Presence of the Hmq system and production of 4-hydroxy-3-methyl-2-alkylquinolines is heterogeneously distributed between *Burkholderia cepacia* complex species and more prevalent among environmental than clinical isolates.

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Abstract

Some *Burkholderia cepacia* complex (Bcc) strains have been reported to produce 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs), analogous to the 4-hydroxy-2-alkylquinolines of *Pseudomonas aeruginosa*. Using *in silico* analyses, we previously showed that the *hmqABCDEFG* operon, which encodes enzymes involved in the biosynthesis of HMAQs, is carried by about one-third of Bcc strains, with considerable inter- and intra-species variability. In the present study, we investigated by PCR, using consensus primers, the distribution of *hmqABCDEFG* in a collection of 313 Bcc strains (222 of clinical and 91 of environmental origins) - belonging to 18 Bcc species. We confirmed that the distribution is species-specific, although not all strains within a species carry the *hmqABCDEFG* operon. Among the 30% of strains bearing the *hmqABCDEFG* operon, we measured the total HMAQs production and showed that 90% of environmental isolates and 68% of clinically isolated Bcc produce detectable levels of HMAQs when cultured in TSB medium. For the strains having the *hmqABCDEFG* operon but not producing HMAQs, we studied the transcription and showed that none expressed the *hmqA* gene under the specified culture conditions. Interestingly, the *hmqABCDEFG* operon is more prevalent among plant root environment species (e.g. *B. ambifaria, B. cepacia*) and absent in species commonly found in chronically colonized individuals with cystic fibrosis (e.g. *B. cenocepacia, B. multivorans*), suggesting that the Hmq system could play a role in niche adaptation by influencing rhizosphere microbial community and could have been lost through evolution. Understanding the Hmq system and its regulation will provide clues concerning the production of HMAQs and their functions in Bcc.
Keywords: hmqABCDEFG operon, 4-hydroxy-2-alkylquinolines (HAQs), pqsABCDEFG, Pseudomonas Quinolone Signal (PQS), quorum sensing (QS)

Introduction

The environmental species of Burkholderia can be divided into two phylogenetic groups: (1) pathogenic species and (2) plant-beneficial species (Eberl and Vandamme, 2016). Based on this still controversial separation (Vandamme et al., 2017), the latter group was reclassified as Paraburkholderia, based on lower %GC and lack of virulence in Caenorhabditis elegans (Angus et al., 2014; Sawana et al., 2014). The pathogenic Burkholderia group comprises (1) plant pathogens (e.g.: Burkholderia andropogonis causing leaf streak on Sorghum (Ramundo and Claflin, 2005) and B. glumae causing bacterial panicle blight on rice (Azegami et al., 1985; Nandakumar et al., 2009; Ham et al., 2010)); (2) the “pseudomallei” group, comprised on B. pseudomallei (the causative agent of melioidosis), B. thailandensis (avirulent model) and B. mallei (causing glanders in equids) species (Howe, 1950; Smith et al., 1987; Chaowagul et al., 1989; Sandford, 1990) - and finally (3) the Burkholderia cepacia complex (Bcc), comprising at least 26 different species [(Bach et al., 2017; Martina et al., 2017; Weber and King, 2017); reviewed by (Eberl and Vandamme, 2016)], most considered opportunistic pathogens.

Bcc bacteria have been used in (1) agriculture for biocontrol of phytopathogens and plant growth-promoting properties [e.g.: pea protection by B. ambifaria against Pythium and Aphanomyces (Parke, 1991; Mullins et al., 2019)] and (2) bioremediation [e.g.: B. vietnamiensis with its trichloroethylene degradation abilities (Gillis et al., 1995; O’Sullivan and Mahenthiralingam, 2005)]; reviewed in Vial et al. (2011). Bcc bacteria are also well known for secondary metabolite production, including antibiotics [reviewed by Depoorter et al., (2016)].
However, in the 1980s, Bcc opportunistic pathogens have emerged as a serious issue among certain immunocompromised individuals (e.g. with the chronic granulomatous disease) and people with cystic fibrosis (CF) causing the ‘cepacia syndrome’, pushing authorities to prohibit their use in biotechnological applications. It is now generally accepted that their high transmissibility and intrinsic resistance to clinically relevant antibiotics makes them particularly problematic (Gilligan, 1991; Speert et al., 1994; Govan and Deretic, 1996).

