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Elucidation of the *Burkholderia cenocepacia* hopanoid biosynthesis pathway uncovers
functions for conserved proteins in hopanoid-producing bacteria

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Running title: Hopanoid Biosynthesis in *B. cenocepacia* K56-2

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Summary

Hopanoids are bacterial surrogates of eukaryotic membrane sterols and among earth's most abundant natural products. Their molecular fossils remain in sediments spanning more than a billion years. However, hopanoid metabolism and function are not fully understood. *Burkholderia* species are environmental opportunistic pathogens that produce hopanoids and also occupy diverse ecological niches. We investigated hopanoids biosynthesis in *B. cenocepacia* by deletion mutagenesis and structural characterization of the hopanoids produced by the mutants. The enzymes encoded by *hpnH* and *hpnG* were essential for production of all C₃₅ extended hopanoids, including bacteriohopanetetrol (BHT), BHT glucosamine and BHT cyclitol ether. Deletion of *hpnI* resulted in BHT production, while $\Delta hpnJ$ produced only BHT glucosamine. Thus, HpnI is required for BHT glucosamine production while HpnJ is responsible for its conversion to the cyclitol ether. The $\Delta hpnH$ and $\Delta hpnG$ mutants could not grow under any stress condition tested, whereas $\Delta hpnI$, $\Delta hpnJ$, and $\Delta hpnK$ displayed wild type growth rates when exposed to detergent, but varying levels of sensitivity to low pH and polymyxin B. This study not only elucidates the biosynthetic pathway of hopanoids in *B. cenocepacia*, but also uncovers a biosynthetic role for the conserved proteins HpnI, HpnJ, and HpnK in other hopanoid-producing bacteria.

Keywords: hopanoids/*Burkholderia cenocepacia*/membrane permeability

Introduction

Hopanoids are bacterial membrane lipids ubiquitous in modern day environments dominated by microbes and readily preserved in ancient sedimentary rock (Ourisson and Albrecht, 1992), making them ideal tools for reconstructing and understanding microbial ecosystems (Brocks et al., 2004; Summons and Lincoln, 2012). Due to size and structural similarities, hopanoids are considered bacterial surrogates of eukaryotic sterols, possibly regulating the fluidity and permeability of the bacterial membrane (Ourisson et al., 1987; Kannenberg, 1999). Only recently have studies focused on determining the biological function of hopanoids using genetic manipulation and *in vivo* experimentation. Such studies confirm that hopanoids are involved in enhancing the stability and impermeability of the bacterial membrane, conferring resistance to multiple stress conditions including pH, temperature, and exposure to detergents and antibiotics (Doughty et al., 2009; Welander et al., 2009; Schmerk et al., 2011; Malott et al., 2012). However, it remains unclear what functional importance can be ascribed to particular variations in the hopanoid core and side chain structures.

Hopanoids in lower eukaryotes and bacteria are derived from the direct cyclization of squalene (Rohmer et al., 1984; Ochs et al., 1992), which results in a pentacyclic triterpenoid core commonly modified by unsaturation, methylation and/or the addition of diverse C₅ ribose-derived side chains (Flesch and Rohmer, 1988; Rohmer, 1993). To date, few studies have successfully investigated the genes or proteins involved in modifying the hopanoid core. HpnP and HpnR have been identified as the radical S-adenosylmethionine (SAM) enzymes responsible for production of 2- and 3-methylhopanoids, respectively (Welander et al., 2010; Welander and Summons, 2012). The production of C-3 methylated hopanoids is important for late stationary survival and maintenance of intracytoplasmic membranes in *Methylococcus capsulatus* (Welander and Summons, 2012), while the production of C-2 methylated hopanoids is important for stress tolerance (Welander and Summons, 2012; Kulkarni et al., 2013). Hopanoids with C₃₅ extended side chains are common among hopanoid producers, but little is known about their biosynthesis or their function(s). The B-12 binding radical SAM protein HpnH and the nucleoside phosphorylase HpnG catalyze the first and second steps in hopanoid side chain biosynthesis, respectively (Bradley et al., 2010; Welander et al., 2012b). In *Rhodopseudomonas palustris*, the deletion of *hpnH* resulted in a strain only capable of producing C₃₀

hopanoids, and with compromised integrity of the outer membrane (Welandar et al., 2012b). These discoveries have provided much needed insight into the initiation of C₃₅ hopanoid production; however, the additional enzymes involved in side chain biosynthesis and the specific role that different functionalized hopanoids play in membrane stability and stress tolerance remain to be elucidated.

Burkholderia cenocepacia is one of the 17 species of genetically related bacteria known as the *B. cenocepacia* complex (Bcc). These bacteria are widely spread in aquatic and soil environments where they play beneficial roles including the promotion of plant growth and the degradation of pollutants (Coenye and Vandamme, 2003). Bcc bacteria are also opportunistic pathogens, causing infections in cystic fibrosis patients and other immune-compromised individuals (Vandamme et al., 1997; Speert, 2002). Given their particular environmental niche, it is not surprising that *Burkholderia* species are intrinsically resistant to most clinically relevant antibiotics and antimicrobial peptides (Waters and Ratjen, 2006; Loutet and Valvano, 2011). Resistance to antimicrobials depends, in part, on the production of hopanoids (Schmerk et al., 2011). The deletion of both squalene-hopene cyclase (*Shc*) encoding genes in *B. cenocepacia* K56-2 resulted in a strain (Δshc) that could not produce hopanoids and displayed increased sensitivity to low pH, detergent, and various antibiotics. The mutant was also unable to produce flagella, resulting in severely diminished motility (Schmerk et al., 2011). Several *Burkholderia* species produce various C₃₅ extended hopanoids, including bacteriohopanetetrol (BHT), BHT glucosamine, and BHT cyclitol ether; however, the functional importance of these individual hopanoids has not been explored (Cvejic et al., 2000; Talbot et al., 2007b).

