Centre Armand-Frappier Santé - Biotechnologies

L'OPÉRON *hmqABCDEFG* ET SA RÉGULATION, IMPLIQUÉS DANS LA BIOSYNTHÈSE DES 4-HYDROXY-3-MÉTHYL-2-ALKYLQUINOLINES CHEZ LE COMPLEXE *BURKHOLDERIA CEPACIA*

Par
Pauline M.L. Coulon

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Président du jury et examinateur interne	Professeur Charles Dozois Centre Armand-Frappier Santé - Biotechnologies – Institut National de la Recherche Scientifique
Examinateur externe	Professeur Roger Lévesque Département de microbiologie-infectiologie et d'immunologie Institut de biologie Intégrative et des systèmes Université de Laval
Examinateur interne	Docteur Ludovic Vial Département de Biologie Université Claude Bernard Lyon 1
Directeur de recherche	Professeur Eric Déziel Centre Armand-Frappier Santé - Biotechnologies – Institut National de la Recherche Scientifique

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RÉSUMÉ

La communication intercellulaire chez les bactéries, souvent nommée *quorum sensing* (QS), permet la modulation de l'expression de gènes impliqués dans la survie et l'interaction avec un hôte en réponse à la densité de la population. Chez les bactéries appartenant au complexe des *Burkholderia cepacia* (Bcc), de multiples systèmes de QS sont prédits, cependant notre compréhension reste incomplète. Le groupe de Bcc est composé de plus de 26 espèces environnementales dont certaines sont des pathogènes opportunistes majoritairement retrouvés chez les patients immunodéprimés et chez les individus atteints de fibrose kystique.

Mon projet de doctorat portait sur la caractérisation du système Hmq, responsable pour la synthèse d'une famille de molécules de la famille des 4-hydroxy-3-méthyl-2-alkylquinolines (HMAQs) prédites pour agir comme molécules de signalisation chez certaines Bcc. Même si le système Hmq n'appartient pas au QS, il existe un lien étroit entre le QS et le système Hmq. En effet, les HMAQs inhibent le QS et le QS active le système Hmq chez les Bcc. Le rôle primaire des HMAQs restant à être déterminé, j'ai montré que les HMAQs synthétiques ont un effet antifongique contre les levures et champignons et que les NOHMAQs synthétiques ont une activité antimicrobienne plus forte contre les bactéries à Gram positif. En ce qui concerne la prévalence du système Hmq chez les Bcc évaluée par analyses bioinformatiques et expérimentalement, j'ai montré que 1/3 des Bcc possèdent le système Hmq transmis horizontalement. En criblant par PCR – et en quantifiant la production de HMAQs par LC-MS chez une collection de 312 souches, j'ai montré que le système Hmq est plus prévalent chez les souches environnementales que les souches cliniques. Les Bcc *Burkholderia cenocepacia* et *B. multivorans* – pathogènes opportunistes prédominants – ne possèdent pas le système Hmq alors que cet opéron est couramment trouvé chez *B. ambifaria, B. cepacia* et *B. contaminans* – espèces environnementales et pathogènes opportunistes émergents. En étudiant l'effet du système Hmq sur la croissance du pois*, Pisum sativum,* j'ai montré que le système Hmq qui augmente la croissance de racines, soit en utilisant directement les HMAQs comme antimicrobien en modulant la rhizosphère soit indirectement, via le QS en contrôlant différents antimicrobiens, en contrôlant des gènes impliqués dans la nodulation des plantes ou bien encore en favorisant la croissance des racines en présence de nitrate.

Les Bcc modulent leur virulence soit par la variation de phase en régulant l'expression des gènes (variants), soit en éliminant leur mégaplasmide de virulence (pc3-null). En étudiant les protéomes des deux types de variants chez *B. ambifaria*, j'ai montré que les gènes codants pour les principaux facteurs de virulence et localisés sur le pc3 sont sous-exprimés. Chez *B. ambifaria* CEP0996 pc3-null (ayant perdu son pc3), les différences phénotypiques observées dont la formation de biofilm, la motilité, la production de sidérophores semblent être liées à la perte du régulateur transcriptionnel ShvR appartenant à la famille LysR et contrôlant plus de 1000 gènes, dont 13 en commun avec le QS, et codant pour des facteurs de virulence tels que la formation de biofilm, la motilité et les molécules antimicrobiennes dont potentiellement les HMAQs. Chez le variant de *B. ambifaria* HSJ1, aucune variation de production de AHLs et changements génomiques n'ont été observés. La méthylation de l'ADN pouvant être impliquée dans la variation de phase, en modulant l'expression génique, j'ai étudié les méthylomes de *B. ambifaria* HSJ1 sauvage et variant et montré que le variant est plus méthylé que la souche sauvage. Ce résultat indique que la méthylation de l'ADN pourrait être le mécanisme sous-jacent de la sous expression des différents groupes de gènes détectés par analyse protéomique dont celui codant pour le régulateur transcriptionnel ShvR.

Mot-clefs : complexe des *Burkholderia cepacia*, système Hmq, 4-hydroxy-3-méthyl-2-alkylquinolines (HMAQs), quorum sensing, promotion de la croissance des plantes, variation de phase, méthylation de l'ADN, virulence

ABSTRACT

Quorum sensing (QS), the modulation of gene expression in response to cell-population density, has a crucial role in survival and bacterial pathogenesis. Within the *Burkholderia cepacia* complex (Bcc), multiple QS systems have been predicted, yet our understanding of these is still incomplete. Bcc comprises at least 26 environmental species, some of which are opportunist pathogens and are found within immunosuppressed patients and people suffering from cystic fibrosis (CF).

My Ph.D. project focused on characterizing the Hmq system, a homolog of the *P. aeruginosa* PQS system, which we predicted would produce the signaling molecules 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) to regulate virulence in Bcc. The Hmq system is not a QS system *per se* but rather a system with to and for interplay. The HMAQs inhibit the QS while the QS activates the Hmq system within Bcc. The primary role of the Hmq system is still unknown. However, I have demonstrated that synthetics HMAQs and HMAQNOs have an antimicrobial property against fungi for HMAQs and bacteria and fungi for HMAQNOs with a higher efficiency against Gram-positive bacteria.

By assessing the Hmq pathway conservation using bioinformatics analysis and experimentally, I found 1/3 of Bcc possesses this Hmq system. By screening 312 Bcc strains - by PCR - and quantifying the HMAQs produced using LC-MS under different conditions, I have shown that the Hmq system is more prevalent in environmental isolates than clinical ones. Interestingly, *B. cenocepacia* and *B. multivorans* lack the Hmq system, while emerging opportunist species such as *B. ambifaria, B. cepacia,* and *B. contaminans* are the principal carriers. By investigating the effect of the Hmq system on plant growth-promoting, I showed that the Hmq system plays a role in root growth promotion in *Pisum sativum* by (1) potentially using HMAQs as antimicrobial molecules to modulate the rhizosphere or (2) modulating the main Cep QS system resulting in modulating different virulence factors such as other antimicrobial molecules, by controling genes involved in the plant nodulation or by favorizing the growth of roots in presence of nitrates.

Bcc modulate virulence by producing variant colonies through phase variation that regulates gene expression (variant) and genomic variation by ejecting their third chromosome or megaplasmid (pc3-null). By investigating the proteomes of two *B. ambifaria* model strains focusing upon genes encoding the main virulence factors located on the pc3. I found they are downregulated in both variants. In *B. ambifaria* CEP0996 pc3-null (variant having lost its pc3), observed phenotypes such as biofilm formation, motility, and siderophore production, seen to link the loss of the LysR family transcriptional regulator ShvR, known to control over 1000 genes (13 common genes with the QS system) and virulence factors in *B. cenocepacia* – resulting in differences in phenotypes such siderophores, motility, antimicrobial molecules such as HMAQs. Concerning the *B. ambifaria* HSJ1 variant, I have demonstrated that the difference in observed phenotypes between *B. ambifaria* HSJ1 wildtype and variant is not due to a difference in genomes or acyl-homoserine lactones (AHLs) production. DNA methylation being involved in bacterial phase variation by regulating gene expression, I have studied methylomes and showed that *B. ambifaria* HSJ1 variant is more methylated than the wild type. This result suggests that DNA methylation could be involved in phase variation by regulating virulence factors (for which a difference in the production of proteins was observed by proteomics analysis) mainly by modulating the transcriptional expression of *shvR*.

Keywords : *Burkholderia cepacia* complex, Hmq system, 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs), quorum sensing, plant growth promoting, phase variation, DNA methylation, virulence.

TABLE DES MATIÈRES

LISTE DES FIGURES

LISTE DES TABLEAUX

LISTE DES ABRÉVIATIONS

3

3OHC$_6$-HSL : N-(3-oxo-hexanoyl)-homosérine lactone
3-oxo-C$_6$-HSL : N-(3-oxohexanoyl)-homoserine lactone

6

6mA : adénine méthylée en N6

A

ABC : ATP-binding cassette
AHLs : N-acyl-homosérine lactones
AHQs : 4-hydroxy-2-alkylquinolines
AstR : Adhesion and type six secretion system regulator
ATP : adénosine-tri-phosphate

B

BDFS : *Burkholderia* diffusible signal factor
BSCA : milieu d'agar sélectif pour *B. cepacia*

C

c1 : premier chromosome
c2 : deuxième chromosome
C$_6$-HSL : N-hexanoyl-L-homosérine lactone
C$_8$-HSL : N-octanoyl-L-homosérine lactone
Cci : *B. cenocepacia* Pathogenicity Island
CF : cystic fibrosis
CGD : chronic granulomatous disease
c-di-GMP : Di-guanosine monophosphate cyclique

D

DNA MTase : ADN méthyltransférase

E

EPS : exopolysaccharides

F

FAD : flavin adenine dinucleotide
FK : fibrose kystique

G

GSC : granulomatose septique chronique

H

HMAQs : 4-hydroxy-3-méthyl-2-alkylquinolines

L

LB : Bouillon de culture lysogénique
LPS : lipopolysaccharides

M

MHA : Mueller-Hinton agar
MOPS : 3-(*N*-morpholino)propanesulfonic acid
MvfR : multiple virulence factor regulator

N

NOHMAQs : HMAQs N-oxydes

P

pc3 : troisième chromosome
PDA : potato dextrose agar
PQS : *Pseudomoas* Quinolone signal

Q

QS : quorum sensing

S

SARM : *Staphylococcus aureus* résistante à la méthicilline
ScmR : *secondary metabolite regulator*
ShvR : *shiny variant colony regulator*
SNP : polymorphisme du nucléotide simple

T

TEM : transmission electronic microscopy

1. INTRODUCTION

1.1. Le genre *Burkholderia*, des β-protéobactéries ubiquitaires à deux visages

Le genre *Burkholderia* est composé de plus de 60 espèces bactériennes à Gram négatif venant de niches écologiques diverses. Cependant plusieurs espèces ont été reclassifiées dans les nouveaux genres *Paraburkholderia, Caballeronia, Trinickia, Mycetohabitans* et *Robbsia*. Le groupe des *Burkholderia* sensu stricto composés des espèces pathogènes des plantes et des mammifères, comprenant les espèces pathogènes des plantes, le groupe Bcc et le groupe « *pseudomallei* » [**Figure 1.1A** ;(Angus *et al.*, 2014; Dobritsa *et al.*, 2017; Dobritsa & Samadpour, 2016; Estrada-de Los Santos *et al.*, 2018; Lopes-Santos *et al.*, 2017; Suárez-Moreno *et al.*, 2012; Wallner *et al.*, 2019)].

1.1.1 Les différents groupes d'espèces composant le genre *Burkholderia*

1.1.1.1 Les nouveaux genres *Robbsia, Mycetohabitans, Trinickia, Paraburkholderia* et *Caballeronia*

Les espèces de *Burkholderia* bénéfiques aux plantes et à l'environnement ont été reclassifiées dans le genre *Paraburkholderia* (Sawana *et al.*, 2014). Cette séparation de genre est basée sur (1) l'analyse de 22 marqueurs moléculaires hautement spécifiques basés sur des séquences d'insertion-délétion conservées ; (2) le %GC – les *Burkholderia* ont un %GC supérieur à 65%, contrairement aux *Paraburkholderia* dont leur %GC est inférieur à 65% ; (3) l'avirulence chez *Caenorhabditis elegans* (Angus *et al.*, 2014; Sawana *et al.*, 2014).

Cependant, cette nouvelle classification est controversée, car certaines espèces environnementales connues pour être des phytopathogènes ont été génétiquement classifiées en tant que *Paraburkholderia* (*P. andropogonis, P. caryophylli, P. soli, P. endofungorum, P. rhizoxinica, P. sordidicola* et *P. glathei* [**Figure 1.1B ;** (Eberl & Vandamme, 2016; Wallner *et al.*, 2019)]).

Le genre *Caballeronia* représente le clade entre le genre *Bukholderia* et *Paraburkholderia* avec une signature moléculaire spécifique utilisée par Sawana *et al.* (2014) (Dobritsa & Samadpour, 2016).

Les genres *Mycetohabitans* et *Trinickia* ont été déterminés par l'alignement de leurs séquences génomiques, puis par l'analyse de séquences des gènes impliqués dans la diazotrophie et la nodulation, ainsi que ceux impliqués dans la pathogénicité (e.g. système de sécrétion de type III et IV). Ce deux genres sont respectivement composés de symbionts fongiques ayant des petits génomes et de diverses bactéries du sol et associées aux plantes (Estrada-de Los Santos *et al.*, 2018).

Enfin, le genre *Robbsia* est compose de l'espèce *Robbsia andropogonis* qui forme un clade distinct lors de l'analyse phylogénétique basées sur 30 séquences de gènes conservés (Lopes-Santos *et al.*, 2017).

1.1.1.2 Les espèces phytopathogènes

Les espèces phytopathogènes sont divisées en deux clades : les *Paraburkholderia* et les *Burkholderia*.

Celles appartenant au genre *Paraburkholderia* - *P. andropogonis, P. caryophylli, P. soli, P. endofungorum, P. rhizoxinica, P. sordidicola* et *P. glathei* - sont pathogènes de plantes rarement retrouvés au sein des infections humaines ; comme *P. andropogonis* qui est un agent causal de bandes bactériennes chez le *Sorghum* et le maïs (Ramundo & Claflin, 2005; Sawana *et al.*, 2014).

Les phytopathogènes appartenant au clade des *Burkholderia* – *B. glumae, B. gladioli* et *B. plantarii* - sont très connus comme ravageurs du riz (**Figure 1.2**) retrouvés, par exemple, en Chine et aux États-Unis (Azegami *et al.*, 1985; Ham *et al.*, 2010; Nandakumar *et al.*, 2009). Les espèces *B. glumae* et *B. plantarii* ne sont pas retrouvées chez l'Homme, contrairement à *B. gladioli* (Imataki *et al.*, 2014; Kennedy *et al.*, 2007; Segonds *et al.*, 2009), cependant elles sont toutes les trois pourvues des systèmes de sécrétion de type III et de type VI, les rendant capables d'infections animales, ce qui les différencie des *Paraburkholderia* (Angus *et al.*, 2014; Zhou-qi *et al.*, 2016).

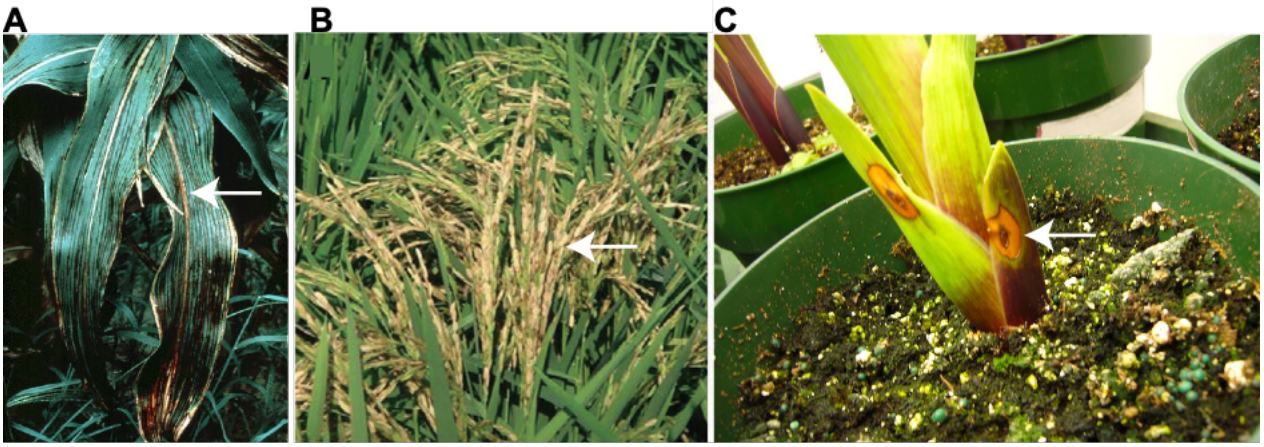


Figure 1.2 Exemple de plantes infectés par des espèces de *Burkholderia* et *Paraburkholderia*
A) *P. andropogonis* infecte les plantes de *Sorghum* ou encore le maïs provoquant des lésions et la nécrose des feuilles, des graines et des tiges (Plantwise knowlegde bank). B) *B. glumae* infecte le riz provoquant une pourriture molle des gaines foliaires, brunissement et imbibition d'eau, accompagnée d'un flétrissement ou d'une pourriture molle des feuilles (image tirée de Ham *et al.*, [2010]). C) *B. gladiolis* infecte les plantes bulbaires entrainant la nécrose des feuilles en forme de cercle, le dépérissement des tiges ainsi que des lésions des organes végétatifs (Plantwise knowlegde bank).

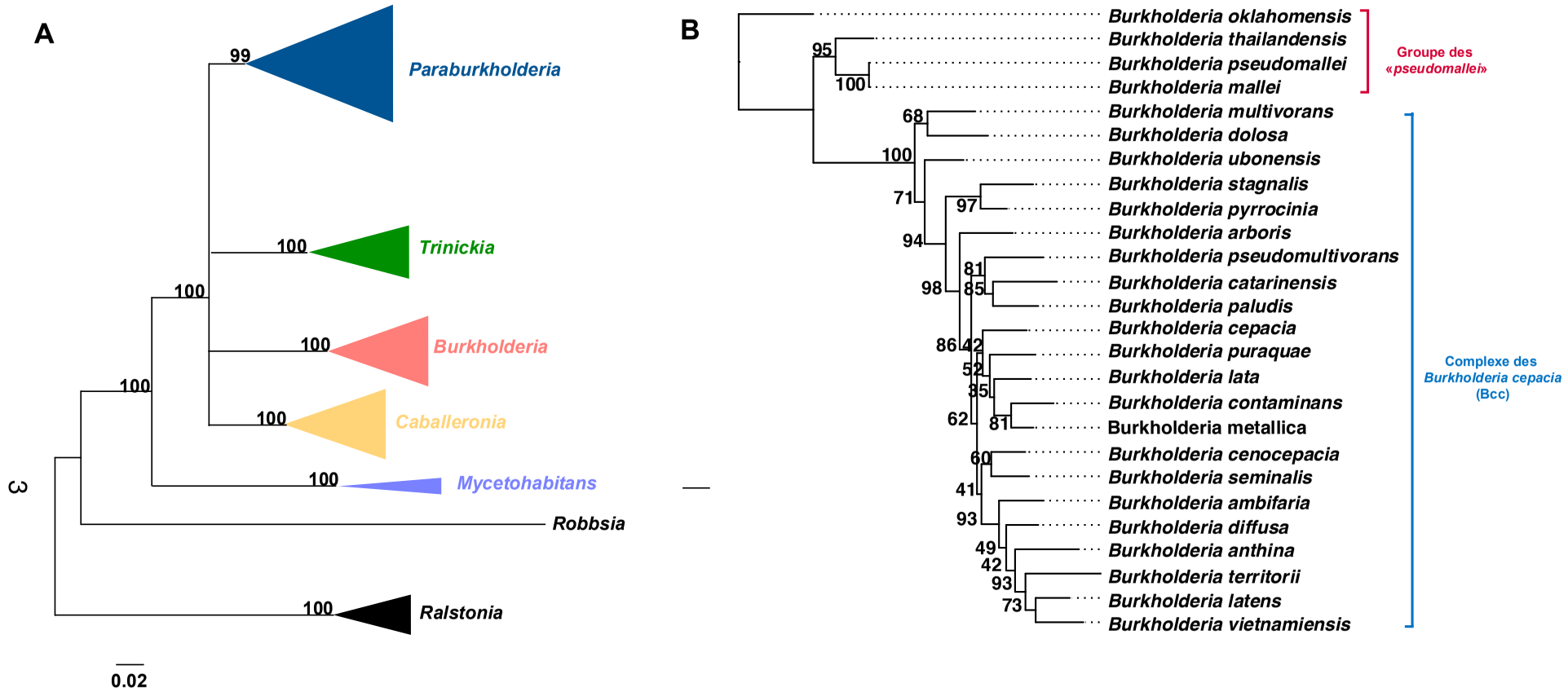


Figure 1.1 Arbre phylogénétique du genre *Burkholderia,* récemment divisé en plusieurs groupes : *Burkholderia sensu stricto*, *Paraburkholderia, Caballeronia, Trinickia, Mycetohabitans et Robbsia.*

A) Nouvelle classification du genre *Burkholderia* basée sur la séquence 16S ARN ribosomal, ainsi que les séquences en acides aminés des protéines impliquées dans la diazotrophie et la pathogenecité. Illustration modifiée de Estrada-de Los Santos *et al.* (2018). B) Classification des espèces au sein du genre *Burkholderia sensu stricto* basée sur les séquences MLST (*gltB, gyrB, lepA, phaC, recA, trpB).*

1.1.1.3 Les espèces appartenant au groupe « *pseudomallei* » sont pathogènes

En plus des pathogènes des plantes, il existe le groupe des « *pseudomallei* » composé de *B. pseudomallei, B. mallei, B. thailandensis*, et *B. oklahomensis*.

B. pseudomallei est un pathogène retrouvé dans les zones humides d'Asie du Sud et du nord de l'Australie causant la mélioïdose chez l'Homme (Chaowagul *et al.*, 1989; Smith *et al.*, 1987). *B. mallei* est un pathogène intracellulaire obligatoire provoquant la maladie de la morve chez les équidés (Howe, 1950; Howe & Miller, 1947; Sandford, 1990). *B. mallei* est une espèce dérivée de *B. pseudomallei* – avec 99% d'identité nucléotidique – qui a subi un réarrangement génomique et une perte de 20% de son génome (Nierman *et al.*, 2004; Song *et al.*, 2010). *B. thailandensis* est une bactérie environnementale, non pathogène à l'Homme – mais pouvant dans de rares cas causer des infections (Brett *et al.*, 1998). Provenant de la même niche écologique que *B. pseudomallei*, *B. thailandensis* est considérée comme un modèle d'étude par sa haute similarité (85% des gènes conservés ; [Haraga *et al.*, 2008; Lertpatanasuwan *et al.*, 1999; Ngamdee *et al.*, 2015; Yu *et al.*, 2006]). *B. oklahomensis*, initialement isolée à partir d'une blessure contaminée par du sol, a longtemps été confondu avec *B. pseudomallei*. Néanmoins, cette espèce est encore moins virulente que *B. thailandensis* chez la souris et le hamster (DeShazer, 2007; Glass *et al.*, 2006).

Basés sur des comparaisons biochimiques, immunologiques et génétiques, nous savons que le groupe *« pseudomallei »* est proche du groupe Bcc (Woods & Sokol, 2006).

1.1.1.4 Les espèces appartenant au complexe *Burkholderia cepacia* pouvant être des pathogènes opportunistes

Le groupe Bcc est constitué d'au moins 22 espèces bactériennes (*Burkholderia ambifaria, Burkholderia anthina, Burkholderia arboris, Burkholderia catarinensis, Burkholderia cenocepacia, Burkholderia cepacia, Burkholderia contaminans, Burkholderia diffusa, Burkholderia dolosa, Burkholderia lata, Burkholderia latens, Burkholderia multivorans, Burkholderia paludis, Burkholderia pseudomultivorans, Burkholderia pyrrocinia, Burkholderia puraquae, Burkholderia mettalica, Burkholderia seminalis, Burkholderia stagnalis, Burkholderia territorii, Burkholderia ubonensis* et *B. vietnamiensis* ; **Figure 1. 1B**) provenant généralement de la rhizosphère, du sol ou de l'eau (Eberl & Vandamme, 2016; Loveridge *et al.*, 2017). Le séquençage du gène codant pour la protéine ribosomale 16S et/ou du gène *recA* a permis d'identifier la majorité des espèces de Bcc. Cependant il y a des défis d'affiliation inter-espèces mais aussi intra-espèces. Depuis quelques années, l'identification *« multilocus sequence typing »* (MLST) a permis de séparer spécifiquement les différentes souches au sein d'une espèce. Les Bcc sont principalement séquencées en Australie (423 isolats), au Canada (341 isolats) et aux États-Unis (500 isolats ; **Figure 1.3**).

Burkholder (1950) a découvert et décrit la première souche de *B. cepacia* – anciennement *Pseudomonas cepacia* - comme étant un pathogène de l'oignon. Depuis, il a été montré que certaines souches de Bcc peuvent aussi avoir des intérêts d'utilisation dans les domaines de l'industrie et de l'agriculture pour leur potentiel : (1) de bio-remédiation – par fixation de l'azote ou dégradation du trichloroéthylène au niveau de la rhizosphère du blé [e.g. : *B. vietnamiensis (Gillis et al., 1995; O'Sullivan & Mahenthiralingam, 2005)*] – (2) bio-contrôle de croissance des plantes – protection des pois contre les champignons *Pythium* et *Aphanomyces* [e.g. : *B. ambifaria (Parke, 1991)*] – et enfin, (3) de synthèse d'un grand nombre d'antibiotiques (Govan & Deretic, 1996).

Les bactéries appartenant aux Bcc ont été pendant longtemps répandues dans les champs agricoles afin de bénéficier de leurs vertus bio-fertilisantes. Cependant une émergence de certains Bcc a été détectée chez (1) les patients immunodéprimés - et plus particulièrement ceux atteints de la granulomatose septique chronique (GSC) – (2) les nouveau-nés, mais aussi chez (3) les individus atteints de fibrose kystique (**Figure 1.4** ; [Gilligan, 1991; Govan & Deretic, 1996; LiPuma, 1998; Speert *et al.*, 1994]). Par exemple, *B. cenocepacia*, *B. multivorans* et *B. vietnamiensis* sont majoritairement isolées chez les patients atteints de FK (Gilligan, 1991; Govan & Deretic, 1996; Speert *et al.*, 1994) contrairement à *B. ambifaria, B. cepacia, B. contaminans, B. pyrrocinia* et *B. ubonensis* qui sont majoritairement isolées dans l'environnement (**Figure 1.4** ; [Balandreau *et al.*, 2001; Cipolla *et al.*, 2018; Mahenthiralingam *et al.*, 2005; Vial *et al.*, 2011; Vidal-Quist *et al.*, 2014]).

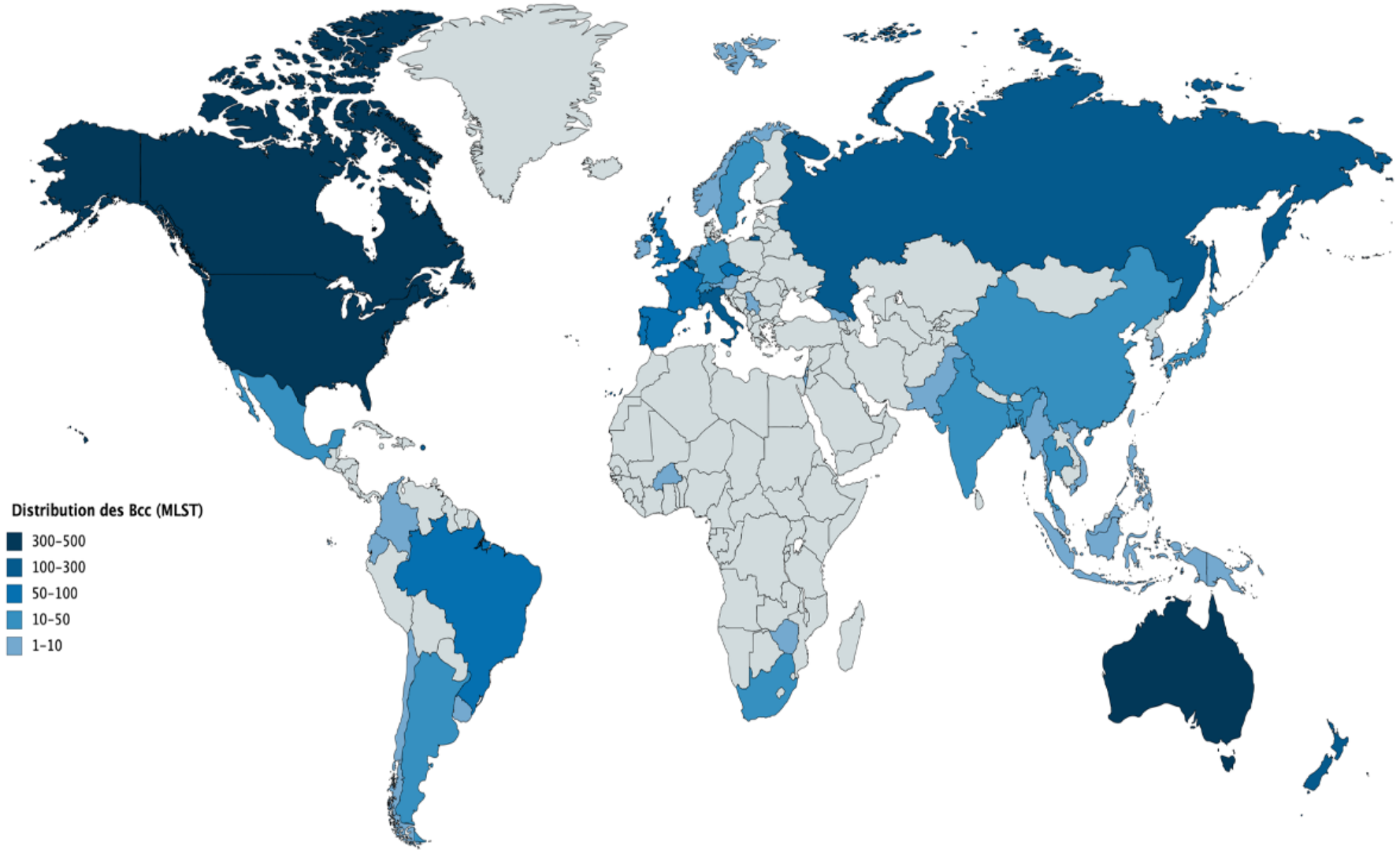


Figure 1.3 Distribution dans le monde des différents isolats du complexe *Burkholderia cepacia* basée sur les séquences disponibles

Carte mondiale représentant la distribution de l'identification et du séquençage des Bcc. Données de 2018, de l'origine des souches valables sur http://www.pubmlst.org

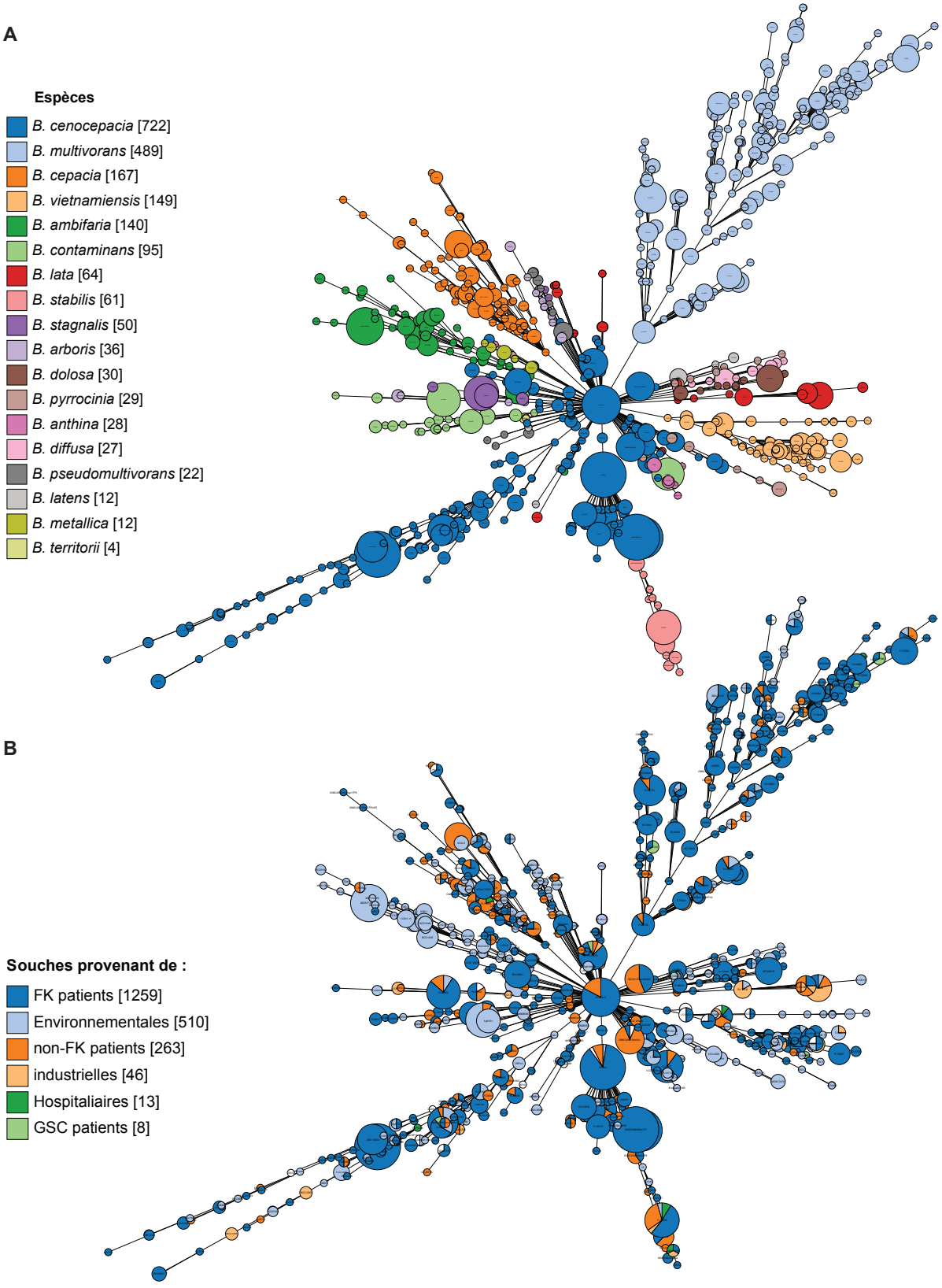


Figure 1.4 Distribution des différents isolats appartenant au complexe *Burkholderia cepacia* en fonction de leurs espèces et origines

Représentation de type arbre à grappes basée sur l'identification de type MSLT ; taille de noeud =336% Kurtosis 100% (Zhou *et al.*, 2018). A) Les espèces sont différenciées par la couleur, un nœud peut comprendre plusieurs isolats, les branches ont leur taille réelle. B) La source de chaque isolat est différenciée par la couleur, un nœud peut comprendre plusieurs isolats, les branches ont leur taille réelle. La grosseur des cercles est proportionnel au nombre de souches dans chaque noeud.

La colonisation des poumons par les Bcc se fait en général après *Staphylococcus aureus* résistante à la méthicilline (SARM) et *P. aeruginosa* (**Tableau 1.1** ; [Fondation, 2018]). Les symptômes liés à une infection par les Bcc étant peu connus, le diagnostic de l'infection est difficile. L'infection provoque soit (1) une détérioration lente de la fonction respiratoire, soit (2) une nécrose pulmonaire se transformant en septicémie et entrainant le décès du patient – connue comme le « *cepacia syndrome* ». La sévérité de l'infection dépendra de si la souche est plus ou moins virulente et hautement résistantes aux antibiotiques (Gold *et al.*, 1983; LiPuma *et al.*, 2001; Mahenthiralingam *et al.*, 2001; Thomassen *et al.*, 2015).

Tableau 1.1 Prévalence des microorganismes chez les patients atteints de fibrose kystique

Bactéries	Infection chez les patients FK (%)	Moyenne de l'âge de la première infection (années)	Caractéristiques des bactéries chez les patients FK
P. aeruginosa	44,6	5,2	•Principale cause d'infection du système respiratoire •Associé avec la détérioration de la fonction pulmonaire •17,9% des souches sont multi-drogues résistantes
Bcc	2,4	19,4	•Petite proportion des patients FK infectés •Peu causer une détérioration rapide de la fonction pulmonaire •Multi-drogues résistantes
SARM	25,2	11,1	•Prévalence chez les personnes avec ou sans FK •Multi-drogues résistantes •Souches provenant des centres hospitaliers et des communautés
Stenotrophomonas maltophila	12,6	9,4	•Retrouvé dans l'eau, le sol, les plantes, les animaux et les environnements hospitaliers •Souvent Multi-drogues résistantes
Achromobacter xylosoxidans	5,8	13,8	•Retrouvé des environnements naturels (sol et eau) •Souvent Multi-drogues résistantes
Mycobacteria non-tuberculeuses	12,6	20,7	•Retrouvé dans l'eau et le sol •Transmission entre patients •Traitement lourd et peu toléré

Adapté du report annuel des données 2017 de la Fondation fibrose kystique (États-unis ; Fondation, 2018)

Contrairement à *P. aeruginosa*, la transmission des Bcc entre patients atteints de FK est possible et il existe aussi des transmissions indirectes via la contamination de l'environnement (aérosols de toux, mains mal lavées, porteur sain, *etc*… [Blanchard *et al.*, 2020; Bodilis *et al.*, 2018; Burns, 2001; Doring *et al.*, 1996]).

1.1.2. Leur composition génomique : deux chromosomes et un mégaplasmide ou chromide de virulence spécifique au complexe *Burkholderia cepacia*

Le génome des Bcc est composé de plusieurs chromosomes dérivé d'un seul chromosome ancestral qui se serait divisé au cours de l'évolution. Les génomes sont donc composés : (1) d'un « *core* » génomique (constitué de gènes retrouvés dans toutes les souches) et (2) d'un « *pan* » génomique (constitué de gènes retrouvés dans au moins une souche ; [Tettelin *et al.*, 2005]). Chez *Burkholderia,* 1000 gènes composent le génome dit « de base » et au moins 40 000 gènes appartiennent au pangénome évoluant plus vites que les gènes essentiels (Cooper *et al.*, 2010; Ussery *et al.*, 2009).

Les espèces appartenant au Bcc ont un génome constitué de deux chromosomes principaux : les gènes essentiels seraient majoritairement regroupés sur le premier chromosome (c1) et les gènes non essentiels se regrouperaient quant à eux, sur le deuxième chromosome (c2) contenant quelques gènes essentiels (Egan *et al.*, 2005). Les Bcc ont un troisième chromosome (pc3; **Tableau 1.2**). Le 3ème réplicon, ou pc3, a été redéfini comme étant un mégaplasmide de virulence, stabilisé par la présence de 18 gènes appartenant au génome dit « de base » d'où sa description en tant que chromide (Agnoli *et al.*, 2011; diCenzo *et al.*, 2019). Le taux d'accumulation de nouveaux gènes dans le pc3, par transferts horizontaux, est supérieur à celui de c1 et c2 - ce qui leur permet une diversité fonctionnelle entre les espèces de Bcc et une meilleure adaptation aux nouveaux environnements. Par ailleurs, le pc3 n'est pas essentiel de par (1) sa perte spontanée en générant des variants phénotypiques - e.g. chez *B. cenocepacia*, *B. stabilis* et *B. ubonensis* – et (2) la possibilité de le supprimer expérimentalement chez au moins sept espèces de Bcc (Agnoli *et al.*, 2011; Price *et al.*, 2017).

Tableau 1.2 Composition génomique de quelques souches de *Burkholderia* dont les génomes complets sont disponibles sur la base de données http://www.*Burkholderia*.com

Groupes	Souches	Chromosomes	Taille (Mb)	Gènes
Groupe de Bcc	*B. ambifaria* AMMD	C1	3,55	3 223
		C2	2,64	2 275
		pc3	1,28	987
	B. cenocepacia J2315	C1	3,87	3 628
		C2	3,21	2 857
		pc3	0,87	782
	B. dolosa AU0158	C1	3,40	3 099
		C2	2,16	1 860
		C3	0,83	682
	B. lata 383	C1	3,69	3 339
		C2	3,58	3 131
		pc3	1,39	1 173
	B. multivorans ATCC 17616	C1	3,44	3 224
		C2	2,47	2 169
		C3	0,91	840
	B. vietnamiensis G4	C1	3,65	3 387
		C2	2,41	2 148
		pc3	1,24	1 171
Phytopathogène	*B. glumae* BGR1	C1	3,90	3 494
		C2	2,82	2 286
	B. mallei ATCC 23344	C1	3,51	3 047
		C2	2,32	2 044
Groupe des « *pseudomallei* »	*B. pseudomallei* K96243	C1	4,07	3 529
		C2	3,17	2 406
	B. thailandensis E264	C1	3,80	3 343
		C2	2,91	2 370

1.1.3. La variation de la morphologie de colonie chez *Burkholderia*

Les bactéries font face à de soudains changements environnementaux exigeant une adaptation rapide pour maintenir leur population (Hallet, 2001; Moxon *et al.*, 1994). Au-delà des réponses physiologiques d'adaptation instantanée (systèmes à deux composantes, facteurs σ et anti-σ, etc...), pour s'adapter, les bactéries peuvent aussi tirer profit de la sélection naturelle et bénéficier de (1) mutations classiques et définitives qui arrivent au sein du génome à basse fréquence – typiquement lors de la réplication du génome, et qui sont transmises verticalement aux descendants – ou à (2) la variation de phase, induisant des changements à plus hautes fréquences ($>10^{-3}$) – spécifiques et réversibles de l'expression génétique (Hallet, 2001; Moxon *et al.*, 1994).

La variation de phase est facilement reconnaissable par un changement morphologique réversible d'une colonie (*e.g.* d'une colonie ayant un aspect rugueux à une colonie ayant un aspect lisse ; **Figure 1.5**).

Chez *Burkholderia*, la variation de phase est principalement décrite chez le groupe *pseudomallei* mais est également retrouvée chez les Bcc et le nouveau groupe *Paraburkholderia* (Agnoli *et al.*, 2011; Al-Maleki *et al.*, 2019; Austin *et al.*, 2015; Bernhards *et al.*, 2017; Chen *et al.*, 2009; Chen *et al.*, 2014; Coutinho *et al.*, 2011; Rondeau *et al.*, 2019; Shea *et al.*, 2017; Vial *et al.*, 2009; Wikraiphat *et al.*, 2015).

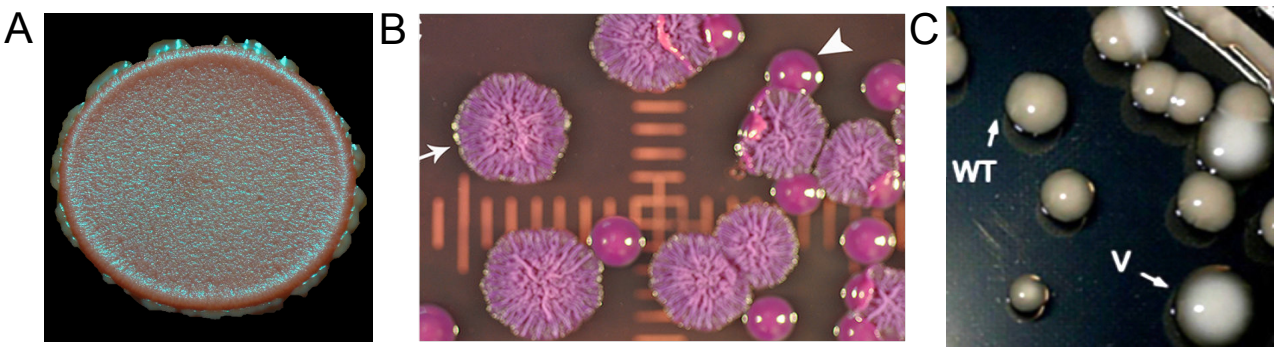


Figure 1.5 Variation de phase : morphologie de colonies de plusieurs espèces de *Burkholderia*.

A) Photographie d'une colonie de *B. ambifaria* HSJ1 - obtenue par microscopie optique (x10) - sur milieu TSA avec 0.1% de rouge Congo ; observation d'une colonie principale de morphologie rugueuse (souche sauvage) et une multitude colonies variantes lisses (décrit par Vial *et al.* [2009]). B) Variation de phase chez *B. pseudomallei* MSHR5848 sur milieu d'agar sélectif pour *B. cepacia* (BSCA) avec des colonies rugueuses (lactose-positive, coloration jaune) et colonies lisses (lactose-négative, coloration rouge ; [illustration tirée de Shea *et al.* (2017)]). C) Variation de phase chez *P. phytofirmans* PsJN sur milieu Lysogeny Broth (LB) avec des colonies beiges (souche sauvage) et des colonies blanches mucoïdes (variants ; [illustration tirée de Rondeau *et al.* (2019)]).

Les colonies variantes ne diffèrent pas seulement au point de vue de la morphologie coloniale, mais aussi au niveau de la morphologie cellulaire, de leur sensibilité ou d'utilisation de source biochimique et bien encore au niveau de leur virulence (Agnoli *et al.*, 2011; Rondeau *et al.*, 2019; Shea *et al.*, 2017; Vial *et al.*, 2009; Wikraiphat *et al.*, 2015). Différents mécanismes régulent la variation de phase tels que : (1) la variation ou le polymorphisme génomique (Shea *et al.*, 2017), ou

(2) des facteurs transcriptionnels ou épigénétiques (Sánchez-Romero *et al.*, 2015; Wikraiphat *et al.*, 2015). Il est important de noter que si ce phénomène induit des changements réversibles, il est alors définit comme variation de phase; au contraire si les changements induits sont irréversibles alors, ce phénomène est considéré comme un simple changement phénotypique.

1.1.3.1. Des variations génomiques peuvent générer la variation de la morphologie de colonie

La variation de phase peut être induite par des changements génomiques mineurs comme des mutations ponctuelles d'un nucléotide ou des réarrangements de l'ADN majeurs (quelques kilobases) - pouvant générer une instabilité de conformation de l'ADN, un arrêt de la transcription ou bien encore de la traduction des protéines (résumé par Wisniewski-Dye & Vial, [2008]; Woude & Bäumler, [2004]).

La présence de séquences répétées peut provoquer lors de la réplication (1) un mauvais alignement des séquences d'ADN mère-fille générant une augmentation ou une diminution du nombre de séquences répétées (**Figure 1.6A ;** [Belkum *et al.*, 1999; Belkum *et al.*, 1998; Levinson & Gutman, 1987]), ou (2) une inversion d'orientation de la région promotrice entre deux séquences inversées répétées (**Figure 1.6B ;** [Klemm, 1986]) - engendrant un effet sur la transcription et traduction du gène associé.

Une recombinaison homologue peut avoir lieu entre deux allèles (ou conversion de gène - l'un silencieux et l'autre exprimé) entre les séquences géniques ou promotrices (ou transposition d'ADN) créant ainsi un échange d'ADN unidirectionnel et pouvant rendre le gène silencieux sur demande (**Figure 1.6C ;** [(Borst & Greaves, 1987; Howell-Adams & Seifert, 2000; Kline *et al.*, 2004)]).

Chez *B. pseudomallei* MSHR5848, la variation de phase est la conséquence d'une insertion-délétion de trois paires de bases dans le promoteur d'une lipoprotéine membranaire faisant partie d'un *cluster* de gènes appartenant au phage ϕE12-2 présent dans le génome de *Burkholderia* - entrainant une surexpression de ce *cluster* chez le variant lisse en comparaison au variant rugueux - engendrant les différences phénotypiques et de virulence (DeShazer *et al.*, 2019; Shea *et al.*, 2017).

Chez *P. phytofirmans* PsJN, les variants phénotypiques (plus mucoïde, avec une réduction de motilité par manque de flagelle, une surproduction d'EPS, et une meilleure production de biofilm) – sont liés à des mutations spontanées de type polymorphisme du nucléotide simple (SNP) ou délétion de quatre paires de bases respectivement dans les gènes *iscS* et *hscA* appartenant au système « *iron-sulfur cluster* » (ISC) codé par le groupe de gènes *iscSUA-hscBA-fdx-iscX* (Rondeau *et al.*, 2019).

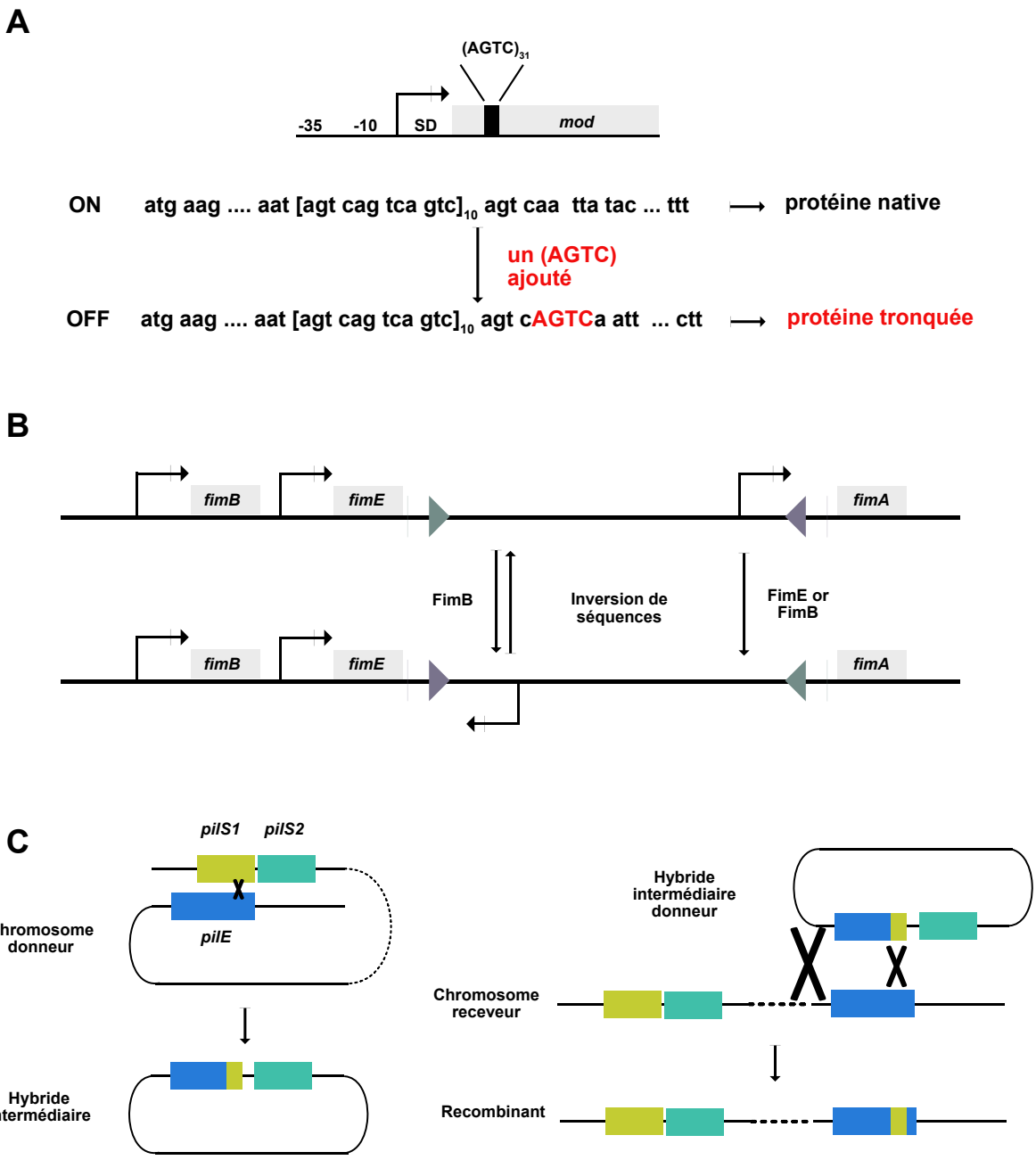


Figure 1.6 Variation de phase générée par des variations génomiques.

A) Variation de phase générée par l'insertion d'un codon d'une séquence répétée lors de la réplication de l'ADN (exemple de la régulation du gène *mod* chez *Haemophilus influenzae* HI056 (décrit par van Ham *et al.* [1993]). B) Variation de phase générée par inversion de séquence (exemple de la région promotrice du gène *fimA* chez *E. coli* ; [décrit par Klemm (1986)]). C) Variation de phase générée par recombinaison homologue (exemple du gène *pilE* chez *Neisseiria gonorrhoeae* ; [décrit par Kline *et al.* (2004) et (Howell-Adams & Seifert, 2000)]). Illustration modifiée de Woude & Bäumler, (2004).

1.1.3.2. Des facteurs transcriptionnels peuvent générer la variation de phase

Chez *B. cenocepacia,* le régulateur transcriptionnel « Shiny colony variant regulator » (ShvR) régulent l'expression de plus de 1000 gènes incluant des protéases, des lipases, la production d'exopolysaccharides (EPS), la production de molécules antimicrobienne ainsi que la production de AHLs et l'expression de *cepR/cepI* du système de quorum sensing CEP (QS [voir **Partie 1.2** ; Gomes *et al.*, 2018; O'Grady *et al.*, 2010; O'Grady & Sokol, 2011; Subramoni *et al.*, 2011]). ShvR corégule 263 gènes en commun avec le QS, mais régule indépendamment la formation de biofilm et la morphologie des colonies (Bernier *et al.*, 2007; O'Grady *et al.*, 2010; Subramoni *et al.*, 2011).

Une étude sur quatre souches de *B. pseudomallei* a reporté que des variants de phase de type mucoïde et non-mucoïde produisent différents types d'antigènes de lipopolysaccharides (LPS) (Wikraiphat *et al.*, 2015). Cette différence d'antigène n'est pas due à une différence d'expression ou une mutation de *wbiA* – permettant la production de l'antigène O ayant un effet protecteur chez *B. pseudomallei* et impliqué dans sa virulence (Brett *et al.*, 2003). Les auteurs supposent que la production de LPS chez *B. pseudomallei* pourrait être sous le contrôle d'un régulateur de type ShvR (Wikraiphat *et al.*, 2015).

1.1.3.3. Des facteurs épigénétiques peuvent générer la variation de phase

L'épigénétique réfère aux changements d'expression de gènes sans modifications de l'ADN et qui est un phénomène héritable. Chez les bactéries, le facteur épigénétique le plus connu est la méthylation d'ADN (revu par Casadesús and Low (2006) et Sánchez-Romero and Casadesús [2020]).

Lors de la méthylation de l'ADN un groupement méthyle est ajouté au niveau de l'azote N6 de l'adénine (6mA) et du N4 ou C5 de la cytosine (4mC ou 5mC ; [Cheng, 1995; Løbner-Olesen *et al.*, 2005; Wion & Casadesús, 2006]). La méthylation de l'ADN se fait grâce à des systèmes de restriction et de modification de l'ADN constitués, pour la majorité, d'une endonucléase de restriction et d'une ADN méthyltransférase (DNA MTase; **Tableau 1.3** ; [Jeltsch, 2002; Loenen & Raleigh, 2014; Wilson & Murray, 1991]). La méthylation de l'ADN de type 6mA est utilisée lors de la réplication de l'ADN afin de différencier l'ADN *de novo* de l'ADN mère (Reisenauer *et al.*, 1999). Cependant, les 6mA jouent un rôle important dans l'expression génique et spécialement dans la variation de phase - d'autant plus si la méthylation est située dans la région promotrice des gènes (Low *et al.*, 2001; Srikhanta *et al.*, 2010). D'autre part, la méthylation des cytosines de type 5mC protège les génomes bactériens contre les enzymes de restriction (Roberts *et al.*, 2005) alors que le type 4mC est de plus en plus reconnu comme jouant un rôle important dans la. Modulation de

l'expression génique, par exemple celle de facteurs de virulence chez les bactéries (Gaultney *et al.*, 2020; Kahramanoglou *et al.*, 2012; Kumar *et al.*, 2018a).

Tableau 1.3 Les différents systèmes de restriction-modification de l'ADN – Classification tirée de Casadesús et *al.*, (2016)

Type	Composition	Cofacteurs	Modèle de restriction de l'ADN
I	Multiple sous-unités	Mg^{++} AdoMet ATP	Clivage aléatoire éloigné du site de reconnaissance asymétrique
II	Enzymes de restriction et de modification séparées	Mg^{++}	Clivage au sein du site de reconnaissance symétrique
IIs	Enzymes de restriction et de modification séparées	Mg^{++}	Clivage à une distance fixe du site de reconnaissance symétrique
III	Enzymes de restriction et de modification séparées	Mg^{++} ATP	Clivage à une distance fixe du site de reconnaissance symétrique
IV	Plusieurs sous-unités	Mg^{++} GTP	Clivage de l'ADN contenant des nucléotides méthylés

Chez *B. cenocepacia* H111, l'apparition de variants ayant perdu le 3ème réplicon est plus fréquente en cas de perte de la méthylation des adénines (Mannweiler *et al.*, 2020). En effet, le système de réplication chromosomique *parABS* est hautement méthylé, ce qui suggère que la méthylation de l'ADN est requise pour la stabilité du pc3 (Mannweiler *et al.*, 2020). La méthylation permettrait à la souche de reconnaitre l'ADN étranger rendant ainsi la transmission du pc3 impossible entre les espèces de Bcc (Agnoli *et al.*, 2011; Mannweiler *et al.*, 2020). Cependant, le mécanisme induisant la perte du pc3 reste toujours indéterminé.

Chez *B. cenocepacia*, la méthylation de l'ADN joue un rôle important dans la régulation de certains facteurs de virulences - de type Marche/Arrêt (**Figure 1.7** ; [Vandenbussche *et al.*, 2020]). En effet, l'absence de DNA MTase engendre l'augmentation de l'agrégation des cellules, la production de biofilm et la diminution de la motilité et de la virulence pour les larves de *Galleria mellonella* (Vandenbussche *et al.*, 2020).

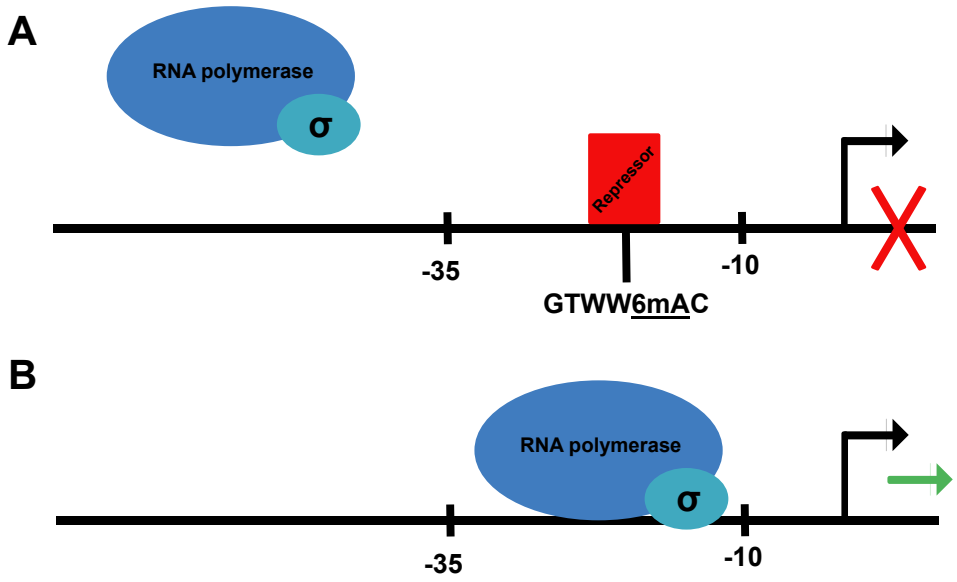


Figure 1.7 Modèle proposé de la régulation des facteurs de virulence par la méthylation de l'ADN chez *B. cenocepacia*.

A) En présence de méthylation, le promoteur est reconnu par le répresseur qui se fixe sur l'ADN, empêchant l'ARN polymérase de transcrire le gène associé. B) En l'absence de méthylation, le répresseur ne se fixe plus sur l'ADN et l'ARN polymérase peut transcrire le gène. Illustration modifiée de Vandenbussche *et al.* (2020).

1.1.3.4. La variation de morphologie de colonie peut aussi être induite par des changements génomiques irréversibles

Chez les Bcc, les souches cliniques *B. cenocepacia* H111 et *B. stabilis* R3338 produisent des variants de morphologie de colonie induits par la perte de leur pc3 (Agnoli *et al.*, 2011). Cette perte de ce mégaplasmide engendre un changement phénotypique irréversible des colonies bactériennes en plus de diminuer l'activité antifongique, la virulence, l'activité protéolytique ou encore le métabolisme de certains sucres et acides gras (Agnoli *et al.*, 2014; Agnoli *et al.*, 2017; Agnoli *et al.*, 2011). Les mêmes changements phénotypiques ont été aussi observés chez plusieurs souches cliniques de *B. ambifaria* - dont la souche *B. ambifaria* HSJ1 dont le variant possède son pc3 – leur permettant de mieux s'adapter à la rhizosphère (Vial *et al.*, 2009).

1.2. La communication intercellulaire chez *Burkholderia*

1.2.1. Le *quorum sensing* chez les bactéries

Les bactéries sont capables d'établir une communication intercellulaire – ou QS au sein de leur population. Chaque bactérie synthétise et sécrète des molécules de signalisation diffusible (aussi nommés autoinducteurs) permettant d'évaluer la densité de sa propre population dans un environnement. Une fois le quorum atteint, l'ensemble de la population régule simultanément l'expression de gènes qui sont bénéfiques dans l'environnement actuel (Fuqua, Winans, and Greenberg 1994).

Le QS a été découvert chez la bactérie *Vibrio fischeri* qui vit en symbiose au sein des photophores du calamar *Euprymna scolopes*. Cette bactérie possède un système de QS appelé Lux qui est composé d'une autoinducteur synthase LuxI et d'un régulateur transcriptionnel LuxR. Ce système permet la synthèse de la molécule de signalisation N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C_6-HSL) qui forme un complexe avec le régulateur LuxR (Greenberg, 1999). Ce complexe génère une rétroaction positive sur *luxI* et régule les gènes dont l'opéron *luxCDABE,* dont les protéines synthétisent de la lumière à partir de l'adénosine-tri-phosphate (ATP ; [Engebrecht & Silverman, 1984; Hastings *et al.*, 1973; Miyamoto *et al.*, 1988]).
On sait maintenant que la majorité des bactéries emploient des systèmes de régulation globaux de type *quorum sensing* (Bassler, 1999).

1.2.1.1. Les molécules de communication

Les molécules de communication du QS les plus courantes et connues sont de deux types : (1) des oligopeptides auto-inducteurs chez les bactéries à Gram positif ; et (2) des N-acyl-homosérine lactones (AHLs) chez les bactéries à Gram négatif.

a. Le QS chez les bactéries à Gram positif

Typiquement, les oligopeptides auto-inducteurs, nouvellement synthétisés par la synthase, sont transportés en dehors de la cellule par un transporteur de type ATP-binding cassette (ABC). Ils induisent une cascade de phosphorylation/déphosphorylation via des systèmes à deux composants - permettant aux régulateurs, des gènes contrôlés par le QS, de se lier à leurs cibles (**Figure 1.8** ; [Kleerebezem *et al.*, 1997]).

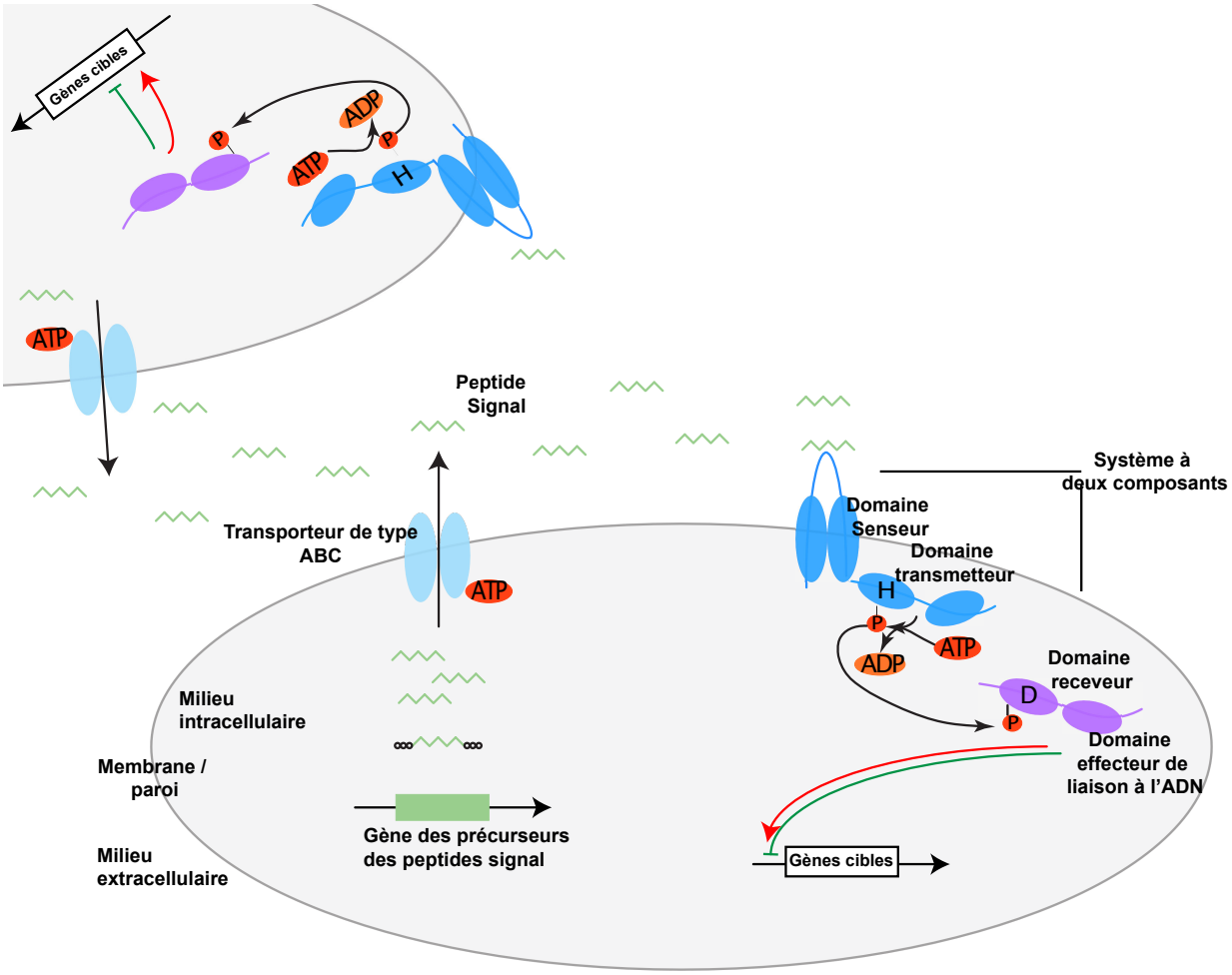


Figure 1.8 **La communication bactérienne chez les bactéries à Gram positif**

Le *quorum sensing* dépend de la concentration de peptide signal extracellulaire que les bactéries synthétisent et qui sont transportés par un transporteur ABC vers le milieu extracellulaire. Le signal externe est détecté par le domaine senseur d'un système à deux composantes induisant une cascade de phosphorylation/déphosphorylation au sein du système ; permettant ainsi la fixation du régulateur et l'activation de gènes cibles du QS. Illustration modifiée de Miller & Bassler (2001).

b. Le QS chez les bactéries à Gram négatif

Les N-acyl-homosérine lactones (AHLs), synthétisées par une synthase LuxI, ont un cycle lactone commun - couplé à une chaine acyle par une liaison amide. La chaine acyle des AHLs a des substitutions et une longueur variable en fonction de l'espèce bactérienne (**Figure 1.9** ; [Fuqua & Greenberg, 1975; Fuqua & Greenberg, 1998]).