Cell-to-cell communication mechanisms, e.g. quorum sensing (QS), act by (1) controlling gene transcription at the population level (Fuqua and Greenberg, 1975), (2) promoting colonization, and (3) optimizing interaction with hosts and increasing resistance to stress (Stewart and Costerton, 2001; Juhas et al., 2005). CepR/CepI is the primary QS system in Bcc species. The CepI synthase produces the autoinducer ligand N-octanoyl-homoserine lactone (C8-HSL) which interacts with the transcriptional regulator CepR to activate the transcription of several target genes, such as genes involved in the production of pyrrolnitrin (tryptophan halogenase (prnA); BAMB_RS23660), enacyloxins (LuxR family transcriptional regulator; BAMB_RS29445), and occidiofungins (amino acid adenylation domain-containing protein; BAMB_RS32210) (McKenney et al., 1995; Lewenza et al., 1999; Lewenza and Sokol, 2001; Chapalain et al., 2013). Depending on the Bcc species, at least two other Cep-like systems may be present, synthesizing different acyl-homoserine lactones (AHLs) as ligands and regulating each other (Choudhary et al., 2013).

The bacterium Pseudomonas aeruginosa carries a distinct QS system whose ligands are not AHLs but instead 4-hydroxy-2-alkylquinolines (HAQs), such as the Pseudomonas Quinolone Signal (PQS) and 4-hydroxy-2-heptylquinoline (HHQ) (Pesci et al., 1999; Déziel et al., 2004; Heeb et al., 2011). Interestingly, some strains of Bcc (B. ambifaria and B. cepacia), as well as B.
*pseudomallei* and *B. thailandensis*, produce homologous molecules, that we call 4-hydroxy-3-methyl-2-alkylquinolines (Diggle et al., 2006; Vial et al., 2008; Ritzmann et al., 2019), that are synthesized by enzymes encoded by the *hmqABCDEFG* operon (Vial et al., 2008). In contrast with *P. aeruginosa* HAQs, the main HMAQs produced by *Burkholderia* bear a methyl group at the 3’ position and an unsaturation of the alkyl side chain. An increasing number of Bcc strains are being reported to produce some HMAQs congeners (Mori et al., 2007; Vial et al., 2008; Kilani-Feki et al., 2011, 2012; Mahenthiralingam et al., 2011; Li et al., 2018), but this remains mostly anecdotal. In contrast with the HAQ/PQS system of *P. aeruginosa*, the *Burkholderia* Hmq system does not appear to be a QS system *per se*, although we have shown that it is closely interrelated with the Cep system in *B. ambifaria* HSJ1 and the three Bta QS systems in *B. thailandensis* E264 (Vial et al., 2008; Chapalain et al., 2017; Le Guillouzer, 2018).

Functions of HHQ and PQS in *P. aeruginosa* as QS inducers, immunomodulators, antimicrobials have been described (Lin et al., 2018). Several studies report novel molecules belonging to the HAQ family and various bacterial species having antimicrobial activities (Wratten et al., 1977; Hamasaki et al., 2000; Whalen et al., 2014; Meyer et al., 2017; Dow et al., 2019). Only a few functions of HMAQs are known, apart from intra and interspecies QS signal (Vial et al., 2008; Chapalain et al., 2017; Le Guillouzer, 2018), especially as antimicrobials - having a lower activity than antibiotics - however, their main function remains enigmatic (Mori et al., 2007; Kilani-Feki et al., 2011, 2012; Mahenthiralingam et al., 2011; Li et al., 2018; Piochon et al., 2020). Nonetheless, given the demonstrated role of HAQs and PQS in *P. aeruginosa*, HMAQs may also play a role in the virulence and pathogenicity of opportunistic *Burkholderia* pathogens (Vial et al., 2008, 2009; Chapalain et al., 2017). We have previously characterized (Vial et al. (2008, 2009)) a series of clinical *B. ambifaria* strains able to produce
HMAQs and that phenotypic variant of these *B. ambifaria* strains had lost their ability to produce several secondary metabolites, including HMAQs, similar a set of environmental isolates. Therefore, to better understand these metabolites, we posited the hypotheses that (1) the *hmqABCDEFG* operon is more frequently found among clinical Bcc strains and that (2) the clinical Bcc strains produce higher concentrations of HMAQs than environmental ones. Our previous bioinformatic study of the distribution of the *hmqABCDEFG* operon in the Bcc, based on available 1,257 whole-genome sequences belonging to 21 Bcc species, showed that strains belonging to the *B. ambifaria*, *B. cepacia*, *B. contaminans* *B. pyrrocinia*, *B. stagnalis*, *B. territorii*, and *B. ubonensis* species carry the *hmqABCDEFG* operon, but not all strains within a species, while *B. anthina*, *B. arboris* *B. cenocepacia*, *B. diffusa*, *B. latens*, *B. metallica*, *B. multivorans*, *B. pseudomultivorans*, *B. seminalis*, *B. stabilis* and *B. vietnamiensis* - species mainly found in clinical cases - are lacking *hmqABCDEFG* operon (Coulon *et al.*, 2019). To experimentally validate our *in silico* study and verify the ability of Bcc isolates carrying the *hmqABCDEFG* operon to actually produce HMAQs, a collection of 313 Bcc strains, comprising 222 clinical and 91 environmental isolates belonging to 18 different Bcc species, was analyzed to first determine the presence of the *hmqABCDEFG* operon in their genome. We then directly determined, by liquid chromatography coupled to mass spectrometry (LC/MS) analyses, the ability of all the strains bearing the *hmqABCDEFG* operon to produce HMAQs. Finally, we verified the expression of *hmqA* in Bcc strains having the *hmqABCDEFG* and not producing HMAQs to investigate this lack of HMAQ production. Our data confirm that the Hmq system is heterogeneously distributed between Bcc species, with high prevalence in some species (e.g. *B. cepacia*) and near absence in other (e.g. *B. cenocepacia* and *B. multivorans*). Globally, higher
frequency among strains of environmental origins vs clinical isolates suggests that the
*hmqABCDEFG* operon and HMAQ production could play a role in Bcc niche adaptation.

