RESEARCH LETTER

Characterization of melanin-overproducing transposon mutants of *Pseudomonas putida* F6

Jasmina Nikodinovic-Runic[1], Leona B. Martin[1], Ramesh Babu[2], Werner Blau[2] & Kevin E. O'Connor[1]

[1]School of Biomolecular and Biomedical Sciences, University College Dublin, Dublin, Ireland; and [2]Polymer Research Centre, School of Physics, Trinity College Dublin, Dublin, Ireland

Correspondence: Kevin E. O'Connor, School of Biomolecular and Biomedical Sciences, Ardmore House, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: +353 1 716 1307; fax: +353 1 716 1183; e-mail: kevin.oconnor@ucd.ie

Received 5 March 2009; accepted 28 June 2009.
Final version published online 17 July 2009.

DOI:10.1111/j.1574-6968.2009.01716.x

Editor: Alexander Steinbüchel

Keywords
melanin; tyrosinase; *Pseudomonas putida* F6.

Abstract

Two melanin-overproducing *Pseudomonas putida* F6 mutants were generated using transposon (Tn5) mutagenesis. Mutants were disrupted in a transcriptional regulator (TR) and a homogentisate 1,2-dioxygenase (HDO) gene. Colonies of mutant F6-TR overproduced a black pigment on solid medium. The same mutant (F6-TR) had a 3.7-fold higher tyrosinase activity compared with the wild-type strain when induced with ferulic acid. However in tyrosine uptake assays whole cells of the mutant strain F6-TR consumed eight times less tyrosine compared with the wild-type strain. Mutant F6-HDO produced a diffusible red pigment into the growth medium. Pigment production by mutant F6-HDO is sixfold higher than the wild-type strain. The biomass yield of mutant F6-HDO grown on tyrosine as the sole source of carbon and energy was 1.2-fold lower than the wild-type strain. While the growth of the wild-type strain was completely inhibited by 5 min of exposure to UV light (254 nm) both mutant strains showed survival rates $> 30\%$. Mutant F6-HDO was able to tolerate higher concentrations of hydrogen peroxide (H_2O_2) exhibiting 1.5 times smaller zones of inhibition at 10 mM H_2O_2 compared with mutant F6-TR and the wild-type strain. The pigments produced by all strains were purified and confirmed to be melanins.

Introduction

Melanins are pigments that are produced by a broad range of microorganisms. They are not considered essential for growth and development of cells, but are required in order to enhance the ability of the producing species to compete and survive under certain environmental conditions, such as in the presence of UV radiation (Lopez-Serrano *et al.*, 2004). Melanins have been reported to have strong affinity for metals and to be efficient scavengers of free radicals (Sichel *et al.*, 1991). In *Azotobacter chroococcum* and *Burkholderia cenocepacia* they protect against reactive oxygen species (ROS) (Shivprasad & Page, 1989; Keith *et al.*, 2007). Melanins tend to be either black or brown pigments although other colours may occur (Hill, 1992). Melanins derived from L-3,4-dihydroxyphenylalanine (L-DOPA) are referred to as eumelanins and are black or brown (Hill, 1992). Reddish or yellow melanins that incorporate cysteine with L-DOPA are called pheomelanins (Wakamatsu & Ito, 2002). Red–brown, water-soluble melanins formed from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate (PHPPA) and homogentisic acid (HGA), are called pyomelanins (Yabuuchi & Ohyama, 1972; Kotob *et al.*, 1995). Melanin synthesis in bacteria is carried out in the majority of cases by phenoloxidases (tyrosinases, laccases or catecholases) and/or via the polyketide synthase pathway (Jacobson, 2000). However, other enzymes such as *p*-hydroxyphenylacetic acidPHPA hydroxylase when heterologously expressed in *Escherichia coli* have also been shown to produce melanin (Gibello *et al.*, 1995). Tyrosinase (EC 1.14.18.1) is an enzyme that is ubiquitously distributed in microorganisms, animals and plants. Most of the information on the structure and function of tyrosinase has been obtained from studies on *Agaricus bisporus* (mushroom) and *Streptomyces antibioticus* (Claus & Decker, 2006; Matoba *et al.*, 2006). Tyrosinase from *Pseudomonas putida* F6 has been previously purified and biochemically characterized (McMahon *et al.*, 2007). Tyrosinase catalyses the *ortho* hydroxylation of monophenols to *o*-diphenols (monophenolase, cresolase activity), followed by the subsequent oxidation of the *o*-diphenol to

the corresponding *o*-quinone derivative (Sanchez-Ferrer *et al.*, 1995). The latter products polymerize to form melanin-like pigments.

We wish to gain an insight into the role of tyrosinase and other factors affecting melanin synthesis in *P. putida* F6. Transposon mutagenesis, a powerful tool for the genetic, physiological and biochemical analysis of bacteria (deLorenzo & Timmis, 1994) was utilized to generate two mutants exhibiting increased pigmentation.

