Microbiology (2015), **161**, 1211–1218 DOI 10.1099/mic.0.000030

Pyomelanin production in *Penicillium chrysogenum* is stimulated by L-tyrosine

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Received 22 October 2014
Accepted 1 June 2015

From a tomb in Upper Egypt we isolated a strain of *Penicillium chrysogenum* that was capable of producing brown pigment *in vitro* when grown in a minimal salts medium containing tyrosine. We present evidence that this pigment is a pyomelanin, a compound that is known to assist in the survival of some micro-organisms in adverse environments. We tested type strains of *Pe. chrysogenum,* which were also able to produce this pigment under similar conditions. Inhibitors of the DHN and DOPA melanin pathways were unable to inhibit the formation of the pigment. Fourier transform IR analysis indicated that this brown pigment is similar to pyomelanin. Pyrolysis-GC/MS revealed the presence of phenolic compounds. Using LC/MS, homogentisic acid, the monomeric precursor of pyomelanin, was detected in supernatants of *Pe. chrysogenum* cultures growing in tyrosine medium but not in cultures lacking tyrosine. Partial regions of the genes encoding two enzymes in the homogentisic acid pathway of tyrosine degradation were amplified. Data from reverse-transcription PCR demonstrated that *hmgA* transcription was increased in cultures grown in tyrosine medium, suggesting that tyrosine induced the transcription.

INTRODUCTION

Microbial melanins are polymers that may aid in the survival of some micro-organisms under adverse conditions. Different classes of melanins exist, including eumelanin, phaeomelanin, allomelanin and pyomelanin (Butler & Day, 1998; Eisenman & Casadevall, 2012). Dihydroxynaphthalene melanin (DHN-melanin) and dihydroxyphenylalanine (DOPA) are two examples of melanins produced by a wide range of fungi (Butler & Day, 1998). In the former pathway, 1,3,6,8-tetrahydroxynaphthalene undergoes a series of reduction and dehydration reactions to form DHN-melanin. This melanin is responsible for the characteristic brown or black colour of fungal spores (Bell & Wheeler, 1986). DOPA melanin, primarily studied in humans, has also been found in fungi. This pathway of melanin production relies on tyrosine oxidation (Butler & Day, 1998).

Chemically, melanins are heterogeneous polymers consisting of phenolic compounds, usually containing carbohydrates and proteins in bound form. These complex mixtures are difficult to analyse (Hamilton & Gomez, 2002). Identification and analysis methods have traditionally been based on properties such as solubility in various solvents.

Pyomelanin is a water-soluble melanin that is formed when homogentisic acid, an intermediate in the tyrosine degradation pathway, accumulates and subsequently polymerizes (Fig. 1). This extracellular melanin was first described in *Pseudomonas aeruginosa* (Yabuuchi & Ohyama, 1972). It has since been described in the filamentous fungus *Aspergillus* (Schmaler-Ripcke *et al.*, 2009; Keller *et al.*, 2011), the yeast *Yarrowia* (Carreira *et al.*, 2001), the dimorphic fungus *Sporothrix* (Almeida-Paes *et al.*, 2012) and many bacterial genera including *Shewanella*, *Legionella*, *Vibrio*, *Alcaligenes*, *Hyphomonas*, *Streptomyces* and *Rhizobium* (Arias-Barrau *et al.*, 2004; Méndez *et al.*, 2011; Turick *et al.*, 2002, 2009).

In micro-organisms, several functions contributing to survival in adverse conditions have been ascribed to pyomelanin (Turick *et al.*, 2009). A brown pigment in *Legionella pneumophila* was observed to confer protection from UV radiation (Steinert *et al.*, 2001). Under conditions of low dissolved oxygen levels in the environment, pyomelanin, owing to its ability to accelerate solid-phase metal reduction, is hypothesized to aid in the survival of *Shewanella oneidensis* MR-1 (Turick *et al.*, 2009). *Ps. aeruginosa* strains in patients with chronic lung infections produce a brown pigment that helps them tolerate

Abbreviations: DHN, dihydroxynaphthalene; DOPA, dihydroxyphenylalanine; FTIR, Fourier transform IR; ITS, internal transcribed spacer; Py-GC/MS, pyrolysis–GC/MS; RT-PCR, reverse transcription-PCR.

