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# A Putative ABC Transporter, HatABCDE, Is among Molecular Determinants of Pyomelanin Production in *Pseudomonas aeruginosa*▽†

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**Pyomelanin overproduction is a common phenotype among *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis and urinary tract infections. Its prevalence suggests that it contributes to the persistence of the producing microbial community, yet little is known about the mechanisms of its production. Using transposon mutagenesis, we identified factors that contribute to melanogenesis in a clinical isolate of *P. aeruginosa*. In addition to two enzymes already known to be involved in its biosynthesis (homogentisate dioxygenase and hydroxyphenylpyruvate dioxygenase), we identified 26 genes that encode regulatory, metabolic, transport, and hypothetical proteins that contribute to the production of homogentisic acid (HGA), the monomeric precursor of pyomelanin. One of these, PA14_57880, was independently identified four times and is predicted to encode the ATP-binding cassette of an ABC transporter homologous to proteins in *Pseudomonas putida* responsible for the extrusion of organic solvents from the cytosol. Quantification of HGA production by *P. aeruginosa* PA14 strains missing the predicted subcomponents of this transporter confirmed its role in HGA production: mutants unable to produce the ATP-binding cassette (PA14_57880) or the permease (PA14_57870) produced substantially less extracellular HGA after growth for 20 h than the parental strain. In these mutants, concurrent accumulation of intracellular HGA was observed. In addition, quantitative real-time PCR revealed that intracellular accumulation of HGA elicits upregulation of these transport genes. Based on their involvement in *h*omogentisic *a*cid *t*ransport, we rename the genes of this operon *hatABCDE*.**

*Pseudomonas aeruginosa* is a metabolically versatile, opportunistic pathogen that is a major cause of life-threatening infections in patients with burn wounds, compromised immunity, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) (23, 41). A major contributor to *P. aeruginosa*'s pathogenicity is its remarkable genomic plasticity, which often results is a wide range of phenotypic variation among isolates obtained from both acute and chronic infections. These phenotypes include small colony variant formation (24), alginate overproduction (36), hyperpigmentation (22), autoaggregation (13), and autolysis (64). Many of these phenotypes evolve as infections progress, and most have been ascribed to "loss-of-function" genome diversification that promotes long-term survival in the host environment (54). In this regard, recent studies have stimulated interest in another example of a loss-of-function phenotype, the mutation or deletion of *hmgA*, which encodes the homogentisate 1,2-dioxygenase enzyme. The absence of this protein leads to the accumulation and subsequent export of homogentisic acid (HGA), which ultimately aggregates into the pyomelanin polymer that manifests as a reddish brown pigmentation of *P. aeruginosa* colonies and their surrounding milieu (Fig. 1A) (5, 49).

Production of pyomelanin (and other forms of melanin) has been described to occur in a wide range of bacterial species, including *Aeromonas* (4), *Azotobacter* (51), *Azospirillum* (50), *Bacillus* (3), *Legionella* (8), *Marinomonas* (33), *Micrococcus* (40), *Mycobacterium* (45), *Proteus* (1), *Rhizobium* (12), *Shewanella* (61), *Sinorhizobium* (38), *Streptomyces* (67), and *Vibrio* (63) species. Notably, isolates of other bacterial species associated with chronic infections of the CF lung, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*, can also be melanogenic (28, 58), suggesting a possible role for this pigment in the establishment and/or persistence of infection. Some genera produce melanin under normal conditions via polyphenol oxidases or laccases, while others synthesize the pigment only in response to specific environmental conditions (17, 35). Many species, however, including *P. aeruginosa*, overproduce pyomelanin as a result of a point mutation in *hmgA* or large chromosomal deletions of loci containing the homogentisate operon (2, 19). While these genetic variations have been frequently reported, there is little understanding of the competitive advantage, if any, that this pigment confers to the producing bacterium.

Proposed roles for pyomelanin include the enhancement of bacterial surface attachment (20), extracellular electron transfer (61), iron reduction/acquisition (8), induction of virulence factor expression (63), heavy metal binding (21), and protection from environmental stress (11, 28, 32, 44, 53, 65). A protective role has also been proposed to occur in *P. aeruginosa* PA14, where pyomelanin was shown to contribute to the persistence of an overproducing strain in a chronic CF infection model in mice (49). However, given that melanogenic isolates have been recovered from laboratory-grown communities of *P. aeruginosa* PAO1 (5, 56), it is probable that pyomelanin plays other roles in addition to protection against host defense mechanisms.

As a first step toward gaining a better understanding of

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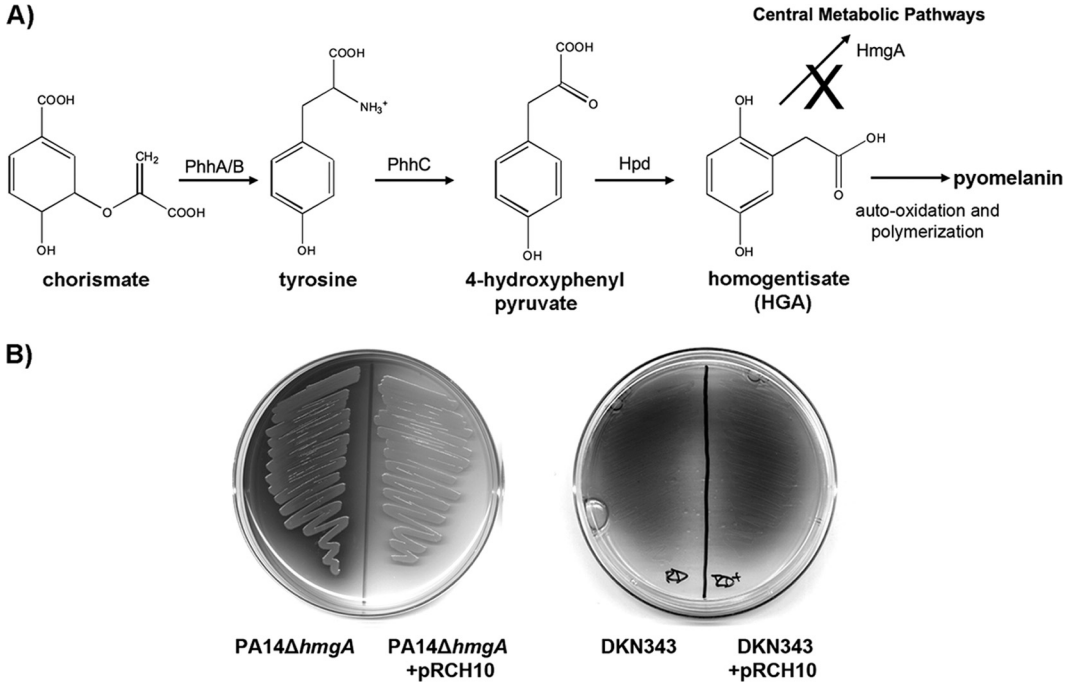


