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Single amino acid substitution in homogentisate 1,2-dioxygenase is responsible for pigmentation in a subset of *Burkholderia cepacia* complex isolates

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Summary

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacilli that are ubiquitous in the environment and have emerged over the past 30 years as opportunistic pathogens in immunocompromised populations, specifically individuals with cystic fibrosis (CF) and chronic granulomatous disease. This complex of at least 18 distinct species is phenotypically and genetically diverse. One phenotype observed in a subset of *Burkholderia cenocepacia* (a prominent Bcc pathogen) isolates is the ability to produce a melanin-like pigment. Melanins have antioxidant properties and have been shown to act as virulence factors allowing pathogens to resist killing by the host immune system. The melanin-like pigment expressed by *B. cenocepacia* is produced through tyrosine catabolism, specifically through the autoxidation and polymerization of homogentisate. *Burkholderia cenocepacia* J2315 is a CF clinical isolate that displays a pigmented phenotype when grown under normal laboratory conditions. We examined the amino acid sequences of critical enzymes in the melanin synthesis pathway in pigmented and non-pigmented Bcc isolates, and found that an amino acid substitution of glycine for arginine at amino acid 378 in homogentisate 1,2-dioxygenase correlated with pigment production; we identify this as one mechanism for expression of pigment in Bcc isolates.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacilli that are found ubiquitously in the environment and have emerged over the past 30 years as opportunistic pathogens in immunocompromised populations, specifically cystic fibrosis (CF) and chronic granulomatous disease (CGD). This complex of at least 18 distinct species is the leading cause of bacterial infections in CGD patients, who are characterized by increased susceptibility to bacterial and fungal infections caused by genetic defects in phagocyte Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Phox) (Winkelstein *et al.*, 2000). Bcc is a significant cause of morbidity and mortality in both CGD and CF (Winkelstein *et al.*, 2000; Courtney *et al.*, 2004; Holland, 2010) and reviewed in

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Mahenthiralingam and colleagues (2005). During the course of an infection, Bcc must combat the innate immune system in order to persist. Because of the prevalence of Bcc as CGD pathogens and the fact that CGD patients have defects in oxidative killing mechanisms, it is hypothesized that this mechanism of killing by host phagocytes is essential for Bcc clearance in healthy individuals (Zelazny *et al.*, 2009; Greenberg *et al.*, 2010) and reviewed in Porter and Goldberg (2011). Superoxide dismutases and catalases are enzymes commonly used to protect bacteria from oxidative damage during normal respiration and in interactions with the host innate immune response, but pathogens can also utilize additional factors that assist in protection from host oxidative killing mechanisms.

Melanins are produced by all kingdoms of life and are a normal component of human skin and hair, but they can also be exploited by pathogens to promote their own survival in human hosts. Melanins are characterized as structurally diverse, high molecular weight polymers that are composed of quinolines, which can exist in three different oxidation states (White, 1958; Nosanchuk and Casadevall, 1997; Nosanchuk *et al.*, 1999; Jacobson, 2000). Melanins act as a trap for unpaired electrons and this activity contributes to the antioxidant role in the presence of reactive oxygen species (Sichel *et al.*, 1991). Different types of melanins are characterized by the pathways from which they are derived. Eumelanins are synthesized from 3,4-dihydroxyphenylalanine (DOPA) by phenoloxidases. Yellow or reddish melanins incorporate cysteine with DOPA and are called pheomelanins. Melanins derived from homogentisic acid (HGA) by tyrosinases are called pyomelanin (Yabuuchi and Ohyama, 1972). Melanins formed from acetate via the polyketide synthesis pathway are called dihydroxynaphthalene melanins (Nosanchuk and Casadevall, 2006).

