TyrR Regulates the Ability of Enterobacter hormaechei to Induce Melanin Production in Cryptoccus neoformans

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TyrR regulates the ability of *Enterobacter hormaechei* to induce melanin production in *Cryptococcus neoformans*

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Abstract

The yeast *Cryptococcus neoformans* is present in the microbiota of many plants and animals and produces melanin, a compound that protects cells against UV light and promotes virulence. Melanin production can be induced by exogenous tyrosine, homogentisic acid, catecholamines, or their precursors such as L-DOPA, all of which may be produced by bacteria. The goal of this study was to investigate the interaction between *Enterobacter hormaechei* (a mosquito gut isolate) and *C. neoformans* with respect to the production of melanin by *C. neoformans* in the presence of *E. hormaechei*, potentially due to the production of L-DOPA or other metabolites by this bacterium. After performing transposon mutagenesis, over 7000 *E. hormaechei* mutants were screened, resulting in the transcription factor TyrR being identified as necessary for *E. hormaechei* to promote *C. neoformans* melanization. TyrR is a transcription factor known to regulate genes involved in amino acid metabolism and L-DOPA synthesis. In future studies, TyrR will be characterized in *E. hormaechei* for its role in inducing *C. neoformans* melanogenesis through gene complementation, melanization assays, mass spectrometry, and melanin particle analysis.
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Introduction

_Cryptococcus neoformans_ is a fungus that is found globally in a wide variety of niches, including mammalian, avian, and arboreal hosts [1]. This fungus is categorized into three strains, _C. neoformans var. neoformans_, _C. neoformans var. gattii_, and _C. neoformans var. grubii_, which are dissimilar enough that they have been described as three separate species [1]. _C. neoformans var. grubii_ (referred to simply as _C. neoformans_ in this study) is the best studied among these strains, a common fungus capable of producing several virulence factors that affect its pathogenicity in immunocompromised hosts [1]. These virulence factors allow this pathogen to produce a capsule, grow at 37°C, and produce melanin [1].

Melanogenesis is closely associated with increased virulence in _C. neoformans_ due to protection against host defense mechanisms by these negatively charged, hydrophobic pigments [2]. Melanin particles can confer protection from UV radiation, but they also play a role in protection against host immune systems [1]. Studies have shown that compared to mice infected with non-melanin producing _C. neoformans_ strains, mice infected with melanin-producing strains had reduced tumor necrosis factor-α (TNF-α) production and decreased clonal expansion of lymphocytes [2]. Non-melanized cells have been shown to completely dissolve when exposed to acid digestion, whereas melanized cells are resistant, allowing for them to bypass many of the innate immune defenses of the host [2]. By weakening the host immune system through melanogenesis, especially in immunocompromised hosts, the virulence of the fungus increases.
Unlike many organisms that are capable of intrinsic melanogenesis, *C. neoformans* requires the conversion of an exogenous substrate via the enzyme laccase, a phenol or diphenol oxidase [1]. There are several types of melanin, including eumelansins, phaeomelansins, allomelansins, and pyomelansins, each of which is derived from a different set of precursors. Allomelansins are derived from nitrogen-free precursors, while phaeomelansins are derived from the amino acids tyrosine and cysteine [1]. Pyomelansins, typically dark red or brown in color, are derived from *p*-hydroxyphenylpyruvate and homogentisic acid (HGA), known metabolites of tyrosine [1]. Eumelansins, which are typically black in color, are derived from catecholamines such as epinephrine, norepinephrine, and dopamine, or their precursors such as L-DOPA [1]. These catecholamine precursors are readily available in the central nervous system of mammalian hosts and induce eumelanin production in *C. neoformans*, increasing its virulence. This process may explain why the central nervous system is such a common infection site for *C. neoformans*, which causes meningoencephalitis in immunocompromised individuals [2].

The sources of these exogenous substrates are still being investigated, but the results of studies have indicated that bacterial interactions with *C. neoformans* may be involved in the induction of melanogenesis [3]. This phenomenon was first discovered when *C. neoformans*, which forms opaque and creamy colonies when cultured in isolation on SM medium, turned dark brown in the presence of *Klebsiella aerogenes* [3]. We chose to study this phenomenon using *Enterobacter hormaechei*, a bacterium known to be capable of HGA production and previously shown by the Casadevall Lab to produce catecholamine precursors such as L-DOPA via High Performance Liquid
Chromatography (HPLC) analysis (Table 2). Since *E. hormaechei* can produce known melanin precursors, it can be used as a model for further bacterial-fungal interaction studies.

Initial melanization assays were able to show that *C. neoformans*, which is unable to melanize when cultured in isolation, developed dark pigmentation when grown in the presence of *E. hormaechei*, indicating that *E. hormaechei* can produce and secrete melanin precursors to induce fungal melanogenesis. However, to identify the associated precursors produced by *E. hormaechei*, this bacterium must be characterized in terms of the genes and pathways involved in the production of an exogenous substrate that can induce *C. neoformans* melanization.

