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Linda Wu December 2, 2019

Effect of pyomelanin pigment production by *Burkholderia cenocepacia* on virulence and resistance to reactive nitrogen and oxygen species *in vitro* and *in vivo.*

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology

2020

Abstract

Effect of pyomelanin pigment production by *Burkholderia cenocepacia* on virulence and resistance to reactive nitrogen and oxygen species *in vitro* and *in vivo.*

By Linda Wu

Burkholderia cenocepacia is a Gram-negative bacilli that is known to infect patients with immunocompromising diseases, particularly those with cystic fibrosis (CF) and chronic granulomatous disease (CGD). Some strains of *B. cenocepacia* are known to naturally produce a pigment known as pyomelanin. Previous research on *B. cenocepacia* C5424 strain has shown a decrease in the resistance to oxidative stress when the pyomelanin pigment was absent. This project aims to investigate the role of pyomelanin presence on the resistance and virulence of the naturally pigmented strain J2315 and naturally non-pigmented strain K56-2.

To investigate the impact of pigmentation on the resistance of *B. cenocepacia* strains to oxidative stress, a non-pigmented isogenic strain was generated from the naturally pigmented strain J2315 utilizing an allelic exchange of a previously known gene in the pyomelanin production pathway. In the same manner, a pigmented isogenic strain was generated from the naturally non-pigmented strain K56-2. These isogenic mutant strains were developed prior to the start of this project by a former lab member of the Goldberg lab. These strains were subjected to oxidative stress with H_2O_2 and NO oxidants at various concentrations *in vitro*. The results reflected an inherent difference in the resistance of J2315 and K56-2 to oxidative stress. However, at particular concentrations of reactive oxygen and nitrogen species, the presence of the pigment demonstrated a slight protective advantage for the naturally non-pigmented K56-2 strain.

To determine the impact of pyomelanin on the virulence of *B. cenocepacia* strains *in vivo*, we performed an intratracheal infection in CGD mice models and observed the colonization levels and survival of the mice post-infection. We detected no significant differences between the colonization levels of the wild-type strains and their isogenic mutants, indicating that within the CGD mice model, the presence or absence of the pigment does not impact the virulence of the strain.

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Acknowledgements

I would like to thank all the members of the Goldberg lab for taking the time to help me through this project, especially my mentor Dr. Dina Moustafa, who was with me every step of the way, former lab member, Kathryn MacGillivray (Wendorf), who taught me the experimental foundations of everything I did in lab as well as introducing me to the *Burkholderia* bacteria and its impact on the human body, and my advisor Dr. Joanna Goldberg, who gave me this incredible opportunity and a wonderful laboratory environment to learn and mature my science.

I want to also acknowledge the other members of my committee, Dr. Jen Heemstra, Dr. William Kelly, Dr. Sarah Fankhauser, Dr. Dina Moustafa, and Dr. Joanna Goldberg for their time, patience, and support. Additional thanks to Dr. Eisen and Dr. Escobar, the Biology Honors Coordinators, for their support in the process of completing honors.

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Introduction

Burkholderia cenocepacia is a Gram-negative bacilli and is a member of a bacterial group collectively referred to as *Burkholderia cepacia* complex (Bcc), a group of at least 20 genetically and closely related bacterial species. Bcc is naturally found in the soil and has emerged as important opportunistic pathogens, causing morbidity and mortality in immunocompromised patients, particularly those with cystic fibrosis (CF) and chronic granulomatous disease (CGD) (1). CF is an autosomal recessive genetic disease caused by mutations in the CF transmembrane conductance regulator, resulting in complications in various organ systems; however, the major cause of morbidity and mortality is attributed to extensive lung damage due to chronic respiratory bacterial infections and inflammation (2). Bcc infections in cystic fibrosis (CF) patients can be associated with rapid decay of lung function which may further develop into a life-threatening systemic infection known as cepacia syndrome (4). During *B. cenocepacia* colonization, the airways of CF patients exhibit a pronounced inflammatory response that results in the release of reactive oxygen and reactive nitrogen species. CGD is primarily a hereditary, immunodeficiency disease caused by defects in any of the five subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex responsible for the respiratory burst in phagocytic leukocytes. Normal phagocytic cells, such as macrophages and neutrophils, have the ability to kill bacteria via oxidative burst. However, due to this defect in CGD patients, phagocytic cells are unable to produce reactive oxygen species (ROS), which results in the ineffective clearance of some pathogens. This renders CGD patients susceptible to recurrent bacterial and fungal infections, which are frequently accompanied by severe inflammation and

granuloma formation (3). Both CF and CGD patients, with immunocompromising conditions, become highly susceptible to Bcc infections.

Some Bcc isolates have developed defense mechanisms against the oxidative stress imposed by host organisms. Previous studies have demonstrated that some Bcc isolates have shown the ability to survive intracellularly within murine and human phagocytic cells, particularly within cultured macrophages as well as in human hosts. A more recent study using a resected lung from a CF-patient undergoing lung transplant demonstrated Bcc capabilities to survive intracellularly within human hosts (5). Inside the macrophage, Bcc resides within specialized vacuoles and avoids fusion with the lysosome. The mechanism Bcc uses to persist in the host organism has been demonstrated in zebrafish embryos: Bcc was able to survive in macrophages, despite being in a highly oxidative environment with high concentrations of hydrogen peroxide (H_2O_2) and nitric oxide (NO) (6). A previous study using *B. cenocepacia* strain P1 (Cardiff epidemic strain) demonstrated the strain was capable of attenuating the oxidative burst of human monocyte cell line MonoMac-6. This ability was attributed to the presence of dark brown pigment melanin-like pigment known as pyomelanin (7).

