3,4-Dihydroxyphenylacetate 2,3-dioxygenase modulates the ability of Enterobacter hormaechei to induce Cryptococcus neoformans melanization

Amy Nelson
amy.n.nelson@uconn.edu

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3,4-Dihydroxyphenylacetate 2,3-dioxygenase modulates the ability of Enterobacter hormaechei to induce Cryptococcus neoformans melanization

Amy Nelson
Dr. Joerg Graf
Dr. Jeremiah Marden
Department of Molecular and Cell Biology
University of Connecticut
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Abstract

In this study, we performed transposon mutagenesis to create a library of *Enterobacter hormaechei* mutants and developed a melanization assay to identify mutants that could not induce the typical melanization phenotype in *Cryptococcus neoformans*. Relevant phenotype labels included: “hypomelanizer”, a total lack of melanization; “hypermelanizer”, an increased melanization; or “abnormal”, an increased melanization with complete color change of the assay plate to a reddish-brown. Genomic sequencing of 47 mutants and bioinformatic analysis allowed us to pinpoint the transposon insertion site in each *E. hormaechei* mutant to identify the genes that were affected. A single mutant that induced the abnormal phenotype in *C. neoformans* was further analyzed and observed to harbor a disruption of the gene that encodes the enzyme 3,4-dihydroxyphenylacetate 2,3-dioxygenase. This phenotype may be attributable to the excess production of homogentisic acid, a byproduct of tyrosine catabolism. However, additional biochemical tests to demonstrate this possibility are necessary, and complementation of the mutant should be performed. Additionally, the detection of dopamine in all mutant cultures should be performed to determine if any genes with a transposon insertion contribute to dopamine production in *E. hormaechei*. 
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Introduction

*Enterobacter hormaechei* is a member of the *Enterobacter cloacae* complex of the *Enterobacteriaceae* bacterial family. Often found in the gut of animals, it is a gram negative, rod-shaped, facultative anaerobic, enteric bacterium (1). *Cryptococcus neoformans* is an encapsulated yeast and opportunistic pathogen that often causes meningoencephalitis in immunocompromised individuals (2, 3). When grown in the presence of specific bacteria, this yeast can use exogenous metabolites to produce melanin particles, thereby becoming light brown in color. Melanins increase the virulence of *C. neoformans* by reducing its susceptibility to host immune responses and other environmental stressors such as UV radiation (4, 5). The biosynthesis of melanins requires a diphenol oxidase, which is typically a laccase, tyrosinase, or catecholase (4). Melanins are not produced if such an enzyme is nonfunctional, which results in *C. neoformans* remaining white in color and having reduced virulence (6). As a negative control in visual assays, a strain of *C. neoformans* with a deletion of the gene encoding the Lac1 laccase is commonly utilized.

*E. hormaechei* appears to produce metabolites that can be used as precursors for melanin synthesis in *C. neoformans*. Such substrates include diphenolic compounds such as catecholamines or their precursors such as L-DOPA and tyrosine, or other bacterial metabolites such as homogentisic acid. By creating *E. hormaechei* knockout mutants and observing the phenotype they induce in *C. neoformans*, it is possible to identify relevant factors involved in this interaction. For example, by creating a knockout that fails to induce melanization of *C. neoformans*, the nonfunctional gene(s) can be identified and investigated for a role in melanin synthesis. Further insight could come from characterizing mutants that increase the melanization of *C. neoformans*, in which the lack of a product could shift the equilibrium of the pathway in *E.*
hormaechei into overdrive or cause this bacterium to utilize a different substrate and produce a different metabolite that interacts with C. neoformans. Ideally, relevant enzymes would be discovered in E. hormaechei, since such information is currently unknown.

In previous studies, the Casadevall lab at Johns Hopkins University observed that the mosquito species Anopheles gambiae had increased resistance to infection by Plasmodium falciparum, a parasite that causes malaria (Fig. 1). However, whether E. hormaechei secretes dopamine or other diphenolic compounds itself has not been fully proven. Verifying this activity was the goal of this project as well as discovering which enzymes in E. hormaechei are responsible for producing such compounds.

Materials and Methods

Strains and Media

E. hormaechei strain PA, C. neoformans serotype D strain B3501, and C. neoformans Δlac were used in this study, as outlined in Table 1. The major strain of interest for this project was an E. hormaechei mutant with a disruption in the HpcB enzyme produced by transposon mutagenesis and designated as E. hormaechei Tn-hpcB.

