Ecotoxicology and Environmental Safety 159 (2018) 127–135

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Anthracene drives sub-cellular proteome-wide alterations in the degradative system of *Penicillium oxalicum*

R. Lucero Camacho-Morales[a,1], Cristina García-Fontana[a,b], Joaquín Fernández-Irigoyen[c], Enrique Santamaría[c], Jesús González-López[a,b], Maximino Manzanera[a,b], Elisabet Aranda[a,b,*]

[a] Institute of Water Research, University of Granada, Ramón y Cajal, Fray Luís 4, Granada 18071, Spain
[b] Department of Microbiology, Pharmacy Faculty, University of Granada, Spain
[c] Proteored-ISCIII, Proteomics Unit, Navarrabiomed, Complejo Hospitalario de Navarra (CHN), Universidad Pública de Navarra (UPNA), IdiSNA, Irunlarrea 3, 31008 Pamplona, Spain

ARTICLE INFO

Keywords:
Biodegradation
Subproteome
Penicillium oxalicum
Polycyclic aromatic hydrocarbons
2D-gel proteomics
Shotgun proteomics

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in polluted environments and are included in the priority list of toxic compounds. Previous studies have shown that the fungus *Penicillium oxalicum,* isolated from a hydrocarbon-polluted pond, has a great capability to transform different PAHs in short periods under submerged fermentation conditions. Although cytochrome p450s (CYPs) seems to be the main responsible enzyme in this process, changes in proteome profile remains poorly understood. The aim of this work was to characterise molecular disturbances in the cytosolic and microsomal sub-proteomes of *P. oxalicum* by applying two-dimensional (2D) gel electrophoresis and label-free quantitative proteomics during anthracene biodegradation. Our results showed that by using 2D-gels, 10 and 8 differential proteins were over-expressed in the cytosolic and microsomal fractions, respectively. Most of them were related to stress response. Shotgun proteomics allowed the identification of 158 and 174 unique protein species that differentially accumulated during anthracene biotransformation, such as CYPs, epoxide hydrolases and transferases enzymes, belonging to Phase I and Phase II of the metabolism of xenobiotics, contributing to the anthracene biodegradation pathway. These results confirm the biological significance of ascomycetes fungi the rol of CYPs on biodegradation and the need of a deeper knowledge on fungal proteomics for the application of the appropriate microorganisms in biodegradation processes.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of chemical compounds widely distributed in the environment, with teratogenic and carcinogenic effects. Both their chemical structure and their absorption onto particles surfaces makes them persistent to chemical and biological degradation and, therefore, highly resistant under natural conditions (Bamforth and Singleton, 2005; Lebrun et al., 2011; Samanta et al., 2012). Anthracene is a PAH listed as one of the priority compounds by the European Commission and also by the US Environmental Protection Agency (EPA). Like other PAHs, anthracene pollution is still of great concern due to its toxicity, and the relative high concentration in the environment, which can range from 2 ppb to 70 ppb, on unpolluted and aged-PAH polluted soils, respectively, or 100 ppm on coal tar (Wise et al., 1988; Nadal et al., 2004; García-Sánchez et al., 2018). Although anthracene may undergo adsorption, volatilisation, photolysis and chemical degradation in the environment, microbial degradation represents the major degradation process (Haritash and Kaushik, 2009).

Over the last decades, there has been an increasing interest in the capability of fungi to degrade organic pollutants, which has resulted in the characterisation of strategies and biochemical pathways that lead to partial or complete removal of these toxic substances (Dua et al., 2002; Gray et al., 2010; Das and Chandran, 2011). White-rot fungi are considered the most effective PAH degraders (Eibes et al., 2006; Rufyikiri et al., 2004; Harms et al., 2011), and this capability is due mainly to the secretion of extracellular ligninolytic enzymes that can degrade a myriad of substances. Generally, these enzymes oxidize PAHs via a non-specific, radical-based reaction with the formation of the corresponding quinones. However, the intracellular enzymatic systems greatly contribute to these transformations (Bamforth and Singleton, 2005; Chigu et al., 2010). In fact, fungi lacking of ligninolytic activity with