Melanin constitutes a general class of complex high molecular weight polyphenolic heteropolymers that include eumelanin, pheomelanin, and pyomelanin. The melanin is capable of serving as electron traps for reactive oxygen species due to its ability to exist in multiple oxidation states (8). Pyomelanin is a pigment that is commonly found in many systems of life, particularly bacteria and fungi. It is hypothesized that the pigment presence could promote the survival of these pigmented organisms in human hosts. One phenotype observed in a subset of *B. cenocepacia* isolates is their ability to

produce pyomelanin under normal laboratory conditions. In previous *in vitro* studies, pyomelanin has been demonstrated to contribute to the increased resistance of *B. cenocepacia* strain C5424 to extracellular hydrogen peroxide, which is the main type of reactive oxygen species found in macrophages (9). In the same study, a non-pigmented isogenic mutant of C5424 was found to exhibit a higher sensitivity to reactive oxygen species killing. Another study on the resistance of *Pseudomonas aeruginosa* strains PAO1 and PA14 to extracellular hydrogen peroxide found that the lack of pigment leads to decreased host killing in mouse lungs infected with P. aeruginosa strains (10). While potentially advantageous, the pyomelanin production phenotype is not found in every member of Bcc; in particular, it is present in *B. cenocepacia* J2315 and absent in *B. cenocepacia* K56-2, two wild-type CF clinical isolates. *B. cenocepacia* J2315 and K56-2 were isolated from CF patients.

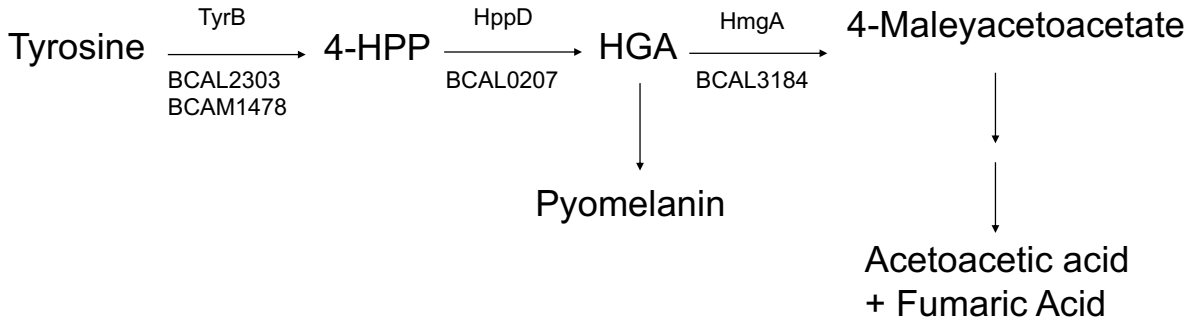


Figure 1. Pyomelanin synthesis pathway. Adopted from Gonyar et al (8).

Pyomelanin is produced through tyrosine catabolism, specifically due to the accumulation of homogentisic acid (HGA), which is synthesized via 4-hydroxyphenylpyruvate dioxygenase (4-HDDP) (Figure 1). HGA is secreted extracellularly and spontaneously auto-oxidizes to form benzoquinoneacetic acid, which

undergoes self-polymerization to produce pyomelanin (8). Two essential enzymes in the synthesis of pyomelanin includes HppD and HmgA. In the melanin production pathway, the *hppD* gene codes for a protein that is responsible for the conversion of 4-hydroxyphenylpyruvate to homogentisic acid. This protein produces the precursor molecule HGA that HmgA acts on to produce melanin (9). Homogentisate 1,2-dioxygenase (HmgA) converts HGA to maleylacetoacetate. Our lab has previously reported that a single amino acid change from glycine (non-pigmented phenotype) to arginine (pigmented phenotype) at residue 378 of HmgA contributes to the pigment production phenotype. When this amino acid residue change occurs, this renders the HmgA protein nonfunctional and the pathway stops at the intermediate molecule HGA, which ultimately results in pyomelanin production (8). While numerous studies had investigated the role of pyomelanin *in vitro*, particularly through manipulating *hppD*, crucially, there has been a gap in the literature about the role of *B. cenocepacia* melanin pigment *in vivo*. The objective of this study is to investigate whether pyomelanin contributes to the protection of pigmented strains against oxidative stress *in vitro* as well as *in vivo*. We investigated whether pyomelanin is associated with increased virulence *in vivo* using a CGD mouse model of lung infection and a wax worm (*Galleria mellonella*) model system. We infected mice with J2315 (pigmented) and K56-2 (non-pigmented) as well as their respective isogenic mutants J2315::K*hmgA* (non-pigmented) and K56-2::J*hmgA* (pigmented) and observed their relative survival over a period of time.

Materials and methods

Table 1. *Burkholderia cenocepacia* strains used in this project.