This study identifies the types of extended hopanoids produced by *B. cenocepacia* and utilizes genetic tools to generate a collection of mutants in predicted hopanoid biosynthetic genes. Environmental stress tests performed with the mutants lead to new suggestions about the biological roles of C₃₅ extended hopanoids. Our results are the first to define the biosynthetic pathway of hopanoids in *Burkholderia* and to define a biosynthetic role for the proteins HpnI, HpnJ and HpnK in any hopanoid producing bacterium.

Results and Discussion

B. cenocepacia produces a mixture of hopanoids

The biosynthesis of hopanoids requires the cyclization of squalene, a reaction catalyzed by squalene-hopene cyclase (Ochs et al., 1992), followed by the functionalization of the basic hopanoid structure through the addition of various side chains and additional modifications. Previously (Schmerk et al., 2011), we reported the identification and characterization of two paralogs of the squalene-hopene cyclase gene (*shc*) in *B. cenocepacia*, which are located on chromosomes 2 (BCAM2831) and 3 (BCAS1067), herein designated as *hpnF2* and *hpnF3*, respectively (Fig. 2). GC analysis of lipid extracts obtained from the deletion mutant lacking both *hpnF* genes (Δshc) demonstrated that this strain did not produce hopanoids but at the same time accumulated an unidentified compound, which was also produced by the *hpnF2* single deletion mutant (Schmerk et al., 2011). This compound was subsequently identified as squalene (Figure S1). We next examined lipid extracts from stationary phase liquid cultures to determine the species of functionalized hopanoids produced by *B. cenocepacia*. These experiments revealed that *B. cenocepacia* produces various hopanoid structures including the C₃₀ hydrocarbon diploptene and three functionalized bacteriohopanepolyols: bacteriohopanetetrol (BHT), BHT cyclitol ether, and BHT glucosamine (Figure 1a and Figure S2). *B. cenocepacia* also produced an unsaturated hopanoid. The mass spectral data was insufficient to distinguish if it is an unsaturated BHT cyclitol ether or BHT glucosamine, and whether the unsaturation is at C-6 or C-11 positions of the ring system (Figure 1a). However, our findings agree with previous studies reporting that a strain of *B. cepacia* produces these same saturated hopanoid species and also a C-6 unsaturated BHT cyclitol ether (Cvejic et al., 2000; Talbot et al., 2007a).

Characterization of hopanoid biosynthesis genes in *B. cenocepacia*

The hopanoid biosynthesis associated genes of *B. cenocepacia* are located in one cluster on chromosome 1 and two clusters on chromosome 2 (Figure 1b). In addition, the second squalene-hopene cyclase gene (*hpnF3*) is present as a single gene on chromosome 3, as discussed above. Homologs of *ispH/lytB* (BCAM2738 and BCAL2710), *hpnE* (BCAM2832) and *hpnC* (BCAM2833) are required for squalene production (Perzl et al., 1998; Wolff et al., 2003). *ispH*_{BCAL2710} and *ispH*_{BCAM2738} were deleted in a previous study (Loutet et al., 2011), while *hpnC* and *hpnE* could both

be essential for bacterial viability, as their homologs could not be deleted in *R. palustris* (Welander et al., 2012b). In the present study, we focus on the genes required for the synthesis of the hopanoid chain (Table 1).

The *hpnA* homolog (BCAM2735) could not be deleted; repeated attempts to delete this gene resulted in exconjugants reverting to the wild type background. To confirm whether this gene was essential for bacterial viability, we created a conditional mutant by placing *hpnA* under the control of a rhamnose-inducible promoter (Ortega et al., 2007). The conditional *P_{rhaB}::hpnA* mutant could grow in the presence of rhamnose (permissive condition) and glucose (non-permissive condition), indicating that *hpnA* is not essential for *B. cenocepacia* (data not shown). The reasons why this gene could not be deleted remain unknown. A similar case was encountered when attempting to delete *hpnH* (BCAM2739). To successfully remove *hpnH* from the chromosome the deletion had to be constructed in a Δshc background strain (Schmerk et al., 2011). After *hpnH* was successfully deleted, hopanoid production was restored by introducing pBCAM2831, which encodes the HpnF2 squalene-hopene cyclase.

Chemical analyses of the hopanoids produced by the deletion mutants were performed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), and demonstrated that five of the ten genes play roles in the production of extended hopanoids (Table 2). The other five genes did not demonstrate a clear role in hopanoid biosynthesis. It is possible that these genes are involved in the regulation of hopanoid production or their transport to specific locations within the bacterial cell envelope, both of which were not evaluated in this study.

HpnI and HpnK are required for bacteriohopanetetrol glucosamine production

The B-12 binding radical SAM protein HpnH is responsible for the primary step in producing extended hopanoids (Bradley et al., 2010; Welander et al., 2012b). The C₅ unit of the extended hopanoid side chain is derived from a ribosugar, whose addition is catalyzed by HpnH (Figure 2) (Bradley et al., 2010). The deletion of *hpnH* (BCAM2739) in *B. cenocepacia* resulted in a similar effect to that observed in *M. extorquens* and *R. palustris*, with only the C₃₀ hopanoid diploptene being produced (Figure S3). Deletion of *hpnG* also prevented the production of any functionalized