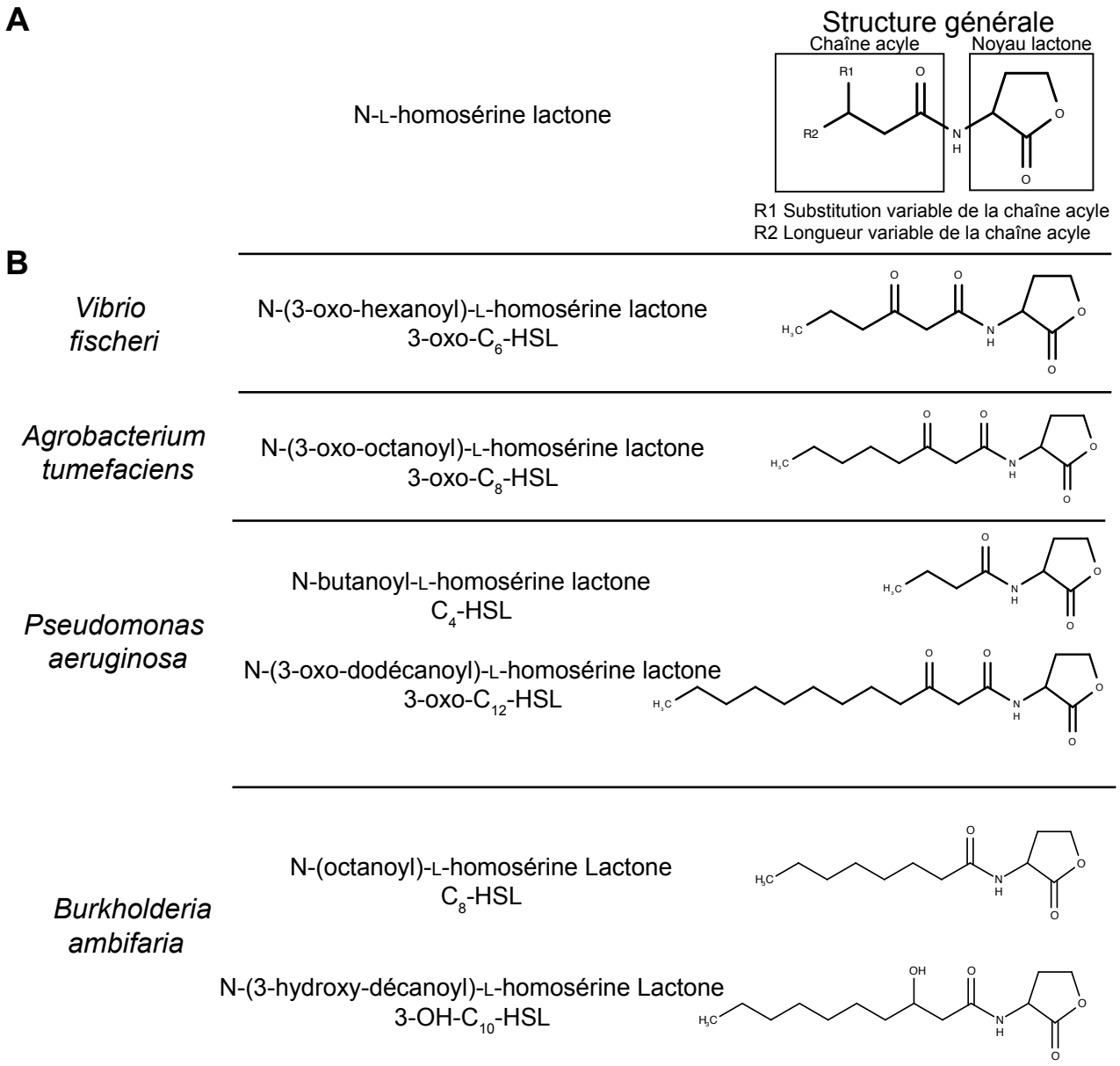


Figure 1.9 Structure chimique de quelques AHLs retrouvées chez les bactéries à Gram négatif

Les AHLs sont synthétisées par des synthases homologues à LuxI. A) Structure commune des AHLs. B) Les AHLs sont espèces-spécifiques et permettent la communication intra-espèces et inter-espèces. Illustration modifiée de Le Guillouzer (2018).

Ces molécules diffusent librement ou utilisent un transporteur actif vers l'extérieur de la cellule les rendant détectables par l'ensemble de la population. Les AHLs se fixent alors sur le régulateur transcriptionnel de type LuxR générant ainsi une boucle d'autorégulation et le contrôle des gènes cibles comme chez *V. fischeri* (**Figure 1.10**).

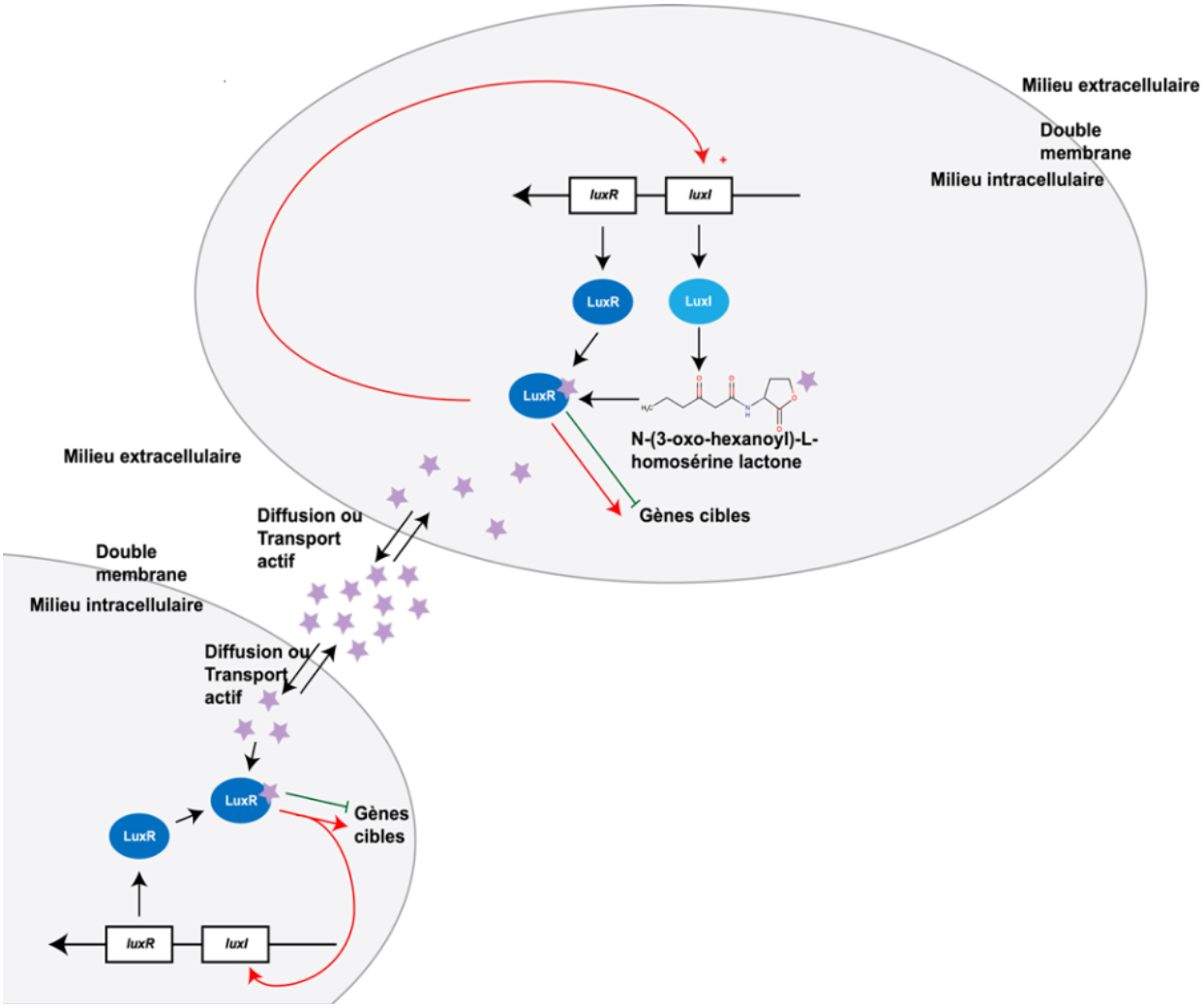
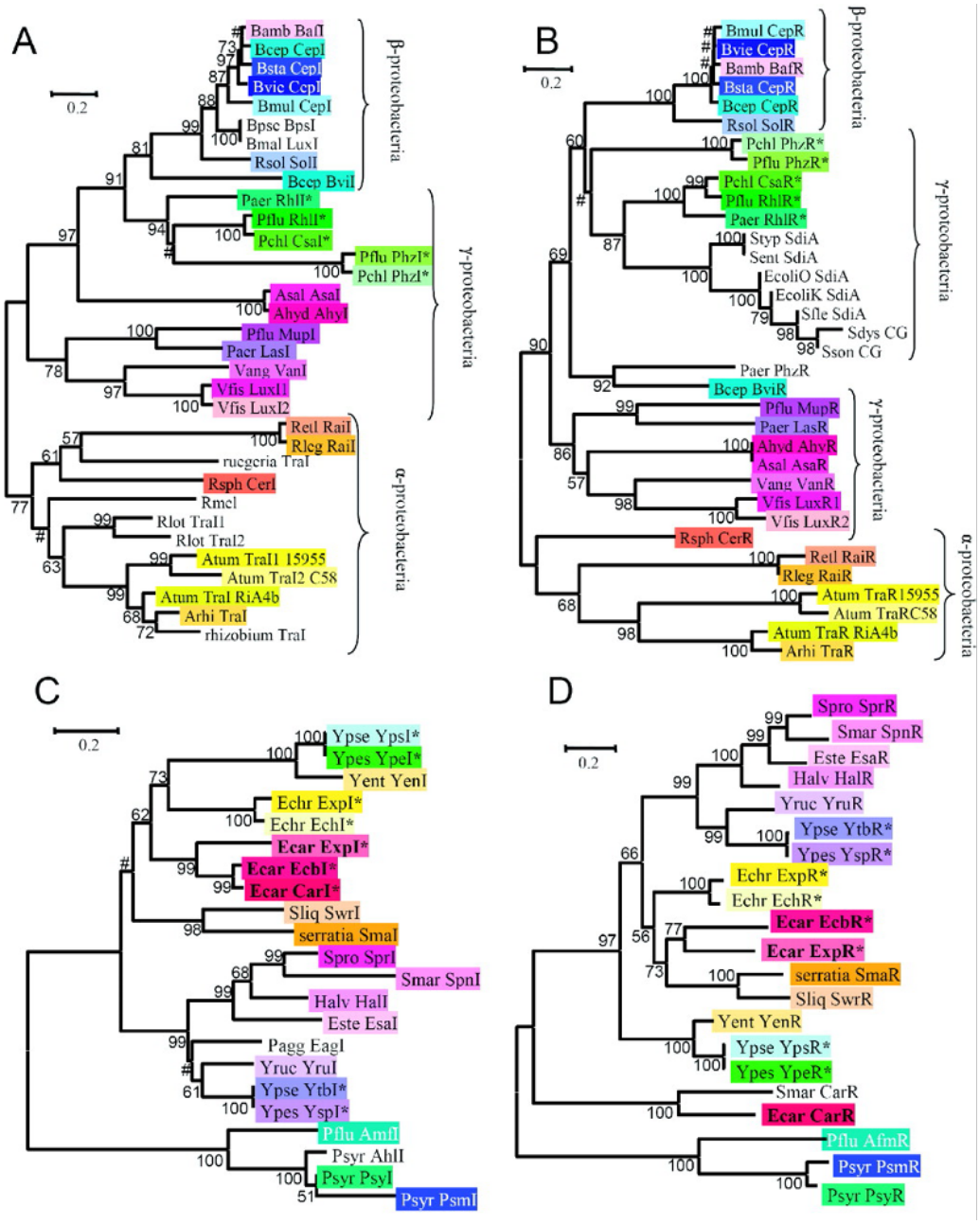


Figure 1.10 Modèle simplifié du *quorum sensing* à base de signaux de type N-acyl-homoserine lactones chez les bactéries à Gram négatif – exemple de *V. fischeri*

L'auto-inducteur N-(3-oxo-hexanoyl)-homosérine lactone (3OHC$_6$-HSL) est synthétisé par l'enzyme LuxI. Les 3OHC$_6$-HSL se lient sur le régulateur transcriptionnel LuxR et le complexe LuxR-3OHC$_6$-HSL forme ainsi une boucle d'autorégulation et régule les gènes cibles de QS. Illustration modifiée de Miller and Bassler (2001).

1.2.1.2. La répartition du système Lux au sein des bactéries

Le système *luxI/lux*R, permettant la synthèse des AHLs, est retrouvés parmi les α-proteobactéries (e.g. : *traI/traR* chez *Agrobacterium tumefaciens* [White & Winans, 2007]), ɣ-proteobactéries (e.g. : *rhlI/rhlR* chez *Pseudomonas aeruginosa* [Fuqua *et al.*, 1994]) et des homologues de type LuxS, permettant la synthèse des auto-inducteurs (autres qu'AHLs), qui sont retrouvés chez les ε-proteobactéries (e.g. : *Campylobacter jejuni* [Elvers & Park, 2002]), Firmicutes (e.g. : *Clostridium perfringens* [Ohtani *et al.*, 2002]), *Actinobacteria*, (e.g : *Bifidobacterium longum* [Sun *et al.*, 2014])



Deinococcus (e.g. : *Deinococcus radiodurans* [Lin *et al.*, 2016]), *Spirochaete* (e.g : *Borrelia burgdorferi* [Stevenson & Babb, 2002]).

Figure 1.11 Homologies de *luxI/luxR* chez les bactéries

(A) Arbre base sur l'alignement protéique de LuxI des membres du groupe A. (B) Arbre base sur l'alignement protéique de LuxR des members du groupe A. (C) Arbre base sur l'alignement protéique de LuxI des membres du groupe B. (D) Arbre base sur l'alignement protéique de LuxR des membres du groupe B. La méthode phylogénétique utilisée est la méthode "Neighbour-Joining" avec une correction ɣ correction. Abréviation disponible en annexe I. Le symbole (#) indique les nœuds dans la valeur de bootstrap est inférieure à 50%. Illustration tirée de Lerat & Moran, (2004).

1.2.1.3. Quelques mécanismes contrôlés par le *quorum sensing*

Chez un grand nombre de bactéries, le QS contrôle divers mécanismes comme : (1) la biosynthèse d'antibiotique chez les *Streptomyces* (Takano, 2006), (2) la conjugaison bactérienne chez *Enterococcus faecalis* (Dunny *et al.*, 1978), (3) la compétence génétique chez *Streptococcus pneumoniae* (Morrison, 1997), (4) la sporulation chez *Bacillus subtilis (Silhavy & Hoch, 1995)*, (5) de nombreux facteurs de virulence notamment chez *Pseudomonas aeruginosa (Miller & Bassler, 2001)*, (6) la formation de biofilm - dont ceux composés de *P. aeruginosa* et *Burkholderia cepacia* au sein des poumons des individus atteints de fibrose kystique (FK), mais aussi des personnes immunodéprimées comme les patients atteints de granulomatose septique chronique (GSC; [Riedel *et al.*, 2001]) – (7) d'optimiser la colonisation et d'intervenir dans les interactions hôte-pathogène (Juhas *et al.*, 2005) ou encore (8) de résister au stress environnemental comme les antibiotiques (Stewart & Costerton, 2001).

1.2.2. Le complexe *Burkholderia cepacia* possède différents systèmes de *quorum sensing*

1.2.2.1. Le système Cep du complexe *Burkholderia cepacia* est homologue aux systèmes Las et Rhl de *Pseudomonas aeruginosa*

La production de facteurs de virulence des Bcc (*e.g.* : sidérophores, protéases, lipases etc…) est contrôlée par le QS en produisant des AHLs. Ceci a été premièrement démontré que le produit extracellulaire de *B. cepacia* 10661 active les rapporteurs transcriptionnels *luxR::lux vsmR::lux* et *Chromobacterium violaceum* CVO26 - connus pour être activés en présence de AHLs de différentes longueurs (McKenney *et al.*, 1995; Pesci & Iglewski, 1997).

Le système Cep - situé sur le c2 - est le système principal de QS des Bcc et est constitué de : (1) la synthase CepI synthétisant en majorité du N-octanoyl-L-homosérine lactone (C_8-HSL) mais aussi du N-hexanoyl-L-homosérine lactone (C_6-HSL; **Tableau 1.4**) ; et (2) du régulateur CepR qui en se liant au C_8-HSL autorégule le système Cep et les gènes contrôlés par le QS (Lewenza *et al.*, 1999).

Les Bcc comptent deux ou trois systèmes de QS, dépendamment des souches. Par exemple, en plus du système CepR/CepI, *B. cenocepacia* possède le système « *B. cenocepacia* Pathogenicity Island » (Cci), qui synthétise des C_6-HSL (majorité) et des C_8-HSL (minorité) et qui inhibe le système Cep ; pour sa part, *B. vietnamiensis* G4 possède le système Bvi qui synthétise du N-decanoyl-L-homosérine lactone (C_{10}-HSL) et qui est activé entre autres par le système Cep ; *B. ambifaria* HSJ1 possède le système Cep*2* qui synthétise du N-(3-oxo-décanoyl)-L-homosérine

lactone ($3OHC_{10}$-HSL) et qui est activé par le système Cep (Chapalain *et al.*, 2017; Conway & Greenberg, 2002; Gotschlich *et al.*, 2001; Malott *et al.*, 2005).

Tableau 1.4 Le *quorum sensing* chez les Bcc

Espèce	Système de QS	Chromosome	AHL majoritaire	Commentaire
B. ambifaria	*cepR/rsaM/cepI*	2	C_8-HSL	Retrouvé chez toutes les *B. ambifaria*
	cepR2 //rsaM2/ cepI2	3	$3OHC_{10}$-HSL	Retrouvé chez *B. ambifaria* IOP40
	-/-	2		
B. *cenocepacia*	*cepR/rsaM/cepI*	2	C_8-HSL	Retrouvé chez toutes les *B. cenocepacia*
	cciR/cciI	2	C_6-HSL	
B. multivorans	*bmuR/rsaM/bmuI*	2	C_8-HSL	*B. multivorans* ATCC 17616
B. vietnamiensis	*cepR/rsaM/cepI*	2	C_8-HSL	*B. vietnamiensis* G4
	bviR/-/BviI	3	C_{10}-HSL	
B. cepacia	*cepR/rsaM/cepI*	2		*B. cepacia* ATCC 25416, *B. cepacia* DBOI, *B. cepacia* 10661

« - » indique le manque de données

1.2.2.2. Le système de *quorum sensing* basé sur le « *Burkholderia* diffusible signal factor » du complexe *Burkholderia cepacia*

Le système *« Burkholderia diffusible signal factor »* (BDFS) est impliqué dans une potentielle communication inter-espèce puisque ce système de type « *diffusible signal factor* » (DSF) est aussi retrouvé chez *Xanthomonas campestris, Candida albicans* ou encore *Stenotrophomonas maltophilia* (Boon *et al.*, 2007; de Rossi *et al.*, 2014; Deng *et al.*, 2013). Tout comme le QS à base de AHL, le système BDSF contrôle la motilité, l'activité protéolytique, la virulence et la formation du biofilm chez 12 espèces de Bcc (Boon *et al.*, 2007; Suppiger *et al.*, 2015). La synthase RpfF synthétise des acides gras de type acide *cis*-*cis*-11-méthyldodéca-2,5-diénoique ou BDSF qui diffusent à travers la membrane cellulaire et se fixent sur le senseur RpfR ; le complexe ainsi formé se fixe sur la région promotrice des gènes de virulence cibles dont certains sont co-régulés par le système Cep (McCarthy *et al.*, 2010; Schmid *et al.*, 2012). En dégradant le di-guanosine monophosphate cyclique (c-di-GMP), le système BDSF active indirectement le système Cep et

induit aussi la maturation du biofilm (**Figure 1.12** ; [Boon *et al.*, 2007; Deng *et al.*, 2013; McCarthy *et al.*, 2010; Suppiger *et al.*, 2015; Traverse *et al.*, 2013]).

1.2.2.3. Les systèmes de *quorum sensing* sont régulés par divers régulateurs chez le complexe *Burkholderia cepacia*

Les systèmes de QS s'autorégulent et sont capables d'interagir entre eux, sont sous le contrôle de divers régulateurs (**Figure 1.12**).

Le régulateur transcriptionnel RsaM – dont le gène *rsaM* se situe en général entre les gènes *cepR* et *cepI* chez la majorité des Bcc – régulerait indirectement l'abondance ou l'activité des protéines du système Cep en plus d'être activé par le système Cep (Choudhary *et al.*, 2013; Michalska *et al.*, 2014).

Bien qu'ils soient principalement décrits chez *B. cenocepacia*, les gènes codant pour (1) le régulateur transcriptionnel de type LysR « Shiny colony variants » ShvR, (2) le senseur kinase « *Adhesion and type six secretion system regulator* » (AstR) et (3) l'hypothétique protéine cytoplasmique codée par le gène BCAM1871 sont présents chez la majorité des Bcc (**Tableau 1.5**) et régulent activement le système Cep (**Figure 1.12** ; revu par Suppiger *et al.*, [2013]). En effet, ShvR inhibe l'expression des régulateurs CepR et CciR (Bernier *et al.*, 2007; O'Grady *et al.*, 2010; O'Grady & Sokol, 2011). AstR modulerait la production des AHLs en inhibant les systèmes Cep et Cci par une cascade de signaux de transduction dont le mécanisme reste inconnu (Aubert *et al.*, 2008). La protéine cytoplasmique putative codée par le gène BCAM1871 et co-transcript avec le gène *cepI*, augmente l'activité des AHLs et active l'expression des régulateurs CepR et CciR (O'Grady *et al.*, 2012).

Le système Hma, récemment décrit, produit des molécules de signalisation de type « valdiazen » qui sont synthétisées par les enzymes encodées par les opérons *hmaABCDE* et *hamFG* (Jenul *et al.*, 2018). Ces opérons sont inhibés par le système Cep et les valdiazen activent (1) l'autoinducteur orphelin CepR2 – lui-même inhibé par le système Cci – (2) le régulateur transcriptionnel CepS et (3) différents gènes impliqués dans la virulence de *B. cenocepacia* dont les sidérophores nommés ornibactines (Jenul *et al.*, 2018; Malott & Sokol, 2007).

Tableau 1.5 **Les principaux régulateurs du système Cep de *B. cenocepacia* dont les gènes orthologues sont retrouvés chez la majorité des espèces de Bcc**

Espèce	*shvR*	*atsR*	*BCAM1871*
B. ambifaria AMMD	BAMB_RS30675	BAMB_RS25715	BAMB_RS20650
B. lata 383	BCEP18194_RS04320	BCEP18194_RS03750	BCEP18194_RS28035
B. multivorans ATCC 17616	-	Bmul_5222	Bmul_3968
B. vietnamiensis G4	-	Bcep1808_3924	Bcep1808_5263

1.2.3. Le système Hmq chez *Burkholderia* : homologue du système de *quorum sensing* PQS de *P. aeruginosa* et de la bactérie marine *Microbulbifer* sp

En 2006, *Diggle et al.* ont remarqué que certaines souches cliniques de *Burkholderia* (quatre *B. pseudomallei* et une *B. cenocepacia*) - en plus du modèle d'étude *B. thailandensis* - ont des gènes homologues à ceux de l'opéron *pqsABCDE* chez *P. aeruginosa*, leur permettant de produire des 4-hydroxy-2-alkylquinolines (HAQs) sans produire du 3,4-dihydroxy-2-heptylquinoline (*Pseudomonas* Quinolone Signal ou PQS) comme *P. aeruginosa*.

En 2008, Vial *et al.* ont découvert que l'opéron homologue chez *Burkholderia* possède deux gènes supplémentaires, nommés *hmqF* et *hmqG* – formant ainsi l'opéron *hmqABCDEFG* - qui permettent la production de HAQs insaturés (Agarwal *et al.*, 2012) et de 4-hydroxy-3-méthyl-2-alkylquinolines (HMAQs) chez *B. thailandensis* E864, *B. pseudomallei* 1086b, *B. ambifaria* HSJ1 (**Figure 1.13** ; **Tableau 1.6**).

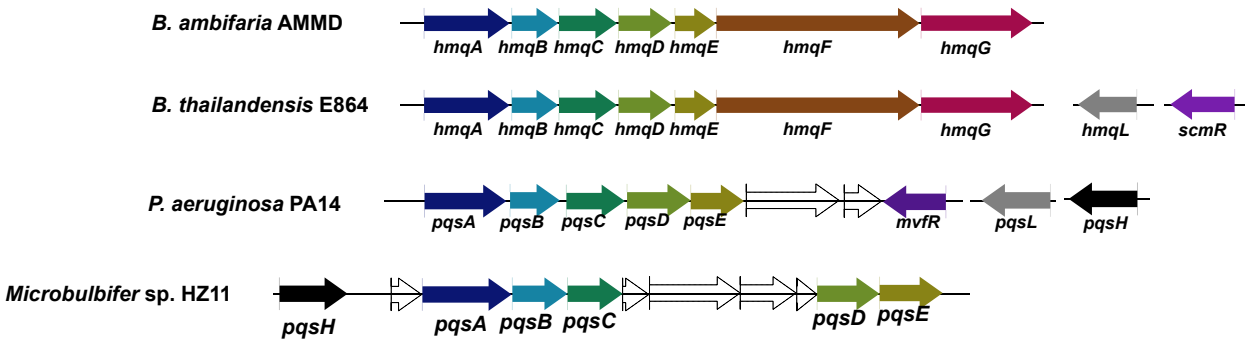


Figure 1.13 **Homologie entre l'opéron *hmqABCDEFG* chez *Burkholderia* et l'opéron *pqsABCDE* chez *P. aeruginosa* et dernièrement *Microbulbifer* sp**

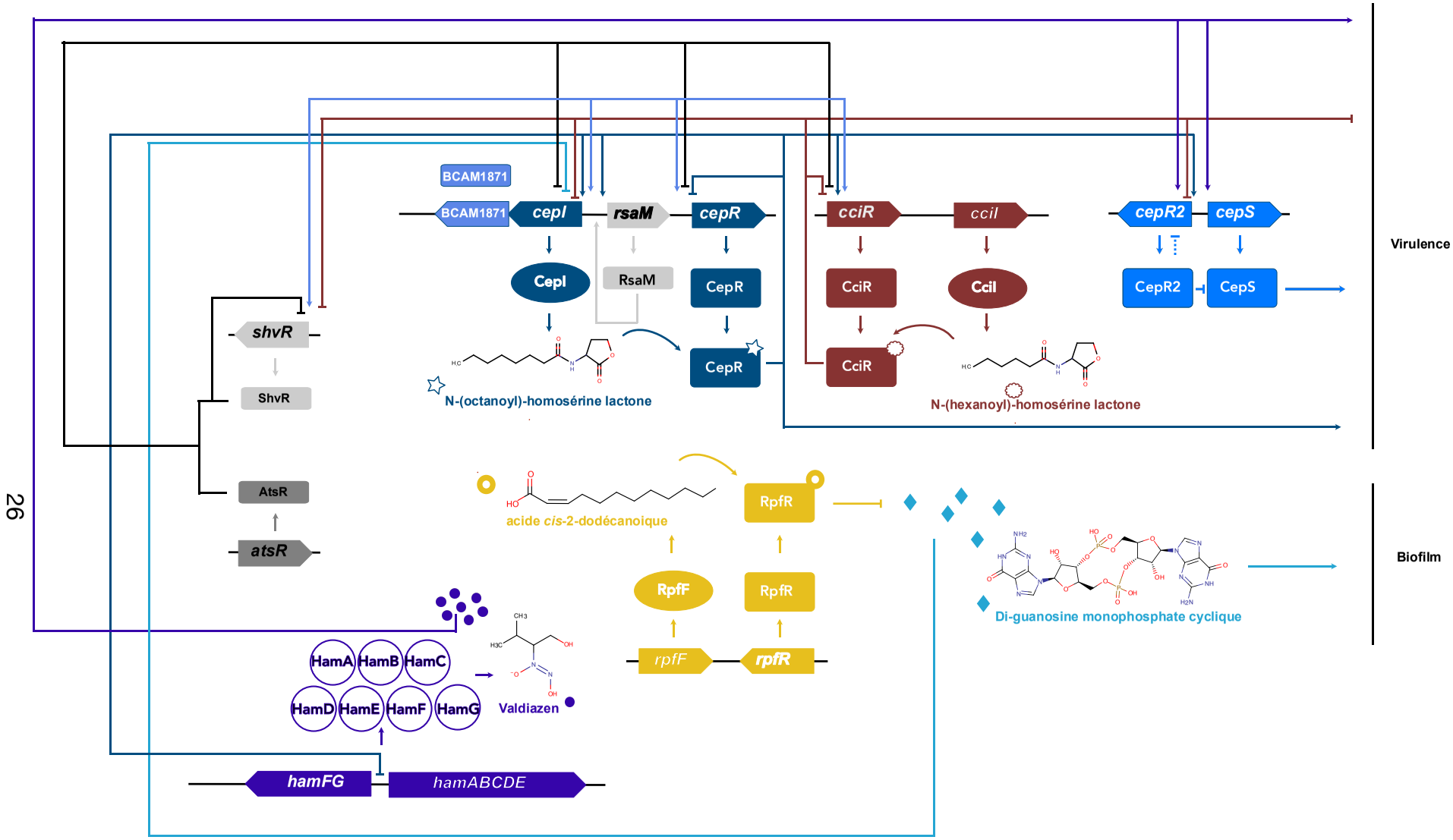


Figure 1.12 Inter-régulation des systèmes de QS chez *B. cenocepacia*

B. thailandensis E864 possède les gènes *hmqL* et *scmR* contrairement à *B. ambifaria* AMMD. Les gènes *hmqF* et *hmqG* sont spécifiques aux bactéries du genre *Burkholderia*. Les gènes colorés de la même couleur sont homologues entre eux. Les gènes externes à l'opéron *pqsABCDE* sont représentés en transparent. Illustration modifiée de (Ritzmann *et al.*, 2019).

Tableau 1.6 Homologie de séquence protéiques des enzymes codées par l'opéron *pqsABCDE* de *P. aeruginosa* PA14 et l'opéron *hmqABCDEFG* chez *B. ambifaria* AMMD

	Identité (%)	*Similarité (%)*	
PqsA	31	45	HmqA
PqsB	42	57	HmqB
PqsC	40	61	HmqC
PqsD	54	71	HmqD
PqsE	33	48	HmqE

1.2.3.1. La production de HMAQs chez *Burkholderia*

Burkholderia sp. QN15488 a été rapportée comme étant la première souche productrice de HMAQ-C_8:2', aussi nommé burkholone (Mori *et al.*, 2007). Les souches de *Burkholderia*, capables de produire des AHQs et HMAQs, produisent une diversité de HMAQs regroupées en trois familles (insaturés, saturés et N-oxides ; **Tableau 1.7** ; [Vial *et al.*, 2008]). Les caractéristiques principales différenciant les HMAQ des HAQ sont la présence d'une insaturation sur la chaine aliphatique et d'un groupe méthyle en position 3'.

Les mêmes HMAQs étant produites par les différentes espèces de *Burkholderia* possédant un opéron *hmqABCDEFG*, l'abondance des différents congénères varie entre les espèces appartenant au groupe « *pseudomallei* » vs au groupe Bcc. Par exemple, *B. ambifaria* HSJ1 – appartenant aux Bcc – produit préférentiellement des HMAQ-C_7:2' aux HMAQ-C_9:2' qui s'accumulent dans le surnageant de culture. Seuls, *B. pseudomallei* 1026B et *B. thailandensis* E264 produisent des NOHMAQ-C_9:2' et préférentiellement des HMAQ-C_9:2' à des HMAQ-C_7:2' qui peuvent être dégradées puis reproduites (**Figure 1.14** ; [Vial *et al.*, 2008]). Les trois souches sont capables de produire des congénères de HMAQs saturées mais en plus faible quantité que les HMAQs insaturées.

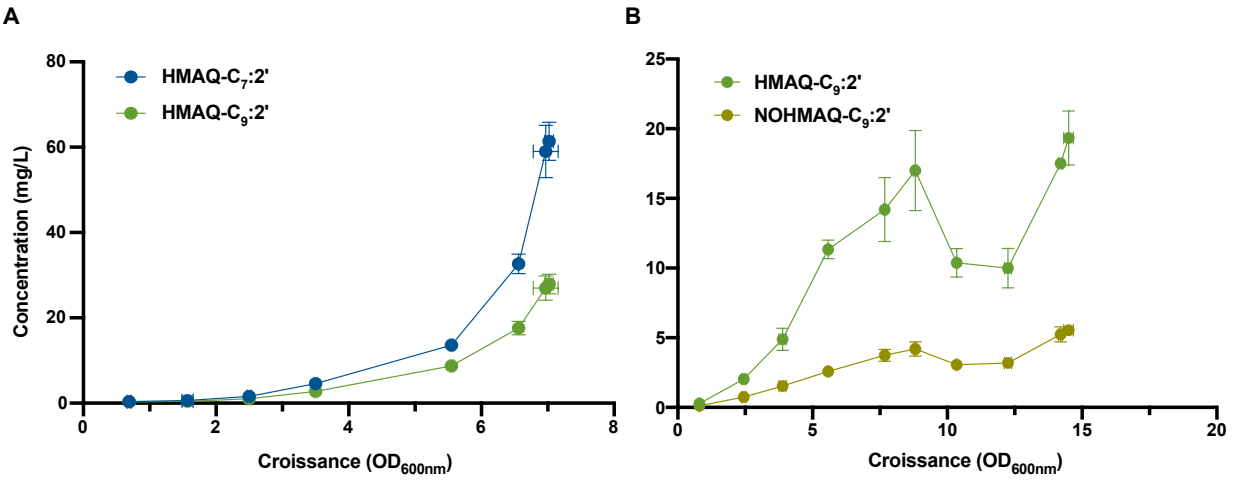


Figure 1.14 La cinétique de production de HMAQ est différente chez *B. ambifaria* HSJ1 et *B. thailandensis* E264.

Les deux HMAQs les plus abondantes sont présentées pour chaque souche. A) La production de HMAQ-C_7:2'et de HMAQ-C_9:2' suivent la tendance de la courbe de croissance chez *B. ambifaria* HSJ1, résultant en une accumulation des molécules. B) La production de HMAQ-C_9:2' et de NOHMAQ-C_9:2' forment deux pics chez *B. thailandensis* E264, laissant penser à une dégradation des molécules. Figure modifiée de Vial *et al.* (2008).

Vial *et al.* (2009) ont reporté que les souches cliniques de *B. ambifaria* produisent des HMAQs contrairement à leur variant phénotypique de type environnemental et des souches environnementales. Puisque les souches ne produisant pas de HMAQs, possèdent néanmoins l'opéron *hmqABCDEFG*, ces informations suggèrent qu'il existerait potentiellement des régulateurs agissants sur l'opéron *hmqABCDEFG* ou une régulation post-transcriptionnelle de cet opéron.

Depuis, la production de HMAQs a été identifiée chez les souches environnementales *B. cepacia* Cs5, *B. ambifaria* AMMD et *Burkholderia sp* MBAF1239 lors de la recherche de la production de nouvelles molécules antimicrobiennes (Kilani-Feki *et al.*, 2011; Li *et al.*, 2018; Mahenthiralingam *et al.*, 2011).

Tableau 1.7 Les différentes familles de HMAQs

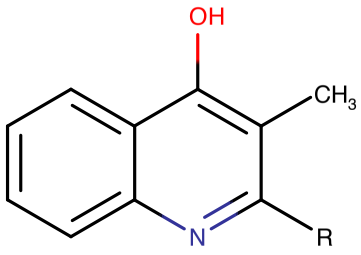
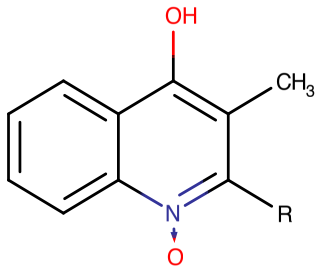
Familles		Composés	*B. ambifaria*	*B. thailandensis*	*B. pseudomallei*
	4-Hydroxy-3-méthyl-2-alkylquinolines insaturées (HMAQ-Cx:2')	HMAQ-C_5:2'	++	ND	+
		HMAQ-C_6:2'	+	ND	ND
		HMAQ-C_7:2'	+++++	+	+
		HMAQ-C_8:2'	++	ND	+
		HMAQ-C_9:2'	++++	++++	++
		HMAQ-C_{10}:2'	+	ND	+
		HMAQ-C_{11}:2'	ND	+	+
	4-Hydroxy-3-méthyl-2-alkylquinolines saturées (HMAQ-Cx)	HMAQ-C_5	+	ND	ND
		HMAQ-C_6	+	ND	ND
		HMAQ-C_7	++	+	+
		HMAQ-C_8	+	+	ND
		HMAQ-C_9	++	+	ND
		HMAQ-C_{10}	+	ND	ND
	4-Hydroxy-3-methyl-2-alkylquinolines N-oxides insaturées (NOHMAQ-Cx:2')	NOHMAQ-C_6:2'	ND	ND	+
		NOHMAQ-C_7:2'	ND	ND	+
		NOHMAQ-C_8:2'	ND	++	+
		NOHMAQ-C_9:2'	ND	+++	++
		NOHMAQ-C_{10}:2'	ND	+	+
		NOHMAQ-C_{11}:2'	ND	+	+

Tableau modifié de Vial *et al.* (2008)

1.2.3.2. La biosynthèse des 4-hydroxy-3-méthyl-2-alkylquinolines par les enzymes codées par l'opéron *hmqABCDEFG*

Les gènes *hmqABCDE* de l'opéron *hmqABCDEFG* chez *Burkholderia* et ceux de l'opéron *pqsABCDE* chez *P. aeruginosa* étant homologues, ils coderaient pour les mêmes protéines putatives, nécessaires á la biosynthèse des HAQs et des HMAQs (**Figure 1.15**).

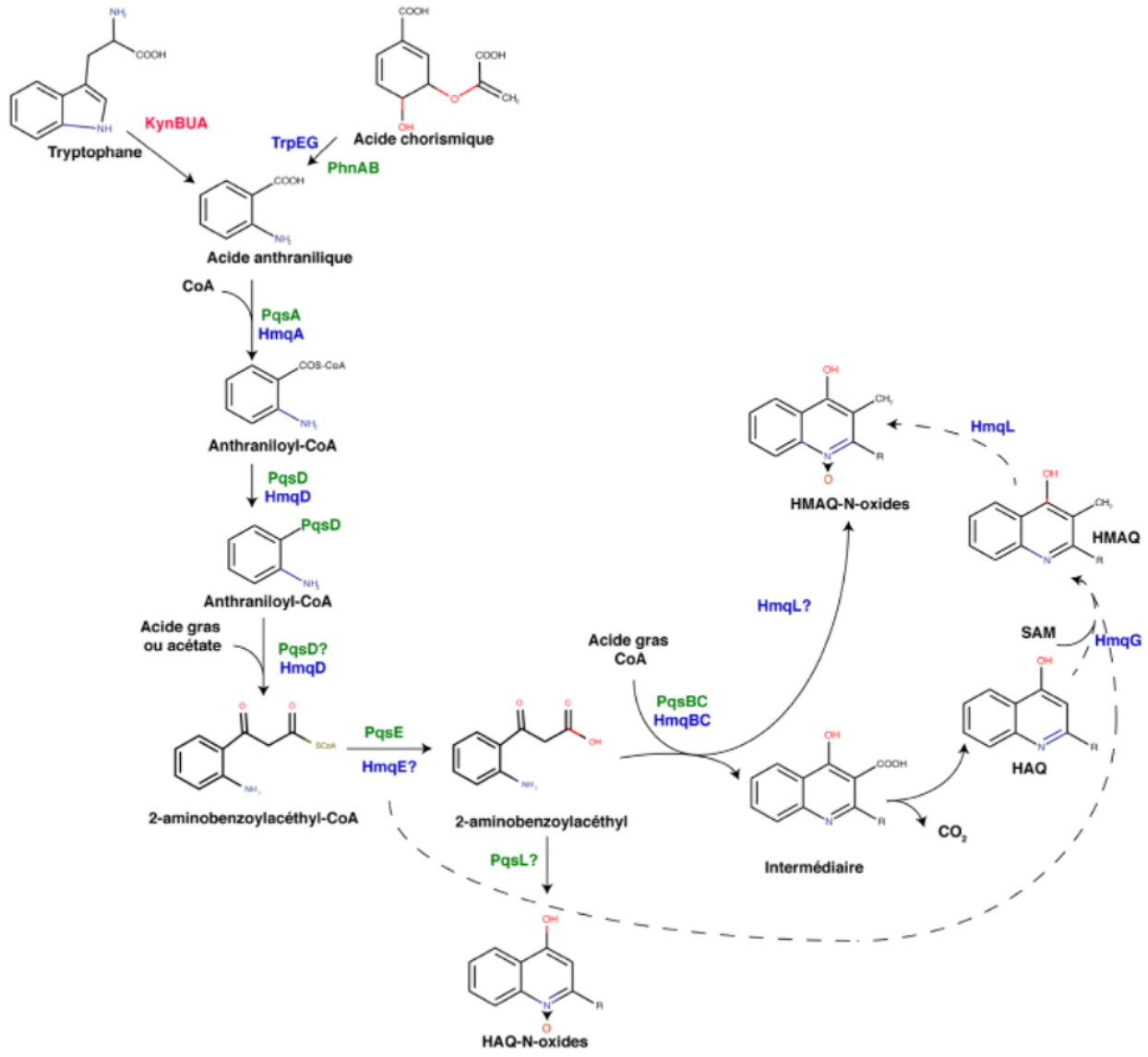


Figure 1.15 La biosynthèse des HAQ et HMAQ chez *Burkholderia* : homologie de la voie de synthèse des HAQs et PQS chez *P. aeruginosa*

Les enzymes permettant la synthèse de AHQs chez *P. aeruginosa* PA14 sont illustrées en vert. Celles en bleues correspondent aux enzymes retrouvées chez *Burkholderia*. Celles en rouge sont retrouvées chez les deux genres bactériens. Les HMAQs sont retrouvées uniquement chez *Burkholderia*. Illustration tirée de Vial *et al.* (2008).

L'anthraniloyl-coenzyme A synthase ou HmqA permettrait ainsi la synthèse de l'anthraniloyl-coenzyme A à partir de l'acide anthranilique (Coleman *et al.*, 2008). La 3-oxoacyl-ACP-synthase ou HmqD formerait du 2-aminobenzoylacétyl-Coenzyme A en transférant l'anthraniloyl sur un malonyl-Coenzyme A (Dulcey *et al.*, 2013; Zhang *et al.*, 2008). Le complexe de type transférase HmqB-C – formé comme le complexe PqsB-C de par la similarité conservée entre la protéine PqsB et la partie N-terminale de la protéine HmqB (5DWZ ; www.rcsb.org ; [Drees *et al.*, 2016]) - permettrait la synthèse de HHQ à partir de 2-aminobenzoylacétyl-Coenzyme A et d'octanoate (Dulcey et al. 2013; Drees et al. 2016). La métallo-β-lactamase ou HmqE favoriserait la voie de synthèse des HAQs contrairement à PqsE (Diggle *et al.*, 2006; Drees & Fetzner, 2015; Farrow *et al.*, 2008; Folch *et al.*, 2013; Rampioni *et al.*, 2016; Rampioni *et al.*, 2010).

Les deux gènes supplémentaires *hmqF* et *hmqG* – sans correspondance chez *P. aeruginosa* – sont respectivement responsables de l'insaturation de la chaine alkyl et de la méthylation des HAQs donnant des HAQs insaturées et des HMAQs insaturées et saturées. Aucun analogue de PqsH étant présent chez *Burkholderia,* la synthèse de congénères PQS est absente. Ces trois différences expliquent la synthèse ainsi des HMAQs au lieu des HAQs tels le PQS (Agarwal *et al.*, 2012; Vial *et al.*, 2008).

La mono-oxygénase « flavin adenine dinucleotide » - dépendante (FAD) ou HmqL est présente chez les bactéries des espèces *B. pseudomallei* et *B. thailandensis* - mais absente chez les espèces appartenant aux Bcc - leur permettant ainsi la synthèse de HMAQ N-oxydes (NOHMAQs ; [Klaus *et al.*, 2020; Vial *et al.*, 2008]).

1.2.3.3. La régulation du système Hmq chez *Burkholderia*

Un système de QS est défini par son autorégulation via ses molécules de communication et par régulation entre les systèmes de QS (Fuqua, Winans and Greenberg, 1994).

De par la similarité de système Hmq chez *Burkholderia* et du système PQS chez *P. aeruginosa*, leur régulation devrait être similaire : (1) en étant régulé par un homologue du régulateur transcriptionnel « *multiple virulence factor regulator* » (MvfR ou PqsR) de *P. aeruginosa* et (2) serait un système appartenant au QS en jouant un rôle actif dans le système de QS chez *Burkholderia* (Wade et al. 2005; Xiao et al. 2006a ; Xiao et al. 2006b; Sams et al. 2015)

a. La régulation du système PQS chez *P. aeruginosa*

Le système PQS est le troisième système de QS chez *P. aeruginosa*. En plus de leur propre boucle de régulation, il existe des inter-régulations entre les systèmes Las, Rhl et PQS (**Figure 1.16** ; revu par García-Reyes *et al.*, [2019]). Le système PQS*,* dépendant des gènes *pqsABCDE*, *pqsH* et *pqsL*

produit des HAQs dont le HHQ et le HQNO, et PQS jouant un rôle important dans le système général du QS chez cette espèce (Sams *et al.*, 2015). Le régulateur transcriptionnel MvfR – lié au ligand PQS ou HHQ (Wade *et al.*, 2005; Xiao *et al.*, 2006a ; Xiao et al. 2006b) - active sa propre expression et celle de l'opéron *pqsABCDE* en plus de réguler au moins 35 gènes, dont ceux impliqués dans la virulence (Cao *et al.*, 2001). Le système PQS est régulé positivement par le système Las - au niveau de l'expression des gènes *pqsH* et *mvfR* - et par le système Rhl – en inhibant l'expression de *mvfR* et en activant celle de l'opéron *pqsABCDE* (Wade *et al.*, 2005; Xiao *et al.*, 2006a ; Xiao et al. 2006b).

Cependant, l'inter-régulation entre les trois systèmes reste complexe. PqsE n'est pas essentiel à la production de HHQ/PQS mais à un rôle fondamental dans l'activité du régulateur transcriptionnel RhlR par un mécanisme encore inconnu (Farrow *et al.*, 2008; Groleau *et al.*, 2020).

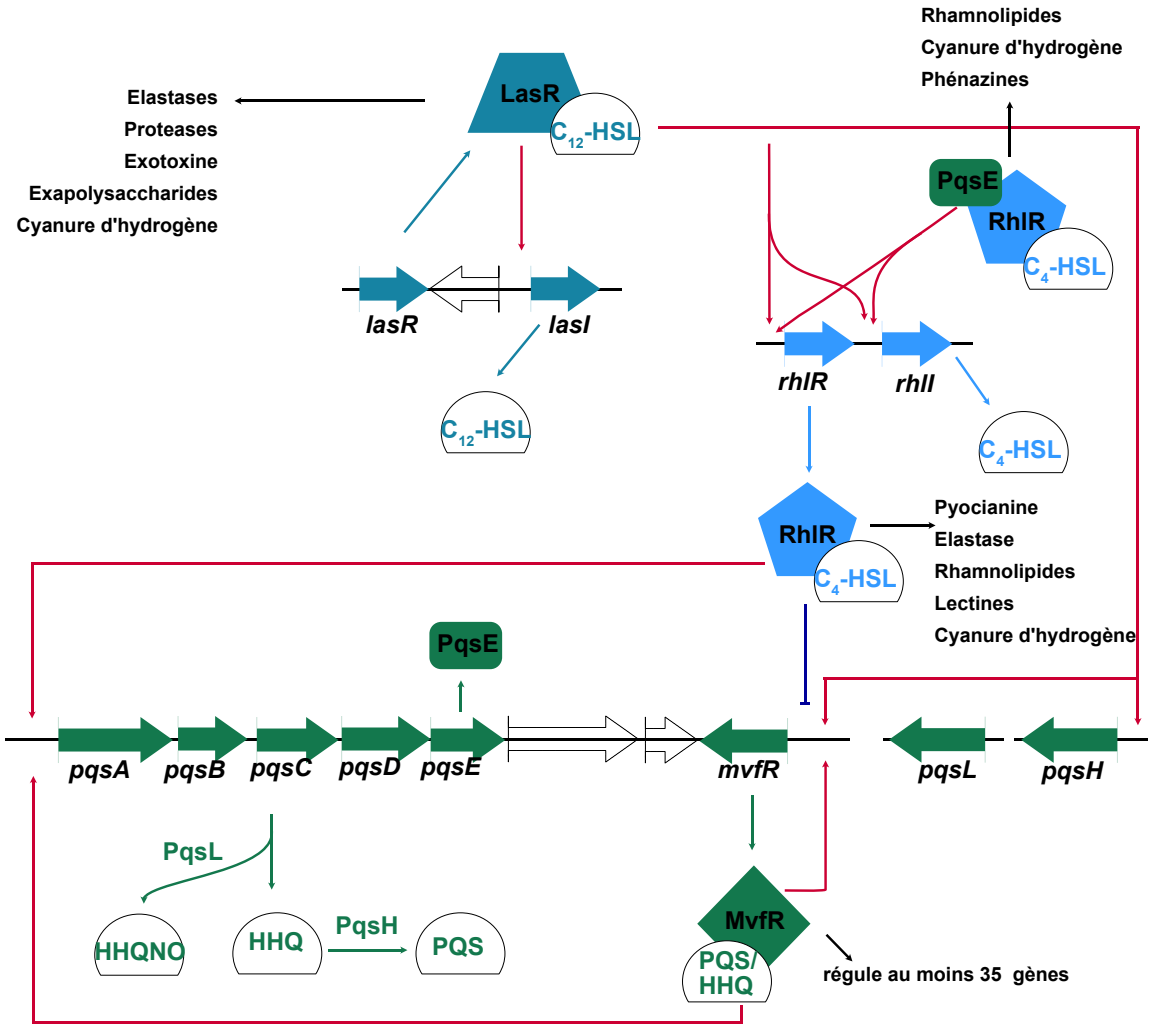


Figure 1.16 Inter-régulation des trois systèmes de QS chez *Pseudomonas aeruginosa*

Le complexe LasR-C_{12}HSL active le système Rhl et la transcription de *pqsH* et *mvfR*. Le complexe RhlR-C_4HSL inhibe la transcription de *mvfR* et active l'opéron *pqsABCDE*, cependant le complexe RhlR-C_4HSL est dépendant de PqsE pour activer la transcription de ces propres gènes et celle de quelques gènes cibles. Le complexe MvfR-HHQ/PQS active sa propre transcription et celle de l'opéron *pqsABCDE*. Illustration modifiée de García-Reyes *et al.* (2019) et Groleau *et al.* (2020).

b. Le système Hmq n'appartient pas au QS

Contrairement aux attentes initiales, les HMAQs ont un très faible effet sur l'expression de l'opéron *hmqABCDEFG* chez *Burkholderia* (**Figure 1.17** ; (Chapalain et al. 2017; Vial et al. 2008; Guillouzer 2018]).

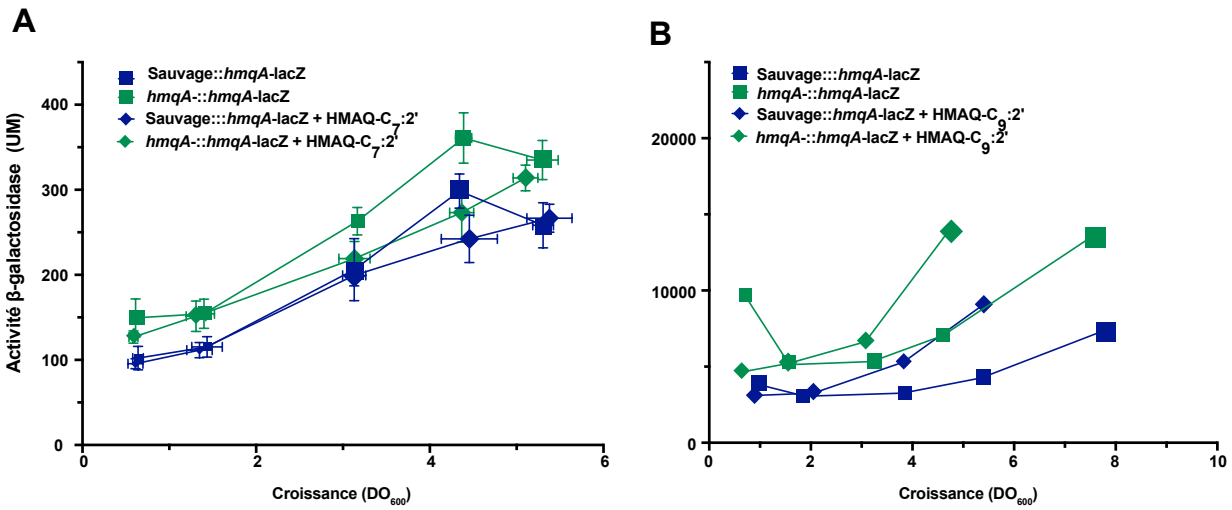


Figure 1.17 Les HMAQs n'autorégulent pas l'opéron *hmqABCDEFG* chez *Burkholderia*.

A) L'ajout de 50 µM de HMAQ-C_7:2' n'affecte pas l'expression de l'opéron *hmqABCDEFG* chez le mutant *hmqA*- de *B. ambifaria* HSJ1. B) L'ajout de 50 µM de HMAQ-C_9:2' n'affecte pas l'expression de l'opéron *hmqABCDEFG* chez le mutant *hmqA*- de *B. thailandensis* E264. Figure modifiée de Chapalain *et al.* (2017) et Le Guillouzer (2018).

c. Le système Hmq régule le QS

Contrairement à la situation chez *P. aeruginosa* (Déziel *et al.*, 2004), chez *Burkholderia*, le système Hmq régule le QS à base de AHLs mais cette régulation est différente entre les Bcc et le groupe « *pseudomallei* ». Chez *B. ambifaria* HSJ1, le système Hmq inhiberait indirectement le système Cep soit sur l'activité de CepI soit sur la fonction de C_8-HSL - et donc indirectement la production de 3OHC_{10}-HSL (**Figure 1.18**).

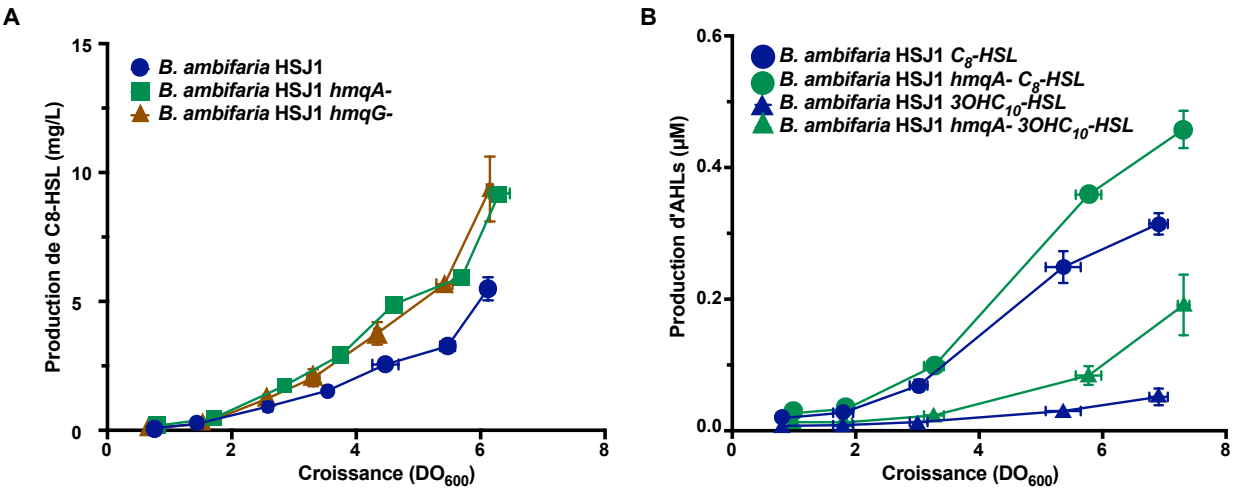


Figure 1.18 Le système Hmq inhibe la production d'AHLs chez *B. ambifaria* HSJ1

A) Production de C_8-HSL chez les mutants *hmqA-* et *hmqG-* de *B. ambifaria* HSJ1 (Figure modifiée de Vial *et al.* [2008]). B) Production de C_8-HSL et de 3OH-C_{10}-HSL du mutant *hmqA-* chez *B. ambifaria* HSJ1 (Figure modifiée de Chapalain *et al.*, [2017]).

Cependant chez *B. thailandensis* E264, la production des AHLs est activée par le système Hmq pendant que l'expression des synthases des trois systèmes de QS n'est pas affectée (**Figure 1.19**).

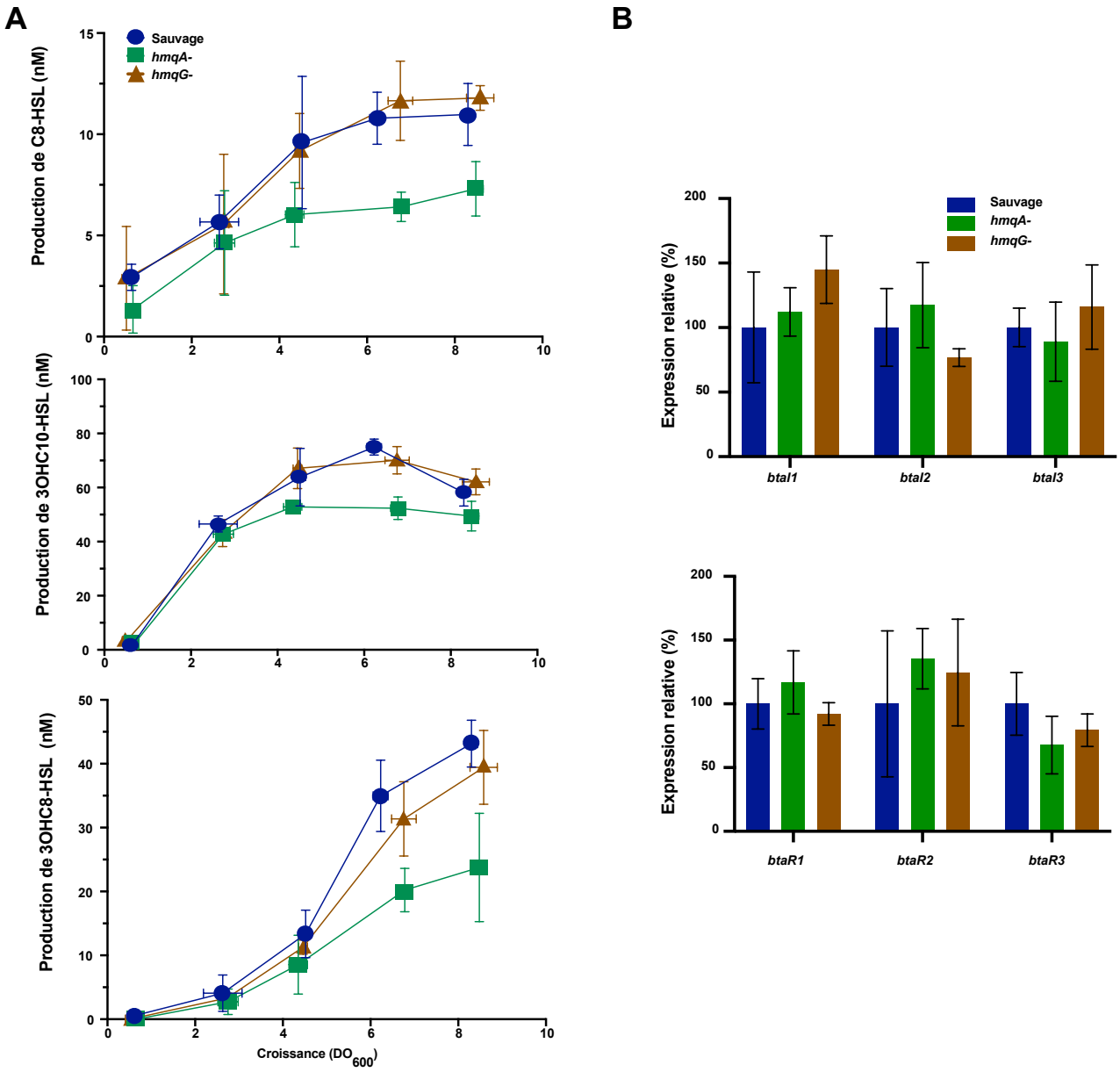


Figure 1.19 Le système Hmq active la production d'AHLs chez *B. thailandensis* E264

A) Production des différentes AHLs chez les mutants *hmqA-* et *hmqG-* chez *B. thailandensis* E264. B) Expression relative des synthases *btaI1*, *btaI2* et *btaI3* et des régulateurs *btaR1, btaR2* et *btaR3* chez les mutants *hmqA-* et *hmqG-* chez *B. thailandensis* E264. Figures modifiées de Le Guillouzer (2018).

d. Le QS régule le système Hmq

Chez *Burkholderia*, le QS régule le système Hmq mais cette régulation est différente chez les Bcc et le groupe « *pseudomallei* ». Le système Cep activerait l'expression de l'opéron *hmqABCDEFG* et la production de HMAQ chez *B. ambifaria* HSJ1 (**Figure 1.20** ; [Chapalain *et al.*, 2017; Vial *et al.*,

2008]). Cependant, aucune séquence consensus Cep ou Cep-box n'a été trouvée, laissant penser que la régulation par CepR sur le système Hmq est indirecte (Chapalain *et al.*, 2017).

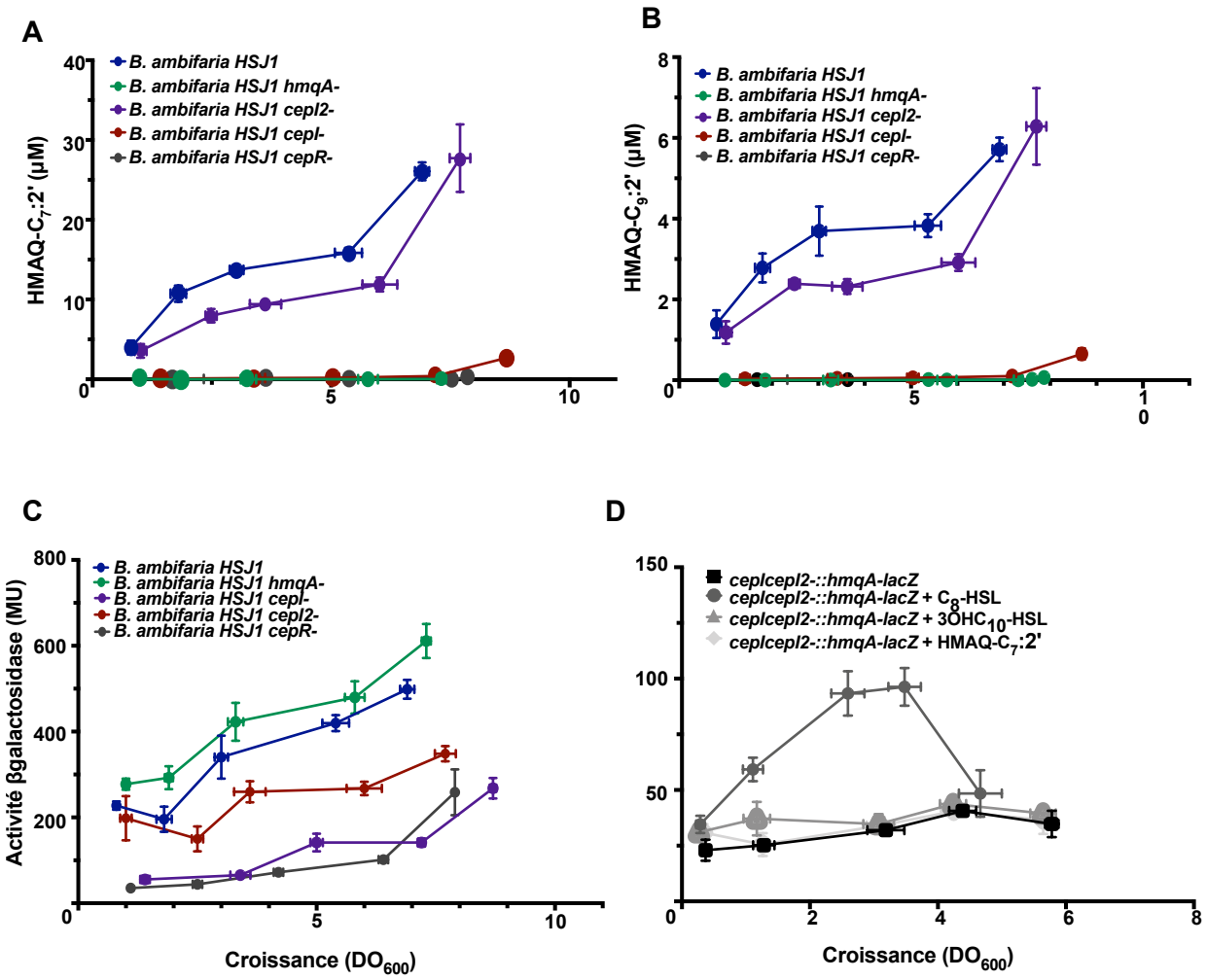


Figure 1.20 **Le système Cep active la production de HMAQ chez *B. ambifaria* HSJ1**

A) Production de HMAQ-C_7:2' des mutants *cepI-* et *cepI2-* chez *B. ambifaria* HSJ1. B) Production de HMAQ-C_9:2' des mutants *cepI-* et *cepI2-* chez *B. ambifaria* HSJ1. C) Activée β-galactosidase du gène rapporteur P*hmqA-lacZ* des mutants *cepI-* et *cepI2-* chez *B. ambifaria* HSJ1. D) Activée β-galactosidase du gène rapporteur P*hmqA-lacZ* du double mutant *cepI-cepI2-* supplémenté ou non de AHLs chez *B. ambifaria* HSJ1. Figures modifiées de Chapalain *et al.* (2017).

Chez *B. thailandensis* E264, l'expression de l'opéron *hmqABCDEFG* et la production des HMAQ sont inhibées par les systèmes de QS (**Figure 1.21** ; [Le Guillouzer, 2018; Majerczyk *et al.*, 2014; Mao *et al.*, 2017]).

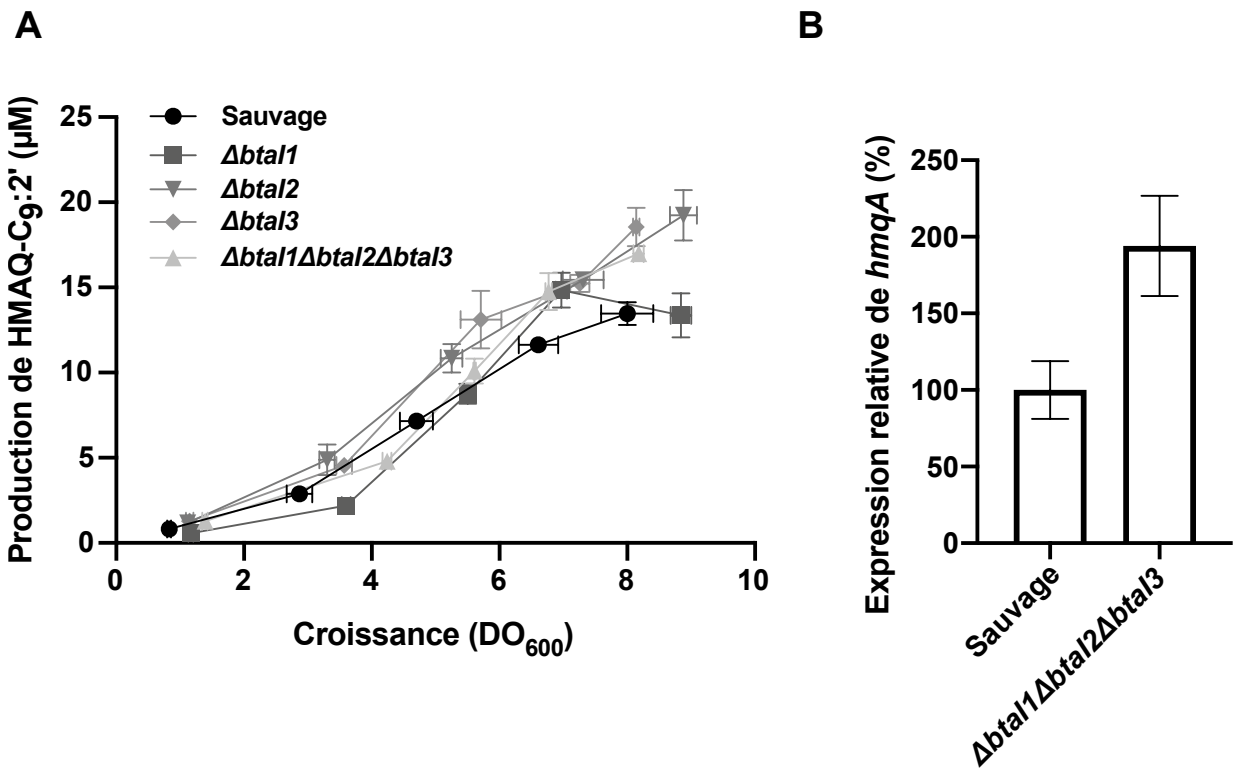


Figure 1.21 Le QS inhibe la production de HMAQ et l'expression de l'opéron *hmqABCDEFG* chez *B. thailandensis* E264

A) Production de HMAQ-C_9:2' chez les mutants simples et le triple mutant du QS chez B. thailandensis E264. B) Expression du gène hmqA chez le triple mutant du QS chez B. thailandensis E264. Figures modifiées de Le Guillouzer (2018).

e. Les autres régulateurs du système Hmq

Contrairement aux HHQ et PQS, qui autorégulent le système PQS chez *P. aeruginosa* PA14 via la régulation transcriptionnelle MvfR, le système Hmq est dépourvu d'homologue de MvfR (Cao *et al.*, 2001; Déziel *et al.*, 2004). Dumais (2010) a découvert, par mutagenèse aléatoire, que HmqR – un régulateur transcriptionnel de type LysR - active le système Hmq chez *B. thailandensis* E264 (**Figure 1.22 ;**[Le Guillouzer, 2018]). Renommé « *secondary metabolite regulator* » ou ScmR, ce régulateur, en plus d'activer le système Hmq et la production de HMAQs, active les systèmes de QS et inhibe la production de métabolites secondaires chez *B. thailandensis* (Le Guillouzer *et al.*, 2020; Mao *et al.*, 2017; Martinez *et al.*, 2020). Les régulateurs directs du système Hmq chez les Bcc restent encore inconnus à ce jour.