The GenBank/EMBL/DDBJ accession numbers for the ITS sequences of *Penicillium chrysogenum* obtained in this study are KP280083 and KP280084.

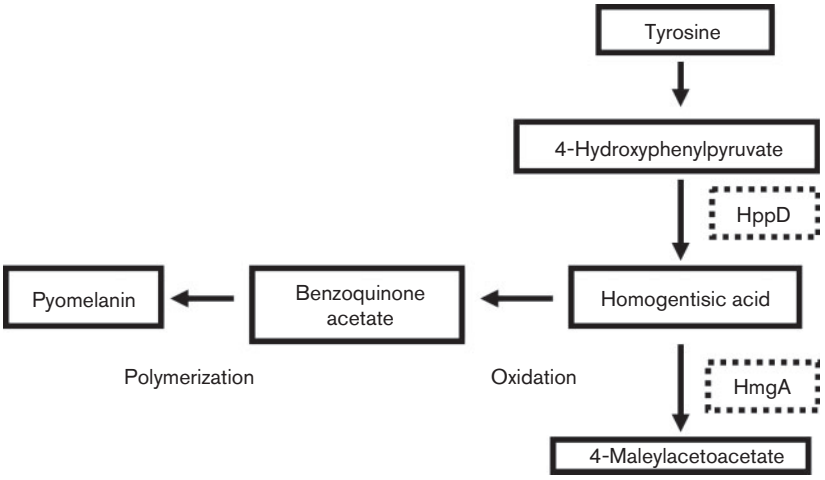

Fig. 1. Pathway for pyomelanin formation. When homogentisic acid accumulates, it auto-oxidizes and polymerizes to form pyomelanin. The enzymes HppD and HmgA catalyse the formation and breakdown, respectively, of homogentisic acid.

oxidative stress *in vitro* (Rodríguez-Rojas *et al.*, 2009). Turick and colleagues suggested that pyomelanin was important in the life cycle of *Shewanella algae*, a facultative anaerobe, since this organism can use the pigment to accelerate the rate of dissimilatory iron mineral reduction in addition to using it as a terminal electron acceptor (Turick *et al.*, 2002, 2003, 2008a). Recent research on *Aspergillus fumigatus* identified the production of pyomelanin (Schmaler-Ripcke *et al.*, 2009). Deletion mutants in which pyomelanin production was abolished were more susceptible to reactive oxygen species. This finding suggests that pyomelanin protects *Aspergillus* from reactive oxygen species.

Penicillium chrysogenum is a filamentous fungus of enormous medical significance, owing to its ability to produce penicillin, the first antibiotic to be described (Fleming, 1929). It is a ubiquitous fungus and has been isolated from a range of different environments, such as soil and caves (Alexopoulos, 1996). We recently isolated from a tomb in Upper Egypt a fungus that was identified by internal transcribed spacer (ITS) gene sequencing as a strain of *Pe. chrysogenum* (Vasanthakumar *et al.*, 2013). We describe in this report our data showing that both this isolate and the type strain of *Pe. chrysogenum* produce a brown pigment when grown in a minimal salts medium containing tyrosine. We present evidence that this pigment is pyomelanin. We also describe the results of our investigation into the chemical and genetic pathways underlying the formation of this pigment by *Pe. chrysogenum*.

METHODS

Isolation and growth of the fungal strains. Swabs moistened with sterile deionized water were used to collect samples from the tomb. A dilution series of samples was spread onto microbiological growth media such as tryptic soy agar, potato dextrose agar and malt extract agar (Becton Dickinson). Pure cultures of fungal isolates were obtained. Type strains of *Pe. chrysogenum* and *Aspergillus niger* were obtained from ATCC (*Pe. chrysogenum* strains Wisconsin 54-1255, ATCC 28089, NRRL 811 and ATCC 10107; *A. niger*, ATCC 10535).

Identification of fungi by ITS sequencing. DNA was extracted from fungal material using the UltraClean Microbial DNA extraction kit (MoBio). The ITS region was amplified as previously described (Vasanthakumar *et al.*, 2013), using the primers ITS1 and ITS4 (White *et al.*, 1990), and amplicons were sequenced using ITS1 or ITS4 at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Sequences were trimmed and edited using FinchTV version 1.5.0 (Geospiza) and checked against GenBank and UNITE databases (Abarenkov *et al.*, 2010). ITS sequences were submitted to GenBank.