**Results**

The *hmqABCDEFG* operon is heterogeneously distributed across and within Bcc species

We previously examined 1,257 whole-genome sequences belonging to 21 different Bcc species to assess the distribution of the *hmqABCDEFG* operon (Coulon *et al.*, 2019). We found that at least one sequenced strain belonging to 7 out of 21 species carries the *hmqABCDEFG* operon (*B. ambifaria, B. cepacia, B. contaminans B. pyrrocinia, B. stagnalis, B. territorii*, and *B. ubonensis*); one striking initial finding was that prevalence of the *hmqABCDEFG* operon within a species appeared highly variable (Coulon *et al.*, 2019). Here, to validate our *in silico* analyses of the distribution of the *hmqABCDEFG* operon based on homology and orthology (Coulon *et al.*, 2019) and to globally determine the ability of Bcc to produce HMAQs, we screened a collection of 313 Bcc strains (222 of clinical and 91 of environmental origins; listed in Table S1), belonging to 18 Bcc species: *B. ambifaria, B. anthina, B. arboris, B. cenocepecia, B. cepacia, B. contaminans, B. diffusa, B. dolosa, B. lata, B. metallica, B. multivorans, B. pyrrocinia, B. seminalis, B. stabilis, B. stagnalis, B. territorii, B. ubonensis, B. vietnamiensis*, plus a few more classified in the ‘other Bcc’ group (PubMLST database; https://pubmlst.org/bcc/info/protocol.shtml) - for the presence of *hmqABCDEFG* by PCR using consensus primers targeting *hmqA* and *hmqG*. We had previously determined that the presence of a *hmqG* orthologue correlates with the presence of a complete *hmqABCDEFG* operon (Coulon *et al.*, 2019). Here, we found that 30% of the tested strains possess an *hmqABCDEFG* operon,
including 53% of environmental but only 21% of clinical strains (Figure 1A). Among the 18 different species investigated, 14 comprise at least one strain carrying the operon (Figure 1B).

The $hmqABCDEFG$ operon is more prevalent among clinical strains for species $B. ambifaria$, $B. anthina$, $B. contaminans$, $B. diffusa$, $B. ubonensis$ and $B. vietnamiensis$. However, it is more prevalent among environmental strains for $B. contaminans$, $B. cepacia$, $B. dolosa$, $B. lata$, $B. metallica$, $B. pyrrocinia$, and the ‘other Bcc’ group. Clinical $B. seminalis$ and environmental $B. stagnalis$ and $B. territorii$ species carry $hmqABCDEFG$.

We found that isolates of $B. dolosa$, $B. anthina$, and $B. vietnamiensis$ carry the $hmqABCDEFG$ operon, which was not predicted in our previous analysis of available genomic data (Coulon et al., 2019), we confirmed here our PCR results by sequencing of the amplicons using primers listed in Table S2.

