 4861, classified as Microascacea sp.2, were selected for LC–HRMS analysis. Chemical profiling of antibacterial extracts from MUT 4861 and MUT 4865 by LC HRMS allowed identification of the main components of the crude extracts. Several sphingosine bases were identified, including a compound previously unreported from natural sources, which gave a rationale to the broad spectrum of antibacterial activity exhibited.

Keywords: Antimicrobial compounds; Bioactive fungal compounds; Marine fungi; Marine natural products; Multidrug-resistant bacteria; Sphingosine bases

1. Introduction

The worldwide diffusion of antibiotic-resistant microorganisms requires development of new efficient antimicrobial molecules. For more than half a century, the main strategy for obtaining new antimicrobial agents has consisted of semi-synthetic remodeling of natural products. However, drugs obtained in this way are only temporarily effective against pathogenic microorganisms, which develop antibiotic resistance [1]. The problem regarding microbial resistance to antibiotics may be overcome by the discovery of new natural products which, due to their chemical novelty, could inhibit unknown single or multiple microbial targets.

The search for natural products of pharmaceutical interest in the marine environment has been progressing at an

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unprecedented rate, resulting in the discovery of a number of molecules, many of which have new carbon skeletons and interesting biological activities [2,3].

Among marine microorganisms, fungi play a crucial role, as they are a reservoir of biologically active secondary metabolites [4–6]. Recently, several new metabolites from marine fungi have been reported to display notable antibacterial activities [7–9]. Despite their proven biosynthetic potential, scientific research has not intensively focused on marine fungi for seeking new drugs [10]. However, promising fungi are equipped with gene clusters potentially involved in biosynthesis of secondary metabolites [11]. Therefore, research into the isolation, identification and characterization of new fungal strains capable of producing useful bioactive natural compounds should be carried out.

Hence, the aim of this work was to assess the antibacterial potential of nine sterile mycelia isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea, against some representative multidrug-resistant (MDR) bacteria, relevant in cystic fibrosis and nosocomial infections, and to analyze the chemical profiles of the most active fungal crude extracts.

2. Materials and methods

2.1. Fungal strains

Fungi were isolated and roughly identified from the green alga *F. petiolata* collected in March 2010 near Elba Island in the Mediterranean Sea [12], and are preserved at the *Mycotheca Universitatis Taurinensis* – MUT (DBIOS – University of Turin). All selected fungi were revealed to be sterile mycelia and were identified by molecular analysis (Table 1).

Table 1
MUT code, taxonomic assessment of sterile mycelia isolated from *F. petiolata* and GenBank accession numbers.

MUT code	Fungal taxa	GenBank accession number ITS and LSU
4883	*Biatriospora* sp.	KR014352 KP671728
4865	*Beauveria bassiana* (Bals.-Criv.) Vuill.	KR014380 KP671729
4860	*Massarina* sp.	KR014362 KP671730
4885	Microascacea sp.1	KR014356 KP671717
4861	Microascacea sp.2	KR014360 KP671746
4859	Roussoellacea sp.1	KR014355 KP671716
4886	Roussoellacea sp.2	KR014358 KP671720
4966	Roussoellacea sp.3	KR014366 KP671740
4979	*Knufia petricola* (U. Wollenzien & de Hoog) Gorbushina & Gueidan	KR014376 KP671749

2.2. Molecular, bioinformatics and phylogenetic analyses

Genomic DNA was extracted using cetyl trimethyl ammonium bromide (CTAB, Sigma–Aldrich St. Louis, USA) according to the protocol of Graham et al. [13].

The nrDNA internal transcribed spacer (ITS) and large ribosomal subunit (LSU) partial regions were amplified using universal primers ITS1F/ITS4 (Sigma–Aldrich St. Louis, USA) and LR0R/LR7, as previously described [14].

Amplification products were sequenced at Macrogen Europe (The Netherlands). Sequences were checked and assembled using Sequencher 4.9 software and compared to those available in the GenBank database using the BLASTn option of the BLAST program (www.blast.ncbi.nlm.nih.gov) and CBS Mycobank pairwise sequence alignment (www.mycobank.org). Newly generated sequences were deposited in the GenBank database and were assigned accession numbers reported in Table 1.

Phylogenetic analysis was performed only on LSU sequences, as comparable ITS sequences of fungi studied in this article are rarely found in public databases and/or are poorly informative. LSU sequences were selected for phylogenetic analysis on the basis of BLASTn and CBS results. Two sequence datasets were composed, following reference [14] for Pleosporales and reference [15] for Sordariomycetes.

Alignments were generated using MEGA 5.10 [16] and manually refined. Phylogenetic analyses were performed using both Bayesian inference (BI; MrBayse3.2.2) [17] and maximum likelihood (ML; RAxML v.7.3.2) [18] approaches, as previously described [14]. Bayesian posterior probability (BPP) values over 0.6 (with MLB over 50%) are reported in the resulting trees.

2.3. Fungal growth conditions

Preliminary growth condition tests were performed in order to define the most effective and appropriate medium for inducing production of bioactive secondary metabolites in the selected fungal strains. Each fungal strain was inoculated in duplicate by 10 agar plugs of 5 mm diameter cut from the edge of actively growing culture onto malt extract agar in 150 mL flasks containing 100 mL of three different media: PCB (10 g of crushed potatoes and 10 g of crushed carrots in 1 L of ddH_2O), MeCl (20 g malt extract, 17 g NaCl in 1 L of ddH_2O) and WST30 (10 g glucose monohydrate, 5 g soya peptone, 3 g malt extract, 3 g yeast extract, 30 g NaCl). Flasks were incubated in the dark at 24 °C and rotated at 150 rpm. The broth and mycelium of each strain were collected after 2 and 4 weeks and submitted to an extraction procedure for preliminary biochemical analysis (see below). MeCl medium and 4 week incubation were selected as the best conditions (24 °C in the dark). Hence, each fungus was inoculated (100 agar plugs of 5 mm diameter) in 2 L flasks containing 1.5 L of MeCl, which was incubated in the dark at 24 °C at 180 rpm for 4 weeks.

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