Pigment production. Fungi were grown in a minimal salts medium [0.22 g $(NH_4)_2SO_4$ l^{-1}, 1.20 g KH_2PO_4 l^{-1}, 0.23 g $MgSO_4 . 7H_2O$ l^{-1}, 0.25 g $CaCl_2 . 2H_2O$ l^{-1}] containing 60 % (w/v) sodium lactate (9 ml l^{-1}) and tyrosine (2 g l^{-1}) as well as in growth medium without tyrosine. In addition to the fungi isolated, *Pe. chrysogenum* strains Wisconsin 54-1255 and NRRL 811 (Table 1) were also tested for pigment production. Inoculated strains were incubated at 37 °C. Synthetic pyomelanin was generated by treating homogentisic acid with NaOH under aerobic conditions.

To identify the putative pathway of melanin production, fungal cultures were grown on tyrosine medium amended with inhibitors of melanin synthesis. Briefly, 25 μg tricyclazole ml^{-1} (an inhibitor of the DHN-melanin pathway) (Liu *et al.*, 2014) and 50 μg kojic acid ml^{-1} (an inhibitor of the DOPA melanin pathway) were included in tyrosine medium. Fungal cultures were observed every day for pigment production both in the fungal biomass and in the medium. The effect of sulcotrione, a known inhibitor of pyomelanin production (Almeida-Paes *et al.*, 2012), was not tested in this study. However, the effect of including ascorbic acid, an antioxidant, which would presumably inhibit auto-oxidation of homogentisic acid, is reported.

Fourier transform IR (FTIR) analysis. The pigmented growth medium was filtered to exclude all fungal material. A representative sample particle was placed on a diamond window, and analysed by a transmitted IR beam with an aperture of approximately 100 × 100 μm, using a × 15 objective. Each spectrum was the sum of 200 scans at a resolution of 4 cm^{-1}. Based on the initial analysis results of bulk material, extraction was done by placing a microdroplet of solvent on the sample, and analysis was performed on the resultant extracted dried solvent ring.

Analysis of brown pigment using pyrolysis–GC/MS (Py-GC/MS). The dialysed, cell-free fungal growth medium was analysed by (i) a Frontier Lab PY-2020D double-shot pyrolyser with Agilent Technologies 5975C inert MSD/7890A GC; (ii) 320 °C pyrolysis

Table 1. Isolates of *Pe. chrysogenum* tested for brown pigment production

Isolate ID	Origin	Depository ID*	Reference
Wis 54-1255	Cantaloupe melon	ATCC 28089/NRRL 1951	O'Sullivan & Pirt (1973)
NRRL 811	Cheese	ATCC 10107/NRRL 811	Moyer *et al.* (1936)
T04C	Limestone wall – sealed tomb	NA	Vasanthakumar *et al.* (2013)
Fb	Limestone wall – open tomb	NA	Vasanthakumar *et al.* (2013)

*NA, Not applicable.

interface; (iii) 30 m × 0.25 mm × 0.25 μm J&W DB-5MS-UI column with Frontier Vent-Free adaptor (40 m effective column length); (iv) 1 ml helium min^{-1}; (v) 320 °C injector with 50 : 1 split ratio; (vi) oven programme: 2 min at 40 °C, 6 °C min^{-1} to 320 °C, 9 min isothermal; (vii) 320 °C MS transfer line; (viii) 230 °C source; (ix) 150 °C MS quad; (x) 10–600 Da scanned at 2.59 scans s^{-1}. Samples were pyrolysed at 550 °C in 50 μl stainless steel Eco-Cups (Frontier Laboratories).

Detection of homogentisic acid using LC/MS. The filtered growth medium was analysed in an Agilent 6210 ESI-TOF LC/MS. Samples were run in negative-ion mode for 20 min, using an Agilent Zorbax C-18 reverse-phase column (3.5 μm particle size, 2.1 × 100 mm). A formula confirmation method was used in the Agilent MassHunter software to detect homogentisic acid (formula $C_8H_8O_4$). Retention times and mass spectra were compared with those of the standard. Homogentisic acid (50 mM) and water were used as the standard and blank, respectively.

