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Protective Effects of Pigmentation in Pseudomonas Aeruginosa: Insights on Pyomelanin Production and Inhibition By Ntbc

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PROTECTIVE EFFECTS OF PIGMENTATION IN *PSEUDOMONAS AERUGINOSA*: INSIGHTS ON PYOMELANIN PRODUCTION AND INHIBITION BY NTBC

by

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ABSTRACT

PROTECTIVE EFFECTS OF PIGMENTATION IN *PSEUDOMONAS AERUGINOSA*: INSIGHTS ON PYOMELANIN PRODUCTION AND INHIBITION BY NTBC

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Pyomelanin is an extracellular, reddish-brown pigment produced by several environmental and pathogenic bacteria and fungi. It is derived from the phenylalanine/tyrosine catabolism pathway and is produced when homogentisate (HGA) is secreted from the cell, auto-oxidized, and self-polymerized. Point mutations or chromosomal deletions in *hmgA*, which encodes homogentisate 1,2-dioxygenase, result in the accumulation of HGA and subsequent pyomelanin production. My work showed that the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111 had a chromosomal deletion of *hmgA*, while a second pyomelanogenic clinical isolate, DKN343, had a loss of function mutation in HmgA. The 4-hydroxyphenylpyruvate dioxygenase (Hpd) inhibiting compound 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) has been proposed as a treatment for microbial infections by pyomelanin producers. NTBC inhibits Hpd by binding irreversibly to the ferrous metal center in the active site of the enzyme, which should prevent HGA formation and subsequent pyomelanin production. NTBC reduced pyomelanin production in a dose dependent manner in both PA1111 and DKN343; DKN343, however, was more

resistant to NTBC than PA1111. NTBC resistance studies revealed that the multi-drug efflux pump MexAB-OprM was responsible for the inherent resistance to NTBC observed in DKN343. One of the functions of pyomelanin is resistance to oxidative stress, and treatment of PA1111 with NTBC resulted in increased sensitivity to H_2O_2 compared to the untreated strain. Additionally, reduction in pyomelanin production with NTBC had no effect on PA1111 aminoglycoside minimum inhibitory concentrations, which demonstrated that NTBC could possibly be used in conjunction with antibiotics. Pyomelanin was involved in persistence of *P. aeruginosa* in mouse models of infection, so I investigated macrophage-based killing of pyomelanogenic *P. aeruginosa*. My results showed little difference in bacterial survival during RAW 264.7 infection between pyomelanin producers and non-producers under different test conditions. My work provided some data for the potential development of NTBC as a treatment for pyomelanogenic microbial infections. NTBC shows some promise as a therapeutic agent, but its effectiveness may be limited in organisms with high levels of antimicrobial resistance such as *P. aeruginosa*. Future studies should focus on the prevalence of NTBC resistance in other pyomelanogenic organisms to determine the future applications of NTBC as an anti-microbial therapy.

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Chapter One

Overview of pyomelanin production and anti-virulence treatment in *Pseudomonas aeruginosa*

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative bacterium in the Gamma Proteobacteria class. It is rod shaped and has a single polar flagellum for swimming motility. *P. aeruginosa* is found in diverse ecological environments including soil, organic matter, water, plants, animals, and moist surfaces, and has the ability to form biofilms (1). Additionally, *P. aeruginosa* is an opportunistic pathogen and is a common cause of both acute and chronic infections in individuals who are compromised. *P. aeruginosa* frequently colonizes the lungs of cystic fibrosis (CF) patients and forms biofilms that are very difficult to eradicate, leading to persistent bacterial infections (2). In addition, *P. aeruginosa* is frequently isolated from burn patients (3, 4), where it causes an acute infection. *P. aeruginosa* has been implicated in a variety of other infections, including pneumonia and upper respiratory tract infections, urinary tract infections, eye infections such as keratitis, and infections in various other parts of the body (1).

The persistence of *P. aeruginosa* infections is due, in part, to different mechanisms of resistance to antimicrobial agents. Low outer membrane permeability can prevent antimicrobial agents from entering the cell (5). Chromosomally encoded β-lactamases can also inactivate β-lactam antibiotics (1). Biofilm formation results in increased resistance to various antimicrobial agents (1, 6). This is likely due to the fact that cells in a biofilm display heterogeneous physiology depending on where they are located (7), and slow growing or dormant cells in a biofilm may be a resistant population because many antimicrobial agents are dependent on actively metabolizing cells to be effective (8). Additionally, multidrug efflux pumps have broad specificity for antibiotics

and other antimicrobial agents and function in transporting these compounds from the cell (9). These different resistance mechanisms make it difficult to treat *P. aeruginosa* infections.

P. aeruginosa has 12 different resistance nodulation division (RND) efflux pumps, four of which are known to contribute to antibiotic resistance (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) (1). MexAB-OprM is constitutively expressed in *P. aeruginosa*, and hyperexpression of *mexAB-oprM* has been observed in multi-drug resistant clinical isolates (6). This hyperexpression is frequently due to mutations in *mexR*, which encodes the MexR repressor for the *mexAB-oprM* operon (10). Two other regulators of *mexAB-oprM* transcription have been identified. Mutations in *nalD* can lead to hyperexpression of MexAB-OprM and multi-drug resistance in both laboratory and clinical isolates of *P. aeruginosa* (11). NalD has also been shown to bind to a second *mexAB-oprM* promoter, and a *nalD* mutant showed increased expression from that promoter (12). Novobiocin can bind to NalD, causing it to dissociate from the *mexAB-oprM* promoter, and result in increased *mexAB-oprM* expression (13) Additionally, *nalC* mutants showed hyperexpression of *mexAB-oprM* (14). NalC represses expression of *armR*, which encodes the anti-MexR repressor that binds to MexR and prevents MexR from binding to the *mexAB-oprM* promoter, thereby resulting in hyperexpression of *mexAB-oprM* (15). Pentachlorophenol induces expression of *armR* via modulation of NalC repressor activity (15). Additionally, oxidative stress can increase expression of *mexAB-oprM* (16). Since MexAB-OprM is constitutively expressed, it contributes to intrinsic resistance to antimicrobial agents. It is able to extrude several classes of antibiotics, as well as dyes, detergents, organic solvents,

fatty acid synthesis inhibitors, and homoserine lactone (13), which demonstrates that it has broad substrate specificity.

Other multi-drug efflux pumps can contribute to antibiotic resistance. MexXY is induced in response to aminoglycoside, tetracycline, and macrolide antibiotics, as well as oxidative stress (16, 17). Additionally, clinical isolates can express both MexAB-OprM and MexXY multi-drug efflux pumps (17). Nitrosative stress and chloramphenicol are capable of inducing expression of *mexEF-oprN* (16). Induction of *mexCD-oprJ* expression occurs in response to membrane damaging agents including biocides, dyes, detergents, organic solvents, and cationic antimicrobial peptides such as polymyxin B and colistin (16). MexCD-OprJ and MexEF-OprN are not usually expressed in wild type *P. aeruginosa* under normal laboratory conditions, but are expressed in *nfxB* and *nfxC* mutants, respectively (18). The fact that multi-drug efflux pump expression can be induced by different stressors indicates that these pumps may not have evolved to extrude antimicrobial agents and they likely play a role in stress response.

P. aeruginosa has a large genome, with several variable sections containing genes that are involved in producing a variety of virulence factors, including secreted proteins, toxins, and pigments (19). The four different pigments that can be produced by *P. aeruginosa* are pyocyanin, pyoverdine, pyorubrin, and pyomelanin. Pyocyanin is a blue-green pigment and is produced in 90-95 percent of *P. aeruginosa* isolates (20). This pigment is a phenazine and redox active secondary metabolite that functions as a virulence factor (20). Pyoverdine is a yellow-green pigment that functions as a siderophore for iron acquisition (21). This pigment is widely distributed in *Pseudomonas* species and over 60 pyoverdines have been identified to date (22). Pyorubrin is a red

pigment that is rarely isolated and infrequently studied in *P. aeruginosa* (23, 24). Pyomelanin is a reddish-brown pigment produced in some strains of *P. aeruginosa* (25). This pigment is one of four types of melanin and will be described in detail in this work.

1.2 Types of melanin

Melanin production has been identified in many organisms, including animals, bacteria, and fungi. In general, melanin pigments are composed of polymerized phenolic or indolic compounds with an undefined structure that are dark in color (26, 27). These pigments are negatively charged and hydrophobic with a high molecular weight (26, 27). Melanins are also susceptible to bleaching by oxidizing agents, resistant to concentrated acid, and insoluble in aqueous or organic fluids (26, 27). Melanin synthesis occurs through either phenoloxidases, such as tyrosinases, laccases, and catacholases, or the polyketide synthase pathway (27). In bacteria, tyrosinases are more common in melanin production than laccases (27). Melanin may provide a survival advantage in the various environments encountered by bacteria and fungi and has been associated with virulence of pathogenic organisms in hosts (26, 27).

Four types of melanin can be produced including eumelanin, pheomelanin, dihydroxynaphthalene (DHN) melanin, and pyomelanin. Eumelanin is usually black or brown and is formed from 3,4-dihydroxyphenyalanine (DOPA) by phenoloxidases (26, 28). This type of melanin can be found in bacteria, fungi, and animals (28-32). Pheomelanin is red or yellow and is formed by the incorporation of cysteine with DOPA (26, 28). Animals typically produce this type of melanin (28, 32). DHN-melanin is formed from acetyl-CoA and malonyl-CoA via the polyketide synthase pathway and is usually

black or brown (26, 33). This type of melanin is commonly found in fungi (31). Pyomelanin is generally reddish-brown and is formed from homogentisate during tyrosine catabolism (34). Both fungi and bacteria produce this type of melanin (27, 35).

1.3 Pyomelanin production

Pyomelanin has been reported in many clinical and environmental bacterial and fungal species, including *P. aeruginosa*, *Burkholderia cepacia* complex, *Vibrio cholerae*, *Legionella pneumophila*, *Shewanella algae*, *Bacillus anthracis*, *Aeromonas media*, *Acinetobacter baumannii*, *Sinorhizobium meliloti, Streptomyces coelicolor*, *Alteromonas stellipolaris*, *Aspergillus fumigatus*, *Sporothrix* sp., and *Penicillium chrysogenum* (25, 36-49). In these strains, pyomelanin is either naturally produced in the wild type strain or is the result of mutation or deletion of genes involved in tyrosine catabolism. Natural production of pyomelanin in bacterial and fungal species is important in allowing us to study how the pigment is produced under different environmental conditions. Inactivation of genes involved in pyomelanin production via mutation or deletion can allow us to elucidate the functions of pyomelanin, as well as examine the individual contributions of genes in tyrosine catabolism to pigment production.

1.3.1 Phenylalanine and tyrosine catabolism pathway

Pyomelanin is a negatively charged, extracellular, reddish-brown pigment derived from the phenylalanine and tyrosine catabolism pathway (Figure 1.1) (34, 50). The tyrosine catabolism pathway is conserved in almost all aerobic organisms and is used to acquire energy from tyrosine breakdown (51). In this pathway, phenylalanine is first

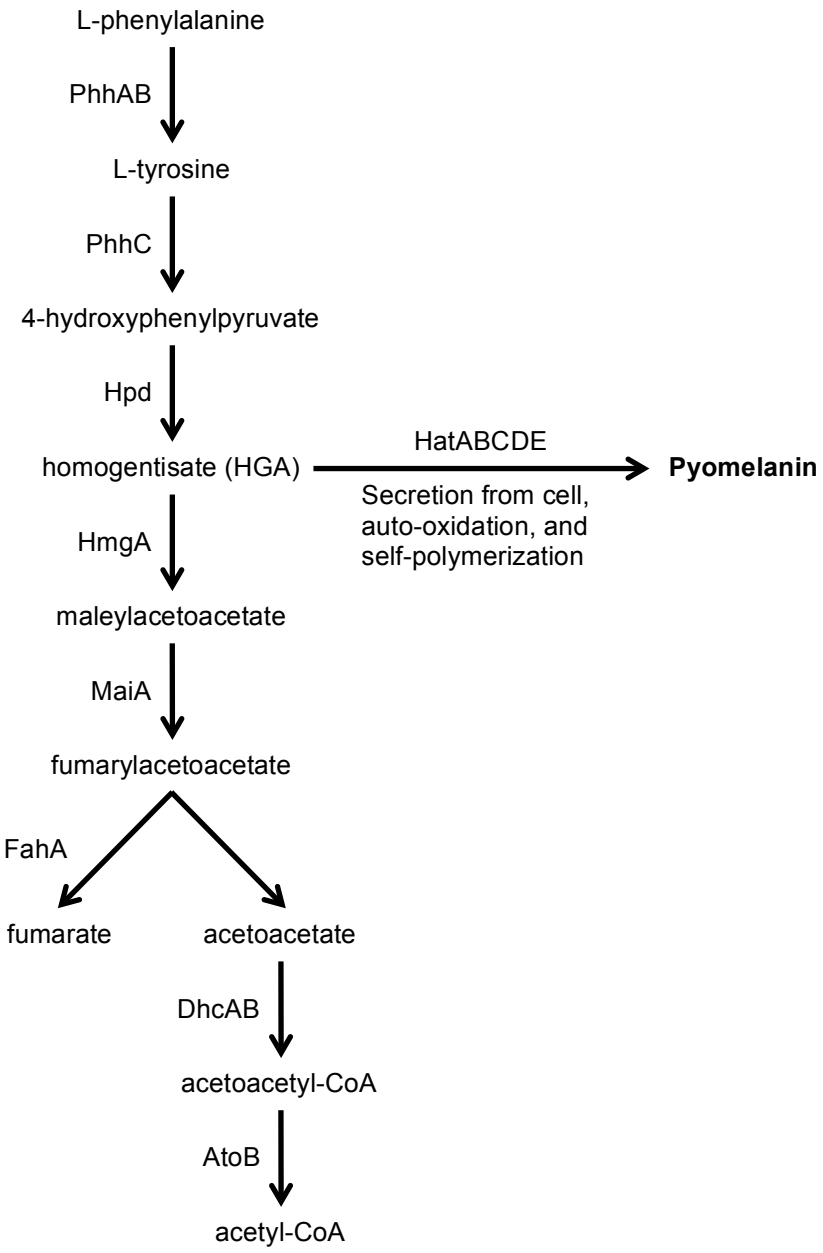

Figure 1.1. Phenylalanine/tyrosine catabolism pathway in *P. aeruginosa*. PhhA, phenylalanine-4-hydroxylase; PhhB, pterin-4-α-carbinolamine dehydratase; PhhC, aromatic amino acid aminotransferase; Hpd, 4-hydroxyphenylpyruvate dioxygenase; HmgA, homogentisate 1,2-dioxygenase; MaiA, maleylacetoacetate isomerase; FahA, fumarylacetoacetase; DhcAB, dehydrocarnitine CoA transferase, subunits A and B; AtoB, acetyl-CoA acetyltransferase; HatABCDE, HatABCDE ABC transporter. *P. aeruginosa* nomenclature is used (Pseudomonas Genome Database http://www.pseudomonas.com).

converted to tyrosine by phenylalanine-4-hydroxylase (PhhA) and pterin-4-alpha-carbinolamine dehydratase (PhhB). Tyrosine is then converted to 4-hydroxyphenylpyruvate by an aromatic amino acid aminotransferase (PhhC or TyrB). Next, 4-hydroxyphenylpyruvate is converted to homogentisate (HGA) by 4-hydroxyphenylpyruvate dioxygenase (Hpd). After HGA is formed, it is converted to maleylacetoacetate by homogentisate 1,2-dioxygenase (HmgA). Maleylacetoacetate is converted to fumarylacetoacetate by maleylacetoacetate isomerase (MaiA or HmgC). In the next step in the pathway, fumarylacetoacetate is split into fumarate and acetoacetate by fumarylacetoacetase (FahA or HmgB). The last steps of the pathway involve conversion of acetoacetate into acetoacetyl-CoA by the dehydrocarnitine CoA transferase, subunits A and B (DhcA and DhcB), and finally the formation of acetyl-CoA from acetoacetyl-CoA by the acetyl-CoA acetyltransferase (AtoB) (52). The end products of tyrosine catabolism go into the TCA cycle (45, 53).

Pyomelanin production occurs when there is a defect in the tyrosine catabolism pathway, either through mutations or deletions of key genes, or reduced enzyme activity for the enzymes in the latter part of the pathway (HmgA, MaiA, and FahA) compared to enzymes in the upper part of the pathway (54-56). In *P. aeruginosa*, it was found that strains containing mutations in *maiA* and *fahA* did not produce pyomelanin, which suggested that inactivation of *hmgA* was the only cause of pyomelanin production in that organism (56). Several proteins in the phenylalanine and tyrosine catabolism pathway have been implicated in pyomelanin production, and these proteins will be discussed in detail below.

1.3.2 Homogentisate 1,2-dioxygenase (HmgA)

One of the major proteins that impacts pyomelanin production is HmgA, which is responsible for converting HGA to maleylacetoacetate. In *P. aeruginosa*, defects in the *hmgA* gene, either through chromosomal deletions or point mutations, result in pyomelanin production because the tyrosine catabolism pathway stalls and HGA accumulates (55, 56). Pyomelanin is produced when HGA is secreted from the cell, leading to auto-oxidation and self-polymerization (34, 56, 57). Secretion of HGA occurs via the HatABCDE transport system, and defects in this transport system result in reduced pyomelanin production (57).

Deletions of *hmgA* and potential defects in regulation of *hmgA* expression have been identified in various strains of *P. aeruginosa* that produce pyomelanin. In the *P. aeruginosa* pyomelanogenic clinical isolate PA1111, which was isolated from an acute infection in a burn patient, a chromosomal deletion of *hmgA* was identified and determined to be the cause of pyomelanin production in that strain (58, 59). Additional studies of two clinical pyomelanogenic *P. aeruginosa* strains from CF patients showed that one strain contained a deletion of *hmgA*, while the other strain had no mutations or deletions of *hmgA*, which indicated that mutations in a transcriptional regulator may be responsible for pigment production in that strain (56). Interestingly, downregulation of *hmgA* in a chronic isolate of *P. aeruginosa* Australian epidemic strain-1 (AES-1) from a CF lung did not result in pyomelanin production (60), which indicated that there was enough functional HmgA produced to prevent pigment formation even when *hmgA* expression was downregulated. These results demonstrate that chromosomal deletions of *hmgA* may be a frequent cause for pyomelanin production in *P. aeruginosa*, and it

can occur in isolates from both acute and chronic infections. Regulation of *hmgA* expression may also be important in pigment production, as downregulation of *hmgA* did not result in pigment production, while a strain with a possible defect in a transcriptional regulator did show pyomelanin production.

Studies in several other bacteria have revealed defects in *hmgA* as a cause for pyomelanin production. In some strains of *V. cholerae*, pyomelanin can be produced under normal experimental growth conditions. Analysis of these strains revealed that they contained either a 15 or 10 base pair (bp) deletion mutation in homogentisate oxygenase, and the 15 bp deletion was conserved in six O139 strains that were studied (61). Additionally, in a *Burkholderia cenocepacia* pyomelanogenic CF clinical isolate a single point mutation in *hmgA* resulted in an amino acid change from a glycine to an arginine at residue 378, and this mutation was conserved in three of four pigmented *B. cepacia* complex strains (62). The G378R mutation in *B. cenocepacia* was located in the iron cofactor binding region of HmgA, which could affect iron binding and subsequent enzyme function (62). In *A. media* WS, it was recently reported that pyomelanin is the major melanin produced in this strain, not L-DOPA melanin as previously thought, and the *hmgA* gene was disrupted by an insertion of a different gene in the opposite orientation (42). Conservation of deletions and mutations in *hmgA* is important because it demonstrates stability of a defect across several strains of bacteria, and could also represent a hot spot for mutations. Additionally, conservation of mutations could represent horizontal gene transfer between different bacteria, which would explain why they have the same mutation or deletion.

Pyomelanin production has also been observed in several other bacterial and fungal strains after transposon mutagenesis or in-frame deletion of *hmgA*, including *B. anthracis*, *Vibrio campbellii*, *S. meliloti*, *Pseudomonas chlororaphis*, and *A. fumigatus* (41, 44, 47, 63, 64). Creation of stable mutations in *hmgA* for bacteria that do not produce pyomelanin in the wild type strain can allow us to study mechanisms of pigment production in different bacteria, as well as allow us to study the functions of pyomelanin in these bacteria.

1.3.3 4-hydroxyphenylpyruvate dioxygenase (Hpd)

The second major protein involved in pyomelanin production is Hpd, the enzyme responsible for the production of HGA from 4-hydroxyphenylpyruvate. Transposon mutagenesis or deletion of *hpd* led to an abolishment of pyomelanin production in *B. cenocepacia*, *L. pneumophila*, *P. aeruginosa*, *A. media* WS, and *A. fumigatus* (39, 42, 47, 57, 62, 65). The lack of pigmentation in these *hpd* mutant strains demonstrates the importance of Hpd in the production of the pyomelanin precursor molecule HGA. If Hpd is not present, HGA cannot form, and pyomelanin production will not occur.

Additionally, expression of Hpd from the bacterial species *A. stellipolaris* LMG 21856, *L. pneumophila*, *B. cenocepacia*, and *Streptomyces avermitilis*, as well as the archaeon *Picrophilus torridus*, in *Escherichia coli* resulted in pyomelanin production (36, 46, 66-68). *E. coli* does not possess genes for the tyrosine catabolism pathway; therefore, expression of recombinant Hpd in *E. coli* produces HGA, which can oxidize and polymerize to form pyomelanin. These results emphasize the importance of Hpd in pyomelanin production and also show that Hpd from different microbial species are

functional in *E. coli*. The ability to produce functional recombinant Hpd in *E. coli* can allow researchers to further examine the functions and characteristics of this protein independent of the organism from which it was originally isolated.

1.3.4 Other proteins implicated in pyomelanin production

Additional proteins involved in pyomelanin production include phenylalanine-4-hydroxylase (PhhA) and pterin-4-alpha-carbinolamine dehydratase (PhhB), which convert phenylalanine to tyrosine, and the aromatic amino acid aminotransferase (PhhC/TyrB), which converts tyrosine to 4-hydroxyphenylpyruvate. *phhA*, *phhB*, and *phhC* transposon mutants in a pyomelanogenic *P. aeruginosa* strain showed no pigment production (57). In *L. pneumophila*, *phhA* mutants also showed reduced pigmentation during growth (69). Additionally, *phhA* and *tyrB* mutants in *A. media* WS showed reduced pigment production (42). These results show a functional role for proteins in the upper part of the phenylalanine/tyrosine catabolism pathway in pyomelanin production.