SM agar plates supplemented with L-tyrosine (ThermoFisher Scientific, Waltham, Massachusetts, USA) were used for the melanization assays. Since tyrosine is a known precursor in certain melanization pathways, it was added to the base SM medium at 0.25% to enhance the melanization induced in C. neoformans. The modified SM medium contained (per liter) 10 g Bacto Proteose Peptone, 1 g yeast extract, 2.05 g MgSO₄·7H₂O, 1.9 g KH₂PO₄, 0.6 g K₂HPO₄, and 20 g agar. Deionized water was added to bring volume to 950 ml after which the pH of the medium was adjusted to 6.5 using potassium hydroxide. Then, the medium was autoclaved,
cooled in a water bath to 55°C, and supplemented with 25 ml of a 40% glucose solution and 25 ml of a 10% tyrosine solution, both of which were filter sterilized. The melanization assays were performed using 60 × 15 mm Petri dishes (ThermoFisher Scientific), and each dish received 10 ml of medium. To guide streaking during melanization assays, a permanent marker was used to draw two lines, 13 mm apart, on the bottom of the plates. Additionally, the plates were labeled with a number and letter combination for identification that corresponded to wells in a 96-well plate, such that the alphanumerical combination consisted of the assay set number and the well number. For example, for the first set of melanization assays, a plate labeled 1-C5 corresponded to the well in the third row and fifth column of the 96-well plate (Fig. 2). The plates were stored at 4°C.

The C. neoformans strains were grown on Yeast Peptone Dextrose (YPD) agar plates that contained 10 g of yeast extract, 20 g of peptone, 20 g of dextrose, and 20 g of Bacto Agar per liter of medium. Hygromycin B was added at a final concentration of 200 µg/ml to selectively grow the Δlac strain.

Lysogeny broth (LB) medium was used for overnight cultures of E. hormaechei and consisted of 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of medium, with 15 g of Bacto Agar added for plates and kanamycin was added at a final concentration of 50 µg/ml (Km50) to select for the transposon mutants (transformants).

**Genomic Extraction**

The E. hormaechei PA strain was streaked on an LB plate and incubated for 24 hours at 30°C. Then, a 10 ml LB culture was prepared from an isolated colony and incubated for 24 hours at 30°C with shaking at 200 rpm. Genomic DNA was prepared using a Nextera XT DNA Library
Preparation Kit (Illumina, San Diego, California, USA), then extracted using a MasterPure Complete DNA and RNA Purification kit (Lucigen Corporation, Middletown, Wisconsin, USA). Next, fragment length of the DNA was evaluated using a Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Once diluted, the genome was sequenced by Miseq (Illumina). The reads were quality filtered, then the genome was assembled using CLC Sequence Viewer 8.0.0 (Qiagen, Germantown, Maryland, USA) and annotated using the RAST server.

**Transposon mutagenesis**

*E. hormaechei* mutants were generated by transposon mutagenesis using an EZ-Tn5™<R6Kγori/KAN-2>Tnp Transposome Kit (Lucigen) following a previously described protocol (7). *E. hormaechei* was cultured overnight in LB medium and then subcultured to an OD (600 nm) of 0.6. The cells were chilled on ice, washed, and centrifuged at 4°C and 7,000 × g several times with ice cold water. All materials were chilled prior to coming into contact with the cells, including tubes, pipette tips, and cuvettes. The transposome (0.5 µl) was added to 40 µl of electrocompetent *E. hormaechei* cells, which was transferred to a 2-mm gap electroporation cuvette. A MicroPulser electroporator (Bio-Rad Laboratories, Hercules, California, USA) was used to shock the cells (25 µF, 200 Ω, and 2.5 kV), after which 960 µl of super optimal broth with catabolite repression (SOC) media was immediately added to the cuvette. Next, the cells were transferred to a microcentrifuge tube and incubated at 30°C for 45 minutes with shaking at 120 rpm. As a control, 40 µl of electrocompetent *E. hormaechei* cells and 960 µl of SOC broth were added to a microcentrifuge tube without any transposome and incubated at the same conditions as the electroporated cells. 1:10 and 1:100 dilutions of the cells were prepared, then
the cells were spread on LB-Km50 agar plates (100 µl per plate). The plates were incubated at 30°C overnight, then stored at 4°C for later use in melanization assays.