hopanoids and LC-MS analysis demonstrated the sole accumulation of adenosyl hopane (Figure S4, Figure 2, step 2), an intermediate also found accumulated in *R. palustris* and *M. extorquens hpnG* mutants (Bradley et al., 2010; Welander et al., 2012b). With the adenine cleaved off the hopanoid compound by the nucleosidase HpnG (Figure 2, step 3), the resulting intermediate would be free to form bacteriohopanetetrol (BHT; Figure 2, step 4). In earlier studies, the final BHT intermediate has been suggested to be ribosyl hopane or derivatives such as ribonyl hopane and formyl hopane, and also phosphoribohopane rapidly cleaved to form BHT (Duvold and Rohmer, 1999; Seemann et al., 1999; Bradley et al., 2010; Welander et al., 2012b). However, the hopanoid biosynthesis associated gene clusters of *B. cenocepacia* do not encode any enzymes that could catalyze the formation of BHT from such intermediates, despite the common belief that one more enzyme is required for its production. Because monosaccharides such as ribose and glucose naturally exist in both cyclic and open chain forms, it is conceivable that the ribose opens up without the aid of an enzyme (Bradley et al., 2010). Therefore, we propose that the conversion of the ribose hopanoid to BHT occurs nonenzymatically.

LC-MS results revealed that the next enzyme for the formation of extended hopanoids is the glycosyltransferase HpnI (Figure 2, step 5, and Figure 3b). Labelled sugar incorporation experiments previously performed in *Zymomonas mobilis* demonstrated that the cyclopentane group added to the extended hopanoid side chain is derived from *N*-acetyl-D-glucosamine (GlcNAc) (Vincent et al., 2003). Bradley *et al.* have proposed that HpnI catalyzes the addition of GlcNAc to the hopanoid side chain (Bradley et al., 2010). Deletion of *hpnI* (BCAL1050) resulted in a mutant that only produced BHT (Figure 3b). However, it is still uncertain whether this enzyme utilizes the ribosylhopane intermediate or BHT as its substrate.

A deacetylation step (Figure 2, step 6), prior to ring contraction, is predicted to follow the glycosylation of the hopanoid side chain (Vincent et al., 2003). HpnK (BCAL1052) contains a YdjC protein motif which is thought to be involved in cellulose metabolism (Lai and Ingram, 1993). In *Citrobacter rodentium* the *hpnK* homolog is annotated as a chitobiose-phosphate hydrolase (*chbG*). A study of *chbG* in *E. coli* demonstrates that this gene encodes a monodeacetylase that acts on chitooligosaccharide substrates similar in structure to acetylglucosamine (Verma and Mahadevan,

2012). BLASTp analysis of *E. coli* ChbG against the *Burkholderia cenocepacia* J2315 protein database identifies HpnK as the polypeptide providing the most significant alignment (E-value $4e^{-15}$). Deletion of *hpnK* in *B. cenocepacia* resulted in production of BHT glucosamine but not BHT cyclitol ether (Figure 3c), indicating deacetylation is required for the subsequent production of BHT cyclitol ether. Because all lipid samples have to be acetylated as part of the LC-MS analysis protocol we were not able to directly detect the deacetylated BHT glucosamine.

HpnJ is essential for the production of bacteriohopanetetrol cyclitol ether

Deletion of *hpnJ* (BCAL1051) resulted in the loss of BHT cyclitol ether production while the production of the other extended hopanoids was maintained (Figure 3d). A ring contraction may be necessary to produce BHT cyclitol ether from BHT glucosamine (Figure 2, step 7), but the exact mechanism of the ring contraction reaction remains unknown (Vincent et al., 2003; Pan and Vincent, 2008). Like HpnH, HpnJ is annotated as a radical SAM protein. This family of enzymes catalyzes a wide range of reactions including RNA modifications and the synthesis of cofactors and antibiotics (Sofia et al., 2001), and utilize an enzyme-bound [4Fe-4S] cluster (Frey et al., 2008; Duschene et al., 2009; Shisler and Broderick, 2012). The iron-sulfur cluster is active in its reduced state and from this state it can transfer an electron to the sulfonium of SAM. This electron transfer promotes the homolytic cleavage of SAM, producing methionine and a 5'-deoxyadenosyl (5'-dAdo) radical intermediate (Nicolet et al., 2009). This highly reactive radical can abstract a hydrogen atom from its substrate, often from unreactive positions (Hioe and Zipse, 2012). Of the thousands of predicted radical SAM enzymes only a small number have been biochemically characterized (Sofia et al., 2001; Frey et al., 2008). However, a recent study provided mechanistic details for ring contraction by QueE, the radical SAM enzyme of *Bacillus subtilis* (McCarty et al., 2013). QueE utilizes SAM to abstract a hydrogen atom from 6-carboxy-5,6,7,8-tetrahydropterin (CPH₄) and perform a ring contraction similar to that observed in the conversion of BHT glucosamine to BHT cyclitol ether (McCarty et al., 2013). Therefore, it is possible that the ring contraction that converts the BHT glucosamine to the cyclitol ether also occurs through a radical SAM mechanism.