Neither the phylogeny of Bcc species nor the loss of the third chromosome explain the distribution of the $hmqABCDEFG$ operon

Since not all Bcc species carry the $hmqABCDEFG$ operon, we asked whether the distribution of the operon could be related to the phylogenetic distribution of Bcc species. Based on MultiLocus Sequence Typing (MLST) sequences, we found that $B. ambifaria$, $B. cepacia$, $B. contaminans$, $B. pyrrocinia$ and $B. stagnalis$ species in which $hmqABCDEFG$ is the most prevalent, are not clustered (Figure 2). The same was observed for $B. cenocepacia$ and $B. multivorans$ which both do not possess the $hmqABCDEFG$ operon (Figure 2).

Bcc bacteria are known to lose their third chromosome (c3) which is a virulence mega-plasmid containing a few core genes (Agnoli et al., 2011; diCenzo et al., 2019). The $hmqABCDEFG$ operon being generally located on the c3 replicon, we investigated whether the
absence of the \textit{hmqABCDEFG} operon was related to the loss of the c3, but it is not the case (Table S3).

A majority of Bcc strains carrying the \textit{hmqABCDEFG} operon also produce HMAQs

We then verified whether the presence of the biosynthetic genes is indicative of known HMAQ production. We cultured the 94 strains that were PCR-positive for \textit{hmqA} and \textit{hmqG} in Tryptic Soy Broth (TSB) medium at 30°C, 250 rpm for overnight under the tested conditions, we could detect the production of HMAQ in 72\% of the strains - 65\% clinical and 79\% environmental - carrying the \textit{hmqABCDEFG} operon (Figure 3). None of the PCR-positive \textit{B. anthina}, \textit{B. diffusa}, \textit{B. dolosa}, \textit{B. metallica}, \textit{B. seminalis} and \textit{B. ubonensis} strains produced HMAQ under our conditions.

To validate our LC-MS method, we measured the production of HMAQs by 31 Bcc strains determined not to carry the \textit{hmqABCDEFG} operon; all the 31 strains – belonging to \textit{B. ambifaria}, \textit{B. anthina}, \textit{B. arboris}, \textit{B. cenocepacia}, \textit{B. multivorans}, \textit{B. pyrrocinia}, \textit{B. stabilis}, \textit{B. ubonensis} and \textit{B. vietnamiensis} species - did not produce detectable HMAQs (Table S4).

HMAQ production was slightly more prevalent among clinical strains for \textit{B. cepacia} and \textit{B. vietnamiensis}. However, it was also more prevalent among environmental strains for \textit{B. ambifaria}, \textit{B. contaminans}, and \textit{B. lata}. Most of the environmental strains of \textit{B. pyrrocinia}, \textit{B. stagnalis}, \textit{B. territorii} and the ‘other Bcc’ group species also produced HMAQ.

All HMAQ-producing Bcc strains mainly produce the HMAQ-C$_{7}:2'$ and HMAQ-C$_{9}:2'$ congeners
To verify which HMAQ congeners are produced by the various Bcc, we scanned by LC/MS for the 14 congeners of HAQs and HMAQs we had previously identified (Table S5) (Vial et al., 2008). We found that the major congeners produced were HMAQ-C7:2’ and HMAQ-C9:2’, as previously observed for *B ambifaria* HSJ1 (Vial et al., 2008). Most of the strains were able to produce other HMAQs such as HMAQ-C7 and HMAQ-C8:2’ (also known as burkholone). HMAQ-C8, HMAQ-C6 were the most abundant molecules after HMAQ-C7:2’ and HMAQ-C9:2’ and HHQ-C9 was also detected (Table S6). Concentrations of HMAQ-C7:2’ and HMAQ-C9:2’ as well as all identified congeners are listed in Tables S5 and S6.

We also found that the concentration of HMAQs produced was variable among the various species. A Kruskal-Wallis test confirmed that there was no statistical difference in the concentrations produced between the clinical and environmental strains (*p*-value of 0.19 for HMAQ-C7:2’ and 0.22 for HMAQ-C9:2’). However, clinical strains of *B. ambifaria* and *B. vietnamiensis* strains produced more HMAQs than environmental ones. The opposite was observed for *B. cepacia* and *B. contaminans* strains (Table S6).