Materials and methods

Reagents

Phenylacetic acid, PHPA, PHPPA, L-tyrosine, L-DOPA, ferulic acid, homogentisic acid, 4-fluorophenol, synthetic melanin, *o*-dianisidine, 6-hydroxydopamine hydrobromide, deuterated dimethyl sulphoxide (d-DMSO) and Luria–Bertani (LB) broth were purchased from Sigma-Aldrich, Ireland. Tri-sodium citrate was from BDH Laboratories, UK.

Bacterial strains and plasmids

Pseudomonas putida F6 was isolated from soil for its ability to utilize PHPA as a sole source of carbon and energy (O'Connor *et al.*, 2001). *Escherichia coli* CC118λ*pir* hosted the mini-Tn5 derivative pUT-Km1(Herrero *et al.*, 1990). This suicide plasmid has the R6K origin of replication and encodes resistance to kanamycin and ampicillin (de Lorenzo *et al.*, 1990). Plasmid pRK600 (Cm^r, mob^+, tra^+) hosted in *E. coli* HB101 (Invitrogen) was used as a helper in triparental mating experiments. *Escherichia coli* TOP 10 (Invitrogen) was a general cloning host. TOPO® pCR® 2.1 (Invitrogen) was used for cloning of PCR products according to the manufacturer's instructions.

Mutagenesis of *P. putida* F6 by the mini-Tn5Km1 transposon

Triparental matings involving *E. coli* CC118λ*pir* (pUT-Km1) as the transposon donor strain, *P. putida* F6 as the recipient and *E. coli* HB101 (pRK600) as the helper strain were carried out in a 1-mL volume in the ratio 7 : 2 : 1, respectively (Vilchez *et al.*, 2000; O'Leary *et al.*, 2005).

Transconjugants of *P. putida* F6 were selected on E2 agar plates (Vogel & Bonner, 1956) supplemented with tri-sodium citrate (15 mM), kanamycin and L-tyrosine (5 mM). Approximately, 10^5 independent mutants colonies were generated and were individually transferred to sterile 96-well microtitre plates containing 200 μL of E2/citrate/km (50 μg mL^{-1}) in each well (O'Leary *et al.*, 2005). Following a 24-h incubation colonies were transferred onto E2 agar plates containing L-tyrosine (5 mM) (System Duetz, Kuhner AG, Switzerland). Mutants were screened and selected based on the increased melanin production by visual inspection of the pigmentation of the colonies (Solano *et al.*, 2000). Two mutants were selected and subcultured for sequencing and further characterization.

Culture media and growth conditions

Pseudomonas putida F6 wild type as well as the *P. putida* F6 mutants were grown in liquid culture in 50 mL of E2 medium (Vogel & Bonner, 1956) in 250-mL Erlenmeyer flasks. Media contained tri-sodium citrate (15 mM) as carbon source and when necessary kanamycin was added (50 μg mL^{-1}). Cultures were incubated at 30 °C with shaking at 200 r.p.m. for 24 h. In melanin quantification experiments cells were grown as above in E2 broth supplemented with L-tyrosine (5 mM). Samples were taken periodically and melanin production was monitored at 400 nm (Ruzafa *et al.*, 1995; Chatfield & Cianciotto, 2007).

Identification of DNA sequences of transposon mutants

Identification of the genes disrupted by Tn5 was carried out using a PCR method and arbitrary primers described previously (Caetano-Anolles, 1993; Espinosa-Urgel *et al.*, 2000). Obtained PCR products were cloned into pCR 2.1-TOPO (Invitrogen) before sequencing. Sequencing was carried out by GATC biotech (Konstanz, Germany). Sequences were analysed and compared with the GenBank database using the BLAST program (Altschul *et al.*, 1997).

Enzyme assays in cell extracts

Pseudomonas putida F6 and mutants were grown in flasks (50-mL culture in 250-mL flasks) in E2/citrate medium, E2/citrate supplemented with L-tyrosine (1 mM) and E2/citrate supplemented with ferulic acid (1 mM) at 30 °C for two 24 h (Brooks *et al.*, 2004). The cells were harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C and washed twice in ice-cold 50 mM potassium phosphate buffer, pH 7 (10 mL). Cell-free extract (CE) was prepared using the BugBuster amine-free reagent (Novagen) according to the instruction manual.

Tyrosinase enzyme assays were performed in 96-well microtitre plates with L-tyrosine (1 mM) as the sole substrate. Assays were performed in air-saturated 50 mM phosphate buffer, pH 7, at 30 °C. The total reaction volume was 200 μL and contained 0.015 mg protein. The reaction was monitored at 475 nm (Espin *et al.*, 1995) and recorded using a SpectraMax-340 microtitre plate reader (Molecular Devices, Sunnyvale). Reduced nicotinamide cofactors (NAHD) were not added to the cell extract to ensure that any hydroxylase enzymes potentially present in the cell extract

would not be able to oxidize tyrosine and thus any activity present can be attributed to tyrosinase.