Phylogenetic analysis of HpnI and HpnJ homologs

The production of BHT glucosamine has been documented in several species, including *Z. mobilis*, *Geobacter sulfurreducens*, *G. metallireducens* and *B. cepacia* (Flesch and Rohmer, 1989; Talbot et al., 2007b; Eickhoff et al., 2013). Since the enzyme responsible for the synthesis of this hopanoid has been identified, bioinformatics analysis can be used to predict which bacteria could produce BHT glucosamine. An unrooted maximum likelihood tree was created using the top 250 sequences retrieved via a protein BLAST search of the *B. cenocepacia* HpnI sequence against the KEGG and NCBI databases (Figure 4). All bacteria contained within this tree encode at least one copy of the squalene-hopene cyclase HpnF, indicating that all of these strains have the capacity to produce hopanoids. The ability to produce BHT glucosamine appears to be widespread among hopanoid producers, with the gene encoding HpnI being present in α -, β -, δ -, and γ -proteobacterial species, as well as in various cyanobacteria and the poorly characterized phylum of acidobacteria. The bacterial taxa in this tree reside in a wide range of water and soil associated environments and must endure variations in temperature, pH and exposure to a variety of chemical and metal stresses (Diels et al., 2009; Loutet and Valvano, 2010; Roger et al., 2012; Mamlouk and Gullo, 2013). It is possible that complex extended hopanoids, like BHT glucosamine are important in maintaining bacterial membrane stability in these constantly changing environments. Most *Burkholderia* species, including *B. cenocepacia*, contain only one copy of *hpnI*, found within the beta proteobacteria clade with the closely related *Ralstonia* and *Cupriavidus* spp. However, several *Burkholderia* species, including *B. pseudomallei* and *B. thailandensis*, contain 2 copies of *hpnI*. This second copy of the gene lies within an alpha proteobacterial clade and may have been acquired through horizontal gene transfer. *Hyphomicrobium* spp. and *Phaeospirillum molischianum* also contain 2 copies of *hpnI* which are found within clades belonging to 2 different phyla, indicating that they too have likely acquired an extra copy of the gene via horizontal gene transfer.

Many bacterial taxa, including *M. fujisawaense* (Talbot et al., 2007b), *M. extoquorens* (Bradley et al., 2010), *G. sulfurreducens*, *G. metallireducens* (Eickhoff et al., 2013), *B. pseudomallei*, *B. gladioli*, *B. cepacia* (Cvejic et al., 2000; Talbot et al., 2007b) and *Candidatus Chloracidobacterium thermophilum* (Costas et al., 2012) produce BHT cyclitol ether. We created a maximum likelihood

tree of the top 250 sequences retrieved via a protein BLAST search of the *B. cenocepacia* HpnJ sequence against the KEGG and NCBI databases (Figure 5). As mentioned previously, HpnJ is a radical SAM protein and BLAST searches of radical SAM hopanoid biosynthesis proteins generally pick up other radical SAM proteins that are not associated with hopanoid biosynthesis. To differentiate between these non-hopanoid biosynthesis radical SAM proteins and true HpnJ homologs the e-value for a bona fide HpnJ was set to e^{-100} or lower, as these was the lowest e-value for which a homolog of known but different function could be identified. The majority of HpnJ homologs were found in species that also contained an HpnI homolog, thereby suggesting that the production of BHT cyclitol ether depends on the production of a BHT glucosamine precursor, as we have observed in *B. cenocepacia*. As *B. cenocepacia* appears to produce much more BHT cyclitol ether than BHT glucosamine (Figure 3a), we speculate that the former plays the dominant role in maintaining membrane stability in response to environmental stresses.

Extended hopanoids are important in protecting B. cenocepacia from environmental stresses

Our previous work (Schmerk et al., 2011) demonstrated that hopanoid production plays an important role in the ability of *B. cenocepacia* to grow under diverse stress conditions, including low pH, the detergent sodium dodecyl sulfate (SDS), and the antimicrobial lipopeptide polymyxin B. This is likely due to the capacity of hopanoids to maintain membrane stability, a notion that was consistent with the observed retraction of the inner membrane from the outer membrane in the Δshc strain, and the mutant's inability to produce flagella (Schmerk et al., 2011). To determine whether extended hopanoids play any role in the ability of *B. cenocepacia* to resist stress conditions we monitored the growth of all mutants over a period of 24 h in LB buffered to pH 7.0 or pH 4.0, as well as LB supplemented with 0.03% SDS or 1 mg ml⁻¹ polymyxin B. $\Delta hpnH$, which only produces the C₃₀ hopanoid diploptene, behaved like Δshc , as it was unable to grow in all conditions tested, except in the pH 7.0 control medium (Figure 6). Therefore, we conclude that the production of diploptene alone is not sufficient for *B. cenocepacia* to fully adapt to the stress conditions tested. The susceptibility of $\Delta hpnH$ to both SDS and polymyxin B indicates that this strain likely suffers from increased membrane permeability, as increased sensitivity to detergents and antibiotics are indicators of

membrane damage in Gram-negative bacteria (Begley et al., 2005; Ruiz et al., 2005; Welander et al., 2009; Loutet and Valvano, 2011). We were unable to determine if this membrane damage results in decreased motility, as the construction of the *hpnH* strain required the presence of a complementing plasmid expressing *hpnF2* (pBCAM2831). The pSCRhaB2 vector used for this complementation severely alters motility patterns observed in swarming and swimming assays. There was no significant motility defect observed for any of the other nine mutants tested indicating that diploptene and/or adenosyl hopane, which is produced by $\Delta hpnG$, alone can confer sufficient membrane integrity or stability to properly assemble the flagellar apparatus.

The remaining mutants with defects in hopanoid side chain assembly demonstrated a range of phenotypes under stress (Figure 7). $\Delta hpnG$, which only produces adenosylhopane, had a phenotype similar to $\Delta hpnH$ and was unable to grow in all conditions tested aside from the pH 7.0 control. The minimum inhibitory concentration (MIC) of polymyxin B for $\Delta hpnG$ was $64 \mu\text{g ml}^{-1}$ (data not shown), a significantly lower value than that of the wild type ($>1024 \mu\text{g ml}^{-1}$), and comparable to that of Δshc ($128 \mu\text{g ml}^{-1}$). Therefore, lack of hopanoids (Δshc) and production of adenosyl hopane ($\Delta hpnG$) are detrimental to the bacterium. Introducing a functional *hpnG* gene by complementation with pHpnG restored growth to near wild type levels in the presence of 0.03% SDS, and partially restored growth in pH 4.0 and in the presence of polymyxin B (Figure S5). Since the hopanoid intermediate produced by $\Delta hpnG$ does not accumulate in the wild type, it is difficult to determine whether the phenotypes observed in the mutant are due to the lack of C_{35} extended hopanoids or to the build-up of adenosylhopane itself. It is also possible that adenosylhopane intermediate may be somehow mislocalized within the bacterial cell.