Strains	Phenotype	Source
J2315	Pigmented	CF Isolate
J2315::K*hmgA*	Non-Pigmented	Constructed by allelic exchanged *hmgA* from K56-2 (Constructed by Dr. Sarah Fankhauser)
K56-2	Non-Pigmented	CF Isolate
K56-2::J*hmgA*	Pigmented	Constructed by allelic exchanged *hmgA* from J2315 (Constructed by Dr. Sarah Fankhauser)

***In vitro* sensitivity to reactive oxygen and nitrogen species:** To monitor the survival of the mutant strains compared to the parent strains, J2315, K56-2, and their isogenic mutants were grown overnight at 37°C, shaking in Luria broth (LB) medium. Late stationary phase cultures were back diluted to an OD_{600} = 0.2 and were incubated with various concentrations of H_2O_2 (Sigma-Aldrich) or sodium nitrosprusside (SNP), a nitric oxide (NO) donor (10) (Chem-ImpexInt'l Inc), in 96-well plates at 37°C for 18 to 20 hours and read in 20 min increments using microplate reader (BioTek instruments, Inc). We used various concentrations of H_2O_2 and SNP ranging from 1 mM to 200 mM in a 96-well plate and measured the absorbance of the cultures over 20 hours at an optical

density of 600. The concentrations were achieved by serially diluting the reactive oxygen or nitrogen compounds from 200 mM down to 1 mM in a 96-well plate. The strains were incubated in duplicates and the growth curves were analyzed based on relative survival at each concentration of H_2O_2 and SNP.

***In vitro* survival of strains in various concentrations reactive oxygen and nitrogen species:** To observe the survival of the mutant strains compared to the parent strains under various concentrations of reactive oxygen and nitrogen species, they were treated with high concentrations of H_2O_2 and SNP and plated for survival at fixed endpoints. The cultures were grown the strains until late stationary phase and back diluted to an OD_{600} = 1.0. Strains were treated with 100 mM of either H_2O_2 or SNP. Aliquots were removed at 0, 30, 60, and 120 min post-treatment and serially diluted and were plated in duplicate on LB agar plates and incubated up to 48 hours at 37°C and counted for colony-forming units (CFU/mL) determination.

Mouse lung infection: All animal procedures were conducted according to the guidelines of the Emory University Institutional Animal Care and Use Committee (IACUC), under approved protocol number DAR-2003421-042219BN. The study was carried out in strict accordance with established guidelines and policies at Emory University School of Medicine, and recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, as well as local, state, and federal laws. gp91phox-/- (B6.129S-Cybbtm1Din/J). Mice were purchased from the Jackson laboratory and are bred and maintained with the Emory Division of Animal Resources. Bacterial strains were inoculated on LB agar plates from frozen glycerol stocks and was incubated for 48 hours at 37°C. 24 hours before infection, 3 mL cultures

in LB were started from these plates. The day of infection, cultures were back diluted to OD_{600} 0.01 in 50 mL LB. Cultures were incubated with shaking at 37°C for 4-5 hours until OD_{600} of 0.08 was reached. Cultures were spun down and washed twice with PBS and resuspended in PBS to OD_{600} 0.08. Cultures were diluted in phosphate buffer saline (PBS) to obtain a concentration of $1x10^7$ CFU/mouse in a 50 µL volume. For infections, 8-10-week-old gender matched mice were infected via intratracheal instillation as previously described (13). Mice were euthanized at indicated time points, and whole lungs were collected aseptically and homogenized in 1 mL phosphate buffered saline. Lung homogenates were serially diluted and plated on appropriate culture medium for colony-formation units (CFU) determination.

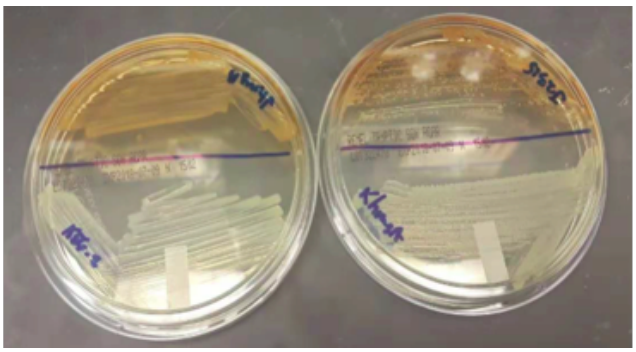
Results and Discussion

Single amino acid change in HmgA is sufficient for pyomelanin production in *B. cenocepacia*

Our group had previously reported that glycine at residue 378 in HmgA encoding homogentisate 1,2-dioxygenase is present in mostly all pyomelanin-producing Bcc isolates (8). In order to determine whether this amino acid substitution in HmgA was sufficient for pyomelanin production in *B. cenocepacia, a* previous lab member (Dr. Sarah Fankhauser) used allelic exchange to construct a J2315 strain containing the *hmgA* from K56-2 and the K56-2 strain containing the *hmgA* from J2315. This gave us two pairs of isogenic strains (J2315, J2315::K*hmgA* and K56-2, K56-2::J*hmgA*). After 48 hours of growth on solid LB media J2315::K*hmgA* did not produce visible pigment, as compared to J2315; while K56-2::J*hmgA* produced pigment as compared to K56-2 (Figure 2).

K56-2::J*hmgA*

K56-2



J2315

J2315::K*hmgA*

Figure 2. **Phenotypes of wild-type (J2315 and K56-2) and isogenic mutant strains (J2315::K*hmgA* and K56-2::J*hmgA*).** All four strains streaked on Luria agar plates (LA) and incubated at 37°C for 48 hours. The allelic exchange produced isogenic mutants that was either pigmented (K56-2::J*hmgA*) or non-pigmented (J2315::K*hmgA*) from their respective pigmented (J2315) or non-pigment (K56-2) wild-type parental strains.