FIG. 1. Pyomelanin production by the PA14 Δ*hmgA* and DKN343 strains. (A) Homogentisate pathway for the catabolism of chorismate and aromatic amino acids. Enzyme names are shown above the arrows for each step. A mutation or deletion of the *hmgA* gene (encoding homogentisate 1,2-dioxygenase) leads to the accumulation of pyomelanin. (B) Pyomelanin overproduction by the PA14 Δ*hmgA* mutant is abolished when complemented with an intact *hmgA* gene. Complementation of a melanogenic clinical *P. aeruginosa* isolate, DKN343, with *hmgA* results in no phenotypic change, indicating that other factors contribute to its pigmentation.

pyomelanin function, we sought to identify the molecular determinants of its production in *P. aeruginosa*. By screening a library of pTnTet/minimariner transposon mutants of a pyomelanin-overproducing clinical isolate for alterations in pigmentation, we identified several genes whose products are involved in tyrosine catabolism, central metabolic pathways, nucleotide biosynthesis, regulation, and membrane transport, in addition to hypothetical proteins of unknown function. We chose to further characterize the gene identified most frequently in our screen, one annotated as encoding a putative ATP-binding cassette of an ABC-type transporter. Here, we demonstrate that this transporter is involved in HGA transport and the subsequent extracellular formation of pyomelanin.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids that were used in this study are listed in Table 1. *Pseudomonas aeruginosa* and *Escherichia coli* were grown in lysogeny broth (LB) at 37°C unless otherwise specified. When necessary, medium was supplemented with gentamicin at 20 μg/ml (*E. coli*) or 100 μg/ml (*P. aeruginosa*), ampicillin at 100 μg/ml (*E. coli*) or 300 μg/ml (*P. aeruginosa*), carbenicillin (300 μg/ml), tetracycline (300 μg/ml), or chloramphenicol (30 μg/ml). *E. coli* strain β2155 was supplemented with 60 μM diaminopimelic acid (DAP). For homogentisate quantification experiments, *P. aeruginosa* was grown in morpholinepropanesulfonic acid (MOPS)-buffered medium (50 mM MOPS [pH 7.2], 93 mM $NH_4Cl$, 43 mM NaCl, 3.7 mM $KH_2PO4$, 1 mM $MgSO_4$, and 3.5 μM $FeSO_4 \cdot 7H_2O$) supplemented with 20 mM glucose, 5 mM tyrosine, and 10 mM ascorbic acid (MTA medium). *Saccharomyces cerevisiae* was grown in YPD broth (2% yeast extract, 4% peptone, and 4% dextrose), and selections were performed using a uracil dropout medium.

**Transposon mutagenesis.** A hyperactive mariner transposon was used for the mutagenesis (9). The transposon delivery vector pTnTet (National Center for Biotechnology Information accession no. AY115560) was introduced by transformation into the *E. coli* β2155 donor bacterial strain (15, 16), which can grow only in the presence of DAP. The hypermelanogenic strain DKN343 and β2155 (carrying pTnTet) were grown overnight in LB medium and LB-chloramphenicol medium containing 60 μM DAP, respectively. Cells were combined in a donor-to-recipient ratio of 5:1, centrifuged at 8,000 × *g* for 5 min, resuspended in 25 μl of LB-DAP, and spotted onto LB agar. Mating proceeded for 8 h at 37°C, at which point cells were harvested and resuspended in 1 ml of LB. One hundred microliters of culture was then plated on LB-tetracycline agar, and transposon mutants were allowed to grow for 24 h at 37°C. Colonies were randomly picked, transferred to 96-well plates containing 100 μl LB-tetracycline, and grown overnight at 37°C. One hundred microliters of a 40% (vol/vol) glycerol solution was then added to each culture, and plates were stored at −80°C for subsequent screening.

To identify potential gene products involved in melanogenesis, frozen stocks of transposon mutants were inoculated into 96-well deep-well plates (Greiner Bio-One, Inc.) containing 1 ml LB-tetracycline medium. Plates were incubated at 37°C with shaking at 250 rpm for 36 h and screened for pyomelanin production. Strains showing a decrease in pyomelanin production (in duplicate assays) were then grown for an additional 48 h in LB. Strains with no detectable growth defect in LB medium (compared to the level for the parent strain) were selected for further analysis.

**Arbitrary PCR and mutant sequencing.** For sequence analysis of transposon junctions, an arbitrary-PCR-based technique (6, 43) was used to identify sequences flanking the transposon insertion sites. The arbitrary method involved two rounds of reactions, with the first using a primer unique for the minimariner transposon and one degenerate primer (primer pair 1) (see Table S1 in the supplemental material). The degenerate primer contained a 7-nucleotide sequence at the 3′ end that commonly occurs in *P. aeruginosa*. The second round included nested primers (primer pair 2) unique to the transposon as well as the 5′ end of the arbitrary primer for specific amplification of PCR products obtained in the first round. Sequences of PCR products were determined by the Biopolymers Laboratory in the Massachusetts Institute of Technology Center for Cancer Research. Sequences were then either submitted to a BLAST search of the *Pseudomonas aeruginosa* PA14 genome on the *Pseudomonas* website (http://www.pseudomonas.com/) or submitted to a GenBank search to identify insertion loci.