Melanins confer a survival advantage in the environment as well as the host. Melanins have been shown to increase microbe survival through protection from oxidative stress, digestive enzymes (in amoeboid and nematode predators, as well as phagocytes), radiation, extreme temperature and heavy metal toxicity (Nosanchuk and Casadevall, 2006). *Cryptococcus neoformans*, *Aspergillus* sp., *Wangiella dermatitidis*, *Sporothrix schenckii* and *Burkholderia cepacia* are all pathogens that produce melanin, but the role of melanin in defence against the host is best characterized in *C. neoformans* and *Aspergillus* species (Liu and Nizet, 2009).

The melanin-like pigment produced by *B. cenocepacia* C5424 was characterized in a study by Keith and colleagues, and it was shown *in vitro* that absence of the pigment led to an increase in sensitivity to hydrogen peroxide and extracellular superoxide (Keith *et al.*, 2007). This study also showed that the pigment-deficient mutant colocalized to a higher extent than the wild-type strain with more degradative compartments in macrophages and that this increase in colocalization was dependent on Phox activity (Keith *et al.*, 2007). This suggested that pigment-deficient bacteria were less protected from oxidative killing in the phagosome, and the inability to delay progression to a more degradative compartment was a potential reason for the sensitivity. The study by Keith and colleagues also began to describe the distribution of this pigmented phenotype among Bcc strains. They reported that out of the 22 *B. cenocepacia* isolates in their collection, only four were pigmented after 72 h of culture on L-agar. They attempted to complement the non-pigmented *B. cenocepacia* strain K56-2 with the 4-hydroxyphenylpyruvate dioxygenase (*hppD*) gene from the pigmented *B.*

cenoepectacia C5424 strain; however, this did not confer a pigmented phenotype to *B. cenoepectacia* K56-2 (Keith *et al.*, 2007). These data demonstrate that the pigment is important for *B. cenoepectacia* survival in interactions with host immune cells, but it is still not understood what mechanisms allow for pigmentation in a subset of Bcc isolates.

We have identified homogentisate 1,2-dioxygenase (HmgA) as a potentially critical enzyme for regulation of pigment production. We have observed a correlation between a glycine to arginine change at residue 378 of HmgA and pigment production in members of the Bcc. This study tests the hypothesis that one mechanism for pigmentation in Bcc isolates is defective HmgA activity due to a G378R amino acid substitution.

Results and discussion

Burkholderia cenoepectacia J2315 produces a melanin-like pigment

Because of the established role of the pigment produced by Bcc as a potential virulence factor, we began to characterize the expression of this pigment by *B. cenoepectacia* J2315. *Burkholderia cenoepectacia* J2315 is the sequenced type strain and a member of the ET-12 lineage. *Burkholderia cenoepectacia* J2315 is a CF clinical isolate that displays a pigmented phenotype when grown under normal laboratory conditions; pigment becomes visually detectable at stationary phase.

Burkholderia cenoepectacia J2315 encodes homologous genes for all enzymes in the pyomelanin synthesis pathway (Fig. 1A) but does not encode any genes required for eumelanin or pheomelanin synthesis (Kanehisa and Goto, 2000) (data not shown). Pyomelanins are synthesized through tyrosine catabolism with HGA as an intermediate. Tyrosine is converted to 4-hydroxyphenylpyruvate (4HPP) by the aromatic aminotransferase TyrB. There are two homologues to TyrB encoded in the genome of *B. cenoepectacia* J2315: BCAL2303 and BCAM1478. 4HPP is then modified by HppD (BCAL0207) to form HGA. HGA is then released from the cell, oxidized and polymerized to form pyomelanin. HmgA facilitates diversion of HGA into an alternative pathway, resulting in the production of acetoacetic acid and fumaric acid.