Genetic analysis of enzymes in the tyrosine degradation pathway. DNA was isolated from *Pe. chrysogenum* strains using the MoBio Microbial DNA Isolation kit. Primers were designed for genes encoding the enzymes homogentisate dioxygenase (HmgA) and 4-hydroxyphenylpyruvate dioxygenase (HppD), based on the published genome sequence of AM920427 *Pe. chrysogenum* Wisconsin 54-1255 (van den Berg *et al.*, 2008; Table 2). These primers enabled amplification of partial regions of these genes using PCR. The conditions for PCR were as follows: an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 7 min. Amplicons were sequenced in both directions using the forward and reverse primers at the Dana-Farber/Harvard Cancer Center DNA Resource Core.

Transcription analysis of HmgA and HppD. Total RNA was extracted from fungi using the RNeasy kit (Qiagen). DNase (Qiagen) treatment was performed to exclude possible contamination by small amounts of lingering DNA. Reverse transcription-PCR (RT-PCR) was performed on the RNA using *hmgA*, *hppD* and ITS primers. Conditions for RT-PCR were as follows: 50 °C for 30 min, 25–30 cycles of 94 °C for 30 s, 52 °C for 45 s and 72° for 120 s, followed by an extension cycle at 72 °C for 7 min.

RESULTS

Identification and description of *Pe. chrysogenum* isolated in this study

ITS sequencing confirmed the identity of the isolates as *Pe. chrysogenum*, with 98–99 % similarity to GenBank sequences (accession numbers JQ422624 and AM948960). Sequences obtained in this study were deposited in GenBank under the accession numbers KP280083 and KP280084. *Pe. chrysogenum* colonies of the isolated strains contained white mycelia with olive green spores on rich media and greyish spores on minimal salts media.

Production of brown pigment in microbiological growth medium amended with tyrosine

Growth of *Pe. chrysogenum* isolates in tyrosine medium was apparent within 48 h. However, the brown pigment was apparent only 10–15 days after inoculation. All *Pe. chrysogenum* strains grown in a growth medium containing tyrosine produced a brown pigment whereas those grown in medium lacking tyrosine did not (Fig. 2a, b). The pigment accumulated extracellularly. However, over time, the fungal cell walls appeared to also accumulate pigment, as observed using light microscopy (data not shown). It was not clear whether this pigment was pyomelanin or some other melanin.

Pe. chrysogenum cultures grown on kojic acid-amended tyrosine medium were identical to cultures grown on

Table 2. Description of primers used in PCR amplification of genes for enzymes (*hmgA* and *hppD*) active in the pyomelanin pathway in *Pe. chrysogenum*

Gene	Primer sequence	% Identity gene	GenBank accession no. for gene	% Identity predicted protein	GenBank accession no. for protein
hmgA-F	GCGGGGATACATCTGTGAAT	99	AM920427.1	95	XP_002557732
hmgA-R	CCGACCATGTGAGCACTAAA				
hppD-F	CAGTCAAGGACGTTGCCTTT	99	AM920427.1, XM_002557688	58	XP_002557734
hppD-R	TAGCCACCCTCATCGAAATC				

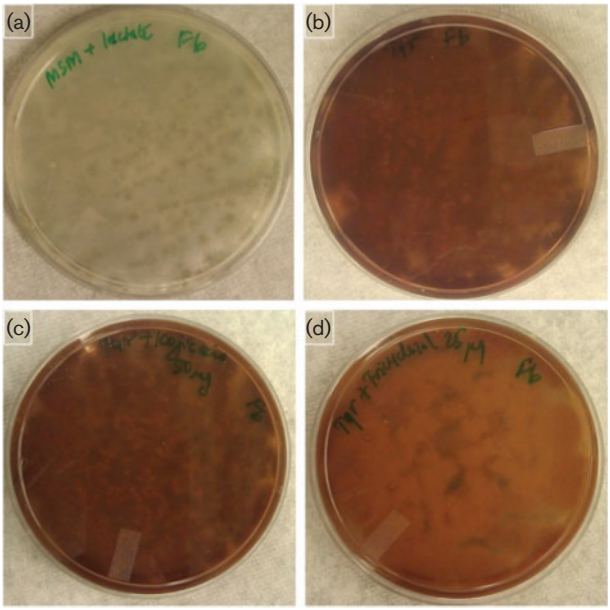

Fig. 2. Growth of *Pe. chrysogenum* in minimal salts medium without tyrosine (a) or with tyrosine (b). Brown pigment is formed only when tyrosine is present. Inclusion of DOPA melanin inhibitor kojic acid (c) and DHN-melanin inhibitor tricyclazole (d) in the tyrosine medium did not inhibit extracellular brown pigment formation.