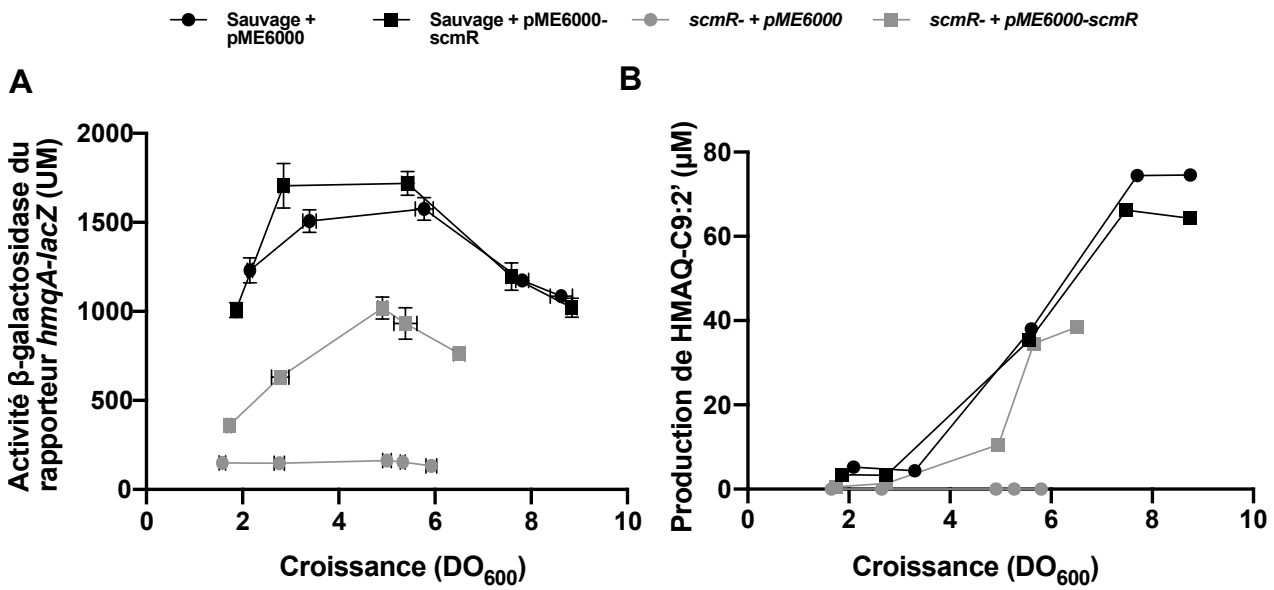


Figure 1.22 Le régulateur HmqR/ScmR active l'expression de l'opéron *hmqABCDEFG* et la production de HMAQ-C_9:2' chez *B. thailandensis* E264.

A) Activité β-galactosidase du gène rapporteur P*hmqA*-lacZ chez le mutant *scmR*-. B) Production de HMAQC_9-2' chez le mutant *scmR*-. Figure tirée de Le Guillouzer (2018).

Les différences retrouvées au niveau de la production des HMAQs, de la régulation du système Hmq et son implication dans la régulation du QS, suggèrent que le système Hmq joue un rôle différent chez les Bcc et chez les espèces appartenant au groupe « *pseudomallei* » (**Figures 1.23-1.24**).

1.2.3.4. La fonction des HAQs chez *P. aeruginosa* et des HHQ et HMAQ chez *Burkholderia*

De par la présence de l'opéron *hmqABCDEFG* sur le mégaplasmide de virulence, sa distribution retrouvée principalement au sein des souches cliniques des Bcc qui sont capables de produire des HMAQs (Diggle et *al.*, 2006; Vial et *al*., 2008), et son homologie avec le système PQS de *P. aeruginosa*, le système Hmq et les HMAQs pourraient jouer un rôle dans la virulence de certaines Bcc (Agnoli *et al.*, 2011; diCenzo *et al.*, 2019).

Chez *P. aeruginosa* les HAQs sont tout d'abord connues pour être des molécules de communication. De plus, le HHQ et le PQS moduleraient le système immunitaire de l'hôte et le HQNO serait un antimicrobien en plus d'avoir un effet immuno-modulateur (Heeb *et al.*, 2011; Saalim *et al.*, 2020; van Kessel, 2019).

De par la présence de HHQ et PQS dans les sécrétions pulmonaires et dans le liquide broncho-alvéolaire des personnes atteintes de fibrose kystique, ces molécules permettraient le développement de la niche écologique de *P. aeruginosa* par compétition avec les autres microorganismes (Guina *et al.*, 2003; Machan *et al.*, 1992; Royt *et al.*, 2001; Royt *et al.*, 2007) . Lors de l'infection, le HHQ et le PQS dérégulent le système immunitaire de l'hôte et interagissent avec le facteur de transcription NF-κB, induisant la suppression du système immunitaire inné de l'hôte (Kim *et al.*, 2010). Le PQS inhibe la prolifération des cellules T ainsi que la sécrétion de l'interleukine-2 par les cellules mononuclées du sang périphérique (Hooi *et al.*, 2004; Skindersoe *et al.*, 2009).

Le HQNO a des propriétés antimicrobiennes en inhibant la respiration en bloquant (1) les ubiquinones, les ménaquinones, (2) le cytochrome *bc1* de la chaine respiratoire chez les bactéries à Gram positif, (3) l'enzyme du transporteur sodium NaDH-quinone oxydoréductase chez les bactéries à Gram négatif et (4) le cytochrome *c* de la chaine respiratoire chez les eucaryotes (Ark & Berden, 1977; Déziel *et al.*, 2004; Hacker *et al.*, 1993; Hase *et al.*, 2001; Machan *et al.*, 1992).

Le rôle principal des HMAQs chez les Bcc reste à être déterminé. À ce jour, les HMAQs auraient (1) un effet indirect sur les phénotypes relatifs au QS (e.g. : production de sidérophores, activité protéolytique ; [Vial *et al.*, 2008]) et (2) une activité antifongique contre *Alternaria alternata, Aspergillus niger, Fusarium culmorum, F. graminearum, F. oxysporum* et *Rhizoctonia solani* en plus (3) d'un potentiel rôle d'antibiotique cytotoxique contre des cellules cancéreuses dépendantes IGF-I montrant le fort potentiel pharmaceutique des HMAQs (Kilani-Feki *et al.*, 2011; Li *et al.*, 2018; Mori *et al.*, 2007). Les HMAQs N-oxydes ayant une activité antimicrobienne contre *B. subtilis* (Klaus *et al.*, 2020), et étant seulement produites par le groupe « *pseudomallei* », ceci laisse penser que le rôle antimicrobien des HMAQs et HMAQs N-oxydes n'est pas primaire. Chez le groupe « *pseudomallei* » trois souches FK de *B. pseudomallei* surexpriment l'opéron *hmqABCDEFG* sur le

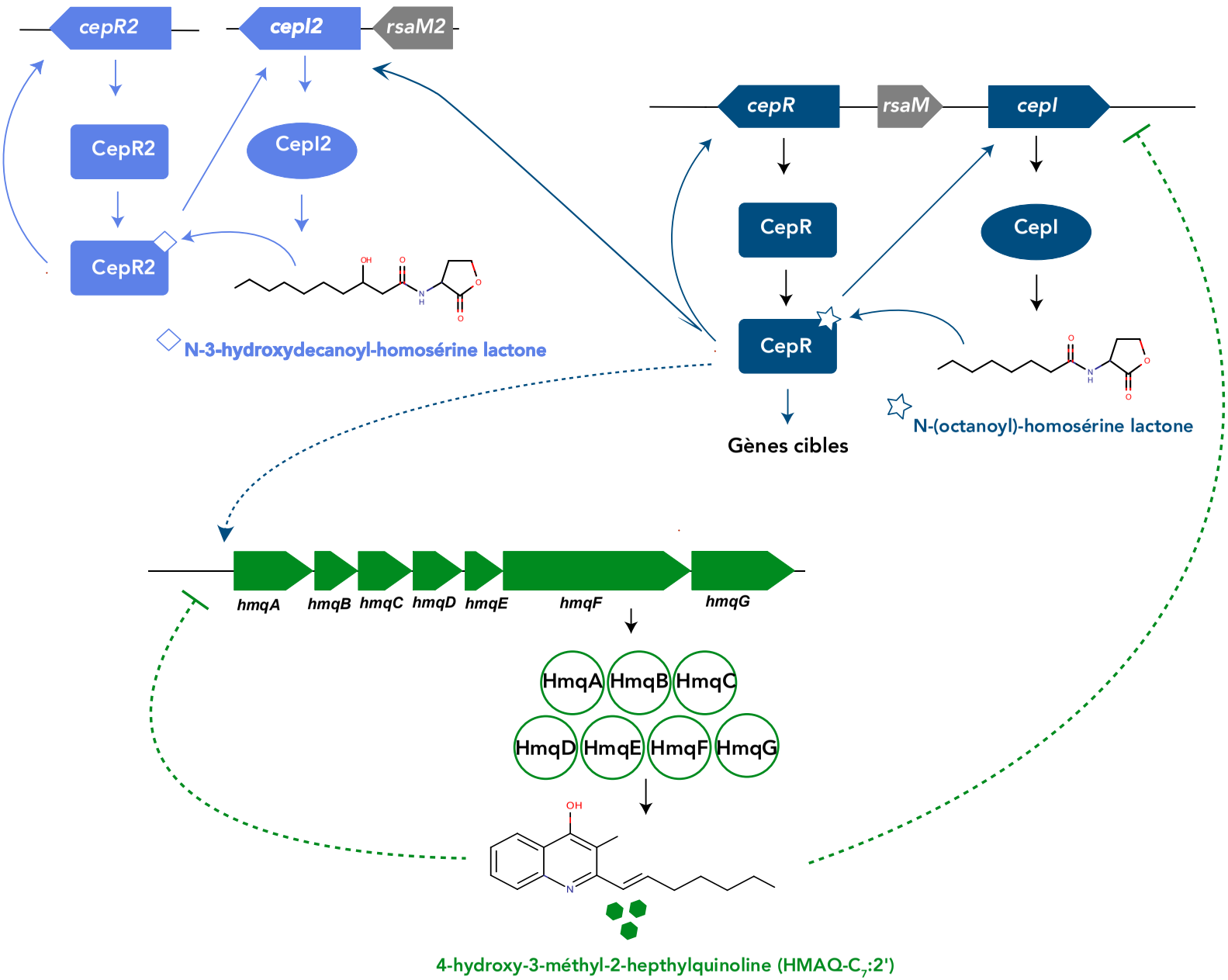


Figure 1.23 Interaction du QS avec le système Hmq chez *B. ambifaria* HSJ1.

Les deux systèmes du QS de *B. ambifaria* HSJ1 interagissent avec le système *Hmq*. En plus de réguler négativement la production de C_8-HSL, la production de HMAQs est induite indirectement par le système *Cep*. Illustration modifiée de Chapalain *et al.* (2017); Vial *et al.* (2008).

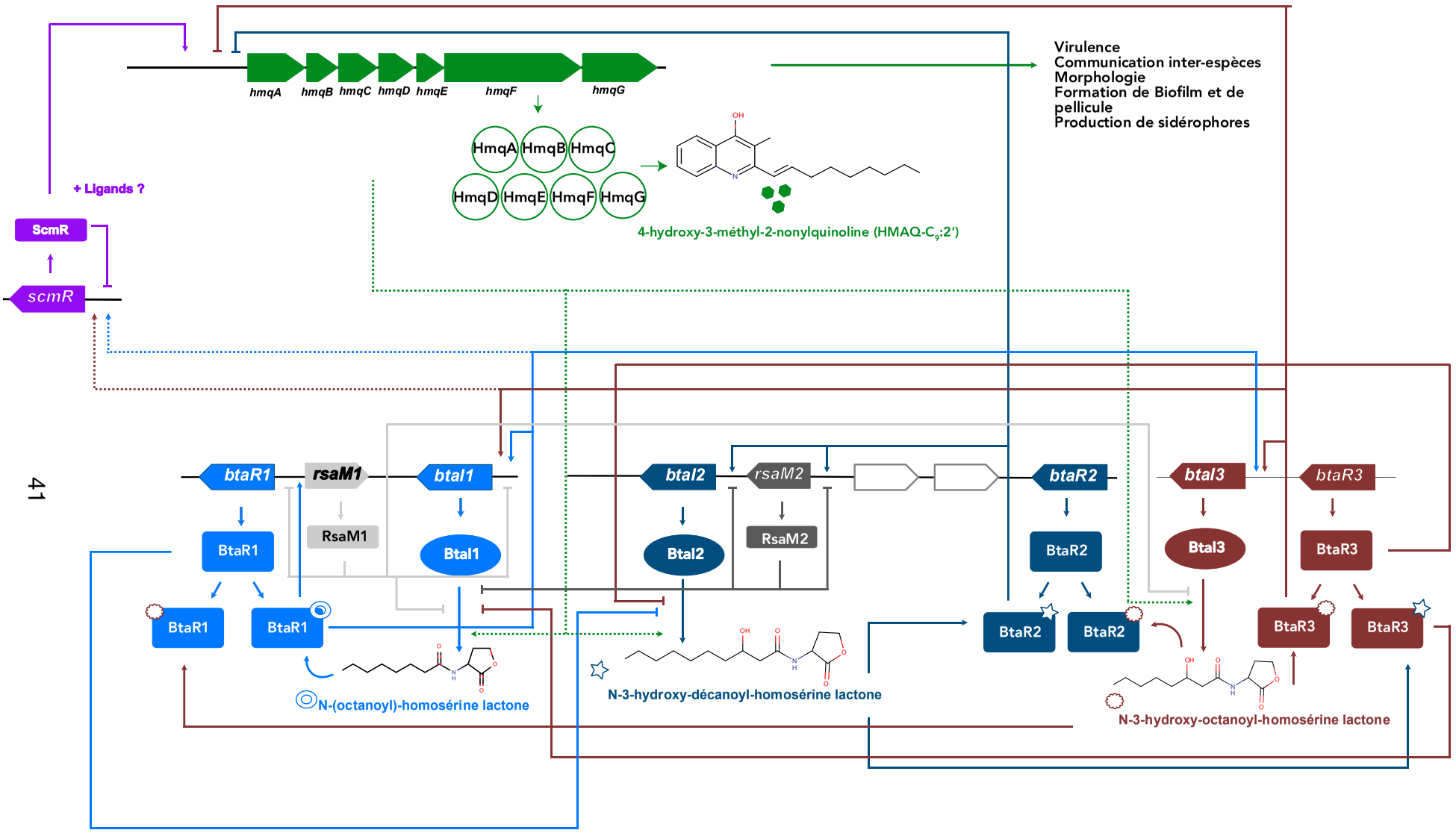


Figure 1.24 Interaction du QS avec le système *Hmq* chez *B. thailandensis* E264.

Les systèmes BtaI2 et BtaI3 inhibent le système Hmq pendant que le système Hmq active les trois systèmes de QS chez *B. thailandensis* E264 (Le Guillouzer, 2018).

long terme de l'infection et les HMAQs activent faiblement le système PQS chez *P. aeruginosa* signifiant que les HMAQs permettrait la communication inter-espèces et agiraient comme agents antimicrobiens afin d'établir la niche écologique de ces bactéries au sein des poumons des personnes atteintes de Fibrose Kystique ou bien moduleraient le système immunitaire de l'hôte pendant l'infection (Le Guillouzer, 2018; Price *et al.*, 2018).

2. HYPOTHÈSE ET OBJECTIFS

2.1. Hypothèse

Certaines souches de *Burkholderia* sont connues pour produire des HMAQ, des métabolites extracellulaires qui ont une forte homologie avec les HAQ produites par *P. aeruginosa*. Chez cette dernière espèce, certains HAQs agissent comme signaux intercellulaires dans un système de *quorum sensing* et sont responsables de la régulation d'un grand nombre de facteurs de virulence et ont un rôle dans la pathogénicité. Une autre famille de HAQs, ayant une substitution N-oxyde, possède une activité antimicrobienne.

Seules quelques rares souches de Bcc (majoritairement cliniques) ont été rapportées comme productrices de HMAQs. Concernant la régulation du système Hmq, des études ont été rapportées chez *B. ambifaria* HSJ1 montrant que les HMAQs inhibent : (1) la production de C_8-HSL en inhibant l'expression de CepI – synthase du système de *quorum sensing* Cep et (2) l'expression de l'opéron *hmqABCDEFG.*

2.2. Objectifs

Afin de répondre à la problématique posée et de confirmer les hypothèses, l'objectif général de ma thèse est de déterminer la prévalence et le rôle des HMAQ synthétisées par les enzymes codées par l'opéron *hmqABCDEFG* chez le complexe *Burkholderia cepacia* ainsi que la régulation de ce système.

Les objectifs spécifiques de mon projet sont de :

1 – L'dentification de la distribution de l'opéron *hmqABCDEFG* chez les Bcc par analyses bioinformatiques

2 – De cribler les souches de Bcc pour la présence de l'opéron *hmqABCDEFG* et la production de HMAQs

3 – De caractériser la régulation de l'opéron *hmqABCDEFG*, via la variation de la morphologie des colonies chez *B. ambifaria*

4 – De déterminer le rôle des HMAQ chez les Bcc

3. POTENTIAL OF THE BURKHOLDERIA CEPACIA COMPLEX TO PRODUCE 4-HYDROXY-3-METHYL-2-ALKYQUINOLINES

Le Potentiel du complexe *Burkholderia cepacia* à produire des 4-hydroxy-3-méthyl-2-alkyquinolines

Auteurs :
Pauline M.L. Coulon[1], Marie-Christine Groleau[1] and Eric Déziel[1]*
[1]INRS - Institut Armand Frappier, Laval, Québec, Canada

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Contribution des auteurs :
Pauline M.L. Coulon a conçu et mis en page l'article ; acquis, analysé et interprété des données
Marie-Christine Groleau a réalisé les expériences donnant la nouvelle hypothèse de la biosynthèse des 4-hydroxy-3-méthyl-2-alkyquinolines (HMAQs)
Eric Déziel a fourni les ressources nécessaires ainsi que le financement.
Les trois auteurs ont révisé l'article.

Conflits d'intérêt :
Les auteurs déclarent que la recherche a été conduite en l'absence de relations commerciales ou financières qui pourraient conduire à un potentiel conflit d'intérêt.

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ED détient la chaire de la recherche Canadienne en sociomicrobiologie.

3.1 Abstract

A few *Burkholderia* species, especially *Burkholderia pseudomallei, Burkholderia thailandensis, Burkholderia ambifaria* and *Burkholderia cepacia*, are known to produce and release various 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs), a family of molecules analogous to the 4-hydroxy-2-alkylquinolines (aka 2-n-alkyl-4(1*H*)-quinolones) of *Pseudomonas aeruginosa*, which include the *Pseudomonas* quinolone signal (PQS). However, while these exoproducts play several roles in *P. aeruginosa* virulence and survival, the available literature is very limited on their distribution and function in *Burkholderia*. In this perspective article, we studied the distribution of the *hmqABCDEFG* operon, which encodes the enzymes involved in the biosynthesis of HMAQs, in the *Burkholderia cepacia* complex (Bcc) group. Based on the available sequence data, about one third of Bcc species carry a homolog of the *hmqABCDEFG*, and not all sequenced strains in a given species possess this operon. Looking at the synteny of genes surrounding the *hmqABCDEFG* operon, we found that for some species, the operon seems to have been deleted or replaced by other genes. Finally, we review the literature on the possible function of HMAQs. Understanding the Hmq system may provide clues concerning their functions in Bcc.

Keywords: *hmqABCDEFG* operon (*hmq*), quorum sensing, synteny, 4-hydroxy-2-alkylquinolines (HMAQ), alkylquinolones, *Pseudomonas aeruginosa*, *pqsABCDE* operon, biosynthesis.

3.2 Introduction

In 1992 several species originally classified as *Pseudomonas* defined the new *Burkholderia* genus (Yabuuchi et al., 1992). This genus now comprises more than 60 Gram-negative bacterial species, which fit into two clades: the plant-associated beneficial and environmental one or the pathogenic one (Suárez-Moreno et al., 2012;Eberl and Vandamme, 2016). The plant-associated beneficial and environmental clade has recently been renamed *Paraburkholderia* because these bacteria lack biomolecular markers specific to pathogenic strains belonging to *Burkholderia* genus (Sawana et al., 2014). However, this split is controversial since some strains can be both pathogenic to animals and beneficial to plants (Eberl and Vandamme, 2016). The *Burkholderia* pathogenic clade is composed of plant, animal and human pathogens separated in two well-known groups: the "*pseudomallei*" group (*B. pseudomallei, B. mallei* and the environmental strain and study model *B. thailandensis*) and the opportunist pathogen species forming the *Burkholderia cepacia* complex [Bcc; (Suárez-Moreno et al., 2012)].

Bacteria belonging to the Bcc are mostly found in the rhizosphere, soil and water (Eberl and Vandamme, 2016;Loveridge et al., 2017). Bcc species are of interest in the industrial and

agricultural fields, for instance for their potential in bioremediation (e.g. *Burkholderia vietnamiensis*), in plant growth promotion (e.g. *Burkholderia ambifaria*) and also for their capacity to produce an array of secondary metabolites [reviewed by Vial et al. (2011)]. However, the realisation that many Bcc species are responsible for serious chronic infections among immunosuppressed patients in general, notably those suffering from cystic fibrosis (CF) or chronic granulomatous disease (CGD), has put a hold on their biotechnological use, especially in agriculture (Vial et al., 2011). Indeed, because such infections are highly transmissible between patients and typically highly antibiotic resistant, they are often fatal (Gold et al., 1983;Gilligan, 1991;Govan et al., 1993;Speert et al., 1994;Govan and Deretic, 1996;LiPuma, 1998).

Bcc species produce various virulence determinants such as exopolysaccharides, siderophores and antimicrobials and can adopt several social behaviours such as swarming motility and biofilm formation. These are controlled by quorum-sensing (QS)(Kang et al., 1998;Lewenza et al., 1999;Richau et al., 2000;Huber et al., 2001;El-Banna and Winkelmann, 2002;Aguilar et al., 2003). QS is a cell-to-cell communication system used by bacterial populations to sense their density and thus control the transcription of certain genes in a coordinated manner (Fuqua and Greenberg, 1998). In fact, this signaling system allows bacteria to optimize colonisation, to interact with their hosts and to better resist to stresses (Stewart and Costerton, 2001;Juhas et al., 2005).
In the Bcc, CepR is the main QS transcriptional regulator, which is activated by the autoinducing signal N-octanoyl-homoserine lactone (C_8-HSL), the product of CepI (McKenney et al., 1995;Lewenza et al., 1999;Lewenza and Sokol, 2001). Depending on the species, Bcc have at least two *cep* (*lux*) systems (Choudhary et al., 2013), similar to the *las* and *rhl* systems in *Pseudomonas aeruginosa*. Interestingly, Pesci et al. (1999) reported a third signal they called the *Pseudomonas* quinolone signal (PQS) also produced in the latter bacterial species. It was then found that PQS actually belongs to a large family of extracellular molecules called 4-hydroxy-2-alkylquinolines (HAQ), also referred to as 2-n-alkyl-4(1*H*)-quinolones (Déziel et al., 2004). Gallagher et al. (2002) identified the *pqsABCDE* polycistronic operon as required for the production of these HAQs in *P. aeruginosa*. The *pqs* system was confirmed as a *bona fide* QS system when PQS, and its biosynthetic precursor 4-hydroxy-2-heptylquinoline (HHQ), were shown to act as autoinducing ligands of the transcriptional regulator MvfR (also known as PqsR) (Wade et al., 2005;Xiao et al., 2006). MvfR controls the expression of *pqsABCDE* (Déziel et al., 2004) resulting in an autoinducing loop. Unexpectedly, a few *Burkholderia* strains were then reported to produce minute levels of HHQ (but not PQS) and to carry a cluster of *pqsABCDE* homologues (Diggle et al., 2006). Subsequently, it was found that the main products in these *Burkholderia* strains are actually 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs), principally distinct from *P. aeruginosa* HAQs by the presence of a methyl at the 3' position and the predominance of an unsaturated alkyl side chain, among congeners (Vial et al., 2008), explaining why Diggle et al. (2006) had only detected very low concentrations of HHQ (Figure 3.1). This biosynthetic operon was accordingly named

hmqABCDEFG, with the additional encoded HmqF and HmqG respectively responsible for the unsaturation (Agarwal et al., 2012) and the methylation (Vial et al., 2008) of HMAQs. It should be mentioned that no congener corresponding to PQS, thus with a hydroxy substitution at the 3 position, has been detected in any *Burkholderia* culture, in agreement with the absence of a *pqsH* homologue. As with the *pqs* system in *P. aeruginosa*, interactions between the *hmq* and *cep* systems have been reported in *B. ambifaria* (Vial et al., 2008;Chapalain et al., 2017). While the production of some HMAQ congeners by a few *Burkholderia* isolates has been reported (Diggle et al., 2006;Mori et al., 2007;Vial et al., 2008;Kilani-Feki et al., 2011;Mahenthiralingam et al., 2011;Kilani-Feki et al., 2012;Li et al., 2018), no studies have yet systematically investigated the prevalence of the *hmq* system and the capacity to produce HMAQs in the genus *Burkholderia*. In this perspective article, we review the knowledge on the *hmq* system, update the HMAQ biosynthesis pathway and investigate the distribution of the *hmqABCDEFG* operon in *Burkholderia* and especially in Bcc.

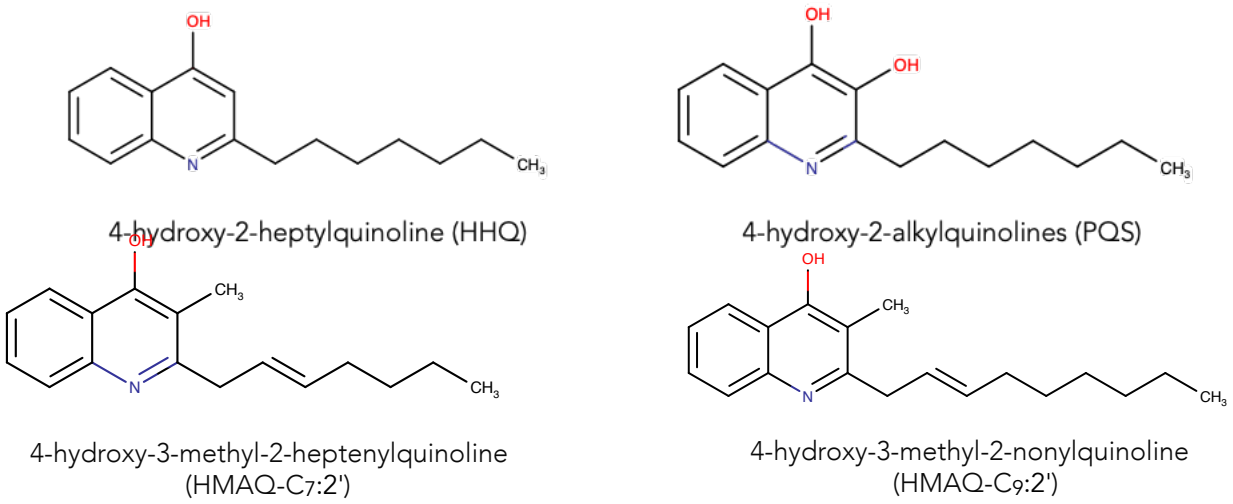

Figure 3.1 HAQ molecules produced by *Pseudomonas aeruginosa* and *Burkholderia* cepacia complex species.
Both species synthetize 4-hydroxy-2-heptylquinoline (HHQ). *P. aeruginosa* produces 4-hydroxy-2-alkylquinoline (PQS) and Bcc species produce 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs).

3.3. Distribution of the *hmqABCDEFG* operon in the *Burkholderia* genus

Based on complete and draft genome sequences available in the *Burkholderia* Genome database **(http://www.burkholderia.com)**, we have performed two complementary analyses with *hmqABCDEFG* of *B. ambifaria* strain AMMD as a reference: (1) an alignment sequence analysis () and (2) an orthology analysis. The BLAST analysis was run for the nucleic acid sequence of each gene composing the *hmq* operon, using the default settings (Johnson et al., 2008;Winsor et al., 2008) (Johnson et al. 2008; Winsor et al. 2008). The orthology analysis consisted in examining the similarity of the protein sequence and the flanking gene regions by the pair-wise Diamond searches

(Johnson et al., 2008;Winsor et al., 2008). While that method may be more accurate than a BLAST analysis, an orthology analysis could miss some positive results if the *hmqABCDEFG* operon is in a different genomic location than the reference. Thus, a combination of both methods was selected.

Using the two analyses, we screened: (1) the seven genes composing the *hmqABCDEFG* operon based on the percentage of identity and (2) the species having all seven genes present in their genome. Sequences of the *hmqABCDEFG* operon are well conserved, with a nucleotide identity between 70 and 100%. Importantly, we only found instances of a *hmqG* homologue in a genome if it belonged to a *hmqABCDEFG* operon.

Based on both analysis methods, the *hmqABCDEFG* operon was found in six out of 21 Bcc species: *B. cepacia, B. ambifaria, B. pyrrocinia, B. ubonensis, B. contaminans*, and *B. stagnalis*, plus in some still unclassified *Burkholderia* spp. - having at least one sequenced genome present in the Burkholderia.com database (**Table 3.1**). Furthermore, some strains of *B. lata,* and *B. territorii* appear to have the *hmqABCDEFG* operon too, but this was only found via the BLAST analyses. Actually, the percentage of strains having the putative operon is higher by BLAST analysis than by orthology analysis. The *hmqABCDEFG* operon shows strong nucleic acid identity between the strains and within the genus *Burkholderia*. In fact, the average nucleic identities for each gene are between 78.5 and 83.9% compared to the *B. ambifaria* AMMD operon sequences (**Table S3.1**). All these data show that (1) about one third of Bcc species carry the *hmqABCDEFG* operon and (2) within a species not all of the strains carry it.

A Bayesian phylogeny analysis, based on the *hmqABCDEFG* genes, shows extensive concordance with strain speciation, implying that this operon has been inherited from the common ancestor of *Burkholderia* rather than the results of recent gene transfer (**Figure S3.1**). The presence of homologous operons in the evolutionary distant genera *Burkholderia* and *Pseudomonas* suggests a possible past horizontal gene transfer event, typically characterised (1) by a different %GC, (2) mobile elements insertion and (3) genomic islands (Lawrence and Ochman, 2002;Juhas et al., 2009;Ravenhall et al., 2015). A similarly high %GC between *Burkholderia* (65%) and *P. aeruginosa* (67%) prevents the use of %GC to infer lateral gene transfer between these species. ISfinder (http://www-is.biotoul.fr) was used to find already known repeat sequences as indications of mobile elements insertion (Siguier, 2006). Islandviewer 4 (http://www.pathogenomics.sfu.ca/islandviewer) was used to predict genomic island on the third chromosome of *B. ambifaria* AMMD genome, and the second chromosome of *B. thailandensis* E264 and *B. pseudomallei* K96243 genomes (Bertelli et al., 2017). None of these methods revealed clear indications of horizontal gene transfer of the *hmqABCDEFG* operon and its surrounding genes.

Tableau 3.1 The *hmqABCDEFG* operon distribution in *Burkholderia cepacia* complex

	Species	*Bcc strains included in Burkholderia Genome DB* [Complete genomes]	*Analysis of the* hmqABCDEFG *operon's distribution by* Orthology analysis (DIAMOND)	Alignment analysis (BLASTN)
Bcc	*Burkholderia cepacia*	337 [24]	42	77
	(genomovar I) *Burkholderia multivorans*	56 [7]	-	-
	(genomovar II) *Burkholderia cenocepacia*	243 [15]	-	-
	(genomovar III) *Burkholderia stabilis*	-	-	-
	(genomovar IV) *Burkholderia vietnamiensis*	41 [6]	-	-
	(genomovar V) *Burkholderia dolosa*	2 [1]	-	-
	(genomovar VI) *Burkholderia ambifaria*	6 [2]	2	3
	(genomovar VII) *Burkholderia anthina*	8 [-]	-	-
	(genomovar VIII) *Burkholderia pyrrocinia*	4 [1]	1	3
	(genomovar IX) *Burkholderia ubonensis*	292 [6]	75	283
	(genomovar X) *Burkholderia latens* (BCC1)	2 [1]	-	-
	Burkholderia diffusa (BCC2)	12 [1]	-	-
	Burkholderia arboris (BCC3)	-	-	-
	Burkholderia seminalis (BCC7)	3 [1]	-	-
	Burkholderia metallica (BCC8)	1 [1]	-	-
	Burkholderia lata (group K)	4 [2]	-	2
	Burkholderia contaminans	7 [1]	2	3
	(group K, BCCAT) *Burkholderia pseudomultivorans*	9 [1]	-	-
	Burkholderia stagnalis (BCC B)	64 [1]	32	63
	Burkholderia territorii (BCC L)	33 [1]	-	2
	Burkholderia paludis	-		
	Burkholderia sp.	59 [18]	2	11
Total of Bcc strains		**1257 [91]**	**166**	**447**
	B. pseudomallei	677 [75]	27	655
	B. thailandensis	28 [15]	13	22
Total of *pseudomallei* group strains		**705 [90]**	**284**	**677**
Total of strains		**1962 [181]**	**450**	**1123**

The distribution of the *hmqABCDEFG* operon has been determined by using the orthology analysis available on *Burkholderia* genome DB and BLAST from *B. ambifaria* AMMD nucleic acid sequence. Both methods have been used individual gene, and only the strains having all seven genes in their genome have been kept. For the BLAST analysis, only genes having a high identity with the reference have been kept. The sign "-" means that no sequences were available

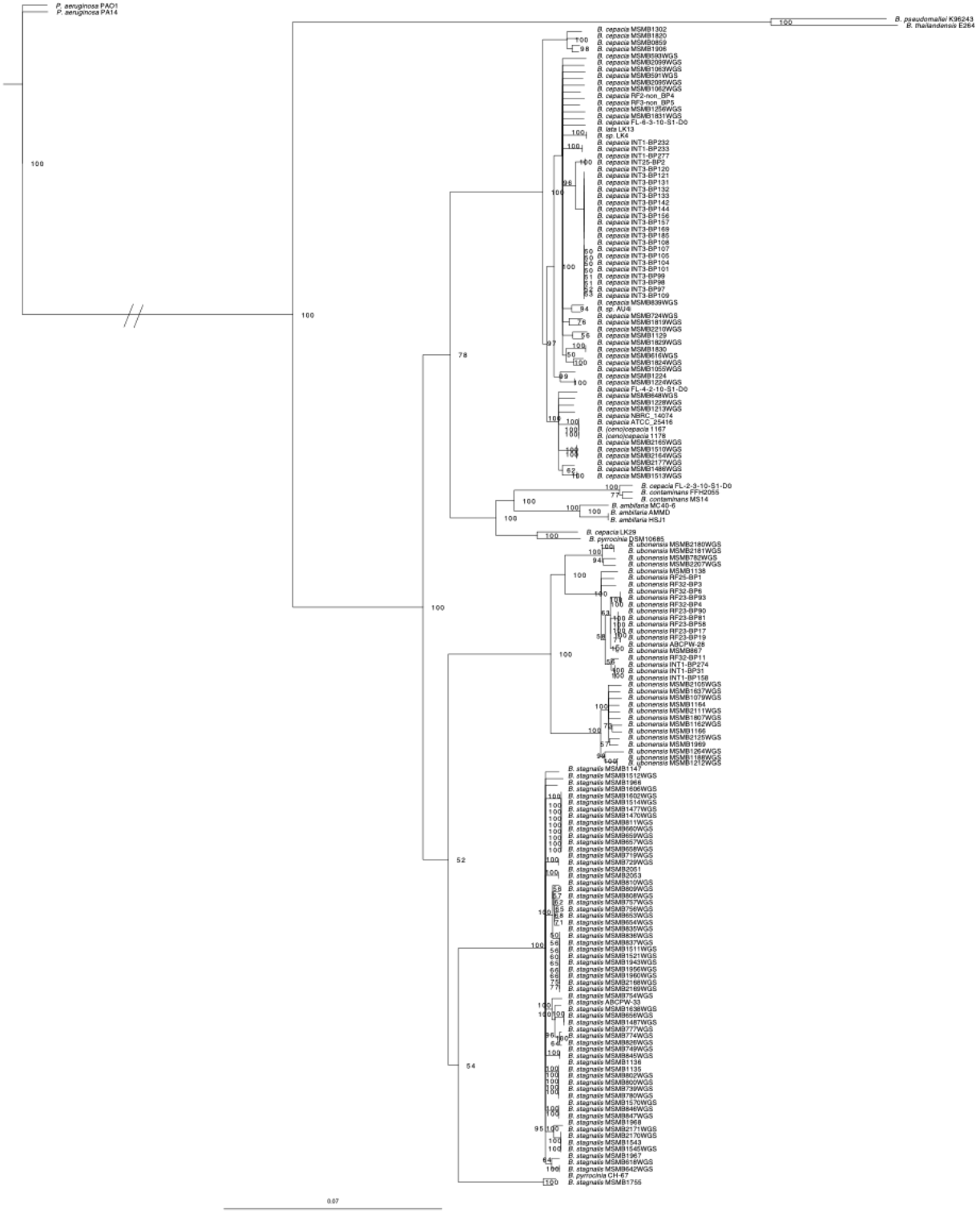

Figure S 3.1 Phylogenetic tree of strains of *Burkholderia* and the related species *P. aeruginosa* generated in Mr Bayes based on the nucleic acids sequence of the complete *hmq* operon.
A BLAST has been run from *B. ambifaria* AMMD genome. the sequences have been aligned by MUSCLE before to have been analyzed by Mr Bayes using the quick start settings on 9.000.000 generations. The p-value of the branches spilts was approximately 0.03.

3.4. The synteny of the *hmqABCDEFG* operon in *Burkholderia*

Given that the *hmqABCDEFG* operon has a heterogeneous distribution in Bcc, we decided to examine its genomic context. The number of complete genome sequences being limited, the analyses was performed on *B. ambifaria* AMMD, *B. ambifaria* MC40-10, *B. cepacia* ATCC25416, *B. contaminans* MS14, *B. pyrrocinia* DSM 10685, *B. ubonensis* MSMB22, *B. pseudomallei* K96243 and *B. thailandensis* E264, all available in the *Burkholderia* Genome database (Winsor et al., 2008).

A multiple whole-genome alignment using progressiveMAUVE software was done to view conserved regions surrounding the *hmqABCDEFG* operon [**Figure 3.2**; (Darling et al., 2010)]. By this analysis, we defined three groups: (1) *B. ambifaria* (2) *B. contaminans, B. pyrrocinia, B. cepacia* and (3) *B. ubonensis*, *B. cenocepacia* H111 and *B. dolosa* AU158 strains. Members of this third group lack the *hmqABCDEFG* operon in their genomes, are also included in the comparison to show a possible gene rearrangement. Synteny in genes surrounding the operon confirms the absence of the operon in *B. cenocepacia* H111 in which it is replaced by a number of metabolic genes. Also, a high similarity exists between the neighbouring genes of *B. dolosa* AU158 and *B. ubonensis* MSMB22. In fact, in *B. dolosa* AU158 the *hmqABCDEFG* seems to have been deleted from or not yet integrated into the genome of this strain. The lack of available complete sequences limits the synteny analysis, more comparison could help us to have a better understanding of the *hmqABCDEFG* distribution.

3.5. The Hmq synthesis pathway in *Burkholderia*

In *Burkholderia,* the *hmq* system was found first on the second chromosome of four *Burkholderia pseudomallei* (K96243, 576, 10276, 844)*, B. thailandensis* E30 and on the third chromosome of *B. cenocepacia* J415, to be homologous to the *Pseudomonas* Quinoline Signal (PQS) system found in *P. aeruginosa* (Diggle et al., 2006). The *hmq* system produces 4-hydroxy-2-heptylquinoline (HHQ) but not 3,4-dihydroxy-2-heptylquinoline (PQS) signaling molecules (Diggle et al., 2006). In fact, the strains produce the methylated 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) instead of PQS molecules (Vial et al., 2008). The *hmqABCDEFG* operon encodes the *hmq* system and has two more genes than the *pqsABCDE* operon in *P. aeruginosa* meaning that the HMAQs biosynthesis and PQS biosynthesis pathways are similar except for the HmqF and HmqG additional functions [**Figure 3.3**; (Vial et al., 2008)].

In fact, the putative anthraniloyl-CoA synthase encoded by *hmqA*, allows anthraniloyl-CoA synthesis from anthranillic acid, coming from tryptophan or chorismic acid by the KynABU catabolism pathway and the TrpEG biosynthesis pathway, respectively (Farrow and Pesci, 2007). The putative 3-oxoacyl-ACP-synthase (HmqD), binding the anthraniloyl-CoA to transfer a SCoA fatty acid or an acetate to form 2-aminobenzoylacetate-CoA (Zhang et al., 2008;Dulcey et al., 2013). In addition, playing an active role in the QS, the putative metallo- *β* - lactamase HmqE should favor the HHQ pathway production instead the DHQ one, because of the phenotype restoring in a *pqsE* mutant by adding *hmqE* (Diggle et al., 2006;Farrow et al., 2008;Rampioni et al., 2010;Folch et al., 2013;Drees and Fetzner, 2015;Rampioni et al., 2016). However, a *pqsE* mutant is still able to produce HHQ/ PQS, showing that PqsE is not essential for PQS biosynthesis. Moreover, the *β* -ceto-decanoyl-ACP synthases HmqB and the HmqC should form a complex like PqsBC [5DWZ; www.rcbs.org ; (Drees et al., 2016)] because of the high similarity between the N-terminal amino acid sequence between HmqB and PqsB. By protein sequence analysis performed with Chimera [UCSF Chimera; (Pettersen et al., 2004)], this conserved amino acid sequence should allow the interaction between HmqB and HmqC by forming the same structure PqsB/PqsC putatively. The two supplementary genes *hmqF* and *hmqG* encode, respectively, for putative AMP dependant synthase ligase and methylase. HmqF is responsible for the unsaturation on the alkyl chain and HmqG is responsible for the methylation of the molecule (Vial et al., 2008;Agarwal et al., 2012).

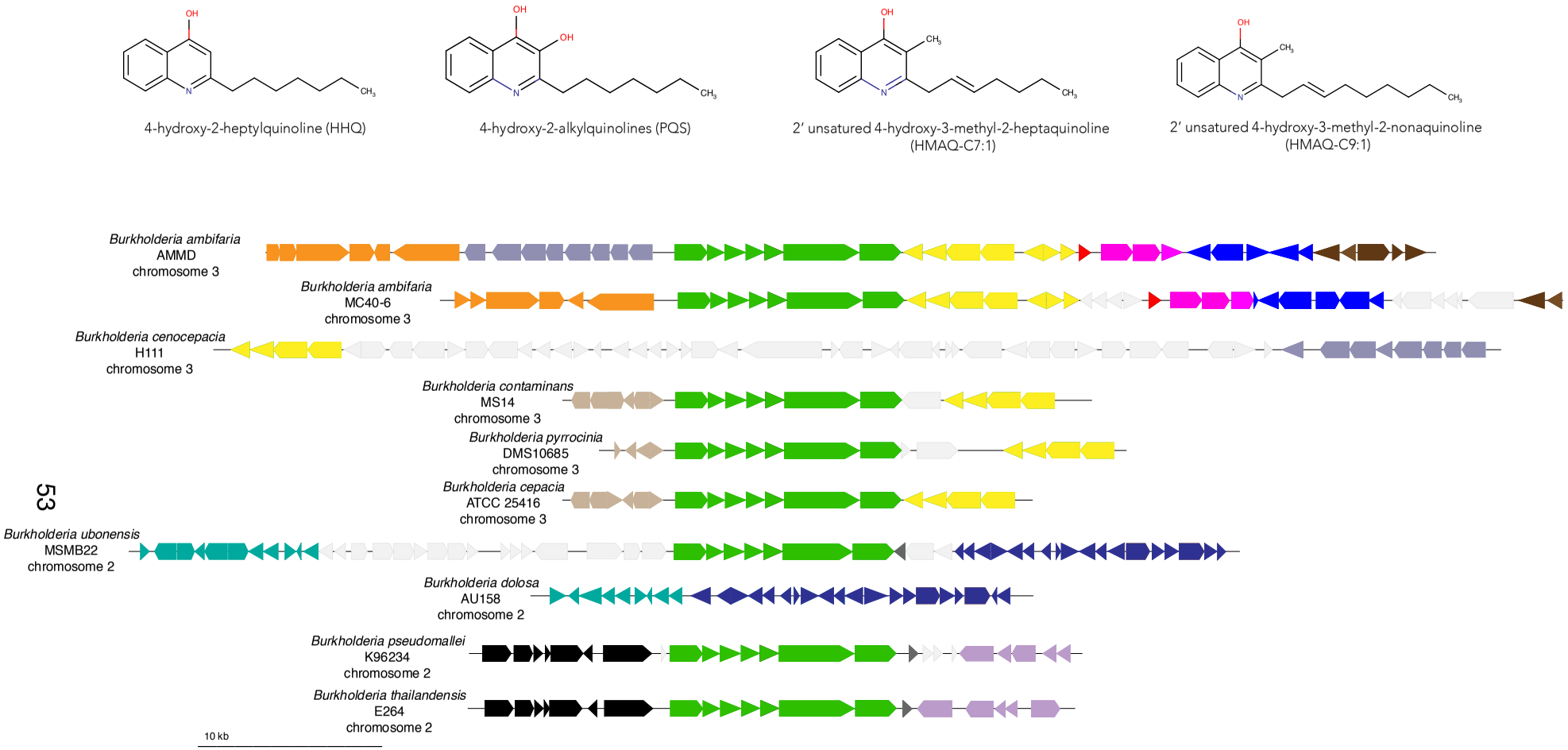

Figure 3.2 HAQ molecules and the synteny of genes surrounding the *hmqABCDEFG* operon in *Burkholderia* cepacia complex species.

A) Representation of HAQ molecules found in *P. aeruginosa* or/and in *Burkholderia*. B) Synteny of *hmqABCDEFG* operon in Bcc: Each colour represents a different synteny group determined by MAUVE software. Only the closest genes of the *hmqABCDEFG* operon have been shown. The *hmqABCDEFG* operon is represented in green colour. Light grey colour represents variable metabolic genes without synteny.

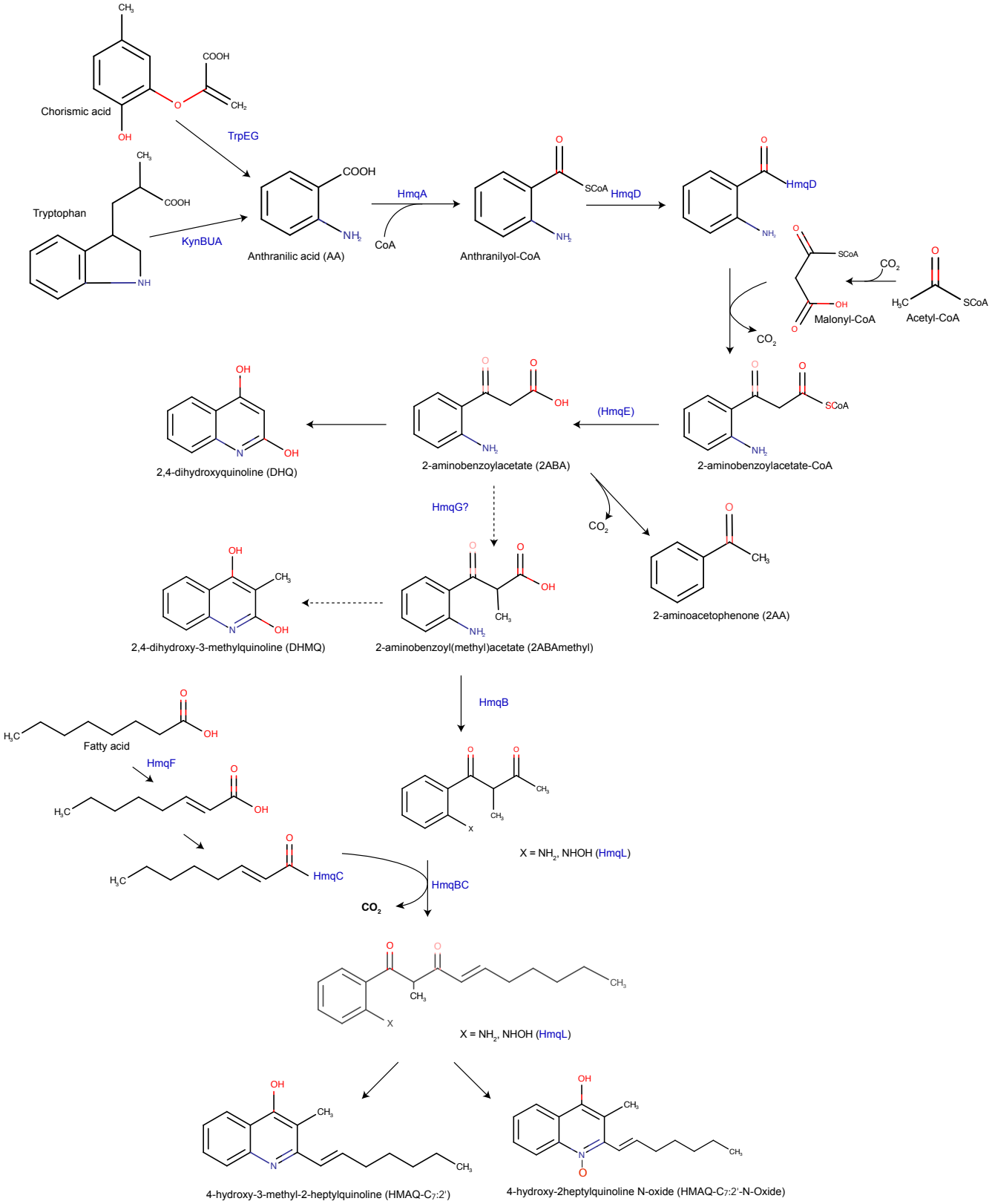

Figure 3.3 An updated version of the Hmq biosynthesis pathway based on Vial et al. (2008).
This Hmq biosynthesis pathway, homologous to PQS biosynthesis in *P. aeruginosa*, produces HHQ and HMAQs both with a saturated or unsaturated acyl chain. HmqF adds the unsaturation on the fatty acid. HmqG may methylate 2-ABA to form 2-ABAmethyl which will form either HMAQs and HMAQ-N-oxides but also DHMQ in lower quantity. Note that only *B. pseudomallei* and *B. thailandensis* are able to produce HQNO molecules.

Burkholderia species do not have PqsH homolog in their genomes which explains the absence of the synthesis of hydroxyl HHQ molecules including PQS (Déziel et al., 2004). However, HmqL, a homolog PqsL, has been found in *B. thailandensis* E264. This protein is a putative flavin adenine dinucleotide mono-oxygenase allowing the HQNO production (Vial et al., 2008; Butt et al., 2016). HmqL might also present in the strain *B. ambifaria* AMMD by BLAST analysis on *Burkholderia* Genome BD. An experimental analysis of HMAQs production would confirm this hypothesis.

The first HMAQ congener reported, named burkholone, was identified in culture broth of the environmental strain *Burkholderia* sp. QN15488 (Mori et al., 2007). Vial et al. (2008) described the production of three HMAQ families in *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. ambifaria* HSJ1, for a total of 19 different HMAQ congeners. This is without mentioning congeners without a methyl at the 3 position, thus similar to those produced by *P. aeruginosa* (Déziel et al., 2004). While it was originally believed that among Bcc species, only clinical *B. ambifaria* strains produce these molecules (Vial et al., 2008;Vial et al., 2009), it was later reported that environmental *B. ambifaria* AMMD and a few *B. cepacia* strains also produce HMAQs (Kilani-Feki et al., 2011;Mahenthiralingam et al., 2011;Kilani-Feki et al., 2012). However, these latter studies required large concentrations of culture extracts, maybe explaining the absence of detection in the original studies. This suggests disparities in production levels between strains, possibly reflecting the lack of knowledge on the culture conditions directing the production of these secondary metabolites. Recently, Li et al. (2018) isolated new HMAQs from the culture broth of the environmental strain *Burkholderia* sp. MBAF1239. It is expected that more HMAQs congeners will be reported, with advances in metabolomic studies [e.g. see Okada et al. (2016)].

3.6. Regulation of Hmq system and function of HMAQs

Recently, a LysR-type transcriptional regulator named ScmR was found to positively influence the transcription of the *hmqABCDEFG* operon in *B. thailandensis* E264 (Mao et al., 2017). We found that ScmR has a potential ortholog with a portion of the gene being 91.6% homologous in *B. ambifaria* AMMD strain (BAMB_RS03575). While HAQs such as HHQ and PQS are QS autoinducers in *P. aeruginosa*, no *hmqABCDEFG* operon induction by HMAQs has been seen in *B. ambifaria* HSJ1 and *B. thailandensis* E264 (Pesci et al., 1999;Vial et al., 2008;Chapalain et al., 2017). However, the *hmqABCDEFG* operon appears indirectly repressed by the *cep* system, while production of acyl-HSL is increased in a HMAQ-null mutant (Chapalain et al., 2017).

When present on the genome of Bcc strains, the *hmqABCDEFG* operon is located on the third chromosome except in *B. ubonensis* in which it is similar to the "*pseudomallei*" group, where it is located on chromosome 2. Its location on the third chromosome, defined by (Agnoli et al., 2011) as a virulence megaplasmid, suggests that the *hmqABCDEFG* operon is involved in virulence in some

Bcc species. For now, the only identified function of HMAQs is as antimicrobials (Kilani-Feki et al., 2011;Mahenthiralingam et al., 2011). The non-methylated HQNO plays a role in *B. thailandensis* E264 by inhibiting cytochrome *bc1* and pyrimidine biosynthesis like in *P. aeruginosa* (Wu and Seyedsayamdost, 2017). Also, HMAQ-C_9:2', the main congener produced by *B. thailandensis*, seems to dissipate the proton motive force and also inhibits pyrimidine biosynthesis acts synergistically with HQNO to inhibit bacterial growth (Wu and Seyedsayamdost, 2017). The question remains open for the Bcc; in our previous work, HQNO family congeners were absent from *B. ambifaria* cultures, and we proposed this was explained by the absence of a PqsL homologue, the enzyme required for the biosynthesis of this family (Lépine et al., 2004;Vial et al., 2008). All our analyses performed since then on HMAQs produced by a range of Bcc strains support this assertion: the Bcc does not produce HQNO family H(M)AQs (Vial et al., 2008). Since HMAQs affect C_8-HSL production, this impacts QS phenotypes such as the production of siderophores and proteolytic activity (Vial et al., 2008). Because of the analogy with the HAQs, HMAQs might act as signaling molecules, be required to control many virulence factors and even have an immuno-modulating activity as described for other HAQs (Heeb et al., 2011;Price et al., 2018). In fact, in *P. aeruginosa,* HHQ synchronizes the bacterial population by inducing the production of PQS (Déziel et al., 2004). Furthermore, PQS and HHQ act as immunomodulator interacting with the peripheral blood mononuclear cells and the dendritic cells (Hooi et al., 2004;Skindersoe et al., 2009;Kim et al., 2010a;Kim et al., 2010b). Price et al. (2018) have shown that three *B. pseudomallei* strains isolated from CF patients overexpressed the *hmqABCDEFG* operon in long term infection. These strains could use HMAQs as antimicrobials in niche protection or to modulate the host immune response during infection (Price et al., 2018). Apart from signaling properties, HMAQ might have pharmaceutical potential by acting as an antimicrobial and as a cytotoxic antibiotic against IGF-I dependant cells in cancer progression (Mori et al., 2007;Li et al., 2018).

3.7. Conclusion and Futures Directions

Taking all these elements into consideration, it is important to understand the distribution of the *hmqABCDEFG* operon and its evolution within the Bcc. The *hmqABCDEFG* operon's presence is distributed within some species in the Bcc but not all of the sequenced strains in a given species have the operon. More sequencing data will be required to reach broader conclusions on the *hmqABCDEFG* operon distribution trends. Moreover, because available data suggest that clinical strains produce more HMAQs than the environmental ones, it will be necessary to assess this characteristic in more Bcc species, and in more strains by species.

Variant strains of *B. ambifaria* HSJ1 produce much less HMAQ than the wild type (Vial et al., 2009) even if they have 99.8 % nucleotide identity with *B. ambifaria* HSJ1 and *B. ambifaria* AMMD genomes, suggesting that a negative regulator could control the *hmq* system. It will be interesting to

know if the QS regulation of HMAQ production could be a conserved feature in Bcc, as previously reported in *B. ambifaria* HSJ1, by testing different species (Chapalain et al., 2017).

Our understanding of the prevalence, regulation and functions of HMAQs is limited. Apart from the antimicrobial activity, no function has been discovered yet. However, HMAQs may have similar roles to HAQs produced by *P. aeruginosa* based on their analogy (Vial et al., 2008; Price et al., 2018).
Future studies of the regulation of the *hmq* system and the role of HMAQs are necessary to know if they play a role in virulence. This knowledge should provide a better understanding of *Burkholderia* especially in pathogens *B. pseudomallei* and Bcc strains.

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4. 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

La présence du système Hmq et la production des 4-hydroxy-3-méthyl-2-alkylquinolines sont distribuées hétérogènement entre les espèces du complexe *Burkholderia cepacia* et sont plus prévalents parmi les souches environementales que cliniques

Auteurs :

Pauline M.L. Coulon[A], James E.A. Zlosnik[B], & Eric Déziel[A*]

[A]Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique (INRS), Laval, QC, Canada

[B]Canadian *Burkholderia cepacia* Complex Research and Referral Repository, University of British Columbia, Vancouver, BC, Canada

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PC : conception et réalisation du manuscrit, et acquisition des données ; JZ : donation et identification de 200 souches cliniques de Bcc; ED : conception du projet, ressources et financement ; PC, JZ et ED : analyse et interprétation des données, révision du manuscrit.

Lien entre l'article ou les articles précédents et le suivant :

L'article précédent présente la prévalence de l'opéron *hmqABCDEFG* chez les Bcc en ayant réalisé une étude par bioinformatique basée sur 1 256 génomes disponibles appartenant à 21 espèces de Bcc. Cette étude *in sillico* a montré que le système Hmq est présent chez *B. ambifaria, B. cepacia, B. contaminans B. pyrrocinia*, *B. stagnalis, B. territorii* et *B. ubonensis* et absent chez *B. anthina, B. arboris B. cenocepacia, B. diffusa, B. latens, B. metallica, B. multivorans, B. pseudomultivorans, B. seminalis, B. stabilis* et *B. vietnamiensis* – qui sont des espèces majoritairement isolées de cas cliniques. Le séquençage n'étant homogène il reste à savoir si les résultats obtenus ne sont pas baisés par le manque de séquences pour certaines espèces de Bcc. Par ailleurs, l'étude de présence de l'opéron ne prédit en aucun cas si le système Hmq est fonctionnel et donc s'il y a production de HMAQs. C'est pourquoi j'ai déterminé la présence de l'opéron *hmqABCDEFG* chez 312 souches de Bcc comprenant 222 souches cliniques et 90 souches environnementales. J'ai ensuite étudié la production de HMAQs chez les souches porteuses de l'opéron. Cet article fait l'objet de ces deux points et a permis de déterminer que le système Hmq est plus prévalent chez les souches environnementales que les souches cliniques de Bcc, qui semblerait être impliqué dans la promotion de croissance des plantes qui est connue pour être une propriété biologique de certaines souches de Bcc.

4.1. Abstract

The *Burkholderia cepacia* complex (Bcc) comprises several species of closely related, versatile bacteria. Some Bcc strains produce 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs), analogous to the 4-hydroxy-2-alkylquinolines of Pseudomonas aeruginosa. Using *in silico* analyses, we previously estimated 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 intraspecies variability. In the present study, we investigated by PCR, using consensus primers, the distribution of *hmqABCDEFG* in a collection of 312 Bcc strains (222 of clinical and 90 of environmental origins) belonging to 18 Bcc species. We confirmed that this operon is not distributed evenly among Bcc species. Among the 30% of strains bearing the *hmqABCDEFG* operon, we found that 92% of environmental isolates and 82% of clinically isolated Bcc strains produce levels of HMAQs detectable by liquid chromatography-mass spectrometry in at least one of the tested culture conditions. Among the *hmqABCDEFG*-positive but HMAQ-negative strains, none expressed the *hmqA* gene under the specified culture conditions. Interestingly, the *hmqABCDEFG* operon is more prevalent among plant root environment species (e.g., *Burkholderia ambifaria* and *Burkholderia cepacia*) and absent in species commonly found in chronically colonized individuals with cystic fibrosis (e.g., *Burkholderia cenocepacia* and *Burkholderia multivorans*), suggesting a role for the Hmq system in niche adaptation. We investigated the impact of the Hmq system on plant growth promotion and found that *Pisum sativum* root development by *B. ambifaria* required a functional HMAQ system.

4.2. Importance

Environmental bacteria belonging to the various closely related species forming the *Burkholderia cepacia* complex (Bcc) can infect plants and animals, including humans. Their pathogenicity is regulated by intercellular communication, or quorum sensing, allowing them to collaborate instead of acting individually. Bcc organisms generally exploit interacting quorum sensing systems based on N-acyl-homoserine lactones as signaling molecules. Several Bcc strains also carry an *hmqABCDEFG* operon responsible for the biosynthesis of 4-hydroxy-3-methyl-2alkylquinolines (HMAQs), molecules analogous to the Pseudomonas quinolone signal (PQS) system of *P. aeruginosa*. Our finding that the prevalence of the Hmq system and HMAQ production are very different between various Bcc species suggests a key role in niche adaptation or pathogenicity. This is supported by a significant reduction in plant growth promotion in the absence of HMAQ production for a beneficial Bcc strain.

Keywords: *hmqABCDEFG* operon, 4-hydroxy-2-alkylquinolines (HAQs), *pqsABCDE, Pseudomonas* Quinolone Signal (PQS)

4.3. Introduction

The environmental species of *Burkholderia* can be divided into two phylogenetic groups: (i) pathogenic species and (ii) plant-beneficial species (1). Based on this still-controversial separation (2), the latter group was reclassified as *Paraburkholderia*, based on lower GC content and lack of virulence in *Caenorhabditis elegans* (3, 4). The pathogenic *Burkholderia* group comprises (i) plant pathogens (e.g., *Burkholderia andropogonis*, which causes leaf streak on sorghum [5], and *B. glumae*, which causes bacterial panicle blight on rice [6–8]); (ii) the pseudomallei group, composed of *B. pseudomallei* (the causative agent of melioidosis), *B. thailandensis* (closely related to B. pseudomallei but avirulent), and *B. mallei* (causing glanders in equids) (9–11); and (iii) the *Burkholderia cepacia* complex (Bcc), comprising at least 26 different species (12–14; reviewed in reference 1), most considered opportunistic pathogens. Bcc bacteria have been used (i) in agriculture for the biocontrol of phytopathogens and for their plant growth-promoting properties (e.g., pea protection by *B. ambifaria* against *Pythium* and *Aphanomyce*s [15, 16]) and (ii) in bioremediation (e.g., *B. vietnamiensis* with its trichloroethylene degradation abilities [17, 18; reviewed in reference 19]). Bcc bacteria are also well known for secondary-metabolite production, including antibiotics (reviewed in reference [20]). However, since the 1980s, Bcc opportunistic pathogens have emerged as a serious threat among certain immunocompromised individuals (e.g., those with chronic granulomatous disease [CGD]) and people with cystic fibrosis (CF), causing the *cepacia* syndrome and 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 make them particularly problematic (21–23).

Cell-to-cell communication mechanisms, e.g., quorum sensing, act by (i) controlling gene transcription at the population level (24), (ii) promoting colonization, and (iii) optimizing interaction with hosts and increasing resistance to stress (25, 26). Depending on the Bcc species, at least two Cep-like quorum sensing systems may be present, synthesizing different acyl-homoserine lactones (AHLs) as ligands and regulating each other and genes implicated in virulence (27–31).

The bacterium *Pseudomonas aeruginosa* carries a quorum sensing system based on signaling using 4-hydroxy-2-alkylquinolines (HAQs), such as the *Pseudomonas* quinolone signal (PQS) and 4-hydroxy-2-heptylquinoline (HHQ) (32–34). Interestingly, some species of the Bcc (*B. ambifaria* and *B. cepacia*), as well as *B. pseudomallei* and *B. thailandensis*, produce analogous molecules called 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) (35–37) that are synthesized by enzymes encoded by the *hmqABCDEFG* operon (36). In contrast with *P. aeruginosa* HAQs, the main HMAQs produced by *Burkholderia* bear a methyl group at the 39 position and lack saturation of their alkyl side chain. Although an increasing number of Bcc strains are being reported to produce some HMAQ congeners (36, 38–44), this remains mostly anecdotal. In contrast with the HAQ/PQS quorum sensing system of *P. aeruginosa*, the *Burkholderia* Hmq system does not appear to form a quorum sensing system per se (specific transcriptional regulation of target genes in

response to concentration of signaling molecules), and instead, we and Le Guillouzer have shown that it is closely interrelated with the Cep quorum sensing system in *B. ambifaria* HSJ1 and with the three Bta quorum sensing systems in *B. thailandensis* E264 (36, 45, 46), as HMAQs impact AHL production in both species (36, 45, 46).

Functions of *P. aeruginosa* HHQ and PQS as quorum sensing autoinducers, immunomodulators, and antimicrobials have been described (47). Several studies report novel molecules belonging to the HAQ family and various bacterial species having antimicrobial activities (48–52). Apart from some intra- and interspecies activity as signals (36, 45, 46) and as low-activity antimicrobials, the primary function of HMAQs remains enigmatic (38–41, 53, 54). Nonetheless, given the demonstrated role of HAQs and PQS in *P. aeruginosa*, HMAQs may also play roles in niche competition and virulence of producing *Burkholderia* strains (36, 46). We previously characterized a series of clinical *B. ambifaria* strains able to produce HMAQs (36, 43). Their respective colony morphotype variant had lost the ability to produce several secondary metabolites, including HMAQs, similarly to a set of environmental *B. ambifaria* isolates (36, 43). Therefore, to better understand HMAQs, we postulated that (i) the h*mqABCDEFG* operon is more frequently found among clinical Bcc strains and (ii) among HMAQ-producing Bcc isolates, clinical strains produce higher concentrations than environmental ones. Our previous bioinformatic study on the distribution of the *hmqABCDEFG* operon in the Bcc, based on available whole-genome sequences for 1,257 strains belonging to 21 Bcc species, showed that *B. ambifaria, B. cepacia, B. contaminans, B. pyrrocinia, B. stagnalis, B. territorii,* and *B. ubonensis* strains carry the *hmqABCDEFG* operon, although not all strains within a species do (55). On the other hand, all sequenced genomes of *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 isolates from clinical cases—that have been investigated are lacking the *hmqABCDEFG* operon (55). 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 312 Bcc strains, comprising 222 clinical and 90 environmental isolates belonging to 18 different Bcc species, was analyzed. We first assessed the presence of the *hmqABCDEFG* operon in their genomes and then directly determined, by liquid chromatography coupled to mass spectrometry (LC-MS), the ability of all the strains bearing the *hmqABCDEFG* operon to produce HMAQs. Our data show that the Hmq system is heterogeneously distributed between Bcc species, with high prevalence in some species (e.g., *B. cepacia*) and near absence in others (e.g., *B. cenocepacia* and *B. multivorans*). Finally, we investigated the impact of the Hmq system on plant growth using *hmqA* and *hmqG* mutants and found that *Pisum sativum* root growth promotion by a *B. ambifaria* strain was lost in the absence of HMAQ production.