For determination of other enzyme activities, cells were grown in E2 medium, with citrate (15 mM) and L-tyrosine (1 mM) for 48 h at 30 °C. CEs were prepared as above, and supplemented with 40% glycerol (v/v) for storage at −20 °C.

Laccase activity was measured spectrophotometrically, by following the rate of oxidation of syringaldazine at 525 nm (Solano *et al.*, 2000) in the reaction buffer containing 50 mM potassium phosphate and 0.05 mM syringaldazine upon addition of CE (20 μL in 0.2-mL total volume). The molar extinction coefficient of the oxidation product, $\varepsilon = 65\,000\ M^{-1}\ cm^{-1}$, was used to calculate laccase activity (Harkin & Obst, 1973). One unit of laccase activity is defined as the amount of the enzyme that catalysed the oxidation of 1 μmol of syringaldazine per min at 30 °C.

Catalase activity in CEs was determined spectrophotometrically by following the disappearance of H_2O_2 over time at 240 nm (Beers & Sizer, 1952). One unit of catalase decomposed 1 μmol H_2O_2 mg^{-1}protein at 25 °C. The assay was performed in 1-mL volume in 50 mM phosphate buffer, pH 7, containing 10 mM H_2O_2 upon addition of CE (0.1 mL).

Peroxidase activity was assayed by following the oxidation of *o*-dianisidine at 460 nm in a reaction buffer containing 50 mM potassium phosphate, 1 mM H_2O_2, 0.34 mM *o*-dianisidine (Sigma) upon addition of CE (20 μL). One unit of peroxidase activity equals 1 micromole H_2O_2 reduced per minute, with $\varepsilon = 113\,000\ M^{-1}\ cm^{-1}$ (Schnell & Steinman, 1995).

Superoxide dismutase (SOD) activity in CEs was determined spectrophotometrically by measuring the inhibition of the initial rate of auto-oxidation of 6-hydroxydopamine at 490 nm, in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM 6-hydroxydopamine hydrobromide upon addition of CEs (20 μL in 0.2-mL total assay volume). One unit of SOD activity corresponded to 50% inhibition of the initial rate (Heikkila & Cabbat, 1976). A standard curve using known units of SOD activity was obtained in order to determine the units of activity in each sample. We used SOD from *E. coli* (Sigma) as a positive control and for the generation of the standard curve.

Tyrosine uptake monitoring by HPLC analysis

Pseudomonas putida F6 and mutant F6-TR were grown in E2/citrate broth as described previously. Cultures were centrifuged at 13 000 ***g*** and cell pellets were resuspended in 50 mM phosphate buffer, pH 7, to an $OD_{540\,nm}$ of 10, supplemented with 2 mM L-tyrosine and incubated at 30 °C shaking at 200 r.p.m. Samples of 0.45 mL were removed every 30 min, acidified with 1 M HCl (50 μL) centrifuged at 13 000 ***g*** for 5 min and filtered for HPLC analysis.

HPLC analysis was performed using C-18 Hyperclone ODS 5-μm column (250 × 4.6 mm) (Phenomenex) on a Hewlett Packard HP1100 instrument equipped with an Agilent 1100 series diode array detector. Mobile phase was a solution of methanol and (0.1%) phosphoric acid in the ratio of 15 : 85, with a flow rate of 0.8 mL min^{-1}.

UV sensitivity assay

UV sensitivity assays were performed using the previously published protocol with slight alterations (Simonson *et al.*, 1990). Because of delayed melanin production by mutant F6-TR in liquid cultures (5–7 days), cell suspensions were made by scraping the bacterial colonies producing pigment from the E2/citrate/L-tyrosine plates. Sterile 50 mM phosphate buffer (500 μL) was applied to the surface of the plate and sterile cotton swab was used to gently detach the cells making bacterial suspension. Suspensions were adjusted to the $OD_{600\,nm}$ of 0.2 using sterile phosphate buffer. Cell suspension (2.5 mL) was placed in the Petri plate and illuminated with the UV lamp (254 nm, Mineralight® Lamp R-52) for 1, 2 and 5 min. Serial dilutions of untreated cell suspensions as well as serial dilutions for the treated samples were plated for each time point and colonies counted and represented as percentage survival.

Hydrogen peroxide (H_2O_2) disc diffusion assay

Cell suspension prepared as described above (100 μL) was spread onto fresh plates (LB and E2/citrate/L-tyrosine), and sterile paper discs (Oxoid) were applied to the surface. H_2O_2 (8 μL) of 1, 5 and 10 mM were applied to discs. The plates were incubated at 30 °C for 48 h, and the zones of inhibition were measured.

Melanin purification and analysis

Pseudomonas putida F6, F6-HDO and F6-TR were grown in E2/citrate/L-tyrosine (5 mM) and incubated at 30 °C for 72 h to allow melanin to accumulate. Cells were removed by centrifugation (13 000 ***g*** for 15 min). Supernatant was acidified (pH 2) using 6 M HCl and melanin was allowed to precipitate for 4 h at 20 °C. The precipitated melanin suspension was dialyzed (8 kDa) for 24 h with four changes of water. After 24 h, the melanin samples were freeze dried.