The conversion of phenylalanine to tyrosine by PhhAB is important in pyomelanin production because in the absence of tyrosine in the environment, phenylalanine must be converted to tyrosine for the phenylalanine/tyrosine catabolism pathway to eventually produce the HGA needed for pigment production. The *phhA* mutants that showed reduced pigmentation may have the ability to obtain tyrosine from the environment when PhhA is non-functional, thereby allowing them to produce some HGA through tyrosine catabolism. The *phhA* mutants that showed no pigmentation could exhibit reduced uptake of tyrosine from the environment compared to the *phhA* mutants that

did show some pigmentation, which would account for the differences in pyomelanin production that were observed. It is also possible that the *phhA* mutants can obtain tyrosine through aromatic amino acid biosynthesis, which could then allow them to produce some pigment. *Pseudomonas putida* is capable of phenylalanine and tyrosine biosynthesis from chorismate via chorismate mutase (PheA) and bifunctional cyclohexadienyl dehydrogenase/3-phosphoshikimate 1-carboxyvinyltransferase (PP1770/TyrA) (70). *P. aeruginosa* has a homolog for PheA (87.19 percent identity), but 3-phosphoshikimate 1-carboxyvinyltransferase prephenate dehydrogenase is a pseudogene (71).

The conversion of tyrosine to 4-hydroxyphenylpyruvate by PhhC/TyrB is important because 4-hydroxyphenylpyruvate is needed to produce HGA. The lack of pigmentation in *phhC* mutants suggested that the bacteria do not possess additional aromatic amino acid aminotransferases that can convert tyrosine into 4-hydroxyphenylpyruvate. Reduced pigmentation in *tyrB* mutants, however, suggested that there could be an additional enzyme functioning as an aromatic amino acid aminotransferase that can contribute to the conversion of tyrosine to 4-hydroxyphenylpyruvate, which would allow some HGA to be produced.

Other proteins may also be involved in pyomelanin production. In *P. putida*, deletion of two genes encoding an acyl-CoA transferase involved in acetoacetate degradation resulted in pigment production (53), which demonstrated that the proteins encoded by those genes are important for a functional tyrosine catabolism pathway. The homologous proteins for the *P. putida* acyl-CoA transferase in *P. aeruginosa* are dehydrocarnitine CoA transferase, subunits A and B (DhcA, 85.78 percent identity;

DhcB, 90.37 percent identity) (71). Although it has not been demonstrated that DhcA and DhcB are involved in pyomelanin production in *P. aeruginosa*, it is certainly possible that they could be involved since the homologous proteins in the closely related organism *P. putida* have been implicated in pigmentation.

1.3.5 Laboratory growth conditions

Laboratory growth conditions may also affect pyomelanin production. Many organisms require the addition of tyrosine to growth medium in order to produce pyomelanin. In *S. meliloti*, addition of tyrosine to the medium enhanced pyomelanin production (44). Additionally, in *V. cholerae*, pyomelanin was produced in low nutrient conditions when tyrosine was supplemented, but never in rich media (54). Tyrosine addition to media enhanced pigment production in *L. pneumophila* (66). In *P. chrysogenum*, pyomelanin was produced when tyrosine was included in the growth medium, and HGA was detected only in cultures grown with tyrosine (49). Pyomelanin production in *A. fumigatus* occurred when strains were grown on media containing tyrosine (47). *Sporothrix* complex species were also able to produce pyomelanin at varying levels when they were grown in minimal media containing tyrosine (48). These results demonstrate the importance of including tyrosine in growth medium. Additionally, adding a specific concentration of tyrosine to a minimal growth medium can help eliminate some of the variability in experimental results and show that pigment is produced only under certain conditions. It is also possible that the concentration of tyrosine in a rich medium is not high enough to induce pigment production in some strains of bacteria and fungi.

1.4 Regulation of pyomelanin production

In *P. aeruginosa*, the genes for phenylalanine/tyrosine catabolism are found in several operons that are scattered in the genome (Figure 1.2). The *phhABC* genes are found in an operon, and the transcriptional regulator *phhR* is divergently transcribed (50, 71). *hpd* is not in an operon, but it is located in close proximity to *phhABC* (71). The *hmgA-fahA-maiA* genes are also found in an operon, and the probable transcriptional regulator gene PA2010, which is homologous to the *P. putida* repressor *hmgR* that is known to regulate the *hmg* operon, is divergently transcribed (50, 71). The *dhcAB* genes form an operon, with *atoB*, which encodes the acetyl-CoA acetyltransferase, directly downstream but not part of the operon, and the transcriptional regulator *dhcR* is divergently transcribed (71). Organization of operons and names of phenylalanine/tyrosine catabolism genes vary in different organisms, including in other *Pseudomonas* species (50).

Several proteins have been implicated in the regulation of phenylalanine/tyrosine catabolism in various organisms, including Crc, TyrR/PhhR, and HmgR. Both global and operon specific regulators have been identified that control phenylalanine/tyrosine catabolism, which may be important in pyomelanin production. Regulators found in *Pseudomonas* species will be discussed in the following sections.

1.4.1 Crc global regulator

Crc is a global regulator that controls expression of genes from aromatic compound catabolism pathways. In *P. putida* and *P. aeruginosa*, Crc acts as a master regulator of carbon metabolism (72). Studies in *P. putida* have shown that Crc

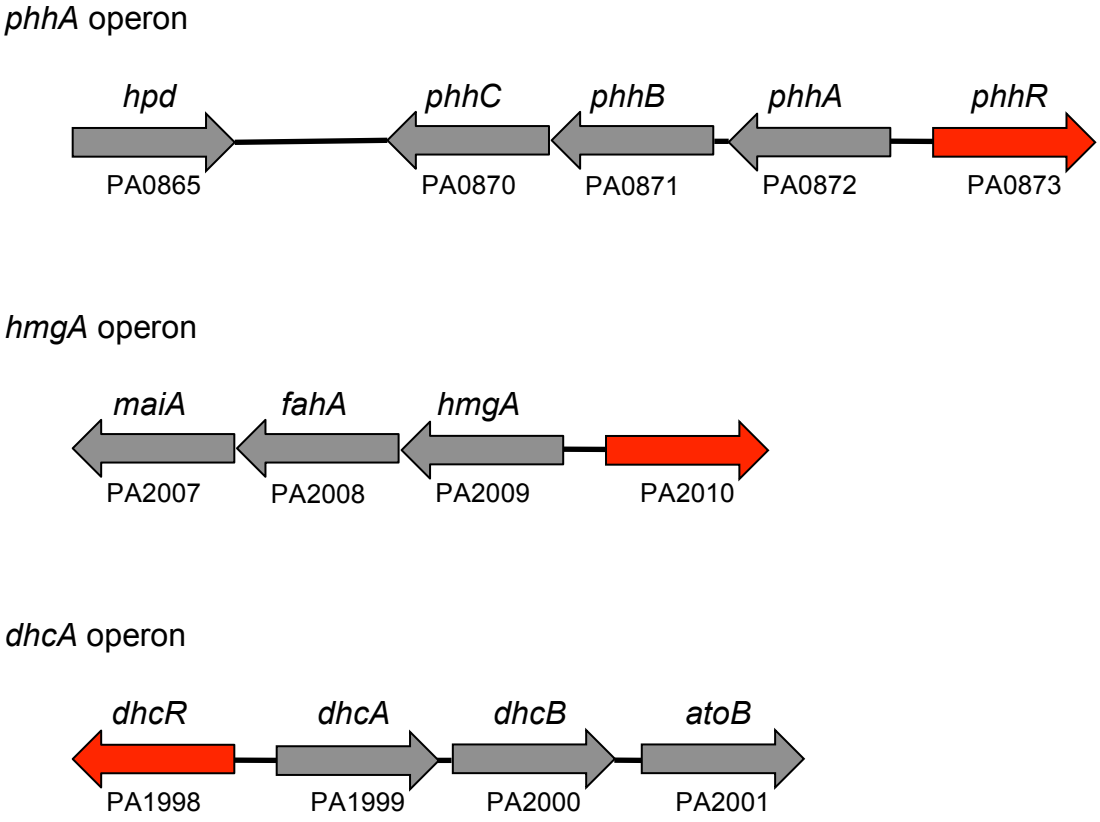

Figure 1.2. Organization of genes for phenylalanine/tyrosine catabolism in *P. aeruginosa*. Operons are formed for *phhABC*, *hmgA-fahA-maiA*, and *dhcAB*. *phhR* and *dhcR* are genes for regulatory proteins of their respective operons. PA2010 is predicted to be a homolog of *hmgR* from *P. putida*, which is a gene for a regulatory protein of the *hmgA* operon. Genes for regulatory proteins are in red. *P. aeruginosa* nomenclature and PA numbers from PAO1 are used (Pseudomonas Genome Database http://www.pseudomonas.com). Arrow size and distance between genes is not drawn to scale.

represses expression of *hpd*, *hmgA*, and *mai*, which was indicated by increased mRNA levels for those genes in a *crc* mutant compared to the wild type strain (72). As Crc is not known to bind DNA, it is unknown if Crc affects transcription of the genes it regulates directly or indirectly (72). Conflicting reports about the binding target for Crc have been reported. In one study, Crc from *P. putida* was found to bind to mRNA and repress translation (73), while a later study showed that Crc from *P. aeruginosa* did not bind to RNA (74). Crc appears to be an important regulator of gene expression in *P. putida*, and it is likely that similarities exist in *P. aeruginosa*. Additionally, in a pyomelanogenic *P. aeruginosa* strain, a *crc* transposon mutant showed reduced pyomelanin production (57), which indicates that Crc may have a role in pigment formation by regulating expression of genes involved in tyrosine catabolism.

1.4.2 TyrR and PhhR transcriptional regulators

Another regulator for phenylalanine/tyrosine catabolism genes is TyrR/PhhR. In *E. coli*, TyrR acts as both a repressor and an activator of DNA transcription. To activate transcription, TryR interacts with the C-terminal region of the α-subunit of RNA polymerase (αCTD) when aromatic amino acids are present and binds to DNA upstream of the promoters for genes that encode aromatic amino acid transporters (75). In *E. coli*, TyrB is an aromatic amino acid aminotransferase involved in the biosynthesis of tyrosine and phenylalanine (76). TyrR represses *tyrB* transcription in the presence of tyrosine or phenylalanine. TyrR forms hexamers in the presence of tyrosine, which bind to DNA and prevent open complex formation during initiation of transcription (76). When phenylalanine is present, TyrR dimers or tetramers bind to DNA and an open complex

can form, but RNA polymerase cannot exit the promoter (76). Both of these mechanisms of repression prevent *tyrB* transcription. The *Pseudomonas* TyrR homolog is PhhR (77), which has similar regulatory functions in both *P. putida* and *P. aeruginosa*, with a few differences.

PhhR is a global regulator in *P. putida* that activates genes for phenylalanine degradation (53). In both *P. aeruginosa* and *P. putida*, PhhR induced the *phhA*, *hpd*, *hmgA*, and *dhcA* (PP3122 in *P. putida*) transcriptional units in response to phenylalanine (52, 53, 77). Additionally, the expression of phenylalanine/tyrosine catabolism genes was downregulated in a *phhR* mutant strain compared to the wild type strain (52, 53). *phhR* expression was also induced in the presence of aromatic amino acids, and *P. putida* PhhR appeared to negatively regulate *phhR* gene expression (52, 53). These results show that PhhR is necessary for transcription of genes involved in phenylalanine/tyrosine catabolism, and aromatic amino acids are necessary for induction of gene transcription. In addition, a *phhR* transposon mutant in a *P. aeruginosa* pyomelanin producer showed abolishment of pigmentation (57), which indicates a functional role for PhhR in pyomelanin production. This is likely due to downregulation of phenylalanine/tyrosine catabolism gene expression in *phhR* mutants, which could prevent pigment production because the pyomelanin precursor molecule HGA would not be produced.

Experimental evidence has shown that PhhR binds to the *phhA*, *hpd*, and *dhcA* (PP3122) promoters in *P. aeruginosa* and *P. putida*, and also to the *hmgA* promoter in *P. putida*, but not in *P. aeruginosa* (52, 53). The experimental data for PhhR promoter binding matches the predictions for PhhR binding sites in the promoters of those

transcriptional units. Additionally, in *P. aeruginosa* it was found that PhhR mainly regulates transcription from σ^{54} promoters, not σ^{70} promoters as seen in *E. coli* TyrR (52, 77). Potential σ^{54} binding sites were identified in *P. aeruginosa* upstream of *phhA*, *hpd*, and *dhcA*, but not *hmgA* (52), which corresponds with the identification of PhhR binding sites upstream of the same genes and provides further evidence that those transcriptional units are regulated by PhhR.

Additional differences between PhhR from *P. putida* and *P. aeruginosa* have been identified. In *P. putida*, genes for aromatic amino acid biosynthesis were repressed in the presence of phenylalanine (53). In *P. aeruginosa*, PhhR did not control expression of biosynthesis genes for aromatic amino acids (52). Overall, there are many similarities between PhhR from the closely related species *P. aeruginosa* and *P. putida*, but there are some differences.

1.4.3 HmgR transcriptional repressor

A third regulator for tyrosine catabolism genes is HmgR. HmgR is an IclR-type regulator that functions as a repressor for *hmgABC* gene expression in *P. putida*, and the homologous protein PA2010 has been identified in *P. aeruginosa* (50). Studies of HmgR function have shown that the protein binds to the *hmg* promoter in a region that partially overlaps the ribosome binding site for *hmgA* to repress transcription of *hmgABC* in the absence of HGA (50). HGA inhibited HmgR binding to the *hmg* promoter, and a *hmgR* mutant strain constitutively produced HmgA in normal amounts (50). These studies show that HmgR is important for repression of *hmgABC* gene expression in *P. putida*, and is likely to be important in *P. aeruginosa* as well, as the

hmgA promoter in *P. aeruginosa* contains a potential HmgR binding site (52). Because *P. putida* and *P. aeruginosa* are closely related organisms, it is expected that HmgR would have a similar function in *P. aeruginosa*.

HmgR repression can potentially explain PhhR regulation of *hmgA*. As described above, PhhR regulated *hmgA* expression in *P. aeruginosa*, but there was no PhhR binding site (52). This phenomenon can be explained by the presence or absence of Hpd and HGA in the cell, as well as the HmgR binding site in the *hmgA* promoter. Since *hpd* expression is controlled by PhhR, if PhhR activates *hpd* when phenylalanine is present, the Hpd protein will be produced and HGA will subsequently be formed (52). HGA can then bind to HmgR, which prevents HmgR from binding to the *hmgA* promoter, and finally allows for expression of *hmgA* (52). PhhR indirectly regulates *hmgA* expression through HmgR in *P. aeruginosa*, which adds to the complexity of regulation of phenylalanine/tyrosine catabolism gene expression, and also pyomelanin production. Interestingly, in *P. putida*, both PhhR and HmgR directly regulate *hmgA* expression, as binding sites for both regulators have been identified in the promoter for *hmgA* and experiments have shown regulation of *hmgA* transcription by PhhR and HmgR. This demonstrates that there can be differences in regulation by two closely related species.

1.5 Functions of pyomelanin

Microbes encounter a variety of conditions in their natural habitats, so it is necessary to have mechanisms that will allow them survive variations in the environment. One of these mechanisms is to produce pigments, such as pyomelanin.

Pyomelanin has several functions that may help bacteria or fungi survive in host organisms or the environment. These functions will be discussed in detail in the following sections.

1.5.1 Protection from oxidative and UV stress

Pyomelanin provides protection from oxidative stress in some bacterial and fungal species. In *P. aeruginosa*, pyomelanin protected cells from H_2O_2 induced oxidative stress in both a plate based assay and a viable count assay (56, 58). Additionally, *P. aeruginosa* pyomelanin containing supernatant was able to protect non-pigmented wild type cells from H_2O_2 (56). Pyomelanin produced by *P. aeruginosa* was also able to protect cells from photodynamically induced oxidative stress, which can generate singlet oxygen, superoxide anion radical, H_2O_2, and hydroxyl radical, by scavenging radicals and quenching singlet oxygen (78). These results demonstrate that pyomelanin produced by *P. aeruginosa* is able to provide protection from oxidative stress under a variety of different test conditions, which is important because bacteria may encounter different conditions in the various environments they inhabit.

Other bacterial and fungal species have also demonstrated resistance to oxidative stress when pyomelanin was present. In *V. campbellii*, pyomelanin containing supernatant was able to protect non-pigmented wild type cells from H_2O_2 induced oxidative stress (63); this protective effect was also seen in *P. aeruginosa*. Non-pigmented strains of *B. cenocepacia* were more sensitive to H_2O_2 in a disc diffusion assay, as well as to extracellular superoxide generated by the xanthine/xanthine oxidase reaction, compared to strains that produced pyomelanin (36). In *A. fumigatus*,

pyomelanin producers were more resistant to H_2O_2 induced oxidative stress than non-producers in an agar plate diffusion assay (47). Pyomelanin provided protection from both oxygen and nitrogen derived oxidants in *Sporothrix* sp. (48). In *B. anthracis*, however, pyomelanin did not provide protection from oxidative stress in a H_2O_2 disc diffusion assay, or when a non-pigmented strain was suspended in supernatants from a pyomelanin producer followed by exposure to H_2O_2 (41). These results show that pyomelanin frequently provides protection from oxidative stress in bacteria and fungi, but this protection is not universal in all organisms that produce pyomelanin.

Additionally, pyomelanin can provide resistance to UV stress that may be encountered by bacteria and fungi in the environment. In *B. anthracis*, pyomelanin producers showed increased survival compared to non-pigmented strains after UV irradiation (41). *L. pneumophila* with a mutation in the *hpd* homolog *lly*, which eliminated pyomelanin production in the bacteria, was more sensitive to light than a pigmented strain (66). In *Sporothrix* sp., pyomelanin production provided protection from UV light (48). The ability of pyomelanin to protect against UV stress is important because UV radiation can cause mutations in DNA that can be detrimental for the organism if they cannot be repaired (79). These results show that pyomelanin can protect bacteria and fungi from stresses they may encounter in the environment, whether it is in a host during infection (oxidative stress) or in the natural environment (UV stress), which could be detrimental for their survival.

1.5.2 Iron reduction and acquisition

Pyomelanin also functions in electron transfer and metal reduction. Studies in *S. algae* BrY have shown that pyomelanin can act as a soluble electron shuttle for iron oxide reduction and as a terminal electron acceptor (40). *S. algae* BrY cells that produced pyomelanin, as well as non-pyomelanogenic cells with added pyomelanin, were able to reduce more ferric oxide than cells that did not produce pyomelanin (80). These results indicate that it is the presence of pyomelanin that is important for increased iron reduction, not necessarily the ability of the organism to produce pyomelanin itself. In *L. pneumophila*, pyomelanin had ferric reductase activity (39). A *hmgA* mutant that produced increased amounts of pyomelanin had higher ferric reductase activity than wild type *L. pneumophila*, which produced low levels of pyomelanin, while a *lly* mutant (*hpd* mutant) that was null for pyomelanin production had lower ferric reductase activity than wild type (39). This data indicates that increased amounts of pyomelanin production resulted in increased iron reduction.

Pyomelanin is also involved in iron uptake, as well as enhancement of bacterial growth in low iron conditions. Studies of pyomelanin in *L. pneumophila* have shown that both pyomelanin and the pyomelanin precursor molecule HGA are involved in iron reduction, and the bacteria can take up the reduced iron (81). Pyomelanin also increased growth of *L. pneumophila* in low iron conditions, which was likely due to the association of iron with pyomelanin, which could lead to increased iron assimilation (81). *L. pneumophila* also produces siderophores for acquiring iron, and a hyperpigmented *hmgA* transposon mutant produced lower levels of siderophores than the wild type, while a siderophore mutant produced more pyomelanin than wild type, which indicates

that pyomelanin is involved in iron assimilation (81). These data demonstrate that *L. pneumophila* has different methods for acquiring iron, and if one mechanism is inactive, the other mechanism can compensate. Although it has not been tested in *P. aeruginosa*, it is possible a similar mechanism for acquiring iron could exist in that organism since it is capable of producing both pyomelanin and siderophores.

Enzymes involved in pyomelanin production are also affected by iron concentrations. In *L. pneumophila*, PhhA was involved in pyomelanin production and growth in low tyrosine media (69). PhhA has an iron cofactor in its active site and its enzymatic activity required iron, but high concentrations of iron also inhibited activity (69). In *S. algae* BrY the rate of pyomelanin production and the amount of pyomelanin produced decreased when iron concentrations were higher than the optimum (82). These results demonstrate that iron is necessary for pyomelanin production and that pyomelanin itself is capable of providing bacteria with iron needed for growth and other functions. The concentration of iron is important, however, since the presence of too much iron can inhibit enzymatic function.

1.5.3 Antibiotic resistance

Resistance to antibiotics and other antimicrobial drugs can be a major problem when treating bacterial and fungal infections. Various studies have shown that melanin can bind to some antibiotics and antifungal drugs, thereby reducing the activity of those drugs (26). Isotherm analysis of gentamicin and synthetic melanin revealed high levels of binding between those compounds (83). Additionally, interactions between tobramycin and synthetic melanin resulted in a decrease in antibiotic activity under

certain conditions (84). Increased resistance to specific antibiotics was observed in pyomelanogenic *Stenotrophomonas maltophilia* compared to non-pigmented strains (85). In *Sporothrix* sp., pyomelanin production resulted in increased resistance to the antifungal antibiotic amphotericin B (48). In a second study in *Sporothrix* sp., pyomelanin provided protection from the antifungal drug terbinafine during a time-kill assay (86). These studies all reveal a role for melanin in antimicrobial resistance.

In *P. aeruginosa*, it was reported that pyomelanin producing strains were more sensitive to antibiotics than non-pyomelanogenic strains (24, 25, 87). In these studies, several different classes of antibiotics were tested, including aminoglycosides, β-lactams, and polymyxins. These data conflict with the studies presented above that showed reduced efficacy of antimicrobials in the presence of both synthetic melanin and pyomelanin.

Recent studies in *P. aeruginosa*, however, reported no differences in sensitivity to aminoglycoside, cephalosporin, β-lactam, and fluoroquinolone antibiotics in pyomelanin producers compared to non-producers (56, 58). In *A. baumannii*, pyomelanogenic clinical isolates were resistant to antibiotics, and treatment with sulcotrione, an inhibitor of the tyrosine catabolism pathway, reduced pigment production but this reduction in pigment production had no effect on antibiotic resistance (43). These results show that pyomelanin production in *A. baumannii*, like *P. aeruginosa*, does not appear to have an effect on antibiotic resistance.

These conflicting reports about the effects of pyomelanin and other types of melanin on antibiotic sensitivity make it difficult to draw conclusions. Taken together, these results suggest that pyomelanin production has little effect on antibiotic resistance

in bacteria, but may have an effect on antibiotic resistance in fungi. A limitation of antibiotic sensitivity assays is that many of them are performed by broth microdilution, which uses diluted bacterial cultures, so little preformed pyomelanin may be present at the start of the assay, which could affect the results if one wants to determine the effects of pyomelanin on antibiotic resistance. Broth microdilution may show that pyomelanin has no effect on antimicrobial resistance when there could actually be resistance if a different type of assay, such as a time-killing assay, was performed. In a time-killing assay, microbes are exposed to 2x minimum inhibitory concentration (MIC) of antimicrobial agents and viable counts are determined at several time points (86). This type of assay could potentially allow the researcher to start with higher levels of preformed pyomelanin at the beginning of the assay, which may have an effect on the antibiotic resistance results.

