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The old 3-oxoadipate pathway revisited: New insights in the catabolism of aromatics in the saprophytic fungus *Aspergillus nidulans*

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ABSTRACT

Aspergilli play major roles in the natural turnover of elements, especially through the decomposition of plant litter, but the end catabolism of lignin aromatic hydrocarbons remains largely unresolved. The 3-oxoadipate pathway of their degradation combines the catechol and the protocatechuate branches, each using a set of specific genes. However, annotation for most of these genes is lacking or attributed to poorly- or un-characterised families. *Aspergillus nidulans* can utilise as sole carbon/energy source either benzoate or salicylate (upstream aromatic metabolites of the protocatechuate and the catechol branches, respectively). Using this cultivation strategy and combined analyses of comparative proteomics, gene mining, gene expression and characterisation of particular gene-replacement mutants, we precisely assigned most of the steps of the 3-oxoadipate pathway to specific genes in this fungus. Our findings disclose the genetically encoded potential of saprophytic Ascomycota fungi to utilise this pathway and provide means to untie associated regulatory networks, which are vital to heightening their ecological significance.

1. Introduction

Aspergillus species are widespread in terrestrial habitats where they have an important role in the natural cycling of chemical elements, particularly in the decomposition of plant litter. These fungal saprophytes can, in general, efficiently degrade complex plant polymers including starches, hemicelluloses, celluloses, pectins and other sugar polymers. Quinate and shikimate, comprising more than 10 wt% of decaying leaf litter, are other abundant carbon sources for aspergilli. The aromatic protocatechuate is the end intermediate of their utilisation pathways – well studied in both *Aspergillus nidulans* and *Neurospora crassa* (Hawkins et al., 1993; Tang et al., 2011). Plant lignin-related aromatic hydrocarbons, which constitute also an abundant carbon source for saprophytic fungi, are usually considered inhibitory compounds in the decomposition of leaf litter and fermentation of lignocellulosic hydrolysates (Palmqvist and Hahn-Hägerdal, 2000).

The peripheral (upper) pathways of the aerobic catabolism of aromatic hydrocarbons have been extensively investigated in fungi but the subsequent central pathways are still poorly understood (Wright, 1993). They usually occur ultimately via one of five intermediates, either catechol, protocatechuate, homogentisate, gentisate or hydroquinone (Harwood and Parales, 1996). The homogentisate degradation pathway (involved *e.g.* in the phenylalanine metabolism) has been recently resolved in *Penicillium chrysogenum* (Veiga et al., 2012). On the contrary, the 3-oxoadipate pathway that combines the protocatechuate and the catechol branches, remains largely overlooked, notwithstanding its significance in the end degradation of numerous exogenous aromatic hydrocarbons (Harwood and Parales, 1996). It is generally accepted that the composing biochemical reactions of this pathway in bacteria and fungi are rather well conserved, despite some distinguishable features (Harwood and Parales, 1996). The two branches of the pathway progress mostly through identical reaction steps that yield chemically analogous intermediates. They converge with the formation of 3-oxoadipate (β-ketoadipate) which is channelled into the tricarboxylic acid (TCA) cycle. Established knowledge on aspergilli revealed that they are able to use both branches of the 3-oxoadipate pathway using exclusively branch-specific genes (Boschloo et al., 1990; Kuswandi and Roberts, 1992). However, most reaction steps of this pathway either lack the assigned gene or are functionally assigned to multi-

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ple genes that encode poorly- or un-characterised protein families often predicted based on "nebulous" homologies to their bacterial counterparts. For example, the 3-carboxy-*cis*,*cis*-muconate intermediate (protocatechuate branch) undergoes specific cycloisomerisation in bacteria and fungi (Harwood and Parales, 1996) but the genes currently associated to the downstream reactions in fungi disregards this fact. Better understanding of the aromatic degradation pathways in fungi can push the technological development of novel biorefinery and bioremediation strategies (Palmqvist and Hahn-Hägerdal, 2000).

The present contribution aims to elucidate the 3-oxoadipate pathway in the model fungus *A. nidulans*. Combined comparative proteomics, gene expression and functional studies were undertaken using either benzoate or salicylate as sole carbon/energy source, in comparison with growth on acetate (control). Benzoate and salicylate constitute, respectively, the upstream aromatic metabolites of the protocatechuate and the catechol branches of the 3-oxoadipate pathway. This strategy allowed to assigning most of the steps of this pathway to specific genes. Our findings spotted a few incorrect conceptions predicted from bacterial studies and strengthened the significance of fundamental studies on model fungi/yeast.

2. Materials and methods

2.1. Strain and growth conditions

A. nidulans FGSC A4 asexual spores were harvested and maintained as frozen suspensions at −80 °C (Martins et al., 2014b). Cultures were initiated with 10^5 spores/mL and incubated with orbital agitation (160 rpm) in the dark at 27 °C. Batch cultivations were performed in 250 mL screw thread flasks with a working volume of 50 mL. A low nitrogen minimal medium was used containing per litre 1 g K_2HPO_4, 3 g $NaNO_3$, 0.01 g $ZnSO_4{\cdot}7H_2O$, 0.005 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.5 g KCl. Filter sterilised salts were added to an autoclave sterilised phosphate (pH 7.0) solution. The carbon sources were added either directly to the phosphate solution for sterilisation (150 mM sodium acetate – control) or to the mineral media after filter sterilisation (50 mM sodium benzoate or sodium salicylate). *A. nidulans* FGSC A1145 and A1147 (as well as the gene replacement mutants) used similar cultivation conditions but media were supplemented with the appropriate nutrients requirements (Table 1).