**Auxotroph testing**

Transformants were patch plated on LB-Km50 and M-9 minimal medium using a sterile toothpick. All mutants are able to grow on LB-Km50, but auxotrophs cannot grow on M-9 medium. The plates were incubated at 30°C for 24 hours, then patches were evaluated for growth. Auxotroph testing was initially performed on the transformants from the first electroporation to roughly estimate the percentage at which they were produced. Identified auxotrophs were not tested in melanization assays because the interrupted gene was not expected to be involved in a pathway of interest. Subsequent auxotroph testing was not performed until melanization assays were completed and *E. hormaechei* mutants that had induced atypical melanization phenotypes in *C. neoformans* had been identified. These relevant mutants were then tested and auxotrophs were omitted from further investigation.

**Southern blot**

To ensure that the transposon only inserted into the genome of each mutant once, Southern blot analysis was performed. Genomic DNA of 10 transformants was extracted using a MasterPure Complete DNA and RNA Purification kit (Lucigen) following the manufacturer’s instructions. The concentration of the DNA samples was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific) and Qubit fluorometer (ThermoFisher Scientific), after which the DNA was stored at -80°C.
Primers for the kanamycin resistance gene in the transposon were designed (Table 2), then the gDNA of one transformant was used as the template for PCR amplification of the gene using Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, Massachusetts, USA). The annealing temperature was set to 63°C, as suggested by the NEB Tm calculator and 33 cycles ran. The identity of the amplicon was confirmed by running a 1% agarose gel. The kanamycin resistance gene is 806 base pairs and a band of this size was observed. A Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) was used to purify the amplicon DNA. Subsequently, the concentration of the PCR product was quantified using a Nanodrop spectrophotometer and a Qubit fluorometer then the DNA was stored at -80°C.

A North2South Biotin Random Prime Labeling Kit (ThermoFisher Scientific) was used to generate a biotinylated probe complementary to the kanamycin gene in the transposon. The kanamycin gene amplified from the selected transformant served as the template DNA. DNA from wild-type *E. hormaechei* and 10 transformants was digested with *Eco*RI (NEB #R3101) overnight. Next, the digested DNA was electrophoresed on a 0.6% agarose gel for 45 minutes at 100 V. The gel was treated with several buffers, and the DNA was transferred to a positively-charged nylon membrane and exposed to UV radiation to fix the DNA.

A North2South Chemiluminescent Hybridization and Detection Kit (ThermoFisher Scientific) was used to detect the transposon in the transformants. Then, the kanamycin gene probe was added to the membrane and incubated at 55°C overnight. The membrane was washed with stringency wash buffer and incubated for 20 minutes with agitation three times. Blocking buffer was added to the membrane, which was then incubated for 15 minutes with agitation. Blocking buffer was decanted from the membrane and Streptavidin-HRP was added to it. This
mixture was then poured on the membrane, which was incubated for another 15 minutes with agitation. The membrane was washed four times with diluted wash buffer and incubated for 5 minutes with agitation each time. Next, the membrane was transferred into a fresh wash container and covered with substrate equilibration buffer, then incubated for 5 minutes with agitation. The membrane was transferred onto a piece of plastic wrap and covered with a solution of equal volumes luminol and stable peroxide solution, then incubated for 5 minutes. The solution was decanted from the membrane and covered in plastic wrap, after which the membrane was then imaged.

**Melanization assay**

*C. neoformans* was streaked for isolation on a YPD agar plate and *C. neoformans Δlac* was streaked on YPD + hygromycin B. Both plates were incubated at 30°C for 48 hours. *E. hormaechei* was streaked for isolation on LB agar and incubated at 30°C for 24 hours.

Melanization assays were performed in sets of 96 mutants, which corresponded to a 96-well plate. For each melanization plate, an individual colony from a spread plate was picked using a sterile disposable loop. The colony was evenly distributed within both delineated halves of the plate, then that same loop was used to inoculate the appropriate well of the 96-well plate. Sterile flat-ended toothpicks were used to streak *C. neoformans* down the center of each melanization plate in the gap between the two regions streaked with bacteria. As a control, wild-type *E. hormaechei* was streaked on a melanization plate with *C. neoformans* down the center. As a negative control, wild-type *E. hormaechei* was streaked on a melanization plate with *C. neoformans Δlac* down the center. The edges of 96-well plate were covered with Parafilm to reduce evaporation and the plate was incubated at 30°C for 24 hours. The melanization plates
were stacked on a tray, covered in a black plastic bag, and incubated at 30°C for 72 hours. The bag reduced light exposure and created a reduced aerobic environment, though the bag was not sealed tightly over the tray.