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristic(s) | Reference or source |
|---|---|---|
| Strains | | |
| *P. aeruginosa* | | |
| PA14 | Clinical isolate UCBPP-PA14 | 47 |
| PA14 *hmgA*::MAR2xT7 | PA14 with a mariner transposon insertion in *hmgA* | 31 |
| PA14 Δ*hmgA* | PA14 with a deletion of *hmgA* | This study |
| PA14 Δ*hmgA* Δ*hatA-E* | PA14 Δ*hmgA* with a deletion of operon *hatABCDE* | This study |
| PA14 Δ*hmgA* Δ*hatA* | PA14 Δ*hmgA* with a deletion of PA14_57880 (*hatA*) | This study |
| PA14 Δ*hmgA* Δ*hatB* | PA14 Δ*hmgA* with a deletion of PA14_57870 (*hatB*) | This study |
| PA14 Δ*hmgA* Δ*hatC* | PA14 Δ*hmgA* with a deletion of PA14_57850 (*hatC*) | This study |
| PA14 Δ*hmgA* Δ*hatD* | PA14 Δ*hmgA* with a deletion of PA14_57840 (*hatD*) | This study |
| PA14 Δ*hmgA* Δ*hatE* | PA14 Δ*hmgA* with a deletion of PA14_57830 (*hatE*) | This study |
| PA14 Δ*phhC* | PA14 with a deletion of *phhC* | This study |
| DKN343 | Pyomelanin-overproducing clinical isolate | M. Schobert, Hannover Medical School |
| *E. coli* | | |
| β2155 | *thrB1004 pro thi strA hsdS lacZ*ΔM15 (F′ *lacZ*ΔM15 *lacI*$^q$ *traD36 proA*$^+$ *proB*$^+$) *dap*::*erm recA*::RP4-2-*tet*::Mu-km λ[ι] *pir*; Erm$^r$ Tet$^r$ Kan$^r$ | 15 |
| DH5α | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lacU169* (ϕ80*lacZ*ΔM15) | Bethesda Research Laboratories |
| *S. cerevisiae* InvSc1 | Diploid *ura3-52*/*ura3-52* | Invitrogen |
| Plasmids | | |
| pTnTet | pSC123 with the Km$^r$ cassette replaced with the MluI fragment carrying a Tet$^r$ cassette from pBSL199. | 48 |
| pMQ30 | *ori* ColE1 CEN6 ARSH4 *aacC1 lacZ*α *oriT* URA3 *sacB* | 52 |
| pMQ64 | *ori* ColE1 PoriV *rep gacC1 lacZ*α *oriT* URA3 2μm | 52 |
| pRCH1 | 2-kb fusion PCR fragment containing Δ*hmgA* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* mutant | This study |
| pRCH2 | 2-kb fusion PCR fragment containing Δ*hatABCDE* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatABCDE* mutant | This study |
| pRCH3 | 2-kb fusion PCR fragment containing Δ*hatA* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatA* mutant | This study |
| pRCH4 | 2-kb fusion PCR fragment containing Δ*hatB* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatB* mutant | This study |
| pRCH5 | 2-kb fusion PCR fragment containing Δ*hatC* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatC* mutant | This study |
| pRCH6 | 2-kb fusion PCR fragment containing Δ*hatD* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatD* mutant | This study |
| pRCH7 | 2-kb fusion PCR fragment containing Δ*hatE* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*hmgA* Δ*hatE* mutant | This study |
| pRCH8 | 2-kb fusion PCR fragment containing Δ*phhC* cloned into the BamHI/EcoRI site of pMQ30; used to make the PA14 Δ*phhC* mutant | This study |
| pRCH10 | 1.5-kb *hmgA* complementation fragment cloned into pMQ64; used to complement the PA14 Δ*hmgA* mutant | This study |
| pRCH11 | 3.3-kb *hepABCDE* fragment cloned into pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatABCDE* mutant | This study |
| pRCH12 | 1.3-kb fragment containing *hatA* and its promoter cloned into the BamHI/EcoRI site of pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatA* mutant | This study |
| pRCH13 | 1.3-kb fragment containing *hatB* and its promoter cloned into pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatB* mutant | This study |
| pRCH14 | 967-bp fragment containing *hatC* and its promoter cloned into pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatC* mutant | This study |
| pRCH15 | 1.1-kbp fragment containing *hatD* and its promoter cloned into pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatD* mutant | This study |
| pRCH16 | 794-bp fragment containing *hatE* and its promoter cloned into pMQ64; used to complement the PA14 Δ*hmgA* Δ*hatE* mutant | This study |

**Generation of deletion mutants.** We generated an unmarked deletion mutant of strain PA14, designated PA14_38510 (*hmgA*), and a double mutant, the Δ*hmgA* ΔPA14_57880-57830 mutant, which also contained an unmarked deletion of genes PA14_57880 through PA14_57830 (*hatABCDE*). For the *hmgA* mutant, the ~1-kb sequences flanking the *hmgA* gene were amplified using primer pairs 3 and 4 (see Table S1 in the supplemental material). These flanking fragments were combined, and deletion plasmid pRCH1 was constructed using yeast homologous recombination methods (52). Briefly, 0.5 ml of *S. cerevisiae* was centrifuged at low speed for 30 s, washed with Tris-EDTA (TE) buffer, and resuspended in 0.5 ml of "Lazy Bones" transformation solution (40% polyethylene glycol 3350 [PEG 3350], 0.1 M lithium acetate, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5). To this mixture, we added 20 μl of boiled salmon sperm DNA (2 mg/ml), 5 μl of BamHI/EcoRI linearized pMQ30 vector, and 20 μl of each oligonucleotide described above (TE buffer was used in place of PCR-generated amplicons as a control). This mixture was vortexed for 1 min, incubated overnight at room temperature, heat shocked at 42°C, and centrifuged at low speed for 30 s to remove the transformation mixture. Cells were resuspended in TE and plated onto uracil dropout selection agar. Uracil-prototrophic colonies arising after 72 h of growth were pooled, and the resulting deletion plasmid, pRCH1, was liberated and was subsequently introduced by transformation into chemically

competent *P. aeruginosa* PA14 cells prepared by a rapid sucrose-based method (10). Single recombinants (merodiploid containing the intact *hmgA* gene and the deletion) were selected on LB gentamicin agar. Potential deletion mutants were generated by selecting for a resolved merodiploid (double recombinant) by identifying strains that grew in the presence of 10% sucrose. Double recombinants were confirmed by PCR to determine that the *hmgA* gene had been deleted, and one was chosen for further study. To construct the double mutant, an unmarked deletion of PA14_57880-PA14_57830 (*hatABCDE*) was constructed in the same way in the Δ*hmgA* background, using primer pairs specific for the transporter operon (see Table S1 in the supplemental material). With this same approach, individual gene (*hatA-hatE*) deletion mutants in the Δ*hmgA* background and a PA14 Δ*phhC* strain were also created.

**Complementation.** For complementation constructs, the *hmgA* and *hatABCDE* genes were PCR amplified from *P. aeruginosa* PA14 chromosomal DNA. These fragments included ~300 bp upstream of the start codons, so that native promoters would be incorporated into the complementing construct. PCR products were incorporated into shuttle vector pMQ64 using the homologous recombination methods described above. The resulting plasmids, pRCH10 and pRCH11, were then introduced by electroporation into chemically competent cells of their corresponding deletion mutant. Recipients were selected for on LB agar supplemented with gentamicin (100 μg $ml^{-1}$). For complementation of individual ABC transporter protein mutants, genes PA14_57880 through PA14_57830 were amplified, combined with the promoter region immediately upstream of the transporter operon, and recombined into pMQ64. The resulting plasmids (pRCH12 to -16) were mobilized into their corresponding mutants. In each case, vector pMQ64 without a complementing construct was used as a control.