2.2. Metabolites identification and quantification

Culture media were analysed by ultra-performance liquid chromatography (UPLC) for the identification and/or quantification of the aromatic compounds and their degradation intermediates (Martins et al., 2014b). The chromatographic profiles were acquired at 228 nm and 204 nm, respectively for the quantification of benzoate (retention time, Rt: 3.20 min) and salicylate (Rt: 3.44 min). Further identifications, usually at 212 nm or 260 nm, used commercial standards of protocatechuate, 4-hydroxybenzoate, catechol, 2,3-dihydroxybenzoate and *cis*,*cis*-muconate (Rt: 1.81; 2.18; 2.40; 2.54 and 1.83, respectively), as well as standards of *cis*,*trans*-muconate, *trans*,*trans*-muconate and muconolactone prepared using standard synthesis methods (Elvidge et al., 1950) (Rt: 2.04; 1.71 and 1.40 min, respectively). 3-Carboxymuconolactone (Rt: 1.24) and 3-carboxy-*cis*,*cis*-muconate (Rt: 1.34) were putatively identified based on their absorption spectra.

Table 1
List of *Aspergillus nidulans* strains used in this study.

Strain	Relevant phenotype	Source
A4	Glasgow wild type; *veA*+	FGSC[a]
A1145	*pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	FGSC[a]
A1147	*pyrG89*; *argB2*; *pabaB22*; *nkuA*::*argB*; *riboB2*	FGSC[a]
DOH4.7	ΔAN4531::*pyrG*^{Af}; *pyrG89*; *argB2*; *pabaB22*; *nku*::*argB*; *riboB2*	This study
DOH9.1	ΔAN3895::*pyrG*^{Af}; *pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	This study
DOH11.2	ΔAN4061::*pyrG*^{Af}; *pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	This study
DOH12.2	ΔAN1151::*pyrG*^{Af}; *pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	This study
DOH13.5	ΔAN5232::*pyrG*^{Af}; *pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	This study

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2.3. Protein samples preparation

Mycelial and extracellular proteins were obtained and quantified as previously described (Carvalho et al., 2013; Martins et al., 2014a). Mycelial (100 mg powder mixed with 20 mg poly(vinyl)polypyrrolidone) and extracellular (concentrated using 10 kDa centrifugal devices) proteins were precipitated with cold acetone containing 10% w/v trichloroacetic acid and 60 mM dithiothreitol (DTT). The washed pellets were dissolved in similar buffers that contain 7 M urea, 2 M thiourea, 4% w/v CHAPS, 60 mM DTT and 1.0% v/v of matching IPG buffer (GE Healthcare), with addition of 1% (w/v) Triton X-100 in the case of extracellular proteins, and finally clarified by centrifugation.

2.4. Two-dimensional gel electrophoresis (2DE)

200 μg of mycelial and 50 μg of extracellular proteins were loaded to 13 cm IPG strips pH 3–10 NL and pH 3–5.6 NL, respectively. Protein isoelectric focusing, electrophoresis, staining, image acquisition and analysis followed previously described methods (Carvalho et al., 2013; Martins et al., 2014a). The gels were stained with flamingo dye (Bio-Rad) or colloidal coomassie blue for image acquisition or spot excision, respectively. Average values of the normalised volumes from the biological triplicates of each growth condition determined the up-accumulation level of each protein and were used for one-way ANOVA analysis with Progenesis SameSpots software v2.0 (Nonlinear Dynamics, UK). The level of significance was defined at p-value < 0.05.

2.5. Protein identification

The differential protein spots were excised from gels manually, and processed using the Ettan Digester robot of the Ettan Spot Handling Workstation (GE Healthcare) (Carvalho et al., 2013; Martins et al., 2014a). Samples (0.7 μL) were then spotted on MALDI-TOF target plates (Applied Biosystems), before the deposit of 0.7 μL CHCA (7 mg mL^{-1} in ACN 50%, TFA 0.1%). Peptide mass determinations were carried out using the AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). Both peptide mass fingerprinting and tandem MS in reflectron mode analyses were performed. The ten most intense peaks were selected automatically and used for MS/MS. Calibration was carried out with the peptide mass calibration kit for 4700 (Applied Biosystems). Proteins were identified, with ProteinPilot, by searching against the NCBI nr database downloaded on the 18/01/2013 (restricted to fungi taxa, 1635212 sequences) with Mascot v2.3 (Matrix Science). All searches were executed allowing for a mass window of 100 ppm for the precursor mass and 0.5 Da for fragment ion masses. Search parameters allowed for carboxyamidomethylation of cysteine as fixed modification. Methylation of aspartate or glutamate, and oxidation of methionine and tryptophan (single oxidation, double oxidation and kynurenin) were set as variable modifications. Homology identification was retained with probability set at 95%. All identifications were confirmed manually.