After 24 hours of incubation, 100 µl of 20.8% glycerol was added to each well of the 96-well plate, which was then stored at -80°C. After 72 hours of incubation, the melanization plates were imaged on a light box and evaluated for the phenotypes they induced in *C. neoformans*. Plates that induced the same phenotype compared to the wild-type *E. hormaechei* were discarded. Plates that induced a different phenotype, whether a higher or lower degree of melanization, were retained and streaked for isolation for a second melanization assay.

**Secondary melanization assay**

To confirm that an *E. hormaechei* mutant induced an atypical melanization phenotype in *C. neoformans*, these mutants were tested a second time. A wooden stick was used to spot bacteria from the original melanization plate and streak it for isolation on an LB-Km50 plate. These plates were incubated at 30°C for 24 hours. Next, a sterile disposable loop was used to pick an isolated colony for each mutant and streak it on a SM + 0.25% L-tyrosine plate using the same technique as in the original melanization assay. *C. neoformans* was streaked down the middle of each plate. The plates were stored at 30°C for 72 hours, then imaged and evaluated for the phenotype they induced in *C. neoformans*.

In the secondary assays, 96-well plates were not utilized to make stocks of the mutants. Instead, each loop was used to inoculate a 5 ml LB-Km50 culture. The cultures were incubated for 24 hours at 30°C with shaking at 200 rpm. Stocks of each mutant was made by adding 792 µl of culture and 208 µl of 50% glycerol to a 1.5 ml cryogenic vial and vortexing vigorously for 8-
10 seconds. The vials were stored at -80°C. Note: these stocks were utilized for auxotroph testing.

*Library preparation and gene identification*

There were 47 mutants of interest identified by melanization assays whose genomes were subsequently sequenced. The 47 mutants were grown on LB Km50 at 30°C for 24 hours. Isolated colonies from each plate were used to prepare 5 ml cultures, incubated at 30°C for 12 hours. Genomic DNA from each mutant was extracted using MasterPure Complete DNA and RNA Purification Kit (Lucigen) and was stored at -20°C. A Nextera DNA Flex Library Prep Kit from Illumina was used to prepare genomic libraries of the 47 samples, using input DNA of 100-500 ng. The quality of the libraries was checked using a Tapestation instrument (Agilent Technologies), then the libraries were pooled to 2 nM and sequenced by MiSeq (Illumina). Using CLC sequence viewer, the reads were quality filtered and trimmed to eliminate adapter sequences. The genomes were assembled *de novo* and the reads were mapped to contigs to identify where the transposon inserted in each mutant. Subsequently, 100 base pairs upstream and downstream of the transposon were recorded and Unix was used to BLAST these sequences against the wild-type *E. hormaechei* PA genome. The output gave us a contig and protein encoding gene (PEG) number for each gene interrupted. Next, the annotated genome on RAST was referenced to identify the functions of the affected genes. The NCBI Conserved Domain Database was also used to obtain a better sense of gene function and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to learn about pathways that the genes were involved in.
Results

*E. hormaechei* genome assembly

The *E. hormaechei* PA genome was 4,646,605 base pairs, assembled into 29 contigs with an N50 of 300,865. This genome served as a reference for the 47 mutant genomes sequenced.

Auxotroph testing

Auxotrophs are mutants that require supplementation in the medium to grow because the transposon interrupted a gene required for the biosynthesis of an essential product, such as a nucleic acid or amino acid. If the transposon inserted into each genome randomly, 2-3% of the transformants were expected to be auxotrophs. From the first round of *E. hormaechei* transformants, 224 were patch plated and 5 were identified as auxotrophs (2.2%), indicating that the transposon randomly inserted into each genome.

Southern blot

The Southern blot indicated the transposon inserted each genome only once. The *EcoRI* overnight digestion of 10 transformants and wild-type *E. hormaechei* was incomplete (Fig. 3). The ideal result would have been a smear along the length of the gel rather than a larger sized band at the top, which may be due to a lower probe concentration than the protocol recommended and failure to denature the DNA prior to adding the probe. Nonetheless, the Southern blot itself appears to have one band per mutant (Fig. 4).