4.4. Results

4.4.1. 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 by orthology and homology analyses (55). We found that at least one sequenced strain belonging to 7 of the 21 Bcc 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 (55). Here, to validate our in silico analyses (55) and to globally determine the ability of Bcc to actually produce HMAQs, we screened a collection of 312 Bcc strains (222 of clinical and 90 of environmental origins; listed in **Table S4.1**), belonging to 18 Bcc species: *B. ambifaria, B. anthina, B. arboris, B. cenocepacia, 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,* and *B. vietnamiensis*, plus a few more classified in the "other Bcc" group (PubMLST database [https://pubmlst.org/organisms/burkholderia-cepacia-complex]).

Table S 4.1 List of strains investigated in this study

Strains	Type	References	Other names
Burkholderia ambifaria			
B. ambifaria AMMD	Soil, rhizosphere (USA)	Coenye et *al*., 2001	LMG19182/ FC0768/ BCC0588
B. ambifaria AU0212	CF isolate (USA)	Payne et *al*., 2005	
B. ambifaria AU4157	Clinical isolate	BcRLR	
B. ambifaria AU7994	Clinical isolate		
B. ambifaria AU8235	Clinical isolate	BcRLR	
B. ambifaria CEP0516	CF isolate (Australia)	Coenye et *al*., 2001	
B. ambifaria CEP0617	Clinical isolate	Coenye et *al*., 2001	LMG-P 24636
B. ambifaria CEP0958	CF isolate (Australia)	Coenye et *al*., 2001	
B. ambifaria CEP0990	Clinical isolate		
B. ambifaria CEP0996	CF isolate (Australia)	Coenye et *al*., 2003	LMG 19467
B. ambifaria CEP1231	Clinical isolate		
B. ambifaria ES0020	Environmental isolate, Oregon (USA)	BcRLR	

B. ambifaria HI2425	Soil, New York (USA)	BcRLR	
B. ambifaria HI2468	Pea rhizosphere, Wisconsin (USA)	BcRLR	
B. ambifaria HI2482	Soil, New York (USA)	BcRLR	
B. ambifaria HI2626	Soil, rice field, New York (USA)	BcRLR	
B. ambifaria HI2672	Soil, rice field, New York (USA)	BcRLR	
B. ambifaria HI3544	Soil, North Carolina (USA)	BcRLR	
B. ambifaria HI3590	Environmental isolate (USA)	BcRLR	
B. ambifaria HI3687	Soil, North Carolina (USA)	BcRLR	
B. ambifaria HI3709	Soil, North Carolina (USA)	BcRLR	
B. ambifaria HI3738	Soil, Michigan (USA)	BcRLR	
B. ambifaria HI3890	Soil, Illinois (USA)	BcRLR	
B. ambifaria HSJ1	CF isolate (Canada)	Vial et *al*., 2008	
B. ambifaria HSJ1 pKnock::*hmqA* Cm			
B. ambifaria HSJ1 pKnock::*hmqG* Cm			
B. ambifaria IOP40-10	Environmental isolate	J. Tiedje	
B. ambifaria LMG17828		Coenye et *al*., 2001	ATCC 53266 /FC0662
B. ambifaria MW2073	Environmental isolate		
B. ambifaria PC736	Environmental isolate (USA)	BcRLR	
B. ambifaria PHP7	Environmental isolate	Coenye et *al*., 2001	
B. ambifaria VC11631	CF isolate, Quebec (Canada)	CBCCRRR	
B. ambifaria VC15422	CF isolate, Ontario (Canada)	CBCCRRR	
B. ambifaria VC16196	Clinical isolate, Quebec (Canada)	CBCCRRR	
Burkholderia anthina			
B. anthina HI3538	Soil, North Carolina (USA)	BcRLR	Bcc indeterminate 7 HI3538
B. anthina HI3655	Soil, North Carolina (USA)	BcRLR	
B. anthina VC15382	Clinical isolate, Quebec (Canada)	CBCCRRR	
B. anthina VC16083	Clinical isolate, Quebec (Canada)	CBCCRRR	
Burkholderia arboris			
B. arboris ES0222	Environmental isolate, Pennsylvania (USA)	BcRLR	
B. arboris ES0263	Soil, Pennsylvania (USA)	BcRLR	
B. arboris VC10224	Clinical isolate (Canada)	CBCCRRR	
B. arboris VC8833	CF isolate, New Brunswick (Canada)	CBCCRRR	
Burkholderia cenocepacia			

B. cenocepacia CEP024	CF isolate isolate (Canada)	Speert collection	
B. cenocepacia CEP0511	CF isolate isolate (Australia)	Baldwin et *al*., 2005	LMG 18830
B. cenocepacia CEP0565	Clinical isolate		
B. cenocepacia ES1405	Environmental isolate, Ontario (Canada)	BcRLR	
B. cenocepacia HI2424	Soil, New York (USA)	BcRLR	
B. cenocepacia HI2606	Soil, New York (USA)	BcRLR	
B. cenocepacia HI2876	Dialysis water, Missouri (USA)	BcRLR	
B. cenocepacia HI2976	Sink, North Carolina (USA)	BcRLR	
B. cenocepacia HI3540	Soil, North Carolina (USA)	BcRLR	
B. cenocepacia HI3855	Contaminated mouthwash, South Carolina (USA)	BcRLR	
B. cenocepacia HI4004	Plumbing biofilm, Washington (USA)	BcRLR	
B. cenocepacia HI4101	Doctor examination room, Illinois (USA)	BcRLR	
B. cenocepacia HI4143	Temperature probe, Arizona (USA)	BcRLR	
B. cenocepacia HI4261	Chlorhexidine body wipe, Kansas (USA)	BcRLR	
B. cenocepacia HI4437	Contaminated lotion, Massachusetts (USA)	BcRLR	
B. cenocepacia HI4904	Contaminated cleansing foam, Pennsylvainia (USA)	BcRLR	
B. cenocepacia K56-2	CF isolate isolate (Canada)	Lipuma et *al*., 2001	
B. cenocepacia IIIA VC10277	CF isolate, Alberta (Canada)	CBCCRRR	
B. cenocepacia IIIA VC12308	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIA VC13139	CF isolate, Quebec (Canada)	CBCCRRR	
B. cenocepacia IIIA VC14610	CF isolate, New Brunswick (Canada)	CBCCRRR	
B. cenocepacia IIIA VC15419	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIA VC15451	CF isolate, Ontario (Canada)	CBCCRRR	
B. cenocepacia IIIA VC16156	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIA VC16199	CF isolate, Alberta (Canada)	CBCCRRR	
B. cenocepacia IIIA VC16873	CF isolate, New Brunswick (Canada)	CBCCRRR	
B. cenocepacia IIIA VC16874	CF isolate, New Brunswick (Canada)	CBCCRRR	
B. cenocepacia IIIA VC17671	CF isolate, Quebec (Canada)	CBCCRRR	
B. cenocepacia IIIA VC17819	CF isolate, Alberta (Canada)	CBCCRRR	
B. cenocepacia IIIA VC18585	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIA VC18609	CF isolate, Quebec (Canada)	CBCCRRR	
B. cenocepacia IIIA VC18996	CF isolate, New Brunswick (Canada)	CBCCRRR	
B. cenocepacia IIIA VC18999	CF isolate, New Brunswick (Canada)	CBCCRRR	

B. cenocepacia IIIA VC3917	CF isolate, New Brunswick (Canada)	CBCCRRR
B. cenocepacia IIIA VC5069	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIA VC5621	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIA VC6356	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIA VC6553	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIA VC8286	CF isolate, Alberta (Canada)	CBCCRRR
B. cenocepacia IIIA VC8356	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIA VC8426	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIA VC8607	CF isolate, Alberta (Canada)	CBCCRRR
B. cenocepacia IIIA VC8611	CF isolate, Alberta (Canada)	CBCCRRR
B. cenocepacia IIIA VC8614	CF isolate, Alberta (Canada)	CBCCRRR
B. cenocepacia IIIA VC9080	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIA VC9296	CF isolate, Newfoundland and Labrador (Canada)	CBCCRRR
B. cenocepacia IIIB VC11311	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIB VC11653	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC13104	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC13187	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC14376	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC14524	CF isolate, Alberta (Canada)	CBCCRRR
B. cenocepacia IIIB VC14529	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC15122	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIB VC15240	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC15241	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC16932	CF isolate, Nova Scotia (Canada)	CBCCRRR
B. cenocepacia IIIB VC17657	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC18097	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC18107	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIB VC18236	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIB VC18454	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC18569	CF isolate, Quebec (Canada)	CBCCRRR
B. cenocepacia IIIB VC18658	CF isolate, British Columbia (Canada)	CBCCRRR
B. cenocepacia IIIB VC5625	CF isolate, Ontario (Canada)	CBCCRRR
B. cenocepacia IIIB VC6598	CF isolate, British Columbia (Canada)	CBCCRRR

B. cenocepacia IIIB VC7349	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIB VC7849	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIB VC7911	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIB VC8340	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cenocepacia IIIB VC8870	CF isolate, Manitoba (Canada)	CBCCRRR	
B. cenocepacia IIIB VC9859	CGD, Ontario (Canada)	CBCCRRR	
Burkholderia cepacia			
B. cepacia ATCC25416	Onion root	Yabuuchi et *al*., 1992	LMG1222/CEP0031
B. cepacia CEP0509	CF isolate (Australia)	Vandamme et *al*., 2008	LMG18821
B. cepacia BTS13	CF isolate (Italy)	Lagotolla et *al*., 2002	
B. cepacia HI2430	Soil, New York (USA)	BcRLR	
B. cepacia HI2563	Soil, New York (USA)	BcRLR	
B. cepacia HI2578	Soil, New York (USA)	BcRLR	
B. cepacia HI2615	Soil, celery field, New York (USA)	BcRLR	
B. cepacia HI2671	Soil, rice field, New York (USA)	BcRLR	
B. cepacia HI2741	Contaminated mouthwash, New York (USA)	BcRLR	
B. cepacia HI3312	Dialysis machine, Florida (USA)	BcRLR	
B. cepacia HI3551	Soil, North Carolina (USA)	BcRLR	
B. cepacia HI3708	Soil, North Carolina (USA)	BcRLR	
B. cepacia HI3895	Soil, Illinois (USA)	BcRLR	
B. cepacia HI4577	Hospital sink drain (USA)	BcRLR	
B. cepacia VC13132	CF isolate, Ontario (Canada)	CBCCRRR	
B. cepacia VC13196	CF isolate, Alberta (Canada)	CBCCRRR	
B. cepacia VC13394	CF isolate, Alberta (Canada)	CBCCRRR	
B. cepacia VC13575	CF isolate, Ontario (Canada)	CBCCRRR	
B. cepacia VC14106	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cepacia VC14457	CF isolate, British Columbia (Canada)	CBCCRRR	
B. cepacia VC16383	Clinical isolate, Quebec (Canada)	CBCCRRR	
B. cepacia VC16708	CF isolate, Quebec (Canada)	CBCCRRR	
B. cepacia VC17333	CF isolate, Alberta (Canada)	CBCCRRR	
B. cepacia VC17746	Non-CF isolate, Quebec (Canada)	CBCCRRR	
B. cepacia VC17928	CF isolate, Quebec (Canada)	CBCCRRR	
B. cepacia VC18315	CF isolate, British Columbia (Canada)	CBCCRRR	

B. cepacia VC18839	Non-CF isolate, Quebec (Canada)	CBCCRRR
B. cepacia VC18842	Non-CF isolate, Quebec (Canada)	CBCCRRR
B. cepacia VC19225	CF isolate, British Columbia (Canada)	CBCCRRR
B. cepacia VC19276	CF isolate, Quebec (Canada)	CBCCRRR
B. cepacia VC9490	CF isolate, British Columbia (Canada)	CBCCRRR
Burkholderia contaminans		
B. contaminans FFH2055	CF isolate (Argentina)	Nunvar et *al*., 2016
B. contaminans HI3422	Contaminated nasal spray, Colorado (USA)	BcRLR
B. contaminans HI3570	Soil, North Carolina (USA)	BcRLR
B. contaminans HI3852	Trypan blue opthalmic solution (USA)	BcRLR
B. contaminans HI3887	Pharmacy IV fluid, Missouri (USA)	BcRLR
B. contaminans HI4067	Environmental isolate (Argentina)	BcRLR
B. contaminans HI4232	Contaminated mouthwash, Arizona (USA)	BcRLR
B. contaminans HI4402	Water, Kentuky(USA)	BcRLR
B. contaminans VC14347	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC15406	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC16087	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC16848-b	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC16897	Clinical isolate, Quebec (Canada)	CBCCRRR
B. contaminans VC16948	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC19056	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC19124	CF isolate, British Columbia (Canada)	CBCCRRR
B. contaminans VC9624	CF isolate, Ontario (Canada)	CBCCRRR
Burkholderia diffusa		
B. diffusa HI2617	Soil, celery field, New York (USA)	BcRLR
B. diffusa HI3576	Soil, North Carolina (USA)	BcRLR
B. diffusa HI3672	Soil, North Carolina (USA)	BcRLR
B. diffusa HI3740	Soil, Michigan (USA)	BcRLR
B. diffusa VC14008	CF isolate, Ontario (Canada)	CBCCRRR
B. diffusa VC15063	CF isolate, Quebec (Canada)	CBCCRRR
B. diffusa VC6752	CF isolate, Alberta (Canada)	CBCCRRR
B. diffusa VC6966	Non-CF isolate, Quebec (Canada)	CBCCRRR
B. diffusa VC7394	CF isolate, Quebec (Canada)	CBCCRRR

B. diffusa VC7913	CF isolate, Quebec (Canada)	CBCCRRR	
Burkholderia dolosa			
B. dolosa CEP0021	CF isolate (Canada)	CBCCRRR	
B. dolosa LMG21443	*Alysicarpus glumaceus* root nodule	Vandamme et *al*., 2002	
B. dolosa VC14902	CF isolate, Alberta (Canada)	CBCCRRR	
B. dolosa VC17647	CF isolate, Quebec (Canada)	CBCCRRR	
Burkholderia lata			
B. lata BC01	River water, South Carolina (USA)	BcRLR	
B. lata VC19230	Clinical isolate, British Columbia (Canada)	CBCCRRR	
B. lata VC6377	CF isolate, Ontario (Canada)	CBCCRRR	
B. lata VC8171	CF isolate, New Brunswick (Canada)	CBCCRRR	
Burkholderia metallica			
B. metallica ES0559	Environmental isolate, Oregon (USA)	BcRLR	
B. metallica HI3647	Soil, North Carolina (USA)	BcRLR	
B. metallica VC15467	CF isolate, Ontario (Canada)	CBCCRRR	
B. metallica VC8135	Clinical isolate, British Columbia (Canada)	CBCCRRR	
Burkholderia multivorans			
B. multivorans LMG16660	CF isolate isolate (Canada)	CBCCRRR	CEP0781
B. multivorans HI2790	Doctor office (USA)	BcRLR	
B. multivorans LMG17588	Soil (USA)	Vandamme et *al*., 2008	ATCC17616/ CEP0144
B. multivorans VC12152	Clinical isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC12258	CF isolate, Saskatchewan (Canada)	CBCCRRR	
B. multivorans VC12539	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC12675	Clinical isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC13125	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC13145	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC13162	CF isolate, Ontario (Canada)	CBCCRRR	
B. multivorans VC13451	Non-CF isolate, Nova Scotia (Canada)	CBCCRRR	
B. multivorans VC13673	CF isolate, Alberta (Canada)	CBCCRRR	
B. multivorans VC13702	CF isolate, Alberta (Canada)	CBCCRRR	
B. multivorans VC13776	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC14090	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC14422	NON CF isolate, Saskatchewan (Canada)	CBCCRRR	

B. multivorans VC14443	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC14749	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC14757	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC15002	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC15085	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC15268	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC15273	CF isolate, Ontario (Canada)	CBCCRRR	
B. multivorans VC15814	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC15834	CF isolate, Saskatchewan (Canada)	CBCCRRR	
B. multivorans VC15873	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC15952	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC15953	CF isolate, Ontario (Canada)	CBCCRRR	
B. multivorans VC15977	CF isolate, Alberta (Canada)	CBCCRRR	
B. multivorans VC16475	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC16487	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC16759	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC16959	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC17546	Clinical isolate, Saskatchewan (Canada)	CBCCRRR	
B. multivorans VC18625	CF isolate, Newfoundland and Labrador (Canada)	CBCCRRR	
B. multivorans VC3419	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC4282	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC6534	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC6564	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC7102	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC7704	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC7870	CF isolate, Quebec (Canada)	CBCCRRR	
B. multivorans VC7960	CF isolate, British Columbia (Canada)	CBCCRRR	
B. multivorans VC9159	CF isolate, British Columbia (Canada)	BcRLR	
B. multivorans VC9858	CF isolate, British Columbia (Canada)	CBCCRRR	
Burkholderia pyrrocinia			
B. pyrrocinia CH-67	Soil, rhizosphere (Korea)	Lee et *al.*, 2011	
B. pyrrocinia ES0490	Environmental isolate, Ohio (USA)	BcRLR	Bcc indeterminate 1 ES0490
B. pyrrocinia BC02	River water, South Carolina (USA)	BcRLR	Bcc indeterminate 2 BC02

B. pyrrocinia HI2575	Soil, New York (USA)	BcRLR	Bcc indeterminate 5 HI2575
B. pyrrocinia HI2690	Soil, rice field, New York (USA)	BcRLR	Bcc indeterminate 5 HI2690
B. pyrrocinia HI2701	Soil, New York (USA)	BcRLR	Bcc indeterminate 5 HI2701
B. pyrrocinia HI3892	Soil, Illinois (USA)	BcRLR	Bcc indeterminate 5 HI3892
B. pyrrocinia ES0209	Environmental isolate, Pennsylvania (USA)	BcRLR	Bcc indeterminate 9 ES0209
B. pyrrocinia LMG21824	CF isolate (UK)	Coenye et *al*., 2003	
Burkholderia seminalis			
B. seminalis HI2490	Soil, New York (USA)	BcRLR	
Burkholderia stabilis			
B. stabilis C7322	CF isolate (Canada)	Mahenthiralingam et al., 2000	
B. stabilis HI2462	Contaminated shampoo (USA)	BcRLR	
B. stabilis VC10097	Non-CF isolate, Quebec (Canada)	CBCCRRR	
B. stabilis VC12344	CF isolate, Quebec (Canada)	CBCCRRR	
B. stabilis VC12965	Environmental isolate, Ontario (Canada)	CBCCRRR	
B. stabilis VC17755	Cancer, Alberta (Canada)	CBCCRRR	
B. stabilis VC6296	CF isolate, Alberta (Canada)	CBCCRRR	
B. stabilis VC6482	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC6747	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC6749	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC6753	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8622	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8623	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8629	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8636	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8638	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC8967	Non-CF isolate, Ontario (Canada)	CBCCRRR	
B. stabilis VC8971	CF isolate, Ontario (Canada)	CBCCRRR	
B. stabilis VC9042	CF isolate, British Columbia (Canada)	CBCCRRR	
B. stabilis VC9562	CF isolate, Ontario (Canada)	CBCCRRR	
B. stabilis VC9945	CF isolate, Quebec (Canada)	CBCCRRR	
Burkholderia stagnalis			
B. stagnalis HI2720	Soil, tabasco, (Mexico)	BcRLR	

B. stagnalis HI3537	Soil, North Carolina (USA)	BcRLR	Bcc indeterminate 6 HI3537
B. stagnalis MSMB1956WGS	Environmental isolate, North Carolina (USA)	MSHR-NAU	
Burkholderia territorii			
B. territorii MSMB1301WGS	Environmental isolate, North Carolina (USA)	MSHR-NAU	
B. territorii MSMB1502WGS	Environmental isolate, North Carolina (USA)	MSHR-NAU	
Burkholderia ubonensis			
B. ubonensis LMG20358	Environmental isolate (Thailand)	Coenye et *al*., 2001	BCC1603
B. ubonensis LMG24263	Nosocomial (Thailand)	Vanlaere et *al*., 2008	
Burkholderia vietnamiensis			
B. vietnamiensis CEP0040	CF isolate (Canada)	Mahenthiraligham collection	LMG 18835
B. vietnamiensis G4	Trichloroethene enrichment	Nelson et *al*., 1987	
B. vietnamiensis HI3534	Barium, Texas (USA)	BcRLR	
B. vietnamiensis VC0024	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC10362	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC10442	Non-CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC10676	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC11253	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC11275	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC11668	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC12002	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC13138	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC13308	CF isolate, Alberta (Canada)	CBCCRRR	
B. vietnamiensis VC13830	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC13984	CF isolate, Ontario (Canada)	CBCCRRR	
B. vietnamiensis VC14091	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC14473	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC14737	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC15208	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC15292	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC15774	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC16431	CF isolate, British Columbia (Canada)	CBCCRRR	
B. vietnamiensis VC17180	CF isolate, Quebec (Canada)	CBCCRRR	
B. vietnamiensis VC17270	CF isolate, Alberta (Canada)	CBCCRRR	

B. vietnamiensis VC17399	CF isolate, Quebec (Canada)	CBCCRRR
B. vietnamiensis VC17834	CF isolate, Newfoundland and Labrador (Canada)	CBCCRRR
B. vietnamiensis VC18210	CF isolate, British Columbia (Canada)	CBCCRRR
B. vietnamiensis VC18530	CF isolate, Newfoundland and Labrador (Canada)	CBCCRRR
B. vietnamiensis VC18712	CF isolate, Alberta (Canada)	CBCCRRR
B. vietnamiensis VC18844	CF isolate, Quebec (Canada)	CBCCRRR
B. vietnamiensis VC2824	CF isolate, British Columbia (Canada)	CBCCRRR
B. vietnamiensis VC5914	CF isolate, Ontario (Canada)	CBCCRRR
B. vietnamiensis VC8245	CF isolate, British Columbia (Canada)	CBCCRRR
B. vietnamiensis VC8613	CF isolate, Alberta (Canada)	CBCCRRR
B. vietnamiensis VC9237	CF isolate, British Columbia (Canada)	CBCCRRR
B. vietnamiensis VC9752	CF isolate, British Columbia (Canada)	CBCCRRR
Other Bcc group		
B. sp LMI-SB2		
B. sp VC14128	CF isolate, British Columbia (Canada)	CBCCRRR
B. sp VC15804	CF isolate, Ontario (Canada)	CBCCRRR
B. sp VC16512	Clinical isolate, Alberta (Canada)	CBCCRRR
B. sp VC18848	CF isolate, Quebec (Canada)	CBCCRRR
Other Bcc - Bcc indeterminate 1 BC06	River water, South Carolina (USA)	BcRLR
Other Bcc - Bcc indeterminate 3 BC13	River water, South Carolina (USA)	BcRLR
Other Bcc - Bcc indeterminate 3 ES0139	Environmental isolate, Pennsylvania (USA)	BcRLR
Other Bcc - Bcc indeterminate 4 BC04	River water, South Carolina (USA)	BcRLR
Other Bcc - Bcc indeterminate 5 BC03	River water, South Carolina (USA)	BcRLR
Other Bcc - Bcc indeterminate 8 HI4407	Water, Kentucky (USA)	BcRLR

CBCCRRR: Strains were provided by Canadian *Burkholderia cepacia* complex Research and Referral Repository, University of British Colombia, Canada

BcRLR: *Burkholderia cepacia* Research Laboratory and Repository, University of Michigan

MSHR-NAU: Menzies School of Health Research and Northern Arizona University, USA

Presence of *hmqABCDEFG* was determined by PCR using consensus primers targeting *hmqA* and *hmqG* (based on highly conserved regions, as shown in **Figure S4.1**). We had previously shown that presence of a *hmqG* orthologue correlates with the presence of a complete *hmqABCDEFG* operon (55).

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Consensus                 TGCTTTCCGSAGCAYGTGCTCMRGCCYACSGCCGAHGACGTSTTCTGCGGRTCGCCRCYG   720
B_ubonensis_MSMB22        .........C....C......AA...C..G.....C.....C........A.....G.C.   720
B_stagnalis_MSMB777WGS    .........C....T......AA...C..G.....T.....C........A.....G.C.   720
B_stagnalis_MSMB1956WGS   .........C....C......AA...C..G.....T.....C........A.....G.C.   720
B_territorii_MSMB1502WGS  .........C....C......CG...C..G.....A.....C........A.....G.T.   720
B_territorii_MSMB1301     .........C....C......CG...C..G.....A.....C........A.....G.C.   720
B_cepacia_ATCC25416       .........C....C......CG...C..G.....C.....C........A.....G.C.   720
B_lata_LK13               .........C....C......CG...C..G.....T.....C........A.....G.C.   720
B_ubonensis_MSMB867       .........C....C......AA...C..G.....C.....C........A.....G.C.   720
B_stabilis_EB159          .........C....C......CG...C..G.....C.....C........G.....A.C.   720
B_stabilis_LA20W          .........C....C......AG...C..G.....T.....C........G.....A.C.   720
B_pyrrocinia_CH-67        .........C....C......AA...C..G.....T.....C........A.....G.C.   720
B_contaminans_FFH2055     .........C....C......AA...C..G.....T.....C........G.....G.C.   720
B_contaminans_MS14        .........C....C......AA...T..G.....T.....C........G.....G.C.   720
B_ambifaria_AMMD          .........G....C......CG...C..C.....T.....G........A.....G.C.   720
B_ambifaria_CEP0996       .........G....C......CG...C..C.....T.....G........A.....G.C.   720
B_ambifaria_HSJ1          .........G....C......CG...C..C.....T.....G........A.....G.C.   720
B_cepacia_LK29            .........C....C......AG...C..G.....T.....C........G.....A.C.   720
B_pyrrocinia_DSM10685     .........C....C......AG...C..G.....T.....C........G.....A.C.   720

Consensus                 CTCGCGTTYACGTTYGGSCTCGGCGCRCTGYTGCTGTTTCCRRTCAGYGTCGGCGCRAGC   780
B_ubonensis_MSMB22        ........C.....T..C........G...C..........GG....C........G...   780
B_stagnalis_MSMB777WGS    ........C.....C..C........G...C..........GG....C........G...   780
B_stagnalis_MSMB1956WGS   ........C.....C..C........G...C..........GG....C........G...   780
B_territorii_MSMB1502WGS  ........C.....C..C........G...C..........GG....C........G...   780
B_territorii_MSMB1301     ........C.....C..C........G...C..........GG....C........G...   780
B_cepacia_ATCC25416       ........C.....C..C........G...C..........AG....C........G...   780
B_lata_LK13               ........C.....C..C........G...C..........GG....C........A...   780
B_ubonensis_MSMB867       ........C.....T..C........G...C..........GG....C........G...   780
B_stabilis_EB159          ........C.....C..C........A...C..........GA....T........A...   780
B_stabilis_LA20W          ........C.....C..C........G...C..........GA....C........G...   780
B_pyrrocinia_CH-67        ........C.....C..C........G...C..........GG....C........G...   780
B_contaminans_FFH2055     ........T.....C..C........G...C..........GA....T........G...   780
B_contaminans_MS14        ........T.....C..C........G...C..........GA....T........G...   780
B_ambifaria_AMMD          ........C.....C..G........G...C..........GA....T........G...   780
B_ambifaria_CEP0996       ........C.....C..G........G...C..........GA....T........G...   780
B_ambifaria_HSJ1          ........C.....C..G........G...C..........GA....T........G...   780
B_cepacia_LK29            ........C.....C..C........G...C..........GA....T........A...   780
B_pyrrocinia_DSM10685     ........C.....C..C........G...T..........GA....C........G...   780

Consensus                 GACRCGCGRCAACGYGACTACGTSARGYACGGYTGGAACCTGACGGGSGAYRSCGCSTAY  1260
B_ubonensis_MSMB22        ...G....G.....C........C.G.C....C..............G..TGC...C..C  1251
B_stagnalis_MSMB777WGS    ...G....G.....C........G.A.C....C..............C..CAG...C..C  1251
B_stagnalis_MSMB1956WGS   ...G....G.....C........G.A.C....C..............C..CAG...C..C  1251
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B_territorii_MSMB1502WGS    ...A....G.....C........C.A.C....T.............C..CAG...C..C   1260
B_territorii_MSMB1301       ...A....G.....C........C.A.C....T.............C..CAG...C..C   1260
B_cepacia_ATCC25416         ...A....G.....C........C.A.C....C.............C..CAG...C..C   1260
B_lata_LK13                 ...A....A.....C........C.A.C....C.............C..CAG...C..C   1260
B_ubonensis_MSMB867         ...G....G.....C........C.G.C....C.............C..TGC...C..T   1251
B_stabilis_EB159            ...G....G.....C........G.A.C....C.............C..CAG...C..C   1251
B_stabilis_LA20W.           ...G....G.....C........G.A.C....C.............C..CAG...C..C   1251
B_pyrrocinia_CH-67          ...G....G.....T........G.A.C....C.............C..CAG...G..C   1251
B_contaminans_FFH2055       ...G....G.....C........C.A.C....C.............C..CAG...G..C   1251
B_contaminans_MS14          ...G....G.....C........C.A.C....C.............C..CAG...G..C   1251
B_ambifaria_AMMD            ...A....G.....C........C.A.T....C.............C..CAG...C..C   1251
B_ambifaria_CEP0996         ...A....G.....C........C.A.T....C.............C..CAG...C..C   1251
B_ambifaria_HSJ1            ...A....G.....C........C.A.T....C.............C..CAG...C..C   1251
B_cepacia_LK29              ...G....G.....C........C.A.C....C.............C..CAG...C..C   1251
B_pyrrocinia_DSM10685       ...G....G.....C........G.A.C....C.............C..CAG...C..C   1251

Consensus                   TGCGTCGAGCGGCGCCGGVTCBGGGCCSGCGTCGCASGAAATCACGACVAARATCCTGCR  10766
B_ubonensis_MSMB22          ..................C..G.....G........G...........G..A.......G  10716
B_stagnalis_MSMB777WGS      ..................G..G.....G........G...........G..G.......G  10620
B_stagnalis_MSMB1956WGS     ..................G..G.....C........G...........G..G.......G  10608
B_territorii_MSMB1502WGS    ..................G..T.....G........G...........G..G.......G  10630
B_territorii_MSMB1301       ..................G..T.....G........G...........G..G.......G  10640
B_cepacia_ATCC25416         ..................A..G.....G........G...........G..G.......G  10649
B_lata_LK13                 ..................G..G.....G........G...........G..G.......G  10667
B_ubonensis_MSMB867         ..................G..G.....G........G...........C..A.......G  10653
B_stabilis_EB159            ..................G..C.....G........G...........G..A.......G  10624
B_stabilis_LA20W            ..................G..C.....G........G...........G..A.......A  10621
B_pyrrocinia_CH-67          ..................G..G.....G........G...........G..G.......G  10637
B_contaminans_FFH2055       ..................G..C.....G........G...........G..A.......G  10614
B_contaminans_MS14          ..................G..C.....G........G...........G..A.......G  10611
B_ambifaria_AMMD            ..................G..C.....G........G...........A..A.......G  10637
B_ambifaria_CEP0996         ..................G..C.....G........G...........A..A.......G  10637
B_ambifaria_HSJ1            ..................G..C.....G........G...........A..A.......G  10637
B_cepacia_LK29              ..................G..C.....C........C...........G..A.......G  10628
B_pyrrocinia_DSM10685       ..................G..C.....G........G...........G..A.......A  10628

Consensus                   VCCSSAVRSGTGGYTGCGCACCGARCAGCTKCCGTCGGTRTTCAAYTGGGGCATGCAYTT  11546
B_ubonensis_MSMB22          A..GG.AGG....T..........G.....G........G.....T...........T..  11496
B_stagnalis_MSMB777WGS      G..CG.CGC....C..........G.....T........G.....C...........C..  11400
B_stagnalis_MSMB1956WGS     G..CC.CGC....C..........G.....T........G.....C...........C..  11388
B_territorii_MSMB1502WGS    G..CG.CGC....C..........G.....G........G.....C...........C..  11410
B_territorii_MSMB1301       G..CG.CGC....C..........G.....G........G.....C...........C..  11420
B_cepacia_ATCC25416         C..GG.CAC....T..........G.....T........G.....C...........T..  11429
B_lata_LK13                 C..GG.CAC....T..........G.....T........G.....C...........T..  11447
B_ubonensis_MSMB867         A..GG.CGC....T..........G.....G........G.....C...........T..  11433
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B_stabilis_EB159            G..GG.CAC....T.........G.....T........G.....C..........T..  11404
B_stabilis_LA20W            G..GG.CAC....T.........G.....T........G.....C..........T..  11401
B_pyrrocinia_CH-67          G..CG.GGC....C.........G.....T........G.....C..........C..  11417
B_contaminans_FFH2055       G..GG.CAC....T.........A.....T........G.....C..........T..  11394
B_contaminans_MS14          G..GG.CAC....T.........G.....T........G.....C..........T..  11391
B_ambifaria_AMMD            G..GG.CAC....T.........G.....T........A.....C..........T..  11417
B_ambifaria_CEP0996         G..GG.CAC....T.........G.....T........A.....C..........T..  11417
B_ambifaria_HSJ1            G..GG.CAC....T.........G.....T........A.....C..........T..  11417
B_cepacia_LK29              G..GG.CAC....T.........G.....T........G.....C..........T..  11408
B_pyrrocinia_DSM10685       G..GG.CAC....T.........G.....T........G.....C..........T..  11408

Consensus                   YGTGTCGCGBCARTTCATGGCGCCGTTCAACGARTTCAYRCTYGCRYTGACSCTSGCYGG  11606
B_ubonensis_MSMB22          C........C..G.....................A....TG..C..GT....G..G..C..  11556
B_stagnalis_MSMB777WGS      C........G..G.....................A....TG..C..GT....G..G..C..  11460
B_stagnalis_MSMB1956WGS     C........G..G.....................A....TG..C..GT....G..G..C..  11448
B_territorii_MSMB1502WGS    T........T..G.....................G....CA..T..AC....G..G..C..  11470
B_territorii_MSMB1301       T........T..G.....................G....CA..T..AC....G..G..C..  11480
B_cepacia_ATCC25416         C........C..G.....................A....CG..C..GC....C..G..C..  11489
B_lata_LK13                 C........C..G.....................A....CG..C..GC....C..G..C..  11507
B_ubonensis_MSMB867         C........C..G.....................A....TG..C..GT....G..G..C..  11493
B_stabilis_EB159            C........T..G.....................A....CG..C..GT....G..G..C..  11464
B_stabilis_LA20W            C........T..G.....................A....CG..C..GT....C..C..C..  11461
B_pyrrocinia_CH-67          C........C..G.....................A....CG..C..GC....C..G..C..  11477
B_contaminans_FFH2055       C........T..A.....................A....CG..T..GT....C..G..C..  11454
B_contaminans_MS14          C........T..A.....................A....CG..T..GT....C..G..C..  11451
B_ambifaria_AMMD            C........C..A.....................A....CA..C..GT....C..G..T..  11477
B_ambifaria_CEP0996         C........C..A.....................A....CA..C..GT....C..G..T..  11477
B_ambifaria_HSJ1            C........C..A.....................A....CA..C..GT....C..G..T..  11477
B_cepacia_LK29              C........T..G.....................A....CG..C..GT....G..G..C..  11468
B_pyrrocinia_DSM10685       C........T..G.....................A....CG..C..GT....C..C..C..  11468
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Figure S 4.1 Partial alignment of *hmqA* and *hmqG* genes and primers' binding sequences

Sequences were aligned using Clustal Omega .Consensus sequences corresponding to hmqA primers are in red and in green for hmqG primers. These primers were used for the screening.

Here, we found that 30% of the 312 tested strains possess an *hmqABCDEFG* operon, including 53% of the environmental strains but only 21% of clinical strains (**Figure 4.1A**). A Fisher test with a Pvalue of $2.13 \cdot 10^{-10}$ indicates that the prevalence of *hmqABCDEFG* is significantly different depending on the strains' sampling origin (environmental vs. clinical). Among the 18 different Bcc species investigated, 14 species comprise at least one strain carrying the operon (**Figure 4.1B** and **Figure S4.2**). However, *B. cenocepacia*, *B. multivorans*, and *B. vietnamiensis* are overrepresented among clinical strains compared to the other species. We thus decided to subsample these three species with only 20 clinical strains, increasing the prevalence of the *hmqABCDEFG* operon in

clinical Bcc strains to 30% (46 strains carrying the operon among the 151 subsampled clinical strains), which is still statistically significantly lower than the prevalence of the *hmqABCDEFG* operon within environmental strains (Fisher test with a Pvalue of $1.324 \cdot 10^{-26}$).

More precisely, the *hmqABCDEFG* operon is more prevalent among clinical strains of *B. ambifaria, B. anthina, B. contaminans, B. diffusa, B. ubonensis,* and *B. vietnamiensis.* While 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.*

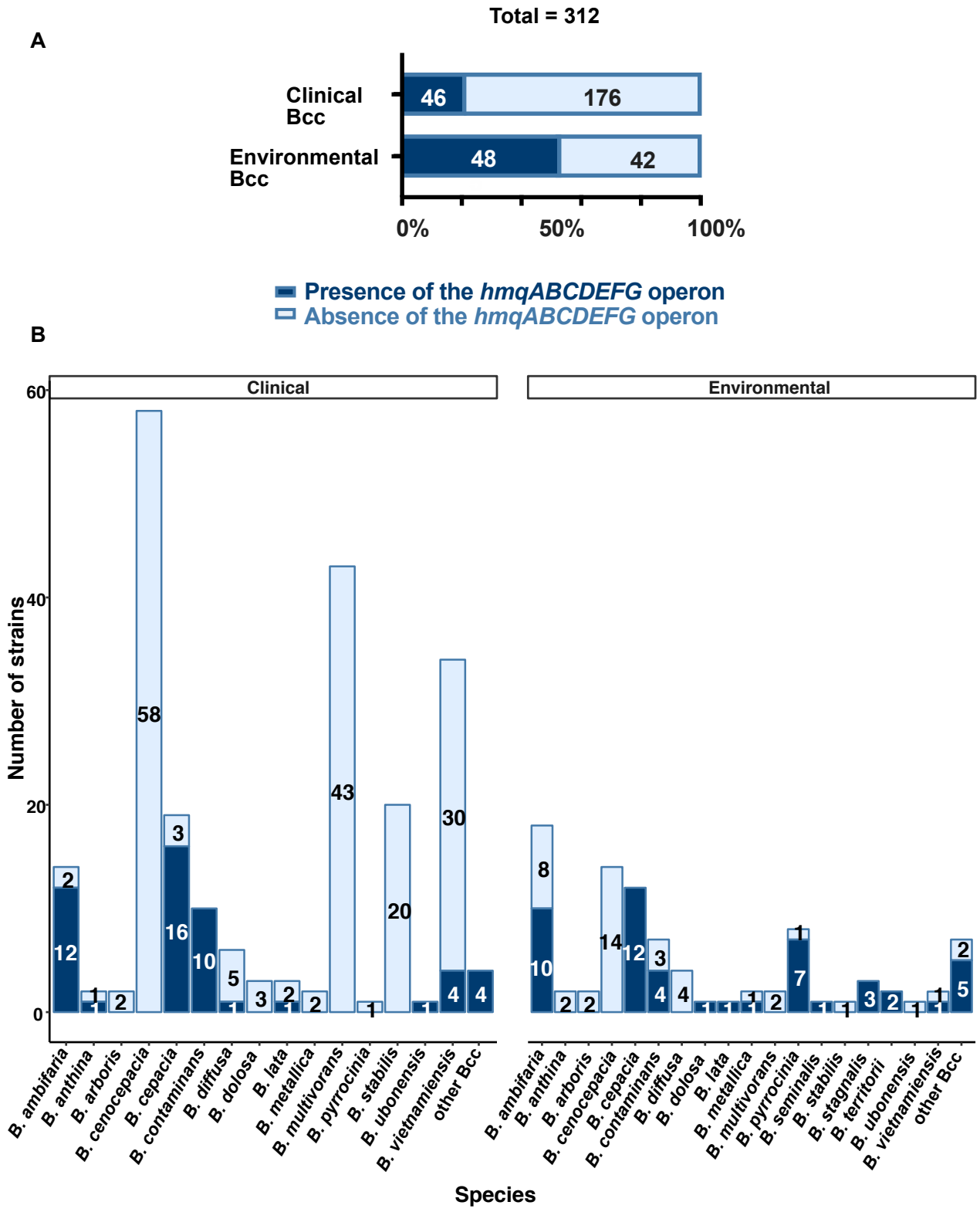

Figure 4.1 **Bcc strains screening for presence of the hmqABCDEFG operon in their genomes**

(A) Distribution of the operon between environmental and clinical Bcc strains investigated in this study. (B) Distribution of the *hmqABCDEFG* operon within tested Bcc species.

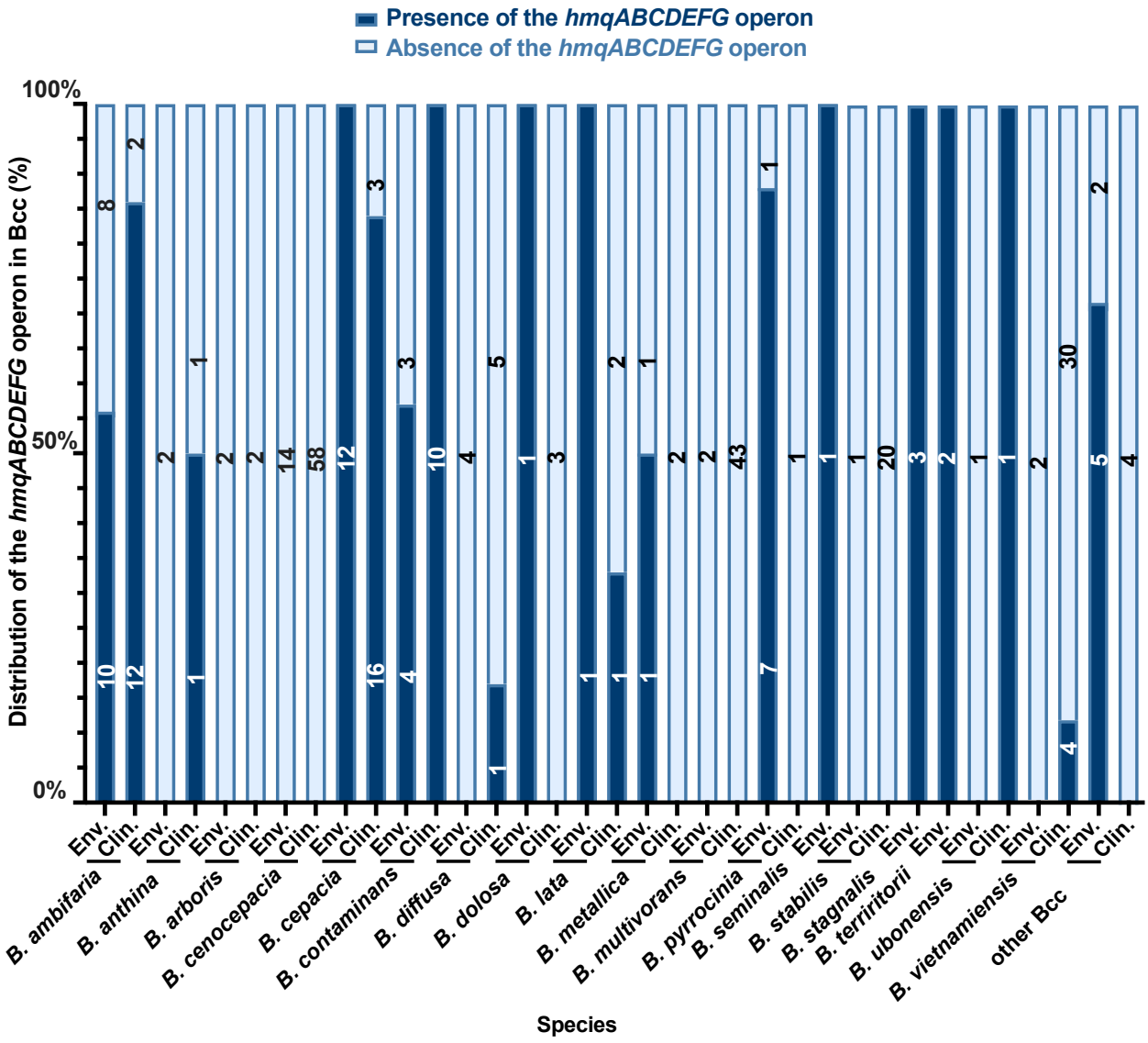

Figure S 4.2 Presence of the *hmqABCDEFG* operon in the genomes of Bcc strains

The results are in percentages of total strains for each origin. Numbers represent the number of strains in each species and for each origin, carrying (white) or not (black) the *hmqABCDEFG* operon.

We found isolates of *B. dolosa*, *B. anthina*, and *B. vietnamiensis* carrying the *hmqABCDEFG* operon, which was not predicted in our previous analysis of available genomic data (55); we confirmed these PCR results by sequencing the amplicons using primers listed in **Table S4.2**.

Table S 4.2 Comparison of *in silico* and *in vitro* results of the distribution of the *hmqABCDEFG* operon

Strains	Prevalence of *hmqABCDEFG* operon (%)	
	Bioinformatics analysis by homology [total genome sequences]	Experimental analysis by PCR [total screened strains]
B. cepacia **(genomovar I)**	23 [337]	90 [31]
B. multivorans **(genomovar II)**	0 [56]	0 [45]
B. cenocepacia **(genomovar III)**	0 [243]	0 [72]
B. stabilis **(genomovar IV)**	-	0 [21]
B. vietnamiensis **(genomovar V)**	0 [41]	13 [36]
B. dolosa **(genomovar VI)**	0 [2]	25 [4]
B. ambifaria **(genomovar VII)**	50 [6]	68 [32]
B. anthina **(genomovar VIII)**	0 [8]	25 [4]
B. pyrrocinia **(genomovar IX)**	75 [4]	78 [9]
B. ubonensis **(genomovar X)**	97 [292]	50 [2]
B. latens **(BCC1)**	0 [2]	-
B. diffusa **(BCC 2)**	0 [12]	10 [10]
B. arboris **(BCC 3)**	-	0 [4]
B. seminalis **(BCC 7)**	0 [3]	100 [1]
B. metallica **(BCC 8)**	0 [1]	25 [4]
B. lata **(group K)**	50 [4]	50 [4]
B. contaminans **(group K, BCCAT)**	43 [7]	82 [17]
B. pseudomultivorans	0 [9]	-
B. stagnalis **(BCC B)**	98 [64]	100 [3]
B. territorii **(BCC I)**	6 [33]	100 [2]
B. paludis	-	-
Other Bcc group	18 [59]	45 [11]
Total	35 [1257]	30 [312]

Not available data are represented by “-“. Kendall's rank test with a p-value of 0.01958

4.4.2. Considering that horizontal gene transfer and pc3 chromosomal rearrangement could explain the heterogeneous distribution of the *hmqABCDEFG* operon in the Bcc

Since not all Bcc species carry *hmqABCDEFG*, we asked whether the distribution of the operon could be related to a loss of the third chromosome in Bcc or to horizontal gene transfer. Bcc bacteria can lose their third chromosome (pc3), a virulence mega-plasmid containing a few core genes (56, 57). The *hmqABCDEFG* operon being generally located on the pc3 replicon, except in *B. ubonensis* which carries this operon on its second chromosome like *B. pseudomallei* and *B. thailandensis*, and because the synteny of the *hmqABCDEFG* operon is conserved within a species (55), we verified if the strains missing the operon were also missing their pc3 replicon. Our analysis showed that the absence of the *hmqABCDEFG* operon does not correlate with the loss of pc3 (**Table S3**).

Table S 4.3 Study of the presence of the third replicon in Bcc strains carrying or not the *hmqABCDEFG* operon

Strains	Number of chromosomes	Presence of the *hmqABCDEFG* operon
Burkholderia ambifaria AMMD	3	+
Burkholderia ambifaria MC40-6	3	+
Burkholderia cenocepacia 842	3	-
Burkholderia cenocepacia 895	2	-
Burkholderia cenocepacia AU 1054	3	-
Burkholderia cenocepacia CR318	3	-
Burkholderia cenocepacia DDS 22E-1	3	-
Burkholderia cenocepacia DWS 37E-2	3	-
Burkholderia cenocepacia H111	3	-
Burkholderia cenocepacia HI2424	3	-
Burkholderia cenocepacia J2315	3	-
Burkholderia cenocepacia MC0-3	3	-
Burkholderia cenocepacia MSMB384WGS	3	-
Burkholderia cenocepacia VC12308	3	-
Burkholderia cenocepacia VC12802	2	-
Burkholderia cenocepacia VC7848	1	-
Burkholderia cepacia ATCC 25416	3	+
Burkholderia cepacia DDS 7H-2	3	-

Burkholderia cepacia FDAARGOS_345	3	+
Burkholderia cepacia FDAARGOS_388	3	+
Burkholderia cepacia GG4	2	-
Burkholderia cepacia INT3-BP177	2	-
Burkholderia cepacia JBK9	3	-
Burkholderia cepacia LO6	1	-
Burkholderia cepacia MSMB1184WGS	3	+ (c2)
Burkholderia contaminans MS14	3	+
Burkholderia diffusa RF2-non-BP9	3	-
Burkholderia dolosa AU0158	3	-
Burkholderia lata 383	3	-
Burkholderia lata FL-7-5-30-S1-D0	3	+
Burkholderia latens AU17928	3	-
Burkholderia metallica FL-6-5-30-S1-D7	3	-
Burkholderia multivorans ATCC 17616	3	-
Burkholderia multivorans ATCC 17616	3	-
Burkholderia multivorans ATCC BAA-247	3	-
Burkholderia multivorans AU1185	3	-
Burkholderia multivorans MSMB1640WGS	3	-
Burkholderia pyrrocinia DSM 10685	3	+
Burkholderia seminalis FL-5-4-10-S1-D7	3	-
Burkholderia stabilis ATCC BAA-67	3	-
Burkholderia stabilis FERMP-21014	3	-
Burkholderia stagnalis MSMB735WGS	3	-
Burkholderia territorii RF8-non-BP5	3	-
Burkholderia ubonensis MSMB0783	3	-
Burkholderia ubonensis MSMB1189WGS	3	+
Burkholderia ubonensis MSMB1471WGS	2	+
Burkholderia ubonensis MSMB2035	3	+
Burkholderia ubonensis MSMB22	3	+
Burkholderia ubonensis RF23-BP41	3	+
Burkholderia vietnamiensis AU1233	2	-
Burkholderia vietnamiensis FL-2-3-30-S1-D0	3	-

Burkholderia vietnamiensis G4	3	-
Burkholderia vietnamiensis HI2297	3	-
Burkholderia vietnamiensis LMG 10929	3	-
Burkholderia vietnamiensis MSMB608WGS	3	-

By comparing the phylogenetic tree based on Multilocus Sequence Typing (MLST) sequences and the distribution of strains possessing the *hmqABCDEFG* operon, we found that the resulting MLST and *hmqABCDEFG* phylogenies mostly match except for two groups which are inversed (**Figure 4.2).** The Hmq system comes from a common ancestor with possible chromosomic rearrangement for some strains.

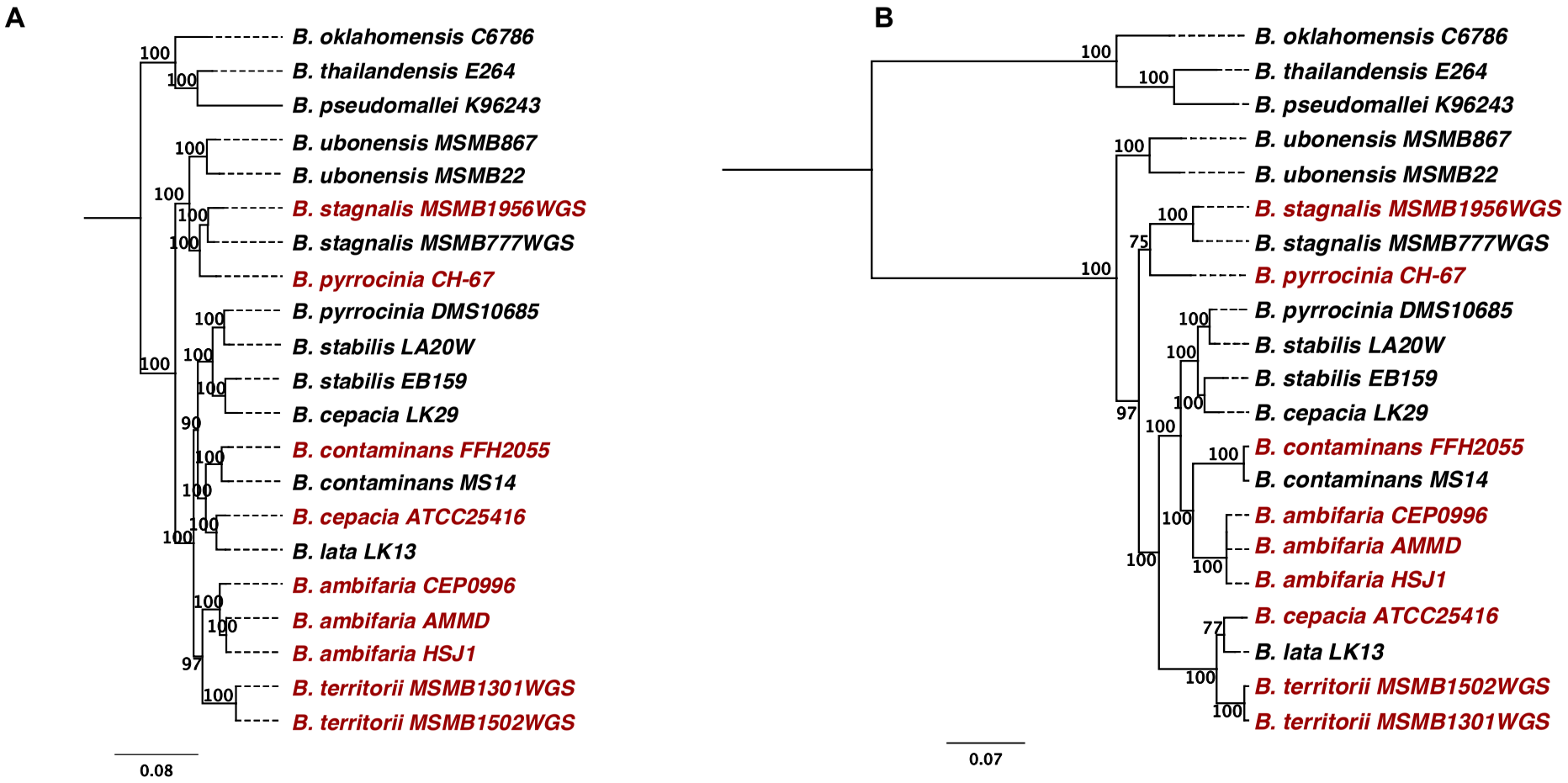

Figure 4.2 Phylogeny indicates that acquisition of the *hmqABCDEFG* operon results from horizontal gene transfer within Bcc species.

A) Phylogeny of Bcc strains carrying the *hmqABCDEFG* operon, based on MLST genes (*atpD, gltB, gyrB, recA, lepA, phaC* and *trpB*) (8) B) Phylogeny of the Bcc species based on their *hmqABCDEFG* operon. Trees were generated by RAxML using GTRGAMMA model and 1000 bootstraps. The branches are labelled where bootstrap values are >50%. Strains in black were chosen as model and were not a part of our study. Strains used in this study are labelled in red.

4.4.3. Most Bcc strains carrying the *hmqABCDEFG* operon 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 *hmqA* and *hmqG* in Tryptic Soy Broth (TSB) medium at 30°C, 60 rpm overnight and we detected the production of HMAQ in 72% of the PCR-positive strains - 65% clinical and 79% environmental - carrying the *hmqABCDEFG* operon (**Table S4.4**). None of the PCR-positive *B. anthina*, *B. diffusa*, *B. dolosa*, *B. metallica*, *B. seminalis,* and *B. ubonensis* strains produced detectable HMAQs under these culture conditions.

Table S 4.4 HMAQ production in different culture media at 30°C for strains having the *hmqABCDEFG* operon in their genome

Strains	Type	*hmq* operon		HMAQ production		
		hmqA	*hmqG*	TSB	ASM	TSA
B. ambifaria AMMD	Environmental	+	+	-	+	+
B. ambifaria AU0212	Clinical	+	+	+	+	+
B. ambifaria AU4157	Clinical	+	+	+	+	-
B. ambifaria AU7994	Clinical	+	+	-	-	-
B. ambifaria CEP0617	Clinical	+	+	+	+	-
B. ambifaria CEP0958	Clinical	+	+	+	+	+
B. ambifaria CEP0990	Clinical	+	+	+	-	-
B. ambifaria CEP0996	Clinical	+	+	+	+	+
B. ambifaria CEP1231	Clinical	+	+	-	+	-
B. ambifaria ES0020	Environmental	+	+	+	+	+
B. ambifaria HI2425	Environmental	+	+	-	+	+
B. ambifaria HI2468	Environmental	+	+	+	+	+
B. ambifaria HI2482	Environmental	+	+	+	+	+
B. ambifaria HI2626	Environmental	+	+	+	+	+
B. ambifaria HI2672	Environmental	+	+	+	+	+
B. ambifaria HI3709	Environmental	+	+	+	+	+
B. ambifaria HI3738	Environmental	+	+	+	+	+
B. ambifaria HSJ1	Clinical	+	+	+	+	+
B. ambifaria PC736	Environmental	+	+	+	+	+
B. ambifaria VC11631	Clinical	+	+	-	-	-
B. ambifaria VC15422	Clinical	+	+	+	+	+
B. ambifaria VC16196	Clinical	+	+	+	+	+

B. anthina VC15382	Clinical	+	+	-	-	-
B. cepacia BTS13	Clinical	+	+	+	-	-
B. cepacia HI2430	Environmental	+	+	+	+	+
B. cepacia HI2563	Environmental	+	+	+	+	+
B. cepacia HI2578	Environmental	+	+	+	+	+
B. cepacia HI2615	Environmental	+	+	+	+	+
B. cepacia HI2671	Environmental	+	+	+	+	+
B. cepacia HI2741	Environmental	+	+	+	+	+
B. cepacia HI3312	Environmental	+	+	+	+	+
B. cepacia HI3551	Environmental	+	+	+	+	+
B. cepacia HI3708	Environmental	+	+	+	+	+
B. cepacia HI3895	Environmental	+	+	+	+	+
B. cepacia HI4577	Environmental	+	+	+	-	+
B. cepacia ATCC25416	Environmental	+	+	-	-	-
B. cepacia VC13132	Clinical	+	+	+	+	+
B. cepacia VC13196	Clinical	+	+	-	-	-
B. cepacia VC13394	Clinical	+	+	+	+	+
B. cepacia VC13575	Clinical	+	+	-	-	-
B. cepacia VC14106	Clinical	+	+	+	+	+
B. cepacia VC14457	Clinical	+	+	+	+	+
B. cepacia VC17333	Clinical	+	+	+	+	+
B. cepacia VC17746	Clinical	+	+	+	+	+
B. cepacia VC17928	Clinical	+	+	+	+	+
B. cepacia VC18315	Clinical	+	+	+	+	+
B. cepacia VC18839	Clinical	+	+	+	+	+
B. cepacia VC18842	Clinical	+	+	+	+	+
B. cepacia VC19225	Clinical	+	+	-	+	+
B. cepacia VC19276	Clinical	+	+	+	+	+
B. cepacia VC9490	Clinical	+	+	+	+	+
B. contaminans FFH2055	Clinical	+	+	+	-	-
B. contaminans HI3570	Environmental	+	+	+	+	+
B. contaminans HI3852	Environmental	+	+	+	+	+
B. contaminans HI4067	Environmental	+	+	+	+	+

B. contaminans HI4232	Environmental	+	+	+	+	+
B. contaminans VC14347	Clinical	+	+	+	+	+
B. contaminans VC15406	Clinical	+	+	-	-	+
B. contaminans VC16087	Clinical	+	+	+	+	+
B. contaminans VC16848-b	Clinical	+	+	+	+	+
B. contaminans VC16897	Clinical	+	+	-	-	-
B. contaminans VC16948	Clinical	+	+	-	-	+
B. contaminans VC19056	Clinical	+	+	+	+	+
B. contaminans VC19124	Clinical	+	+	+	+	+
B. contaminans VC9624	Clinical	+	+	+	+	+
B. diffusa VC14008	Clinical	+	+	-	-	-
B. dolosa LMG21443	Environmental	+	+	-	-	-
B. lata BC01	Environmental	+	+	+	+	+
B. lata VC6377	Clinical	+	+	-	+	+
B. metallica ES0559	Environmental	+	+	-	-	-
B. pyrrocinia Bcc indeterminate 2 BC02	Environmental	+	+	+	+	-
B. pyrrocinia Bcc indeterminate 5 HI2575	Environmental	+	+	+	+	+
B. pyrrocinia Bcc indeterminate 5 HI2690	Environmental	+	+	+	+	+
B. pyrrocinia Bcc indeterminate 5 HI2701	Environmental	+	+	+	+	+
B. pyrrocinia Bcc indeterminate 5 HI3892	Environmental	+	+	+	+	+
B. pyrrocinia Bcc indeterminate 9 ES0209	Environmental	+	+	-	+	+
B. pyrrocinia CH-67 (LMG14191)	Environmental	+	+	+	+	+
B. seminalis HI2490	Environmental	+	+	-	+	-
B. stagnalis Bcc indeterminate 6 HI3537	Environmental	+	+	-	+	+
B. stagnalis HI2720	Environmental	+	+	-	+	-
B. stagnalis MSMB1956WGS	Environmental	+	+	+	+	-
B. territorii MSMB1301WGS	Environmental	+	+	+	+	+
B. territorii MSMB1502WGS	Environmental	+	+	+	+	+
B. ubonensis LMG24263	Clinical	+	+	-	-	-
B. vietnamiensis CEP0040	Clinical	+	+	-	-	-
B. vietnamiensis HI3392	Environmental	+	+	-	+	+
B. vietnamiensis VC17180	Clinical	+	+	-	-	-
B. vietnamiensis VC8245	Clinical	+	+	+	+	+

B. vietnamiensis VC9237	Clinical	+	+	-	-	-
other Bcc - Bcc indeterminate 1 BC06	Environmental	+	+	+	+	+
other Bcc - Bcc indeterminate 3 BC13	Environmental	+	+	+	+	+
other Bcc - Bcc indeterminate 3 ES0139	Environmental	+	+	+	+	+
other Bcc - Bcc indeterminate 4 BC04	Environmental	+	+	+	+	+
other Bcc - Bcc indeterminate 5 BC03	Environmental	+	+	+	+	+

Assuming that the absence of HMAQ production in 26 of the 94 PCR-positive strains could simply be the result of unfavorable culture conditions, we assayed the production of these metabolites in TSB again but at 37°C, in Artificial Sputum Medium (ASM; at 30°C at 57 rpm, overnight) and on Tryptic Soy Agar plates (TSA; incubated at 30°C for four days). As shown in **Table 4.1**, 4 out of 26 previously negative strains produced HMAQs when grown in TSB 37°C, while ASM growth induced production levels allowing detection of HMAQs in 10 out of the 26 Bcc strains - seven environmental and three clinical strains.

Table 4.1 Production of HMAQs under alternative culture conditions for hmqABCDEFG-positive strains not producing HMAQs in TSB at 30°C

Strains	Type	HMAQ production		
		TSB (37°C)	ASM (30°C)	TSA (30°C)
***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. pyrrocinia* 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. vietnamiensis* CEP0040**	Clinical	-	-	-
***B. vietnamiensis* VC17180**	Clinical	+	-	-
***B. vietnamiensis* VC9237**	Clinical	-	-	-

Surface growth on TSA plates induced the detectable production of HMAQs for five environmental and four clinical strains. Among the strains already producing HMAQs in TSB at 30°C, most of them also produce HMAQs in ASM and TSA (**Table S4.4**). Interestingly, two *B. contaminans* strains that were negative in both TSB and ASM produced detectable HMAQs when cultured on TSA plates. These additional culture conditions reduced the number of HMAQ-negative strains from 26 to 15,

increasing the number of Bcc isolates able to produce HMAQs to a total of 82, that is, 87% of strains carrying the *hmqABCDEFG* operon including 92% environmental and 82% clinical isolates (**Figure 4.3 – Figure S4.3**).

To validate our strategy, we assessed the production of HMAQs by 31 Bcc strains determined by PCR not to carry the *hmqABCDEFG* operon; all the 31 strains – belonging to *B. ambifaria, B. anthina, B. arboris, B. cenocepacia, B. multivorans, B. pyrrocinia, B. stabilis, B. ubonensis,* and *B. vietnamiensis* species – indeed did not produce detectable HMAQs (**Table S4.5**).

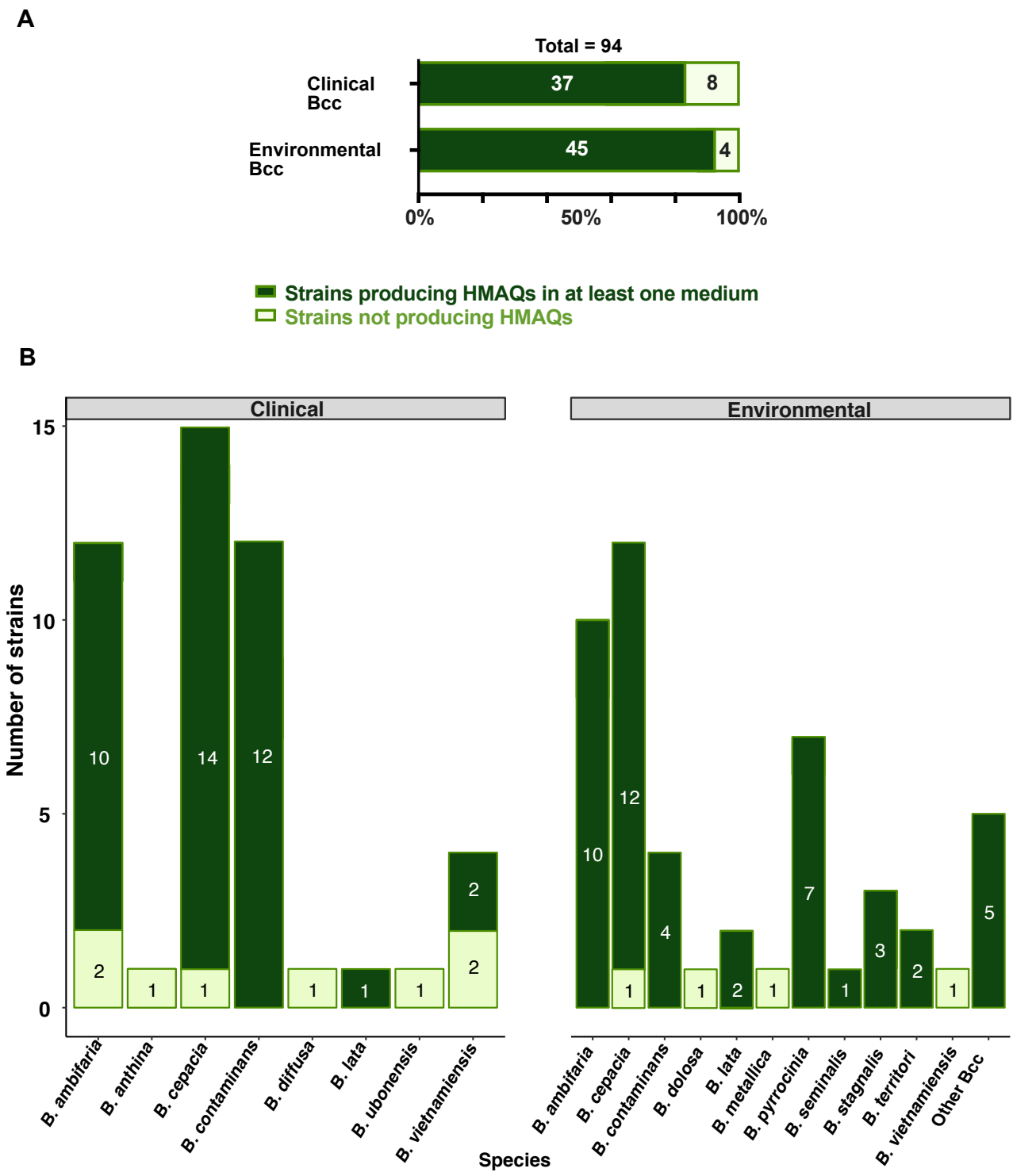

Figure 4.3 **Distribution of HMAQ production among Bcc strains carrying the *hmqABCDEFG* operon**

(A) The distribution of environmental and clinical Bcc strains regarding their ability to produce HMAQs in at least one of the tested culture conditions (B) Distribution of HMAQ producing-Bcc species. HMAQs were detected and quantified by LC/MS with a limit of detection of 50 µg/L for each molecule in the total culture.