Over 7000 *E. hormaechei* transposon mutants were generated and tested for their ability to induce fungal melanogenesis via melanization assays. We were interested in identifying mutants that were unable to induce melanogenesis, which indicated that the disrupted gene in each of these mutants is necessary for the production of melanin precursor molecules. The results of these assays and subsequent bioinformatics analyses showed that the *E. hormaechei* transcription factor TyrR is necessary for the induction of *C. neoformans* melanization. The observed decrease in melanization of *C. neoformans* when interacting with the tyrR-disrupted transposon mutant may be due to the loss of *tpl* expression, which in other bacteria has been shown to modulate the production of L-DOPA, a known eumelanin precursor.

In future studies, the potential role of TyrR in L-DOPA production can be further assayed through gene complementation, mass spectrometry (MS) analysis to assess L-
DOPA production, and melanin particle studies to determine if the dark pigment formed by \textit{C. neoformans} mediated by \textit{E. hormaechei} is due to the presence of eumelanin.

\textbf{Materials and Methods}

\textit{Strains, Growth, and Storage Conditions}

The strains used in this study are presented in Table 1. \textit{E. hormaechei} strain PA was grown on agar-solidified Lysogeny Broth (LB) medium consisting of (per L): 10 g of tryptone (BD: Becton, Dickinson & Co, Sparks, Maryland, USA), 5 g of yeast extract (BD), 10 g of NaCl, and 15 g of Bacto agar (1.5%) (BD). This strain was grown on LB at 30°C for approximately 24 hours.

\textit{E. hormaechei} transposon mutants were grown on agar-solidified LB medium supplemented with 50 µg/mL of kanamycin (Km) to select for kanamycin-resistant mutants. These strains were incubated at 30°C for approximately 24 hours.

Yeast extract-peptone-dextrose (YPD) medium was used for fungal growth and consisted of (per L): 10 g of yeast extract (BD), 20 g of peptone (BD), 20 g of dextrose (BD), and 20 g of Bacto agar (BD). \textit{C. neoformans} was grown on agar-solidified YPD for 48 hours at 30°C, while \textit{C. neoformans} \textit{Δlac var. grubii} (referred to as \textit{C. neoformans} \textit{Δlac} in this study) was grown on YPD supplemented with 200 U/mL of hygromycin B for 48 hours at 30°C.

SM medium was used for melanization assays and consisted of (per L): 10 g proteose peptone (BD), 1 g of yeast extract (BD), 1 g anhydrous MgSO\textsubscript{4}, 1.9 g of KH\textsubscript{2}PO\textsubscript{4}, 0.6 g of K\textsubscript{2}HPO\textsubscript{4}, 20 g of agar (BD), and 25 mL of 40% glucose (Alfa Aesar, Ward Hill, Massachusetts, USA), with the pH adjusted to 6.5 [3]. The medium was
supplemented with 25 mL of L-tyrosine (Research Products International (RPI), Mt. Prospect, Illinois, USA).

M9 minimal medium plates, used to assess the percentage of auxotrophs among the mutants, consisted of (per L): 64 g of Na₂HPO₄·7 H₂O, 15 g of KH₂PO₄, 2.5 g of NaCl, 5 g of NH₄Cl, 2 mL of 1 M MgSO₄, 20 mL of glucose (AA), 0.1 mL of 1 M CaCl₂, and 15 g of agar (BD).

Super optimal broth with catabolite repression (SOC) medium was used for *E. hormaechei* and *Escherichia coli* transformations and consisted of (per L) 20 g of tryptone (BD), 5 g of yeast extract (BD), 0.5 g of NaCl, 0.186 g of KCl, 0.95 g of MgCl₂, and 4 g of glucose (AA).

**Bioinformatics**

The genes and proteins involved in the dopamine synthesis pathway have been characterized in other bacterial species. *E. hormaechei* was sequenced using a MiSeq Illumina platform to identify putative genes involved in inducing *C. neoformans* melanization using the NCBI Basic Local Alignment Search Tool (BLAST).

Using a Lucigen DNA isolation kit (Lucigen Corporation, Middleton, Wisconsin, USA), *E. hormaechei* genomic DNA was isolated and then used for library preparation with a Nextera XT DNA Sample Preparation kit (Illumina, San Diego, California, USA) and the Illumina protocols FC-131-1024 and FC-131-1001 [4]. These protocols were followed for the tagmentation of genomic DNA (gDNA), PCR amplification of fragments, and PCR cleanup using AMPure XP beads to purify the library and remove short fragments. The tagmented, amplified, and purified gDNA was quantified for library validation using a Qubit fluorometer (ThermoFisher Scientific, Waltham, Massachusetts,
USA). Using a bioanalyzer, the average base pair length of the gDNA fragments was determined for subsequent MiSeq Illumina sequencing.

Following Illumina sequencing, the sequences were quality filtered, assembled using CLC (CLC bio, Aarhus, Denmark), and annotated with Rapid Annotation using Subsystem Technology (RAST).