Previous work by Keith and colleagues defined the pigment produced by *B. cenoepectacia* strain C5424 as a pyomelanin synthesized by this pathway (Keith *et al.*, 2007), and we predicted that the pigment produced by J2315 would be synthesized via the same mechanism. As expected with a pigment synthesized through tyrosine catabolism, growth in tyrosine (Luria broth supplemented with 1 mg ml⁻¹ L-tyrosine) leads to increased pigment production (Fig. 1B). Additionally, we created a deletion mutant in *hppD* in *B. cenoepectacia* J2315 and this mutant, J2315 *hppD*, exhibited a non-pigmented phenotype (Fig. 1C) that corroborates what was shown by Keith and colleagues (2007) in *B. cenoepectacia* C5424. Since HppD is required for pyomelanin production and not involved with other melanin synthesis pathways, we concluded that *B. cenoepectacia* J2315 is producing a pigment through the pyomelanin synthesis pathway, which had not previously been established.

Identification of amino acid substitution in HmgA

Several studies using other pathogenic bacteria have associated defects in HmgA function with a pigmented or hyperpigmented phenotype (Rodriguez-Rojas *et al.*, 2009; Schmalzer-Ripcke *et al.*, 2009; Valeru *et al.*, 2009; Wang *et al.*, 2013). Since HmgA catalyses an enzymatic step that shuttles HGA into another pathway where it is further degraded to acetoacetic acid and fumaric acid, a decrease in HmgA activity would lead to accumulation of HGA and to an increase in pigment production. After aligning the inferred amino acid sequence of HmgA from publicly available Bcc sequences (NCBI, 8 September 2014), we observed a glycine to arginine amino acid change at residue 378 that only occurred in pigmented Bcc strains (Fig. 2). The glycine at this residue was conserved among Bcc isolates and in other bacterial genera (Fig. 2). To further investigate this correlation, we characterized the pigmentation phenotype and sequenced HmgA in two additional Bcc isolates that did not have public sequence data available (*B. cenocepacia* C3865 and C5424). The *hmgA* genes from *B. cenocepacia* C5424 and C3865 were amplified from genomic DNA using *hmgA* seq Fd (GCGCGATCCACCTGTATG) and *hmgA* seq Rv (GTTGCTCCGATTGAAGTGT). All non-pigmented strains encoded a glycine at residue 378 (Table 1). Out of the four pigmented strains in our collection, three encoded an arginine at residue 378 (Table 1). *Burkholderia cenocepacia* C3865 is pigmented and encodes a glycine at residue 378. We observed differences in this strain in both the timing of pigment production and the hue of the pigment, and we hypothesize that this strain may produce pigment through an alternative, not yet defined mechanism.

The human enzyme (termed HGO) has a crystal structure available (Titus *et al.*, 2000) and is 52% identical at the protein level to HmgA encoded in *B. cenocepacia* J2315. HGO assembles as a dimer of trimers, and the active site iron ion is coordinated between subunits in the trimer. Two histidines and one glutamic acid are involved in iron binding, and they correspond to H341, E347 and H377 in the *B. cenocepacia* protein (Fig. 2). The G378R change occurs in this region, and this substitution could potentially disrupt iron binding and therefore enzyme function. Additionally, mutations in this region have previously been shown to inactivate HGO in the human disease alkaptonuria (Rodriguez *et al.*, 2000).

Expression of *hmgA* from a non-pigmented strain results in a non-pigmented phenotype in *B. cenocepacia* J2315

Burkholderia cenocepacia J2315 and *B. cenocepacia* K56-2 both belong to the ET-12 lineage and are closely related. The *hmgA* gene in *B. cenocepacia* J2315 and K56-2 is identical except for a one base pair change (G to C) that results in the glycine (GGA) to arginine (CGA) change at residue 378 in the HmgA protein in J2315. To begin to test whether this amino acid change was responsible for the observed pigmented phenotype, we amplified the *hmgA* genes from *B. cenocepacia* J2315 (a pigmented strain that encodes an arginine at residue 378 of HmgA) and *B. cenocepacia* K56-2 (a non-pigmented strain that encodes a glycine at residue 378 of HmgA), referred to as *JhmgA* and *KhmgA* respectively. The amplified *hmgA* gene from both of these strains was then cloned into the pUCP18Tc vector and transferred to *B. cenocepacia* J2315 and K56-2. *Burkholderia cenocepacia* J2315 complemented with both its own gene (pUCP18TC-*JhmgA*) or the empty vector (pUCP18Tc) maintained a pigmented phenotype as measured by absorbance of the