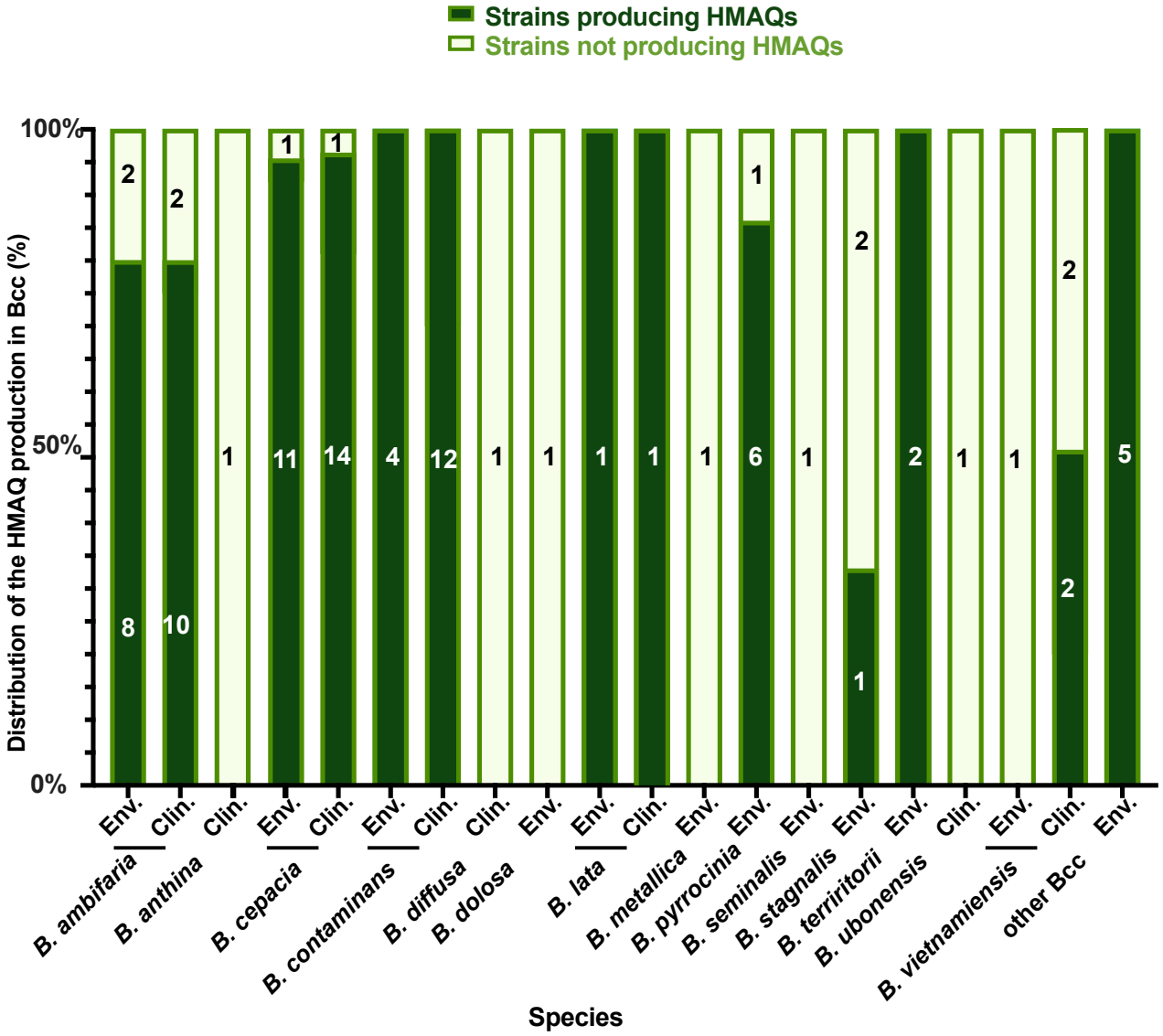

Figure S 4.3 Distribution of HMAQ production among Bcc species

HMAQs have been quantified by LC/MS with a limit of detection of 50 µg/L for each molecule in the total culture. Results are presented as percentages of total strains for each origin. Numbers represent the number of strains in each species and for each origin, producing (white) or not (black) HMAQs.

Table S 4.5 Quantification of the production of HMAQs for the Bcc strains which do not carry the *hmqABCDEFG* operon in their genome

Strains	Type	*hmqA*	*hmqG*	HMAQ production (TSB)
B. ambifaria CEP0516	Clinical	-	-	-
B. ambifaria HI3590	Environmental	-	-	-
B. ambifaria HI3687	Environmental	-	-	-
B. ambifaria IOP40-10	Environmental	-	-	-
B. ambifaria LMG17828	Environmental	-	-	-
B. ambifaria PHP7	Environmental	-	-	-
B. anthina VC16083	Clinical	-	-	-
B. arboris VC8833	Clinical	-	-	-
B. cenocepacia CEP024	Clinical	-	-	-
B. cenocepacia CEP0511	Clinical	-	-	-
B. cenocepacia CEP0565	Clinical	-	-	-
B. cenocepacia IIIA VC16156	Clinical	-	-	-
B. cenocepacia IIIB VC11311	Clinical	-	-	-
B. cenocepacia IIIB VC15122	Clinical	-	-	-
B. cenocepacia K56-2	Clinical	-	-	-
B. cenocepacia LMG19240	Environmental	-	-	-
B. multivorans CEP0781	Clinical	-	-	-
B. multivorans LMG17588	Environmental	-	-	-
B. multivorans VC14090	Clinical	-	-	-
B. multivorans VC16759	Clinical	-	-	-
B. pyrrocinia LMG21824	Clinical	-	-	-
B. stabilis LMG18870	Clinical	-	-	-
B. stabilis VC6749	Clinical	-	-	-
B. stabilis VC6753	Clinical	-	-	-
B. ubonensis LMG20358	Environmental	-	-	-
B. vietnamiensis G4	Environmental	-	-	-
B. vietnamiensis VC13984	Clinical	-	-	-
B. vietnamiensis VC18210	Clinical	-	-	-
B. vietnamiensis VC18712	Clinical	-	-	-
B. vietnamiensis VC18844	Clinical	-	-	-
B. vietnamiensis VC2824	Clinical	-	-	-

4.4.4. All HMAQ-producing Bcc strains mainly produce the HMAQ-C_7:2' and HMAQ-C9:2' 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 S4.6**; [36]). We found that the main congeners produced were HMAQ-C_7:2' and HMAQ-C_9:2', as previously observed for *B. ambifaria* HSJ1 (36), followed by HMAQ-C_8 and HMAQ-C_6 (**Figure 4.4**; **Table S4.6**). Most of the strains produced other HMAQs such as HMAQ-C_7 and HMAQ-C_8:2' (also known as burkholone).

We also found that the concentration of HMAQs produced was variable among the various species when grown in TSB at 30°C. A Kruskal-Wallis test confirmed the absence of statistical difference in the concentrations produced between the clinical and environmental strains (*p*-value of 0.19 for HMAQ-C_7:2' and 0.22 for HMAQ-C_9:2'). However, clinical strains of *B. ambifaria* and *B. vietnamiensis* produced more HMAQs than environmental ones. The opposite was observed for *B. cepacia* and *B. contaminans* strains (**Figure 4.4**).

Table S 4.6 HMAQs and HAQs congeners produce by the Bcc strains

HMAQs production (mg/L/OD600nm)

Strains	HMAQ-C7:2'		HMAQ-C9:2'		HMAQ-C5		HHQ-C7		HMAQ-C6		HMAQ-C7	
	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
B. ambifaria AU0212	8.7E-02	6.7E-02	4.1E-02	3.1E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria AU4157	1.7E+00	1.4E+00	1.3E+00	1.0E+00	5.8E-04	5.1E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.5E-03	4.0E-03
B. ambifaria CEP0617	4.7E+00	4.9E-01	4.4E+00	3.5E-01	1.8E-03	2.5E-04	5.6E-03	4.4E-04	1.7E-03	2.3E-04	4.1E-02	6.1E-03
B. ambifaria CEP0958	2.9E+00	5.8E-02	2.8E+00	1.5E-01	1.3E-03	0.0E+00	1.5E-03	2.3E-04	0.0E+00	0.0E+00	8.5E-03	2.1E-04
B. ambafaria CEP0990	2.3E+00	1.1E+00	1.8E+00	9.2E-01	2.7E-03	1.4E-03	2.0E-03	1.5E-04	1.6E-03	5.8E-04	1.2E-02	6.0E-03
B. ambifaria CEP0996	2.6E+00	1.3E+00	1.4E+00	5.8E-01	1.3E-03	1.4E-03	2.8E-03	2.1E-04	0.0E+00	0.0E+00	6.0E-03	3.5E-03
B. ambifaria ES0020	2.1E+00	3.5E-01	1.8E+00	3.2E-01	9.9E-04	4.7E-04	1.0E-02	2.8E-03	3.7E-05	2.7E-05	3.6E-02	7.2E-03
B. ambifaria HI2468	6.0E-01	2.5E-02	4.2E-01	4.6E-02	2.6E-04	3.5E-05	1.7E-03	1.5E-04	3.3E-04	2.0E-04	6.2E-03	7.1E-04
B. ambifaria HI2482	7.0E-01	7.8E-02	4.1E-01	3.2E-02	8.1E-04	1.4E-04	3.3E-03	4.0E-04	5.6E-04	3.8E-04	6.3E-03	7.0E-04
B. ambifaria HI2626	8.6E-01	7.1E-02	6.1E-01	4.4E-02	9.0E-04	8.5E-05	3.0E-03	6.1E-04	2.7E-04	2.7E-04	7.3E-03	6.1E-04
B. ambifaria HI2672	1.1E+00	5.8E-02	8.1E-01	1.5E-02	1.3E-03	2.1E-04	2.1E-03	1.2E-04	4.0E-04	3.5E-04	1.2E-02	5.8E-04
B. ambifaria HI3709	4.3E-01	3.3E-01	3.1E-01	2.8E-01	5.2E-04	4.3E-04	1.9E-03	1.4E-03	1.8E-03	9.9E-04	6.9E-03	4.6E-03
B. ambifaria HI3738	9.3E-01	6.6E-01	6.1E-01	4.8E-01	1.3E-03	9.8E-04	2.7E-03	1.3E-03	9.6E-04	3.0E-04	1.2E-02	8.3E-03
B. ambifaria HSJ1	2.5E+00	1.1E+00	2.4E+00	1.1E+00	4.0E-04	6.9E-04	1.4E-03	0.0E+00	0.0E+00	0.0E+00	1.0E-02	5.3E-03
B. ambifaria PC736	9.8E-01	2.3E-01	6.1E-01	1.8E-01	1.0E-03	3.2E-04	2.1E-03	4.2E-04	4.5E-04	3.3E-04	6.0E-03	1.9E-03
B. ambifaria VC15422	5.5E-01	6.5E-01	3.8E-01	4.9E-01	0.0E+00	0.0E+00	2.1E-03	3.6E-03	3.3E-04	5.8E-04	1.5E-03	2.0E-03
B. ambifaria VC16196	1.1E+00	1.8E-01	9.5E-01	5.2E-02	0.0E+00	0.0E+00	2.1E-03	5.2E-04	2.0E-04	3.5E-04	2.0E-03	1.2E-04
B. cepacia BTS13	2.8E-01	4.3E-01	1.3E-01	1.8E-01	0.0E+00	0.0E+00	3.2E-04	5.5E-04	0.0E+00	0.0E+00	2.2E-03	3.9E-03
B. cepacia HI2430	3.2E+00	3.1E-01	2.3E+00	3.0E-01	7.8E-03	4.0E-04	1.9E-03	3.2E-04	2.8E-03	2.4E-03	2.1E-02	2.6E-03
B. cepacia HI2563	1.2E+00	3.5E-01	7.3E-01	2.3E-01	5.4E-03	1.0E-03	3.7E-03	1.8E-03	9.7E-05	1.4E-04	2.6E-02	8.4E-03
B. cepacia HI2578	5.5E-01	3.0E-01	2.6E-01	2.3E-01	1.0E-03	8.5E-04	7.1E-04	3.2E-04	0.0E+00	0.0E+00	7.9E-03	3.8E-03
B. cepacia HI2615	1.0E+00	5.8E-01	6.9E-01	4.4E-01	4.1E-03	2.1E-03	7.8E-03	3.1E-03	1.0E-03	6.9E-04	3.1E-02	1.5E-02
B. cepacia HI2671	4.9E-01	2.5E-01	2.8E-01	1.7E-01	7.9E-04	6.2E-04	3.1E-04	2.2E-04	5.2E-04	4.0E-04	2.2E-03	1.3E-03
B. cepacia HI2741	3.6E+00	7.6E-01	2.1E+00	5.0E-01	8.1E-03	2.9E-03	1.8E-03	7.0E-04	1.2E-04	1.8E-04	2.9E-02	9.8E-03
B. cepacia HI3312	6.3E-01	7.1E-02	4.1E-01	5.6E-02	1.4E-03	1.5E-04	2.8E-04	5.6E-05	3.7E-04	1.1E-04	5.0E-03	5.9E-04
B. cepacia HI3551	7.6E-01	3.2E-01	5.1E-01	2.6E-01	1.5E-03	7.0E-04	6.9E-04	2.5E-04	1.7E-03	1.3E-03	6.7E-03	2.9E-03
B. cepacia HI3708	1.3E+00	1.7E+00	6.1E-01	8.6E-01	5.0E-04	8.7E-04	5.3E-04	9.2E-04	2.7E-02	3.0E-02	1.3E-02	1.8E-02
B. cepacia HI3895	4.2E-01	0.0E+00	2.2E-01	1.7E-02	9.4E-04	9.8E-05	7.1E-04	6.1E-05	6.9E-04	6.0E-04	5.4E-03	2.5E-04
B. cepacia VC13132	1.9E-01	1.2E-01	1.3E-01	7.2E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	6.3E-05	1.1E-04
B. cepacia VC13394	1.1E+00	1.2E-01	9.1E-01	9.0E-02	1.3E-03	2.1E-04	2.7E-03	5.5E-04	7.1E-04	1.6E-04	2.0E-03	2.6E-04
B. cepacia VC14106	8.9E-01	1.1E-01	7.8E-01	1.1E-01	5.8E-03	5.1E-03	0.0E+00	0.0E+00	2.3E-03	4.0E-03	4.0E-02	1.9E-02
B. cepacia VC14457	5.3E-01	6.0E-02	3.6E-01	2.0E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC17333	5.1E-01	1.2E-01	4.8E-01	1.7E-01	0.0E+00	0.0E+00	6.5E-04	7.1E-04	0.0E+00	0.0E+00	5.2E-04	4.8E-04
B. cepacia VC17746	3.7E-01	3.3E-01	2.6E-01	2.3E-01	0.0E+00	0.0E+00	1.7E-04	2.9E-04	0.0E+00	0.0E+00	2.0E-04	3.5E-04
B. cepacia VC17928	4.5E-01	3.8E-01	4.2E-01	4.1E-01	3.3E-04	5.8E-04	9.3E-04	1.6E-03	2.3E-04	4.0E-04	5.3E-04	9.2E-04
B. cepacia VC18315	2.8E+00	1.5E-01	1.6E+00	5.8E-02	2.0E-03	3.2E-03	4.6E-04	5.1E-04	7.7E-04	1.3E-03	8.1E-03	8.2E-03
B. cepacia VC18839	1.1E-01	2.9E-02	4.8E-02	4.4E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.4E-03	1.2E-03
B. cepacia VC18842	1.1E+00	7.7E-01	8.6E-01	6.0E-01	1.2E-03	1.1E-03	2.4E-03	2.1E-03	5.1E-04	4.5E-04	2.1E-03	1.8E-03
B. cepacia VC19276	4.2E-01	2.3E-01	3.4E-01	2.9E-01	0.0E+00	0.0E+00	4.5E-04	5.0E-04	0.0E+00	0.0E+00	5.3E-04	9.2E-04
B. cepacia VC9490	9.1E-01	1.9E-01	7.0E-01	7.5E-02	1.1E-03	2.6E-04	2.0E-03	6.8E-04	2.6E-04	4.4E-04	1.9E-03	5.2E-04
B. contaminans FFH2055	7.5E-01	4.8E-01	4.6E-01	4.1E-01	2.0E-04	3.5E-04	1.1E-03	3.2E-04	0.0E+00	0.0E+00	6.0E-03	3.3E-03
B. contaminans HI3570	7.5E-01	2.0E-01	2.9E-01	2.6E-01	1.0E-03	8.8E-04	6.9E-04	1.9E-04	2.0E-04	1.6E-04	9.3E-03	2.5E-03
B. contaminans HI3852	1.3E-01	7.7E-02	0.0E+00	0.0E+00	3.2E-05	3.4E-05	1.0E-04	2.3E-05	6.7E-04	5.8E-04	1.6E-03	6.6E-04
B. contaminans HI4067	4.6E-01	4.6E-02	0.0E+00	0.0E+00	3.5E-04	7.5E-05	5.4E-04	5.3E-05	5.4E-04	9.2E-04	6.2E-03	9.5E-04
B. contaminans HI4232	4.6E-01	8.6E-02	6.3E-02	1.1E-01	4.3E-04	7.8E-05	6.6E-04	4.7E-04	4.7E-04	3.0E-04	6.7E-03	1.3E-03
B. contaminans VC14347	1.5E-01	5.2E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC16087	1.6E-01	2.9E-02	4.0E-02	6.9E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.8E-03	1.4E-03
B. contaminans VC16087	2.0E-01	1.8E-01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC16848-b	5.0E-01	9.2E-02	1.1E-02	1.2E-02	1.2E-04	2.1E-04	4.3E-04	4.8E-04	0.0E+00	0.0E+00	5.1E-04	5.0E-04
B. contaminans VC19056	4.5E-01	4.5E-02	7.7E-03	1.3E-02	7.7E-05	1.3E-04	1.1E-04	2.0E-04	0.0E+00	0.0E+00	5.4E-04	9.3E-05
B. contaminans VC19124	3.5E-01	1.5E-01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.6E-02	6.4E-03
B. contaminans VC9624	1.5E-01	1.7E-01	2.1E-02	2.1E-02	2.6E-02	2.3E-02	5.9E-03	4.9E-03	5.2E-03	4.6E-03	7.0E-02	6.0E-02
B. lata BC01	2.2E+00	3.6E-01	2.9E-02	5.9E-03	2.5E-03	4.6E-04	1.1E-02	5.8E-04	9.0E-04	1.9E-04	5.0E-02	8.7E-03
B. pyrrocinia BC02	1.8E+00	6.4E-01	2.8E-02	1.2E-02	1.7E-03	7.6E-04	1.4E-02	3.5E-03	2.2E-05	2.2E-05	4.8E-02	1.5E-02
B. pyrrocinia CH-67	2.0E-01	1.0E-02	1.3E+00	1.2E-01	0.0E+00	0.0E+00	2.2E-03	3.5E-04	0.0E+00	0.0E+00	4.5E-03	2.6E-04
B. pyrrocinia HI2575	9.0E-01	2.1E-01	8.2E-01	2.5E-01	1.8E-03	9.5E-04	3.5E-03	5.1E-04	9.4E-04	1.4E-04	1.6E-02	4.7E-03
B. pyrrocinia HI2690	7.2E-01	2.5E-01	6.8E-01	2.8E-01	1.0E-03	1.5E-04	3.8E-03	9.0E-04	2.0E-04	3.4E-04	7.4E-03	3.2E-03
B. pyrrocinia HI3892	7.0E-01	1.5E-01	6.0E-01	1.7E-01	9.6E-04	2.4E-04	2.8E-03	6.6E-04	5.2E-04	1.3E-04	1.1E-02	3.0E-03
B. stagnalis MSMB1956WGS	2.9E-03	3.5E-04	1.4E-02	2.0E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. territorii MSMB1301WGS	1.2E+00	5.3E-01	7.6E-01	2.2E-01	4.7E-04	8.1E-04	1.1E-03	1.2E-04	2.5E-04	4.3E-04	1.0E-02	7.7E-03
B. territorii MSMB1502WGS	5.1E-01	1.7E-01	3.1E-01	1.7E-01	0.0E+00	0.0E+00	1.3E-04	2.2E-04	0.0E+00	0.0E+00	2.5E-03	1.1E-03
B. vietnamiensis VC8245	5.6E-01	1.9E-01	5.0E-01	2.3E-01	5.7E-04	6.0E-04	9.7E-04	1.2E-03	2.2E-04	3.8E-04	8.6E-04	9.0E-04
Other Bcc BC06	8.8E-01	3.2E-01	0.0E+00	0.0E+00	1.3E-03	8.2E-04	1.1E-03	6.5E-04	5.0E-04	2.9E-04	8.3E-03	7.5E-03
Other Bcc BC13	1.9E+00	1.5E-01	3.4E-02	9.3E-03	4.0E-03	7.4E-04	1.1E-03	1.2E-04	8.8E-04	9.0E-04	2.5E-02	4.6E-03
Other Bcc ES0139	1.6E+00	5.7E-01	3.6E-02	1.6E-02	1.2E-03	4.6E-04	2.1E-03	7.6E-04	4.8E-04	5.4E-04	9.5E-03	4.3E-03
Other Bcc BC04	1.0E+00	3.6E-01	0.0E+00	0.0E+00	1.8E-03	5.5E-04	1.4E-03	4.0E-04	2.1E-03	1.8E-03	1.1E-02	4.6E-03
Other Bcc BC03	4.6E-02	3.1E-03	1.8E-01	1.2E-02	0.0E+00	0.0E+00	4.8E-05	3.0E-05	7.6E-05	7.1E-05	3.8E-04	5.1E-05
Other Bcc HI2701	1.2E+00	3.5E-01	1.2E+00	3.7E-01	1.6E-03	4.5E-04	2.3E-03	7.4E-04	1.9E-04	2.2E-04	1.5E-02	5.2E-03

Strains	HHQ-C9:2'		HMAQ-C8:2'		HHQ-C9		HMAQ-C8		HMAQ-C9		HMAQ-C10:2'		HMAQ-C10		HMAQ-C11	
	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
B. ambifaria AU0212	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria AU4157	3.7E-03	3.2E-03	0.0E+00	0.0E+00	4.6E-03	4.0E-03	4.6E-03	4.0E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria CEP0617	2.9E-02	3.6E-03	2.4E-03	2.3E-04	4.0E-02	3.2E-03	4.0E-02	3.2E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria CEP0958	2.2E-02	1.7E-03	6.0E-04	5.2E-04	1.3E-02	5.8E-04	1.3E-02	5.8E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.0E-03	8.1E-05	0.0E+00	0.0E+00
B. ambafaria CEP0990	1.0E-02	8.5E-04	0.0E+00	0.0E+00	9.4E-03	4.0E-03	9.4E-03	4.0E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria CEP0996	1.9E-02	2.6E-03	0.0E+00	0.0E+00	3.7E-03	1.7E-03	3.7E-03	1.7E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria ES0020	6.5E-02	4.6E-03	4.5E-03	3.5E-04	0.0E+00	0.0E+00	7.7E-02	1.8E-02	1.0E-03	1.5E-03	5.3E-04	2.0E-04	4.0E-03	1.5E-03	4.1E-03	1.6E-03
B. ambifaria HI2468	1.7E-02	1.7E-03	5.7E-04	7.2E-05	0.0E+00	0.0E+00	7.4E-03	1.0E-03	1.1E-05	1.2E-05	1.1E-04	1.8E-05	4.1E-04	2.0E-05	3.4E-04	3.2E-05
B. ambifaria HI2482	2.0E-02	1.2E-03	6.7E-04	7.0E-05	0.0E+00	0.0E+00	6.3E-03	6.8E-04	2.5E-05	1.4E-05	1.3E-04	0.0E+00	5.3E-04	8.9E-05	5.0E-04	4.6E-05
B. ambifaria HI2626	2.8E-02	4.9E-03	8.8E-04	1.1E-04	0.0E+00	0.0E+00	9.0E-03	7.0E-04	4.6E-05	2.5E-06	1.8E-04	2.0E-05	8.1E-04	6.9E-05	7.8E-04	9.7E-05
B. ambifaria HI2672	2.2E-02	2.5E-03	8.2E-04	6.1E-05	0.0E+00	0.0E+00	1.7E-02	5.8E-04	3.7E-05	3.4E-05	4.6E-04	4.5E-05	1.8E-03	1.5E-04	1.6E-03	5.8E-05
B. ambifaria HI3709	1.3E-02	9.1E-03	6.0E-04	4.7E-04	0.0E+00	0.0E+00	9.6E-03	9.2E-03	1.2E-05	1.3E-05	8.3E-05	1.3E-04	4.0E-04	6.0E-04	4.4E-04	6.6E-04
B. ambifaria HI3738	1.7E-02	1.1E-02	9.4E-04	6.8E-04	0.0E+00	0.0E+00	1.8E-02	1.5E-02	6.1E-05	4.6E-05	2.8E-04	2.7E-04	1.6E-03	1.6E-03	1.3E-03	1.2E-03
B. ambifaria HSJ1	1.5E-02	5.8E-04	0.0E+00	0.0E+00	1.1E-02	5.3E-03	1.1E-02	5.3E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria PC736	2.4E-02	5.6E-03	7.1E-04	1.5E-04	0.0E+00	0.0E+00	8.0E-03	2.8E-03	0.0E+00	0.0E+00	2.2E-04	2.6E-05	8.8E-04	1.8E-04	3.6E-04	9.2E-05
B. ambifaria VC15422	1.8E-02	3.0E-02	5.3E-04	9.2E-04	1.3E-04	2.3E-04	9.0E-04	1.6E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. ambifaria VC16196	3.0E-02	2.3E-03	2.7E-04	4.7E-04	0.0E+00	0.0E+00	2.1E-03	3.5E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia BTS13	2.5E-03	3.5E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.2E-03	2.1E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia HI2430	2.5E-02	4.0E-03	8.8E-04	1.9E-04	0.0E+00	0.0E+00	4.5E-02	7.5E-03	1.2E-04	1.6E-05	2.0E-03	4.6E-04	4.0E-03	9.7E-04	1.9E-03	4.6E-04
B. cepacia HI2563	1.3E-02	2.1E-03	1.0E-03	3.3E-04	0.0E+00	0.0E+00	3.4E-02	1.1E-02	3.0E-05	3.2E-05	3.1E-04	1.7E-04	1.5E-03	6.4E-04	8.8E-04	2.1E-04
B. cepacia HI2578	3.5E-03	2.5E-03	1.5E-04	4.5E-05	0.0E+00	0.0E+00	5.4E-03	4.4E-03	5.7E-06	9.8E-06	1.0E-04	1.2E-04	1.8E-04	1.7E-04	1.0E-04	8.3E-05
B. cepacia HI2615	2.4E-02	1.1E-02	2.2E-03	1.0E-03	0.0E+00	0.0E+00	4.1E-02	2.6E-02	0.0E+00	0.0E+00	2.3E-04	1.9E-04	1.6E-03	1.1E-03	9.3E-04	7.5E-04
B. cepacia HI2671	4.0E-03	2.9E-03	8.2E-05	6.9E-05	1.6E-05	2.8E-05	2.2E-03	1.8E-03	1.0E-05	8.5E-06	7.7E-05	5.5E-05	1.2E-04	1.3E-04	6.7E-05	8.1E-05
B. cepacia HI2741	1.6E-02	1.7E-03	7.1E-04	1.9E-04	0.0E+00	0.0E+00	4.0E-02	1.7E-02	1.5E-04	6.3E-05	1.2E-03	3.4E-04	2.3E-03	9.5E-04	9.4E-04	3.7E-04
B. cepacia HI3312	3.8E-03	6.1E-04	1.3E-04	1.0E-05	0.0E+00	0.0E+00	9.1E-03	1.8E-03	2.0E-05	1.8E-05	2.6E-04	7.2E-05	5.8E-04	1.3E-04	2.5E-04	8.4E-05
B. cepacia HI3551	5.2E-03	2.1E-03	1.3E-04	1.0E-04	0.0E+00	0.0E+00	5.2E-03	3.0E-03	2.6E-06	4.5E-06	2.3E-04	1.5E-04	2.5E-04	1.8E-04	1.6E-04	1.0E-04
B. cepacia HI3708	8.3E-03	8.4E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	9.5E-03	1.2E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia HI3895	3.2E-03	3.2E-04	1.2E-04	2.2E-05	0.0E+00	0.0E+00	3.2E-03	1.5E-04	5.7E-06	9.8E-06	7.5E-05	2.2E-05	1.2E-04	2.3E-05	4.9E-05	1.7E-05
B. cepacia VC13132	1.8E-03	8.6E-04	0.0E+00	0.0E+00	5.7E-05	9.8E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC13394	3.1E-02	3.0E-03	6.9E-04	7.8E-05	0.0E+00	0.0E+00	2.1E-03	2.1E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC14106	1.7E-02	1.7E-03	5.2E-02	1.1E-02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	7.3E-02	1.3E-02	0.0E+00	0.0E+00	7.3E-03	6.7E-04	0.0E+00	0.0E+00
B. cepacia VC14457	7.3E-03	6.8E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC17333	8.6E-03	3.2E-03	1.6E-04	2.7E-04	0.0E+00	0.0E+00	6.6E-04	5.8E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC17746	5.5E-03	4.9E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.1E-04	3.6E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC17928	1.3E-02	1.5E-02	2.6E-04	4.5E-04	0.0E+00	0.0E+00	6.7E-04	1.2E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC18315	1.3E-02	2.0E-02	1.7E-04	2.9E-04	9.7E-03	1.6E-02	9.9E-03	1.6E-02	2.4E-03	4.1E-03	2.9E-03	5.0E-03	2.4E-03	4.1E-03	1.1E-03	1.8E-03
B. cepacia VC18839	0.0E+00	0.0E+00	9.7E-04	1.7E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC18842	2.8E-02	2.2E-02	3.0E-04	5.1E-04	0.0E+00	0.0E+00	1.6E-03	1.4E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC19276	7.1E-03	6.9E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	5.0E-04	8.7E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. cepacia VC9490	1.9E-02	3.8E-03	2.2E-04	3.8E-04	0.0E+00	0.0E+00	1.1E-03	6.4E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans FFH2055	4.3E-03	2.3E-04	1.7E-04	3.0E-04	0.0E+00	0.0E+00	3.7E-03	1.9E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans HI3570	3.4E-03	2.9E-03	2.1E-04	9.0E-05	1.7E-06	3.0E-06	5.4E-03	4.9E-03	4.7E-06	8.1E-06	1.4E-04	1.3E-04	3.4E-04	3.5E-04	1.7E-04	1.5E-04
B. contaminans HI3852	2.3E-05	1.7E-05	1.6E-05	1.7E-05	1.7E-05	1.5E-05	3.2E-06	5.5E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans HI4067	8.6E-05	1.2E-05	1.0E-04	5.7E-05	1.1E-05	4.2E-06	4.8E-05	1.7E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.6E-06	4.7E-06
B. contaminans HI4232	1.4E-03	2.2E-03	1.1E-04	9.5E-05	1.3E-05	1.2E-05	1.4E-03	2.3E-03	0.0E+00	0.0E+00	1.2E-05	2.0E-05	8.0E-05	1.4E-04	6.0E-05	1.0E-04
B. contaminans VC14347	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC16087	0.0E+00	0.0E+00	3.1E-03	2.3E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.2E-03	2.0E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC16087	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC16848-b	5.7E-05	9.8E-05	0.0E+00	0.0E+00	9.3E-05	1.6E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC19056	0.0E+00	0.0E+00	0.0E+00	0.0E+00	8.3E-05	1.4E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC19124	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. contaminans VC9624	5.4E-02	4.7E-02	2.7E-03	2.4E-03	1.3E-01	1.1E-01	1.3E-01	1.1E-01	5.5E-03	9.1E-03	3.9E-03	3.6E-03	1.1E-02	9.2E-03	4.6E-03	4.1E-03
B. lata BC01	1.3E-03	1.7E-04	9.2E-03	1.6E-03	0.0E+00	0.0E+00	3.4E-03	7.5E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.9E-05	1.2E-05	6.1E-05	5.6E-06
B. pyrrocinia BC02	6.4E-04	3.3E-04	3.9E-03	2.6E-03	0.0E+00	0.0E+00	1.6E-03	6.2E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.9E-05	1.1E-05	4.5E-05	1.9E-05
B. pyrrocinia CH-67	1.0E-01	1.1E-02	4.5E-03	6.0E-04	0.0E+00	0.0E+00	2.9E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. pyrrocinia HI2575	3.1E-02	1.4E-02	2.0E-03	9.5E-04	0.0E+00	0.0E+00	4.3E-02	1.9E-02	3.2E-05	3.6E-05	1.5E-04	5.9E-05	1.6E-03	1.2E-03	3.3E-03	2.9E-03
B. pyrrocinia HI2690	4.7E-02	1.9E-02	3.1E-03	1.3E-03	0.0E+00	0.0E+00	3.5E-02	1.8E-02	1.7E-05	2.9E-05	1.3E-04	1.7E-05	2.0E-03	7.4E-04	1.5E-03	1.1E-03
B. pyrrocinia HI3892	1.9E-02	2.1E-03	1.2E-03	3.8E-04	0.0E+00	0.0E+00	1.9E-02	7.9E-03	2.6E-05	1.2E-05	4.4E-05	1.2E-05	5.4E-04	2.7E-04	2.3E-04	1.2E-04
B. stagnalis MSMB1956WGS	9.7E-05	1.7E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. territorii MSMB1301WGS	5.6E-03	1.4E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	7.2E-03	5.9E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. territorii MSMB1502WGS	2.2E-03	2.5E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.5E-03	7.6E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
B. vietnamiensis VC8245	1.0E-02	7.0E-03	1.3E-04	2.3E-04	0.0E+00	0.0E+00	6.8E-04	7.6E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Other Bcc BC06	4.6E-04	1.6E-04	6.5E-04	9.9E-04	1.7E-05	1.5E-05	3.4E-04	3.7E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.7E-06	8.1E-06
Other Bcc BC13	6.8E-04	1.4E-04	7.3E-04	1.8E-04	2.0E-05	3.4E-05	1.4E-03	3.6E-04	0.0E+00	0.0E+00	1.2E-05	1.6E-05	2.0E-06	3.0E-06	1.3E-05	6.0E-06
Other Bcc ES0139	8.9E-04	2.7E-04	2.2E-04	9.7E-05	1.8E-05	1.4E-05	3.6E-04	1.7E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Other Bcc BC04	3.8E-04	1.9E-04	3.1E-04	1.7E-04	2.4E-05	8.7E-06	2.3E-04	1.4E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.3E-06	5.7E-06	1.5E-05	1.2E-05
Other Bcc BC03	3.7E-03	5.3E-04	9.9E-05	1.2E-06	0.0E+00	0.0E+00	2.6E-03	5.8E-05	3.7E-06	6.4E-06	6.8E-05	9.8E-06	6.3E-05	2.0E-05	6.2E-06	5.4E-06
Other Bcc HI2701	3.3E-02	9.0E-03	1.9E-03	6.2E-04	0.0E+00	0.0E+00	5.6E-02	1.9E-02	4.0E-05	6.9E-05	4.2E-04	1.2E-04	4.2E-03	1.4E-03	5.1E-03	1.9E-03

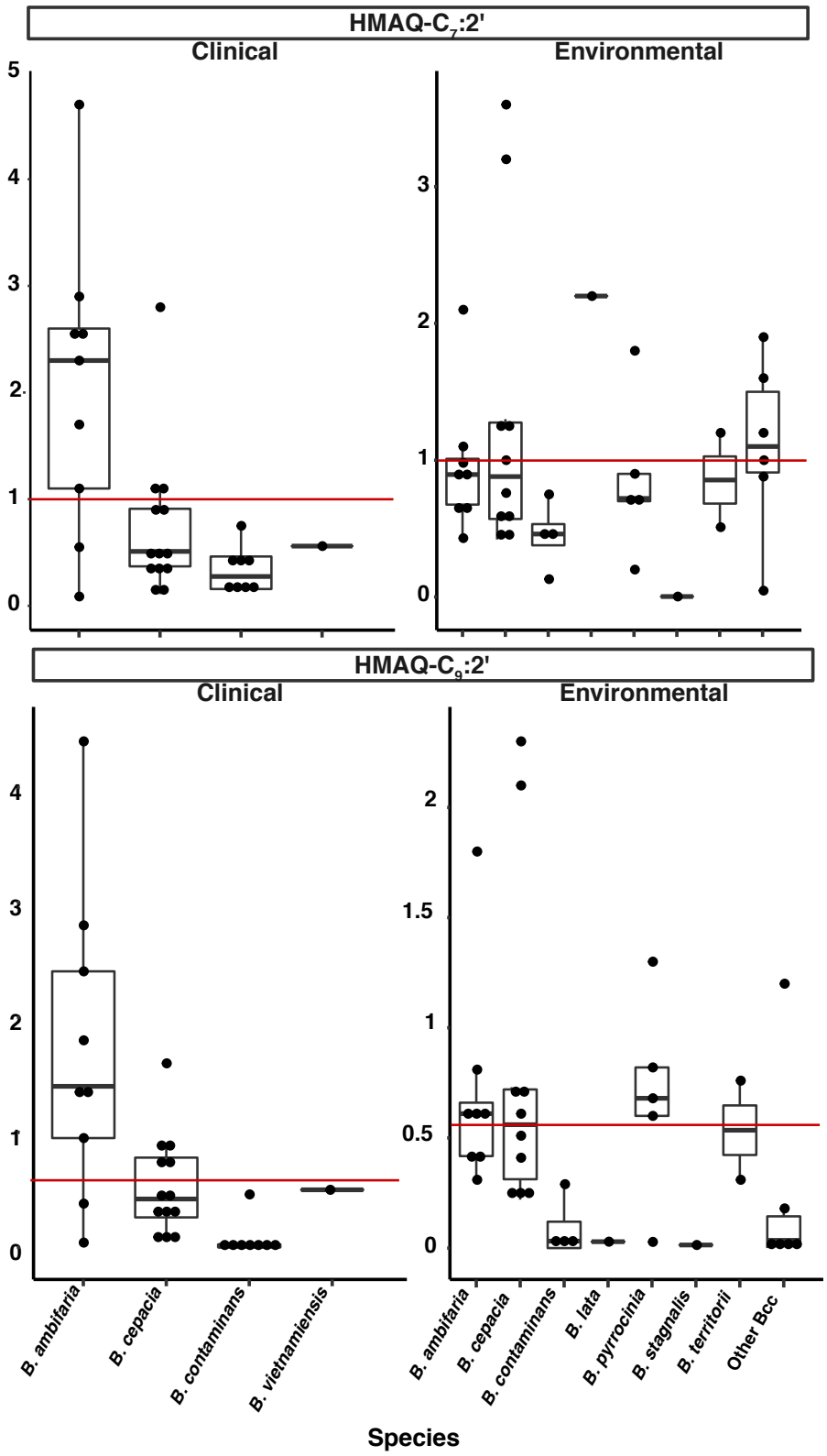

Figure 4.4 Concentration of the main produced HMAQs among Bcc species when cultured in tryptic soy broth

HMAQ-C_7:2' and HMAQ-C_9:2' were quantified by LC-MS with a lower limit of detection of 50 mg/liter for each molecule in the total culture. Tested strains are grouped by species. The production of HMAQs was quantified in three biological replicate cultures for each strain, and each dot represents the average HMAQ production for each strain, in milligrams per liter. Lines represent the production average for the clinical and environmental strains.

4.4.5. The presence of the *hmqABCDEFG* and production of HMAQs do not correlated 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 asked if HMAQ production 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 with the origin of the sample (sputum, throat, sinus *etc.*). Information was only available for 53 out of 222 clinical strains (**Table S4.7**). 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 4.2**).

Table S 4.7 Correlation data on the presence of the *hmqABCDEFG* operon and the production of HMAQs in clinical Bcc isolates

Species	*hmq* operon	HMAQ production	Co-isolated with *Pseudomonas* +/- 7 days	*Pseudomonas* in the previous year	Source
***B. cenocepacia* IIIA VC12308**	no	no	yes	yes	sputum
***B. cenocepacia* IIIA VC15419**	no	no	no	no	sputum
***B. cenocepacia* IIIA VC18585**	no	no	yes	no	respiratory
***B. cenocepacia* IIIA VC18996**	no	no	no	no	sputum
***B. cenocepacia* IIIA VC18999**	no	no	no	no	throat
***B. cenocepacia* IIIA VC3917**	no	no	yes	yes	sputum
***B. cenocepacia* IIIA VC6356**	no	no	no	yes	sputum
***B. cenocepacia* IIIA VC6553**	no	no	no	yes	sputum
***B. cenocepacia* IIIB VC11311**	no	no	yes	yes	sputum
***B. cenocepacia* IIIB VC15122**	no	no	no	no	throat
***B. cenocepacia* IIIB VC6598**	no	no	yes	yes	sputum
***B. cenocepacia* IIIB VC7349**	no	no	no	yes	respiratory
***B. cenocepacia* IIIB VC7849**	no	no	no	yes	sputum
***B. cenocepacia* IIIB VC7911**	no	no	no	no	respiratory
***B. cenocepacia* IIIB VC8340**	no	no	yes	yes	respiratory
***B. cepacia* VC18315**	yes	yes	no	yes	respiratory
***B. cepacia* VC9490**	yes	yes	yes	no	sputum
***B. contaminans* VC14347**	yes	yes	yes	yes	respiratory
***B. contaminans* VC15406**	yes	no	no	yes	sinus
***B. contaminans* VC16087**	yes	yes	no	no	respiratory
***B. lata* VC19230**	no	no	no	no	throat
***B. metallica* VC8135**	no	no	no	yes	sputum

***B. multivorans* VC12152**	no	no	yes	no	respiratory
***B. multivorans* VC12539**	no	no	no	yes	respiratory
***B. multivorans* VC12675**	no	no	no	no	sputum
***B. multivorans* VC13125**	no	no	no	no	respiratory
***B. multivorans* VC13145**	no	no	no	no	sputum
***B. multivorans* VC14443**	no	no	no	no	sputum
***B. multivorans* VC14749**	no	no	no	yes	sputum
***B. multivorans* VC14757**	no	no	no	yes	respiratory
***B. multivorans* VC15814**	no	no	no	no	respiratory
***B. multivorans* VC16959**	no	no	no	no	sputum
***B. multivorans* VC3419**	no	no	yes	yes	sputum
***B. multivorans* VC4282**	no	no	no	yes	sputum
***B. multivorans* VC6564**	no	no	yes	yes	sputum
***B. multivorans* VC7102**	no	no	yes	yes	throat
***B. multivorans* VC7704**	no	no	yes	yes	sputum
***B. multivorans* VC7960**	no	no	no	no	respiratory
***B. multivorans* VC9159**	no	no	no	no	respiratory
***B.* sp VC14128**	no	no	no	no	respiratory
***B. stabilis* VC6482**	no	no	yes	yes	sputum
***B. stabilis* VC9042**	no	no	no	no	respiratory
***B. vietnamiensis* VC10362**	no	no	no	no	respiratory
***B. vietnamiensis* VC11253**	no	no	no	no	sputum
***B. vietnamiensis* VC11275**	no	no	no	yes	respiratory
***B. vietnamiensis* VC12002**	no	no	no	no	respiratory
***B. vietnamiensis* VC15208**	no	no	no	no	sputum
***B. vietnamiensis* VC15774**	no	no	no	yes	sputum
***B. vietnamiensis* VC16431**	no	no	no	no	respiratory
***B. vietnamiensis* VC18210**	no	no	no	no	respiratory
***B. vietnamiensis* VC2824**	no	no	yes	yes	sputum
***B. vietnamiensis* VC9237**	yes	no	yes	yes	respiratory
***B. vietnamiensis* VC9752**	no	no	no	no	respiratory

Table 4.2 Correlation between the presence of the *hmqABCDEFG* operon and the production of HMAQs in clinical Bcc, along with sampling data

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

Raw data from Table S8. P-values are representative of the correlation (tested by Fisher's exact test for count data) and are considered significant under 0.05. *Possible origins: sputum, respiratory, throat, or sinus

4.4.6. Lack of expression of the *hmqABCDEFG* operon explains the absence of HMAQ detection in some Bcc strains cultures

Our screening revealed that 26 strains carrying the *hmqABCDEFG* operon do not produce HMAQs in TSB at 30°C. To investigate the possibility that too low transcription of the biosynthetic genes would explain this absence of detectable production, which is compatible with the induction seen when changing the culture conditions, we measured the transcription of the *hmqABCDEFG* operon for one HMAQ-negative and one HMAQ-positive strain per species of *B. ambifaria, B. cepacia, B. contaminans* and *B. vietnamiensis* by targeting the *hmqA* gene by RT-PCR.

Results show that *hmqABCDEFG*-positive but HMAQ-negative strains *B. ambifaria* AMMD*, B. cepacia* ATCC25416, *B. contaminans* VC15406, and *B. vietnamiensis* VC9237 do not express the *hmqA* gene when grown in TSB at 30°C, 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 (**Fig. S4.4**). Thus, we decided to investigate if a mutation in the promotor region could explain the lack of expression of the *hmqABCDEFG* operon, and we showed no difference between *B. ambifaria* HSJ1 and AMMD promotor sequences (**Figure S4.5**). The lack of expression of *hmqABCDEFG* is a matter of regulation especially since strain AMMD produced HMAQs in ASM and TSA. 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 under the specified culture conditions. This is supported by the finding that several of these strains could eventually produce measurable levels of HMAQs when changing culture conditions (as presented above, **Table 4.1**).

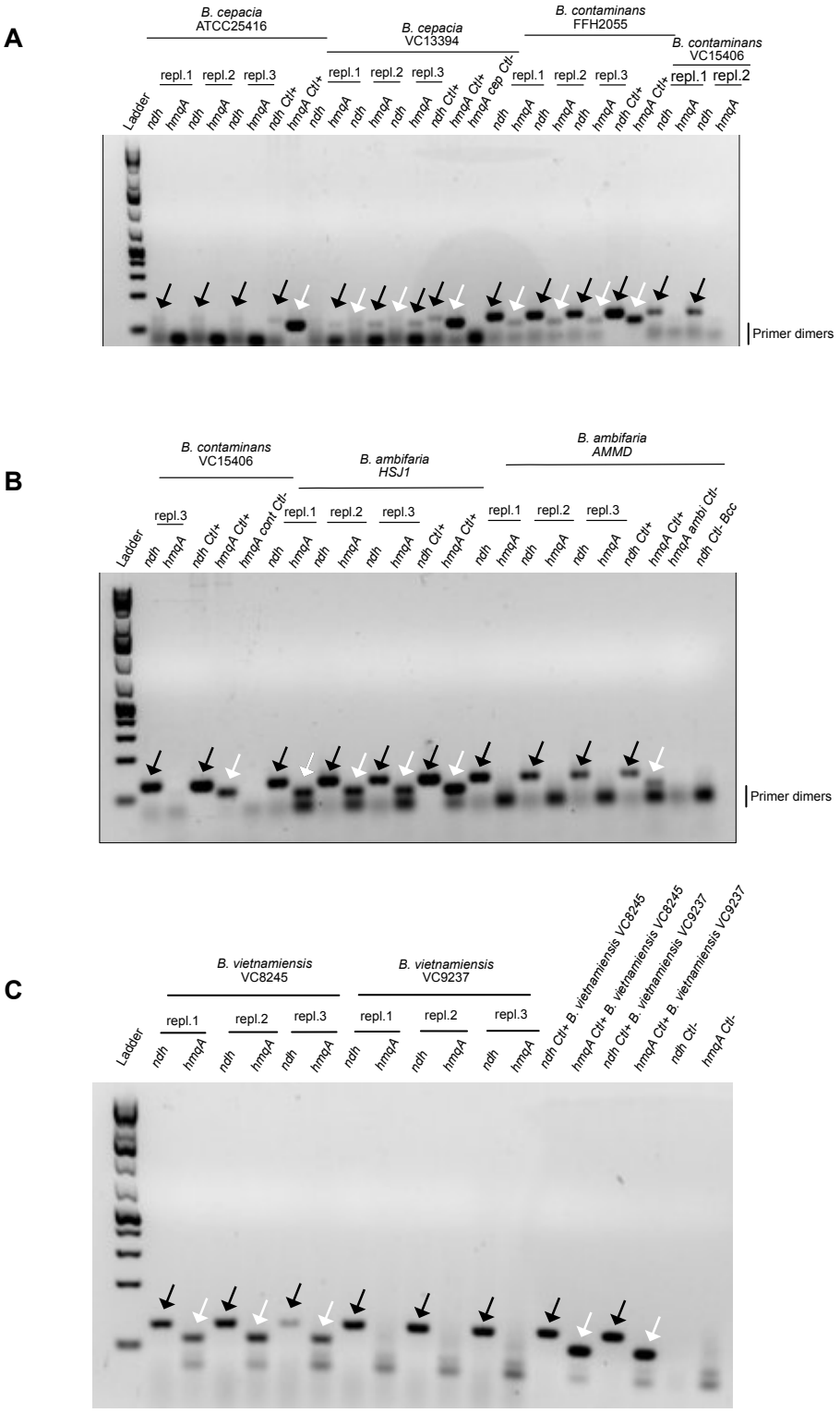

Figure S 4.4 Expression of the hmqA gene in strains the main species of Bcc having the hmqABCDEFG operon but for which HMAQ production was not detected.

A) RT-PCR on *B. cepacia* and *B. contaminans* strains. B) RT-PCR *on B. contaminans* and *B. ambifaria* strains. C) RT-PCR on *B. vietnamiensis* strains. Black and white arrows indicate ndh and *hmqA* amplicons respectively.

```
Consensus        TCTGTCATCCCGACTAGCTGGTGATGGAAACCGCGAACTTCAGACGAGTCGCCTTCGATT    60
phmq_Bambi_AMMD+ ............................................................    60
Phmq_HSJ1        ............................................................    60

Consensus        GCCCGCTTGCCGCGCGGGCTCGATGCGCTCCTGCGTTGGTTGACGGCGCTGATTCAGTGA   120
phmq_Bambi_AMMD+ ............................................................   120
Phmq_HSJ1        ............................................................   120

Consensus        ACGCAGCGTATCAATCCAATTACATCGGTCCCGATACATTTTCTGTGCGCTTTTCCATAC   180
phmq_Bambi_AMMD+ ............................................................   180
Phmq_HSJ1        ............................................................   180

Consensus        CTTTGGTAATGCATCGAAAACGTGCGGCAAAGCGGGAATGGCGCGGAATGGTGCGAACGA   240
phmq_Bambi_AMMD+ ............................................................   240
Phmq_HSJ1        ............................................................   240

Consensus        CGCGAAAACGCAATGCCTGCGCGGATGTGCGGGATGGCGCGGTGACTGAGGCACGGTGGG   300
phmq_Bambi_AMMD+ ............................................................   300
Phmq_HSJ1        ............................................................   300

Consensus        GCGTGCGGTGCGCGGGATGGTGCGGTAGGGGAAAATCCTGACGGGGGGTGCGTAGGGGAA   360
phmq_Bambi_AMMD+ ............................................................   360
Phmq_HSJ1        ............................................................   360

Consensus        ACTACTGATAACGGCGGCGCGCGACGCGGGCGGCGCGGCAGATGGCTGATGGCTGATGGC   420
phmq_Bambi_AMMD+ ............................................................   420
Phmq_HSJ1        ............................................................   420

Consensus        TGATGGCTGATGGCTGATGATGATCGCGGCGGCCCGTGCGCCACGCGATCACGCGCGGCA   480
phmq_Bambi_AMMD+ ............................................................   480
Phmq_HSJ1        ............................................................   480

Consensus        GATCTTCGCGATGTTCAAGCGGGCGTTGCCAGAAGATTTATCGGTGCTTGACAGCCAGGA   540
phmq_Bambi_AMMD+ ............................................................   540
Phmq_HSJ1        ............................................................   540

Consensus        TGTGCTCGAATCCTCGAACCGAGACATTCCGATTTCTCACTTAAAATAAAATATTCGTTA   600
phmq_Bambi_AMMD+ ............................................................   600
Phmq_HSJ1        ............................................................   600

Consensus        ATACAATTACGCAAATCCATATGGGCGCAATCCGAGACCCCGGCGCCCGAACCCGTGATT   660
phmq_Bambi_AMMD+ ............................................................   660
Phmq_HSJ1        ............................................................   660

Consensus        TTCCATCCAGATCGACTCAAGCCCCGCTGGTCATGAGAGCGCGGGTCTTTCGCCTTTCGC   720
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phmq_Bambi_AMMD+ ..........................................................  720
Phmq_HSJ1        ..........................................................  720

Consensus        GCGGAGCAATCTACTTCGTGACGCGATGCTCGTTTCGCCTTGATGAACTTCCCGACGCTT  780
phmq_Bambi_AMMD+ ..........................................................  780
Phmq_HSJ1        ..........................................................  780

Consensus        TATTCAGTGCTCCGACAACCAGCTCCACCGAAGTCAACAAATCGCAAGATGGTTAACCGA  840
phmq_Bambi_AMMD+ ..........................................................  840
Phmq_HSJ1        ..........................................................  840

Consensus        AATCGATTAATTCAATAAAACGTCGGCGCTTAATGGAATCGATTTCAACAAATAATTCCA  900
phmq_Bambi_AMMD+ ..........................................................  900
Phmq_HSJ1        ..........................................................  900

Consensus        ATCACCCCCTCATATGACCGTTTACTTTCCAAGTTTACTTCCGCTAAGCTTACGGCACAC  960
phmq_Bambi_AMMD+ ..........................................................  960
Phmq_HSJ1        ..........................................................  960

Consensus        TTTCTAAACCAACCGAATCGATCGACGCATTTCCAACTTCAAGCAATCCGCACTAAATAG 1020
phmq_Bambi_AMMD+ .......................................................... 1020
Phmq_HSJ1        .......................................................... 1020

Consensus        CTCGATTATTGTTTATTTTTAAAATCAAATGAGACGGAATCAATTCATAAACACCGGCAA 1080
phmq_Bambi_AMMD+ .......................................................... 1080
Phmq_HSJ1        .......................................................... 1080

Consensus        GCCATCATGTGATTGACCGGGCTCTCCGTGCGTCGGCCGTCGACATGTGCCTGCCGCCCC 1140
phmq_Bambi_AMMD+ .......................................................... 1140
Phmq_HSJ1        .......................................................... 1140

Consensus        GACGCCGTCTCTTTGGCATCGCGATTCAGGTCGGGCGGGCCGGGCCCGATCATCTGGCCC 1200
phmq_Bambi_AMMD+ .......................................................... 1200
Phmq_HSJ1        .......................................................... 1200

Consensus        GGCCCGCGCCGCACCGCTATCGCGAACTTCAAACTCACCCTCTTGCGAGCGCTGACG    1257
phmq_Bambi_AMMD+ ..........................................................    1257
Phmq_HSJ1        ..........................................................    1257
```

Figure S 4.5 Alignment of the hmqABCDEFG operon promoter regions of B. ambifaria HSJ1 and B. ambifaria AMMD

Sequences were aligned using Clustal Omega.

4.4.7. The capacity to produce HMAQs is required for root growth promotion

Since *B. ambifaria* is a good plant-growth promoter ([15, 16]; reviewed by Vial *et al*. 2011 [19]), and the Hmq system is more prevalent among environmental strains, we investigated the impact of the Hmq system on growth of the common pea (*P. sativum*). While *B. ambifaria* HSJ1 promoted *P. sativum* roots' development, the isogenic *hmqA*::pKnock-Cm and *hmqG*::pKnock-Cm mutants lost this effect **(Figure 4.5).**

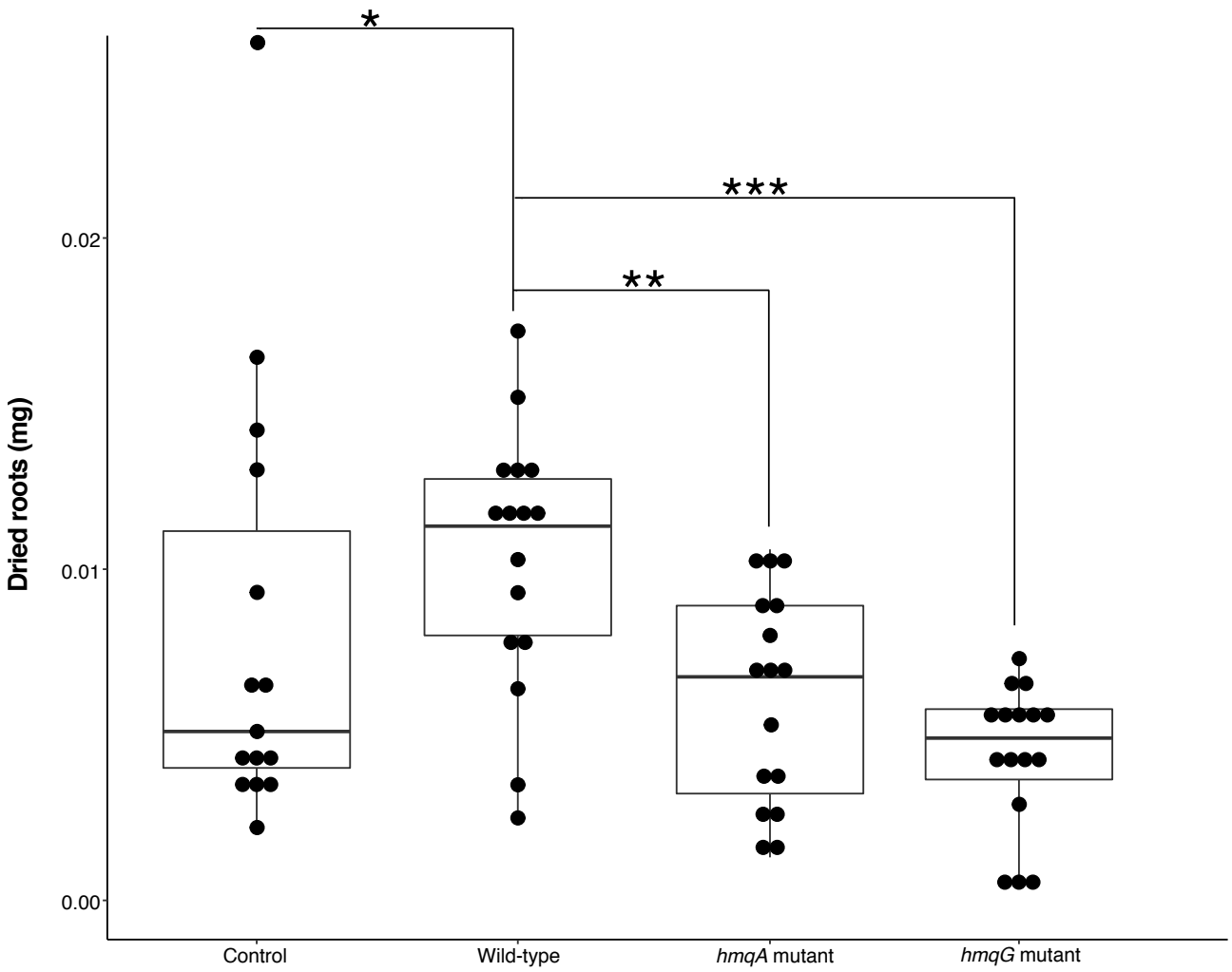

Figure 4.5 Presence of the intact *hmqABCDEFG* operon in *B. ambifaria* promotes the development of roots in a *P. sativum* plant model

The impact of *hmqA*- and *hmqG*- mutants of *B. ambifaria* HSJ1 were measured on root development of *P. sativum* compared to the wild-type strain. Weight of dried roots was measured after 5 days of cultivation. A Dunn test was performed, and *p*-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, and *** 0.001 and 0.0001.

4.5. Discussion

This study aimed at understanding the prevalence of the Hmq system and corresponding HMAQ production among the various species belonging to the Bcc to experimentally validate and extend our previous *in silico* analyses (55). Besides non-Bcc species *B. pseudomallei* and *B. thailandensis*, only a few strains of *B. ambifaria* and *B. cepacia* were already known to carry the *hmqABCDEFG* operon and to produce HMAQs (16, 36, 38–44). Indeed, the presence of the *hmqABCDEFG* operon is well-conserved in *B. pseudomallei* and *B. thailandensis* but remains unclear within the Bcc (55). To understand the ecological role of the Hmq system, we first needed to evaluate its distribution and prevalence. Since available genomes are not equally distributed between Bcc species, we screened a panel of 312 Bcc strains for the presence of the *hmqABCDEFG* operon by PCR. Since the *hmqG* gene is only detectable when the complete *hmqABCDEFG* operon is present and that no significant variation of the *hmqABCDEFG* operon was observed in the 447 positive genomes analyzed by bioinformatic (55), we considered the operon to be present when both *hmqA* and *hmqG* genes were amplified. While it cannot be excluded that that some strains carry a variation of the *hmqABCDEFG* operon, we did not detect any in our previous bioinformatic analysis comparing whole genomes (55). Prevalence of the *hmqABCDEFG* operon found here for the Bcc followed the same distribution than when analyzed *in silico*. Our PCR-based screening could miss some strains carrying the operon due to the limited availability of whole-genome sequences for some Bcc species (e.g., *B. arboris, B. metallica, B. stabilis*). Nevertheless, our primers could amplify *hmqA* and *hmqG* targets in species previously unknown to carry a *hmqABCDEFG* operon (e.g., *B. vietnamiensis*), which was expected due to the high level of identity (>88%) of *hmqA* and *hmqG* among identified and sequenced Bcc species (55). Furthermore, testing the production of HMAQs in a group of 31 PCR-negative strains confirmed the absence of production of these metabolites. Taken together, we are confident that we identified the strains carrying the genetic ability to synthesize HMAQs.

Based on our previous results obtained with a few *B. ambifaria* isolates, we hypothesized that the Hmq system would be more prevalent among clinical isolates and produce more HMAQs than environmental ones (36, 43, 46). Unexpectedly, we uncovered that *B. cenocepacia*, *B. multivorans,* and *B. vietnamiensis* - the prominent Bcc species colonizing immunosuppressed and CF individuals, transmitted between patients (22, 23, 58), do not or rarely (0 out of 72 *B. cenocepacia*, 0 out of 35 *B. multivorans* and 5 out of 35 *B. vietnamiensis*) carry the *hmqABCDEFG* operon. In contrast, 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 (19, 59–62). The presence of the Hmq system in clinical strains of these five common environmental species is

compatible with a recent or direct environmental acquisition in CF patients due to the horizontal transfer of the *hmqABCDEFG* operon among Bcc species (60, 63, 64).

Among the strains carrying the *hmqABCDEFG* operon, not all produced HMAQs under the tested conditions. Since the limit of detection for these molecules was 50 µg/L, we cannot exclude that some of these strains actually produced lower levels. For those strains producing HMAQs in TSB, growth in ASM did not inhibit HMAQs production and stimulated a detectable production in ten additional strains, which could be explained by differences in regulation of the biosynthetic operon. Further, an increase in the number of HMAQ-positive strains when testing a third culture condition (4 days on TSA plates) highlights the need to use nutritionally appropriate media when investigating the production of secondary metabolites (53). For this purpose, we tried to optimize a medium for HMAQs production. However, it was impossible to identify a single appropriate carbon source due to the low production of the molecules in the minimal medium, whatever the tested culture duration (data not shown). Nevertheless, our results show that the production of HMAQs in the Bcc is dependent on the conditions of growth and that *hmqABCDEFG*-positive strains not producing detectable HMAQs under our tested conditions could produce enough HMAQs in different ones.

Lack of transcription of the *hmqABCDEFG* operon seemed to explain that 28% of Bcc strains carrying the genes did not produce HMAQs when grown in TSB at 30°C. Indeed, production could be detected simply by changing the culture conditions, suggesting that the promotor of the *hmqABCDEFG* was not expressed, although we cannot exclude that it could be nonfunctional in some strains. We thus believe the same explanation (poor *hmqABCDEFG* transcription) could apply to the remain 13% strains for which we could not yet identify appropriate culture conditions. Future work will involve a better understanding of the nutritional and regulatory elements controlling the expression of the *hmqABCDEFG* operon and production of HMAQs.

Because the *hmqABCDEFG* operon is more prevalent in environmental strains, which are often isolated from the rhizosphere, we investigated the impact of the Hmq system on the growth promotion ability of a *B. ambifaria* strain of a model plant, *P. sativum*. The inactivation of *hmqA* or *hmqG* prevented root growth promotion, presumably because of the inability to produce HMAQs. As it is the case for cepacin or pyrrolnitrin, HMAQs could influence the rhizosphere microbial community, for instance, due to their antimicrobial activity (16, 36, 39–41, 45, 46, 50, 54, 65) or else. As we have previously reported that the HMAQs influence the Cep regulatory system, the molecules could indirectly impact the production of other plant beneficial metabolites and functions regulated by quorum sensing (e.g. enacyloxin, pyrrolnitrin, cepacin, AFC lipopeptide) (16, 30, 36, 39–41, 45, 46, 50, 54, 65–67). Further experiments with other HMAQ producers and pure HMAQs will provide exciting insights.

4.6. Material and Method

4.6.1. Strains and culture conditions

A total of 312 strains isolated from either clinical or environmental settings were used in this study and listed in **Table S4.1**). Uncertain identification was confirmed by amplifying and sequencing the *recA* and *gyrB* genes at the IRCM Sequencing platform (Montreal) following the directions on the PubMLST database (https://pubmlst.org/bcc/info/protocol.shtml; **Table S4.9**).

Strains were cultured in borosilicate tubes containing 3 mL tryptic soy broth (TSB; Difco) from stocks frozen at -80°C in 15% glycerol and incubated at 30°C with 57 rpm rotative shaking overnight (~16h).

Table S 4.9 Primers used in this study

Name	Sequence	Function	Reference
hisA_Bcc_F	GGTCGACCTGAACGGCGC	Reference gene for PCR screening	Papaleo et *al.*, 2011
hisA_Bcc_R	CGTCGGTCGCGACCTTGCC		Papaleo et *al.*, 2011
hmqA_Bcc_F	CCGCTCGCGTT**Y**ACGTT**Y**GG	Amplification of *hmqA* gene in Bcc - degenerated	This study
hmqA_Bcc_R	CCCGTCAGGTTCCAGCCG		This study
hmqG_Bcc_F	GGCGTCGCAGGAAATCACG	Amplification of *hmqG* gene in Bcc - degenerated	This study
hmqG_Bcc_R	CGCGACACGAA**R**TGCATGCC		This study
recA_Bcc_F	GATAGCAAGAAGGGCTCC	Identification of Bcc	Pubmlst
recA_Bcc_R	CTCTTCTTCGTCCATCGCCTC		Pubmlst
gyrB_Bcc_F	CGACAACTCGATCGACGA		Pubmlst
gyrB_Bcc_R	GACAGCAGCTTGTCGTAG		Pubmlst
recA_Bcc_seq_F	TGACCGCCGAGAAGAGCAA	Sanger sequencing	Pubmlst
recA_Bcc_seqR	GACCGAGTCGATGACGAT		Pubmlst
gyrB_Bcc_seq_F	ATCGTGATGACCGAGCTG		Pubmlst
gyrB_Bcc_seqR	CGTTGTAGCTGTCGTTCC		Pubmlst
ndh_deb_F	GCTCGGCTACGACGATCT	Reference gene for RT-PCR	This study
ndh_ deb_R	GGCCTGGTCGAGGTTTTC		This study
hmqA _RTPCR_F	CTTGCCCCCTGCCGAAGATT	Expression of *hmqA* by RT-PCR	This study
hmqA _RTPCR_R	CGGCGCAATTGAGAAACGG		This study

4.6.2. Detection of the presence of *hmqABCDEFG* operon by PCR

Genomic DNA was extracted following a previously described method (68). Briefly, cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 5 mM EDTA-2Na pH 8, 3% SDS) and transferred to a tube containing glass beads. The cells were lysed in a Fast-Prep-24 instrument (MP Biomedicals). Then the lysates were centrifuged at 8,000 x *g* for 5 min, the supernatants were transferred to a new tube, and 2.5 N ammonium acetate was added. The supernatant was transferred to a new tube, and one volume of isopropanol was added. The pellets were washed with 75% ethanol and dried before resuspension into 50 µL water.