Role of pyomelanin in protection against specific immune defense molecules *in vitro*

To determine whether pyomelanin production would alter resistance characteristics to specific immune response molecules such as hydrogen peroxide and nitric oxide, we incubated the mutants (J2315::K*hmgA* and K56-2::J*hmgA*) and wild-type (J2315 and K56-2) with various concentrations of H_2O_2. The J2315::K*hmgA* strain did not demonstrate any difference in growth compared to the wild-type J2315 strain under any of these conditions, suggesting that the lack of pigment in J2315::K*hmgA* did not impact its resistance to H_2O_2 at the experimental concentrations (Figure 3A). However, K56-

2::J*hmgA* demonstrated a significantly higher growth compared to K56-2 at a concentration of 12.5 mM H_2O_2, as this concentration completely abolished the ability of K56-2 strain to grow. This suggests that at a concentration of 12.5 mM H_2O_2, the pigment may contribute to an increased resistance of K56-2::J*hmgA* to H_2O_2 treatment (Figure 3B).

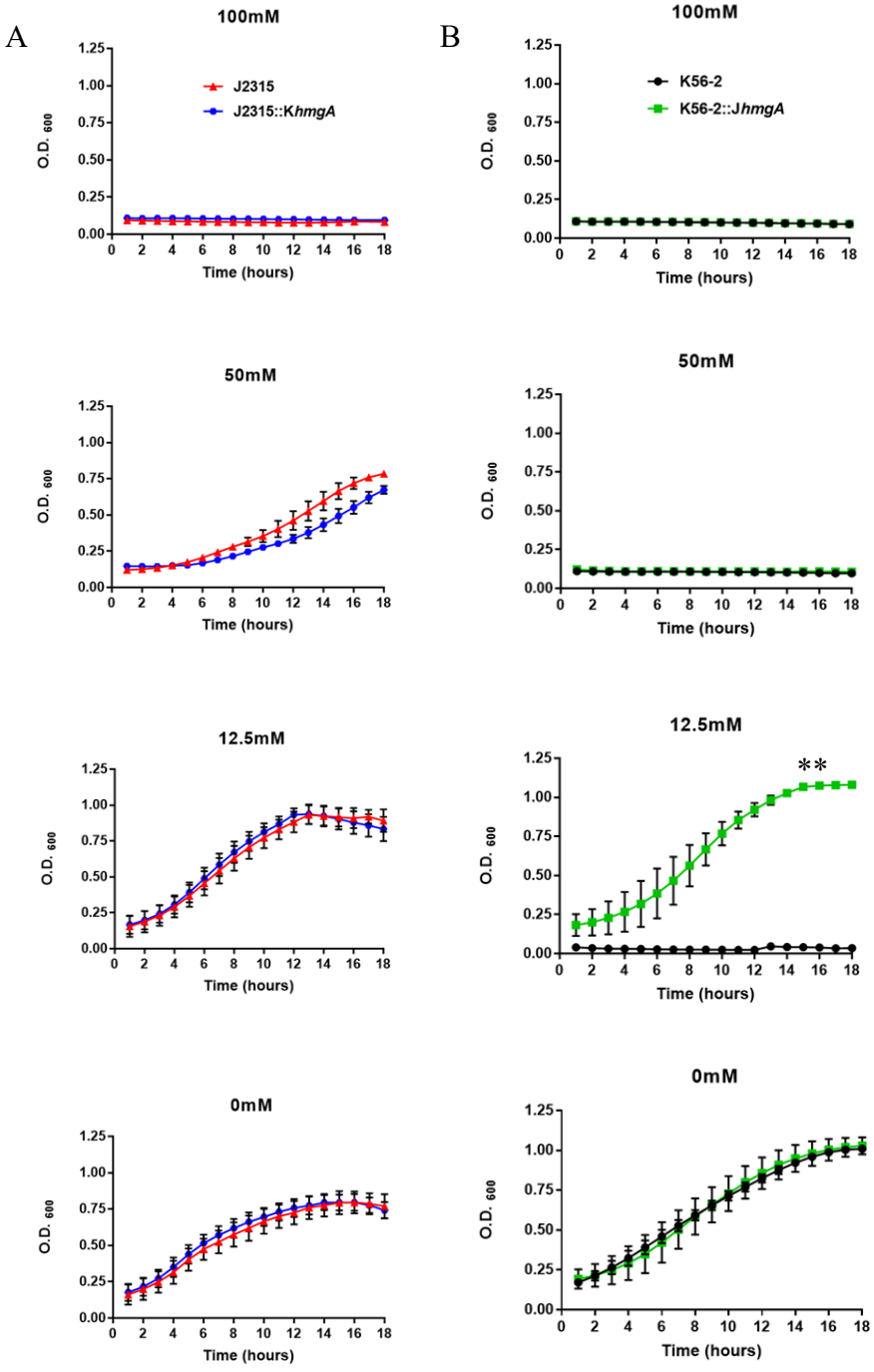

A
B
100mM
J2315
J2315::KhmgA
K56-2
K56-2::JhmgA
50mM
12.5mM
**
0mM
O.D. 600
Time (hours)

Figure 3. Growth curves of J2315::K*hmgA*, J2315, K56-2::J*hmgA*, and K56-2 treated with various concentrations of H_2O_2 over an 18 hours incubation period. The data shows results of 4 replicates. The asterisk indicates significant difference between the two strains. Data were analyzed by student's *t* test. ** *P<0.005*. Data are pooled from two independent experiments. Error bars represent standard errors of the mean.