Transposon Mutagenesis

The following procedure followed a previously described protocol by Jonathan James Caguiat [5] using an EZ-Tn5<R6Kyori/Kan-2> Tnp Transposome kit from Lucigen.

*E. hormaechei* was cultured overnight from a single colony in LB at 30°C and then was diluted to an optical density at 600 nm (OD_{600}) of approximately 0.4-0.6. Approximately 100 mL of cell culture was incubated in an ice water bath for approximately 15 minutes with periodic swirling to ensure the cells were in a metabolically inactive state. All tubes, pipette tips, and electroporation cuvettes were stored at -20°C prior to usage and were then stored on ice.

The 100 mL culture was then split into four 25 mL aliquots in 50 mL conical tubes and centrifuged at 4°C for 5 minutes at 7000 rcf. Then the supernatants were decanted, and the cell pellets were combined in 25 mL of ddH₂O. The cells then underwent multiple wash steps, after which the cell pellet was resuspended in an approximately equivalent volume of ddH₂O.

Once competent, 40 µL of cells were electroporated with 0.5 µL of transposome in an ice-cold 0.2-mm electroporation cuvette at 25 µF, 200 Ω, and 2.5 kV, after which the cells were immediately suspended in 960 µL of filter-sterilized SOC medium. The
cells were then incubated on a shaker at 30°C for 45 minutes at 120 rpm. A negative control was also assayed in which 40 µL of cells were mixed with 960 µL of SOC.

After 45 minutes of recovery, 10- and 100-fold dilutions of the recovered cells and the negative control were spread onto LB plates supplemented with 50 µg/mL of Km (LB-Km50) to select for cells with an incorporated transposon and were incubated at 30°C for approximately 24 hours. The thousands of mutants generated from this process were then analyzed and used in melanization assays to identify genes involved in the synthesis of melanin precursors such as L-DOPA.

*Testing for Auxotrophs*

To verify that the transposon randomly inserted into the genome of *E. hormaechei*, an auxotrophy test was performed. Two hundred and twenty-four colonies from the 10-fold dilution spread plates of electroporated *E. hormaechei* were patched in duplicates onto LB-Km50 and M9 minimal medium plates. The patch plates were then analyzed to ensure that 2-3% of the mutants were auxotrophs.

*Southern Blot Analysis*

A Southern Blot Analysis was performed to ensure that the transposon inserted only once into the *E. hormaechei* genome. A Thermo Scientific North2South™ Biotin Random Prime Labeling kit was used to prepare biotin-labeled DNA probes needed following the manufacturer’s instructions [6].

A DNA probe complementary to the Km resistance gene present on the transposon was generated and labeled with biotin-11-dUTP. A mutant from the previous transposon mutagenesis containing the transposon, and therefore the Km resistance gene, was used to PCR amplify a fragment from the Km resistance gene using primers Kan_F
and Kan_R (Table 3). This fragment, Klenow fragment, and a dNTP mix containing Biotin-11-dUTP were combined to generate the labeling reaction of the probe. During this labeling reaction, many short fragments of nucleotides complementary to the Km resistance gene were generated all labeled with Biotin.

To prepare the Southern Blot, DNA from *E. hormaechei* mutants were digested with *Eco*RI-HF® (NEB #R3101) and run on a 0.6% Tris-Borate-EDTA (TBE) Buffer (5×) agarose gel. To this end, 2 µL of DNA, 8 µL of ddH₂O, 2 µL of loading buffer, and 0.5-1 µL of *Eco*RI were combined and incubated for 3 hours. The digested DNA was then loaded onto a gel and run at 80 V for approximately 2 hours. The gel was then stained with 10 µL of SYBER Safe in 100 mL of ddH₂O, destained with 100 mL of ddH₂O, and then immediately transferred to a nylon membrane to proceed with the Southern blot.

To then visualize the location of the transposon within the digested DNA, a Thermo Scientific North2South™ Chemiluminescent Hybridization and Detection kit was used following the manufacturer’s protocol [7].

Following the transfer of the DNA to the nylon membrane, the membrane was placed in a 50 mL centrifuge tube, and enough hybridization buffer was added to completely cover the blot. The sealed container was then rotated for approximately 30 minutes at 55°C. During this time, the biotinylated DNA probe was denatured at 100°C for 10 minutes and placed on ice for 5 minutes. Then, approximately 30 ng of the biotinylated probe per milliliter of hybridization buffer was added and then incubated overnight while rotating at 55°C. The blot underwent multiple wash steps before a 1:300 dilution of streptavidin-horseradish peroxidase was added to the nylon membrane and
incubated while rotating. Following multiple wash steps, the nylon membrane was removed from the 50 mL centrifuge tube and enough Substrate Working Solution (containing equal volumes of Luminol/Enhancer Solution and Stable Peroxide Solution) to cover the membrane was added. The reaction between this substrate solution and horseradish peroxidase bound to the probe emits light and was visualized with a Gel Documentation System (ThermoFisher Scientific) under UV light to determine the location of the probe and ultimately the location of the transposon.