The melanin samples (10 mg) were dissolved (or suspended) in 1 mL of d-DMSO. Solution nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX400 (Bruker BioSpin Limited) with ^1H at 400.13 MHz and d-DMSO solvent was used as internal reference for chemical shifts in ^1H NMR.

The samples for Fourier transform infrared spectroscopy (FT-IR) analysis were prepared by mixing the samples with KBr powder. Analysis was carried out on a Nexus Nicolet

FT-IR Spectrometer (Thermo-Electron, Waltham) in main bench mode. The spectra recorded over a range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 128 scans per sample.

Protein determination

Total protein amount in CEs were determined using the bicinchoninic acid method as described previously (Smith *et al.*, 1985).

Results

Identification of gene disruption in transposon mutants

Pseudomonas putida F6 produces a pigment into the growth medium when supplied with tyrosine (Fig. 1). Two transposon mutants were selected for study based on altered pigmentation relative to the wild-type strain on agar plates. Sequencing of the disrupted gene, using primers based on the sequence of the mini-transposon was performed and a subsequent BLAST analysis carried out as described previously (Espinosa-Urgel *et al.*, 2000).

The gene disrupted in mutant F6-TR had 84% homology to a transcriptional regulator (TR) from the Crp/Fnr family (Table 1). This family is the most versatile group of DNA-binding TRs that usually act as positive regulators, but repression has also been reported (Korner *et al.*, 2003). The

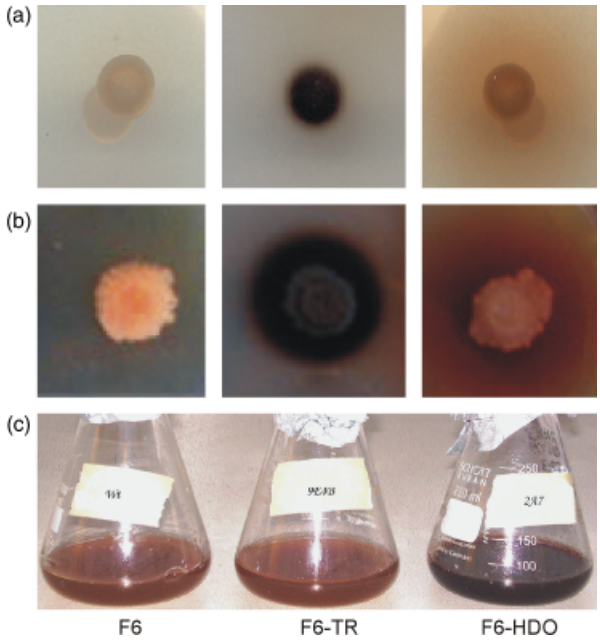

Fig. 1. Melanin production by *Pseudomonas putida* F6 and mutants on E2/citrate/L-tyrosine (5 mM) agar (a) at 24 h, (b) at 72 h and (c) broth at 48 h.

Table 1. Summary of transposon mutants generated in this study

Mutant	Gene identification	Homology (%)	BLAST sequence reference no.
F6-HDO	HDO	92	YP_001269785
F6-TR	Transcriptional regulator Crp/Fnr family, cAMP	84	YP_001267781

colony of F6-TR was much darker than that of the wild-type strain and a dark black halo surrounded the colony (Fig. 1). Mutant F6-HDO was disrupted in a gene with 92% homology to homogentisate 1,2-dioxygenase (HDO) (EC 1.13.11.5) (Table 1). HDO is a ring cleaving dioxygenase that converts HGA to 4-maleyl-acetoacetate. A diffuse reddish-brown pigmented halo appears around the mutant colony but the colony itself does not appear much darker than the wild-type strain (Fig. 1).

Mutant F6-HDO exhibited a longer lag phase (1 h) and achieved a 1.2-fold lower final biomass compared with the wild-type strain (data not shown). To ensure that the mutants, altered in their melanin production, are unaffected in their growth, citrate as a carbon source was added to the medium. Citrate did not repress the production of melanin in the wild-type or mutant strain.

Monitoring melanin production over time

In order to quantify melanin production by wild type and mutants of *P. putida* F6, the $OD_{400\,nm}$ of culture supernatant (E2/citrate/L-tyrosine 5 mM) was monitored over time (Ruzafa *et al.*, 1995; Chatfield & Cianciotto, 2007). *Pseudomonas putida* F6 wild-type cells did not produce pigment for the first 4 h after inoculation and produced low levels up to 8 h of growth. The culture medium of mutant F6-HDO was visibly pigmented within 6 h of inoculation. Melanin production ($OD_{400\,nm}$) occurs rapidly between 8 and 12 h in mutant F6-HDO cultures, but the production rate rapidly decreases after this time. Pigment production is approximately sixfold higher in liquid cultures of mutant F6-HDO compared with the wild-type strain after 20 h (Fig. 2). Despite being much darker than the wild type on agar plates (Fig. 1), liquid cultures of mutant F6-TR produced similar levels of melanin to wild-type strain in liquid medium after 48 h of growth (Fig. 2). Extended incubation (5–7 days) did result in higher levels of melanin production (data not shown).