For the strains acquired from the *Burkholderia cepacia* Research Laboratory and Repository and from the Canadian *Burkholderia cepacia* complex Research and Referral Repository, genomic DNA was extracted using a 96-well 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 S4.9**). A strain was considered to have a complete *hmqABCDEFG* operon when amplification for both *hmqA* and *hmqG* targets was obtained, based on our previous analyses (55).

4.6.3. Detection of the presence of the pc3 chromosome and the *hmqABCDEFG* operon in Bcc strains

Assembled genomes are available on DB Burkholderia (http://www.Burkholderia.com). We have selected only complete assembled genomes and searched for the presence of the *hmqABCDEFG* operon by orthology and homology directly on DB Burkholderia. Results are listed in **Table S4.3**.

4.6.4. Phylogeny of the Bcc based on MLST and *hmqABCDEFG* sequences

First, *atpD, gltB, gyrB, recA, lepA, phaC,* and *trpB* gene sequences were used to generate a Bcc phylogeny based on MSLT. Second, the *hmqABCDEFG* operon sequence was used to determine the co-evolution or horizontal transfer of the Hmq system within Bcc species. Sequences were found on DB Burkholderia (http://www.Burkholderia.com) and concatenated in the following order: *atpD, gyrB, recA, gltB, lepA, phaC* and *trpB;* and *hmqABCDEFG* in parallel. The resulting concatenated sequences were aligned using Clustal Omega (69), and trees were generated by RAxML using the GTRGAMMA model and 1000 bootstraps. The branches are labelled where bootstrap values are >50.

4.6.5. Quantification of HMAQ production by LC-MS/MS

Bcc strains were cultured in 5 mL tryptic soy broth (TSB) at a starting OD_{600} of 0.05 and incubated at 30°C with shaking for an overnight. 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4)

was used as an internal standard (70). The total HMAQs were extracted from 4 mL culture with one volume of ethyl acetate. After nitrogen evaporation, the residues were dissolved in 400 µL 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 (70). 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 (36). This experiment was conducted with three independent biological replicates.

4.6.6. Artificial Sputum medium (ASM), Tryptic soy Broth (TSB), and Tryptic soy Agar (TSA) medium assays

The strains were grown in ASM liquid medium (71) and TSA agar plates out from overnight cultures and incubated at 30°C for 24h and four days, respectively, or in TSB and incubated at 37°C for 24h.

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

The experiments were performed in two independent biological replicates.

4.6.7. Detection of the expression of the *hmqABCDEFG* operon by RT-PCR

Total RNA was extracted from cultures grown in TSB to an OD_{600} of 3.0 using TransZol (Transgene, Canada) by following the manufacturer's instructions. Residual DNA was removed using the Turbo DNAse (Thermo Fisher, Canada). Reverse-transcription was performed using the iScript kit (BioRad, Canada). The expression of *hmqABCDEFG* operon was determined by PCR targeting the *hmqA.* The *ndh* gene served as a reference gene (**Table S4.9**; [72]).

4.6.8. Correlation between the presence of the *hmqABCDEFG* operon and the production of HMAQs in Bcc with different characteristics

Based on our qualitative data (**Tables 4.2 and S4.8**), we studied the correlation by Fisher's exact test for count data using R software (http://www.R-project.org ; [73]).

4.6.9. *Pisum sativum* growth promotion

Pisum sativum seeds were decontaminated using successive bleach and 70% ethanol treatments (10 min each, under shaking [57 rpm], 3 times) and then germinated for four days at room

temperature on 0.8% agar plates. *B. ambifaria* HSJ1 and isogenic *hmqA::*pknock-Cm, and *hmqG::*pknock-Cm mutants were incubated at 30°C overnight in TSB with shaking at 57 rpm. Seeds were exposed by adding 10^5 bacteria/mL in Murashige and Skoog (MS) basal medium (Sigma; [(43)]). Plants were grown at room temperature for five days. Roots were then cut, dried at 52°C overnight, and weighted. A Dunn test was used for statistical analyses. This experience was repeated twice, and the same conclusions.

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5. SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF BURKHOLDERIA-RELATED 4-HYDROXY-3-METHYL-2-ALKENYLQUINOLINES (HMQAS) AND THEIR N-OXIDE COUNTERPARTS

Synthèse et activité antimicrobienne des 4-Hydroxy-3-méthyl-2-alkenylquinolines (HMAQs) et des composés *N*-oxides produits par *Burkholderia*

Auteurs :

Marianne Piochon,† Pauline M. L. Coulon,† Armand Caulet,† Marie-Christine Groleau,† Eric Déziel,*,† et Charles Gauthier*,†

†Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique (INRS), 531, boul. des Prairies, Laval (Québec), Canada, H7V 1B7

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Lien entre l'article ou les articles précédents et le suivant :

Les deux articles précédents présentent la prévalence de l'opéron *hmqABCDEFG* chez les Bcc en ayant réalisé une étude par bioinformatique et une étude expérimentale. Les espèces de Bcc *B. cenocepacia, B. multivorans* et *B. vietnamensis* (prédominantes chez les patients immunodéprimés et les personnes atteintes de fibrose kystique) ne portent pas ou très rarement l'opéron *hmqABCDEFG* au sein de leur génome, contrairement aux espèces majoritairement environnementales *B. ambifaria*, *B. cepacia*, *B. contaminans*, *B. pyrrocinia,* et *B. ubonensis* – invalidant notre hypothèse de départ. Ce qui est intéressant c'est que la majorité des souches ayant le système Hmq produisent des HMAQs qui sont tous deux impliqués dans la promotion des plantes qui est connue pour être une propriété biologique de certaines souches de Bcc. La fonction des HMAQs reste encore inconnue, cependant par leur analogie avec le HHQ et PQS de *P. aeruginosa* dont les propriétés sont très étudiées, les HMAQs pouvaient avoir les mêmes caractéristiques. L'équipe de recherche du Professeur Charles Gauthier a réussi à synthétiser les HMAQs me permettant d'étudier l'effet antimicrobien des HMAQs et de leurs congénères NOHMAQs – faisant l'objet de cet article.

5.1. Abstract

The *Burkholderia* genus offers a promising potential in medicine because of the diversity of biologically active natural products encoded in its genome. Some pathogenic *Burkholderia* spp. biosynthesize a specific class of antimicrobial 2-alkyl-4(1*H*)-quinolones, *i.e.*, 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs) and their *N*-oxide derivatives (HMAQNOs). Herein, we report the synthesis of a series of six HMAQs and HMAQNOs featuring a *trans*-^{2} double bond at the C2-alkyl chain. The quinolone scaffold was obtained *via* the Conrad-Limpach approach while the (*E*)-2-alkenyl chain was inserted through Suzuki-Miyaura cross-coupling under microwave radiation without noticeable isomerization according to the optimized conditions. Subsequent oxidation of enolate-protected HMAQs cleanly led to the formation of HMAQNOs following cleavage of the ethyl carbonate group. Synthetic HMAQs and HMAQNOs were evaluated *in vitro* for their antimicrobial activity against different Gram-negative and Gram-positive bacteria as well as against molds and yeasts. The biological results support and extend the potential of HMAQs and HMAQNOs as antimicrobials, especially against Gram-positive bacteria. We also confirm the involvement of HMAQs in the autoregulation of the Hmq system in *Burkholderia ambifaria*.

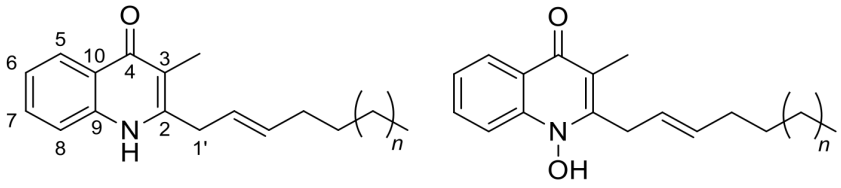
5.2. Introduction

The *Burkholderia* genus includes a vast group of Gram-negative bacteria found in diverse ecological niches [1, 2]. Some *Burkholderia* spp. are of serious pathogenic concerns, such as the Centers for Disease Control and Prevention (CDC) Tier 1 select agents [3, 4], *Burkholderia pseudomallei* and *B. mallei* – the infectious agents of melioidosis [5, 6] and glanders [7] respectively – and the devastating plant crop pathogens *B. glumae* and *B. gladioli*, which cause major yield losses in rice productions [8, 9]. Others, like those forming the *B. cepacia* complex, include species that can both live in beneficial associations with their eukaryotic hosts (mammals, plants, and fungi; [2]) and cause several hard-to-treat opportunistic infections, such as the cepacian syndrome in individuals suffering from cystic fibrosis [10].

Burkholderia spp. offer a tantalizing potential in medicine because of their capacity to produce highly potent and structurally diverse metabolites [11, 12]. A plethora of natural products exhibiting various biological functions have been identified from *Burkholderia* spp [11]. To name a few examples for *B. pseudomallei*, cytotoxic siderophores (*e.g.*, malleilactone; [13]), proteasome inhibitors (*e.g.*, deoxyglidobactin C; [14]), tensioactive lipopeptides (*e.g.*, malleipeptin A; [14]), and rhamnolipids [15]) as well as quorum sensing signal and modulators such as *N*-acyl homoserine lactones (AHLs; [16]) and 2-alkyl-4(1*H*)-quinolones (AQs; [17, 18]) have been reported.

Burkholderia spp. biosynthesize a specific class of AQs, namely 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQ, **1**–**3**) or 2-alkenyl-3-methylquinolin-4(1*H*)-ones according to IUPAC, which feature a methyl group at C3 and a *trans*-^{2} unsaturation at the C2-alkyl chain (Figure 5.1).[18-28]

HMAQs **1** and **3**, respectively the heptenyl and nonenyl congeners, exhibit potent antifungal [20, 21, 23, 25, 26] and antibacterial [20, 28] activities against diverse microorganisms as well as plant growth promoting activities [23]. Furthermore, HMAQs **1** and **3** act as potent modulators of quorum sensing in *B. ambifaria* HSJ1 by decreasing the production of AHLs[18]. HMAQ **2** (burkholone), the 2-octenyl congener, is cytotoxic against a murine hemopoietic cell line devoid of the insulin receptor substrate 1 [29]. HMAQs are also found in the form of their *N*-oxide counterparts (HMAQNOs **4**–**6**, **Figure 5.1**; [30, 31]). HMAQNO **6** (YM-30059), identified from both *Burkholderia* and *Arthrobacter* spp., is a potent antibiotic agent against multi-drug resistant *Staphylococcus aureus*, *S. epidermidis*, and *Bacillus subtilis* strains [32]. Interestingly, 2-heptyl-4(1*H*)-quinolone *N*-oxide (HQNO) – the unmethylated and saturated analogue of HMAQNO **4** – is able to act synergistically with HMAQ **3** enabling inhibition of bacterial growth while showing divergent biological functions when tested alone [33]. Taken together, these studies show that subtle structural modifications can profoundly impact the biological activities of AQs.



1 *n* = 1
2 *n* = 2 (**burkholone**)
3 *n* = 3

4 *n* = 1
5 *n* = 2
6 *n* = 3

quinolone	concentration (mgL^{-1})		
	Ba[a]	***Bt***[b]	***Bp***[c]
1 (AMQ)	58.80	0.79	0.10
2 (AMQ)	1.50	nd	0.04
3 (AMQ)	27.73	17.59	1.87
4 (AMQNO)	nd[d]	nd	0.02
5 (AMQNO)	nd	0.22	0.07
6 (AMQNO)	nd	4.29	0.68

[a]*B. ambifaria* HSJ1. [b]*B. thailandensis* E264. [c]*B. pseudomallei* 1026b. [d]Not detected.

Figure 5.1 Chemical structures of *Burkholderia*-related HMAQs 1–3 and HMAQNOs 4–6. The keto – rather than the enol – form of the quinolone ring is arbitrarily drawn throughout the paper.

Although an obvious evolutionary advantage for bacteria, the large number of closely related AQ congeners (>29; [18]) of similar polarities can complicate the purification process from bacterial cultures. Highly potent AQs could be found as minor contaminants within samples of other less potent AQs isolated from bacterial extracts, generating experimental bias regarding the biological activity of single compounds. Chemical synthesis has a long-standing record of success regarding the preparation of pure and homogeneous natural products[34-36]. Well-designed synthetic pathways can allow the preparation of natural products and analogues in sufficient amounts for enabling the confirmation of structural identities and biological activities as well as for sustaining drug discovery and pharmaceutical development.[36, 37] Within this framework, we herein report the synthesis, antifungal, and antibacterial activities of HMAQs **1**–**3** and HMAQNOs **4**–**6** identified from *Burkholderia* spp. We also confirm their involvement in the autoregulation of the Hmq system, which is responsible for synthesizing HMAQs in *B. ambifaria* [38, 39].

5.3. Results and Discussion

5.3.1. Retrosynthetic analysis

Two important challenges had to be addressed for the total synthesis of HMAQs [40, 41], *i.e.*, the formation of the 2,3-dialkylatedquinolin-4(1*H*)-one core along with the presence of an unconjugated *trans*-^{2} unsaturation at the 2-alkyl chain. Synthetic routes in which the 2-alkenyl chain would be attached to the molecule prior to the acid- or base-catalyzed cyclization for the formation of the quinolone core [42] would possibly result in failure because of the thermodynamically favourable isomerization of the double bond occurring from the *trans*-^{2} to the *trans*-^{1} position.

As depicted in our retrosynthetic strategy (**Figure 5.2**), we envisioned to study two alternative approaches to construct the quinolone scaffold. In both synthetic routes, the (*E*)-2-alkenyl chain would be attached to the quinolone core at the final step *via* either a Wittig reaction or a Suzuki-Miyaura cross-coupling, theoretically avoiding the risk of double bond isomerization. For the first route, aldehyde **I** would serve as an advanced intermediate for the Wittig reaction. It would come from the reduction of corresponding ester **III**, which would be generated *via* the PTSA-catalyzed reaction of 2-(4,4-dimethyl-1,3-oxazolin-2-yl)phenylamine with ethyl 3-oxopentanoate. As the opposite regioisomer could also be formed during the formation of the quinolone core **III**, *i.e.*, the alkyl chain at C2 rather than at C3,[43] a second route was planned in which HMAQ scaffold **IV** would be obtained *via* the Conrad-Limpach approach. Functionalization of derivative **IV** into 2-methylchloride **II** followed by Suzuki-Miyaura cross-coupling with boronic ester alkenyl chains would provide HMAQs. Finally, regioselective oxidation of HMAQs would yield HMAQNOs.

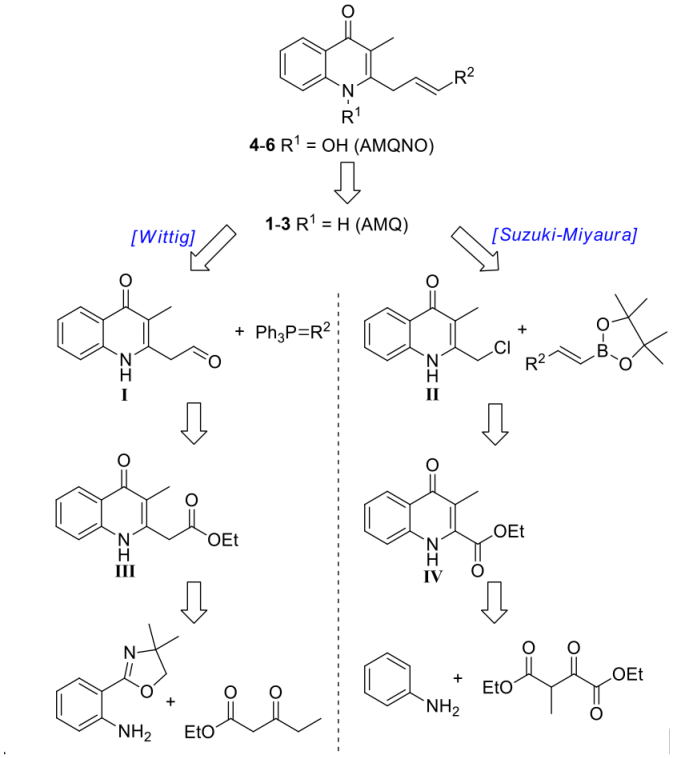


Figure 5.2 **Retrosynthetic analysis of HMAQs 1–3 and HMAQNOs 4–6**

5.3.2. Synthesis of HMAQs

The synthesis of quinolone scaffold **S6** *via* the Luo, Ravi and Xue [43] procedure was first attempted (see **Figure S5.1** in the supplementary material section 5.6). Oxazolinylphenylamine **S3** was synthesized beforehand through zinc(II) chloride-promoted condensation of isatoic anhydride (**S1**) with 2-amino-2-methylpropanol (**S2** ; [44]). Then, oxazoline **S3** was subjected to acid-catalyzed condensation-cyclization with ethyl 3-oxopentanoate (**S4**) in refluxing *n*-butanol,[43] which indeed led to a quinolone derivative. However, NMR analyses proved that the opposite regioisomer **S5**[45] was exclusively formed instead of target derivative **S6**. This result was rationalized by the formation of an imine, which, once tautomerized into the most stable enamine intermediate, would favoured 6-exo-trig cyclization [43]. In our case, as regioisomer **S5** was formed, the most stable enamine would come from proton transfer tautomerization at the C2 rather than at the C4 position of the pentanoate chain.

We then turned our attention to the Conrad-Limpach approach [46-48]. This route was straightforward and rather high-yielding for the formation of the quinolone scaffold (**Figure 5.3**). Aniline (**7**) was condensed with diethyl 2-methyl-3-oxosuccinate (**8**) in the presence of acetic acid to furnish diester **9**, which underwent polyphosphoric acid (PPA)-promoted intramolecular Friedel-Crafts acylation.[49] Resulting quinolone ester **10** was then reduced under the action of $LiAlH_4$ and the primary alcohol

chlorinated with $SOCl_2$. Using this synthetic route 5 g of chloride **11** in convenient yields and purity (as indicated by NMR) was prepared. It is noteworthy that partial insolubility of most of the intermediates in common organic solvents rendered the work-ups and purification procedures of this synthetic route challenging (see experimental procedure for details).

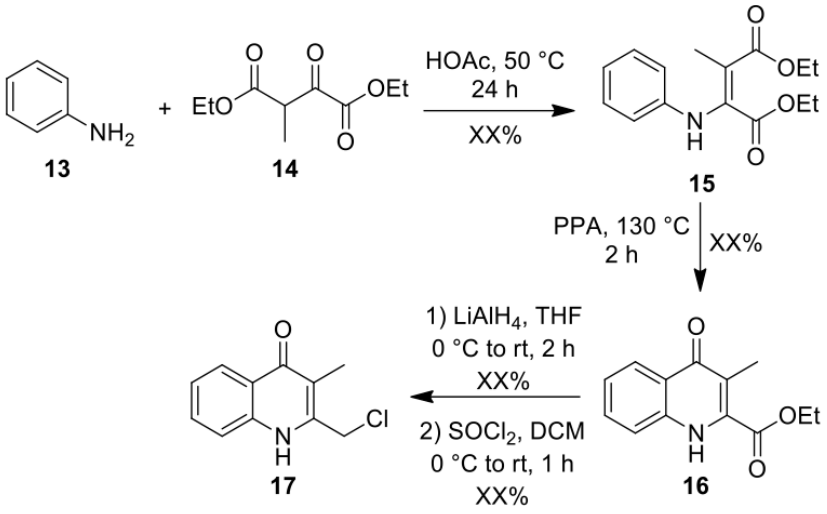
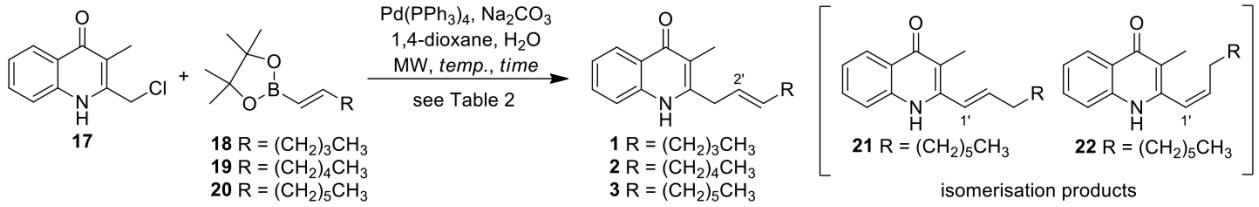


Figure 5.3 Synthesis of 2-(Chloromethyl)-quinolone 11 *via* Conrad-Limpach Approach

With large amounts of 2-methylchloridequinolone **11** in hand, we next investigated the synthesis of the target HMAQs **1**–**3** *via* Suzuki-Miyaura cross-coupling [48, 50]. Commercially available *trans*-alkenylboronic acid pinacol esters **12**–**14** were reacted with chloride **11** under the catalytic action of tetrakis(triphenylphosphine)palladium(0) using sodium carbonate as a base in a microwave-heated 1,4-dioxane/water solution [48]. As depicted in Table 5.1, although coupling yields were better in microwave-heated than in classic refluxing conditions (entry 1), isomerization occurred when prolonged reaction times were employed (entries 2 and 3). As revealed by ^{1}H NMR, the thermodynamically stable *trans*-[1] (**15**) and *cis*-[1] (**16**) derivatives were formed through isomerization of *trans*-[2] HMAQ **1**. Pleasingly, we found that decreasing the reaction time and temperature to one minute and 70 °C, respectively, avoided isomerization while still allowing full conversion of the starting material (entries 4 to 6). Analytical (HRMS) and spectral (^{1}H and ^{13}C NMR, Tables 2 and 3) data of HMAQ **1** [19-23, 25, 26, 28], HMAQ **2** (burkholone; [29]) and HMAQ **3** [20, 21, 27, 28] were in agreement with those published for the isolated natural products.

Table 5.1 Synthesis of Target 2-Alkenylquinolones *via* Suzuki-Miyaura Reaction



entry	Bpin alkene	temp. (° C)	time (min)	product (yield, %)[a]	alkene selectivity ratio (*E*-2:*E*-1:*Z*-1)[b]
1	**14**	120	120	**3** (32)[c]	1:0:0
2	**14**	120	120	**3** (78)[d]	2.6:1.0:1.5
3	**14**	120	10	**3** (58)[d]	2.7:1.0:1.4
4	**14**	70	1	**3** (78)	1:0:0
5	**12**	70	1	**1** (70)	1:0:0
6	**13**	70	1	**2** (74)	1:0:0

[a]Isolated yields. [b]Determined by ^{1}H NMR analysis of the crude reaction mixture. [c]Classical refluxing conditions. [d]Isolated as an inseparable mixture of three isomers.

Table 5.2 ^{1}H NMR Data [600 MHz, δ (ppm)] for HMAQs 1–3 in $CDCl_3$

H no.	1	2	3
N-H	9.60 (1H, br s)	10.17 (1H, br s)	9.89 (1H, br s)
Ar-H	8.40-8.34 (1H, m)	8.41-8.34 (1H, m)	8.40-8.34 (1H, m)
	7.32-7.23 (1H, m)	7.30-7.24 (1H, m)	7.32-7.23 (1H, m)
	7.56-7.79 (1H, m)	7.56-7.45 (2H, m)	7.56-7.49 (2H, m)
	7.43-7.36 (1H, m)		7.46-7.42 (1H, m)
1’	3.48 (2H, d (*J* = 6.5 Hz))	3.50 (2H, d (*J* = 6.4 Hz))	3.49 (2H, d (*J* = 6.6 Hz))
2’	5.47-5.72 (2H, m)	5.48-5.68 (2H, m)	5.48-5.69 (2H, m)
3’			
4’	2.06 (2H, dd (*J* =13.8, 7.0 Hz))	2.02 (2H, dd (*J* =14.3, 7.1 Hz))	2.03 (2H, dd (*J* =14.2, 7.0 Hz))
5’	1.39-1.25 (4H, m)	1.38-1.17 (6H, m)	1.39-1.19 (8H, m)
6’			
7’	0.89 (3H, t (*J* = 7.1 Hz)		
8’	n.a.	0.87 (3H, t (*J* = 7.1 Hz)	
9’	n.a.	n.a.	0.87 (3H, t (*J* = 7.0 Hz))
1’’	2.17 (3H, s)	2.18 (3H, s)	2.18 (3H, s)

Table 5.3 **^{13}C NMR Data [150 MHz, δ (ppm)] for HMAQs 1–6**

C no.	1[a]	2[a]	3[a]	4[b]	5[b]	6[b]
Ar-C	147.0	146.8	147.2	155.9	152.2	152.0
	139.0	139.0	139.1	140.1	140.8	140.8
	131.3	131.3	131.3	134.7	133.2	133.1
	126.3	126.3	126.2	128.0	126.2	126.2
	123.8	123.8	123.8	125.3	125.3	125.2
	117.3	123.1	117.4	122.5	124.5	124.6
	115.9	117.2	118.9	117.0	116.3	116.3
		115.9		116.4	116.0	116.0
C=O	178.2	178.2	178.2	169.3	175.6	n.d.
1’	35.6	35.6	35.6	33.3	33.5	33.5
2’	123.2	123.2	123.2	122.9	124.2	124.3
3’	136.6	136.8	136.5	136.4	135.2	135.1
4’	32.4	32.7	32.7	33.0	32.6	32.8
5’	31.4	31.5	31.8	32.4	32.5	32.6
6’	22.4	29.0	29.3	23.2	30.0	30.3
7’	14.0	22.6	29.0	14.2	23.5	29.8
8’	n.a.	14.2	22.7	n.a.	14.4	23.6
9’	n.a.	n.a.	14.2	n.a.	n.a.	14.4
1”	10.6	10.6	10.7	11.8	11.5	11.5

[a] in $CDCl_3$. [b] in CD_3OD.

5.3.3. Synthesis of HMAQNOs

To enable the vinylogous amine oxidation of HMAQs, the quinolone ring needed to be locked into its hydroxyquinoline tautomer. This was performed by converting HMAQs **1**–**3** into their corresponding ethyl carbonates **17**–**19** using potassium *tert*-butoxide as a base in the presence of ethyl chloroformate[30, 51]. The latter derivatives were then oxidized with *m*CPBA at low temperature (0 °C) to afford *N*-oxide ethyl carbonates **20**–**22** (**Figure 5.4** ; [51]) The moderate yields obtained for this reaction can be explained by the concomitant peroxidation of the unconjugated *trans*-[2] double

bond, leading to epoxide by-products. Methyltrioxorhenium was used as another oxidation system to improve the reaction yields but it mainly led to degradation. Deprotection of ethyl carbonates **20**–**22** was cleanly performed under the action of potassium hydroxide in ethanol and, upon acidification, HMAQNOs **4**–**6** precipitated in good yields from the reaction mixture (**Figures 5.4-5.5**). Analytical (HRMS) and spectral (^{1}H and ^{13}C NMR, **Tables 5.3** and **5.4**) data of HMAQNO **6** [32] were in agreement with those published for the isolated natural product. Analytical and spectral data for HMAQNOs **4** and **5** had not been reported until now.

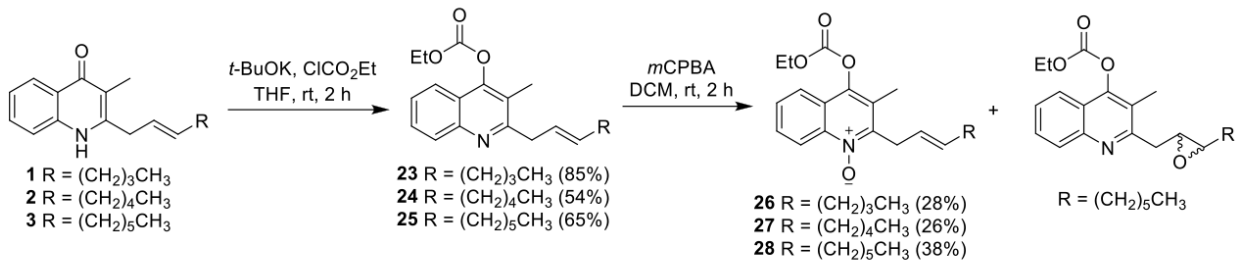


Figure 5.4 **Synthesis of Protected *N*-Oxide Quinolones**

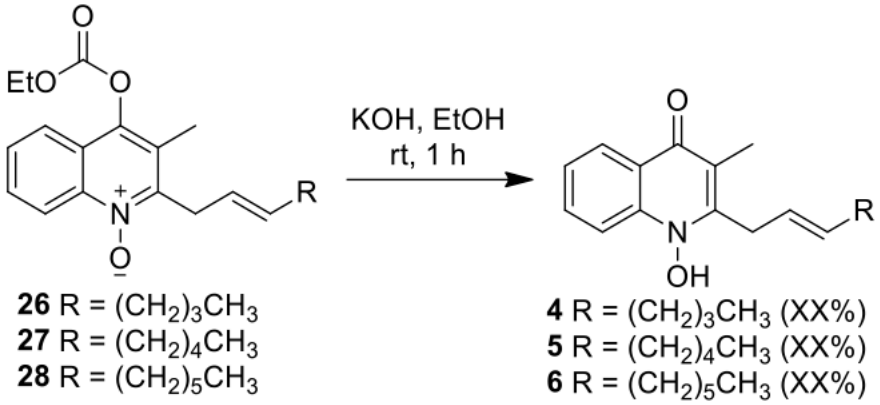


Figure 5.5 **Final Step in the Synthesis of HMAQNOs**

Table 5.4 **^{1}H NMR Data [600 MHz, δ (ppm)] for HMAQNOs 4–6 in CD_3OD**

H no.	4	5	6
Ar-H	8.45-8.39 (1H, m)	8.32-8.26 (1H, m)	8.32-8.26 (1H, m)
	7.73-7.68 (1H, m)	7.48-7.40 (1H, m)	7.48-7.38 (1H, m)
	8.24-8.19 (1H, m)	8.01-7.95 (1H, m)	8.01-7.94 (1H, m)
	8.01-7.94 (1H, m)	8.79-7.71(1H, m)	8.79-7.71(1H, m)
1’	3.96 (2H, dd (*J* = 5.7, 0.8 Hz))	3.81-3.74 (2H, m)	3.83-3.73 (2H, m)
2’	5.69-5.57 (2H, m)	5.64-5.55 (2H, m)	5.64-5.54 (2H, m)
3’			
4’	2.05 (2H, dd (*J* =13.4, 6.5 Hz))	2.06-1.98 (2H, m)	2.08-1.99 (2H, m)
5’	1.46-1.21 (4H, m)	1.39-1.21 (6H, m)	1.41-1.18 (8H, m)
6’			
7’	0.88 (3H, t (*J* = 7.2 Hz)		
8’	n.a.	0.86 (3H, t (*J* = 7.0 Hz)	
9’	n.a.	n.a.	0.85 (3H, t (*J* = 7.0 Hz)
1”	2.39 (3H, s)	2.22 (3H, s)	2.21 (3H, s)

5.3.4. Antimicrobial evaluation of HMAQs and HMAQNOs 1–6

Several studies have proposed that HMAQs act as potent antimicrobial molecules [25, 26, 52]. However, these studies only focused on the extraction of active molecules from bacterial culture supernatants. As previously mentioned, the isolated metabolites could contain contaminants affecting the biological results. To validate previous data and better characterize the biological activity, nine bacteria and ten fungi were tested for their susceptibility to HMAQs **1**–**3** and HMAQNOs **4**–**6** using two different antimicrobial methods, *i.e.*, the Kirby-Bauer disk diffusion test and the broth microdilution assay.

Our results using the Kirby-Bauer method showed that HMAQs **1–3** did not affect any of the bacteria grown on solid medium (**Figure 5.6** and **Figure S5.1**). Among the Gram-negative bacteria, HMAQNOs **4**–**6** only strongly inhibited the growth of *Actinobacillus pleuropneumoniae* and some activity was also noted against *Xanthomonas campestris*. On the other hand, all the tested Gram-positive bacteria, *i.e.*, *B. subtilis*, *S. aureus*, *Streptococcus agalactiae*, and *Paenibacillus peoriae*, were sensitive to HMAQNOs **4**–**6**, but sometimes with significant differences between congeners (**Figure 5.6**). Furthermore, both HMAQs **1**–**3** and HMAQNOs **4**–**6** were able to inhibit the growth of *Cryptococcus neoformans,* whereas they had no effect against *Candida albicans* (**Figure 5.6** and

Figure S5.1). The length of the alkyl chain of HMAQs did not impact the activity against *C. neoformans*, whereas the yeast was more sensitive towards HMAQNOs **5** and **6** than towards HMAQNO **4**. Surprisingly, all eight molds were resistant to HMAQs **1**–**3** and HMAQNOs **4**–**6** meaning that previous reports showing activity of HMAQs against different molds were presumably due to other co-purified metabolites (**Figure 5.6** and **Figure S5.2** ; [25, 26, 52]).

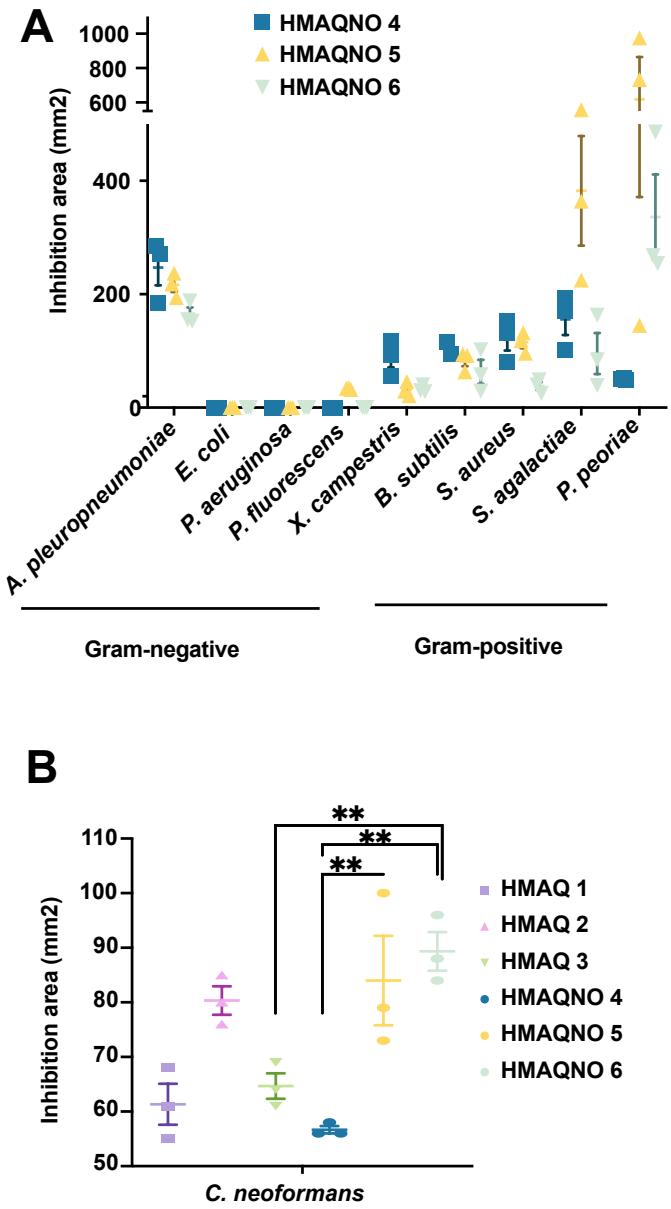


Figure 5.6 Antimicrobial activity of HMAQs 1–3 and HMAQNOs 4–6 using the Kirby-Bauer method

(A) Antimicrobial activity of HMAQNOs 4–6 tested against different Gram-negative and Gram-positive bacteria. (B) Antimicrobial activity of HMAQs 1–3 and HMAQNOs 4–6 against *C. neoformans* H99. MeOH was used as the control in assays with HMAQs 1–3 and DMSO with HMAQNOs 4–6. Means with standard error to the mean are shown. A one-way ANOVA statistical test was performed (** = p value < 0.01).

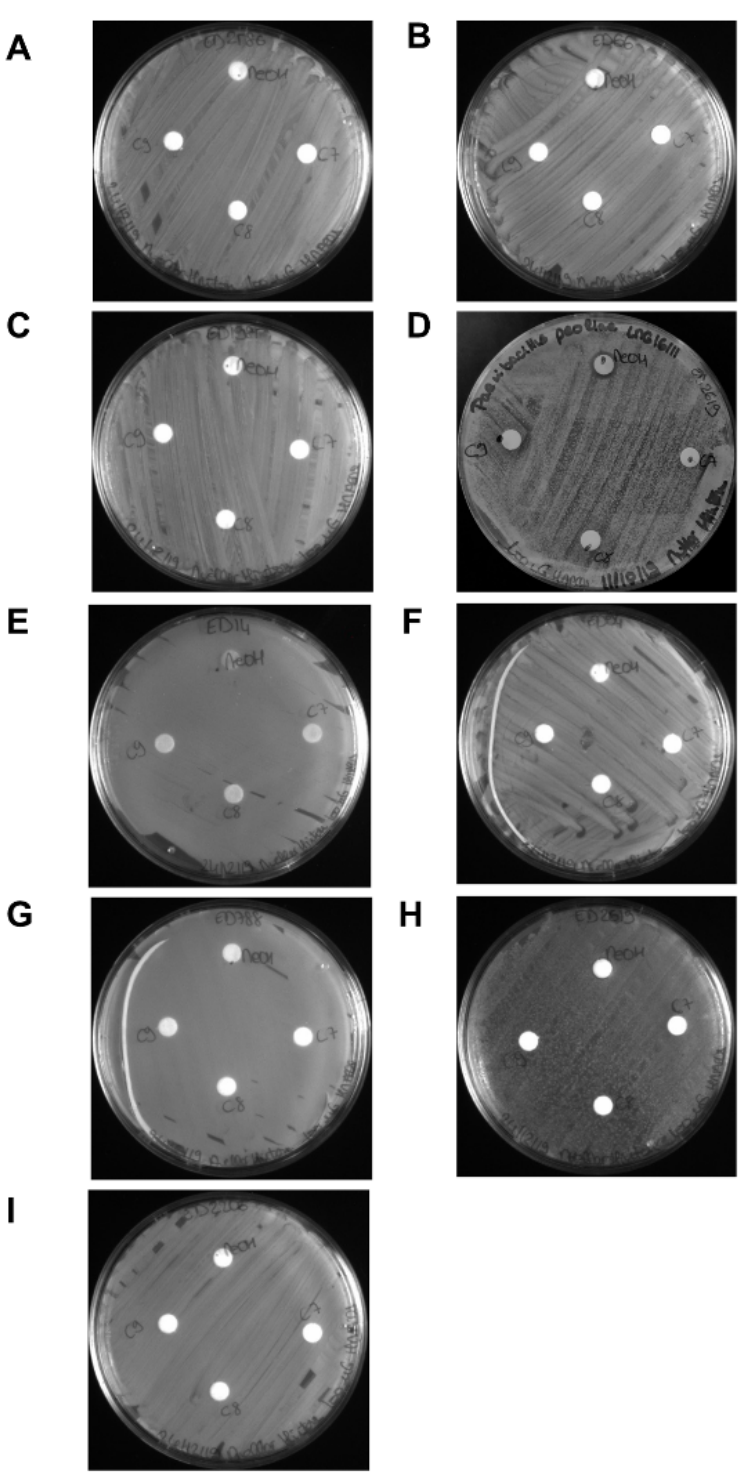


Figure S5.1 HMAQs 1–3 had no effect on bacteria.

Gram-negative bacteria: (A) *A. pleuropneumoniae*, (C) *E. coli*, (E) *P. aeruginosa*, (G) *P. fluorescens*, and (I) *X. campestris.* Gram-positive bacteria: (B) *B. subtilis*, (D) *P. peoriae*, (F) *S. aureus*, and (H) *S. agalactiae*.

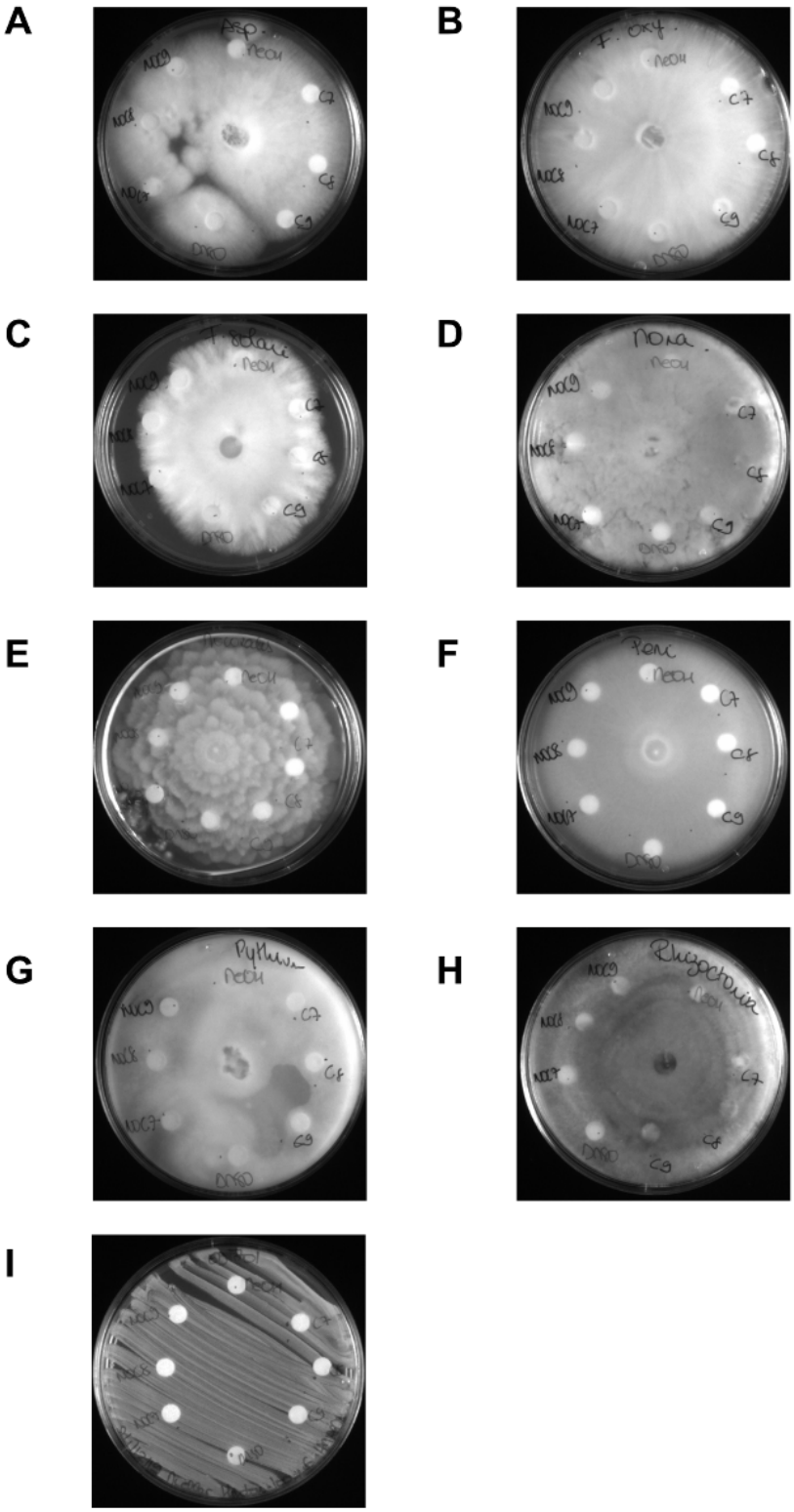


Figure S5.2 HMAQs 1–3 had no effect on fungi

(A) *Aspergillus niger*, (B) *F. oxyporum*, (C) *F. solani*, (D) *Moniliceae*, (E) *Mucorales*, (F) *Penicillum*, (G) *P. ultimum,* (H) *R. solani*, and (I) *C. albicans*.

As we were not able to detect any antimicrobial activity for HMAQs **1**–**3** against bacteria and molds, we decided to use the more sensitive broth microdilution method. Importantly, as HMAQs **1**–**3** and HMAQNOs **4**–**6** are relatively poorly soluble in water, the highest possible tested concentration was 100 µM corresponding to 25.6, 27, 28.4, 27.2, 28.6, and 30 g/mL for respectively HMAQ **1**, HMAQ **2**, HMAQ **3**, HMAQNO **4**, HMAQNO **5**, and HMAQNO **6**. In general, the bacteria were once again more sensitive to HMAQNOs **4**–**6** than to HMAQs **1**–**3** (**Table 5.5**). The growth was completely inhibited at 100 µM − or 27.2, 28.6, and 30 g/mL for HMAQNO **4**, HMAQNO **5**, and HMAQNO **6** respectively − for *S. aureus,* and *B. subtilis* (**Figure S5.4**). These results correlate with the already reported antimicrobial effect of HQNO and the growth inhibition effect of 2-heptyl-3-hydroxy-4-quinolone (PQS) from *Pseudomonas aeruginosa*[53-56]. HMAQNOs **4**–**6** could act like HQNO and target (a) the bacterial respiration by inhibiting the cytochrome b1 in Gram-positive; (b) the Na+–pumping activity of the NADH-quinone reductase system in Gram-negative; and (c) the cytochrome c in eukaryotes, as it has already been shown in *S. aureus*, *B. subtilis*, and *Vibrio alginolyticus* as well as on beef heart mitochondria particles [54, 57, 58].

For *A. pleuropneumoniae*, *B. subtilis*, and *S. aureus*, the observed activity increased with the length of the chain suggesting that the activity of HMAQs **1**–**3** is probably linked to the alkyl chain portion and not to the quinolone ring (**Table 5.5** and **Figure S5.3-5.4**). Wu and Seyedsayamdost [33] demonstrated by bacterial cytological profiling and primary metabolite analysis in *Escherichia coli* that HMAQ **3** acts like monensin on the proton gradient of the proton motive force. They also showed that both HQNO and HMAQ **3** act synergistically by competing with coenzyme Q and inhibiting the synthesis of pyrimidines.[33] Hypothesizing that HMAQNO **4**–**6** act following the same mechanism than HQNO, HMAQs **1**–**3** and HMAQNOs **4**–**6** could also play a synergic role together or with clinically used antibiotics, this needs to be further confirmed.

As expected, *C. neoformans* was more sensitive to HMAQs **1**–**3** than HMAQNOs **4**–**6** also in liquid medium while we found that *C. albicans* was resistant to HMAQs **1**–**3** and HMAQNOs **4**–**6** in liquid medium (Table 5.5 and Figure S5.5). *F. oxyporum*, *F*. *solani*, and *Mucorales* were slightly sensitive to HMAQs **1**–**3** and resistant to HMAQNOs **4–6**, while *A. niger* was resistant to HMAQs **1**–**3** and HMAQNOs **4**–**6** in microdilution (**Table 5.5** and **Figure S5.5**).

Table 5.5 Minimal Inhibitory Concentration of HMAQs 1-3 and HMAQNOs 4-5 against Microorganisms

Microorganism	Minimal inhibitory concentration [µg/mL] (µM)					
	HMAQ **1**	HMAQ **2**	HMAQ **3**	HMAQNO **4**	HMAQNO **5**	HMAQNO **6**
Gram-negative bacteria						
A. pleuropneumoniae	0.80 (3.13)	0.42 (1.56)	0.44 (1.56)	0.42 (1.56)	0.45 (1.56)	0.47 (1.56)
E. coli	25.60 (100)	27.00 (100)	28.40 (100)	6.80 (25)	1.79 (6.25)	1.88 (6.25)
P. aeruginosa	NA[a]	13.50 (50)	NA	13.60 (50)	7.15 (25)	3.75 (12.5)
P. fluorescens	6.40 (25)	6.75 (25)	14.20 (50)	13.60 (50)	7.15 (25)	7.50 (25)
X. campestris	25.60 (100)	27.00 (100)	28.40 (100)	1.70 (6.25)	0.90 (3.13)	3.75 (12.5)
Gram-positive bacteria						
B. subtilis	0.40 (1.56)	0.42 (1.56)	0.44 (1.56)	0.42 (1.56)	0.45 (1.56)	0.94 (3.13)
P. peoriae	3.20 (12.5)	3.38 (12.5)	3.55 (12.5)	0.42 (1.56)	0.45 (1.56)	0.47 (1.56)
S. aureus	0.40 (1.56)	0.42 (1.56)	0.44 (1.56)	0.42 (1.56)	0.45 (1.56)	0.47 (1.56)
S. agalactiae	0.40 (1.56)	0.42 (1.56)	0.44 (1.56)	0.42 (1.56)	0.45 (1.56)	0.47 (1.56)
Fungi						
C. neoformans	0.80 (3.13)	0.42 (1.56)	0.44 (1.56)	13.60 (50)	3.58 (12.5)	1.88 (6.25)
C. albicans	NA	NA	NA	NA	NA	NA
A. niger	NA	NA	NA	NA	NA	NA
F. oxyporum	3.20 (12.5)	NA	3.55 (12.5)	NA	NA	NA
F. solani	NA	NA	1.78 (6.25)	NA	NA	NA
Mucorales	1.60 (6.25)	3.38 (12.5)	3.55 (12.5)	NA	NA	NA

[a]NA: Non-active at the maximum tested concentration (MIC > 100 µM for each molecule or 25.6, 27, 28.4, 27.2, 28.6, 30 g/mL for HMAQ 1, HMAQ 2, HMAQ 3, HMAQNO 4, HMAQNO 5, HMAQNO 6 respectively)

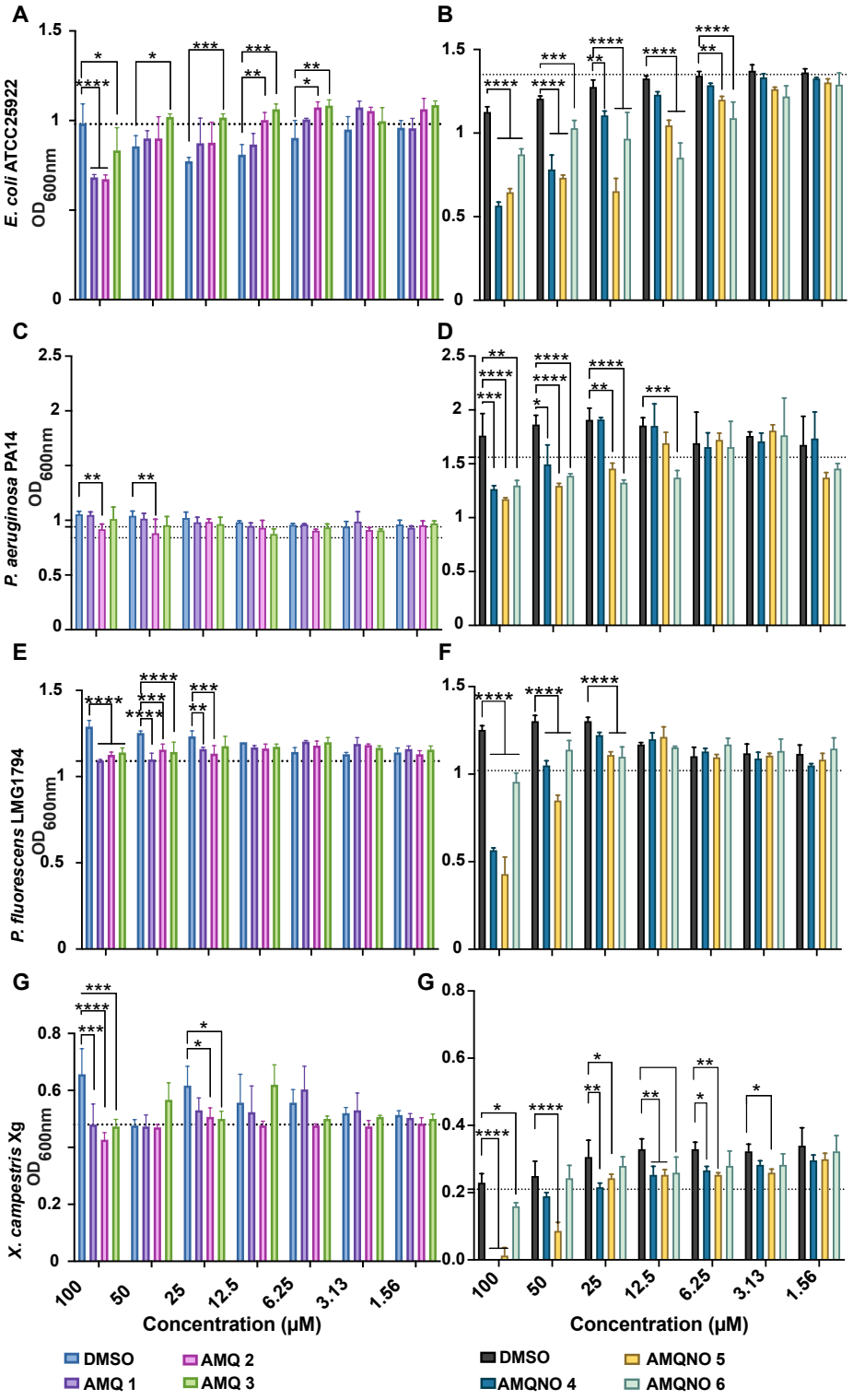


Figure S5.3 Antimicrobial activity of HMAQs 1–3 and HMAQNOs 4–6 against Gram-negative bacteria using the microdilution method with concentrations ranging from 1.56 to 100 µM

Antimicrobial activity of HMAQs 1–3 tested against (A) *A. pleuropneumoniae,* (C) *E. coli*, (E) *P. aeruginosa*, (G) *P. fluorescens*, and (I) *X. campestris.* Antimicrobial activity of HMAQNOs 4–6 tested against (B) *A. pleuropneumoniae,* (D) *E. coli*, (F) *P. aeruginosa*, (H) *P. fluorescens*, and (J) *X. campestris.* Horizontal lines represent the growth of the microorganism without molecule or DMSO in the culture. Two-way ANOVA statistical tests were performed to compare the effect of HMAQs and HMAQNOs with the control DMSO giving: **** p value < 0.0001, *** p value < 0.001, ** p value < 0.01 and * p value < 0.05.

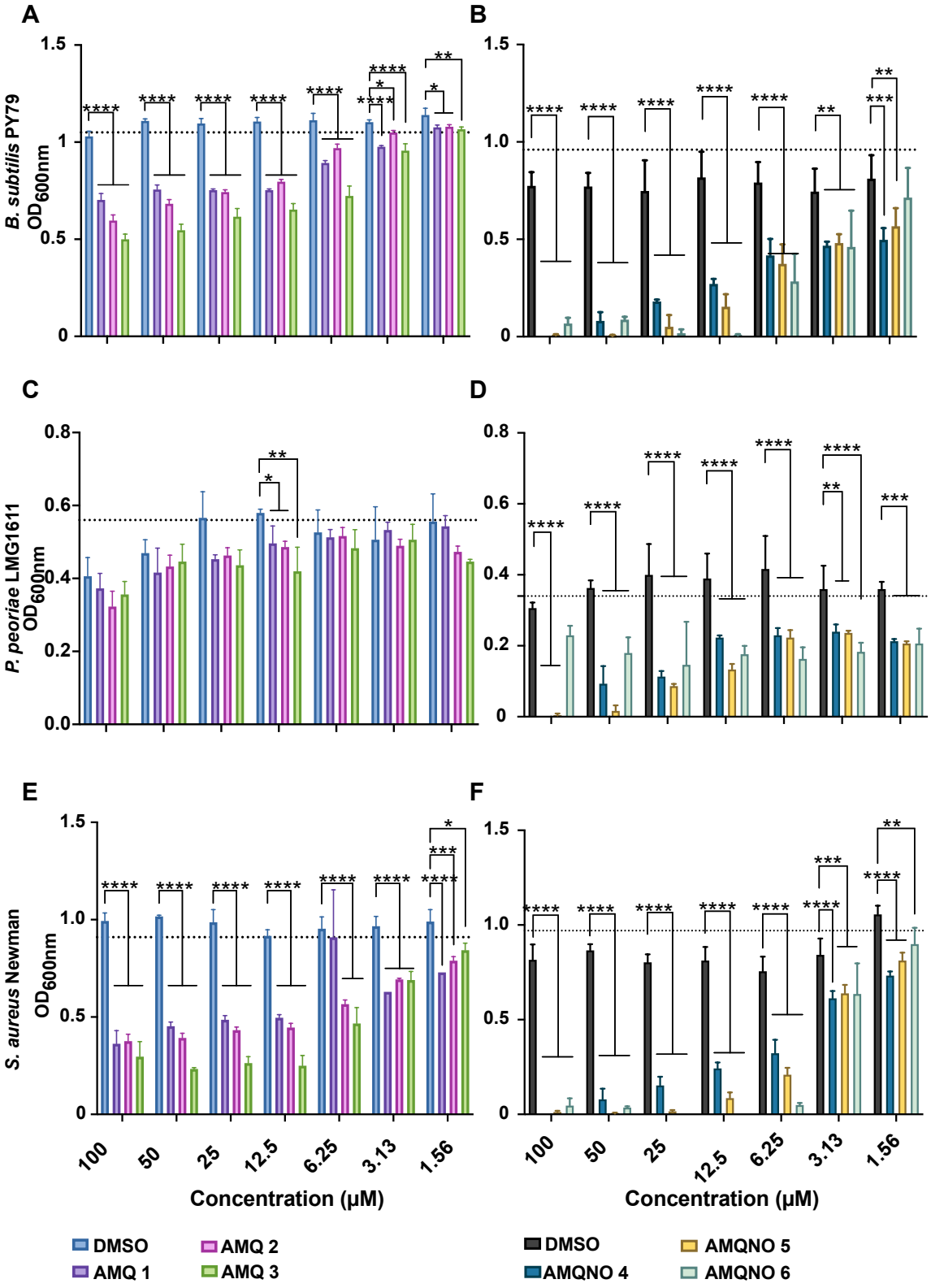


Figure S5.4 Antimicrobial activity of HMAQs 1–3 and HMAQNOs 4–6 against Gram-positive bacteria using the microdilution method with concentrations ranging from 1.56 to 100 µM

Antimicrobial activity of HMAQs 1–3 tested against (A) *B. subtilis*, (C) *P. peoriae*, (E) *S. aureus* and, (G) *S. agalactiae.* Antimicrobial activity of HMAQNOs 4–6 tested against (B) *B. subtilis*, (D) *P. peoriae*, (F) *S. aureus,* and (H) *S. agalactiae.* Horizontal lines represent the growth of the microorganism without molecule or DMSO in the culture. Two-way ANOVA statistical tests were performed to compare the effect of HMAQs and HMAQNOs with the control DMSO giving: **** p value < 0.0001, *** p value < 0.001, ** p value < 0.01 and * p value < 0.05.

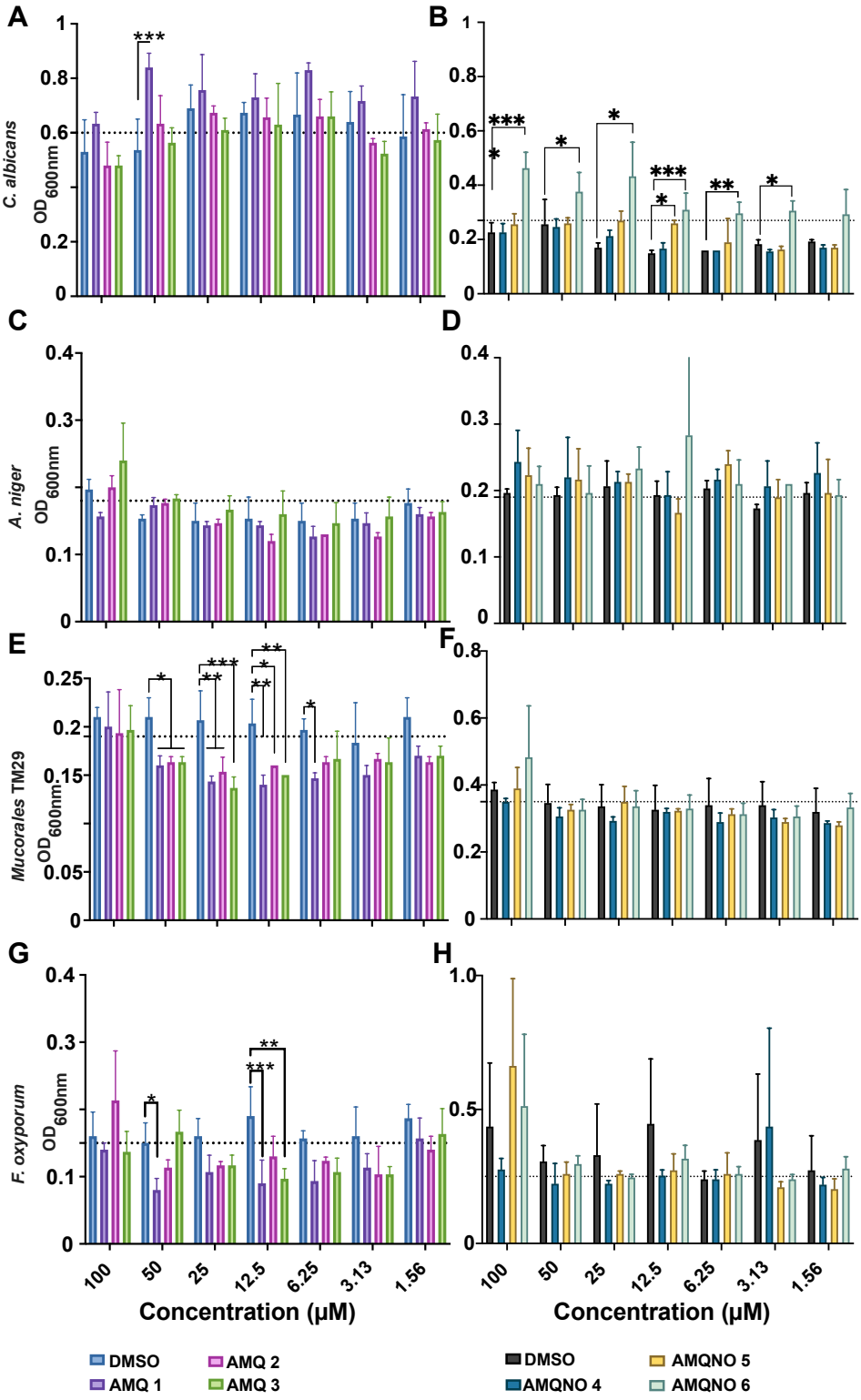


Figure S5.5 Antimicrobial activity of HMAQs 1–3 and HMAQNOs 4–6 against yeast and fungi using the microdilution method with concentrations ranging from 1.56 to 100 µM

Antimicrobial activity of HMAQs 1–3 tested against (A) *C. albicans*, (C) *C. neoformans*, (E) *A. niger*, (G) *F. oxyporum*, (I) *F. solani,* and (K) *Mucorales.* Antimicrobial activity of HMAQNOs 4–6 tested against (B) *C. albicans*, (D) *C. neoformans*, (F) *A. niger*, (H) *F. oxyporum*, (J) *F. solani,* and (L) *Mucorales.* Horizontal lines represent the growth of the microorganism without molecule or DMSO in the culture. Two-way ANOVA statistical tests performed to compare the effect of HMAQs and HMAQNOs with the control DMSO giving: **** p value < 0.0001, *** p value < 0.001, ** p value < 0.01 and * p value < 0.05.

5.5.3. Action of HMAQs 1–3 on *B. ambifaria* quorum sensing

As reported in a previous study, the activity of a *hmqA-lacZ* reporter was higher in an *hmqA*- mutant than in the WT strain. Adding purified HMAQs restored the activity to the WT levels. We thus repeated this experiment with the addition of synthetic HMAQs (**Figure 5.7**). The results confirmed that adding HMAQs lowered the expression from the *hmqABCDEFG* promoter in the *hmqA*- mutant.

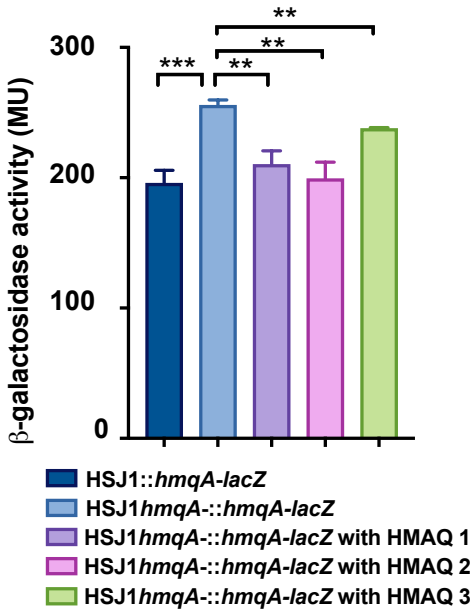


Figure 5.7 β-Galactosidase activity of a *hmqA-lacZ* reporter in *B. ambifaria* HSJ1 *hmqA*- following the addition of HMAQs

DMSO was added in controls. ANOVA statistical tests were performed giving: **** p value < 0.0001, *** p value < 0.001, ** p value < 0.01 and * p value < 0.05.

5.6. Conclusion

In summary, we have accomplished the total synthesis of HMAQs **1**–**3** *via* the Conrad-Limpach approach for the formation of the quinolone core followed by a microwave-heated Suzuki-Miyaura cross-coupling for the insertion of the (*E*)-2-alkenyl chain while avoiding isomerization of the non-conjugated *trans*-$^{2'}$ double bond. Subsequent oxidation of the protected enolate form of HMAQs **1**–**3** cleanly provided HMAQNOs **4**–**6**. This series of AQs (**1**–**6**) produced by *Burkholderia* spp. was obtained in pure and homogeneous forms, which allowed us to confirm and extend their roles as antimicrobials. Strikingly, HMQANOs **4**–**6** were found to be highly active against Gram-positive as compared to Gram-negative bacteria. Future investigations are required to determine if HMAQs **1**–**3** and HMAQNOs **4**–**6** have the same targets in both Gram-negative and Gram-positive bacteria than HQNO and PQS. According to previous studies, we believe that HMAQs and HMAQNOs are promising metabolites to be used in synergy with other antibiotics.

5.7. Experimental section

5.7.1. General Experimental Procedures

All chemicals and starting materials were purchased from commercial sources and used without further purification. Air and water sensitive reactions were performed in flame-dried glassware under argon atmosphere. Anhydrous solvents (dichloromethane, THF) were dried for at least 3 days over 4 Å molecular sieves previously activated by heating with a heat-gun for 15 min under high vacuum. Reactions in microwave were performed on a CEM Discover SP microwave coupled with an Explorer 12 Hybrid autosampler (Matthews, NC, USA). All reactions were monitored by thin-layer chromatography (TLC) with silica gel 60 F_{254} 0.25 mm pre-coated glass plates. Compounds were visualized by UV_{254}. Flash column chromatography was carried out using silica gel 60 Å (15-40 m). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on 600 MHz Bruker Advance III NMR spectrometers. Elucidations of chemical structures were based on ^{1}H and ^{13}C and spectral assignments, which were confirmed by 2D experiments (COSY and HSQC). Chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent peaks or tetramethylsilane (TMS, $\delta_H = \delta_C = 0$ ppm) and coupling constants are expressed in Hertz (Hz). The labile O*H* and N*H* signals appearing sometimes were not listed. High-resolution mass spectra (HRMS) were recorded on an ESI-Q-TOF mass spectrometer (Department of Chemistry, Université de Montréal).