tyrosine medium, indicating that kojic acid did not inhibit melanin production (Fig. 2c). Cultures grown on tricyclazole-amended tyrosine medium, however, were tan-coloured, rather than greenish, indicating that inhibition of DHN-melanin production led to a change in the pigments in the fungal biomass (data not shown). However, the extracellular pigment in the medium was not affected by tricyclazole (Fig. 2d), indicating that the pathway for formation of this pigment was not inhibited by tricyclazole.

Chemical analysis of brown pigment

FTIR analysis demonstrated significant similarity between the *Pe. chrysogenum* pigment and synthetic pyomelanin (Fig. 3). However, since other humic acid polymers also show a similar FTIR spectrum, a more specific method, Py-GC/MS, was used to analyse the pigment. This method utilizes heat to break the complex phenolic compounds into smaller fragments. Our Py-GC/MS analysis demonstrated that the brown pigment was a complex heterogeneous polymer containing multiple ring structures. In particular, 4-methoxybenzene acetic acid, 4-methoxybenzene propanoic acid and other phenolic compounds wereidentified using information in the NIST library (Lindstrom & Mallard). Though Py-GC/MS was more successful in detecting phenolic compounds, the complexity

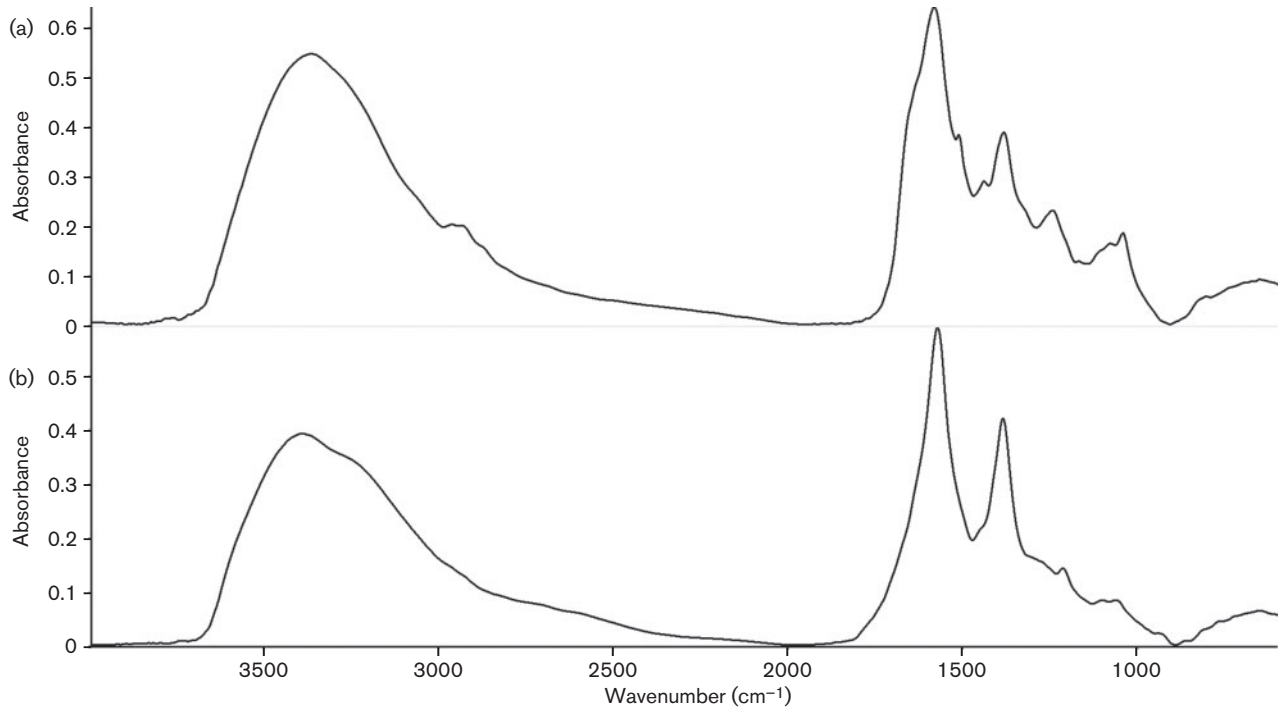

Fig. 3. FTIR analysis of *Pe. chrysogenum* brown pigment (a), and synthetic pyomelanin (generated by treating homogentisic acid with NaOH under aerobic conditions) (b).