**High-performance liquid chromatography (HPLC) quantification of HGA and tyrosine.** For extracellular homogentisate quantification, cultures were grown in 10-ml volumes of MTA over a period of 36 h. At various time points, 1 ml of culture was spun at 8,000 × *g* (to remove bacterial cells), and 700 μl of supernatant was further centrifuged through Spin-X filter columns (0.2-μm filter pore size; Corning) at 10,000 × *g*. Two hundred microliters of filtrate was loaded directly onto a Waters Symmetry $C_{18}$ reverse-phase column (5-μm particle size; 4.6 by 250 mm) in a Beckman SystemGold set up with a photodiode array detector. Filtrates were separated in a gradient of water-0.01% trifluoroacetic acid (TFA) (solvent A) to acetonitrile-0.01% TFA (solvent B) at a flow rate of 1 ml $min^{-1}$ with the following method: a linear gradient from 0 to 15% solvent B from 0 to 2 min, a linear gradient to 83% solvent B from 2 to 22 min, and then a linear gradient to 0% solvent B from 22 to 24 min. The duration of the chromatographic separations was 30 min. The average retention times for tyrosine and homogentisate were 8.2 min and 9.2 min, respectively.

For intracellular HGA quantification, cells were also grown in MTA medium and harvested in parallel with the extracellular samples. Intracellular contents were harvested using a previously described methanol extraction protocol (34, 66). Ten-milliliter aliquots were centrifuged at 8,000 × *g* for 5 min, the supernatant was discarded, and each pellet was resuspended in 300 μl of 80% (vol/vol) methanol stored at −80°C. Cell suspensions were incubated for 15 min at −80°C and centrifuged at 13,000 × *g* for 5 min at 4°C. This time, supernatant was stored in a separate centrifuge tube, and the leftover pellets were twice subjected to a similar treatment in 200 μl of 80% methanol for a total of 700 μl of extract. Methanol was evaporated, and the remaining extract was resuspended in 300 μl of MTA. Extracts were loaded onto Spin-X filter columns and centrifuged at 13,000 × *g* for 30 s. Filtrates were stored at −80°C for further analysis. These samples were separated using the flow rates and gradients described above.

For both sets of samples, standard solutions consisting of HGA and tyrosine at known concentrations in MTA were prepared and analyzed using the same methods. The area under each peak was calculated in absorbance units in the 278-nm (tyrosine) and 290-nm (HGA) channels using System Gold 32 Karat software.

**qRT-PCR.** RNA was extracted from 0.5 ml of cells at mid-exponential (4-h), early stationary (8-h), and late stationary (20-h) growth. One volume of culture was mixed with two volumes of Bacterial RNAProtect (Qiagen), incubated for 5 min at room temperature, and centrifuged for 10 min at 5,000 × *g*. Cell pellets were frozen at −80°C, and RNA was then extracted using an RNeasy minikit (Invitrogen), according to the manufacturer's instructions, including the optional DNase on-column treatment. A second, off-column Turbo DNase (Ambion, Inc.) treatment was also performed. Extracted RNA was then used as a template for an iScript (Bio-Rad) reverse transcriptase reaction in accordance with the manufacturer's protocol, generating cDNA. The cDNA was used as a template for quantitative PCR (7500 fast real-time PCR system; Applied Biosystems), using iTaq SYBR green Supermix with ROX reaction mixture (Bio-Rad). Samples were assayed in triplicate. The signal was standardized to *recA* using the following equation: relative expression = $2(T_{C\ \text{standard}} - T_{C\ \text{sample}})$, where cycle time ($T_C$) was determined automatically by the 7500 real-time PCR system software program (Applied Biosystems). Expression values are reported relative to the expression value of the same gene, in the same strain, in exponential growth phase. The primers used for quantitative real-time PCR (qRT-PCR) are presented in Table S1 in the supplemental material.

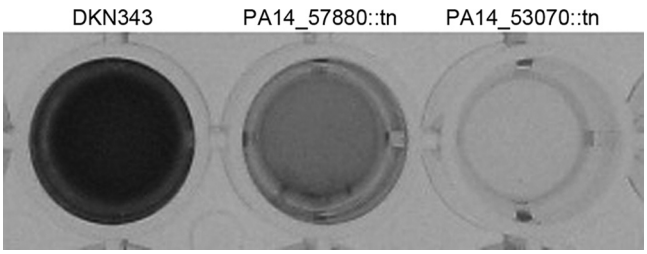


FIG. 2. Identification of genes involved in pyomelanin production. Transposon insertions in genes involved in melanogenesis were identified by defective pigment production. Mutants showing a lack of pyomelanin (PA14_53070::tn; 4-hydroxyphenylpyruvate dioxygenase) or reduced production (PA14_57880::tn; a putative ATP-binding cassette) relative to the level for DKN343 were selected for further analysis.

## RESULTS

**Initial molecular characterization of a melanogenic clinical isolate.** Our first objective was to identify the genetic determinants that contribute to the hyperproduction of pyomelanin. Previous studies reported that only one Mar2xT7 transposon mutant from the PA14 library (31), the *hmgA*::Mar2xT7 strain, produced a reddish brown pigment when grown on LB agar. In addition, Ernst et al. observed that a pyomelanin-producing CF isolate contained a large, 119-kbp chromosomal deletion in the locus containing the *hmgA* gene (19). In both instances, pyomelanin accumulation could be attributed to the lack of a functional homogentisate 1,2-dioxygenase (Fig. 1A). Based on these accounts, we PCR amplified and sequenced the *hmgA* gene in strain DKN343, a melanogenic clinical isolate of *P. aeruginosa*. In addition to a 200-bp upstream promoter region (2), *hmgA* in DKN343 showed a sequence to that of the wild-type PA14 strain, which does not produce pyomelanin when grown in LB. When complemented in *trans* with pRCH10, which contains an intact *hmgA* gene, DKN343 pyomelanin production was not eliminated as it was in a PA14 Δ*hmgA* mutant (Fig. 1B). These observations suggested that a lack of HmgA activity (as in the lone melanogenic PA14 library mutant) is not the cause for the reddish brown pigmentation of strain DKN343. Thus, factors involved in the overproduction of pyomelanin in *P. aeruginosa* in addition to those previously reported (19, 49) remained to be determined.