* Correspondence to: Department of Microbiology, Institute of Water Research, University of Granada, Ramón y Cajal, Fray Luís 4, Granada 18071, Spain.
E-mail address: earanda@ugr.es (E. Aranda).
[1] Present address: Instituto de Ciencias Agrícolas, Universidad Autónoma de Baja California, Carretera a Delta s/n, 21705 Ejido Nuevo León, Baja California, Mexico.

https://doi.org/10.1016/j.ecoenv.2018.04.051
Received 28 February 2018; Received in revised form 23 April 2018; Accepted 26 April 2018

capability to degrade recalcitrant compounds, can perform xenobiotic transformations exclusively via intracellular pathways (Cerniglia, 1997; Camacho-Morales et al., 2014; Martins et al., 2015). The participation of cytochrome P450 monooxygenases (CYPs) in the initial oxidation of PAHs is well established (Cerniglia, 1997; Chigu et al., 2010; Ning and Wang, 2012) which are associated to the microsomal fraction. However, the majority of the studies of the involvement of these intracellular enzymes have been formerly based on indirect measurement, using CYPs inhibitors (Mico et al., 1988).

Penicillium oxalicum is a cosmopolitan fungus that has been isolated from soil and leaves as well as fruits of commercial interest. Previous studies have shown the potential of *P. oxalicum* to degrade PAHs, such as anthracene, over periods of four days, in media supplemented with soybean flour. The analyses on intracellular oxygenases have shown the induction of oxygenase activity in the presence of anthracene (Aranda et al., 2017). A better understanding of enzymatic mechanisms is, however, essential for successful pollutant removal. To achieve this, proteomic studies of biodegradation processes, including proteome-based approaches, are required to identify not only single protein markers, but to reveal protein patterns that respond robustly and specifically to particular pollutants (Lebrun et al., 2011; Wei et al., 2017). Due to the potential of *P. oxalicum* to degrade PAHs, the analysis of proteins differentially expressed during the degradation of anthracene could help to identify the process by which this fungus is able to metabolise anthracene as well as to detect molecular disturbances in the sub-proteome. In this study, we carried out a sub-proteomic analysis of *P. oxalicum* using 2-dimensional gel and label-free proteomics. For that, the differential expression from the cytosolic and membrane fractions of this fungus, using protein and peptide fractionation strategies coupled to nano LC-MS/MS, was performed.

2. Material and methods

2.1. Strain and growth conditions

P. oxalicum was isolated from a contaminated hydrocarbon pond, as described previously by Aranda et al. (2017). Inoculum was obtained from five-day-old malt agar plates. The mycelium was homogenised for 30 s with a sterile Ultra-Turrax homogeniser (Janke & Kunkel KG., West Germany) operated at one-third maximum speed in an Erlenmeyer flask containing 80 mL of distilled water. The homogenised mycelium was used for further inoculations. As inocula, 1 mL of the culture homogenate was used for a total volume of 25 mL of water supplemented with 1% soybean flour (SF medium) in 100-mL Erlenmeyer flasks. The flasks were incubated for 48 h on a rotary shaker (150 rpm) at 28 °C in the dark with three independent replicates.

2.2. Anthracene biodegradation experiments

After 48 h, half of the flasks were sterilised and used as heat-inactivated biotic control. After that, anthracene was added to the cultures (0.1 mM final concentrations) from a stock solution diluted in acetonitrile. The flasks were incubated for an additional 48 h. Anthracene depletion was monitored via HPLC, (HP 1050, Agilent, Germany) and a Synergy Fusion RP C18 column (80 Å; 4 µm, 4.6 × 150 mm; Phenomenex®, Madrid, Spain), according to the methodology described by Aranda et al. (2010).

