INTRODUCTION

Microbial melamins are polymers that may aid in the survival of some micro-organisms under adverse conditions. Different classes of melamins exist, including eumelanin, phaeomelanin, allomelanin and pyomelanin (Butler & Day, 1998; Eisenman & Casadevall, 2012). Dihydroxynaphthalene melanin (DHN-melanin) and dihydroxyphenylalanine (DOPA) are two examples of melamins 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, melamins 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
oxidative stress in vitro (Rodríguez-Rojas et al., 2009). Turick and colleagues suggested that pyomelanin was important in the life cycle of Shewanella algaec, 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 (NH₄)₂SO₄ l⁻¹, 1.20 g KH₂PO₄ l⁻¹, 0.23 g MgSO₄·7H₂O l⁻¹, 0.25 g CaCl₂·2H₂O l⁻¹] containing 60 % (w/v) sodium lactate (9 ml 1⁻¹) 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**

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Origin</th>
<th>Deposition ID*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wis 54-1255</td>
<td>Cantaloupe melon</td>
<td>ATCC 28089/NRRL 1951</td>
<td>O’Sullivan &amp; Pirt (1973)</td>
</tr>
<tr>
<td>NRRL 811</td>
<td>Cheese</td>
<td>ATCC 10107/NRRL 811</td>
<td>Moyer et al. (1936)</td>
</tr>
<tr>
<td>T04C</td>
<td>Limestone wall – sealed tomb</td>
<td>NA</td>
<td>Vasanthakumar et al. (2013)</td>
</tr>
<tr>
<td>Fb</td>
<td>Limestone wall – open tomb</td>
<td>NA</td>
<td>Vasanthakumar et al. (2013)</td>
</tr>
</tbody>
</table>

*NA, Not applicable.

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 x 100 mm). A formula confirmation method was used in the Agilent MassHunter software to detect homogentisic acid (formula C₉H₈O₄). 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 °C 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 (hmgA and hppD) active in the pyomelanin pathway in Pe. chrysogenum**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>% Identity gene</th>
<th>GenBank accession no. for gene</th>
<th>% Identity predicted protein</th>
<th>GenBank accession no. for protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>hmgA-F</td>
<td>GCGGGGATACATCTGGAAT</td>
<td>99</td>
<td>AM920427.1</td>
<td>95</td>
<td>XP_002557732</td>
</tr>
<tr>
<td>hmgA-R</td>
<td>CCGACCATGTGAGCACTAAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hppD-F</td>
<td>CAGTCAAGGACCTGCTTTT</td>
<td>99</td>
<td>AM920427.1</td>
<td>58</td>
<td>XP_002557734</td>
</tr>
<tr>
<td>hppD-R</td>
<td>TAGCCACCCTCATGAAATC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 were identified using information in the NIST library (Lindstrom & Mallard). Though Py-GC/MS was more successful in detecting phenolic compounds, the complexity

![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.](image)

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention time (min)</th>
<th>Relative molecular mass</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium chrysogenum</em> − tyrosine</td>
<td>8.449</td>
<td>168.04235</td>
<td>109</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em> + tyrosine</td>
<td>6.039</td>
<td>168.04235</td>
<td>2 489 666</td>
</tr>
<tr>
<td>Homogentisic acid</td>
<td>5.948</td>
<td>168.04269</td>
<td>661 785</td>
</tr>
</tbody>
</table>

![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.](image)
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 tricyclazole 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 maleylacetate 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 β-Actin

β-Actin

1 kb Ladder

- Tyr

hmgA

Tyr

hpPD

- Tyr

Tyr

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.
Pyomelanin production by *Penicillium chrysogenum*

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 (Schmalmer-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**

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