1.5.4 Infection persistence and virulence

Pyomelanin may also contribute to microbial infection persistence. Pyomelanin and HGA have the ability to release iron from iron chelates that occur in a mammalian host, including ferritin and transferrin, or in the environment, such as ferric hydroxide (81). The ability to obtain iron from the environment may be important in bacterial persistence during infection since iron is necessary for bacterial growth. Many metabolic pathways and cellular functions require iron (88), including the PhhA, Hpd, and HmgA enzymes involved in phenylalanine/tyrosine catabolism that have an iron cofactor in the active site of the enzyme.

Melanin is also known to scavenge superoxide anion produced by macrophages (37). Pyomelanin producing *B. cenocepacia* showed reduced trafficking to RAW 264.7 lysosomes compared to a non-pigmented strain (36), which suggested that the bacteria may be persisting inside the phagocyte. Additionally, in *A. fumigatus*, transcription of tyrosine catabolism genes was induced in response to human neutrophils, which suggested that pyomelanin could be involved in immune system escape and fungal survival in humans (35). The potential ability of pyomelanogenic microbes to obtain iron from host iron chelates, scavenge ROS, reduce trafficking to lysosomes, and induce transcription of tyrosine catabolism genes suggest that pyomelanin may play a role in bacterial persistence during infection of phagocytes.

Finally, in a chronic mouse model of infection, pyomelanin producing *P. aeruginosa* exhibited increased persistence compared to the non-pigmented wild type strain (56). A mouse model of acute infection showed that pyomelanogenic *P. aeruginosa* had increased persistence compared to non-pigmented wild type (60). It is possible that some of the mechanisms described above were used to allow the bacteria to persist in the mouse infection models; however, that remains to be tested since the mechanism for bacterial persistence was not identified in the mouse studies. In *L. pneumophila*, however, pyomelanin was not involved in intracellular survival of bacteria in the amoeba host *Hartmannella vermiformis* (66), which indicates that pyomelanin production is not always involved in infection persistence. These differences in bacterial persistence in host organisms could be due to the differences in mammalian and amoeba hosts.

Several studies, however, have shown that pyomelanin can have a variable effect on virulence. In a *Caenorhabditis elegans* virulence assay, pyomelanogenic *P. aeruginosa* was more virulent than non-pigmented wild type (60). In *B. anthracis*, pyomelanin production did not affect expression of virulence genes (41), which demonstrates the potential for a pyomelanin producer to exhibit normal virulence traits. Tyrosine degradation genes were upregulated in a mouse infection model, but pyomelanin did not appear to have a role in pathogenicity or virulence in *A. fumigatus* (35). Decreased virulence (approximately 2.7 fold) was seen for a *V. campbellii* pyomelanin producer with an in-frame deletion in *hmgA* in a shrimp virulence model, as well as downregulated expression of quorum sensing and bioluminescence genes (63). Additionally, decreased virulence by a *P. aeruginosa* pyomelanin producer was observed in an acute mouse model of infection (56). Overall, pyomelanin may have some impact on virulence during bacterial and fungal infections, but the studies presented above demonstrate that this impact could be microbial species or experimental assay dependent.

Infection persistence and virulence are different but related phenomena in microbes. Persistence can be defined as the ability of the microbe to survive longer in a host organism than another microbe. It can also mean that the microbe has higher numbers of cells than another microbe in a host over a specific time frame. Virulence is related to the ability of the microbe to infect a host organism, since a microbe can produce virulence factors to increase its ability to cause infection. Virulence can also be implicated in microbial killing of the host because a more virulent microbe may cause higher mortality of a host than a less virulent microbe. Additionally, in *P. aeruginosa*,

virulence may actually decrease as an infection switches from acute to chronic (89). This ability to downregulate virulence demonstrates that there is interplay between virulence and persistence in microbial infections, and that there may be advantages to persistence over virulence or vice versa, depending on the situation.

1.6 Anti-virulence treatment and NTBC

Bacteria are becoming more resistant to antimicrobial agents such as antibiotics, which is a significant problem as most bacterial infections are treated with antibiotics. Additionally, very few new antibiotics have been developed in recent years. As an alternative to treating infections with antibiotics, anti-virulence therapies that target microbial virulence factors are being investigated. Anti-virulence therapy targets mechanisms that are beneficial for pathogen interactions with the host, instead of targeting functions that are necessary for basic metabolism, which should increase the specificity of the therapy for pathogens over the normal flora in the host (90). Good targets for anti-virulence therapy include mechanisms involved in bacterial adhesion to cells and colonization, secretion of cell surface proteins, regulatory bacterial functions such as quorum sensing, cell wall components such as LPS, and pigments (19, 90). When selecting a virulence factor for anti-virulence therapy, it is ideal if the target is conserved in many species (90) so there is potential for broad application of the therapy.

Microbial pigments can contribute to disease pathogenesis, due to the fact that pigments can interfere with host immune clearance and can have proinflammatory or cytotoxic properties (91). This can confer a survival advantage for the bacteria when

they are in the host. This makes pigments, such as pyomelanin, good targets for anti-virulence therapy. Since pyomelanin is conserved in a variety of bacterial and fungal pathogens there is potential for broad application of an anti-virulence therapy targeting that pigment. Additionally, pyomelanin production and a functional phenylalanine/tyrosine catabolism pathway are not essential in bacteria, as indicated by unimpaired growth of pathway mutants. This makes it less likely for resistance to develop to an anti-virulence therapy targeting pyomelanin. Recently, pyomelanin production in *L. pneumophila* was inhibited by a compound that targeted phenylalanine hydroxylase (92). Another recent study showed that pyomelanin production in *P. aeruginosa* could be inhibited by 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) treatment, which targets Hpd, and this pigment inhibition led to increased sensitivity to oxidative stress (58). These results demonstrate that there is interest in investigating different compounds that inhibit pyomelanin production.

NTBC was originally developed as an herbicide and binds to Hpd irreversibly at its active site, which contains an iron center (93). The active form of Hpd contains a ferrous center in the C-terminal domain, and binding of NTBC prevents oxidation of the active site (51), thereby inhibiting Hpd function. NTBC has been developed as a treatment for type I tyrosinemia (94). Type I tyrosinemia is caused by the lack of fumarylacetoacetase (FahA) in the tyrosine catabolism pathway, which causes the accumulation of toxic metabolites such as fumarylacetoacetate, leading to cirrhosis and cancer of the liver (95). NTBC inhibition of Hpd activity prevents the accumulation of toxic metabolites and progression of disease in type I tyrosinemia (94). NTBC treatment will also inhibit the formation of HGA, thereby preventing pyomelanin production.

Treatment of pyomelanin producing bacteria with NTBC is a novel application for an already existing drug that has received FDA approval for treatment of type I tyrosinemia in humans (51). Pyomelanin producing bacteria become more sensitive to oxidative stress if pigment production is reduced or abolished, which can be achieved through NTBC treatment (58). Increased sensitivity to oxidative stress via reduced pigmentation is anticipated to increase immune cell mediated killing of bacteria and thereby result in increased clearing of infections by the host immune system. Therefore, bacterial or fungal infections by pyomelanogenic organisms may exhibit reduced persistence after treatment with NTBC, demonstrating the potential for NTBC as an anti-virulence therapy.

1.7 Concluding remarks

Pyomelanin production in bacteria and fungi is caused by a defect in phenylalanine/tyrosine catabolism. This pathway is non-essential in microbes, as demonstrated by unimpaired growth of pathway mutants, but it does allow microbes to use phenylalanine and tyrosine as energy sources. One of the most common defects resulting in pyomelanin production is a loss of HmgA function. Pyomelanin production is regulated by global and operon specific regulators that can repress or activate gene transcription. Importantly, pyomelanin has several functions that allow microbes to survive in different environments, including resistance to oxidative and UV stress, and iron reduction and acquisition. The effects of pyomelanin on antibiotic resistance, infection persistence, and virulence have shown variability depending on the microbe tested and experimental assay conditions.

Since pyomelanin has functions that may contribute to pathogenesis and is conserved in several pathogenic bacteria and fungi, we investigated NTBC, which is a known inhibitor of the tyrosine catabolism pathway, as a potential anti-virulence therapy for *P. aeruginosa* infections. In our studies, we examined mechanisms of pyomelanin production in clinical isolates of *P. aeruginosa* from both acute and chronic infections. We also determined the inhibitory effect of NTBC on pyomelanin production and subsequent sensitivity to oxidative stress and aminoglycoside antibiotics. Additionally, we determined mechanisms for inherent NTBC resistance in clinical isolates of *P. aeruginosa*. Finally, we performed infection assays of the RAW 264.7 murine macrophage-like cell line by pyomelanogenic and non-pyomelanogenic strains of *P. aeruginosa* to determine the effects of pyomelanin on bacterial survival during infection. Our experiments show that NTBC has potential as an anti-virulence therapy, but additional studies must be performed.

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Chapter Two

NTBC treatment of the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111 inhibits pigment production and increases sensitivity to oxidative stress

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2.1 Introduction

Pseudomonas aeruginosa is an environmental bacterium that is capable of causing both acute and chronic infections in compromised patients. This organism is extremely adaptable, has a high level of intrinsic antibiotic resistance, a wide range of virulence factors, and the ability to form biofilms (reviewed in (1)). Antibiotics are an essential part of treating *P. aeruginosa* infections, but the inherent resistance combined with emerging resistance due to selective pressure limits the therapeutic options available. As a new strategy to combat infectious disease, the specific inhibition of virulence factors has been proposed as an alternate treatment mechanism (2). By attenuating bacterial virulence without targeting essential bacterial pathways, it may be possible to aid in the clearing of infections while minimizing selective pressures that perpetuate resistance.

Pyomelanin, a dark brown/black pigment is a potential target for anti-virulence compounds. Pyomelanin production has been reported in *P. aeruginosa* isolates from urinary tract infections and chronically infected cystic fibrosis (CF) patients (3, 4). Pyomelanin is one of many forms of melanin that is produced by a wide variety of organisms. Production of pyomelanin is reported to provide a survival advantage, scavenge free radicals, bind various drugs, give resistance to light and reactive oxygen species, and is involved in iron reduction and acquisition, and extracellular electron transfer (4-9). A number of environmental and pathogenic bacteria have been reported to produce this pigment (3, 8, 10-14). In *Shewanella oneidensis* and *S. algae*, pyomelanin plays a role in biogeochemical cycling of metals, as pigment production enhances hydrous ferric oxide reduction and electron transfer (15-17). In *Legionella*

pneumophilia, pigment production may contribute to pathogenesis as pyomelanin mediates ferric reduction from ferritin and transferrin (8). Non-pyomelanogenic strains of *Burkholderia cepacia* are more sensitive to externally generated oxidative stress and show reduced survival in phagocytic cells (11). In *P. aeruginosa* pyomelanin production results in increased persistence and virulence in mouse infection models (3).

Pyomelanin is a negatively charged extracellular pigment of high molecular weight, derived from the tyrosine catabolism pathway (6, 18, 19). 4-hydroxyphenylpyruvate is converted to homogentisate (HGA) by 4-hydroxyphenylpyruvate dioxygenase (Hpd) (Figure 2.1). HGA is then converted to 4-maleylacetoacetate by homogentisate 1,2-dioxygenase (HmgA). A loss of HmgA activity leads to the accumulation of HGA, which is secreted via the ABC transporter HatABCDE. Defects in either the ATP-binding cassette or the permease components of this transporter result in reduced pyomelanin production (4). Once secreted from the cell, HGA auto-oxidizes and self-polymerizes to form pyomelanin. Both point mutations in *hmgA* and chromosomal deletions have been reported in clinical *P. aeruginosa* isolates producing pyomelanin (3, 10).

Hpd activity is essential for the synthesis of HGA, and ostensibly irreversible binding with 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC) inhibits Hpd activity of *Streptomyces avermitilis* in vitro (20). Although it was originally developed as a herbicide, NTBC is a FDA approved treatment for type I tyrosinemia (21). Type I tyrosinemia is the result of a defect in the tyrosine catabolism pathway, which causes the accumulation of toxic metabolites such as fumarylacetoacetate, leading to cirrhosis and cancer of the liver (22). Binding of NTBC to Hpd prevents the

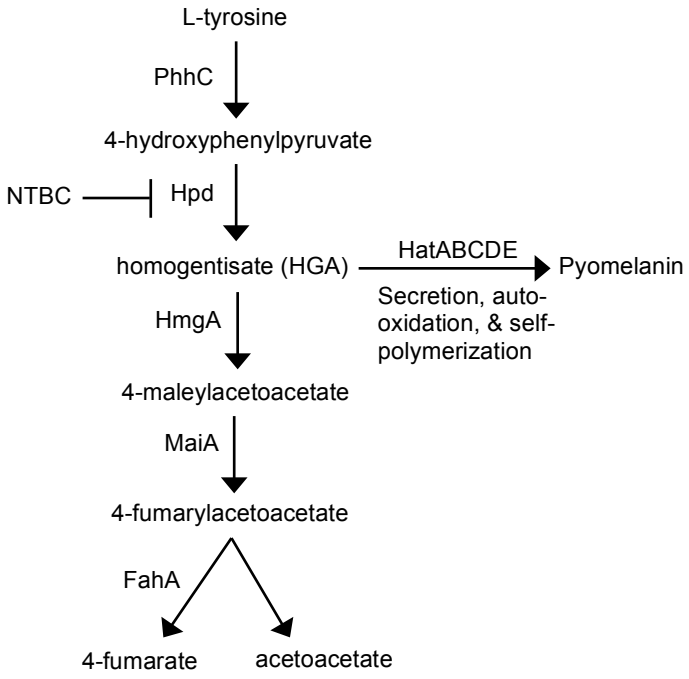

Figure 2.1. Tyrosine catabolism pathway of *Pseudomonas aeruginosa*. Inactivation of HmgA results in the secretion of HGA, which auto-oxidizes and self-polymerizes to form pyomelanin. NTBC inhibits HGA production and pyomelanin formation through interactions with Hpd.

accumulation of toxic metabolites and disease progression (21). We report here on NTBC treatment of pyomelanogenic strains of *P. aeruginosa*, the resulting reduction in pyomelanin production, and the corresponding increase in sensitivity to oxidative stress.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

Laboratory strains of *P. aeruginosa* PAO1 (obtained from Carrie Harwood, University of Washington), transposon mutants *hpd::tn* (PW2577) and *hmgA::tn* (PW4489) and the clinical isolate PA1111 (obtained from Dara Frank, MCW) were grown at 37°C in LB supplemented with tetracycline (60 µg/ml) and gentamicin (50 µg/ml) as appropriate. The transposon mutants were obtained from the University of Washington transposon mutant collection (23). *Escherichia coli* DH5α (NEB) was used as a host for recombinant plasmids, and was grown in LB with gentamicin (10 µg/ml) as appropriate.

2.2.2 Chemicals

NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione), H_2O_2, and tobramycin were purchased from Sigma-Aldrich. Gentamicin and kanamycin were purchased from Gold Bio and Fisher Scientific, respectively.

2.2.3 HmgA overexpression

hmgA (PA2009) was amplified from the PAO1 genome using primers hmgAfor (5’

GGTCTAGAATGAACCTCGACTCCACTGC 3') and hmgArev (5' CCGAGCTCTTATCTCCGTTGCGGGTTG 3') and cloned into the XbaI and SacI sites of pJN105 (24). The PCR product was sequenced to ensure no mutations were introduced. The resulting plasmids were transformed into *P. aeruginosa* strains. Following confirmation of transformation, HmgA expression was induced with arabinose during overnight growth in LB supplemented with gentamicin.

2.2.4 RT-PCR

Strains were grown overnight until pigment production occurred in pyomelanin producers. RNA was isolated from $5x10^8$ cells using RNeasy Mini Kit (Qiagen) and digested with DNaseI (Promega). RT-PCR was performed using OneStep RT-PCR Kit (Qiagen) with equal concentrations of RNA for all strains tested. Primers were designed to amplify 202 base pairs at the 5' end of *hpd*, beginning with the start codon. The following primers were used: hpD-RT-F (5' ATGAACGCCGTGCCAAGATCG 3') and hpD-RT-R (5' CGTTGAGCACGATGTTGATATC 3'). Primers used for the amplification of 200 base pairs of 16S rRNA are as follows: 16S-RT-PCR-F (5' GACTCCTACGGGAGGCAGC 3') and 16S-RT-PCR-R (5' GTATTACCGCGGCTGCTGGC 3'). Relative amounts of RT-PCR products were estimated using ImageJ software.

2.2.5 Southern Hybridization

Chromosomal DNA was isolated from laboratory and clinical isolates of *Pseudomonas aeruginosa*, digested with SalI, electrophoresed, and transferred to

positively charged nylon membranes by a downward capillary transfer method (25). A digoxigenin-labeled probe was generated through amplification of *hmgA* by PCR and the incorporation of digoxigenin-UTP by random priming as recommended by the manufacturer (Roche). Southern hybridizations were performed as previously described (26).

2.2.6 Growth Curves

Overnight cultures were grown in LB + 300 μM NTBC or LB with the corresponding amount of DMSO. The overnight cultures were diluted to OD_{600} 0.05 in LB + 300 μM NTBC or LB + DMSO, and the optical density was measured every hour. Each sample was pelleted and resuspended in LB prior to the optical density reading to ensure the results were not influenced by the presence of pyomelanin.

2.2.7 Oxidative stress assay

Overnight cultures were grown with NTBC (300 μM) or with a corresponding volume of DMSO as a control. Optical densities (OD_{600}) were measured using washed cells, and cultures were diluted to equivalent OD_{600} values (~2.5). Ten-fold serial dilutions were made in PBS containing either 300 μM NTBC or DMSO as appropriate. 5 μL of each serial dilution was spotted onto LB plates containing the indicated concentration of H_2O_2. Laboratory strains were incubated for 24 h and PA1111 was incubated for 45 h at 37°C.

2.2.8 Determination of MICs

Minimal inhibitory concentrations (MICs) were determined by two-fold serial microtiter broth dilution (27), using an inoculum of 2.75 x 10^5 CFU/ml. Inoculum concentration was determined using washed cells to ensure that pyomelanin production did not affect OD_{600} readings. NTBC was included in the appropriate wells at a final concentration of 300 μM. MICs were recorded as the lowest concentration of antibiotic inhibiting growth following 24 h of incubation at 37°C.

2.3 Results and Discussion

2.3.1 Pyomelanin production by a clinical isolate of *P. aeruginosa.*

PA1111, a pyomelanogenic clinical isolate from an acute infection was obtained from Dara Frank (Medical College of Wisconsin). This strain lacked type III secretion proteins but was cytotoxic in a tissue culture assay (28). To determine the cause of pyomelanin production in this isolate, HmgA (PA2009) from PAO1 was expressed from pJN105 (24). Following induction with 0.05% and 0.1% arabinose, pyomelanin production was eliminated in *hmgA::tn* and PA1111 respectively (Figure 2.2A). *P. aeruginosa hmgA::tn* functions as a positive control for pyomelanin production as *hmgA* is interrupted with the IS*phoA/hah* transposon (23). *P. aeruginosa* PAO1 and *hpd::tn* (isogenic to *hmgA::tn*) were included as negative controls; neither strain produces pyomelanin.

Since increased amounts of arabinose were required to eliminate pyomelanin production in PA1111 relative to *hmgA::tn* (compare 0.05 to 0.1%), we assayed *hpd* transcript levels through RT-PCR (Figure 2.2B). Quantification of the relative levels

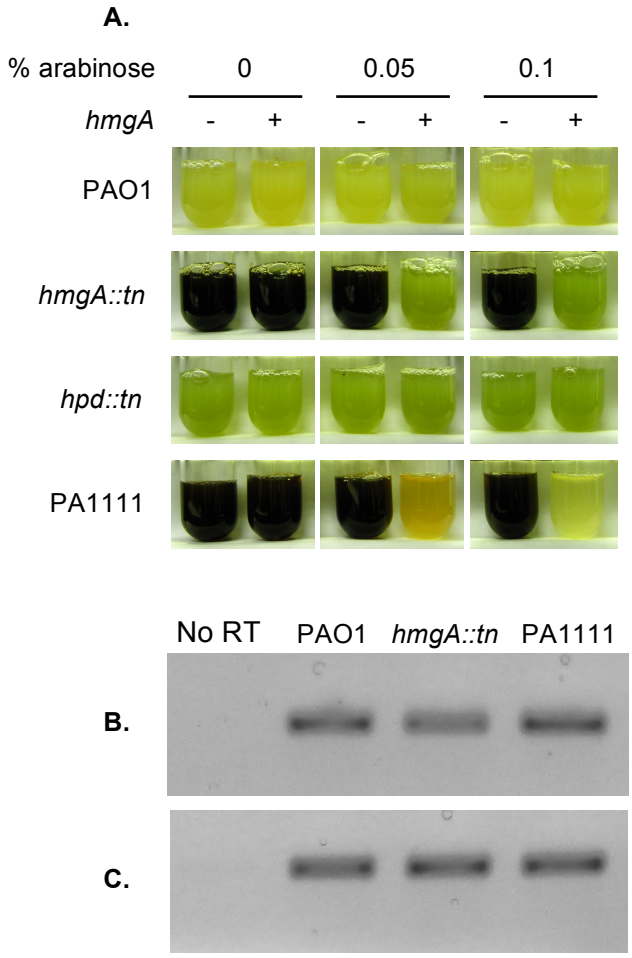

Figure 2.2. HmgA expression alleviates pyomelanin production in lab and clinical isolates in a dose dependent manner. **A)** The indicated *P. aeruginosa* strains containing either *hmgA*-pJN105 (+) or pJN105 (-) were incubated overnight in LB + gentamicin (50 µg/ml) with the indicated concentrations of arabinose. **B)** RT-PCR amplification of *hpd* transcript in PAO1, *hmgA::tn*, and PA1111. **C)** RT-PCR amplification of 16S rRNA from PAO1, *hmgA::tn* and PA1111.

revealed that in both PAO1 and PA1111 *hpd* transcript was approximately 10% more abundant than in *hmgA::tn*. It is unlikely that this subtle increase in *hpd* transcript levels is responsible for the residual pyomelanin production in PA1111 at low levels of induction (0.05% arabinose). This suggests that the clinical isolate may have altered translation or post-translational modification resulting in increased expression or activity of Hpd.