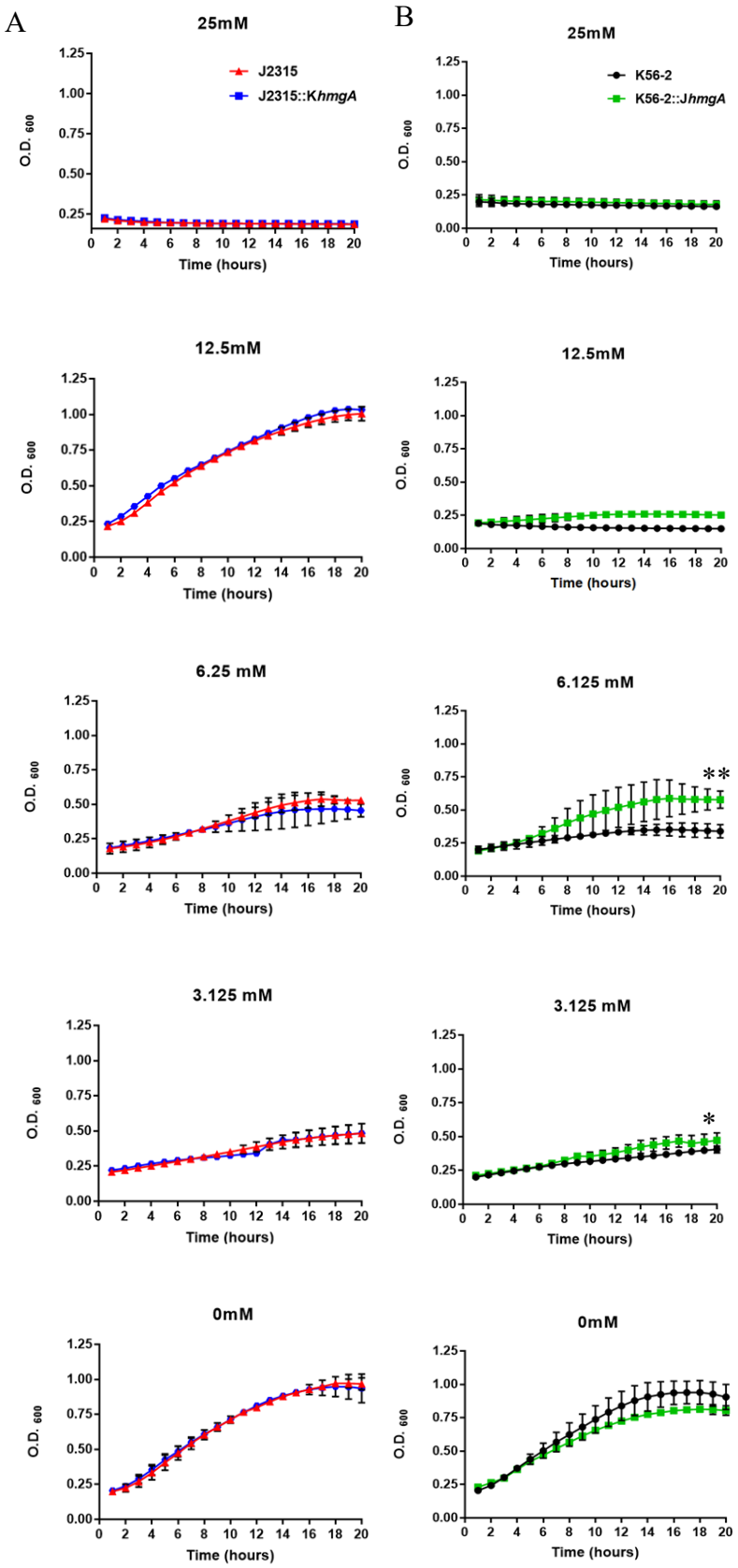

A
B
25mM
J2315
J2315::KhmgA
K56-2
K56-2::JhmgA
12.5mM
6.25 mM
6.125 mM
3.125 mM
0mM
O.D. 600
Time (hours)
**
*

Figure 4. Growth curves of J2315::K*hmgA*, J2315, K56-2::J*hmgA*, and K56-2 treated with various concentrations of SNP over a 20 hours growth period. Data were analyzed by student's *t* test. *$P<0.05$, ** $P<0.005$. Data are pooled from two independent experiments. Error bars represent standard errors of the mean.

In order to test pyomelanin's role in resistance to reactive nitrogen species, we exposed all four strains to SNP and measured their growth over a 20 hrs exposure period. Similar to the H_2O_2 treatment, both J2315 and J2315::K*hmgA* demonstrated similar growth at all the experimental concentrations of SNP (Figure 4A). This suggests that both the pigmented and non-pigmented phenotypes of J2315 were equally resistant to the NO generated by SNP. However, K56-2::J*hmgA* demonstrated higher growth compared to K56-2 at concentrations of SNP lower than 6.25 mM (Figure 4B). This indicates a potential protective role of the pigment at this range of SNP concentration in this strain. It is also notable that at similar concentrations, both J2315 and K56-2 showed similar survival in treatment with SNP, which was different from the much higher resistance to H_2O_2 observed in J2315 and J2315::K*hmgA*. This suggests that the defense mechanism of the two strains against reactive oxygen species may be different from the that of against reactive nitrogen species.