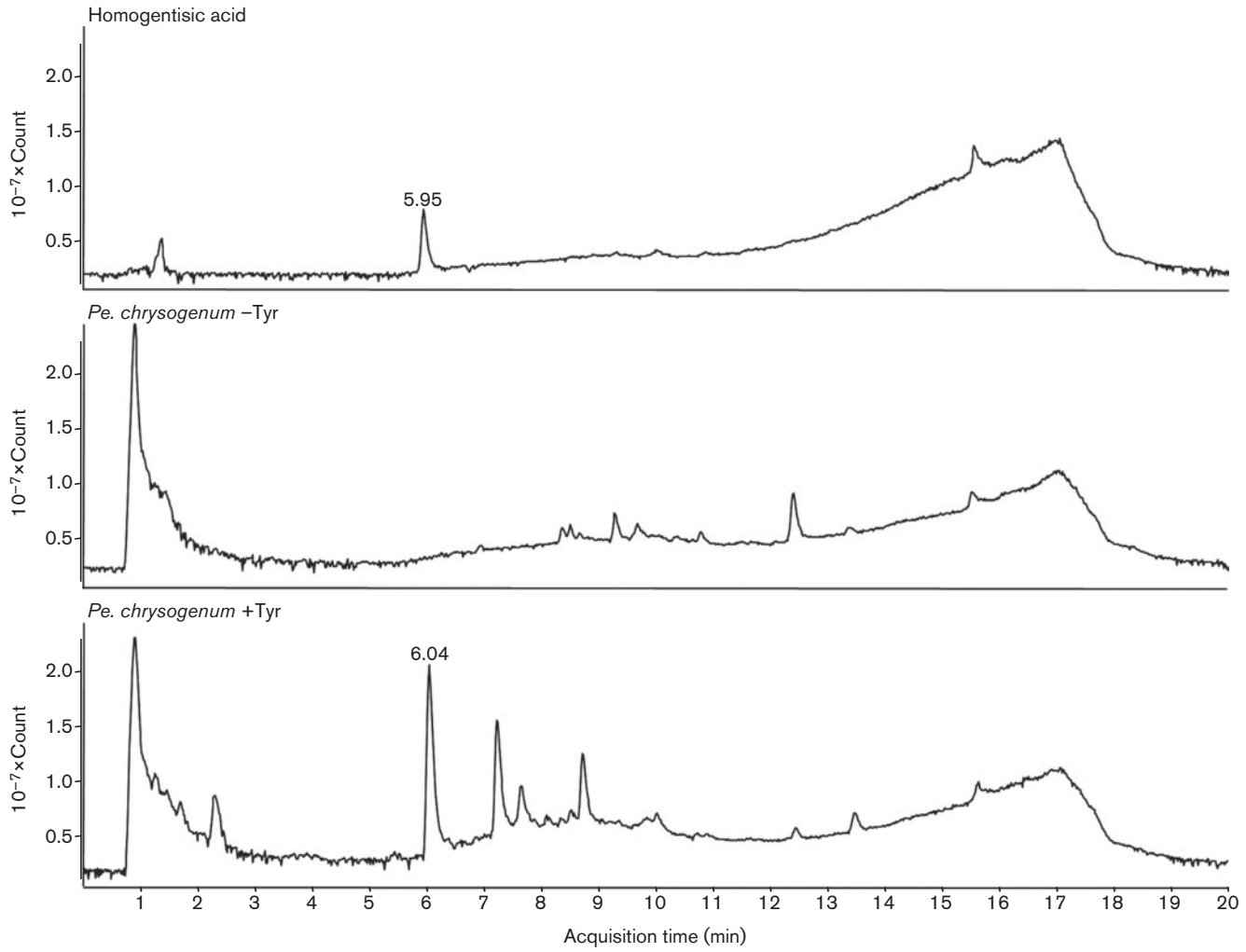

Fig. 4. LC/MS detection of homogentisic acid in *Pe. chrysogenum* culture supernatant (bottom panel). In the absence of tyrosine, no homogentisic acid was detected (middle panel). The top panel demonstrates the retention time of homogentisic acid, which was used as the standard.