The ability to abolish pyomelanin production in PA1111 through expression of wild type HmgA suggested that either a chromosomal deletion or inactivation of the *hmgA* gene occurred, both of which have been reported in clinical isolates of *P. aeruginosa* (3, 10). A third reported cause of pyomelanin production is imbalanced enzyme expression within the L-tyrosine catabolism pathway. In *Vibrio cholerae* (ATCC 14035), homogentisate dioxygenase and the downstream enzymes are expressed at lower levels than hydroxyphenylpyruvate dioxygenase, leading to an accumulation of HGA and pyomelanin production (29). To determine the genetic cause of pyomelanin production in PA1111, we attempted to PCR amplify and sequence *hmgA*. Despite repeated attempts, we were unable to amplify *hmgA* via colony PCR. To verify these results, Southern hybridization was performed with DIG-labeled *hmgA* as a probe. No hybridization was detected between the *hmgA* probe and the PA1111 genome (Figure 2.3). This, combined with our ability to complement the pyomelanin phenotype via induction of *hmgA* expression, suggests that a chromosomal deletion has occurred.

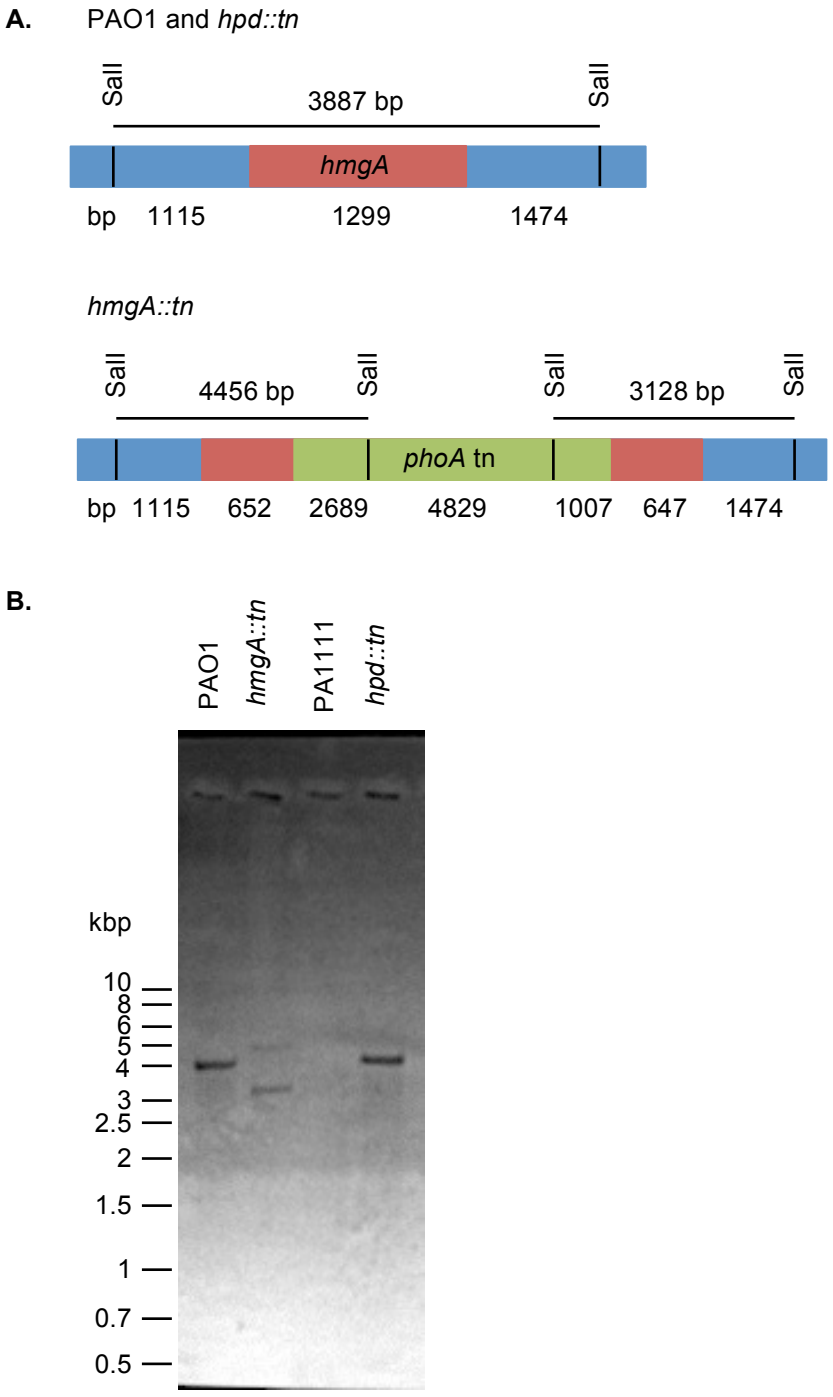

Figure 2.3. PA1111 has a chromosomal deletion of *hmgA*. **A)** Schematic for predicted band sizes with SalI digestion of genomic DNA from PAO1, *hpd::tn*, and *hmgA::tn*. Blue, chromosomal DNA upstream and downstream from *hmgA*; red, *hmgA*; green, *phoA* transposon (tn) in *hmgA*. **B)** Southern blot hybridization of DIG-*hmgA* probe to SalI digested DNA from PAO1, *hmgA::tn*, PA1111, and *hpd::tn*. Bands at the predicted sizes were seen for PAO1, *hmgA::tn*, and *hpd::tn*. PA1111 showed no hybridization with DIG-*hmgA*, indicating that a chromosomal deletion of *hmgA* occurred. kbp, kilo base pairs.

2.3.2 NTBC inhibits pyomelanin production in *P. aeruginosa* without disrupting growth.

NTBC is known to bind Hpd (4-hydroxyphenylpyruvate dioxygenase) and inhibit the conversion of 4-hydroxyphenylpyruvate to homogentisate (20). We, therefore, assayed NTBC treatment for disruption of pyomelanin production in *P. aeruginosa*. The two pyomelanin producing strains (*hmgA::tn* and PA1111) were grown overnight with increasing amounts of NTBC. Following overnight growth, inhibition of pyomelanin production was determined visually (Figure 2.4A). NTBC (300 μM) inhibited pyomelanin production in *hmgA::tn,* while PA1111 required higher concentrations of NTBC to inhibit pyomelanin production (900 μM). Sequencing of hpd_{PA1111} revealed two silent mutations upon comparison with hpd_{PAO1} (PA0865), demonstrating that mutations within Hpd were not responsible for the residual PA1111 pyomelanin production in the presence of 300 μM NTBC. To ensure that NTBC did not alter the growth of *P. aeruginosa,* we measured the optical densities of cultures grown in the presence and absence of NTBC. The laboratory strains and PA1111 grew at the same rate in the presence or absence of 300 μM NTBC (Figure 2.4B), indicating that the reduction in pigmentation was not due to altered growth rates.

2.3.3 NTBC treatment of pyomelanogenic strains does not alter aminoglycoside MICs.

It has been reported that melanin has the ability to non-specifically bind a number of diverse compounds. Isotherm analysis indicated that gentamicin had a high level of binding to synthetic melanin through a series of diverse interactions (30). Melanin-

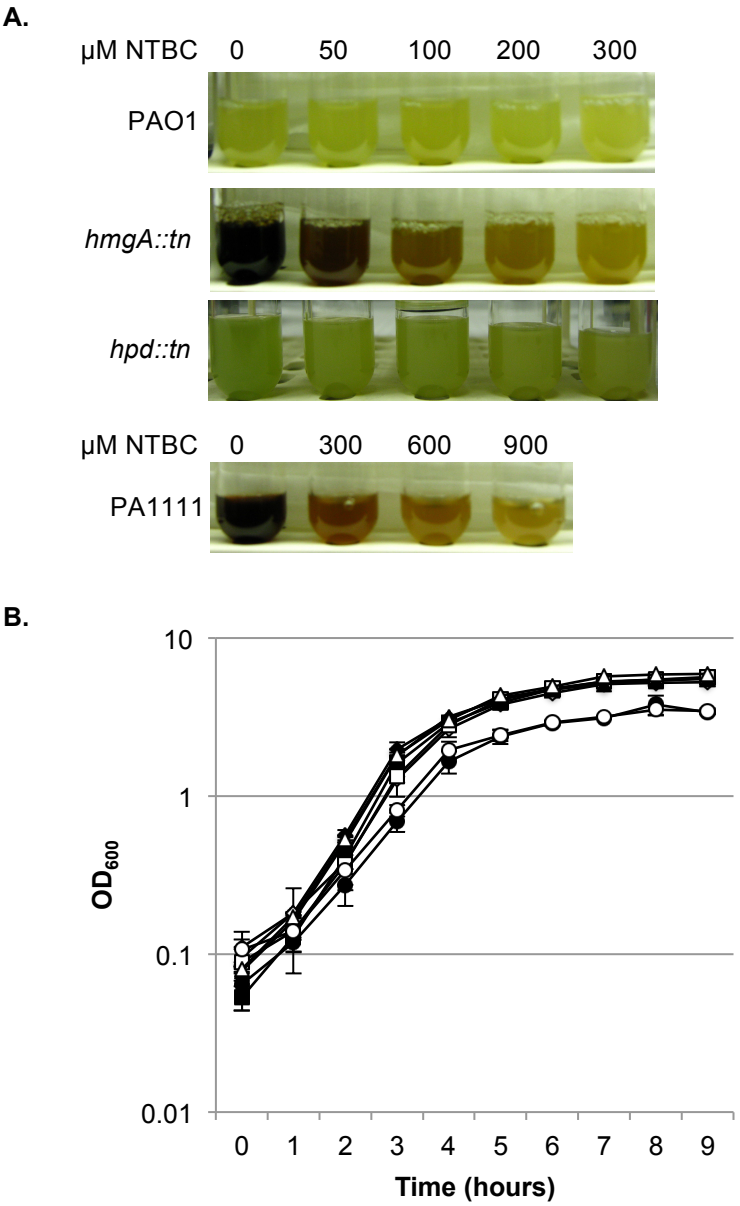

Figure 2.4. NTBC treatment inhibits pyomelanin production without affecting growth. **A)** Pyomelanin production by *P. aeruginosa* with and without NTBC treatment. Laboratory and clinical strains were grown overnight with the indicated concentrations of NTBC. **B)** Growth curves of laboratory and clinical strains of *P. aeruginosa* with and without 300 μM NTBC treatment. Strains grown without NTBC are indicated with closed symbols, while those grown with NTBC are indicated with open symbols. Wild type PAO1 (diamonds), *hpd::tn* (triangles), *hmgA::tn* (squares), PA1111 (circles). The growth curves are compiled from three independent experiments, with error bars indicating standard error of the mean.

tobramycin interactions have resulted in a decrease of antibiotic activity of 80% under certain conditions (31). Aminoglycosides are positively charged at physiological pH, which may contribute to the interactions with negatively charged melanin (6). Furthermore, a significant correlation was seen between pyomelanin production in *Stenotrophomonas maltophilia* and resistance to specific antibiotics (14). We therefore assayed both pyomelanin producing and non-producing strains (with and without NTBC treatment) to determine the minimal inhibitory concentrations (MICs) of aminoglycosides.

Minimal inhibitory concentrations were determined by two-fold serial microtiter broth dilution (27). Our results indicated that, under these conditions, the pyomelanin producing strains (*hmgA::tn* and PA1111) did not show significantly higher aminoglycoside MICs than the non-pyomelanin producing strains (PAO1 and *hpd::tn*, Table 2.1). While treatment of the pyomelanin producing strains with NTBC did inhibit pyomelanin production, the MICs remained unchanged. These data indicated that neither pyomelanin production nor NTBC treatment affect the aminoglycoside MICs for *P. aeruginosa*. This is in agreement with an earlier study wherein MICs were unaltered by pyomelanin production (3), and provides further clarity to the discussion within the literature regarding pyomelanin production and antibiotic resistance. Early studies of pyomelanin production reported that pyomelanogenic *P. aeruginosa* isolates were more sensitive to antibiotics when compared to non-pyomelanogenic strains (32). In contrast, when *Staphylococcus aureus* was incubated in supernatant from pyomelanogenic *P. aeruginosa*, the MIC values remained unchanged (33). When considering the results of these studies it is critical consider the sources of the melanin; the isotherm analysis was

Table 2.1. Aminoglycoside MICs (μg/ml) of laboratory and clinical isolates of *P. aeruginosa*.

	PAO1		*hmgA::tn*		*hpd::tn*		PA1111	
	-NTBC	+NTBC	-NTBC	+NTBC	-NTBC	+NTBC	-NTBC	+NTBC
Gentamicin	1	0.5	2	2	1	1	0.5	0.5
Kanamycin	16	8	32	32	32	32	16	16
Tobramycin	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5

Three independent colonies were tested in triplicate for each strain.

conducted with eumelanin (or synthetic melanin) generated from 3,4-dihydroxyphenylalanine (DOPA), not pyomelanin generated from homogentisate (6). It is possible that the discrepancy between our results and the isotherm studies is due to differences in melanin structures (G. Moran, personal communication). While the *S. maltophilia* studies did correlate pyomelanin production with increased resistance to some β-lactam antibiotics and fluoroquinolones, resistance was not detected to either gentamicin or trimethoprim/sulfamethoxazole (14). Importantly, a direct causal relationship was not tested, and the authors acknowledged that these phenotypes could have resulted from independent mutations.

2.3.4 NTBC treatment of pyomelanin producing *P. aeruginosa* increases sensitivity to oxidative stress.

The antioxidant properties of pyomelanin are proposed to contribute to the increased persistence and virulence of pyomelanogenic bacteria in infection models (3, 11, 12). Since pyomelanogenic strains of *Burkholderia cepacia* and *P. aeruginosa* have increased resistance to hydrogen peroxide, we examined if NTBC treatment increased sensitivity of pyomelanogenic strains of *P. aeruginosa* to oxidative stress.

The H_2O_2 spot plates showed that both pyomelanogenic strains (*hmgA::tn* and PA1111) have increased resistance to hydrogen peroxide relative to the non-pyomelanogenic strains (PAO1 and *hpd::tn*) (Figure 2.5). Importantly, NTBC treatment of pyomelanogenic strains resulted in increased sensitivity to 0.6 mM H_2O_2. This illustrates the potential use of NTBC as an anti-virulence factor. The change in sensitivity to H_2O_2 was smaller for PA1111 than *hmgA::tn*, and resulted in an

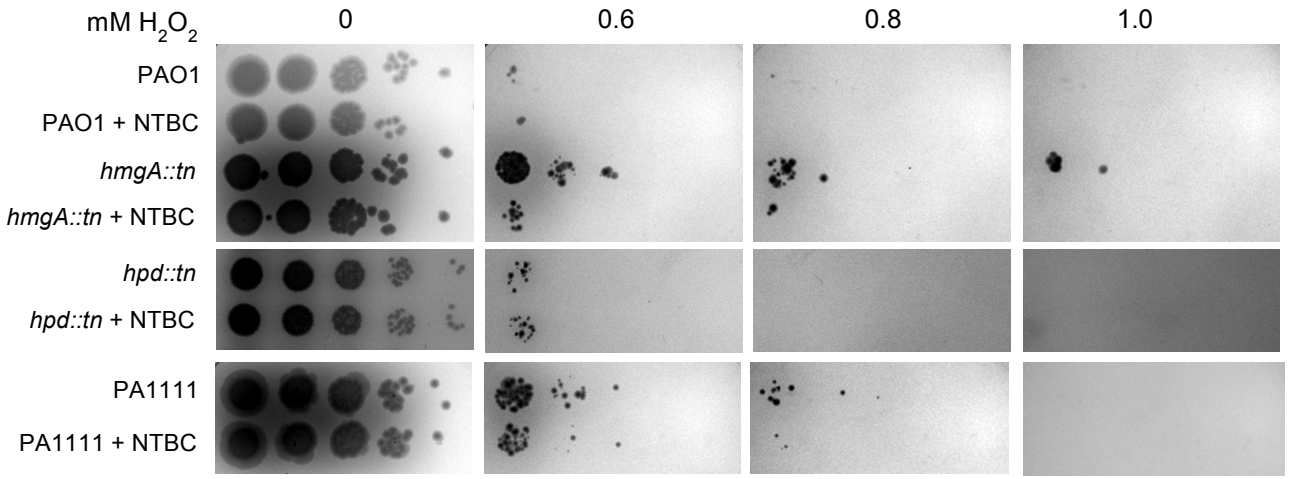

Figure 2.5. NTBC treatment increases H_2O_2 sensitivity in pyomelanin producing strains. 10-fold serial dilutions of the indicated strains were spotted onto LB plates containing the indicated concentrations of hydrogen peroxide.

approximately 24% reduction in number of PA1111 colony forming units (based on 4 independent experiments). It is likely that the residual pyomelanin produced in PA1111 at 300 μM NTBC provides a small level of protection against oxidative stress compared to *hmgA::tn*. As expected, NTBC treatment of either wild type PAO1 or *hpd::tn* did not affect sensitivity to H_2O_2.

In this report, we determined that the pyomelanin production in a strain of *P. aeruginosa* PA1111 isolated from an acute infection was likely due to the loss of HmgA activity resulting from a chromosomal deletion (28). This phenotype has previously been reported for CF isolates and has been shown to decrease clearance/increase persistence in mouse models of chronic infection, suggesting that the development of pyomelanin production may confer an adaptive advantage (3, 10). Given the antioxidant properties of pyomelanin, it is likely that pigment production would provide protection from oxidative stress in both chronic and acute infections.

This study has shown that NTBC treatment inhibited pyomelanin production by *P. aeruginosa*, and in doing so increased the sensitivity of both laboratory and clinical isolates to oxidative stress, as is found in the respiratory burst from macrophages and monocytes. This suggests that NTBC, as an already FDA-approved compound, has potential as an anti-virulence factor that could be used in combination with existing antibiotics. Pyomelanin is made by a wide variety of organisms, and has been reported in both chronic and acute infections. Given the number of organisms that produce pyomelanin, its functions in iron acquisition and as an antioxidant, and the presence of pyomelanin in both acute and chronic infections, there are a high number of potential applications of NTBC as an anti-virulence factor.

2.4 Acknowledgements

The authors thank Dara Frank and Carrie Harwood for their generous contribution of strains. We thank G. Moran, and D. Stafford for helpful comments and discussion. University of Wisconsin Milwaukee Research Foundation holds patent no. 8,354,451; with claims broadly directed to treating or inhibiting the progression of infection of a microorganism in a patient by administering a 4-hydroxyphenylpyruvate dioxygenase-inhibiting compound such as 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). Inventors are Graham Moran and Pang He. This research was supported by the National Institutes of Health (R00-GM083147). The University of Washington *P. aeruginosa* transposon mutant library is supported by NIH P30 DK089507.

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Chapter Three

Resistance to NTBC in the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate DKN343

3.1 Introduction

Pyomelanin has several functions that are involved in helping bacteria and fungi survive in host organisms or the environment. First, pyomelanin can provide protection from oxidative stress and UV light (1-8). Additionally, pyomelanin is involved in electron transfer, iron reduction, and iron acquisition (9-12). Studies have also shown that pyomelanin may be involved in *Pseudomonas aeruginosa* infection persistence (2, 13). Pyomelanin production in *Vibrio campbellii* has also been associated with reduced virulence (14), which could allow for increased infection persistence.

Many clinical and environmental bacterial and fungal species have been reported to produce pyomelanin, including *P. aeruginosa*, *Burkholderia cepacia* complex, *Vibrio cholerae*, *Legionella pneumophila*, *Shewanella algae*, *Bacillus anthracis*, *Aeromonas media*, *Acinetobacter baumannii*, *Sinorhizobium meliloti, Streptomyces coelicolor*, *Alteromonas stellipolaris*, *Aspergillus fumigatus*, *Sporothrix* sp., and *Penicillium chrysogenum* (4-7, 10, 12, 15-23). In these strains, pyomelanin is either naturally produced in the wild type strain or is the result of a mutation or deletion of genes known to be involved in pyomelanin production.

Pyomelanin is a negatively charged, extracellular, reddish-brown pigment derived from the tyrosine catabolism pathway when homogentisate (HGA) is secreted from the cell via the HatABCDE ABC transport system, auto-oxidized, and self-polymerized (Figure 3.1) (2, 24-26). 4-hydroxyphenylpyruvate dioxygenase (Hpd) is required for pyomelanin production as it converts 4-hydroxyphenylpyruvate into HGA. Inactivation of *hpd* via deletion or transposon mutagenesis in pyomelanin producers eliminates pigment production (5, 12, 26, 27). During the breakdown of tyrosine, HGA is converted

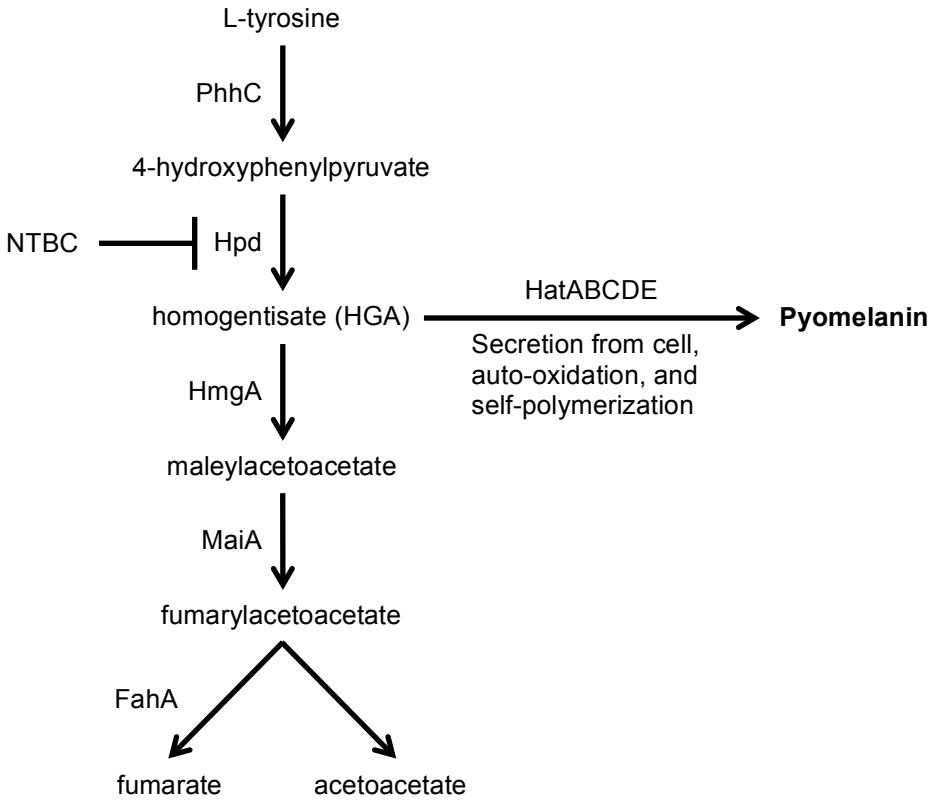

Figure 3.1. Tyrosine catabolism pathway in *P. aeruginosa*. Pyomelanin is produced via inactivation of HmgA, which results in secretion of HGA from the cell and subsequent auto-oxidation and self-polymerization to form pigment. NTBC binds to Hpd to prevent formation of HGA and pyomelanin production. PhhC, aromatic amino acid aminotransferase; Hpd, 4-hydroxyphenylpyruvate dioxygenase; HmgA, homogentisate 1,2-dioxygenase; MaiA, maleylacetoacetate isomerase; FahA, fumarylacetoacetase; HatABCDE, HatABCDE ABC transporter.

into 4-maleylacetoacetate by homogentisate 1,2-dioxygenase (HmgA). Mutations that result in loss of HmgA function lead to an accumulation of HGA and consequently pyomelanin production (1, 2, 28). A relative reduction in enzyme activity in the latter part of the tyrosine catabolism pathway (HmgA, MaiA, and FahA) compared to the upper part of the pathway can also lead to pyomelanin production (29).