**Mutant screen.** To identify these factors, we utilized a transposon mutagenesis approach targeting defective pigmentation in strain DKN343 (Fig. 2). Out of 13,000 mutants screened, 84 exhibited reduced (e.g., PA14_57880, which encodes a putative ATP-binding cassette) or abolished (e.g., PA14_53070, encoding hydroxyphenylpyruvate dioxygenase) pigment production relative to the level for the parent strain. These mutants were screened for general growth defects, after which 48 were identified as potential candidates for the identification of gene products involved in the production of pyomelanin. DNA se-

TABLE 2. Pyomelanin-deficient DKN343 minimariner transposon mutants identified in this study[a]

| Protein group and locus of insertion (PA14 homolog) | Gene name | Function or product name | Polar effect possible | No. of multiple hits | Phenotype |
|---|---|---|---|---|---|
| Amino acid metabolism | | | | | |
| PA14_52990 | *phhA* | Phenylalanine 4-monooxygenase | N | 2 | No pigment |
| PA14_53000 | *phhB* | Pterin-4-alpha-carbinolamine dehydratase | Y | | No pigment |
| PA14_53010 | *phhC* | Aromatic amino acid aminotransferase | N | 2 | No pigment |
| PA14_53070 | *hpd* | 4-Hydroxyphenylpyruvate dioxygenase | N | 4 | No pigment |
| Nucleotide biosynthesis | | | | | |
| PA14_05250 | *pyrC* | Nucleotide metabolism | Y | 2 | Reduced |
| PA14_24640 | *pyrD* | Dihydroorotate dehydrogenase | Y | | Reduced |
| PA14_70370 | *pyrE* | Orotate phosphoribosyltransferase | Y | 2 | Reduced |
| PA14_26890 | *pyrF* | Orotidine 5′-phosphate decarboxylase | N | | Reduced |
| PA14_62910 | *carB* | Carbamoyl phosphate synthase | Y | | Reduced |
| Transcription and regulation | | | | | |
| PA14_52980 | *phhR* | Transcriptional regulator | N | 2 | No pigment |
| PA14_62540 | *cbrB* | Two-component response regulator | Y | | Reduced |
| PA14_67680 | *ntrC* | Two-component response regulator | N | | Reduced |
| PA14_56980 | *crc* | Catabolite repression control protein | N | | Reduced |
| PA14_57940 | *rpoN* | RNA polymerase factor sigma 54 | N | | Reduced |
| PA14_10770 | | Putative sensor/response regulator hybrid | N | | Reduced |
| PA14_29590 | | Putative transcriptional regulator | N | | Reduced |
| Membrane proteins | | | | | |
| PA14_52400 | *kup* | Potassium uptake protein | N | | Reduced |
| PA14_67450 | *blc* | Outer membrane lipoprotein | Y | | No pigment |
| PA14_57880 | | ABC transporter ATP-binding protein | Y | 4 | Reduced |
| Other or unknown function | | | | | |
| PA14_60330 | *ispH* | 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase | N | | Reduced |
| PA14_38480 | *gnyA* | Geranoyl-CoA carboxylase | Y | | Reduced |
| PA14_53260 | | Hypothetical protein | N | | Reduced |
| PA14_25730 | | Hypothetical protein | Y | | No pigment |
| PA14_49050 | | Hypothetical protein | N | | No pigment |
| PA14_27390 | | Hypothetical protein | Y | | Reduced |
| PA14_22260 | | Hypothetical protein | Y | | Reduced |

[a] Eight mutants were sent for sequencing twice and were unable to generate usable sequence data. Three mutants had usable sequences, but no hits were found in any of the *P. aeruginosa* databases. N, no; Y, yes; CoA, coenzyme A.

quences (~200 to 400 bp) flanking the transposon insertion junctions were then determined by arbitrarily primed PCR, which showed insertions mapped to genes predicted to encode various classes of proteins (Table 2). These included amino acid metabolic enzymes (some of which, as expected, are involved in tyrosine catabolism) (Fig. 1A), nucleotide biosynthetic proteins (including several *pyr* genes involved in pyrimidine biosynthesis), transcription factors and regulatory proteins (*phhR*, *cbrB*, *ntrC*, *crc*, *rpoN*, PA14_10770, and PA14_29590), membrane proteins (*kup* and *blc*), and several putative proteins of unknown function (PA14_22260, PA14_25730, PA14_27390, PA14_49050, and PA14_53260). Arbitrary PCR of three transposon insertion loci generated sequences with no significant homology to published gene sequences, and we were unable to obtain transposon-flanking sequences for eight additional mutants.

The most frequent site of insertion of the minimariner transposon (hit four times independently) was in a sequence with homology to PA14_57880, the first gene of a five-gene operon in *P. aeruginosa* PA14 (Fig. 3A). Using BLAST to search against other microbial genomes, we found that the proteins encoded by these genes shared significant identity with those encoded by the *ttg2* operon of *Pseudomonas putida*. This operon has been reported to be involved in toluene tolerance (30). The first gene in the PA14 operon (PA14_57880) is predicted to encode an ATP-binding cassette that shares 83.1% amino acid identity with Ttg2A. The associated permease, encoded by PA14_57870, is 80.6% identical to Ttg2B. PA14_57850 encodes a conserved hypothetical protein with 77% identity with Ttg2C and is a predicted "Mce-like" periplasmic protein found widely distributed in Gram-negative bacteria (7). The PA14_57840 protein, also annotated as a putative toluene tolerance protein (72.3% identity with Ttg2D), is a predicted secreted protein with no known function. PA14_57830, the last gene in the operon, exhibits 61.7% identity with Ttg2E and encodes a protein with a predicted STAS domain (an antisigma factor antagonist) thought to contribute to the transport function of the permease. Given the structural similarity between toluene and homogentisic acid (Fig. 3B), we hypothesized that the gene products of the PA14_57880-PA14_57830 operon constitute an ABC-type transport system involved in the transport of HGA across the cytoplasmic membrane (Fig. 3C). Because the PA14 genome was completely available to us whereas that of the DKN343 clinical isolate is not yet complete, we decided to pursue further characterization of these genes in a *P. aeruginosa* PA14 mutant background.

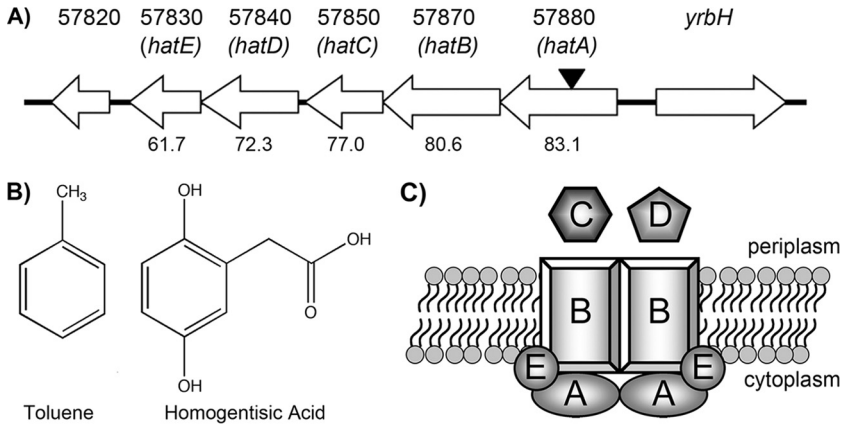


FIG. 3. Characterization of the PA14_57880-PA14_57830 operon. (A) Four mutants with defective pyomelanin production contained transposon insertions in a locus with significant homology to PA14_57880, the first gene of a 5-gene operon encoding a putative ABC-type transporter. We name these genes *hatABCDE* for homogentisic acid transport proteins. Each gene product is homologous to proteins in *P. putida* involved in toluene tolerance. Numbers under the genes indicate the percent amino acid identity (similarity) with the corresponding *P. putida* KT2440 homolog. (B) Structures of toluene and homogentisate. (C) Schematic representing the organization of the putative transport proteins with respect to the cytoplasmic membrane.