The $\Delta hpnI$, $\Delta hpnJ$, and $\Delta hpnK$ mutants could grow similarly to wild type in 0.03% SDS (Figure 7), indicating that the presence of the C_{35} extended hopanoid BHT is sufficient to confer a higher degree of membrane integrity than the C_{30} hopanoid diploptene. $\Delta hpnI$ and $\Delta hpnJ$ were partially resistant to low pH and polymyxin B, and grew slower than wild type (Figure 7). Comparatively, $\Delta hpnJ$ displayed a less severe phenotype than $\Delta hpnI$, demonstrating that the three C_{35} extended hopanoids are likely to play unique roles in enhancing the membrane integrity of *B. cenocepacia*.

Complementation of the $\Delta hpnI$ and $\Delta hpnJ$ mutants with pHpnI and pHpnJ, respectively, restored growth at pH 4.0 and in the presence of polymyxin B to wild type levels (Figure S5).

$\Delta hpnK$, which is thought to produce only acetylated BHT glucosamine, grew more poorly than $\Delta hpnI$ and $\Delta hpnJ$ at pH 4.0 and could not in 1 mg ml⁻¹ polymyxin B (Figure 7). The MIC value of polymyxin B for $\Delta hpnI$, $\Delta hpnJ$, and $\Delta hpnK$ was 256 µg ml⁻¹ (data not shown). The acetylated BHT glucosamine detected in this mutant is a hopanoid intermediate that would not normally be produced in the wild type strain. As proposed for the *hpnG* mutant, it is possible that this intermediate interferes with proper transport or membrane localization causing an increase in membrane permeability when compared to $\Delta hpnI$ and $\Delta hpnJ$ mutants. Complementation of $\Delta hpnK$ via pHpnK was able to partially restore growth at pH 4.0 and restored growth to a level similar to wild type in the presence of polymyxin B (Figure S5).

The remaining mutants tested displayed high variations in their degree of sensitivity to the tested stress conditions (Figure S6 and Table 3). We speculate that since these genes do not play a detectable role in hopanoid side chain biosynthesis they may be involved in the regulation and/or membrane transport of hopanoid molecules. The BCAM2736 and *hpnB* (BCAM2737) genes are highly conserved among *Burkholderia* species and other hopanoid producing bacteria; however, the deletion of these genes did not result in any defect in hopanoid biosynthesis or the ability of these mutants to tolerate membrane stress. *Burkholderia* species are highly adaptive to a wide range of ecological niches (Coenye and Vandamme, 2003), including the ability to colonize various hosts (Loutet and Valvano, 2010). Therefore, the conditions used in our experiments most likely underestimate the full spectrum of situations for which hopanoid production by *B. cenocepacia* could be required, and it is possible that these genes may be required in situations not modeled by our experiments. There is little information concerning the possible function of HpnL (BCAL1053). The loss of this protein resulted in an intermediate phenotype, being able to grow as well as MH1K in the pH 7.0 buffered control and in 0.03% SDS, but exhibiting delayed growth in both low pH medium and medium containing polymyxin B (Figure S6).

$\Delta hpnM$ (BCAM2827) grew slower than MH1K at pH 4.0, and was unable to grow in the presence of both detergent and polymyxin B (Figure S6). HpnM proteins are members of the toluene

tolerance protein Ttg2D family, a group of ABC-like transporters. Ttg2 plays a role in toluene tolerance in *Pseudomonas putida* (Kim et al., 1998), a process that involves increasing the cell membrane rigidity by changing the fatty acid and phospholipid compositions of the outer membrane (Ramos et al., 1997). MlaC is also a member of this protein family and is involved in maintaining lipid asymmetry via the retrograde trafficking of phospholipids from the outer to inner membrane in *E. coli* (Malinverni and Silhavy, 2009). It is likely that HpnM is also involved in the trafficking of lipids, specifically the glucosamine or cyclitol ether hopanoids, in response to environmental cues. HpnN is an RND-family transporter protein shown to be essential in the transport of hopanoids from the inner to outer membrane of *R. palustris* (Doughty et al., 2011). In our previous work, deletion of the *hpnN* gene in *B. cenocepacia* did not result in increased sensitivity to growth in low pH, SDS or polymyxin B but did result in increased sensitivity to other antibiotics (Schmerk et al., 2011). Together these results suggest that multiple transporter proteins may be involved in coordinating the trafficking of different hopanoids within the membrane of *B. cenocepacia* and likely other hopanoid producing bacteria.

Conclusions

In this study, we have defined the majority of the genes involved in the hopanoid biosynthetic pathway of *B. cenocepacia*. This information will illuminate future identification of the unique and specific functions that C₃₅ extended hopanoids, such as BHT cyclitol ether, play in bacterial membrane physiology. Given that BHT provides a much higher degree of membrane integrity than diploptene, it is clear that C₃₅ extended hopanoids, even in their most basic form, play a vital role in the function of the *B. cenocepacia* membrane. Identifying the genes responsible for the modification of extended hopanoids has also provided the tools needed to predict their structures based on genomic and metagenomic sequence information, and will help with the interpretation of geomicrobiological data. This work will also lead the way for future studies of functionalized hopanoids, providing insight into their specific biological functions while also allowing for a more informed interpretation of the hopanoid fossil record. Furthermore, the study of hopanoids in *B. cenocepacia* provides an opportunity to explore novel treatment options for cystic fibrosis patients infected with Bcc species.