Bacteria are becoming more resistant to antibiotics, so investigation into new antimicrobial agents is necessary. Anti-virulence therapy targets mechanisms that are beneficial for pathogen interactions with the host, instead of targeting functions that are necessary for basic metabolism (30). This allows the therapy to have specificity for pathogens over the normal flora in the host (30). Since pyomelanin has several functions that may be advantageous for bacterial or fungal survival in a host, and has been reported to contribute to infection persistence, it is an ideal target for anti-virulence therapy. We have previously shown that the Hpd inhibiting compound 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) reduces pyomelanin production in *P. aeruginosa* clinical isolates and increases sensitivity to oxidative stress (1). NTBC, therefore, shows promise as an anti-virulence treatment.

P. aeruginosa is a Gram negative bacterium that is able to survive in a variety of different ecological environments including soil, water, plants, and animals (31). This organism is also an opportunistic pathogen that frequently colonizes the lungs of cystic fibrosis (CF) patients, where it causes chronic infections by forming biofilms (32). Additionally, it causes acute infections in burn patients (33, 34). *P. aeruginosa* is capable of producing a variety of virulence factors including secreted proteins, toxins, and pigments that may help the bacteria survive during infection (35).

In our current study, we identified the mechanism of pyomelanin production in the clinical *P. aeruginosa* isolate DKN343. DKN343 was previously reported to have a wild type *hmgA* sequence, and expression of *hmgA* from a plasmid did not alleviate pyomelanin production, which suggested that pyomelanin was produced via a different mechanism than inactivation of HmgA (26). We found, however, a mutation in HmgA that is predicted to render the enzyme non-functional causing a defect in tyrosine catabolism and resulting in pyomelanin production. In addition, we discovered a high level of pre-existing resistance to NTBC, which was mediated by the MexAB-OprM multi-drug efflux pump.

3.2 Materials and Methods

3.2.1 Strains, plasmids, and growth conditions

Lists of strains and plasmids are found in Tables 3.1 and 3.2, respectively. Strains were grown on LB agar or in LB broth supplemented with gentamicin, tetracycline, or chloramphenicol where appropriate. Antibiotic concentrations were as follows: gentamicin 10 μg ml^{-1} (*E. coli*), 50 μg ml^{-1} (*hpd::tn, hmgA::tn*, and DKN343 in broth media), and 100 μg ml^{-1} (DKN343 on agar); tetracycline 10 μg ml^{-1} (*E. coli*) and 195 μg ml^{-1} (DKN343); chloramphenicol 5 μg ml^{-1}. Strains were grown at 37°C unless otherwise indicated.

Table 3.1. Strains used in this study.

Strains	Description	Source
P. aeruginosa PAO1	*P. aeruginosa* PAO1 (Iglewski strain)	Carrie Harwood
P. aeruginosa hpd::tn	PW2577, transposon mutant with *lacZ-tn* inserted in *hpd*, Tc^R, pyomelanin non-producer	University of Washington PAO1 transposon mutant collection
P. aeruginosa hmgA::tn	PW4489, transposon mutant with *phoA*-tn inserted in *hmgA*, Tc^R, pyomelanin producer	University of Washington PAO1 transposon mutant collection
P. aeruginosa DKN343	Clinical isolate of *P. aeruginosa* from sputum sample, pyomelanin producer	(26)
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
E. coli S17-1	Tp^R Sm^R *recA thi pro* $hsdR^-$ M^+ RP4 2-Tc::Mu-Km::Tn7 λpir Strain for conjugating plasmids	(36)
*hmgA::tn*Δ*hpd*	In-frame deletion of *hpd* in *hmgA::tn*	This study
DKN343Δ*hpd*	In-frame deletion of *hpd* in DKN343	This study
*hmgA::tn*Δ*mexA*	In-frame deletion of *mexA* in *hmgA::tn*	This study
DKN343Δ*mexA*	In-frame deletion of *mexA* in DKN343	This study
*hmgA::tn*ΔPA0242	In-frame deletion of PA0242 in *hmgA::tn*	This study
DKN343ΔPA0242	In-frame deletion of PA0242 in DKN343	This study

Table 3.2. Plasmids used in this study.

Plasmids	Description	Source
pEX18Tc	Suicide vector for making deletion mutants, Tc^R	Carrie Harwood
pEX19Gm	Suicide vector for making deletion mutants, Gm^R	Carrie Harwood
Δ*hpd*-pEX18Tc	Fusion fragment for *hpd* deletion cloned into pEX18Tc at the BamHI and HindIII sites	This study
Δ*hpd*-pEX19Gm	Fusion fragment for *hpd* deletion cloned into pEX19Gm at the EcoRI and HindIII sites, EcoRI site was from pEX18Tc	This study
Δ*mexA*-pEX18Tc	Fusion fragment for *mexA* deletion cloned into pEX18Tc at the XbaI and KpnI sites	This study
Δ*mexA*-pEX19Gm	Fusion fragment for *mexA* deletion cloned into pEX19Gm at the XbaI and KpnI sites	This study
ΔPA0242-pEX18Tc	Fusion fragment for PA0242 deletion cloned into pEX18Tc at the SacI and HindIII sites	This study
ΔPA0242-pEX19Gm	Fusion fragment for PA0242 deletion cloned into pEX19Gm at the SacI and HindIII sites	This study
pSB109	Derivative of pJN105 with an enhanced ribosome binding site, 6x-His tag, Gm^R	This study
hmgA-pSB109	*hmgA* from PAO1 cloned into pSB109 at the NdeI and SacI sites	This study
$hmgA_{A306T}$-pSB109	*hmgA* from PAO1 with point mutation for A306T	This study
$hmgA_{H330Y}$-pSB109	*hmgA* from PAO1 with point mutation for H330Y	This study
hpd-pSB109	*hpd* from PAO1 cloned into pSB109 at the NdeI and SacI sites	This study
mexA-pSB109	*mexA* from PAO1 cloned into pSB109 at the NdeI and SacI sites	This study
PA0242-pSB109	PA0242 from PAO1 cloned into pSB109 at the EcoRI and SacI sites	This study

3.2.2 Generation of deletion mutants

In-frame deletion mutants of *hpd, mexA* and PA0242 in *P. aeruginosa* were constructed by splicing by overlap extension (SOE) PCR with PAO1 DNA as template. Primers are listed in Table 3.3. The in-frame fusions were sequenced to ensure no mutations were introduced. The deletion alleles were cloned into pEX19Gm or pEX18Tc and transformed into *E. coli* S17-1 for mating with *P. aeruginosa hmgA::tn* and DKN343, respectively. These constructs were introduced into *P. aeruginosa* by conjugation, and merodiploids were selected on chloramphenicol and tetracycline or gentamicin as appropriate and incubated overnight (*hmgA::tn*) or two days (DKN343) to isolate merodiploids. Resolution of the merodiploids was achieved through 10% sucrose counter selection. Following screening on tetracycline or gentamicin and sucrose, the deletions were confirmed by PCR or Southern blot.

3.2.3 NTBC titrations

NTBC titrations were performed as previously described (37) with gentamicin and arabinose supplementation where appropriate. NTBC (Sigma-Aldrich) was dissolved in DMSO and stored at -20°C until use. Cultures were incubated for approximately 24 hours before photos were taken for analysis.

Table 3.3. Primers used in this study.

Primer name	**Primer Sequence** (5' – 3')[a,b]
Primers for in-frame deletions	
Δ*hpd*-up-For	GTAGCGGATCCCGATGCCTGCCACCGGAC
Δ*hpd* -up-Rev	GAGGCTGGCGGCAGCGGGACCGGCCTCCTCGTTGTTC
Δ*hpd* -dn-For	GAACAACGAGGAGGCCGGTCCCGCTGCCGCCAGCCTC
Δ*hpd* -dn-Rev	CGCCGAAGCTTGCCGCGGTGAAGCCGAGC
Δ*mexA*-up-For	CCTCTAGACATCACCGGCAACCTGACCC
Δ*mexA* -up-Rev	CCCCTTGATCAGCCCTTGCTTCGTTGCATAGCGTTGTCCTC
Δ*mexA* -dn-For	GAGGACAACGCTATGCAACGAAGCAAGGGCTGATCAAGGGG
Δ*mexA* -dn-Rev	GGGGTACCTACGGGTAGACCACCTTC
ΔPA0242-up-For	CCAAGAGCTCAACCACAGGCGGGTGGTC
ΔPA0242-up-Rev	GACAATTCTCACAGCTTGACACGCTGCATGCTTCGACTC
ΔPA0242-dn-For	GAGTCGAAGCATGCAGCGTGTCAAGCTGTGAGAATTGTC
ΔPA0242-dn-Rev	GTCCAAGCTTCCTGTGGGTCGCCGAGGTG
Primers for gene sequencing	
hmgA-external forward	CGATAAAAATAACGCAGCCAGC
hmgA-external reverse	GGTTTTGCAGGGGAAAGTCG
hpd-external forward	GGCATCTCCCATGTCGTCGGC
hpd-external reverse	GCCGCTGGAACGGAAACGC
Primers for complementation	
hmgA-for-pSB109	GGCATATGATGAACCTCGACTCCACTGC
hmgA-rev	CCGAGCTCTTATCTCCGTTGCGGGTTG
hpd-for-pSB109	GGCATATGATGAACGCCGTGGCCAAG
hpd-rev	GGGAGCTCGGATCAGATCACGCCGCG
mexA-for-pSB109	CCCATATGATGCAACGAACGCCAGCC
mexA-rev	GGGAGCTCTCAGCCCTTGCTGTCGGTTTTC
PA0242-for-pSB109	GGGAATTCATGCAGCGTTCGATCGCC
PA02424-rev	GGGAGCTCATTCTCACAGCTTGACCC
Primers for site directed mutagenesis	
hmgA-A306T-for	CAGCGTCCCCGGCCTG**ACC**AACATCGACTTCGTG
hmgA-A306T-rev	CACGAAGTCGATGTT**GGT**CAGGCCGGGGACGCTG
hmgA-H330Y-for	CCGTCCGCCATGGTTC**TAC**CGCAACCTGATGAACG
hmgA-H330Y-rev	CGTTCATCAGGTTGCG**GTA**GAACCATGGCGGACGG

[a]Restriction enzyme sites are underlined.
[b]Mutagenic codons are in bold.

3.2.4 Sequencing of *hmgA* and *hpd* from DKN343

hmgA and *hpd* from DKN343 were PCR amplified using the appropriate primers (Table 3.3), purified, and sequenced. The sequencing results were compared to *P. aeruginosa* PA14 to identify any mutations.

3.2.5 Complementation studies

Plasmids for complementation were created by amplifying *hmgA*, *hpd*, *mexA*, and PA0242 from PAO1 using the appropriate primers (Table 3.3). Gene fragments were sequenced and cloned into pSB109. Plasmids were maintained in *E. coli* DH5α and transformed into the appropriate *P. aeruginosa* strain. Strains containing pSB109 empty vector were used as controls. Test tubes containing LB and gentamicin were inoculated with washed overnight cultures to a concentration of OD_{600} 0.05 (*hpd::tn* and *hmgA::tn*) or 0.1 (DKN343) and grown to OD_{600} 0.2-0.3 before induction with arabinose (0-0.5%) for 1.5 h. Whole cell lysates were separated by SDS-PAGE. The remaining cultures were incubated for a total of 24 hours and photos were taken for analysis.

3.2.6 Site directed mutagenesis

Single point mutations of A306T and H330Y in *hmgA* were generated by site directed mutagenesis. PCR was performed with the appropriate mutagenic primers (Table 3.3) using the *hmgA*-pSB109 plasmid as template. The PCR product was digested with DpnI (New England Biolabs) and transformed into DH5α cells. The

plasmid DNA was sequenced to confirm the introduced mutation was present and transformed into *hmgA::tn* for complementation studies as described above.

3.2.7 SDS-PAGE and Western blots

Whole cell lysates from complementation studies were analyzed by SDS-PAGE on 10% gels. Proteins were transferred to PVDF and probed using mouse-α-His antibody (1:3000) and sheep-α-mouse horseradish peroxidase antibody (1:10000). Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate and a CCD camera with Fotodyne software.

3.2.8 Nitrocefin hydrolysis assay

The nitrocefin assay to determine outer membrane permeability was modified from previously described procedures (38, 39). Overnight cultures were diluted 1:60 in 30 ml LB and incubated for 2 h at 37°C with shaking. Imipenem (0.25 μg ml^{-1}) was added to induce β-lactamase expression. Cultures were incubated for an additional 3 h before cells were harvested by centrifugation (5000 xg, 10 min). Cell concentration was normalized based on OD_{600}, and cell pellets were washed and concentrated in 50 mM sodium phosphate buffer (pH 7.2). Aliquots of cells were treated with 0 and 0.1 mM EDTA for 5 min. Samples were centrifuged at 15000 xg for 30 min at room temperature and β-lactamase containing supernatant was saved. β-lactamase containing supernatant was diluted in 50 mM sodium phosphate buffer (pH 7.2) and nitrocefin (Calbiochem) was added at a final concentration of 100 μM in a total volume of 1 ml.

Nitrocefin hydrolysis was measured spectrophotometrically at 482 nm and β-lactamase activity (U/L) was calculated using the extinction coefficient of 17.4 mM^{-1} cm^{-1} for nitrocefin. The enzyme activity per OD_{600} of cells was calculated and averaged over five biological replicates. Statistical analysis was performed by ANOVA, followed by Tukey HSD post-hoc analysis using R (version 3.2.4).

3.2.9 Measurement of pyomelanin production in strains treated with EDTA and NTBC

LB containing 0 and 0.1 mM EDTA with 0 and 50 μM NTBC for *hpd::tn* and *hmgA::tn*, or 0 and 900 μM NTBC for DKN343, were inoculated to an OD_{600} 0.05 with washed overnight cultures. Cultures were grown for approximately 24 h, then cells were pelleted by centrifugation (2 min, 16000 xg), supernatants were collected, and the cell pellet was suspended in an equivalent volume of LB. OD_{600} measurements of the pyomelanin containing supernatant and the resuspended cells were used to calculate the ratio of pyomelanin to cells. This ratio was averaged for three colonies tested in triplicate and percent pyomelanin produced relative to the untreated strain was calculated.

3.2.10 Photo analysis and image manipulation

All photos were taken on a Canon PowerShot A480 digital camera. Photos were cropped in Adobe Photoshop and image resolution was adjusted to 600 pixels/inch.

3.3 Results

Previous studies in our laboratory have focused on the ability of NTBC to inhibit pyomelanin production in clinical isolates of *P. aeruginosa* (1). During these studies we determined that the clinical isolate DKN343 required increased concentrations of NTBC to inhibit pyomelanin production when compared to the pyomelanogenic control *hmgA::tn* (Figure 3.2). DKN343 was isolated from a sputum sample (D. Newman, personal communication) and was reported to have wild type PA14 sequence in *hmgA* (26). Pyomelanin production typically results from either point mutations or deletions of *hmgA*, or an imbalance in enzyme activity within the tyrosine catabolism pathway (2, 28, 29). We therefore investigated the mechanism of pyomelanin production and resistance to NTBC in this clinical isolate.

3.3.1 Pyomelanin production in DKN343 results from a loss of function mutation in HmgA.

To determine the mechanism for pyomelanin production, $hmgA_{DKN343}$ was PCR amplified and sequenced, revealing two mutations when compared to PA14 *hmgA*; in the resulting protein the mutated residues were A306T and H330Y (Figure 3.3). These mutations suggested to us that, contrary to previous reports, DKN343 may produce pyomelanin due to a loss of function mutation in HmgA. Additionally, the H330Y mutation occurred in the HmgA iron co-factor binding site, which we hypothesized would be required for functional HmgA. We therefore assayed the role of the tyrosine catabolism pathway in DKN343 pyomelanin production.

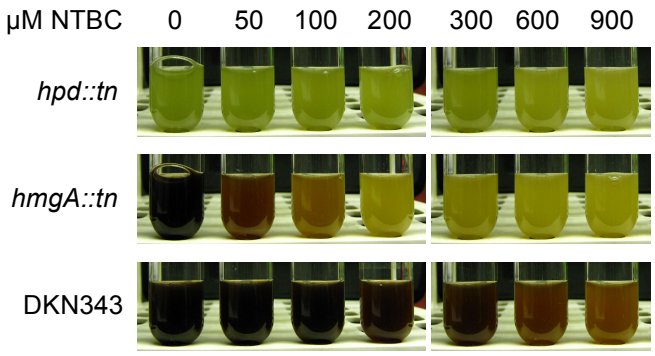

Figure 3.2. NTBC treatment reduced pigment production in pyomelanogenic *P. aeruginosa* strains. Strains were grown in LB with the indicated concentrations of NTBC. The non-pyomelanogenic control *hpd::tn* showed no pigment change in response to NTBC treatment. The pyomelanin producers *hmgA::tn* and DKN343 showed dose dependent reductions in pigmentation with increasing concentrations of NTBC. *hmgA::tn* was the positive control for pyomelanin production. The clinical isolate DKN343 was less responsive to NTBC than the laboratory strain *hmgA::tn*, as indicated by the requirement for higher concentrations of NTBC to reduce pyomelanin production.

```
PA14        MNLDSTALAYQSGFGNEFSSEALPGALPVGQNSPQKAPYGLYAELLSGTAFTMARSEARR 60
DKN343      MNLDSTALAYQSGFGNEFSSEALPGALPVGQNSPQKAPYGLYAELLSGTAFTMARSEARR 60
            ************************************************************

PA14        TWLYRITPSAKHPPFRRLERQIAGAELDAPTPNRLRWDPLALPEQPTDFLDGLLRMAANA 120
DKN343      TWLYRITPSAKHPPFRRLERQIAGAELDAPTPNRLRWDPLALPEQPTDFLDGLLRMAANA 120
            ************************************************************

PA14        PGDKPAGVSIYQYLANRSMERCFYDADGELLLVPQLGRLRLCTELGALQVEPLEIAVIPR 180
DKN343      PGDKPAGVSIYQYLANRSMERCFYDADGELLLVPQLGRLRLCTELGALQVEPLEIAVIPR 180
            ************************************************************

PA14        GMKFRVELLDGEARGYIAENHGAPLRLPDLGPIGSNGLANPRDFLAPVARYEDSRQPLQL 240
DKN343      GMKFRVELLDGEARGYIAENHGAPLRLPDLGPIGSNGLANPRDFLAPVARYEDSRQPLQL 240
            ************************************************************

PA14        VQKYLGELWACELDHSPLDVVAWHGNNVPYKYDLRRFNTIGTVSFDHPDPSIFTVLTSPT 300
DKN343      VQKYLGELWACELDHSPLDVVAWHGNNVPYKYDLRRFNTIGTVSFDHPDPSIFTVLTSPT 300
            ************************************************************

PA14        SVHGLANIDFVIFPPRWMVAENTFRPPWFHRNLMNEFMGLIQGAYDAKAGGFVPGGASLH 360
DKN343      SVHGLTNIDFVIFPPRWMVAENTFRPPWFYRNLMNEFMGLIQGAYDAKAGGFVPGGASLH 360
            *****:**********************:****************************

PA14        SCMSAHGPDAESCDKAIAADLKPHRIDQTMAFMFETSQVLRPSRAALETPALQNDYDACW 420
DKN343      SCMSAHGPDAESCDKAIAADLKPHRIDQTMAFMFETSQVLRPSRAALETPALQNDYDACW 420
            ************************************************************

PA14        ASLVSTFNPQRR   432
DKN343      ASLVSTFNPQRR   432
            ************
```

Figure 3.3. Two amino acid changes were identified in the HmgA protein sequence from the clinical isolate DKN343 compared to the HmgA sequence from *P. aeruginosa* PA14. The two amino acid changes, A306T and H330Y, are highlighted in red. The H330Y change occurred in the iron cofactor binding site of HmgA. Clustal O (1.2.1) multiple sequence alignment of the HmgA protein from PA14 and DKN343 was used to identify the amino acid changes in DKN343.

Because pyomelanin production requires an accumulation of HGA, we created an in-frame deletion of *hpd* in DKN343, as well as in the pyomelanogenic control *hmgA::tn*. Deletion of *hpd* resulted in a loss of pyomelanin production in both *hmgA::tn* and DKN343, while complementation studies restored pyomelanin production (Figure 3.4A). These data indicate that Hpd is required for pyomelanin production in DKN343.

In order to determine if pyomelanin production is due to a loss of function mutation in $HmgA_{DKN343}$, wild type $hmgA_{PAO1}$ was expressed from an arabinose inducible promoter in DKN343 and the positive control *hmgA::tn* (Figure 3.4B). Low levels of HmgA expression alleviated pyomelanin production in both strains, which demonstrated that pigment production results from a defect in $HmgA_{DKN343}$ function. To ascertain whether the A306T or the H330Y mutation was responsible for this loss of function, site directed mutagenesis was used to introduce each mutation in $hmgA_{PAO1}$, and these constructs were tested for their ability to complement the *hmgA::tn* strain (Figure 3.4B). Expression of $HmgA_{A306T}$ eliminated pigmentation, demonstrating that this mutation is still functional. $HmgA_{H330Y}$ did not affect pyomelanin production, which indicated that the H330Y mutation is responsible for loss of function in $HmgA_{DKN343}$. Western blots indicated similar levels of protein expression with both point mutations, thereby eliminating the possibility that $HmgA_{H330Y}$ fails to complement due to protein instability.