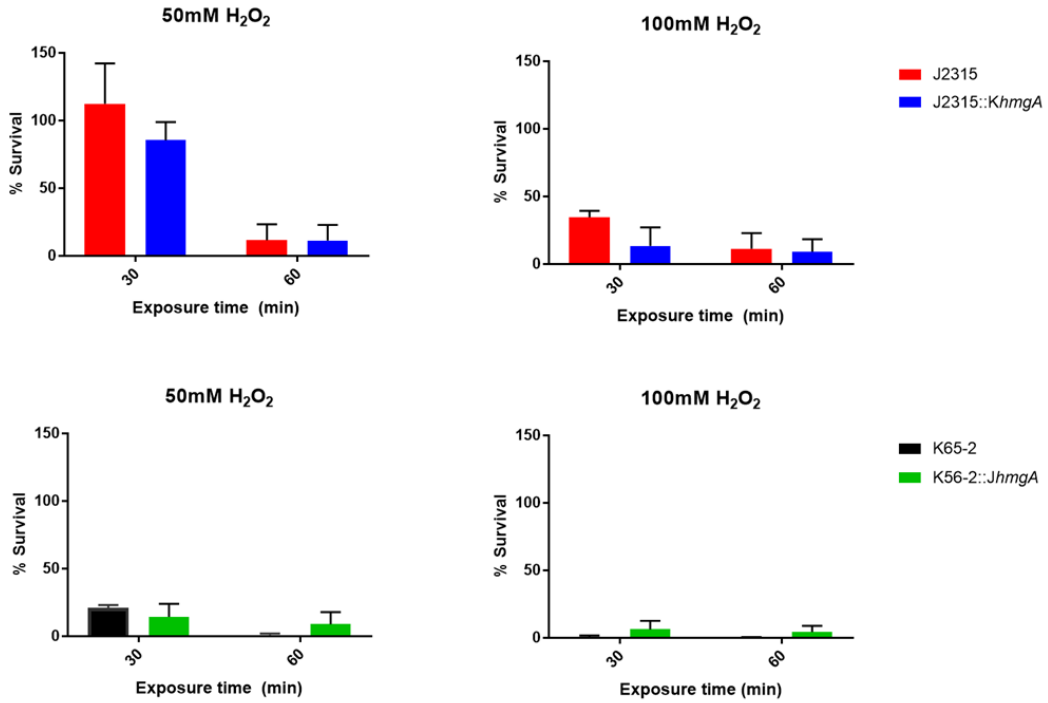


Figure 5. Survival of wild-type strains J2315, J2315::K*hmgA*, K56-2, K56-2::J*hmgA* and treated with 50 mM and100 mM H_2O_2 monitored over 1 hr. The percent survival was calculated by dividing the CFU/mL at fixed timepoints (30 min or 60 min) by the input dosage. Statistics analysis show that none of the mutants are statistically different from the wild-type parents in susceptibility against H_2O_2.

Based on data observed in Figure 2 and 3, we observed a relative impact of pigment in overcoming the oxidative stress after prolonged exposure to oxidative species. However, the short-term (2 hrs or less) effect of pyomelanin in the survival of the bacteria was still unknown. To determine the impact of pyomelanin on the survival of the strains after short-term exposure to reactive oxygen and nitrogen species, we treated the strains with high doses of oxidative species and plated for survival at fixed

timepoints post-exposure. When the strains were treated with 100 mM H_2O_2, we observe that compared to K56-2, the pigmented strain K56-2::J*hmgA* demonstrate higher survival, potentially providing an advantage for the bacteria in survival against the high concentration of oxidative stress (Figure 5). The pigment is hypothesized to be acting as an electron trap, quenching the degradation reaction of $H_2O_2 \rightarrow H_2O + O_2$. Since the pigment is secreted extracellularly, it could be potentially catalyzing this process for the cells in the beginning stages of exposure to H_2O_2, thus providing a defense for the pigmented strains. We hypothesize that towards the later stages of exposure, the bacterial enzymes become saturated with the high concentration of reactive oxygen species. This weakens their defense mechanism and the cell becomes vulnerable to oxidative stress damage by the residual H_2O_2 molecules.

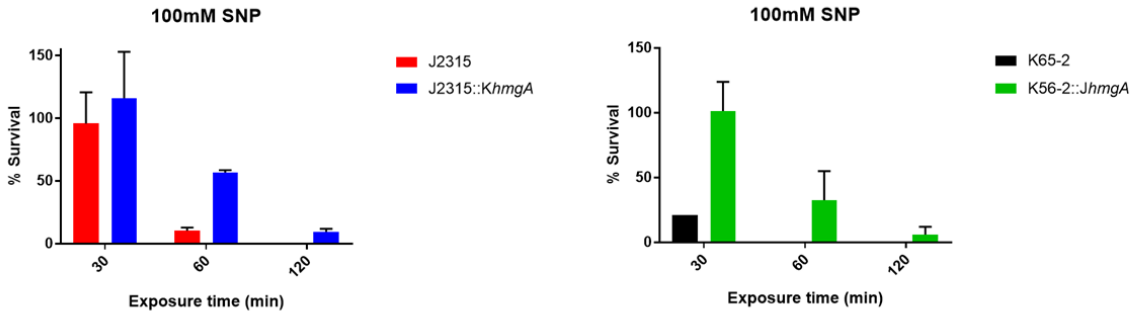


Figure 6. Survival of wild-type strains J2315, J2315::K*hmgA*, K56-2, K56-2::J*hmgA* and treated with 100 mM SNP monitored over 2 hrs. Data are pooled from two independent experiments. Error bars represent standard errors of the mean.

When treated with 100 mM SNP for 2 hrs, K56-2::J*hmgA* show a higher survival than K56-2, however J2315::K*hmgA* demonstrates a higher survival compared to J2315. This experiment supports a slight advantage of the pigment *in vitro*, however the higher survival of J2315::K*hmgA* suggests an alternative mechanism for survival may be playing a larger role for bacterial defense against reactive nitrogen species (Figure 6).