5.7.2. Synthesis of 2-(Chloromethyl)-3-methylquinolin-4(1*H*)-one (11)

A mixture of aniline (7, 6.8 mL, 74 mmol, 1 equiv) and diethyl 2-methyl-3-oxosuccinate (8, 14 mL, 74 mmol, 1 equiv) in glacial acetic acid (37 mL) was refluxed at 50 °C for 24 h under argon. After cooling down to rt, water (100 mL) was gently poured, and the mixture was transferred into a separatory funnel. The aqueous phase was extracted with DCM (3 × 100 mL), the combined organic layers were dried over anhydrous MgSO4, and evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (hexanes-EtOAc, 100:0 to 95:5) to give diester 9 as a yellow oil (13.9 g, 68%). Spectral and analytical data of diester 9 were in accordance with those published.[48] Diester 9 (13.6 g, 49.1 mmol) and polyphosphoric acid (68 g) were refluxed at 130 °C for 2 h under argon with strong stirring. The resulting slurry was poured slowly into a large beaker containing an iced solution of saturated aqueous NaHCO3. The precipitate was filtered and washed with cold water and cold diethyl ether. The residue was then recovered with acetone and concentrated under reduced pressure to give ester 10 (10.5 g, 93%) as a beige amorphous powder. Spectral and analytical data of ester 10 were in accordance with those published.[48] Ester 10 (8.58 g, 37.1 mmol) was dissolved in anhydrous THF (408 mL) and the mixture was cooled down at 0 °C. The reducing agent LiAlH4 (2.81 g, 74.2 mmol) was then slowly added and the reaction was stirred

at rt for 2 h. To quench the reaction, the mixture was cooled down in an ice/water bath and cold EtOAc (40 mL) was poured very slowly using an addition funnel. MeOH (10 mL) was then added dropwise and the mixture was evaporated under reduced pressure. The residue (dry pack) was purified on silica gel flash chromatography (DCM-MeOH, 10:0 to 8:2) to obtain the desired alcohol as a yellow amorphous powder (5.58 g, 78%). The latter compound (5.58 g, 29.5 mmol) was dissolved in anhydrous DCM (221 mL) and cooled down to 0 °C. Thionyl chloride (21.4 mL, 295 mmol) was then added dropwise. The reaction was stirred at rt for 90 min. The mixture was evaporated under reduced pressure and resuspended in MeOH. Toluene was added, and the mixture was co-evaporated three times under reduced pressure in the presence of silica (ratio 3:1). The resulting dried pack was purified by silica gel flash column chromatography (DCM-MeOH, 10:0 to 9:1) to afford chloride 11 as a white amorphous solid (5.06 g, 83%). Spectral and analytical data of chloride 11 were in accordance with those published.[48]

5.7.3. General Procedure for the Synthesis of HMAQs

To a solution of chloride **11** (1.0 equiv) and corresponding *trans*-alkenylboronic acid pinacol ester (1.3 equiv) in 1,4-dioxane (8.3 mLmmol^{-1}) was added aqueous sodium carbonate (2 M, 8.3 mLmmol^{-1}). After bubbling with nitrogen for 5 min, $Pd(PPh_3)_4$ (0.10 equiv) was added and the resulting suspension was heated at 120 °C under microwave irradiation for 1 min. After cooling, the mixture was filtered through Celite® to remove the catalyst and the cake was rinsed with EtOAc. The organic phase was then washed twice with brine, dried over anhydrous $MgSO_4$, and the solution was evaporated under reduced pressure. The crude material was purified by flash chromatography to give HMAQs **1**–**3**.

5.7.4. Synthesis of (*E*)-2-(Hept-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one (1)

According to the general procedure for the synthesis of HMAQs, chloride **11** (160 mg, 0.771 mmol) was reacted with *trans*-hexen-1-ylboronic acid pinacol ester **12** (257 L, 1.00 mmol). The crude material was purified by silica gel flash column chromatography (100% hexanes to 100% EtOAc) to give HMAQ **1** (399 mg, 70%) as a white amorphous powder. ^{1}H and ^{13}C NMR data are reported in Tables 2 and 3, respectively. HRMS (ESI-TOF) *m/z m/z* $[M + H]^+$ calcd for $C_{17}H_{22}NO$ 256.16959; found 256.16938; *m/z* $[M + Na]^+$ calcd for $C_{17}H_{21}NNaO$ 278.15154; found 278.15152.

5.7.5. Synthesis of (*E*)-2-(Oct-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one (2)

According to the general procedure for the synthesis of HMAQs, chloride **11** (184 mg, 0.886 mmol) was reacted with *trans*-hepten-1-ylboronic acid pinacol ester **13** (296 L, 1.15 mmol). The crude material was purified by silica gel flash column chromatography (100% hexanes to 100% EtOAc) to give HMAQ **2** (176 mg, 74%) as a white amorphous powder. ^{1}H and ^{13}C NMR data are reported in

Tables 2 and 3, respectively. HRMS (ESI-TOF) *m/z* [M + H]$^+$ calcd for $C_{18}H_{24}NO$ 270.18524; found 270.18416.

5.7.6. Synthesis of (*E*)-2-(Non-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one (3)

According to the general procedure for the synthesis of HMAQs, chloride **11** (307 mg, 1.48 mmol) was reacted with *trans*-octen-1-ylboronic acid pinacol ester **14** (519 L, 1.92 mmol). The crude material was purified by silica gel flash column chromatography (100% hexanes to 100% EtOAc) to give HMAQ **3** (316 mg, 75%) as a white amorphous powder. ^{1}H and ^{13}C NMR data are reported in Tables 2 and 3, respectively. HRMS (ESI-TOF) *m/z* [M + H]$^+$ calcd for $C_{19}H_{26}NO$ 284.20089; found 284.20121; *m/z* [M + Na]$^+$ calcd for $C_{19}H_{25}NNaO$ 306.18284; found 306.18269.

5.7.7. General Procedure for the Protection of HMAQs

HMAQ (**1**–**3**, 1.0 equiv) was reacted with *t*-BuOK (1.25 equiv) in anhydrous THF (7.0 mLmmol^{-1}) at rt for 1 h. Ethyl chloroformate (2.15 equiv) was then added and the reaction was left stirring at rt for 1.0–1.5 h. When the reaction was completed (as revealed by TLC), water was added, and the mixture was evaporated under reduced pressure to remove THF. The aqueous phase was diluted in water and extracted with EtOAc (3). The combined organic phases were dried over anhydrous $MgSO_4$, filtered through Celite®, and evaporated under reduced pressure. The crude material was purified by silica gel flash chromatography to give HMAQ ethyl carbonates **17**–**19**.

5.7.8. Synthesis of (*E*)-2-(Hept-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one Ethyl Carbonate (17)

According to the general procedure for the protection of HMAQs, quinolone **1** (20 mg, 0.078 mmol) was reacted with *t*-BuOK (11 mg, 0.098 mmol) and ethyl chloroformate (16 L, 0.17 mmol). The crude material was purified by silica gel flash column chromatography (hexanes-EtOAc, 90:10 to 80:20) to give ethyl carbonate **17** (22 mg, 85%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.10–8.01 (m, 1H, H_{Ar}), 7.83–7.76 (m, 1H, H_{Ar}), 7.68–7.62 (m, 1H, H_{Ar}), 7.55–7.48 (m, 1H, H_{Ar}), 5.74–5.44 (m, 2H, H-2' and H-3'), 4.39 (dd, *J* = 14.2 Hz, *J* =7.1 Hz, 2H), 3.75 (br d, *J* = 5.8 Hz, 2H, H-1'), 2.34 (s, 3H, H-1''), 2.01 (dd, *J* = 13.5 Hz, *J* = 6.8 Hz, 2H, H-4'), 1.46–1.41 (m, 3H), 1.39–1.24 (m, 4H, H-5' and H-6'), 0.93–0.84 (m, 3H, H-7'); ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 161.9, 152.5, 151.9, 147.6, 133.3 (C-3'), 129.2 (C_{Ar}), 129.1 (C_{Ar}), 126.6 (C_{Ar}), 125.8 (C-2'), 121.8 (C=N), 121.5 (C=N), 120.6 (C_{Ar}), 65.7 (CH_2), 40.8 (C-4'), 32.5 (C-1'), 31.6 (C-5'), 22.3 (C-6'), 14.4 (CH_3), 14.1 (C-7'), 12.0 (C-1''). HRMS (ESI-TOF) *m/z* [M + H]$^+$ calcd for $C_{20}H_{26}NO_3$ 328.19072; found 328.18946.

5.7.9. Synthesis of (*E*)-2-(Oct-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one Ethyl Carbonate (18)

According to the general procedure for the protection of HMAQs, quinolone **2** (302 mg, 1.12 mmol) was reacted with *t*-BuOK (175 mg, 1.56 mmol) and ethyl chloroformate (293 L, 3.06 mmol). The crude material was purified by silica gel flash column chromatography (hexanes-EtOAc, 100:0 to 80:20) to give ethyl carbonate **18** (207 mg, 54%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.08–8.01 (m, 1H, H_{Ar}), 7.83–7.79 (m, 1H, H_{Ar}), 7.69–7.62 (m, 1H, H_{Ar}), 7.53–7.48 (m, 1H, H_{Ar}), 5.72–5.45 (m, 2H, H-2' and H-3'), 4.42–4.34 (m, 2H), 3.75 (m, 2H, H-1'), 2.34 (s, 3H, H-1''), 2.01 (dd, *J* = 14.0 Hz, *J* =7.1 Hz, 2H, H-4'), 1.53–1.40 (m, 3H), 1.38–1.19 (m, 4H, H-5',H-6'and H-7'), 0.83–0.91 (m, 3H, H-8'); ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 161.9, 152.5, 151.9, 147.6, 133.3 (C-2'), 129.2 (C_{Ar}), 129.1 (C_{Ar}), 126.6 (C_{Ar}), 125.8 (C-3'), 121.8 (C=N), 121.5 (C=N), 120.6 (C_{Ar}), 65.7 (CH_2), 40.8 (C-1'), 32.7 (C-4'), 31.5 (CH_2), 29.1 (CH_2), 22.7 (CH_2), 14.4 (CH_3), 14.2 (C-9'), 12.0 (C-1''). HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{21}H_{28}NO_3$ 342.20637; found 342.20514.

5.7.10. Synthesis of (*E*)-2-(Non-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one Ethyl Carbonate (19)

According to the general procedure for the protection of HMAQs, quinolone **3** (290 mg, 1.03 mmol) was reacted with *t*-BuOK (150 mg, 1.34 mmol) and ethyl chloroformate (244 L, 2.55 mmol). The crude material was purified by silica gel flash column chromatography (hexanes-EtOAc, 100:0 to 80:20) to give ethyl carbonate **19** (238 mg, 65%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.11–8.00 (m, 1H, H_{Ar}), 7.86–7.76 (m, 1H, H_{Ar}), 7.69–7.62 (m, 1H, H_{Ar}), 7.54–7.47 (m, 1H, H_{Ar}), 5.76–5.45 (m, 2H, H-2' and H-3'), 4.38 (dd, *J* = 14.2 Hz, *J* =7.1 Hz, 2H), 3.75 (br d, *J* = 6.1 Hz, 2H, H-1'), 2.34 (s, 3H, H-1''), 2.01 (dd, *J* = 14.0 Hz, *J* =7.1 Hz, 2H, H-4'), 1.48–1.40 (m, 3H), 1.37–1.18 (m, 8H, H-5', H-6', H-7' and H-8'), 0.90–0.81 (m, 3H, H-9'); ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 161.9, 152.5, 151.9, 147.6, 133.3 (C-2'), 129.2 (C_{Ar}), 129.0 (C_{Ar}), 126.6 (C_{Ar}), 125.8 (C-3'), 121.8 (C=N), 121.5 (C=N), 120.6 (C_{Ar}), 65.7 (CH_2), 40.8 (C-1'), 32.7 (C-4'), 31.8 (CH_2), 29.4 (CH_2), 28.9 (CH_2), 22.7 (CH_2), 14.4 (CH_3), 14.2 (C-9'), 12.0 (C-1''). HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{22}H_{30}NO_3$ 356.22202; found 356.22144.

5.7.11. General Procedure for the Oxidation of HMAQ Ethyl Carbonates

Protected quinolone (**17**–**19**, 1.0 equiv) was reacted with *m*CPBA (1.1 equiv) in anhydrous DCM (40 Lmg^{-1}) at rt for 3 h. The reaction was washed twice with aqueous sodium carbonate (0.5 M) and once with water. The organic phase was dried over anhydrous $MgSO_4$, filtered through Celite®, and evaporated under reduced pressure. The crude material was purified by silica gel flash chromatography to give ethyl carbonate *N*-oxides (**20**–**22**).

5.7.12. Synthesis of (*E*)-2-(Hept-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-yl) Ethyl Carbonate *N*-Oxide (20)

According to the general procedure for the oxidation of HMAQ ethyl carbonates, quinolone **17** (192 mg, 0.586 mmol) was reacted with *m*CPBA (126 mg, 87% NMR purity, 0.645 mmol). The crude material was purified by flash silica gel flash column chromatography (hexanes-EtOAc, 95:5 to 70:30) to give *N*-oxide **20** (51 mg, 26%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.80–8.75 (m, 1H, H_{Ar}), 7.86–7.80 (m, 1H, H_{Ar}), 7.77–7.70 (m, 1H, H_{Ar}), 7.66–7.59 (m, 1H, H_{Ar}), 5.72–5.57 (m, 2H, H-2' and H-3'), 4.39 (dd, J = 14.2 Hz, J =7.1 Hz, 2H), 3.95 (br d, J = 5.8 Hz, 2H, H-1'), 2.35 (s, 3H, H-1''), 2.04–1.95 (m, 2H, H-4'), 1.44 (t, J = 7.1 Hz, 3H), 1.37–1.18 (m, 4H, H-5' and H-6'), 0.86 (t, J = 7.1 Hz, 3H, H-7'); ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 152.6, 148.6, 142.0, 140.9, 134.1 (C-3'), 130.1 (C_{Ar}), 128.7 (C_{Ar}), 123.4 (C=N), 123.1 (C=N), 122.0 (C-2'), 121.4 (C_{Ar}), 120.6 (C_{Ar}), 66.0 (CH_2), 33.3 (C-4'), 32.1 (C-1'), 31.5 (CH_2), 22.4 (CH_2), 14.4 (CH_3), 14.1 (C-7'), 12.6 (C-1''). HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{20}H_{26}NO_4$ 344.1856; found 344.1861.

5.7.13. Synthesis of (*E*)-2-(Oct-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-yl) Ethyl Carbonate *N*-Oxide (21)

According to the general procedure for the oxidation of HMAQ ethyl carbonates, quinolone **18** (207 mg, 0.607 mmol) was reacted with *m*CPBA (130 mg, 87% NMR purity, 0.668 mmol). The crude material was purified by silica gel flash column chromatography (hexanes/EtOAc, 95:5 to 70:30) to give *N*-oxide **21** (61 mg, 28%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.80–8.74 (m, 1H, H_{Ar}), 7.86–7.79 (m, 1H, H_{Ar}), 7.77–7.70 (m, 1H, H_{Ar}), 7.65–7.59 (m, 1H, H_{Ar}), 5.72–5.57 (m, 2H, H-2' and H-3'), 4.39 (dd, J = 14.2 Hz, J =7.1 Hz, 2H), 3.95 (br d, J = 5.8 Hz, 2H, H-1'), 2.35 (s, 3H, H-1''), 2.03–1.95 (m, 2H, H-4'), 1.44 (t, J = 7.1 Hz, 3H), 1.39–1.18 (m, 6H, H-5', H-6' and H-7'), 0.85 (t, J = 7.1 Hz, 3H, H-8'); ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 152.6, 148.6, 142.0, 140.9, 134.1 (C-3'), 130.1 (C_{Ar}), 128.7 (C_{Ar}), 123.4 (C=N), 123.1 (C=N), 121.9 (C-2'), 121.4 (C_{Ar}), 120.6 (C_{Ar}), 66.0 (CH_2), 32.6 (C-4'), 32.1 (C-1'), 31.5 (CH_2), 29.0 (CH_2), 22.6 (CH_2), 14.4 (CH_3), 14.2 (C-8'), 12.6 (C-1''). HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{21}H_{28}NO_4$ 358.2013; found 358.2013.

5.7.14. Synthesis of (*E*)-2-(Non-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-yl) Ethyl Carbonate *N*-Oxide (22)

According to the general procedure for the oxidation of HMAQ ethyl carbonates, quinolone **19** (92 mg, 0.26 mmol) was reacted with *m*CPBA (110 mg, 87% NMR purity, 0.563 mmol). The crude material was purified by silica gel flash column chromatography (hexanes-EtOAc, 100:0 to 70:30) to give *N*-oxide **22** (37 mg, 38%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 8.81–8.74 (m,

1H, H_{Ar}), 7.85–7.80 (m, 1H, H_{Ar}), 7.76–7.69 (m, 1H, H_{Ar}), 7.66–7.60 (m, 1H, H_{Ar}), 5.72–5.55 (m, 2H, H-2' and H-3'), 4.39 (dd, *J* = 14.2 Hz, *J* =7.1 Hz, 2H), 3.95 (br d, *J* = 5.8 Hz, 2H, H-1'), 2.35 (s, 3H, H-1''), 2.03–1.95 (m, 2H, H-4'), 1.44 (t, *J* = 7.1 Hz, 3H), 1.37–1.17 (m, 8H, H-5', H-6', H-7', H-8'), 0.85 (t, *J* = 7.1 Hz, 3H, H-9'); ^{13}C NMR (150 MHz, $CDCl_3$) *δ* (ppm) 152.6, 148.6, 142.0, 140.9, 134.1 (C-3'), 130.1 (C_{Ar}), 128.7 (C_{Ar}), 123.4 (C=N), 123.1 (C=N), 121.9 (C-2'), 121.4 (C_{Ar}), 120.6 (C_{Ar}), 66.0 (CH_2), 32.6 (C-4'), 32.1 (C-1'), 31.8 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 22.7 (CH_2), 14.4 (CH_3), 14.2 (C-9'), 12.6 (C-1''). HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{22}H_{30}NO_4$ 372.21693; found 372.21696; *m/z* $[M + Na]^+$ calcd for $C_{22}H_{29}NNaO_4$ 394.19888; found 394.19824.

5.7.15. General Procedure for the Synthesis of HMAQNOs

Protected quinolone *N*-oxide (**20**–**22**, 1.0 equiv) was dissolved in ethanol (95%, 33 Lmg^{-1}) and aqueous KOH was added (5.0 M, 17 equiv). The reaction was stirred at rt for 1 h and then quenched with the addition of cold water (same volume as the solvent). Concentrated HCl (12 N) was added until acidic pH (1–2) was reached. A milky suspension was formed, and cold water was then added to help the precipitation process. The product was collected by filtration under vacuum and washed with cold water to give target HMAQNOs (**4**–**6**).

5.7.16. Synthesis of (*E*)-2-(Hept-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one *N*-Oxide (4)

According to the general procedure for the synthesis of HMAQNOs, protected *N*-oxide **20** (47 mg, 0.14 mmol) was reacted with KOH to give HMAQNO **4** (27 mg, 72%) as a beige amorphous powder. ^{1}H and ^{13}C NMR data are reported in Tables 3 and 4, respectively. HRMS (ESI-TOF) *m/z* $[M + Na]^+$ calcd for $C_{17}H_{21}NNaO_2$ 272.1645; found 272.1631.

5.7.17. Synthesis of (*E*)-2-(Oct-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one *N*-Oxide (5)

According to the general procedure for the synthesis of HMAQNOs, protected *N*-oxide **21** (56 mg, 0.16 mmol) was reacted with KOH to give HMAQNO **5** (34 mg, 77%) as a beige amorphous powder. ^{1}H and ^{13}C NMR data are reported in Tables 3 and 4, respectively. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{18}H_{24}NO_2$ 286.18016; found 286.17984.

5.7.18. Synthesis of (*E*)-2-(Non-2'-en-1'-yl)-3-methylquinolin-4(1*H*)-one *N*-Oxide (6)

According to the general procedure for the synthesis of HMAQNOs, protected *N*-oxide **22** (51 mg, 0.14 mmol) was reacted with KOH to give HMAQNO **6** (31 mg, 76%) as an amorphous beige powder. ^{1}H and ^{13}C NMR data are reported in Tables 3 and 4, respectively. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{19}H_{26}NO_2$ 300.19581; found 300.19555; *m/z* $[M + Na]^+$ calcd for $C_{19}H_{25}NNaO_2$ 322.17775; found 322.17738.

5.7.19. Bacteria and Fungi

Bacillus subtilis PY79, *Paenibacillus peoriae* LMG1611, *Staphylococcus aureus* Newman, *Streptococcus agalactiae* ATCC27956, *Actinobacillus pleuropneumoniae, Burkholderia ambifaria* HSJ1 *hmqA*-lacZ, *Burkholderia ambifaria* HSJ1 *hmqA*-::*hmqA*-lacZ, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PA14, *Pseudomonas fluorescens* LMG1794, *Xanthomonas campestris* Xg, *Cryptococcus neoformans* H99, and *Candida albicans* were cultured in tryptic soy broth (TSB) at 30°C with shaking overnight. *Aspergillus niger*, *Fusarium solani*, *Fusarium oxyporum*, *Mocurales* TM29, *Moniliceae* 1(22), *Penicillum* sp*, Pythium ultimum*, and *Rhizoctonia solani* were grown on potato dextrose agar (PDA) plates at 25 °C for 4 days.

5.7.20. Antimicrobial Disk Susceptibility Tests

Antimicrobial susceptibility disks were used to determine the antimicrobial activity of HMAQs on bacterial and fungal strains using the Kirby-Bauer method [59, 60]. Cultures of bacteria and yeasts were grown overnight at 30°C with shaking in tryptic soy broth (TSB). Then, cells were spread onto Mueller-Hinton agar (MHA) plates using a sterile cotton swab. A total of 10 µL (10,000 $\mu g mL^{-1}$ in MeOH for HMAQs or DMSO for HMAQNOs) for each HMAQ was added on blank antimicrobial susceptibility disks. A 10 µL MeOH or DMSO blank antimicrobial susceptibility disk was used as control. Once MeOH or DMSO was evaporated, the disks were deposited to the surface of an agar plate. Molds were first grown on potato dextrose agar (PDA) plates at 25°C for 4 days. An agar plug was transferred in the middle of a new PDA plate cultured at 25°C for 4 days. Dried antimicrobial susceptibility disks containing 100 µg of compounds and the control were added around the plug of fungus on the PDA plate. The plates were incubated at 25°C for seven more days.

5.7.21. Antimicrobial Susceptibility Assay by Broth Microdilution

Based on the Clinical and Laboratory Standards Institute protocol, bacterial cells were suspended into Mueller-Hinton broth at an OD_{600} of 0.02. A stock of 20 mM of each HMAQ was prepared in DMSO. Serial dilutions were performed to reach concentrations of HMAQs ranging from 100 µM to 0.78 µM. Bacteria were added at a final OD_{600} of 0.01. The plates were incubated for 48 h at 30 °C with shaking at 250 rpm. The OD_{600} was measured using a Cytation 3 plate reader (Biotek). *A. niger*, *F.oxyporum, F. solani*, and *Mocurales* TM29 were grown at 25 °C for 4 days on PDA plates and harvested by adding sterile water onto the plate and transferring into a sterile tube. Then, fungal spores were counted using a hemacytometer cell counting chamber. As described by Pujol *et al.*,[61] a $2 10^4$ $sporesmL^{-1}$ suspension was prepared into RPMI 1640 medium containing with l-glutamine and buffered to pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid (MOPS) . The spores

were added to RPMI 1640 medium containing serially diluted HMAQs and the plates were incubated for two days at rt. The OD_{600} was measured using a Cytation 3 plate reader.

5.7.22. Measurement of *hmqA-lacZ* Activity

As previously described by Chapalain, Groleau, Le Guillouzer, Miomandre, Vial, Milot and Déziel [39] the levels of expression from the *hmqABCDEFG* promoter were assessed using *B. ambifaria* HSJ1 strains carrying the chromosomal *hmqA-lacZ* transcriptional fusion. The β-galactosidase assays were performed as previously described [62]. HMAQs were added to cultures to a final concentration of 50 µM from stocks prepared in DMSO. DMSO was added as a control.

5.8. References

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6. PHENOTYPIC VARIATION IN *BURKHOLDERIA AMBIFARIA*: TWO GROUPS OF COLONY VARIANTS, ONE DEFINED BY GENOME REDUCTION, AND THE OTHER BY DNA METHYLATION

Variation phenotypique chez *Burkholderia ambifaria* : deux groupes de variants de colonies, un défini par une réduction génomique, l'autre par la méthylation de l'ADN

Auteurs :

Pauline M.L. Coulon & Eric Déziel*

Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique (INRS), Laval, QC, Canada

Titre de la revue ou de l'ouvrage :
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Lien entre l'article ou les articles précédents et le suivant :

Les Bcc et surtout des souches cliniques de *B. ambifaria* ont été rapportées pour posséder l'opéron *hmqABCDEFG* et produire des HMAQs. Parmi ces souches cliniques certaines sont capables de produire des variants phénotypes leur permettant de retrouver des propriétés retrouvées au sein des souches environnementales dont la perte de production de HMAQs. En réalisant le criblage expérimentale des Bcc précédent (Chapitre 4), j'ai découvert que chez 6 des 8 variants chez *B. ambifaria,* la perte de production des HMAQs est liée à l'absence de l'opéron hmqABCDEFG en testant les amorces ciblant les gènes *hmqA* et *hmqG* (données figurant dans ce chapitre). L'opéron *hmqABCDEFG* des deux autres variants est toujours présent malgré la perte de production des HMAQs – impliquant une régulation du système Hmq dont le mécanisme pourrait être impliqué dans la perte de production d'autres types de métabolites secondaires. Cet article complète la caractérisation des phénotypes liés aux deux différents types de variants et l'étude du mécanisme impliqué dans la variation de phase chez le variant gardant son pc3.

6.1. Abstract

The *Burkholderia* genus comprises plant-beneficial (mostly *Paraburkholderia*) and pathogenic species (such as the "*pseudomallei*" group and *Burkholderia cepacia* complex [Bcc]). *Burkholderia* use phenotypic variation to readily adapt to environmental changes. Most studies reported that colony variation in *Burkholderia pseudomallei* is due to genomic variation or modulation of gene expression induced by common transcriptional regulators. A few studies have reported that phase variation is observed in Bcc by losing their third replicon or virulence mega plasmid (pc3). Interestingly, in this study, we report that not all *Burkholderia ambifaria* variants have lost their pc3 – and that two types of variant of *B. ambifaria exist*: (1) pc3-null that have lost their pc3 and (2) pc3-positive by keeping their c3. This discovery suggests that another mechanism is involved in colony morphotype variation in *B. ambifaria*.

We first investigated differences in phenotypes of *B. ambifaria* CEP0996 wildtype (WT) and pc3-null variant, and *B. ambifaria* HSJ1 WT and pc3-positive variant. Then, we compared the proteome of colony variants of these two *B. ambifaria* in order to complete their phenotypic characterisation.

Phase variation is generally due to genomic variations, modulation of gene expression or by epigenetic factors. The difference of production of virulence factors, involved in phase variation, in *B. ambifaria* CEP0996 pc3-null and HSJ1v was not related to a difference of production of QS signal molecules (controlling virulence factors) nor genomic variations. Thus, we hypothesized that DNA methylation – main epigenetic factor studied in bacteria - could regulate phase variation and we studied the methylome of both *B. ambifaria* HSJ1 wildtype and variant. We found the genomic DNA of the latter to be more methylated than its WT parent strain.

6.2. Introduction

The main phylogenetic groups of the *Burkholderia* genus are mostly divided between plant-beneficial and pathogenic species (Eberl & Vandamme, 2016). The Burkholderia pathogenic group comprises (i) plant pathogens (such as *B. gladioli* and *B. glumae Burkholderia* pathogenic group and *Burkholderia andropogonis,* the causative agent of leaf streak on Sorghum) (Azegami *et al.*, 1985; Ham *et al.*, 2010; Nandakumar *et al.*, 2009; Ramundo & Claflin, 2005), (ii) "*pseudomallei*" group (with the causative agent of melioidosis: *B. pseudomallei*, the avirulent model: *B. thailandensis* and the causative agent of glanders: *B. mallei* [Chaowagul *et al.*, 1989; Howe, 1950; Sandford, 1990]) - and finally (iii) opportunistic pathogens *Burkholderia cepacia* complex (Bcc, comprising at least 26 different species [Bach *et al.*, 2017; Eberl & Vandamme, 2016; Martina *et al.*, 2017; Weber & King, 2017]).

There is interest in exploiting features of Bcc bacteria such as: (i) secondary metabolite production, including antibiotics (Depoorter *et al.*, 2016), (ii) biocontrol of phytopathogens and plant growth-promoting properties (e.g., *B. ambifaria* protects *Pisum sativum* against *Pythium* and *Aphanomyces* [Mullins *et al.*, 2019; Parke, 1991]) and (ii) bioremediation (e.g., degradation of trichloroethylene by *B. vietnamiensis* [Gillis *et al.*, 1995; O'Sullivan & Mahenthiralingam, 2005; reviewed by Vial *et al.* [2011]]). However, biotechnological applications with Bcc have been prohibited due to their emergence as opportunistic pathogens among specific immunocompromised individuals (e.g., with the chronic granulomatous disease) and people with cystic fibrosis (CF) - causing the 'cepacia syndrome' and leading to death due to the high intrinsic resistance of *Burkholderia* to clinically relevant antibiotics (Gilligan, 1991; Govan & Deretic, 1996; Speert *et al.*, 1994).

Variations in colony morphotype have been reported for *Burkholderia* species – mostly in *B. pseudomallei*. Variants do not only differ in colony/cell morphology but also in biochemical sensitivity or utilization, and virulence (Agnoli *et al.*, 2011; Rondeau *et al.*, 2019; Shea *et al.*, 2017; Vial *et al.*, 2009; Wikraiphat *et al.*, 2015). Variants of the same strain can switch from one morphotype to another – a phenomenon called phase variation. Phase variation is described as a mechanism of environmental adaptation (Vial *et al.*, 2009) is generally due to genomic variations (e.g.: single-nucleotide polymorphism (SNP), insertion-deletions (indels) or sequence inversion) (Shea *et al.*, 2017), modulation of gene expression (Austin *et al.*, 2015; Bernier *et al.*, 2007; O'Grady & Sokol, 2011; Subramoni *et al.*, 2011) or by epigenetic factors - mainly DNA methylation in bacteria (Sánchez-Romero *et al.*, 2015; Wikraiphat *et al.*, 2015).

In the Bcc, phase variation has been mostly described among clinical strains for switching to a phenotype better adapted to the rhizosphere by regulating their virulence factors (Agnoli *et al.*, 2011; Shea *et al.*, 2017; Vial *et al.*, 2009; Wikraiphat *et al.*, 2015). Interestingly, *B. cenocepacia* H111 generates variants which have lost their megaplasmid of virulence (pc3). When other Bcc species were cured of their pc3, it resulted in phenotypes associated with environmental isolates (Agnoli *et al.*, 2011): In *B. cenocepacia* H111 pc3-null missing ShvR (located on the c3), biofilm formation, protease production and colony morphotype were affected: *B. cenocepacia* H111 wildtype has a rough colony morphotype, produces more biofilm and less proteases than *B. cenocepacia* H111 Δ*shvR* which has a smooth colony morphotype (O'Grady *et al.*, 2010; Subramoni *et al.*, 2011). Recently, it was demonstrated that DNA methylation plays a role in (i) the stability of the pc3 by showing that pc3-null variants are more frequent in adenosine DNA methyltransferase (DNA MTase) mutants of *B. cenocepacia* J2315 and K56-2 (Mannweiler *et al.*, 2020); and (ii) the ON/OFF regulation of transcriptional regulators and virulence functions such as biofilm, cell aggregation, motility and virulence (Vandenbussche *et al.*, 2020).

In the present study, we report that *B. ambifaria* produces two groups of colony morphotypes including *B. ambifaria* CEP0996 pc3-null where the pc3 has been lost, as already previously described in *B. cenocepacia* species (Agnoli *et al.*, 2011). We first investigated the proteome of colony variants of two *B. ambifaria* in order to complete their phenotypic characterisation. We found that the transcriptional regulator “Shiny Colony Variation Regulator” (ShvR), which controls colony morphology, virulence factors (such as biofilm formation and the production of antimicrobial molecules), in *B. cenocepacia* K56-2 (Bernier *et al.*, 2007; Gomes *et al.*, 2018; O'Grady *et al.*, 2010; O'Grady & Sokol, 2011; Subramoni *et al.*, 2011) was underproduced in *B. ambifaria* HSJ1v. Then, we demonstrated that lower levels of ShvR in *B. ambifaria* HSJ1v were not related to a difference of AHLs production nor SNPs or genomic variations. Thus, we hypothesized that DNA methylation could regulate phase variation and we studied the methylome of both *B. ambifaria* HSJ1 wildtype and variant. We found the latter to be more methylated by comparing phenotypes and transcriptome of DNA MTases to the wild type in *B. ambifaria* HSJ1v background. Results suggest that DNA MTases play a role in phase variation or regulation

6.3. Material and Methods

6.3.1. Screening for the presence of the third chromosome in wild type and variants

All primers and strains are listed in **Table 6.1-2**. Overnight cultures were set up from -80°C glycerol stocks in tryptic soy broth (TSB) at 30°C under shaking at 57 rpm. DNA was extracted from 1mL culture following a previously described method using Fastprep technique with beads (Durand *et al.*, 2015). PCR amplification using EasyTaq DNA polymerase (Transgen) with specific primers targeting the *hisA* gene which is present on the first chromosome and seven different genes present on the pc3 in *B. ambifaria* (**Table 6.3**).

Table 6.1 List of strains used in this article

Strains	Reference
B. ambifaria CEP0516	Coenye *et al.* (2001)
B. ambifaria CEP0516 pc3-null	Vial *et al.* (2009)
B. ambifaria CEP0617	Coenye *et al.* (2001)
B. ambifaria CEP0617 pC3-null	Vial *et al.* (2009)
B. ambifaria CEP0958	Coenye *et al.* (2001)
B. ambifaria CEP0958 pC3-null	Vial *et al.* (2009)
B. ambifaria CEP0996	Coenye *et al.* (2003)
B. ambifaria CEP0996 pC3-null	Vial *et al.* (2009)
B. ambifaria AU4157	J. Lipuma
B. ambifaria AU4157 pC3-null	Vial *et al.* (2009)
B. ambifaria AU8235	J.Lipuma
B. ambifaria AU8235 pC3-null	Vial *et al.* (2009)
B. ambifaria HSJ1	Vial *et al.* (2008)
B. ambifaria HSJ1v	Vial *et al.* (2009)
B. ambifaria AU0212	Payne *et al.* (2005)
B. ambifaria AU0212v	Vial *et al.* (2009)
B. ambifaria AMMD	Coenye *et al.* (2001)
B. ambifaria PHP7	Coenye *et al.* (2001)
B. ambifaria HI3590	J. Lipuma
B. ambifaria HI3687	J. Lipuma
B. ambifaria IOP40-10	J. Tiedje
B. ambifaria MW2073	

B. ambifaria LMG17828	Coenye *et al.* (2001)
B. ambifaria HSJ1 Δ*shvR*	This study
B. ambifaria HSJ1 Δ*shvR* + pMLC45	This study
B. ambifaria HSJ1 Δ*shvR* + pMLS7	This study
B. ambifaria HSJ1v + pMLC45	This study
B. ambifaria HSJ1 + pMLC45	This study
B. ambifaria HSJ1 ΔHSJ1_RS14940	This study
B. ambifaria HSJ1v ΔHSJ1_RS14940	This study
B. ambifaria HSJ1 ΔHSJ1_RS14940 + XXX	This study
B. ambifaria HSJ1v ΔHSJ1_RS14940 + XXX	This study
B. ambifaria HSJ1 ΔHSJ1_RS14940 + pMLS7	This study
B. ambifaria HSJ1v ΔHSJ1_RS14940 + pMLS7	This study
B. ambifaria HSJ1 ΔHSJ1_RS26905	This study
B. ambifaria HSJ1v ΔHSJ1_RS26905	This study
B. ambifaria HSJ1 ΔHSJ1_RS26905 + XXX	This study
B. ambifaria HSJ1v ΔHSJ1_RS26905 + XXX	This study
B. ambifaria HSJ1 ΔHSJ1_RS26905 + pMLS7	This study
B. ambifaria HSJ1v ΔHSJ1_RS26905 + pMLS7	This study

Table 6.2 List of plasmids used in this article

Plasmids	**Reference**
pUC18 miniT7 gfp tp mut3	Choi and Schweizer (2006)
pMLS7	Lefebre and Valvano (2002)
pMLC45 (pMLS7+*shvR*)	This study

Table 6.3 List of primers used in this article

Name	Sequence	Function	Reference
*hisA*_Bcc_F	GGTCGACCTGAACGGCGC	Located on the c1	Papaleo *et al.* (2010)
*hisA*_Bcc_R	CGTCGGTCGCGACCTTGCC		Papaleo *et al.* (2010)
*hmqA*_Bcc_F	CCGCTCGCGTT**Y**ACGTT**Y**GG	*Amplification of hmqA gene in Bcc - degenerated*	Coulon *et al.* (2020)
*hmqA*_Bcc_R	CCCGTCAGGTTCCAGCCG		Coulon *et al.* (2020)
*hmqG*_Bcc_F	GGCGTCGCAGGAAATCACG	Amplification of *hmqG* gene in Bcc - degenerated	Coulon *et al.* (2020)
*hmqG*_Bcc_R	CGCGACACGAA**R**TGCATGCC		Coulon *et al.* (2020)
BAMB_RS28665_F	CGAATCCAGTTTCGTCGAGC	Molybdopterin binding aldehyse oxidase and xanthine dehydrogenase	This study
BAMB_RS28665_R	CCTGTCGAGATGCCAGGC		This study
BAMB_RS29710_F	CGAGCATCATCGACTTCGTG	Phenyldantoinase	This study
BAMB_RS29710_R	GAGAAGCTGTTCACGAGCAC		This study
BAMB_RS29740_F	CGAGGACGCATACGACAAGG	AcetylCoA acetyl transferase	This study
BAMB_RS29740_R	CGTCGGAATCTTGTCGAGCT		This study
BAMB_RS31530_F	GACTACACGCTCGACCGC	aldose 1 epimerase	This study
BAMB_RS31530_R	GATTCGTCAGGTTCAACGGC		This study
*ndh*_deb_F	GCTCGGCTACGACGATCT	Reference gene for RT-PCR	Coulon *et al.* (2020)
*ndh*_deb_R	GGCCTGGTCGAGGTTTTC		Coulon *et al.* (2020)
*hmqA*_RTPCR_F	CTTGCCCCCTGCCGAAGATT	Expression of *hmqA* by RT-qPCR	Coulon *et al.* (2020)
*hmqA*_RTPCR_R	CGGCGCAATTGAGAAACGG		Coulon *et al.* (2020)
HSJ1_31950_qPCR_F	GCGCCTGATCTGTTTTCCCT	Expression of oleoyl-ACP hydrolase - Occidiofungin cluster	This study
HSJ1_31950_qPCR_R	CGAAGGGACGATCGAAGCAG		This study
HSJ1_RS14940_qPCR_F	GGCAAGGTGAAGCTCGTCT	Expression of adenosine DNA MTase	This study
HSJ1_RS14940_qPCR_R	TCAGGCGCGGATACATCATG		This study
HSJ1_RS26905_qPCR_F	CCGCGATTTCATGACCGAAG	Expression of cytosine DNA MTase	This study
HSJ1_RS26905_qPCR_R	AGAAGATGTACATCGACCCGC		This study
*shvR*_qPCR_F	CAAAACGGTTGCGAGGGATG	Expression of LysR regulator ShvR - Afc lipopeptide cluster	This study
*shvR*_qPCR_R	GGTCAATGTCAGCGAACGGA		This study
*shvR*_overexpression_F	AGCTAGGATCCATGGCGAATGTGAGA TTGGCAA	Overexpression of shvR using pMLS7 backbone digested by BamHI and HindIII	This study
*shvR*_overexpression_R	CTAGACAAGCTTCTAGCCGACGCGAT ACAGCG		This study
pMLS7_Tp_F	ACCTTTGGCTTAGGGGATCG	Trimethoprim resistance gene	This study
pMLS7_Tp_F	TTCAAGTGCAGCCACAGGAT		This study

6.3.2. Whole Genome Sequencing and representation

WT and variants of *B. ambifaria* CEP0996 (ED332) and *B. ambifaria* HSJ1 (ED336) were set up as overnight cultures from -80°C glycerol stock in TSB at 30°C under shaking at 57 rpm. Genomic DNA (gDNA) was extracted from 1 mL of culture using the Genomic DNA extraction kit (Transgen). *B. ambifaria* CEP0996 was sequenced using Oxford Nanopore Technology (ONT) and *B. ambifaria* HSJ1 wildtype and variant genomes were sequenced using Pacific Biosciences technology (PacBio), and all three were also sequenced using Illumina technology. *B. ambifaria* CEP0996 pc3-null was sequenced by only using Illumina technology.

Wild type genomes were assembled using the Flye plugin on Geneious software while the Illumina reads were trimmed, paired and mapped on assembly - obtained from long reads sequencing - using the Bowtie2 plugin both available in the Geneious software [(Kolmogorov *et al.*, 2019; Langmead & Salzberg, 2012); Geneious Prime 2020.1.2 (https://www.geneious.com)]. WT genomes were then annotated using the annotation transfer function with 80% similarity from *B. ambifaria* AMMD reference by adjusting coding DNA sequences (CDS) boundaries and by setting the nucleotide search at 15 and by manually adding missing “open reading frame” (orf). Then reads from *B. ambifaria* CEP0996 pc3-null and *B. ambifaria* HSJ1v Illumina sequencing were mapped on their corresponding wildtype genomes. Coverage of reads for each chromosome was represented using one every 100 nucleotides using Shinycircos R package (Yu *et al.*, 2018).

Variations and SNPs between the DNA of *B. ambifaria* HSJ1 WT and variant strains were identified inside and outside CDS using the default settings of Geneious plugin.

6.3.3. Proteomic Analysis

Colonies were isolated on tryptic soy agar plates containing 0.1% Congo Red (CRTSA) incubated at 30°C for 48h. – Cultures for both colony-morphotype of *B. ambifaria* CEP0996 and *B. ambifaria* HSJ1 were set up for overnight culture in TSB at 30°C under shaking at 57 rpm from three replicates of a single colony. Fresh cultures were set up at an OD_{600} of 0.05 in 5 mL of TSB until the OD_{600} reached 1 at 30°C under shaking at 57 rpm. Total proteins were extracted as previously described (Mohamed *et al.*, 2019). Briefly, cell pellets were washed three times with phosphate-buffered saline (PBS) and centrifuged at 10,000 x g at 4°C then frozen in liquid nitrogen. Frozen cells were suspended in Lysis Buffer (4% SDS, 100mM Tris pH8 and 20 mM DTT) and boiled for 10 min at 95°C at 1800 rpm in a shaking drybath (Thermofisher). After centrifugation 10 min at 17,000 xg, the supernatant was mixed with four volumes of ice-cold acetone and kept overnight at -20°C. After a final centrifugation at 17,000 x g for 10 min, protein pellets were resuspended in 80% ice-cold acetone for 4 additional hours. Finally, protein pellets were put at 65°C to remove excess acetone.

Samples were analysed by LC-MSMS on an Orbitrap Fusion Tribrid system (Thermo) using the label-free technique at the Proteomic Platform of the CHU Québec Research Center (Université de

Laval, Quebec City, Canada). The raw data were treated by MaxQuant software using the settings for label-free quantification (Cox & Mann, 2008). Identification of proteins was made using *B. ambifaria* CEP0996 and *B. ambifaria* HSJ1 protein annotations obtained from genomic analysis described in this paper. Relative label-free quantification of proteins and normalization were set up to run the MaxQuant analysis. Data from *B. ambifaria* CEP0996 variant analysis were treated by removing “by matching” identification for the proteins encoded on the pc3 replicon. Then data were analyzed using Perseus to filter, normalize by subtracting the most frequent values and inpute missing values based on a normal distribution (with downshifting of 2.5σ with a spread of 0.3σ) – except when three missing values were detected in one condition for *B. ambifaria* CEP0996 which were replaced by a constant value based on the lower value = -11.13870621 (Tyanova *et al.*, 2016). The differential protein production between WT and their variant was analyzed by using a |z-score| > 1.5 and a Welch’s t-test with Limma adjusted p-values (Coombs *et al.*, 2010; Kammers *et al.*, 2015). It is important to note that a t-test was also performed for the data where the three missing values in one condition were replaced by a constant even if they are not normally distributed because the Wilcoxon test needs at least 9 replicates (Klammer *et al.*, 2014).

6.3.4. Phenotypic Assays

In this study, we determined colony morphotype, biofilm production, swimming motility, production of siderophores, AHLs and HMAQs as previously described (Chapalain *et al.*, 2013; Vial *et al.*, 2008).

Briefly, morphotypes of strains were obtained by spotting 5 µL of TSB overnight culture on 0.1% Congo red tryptic soy agar (CRTSA) plates. Plates were incubated two days at 30°C.

Biofilm production was realized in 96-well plates by inoculating 200 µL TSB with an overnight culture to a starting OD_{600} = 0.01. Five wells were used for each condition. Plates were incubated at 30°C for 14h and 24h. After incubation, OD_{600} was read using a plate reader (Cytation 3, BoiTek). Then, cultures were removed and rinsed twice with distilled water. When dried, the biofilms were stained by adding a 0.1% crystal violet solution for ten minutes, then rinsed four times with distilled water. When dried, the dye was suspended in 95% ethanol and the OD_{590} was read using a microplate plate reader (Cytation 3, BioTek).

Swimming motility assay was performed on LB plates solidified with 0.25% agar. When plates were dried, a bacterial culture adjusted to OD_{600} = 5 was inoculated at the center of each plate by using a toothpick which was immerged in the bacterial suspension. Plates were inoculated overnight at 30°C or at room temperature. Swimming areas were then measured and converted in cm^2. The experiment was performed using 5 replicates and repeated at least twice.

Siderophore production was determined by Chrome-Azurol S (CAS) assay (Lynne *et al.*, 2011). A 5 µL bacterial culture at OD_{600} = 5 was spotted on CAS plates - in five technical replicates. Plates

were incubated one overnight at 30°C. Siderophore production halos were then measured and converted in cm^2.

Bacterial flagella were observed by transmission electron microscopy (TEM). *B. ambifaria* CEP0996 strains were culture for 48h on CRTSA plates and *B. ambifaria* HSJ1 strains were cultured in 5 mL TSB at 30°C overnight with agitation. A grid was put on individual colonies or inoculated into the liquid culture for 30 seconds and dried for 1 min. Then, 1% paraformaldehyde solution (PFA) was used to stain cells for 1s.

AHLs and HMAQs were extracted - from 4 mL culture supplemented 4 ppm 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4, Sigma) used as an internal standard - with ethyl acetate and concentrated 10 times before resuspending the extracts into acetonitrile (Vial *et al.*, 2008). Quantifications were performed using an LC-MS in positive electrospray ionization using a Kinetex 5 µM EVO C18 100 Å 100x3 mm reverse phase column as previously described (Vial *et al.*, 2008) . A Quattro Premier XE triple quadrupole mass spectrometer 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 AHLs and HMAQs (Vial *et al.*, 2008). This experiment was conducted with three independent biological replicates.

6.3.5. qRT-PCR analyses

Total RNA was extracted from OD_{600} = 1 cultures grown in TSB, using TransZol (TransGen Biotech, Canada) by following the manufacturer's instruction with a supplemental chloroform wash and an RNA precipitation step with ethanol and sodium acetate to completely remove the remaining isopropanol from RNA. Residual DNA was removed using the Turbo DNAse (Thermo Fisher, Canada). Reverse-transcription was performed using the iScript kit (Bio-Rad, Canada). The expression of targeted genes was determined by qPCR and using *ndh* as reference gene (**Table 6.3**; [Subsin *et al.*, 2007]). Log_2 fold-change is represented, and t-test was made on the ΔCT values. The analysis was performed on three biological replicates.

6.3.6. Overexpression of *shvR* in *B. ambifaria* HSJ1v

The HSJ1_RS30385 gene was amplified using EasyTaq (Transgen) and by adding BamHI and HindIII restriction sites in primers. pMLS7 plasmid was digested by BamHI and HindIII enzymes. Both resulting PCR fragment and digested plasmid were purified on an agarose gel and ligated using the T4 DNA ligase (NEB). The resulting plasmid pMLC45 was transformed in *E. coli* χ7213 which was used to transfer the plasmid into *B. ambifaria* HSJ1v by conjugation.

6.3.7. DNA methylome of *B. ambifaria* HSJ1 wild type and variant

Single-Molecule Real-Time (SMRT) was used to determine the methylome of *B. ambifaria* HSJ1 WT and variant strains with PacBio data sequencing. Data were first analyzed using Base Modification and Motifs Analysis pipeline using KineticTools (https://github.com/PacificBiosciences/kineticsTools) and MotifMaker (https://github.com/PacificBiosciences/MotifMaker) with a modification QV threshold of 30. Based on the mean of scores a cut-off of 60 was used for adenine modifications. Then data were visualized using Geneious Prime 2020.1.2 (https://www.geneious.com)]. The methylome in both *B. ambifaria* HSJ1 WT and variant strains were compared based on the motif CAC**A**G and G**T**WW**A**C, m6A and m4C putatively methylated by probable methyltransferases encoded by HSJ1_RS14940 and HSJ1_RS26905, respectively determined by blasting genes available on Rebase databased PacBio analyses (http://rebase.neb.com/cgi-bin/pacbiolist?B).

6.3.8. Inhibition of the DNA methylation assay

Inhibition of the DNA methylation of *B. ambifaria* HSJ1 was achieved following a protocol using sinefungin, as reported by Vandenbussche *et al.* (2020). Briefly, after determining the effect of the sinefungin on growth and its minimal inhibitory concentration (MIC≥ 200 µg/mL) on *B. ambifaria* HSJ1 and variant HSJ1v, we added 50 µg/mL in cultures to investigate the sinefungin effect on phase variation (Vandenbussche *et al.*, 2020).

B. ambifaria HSJ1 and its variant HSJ1v were cultured from colonies obtained on CRTSA after 2 days at 30°C. Individual colonies served to each inoculate one well of a 96-well plate containing 100 µL TSB supplemented or not with 50 µg/mL sinefungin in six replicates for *B. ambifaria* HSJ1 and three replicates for *B. ambifaria* HSJ1v. The plates were then incubated at 30°C with 250 rpm shaking for 24h. The next day, 1 µL of the previous culture was transferred to a new well containing fresh medium with or without sinefungin, – and this was repeated for ten days.

6.4. Results

6.4.1. Clinical *B. ambifaria* generates two types of variants, including those which have lost its third chromosome (pc3)

During our recent screenings for the prevalence of the *hmqABCDEFG* operon in the Bcc (Coulon *et al.*, 2019; Coulon *et al.*, 2020) we noticed that in six out of eight *B. ambifaria* phenotypic variants, we were unable to amplify the *hmqABCDEFG* operon, in contrast with their corresponding WT strains. Indeed, it was impossible to amplify both the *hmqA* (BAMB_RS28725) *and hmqG* (BAMB_RS28755) genes in these variants. This was interesting as we had previously reported that phenotypic variants of *B. ambifaria* generally lose the ability to produce HMAQs (Vial *et al.*, 2009).

Since phenotypic variants of another Bcc species, *B. cenocepacia,* have been reported when their pc3 replicon is absent (Agnoli *et al.*, 2011), we asked whether *B. ambifaria* might similarly lose its pc3. Indeed, five other genes (BAMB_RS28665, BAMB_RS29710, BAMB_RS29740, BAMB_RS31530, BAMB_RS30185) located on the pc3 - based on *B. ambifaria* AMMD genome - could not be amplified. Only the variants of strains *B. ambifaria* HSJ1 and *B. ambifaria* AU0212 retained the seven above genes, although they could not produce HMAQs either (**Table 6.4**; [Vial *et al.*, 2009]).

Table 6.4 Phenotypic variants from eight clinical B. ambifaria investigated to verify the loss of the third chromosome (pc3)

B. ambifaria Strains	BAMB_RS01790 (*hisA*)	BAMB_RS28725 (*hmqA*)	BAMB_RS28755 (*hmqG*)	BAMB_RS28665	BAMB_RS29710	BAMB_RS29740	BAMB_RS31530	BAMB_RS30185	Classification
CEP0516	+	-	-	+	+	+	+	+	WT - clinical
CEP0516 variant	+	-	-	-	-	-	-	-	pc3-null
CEP0617	+	+	+	+	+	+	+	+	WT - clinical
CEP0617 variant	+	-	-	-	-	-	-	-	pc3-null
CEP0958	+	+	+	+	+	+	+	+	WT - clinical
CEP0958 variant	+	-	-	-	-	-	-	-	pc3-null
CEP0996	+	+	+	+	+	+	+	-	WT - clinical
CEP0996 variant	+	-	-	-	-	-	-	-	pc3-null
AU4157	+	+	+	+	+	+	+	+	WT - clinical
AU4157 variant	+	-	-	-	-	-	-	-	pc3-null
AU8235	+	-	-	+	+	+	+	+	WT - clinical
AU8235 variant	+	-	-	-	-	-	-	-	pc3-null
HSJ1	+	+	+	+	+	+	+	+	WT - clinical
HSJ1 variant	+	+	+	+	+	+	+	+	pc3-positive
AU0212	+	+	+	+	+	+	+	+	WT - clinical
AU0212 variant	+	+	+	+	+	+	+	+	pc3-positive
AMMD	+	+	+	+	+	+	+	+	WT - environmental
PHP 7	+	-	-	-	-	-	-	-	WT - environmental
HI3590	+	-	-	+	+	+	+	-	WT - environmental
HI3687	+	-	-	+	+	+	+	+	WT - environmental
IOP40-10	+	-	-	+	+	+	+	+	WT - environmental
MW2073	+	-	-	-	-	-	-	-	WT - environmental
LMG17828	+	+	+	-	+	+	-	-	WT - environmental

To confirm our PCR results, we sequenced the genomes of *B. ambifaria* CEP0996 and *B. ambifaria* HSJ1 using long and short-read sequencing (**Figure 1**) and short-read sequencing for their respective variants. By mapping variant reads on the whole genome sequence of wild type strains, we confirmed that the variant of *B. ambifaria* CEP0996 had lost its c3 (making it a pc3-null strain;

Figure 1A) but not *B. ambifaria* HSJ1 variant (HSJ1v; making it a pc3-positive strain; **Figure 1B**). We also confirmed that *B. ambifaria* AU0212v had kept its pc3 like HJS1v by mapping its short reads sequences on the *B. ambifaria* AMMD genome, an environmental clone of *B. ambifaria* AU0212 (**Figure S1; [**Baldwin *et al.*, 2007]).

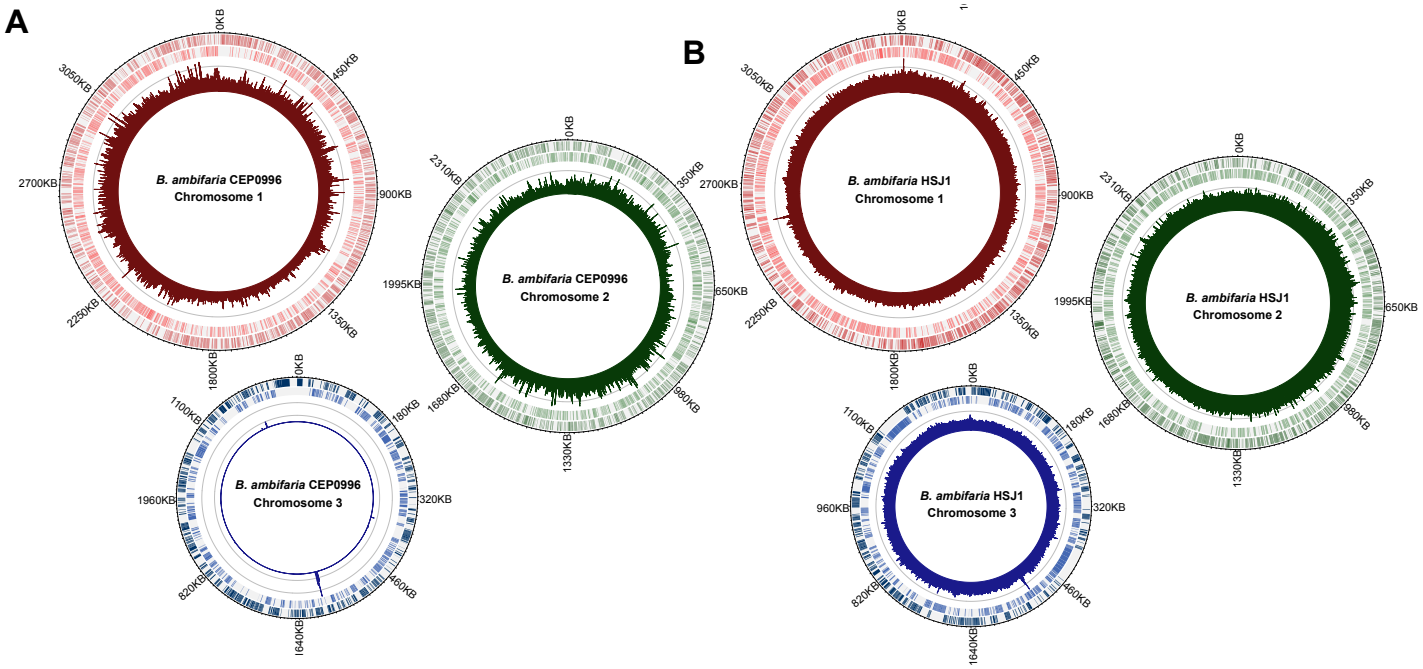

Figure 6.1 Genomic profiles of both types of *B. ambifaria* variants
Illumina reads from variant DNA were mapped to *B. ambifaria* CEP0996 assembly or *B. ambifaria* HSJ1 assembly genomes. The first inside circle represents gene coverage on the positive strand of DNA; the second circle brand represents genes on the negative strand of DNA; the innermost circle represents the coverage of the reads every 100 bp. A) *B. ambifaria* CEP0996 generates a phenotypic variant losing its pc3 replicon (total identified orf 5979). B) *B. ambifaria* HSJ1generates a phenotypic variant retaining its pc3 (total identified orf 6529).

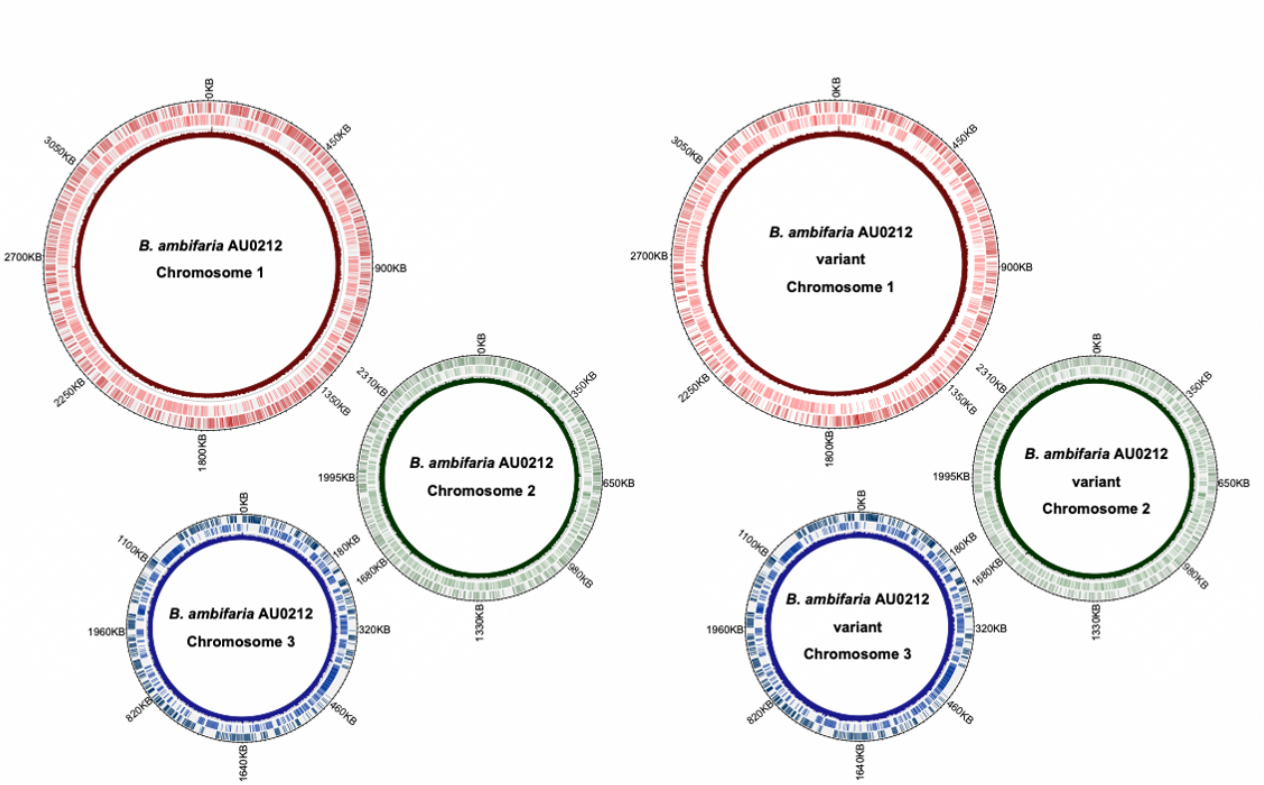

Figure 6.2 ***B. ambifaria*** **AU0212v also retains its pc3 replicon**
Illumina reads were mapped to *B. ambifaria* AMMD assembled genome using Geneious. The coverage of the reads is represented with Shinycircos available on R studio: the first inside circle represents genes on the positive strand of DNA; the second circle brand represents genes on the negative strand of DNA; the innermost circle represents the reads coverage every 100 bp.

6.4.2. The *B. ambifaria* HSJ1v phenotypes are not due to genomic variations

To understand the difference in regulation of *B. ambifaria* HSJ1 WT vs its variant, we first sequenced their genomes to determine the presence of single nucleotide polymorphisms (SNPs) and variations. When variations and SNPs were determined in the Illumina reads of *B. ambifaria* wild type aligned to *B. ambifaria* HSJ1v assembly, no SNPs were significantly observed, except one G->T at position 2,280,894 which is likely explained by low coverage of Illumina sequencing (**Table 6.5**). Thus, we tried to amplify the region of these SNPs by PCR in *B. ambifaria* HSJ1 to Sanger sequenced the obtained product, however, the specific region was not readily amplifiable, suggesting that this region is hard to target, compatible with the low coverage in Illumina reads.

Table 6.5 Sequence variations between *B. ambifaria HSJ1* wild type and variant

	Position	Modification		Variation frequency (%)	Approximative p-value	Genomic region
Chromosome 1						
C	334,574	M -> C	SNP	100	$6.3\ 10^{-26}$	Intergenic region HSJ1_RS14975 - HSJ1_RS14980
G	1,065,717	Y-> T	SNP	100	$4.0\ 10^{-34}$	Type VI secretion protein VasK HSJ1_RS02000
Chromosome 2						
*T	2,280,894	G -> T	SNP	100.00	$3.20\ 10^{-09}$	Peptide-binding protein HSJ1_RS17850
A	2,280,907	R -> A	SNP	100.00	$4.00\ 10^{-13}$	Peptide-binding proteinHSJ1_RS17850

*Low coverage of this area during Illumina sequencing

6.4.3. Phenotypic characterization shows that QS-regulated proteins are differentially produced in variants compared to wild type strains

We next compared the phenotypes between WT strains and their corresponding variants (Agnoli *et al.*, 2011; Vial *et al.*, 2009). Colony morphotype on CRTSA plates, biofilm production in 96 well-plates, motility on 0.25% agar plates, and siderophore production on CAS plates were assessed (**Figure 6.3A-D**). The colony morphotype is different for smooth white variants producing less EPS compared to the rough red wild types (**Figure 6.3A**) (Chung *et al.*, 2003). Interestingly, *B. ambifaria* CEP0996 pc3-null produces less biofilm than its WT while *B. ambifaria* HSJ1v formed less biofilm in the first 14h and then overproduced biofilm at 24h of growth compared to its WT, which could be explained by a difference in the maturation of biofilm (**Figure 6.3B**). Another difference between the variants is that *B. ambifaria* CEP0996 pc3-null is more motile than its WT while it is the opposite for *B. ambifaria* HSJ1v – which seems explained by a difference of the number of flagella (**Figure 6.3C; Figure 6.4**). Concerning siderophore production, both types of variant produce more siderophores than their corresponding WT strain (**Figure 6.3D**). Knowing that virulence factors and antibiotic production are controlled by QS in *B. ambifaria* (Chapalain *et al.*, 2013), we quantified the production of AHLs by LC-MS (**Figure 6.3E-F**). In *B. ambifaria* CEP0996 pc3-null, no production of 3-OH-C_{10}-HSL is detected, as expected, since the Cep2 QS system is located on the pc3 (Chapalain *et al.*, 2017). On the other hand, there is no significant difference in the AHLs production between *B. ambifaria* HSJ1 WT and its variant (**Figure 6.3F**). To verify the hypothesis that QS

signal molecules could restore the wild type phenotype in variants, we tested colony morphotype, siderophores and protease production, known to be affected in *B. ambifaria* HSJ1 *cepI*- mutant, by supplement the media with 2 ppm C_8-HSL and observed that WT phenotypes were not restored (**Figure 6.5**).

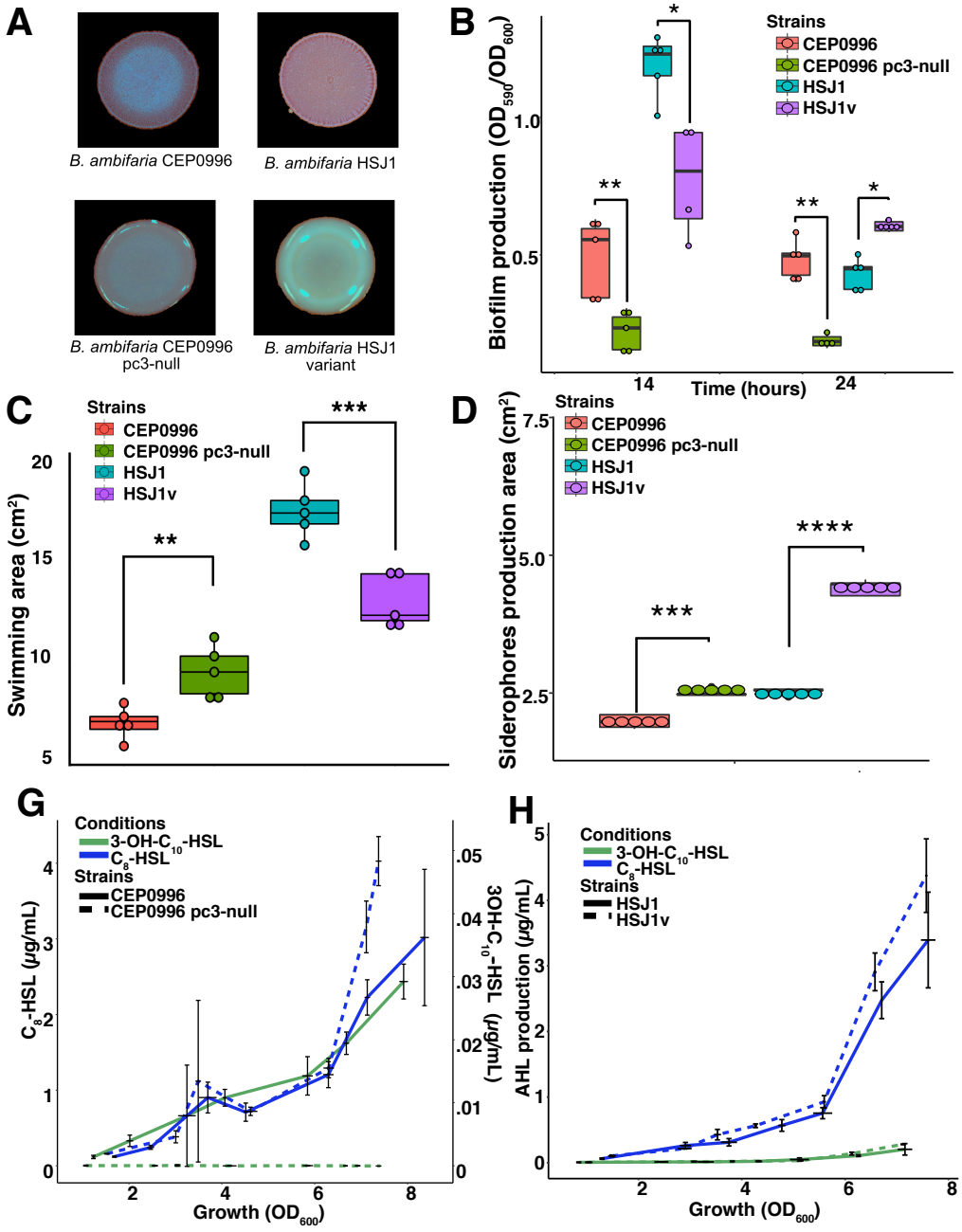

Figure 6.3 Phenotypic assays and quantification of AHLs in both types of variant compared to their respective wild type

A. Morphotypes on CRTSA B. Biofilm production (Wilcoxon signed ranks test) C. Swimming motility (t-test) D. Siderophore production (t-test) E. AHLs production in *B. ambifaria* CEP0996 F. AHLs production in *B. ambifaria* HSJ1. P-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, *** 0.001 and 0.0001, and **** inferior to 0.0001.