Together, these data clearly indicate that pyomelanin production in DKN343 occurs in the traditional manner: HGA is produced by Hpd, but a loss of function mutation in HmgA prevents the conversion of HGA to 4-maleylacetoacetate. The

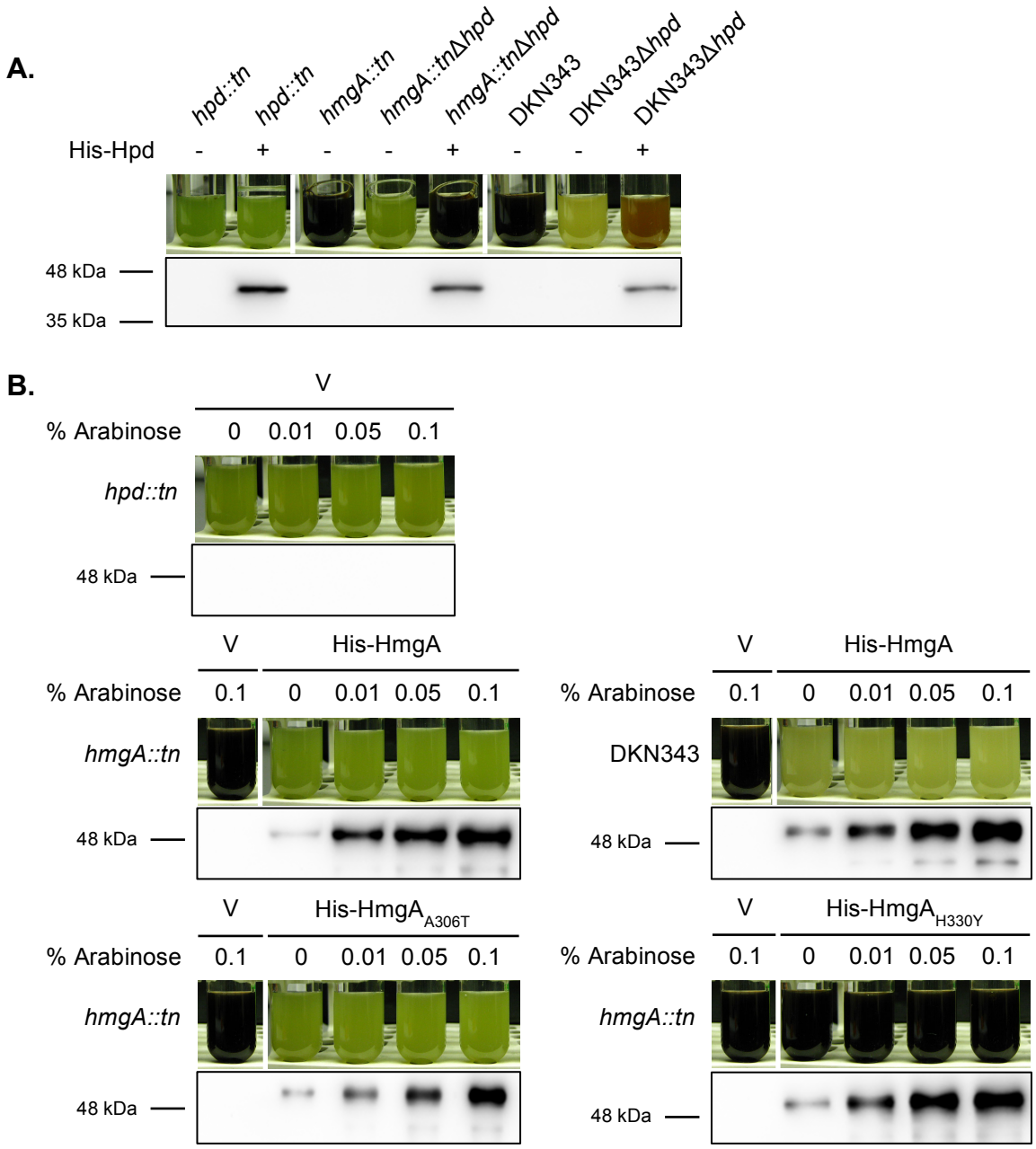

Figure 3.4. Pyomelanin production in DKN343 occurs through the tyrosine catabolism pathway. **A)** Hpd is required for pyomelanin production in DKN343. Deletion of *hpd* resulted in loss of pyomelanin production in *hmgA::tn* and DKN343. Complementation of *hpd* deletion mutants with His-Hpd restored pyomelanin production. The level of His-Hpd expression was determined through anti-His western blotting. **B)** Point mutation in HmgA causes pyomelanin production in the clinical isolate DKN343. Expression of WT His-HmgA *in trans* eliminated pyomelanin production in DKN343, which indicated that a defect in *hmgA* is responsible for pyomelanin production in this strain. The H330Y mutation in HmgA did not alleviate pyomelanin production in *hmgA::tn*, but had no effect on protein expression, which indicated that the H330 residue is important for HmgA function but not protein levels. The A306T mutation in HmgA had no effect on HmgA function, as indicated by the absence of pyomelanin production in *hmgA::tn*. Anti-His western blots for both WT and mutated His-HmgA showed increased protein expression with increasing concentrations of arabinose. Strains were grown in LB containing gentamicin with the indicated arabinose concentrations to induce His-HmgA expression. In panels A and B, *hpd::tn* was the non-pyomelanogenic control, while *hmgA::tn* functioned as a positive control for pyomelanin production resulting from a defect in the tyrosine catabolism pathway.

intracellular HGA is then secreted from DKN343 by the HatABCDE ABC transporter where it is auto-oxidized and self-polymerized into pyomelanin.

3.3.2 Resistance to NTBC is mediated through the MexAB-OprM multi-drug efflux pump.

Studies on the NTBC-mediated inhibition of *Streptomyces avermitilis* 4-hydroxyphenylpyruvate dioxygenase (HppD) revealed that NTBC binds irreversibly to the ferrous metal center in the active site of the enzyme (40). In our previous studies of pyomelanogenic *P. aeruginosa*, NTBC treatment inhibited pyomelanin production in a dose dependent manner (1). Early assays on DKN343 revealed 900 μM NTBC only partially inhibited pyomelanin production (Figure 3.2). This is a notable increase (approximately 12 fold) in the amount of NTBC required compared to the pyomelanogenic laboratory strain *hmgA::tn*, and suggested this clinical isolate possessed pre-existing resistance to NTBC. Because NTBC has been proposed as an anti-virulence agent, it was necessary to ascertain the mechanism of resistance. We investigated several ways Hpd would remain unaffected by NTBC, including mutations in Hpd, possible sequestration of NBTC by the potential Hpd homolog PA0242, the influence of outer membrane impermeability, and the effect of the constitutively active multi-drug efflux pump MexAB-OprM.

A common mechanism of antibiotic resistance in bacteria is through target alterations. We therefore investigated the possibility that the high level of resistance in DKN343 was due to the inability of NTBC to inactivate Hpd_{DKN343}. Analysis of the hpd_{DKN343} sequence failed to identify any mutations relative to the PA14 *hpd* sequence,

suggesting that NTBC should be able to bind and inactivate Hpd_{DKN343}. We also examined the *P. aeruginosa* PAO1 genome for proteins with homology to the active site of Hpd with the possibility that these proteins are binding and sequestering NTBC, thereby increasing the concentration needed for inhibition of pyomelanin production. Using a BlastP alignment, we identified the hypothetical protein PA0242, wherein the C-terminal region of the protein has homology to Hpd, including conservation surrounding the iron co-factor binding sites where NTBC binds (Figure 3.5A). Deletion of PA0242 in *hmgA::tn* and DKN343 did not increase sensitivity of these strains to NTBC (Figure 3.5B), suggesting that PA0242 does not contribute to resistance by sequestering NTBC away from the target enzyme Hpd. These results demonstrate that mutations in Hpd and the presence of the potential Hpd homolog PA0242 do not have an impact on NTBC resistance in DKN343.

P. aeruginosa is known to have low outer membrane permeability, which can contribute to antimicrobial resistance (31). We therefore investigated the effects of increasing outer membrane permeability on NTBC sensitivity. Similar increases in outer membrane permeability were seen in *hpd::tn*, *hmgA::tn*, and DKN343 following treatment with 0.1 mM EDTA when compared to the untreated strain. Nitrocefin hydrolysis by β-lactamase released from the periplasm was used as an indicator of outer membrane permeability (Figure 3.6A). Treatment of bacterial strains with sub-inhibitory concentrations of NTBC (50 μM for *hmgA::tn* and *hpd::tn* and 900 μM for DKN343) and 0.1 mM EDTA revealed that increasing outer membrane permeability did not increase sensitivity to NTBC (Figure 3.6B). In DKN343, the percent of pyomelanin produced in the NTBC treated strains relative to an untreated strain was very similar