**The presence of the hmqABCDEFG and production of HMAQs are not linked to the co-isolation of *P. aeruginosa* nor the origin of samples**

Since the *hmqABCDEFG* operon is homologous to the *pqsABCDE* operon in *P. aeruginosa* we wondered if HMAQ production in TSB condition of clinical Bcc was correlated with a co-isolation or a co-localization with *P. aeruginosa* at some point in the patient as well as the origin of the sample (sputum, throat, sinus *etc.*). Information was only available for 53 strains (Table S7). Using a Fisher's Exact Test for Count Data, we did not find a correlation between the
presence of the hmqABCDEFG operon and the presence of P. aeruginosa – at the sampling time or within the previous year – nor with the origin of the sample (Table 1).

The use of Cystic Fibrosis artificial sputum medium – looking for growth condition relevant for HMAQs production

To induce the production of HMAQs by the 26 Bcc strains carrying the hmqABCDEFG operon but for which we could not detect HMAQs when cultured in TSB (at 30°C at 250 rpm, overnight), we assayed the production of these metabolites in Artificial Sputum Medium (ASM; at 30°C at 250 rpm, overnight) and on Tryptic Soy Agar plates (TSA; incubated at 30°C for four days) As shown in Table 2, growth in ASM allowed detection of HMAQs in 10 out of the 26 Bcc strains - 7 environmental and 3 clinical strains. Surface growth on TSA plates induced the detectable production of HMAQs for 5 environmental and 4 clinical strains. For the strains already producing HMAQ in TSB most of them also produce HMAQs in ASM and TSA (Table S8). These additional culture conditions reduced the number of HMAQ-negative strains to 15 out of 26. Globally, increasing the number of Bcc isolates able to produce HMAQs to a total of 79, that is 84% of strains carrying the hmqABCDEFG operon.

Low express the hmqABCDEFG operon explains absence of HMAQ production in some Bcc strains growing in TSB

Our screening revealed that 26 strains carrying the hmqABCDEFG operon do not produce HMAQ in TSB. To investigate the possibility that low transcription of the biosynthetic genes would explain this absence of production, which is compatible with the induction seen when changing the culture conditions, we measured the expression of the hmqABCDEFG operon for
one HMAQ-negative and one HMAQ-positive strain from each of *B. ambifaria*, *B. cepacia*, *B. contaminans* and *B. vietnamiensis* by targeting the *hmqA* gene by RT-PCR.

The results show that HMAQ-negative strains *B. ambifaria* AMMD, *B. cepacia* ATCC25416, *B. contaminans* VC15406, *B. vietnamiensis* VC9237 strains do not express *hmqA* gene when grown in TSB, while *B. ambifaria* HSJ1, *B. cepacia* VC13394, *B. contaminans* FFH2055 and *B. vietnamiensis* VC8245, which produce HMAQs under these conditions, produce a clear *hmqA* transcript (*Table 3; Figure S1*). Extending these results, we hypothesize that the other strains which carry the *hmqABCDEFG* operon and do not produce HMAQs, do not express the *hmqA* gene, at least when grown in TSB at 30°C.

**Discussion**

This study aimed at understanding the prevalence of the Hmq system and corresponding HMAQ production in the Bcc to complete or confirm our previous *in silico* analyses (Coulon et al., 2019). Only a few strains of *B. ambifaria*, *B. cepacia*, *B. pseudomallei* and *B. thailandensis* species were already known to carry the *hmqABCDEFG* operon and to produce HMAQs (Diggle et al., 2006; Vial et al., 2008; Coulon et al., 2019). Even if the role of HMAQs is still unknown, the presence of the *hmqABCDEFG* operon is well-conserved in *B. pseudomallei* and *B. thailandensis* species but remains unclear within the Bcc (Coulon et al., 2019). To understand the ecological role of the Hmq system, we first needed to evaluate its distribution. Since available Bcc genomes are not equally distributed between species, we screened 313 Bcc strains for the presence of the *hmqABCDEFG* operon by PCR. We confirmed that the *hmqABCDEFG* operon among the Bcc followed the same distribution when analyzed *in silico* - meaning that a laboratory screening is necessary to complete a bioinformatics study - especially when available
whole-genome sequencing is limited. However, we have to take into consideration that our screening could have false-negative results due to the limited availability of whole-genome sequences for some Bcc species (e.g. B. arboris, B. metallica, B. stabilis). Nevertheless, our primers were able to amplify hmqA and hmqG targets in species previously unknown to carry a hmqABCDEFG operon (e.g. B. vietnamiensis).