As these species are intrinsically resistant to most clinically relevant antibiotics, the increased antibiotic sensitivity of the various hopanoid mutants raises the possibility of utilizing unique inhibitors, in combination with current antibiotic treatments, to better control infection in these patients.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in supplemental Table S1. Bacteria grew on LB agar plates or in LB broth with shaking at 37°C. When necessary, *Escherichia coli* cultures were supplemented with 40 µg ml⁻¹ kanamycin, and 30 µg ml⁻¹ tetracycline. *B. cenocepacia* cultures were supplemented, as needed, with 100 µg ml⁻¹ trimethoprim, and 150 µg ml⁻¹ tetracycline.

Construction of deletion strains and complementing plasmids

The construction of unmarked, nonpolar mutant strains was performed as previously described by Flannagan *et al.* (Flannagan et al., 2008). The deletion mutagenesis plasmids were created by cloning ~600-800-bp DNA amplicons flanking each of the putative hopanoid biosynthesis associated genes. Amplified fragments were ligated into pGPI-SceI to create the desired deletion plasmids. The mutagenic plasmids were mobilized into *B. cenocepacia* MH1K by triparental mating and cointegrants selected using 100 µg ml⁻¹ trimethoprim. Selection against *E. coli* donor and helper strains after the triparental mating was accomplished using 200 µg ml⁻¹ ampicillin in combination with 25 µg ml⁻¹ polymyxin B. The pDAI-SceI-SacB vector was used in the final stage of mutagenesis to induce the second recombination event, leading to an unmarked gene deletion. This vector was mobilized into *B. cenocepacia* MH1K cointegrants, and exconjugants were selected with 150 µg ml⁻¹ tetracycline. Colonies were screened by PCR to confirm the presence of the appropriate gene deletions.

Complementing plasmids were constructed by amplifying *hpnG* (BCAM2830), *hpnI* (BCAL1050) and *hpnJ* (BCAL1051), and *hpnK* (BCAL1052) with the appropriate primer pairs. PCR products were

cloned into pSCrhaB2, resulting in the creation of pHpnG, pHpnI, pHpnJ, and pHpnK. Complementing plasmids were introduced into the desired mutant strains by triparental mating as described above. For unknown reasons, deletion of *hpnH* (BCAM2739) was not possible using the method described above. To delete this gene the plasmid pDelBCAM2739 was mobilized into the non-hopanoid-producing strain MH1K Δ *shc* (Schmerk et al., 2011), and the Δ *hpnH* mutant strain was then created as outlined above. To restore hopanoid production following *hpnH* deletion, the *hpnF2* gene (BCAM2831) was introduced via the complementing plasmid pBCAM2831 (Schmerk et al., 2011).

Analysis of hopanoids

Lipid extracts from the wild type (MH1K) and mutant strains were prepared using the method of Welander *et al.* (Welander et al., 2012b). Briefly, 200 ml of stationary phase culture were harvested by centrifugation at 5000g for 10 min at 4°C. Cells were disrupted by sonication in 10 ml of 10:5:4 (v:v:v) methanol (MeOH):dichloromethane (DCM):water for 15 min. Samples were centrifuged at 3000g for 10 min, the supernatant was transferred to a new tube and the pellet was treated once more. Combined supernatants were separated into two phases via the addition of 10 ml DCM and 5 ml water followed by centrifugation at 3000g for 10 min. The organic phase was placed in a new tube and the residual aqueous phase was treated once more with 10 ml DCM and 5 ml water. Following centrifugation, the organic phases were combined and evaporated under a stream of N₂ gas and the total lipid extracts (TLE) were then dissolved in 2 ml DCM. To identify the production of the C₃₀ hopene, acetylated TLEs from each strain were analyzed by high temperature gas chromatography-mass spectrometry (GC-MS) as previously described (Welander et al., 2009). Acetylated TLEs were also analyzed by liquid chromatography-mass spectrometry (LC-MS) to identify any functionalized hopanoids (Welander et al., 2012a). Details of the chromatographic analysis can be found in the supplementary information.

Phylogenetic analysis

Homologs of *B. cenocepacia* HpnI and HpnJ were identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and National Center for Biotechnology Information (NCBI) databases via TBLASTN (Altschul et al., 1997) and the top 250 sequences were aligned via clustalW2 using the Geneious R6 software version 6.1.2 (Biomatters Ltd., Auckland, New Zealand, <http://www.geneious.com/>). Maximum likelihood trees were constructed by PhyML (<http://www.atgc-montpellier.fr/phyml/>) (Guindon et al., 2010) using the LG+gamma model, six gamma rate categories, ten random starting trees, SPR+NNI branch swapping, and substitution parameters estimated from the data. The finalized trees were generated by importing the resulting PhyML tree into iTOL for editing (<http://itol.embl.de/>) (Letunic and Bork, 2011).

Environmental stress tests

Strains grew overnight with shaking in unbuffered LB medium at 37°C. Cultures were adjusted to an OD₆₀₀ 0.005 in the appropriate medium. Buffered LB medium was prepared by adding 100mM (final concentration) MES (4-morpholineethanesulfonic acid) for pH 4.0 or 100mM MOPS (4-morpholinepropanesulfonic acid) for pH 7.0. Where appropriate, the pH 7.0 buffered LB medium was supplemented with 0.03% SDS (w/v) or 1 mg ml⁻¹ polymyxin B. Growth was determined in a 100-well disposable plate using a Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems). Growth was monitored over 24 h at 37°C.