supernatant at OD₄₈₀ after 24–30 h of growth (Fig. 3A). On the other hand, complementation of *B. cenocepacia* J2315 with *KhmgA* (pUCP18TC-*KhmgA*) resulted in a non-pigmented phenotype (Fig. 3A). *Burkholderia cenocepacia* K56-2 remained non-pigmented when containing any of the plasmids, but a statistically significant increase in the OD₄₈₀ of the supernatant was observed when expressing *JhmgA* (pUCP18TC-*JhmgA*) in comparison to *KhmgA* (Fig. 3A). This minor increase in OD₄₈₀ in *B. cenocepacia* K56-2 suggests that there may be competition for substrate between the native KHmgA and the cloned JHmgA enzymes when both are expressed. In these experiments, we did not observe any significant differences in colony-forming unit per millilitre at the 24 h time point between wild-type and complemented strains (data not shown). Additionally, we expressed both *JhmgA* and *KhmgA* in another pigmented Bcc isolate, *B. cenocepacia* BC7, which also has arginine at position 378 in HmgA. *Burkholderia cenocepacia* BC7 expressing *JhmgA* retained a pigmented phenotype, but consistent with what we observed with *B. cenocepacia* J2315, expression of *KhmgA* resulted in a loss of pigmentation (data not shown). These results support the hypothesis that the G378R amino acid change in HmgA encoded by *B. cenocepacia* J2315 and BC7 impairs enzyme activity, leading to the accumulation of visibly detectable pigment, and that an otherwise identical enzyme derived from *B. cenocepacia* K56-2 lacking this amino acid change is functional and eliminates visually detectable pigment production.

Complementation of *Pseudomonas aeruginosa* PA14 *hmgA*::Tn with KHmgA but not JHmgA results in restoration of a wild-type non-pigmented phenotype

We transferred the same plasmids expressing either versions of *hmgA* to a *Pseudomonas aeruginosa* PA14 insertional mutant in *hmgA*, PA14 *hmgA*::Tn. Because of this insertion in *hmgA*, *P. aeruginosa* PA14 *hmgA*::Tn displays a hyperpigmented phenotype. Since this mutant has been previously characterized and has known defects in HmgA (Rodriguez-Rojas *et al.*, 2009), we utilized it to test the functionality of JHmgA and KHmgA enzymes. Similar to what we had observed in *B. cenocepacia* J2315, KHmgA was able to compensate for the *hmgA* mutation. PA14 *hmgA*::Tn (pUCP18Tc-*KhmgA*) had a non-pigmented phenotype on agar plates and supernatants were non-pigmented after growth in Luria–Bertani (LB) for 30–35 h in LB (Fig. 3B). On the other hand, expression of *JhmgA* was unable to complement this phenotype (Fig. 3B). These data suggest that the KHmgA enzyme is functional and leads to an absence of pigment due to conversion of excess HGA to acetoacetic acid and fumaric acid.

Expression of *KhmgA* in *B. cenocepacia* J2315 does not alter susceptibility to H₂O₂

In other systems, it has been shown that pyomelanin production protects against H₂O₂ (Keith *et al.*, 2007; Rodriguez-Rojas *et al.*, 2009). We hypothesized that *B. cenocepacia* J2315 pUCP18Tc-*KhmgA* (non-pigmented) would be more sensitive to H₂O₂ than the isogenic pigmented strains (J2315 containing pUCP18Tc or pUCP18Tc-*JhmgA*). We tested *B. cenocepacia* J2315 expressing *KhmgA* or *JhmgA* for sensitivity to H₂O₂ using two different assays. Using a standard disc assay to test sensitivity of stationary phase cultures grown on agar plates, we observed no difference in the diameter of the zones of inhibition with 50, 100, 400 or 800 mM H₂O₂ (data not shown). Using an assay that was previously performed with *P. aeruginosa* PA14 and PA14 *hmgA*::Tn (Rodriguez-Rojas *et al.*, 2009), we