Tyrosinase enzyme activity

The disruption of genes other than tyrosinase may affect tyrosinase enzyme activity and thus we examined the tyrosine-oxidizing capacity of crude cell extracts of *P. putida* F6 and mutants. These assays were performed in the absence of nicotinamide cofactors (NADH) to ensure that only

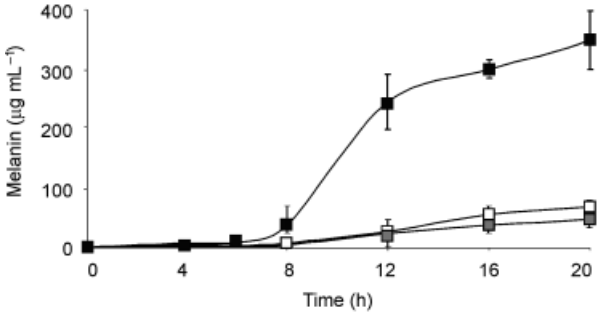

Fig. 2. Melanin production over time by *Pseudomonas putida* F6 and the transposon (Tn5) mutants in liquid culture. (□, wild type; ▨, F6-TR; ■, F6-HDO). Mutant F6-TR can only overproduce melanin on solid medium (see Fig. 1).

Table 2. Tyrosinase activity (U mg^{-1} protein)* of crude cell extracts of wild-type and mutant strains using 1 mM tyrosine as the substrate

Inducer†	*P. putida* F6 wild type	Mutant F6-HDO	Mutant F6-TR
None	1.2 ± 0.1	1.4 ± 0.1	4.2 ± 0.7
Tyrosine (1 mM)	1.0 ± 0.1	ND‡	4.7 ± 0.2
Ferulic acid (1 mM)	46.6 ± 4.5	12.4 ± 0.9	173 ± 11

*A unit is defined as the nanomoles of quinone formed per minute based on the molar extinction coefficient (ε) of dopaquinone of 3700 M^{-1} cm^{-1}.

†Growth medium was E2/citrate (15 mM). 0.015 mg mL^{-1} protein was used in the assay.

‡After prolonged incubation (12 h), the enzyme assay medium went dark indicating melanin formation, but activity was not detected within the assay time frame (2 h).

tyrosinase activity is measured and not any hydroxylase enzymes that could potentially be present in the cell extracts. Three different conditions were used to examine tyrosine-oxidizing (tyrosinase) activity in crude cell extracts of wild type and mutants of *P. putida* F6, i.e. growth on citrate only (noninducing), citrate and tyrosine (1 mM), and citrate and ferulic acid (1 mM). We have previously described ferulic acid as an inducer of tyrosinase activity in *P. putida* F6 (Brooks *et al*., 2004). Crude cell extracts of wild-type *P. putida* F6 cells induced with ferulic acid for 2 h consumed 46.6 nmol tyrosine min^{-1}mg^{-1}protein. Crude cell extracts of mutant F6-TR consumed tyrosine at a rate 3.7-fold higher than the wild-type strain under the same conditions (Table 2). Crude extracts of mutant F6-HDO had 3.8-fold lower tyrosinase activity compared with wild-type cell extracts when induced with ferulic acid (Table 2). Interestingly, tyrosine appears to be a poor inducer of tyrosinase activity in all strains tested with over 40-fold lower activity in the wild type and mutant F6-TR compared with cells induced with ferulic acid. Mutant F6-HDO did not exhibit a detectable activity within the 2-h time frame of the enzyme assay when induced with tyrosine, but did eventually (>12 h) go dark, indicating low levels of tyrosine-oxidizing activity.

Table 3. Laccase, catalase and SOD activities (U mg^{-1} protein)* in *Pseudomonas putida* F6 wild type and mutants (F6-HDO and F6-TR)

Strains†	Laccase activity	Catalase activity	Peroxidase activity	SOD activity
P. putida F6 wild type	6.9 ± 0.1	22.1 ± 0.9	5.6 ± 0.5	0.8 ± 0.1
Mutant F6-HDO	6.6 ± 0.2	34.8 ± 0.5	11.9 ± 0.3	0.8 ± 0.2
Mutant F6-TR	6.8 ± 0.1	24.9 ± 0.4	4.2 ± 0.2	0.7 ± 0.2

*Values are an average of three independent measurements.

†Cells were grown in E2/citrate (15 mM) medium supplemented with 1 mM tyrosine for 48-h shaking at 30 °C.