of the sample made it difficult to separate other fungal products from the brown pigment (data not shown). In addition, the heterogeneity of the complex aromatic compounds made it difficult to obtain uniform results between samples. Therefore, an LC/MS technique was chosen so that the monomeric precursor, homogentisic acid, could be detected in the fungal cultures. The LC/MS analysis was successful in detecting homogentisic acid in *Pe. chrysogenum* cultures that were grown in tyrosine medium (Fig. 4, Table 3). No homogentisic acid was detected when *Pe. chrysogenum* was grown in minimal salts medium lacking tyrosine (Table 3).

Table 3. Retention time and abundance of homogentisic acid detected in supernatant of *Pe. chrysogenum* grown in MSM with or without tyrosine

A formula confirmation program was used in MassHunter software. The formula of the compound is $C_8H_8O_4$ and the target mass is 168.04226.

Sample	Retention time (min)	Relative molecular mass	Abundance
Penicillium chrysogenum−tyrosine	8.449	168.04235	109
Penicillium chrysogenum+tyrosine	6.039	168.04235	2 489 666
Homogentisic acid	5.948	168.04269	661 785

Fungal cultures containing tyrosine and ascorbic acid (an antioxidant) were also tested for homogentisic acid, with the hypothesis that the antioxidant would inhibit the process of auto-oxidation to pyomelanin. Over time, the cultures containing ascorbic acid still contained detectable levels of homogentisic acid whereas cultures without the antioxidant contained very little homogentisic acid. It is likely that the homogentisic acid had been oxidized to pyomelanin.

Analysis of the genetic pathway for pyomelanin formation

Partial *hmgA* and *hppD* genes were amplified in our isolates as well as in the type strain. The *hmgA* and *hppD* gene sequences in our isolate are at least 98 % identical to those in the published genome of the type strain (Table 2). RT-PCR analysis of these genes demonstrated that, in the presence of tyrosine, *hmgA* was expressed at a higher level (Fig. 5). The difference in the transcription of *hppD* was not as obvious.

DISCUSSION

In this study, we present evidence for the production of a water-soluble pigment similar to pyomelanin by *Pe. chrysogenum*. Our data demonstrate that the production of this extracellular brown pigment is not inhibited by tricylazole or kojic acid, inhibitors of the DHN-melanin and DOPA melanin pathways, respectively, and can therefore be attributed to a different pathway. In the current study, we did not test the effect of sulcotrione, a known inhibitor of pyomelanin production (Almeida-Paes *et al.*, 2012).

The pyomelanin group of melanins is characterized by their extracellular nature and water solubility (Turick *et al.*, 2010). The detection of homogentisic acid by LC/MS only in the *Pe. chrysogenum* cultures containing tyrosine and not in the cultures lacking tyrosine demonstrates that the production of homogentisic acid, a precursor of pyomelanin, is dependent on the presence of tyrosine. Though there is genomic information about the homogentisic acid pathway of tyrosine degradation (van den Berg *et al.*, 2008), this is, to our knowledge, the first documented experimental evidence of pyomelanin production by *Pe. chrysogenum*.

The pyomelanin itself was difficult to analyse because of its heterogeneous nature and the presence of complex phenolic structures. When Py-GC/MS was used, some aromatic compounds were identified that could have been derived from tyrosine degradation. However, it was not possible to conclusively identify the constituents of the brown pigment. Moreover, the polymeric nature of pyomelanin makes it extremely heterogeneous (Hunter & Newman, 2010). Therefore, its chemical composition can vary even between replicate tubes of fungal culture (begun from the same stock inoculum) grown in the same medium. However, the LC/MS method was successful in detecting homogentisic acid, the monomeric precursor of pyomelanin. The ability to detect *hmgA* was crucial in conclusively identifying the presence of pyomelanin.