Melanization assay
A total of 7,008 transposon mutants were evaluated by melanization assay. From the initial assays, 183 mutants produced an atypical melanization phenotype in *C. neoformans*. An atypical melanization phenotype included a lack of melanization (hypomelanizer), an increase in melanization (hypermelanizer), or complete color change of the plate to an auburn color (abnormal) (Fig. 5). Note that each plate contained exactly 10 ml of SM + 0.25% L-tyrosine medium to control for the possibility that factors may diffuse through it and contribute to the degree of melanization observed in *C. neoformans* or *E. hormaechei*.

Secondary melanization assays were performed, and mutants with inconsistent phenotypes between initial and secondary assays were eliminated. The growth of the bacteria (e.g., confluent or streaky) and colony size were also noted. Mutants that produced small colonies or that grew streaky on the plate were not further investigated. Mutants of interest had confluent growth and normal colony size compared to wild-type *E. hormaechei* in addition to inducing an atypical phenotype in *C. neoformans*. Auxotrophs were eliminated and ultimately, 47 mutants fit these criteria: 38 hypomelanizers, 5 hypermelanizers, and 4 abnormal. Genomic libraries were created for these mutants and sent for sequencing by NextSeq. Six of the samples failed, most likely due to underrepresentation in the pool, which left 41 sequenced genomes: 33 hypomelanizers, 5 hypermelanizers, and 3 abnormal.

*Transposon insertion site identification*

In the majority of the mutants, the transposon inserted into a unique gene; however, some mutants had the same gene affected. In many hypomelanizers, it is possible that the knockout phenotype produced by transposon insertion could be due to the lack of a protein needed for normal growth. In such cases, the transformant would not be able to grow normally nor induce
wild-type melanization in *C. neoformans*. These mutants do not provide insight into the proteins required for melanization. In two abnormal mutants, the transposon interested into the gene that codes for 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15), also known as homoprotocatechu (HPC) dioxygenase. This enzyme catalyzes the ring-opening step in the catabolism of 4-hydroxyphenylacetate and is implicated in the Tyrosine Metabolism pathway on KEGG (Fig. 6). The conserved domain database shows this gene has homology with other ring-opening dioxygenase genes (Fig. 7A). A local alignment between the *E. hormaechei* gene hit by the transposon and a known sequence for an *hpcB* gene show high similarity and has an E-value of 0 (Fig. 7B). Moving forward, this enzyme was the sole focus of this thesis project due to the stark phenotype it induces in *C. neoformans* and its presence in tyrosine metabolism.

**Discussion**

In collaboration with the Casadevall lab, my lab studied the bacterium *E. hormaechei* strain PA, which was isolated from the gut of the mosquito species *Anopheles arabiensis*. The Casadevall lab observed that several bacteria from the mosquito gut trigger an immune response in *Cryptococcus* fungi. This response is demonstrated by the production of natural pigments known as melanins, which is easily observed by a color change in the yeast from white to light brown. Melanins confer several advantages for the fungal strains such as increased resistance to UV radiation, free radical oxidation, phagocytosis by macrophages, degradation by enzymes, antifungal drugs, and heavy metals (5, 6, 8).

Interactions between different microbial species are ubiquitous in biotic systems, many of which have been studied in previous literature and have provided insight into mutualistic, commensalistic, and parasitic relationships. For example, *Lactobacillus* species colonize the
vaginal microbiota in human females and prevent infections such as bacterial vaginosis when present at healthy levels; lichens are formed when fungi and cyanobacteria or algae unite to form a composite organism; *Pseudomonas aeruginosa* secrete phenazine molecules when grown in close contact with *Candida albicans*, which induce the fungus to produce red pigments and leads to decreased viability of the yeast (9, 10, 11).

My lab focused on *E. hormaechei* strain PA in conjunction with the yeast strain *Cryptococcus neoformans* serotype D strain B3501 and hypothesized that secretion of dopamine by the bacterium caused the melanization response in *C. neoformans*. In dopamine biosynthesis, dihydroxyphenylalanine (DOPA) is the direct precursor of dopamine, which is synthesized from the amino acid tyrosine. Key enzymes in this pathway include tyrosine hydroxylase and dihydroxyphenylalanine (DOPA) decarboxylase. However, because these enzymes have yet to be identified in *E. hormaechei*, a major goal of this project was to identify and characterize the genes responsible for dopamine production in this bacterium.