then tested whether sensitivity to H₂O₂ during exponential phase growth by measuring percent survival after incubation with H₂O₂. We confirmed that PA14 *hmgA*::Tn was more resistant to 25 mM H₂O₂ than PA14 (Fig. 3D). However, *B. cenocepacia* J2315 was much more sensitive to H₂O₂; even at 5 mM H₂O₂, we noted less than 10% survival of J2315 (Fig. 3C). Under these conditions, the expression of *KhmgA* in *B. cenocepacia* J2315 did not increase susceptibility to H₂O₂.

Conclusions

We found that a G378R amino acid substitution in HmgA correlated with pigment production. Complementation of the pigmented phenotype of *B. cenocepacia* J2315 with *hmgA* from K56-2 resulted in a non-pigmented phenotype. These results suggest that the G378R version of HmgA is not functional and we propose that this is one mechanism that allows for pigmentation in some members of the Bcc. Melanins are protective against both oxidative and non-oxidative stresses, which could contribute to increased fitness of Bcc isolates both during infection and in the environment. While we did not observe differences in susceptibility to H₂O₂, this does not exclude the possibility that pyomelanin production in J2315 could be providing protection against other oxidative species. Future exploration into the correlation of HmgA functionality and fitness in different niches could provide important insight into the mechanisms for Bcc survival in the presence of both host and environmental stresses.

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Fig. 1. The pigment produced by *B. cenocepacia* J2315 is a pyomelanin and is synthesized through tyrosine catabolism

A. Genes homologous to enzymes involved in the pyomelanin synthesis pathway were identified in the *B. cenocepacia* J2315 genome using sequence homology and KEGG pathway annotations. 4-HPP, 4-hydroxyphenylpyruvate; HGA, homogentisic acid.

B. Growth of *B. cenocepacia* J2315 in LB with 1 mg ml⁻¹ tyrosine resulted in increased pigment production in comparison to growth in LB without tyrosine as determined by the OD₄₈₀ of the culture supernatant at 24 h.

C. An unmarked deletion mutant in the gene that encodes the last enzyme in the pathway to make the pigment, *hppD* (BCAL0207), was constructed in *B. cenocepacia* J2315 and this strain was non-pigmented. The system for making unmarked deletions through gene splicing by overlap extension described by Flannagan and colleagues (2008) was used to create *B. cenocepacia* J2315 *hppD*. The deletion construct was made by amplifying the flanking regions of the gene using the following four primers: F1-XbaI (GGTCTAGAAATCGGCAACGCCGTCGTTTCCTTGAAGC), R1 (TGCCGCGCGGTGCAAGCGGTCGTGTCTCCTGTGCGG), F2 (CCGCACAGGAGACACGACCGCTTGCACCGCGCGGCA) and R2 EcoRV (GGGATATCTTCGCCGGTTTTACGGGATGGTAGCACTGG).

Fig. 2. A glycine at residue 378 of HmgA is conserved among Bcc strains and is changed to an arginine in *B. cenocepacia* J2315

A. Diagram of the HmgA gene of Bcc showing iron-binding sites. The region marked in brackets corresponds to residues 363–400, which are aligned below.

B. Sequences of inferred amino acid sequence of the *hmgA* gene from Bcc strains from residues 364–400 were aligned to show conservation of a glycine at residue 378. Sequence identity of the entire proteins ranged between 94% and 99%.