Laccase, catalase, peroxidase and SOD enzyme activities

The laccase activity in crude cell extracts of wild-type and mutant strains of *P. putida* F6 were almost identical (Table 3). Catalase activity in CE of all three strains was determined. As catalases can exist as monofunctional and bifunctional enzymes (having additional peroxidase activity), we have also determined peroxidase activity by following the oxidation of *o*-dianisidine at 460 nm. Catalase activity was 1.5-fold higher in F6-HDO mutant relative to the F6 wild type (Table 3). However, mutant F6-TR expressed similar levels of catalase activity to the wild-type strain (Table 3). Peroxidase activity in F6-HDO is 2.1-fold higher than the wild-type strain. While F6-TR mutant exhibited a 1.3-fold lower peroxidase activity compared with the wild-type strain. Interestingly, SOD activity in all three strains was almost identical (Table 3).

Tyrosine uptake by mutant F6-TR

Despite a high level of tyrosinase activity in crude cell extracts of mutant F6-TR harvested from solid medium (agar plates), whole cells grown in liquid culture do not produce more melanin than the wild-type strain. Based on this observation, we postulated that tyrosine uptake may also be affected in this mutant. Both wild-type and mutant F6-TR washed cell suspensions ($OD_{540\,nm}$ of 10), grown under inducing conditions (citrate, 15 mM, and ferulic acid, 1 mM), showed a lag period in tyrosine uptake for the first 3 h of incubation. However, over the next 3 h, whole cells of the wild-type strain consumed eight times more tyrosine (0.32 mM) compared with the mutant F6-TR (0.04 mM).

Cell survival upon exposure to UV light (254 nm)

Melanins are well known to protect cells from the effects of exposure to UV light. The production of higher amounts of melanin should offer extra protection to mutant F6-HDO

and F6-TR. As a result, we exposed the wild-type and mutants strains of *P. putida* F6 to UV light and measured cell survival. Mutant F6-TR does not overproduce melanin in liquid media and thus its survival after UV exposure in liquid was similar to the wild-type strain (data not shown). To overcome this limitation, wild-type and mutants F6-TR and F6-HDO, were grown on solid agar, colonies were scraped from the plate and resuspended in E2 medium to the same OD value at 540 nm (cell density). We plotted the percentage survival as a function of time exposed to UV light (Fig. 3a). Both mutants showed a higher survival rate (73–79%) compared with the wild type (42%) strain after 2 min of exposure. Furthermore the wild-type strain did not survive 5 min of exposure while both mutants exhibited 28–35% survival.

H_2O_2 resistance

Melanin can offer protection against other stresses such as H_2O_2 (Keith *et al.*, 2007). When exposed to various concentrations of H_2O_2 (1–10 mM), mutant F6-HDO exhibited *c.* 1.5 fold smaller zones of clearing at each concentration compared with wild type and mutant F6-TR, which had similar zones of clearing (Fig. 3b).

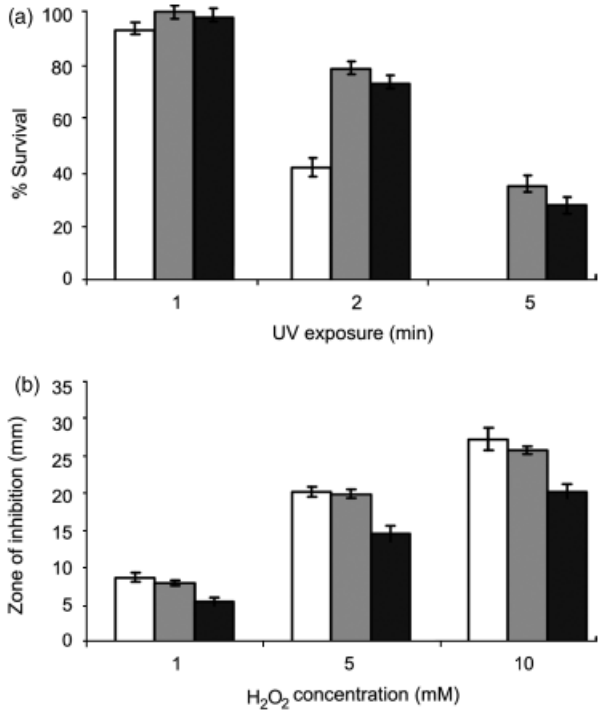

Fig. 3. (a) Cell survival upon exposure to $UV_{254\,nm}$ at 1, 2 and 5 min. (b) The effect of H_2O_2 exposure on cell survival. (□, wild type; ▨, F6-TR; ■, F6-HDO).

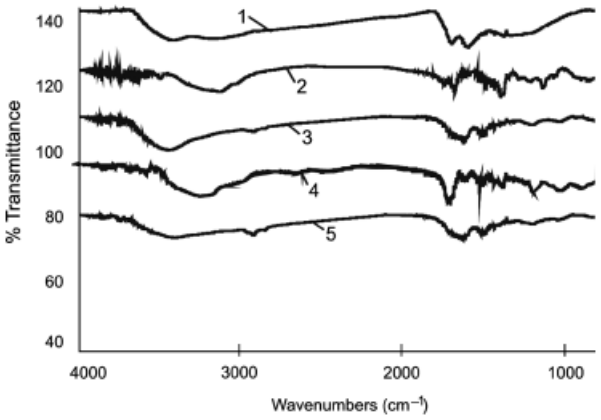

Fig. 4. FT-IR spectra of (1) synthetic melanin (Sigma), (2) auto-oxidized HGA, (3) melanin from *Pseudomonas putida* mutant F6-HDO, (4) melanin from *P. putida* mutant F6-TR and (5) melanin from *P. putida* F6.