Development of the Melanization Assay

Knowing that fungal strains such as *C. neoformans* var. *gattii* and *C. neoformans* melanize in the presence of dopamine and other melanization-inducing metabolites, these fungal strains could be used to assess the induction of melanization by our bacterial model strain, *E. hormaechei*.

Tyrosine is a precursor used by many enzymes in the production of L-DOPA and other melanin inducing metabolites. To optimize the medium for fungal melanization, SM medium was supplemented with varying concentrations of tyrosine, concentrations of glucose, and incubation temperatures. As shown in Figure 1, agar-solidified SM plates were made with normal or reduced concentrations of glucose. Each of those were then supplemented with 0.1, 0.25 or 0.5% of 10% L-tyrosine (RPI). In addition, plates at each L-tyrosine concentration were also incubated at 22, 30, or 37°C. All plates were incubated for 72 hours.

In addition to changes in medium, changes to the format of the assay were optimized. The original style of the melanization assay was adapted from the Casadevall lab, where *E. hormaechei* was struck as a lawn in a half-moon-shape on one half of the
plate (Figure 7A) [3]. The other half of the plate was made up of horizontal streaks of *C. neoformans var. gattii, C. neoformans* and *C. neoformans Δlac* to determine which fungal strain would yield the best melanization phenotype (Figure 7A) [3]. The wild-type *C. neoformans* and *C. neoformans var. gattii* strains melanize in the presence of *E. hormaechei*, whereas the Δlac strain, acting as a negative control, is unable to melanize due to absence of the laccase gene. For further optimization, this format of the assay was altered by streaking two smaller half-moon lawns of mutant *E. hormaechei* on either side of the plate, leaving approximately 2 cm in the middle for a vertical fungal streak (Figure 7B). A negative and positive control were created the same way using wild-type *E. hormaechei* for the half-moons and using the wild-type fungal strain for the positive control and the Δlac fungal strain for the negative control.

Melanization assays were performed with corresponding wells on 96-well plates. Agar-solidified SM-tyrosine plates were labeled according to the number of the 96-well plate and the corresponding well (A1-H12). A plastic loop was used to pick a transposon mutant and spread it onto each side of the plate, creating a lawn of growth. Before discarding the loop, the loop was dipped into the corresponding well on a 96-well plate filled with 100 µL of liquid LB-Km50. The following day, 20.8% glycerol (ThermoFisher Scientific) was added to each well to create a stock of each mutant assayed. Continuing with the assay, after streaking an individual mutant on all 96 plates, a toothpick was used to streak *C. neoformans* down the center of every plate. Positive and negative control plates were also performed as previously described. The plates were covered with a black plastic bag and then incubated for 72 hours at 30°C.
Based on the melanization assay results, mutants that induced hypomelanization, hypermelanization, or abnormal melanization (a dark red melanin) were chosen for further research. These mutants were studied via auxotrophy tests to ensure that the change in phenotype was not due to auxotrophy, after which an additional melanization assay was performed to confirm the phenotype and colony size and shape were assessed to confirm normal growth rates.

**Bioinformatic Analysis of Mutant DNA**

The gDNA of selected mutants was prepped using a QIAcube (QIAGEN, Hilden, Germany) following the QIAamp Mini Kit protocol (QIAGEN) [8] and sequenced using Illumina sequencing.

Sequences were quality filtered and assembled using CLC by mapping the transposon sequence to the mutant genome to determine the site of transposon insertion. Upstream and downstream DNA of the insertion site was obtained, converted into fasta files, and compared against the *E. hormaechei* genome using RAST to map each sequence of the disrupted genes to the *E. hormaechei* genome. Using RAST, the location and function of the gene could be determined. Each gene was characterized using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database and the NCBI Conserved Domain Database to determine which ones may play a role in the induction of *C. neoformans* melanization.

**Plasmid Construction**

The gene of interest (*tyrR*) was cloned into the plasmid pTn7xKS, which contains a gentamicin resistance marker. Four primers were designed to separately amplify the *tyrR* operon promoter and the *tyrR* gene (Table 3). Internal primers were tailed to overlap
each other, and external primers were tailed to overlap at the PstI and KpnI sites in pTn7xKS.

Primers were designed on SnapGene (GSL Biotech, Chicago, Illinois, USA) to be 18-30 nucleotides long, with a melting point around 55-65°C and a guanine-cytosine content of approximately 40-60%. Each primer was designed for use with the Phusion High Fidelity PCR Master Mix with HF Buffer kit (ThermoFisher Scientific), and all annealing temperatures were determined using the New England Biolabs Tm Calculator (New England Biolabs, Ipswich, Massachusetts, USA). The PCR product of tyrR was confirmed by agarose gel electrophoresis.