**Creation and characterization of *hmgA* and transporter mutants.** To determine whether the gene products encoded by the PA14_57880-PA14_57830 operon are involved in HGA transport, we constructed a series of in-frame deletion mutants. First, we made a clean deletion of *hmgA*, which, as expected, resulted in the production of a reddish brown pigment when the mutant was grown in LB for 24 h (Fig. 1B). We used the *hmgA* mutant as our reference strain because it mimicked the constitutive pyomelanin overproduction phenotype of the DKN343 isolate. Second, we made a clean deletion of the entire PA14_57880-PA14_57830 operon in the *hmgA* background, knocking out all five genes of the putative transporter operon to assess its role in pyomelanin production. Following this, we made clean deletions of each individual gene.

Under aerobic conditions, HGA is rapidly oxidized and polymerized into pyomelanin once exported from the cell, making quantification by HPLC challenging due to polymer heterogeneity and variability in elution time. However, by adding 10 mM ascorbic acid to the culture medium (60), we prevented oxidation of HGA and subsequent polymerization, allowing quantification of the pyomelanin precursor. With the use of this approach, comparison of extracellular HGA production in the Δ*hmgA* and Δ*hmgA* ΔPA14_57880-57830 mutants over a 36-h growth period revealed a role for the products of PA14_57880-57830 in HGA transport (Fig. 4A). Extracellular HGA in PA14 Δ*hmgA* cultures was detected after just 4 h of growth (Fig. 4A), corresponding to mid-exponential phase (see Fig. S1 in the supplemental material). HGA production in this mutant reached its maximum (2.15 ± 0.15 mM) after 20 h of growth and remained constant throughout subsequent time points. In contrast, the Δ*hmgA* ΔPA14_57880-57830 mutant showed decreased concentrations of extracellular HGA throughout the exponential and stationary phases. Deletion of the transporter showed no HGA in the extracellular environment until 12 h (0.42 ± 0.01 mM) and smaller amounts than the Δ*hmgA* mutant after 20 h (0.79 ± 0.06 mM). Further growth showed an eventual restoration of the Δ*hmgA* phenotype, which is consistent with the "reduced," but not abolished, pyomelanin production phenotype observed in the transposon screen of DKN343 (Table 2). Complementation with pRCH11 (containing the entire PA14_57880-57830 operon) resulted in partial restoration of the Δ*hmgA* phenotype, as cells amassed 1.30 ± 0.1 mM HGA in the extracellular culture fluid in contrast to 2.15 ± 0.15 mM in the Δ*hmgA* mutant after 20 h of growth.

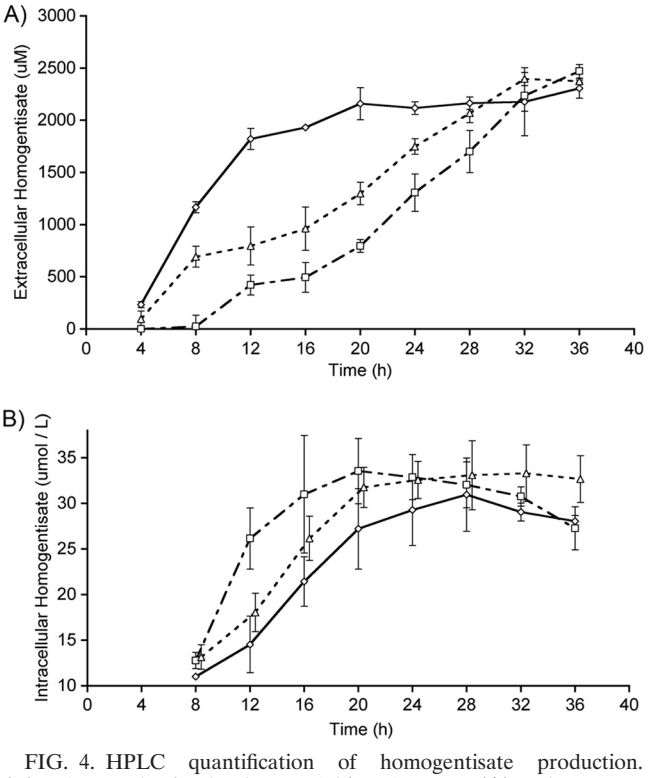


FIG. 4. HPLC quantification of homogentisate production. (A) HGA production by the PA14 Δ*hmgA* mutant (◇) and a mutant containing a full deletion of the PA14_57880-57830 gene cluster (□). Complementation with pRCH11 (△) partially restores the pyomelanin overproduction phenotype. (B) Concurrent quantification of intracellular HGA reveals increased accumulation in the Δ*hmgA* ΔPA14_57880-57830 double mutant (□) relative to the level for the PA14 Δ*hmgA* mutant (◇). Complementation with pRCH11 (△) also results in a partially restored PA14 Δ*hmgA* phenotype. Data represent the averages and standard deviations for biological triplicates.

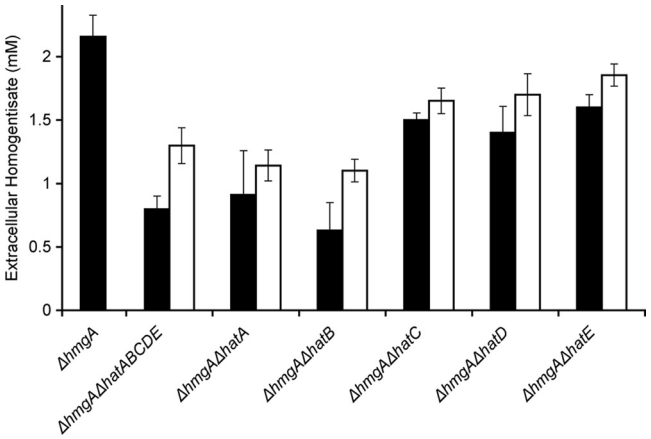


FIG. 5. Functional assessment of single gene deletion transporter mutants. Extracellular HGA quantification for single gene deletion mutants of the PA14_57880 through PA14_57830 genes in the PA14 Δ*hmgA* mutant background (black). Complementation of each transporter mutant with its respective *hat* gene (white) shows a partial restoration of the parent strain (PA14 Δ*hmgA* mutant) phenotype. Data represent the averages and standard deviations for biological triplicates.