Based on our previous results obtained with a few B. ambifaria strains, we hypothesized that the Hmq system would be more prevalent among clinical isolates, and produce more HMAQs than environmental ones (Vial et al., 2008, 2009; Chapalain et al., 2017). Unexpectedly, we uncovered that B. cenocepacia, B. multivorans and B. vietnamiensis - the prominent Bcc species colonizing immunosuppressed and CF individuals, transmitted between patients (Gilligan, 1991; Speert et al., 1994; Govan and Deretic, 1996), do not or rarely (0 out of 72 B. cenocepacia, 0 out of 35 B. multivorans and 5 out of 37 B. vietnamiensis) carry the hmqABCDEFG operon. One possibility is that Bcc species more often found as clinical isolates could have lost the Hmq system through evolution and patient-to-patient selection (Coulon et al., 2019). Our data suggest that the Hmq system could play a beneficial role in niche adaptation to the rhizosphere microbial community due to the large prevalence of the hmqABCDEFG operon among B. ambifaria, B. cepacia, B. contaminans, B. pyrrocinia and B. ubonensis environmental strains, species known for their preference for the plant root environment (Balandreau et al., 2001; Mahenthiralingam et al., 2005; Vial et al. 2011; Vidal-Quist et al., 2014). The presence of the Hmq system in clinical strains of these five common environmental species is compatible with a recent acquisition in CF population (Huang et al., 2001; Mahenthiralingam et al., 2005; Loutet and Valvano, 2010). We can also consider the possibility that the presence of competitive
microorganisms could favor the production of HMAQs in both clinical and environmental strains - e.g.: S. aureus enhances the production of HAQs in P. aeruginosa (Michelsen et al., 2015).

Since phylogeny does not seem to explain the distribution of the $hmq\text{ABCDEFG}$ operon among Bcc species, we considered the hypothesis that the loss of the c3 replicon, sometimes observed in Bcc phenotypic variants (Agnoli et al., 2011), could be related to the absence of the Hmq system, but no correlation was found.

Among the strains having the $hmq\text{ABCDEFG}$ operon, not all produced HMAQs under the tested conditions. It is important to note that the limit of detection of molecules was 0.05 mg/L for each extract which might explain some false-negative results. However, for those strains producing HMAQs in TSB, growth in ASM did not inhibit HMAQs production and stimulated the production in ten additional strains, which could be explained by differences in regulation of the biosynthetic operon. Our results further enforce the need to use nutritionally appropriate media when doing experiments aimed at understanding clinical relevance. For this purpose, we tried to optimize a medium for HMAQs production. However, it was not possible to identify a simple carbon source due to the low production of the molecules in the minimal medium whatever the tested culture time (data not shown).

Lack of transcription of the $hmq\text{ABCDEFG}$ operon seemed to explain that 28% of Bcc strains carrying these genes did not produce HMAQs. Indeed, production could be obtained simply by changing the culture conditions, suggesting that the promotor of the $hmq\text{ABCDEFG}$ was not expressed, although we cannot exclude that it could be nonfunctional in select strains.

Future work will involve a better understanding of the nutritional and regulatory elements controlling the expression of the $hmq\text{ABCDEFG}$ operon and production of HMAQs.
Our results suggest that the Hmq system of Bcc is non-essential for pathogenicity but could be required for adaption to a particular environmental niche by, for example, influencing rhizosphere microbial community (Vial et al., 2008; Kilani-Feki et al., 2011, 2012; Mahenthiralingam et al., 2011; Patten et al., 2012; Chapalain et al., 2013, 2017; Gomes et al., 2018; Le Guillouzer, 2018; Jung et al., 2018; Mullins et al., 2019; Whalen et al., 2019; Piochon et al., 2020). It will be interesting to investigate a possible correlation between the presence of the \textit{hmqABCDEFG} in Bcc species and their evolutionary trajectory.

### Material and Methods

#### Strains and culture conditions

A total of 313 strains isolated from either clinical or environmental settings were used in this study and listed in Table S1). Uncertain identification was confirmed by amplifying and sequencing the of the \textit{recA} and \textit{gyrB} genes at the IRCM Sequencing platform (Montreal) following the protocol available on the PubMLST database (https://pubmlst.org/bcc/info/protocol.shtml; Table S9).

Strains were cultured in borosilicate tubes containing 3mL tryptic soy broth (TSB) from stocks frozen at -80°C in 15% glycerol and incubated at 30°C with 250 rpm rotative shaking overnight (~16h).