To construct the plasmid, pTn7xKS was purified from DH5α following the QIAGEN Plasmid Maxi Kit and protocol [14], and the concentration and quality of the plasmid was determined using Nanodrop (ThermoFisher Scientific) and Qubit instruments. Following the digestion of pTn7xKS by PstI-HF® (NEB #R3140) and KpnI-HF® (NEB #R3142) restriction endonucleases (Figure 11) and gel purification, the plasmid was assembled by Gibson Assembly (GA) [9], a cloning technique used to generate plasmids [9]. A 1:2 vector to insert ratio was used, and dilutions of the tyrR promoter and tyrR gene were prepared accordingly for a 10 µL Gibson reaction consisting of 5 µL 2× Gibson Master Mix, 2 µL of each insert, and 1 µL of plasmid run on the thermocycler.

Following GA, the recombined and assembled plasmids were diluted 4-fold and transformed into E. coli BW25142 and spread plate onto LB-Gentamicin10 to select for transformed gentamicin resistant cells.
Results

Testing for Auxotrophs

During transposon mutagenesis, it is expected that the transposon will be inserted into the genome at random. To confirm the randomness of its insertion, an auxotrophy test was performed. If the transposon inserts randomly into the DNA, it should insert into a gene essential for the survival of the bacterium 2-3% of the time. Therefore, 2-3% of the transposon mutants should be auxotrophs and require supplemented nutrients. Auxotrophs are able to survive on agar-solidified LB medium, which provides supplemented nutrients, but unable to survive on minimal medium, which does not. Five of 224 mutants (2.2%) were identified as auxotrophs, indicating that the transposon inserted into the genome at random (Figure 3).

Southern Blot Analysis

The Lucigen transposome consists of a transposase and a transposon that confers Km resistance. The transposase enzyme is responsible for randomly cutting the genome at insertion sites to insert the transposon. It is important that the transposon inserts only once, and to confirm this, a Southern Blot was performed.

Before performing the Southern Blot, the genome was digested using restriction enzyme EcoRI-HF (Figure 4). The smear that can be visualized for each of the ten samples indicates that the DNA was completely digested by EcoRI producing many different fragment sizes. Therefore, EcoRI is a suitable enzyme to use for the Southern Blot.
After performing the Southern Blot as described in the Materials and Methods, the probe was visualized as binding to only one fragment in each mutant sample, indicating that the transposon inserted only once into each sample (Figure 5).

**Development of the Melanization Assay**

SM medium was used for melanization assays, as this medium was previously shown by the Casadevall lab to allow for optimal growth of both fungi and bacteria [3]. However, the medium was altered for further optimization of fungal melanization. Figure 2 shows the flow chart of all the experimental conditions used, including glucose concentrations, incubation temperatures, and L-tyrosine concentrations. It was determined that there was optimal growth and melanization at 1% glucose and 30°C. As shown in Figure 6, the results of varying the L-tyrosine concentration indicated that 0 and 0.1% L-tyrosine showed very little melanization, while 0.5% L-tyrosine showed high levels of melanization but also a discoloration of the medium itself. This change in medium color at 0.5% L-tyrosine is not optimal, as it can lead to confusion in determining melanization phenotype. Therefore, it was determined that 0.25% L-tyrosine was the best option because it allowed for optimal melanization of the fungal strains, but did not discolor the medium.

Traditionally, the Casadevall Lab performed melanization assays by streaking a half moon of bacterial lawn perpendicular to horizontal streaks of each fungal strain (Figure 7A) [3]. However, to optimize the melanization phenotype, we performed melanization assays by streaking two smaller half moons of bacterial lawn on both sides of the plate leaving approximately 2 cm for a vertical streak of *C. neoformans* (Figure 7B). Using this method, a stronger, more consistent phenotype was observed, because the
fungal strain was able to interact with melanization-inducing metabolites from the bacteria on all sides. The increased uniformity throughout the entire fungal streak makes it optimal for characterization of its phenotype.

Finally, it was also determined which fungal strain would be used as the model for melanization. *C. neoformans* var. *gattii* and *C. neoformans* are both fungal strains that melanize in the presence of L-DOPA and other melanin inducing metabolites. However, because *C. neoformans* showed a much greater production of melanin in the presence *E. hormaechei*, it was selected to assess melanization in all future assays (Figure 6).

When performing melanization assays, each *E. hormaechei* mutant was characterized based on the amount of melanization it induces. Mutants that did not induce fungal melanization were considered “hypomelanizers” (Figure 8C). Mutants that induced more fungal melanization than wild type were considered “hypermelanizers” (Figure 8D). Mutants that induced a dark red melanin were categorized as “abnormal” (Figure 8F). Because characterization based on a visual phenotype can be subjective, any mutant that induced a melanization phenotype that was darker than what would be expected from a “hypomelanizer”, but was not as dark as wild type, was called an “intermediate melanizer” (Figure 8E).