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608 Table 1 Hopanoid biosynthesis genes deleted in *B. cenocepacia*

Gene name	Locus Tag	Annotation	Function	Reference
<i>hpnI</i>	BCAL1050	Hopanoid-associated glycosyl transferase	Transfer of acetylglucosamine from UDP-acetylglucosamine to BHT	This study
<i>hpnJ</i>	BCAL1051	Hopanoid-associated radical SAM superfamily protein	Catalyzes ring contraction to generate BHT cyclitol ether	This study
<i>hpnK</i>	BCAL1052	Hopanoid biosynthesis associated protein (ydcJ)	Deacetylation of BHT acetylglucosamine	This study
<i>hpnL</i>	BCAL1053	Conserved hypothetical protein	Not established	
Unassigned	BCAL1054	Putative lipoprotein (SmpA/OmlA)	Not established	
<i>hpnA*</i>	BCAM2735	Hopanoid-associated sugar epimerase	Not established	
Unassigned	BCAM2736	Conserved hypothetical protein	Not established (only occurs in <i>Burkholderia</i> spp.)	
<i>hpnB</i>	BCAM2737	Hopanoid-associated glycosyl transferase	Not established	
<i>ispH/lytB</i>	BCAM2738	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Not established	
<i>hpnH</i>	BCAM2739	Hopanoid-associated radical SAM superfamily protein	Addition of adenosine to hopane skeleton	Bradley <i>et al.</i> 2010, Welandar <i>et al.</i> 2012b, this study
<i>hpnM</i>	BCAM2827	Hopanoid biosynthesis associated membrane protein	Not established	
<i>hpnN</i>	BCAM2828	Hopanoid-associated RND transporter	Transport of hopanoids to the outer mmebrane	Doughty <i>et al.</i> 2011
<i>hpnG</i>	BCAM2830	Hopanoid-associated nucleosidase	Removal of adenine from adenosyl hopane	Bradley <i>et al.</i> 2010, Welandar <i>et al.</i> 2012b, this study
<i>hpnF2</i>	BCAM2831	Squalene hopene cyclase	Cyclization of squalene	Reipen <i>et al.</i> 1995
<i>hpnF3</i>	BCAS0167	Squalene hopene cyclase	Cyclization of squalene	Reipen <i>et al.</i> 1995
<i>hpnE</i>	BCAM2832	Putative dehydrosqualene reductase	Production of squalene	Perzl <i>et al.</i> 1998
<i>hpnC</i>	BCAM2833	Squalene/phytoene synthase	Production of squalene	Perzl <i>et al.</i> 1998

609 Genes shown in bold were deleted in this study. *Unable to construct this deletion

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Table 2. Hopanoids identified in *B. cenocepacia* K56-2 mutants

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	Diploptene	Bacterio hopanetetrol (BHT)	BHT cyclitol ether	Monounsaturated BHT cyclitol ether or glucosamine	BHT glucosamine	Adenosyl hopane
MH ⁺ :	410	715	1002	1000	1002	746
Base Peak:	191	655	1002	1000	1002	611
Identification of these hopanoids in the following strains:						
K56-2	+	+	+	+	+	-
<i>ΔhpnF2</i> (BCAM2831)	+	+	+	+	+	-
<i>ΔhpnF3</i> (BCAS0167)	+	+	+	+	+	-
<i>Δshc</i> BCAS0167, BCAM2831)	-	-	-	-	-	-
<i>ΔBCAM2736</i>	+	+	+	+	+	-
<i>ΔhpnB</i> (BACM2737)	+	+	+	+	+	-
<i>ΔhpnH</i> (BCAM2739)	+	-	-	-	-	-
<i>ΔhpnM</i> (BCAM2827)	+	+	+	+	+	-
<i>ΔhpnN</i> (BCAM2828)	+	+	+	+	+	-
<i>ΔhpnG</i> (BACM2830)	+	-	-	-	-	+
<i>ΔhpnI</i> (BCAL1050)	+	+	-	-	-	-
<i>ΔhpnJ</i> (BCAL1051)	+	+	-	-	+	-
<i>ΔhpnK</i> (BCAL1052)	+	-	-	-	+	-
<i>ΔhpnL</i> (BCAL1053)	+	+	+	+	+	-
<i>ΔBCAL1054</i> (<i>smgA/omlA</i>)	+	+	+	+	+	-

668 Table 3 Summary of resistance of hopanoid mutants to various environmental stresses

	Hopanoid side chain biosynthesis	Resistance to low pH stress	Resistance to SDS	Resistance to polymyxin B
<i>ΔhpnH</i> (BCAM2739)	+	+	+	+
<i>ΔhpnG</i> (BCAM2830)	+	+	+	+
<i>ΔhpnI</i> (BCAL1050)	+	+	-	+
<i>ΔhpnJ</i> (BCAL1051)	+	+	-	+
<i>ΔhpnK</i> (BCAL1052)	+	+	-	+
<i>ΔhpnM</i> (BCAM2827)	-	+	-	+
<i>ΔhpnL</i> (BCAL1053)	-	+	-	+
<i>ΔBCAM2736</i>	-	-	-	-
<i>ΔhpnB</i> (BCAM2737)	-	-	-	-
<i>ΔBCAL1054 (smpA/omlA)</i>	-	+	+	+

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Figures Legends

Figure 1 (a) Hopanoid compounds detected in *B. cenocepacia* K56-2 lipid extracts at stationary phase. The dotted rectangle contains the structures for C-6 and C-11 monounsaturated BHT cyclitol ethers; however, the mass spectra could not distinguish whether the BHT cyclitol ether or the BHT glucosamine is unsaturated and whether the unsaturation occurs at C-6 or C-11 ring positions. **(b)** The *B. cenocepacia* hopanoid biosynthetic gene clusters are present on chromosome 1, chromosome 2, and chromosome 3. Black arrows indicate genes that have been deleted in this study, white arrows indicate genes that were not deleted, and gray arrows indicate genes that were deleted in a previous study (Schmerk et al. 2011).