#### Detection of the presence of \textit{hmqABCDEFG} operon by PCR

Genomic DNA was extracted following a previously described method (Durand et al., 2015). Briefly, cells were resuspended in lysis buffer (50 mM Tris-HCL pH8, 5 mM EDTA-2Na pH8, 3% SDS) and transferred to a tube containing beads. The cells were lysed in a Fast-Prep-24
instrument (MP Biomedicals, USA). Then the mixes were centrifuged at 8,000 g for 5 min, and the supernatants were transferred to a new tube, and 2.5 N ammonium acetate was added after centrifugation. The supernatant was transferred to a new and one volume isopropanol was added. The pellets were washed by 75% ethanol and dried before to resuspended them into 50 µL water.

For *Burkholderia cepacia* Research Laboratory and Repository and Canadian *Burkholderia cepacia* complex Research and Referral Repository strains, genomic DNA was extracted using a 96-wells plate gDNA extraction kit (Favorgen, Canada).

The *hmqA* and *hmqG* genes were amplified by PCR using the EasyTaq polymerase (Transgen, Canada). Primers were designed based on a consensus sequence of 11 complete Bcc sequences ([Table S9](#))

A strain was considered to have a complete *hmqABCDEFG* operon when amplification for both *hmqA* and *hmqG* were obtained.

**Quantification of HMAQ production by LC/MS/MS**

Bcc strains were cultured in 5 mL tryptic soy broth (TSB) at a starting OD$_{600nm}$ of 0.05 and incubated at 30°C with shaking for an overnight. 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4, Sigma) was used as an internal standard. The total HMAQ were extracted from 4 mL culture with one volume of ethyl acetate. After nitrogen evaporation, the extracts were resuspended in 400 µL of HPLC-grade acetonitrile. Samples were analyzed by liquid chromatography coupled with a mass spectrometer (LC/MS) in positive electrospray ionization using a Kinetex 5 µM EVO C18 100 Å 100x3 mm reverse phase column as previously described by (Vial *et al.*, 2008). A Quattro Premier XE triple quadrupole was used as a detector (Waters). A full scan mode with a scanning range of 130 to 350 Da and a multiple reaction
monitoring (MRM) program were used to detected HMAQ families based on (Vial et al., 2008). This experiment was conducted with three independent biological replicates.

Artificial Sputum medium (ASM) and Tryptic soy Agar (TSA) medium assay

The strains were grown in ASM and TSA plate out from overnight cultures and incubated at 30°C for 24h and four days, respectively.

One mL of ASM culture were extracted as previously described in HMAQ production method. For each TSA plate, 5 mL water was added to be able to extract HMAQs from the agar. For each sample, 1 mL was extracted by one volume ethyl acetate containing 4 ppm HHQ-D4, concentrated 10 times, resuspended in HPLC-grade acetonitrile and analyzed as previously described in HMAQ extraction method.

The experiments were performed in two independent biological replicates.

Detection of the expression of the hmqABCDEFG operon by RT-PCR

Total RNA was extracted from cultures grown in TSB to an OD600 of 3.0 using Transzol (Transgene, Canada) by following the manufacturer’s instruction. Residual DNA was removed using the Turbo DNAse (Thermo Fisher, Canada). Reverse-transcription was performed using the I-Script kit (BioRad, Canada). The expression of the hmqABCDEFG operon gene was determined by PCR targeting the hmqA and ndh as a reference gene (Table S9) (Subsin et al., 2007).

To determine the hmqA gene primers, a semi-random PCR was performed on genomic DNA from B. cepacia VC13394, B. contaminans VC15406, B. vietnamiensis VC8245 and B. vietnamiensis VC9237 using the two specific primers (hmqA_semirandom_3F and
hmqA_semirandom_2F) and sequencing of the PCR product using a third primer (hmqA_semirandom_F; Table S3) by following the protocol of Jacobs et al. (2003).

The link of the presence of the hmqABCDEFG operon and the production of HMAQ in Bcc with different characteristics

Based on our qualitative data (Tables 1 and S7), we studied the correlation by Fisher’s exact test for count data using R software (http://www.R-project.org; (Team, 2018)).