2.3. Protein extraction and purification

The mycelium was separated on Whatman paper, washed with distilled water and transferred to twisted Falcon vials for ultra-congelation with liquid nitrogen. Lytic buffer containing 20 mM Tris HCl pH 7.6, 10 mM NaCl, 1 mM EDTA, 4% SDS, 30% glycerol, 5 mM PMSF, 200 mM DTT and benzonase (250 µL for each 10 g) was added to the sample in 1:2 (w/v). Samples were homogenised using an ultrasonic processor (Q-125 QSonica, USA) with the following program: five times for 10 s with a velocity of 4 m/s and 2 min to brake and cooling while on ice. The homogenate obtained was then centrifuged for 20 min at 4 °C and 20,400*g*. The supernatant was decanted to a new vial and stored as cytosolic fraction at − 70 °C. The precipitated fraction was resuspended in 1:2 Triton X-100 buffer (100 mM Tris-HCl pH 7.4, 0.5% v/v Triton X-100, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA and 5 mM PMSF), incubated on a shaker for 30 min at 4 °C and centrifuged at 125,216*g* for 60 min at 4 °C. The recovered supernatant, labelled as microsomal fraction, was aliquoted and stored at − 70 °C. All reagents used in protein extraction were of high-purity grade. Two controls and two samples were processed: the cytosolic fraction with and without anthracene (Cyt A^+, Cyt A^-, respectively) and the microsomal fraction with and without anthracene (Mic A^+, Mic A^-).

2.4. 2D gel proteomics

Protein precipitation was performed by adding 50% (v/v) ice-cold TCA to obtain a final concentration of 10% (v/v) of TCA in each sample, followed by incubation on ice for 15 min. Subsequently, the samples were centrifuged at 4 °C for 15 min at 20,000*g*. The protein pellets were washed with 1 mL of chilled acetone and centrifuged at 20,000*g*, 4 °C, for 5 min. This washing step was repeated three times to remove traces of TCA. The protein fractions were air-dried until complete removal of the acetone was achieved. The dry samples were then stored at − 20 °C for further use.

2.4.1. Isoelectric focusing (IEF)

The dry protein pellet was dissolved in 300 µL of rehydration buffer (RH) 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5 mM PMSF, 20 mM DTT, 0.5% (v/v) Bio-lyte Ampholyte (BioRad) and trace amounts of bromophenol blue and incubated at 15 °C for 2 h and 200 rpm. The protein samples were then centrifuged at 20,000*g* at 4 °C for 15 min.

Supernatants, containing the protein fraction, were loaded on 17-cm IPG strips (ReadyStrips, BioRad) in a pH range of 3–10 for a wide pH range of protein separation. The IEF was carried out at 20 °C. The strips were first actively rehydrated at 50 V for 14 h and subsequently, IEF was carried out using a Protean i12 IEF System (BioRad) with the following program: an initial step of 100 V for 5 h, followed by four gradient steps of 500 V for 30 min, 500 V for 7 h, 1000 V until 500 V h^{-1} and 8000 V until 13,500 V h^{-1}. Finally, a step of 8000 V was included until 45,000 V h^{-1} were reached. A total of 64,076 V h^{-1} were accumulated.

2.4.2. Two-dimensional electrophoresis

Following IEF, the IPG strips (pH 3–10, GE, Healthcare, Germany) were equilibrated for 15 min in 5 mL of reducing equilibration buffer. This buffer contained 75 mM Tris HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg mL^{-1} of DTT and traces of bromophenol blue. After that, the IPG strips were incubated for 15 min in 5 mL of alkylating equilibration buffer (75 mM Tris HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 25 mg mL^{-1} of iodoacetamide and traces of bromophenol blue). Two-dimension SDS-PAGE was performed on a PROTEAN II xi Basic Electrophoresis System (BioRad). Electrophoresis was performed overnight with 1 mA/gel and 100 V at 15 °C until the bromophenol blue reached the bottom of the gel. The entire electrophoresis unit was covered to protect it from direct light during the run. After electrophoresis, gels were stained for 2 h with fluorescent Oriole Solution (BioRad), protected from direct light and continuously shaken.

2.4.3. Image acquisition and image analysis

The stained gels were immediately observed at excitation/emission wavelengths of 270/604 nm, using the ChemiDoc MP Imaging System (BioRad). The gel images were captured using the ImageLab software and the following protocol: 24.5 cm width, automatic exposure. Images were analysed with PDQuest Basic Software (BioRad). Spots