Because export of HGA was impeded by deleting the five genes encoding the ABC transporter, we reasoned that intracellular HGA should also increase. To test this, we quantified the levels of intracellular HGA in the transporter mutant relative to those in the parent strain (Fig. 4B). As cells entered late exponential phase (12 h), the transporter mutant accumulated a 1.8-fold increase of HGA (26.2 ± 3.4 μmol/liter) relative to the level for the Δ*hmgA* mutant (14.5 ± 2.7 μmol/liter). Intracellular HGA in the transporter mutant peaked at 20 h (33.5 ± 3.6 μmol/liter), after which the concentrations steadily decreased until late stationary phase (36 h), where they were similar to those found in the Δ*hmgA* mutant. Complementation with pRCH11 resulted in partial restoration of the intracellular HGA concentration found in the Δ*hmgA* mutant, mirroring the partial complementation that we observed in the case of extracellular HGA. These similarities implicate the gene products of the PA14_57880-57830 operon in HGA transport.

To assess whether each gene of the transporter operon contributes to the pyomelanin phenotype, we constructed single gene deletion mutants (PA14_57880 through -57830) of the Δ*hmgA* mutant and quantified extracellular HGA after 20 h of growth (when concentration differences were shown to be most pronounced) (Fig. 4A). Relative to what was found for the Δ*hmgA* mutant, each deletion had a defect in HGA production (Fig. 5). A clean deletion of the ATP-binding component (PA14_57880) and the permease (PA14_57870) resulted in the most-significant defects, equaling that of the full operon deletion strain (i.e., less than 40% of the level for the PA14 Δ*hmgA* mutant). Deletions of the other three genes, PA14_57850, -57840, and -57830, also led to decreased HGA concentrations (70, 65, and 74%, respectively), yet their phenotypes were less pronounced. Complementation of these mutants with their respective gene also resulted in partial restoration of HGA production relative to the level for the parent strain. These altered phenotypes thus implicate each gene product of this operon in the extracellular production of HGA. Based on their role as homogentisic acid transport proteins, we propose that the genes of this operon be named *hatABCDE* (Fig. 3A).

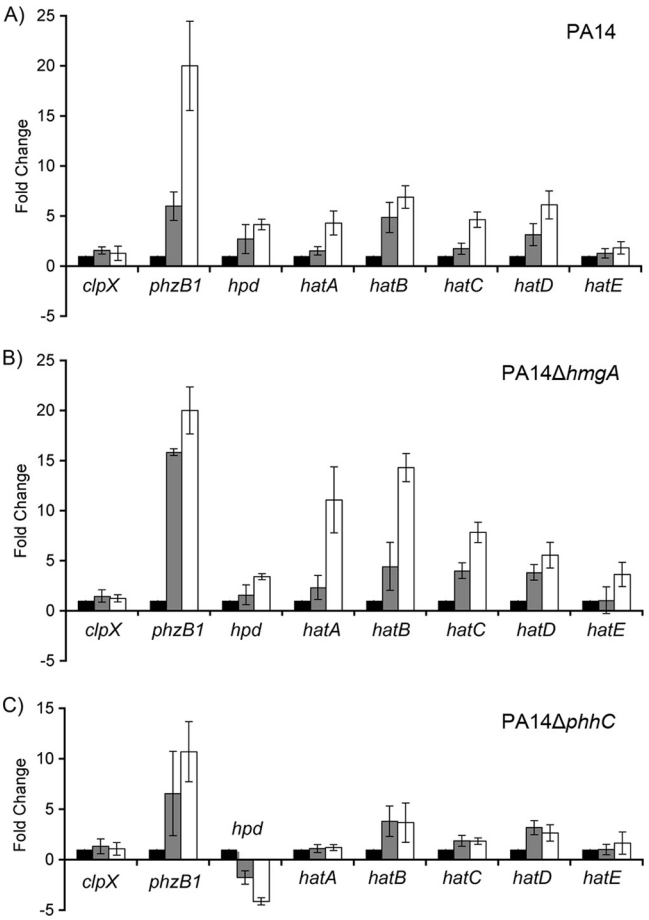


FIG. 6. HGA-dependent regulation of the *hatABCDE* operon. (A) Relative to the level in exponential growth phase (black), the PA14 wild type showed increased expression of the *hat* genes in the early (gray) and late (white) stationary phases. (B) The PA14 Δ*hmgA* mutant showed even greater upregulation of *hatA*, *hatB*, and *hatC*, while the *hatD* and *hatE* expression levels were similar to those in the wild type. (C) The PA14 Δ*phhC* mutant (unable to convert tyrosine into homogentisate) showed little increase in expression in any of the five transporter proteins in stationary phase. Data represent the averages and standard deviations for biological triplicates.

**Regulation of genes in the *hatABCDE* operon is influenced by the accumulation of intracellular homogentisate.** In *P. putida*, there is evidence that the expression of various organic solvent efflux pumps is regulated in response to exposure to toluene (18, 29). Therefore, it seemed possible that the genes in the *hat* operon might be regulated in a similar fashion. To test this, we used qRT-PCR to compare the regulation of the *hatABCDE* genes in the early and late stationary phases relative to the level in exponential phase in response to the accumulation of HGA (Fig. 6). We grew PA14, the Δ*hmgA* mutant, and a third mutant, the Δ*phhC* mutant, which is unable to convert tyrosine into HGA (Fig. 1A), for 4, 8, and 20 h (see Fig. S2 in the supplemental material) and extracted total RNA for qRT-PCR analysis.

In PA14, all five *hatABCDE* genes showed a slight upregu-

lation (4.3-, 6.2-, 4.6-, 6.1-, and 1.8-fold, respectively) in late stationary phase (Fig. 6A) in response to increased tyrosine catabolism, as indicated by an upregulation of *hpd*, which converts 4-hydroxyphenylpyruvate into HGA (Fig. 1A). In contrast, the Δ*hmgA* mutant showed 11- and 14-fold upregulation of *hatA* and *hatB*, respectively, in late stationary phase relative to the levels in exponential phase (Fig. 6B). Since there is no increase in expression of the *hpd* gene in the Δ*hmgA* mutant, the observed increase in *hatAB* expression is likely due to the accumulation of HGA in the cytoplasm as a result of the *hmgA* deletion. There was only a subtle increase in expression of *hatCDE*, suggesting that genes within this cluster might be differentially regulated in response to HGA. Deletions of these three genes also showed a less pronounced HGA export phenotype in the single gene deletion mutants.