Melanin analysis

The pigment from wild-type and mutant strains was isolated, purified and analysed by NMR and FT-IR (Fig. 4). Both NMR and FT-IR analysis of the pigments isolated from *P. putida* F6 and mutants along with synthetic melanin confirmed that the pigments were melanins (Katritzky *et al.*, 2002). The ^{1}H NMR in solution spectra had signals in both the aromatic and aliphatic regions for all of the samples (data not shown). Peaks in the absorption region from 3.70 to 4.20 p.p.m. could be assigned to protons on carbons attached to nitrogen and/or oxygen atoms (Katritzky *et al.*, 2002). Peaks in the region between 7.20 and 8.00 p.p.m. could be assigned to the protons attached to indole and/or other differently substituted aromatic or heteroaromatic rings. The pattern of the ^{1}H NMR melanin spectra of *P. putida* F6 and mutants were very similar to that of commercial melanin sample (data not shown).

In all FT-IR spectra of the melanins isolated from *P. putida* F6 and mutant strains characteristic for melanin can be observed (David *et al.*, 1996; Aghajanyan *et al.*, 2005) (Fig. 4). Despite the fact that the colour of the melanin produced by mutant F6-HDO is different from that from wild type and mutant F6-TR the IR spectral scans of all biologically synthesized melanins were similar. However, the melanin produced by mutant F6-HDO exhibited the highest similarity to the auto-oxidized HGA while the melanin from F6-TR had strong similarities to synthetic melanin (Fig. 4). The melanin produced by *P. putida* F6 wild-type strain appeared to be the mixture of both tyrosine- and HGA-derived melanins.

Discussion

Pseudomonas putida F6 colonies and liquid culture medium are pigmented in the presence of L-tyrosine and other 4-

substituted phenols (Brooks *et al.*, 2004; McMahon *et al.*, 2007; Martin *et al.*, 2008). We wished to investigate factors affecting pigment production in *P. putida* F6 and so random transposon mutants of this bacterial strain were created and characterized.

Melanin overproduction on solid media occurred in two mutants of *P. putida* F6. The overproduction of melanin increased the resistance of both mutants to UV light. Mutant F6-HDO is more resistant to the effects of H_2O_2 compared with the wild-type strain. Mutant F6-TR produces more melanin on solid medium than the wild-type strain and thus it would appear that production of higher levels of melanin does not provide extra protection when H_2O_2 is supplied extracellularly. It is possible that the production of a HGA-based polymer in the growth medium by mutant F6-HDO allows greater resistance to H_2O_2. HGA auto-oxidizes at pH 7 yielding superoxide ($O_2^{\bullet-}$) and H_2O_2 (Martin & Batkoff, 1989; Hiraky *et al.*, 1998). Both of these are highly reactive oxygen species that are able to cause severe cell damage. H_2O_2 is a relatively stable molecule that can diffuse across cell membranes and it is known to induce various cell responses (Bienert *et al.*, 2006). Aerobic bacteria have developed efficient responses for ROS scavenging, which include enzymes such as SODs, catalases and other non-enzymatic antioxidants (Vandenbroucke *et al.*, 2008). Thus accumulation and auto-oxidation of HGA in the growth medium of F6-HDO is likely to yield H_2O_2, which in turn activates a physiological response so that this mutant can more efficiently cope with higher levels of H_2O_2. Indeed, 1.5- and 2.1-fold higher levels of catalase and peroxidase enzyme activity, respectively, were observed in F6-HDO mutant in comparison with the wild type. The production of H_2O_2 as a result of the polymerization of HGA could explain the increased catalase and peroxidase activity as it has been shown previously that both peroxidase and catalase activity in *Pseudomonas* can be directly or indirectly induced by the presence of H_2O_2 (Frederick *et al.*, 2001).

While the pigment produced by wild type and mutant F6-HDO is very different in colour, the analysis by NMR and FT-IR indicated a minor difference between the pigments (Fig. 4). Previous studies on melanin production by *Bacillus thuringiensis* and *B. cenocepacia* showed similar results (Aghajanyan *et al.*, 2005; Keith *et al.*, 2007).