Based on the results, we observed that while J2315 and J2315::K*hmgA* did not show any difference in resistance against reactive oxygen or nitrogen species, K56-2::J*hmgA* offered a protective advantage for the bacteria against specific concentrations of oxidative stress. We hypothesize that J2315::K*hmgA* may be showing higher survival than J2315 due to a different defense mechanism utilized by J2315 and J2315::K*hmgA* against reactive nitrogen species. The tyrosine catabolism pathway for the production of pyomelanin involves an amino acid disruption in the HmgA protein, rendering the protein nonfunctional and ultimately unable to produce the acetoacetic acid and fumaric acid (8). However, J2315::K*hmgA*, is an isogenic mutant of J2315, contains all of the virulence factors that J2315 processes to overcome any oxidative stress as well as the complete catabolic pathway to produce acetoacetic acid and fumaric acid. This may provide a selective advantage for J2315::K*hmgA* in terms of survival against the nitric oxide oxidative stress. In this case, the pyomelanin would not be likely to be contributing to this survival advantage.

Previous studies have examined the transcriptional impact of reactive oxygen treatment to J2315, and they reported an increased expression of genes related to oxidative

stress response via an oxidation of transcription regulator OxyR within 30 min of H_2O_2 treatment (15). The J2315 genome specifically encodes the *katB* genes that breakdown hydrogen peroxide by catalase absent in K56-2 (16,17). This difference in the inherent virulence factors against oxidative stress may be contributing to the higher resistance of J2315 compared to K56-2 found in the growth and fixed-endpoint experiments.

Role of pyomelanin in murine chronic granulomatous disease (CGD) lung infection mode.

In our lab, previous studies with wild-type J2315 and K56-2 strains in CGD mice models showed an inherent difference in response to respiratory infections using these two WT strains as mice infected with J2315 succumbed to infection by 96 hours post infection, while those infected with a similar, sublethal dose of K56-2 strain succumbed to infection at day-10 post infection.

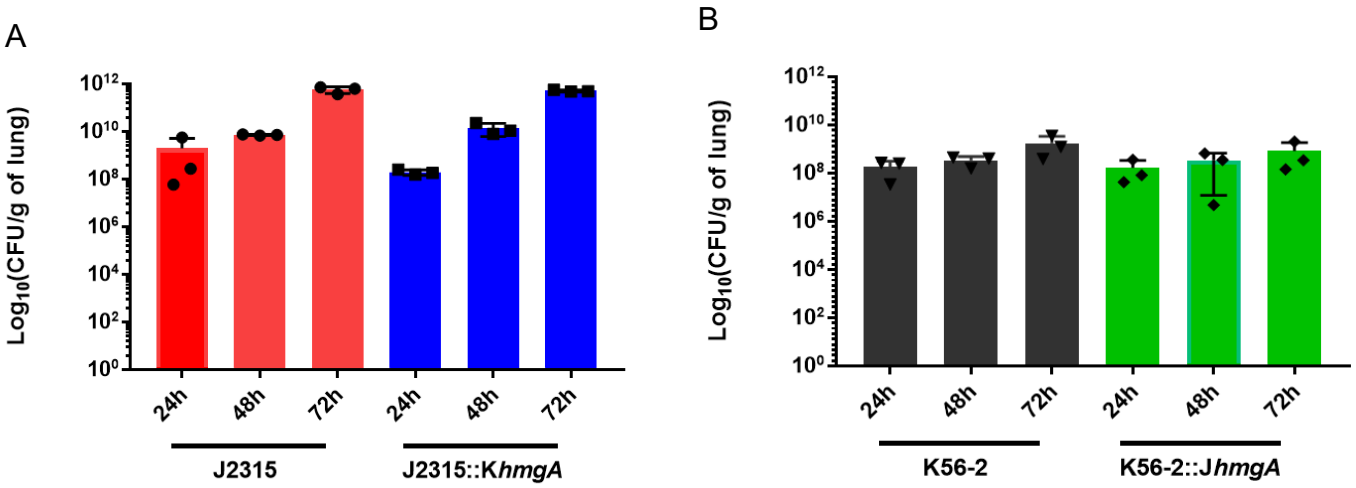


Figure 7. Bacterial load in lung homogenates of CGD mice infected intratracheally with WT *J2315*, and K56-2 and isogenic mutants J2315::K*hmgA*, and K56-2::J*hmgA*. Mice were infected with approximately 10^7 CFU/mouse. They were

euthanized at approximately 24h, 48h, and 72h post-infection. Lung tissues were collected aseptically and homogenized in 1mL of PBS. All samples were plated for viable CFU. Each symbol represents a single mouse. The data is representative of two independent experiments. N=3/strain/time point.

In order to determine whether pyomelanin plays a role during infection into a host, we performed a non-invasive intratracheal infection with 10^7 CFU/mL dosage in CGD mice lungs. After the infection, we harvested lung tissue at fixed time-points post-infection, and we observed no significant difference between the colonization level of J2315 and J2315::K*hmgA* (Figure 7A), as well as between K56-2 and K56-2::J*hmgA* (Figure 7B).