A.

```
Hpd         ------------------------------------MNAVAK-IEQHNPIGTDGFEFVE  22
PA0242      FNDGFRAAPTRANAVDGLRSLLYLEEKTREHLQRQTPHVAVDELFAPPPASLCDGIEFLE  300
                                                 ** : :        **:**:*

Hpd         FTAPDAKGIEQLRQLFNMMGFTETAKHRSKEVFLFQQNDINIVLNGSPTGHVHEFALKHG  82
PA0242      FAVDETLGA-RLGQWLQRLGFARAGEHRSKNVSLLRQGDINLVLNAEPYSFAHGFFEAHG  359
            *:. :: *  :* * :: :**:.:.:****:* *::* ***:***..* ...* *   **

Hpd         PSACAMAFRVKNASQAAAYAESQGAKLVGSHANFGELNIPSLEGIGGSLLYLVDRYG-DR  141
PA0242      PSLCATALCVRDAGQALERARAYGGQPYRGLLGPNEREIPAVRALDGSLLYLVERHTEGR  419
            ** ** *: *::*.**   *.: *.:   .     * :**::..: *******:*:   *

Hpd         SIYDVDFEFIEGRSANDNSVGLTYIDHLTHNVKRGQMDVWSGFYERIANFREIRYFDIEG  201
PA0242      SIYDSDFVTN---DADTSGLGLRRVDHVALALPAEGLDSWVLFYKSLFDFGADDEVVLPD  476
            **** **      .*: ..:**  :**::  :    :* *  **: : :*     . :

Hpd         KLTGLFSRAMTAPCGKIRIPINESADDTSQIEEFIREYHGEGIQHIALTTDDIYATVRKL  261
PA0242      PYGLVTSRAVRSPCGSVRLPLNISEDRNTAIARSLSSYRGSGVHHIAFDCADIFAAVAQA  536
                : ***: :***.:*:*:* * * .: * . : .*:*.*::***:   **:*:* :

Hpd         RDNGVKFMSTPDTYYEKVDTRVAGHGEPLEQLRELNLLIDGAPGDDGILLQIFTDTVIGP  321
PA0242      KEAGVALLEIPLNYYDDLAARFDFDDEFLSELAYYNVLYDRD-AQGGELFHVFTEPFEER  595
            :: ** ::. * .**:.: :*.  . * *.:*   *:* *   .: * *:::**: .

Hpd         IFFEIIQRK-GNQGFGEGNFKALFESIEEDQIRRGVI----357
PA0242      FFFEILQRRHGYAGYGAANVPVRLAAM--AQARRGVRRVKL634
            :****:**: *  *:* .*. . : ::   * ****
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B.

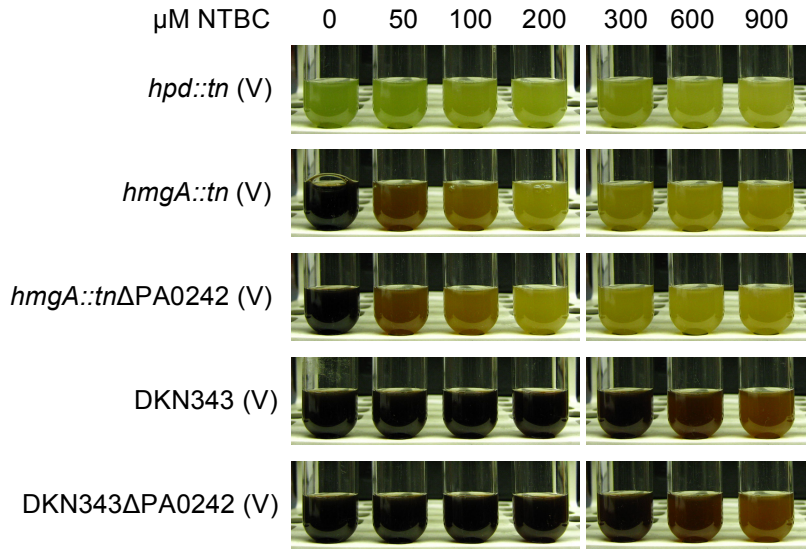

Figure 3.5. PA0242 is not involved in NTBC resistance. **A)** Clustal O (1.2.0) sequence alignment of *P. aeruginosa* PAO1 Hpd and PA0242 amino acid sequence. Hpd has sequence similarity to the C-terminal portion of the hypothetical protein PA0242. The iron cofactor binding sites for Hpd (H168, H246, and E325) and PA0242 (H443, H521, and E599) are highlighted in red. **B)** Deletion of PA0242 had no effect on NTBC sensitivity in the pyomelanin producers *hmgA::tn* and DKN343. *hpd::tn* was the non-pyomelanogenic control, while *hmgA::tn* was a positive control for pyomelanin production. Cultures were grown with the indicated concentrations of NTBC.

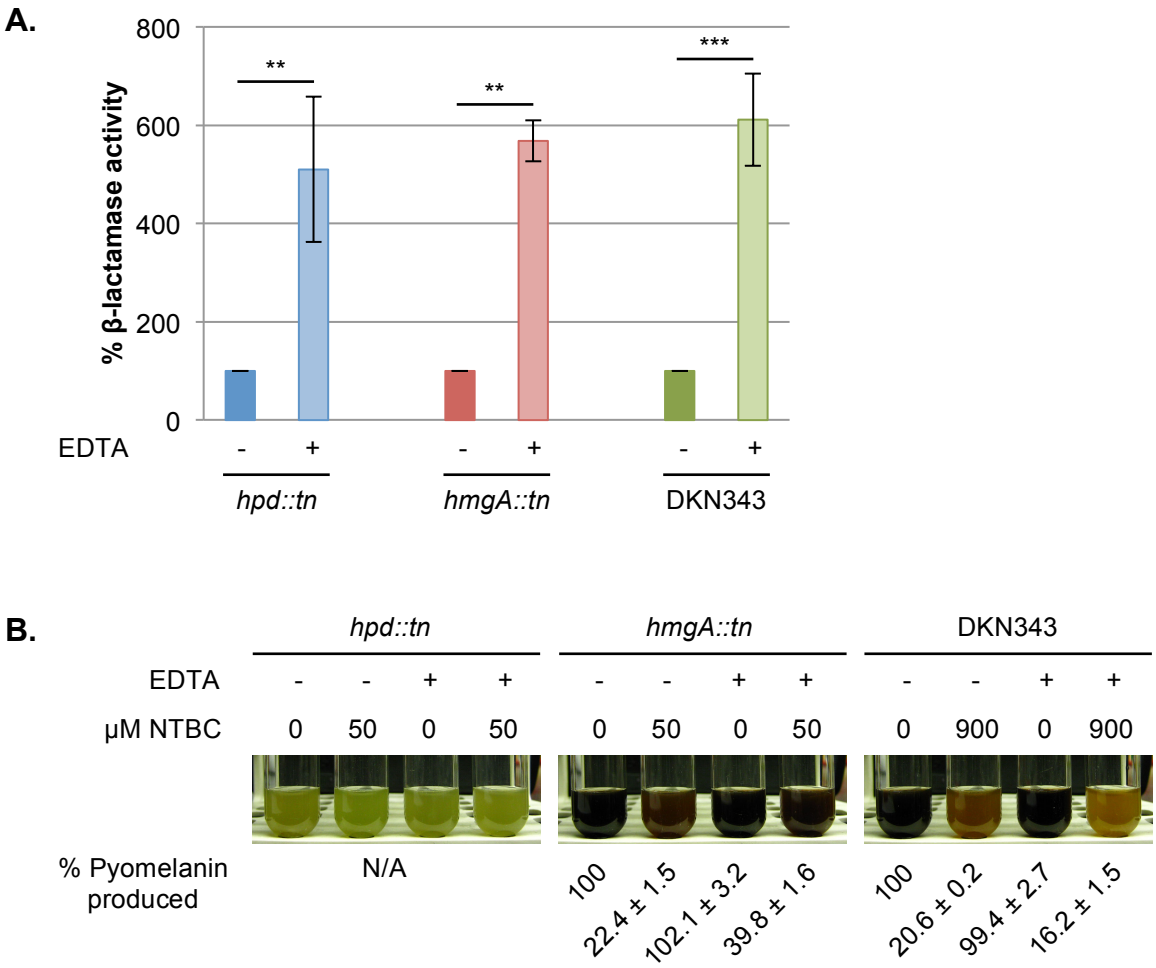

Figure 3.6. An increase in outer membrane permeability does not result in increased sensitivity to NTBC in *P. aeruginosa*. **A)** EDTA treatment of cells increased outer membrane permeability, as measured by extracellular β-lactamase activity via nitrocefin hydrolysis. β-lactamase activity ($U/L/OD_{600}$) for each untreated parent strain (0 mM EDTA) was set to 100% and percent β-lactamase activity was calculated following 0.1 mM EDTA treatment. Five biological replicates were tested. ANOVA followed by Tukey HSD post-hoc analysis: **, $p<0.01$; ***, $p<0.001$. **B)** *hpd::tn*, *hmgA::tn*, and DKN343 were treated with 0 or 0.1 mM EDTA and sub-inhibitory concentrations (0, 50, or 900 μM) of NTBC, as indicated, for 24 hours. *hpd::tn* was the non-pyomelanogenic control strain and *hmgA::tn* was the positive control for pyomelanin production. The percent pyomelanin produced was calculated from the supernatant:culture ratio (OD_{600}) and the untreated strains were set to 100%. Three biological replicates were tested in triplicate.

with and without EDTA treatment, but in *hmgA::tn*, however, the percent of pyomelanin produced in the NTBC treated strains was actually higher in the EDTA treated strain. These results demonstrate that low outer membrane permeability in DKN343 does not contribute to the heightened NTBC resistance.

Multi-drug efflux pumps contribute to antimicrobial resistance by extruding a broad range of compounds from the bacterial cell (41). We therefore tested the contribution of the constitutively active multi-drug efflux pump MexAB-OprM to NTBC resistance by making in-frame deletions of *mexA*, which codes for the membrane fusion protein of the pump. Increased sensitivity to NTBC was observed in the *hmgA::tn* and DKN343 *mexA* mutants, as indicated by reduced levels of NTBC (50 μM for *hmgA::tn* and 100 μM for DKN343) required to inhibit pyomelanin production, and restoration of pigmentation by complementation (Figure 3.7). These results indicate that the MexAB-OprM multi-drug efflux pump mediates resistance to NTBC.

3.4 Discussion

This study determined the mechanism of pyomelanin production in the clinical *P. aeruginosa* sputum isolate DKN343, as well as the mechanism of resistance to NTBC. Pyomelanin was produced through the tyrosine catabolism pathway, not an alternate mechanism as was previously suggested for DKN343 (26). A singe amino acid change in HmgA was identified, and is expected to result in a loss of enzyme function. In addition, we observed that DKN343 was more resistant to NTBC than the laboratory strain *hmgA::tn* (Figure 3.2), and therefore investigated the mechanism of resistance.

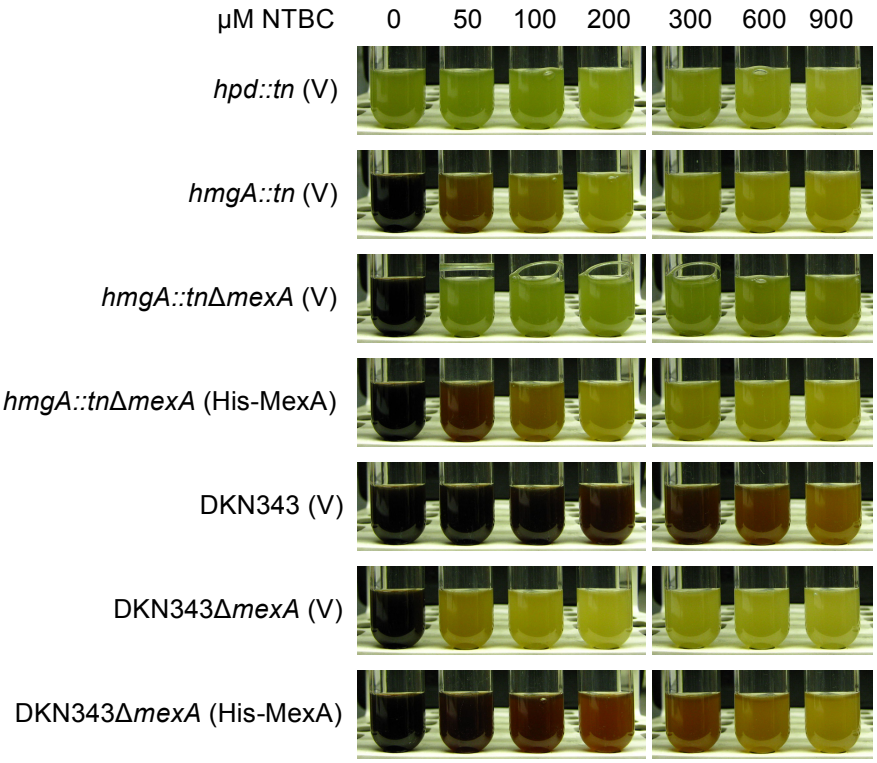

Figure 3.7. Resistance to NTBC is mediated through the MexAB-OprM multi-drug efflux pump. Deletion of *mexA* in *hmgA::tn* and DKN343 increased sensitivity to NTBC as indicated by reduced levels of pyomelanin. Strains complemented with His-MexA showed restoration of pyomelanin production in the presence of NTBC. *hpd::tn* (V) was the pyomelanin non-producer control. Cultures were grown in LB with gentamicin and 0.05% arabinose with the indicated concentrations of NTBC.

After testing several possible mechanisms for NTBC resistance, we determined that the multi-drug efflux pump MexAB-OprM was responsible.

The determination that a mutation in HmgA resulted in pyomelanin production is not unexpected. Previous studies in several bacteria have also identified mutations in *hmgA* as a cause for pyomelanin production. Analysis of several naturally pyomelanogenic strains of *V. cholerae* revealed that they contained either a 15 or 10 base pair (bp) deletion mutation in homogentisate oxygenase, and the 15 bp deletion was conserved in six O139 strains that were studied (42). In a pyomelanogenic strain of *A. media* WS, the *hmgA* gene was disrupted by an insertion of a different gene in the opposite orientation (18). Additionally, in a *Burkholderia cenocepacia* pyomelanogenic CF clinical isolate, a single point mutation in *hmgA* resulted in an amino acid change from a glycine to an arginine at residue 378, and this mutation was conserved in three of four pigmented *B. cepacia* complex strains (27). The G378R mutation in *B. cenocepacia* was located in the iron cofactor binding region of HmgA, which could affect iron binding and subsequent enzyme function (27). The *B. cenocepacia* data corresponds with our results, in which the H330Y mutation in $HmgA_{DKN343}$ was located at one of the iron cofactor binding sites (Figure 3.3) and interfered with protein function, as indicated by a failure of $HmgA_{H330Y}$ to alleviate pyomelanin production when expressed in *hmgA::tn* (Figure 3.4B).

Hpd, the enzyme responsible for the production of HGA from 4-hydroxyphenylpyruvate during tyrosine catabolism, is very important in the production of pyomelanin because HGA is the pyomelanin precursor molecule. Transposon mutagenesis or deletion of *hpd* led to an abolishment of pyomelanin production in

several bacterial and fungal species including *B. cenocepacia*, *L. pneumophila*, *P. aeruginosa*, *A. media* WS, and *A. fumigatus* (5, 12, 18, 26, 27, 43), demonstrating the importance of Hpd in pyomelanin production. Our results showed that deletion of *hpd* in DKN343 abolished pyomelanin production (Figure 3.4A), which further demonstrated that the tyrosine catabolism pathway mediates pyomelanin production in this strain.

P. aeruginosa has high levels of intrinsic and acquired antimicrobial resistance, which can make it difficult to treat infections. Resistance to antimicrobial agents is mediated by several mechanisms, including low outer membrane permeability which can prevent antimicrobials from entering the cell, several multi-drug efflux pumps that can extrude a broad range of compounds, chromosomally encoded β-lactamase which can inactivate β-lactam antibiotics, and biofilm formation (31). We therefore assayed different mechanisms to elucidate the resistance to NTBC observed in DKN343 including target alteration, sequestration by Hpd homologs, outer membrane impermeability, and multi-drug efflux pumps.

In our studies, no mutations in hpd_{DKN343} were identified, indicating that target alteration was not a source of reduced NTBC activity in DKN343. The hypothetical protein PA0242 was identified as a potential Hpd homolog (Figure 3.5A); deletion of PA0242, however, did not increase sensitivity to NTBC in pyomelanin producers (Figure 3.5B), which indicates that PA0242 is likely not sequestering NTBC. Additionally, increasing outer membrane permeability with EDTA did not have an effect on NTBC sensitivity in DKN343 (Figure 3.6).

We also tested the effects of the constitutively expressed multi-drug efflux pump MexAB-OprM on NTBC resistance via deletion of *mexA*. MexAB-OprM contributes to

intrinsic antimicrobial resistance in *P. aeruginosa* by extruding several classes of antibiotics, as well as dyes, detergents, organic solvents, fatty acid synthesis inhibitors, and homoserine lactone (44). This broad substrate specificity makes it possible that NTBC could be a substrate for the pump. Resistance to NTBC was mediated by MexAB-OprM, as indicated by the reduction in pyomelanin production in *mexA* mutants following treatment with reduced levels of NTBC relative to the parent strain (Figure 3.7). Additionally, we observed that complementation of DKN343Δ*mexA* with His-MexA did not restore NTBC resistance to the levels seen in the parent strain DKN343, as indicated by reduced levels of pyomelanin production (Figure 3.7). This could be due to the possibility that MexAB-OprM is hyperexpressed in DKN343, and these induction conditions result in insufficient expression of His-MexA. It has been shown in several studies that mutations in the MexR, NalC, or NalD regulators of *mexAB-oprM* transcription result in hyperexpression of the operon in clinical isolates and laboratory strains of *P. aeruginosa* (45-49). Hyperexpression of MexAB-OprM would lead to increased levels of efflux pumps, contributing to the increased resistance to NTBC observed in DKN343 compared to *hmgA::tn*, and also explain the partial complementation of DKN343Δ*mexA* with His-MexA.

In summary, DKN343 produces pyomelanin because of a point mutation in HmgA located at one of the residues involved in iron co-factor binding. This matches the results seen in several other studies where mutations in HmgA are responsible for pyomelanin production. We also determined that the multi-drug efflux pump MexAB-OprM is responsible for much of the NTBC resistance observed in DKN343, and it is likely that efflux pumps would be a cause for NTBC resistance in other organisms.

Since many bacterial and fungal pathogens produce pyomelanin, inhibition of pyomelanin production is an attractive target for anti-virulence treatment. A recent study in *L. pneumophila* identified a compound that inhibited phenylalanine hydroxylase, which converts phenylalanine to tyrosine, and subsequently inhibited pyomelanin production (50). This study demonstrated that targeting other enzymes in the phenylalanine/tyrosine catabolism pathway may inhibit pyomelanin production in bacteria. Targeting phenylalanine hydroxylase may have limited effectiveness in the inhibition of pyomelanin production, as tyrosine can still be catabolized to the pyomelanin precursor HGA. Because NTBC inhibits a later step in the catabolic pathway, the use of both phenylalanine and tyrosine to synthesize pyomelanin is inhibited. While NTBC shows promise as an anti-virulence therapy, its effectiveness may be limited against an organism with a high level of pre-existing antibiotic resistance such as *P. aeruginosa*. Future studies focused on the prevalence of this resistance in pyomelanogenic microbes are required to determine the future applications of NTBC as an anti-virulence agent.

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Chapter Four

Macrophage-based killing of pyomelanogenic

Pseudomonas aeruginosa

4.1 Introduction

Pseudomonas aeruginosa is a Gram negative rod shaped bacterium that is found in diverse ecological environments including soil, water, plants, and animals (1). It is also an important opportunistic pathogen that causes acute and chronic infections in individuals who are compromised. Chronic *P. aeruginosa* infections are common in the lungs of cystic fibrosis (CF) patients, where the bacterium forms biofilms that are difficult to eradicate, leading to persistence of infection (2). Burn patients are also very frequently colonized by *P. aeruginosa* (3, 4), where the infection is acute. *P. aeruginosa* produces a variety of virulence factors that allow it to colonize a host and cause infection, including secreted proteins, toxins, and pigments (5). Pyomelanin is a reddish-brown pigment produced by some strains of *P. aeruginosa* (6).

Pyomelanin is derived from homogentisate during tyrosine catabolism and is produced when there is a defect in this pathway, either 1) through deletion or mutation of key genes, particularly *hmgA* that encodes homogentisate 1,2-dioxygenase, or 2) a reduction in the activity of enzymes in the lower part of the pathway (7-12). Pyomelanin has a variety of functions including resistance to oxidative and UV stress, electron transfer and metal reduction, iron uptake by the bacterial cell, and persistence during infection (10, 11, 13-20).

During bacterial infections, phagocytic cells such as polymorphonuclear leukocytes (PMNs) and macrophages are recruited to the site of infection (21). These phagocytic cells have several functions that are involved in clearing the infection. Initially, they are involved in phagocytosis of the bacteria, where the bacteria are engulfed into phagosomes that acidify and mature into phagolysosomes (22). Reactive

oxygen species (ROS) are then produced to help bring about bacterial cell death in the phagolysosome (22). ROS production and release into the surrounding tissue leads to inflammation (23). Additionally, phagocytic cells are involved in the production and release of cytokines, which function in the recruitment of additional inflammatory cells to the site of infection (24). In individuals with CF increased PMN recruitment is associated with the release of ROS and proteolytic enzymes that damage tissue and cause prolonged inflammation in the lungs (23, 25, 26).

Because one of the functions of pyomelanin is increased resistance to oxidative stress and studies have shown bacterial persistence during infection by pyomelanin producers, we wanted to determine the effects of pyomelanin on macrophage-based killing of *P. aeruginosa*. We hypothesized that if pyomelanin has a protective effect for the bacteria against ROS produced by macrophages, we would see increased survival of pyomelanin producing bacteria compared to pyomelanin non-producers during infection of macrophages. We used the RAW 264.7 murine macrophage-like cell line for our infection studies.

4.2 Materials and Methods

4.2.1 Bacterial growth conditions

A list of bacterial strains and plasmids is found in Table 4.1. Bacterial strains were grown in LB broth or on LB plates supplemented with antibiotics were appropriate. Antibiotics were used at the following concentrations: gentamicin 10 μg ml^{-1} (*E. coli*) and 50 μg ml^{-1} (*P. aeruginosa*); tetracycline 10 μg ml^{-1} (*E. coli*) and 75 μg ml^{-1} (*P.*

Table 4.1. Strains and plasmids used in this study.

Strains	Description	Source
P. aeruginosa PAO1	*P. aeruginosa* PAO1 (Iglewski strain)	Carrie Harwood
P. aeruginosa Δ*hpd*	In-frame deletion of *hpd* in PAO1, pyomelanin non-producer	This study
P. aeruginosa Δ*hmgA*	In-frame deletion of *hmgA* in PAO1, pyomelanin producer	This study
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
E. coli S17-1	Tp^R Sm^R *recA thi pro* $hsdR^-$ M^+ RP4 2-Tc::Mu-Km::Tn7 λpir Strain for conjugating plasmids	(27)
RAW 264.7	Murine macrophage-like cell line	ATCC

Plasmids	Description	Source
pEX18Tc	Suicide vector for making deletion mutants, Tc^R	Carrie Harwood
pEX19Gm	Suicide vector for making deletion mutants, Gm^R	Carrie Harwood
Δ*hpd*-pEX18Tc	Fusion fragment for *hpd* deletion cloned into pEX18Tc at the BamHI and HindIII sites	This study
Δ*hmgA*-pEX19Gm	Fusion fragment for *hmgA* deletion cloned into pEX19Gm at the EcoRI site, introduced HindIII site was lost during cloning	This study

aeruginosa); chloramphenicol 5 μg ml^{-1}. Strains were grown at 37°C unless otherwise indicated and stored at -80°C in 15% glycerol.

4.2.2 Generation of deletion mutants

In-frame deletion mutants of *hpd* and *hmgA* in *P. aeruginosa* PAO1 were constructed by splicing by overlap extension (SOE) PCR with PAO1 DNA as template. Primers are listed in Table 4.2. The in-frame fusions were sequenced to ensure no mutations were introduced. The deletion alleles were cloned into pEX19Gm or pEX18Tc and transformed into *E. coli* S17-1 for mating with *P. aeruginosa*. These constructs were introduced into *P. aeruginosa* by conjugation, and merodiploids were selected on chloramphenicol and tetracycline or gentamicin as appropriate. Resolution of the merodiploids was achieved through 10% sucrose counter selection. Following screening on tetracycline or gentamicin and sucrose, the deletions were confirmed by PCR.

4.2.3 RAW 264.7 murine macrophage-like cell line growth conditions

RAW 264.7 cells were routinely grown for assays in 25 or 75 cm^2 cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}) at 37°C and 10% CO_2. Cells were grown to 75 – 85% confluence before dissociation from cell culture flasks with TrypLE Express. Aliquots of RAW 264.7 cells from passages 2-5 of the initial stock cell culture were stored at -80°C in Recovery Cell Culture Freezing Medium and thawed as needed. RAW 264.7 cells were discarded after passage 15.

Table 4.2. List of primers used in this study.

Primer name	**Primer Sequence** (5' – 3')[a]
Δ*hpd*-up-For	GTAGCGGATCCCGATGCCTGCCACCGGAC
Δ*hpd* -up-Rev	GAGGCTGGCGGCAGCGGGACCGGCCTCCTCGTTGTTC
Δ*hpd* -dn-For	GAACAACGAGGAGGCCGGTCCCGCTGCCGCCAGCCTC
Δ*hpd* -dn-Rev	CGCCGAAGCTTGCCGCGGTGAAGCCGAGC
Δ*hmgA*-up-For	CTGGAATTCCCTACCCGACGTCGCGCC
Δ*hmgA* -up-Rev	CATGGGGTTATCTCCGTTGGAGGTTCATCTGAGGCCTCCGGGGAG
Δ*hmgA* -dn-For	CTCCCCGGAGGCCTCAGATGAACCTCCAACGGAGATAACCCCATG
Δ*hmgA* -dn-Rev	CTGAAGCTTGGCTGTTGCTCAGGGTCAGG

[a]Restriction enzyme sites are underlined.

4.2.4 RAW 264.7 killing of intracellular bacteria

The infection assay to determine RAW 264.7 killing of intracellular bacteria was modified from previously described procedures (28). RAW 264.7 cells were seeded ($2x10^5$ cells $well^{-1}$) in 12-well cell culture plates and attached for 1 h. Overnight cultures of Δ*hpd* and Δ*hmgA* bacterial strains were diluted in LB to multiplicity of infection (MOI) 54.9 ± 7.0 and added to the wells containing attached RAW 264.7 cells to infect the cells. Infections were synchronized by centrifuging at 130 xg for 5 min, followed by incubation for 30 min at 37°C and 10% CO_2. RAW 264.7 cells were washed 3 times with Dulbecco's phosphate buffered saline lacking $CaCl_2$ and $MgCl_2$ (DPBS) before fresh DMEM containing 0 or 500 μg ml^{-1} gentamicin was added. Infected RAW 264.7 cells were incubated for 2 h, washed 3 times with DPBS, and lysed with 0.1% Triton X-100. Cell lysates were plated on LB to determine bacterial survival. Bacterial killing by RAW 264.7 cells was quantified for each bacterial strain, MOI, and gentamicin condition in triplicate for three independent assays. The recovered bacterial CFU ml^{-1} was normalized to percentage of inoculum to determine total RAW 264.7 associated bacteria (0 μg ml^{-1} gentamicin) and intracellular bacteria (500 μg ml^{-1} gentamicin). Percentage of intracellular bacteria was calculated relative to total cell-associated bacteria. To compile results from three independent assays, viable bacteria or percentage of intracellular bacteria was averaged for each strain and gentamicin condition. Statistical analysis was performed by ANOVA, followed by Tukey HSD post-hoc analysis using R (version 3.2.4).

4.2.5 RAW 264.7 bacterial association and killing of bacteria over time

The time course assay to determine RAW 264.7 bacterial association and killing after a 15 or 30 minute infection time was modified from previously described procedures (29). RAW 264.7 cells suspended in DMEM lacking antibiotics were dispensed into microfuge tubes ($1x10^6$ cells per 900 μl). Overnight cultures of Δ*hpd* and Δ*hmgA* bacterial strains were diluted in LB to MOI 57.2 ± 4.2 (15 minute infection) or MOI 58.3 ± 2.8 (30 minute infection) and added to the RAW 264.7 cells to obtain a total volume of 1 ml. Infections occurred for 15 or 30 min at 37°C with rotation. After infection, samples were incubated at 37°C with rotation and aliquots were taken every 30 min (starting with T=0 min after infection) for viable counts to determine RAW 264.7 total bacterial cell association and killing over time. At each time point, the entire sample was centrifuged at 1500 rpm for 5 min and the pellet was resuspended in an equal volume of fresh DMEM without antibiotics to remove bacteria that were not associated with RAW 264.7 cells. An aliquot of each sample was mixed with water to lyse RAW 264.7 cells, followed by serial dilution in water. Diluted samples were spotted in triplicate on LB to quantify the number of viable bacteria associated with RAW 264.7 cells. The recovered bacterial CFU ml^{-1} for each assay was calculated as a percentage of the inoculum. The percent bacterial survival over time was calculated by dividing the number of viable bacteria at any given time point by the viable bacteria at the beginning of the incubation (T=0 min). Total bacterial association with RAW 264.7 cells at 0 min was used to determine the effect of infection time on bacterial association with RAW 264.7 cells. Three independent assays were tested for 15 and 30 minute infection times. To compile results from three independent assays, viable bacteria or percent survival

was averaged for each strain at each time point. Statistical analysis was performed by ANOVA, followed by Tukey HSD post-hoc analysis using R (version 3.2.4).

4.3 Results

4.3.1 RAW 264.7 cell killing of intracellular bacteria is similar for pyomelanin producers and non-producers.

The *P. aeruginosa* pyomelanin producer Δ*hmgA* and the non-producer Δ*hpd* were tested for survival in the RAW 264.7 murine macrophage-like cell line. A 30 minute infection was followed by incubation with and without gentamicin for 2 hours (Figure 4.1). The CFU ml^{-1} of viable bacteria was normalized as a percentage of the bacterial inoculum. The viable bacteria for Δ*hmgA* isolated from RAW 264.7 cells was approximately two fold higher than the viable bacteria for Δ*hpd* isolated for both total cell-associated bacteria (those exposed to no gentamicin) and for intracellular bacteria (those exposed to gentamicin). The difference between total cell-associated bacteria and between intracellular bacteria when comparing both strains was not statistically significant (Figure 4.1). These results indicated that Δ*hmgA* may associate slightly better with the RAW 264.7 cells than Δ*hpd*, which could be due to the pyomelanin produced by this strain, as Δ*hmgA* and Δ*hpd* are isogenic to each other. Within a strain, there was a fairly large difference between total cell-associated bacteria and intracellular bacteria when viable bacteria were examined (Figure 4.1); this difference, however, was not statistically significant for either strain.

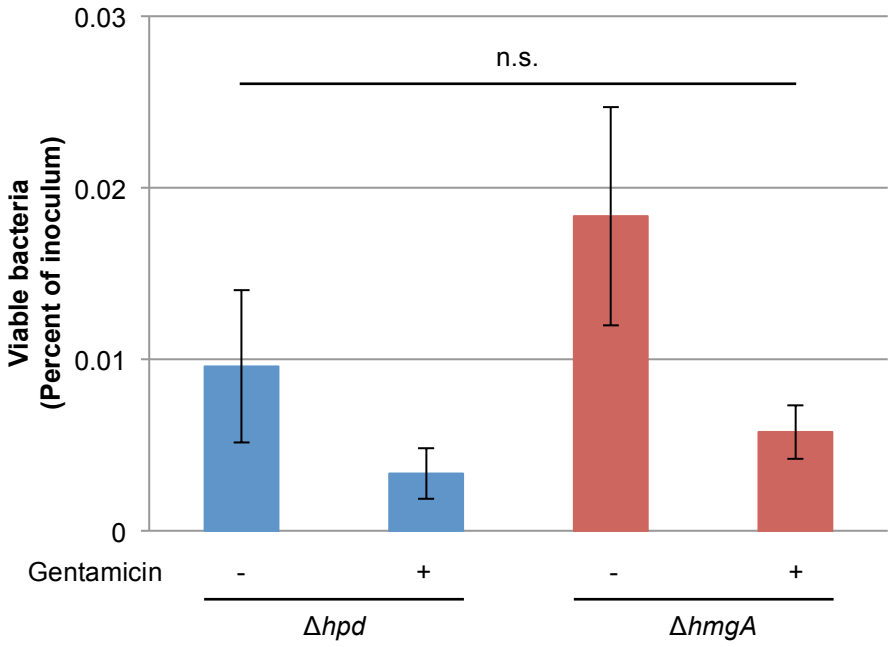