After screening 7008 *E. hormaechei* transposon mutants, mutants that induced melanin phenotypes different from the wild-type strain were further studied. Assays performed included an auxotrophy test, additional melanization assays to confirm their phenotype, and a recording of colony size and shape to confirm regular growth rates. From this data, non-auxotroph mutants were chosen based on the consistency in the melanization phenotype produced and consistency of a wild-type growth rate. From this
data, mutant 2906, a hypomelanizer, was chosen for bioinformatic analysis of its genomic DNA. Mutant 2906, a non-auxotroph, was chosen for having wild-type colony sizes after 24 hours of incubation on LB-Km50 and for having a consistent hypomelanizer phenotype after both primary and secondary melanization assays (Figure 9). This hypomelanizer phenotype indicates that the gene disrupted by the transposon was necessary for the production of melanin precursor molecules and is therefore necessary for the induction of fungal melanogenesis.

*Bioinformatic Analysis of Mutant DNA*

Mutant 2906 was sequenced using Illumina sequencing, and the transposon-interrupted gene was determined using RAST. The interrupted gene was identified as the last gene in a three-gene operon encoding the transcriptional repressor TyrR, a regulator of amino acid metabolism (Figure 10A). Being the last gene of an operon, it is unlikely that its disruption disrupted the expression of any other downstream genes, indicating that this gene disruption alone may be responsible for the hypomelanizer phenotype. The NCBI Conserved Domain Database was used to identify conserved domains in TyrR, resulting in the identification of a TyrR domain (e value = 0e+00), which is involved in the transcriptional regulation of aromatic amino acid metabolism (Figure 10B). The conserved domain ACT_TyrR (e value = 3.41e-36) was also identified, indicating the involvement of this protein in the biosynthesis or transport of aromatic amino acids, as were domains involved in DNA binding at specific TyrR boxes at sigma70 promoters for activation or repression of transcription (Figure 10B). This same conserved domain also indicated that the activity of TyrR fluctuates with fluctuating concentrations of tyrosine within the cell (Figure 10B).
Discussion

Fungal melanogenesis, particularly in *C. neoformans*, has been highly studied with respect to the biosynthesis and uses of melanin in providing an organism with increased virulence and pathogenicity. However, *C. neoformans* is unique in its requirement for an exogenous substrate to induce melanogenesis. Given that this fungus is capable of growth in such a wide variety of niches, the source of these exogenous substrates may be dependent on the particular niche. However, there is evidence that other bacteria in these niches may produce melanization-inducing substrates that can be utilized by *C. neoformans* [3].

Understanding where these melanin precursors are derived from and how they are derived is important in understanding the pathogenicity of *C. neoformans*. Increased melanin is linked to increased virulence. Melanin acts as a protector of fungal cells, not only from UV radiation, but also from the mechanisms of host immune systems, allowing for them to persist in hosts, particularly immunocompromised hosts, inflicting disease [2]. *C. neoformans* causes infection in over 1 million individuals worldwide per year, with 625,000 of those cases resulting in death [15]. However, understanding the mechanism of melanogenesis in non-human hosts, such as vectors like birds, is also crucial in understanding fungal virulence and transmission.

To model bacterial interactions with *C. neoformans* that may result in melanogenesis, *E. hormaechei* was used in this study due to its ability to produce L-DOPA and HGA, which are known eumelanin and pyomelanin precursors, respectively. The melanization assays performed in this study were optimized in terms of medium, growth requirements, and plating format to have consistent and uniform melanization
phenotypes. These assays utilized transposon mutants containing random gene disruptions throughout the \textit{E. hormaechei} genome. Specific gene disruptions interfered with the ability of mutants to induce melanogenesis in \textit{C. neoformans}, indicating that these genes are necessary to produce melanin precursors. One hypomelanizer, mutant 2906, was determined to have a disruption in gene \textit{tyrR}, responsible for expression of transcription factor TyrR.

TyrR has been characterized in \textit{E. coli} as both an activator and repressor of the TyrR regulon, which is composed of eight known transcriptional units that each contain one or more TyrR boxes for TyrR binding [10]. The ability of TyrR to act as a repressor or an activator is determined by both the cofactor it interacts with and the juxtaposition of the binding sites for TyrR and RNA polymerase [10]. TyrR is capable of interacting with all three aromatic amino acids (tyrosine, tryptophan, and phenylalanine), and the presence of these cofactors allows TyrR to bind both strong and weak TyrR boxes [10]. Depending on the amino acid present and the type of TyrR box, this activity can result in either the activation or repression of transcription [10]. In addition, when TyrR is in the presence of cofactors, it can interact with the C-terminal region of the alpha subunit of RNA polymerase to promote transcriptional activation [10].