Fig. 3. Expression of *hmgA* from a non-pigmented strain results in a non-pigmented phenotype in *B. cenocepacia*

A. The *hmgA* genes from *B. cenocepacia* J2315 and K56-2 were amplified from genomic DNA using *hmgA* Fd SacI

(CTAGGAGCTCATGACGCTTGACCTGTGCGAAACCGGCAA) and *hmgA* Rv XbaI (CTAGAGATCTTCATCGTTGCTCCGGATTGA). The PCR products were cloned into TOPO pCR2.1 (Invitrogen) and then digested with SacI and XbaI. Digested inserts were ligated into similarly digested pUCP18Tc (Schweizer, 1991) and sequenced to confirm identity. pUCP18Tc and recombinant *hmgA*-containing plasmids were electroporated into *B. cenocepacia* J2315 and K56-2 using a modified version of a protocol published previously (Dubarry *et al.*, 2010). The resultant plasmid-containing strains were grown in LB with 100 $\mu\text{g ml}^{-1}$ tetracycline at 37°C with shaking for 24–30 h and then evaluated the level of pigmentation by assaying the OD₄₈₀ of the supernatant.

B. Recombinant plasmids containing the *hmgA* genes from J2315 (pUCP18Tc-JhmGA), K56-2 (pUPC18Tc-KhmGA) or the vector (pUCP18Tc) were transferred to *P. aeruginosa* PA14 *hmgA*::Tn. For images of pigmented phenotypes, overnight cultures of bacteria were diluted and spotted onto agar plates, which were incubated at 37°C and visualized after 24–48 hours. Liquid cultures were incubated at 37°C in LB with 50 $\mu\text{g ml}^{-1}$ tetracycline for plasmid-containing strains, and without for non-plasmid containing strains with shaking for 30–35 h and then evaluated for the level of pigmentation by assaying the OD₄₈₀ of the supernatant.

C. Susceptibility to H₂O₂ of J2315 strains expressing pUCP18Tc, pUCP18Tc-KhmGA, pUCP18Tc-JhmGA was measured after exponential phase cultures were incubated for 45 min with 5 mM H₂O₂ and then grown out for 48 h on LB. Susceptibility was measured by

calculating colony-forming units (cfu) after culture sample was incubated with 5 mM H₂O₂ divided by cfu of culture sample incubated in LB.

D. Susceptibility to H₂O₂ of PA14 WT or PA14 *hmgA::Tn* was measured after exponential phase cultures were incubated for 45 min with 25 mM H₂O₂ and then grown out for 24 h on LB. Susceptibility was measured by calculating cfu after culture sample was incubated with 25 mM H₂O₂ divided by cfu of culture sample incubated in LB.

Table 1

Correlation of sequence identify at residue 378 of HmgA and pigment production in Bcc strains.

Strain	Amino acid at residue 378	Produces pigment under normal laboratory conditions	Type of isolate	Lineage	Reference/Assembly ID
<i>Burkholderia cenocepacia</i>					
J2315	R	Yes	CF clinical isolate	ET12	(Holden <i>et al.</i> , 2009)/ #GCF_000009485.1
BC7	R	Yes	CF clinical isolate	ET12	(Varga <i>et al.</i> , 2013)/ #GCF_000333135.2
C5424	R	Yes	CF clinical isolate	ET12	This study
K56-2	G	No	CF clinical isolate	ET12	(Varga <i>et al.</i> , 2013)/ #GCF_000333155.2
H111	G	No	CF clinical isolate	Not assigned	(Carlier <i>et al.</i> , 2014) #GCF_000236215.1
MC0-3	G	No	Environmental isolate	III 3B	#GCF_000019505.1
AU 1054	G	No	CF clinical isolate	PHDC	#GCF_000014085.1
C3865	G	Yes	CF clinical isolate	ET12	This study
<i>Burkholderia multivorans</i>					
ATCC 17616	G	No	Environmental isolate		#GCF_000018505.1
CGD2	G	No	CGD clinical isolate		(Varga <i>et al.</i> , 2012)/ #GCF_000182275.1
CGDI	G	No	CGD clinical isolate		(Varga <i>et al.</i> , 2012)/ #GCF_000182255.1