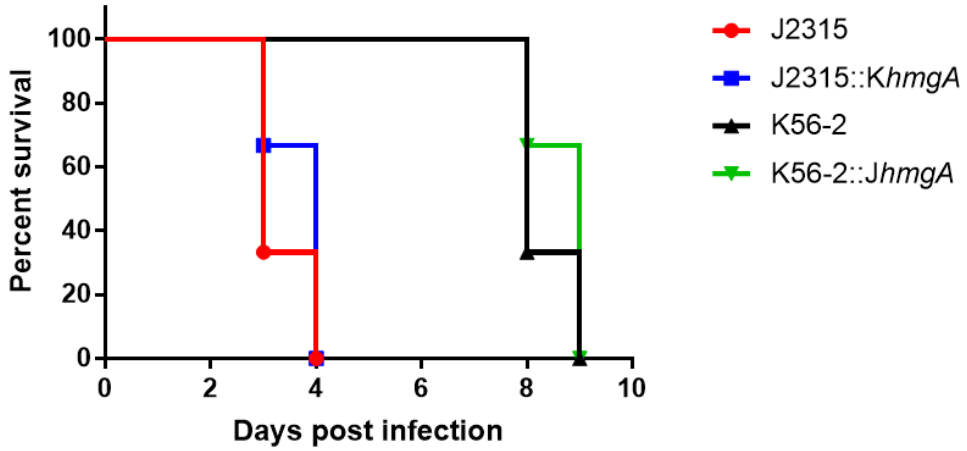


Figure 8. **Survival rates of CGD mice after intratracheal infection with wild-type J2315, K56-2, and isogenic mutants J2315::K*hmgA*, and K56-2::J*hmgA***. Mice were monitored for survival for a period of 9 days post-infection. Input dosage was approximately 10^7 CFU/mouse. Results are represented in Kaplan-Meier survival curves and were analyzed by log-rank test. Log-rank: K56-2 vs. K56-2::J*hmgA* (P = 0.0107);

J2315 vs. J2315::K*hmgA* (P = 0.45). Median survival: J2315, 3 days; J2315::K*hmgA*, 4days; K56-2, 8 days; and K56-2::J*hmgA,* 9 days. Results are representative of two independent experiments. N= 3 mice/group/experiment.

A parallel group of infected mice were monitored for survival. Our results indicate that the absence of the pigment in J2315::K*hmgA* didn't seem to compromise the virulence of that strain. Mice infected with both J2315 and its non-pigmented mutant J2315::K*hmgA* succumbed to infection by day 4 post-infection (Figure 8). Similarly, mice infected with K56-2 or its pigmented mutant K56-2::J*hmgA* showed similar time to death as all mice succumbed to infection day 9 post-infection. The wild-type strains and their isogenic mutants showed no significant difference in the killing of CGD mice upon infection. These results seem to suggest a very minor, if any, contribution of the pigment to the resistance of these strains to mice immune defense in our infection model (Figure 8).

In the CGD mice model, we did not see a difference between the pigmented and non-pigmented strains of J2315 and K56-2. While this model is deficient in producing ROS through NADPH, it is still capable of producing reactive nitrogen species such as NO. In another model with mice defective with the ability to produce NO ($iNOS^{-/-}$), we may be able to determine the potential role of the pyomelanin pigment in the absence of reactive nitrogen species and presence of reactive oxygen species (18). In fungus, melanin has been shown to protect the organism against nitrogen-derived oxidants, suggesting that the pigment may be contributing a slight protective role to the bacteria at certain levels of NO oxidative stress (19). However, although the potential protective

role of the pigment against NO was observed *in vitro*, the CGD mice model did not demonstrate the protective role of the pigment against NO oxidative stress. In this model, we did not observe an impact of the pigment on the colonization and virulence of J2315 and K56-2 strains in CGD mouse lungs.

While the *in vitro* results indicate that there may be some protective advantage offered by the pyomelanin to H_2O_2 exposure and that the pigment may allow for overcoming the oxidative stress at certain concentrations, the *in vivo* data seems contradict those observations. Within the CGD lung infection mouse models, these differences are not detected and the change in pigment phenotype did not play a noticeable role in the virulence of the *B. cenocepacia* J2315 or K56-2 within the host. While the presence of pyomelanin in K56-2::JhmgA provided a protected effect against oxidative stress, the presence of the pigment had no impact in the J2315 background. These observations suggest that the potential protective role of the pigment may be a strain-specific phenomenon. In addition, our experiments show that the pigment does not play a major role in the virulence and colonization of *B. cenocepacia* J2315 and K56-2 in CGD mice hosts. While the presence of pigmented pathogens from clinical and environmental samples may be concerning, our data suggests that pigment alone may not always constitute a virulence factor. However, considerations remain about whether or not the pigment is even produced *in vivo*. It hasn't been demonstrated that pyomelanin is produced when the *B. cenocepacia* strain J2315 infect the lung tissue of host organisms. If the pigment is not produced *in vivo*, it would not be able to perform any protective role for the bacteria even if it could, thus the role of pyomelanin could be dispensable in our experimental *in vivo* model. Overall, although our results did not

indicate whether pyomelanin contributed to the virulence of either strains, the pigment may still play a role in other survival mechanism for the cell apart from defense against oxidative stress.

Future Directions

In the future, we will investigate the potential role of pyomelanin in the virulence and survival of *B. cenocepacia* J2315 and K56-2 strains pigment against different macrophage cell lines. Macrophages are the main immune cells within human hosts where NADPH oxidase participates in oxidative stress killing through H_2O_2 production. This model will further elucidate how the pigment may or may not play a role in physiological concentrations of H_2O_2 or NO. Additionally, other infection model can be used, such as *Galleria melonella* (wax worm), which is another infection model to investigate the role of pyomelanin pigment *in vivo*.

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