The role of TyrR has also been characterized in other bacterial species, including \textit{Erwinia herbicola} [11] and \textit{Citrobacter freundii} [12], and in both species TyrR has been shown to function as a regulator of \textit{tpl}. The gene \textit{tpl} encodes enzyme tyrosine phenol-lyase (Tpl), which is responsible for the degradation of L-tyrosine into pyruvate, ammonium, and a phenol or catechol [13]. If a catechol is produced in the first reaction, Tpl is capable of a reversible reaction that produces L-DOPA (Figure 1) [13]. Studies in
*E. herbicola* have identified a TyrR box in the *tpl* gene promoter, indicating that TyrR is capable of promoting the transcriptional activation of *tpl* in the presence of tyrosine [11]. These studied showed that *tpl* expression increased with increasing levels of tyrosine and decreased with increased glucose concentrations in the medium [11]. Both of these findings are consistent with our results in the optimization of SM medium for melanization assays. In addition, these results were consistent with those of the studies performed using *C. freundii*, which also identified two DNA segments upstream of the *tpl* promoter with high homology to TyrR boxes [12]. Smith and Somerville confirmed the role of TyrR in transcriptional activation by creating a *tpl-lacZ* reporter system. Strains with a deletion of *tyrR* showed a 10-fold decrease in β-galactosidase expression, indicating that TyrR has a large role in the transcriptional activation of the TyrR regulon, which may include *tpl* [12].

With the knowledge that other bacteria are capable of using TyrR as a transcriptional activator for *tpl* expression, the identification of TyrR in *E. hormaechei* as a factor affecting the induction of melanin formation in *C. neoformans* increases our understanding of this process. Future studies are required to confirm the role of TyrR in *E. hormaechei* as an activator of a gene required for the production of a melanin precursor, like L-DOPA, for the possible induction of eumelanin production in *C. neoformans*.

Due to interruptions in research, complementation, MS, and melanin particle analyses were halted. Complementation assays need to be completed to confirm that the disruption in *tyrR* is the reason for the hypomelanizer phenotype of mutant 2906. After complementation of mutant 2906 with *tyrR*, the mutant should induce wild-type
melanization of *C. neoformans* in melanization assays. If this occurs, it indicates that the disrupted *tyrR* is the sole reason for the hypomelanizer phenotype and confirms its role in producing a melanization-inducing metabolite.

To determine which melanization-inducing metabolite TyrR transcription factor plays a role in synthesizing, and therefore which type of melanin production is modulated by TyrR, MS and melanin particle analyses should be performed. Given the role of TyrR as an activator of *tpl* in other bacterial species, it is predicted that TyrR may play a similar role in L-DOPA synthesis in *E. hormaechei*. MS can be used to detect the presence of L-DOPA or dopamine in the supernatant of wild-type and mutant *E. hormaechei* strains to determine if these melanin precursors are being produced. The Casadevall lab confirmed the production of L-DOPA by wild-type *E. hormaechei* via HPLC, showing that 67.5 ng/µL of L-DOPA could be detected over 96 hours (Table 2). L-DOPA can also be detected through MS to compare wild-type L-DOPA production to mutant 2906 L-DOPA production.

*C. neoformans* melanin particles can be isolated and analyzed to characterize the type of melanin that is produced in the presence of *E. hormaechei* and determine if TyrR is in fact playing a role in L-DOPA synthesis and therefore modulating the production of eumelanin. Eumelanin can be distinguished from other melanin particles such as pyomelanin by their diameter and oxidative degradation products. Under transmission electron microscopy, eumelanin particles are observed to have larger diameters at approximately 4–4.5 µm [1]. HPLC chromatographic analysis of oxidation via permanganate can also be used to distinguish eumelanin particles as they should have
oxidative-degradation products with similar chromatographic properties to pyrrole-2,3,5-tricarboxylic acid (PTCA) [1].

Therefore, to further explore the interaction between bacteria and fungus in the induction of melanogenesis as attempted in this study, these interrupted experiments including gene complementation assays, MS, and melanin particle analyses should be completed in the future.
### Tables and Figures

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td><strong>var. grubii H99</strong></td>
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</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Δlac <strong>var. grubii H99</strong></td>
<td>1</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td><strong>var. gattii</strong></td>
<td>1</td>
</tr>
<tr>
<td><em>Enterobacter hormaechei</em></td>
<td>Strain PA; WT; isolated from the midgut of wild-caught <em>Anopheles arabiensis</em> in Zambia, Africa</td>
<td>16</td>
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<tr>
<td><em>E. hormaechei 2906</em></td>
<td>TyrR Transposon Mutant</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli DH5α</em></td>
<td>Carrying plasmid pTn7xKS</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli BW25142</em></td>
<td>Used in Gibson Assembly</td>
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*Table 1 Strains used in this study.*