Since previously published genomic information was available, it was possible to design primers specific for the genes encoding the enzymes homogentisate dioxygenase (*hmgA*) and 4-hydroxyphenylpyruvate dioxygenase (*hppD*). These two enzymes convert homogentisate to maleylacetoacetate and 4-hydroxyphenylpyruvate to homogentisate, respectively (Schmaler-Ripcke *et al.*, 2009). Amplification of partial regions of these two genes revealed that they are 98–99 % similar to the published sequences of Wisconsin 54-1255. Using RT-PCR, it was possible to analyse the role of tyrosine in inducing transcription of the two genes. The increased transcription of *hmgA* in response to tyrosine indicates that tyrosine induces this response. This, in turn, suggests that the presence of tyrosine stimulates the tyrosine

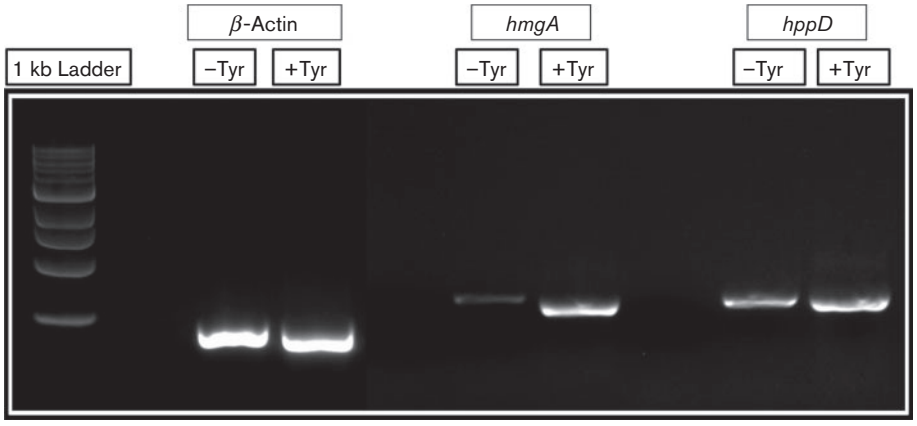

Fig. 5. Transcription of the genes *hmgA* and *hppD* in *Pe. chrysogenum* grown with or without tyrosine. Twelve nanograms of RNA was used in each RT-PCR. A housekeeping gene for β-actin was included as a control.

degradation pathway to be highly expressed. However, the cultures not exposed to tyrosine also demonstrated low levels of *hmgA* transcription, indicating that this gene is constitutively expressed in the fungus even in the absence of tyrosine. The difference observed in *hppD* transcription was subtle. This could be due to the fact that only partial regions of the gene were transcribed in this study.

We demonstrated that *Pe. chrysogenum* isolates from various environments were capable of producing brown pigment *in vitro* when tyrosine was included in the growth medium. This ability suggests that the homogentisic pathway of tyrosine degradation is active in all these isolates. It is possible that pyomelanin plays a role in survival of the fungus under adverse conditions (Turick *et al.*, 2009). In fungi such as *Sporothrix* and *A. fumigatus*, this brown pigment is known to help protect against oxidative stress (Schmaler-Ripcke *et al.*, 2009; Almeida-Paes *et al.*, 2012). In bacteria such as *Ps. aeruginosa* and *Burkholderia cenocepacia*, this pigment performs functions of stress tolerance such as heavy-metal binding (Turick *et al.*, 2003, 2008b), or relief of oxidative stress (Keith *et al.*, 2007). It is conceivable that the *Pe. chrysogenum* isolates found in tombs in Egypt are subjected to multiple stresses due to nutrient deficiency as well as lack of moisture. Pyomelanin might enable the fungus to tolerate these stresses. Future work will be required to determine whether this pigment enables *Pe. chrysogenum* to be an ecologically important component of the microbiome in other stressed environments.

ACKNOWLEDGEMENTS

This research was carried out as a collaborative agreement between the Getty Conservation Institute and Egypt's Supreme Council of Antiquities and was supported by a grant from the Getty Conservation Institute (no. 268237) to the Laboratory of Applied Microbiology, School of Engineering and Applied Sciences, Harvard University. The authors wish to thank Dr Marc Mittelman, Dr Federica Villa and Dr Patricia Sanmartín for insightful discussions, Felix Wu for technical assistance and the anonymous reviewers for useful suggestions.

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Edited by: D. Mattanovich