Using BLAST, enzymes known to be involved in tyrosine metabolism and dopamine biosynthesis in other bacterial species were not identified in the *E. hormaechei* PA genome, including enzymes such as tyrosine phenol lyase, tyrosinase, *p*-hydroxyphenylacetate 3-hydroxylase, tyrosine hydroxylase, and DOPA decarboxylase. Transposon mutagenesis was then pursued as a new approach. A Southern blot was performed to determine that the transposon only inserted once into each genome and the probe designed was complementary to the kanamycin resistance cassette in the transposon. As shown in Fig. 4, some of the bands are thicker than others, which could indicate the presence of more than one band; however, this was deemed unlikely. Southern blot analysis could have been repeated with modifications, but single insertion was later confirmed by genomic sequencing. If repeated, fresh enzyme should have been added.
after a few hours to spike the reaction and the DNA should have been resuspended in water rather than Tris-EDTA (TE) buffer. EDTA is commonly an enzyme inhibitor, which may have lowered the efficiency of the reaction.

Transposon insertion into the \( hpcB \) gene produced the abnormal melanization phenotype in \( C. \) neoformans, but the exact mechanism is unknown. This gene is part of an operon containing genes that code for other enzymes involved in tyrosine metabolism (Fig. 8). In two additional abnormal mutants, the transposon had inserted into different enzymes in this operon. One gene encoded 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60), which was directly upstream of the \( hpcB \) gene and the second gene encoded 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase (EC 4.1.1.68), which was one position further upstream. In phenylalanine metabolism, a component of tyrosine metabolism, HpcB and 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase catalyze successive and reversible steps (Fig. 5). When HpcB catalyzes the reaction that yields 2-hydroxy-5-carboxymethylmuconate semialdehyde, then 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase further converts it to 5-carboxymethyl-2-hydroxymuconate, which undergoes an isomerization before 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase acts.

Since the abnormal phenotype is characterized by the whole agar plate undergoing a color change to auburn, it is likely that \( E. \) hormaechei secretes homogentisic acid (HGA), also known as melanic acid and as a known intermediate in melanin production, which then diffuses through the medium. Melanin subtypes include eumelanins, pheomelanins, allomelanins, and pyomelanins. Eumelanins are produced from catecholamine precursors such as L-DOPA, pheomelanins are derived from tyrosine and cysteine metabolites (5), pyomelanins are formed
during tyrosine catabolism when HGA is oxidized (12), and allomelanins develop from nitrogen-free precursors (5). Melanins produced from catecholamines tend to be dark brown to black in color, while melanins produced from HGA tend to be reddish-brown (13). No biochemical tests were performed to confirm the melanin types produced by the mutants in melanization assays, though this could be an area of further investigation. By observing the color of melanization and taking previous literature into consideration, it was presumed that pyomelanins were produced by *C. neoformans* and led to the abnormal phenotype.

The disruption of HpcB function may cause HGA to be generated in excess because the absence of this enzyme may knock out a viable pathway in tyrosine metabolism, thereby causing more metabolites to be forced towards HGA production. To further ensure that HpcB is necessary for wild-type melanization in *C. neoformans*, complementation should be performed to restore the function of this enzyme in the mutant. Additionally, chemical analysis of cultures should be performed to detect the presence of dopamine or HGA, then compare these quantities to wild-type *E. hormaechei* yield. Mass spectrometry and high performance liquid chromatography (HPLC) are both feasible options to complete this. If dopamine is produced at lower levels in a mutant compared to wild-type *E. hormaechei*, it would be interesting to study the gene(s) affected by transposon insertion and its implications in dopamine production, perhaps by determining if upregulation of this gene causes overproduction of dopamine. The same line of investigation can be performed for HGA in the hopes of understanding which genes in *E. hormaechei* are most crucial for modulating melanization of *C. neoformans*.
Figures and Tables

Figure 1: Data and figure provided by the Casadevall lab, which shows a relationship between mosquito L-DOPA consumption and resistance to *P. falciparum* infection.