Figure 2 Proposed *B. cenocepacia* hopanoid biosynthesis pathway. Dotted arrows in step 8 represent a proposed desaturation reaction.

Figure 3 LC-MS extracted ion chromatograms of acetylated total lipid extracts from **(a)** wild type *B. cenocepacia*, **(b)** $\Delta hpnI$, **(c)** $\Delta hpnK$, and **(d)** $\Delta hpnJ$. The chromatograms are a combination of ions m/z 1002 (I, BHT cyclitol ether and II, BHT glucosamine), 655 (III, bacteriohopanetetrol), and 1000 (IV, monounsaturated BHT cyclitol ether or glucosamine). Hopanoids were identified by comparison of their mass spectra to previously published samples (Talbot et al., 2007a; Talbot et al., 2007b; Talbot et al., 2003a; Talbot et al., 2003b).

Figure 4 Maximum likelihood phylogenetic tree of hopanoid associated glycosyl transferase, HpnI, among sequenced genomes. The *Methylobacterium* spp. and *Burkholderia* spp. (highlighted in red) clades have been collapsed due to the high number of strains present. Some *Burkholderia* species possess 2 copies of HpnI however *B. cenocepacia* strains contain only 1 copy (found in the blue highlighted clade). All organisms present contain at least one copy of *shc* in their genome.

Figure 5 Maximum likelihood phylogenetic tree of hopanoid biosynthesis associated radical SAM protein, HpnJ. The *Methylobacterium* spp., *Cupriavidus* spp., and *Burkholderia* spp. (highlighted in red) clades have been collapsed due to the high number of strains present. With the exception of the collapsed outgroup clades, all organisms contain one or more copies of squalene hopene cyclase in their genome.

Figure 6 A *B. cenocepacia* $\Delta hpnH$ mutant exhibits sensitivity to environmental stresses. Representative growth curves of the wild type (MH1K) and mutant strain in LB buffered to pH 7.0 or pH 4, LB buffered to pH 7.0 supplemented with 0.03% SDS, and LB supplemented with 1 mg ml⁻¹ polymyxin B. Δshc is included as a control that cannot produce any hopanoids. All control strains contain the empty complementing vector pSCRhaB2. The $\Delta hpnH$ mutant had to be created in a Δshc mutant background and contains pBCAM2831 to restore hopanoid biosynthesis. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Figure 7 Mutants involved in the biosynthesis of C₃₅ extended hopanoid side chains display a range of sensitivity to environmental stresses. Representative growth curves of the wild type and mutant strains in LB buffered to pH 7.0, LB buffered to pH 4, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1mg ml⁻¹ polymyxin B. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Supporting information

Supporting Information A. Hopanoids analyses

Table S1. Bacterial strain and plasmids used in this study.

Table S2. Primers used in this study.

Figure S1 Identification of squalene build-up in squalene-hopene cyclase gene deletion mutants. Nonsaponifiable lipids were extracted from the *B. cenocepacia* $\Delta hpnF2$ strain and separated by GC. (a) Total ion chromatogram was compared to that of a squalene standard. (b) Full mass spectrum of the co-eluting peaks from panel (a). The Δshc mutant (lacking both the *hpnF2* and *hpnF3* genes) accumulated an identical peak as in (a) (data not shown).

Figure S2. Mass spectra of functionalized hopanoids produced by wild type *B. cenocepacia* and hopanoid mutants. The top row contains the mass spectra of the hopanoid illustrated above. The bottom row shows the MS-MS spectra of the indicated ion. The unsaturated hopanoid could be either the cyclitol ether or glucosamine hopanoid; it is unclear from the mass spectra which functionalized hopanoid is unsaturated and where the unsaturation occurs (C-6 or C-11).

Figure S3. LC-MS and GC-MS analysis of *B. cenocepacia* $\Delta hpnH$ acetylated total lipid extract. (a) LC-MS combined extracted ion chromatogram (m/z 611, 655, 1000, 1002) demonstrating the lack of functionalized hopanoid production. (b) GC-MS extracted ion chromatogram (m/z 191) demonstrating the production of diploptene.

Figure S4. LC-MS analysis of *B. cenocepacia* $\Delta hpnG$ acetylated total lipid extract. (a) Combined extracted ion chromatogram (m/z 611, 655, 1000, 1002) demonstrating the single adenosyl hopane peak at 29 minutes. (b) Mass spectra of the adenosyl hopane peak showing the 788 ion representing intact adenosyl hopane and the 611 ion representing the loss of an adenine molecule.

Figure S5. Complementation of the hopanoid side chain biosynthesis growth defects. Representative growth curves of the wild type (MH1K), mutant, and complement strains in LB buffered to pH 7.0, LB buffered to pH 4.0, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1mg ml⁻¹ polymyxin B. Δshc is included as a control that cannot produce any hopanoids. All control and mutant strains contain the empty complementing vector pSCRhaB2. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Figure S6. Mutants that are not directly involved in the biosynthesis of hopanoid side chains display an array of sensitivity to different environmental stresses. Representative growth curves of the wild type and mutant strains in LB buffered to pH 7.0, LB buffered to pH 4.0, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1 mg ml⁻¹ polymyxin B. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.