While PA14 does not export detectable quantities of HGA outside the cell, we expect that HGA would still be present as an intermediate of tyrosine catabolism. We therefore used the Δ*phhC* mutant (containing a deletion of an aromatic amino acid aminotransferase) as a control to evaluate the specific contribution of HGA to *hatABCDE* expression (Fig. 6C). An *hpd* mutant was not used, since alternative pathways have been reported for the metabolism of hydroxyphenylpyruvate to HGA (55). Despite a significant downregulation of *hpd* (suggesting that 4-hydoxyphenyl pyruvate induces its expression), there were still increased, albeit low, levels of expression of all *hat* genes in stationary phase relative to the levels in exponential growth. That the *hat* operon is still expressed in the absence of HGA suggests that there may be an additional physiological role(s) for the Hat transporter in stationary phase.

**The HatABCDE transporter is not involved in tyrosine uptake.** While our data support an export role for HatABCDE, the *hat* operon also closely resembles the gene structure typical of ABC uptake systems (26). For example, one recent review proposed that homologous transporters in *Xylella fastidiosa* and *Neisseria meningitidis* serve an amino acid uptake role rather than organic solvent transport (37, 39). Another possibility, therefore, was that the Hat transporter might be involved in the import of tyrosine (an HGA precursor) rather than the export of HGA. To test this, we quantified tyrosine depletion after 12, 24, and 36 h of growth, where differences would be expected based on HGA production at these time points. Despite differences in HGA production by the Δ*hmgA* and Δ*hmgA hatABCDE* mutants, there was little difference in tyrosine depletion from the growth medium between PA14 and the two mutant strains (see Fig. S3 in the supplemental material), indicating that HatABCDE is not involved in tyrosine transport.

## DISCUSSION

Pyomelanin confers a characteristic reddish brown pigmentation upon *Pseudomonas aeruginosa* and other organisms when it is excreted into the extracellular milieu. Because little is understood about pyomelanin processing other than for two proteins involved in its biosynthesis (2, 17), we attempted to identify additional genes involved in melanogenesis. Among those identified was a gene predicted to encode a component of an ABC transporter involved in toluene tolerance. Because toluene is structurally similar to the melanin precursor HGA, we hypothesized that this gene might play a role in HGA transport. Further study of this and other homologous genes in PA14 revealed a significant role for this transporter in the excretion of the pyomelanin precursor.

The generation and screening of a transposon library in a pyomelanin-producing clinical isolate revealed a large number of genetic determinants of pyomelanin production that have not been identified using mutagenesis in a nonpigmented background (49). In addition to the mutation in *hmgA* already described to occur in several bacterial species (8, 49, 63), we identified 26 candidate genes. Statistically, genome coverage in our library was unsaturated (~90%), yet we hit each gene in the homogentisate pathway and some genes multiple times. Therefore, we conclude that a substantial number of genes are involved in *P. aeruginosa* melanogenesis (Table 2). To our knowledge, this is the first report of genes other than *hmgA* and *hpd* (17) that contribute to melanogenesis in any bacterium.

As expected, various genes involved in aromatic amino acid catabolism were identified. Constituting a branch of the chorismic acid pathway, the conversion of chorismate into HGA (via phenylalanine and tyrosine) is dependent on PhhA/B, PhhC, Hpd, and the regulatory protein PhhR (Fig. 1A) (25). Thus, a transposon insertion in any of the genes encoding any of these proteins would be expected to generate an identifiable phenotype; we hit all of these genes in our screen. In addition to PhhR, we also found insertions in genes encoding the regulatory proteins NtrC and CbrB (which together govern the utilization of multiple carbon and nitrogen sources in *Pseudomonas* [42]), the catabolite repression control protein Crc, RpoN (which controls amino acid uptake in the enterobacteriaceae [59]), and the putative response regulator proteins PA14_10770 and PA14_29590. These hits suggest that the regulation of pyomelanin production integrates a complex mix of environmental signals.

Though we identified various putative inner and outer membrane proteins, we focused on a gene predicted to encode an inner membrane protein because it was the one we hit most frequently in our screen. Annotated as a "putative organic solvent tolerance protein" and located proximal to other genes that appeared to encode the components of an ABC transporter, we reasoned that this gene and its neighbors might be involved in the transport of HGA. As expected, when we deleted the *hatABCDE* operon in a melanogenic mutant of strain PA14, the extracellular HGA levels were significantly reduced. After an extended stationary-phase incubation, however, differences in extracellular HGA between the transporter mutant and the parent strain were less pronounced, which indicates that HGA can be exported through multiple pathways. Redundant transport systems are known to exist in many bacterial species. For example, in *E. coli*, mutations in transporters other than the AcrAB-TolC system have been found to generate only minor phenotypic effects, owing to the overlapping substrate specificities of multiple transport proteins (57).

In *P. aeruginosa*, the overlapping functionality of membrane transporters might also explain the differences we observed in the individual *hat* gene deletion mutants. If no redundancy in HGA transport existed, we might expect the loss of each *hatABCDE* gene to result in a similar phenotype. However, this was not the case. We observed the greatest defects for the

*hatA* and *hatB* mutants (encoding the ABC-binding cassette and the permease, respectively), equivalent to that observed for the *hat* operon mutant. In contrast, the *hatC*, *hatD*, and *hatE* mutants were only slightly compromised in HGA excretion (25% less than the parent strain). It is possible that other membrane transport proteins can substitute for HatC, -D, and -E and/or that in their absence, other efflux machinery plays a greater role in exporting HGA. While complementation with the *hat* genes restored some of their function, complementation was only partial. This is not unprecedented, however, as several examples exist where the wild type phenotype could not be fully restored in transport mutants by expressing the complementing gene(s) from a plasmid or chromosomal insertion (27, 46). The reasons for this are typically unexplored; in our case, we speculate that expression of the *hat* genes from the pMQ64 plasmid may have interfered with optimal processing of their gene products.

While most ABC export systems are composed of four functional modules, two transmembrane domains and two nucleotide-binding domains (14), the *hat* operon encodes five gene products. Five-gene operons, which often include a gene encoding a periplasmic binding protein (e.g., HatC), are more typical of ABC uptake systems (26). This led us to question whether the Hat system was exporting HGA or importing an HGA precursor. Based on the facts that no differences in tyrosine uptake were observed for the *hatABCDE* mutant, that intracellular concentrations of HGA increased in this strain background, and that *hat* gene expression was induced by HGA, we favor the interpretation that HatABCDE is involved in HGA export.

In conclusion, HatABCDE appears to make a significant contribution to the production of pyomelanin, a pigment thought to aid in chronic infections of the CF lung. Pyomelanin-producing strains are generally considered to be less virulent, though more persistent, and it is thought that the presence of pyomelanin alone increases resistance to oxidative stress (28, 49, 68). It will be interesting to determine whether mutants impaired in melanogenesis, such as the *hat* mutant and others identified in our screen, might be competitively disadvantaged relative to melanogenic strains for long-term survival.

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