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Dopamine Detection by HPLC after 96 hours (ng/mL)</th>
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<tr>
<td><em>Aeromonas hydrophila</em></td>
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<tr>
<td><em>Enterobacter hormaechei</em></td>
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<td><em>Comamonas sp.</em></td>
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*Table 2 Dopamine detection by HPLC. This data is taken from unpublished work at the Casadevall Lab and shows that dopamine was detected in the supernatant of all three of these strains after 96 hours of incubation.*
<table>
<thead>
<tr>
<th>Primer Name</th>
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<th>Use</th>
</tr>
</thead>
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<td>Kan_F</td>
<td>5’-TGAGCCATATTCAACGGGAAAC-3’</td>
<td>Amplify the Km region of transposon in southern blot</td>
</tr>
<tr>
<td>Kan_R</td>
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</tr>
<tr>
<td>PtyrR_comp_F</td>
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<td>Cloning tyrR promoter for construct</td>
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<tr>
<td>PtyrR_comp_R</td>
<td>cgccgtcctcttgcc</td>
<td>Cloning tyrR promoter for construct</td>
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<tr>
<td>tyrR_comp_F</td>
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<tr>
<td>tyrR_comp_R</td>
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<td>Cloning tyrR construct</td>
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*Table 3 Primers used in this study.*

*Figure 1 Tyrosine Phenol-Lyase catalyzed reaction.* Conversion of a catechol, pyruvate, and ammonium into L-DOPA via tyrosine inducible enzyme Tyrosine Phenol-Lyase [13].
Figure 2 Flow chart of varying conditions for assay confirmation. SM medium was altered to determine the conditions that promote optimal melanization using normal or reduced concentrations of glucose, 0.25 or 0% tyrosine, and varying incubation temperatures. All plates were incubated for 96 hours.
Figure 3 Auxotroph Testing. Seen above, two of the mutants struck onto LB were unable to grow on minimal medium. This exemplifies two auxotroph mutants. This figure shows only 32 struck mutants, but in actuality, 5 of the 224 assayed mutants were auxotrophs, comprising 2.2% of the mutants tested.

Figure 4 EcoRI-digested DNA. EcoRI DNA digest of wild-type E. hormaechei (lane 2) and ten different transposon mutants (lanes 3-12) were run on a 0.6% TBE agarose gel and compared to an NEB 1 kb ladder (MW Markers). The smeared nature of the gel indicates the restriction enzyme cut the mutants into many fragments of varying sizes.
Figure 5 Southern Blot analysis. After completing the Southern Blot, each band in each lane represents the visualization of the biotin-labeled probe binding to a fragment of a mutant genome containing the inserted transposon. There is only one band visualized per mutant indicating that the transposon only inserted once into the genome per mutant.

Figure 6 SM medium plates with varying concentrations of L-Tyrosine. Each plate consists of SM Medium with 1% glucose, incubated for 72 hours at 30°C inside of a bag. Each plate is supplemented with a different concentration of L-tyrosine ranging from 0% to 0.5%. On the left side of the plate, E. hormaechei is struck out in a half moon. On the right side of plate are horizontal fungal streaks of C. gattii (top), C. neoformans Δlac (middle), and C. neoformans (bottom). These plates indicate which L-tyrosine concentration is optimal for the melanization assay, as well as which fungal strain (C. gattii or C. neoformans) is a stronger model.
**Figure 7 Melanization assay style.** Both plates imaged are grown on SM Medium supplemented with 0.25% L-tyrosine. 7a shows *E. hormaechei* struck out as a lawn on the left half of the plate with *C. neoformans* (top) and *C. neoformans Δlac* (bottom). 7b shows *E. hormaechei* struck out in two half moon lawns on either side of the plate with *C. neoformans* (left) or *C. neoformans Δlac* (right) struck down the center of the plate.

**Figure 8 Melanization assay phenotypes.** All plates are grown on SM Medium supplemented with 0.25% L-tyrosine. For comparison, 8a depicts a positive control plate with wild type *E. hormaechei* and wild type *C. neoformans*, and 8b depicts a negative control plate with wild type *E. hormaechei* and *C. neoformans Δlac*. Each category of melanization phenotypes are also depicted including “hypomelanization” (c), “hypermelanization” (d), “intermediate” melanization (e), and “abnormal” melanization (f).
Figure 9 Melanization assay results of mutant 2906. Photos taken after 72 hours of incubation at 30°C depicting positive control wild type E. hormaechei grown with wild type C. neoformans (A), negative control wild type E. hormaechei grown with C. neoformans Δlac (B), and E. hormaechei 2906 growth with C. neoformans (C).

Figure 10 Genomic locations and conserved domains of TyrR. Gene 1 depicted in 12A represents tyrR, the third gene in a three-gene operon preceded by Gene 2 coding for conserved protein Ycjx, and Gene 4 coding for membrane protein YcjF. 12B represents conserved domains found on NCBI Conserved Domain Database for TyrR. Specific hits consist of the TyrR domain (e value = 0e+00) and ACT_TyrR domain (e value = 3.41e-36).
Figure 11 pTn7xKS KpnI/PstI digestion. Purified pTn7xKS digested using restriction enzymes KpnI and PstI was run on a 7% agarose gel electrophoresis and compared to a 1 kb NEB ladder (MW Markers). Lane 2 shows pTn7xKS digested only by KpnI, Lane 3 digested only with PstI, Lane 4 undigested plasmid, and Lane 5 a double digest with both KpnI and PstI.
LITERATURE CITED