Figure 4.1. RAW 264.7 killing of intracellular bacteria is similar for pyomelanin producers and non-producers. Viable bacteria as percent of inoculum was calculated for total cell-associated (no gentamicin treatment) and intracellular (gentamicin treated) bacteria. Δ*hmgA* had a higher percentage of viable bacteria relative to inoculum than Δ*hpd*, which indicated that more of the pyomelanin producing Δ*hmgA* bacteria associated with the RAW 264.7 cells. The bacterial MOI was 54.9 ± 7.0. Three independent assays were tested. Blue bars, Δ*hpd*; red bars, Δ*hmgA*. ANOVA followed by Tukey HSD post-hoc analysis showed no significant differences between the strains and gentamicin treatment.

When the percent of intracellular bacteria was calculated relative to the total cell-associated bacteria, there was a very slight difference between Δ*hmgA* (35.4% ± 5.8%) and Δ*hpd* (37.5% ± 7.1%) in RAW 264.7 cells; this difference was not statistically significant. Additionally, the difference between total cell-associated bacteria and percent of intracellular bacteria was significant for both strains ($p<0.001$). These results indicated that the pyomelanin produced by Δ*hmgA* did not provide protection from RAW 264.7 killing of the intracellular bacteria under these assay conditions.

4.3.2 Association of bacterial pyomelanin producers and non-producers with RAW 264.7 cells is similar after infection for 15 or 30 minutes.

RAW 264.7 cells exposed to Δ*hmgA* and Δ*hpd* for 15 or 30 minute infections showed slight differences in bacterial association with the cells (Figure 4.2). The viable bacterial CFU ml^{-1} that was isolated for each strain was normalized to percentage of bacterial inoculum. The results showed that there were slightly higher percentages of RAW 264.7-associated bacteria for both Δ*hmgA* and Δ*hpd* after the 30 minute infection compared to the 15 minute infection (Figure 4.2); however, this slight increase in bacterial association was not statistically significant for either bacterial strain. The slightly higher association of bacteria with RAW 264.7 cells in the 30 minute infection is likely due to the increased time. There was little difference, however, between Δ*hmgA* and Δ*hpd* association with RAW 264.7 cells within either infection time, and what little difference was present was not statistically significant (Figure 4.2). Overall, these results indicated that pyomelanin did not appear to play a role in bacterial association with the RAW 264.7 cells under the conditions tested.

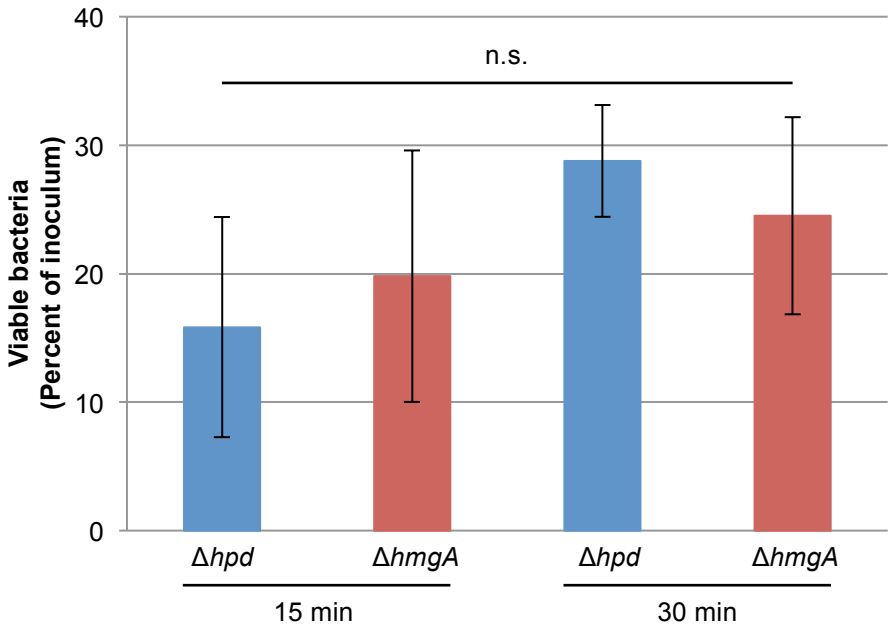

Figure 4.2. Association of bacterial pyomelanin producers and non-producers with RAW 264.7 cells is similar after infection for 15 or 30 minutes. Viable bacteria as percentage of inoculum was calculated for each strain and condition. A 30 minute infection showed slightly higher percentages of RAW 264.7-associated bacteria than a 15 minute infection. The bacterial MOI for the 15 minute infection was 57.2 ± 4.2. The bacterial MOI for the 30 minute infection was 58.3 ± 2.8. Three independent assays were tested for each infection time. Blue bars, Δ*hpd*; red bars, Δ*hmgA*. ANOVA followed by Tukey HSD post-hoc analysis showed no significant differences between the strains and infection times.

4.3.3 Bacterial killing by RAW 264.7 cells over time is similar for pyomelanin producers and non-producers.

The time course assay for RAW 264.7 bacterial killing was determined following a 15 or 30 minute infection with Δ*hmgA* and Δ*hpd* (Figure 4.3). The CFU ml^{-1} for viable bacteria was normalized to percentage of bacterial inoculum. Most of the bacterial killing by RAW 264.7 occurred in the first 30 to 60 minutes after infection. After both the 15 and 30 minute infections, the Δ*hmgA* strain generally had a slightly higher percentage of viable bacteria associated with RAW 264.7 cells than the Δ*hpd* strain (Figures 4.3A and 4.3C); this difference was not statistically significant. These results could be indicative of a slightly higher association of Δ*hmgA* with RAW 264.7 cells during infection, which could be due to the presence of pyomelanin. This observation also corresponds with the data from figure 4.1 in which Δ*hmgA* had an approximately two fold higher percentage of viable bacteria associated with RAW 264.7 cells compared to Δ*hpd*.

The bacterial percent survival over time was slightly higher for Δ*hmgA* compared to Δ*hpd* after both the 15 and 30 minute infection times, but the differences between the two strains was not statistically significant (Figures 4.3B and 4.3D). These results indicated that the presence of pyomelanin in the Δ*hmgA* strain conferred very little survival advantage, if any, during killing by RAW 264.7 cells under the tested conditions. Additionally, increasing or decreasing the infection time had little effect on bacterial survival during the RAW 264.7 killing assay since there were few differences between the bacterial survival curves after different infection times.

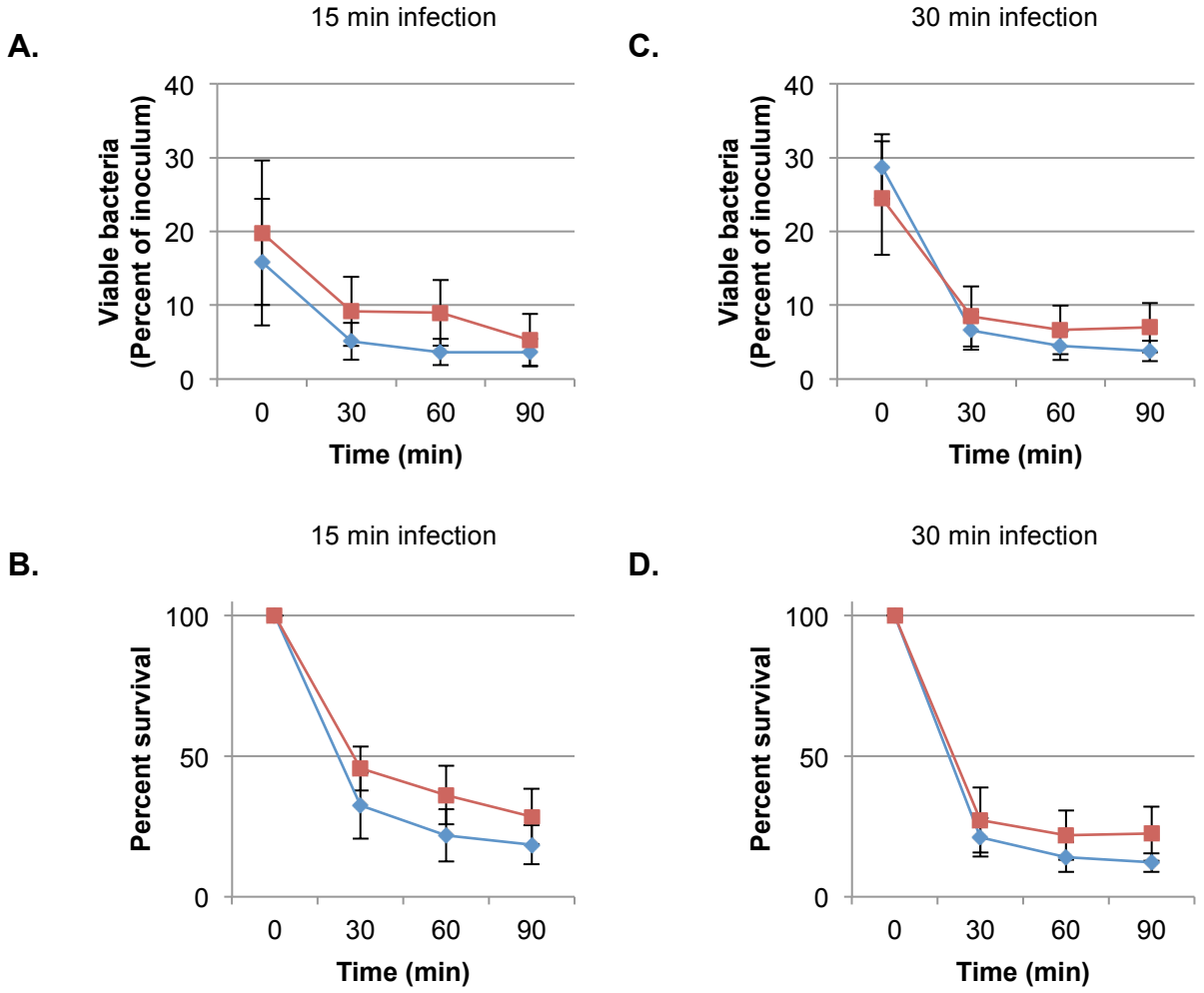

Figure 4.3. Time course of bacterial killing by RAW 264.7 cells following a 15 (A and B) or 30 (C and D) minute infection is similar for pyomelanin producers and non-producers, regardless of infection period duration. **A)** The Δ*hmgA* strain showed a higher percentage of viable bacteria relative to the inoculum concentration than the Δ*hpd* strain after the 15 minute infection. **B)** The percent survival of bacteria isolated from RAW 264.7 cells was slightly higher for Δ*hmgA* than for Δ*hpd* after the 15 minute infection. Percent survival was calculated from the number of viable bacteria at each time point relative to 0 minutes. **C)** The Δ*hmgA* strain showed a slightly higher percentage of viable bacteria relative to the inoculum concentration than the Δ*hpd* strain at every time point except 0 minutes after the 30 minute infection. **D)** The percent survival of bacteria isolated from RAW 264.7 cells was slightly higher for Δ*hmgA* than for Δ*hpd* after the 30 minute infection. Percent survival was calculated from the number of viable bacteria at each time point relative to 0 minutes. Blue diamonds, Δ*hpd*; red squares, Δ*hmgA*. The bacterial MOI for the 15 minute infection was 57.2 ± 4.2. The bacterial MOI for the 30 minute infection was 58.3 ± 2.8. Three independent assays were tested for each infection time. ANOVA followed by Tukey HSD post-hoc analysis showed no significant difference between strains.

4.4 Discussion

It has previously been reported that pyomelanogenic *P. aeruginosa* showed increased persistence in mouse models of infection compared to non-pyomelanogenic strains (11, 20). Melanin also scavenged superoxide anion produced by macrophages (30). Additionally, pyomelanin producing *Burkholderia cenocepacia* showed reduced trafficking to RAW 264.7 lysosomes compared to a non-pigmented strain (14). These studies all suggested that pyomelanin plays a role in bacterial persistence during infection of phagocytes. We therefore decided to investigate the role of pyomelanin produced by *P. aeruginosa* in bacterial killing by the murine macrophage-like cell line RAW 264.7.

Our results for RAW 264.7 killing of *P. aeruginosa* showed that pyomelanin did not provide a survival advantage for the bacteria upon infection of RAW 264.7 cells. Despite testing bacterial survival under several experimental conditions, we found that there was very little, if any, survival advantage from producing pyomelanin by the bacteria. In the RAW 264.7 killing assay, the percentage of surviving intracellular bacteria after a 30 minute infection and subsequent 2 hour treatment with gentamicin was similar for both pyomelanin producers and non-producers (Figure 4.1). Percent survival was also similar for the pyomelanin producer Δ*hmgA* and the non-producer Δ*hpd* in the time course killing assay (Figure 4.3B and 4.3D). These results correlate with experiments done in *Legionella pneumophila* which found that pyomelanin production had no role in intracellular survival of the bacterium in the host organism *Hartmannella vermiformis* (31).

Interestingly, the total percentage of cell-associated Δ*hmgA* was generally higher than that for Δ*hpd* when they were normalized to the amount of inoculum, but the difference between the strains was not statistically significant (Figures 4.1 and 4.3). This result indicated that pyomelanin producers may associate better with RAW 264.7 cells than pyomelanin non-producers. A potential explanation for this observation is that the pyomelanin producers may be slightly more resistant to phagocytosis than non-producers, which could allow the pyomelanogenic bacteria to associate with the RAW 264.7 cells, but not be phagocytized, resulting in higher numbers of cell-associated bacteria. In the fungal species *Penicillium marneffei* and *Paracoccidioides brasiliensis*, melanized cells exhibited reduced phagocytosis by macrophages (32, 33). It should be noted, however, that these organisms produce L-DOPA melanin, and the differences between pyomelanin and L-DOPA melanin may be important when considering these results.

Additionally, we found that there was little difference in bacterial cell association with the RAW 264.7 cells between the Δ*hmgA* and Δ*hpd* strains after a 15 or 30 minute infection (Figure 4.2). Although bacterial cell association was slightly higher after the 30 minute infection compared to the 15 minute infection, the difference was not statistically significant. This difference was likely due to having increased time with which to associate with RAW 264.7 cells in the 30 minute infection. Within an infection time, there was no significant difference between Δ*hmgA* and Δ*hpd* association with RAW 264.7 cells.

One of the differences between our studies and previously published studies is the bacterial strains that were used. In the mouse models of infection, *P. aeruginosa*

PA14 was used (11, 20), while we used *P. aeruginosa* PAO1, which is less virulent than PA14 (34). The difference in virulence between PA14 and PAO1 could contribute to the lack of protection by pyomelanin that was seen in our studies of RAW 264.7 killing of bacteria.

Another factor that could have affected our results is that the bacterial strains were diluted in LB for our experiments and it is unlikely that additional pyomelanin would be produced during the duration of the assay. Therefore, the soluble pyomelanin produced by the Δ*hmgA* strain was diluted, which could have resulted in decreased protection for that strain in the RAW 264.7 killing assays. Cell surface associated pyomelanin would have provided most of the protection for the bacteria in our assays.

In the future, different methods of examining RAW 264.7 killing of *P. aeruginosa* may be investigated. First, it may be possible to quantify RAW 264.7 associated bacterial cells, as well as bacterial uptake, via fluorescence microscopy (35). This method may reveal differences in uptake or cell association between pyomelanin producers and non-producers that could not be determined via viable counts. Flow cytometry could also be used to quantify phagocytosis of pyomelanin producers and non-producers. It is also possible to investigate bacterial survival in RAW 264.7 cells via live/dead staining (35). This technique may give us a better understanding of how many bacteria are taken up and killed by RAW 264.7 cells during infection and would be independent of culturing bacteria on a plate for viable counts. Finally, we could investigate the ability of pyomelanin produced by bacteria to scavenge ROS generated by RAW 264.7 cells (30). This assay could reveal differences in scavenging of ROS in

pyomelanin producers and non-producers and would contribute to our understanding of how the RAW 264.7 cells may be killing the bacteria.

It is possible that our experimental conditions were not ideal to determine if pyomelanin provides protection from macrophage-based killing of bacteria. For example, a different macrophage cell line may have been more appropriate for our experiments. Additionally, infection experiments in cell culture do not always mimic the conditions in an animal infection, so animal based infection assays could give a different result. The previous studies that determined that pyomelanin producers had increased persistence during infection were performed in mouse models of infection (11, 20). Therefore, performing our infection studies in mice may be a more appropriate approach to studying the effects of pyomelanin on bacterial survival during infection. Of course, even after using alternative methods to examine bacterial killing by macrophages or using mouse models of infection, it is possible that the results could correspond with our results and show no protective effect for pyomelanin during infection. If that happens, it suggests that the results presented in this study are valid and not due to experimental error or improper test conditions.

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Chapter Five

Concluding Remarks

In this work, I have investigated mechanisms for pyomelanin production in clinical isolates of *Pseudomonas aeruginosa*. Pyomelanin is an extracellular, reddish-brown pigment derived from homogentisate (HGA), which is produced via the phenylalanine/tyrosine catabolism pathway (1, 2). Previous studies have shown that point mutations or chromosomal deletions in *hmgA*, which encodes homogentisate 1,2-dioxygenase, result in the accumulation of HGA and frequently pyomelanin production (3, 4). My work showed that the pyomelanogenic *P. aeruginosa* clinical isolate PA1111, which was isolated from an acute infection in a burn patient, had a chromosomal deletion of *hmgA* (Chapter 2) (5, 6). A second pyomelanogenic clinical isolate, DKN343, which was isolated from a sputum sample, had previously been reported to have no mutations in *hmgA* (7); I identified two mutations in *hmgA* for this strain, however, and determined that the H330Y mutation in the iron cofactor binding site of HmgA was responsible for pyomelanin production in this strain (Chapter 3). In addition, by deleting *hpd*, which encodes the 4-hydroxyphenylpyruvate dioxygenase that produces HGA, I determined that the phenylalanine/tyrosine catabolism pathway is required for pyomelanin production and that inactivation of HmgA was the sole cause of pyomelanin production in DKN343 (Chapter 3). While these results are in contrast to the previously published study on DKN343, these results are ultimately unsurprising because several pyomelanogenic bacterial species, including *Vibrio cholerae*, *Aeromonas media*, and *Burkholderia cenocepacia* have deletions, insertions, and point mutations in *hmgA*, respectively, as causes for pyomelanin production (8-10).

The Hpd inhibiting compound 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) has been proposed as a treatment for microbial infections by

pyomelanin producers (11). NTBC inhibits Hpd by binding irreversibly to the ferrous metal center in the active site of the enzyme (12). Because NTBC binds to Hpd, which is responsible for producing the pyomelanin precursor molecule HGA, pyomelanin production should be inhibited. I have investigated the effects of NTBC on pyomelanin production in clinical isolates of *P. aeruginosa*, and determined that NTBC reduced pyomelanin production in a dose dependent manner in both PA1111 and DKN343 (Chapters 2 and 3); DKN343, however, was more resistant to NTBC than PA1111. Studies of NTBC resistance in DKN343 revealed that the constitutively active multi-drug efflux pump MexAB-OprM was responsible for the inherent resistance to NTBC seen in that strain (Chapter 3).

I also investigated the effects of NTBC on sensitivity to oxidative stress because one of the functions of pyomelanin is resistance to oxidative stress (4, 13, 14). Treatment of PA1111 with NTBC resulted in increased sensitivity to H_2O_2 oxidative stress compared to the untreated strain (Chapter 2) (6). Additionally, I tested the antibiotic sensitivity of PA1111 with and without NTBC to determine if reducing pyomelanin production had an effect on minimum inhibitory concentrations (MICs). This study was important because conflicting data on the effects of pyomelanin on antibiotic MICs has been published. Various reports in different microbial species have stated that antibiotic MICs were higher in pyomelanogenic organisms, that pyomelanin had no effect on MICs, and that pyomelanin producers were more sensitive to antibiotics (4, 15-21). My work showed that a reduction in pyomelanin production with NTBC had no effect on aminoglycoside MICs (Chapter 2) (6). This demonstrated that NTBC could possibly be used in conjunction with antibiotics.

Finally, I investigated macrophage-based killing of pyomelanogenic and non-pyomelanogenic strains of *P. aeruginosa*. Pyomelanin was found to be involved in persistence of *P. aeruginosa* in mouse models of infection (4, 22). Melanin also scavenged superoxide anion produced by macrophages (23). In *B. cenocepacia*, pyomelanogenic strains showed reduced trafficking to lysosomes than non-pigmented strains (14). In my studies of macrophage-based killing of *P. aeruginosa*, I used the RAW 264.7 murine macrophage-like cell line. My results showed that the pyomelanogenic strain (Δ*hmgA*) showed little difference in bacterial survival during RAW 264.7 infection compared to the non-pyomelanogenic strain (Δ*hpd*) under different test conditions (Chapter 4). A variety of factors could have impacted my results, including the use of *P. aeruginosa* PAO1 instead of the more virulent strain PA14 that was used in the mouse studies, dilution of extracellular pyomelanin in the assay, and the infection assay protocol itself. It is possible that different test or quantification conditions would have been more appropriate for these studies, such as a different way to quantify bacterial viability, a different macrophage cell line, or using a mouse model of infection.

In summary, my work provided some data for the potential development of NTBC as a therapeutic agent for treating pyomelanogenic microbial infections. By elucidating mechanisms of pyomelanin production in clinical isolates of *P. aeruginosa* and testing NTBC sensitivity on those strains, I was able to 1) determine if pyomelanin production was mediated by Hpd and 2) determine if there were any inherent mechanisms of NTBC resistance present in those strains. My studies showed that the clinical isolates PA1111 and DKN343 were more resistant to NTBC than the pyomelanogenic laboratory

strain *hmgA::tn*, and resistance to NTBC in DKN343 was mediated by the MexAB-OprM multi-drug efflux pump. It is possible that other pyomelanogenic organisms could have similar mechanisms for NTBC resistance. NTBC treatment, however, rendered PA1111 more sensitive to H_2O_2 oxidative stress than the untreated strain. Targeting pyomelanin for anti-virulence treatment is attractive because there are many pathogenic bacteria and fungi that produce this pigment. NTBC does show some promise as an anti-virulence agent, but its effectiveness may be limited in organisms with high levels of antimicrobial resistance such as *P. aeruginosa*. Therefore, future studies should focus on the prevalence of NTBC resistance in other pyomelanogenic organisms to determine the future applications of NTBC as an anti-virulence therapy.

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20. **Yabuuchi E, Ohyama A.** 1972. Characterization of "Pyomelanin"-Producing Strains of *Pseudomonas aeruginosa*. Int J Syst Bacteriol **22:**53-64.

21. **Rozhavin M, Sologub V.** 1979. Comparison of the sensitivity of *Pseudomonas aeruginosa* cultures that synthesize melanin and other pigments to 12 antibiotics and 5-nitro-8-quinolinol. Antibiotiki **24:**921-922.

22. **Harmer CJ, Wynn M, Pinto R, Cordwell S, Rose BR, Harbour C, Triccas JA, Manos J.** 2015. Homogentisate 1-2-Dioxygenase Downregulation in the Chronic Persistence of *Pseudomonas aeruginosa* Australian Epidemic Strain-1 in the CF Lung. PLoS One **10:**e0134229.

23. **Zughaier SM, Ryley HC, Jackson SK.** 1999. A Melanin Pigment Purified from an Epidemic Strain of *Burkholderia cepacia* Attenuates Monocyte Respiratory Burst Activity by Scavenging Superoxide Anion. Infect Immun **67:**908-913.

CURRICULUM VITAE

Laura M. Ketelboeter

Place of birth: Madison, WI

EDUCATION

Ph.D., University of Wisconsin-Milwaukee, May 2016
Major area: Microbiology
Secondary area: Molecular Biology

B.S., University of Wisconsin-Oshkosh, June 2007
Major: Biology
Minors: Chemistry, Spanish
Degree Honors: Magna Cum Laude

DISSERTATION TITLE: Protective Effects of Pigmentation in *Pseudomonas aeruginosa*: Insights on Pyomelanin Production and Inhibition by NTBC

HONORS / AWARDS

University of Wisconsin-Milwaukee

2012-2016 Chancellor's Graduate Student Award

2014 Ruth Walker Graduate Grant-in-Aid Award, Department of Biological Sciences

2013 Louise Neitge Mather Scholarship, Department of Biological Sciences

University of Wisconsin-Oshkosh

2004-2007 Dean's List

2003 Honor Roll

PEER REVIEWED PUBLICATIONS

Ketelboeter LM, Bardy SL. Resistance to NTBC in the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate DKN343. In preparation.

Ketelboeter LM, Bardy SL. 2015. Methods to inhibit bacterial pyomelanin production and determine the corresponding increase in sensitivity to oxidative stress. Journal of Visualized Experiments. 102:e53105.

Ketelboeter LM, Potharla VY, Bardy SL. 2014. NTBC treatment of the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111 inhibits pigment production and increases sensitivity to oxidative stress. Current Microbiology. 69:343-8.

Albert RA, Waas NE, Pavlons SC, Pearson JL, **Ketelboeter L**, Rosselló-Móra R, and Busse HJ. 2013. *Sphingobacterium psychroaquaticum* sp. nov., a novel, psychrotrophic bacterium isolated from Lake Michigan water. International Journal of Systematic and Evolutionary Microbiology. 63(Pt 3):952-8.

INVITED LECTURES

Laura M. Ketelboeter. December 4, 2015. The effects of the anti-virulence compound NTBC on pyomelanin production and oxidative stress resistance in clinical isolates of *Pseudomonas aeruginosa*. University of Wisconsin-Milwaukee Department of Biological Sciences Colloquium. Milwaukee, WI.

Laura M. Ketelboeter. November 11, 2015. The effects of the anti-virulence compound NTBC on pyomelanin production and oxidative stress resistance in clinical isolates of *Pseudomonas aeruginosa*. Milwaukee Microbiology Society. Milwaukee, WI.

ABSTRACTS

Laura M. Ketelboeter, Vishwakanth Y. Potharla, Sonia L. Bardy. April 18, 2013. NTBC Treatment Reduces Pyomelanin Production and Increases Sensitivity to Oxidative Stress in *Pseudomonas aeruginosa*. University of Wisconsin-Milwaukee Department of Biological Sciences Symposium. Milwaukee, WI. Poster presentation.

Laura M. Ketelboeter, Sonia L. Bardy. September 7-9, 2012. Effects of NTBC and Antimicrobials on Pyomelanin Producing Strains of *Pseudomonas aeruginosa*. 19th Annual Midwest Microbial Pathogenesis Conference. Milwaukee, WI. Poster Presentation.

Laura M. Ketelboeter, Jessica C. Bach, Alexei V. Medvedev, Sonia L. Bardy. May 21-24, 2011. Methylation Mechanisms of MCPs in *Pseudomonas aeruginosa*. American Society for Microbiology 111th General Meeting. New Orleans, LA. Poster Presentation.

Laura M. Ketelboeter, Jessica C. Bach, Alexei V. Medvedev, Sonia L. Bardy. April 28, 2011. Methylation Mechanisms of MCPs in *Pseudomonas aeruginosa*. University of Wisconsin-Milwaukee Department of Biological Sciences Symposium. Milwaukee, WI. Poster Presentation.

TEACHING EXPERIENCE

University of Wisconsin-Milwaukee

2016	Bio Sci 101	General Survey of Microbiology Guest Lecturer
2010-2016	Bio Sci 101	General Survey of Microbiology – Laboratory Graduate Teaching Assistant

UNDERGRADUATE TRAINEES

University of Wisconsin-Milwaukee

2015 (Spring)	Colin Opay
2014 (Spring)	Michael Mamerow
2013 (Fall)	Zachery Kunkel

PROFESSIONAL DEVELOPMENT

University of Wisconsin-Milwaukee

2012	Animal Use Certification, Animal Care Program
2011	Responsible Conduct of Research Seminar Series