In the wild-type and mutant strains of *P. putida* F6 ferulic acid is a better inducer of tyrosinase activity than tyrosine (McMahon *et al.*, 2007). Interestingly, the difference between tyrosinase activity in induced (ferulic acid) and uninduced cell extracts of the wild type and mutant F6-TR is similar (46.6-fold wild type and 36.8-fold F6-TR). This suggests that the response to induction is similar, but the level of tyrosinase activity in the mutant is higher than wild-type cells. Because the mutant F6-TR exhibits a higher level of tyrosinase activity compared with the wild-type strain under all conditions tested (Table 2), it would appear that increased tyrosinase activity is responsible for the increased pigment production. As we used crude cell extracts to study tyrosine oxidation, it is possible that other enzymes could be responsible for increased pigment production. These enzymes could be laccase and or a phenol hydroxylase (NADH dependent). Laccase and tyrosinase have previously been purified from *P. putida* F6, but laccase does not have activity towards tyrosine (McMahon *et al.*, 2007). However, L-DOPA formed as a result of L-tyrosine oxidation could have acted as a substrate for laccase and thus increased pigment production could be due to the combined activity of both enzymes. We have tested the laccase activity in crude cell extracts of wild-type and mutant strains of *P. putida* F6 using syringaldazine as a substrate as it cannot act as a substrate for tyrosinase (Lucas-Elıo *et al.*, 2002). The laccase activity in crude cell extracts of wild-type and mutant strains of *P. putida* F6 were almost identical (Table 3). While the increased melanin production can still be due to the action of both enzymes, only the tyrosinase activity is increased in the mutant strain. A phenol hydroxylase may exist in *P. putida* F6 and may also contribute to the formation of melanin in whole cells. However, based on these collective observations, it is reasonable to assume that the tyrosinase enzyme known to exist in *P. putida* F6 is a major contributing factor for increased melanin formation in the mutant.

Mutant *P. putida* F6-TR is disrupted in a gene with homology to a TR. This has resulted in a mutant that oxidizes tyrosine at higher rates (Table 2) and hyperpigmentation of colonies on agar plates (Fig. 1). While the exact role of the TR is not known, it is clear that tyrosinase activity is negatively regulated in *P. putida* F6, and overcoming that regulation results in a strain with hyperpigmentation. The lower ability to hyperproduce melanin in liquid cultures is probably linked to a lower rate of tyrosine uptake. In tyrosine uptake experiments, we observed an eightfold lower consumption rate of tyrosine by mutant F6-TR compared with the wild-type strain in liquid cultures. This would support the idea of a wider effect of this mutation on melanin production by cells in suspension. In *E. coli*, tyrosine-specific transport and control system has been described in detail (Wookey *et al.*, 1984; Kwok *et al.*, 1995). TyrR regulator protein has been described that represses and activates transcription of operons required for tyrosine, phenylalanine and tryptophan biosynthesis and uptake (Kwok *et al.*, 1995). Similarly, TyrR TR can be identified in genomes of *Pseudomonas* strains, for example in *P. putida* KT2440 (GenBank accession number PP_4489) (Nelson *et al.*, 2002). However, the TR disrupted by the transposon in mutant F6-TR has no significant sequence similarity to PP_4489. Given that the mutation was observed in a TR in *P. putida* F6 it is possible that a complex regulatory system for tyrosinase expression and tyrosine uptake exists in

P. putida F6. However, polar effects of transposon mutagenesis in mutant F6-TR may also explain the increased tyrosinase expression and decreased tyrosine uptake.

Mutant F6-HDO is disrupted in a gene that has homology with HDO. HGA has been reported to be involved in eumelanin synthesis in a number of bacterial strains (Kotob *et al.*, 1995; Carreira *et al.*, 2001; Keith *et al.*, 2007). Kang *et al.* (2008) have recently reported pigmentation of *Pseudomonas chlororaphis* O6 disrupted in HDO while the authors have not reported the presence of tyrosinase in this strain. *Pseudomonas putida* F6 mutant HDO achieved a final biomass 1.2-fold lower than the wild-type strain with tyrosine as the sole source of carbon and energy, indicating that the tyrosine metabolic pathway through HGA is not critical for growth of *P. putida* F6 but that both are needed for optimal growth of *P. putida* F6 on tyrosine. The metabolism of tyrosine to HGA is known to proceed via PHPPA and PHPA (Coon *et al.*, 1994; David *et al.*, 1996). However, *P. putida* F6 wild type and mutant F6-HDO grew well with PHPPA as the sole source of carbon and energy and did not produce pigment when incubated with this substrate, indicating that it is unlikely to be an intermediate in melanin synthesis in *P. putida* F6. While wild type and mutant F6-HDO grew equally well, only the former produced a pigment when supplied with PHPA as a sole source of carbon. This would suggest that PHPA, a known substrate for tyrosinase (O'Connor *et al.*, 2001), is also not likely to be an intermediate in tyrosine metabolism via HGA in *P. putida* F6.

In conclusion, *P. putida* F6 mutant F6-TR overproduces melanin due to higher levels of tyrosine oxidation compared with the wild-type strain, while mutant F6-HDO produces a melanin based on accumulation of HGA in the growth medium. While both melanins offer increased resistance to UV light, the accumulation of the HGA-based polymer offers additional increased resistance to H_2O_2.

Acknowledgement

This research was funded by the Irish Research Council for Science Engineering and Technology (IRCSET) grant number RS/2003/64.

Authors' contribution

The authors L.B.M. and J.N.-R. contributed equally to this work.

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