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### Table 1. Correlation of the presence of the *hmqABCDEFG* operon and the production of HMAQ in clinical Bcc with sample data

| Presence of the *hmqABCDEFG* operon | Co-isolation with *P. aeruginosa* (previous year) | Co-localization with *P. aeruginosa* | Origin of sample* |
|-------------------------------------|-----------------------------------------------|-------------------------------------|------------------|
| Presence of the *hmqABCDEFG* operon | 0.34                                          | 0.41                                | 0.11             |
| Production of HMAQs                | 0.57                                          | 1                                   | 0.59             |

*Possible origins: sputum, respiratory, throat, or sinus*

Raw data presented in Table S5. *P*-values are representative of the correlation and are considered significant under 0.05.
Table 2. Production of HMAQs in ASM and TSA for strains not producing HMAQs in TSB

| Strains              | Type          | HMAQs production |
|----------------------|---------------|------------------|
|                      |               | TSB  | ASM  | TSA  |
| *B. ambifaria* AMMD  | Environmental | -    | +    | +    |
| *B. ambifaria* AU7994| Clinical      | -    | -    | -    |
| *B. ambifaria* CEP1231| Clinical   | -    | +    | -    |
| *B. ambifaria* HI2425| Environmental | -    | +    | +    |
| *B. ambifaria* VC11631| Clinical    | -    | -    | -    |
| *B. anthina* VC15382 | Clinical      | -    | -    | -    |
| *B. cepacia* ATCC25416| Environmental| -    | -    | -    |
| *B. cepacia* VC13196 | Clinical      | -    | -    | -    |
| *B. cepacia* VC13575 | Clinical      | -    | -    | -    |
| *B. cepacia* VC19225 | Clinical      | -    | +    | +    |
| *B. contaminans* VC15406| Clinical    | -    | -    | +    |
| *B. contaminans* VC16897| Clinical   | -    | -    | -    |
| *B. contaminans* VC16948| Clinical    | -    | -    | +    |
| *B. diffusa* VC14008 | Clinical      | -    | -    | -    |
| *B. dolosa* LMG21443 | Environmental| -    | -    | -    |
| *B. lata* VC6377     | Clinical      | -    | +    | +    |
| *B. metallica* ES0559| Environmental| -    | -    | -    |
| *B. pyrocinia* Bcc indeterminate 9 ES0209 | Environmental | -    | +    | +    |
| *B. seminalis* HI2490 | Environmental | -    | +    | -    |
| *B. stagnalis* Bcc indeterminate 6 HI3537 | Environmental | -    | +    | +    |
| *B. stagnalis* HI2720 | Environmental | -    | +    | -    |
| *B. ubonensis* LMG24263| Clinical     | -    | -    | -    |
| *B. vietnamensis* CEP0040 | Clinical   | -    | -    | -    |
| *B. vietnamensis* HI3392 | Environmental| -    | +    | +    |
| *B. vietnamensis* VC17180 | Clinical    | -    | -    | -    |
| *B. vietnamensis* VC9237 | Clinical    | -    | -    | -    |
Table 3. The expression of the hmqA gene in the main species of Bcc strains having the hmqABCDEFG operon but which do not produce HMAQs.

| Strains          | Type          | HMAQs production in TSB | Expression of hmqA |
|------------------|---------------|-------------------------|---------------------|
| B. ambifaria AMMD | Environmental | -                       | -                   |
| B. ambifaria HSJ1 | Clinical      | +                       | +                   |
| B. cepacia ATCC25416 | Environmental | -                       | -                   |
| B. cepacia VC13394 | Clinical      | +                       | +                   |
| B. contaminans FFH2055 | Clinical     | +                       | +                   |
| B. contaminans VC15406 | Clinical    | -                       | -                   |
| B. vietnamiensis VC8245 | Clinical     | +                       | +                   |
| B. vietnamiensis VC9237 | Clinical   | -                       | -                   |
Figure 1. Bcc strains screening for the \textit{hmqABCDEFG} operon presence in their genome. (A) The distribution of environmental and clinical Bcc strains investigated in this study (B) Distribution of the \textit{hmqABCDEFG} operon within tested Bcc species.
**Figure 2.** Phylogeny of the Bcc species based on MLST genes (*atpD, gldB, gyrB, recA, lepA, phaC and trpB*). The tree was generated by RAxML and is the concatenated MLST sequences (typically of the type strain for a given species) using GTRGAMMA model and 1000 bootstraps. The branches are labelled where bootstrap values are >50%. The presence of the *hmaABCDEFG* operon is correlated between the bioinformatic (Coulon *et al.*, 2019) and PCR methods (Kendall rank test with a p-value of 0.01958, inferior to 5%).
Figure 3. Distribution of the HMAQ production in Bcc. (A) The distribution of environmental and clinical Bcc strains regarding their ability to produce HMAQs (B) Distribution of HMAQ producing-Bcc species. HMAQs have been quantified by LC/MS with a limit of detection of 0.05 mg/L for each molecule in the total culture.