*Anopheles gambiae* resistance to *P. falciparum* infection is drastically increased with L-DOPA diet
Figure 2: A melanization assay plate and a 96-well plate with the corresponding well marked. Regions I and III of the assay plate were evenly streaked with *E. hormaechei* wild-type or transformant by using a sterile loop which was then used to inoculate the corresponding well of the microplate. Region II of the assay plate was streaked with a single line of *C. neoformans* using a sterile toothpick.
Figure 3: Agarose gel with EcoRI overnight digestion of 10 transformants and wild-type *E. hormaechei*. Lane 1: 1 kb ladder (NEB). Lane 2-11: *E. hormaechei* transformants. Lane 12: wild-type *E. hormaechei*.

Figure 4: Southern blot to detect transposon insertion. The probe designed was complementary to the kanamycin resistance gene in the transposon. Lane 1: 1 kb ladder (NEB). Lanes 2-11: *E. hormaechei* transformants. Lane 12: wild-type *E. hormaechei*. 
Figure 5: (A) Top left: wild-type *E. hormaechei* with *C. neoformans*. Bottom left: wild-type *E. hormaechei* with *C. neoformans Δlac*. Top right: *E. hormaechei* transformant (strain 2878) induced hypermelanization in *C. neoformans*. Bottom right: *E. hormaechei* transformant (strain 3025) induced the abnormal phenotype in *C. neoformans*. (B) Top left: wild-type *E. hormaechei* with *C. neoformans*. Bottom left: wild-type *E. hormaechei* with *C. neoformans Δlac*. Top right: *E. hormaechei* transformant (strain 2889) induced hypomelanization in *C. neoformans*. Bottom right: *E. hormaechei* transformant (strain 2906) induced hypomelanization in *C. neoformans*. 
Figure 6: KEGG pathway of tyrosine metabolism. 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15) is boxed and highlighted in red in the lower left region. Related enzymes 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60) and 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase (EC 4.1.1.68) are also present in this region.
Figure 7A: The conserved domains of the gene disrupted in strain 3025 shows high homology with the gene that encodes 3,4-dihydroxyphenylacetate 2,3-dioxygenase. Thus, it was concluded that the gene affected was, indeed, this enzyme.

Figure 7B: Sequence comparison between the gene disrupted by transposon insertion in strain 3025 and the gene known to encode 3,4-dihydroxyphenylacetate 2,3-dioxygenase.
Figure 8: Protein encoding region (PEG) 1374 in *E. hormaechei* from the RAST server. This operon includes the 3,4-dihydroxyphenylacetate 2,3-dioxygenase gene, which is labelled gene 1. Note that transposon insertion into gene 2 or 3 also produced the abnormal phenotype in *C. neoformans*.

Genes:

1) 3,4-dihydroxyphenylacetate 2,3-dioxygenase
2) 5-carboxymethyl-2-oxo-hex-3- ene-1,7-dioate decarboxylase (EC 4.1.1.68)
3) 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60)
4) 2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.--)
5) 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase (EC 4.1.2.52)
6) 5-carboxymethyl-2-hydroxymuconate delta-isomerase (EC 5.3.3.10)
7) Homoprotocatechuate degradative operon repressor
8) 4-hydroxyphenylacetate symporter, major facilitator superfamily (MFS)
9) Transcriptional activator of 4-hydroxyphenylacetate 3-monooxygenase operon, XylS/AraC family
10) 4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.14.9)
11) 4-hydroxyphenylacetate 3-monooxygenase, reductase component (EC 1.6.8.-)
12) 4-hydroxyphenylacetate 3-monooxygenase, reductase component (EC 1.6.8.-)
Table 1: Bacterial and fungal strains used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab strain number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. hormaechei</em> strain PA</td>
<td>2807</td>
</tr>
<tr>
<td><em>C. neoformans</em> serotype D strain B3501</td>
<td>2808</td>
</tr>
<tr>
<td><em>C. neoformans</em> Δlac</td>
<td>2809</td>
</tr>
<tr>
<td><em>E. hormaechei</em> Tn-hpcB</td>
<td>3025</td>
</tr>
</tbody>
</table>

Table 2: Primers used in this study to amplify the kanamycin resistance gene in the transposon, then generate a probe for Southern blot analysis.

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-tgagccatattcaacgggaac-3’</td>
<td>5’-ctcatcgagcatatgaaactg-3’</td>
</tr>
</tbody>
</table>