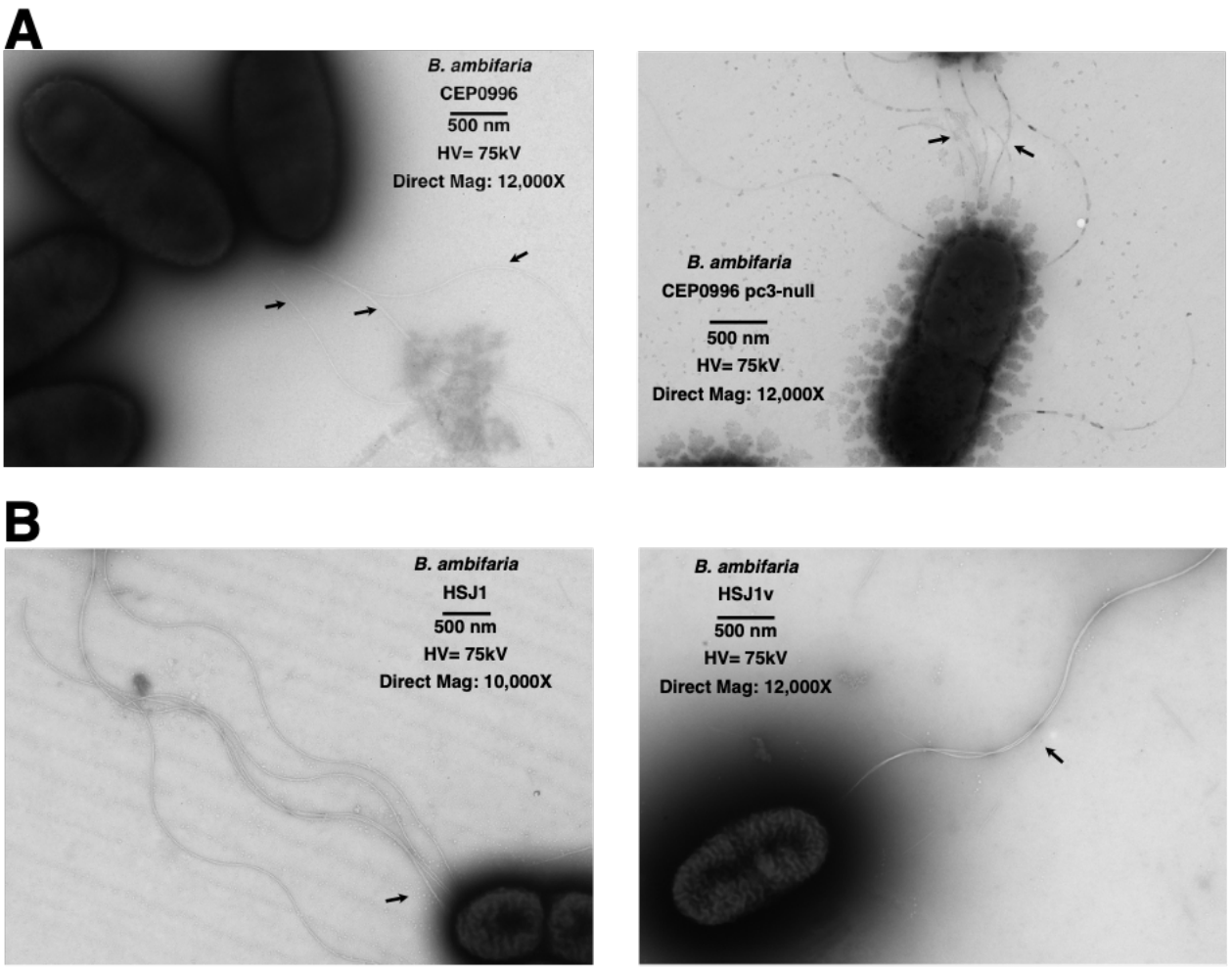

Figure 6.4 Electron microscopy showing the difference in number of flagella between the WT and variant of *B. ambifaria* strains CEP0996 and HSJ1

A. *B. ambifaria* CEP0996 WT produces less flagella than *B. ambifaria* CEP0996 pc3-null. B. *B. ambifaria* HSJ1 WT produces more flagella than *B. ambifaria* HSJ1v.

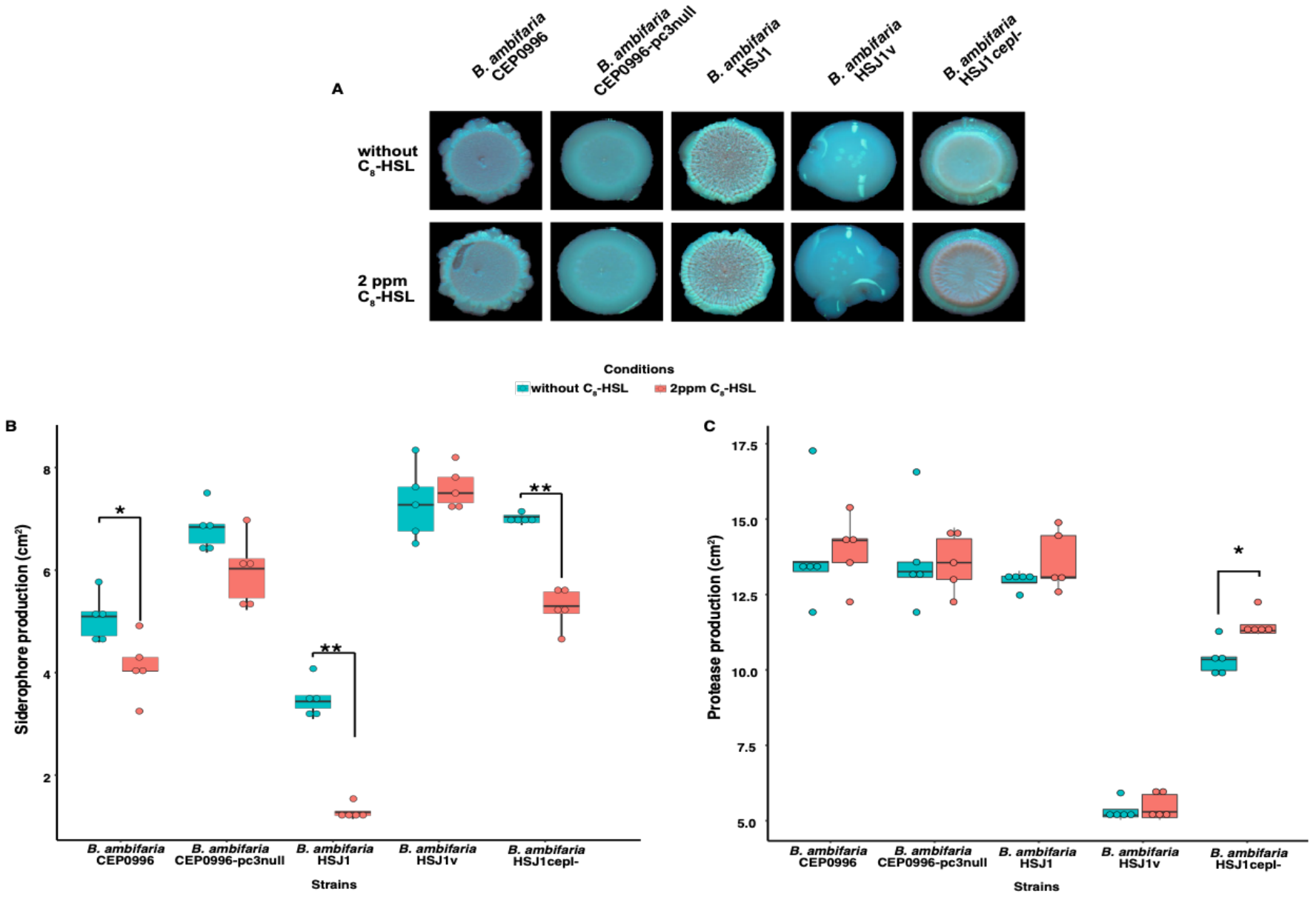

Figure 6.5 **Investigation of the impact of variation of AHLs production on characteristic phenotypes**

A) Colony morphotype on CRLA. B) Siderophore production on CAS plates. C) Protease production on skimmed milk plate. A Wilcoxon test was performed, and P-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, *** 0.001 and 0.0001, and **** inferior to 0.0001.

Since both *B. ambifaria* HSJ1v and AU02212v kept their pc3, in contrast with the other six variant strains, and since Vial et al. (2009) reported several phenotypes in common between both types of variants – such as loss of HMAQs production - we hypothesized that some genes and especially virulence factors located on the pc3 replicon are nevertheless downregulated or shut down in variants retaining their pc3. To tackle this question, we analyzed the proteomes of a *B. ambifaria* CEP0996 WT reference strain and its pc3-null variant, and *B. ambifaria* HSJ1 wildtype and its pc3-positive variant. At OD_{600} = 1, 89% of 3319 identified proteins for *B. ambifaria* CEP0996 and 100% of 3388 identified proteins for *B. ambifaria* HSJ1 were quantified (raw data). Based on a z-score of 1.5 and a Welch's t-test with Limma p-value adjustment, 222 identified proteins were significantly downregulated, with a total of 173 proteins normally encoded on the pc3 replicon, and 48 were upregulated on other chromosomes of *B. ambifaria* CEP0996 pc3-null compare to its WT (raw data). Meanwhile, in *B. ambifaria* HSJ1v, 63 proteins were downregulated, including 45 encoded on pc3 – and 27 proteins were upregulated (raw data).

The identified proteins in common, only for genes present on both genomes, between *B. ambifaria* CEP0996 and *B. ambifaria* HSJ1 belong to primary metabolism, antibiotic biosynthesis clusters, virulence factors and plant growth-promoting factors (raw data**; Table 6.6**). Interestingly, antibiotic biosynthesis clusters, such as the AFP lipopeptide, occidiofungin and the most recently confirmed HMAQs (Piochon *et al.*, 2020), were downregulated in at least one of the type of variants; while virulence factors – such as adhesins, exopolysaccharides (EPS), secretion system and siderophores – were upregulated.

Table 6.6 Main biosynthesis gene clusters differently regulated in the *B. ambifaria* variants

Locus tag (CEP0996)	Locus tag (HSJ1)	Putative function	Log2Fold *B. ambifaria* CEP0996 (pc3-null variant vs WT)	Log2Fold *B. ambifaria* HSJ1 (variant vs WT)	Localization
Oxidation-reduction process					
CEP0996_RS14600	HSJ1_RS15930	Carotenoid 9 10-9' 10' cleavage dioxygenase	-1	**-2**	C1
CEP0996_RS2825	HSJ1_RS1960	1,3-ketoacyl-ACP reductase	0	**-4**	C1
CEP0996_RS2820	HSJ1_RS1955	Acetyl-CoA acetyltransferase	0	**-3**	C1
CEP0996_RS22955	HSJ1_RS26395	Asparaginase	**-5**	**-2**	C2
CEP0996_RS25355	HSJ1_RS23930	Amidohydrolase	**-8**	NQ	C2
NA	HSJ1_RS23345	Hydroxylamine reductase	NA	**3**	C2

CEP0996_RS17640/ CEP0996_RS17645	HSJ1_RS20200	FAD-dependent oxidoreductase CDS	0	**-9**	C2
CEP0996_RS19120	HSJ1_RS18770	4-carboxymuconolactone decarboxylase	NQ	**2**	C2
CEP0996_RS21555	HSJ1_RS16505	Catechol 1,2-dioxygenase	**4**	**2**	C2
CEP0996_RS21575	HSJ1_RS16485	(2Fe-2S)-binding protein	**5**	**3**	C2
CEP0996_RS21580	HSJ1_RS16480	Anthranilate 1,2-dioxygenase small subunit	**6**	**5**	C2
CEP0996_RS28600	HSJ1_RS29135	(2Fe-2S) ferredoxin	NQ	**4**	C3
CEP0996_RS28410	HSJ1_RS29460	Allantoate amidohydrolase	**-7**	**-2**	C3
CEP0996_RS28135	HSJ1_RS29770	MBL fold metallohydrolase	NQ	**-2**	C3
CEP0996_RS30245	HSJ1_RS32430	Catechol 1,2-dioxygenase	NQ	**7**	C3
Fatty acid biosynthetic process					
NA	HSJ1_RS25695	Acyl carrier protein	NA	**2**	C2
Allantoin catabolic process					
CEP0996_RS13180	HSJ1_RS15610	Allantoicase	**-8**	NQ	C1
Amino acid transport					
CEP0996_RS19045	HSJ1_RS18845	Amino acid transporter	NQ	**3**	C2
Aspartate metabolic process					
CEP0996_RS22965	HSJ1_RS26385	Aspartate ammonia-lyase	**-8**	0	C2
Cellulose biosynthetic process					
CEP0996_RS9900	HSJ1_RS9785	Cellulose biosynthesis protein	**-6**	-1	C1
Ubiquinone biosynthetic process					
CEP0996_RS5000	HSJ1_RS4160	Chorismate—pyruvate lyase	**-6**	NQ	C1
Carbohydrate transport					
CEP0996_RS9660	HSJ1_RS9565	D-ribose transporter ATP-binding protein	**-4**	NQ	C1
Lipid glycosylation					
NA	HSJ1_RS21320	Glycosyltransferase family 1	NA	**-3**	C2
Carboxylic acid metabolic process					
CEP0996_RS6480	HSJ1_RS5735	Isocitrate lyase	0	**3**	C1
Sulfate transport					
CEP0996_RS5425	HSJ1_RS4590	Membrane protein	0	**-2**	C1
Proteolysis					
CEP0996_RS20315	NA	Membrane dipeptidase	**8**	NA	C2
Transmembrane transport					
CEP0996_RS20320	HSJ1_RS17625	MFS transporter	**7**	NQ	C2

CEP0996_RS17385	HSJ1_RS20480	MFS transporter	NQ	**2**	C2
Protein folding					
CEP0996_RS26480	HSJ1_RS31520	GroES molecular chaperone	**-8**	**-2**	C3
Cellular amino acid catabolic process					
CEP0996_RS21560	HSJ1_RS16500	Muconate cycloisomerase	**8**	**5**	C2
CEP0996_RS30235	HSJ1_RS32440	Muconate cycloisomerase	NQ	**2**	C3
Protein repair					
CEP0996_RS0235	HSJ1_RS14585	YedY sulfoxide reductase catalytic subunit protein repair	0	**-2**	C1
Regulation of transcription, DNA-templated					
CEP0996_RS11175	HSJ1_RS11270	ArsR family transcriptional regulator	**-5**	NQ	C1
CEP0996_RS1895	HSJ1_RS1000	LysR family transcriptional regulator	2	**4**	C1
CEP0996_RS17540	HSJ1_RS20315	Two-component system response regulators	NQ	**3**	C2
CEP0996_RS20265	HSJ1_RS17680	LuxR family transcriptional regulator	**-7**	NQ	C2
NA	HSJ1_RS30460	AraC family transcriptional regulator	NA	**2**	C3
NA	HSJ1_RS30560	DNA binding protein	NA	**3**	C3
Ribosomal proteins					
CEP0996_RS3895	HSJ1_RS3080	30S ribosomal protein S20	**4**	1	C1
CEP0996_RS0920	HSJ1_RS0005	50S ribosomal protein L34	**4**	1	C1
Unknown					
CEP0996_RS14595	HSJ1_RS15925	Hypothetical protein	1	**-2**	C1
CEP0996_RS0535	HSJ1_RS14245	Hypothetical protein	**3**	1	C1
CEP0996_RS12435	HSJ1_RS12610	Hypothetical protein	**4**	2	C1
CEP0996_RS7860	HSJ1_RS7710	Membrane protein	1	**2**	C1
CEP0996_RS7670	HSJ1_RS7490	Hypothetical protein	1	**-5**	C1
CEP0996_RS22875	HSJ1_RS26480	2-oxoglutarate dehydrogenase	**-7**	-2	C2
CEP0996_RS22880	HSJ1_RS26475	Hypothetical protein	**-12**	**-3**	C2
CEP0996_RS24485	HSJ1_RS24760	Photosystem reaction center subunit H	**-7**	NQ	C2
CEP0996_RS25320	HSJ1_RS23960	Hypothetical protein	**-6**	3	C2
CEP0996_RS14840	HSJ1_RS22960	Hypothetical protein	**6**	NQ	C2
CEP0996_RS16040	HSJ1_RS21895	Phosphoserine phosphatase	**-5**	2	C2
CEP0996_RS16355	HSJ1_RS21585	Purine nucleoside phosphorylase	-1	**-2**	C2
CEP0996_RS18215	HSJ1_RS19585	Hypothetical protein	0	**-2**	C2

CEP0996_RS18880	HSJ1_RS18940	Hypothetical protein	0	**3**	C2
CEP0996_RS19685	HSJ1_RS18235	Oxidoreductase	**7**	1	C2
CEP0996_RS20725	HSJ1_RS17220	Hypothetical protein	-1	**-2**	C2
CEP0996_RS20895	HSJ1_RS17045	Phosphoesterase	**-6**	NQ	C2
CEP0996_RS20900	HSJ1_RS17040	Hypothetical protein	**-7**	0	C2
NA	HSJ1_RS30465	Undecaprenyl phosphate galactose phosphotransferase	NA	**2**	C3
NA	HSJ1_RS30475	Protein tyrosine phosphatase	NA	**2**	C3
CEP0996_RS0545	NA	Hypothetical protein	**5**	NQ	C1
Antimicrobial molecules					
AFC lipopeptide antibiotic biosynthesis					
CEP0996_RS27640	HSJ1_RS30260	Hypothetical protein	ND	**-3**	C3
CEP0996_RS27625	HSJ1_RS30265	Lactate dehydrogenase	ND	**-2**	C3
CEP0996_RS27615	HSJ1_RS30280	Phosphonate ABC transporter ATP binding protein	ND	**-2**	C3
CEP0996_RS27605	HSJ1_RS30290	Methyltransferase type 11	ND	**-2**	C3
NA	HSJ1_RS30305	Hypothetical protein CDS	NA	**-3**	C3
CEP0996_RS27580	HSJ1_RS30315	Pyridoxal dependent decarboxylase	ND	**-2**	C3
CEP0996_RS27570	HSJ1_RS30325	Hypothetical protein	**-10**	**-7**	C3
CEP0996_RS27565	HSJ1_RS30330	Hypothetical protein	NQ	**-2**	C3
CEP0996_RS27560	HSJ1_RS30335	Hypothetical protein	NQ	**-3**	C3
CEP0996_RS27555	HSJ1_RS30340	Acyl carrier protein	**-7**	**-3**	C3
CEP0996_RS27545	HSJ1_RS30350	Hypothetical protein	NQ	**-3**	C3
NA	HSJ1_RS30360	Membrane protein	NA	**-3**	C3
CEP0996_RS27535	HSJ1_RS30365	ABC transporter	NQ	**-3**	C3
CEP0996_RS27515	HSJ1_RS30385	ShvR LysR family transcriptional regulator	NQ	**-3**	C3
Enacyloxin biosynthesis					
NA	HSJ1_RS29185	LuxR family transcriptional regulator	NA	**-2**	C3
NA	HSJ1_RS29190	LuxR family transcriptional regulator	NA	**-3**	C3
NA	HSJ1_RS29195	Xylose isomerase	NA	**-5**	C3
NA	HSJ1_RS29200	Shikimate dehydrogenase	NA	**-6**	C3
NA	HSJ1_RS29225	Oxidoreductase	NA	**-2**	C3
NA	HSJ1_RS29235	Cis-AT modular polyketide synthase	NA	**-10**	C3

NA	HSJ1_RS29260	Cis-AT modular polyketide synthase	NA	**-6**	C3
NA	HSJ1_RS29265	Thioesterase	NA	**-2**	C3
NA	HSJ1_RS29270	Aspartyl beta hydroxylase	NA	**-9**	C3
NA	HSJ1_RS29285	Carbamoyltransferase	NA	**-9**	C3
NA	HSJ1_RS29295	Quinate shikimate dehydrogenase	NA	**-2**	C3
NA	HSJ1_RS29300	MATE family efflux transporter	NA	**-3**	C3
NA	HSJ1_RS29315	6 aminohexanoate dimer hydrolase	NA	**-2**	C3
NA	HSJ1_RS29230	Beta ketoacyl synthase	NA	**-11**	C3
NA	HSJ1_RS29240	Beta ketoacyl synthase	NA	**-5**	C3
NA	HSJ1_RS29280	Cis-AT modular polyketide synthase	NA	**-5**	C3
HMAQ biosynthesis					
CEP0996_RS29320	HSJ1_RS28495	HmqF AMP-dependent synthetase	**-10**	**-12**	C3
CEP0996_RS29315	HSJ1_RS28500	HmqG methyltransferase	**-11**	**-14**	C3
Occidiofungin biosynthesis					
CEP0996_RS26270	HSJ1_RS31885	ATP-binding protein	**-6**	**-2**	C3
CEP0996_RS26265	HSJ1_RS31890	Hypothetical protein	**-13**	**-2**	C3
NA	HSJ1_RS31900	Non-ribosomal peptide synthetase	NA	**-9**	C3
CEP0996_RS26255	HSJ1_RS31905	Non-ribosomal peptide synthetase	**-10**	**-6**	C3
NA	HSJ1_RS31910	Non-ribosomal peptide synthetase	NA	**-10**	C3
NA	HSJ1_RS31915	MBL fold metallo hydrolase	NA	**-9**	C3
CEP0996_RS30800	HSJ1_RS31920	Non-ribosomal peptide synthetase	**-11**	**-10**	C3
CEP0996_RS30790	HSJ1_RS31930	Beta-ketoacyl synthase	**-8**	**-11**	C3
CEP0996_RS30780	HSJ1_RS31940	2,4-diaminobutyrate-4-aminotransferase	**-10**	**-9**	C3
Virulence factors					
Exopolysaccharide biosynthesis					
CEP0996_RS21790	HSJ1_RS16270	Transposase	**-10**	NQ	C2
CEP0996_RS21820	HSJ1_RS16250	BceD exopolysaccharide biosynthesis protein	**-8**	NQ	C2
CEP0996_RS21835	HSJ1_RS16235	BceH glycosyltransferase	-1	**-2**	C2
Proteases					
CEP0996_RS16455	HSJ1_RS21480	ZmpB peptidase CDS	-1	**-3**	C2
Adhesion					
CEP0996_RS19565	HSJ1_RS18350	BamL2 fucose-binding lectin II CDS	0	**-2**	C2

CEP0996_RS21070	HSJ1_RS16890	BamL hypothetical protein CDS	-1	**-7**	C2
Motility					
CEP0996_RS13705	HSJ1_RS15005	Flin flagellar motor switch protein	**9**	NQ	C1
CEP0996_RS14335	HSJ1_RS15610	Hypothetical protein	**6**	NQ	C1
CEP0996_RS8860	HSJ1_RS8705	FkbM family methyltransferase CDS	1	**3**	C1
Secretion system protein (IV/II)					
CEP0996_RS28685	HSJ1_RS29025	Secretion system protein E	**-8**	**-4**	C3
Iron uptake					
CEP0996_RS1985	HSJ1_RS1090	TonB dependent receptor	NQ	**3**	C1
CEP0996_RS23895	HSJ1_RS25365	Iron ABC transporter substrate binding protein	NQ	**4**	C2
CEP0996_RS14920	HSJ1_RS22870	TonB dependent receptor	NQ	**10**	C2
Plant growth-promoting					
Nitrate reductase					
CEP0996_RS29570	HSJ1_RS28215	Peptidyl prolyl cis-trans isomerase	NQ	**3**	C3
CEP0996_RS29550	HSJ1_RS28495	NarZ nitrate reductase	NQ	**7**	C3
Nodulation					
CEP0996_RS8765	HSJ1_RS8610	NodJ nodulation protein	**-5**	NQ	C1

NA: not applicable; NQ: not quantified; **Bold numbers** are significant log2fold values (+ = upregulation; - = downregulation) with both a |z-score| > 1.5 and a Welch's t test Limma pvalue < 0.05.

6.4.4. Genes belonging to the HMAQs and occidiofungin biosynthesis gene clusters are under transcriptional regulation

Interestingly, while HSJ1v retains its pc3, functions encoded on this replicon are nevertheless poorly/not produced. To verify whether the differentially produced proteins are regulated at the transcriptional level, we quantified the expression of two genes by qPCR. The less abundant products of HSJ1_RS28725 (HMAQs biosynthesis) and of HSJ1_RS31950 (occidiofungin biosynthesis) indeed appear both to be explained by their reduced transcription (**Figure 6.6**).

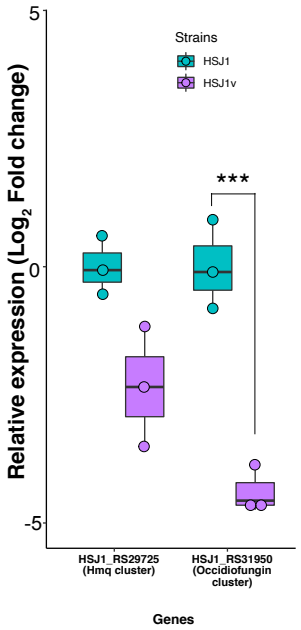

Figure 6.6 Relative expression of genes involved in HMAQs and occidiofungin production in *B. ambifaria* HSJ1 vs its variant

Log_2 Fold change is represented on the graph and a t-test has been performed on the ΔCT values. P-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, *** 0.001 and 0.0001, and **** inferior to 0.0001.

6.4.5. The transcriptional regulator ShvR could control phenotypes observed in *B. ambifaria* HSJ1

The ShvR transcriptional regulator controls over 1,000 genes and especially biofilm formation, protease production, antibiotic synthesis and colony morphotype in *B. cenocepacia* K56-2 (Bernier *et al.*, 2007; Gomes *et al.*, 2018; O'Grady *et al.*, 2010; O'Grady & Sokol, 2011; Subramoni *et al.*, 2011). Since lower levels of the ShvR protein were measured in cultures of *B. ambifaria* HSJ1v compared to its WT (**Figure 6.7A**), we postulated that it could be involved in explaining the variant phenotype. We decided to complement this strain with an expression vector, pMLS7-*shvR* (pMLC45). However, there was no observed recovery of WT phenotypes (**Figure 6.7B-C**). A mutant of *shvR* should confirm that this regulator controls the same phenotype in *B. ambifaria* than in *B. cenocepacia* and contribute to explain the variant phenotype of HSJ1 (and AU02212v).

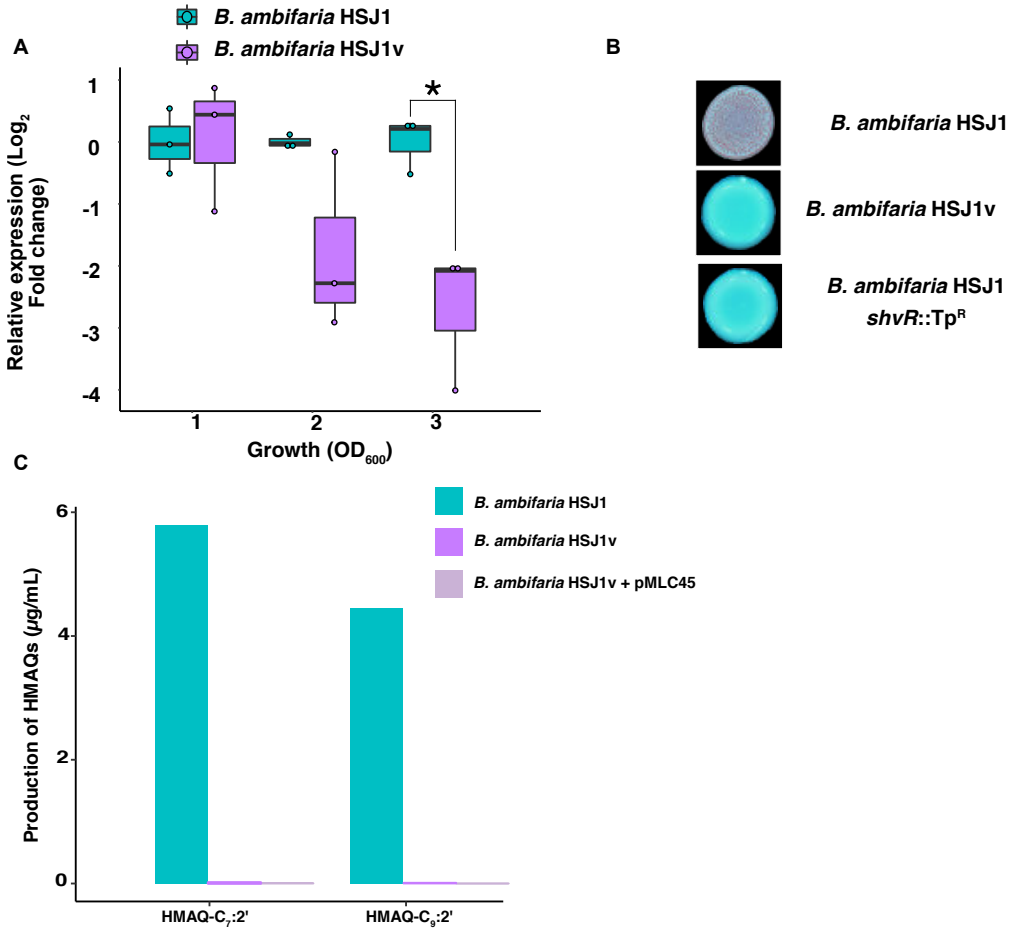

Figure 6.7 Investigation of the implication of ShvR in the phase variation of *B. ambifaria* HSJ1 (preliminary data)

A) Relative expression of *shvR* in *B. ambifaria* HSJ1v and wildtype (Log2 Fold change is represented on the graph and a t-test has been performed on the ΔCT values). B) Colony morphotype on CRLA. C) HMAQs production within an overnight culture. P-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, *** 0.001 and 0.0001, and **** inferior to 0.0001.

6.4.6. DNA methylation could play a role in phase variation of *B. ambifaria* HSJ1 by modulating ShvR production

Since no genetic modifications could explain the major differences in phenotypes observed between *B. ambifaria* HSJ1 and its variant, we hypothesized that an epigenetic mechanism might be involved. Methylation of DNA is a well-known phenotypic variation mechanism. We hypothesized it is involved in phenotype variation by modulating the expression of *shvR*. PacBio methylation analysis revealed that more adenosine and cytosine residues are methylated in *B. ambifaria* HSJ1v than WT: 19,348 6mA against 19,187 (less than 1% difference) and 3,007 4mC against 2,767 (around 8% difference) both at stationary growth phase (**Table 6.7**).

The difference in methylomes was supported by our finding that DNA MTases encoded by genes HSJ1_RS14940 [m6A motifs] and HSJ1_RS26905 [m4C motifs]) are more expressed in *B.*

ambifaria HSJ1v than in the WT in early exponential phase while HSJ1_RS14940 is downregulated in the variant at OD600 = 3 (**Figure 6.8**).

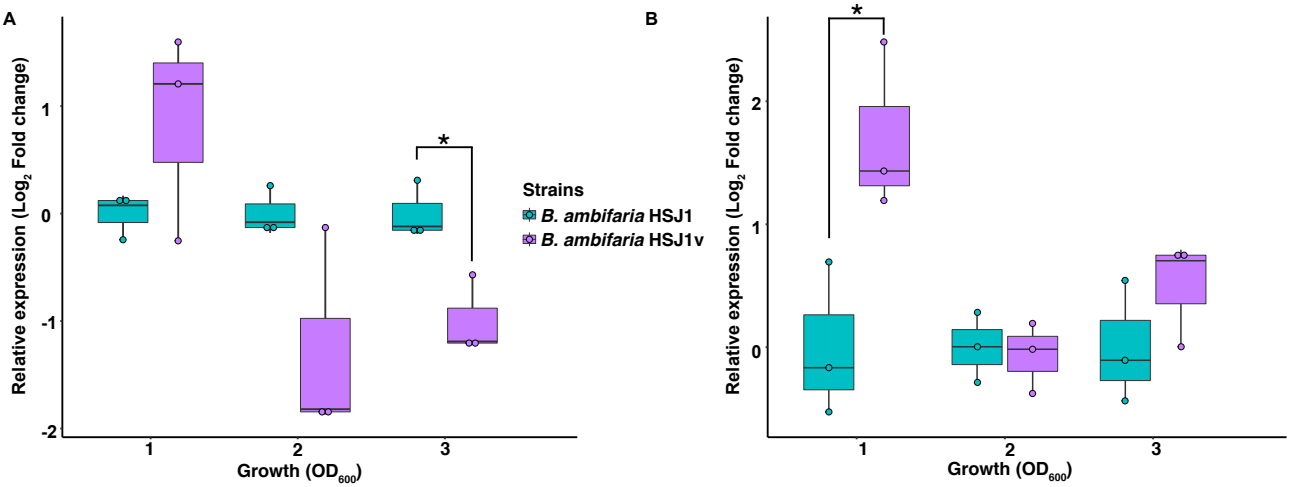

Figure 6.8 Relative expression of genes coding for DNA MTases in *B. ambifaria* HSJ1 wild type and variant.

A) Relative expression of adenosine DNA MTase (HSJ1_RS14940). B) Relative expression of cytosine DNA MTase (HSJ1_RS26905). Log_2 Fold change is represented on the graph and a t-test has been performed on the ΔCT values. P-values are represented with * between 0.5 and 0.01, ** between 0.01 and 0.001, *** 0.001 and 0.0001, and **** inferior to 0.0001.

Table 6.7 DNA methylation in both phenotype of *B. ambifaria* HSJ1

	Motif	Methylation	Strand	Called modified motifs*		Putative Genes and their putative homologs in other Bcc
HSJ1	RG**AT**CY	6mA	F+R	6265/11856		HSJ1_RS14940 (BURCEP_RS0129090 - *B. cepacia* ATCC25416 BCAM0992 - *B. cenocepacia* J2315)
	CAC**A**G	6mA	F	4008/6322		
	G**T**WW**A**C	6mA	F+R	790/1670		
HSJ1v	-	4mC	-		2767	HSJ1_RS26905 (BURCEP_RS0129090 - *B. cepacia* ATCC24516 BCAM0992 - *B. cenocepacia* J2315)
	RG**A**TCY	6mA	F+R	6840/11856		HSJ1_RS14940 (BURCEP_RS0129090 - *B. cepacia* ATCC25416 BCAM0992 - *B. cenocepacia* J2315)
	CAC**A**G	6mA	F	4371/6322		
	GTWW**A**C	6mA	F+R	864/1670		
	-	4mC	-		3007	HSJ1_RS26905 (BURCEP_RS0129090 - *B. cepacia* ATCC24516 BCAM0992 - *B. cenocepacia* J2315)

*modQv>60 for m6A motifs and modQv>25 for m4C motifs

Sinefungin is a well-known DNA MTase inhibitor which can help mimic mutations in DNA MTases, such as found in *B. cenocepacia* H111 and J2315 strains (Vandenbussche *et al.*, 2020). Knowing that the genome of *B. ambifaria* HSJ1v is more methylated than the wild type, we asked if exposure to this inhibitor could inhibit the development of variants in a WT culture or even induce the switch back from variant to WT colony morphotype. We observed that sinefungin indeed slowed down the emergence of variants in a WT colony but did not affect the morphotype of an HSJ1v colony on plate. The evolution of variants in liquid culture was maybe slowed down (see days 4 and 5), but only transiently (**Figures 6.9**).

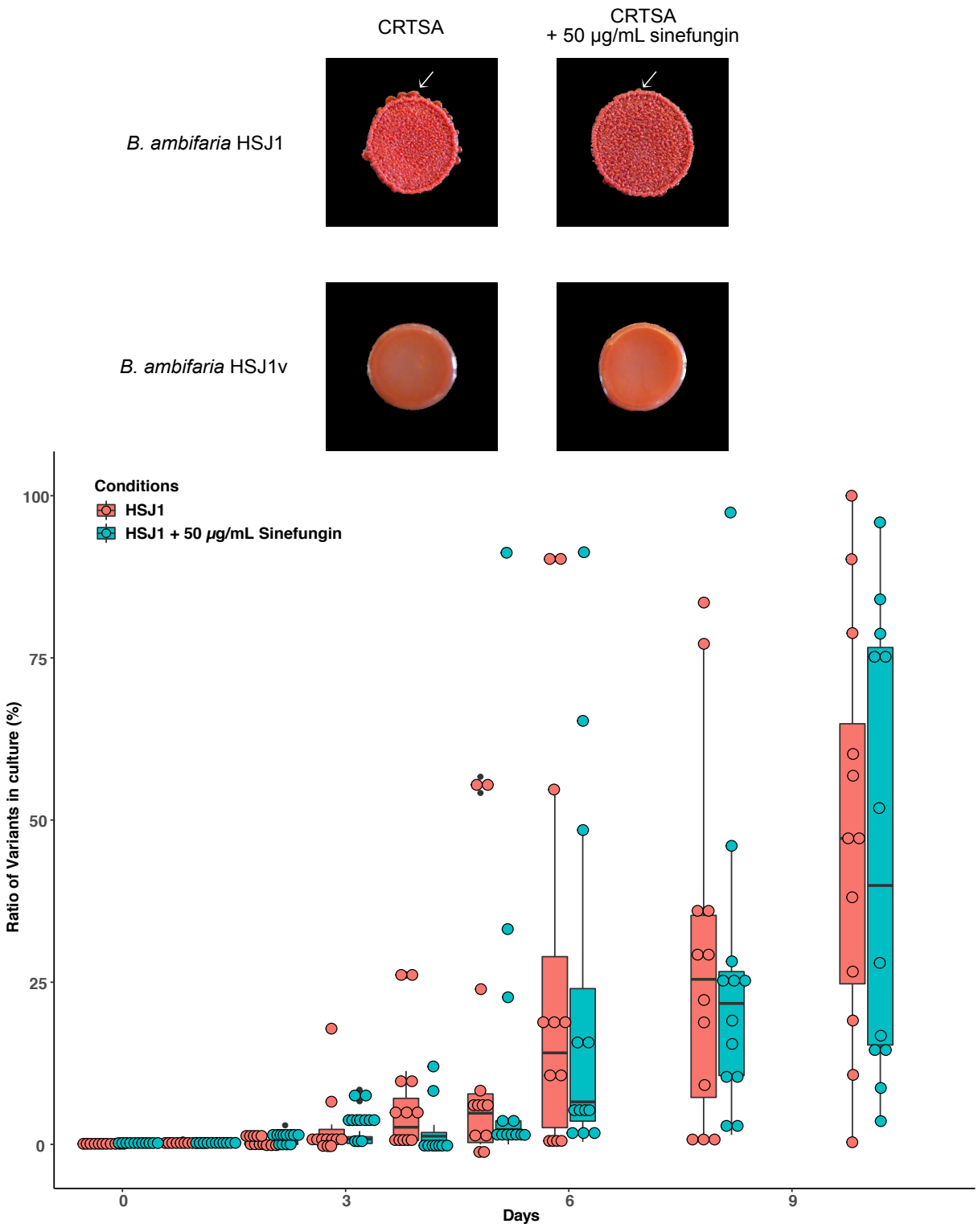

Figure 6.9 **Sinefungin, a methyltransferase inhibitor, slows down the apparition of *B. ambifaria* HSJ1v**

Colony morphotype on CRTSA and the proportion of *B. ambifaria* HSJ1v in the culture of TSB or TBS with 50 µg/mL sinefungin was determined for 10 days.

6.5. Discussion

Diverse colony morphotypes are well described in the *Burkholderia* genus, and mainly through the "*pseudomallei*" group (Agnoli *et al.*, 2011; Al-Maleki *et al.*, 2015; Al-Maleki *et al.*, 2019; Austin *et al.*, 2015; DeShazer *et al.*, 2019; Gierok *et al.*, 2016; Rondeau *et al.*, 2019; Shea *et al.*, 2017; Vial *et al.*, 2009; Vipond *et al.*, 2013; Wang *et al.*, 2019; Wikraiphat *et al.*, 2015). We previously described colony variation in *B. ambifaria* allowing clinical isolates to switch back to an environmental phenotype by decreasing virulence phenotypes such as antibiotic production, mucoidy, cholesterol oxidase activity and virulence in *Dictyostelium discoideum* (Vial *et al.*, 2009). Agnoli *et al.* (2011) have reported that colony variation among Bcc can be a consequence of the loss of the virulence megaplasmid pc3, which is naturally observed in *B. cenocepacia* and *B. stabilis*. We discovered that among eight *B. ambifaria* clinical isolates investigated, described in Vial *et al.* (2009), only six variants were lacking their pc3. First, this showed that loss of pc3 can also occur in *B. ambifaria*. Second, it reveals a second mechanism of phenotypic variation not previously reported in the Bcc. Interestingly, the two variants *B. ambifaria* HSJ1v and AU0212v are both closely related to the environmental isolate *B. ambifaria* AMMD – with a 99.99% identity. We then decided to focus our study on two model strains: *B. ambifaria* CEP0996 (forming pc3-null variant) and *B. ambifaria* HSJ1 (forming a pc3-positive variant). From there, we investigated the regulated proteins between both strains by studying their proteomes and the effect of the variation. Results showed that mainly virulence factors were downregulated in both types of variants – such as EPS and antibiotic production, motility, and siderophore production. By studying these phenotypes, we confirmed the proteomic results and showed that these differences are not due to a difference in AHLs production belonging to the QS system which controls most of the virulence factors – even if C_8-HSL production is increased in *B. ambifaria* CEP0996 pc3-null compared to its WT. This is probably due to the absence of HMAQs molecules (inhibitor of the Cep system in *B. ambifaria* HSJ1) (Chapalain *et al.*, 2017; Vial *et al.*, 2008) or to the absence of an unknown repressor.

The ShvR transcriptional regulator Lys-R family is located on the pc3 in Bcc. ShvR controls biofilm formation, protease production and colony morphotype which are affected in *B. cenocepacia* H111 pc3-null – which are same phenotype being impacted in *B. cenocepacia* K56-2 Δ*shvR*. In fact, *B. cenocepacia* H111 WT has a rough colony morphotype, produces more biofilm and less proteases than *B. cenocepacia* H111 ∆*shvR* which has a smooth colony morphotype (Bernier *et al.*, 2007; Gomes *et al.*, 2018; O'Grady *et al.*, 2010; O'Grady & Sokol, 2011; Subramoni *et al.*, 2011). Interestingly, we confirmed that *shvR* is missing in *B. ambifaria* CEP0996 pc3-null and is down regulated in *B. ambifaria* HSJ1v, which probably explained the differences of observed phenotypes (Mannweiler *et al.*, 2020). However, complementation of *B. ambifaria* HSJ1 variant phenotype was not achieved when *shvR* was overexpressed. ShvR being a LysR-like family regulator, a ligand is likely necessary to activate the regulator (Maddocks & Oyston, 2008). One possibility is that it could be the *afc* cluster for which proteins are downregulated in *B. ambifaria* HSJ1v. However, we still

need to confirm that *shvR* controls the same phenotypes in *B. ambifaria* than in *B. cenocepacia* by inactivating this gene.

The reduced expression of the gene and resulting decreased production of ShvR and other *afc* cluster proteins in *B. ambifaria* HSJ1v is neither explained by a difference of AHL production nor by obvious genomic variations. Thus, we hypothesized that DNA methylation – being a key epigenetic factor implicated in bacterial phase variation and gene expression (Casadesús, 2016; Gaultney *et al.*, 2020; Heusipp *et al.*, 2007; Kumar *et al.*, 2018; Low *et al.*, 2001; Mannweiler *et al.*, 2020; Srikhanta *et al.*, 2010; Vandenbussche *et al.*, 2020; Wion & Casadesús, 2006) - could be involved in *B. ambifaria* phase variation by modulating the expression of virulence factors via ShvR. In the Bcc, the loss of the pc3 in *B. cenocepacia* H111 (including *shvR*) could be a consequence of a chromosome instability controlled by DNA methylation (Agnoli *et al.*, 2011; Mannweiler *et al.*, 2020). Other virulence factors such as biofilm formation, cell aggregation and motility and several transcriptional regulators, and more importantly the *afcC* gene (belonging to *afc* biosynthesis cluster genes) are regulated by DNA Methylation in *B. cenocepacia* K56-2 and J2315 (Vandenbussche *et al.*, 2020). Here, we demonstrated that *B. ambifaria* HSJ1v contains more 6mA and 4mC and that using a DNA Mtases inhibitor, the development of variants from *B. ambifaria* HSJ1 WT slowed down. After five days of culture of *B. ambifaria* HSJ1 WT with sinefungin, an accumulation of variants was observed which could be explained by potential mutation reducing the efficiency of sinefungin or variants already being present. These results suggest that the expression of genes encoding for described phenotypes could be under the control of DNA methylation, such as *shvR*. However, we still need to inactivate both DNA MTases genes to determine if they impact the phenotypes and down regulate the transcription of *shvR* in *B. ambifaria* HSJ1.

6.6. References

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7. DISCUSSION GÉNÉRALE ET CONCLUSION

Mon objectif principal de doctorat était de mieux comprendre le système Hmq chez le groupe des Bcc en déterminant sa prévalence, son mécanisme de régulation ainsi que son rôle dans la pathogénicité et les différents rôles des HMAQs, des éléments qui avaient été principalement étudiés chez le groupe « *pseudomallei* » du genre *Burkholderia*.

Afin de comprendre le rôle écologique du système Hmq, il fut tout d'abord nécessaire de déterminer la prévalence de l'opéron *hmqABCDEFG* au sein des Bcc. Par analyses d'homologie de séquences, j'ai premièrement démontré que le système Hmq est présent chez 79% des souches séquencées de *B. thailandensis* et 97% des souches de *B. pseudomallei,* mais seulement chez 35% des Bcc (Chapitre 3 - [Coulon *et al.*, 2019]). J'ai ensuite confirmé expérimentalement ces données en démontrant que 32% des 312 souches de Bcc criblées par PCR ont l'opéron *hmqABCDEFG* dont 53% des souches environnementales et 21% des souches cliniques (Chapitre 4). Ce résultat était surprenant, car basé sur les travaux précédents du Laboratoire Déziel (Vial *et al.*, 2009; Vial *et al.*, 2008), nous nous attendions à ce que le système Hmq soit plus prévalent chez les souches cliniques de Bcc. Pourtant, les résultats ont clairement démontré que *B. cenocepacia* et *B. multivorans,* et dans une moindre mesure *B. vietnamiensis*, les espèces majoritairement retrouvées chez les individus atteints de fibrose kystique ou bien encore les personnes immunodéprimées, sont dépourvues de système Hmq (Kilani-Feki *et al.*, 2011; Kilani-Feki *et al.*, 2012; Li *et al.*, 2018; Li *et al.*, 2020; Mahenthiralingam *et al.*, 2011; Mori *et al.*, 2007; Vial *et al.*, 2008). Cependant les espèces plus largement environnementales telles que *B. ambifaria*, *B. cepacia* et *B. contaminans* sont plus susceptibles de posséder l'opéron *hmqABCDEFG* dont les isolats cliniques qui sont probablement issus directement de l'environnement. L'opéron Hmq, au sein des Bcc, est potentiellement issu d'un ancêtre commun avec perte de chez certaines espèces probablement par réarrangement chromosomique. En effet, un alignement du pc3 chez les représentants des différentes espèces de Bcc montre qu'il y a très peu de synténie entre les différentes régions du pc3, supposant que la dynamique du transfert des gènes est partielle. D'après l'étude phylogénétique de l'opéron *hmqABCDEFG*, en comparaison à la phylogénie des Bcc basée sur les séquences MLST, il semblerait que l'opéron *hmqABCDEFG* résulterait d'un transfert horizontal entre certaines espèces de Bcc.

La production de HMAQs par les Bcc au laboratoire est dépendante des conditions de culture ; il semble que chaque Bcc ayant l'opéron *hmqABCDEFG* est capable de produire des HMAQs en conditions optimales. Cependant, les facteurs de culture affectant la production de HMAQs devront être déterminés. Au début de mon doctorat, j'ai pré-testé la production de HMAQs chez *B. ambifaria* HSJ1 (souche clinique) et *B. ambifaria* AMMD (souche environnementale) dans le milieu minimal M9 avec différentes sources de carbone (le TSB a servi de témoin positif) et la production de HMAQs chez *B. ambifaria* HSJ1 en présence de champignons (Annexe II). Aucune

production de HMAQs n'a été détectée dans toutes les conditions testées pour *B. ambifaria* AMMD et une quantité moindre a été détectée dans les différentes conditions testées pour *B. ambifaria* HSJ1 en comparaison de de la production de HMAQs en TSB ou en présence de champignons. Après avoir de nouveau analysé des résultats, on observe que (1) les quantités obtenues sont faibles et variables entre les réplicats biologiques – liés à la variation biologique, mais aussi probablement dû au manque de sensibilité du LC-MS - et (2) les DO_{600} atteintes variaient entre 3 et 5. Il serait donc intéressant de recommencer avec des temps de culture différents, et aussi en milieu solide (TSA incubée quatre jours ou encore Mueller-Hinton en présence de champignons) – sachant que *B. ambifaria* AMMD produit des HMAQs en TSA alors qu'aucune production de HMAQs, ni transcription du gène *hmqA* n'ont été observés en TSB (Coulon et al. 2021).

En ce qui concerne le rôle du système Hmq, nous savons que les HMAQs auraient (1) un effet indirect sur les phénotypes relatifs au QS (e.g. production de sidérophores, activité protéolytique ; [Vial *et al.*, 2008]) et (2) une activité antimicrobienne contre les bactéries à Gram-positif et les champignons) et (3) un effet potentiel d'antibiotique cytotoxique contre des cellules cancéreuses dépendantes IGF-I (Kilani-Feki *et al.*, 2011; Klaus *et al.*, 2020 ; Li *et al.*, 2018; Mori *et al.*, 2007). De plus, la surexpression de l'opéron *hmqABCDEFG* est surexprimé chez *B. pseudomallei* est détectée lors d'une infection sur le long terme (Price *et al.*, 2018) ; les HMAQs - analogues structurels du PQS et HHQ chez *P. aeruginosa* (Diggle et *al.*, 2006 ; Vial et *al.*, 2008), active faiblement le système PQS - faisant parti du QS et induisant la virulence - de *P. aeruginosa* (Le Guillouzer, 2018). Ces données montrent les HMAQs permettrait la communication inter-espèces en inhibant par exemple le système PQS de *P. aeruginosa*, et agiraient comme agents anti-microbiens afin d'établir la niche écologique de ces bactéries dans un environnement complexe tel que la rhizosphère.

J'ai pu mettre en évidence l'effet anti-microbien des HMAQs synthétiques en collaboration avec le Pr. Charles Gauthier et la professionnelle de recherche Marianne Piochon (Chapitre 5). De plus, les Bcc étant connues pour être favorables aux plantes en promouvant leur croissance et permettant la bioremédiation (Gillis *et al.*, 1995; Mullins *et al.*, 2019; O'Sullivan & Mahenthiralingam, 2005; Parke, 1991), revue par Vial *et al.* (2011), et de par la plus grande prévalence du système Hmq chez les souches environnementales, j'ai émis l'hypothèse que ces métabolites peuvent être impliqués dans la promotion de la croissance des plantes (Chapitre 4). En ajoutant *B. ambifaria* HSJ1 sauvage ou des mutants du système Hmq (*hmqA*- ne produisant pas de HMAQs et *hmqG*- ne produisant pas uniquement de HMAQ méthylé, c'est-à-dire des HAQ), dans le milieu de croissance de *P. sativum* (ou petit-pois), j'ai démontré que le système Hmq favorisait le développement des racines (masse totale sèche). La différence significative observée entre l'ajout de la souche sauvage et celui des deux mutants démontrent qu'il s'agirait plutôt d'un effet du système Hmq ou des HMAQs. Des études sur l'effet des homosérine lactones sur la promotion de

la croissance des plantes (e.g. *Arabidopsis thaliana*, le blé, l'orge ou bien encore le haricot) ont démontré que les AHLs à chaines courtes (C_6-HSL et C_8-HSL) augmentent la longueur des racines et N-decanoyl-L-homoserine lactone (C_{10}-HSL) induit un raccourcissement des racines avec formation de racines latérales ainsi que de poils (Bai et *al*., 2012 ; Moshynets et *al*., 2019 ; Ortiz-Castro et *al*., 2008 ; Rankl et *al*., 2016; Schenk et al., 2012 ; Von Rad et *al*., 2008). Cependant, Shrestha et *al.* (2020), ont montré que l'ajout un mélange d'homosérine lactones à chaine courte inhibe la croissance de racines et la biomasse de la plante chez *A. thaliana*, mais que la présence d'un mélange d'homosérine lactones à chaine courte et à chaine longue, n'induit aucun changement de ces deux paramètres. Ces résultats suggèrent que l'effet initié par un type d'AHL interfère avec l'effet d'un autre type d'AHLs montrant une complexité de rôle des AHLs sur la promotion de la croissance des plantes. En effet, soit le système Hmq, via les HMAQs en inhibant le QS de *B. ambifaria* HSJ1 : (1) interférait avec l'équilibre et l'effet du C_8-HSL sur la croissance des racines ; (2) modulerait indirectement la production de différentes molécules chez *B. ambifaria*, dont plusieurs antimicrobiens dont la pyrrolnitrine, les enacyloxines, les lipopeptides AFC ou bien encore les occidiofungines (Kilani-Feki *et al.*, 2011; Kilani-Feki *et al.*, 2012; Mahenthiralingam *et al.*, 2011; Vial *et al.*, 2008; Chapalain *et al.*, 2017; Gomes *et al.*, 2018; Jung *et al.*, 2018; Le Guillouzer, 2018; Mullins *et al.*, 2019; Patten *et al.*, 2012; Piochon *et al.*, 2020; Whalen *et al.*, 2019).

Pour vérifier ces deux hypothèses, il sera intéressant de caractériser le microbiome de *P. sativum* dans les conditions suivantes : (1) en présence ou non des mutants *hmqA*- ou *hmqG*- en supplémentant ou non avec des HMAQs, (2) en présence du mutant *cepI*- avec supplémentation ou non de HMAQs et C_8-HSL, et (3) en conditions non stériles afin de permettre à la plante de conserver son microbiome. Enfin, *B. ambifaria* HSJ1 possédant le gène *nodJ* connue pour jouer un rôle dans la nodulation de la plante – cependant, aucun autre gène appartenant à ce groupe n'a encore été identifié. Si en effet, *B. ambifaria* HSJ1 est impliqué dans la production des nodules chez le pois, il y aurait une boucle de croissance : la nodulation de *P. sativum* favoriserait le développement des bactéries bénéfiques à la plante, ce qui augmenterait la dénitrification de la rhizosphère et donc favoriserait le développement des racines (Voisin et *al*., 2003).

La régulation du système Hmq chez les Bcc reste encore à être élucidée. Le système Hmq n'est pas un système de *quorum sensing* en tant que tel, quoique les HMAQs semblent être des molécules de signalisations. En effet, les HMAQs inhibent l'expression du système Hmq, néanmoins aucun régulateur transcriptionnel n'a été au sein de l'opéron *hmqABCDEFG* ou aux alentours du locus. Le système Hmq interagit étroitement avec le QS chez *B. ambifaria* HSJ1 : (1) les HMAQ inhibent le système principal de QS Cep ; (2) le système Hmq est, directement ou non, régulé par le QS (Chapalain *et al.*, 2017; Vial *et al.*, 2008). Ce dernier point devra être vérifié en déterminant si le régulateur du QS, CepR, contrôle directement la transcription de l'opéron *hmqABCDEFG*. Par exemple, un retard sur gel de CepR (en présence de C_8-HSL afin de stabiliser la formation de l'homodimère) en présence de la région non codante de 1500 pb située en amont

de l'opéron *hmqABCDEFG* (découpée en quatre fragments chevauchants) permettrait de déterminer si le système Cep régule directement le système par liaison de CepR sur la région promotrice de l'opéron *hmqABCDEFG*. Mes essais de purification de CepR par surexpression chez *E. coli* BL21 DE3 plysE n'ont pas été conclusifs. En effet en ayant utilisé une purification par affinité basée sur le protocole de Weingart *et al.*, (2005) et une en utilisant un his-tag en position N-terminale. Je n'ai pas réussi à purifier la protéine mais en essayant différentes conditions de cultures. Une solution serait d'utiliser un tag autre que his, car les anticorps anti-his utilisés ont pu révéler les histidines appartenant à d'autres protéines. Cependant, je pense qu'il serait préférable de surexprimer et purifier CepR directement dans *Burkholderia*. En effet, la présence naturelle de C_8-HSL devrait augmenter la stabilité et la production de CepR *in vivo*.

Il serait aussi important d'étudier les interactions QS – système Hmq chez d'autres espèces de Bcc (e.g. *B. cepacia*, *B. contaminans* et *B. vietnamiensis*) en vérifiant (1) l'effet des mutations du système Hmq des gènes *hmqA*, *hmqE* (potentiel régulateur de l'opéron *hmqABCDEFG*), *hmqG* sur l'expression du système Cep et de la production de C_8-HSL, et (2) l'effet du QS sur l'expression du système Hmq et de la production de HMAQs. Par exemple, on pourrait transformer des souches de Bcc avec un plasmide contenant une AHL lactonase afin de dégrader les signaux AHLs, puis de confirmer les résultats en mutant les gènes *cepI* et *cepR*.

D'après l'étude d'expression de *hmqA* chez quatre couples de Bcc, les souches non productrices de HMAQ n'expriment pas le gène *hmqA* en comparaison aux souches produisant des HMAQs. Ce résultat suggère que la production est sous le contrôle d'un régulateur transcriptionnel spécifique puisque aucune mutation n'est détectée dans les séquences promotrices en comparant les souches des quatre espèces étudiées (Chapitre 4). Enfin, une mutagenèse aléatoire (par exemple par transposon) dans *B. ambifaria* HSJ1 en utilisant un rapporteur chromosomique *hmqA-lacZ ou lux ou gfp* permettrait de trouver directement le régulateur du système Hmq, permettant ainsi d'étudier son rôle potentiel. Une autre approche de type « photoaffinitnity labelling », utilisée chez *P. aeruginosa* (Baker *et al.*, 2017), pourrait déterminer les protéines interagissant avec les HMAQs et le HHQ chez *Burkholderia* en utilisant une approche protéomique avec les molécules HMAQs et HHQ comme sondes.

La régulation de l'opéron *hmqABCDEFG* pourrait être aussi étudiée via la variation de phase. Ce mécanisme engendrant une perte de virulence, de production de métabolites (e.g. : molécules antimicrobiennes, sidérophores) mais aussi une perte de production de HMAQs a été observé chez les variants issus des souches cliniques de Bcc (Agnoli *et al.*, 2011; Vial *et al.*, 2009). En étudiant la présence du système Hmq chez les Bcc (Chapitre 4), j'ai découvert que six sur huit variants provenant de souches cliniques de *B. ambifaria* (possédant l'opéron *hmqABCDEFG*),

avaient perdu l'opéron *hmqABCDEFG* donc probablement leur pc3 – démontré par PCR (Chapitre 6) et chez les variants de *B. cenocepacia* et *B. stabilis* (Agnoli *et al.*, 2011; Vial *et al.*, 2009). Par la suite, j'ai étudié le protéome et identifié les phénotypes des souches modèles *B. ambifaria* CEP0996 (donnant un variant pc3-null) et *B. ambifaria* HSJ1 (donnant un variant possédant son c3). Ainsi les protéines, codées par les gènes situés sur le c3, nécessaires à la biosynthèse des molécules antimicrobiennes (e.g. : HMAQS, enacyloxines, lipopeptides AFC, occidiofungines), la production de sidérophores et d'EPS étaient sous-produites, et les protéines impliquées dans la dénitrification ou encore la formation du flagelle nécessaire à la motilité étaient surproduites chez les deux types de variants. Ces gènes (impliqués dans la production de sidérophores, EPS, flagelles, etc…) aussi définis comme facteurs de virulence, sont connus pour être régulés par le QS chez les bactéries. Les phénotypes observés chez *B. ambifaria* CEP0996 pc3-null et chez *B. ambifaria* HSJ1v n'étant pas expliqués par une différence de production de C_8-HSL, un autre mécanisme de régulation est donc impliqué.

Lors de l'analyse protéomique, plusieurs régulateurs ont été identifiés chez *B. ambifaria* CEP0996 pc3-null et *B. ambifaria* HSJ1v. Le régulateur transcriptionnel de type LysR nommé ShvR – localisé sur le c3 - était un candidat intéressant, connu pour : (1) réguler la production des lipopeptides AFC, (2) contrôler quelques phénotypes (e.g. : la morphologie des colonies, la formation de biofilm, la production de protéases) et (3) interagir avec le QS chez *B. cenocepacia* K56-2 (O'Grady *et al.*, 2010; O'Grady *et al.*, 2012). Agnoli *et al.* (2011) ont suggéré que la différence de la morphologie de colonie observée entre *B. cenocepacia* H111 sauvage et variant était indirectement à l'absence de ShvR de par la perte du pc3. La différence de la morphologie de la colonie et les autres phénotypes observés tels que la formation de biofilm et la production de protéase entre *B. ambifaria* CEP0996 sauvage et pc3-null serait probablement due à la perte de *shvR* chez *B. ambifaria* CEP0996 pc3-null.

La sous-expression de *shvR* pourrait être à l'origine de la différence de nombreux phénotypes observés entre *B. ambifaria* HSJ1 sauvage et variant. Une surexpression de *shvR* chez *B. ambifaria* HSJ1 variant ne permet pas de complémenter les phénotypes de la souche sauvage tels que la production de HMAQs et la morphologie de la colonie. Cependant, ShvR étant un régulateur de type LysR, il est probable qu'il ne soit pas fonctionnel sans son ligand. Une hypothèse serait que ce dernier soit produit par le groupe des gènes des lipopeptides AFC – donc rendant la surexpression non possible. C'est pourquoi un mutant *shvR*- dans *B. ambifaria* HSJ1 sauvage sera nécessaire afin de déterminer les facteurs de virulence contrôlés par ShvR. Le système Hmq pourrait être sous le contrôle de ShvR, un peu comme chez *B. thailandensis* où le système Hmq est activé par le régulateur transcriptionnel ScmR – également un régulateur de type LysR, activé par le QS et connu pour réguler la production de différents métabolites secondaires (Le Guillouzer *et al.*, 2020; Mao *et al.*, 2017; Martinez *et al.*, 2020). La production de AHLs étant identique chez *B. ambifaria* HSJ1 sauvage et variant, la régulation de *shvR* et du réseau des

métabolites secondaires déterminés sont sous le contrôle d'un autre mécanisme, tel que (1) la variation ou le polymorphisme génomique (Shea *et al.*, 2017), ou (2) des facteurs épigénétiques (Sánchez-Romero *et al.*, 2015; Wikraiphat *et al.*, 2015).

Une étude génomique entre *B. ambifaria* HSJ1 sauvage et variant a été entreprise et n'a révélé aucune variation de séquences ni de présence de SNP. Une observation similaire a aussi été démontrée chez *B. pseudomallei* MSHR5848, une souche formant deux variants dont les génomes sont identiques (Shea *et al.*, 2017).

La méthylation de l'ADN est le facteur épigénétique le plus connu chez les bactéries et apparait de plus en plus comme mécanisme de régulation de virulence (Casadesús & Low, 2006; Gaultney *et al.*, 2020; Kahramanoglou *et al.*, 2012; Kumar *et al.*, 2018b; Sánchez-Romero & Casadesús, 2020; Srikhanta *et al.*, 2010). C'est pourquoi j'ai décidé d'étudier les méthylomes de *B. ambifaria* HSJ1 sauvage et variant. L'étude globale des méthylomes – en phase stationnaire - a révélé que l'ADN de *B. ambifaria* HSJ1 variant est plus méthylé que celui de la souche sauvage – au niveau des adénosines et des cytosines (Chapitre 6). J'ai utilisé la *« sinefungin* » - l'inhibiteur de DNA MTase générant les mêmes phénotypes que la mutation des DNA MTases chez *B. cenocepacia* K56-2 et J2315 (Vandenbussche *et al.*, 2020) – et démontré une tendance de ralentissement de l'apparition des variants à partir de la souche sauvage, cependant aucune réversion claire n'a été observée chez le variant en présence de *« sinefungin* ». Néanmoins à cause de la variation biologique conséquente il est préférable de muter les deux DNA MTases chez *B. ambifaria* HSJ1 sauvage et variant pour : (1) démontrer le ralentissement ou l'inhibition totale d'apparition de variants, et (2) déterminer si la réversion du variant est contrôlée par la méthylation en étudiant la production de sidérophores, HMAQs, la motilité et la formation de biofilm. Enfin, il serait intéressant de faire un RNAseq des mutants des deux DNA MTases chez *B. ambifaria* HSJ1 sauvage pour déterminer les facteurs de virulence contrôlés via la méthylation de l'ADN.

En conclusion, j'ai déterminé que le système Hmq chez les Bcc est conservé uniquement chez les espèces émergentes cliniques de Bcc mais aussi chez la plupart des espèces environnementales. J'ai aussi démontré que les HMAQs sont des métabolites ayant une action antimicrobienne et qui modulerait la rhizosphère des plants de petit-pois ce qui favoriserait le développement de leurs racines. La partie la plus difficile était de déterminer la régulation du système Hmq. En effet, j'ai peu avancée sur l'étude directe de la régulation chez les différentes espèces de Bcc, mais l'étude de la variation de phase chez *B. ambifaria* montre que le régulateur transcriptionnel ShvR pourrait être un potentiel régulateur du système Hmq en plus de réguler d'autres métabolites secondaires comme démontré chez *B. cenocepacia*.

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9. ANNEXE I: Abréviation utilisée dans la figure 1.11 – tiré de Lerat & Moran, (2004)

Group	Organism	Symbol	Inducer	Receptor
Gamma proteo				
	Vibrio fischeri	Vfis	LuxI	LuxR
			LuxSa	-
	Vibrio anguillarum	Vang	VanI	VanR
	Vibrio harveyi	Vhar	LuxS	LuxP, LuxQ
	Vibrio cholerae	Vcho	LuxS	-
	Vibrio parahaemolyticus	Vpara	LuxS	-
	Vibrio vulnificus	Vvul	LuxS	-
	Photorhabdus luminescens	Plum	LuxS	-
	Proteus mirabilis	Pmir	LuxS	-
	Pseudomonas aeruginosa	Paer	RhlI	RhlR
			LasI	LasR
			?	PhzR
	Pseudomonas fluorescens	Pflu	AfmI	AfmR
			RhlI	RhlR
			PhzI	PhzR
			MupI	MupR
	Pseudomonas chlororaphis = P. aureofaciens	Pchl	PhzI	PhzR
			CsaI	CsaR
	Pseudomonas syringae	Psyr	AhlI	-
			PsmI	PsmR
			PsyI	PsyR
	Salmonella typhimurium	Sty	/	SdiA
			LuxS	-
	Salmonella enterica	Sent	/	SdiA
			LuxS	-
	Shigella flexneri	Sfle	LuxS	-

		/	SdiA
Shigella sonnei	Sson	/	SdiAa
Shigella dysenteriae	Sdys	/	SdiAa
Shewanella	Sone	LuxS	-
oneidensis			
Escherichia	EcoliK12	/	SdiA
***coli* K12**			
		LuxS	-
Escherichia	EcoliO157	/	SdiA
***coli* O157:H7**			
		LuxS	-
Aeromonas	Ahyd	AhyI	AhyR
hydrophila			
Aeromonas	Asalm	AsaI	AsaR/AhyR
salmonicida			
Yersinia pestis	Ypes	LuxS	-
		YpeI	YpeR
		YspI	YspR
Yersinia	Ypseu	YpsI	YpsR
pseudotuberculosis			
		YtbI	YtbR
Yersinia ruckeri	Yruc	YruI/YukI	YruR/YukR
Yersinia	Yent	YenI	YenR
enterocolitica			
		LuxSa	-
Erwinia	Echr	ExpI	ExpR
chrysanthemi = Pect			
obacterium			
chrysanthemi			
		EchI	EchR
Erwinia	Ecar	ExpI/CarI	ExpR CarR<
carotovora = Pectob			
acterium			
carotovorum			
		EcbI	EcbR
Erwinia	Estew	EsaI	EsaR
stewartii = Pantoea			
stewartii			
Serratia	Sliq	SwrI	SwrR
liquenfaciens			
***Serratia* sp**	serratia	SmaI	SmaR
Serratia marcescens	Smar	/	CarR
		SpnI	SpnR

	Serratia	Spro	SprI	SprR
	proteamaculans			
	Hafnia alvei	Halv	HalI	HalR
	Pantoea	Paggl	EagI	No sequence
	agglomerans			
	Pasteurella	Pmult	LuxS	-
	multocida			
	Haemophilus	Hinfl	LuxS	-
	influenzae			
Beta proteo	***Burkholderia***	Bcep	CepI	CepR
	cepacia			
			BviI	BviR
	Burkholderia	Bsta	CepI	CepR
	stabilis			
	Burkholderia	Bvie	CepI	CepR
	vietnamiensis			
	Burkholderia	Bamb	BafI	BafR
	ambifaria			
	Burkholderia	Bmult	CepI	CepR
	multivorans			
	Burkholderia	Bpseu	BspI	
	pseudomallei			
	Burkholderia mallei	Bmal	LuxI	
	Ralstonia	Rsol	SolI	SolR
	solanacearum			
	Neisseria	NmenB	LuxS	-
	***meningitidis* B**			
	Neisseria	NmenA	LuxS	-
	***meningitidis* A**			
	Neisseria	Ngor	LuxSa	-
	gonorrhoeae			
Alpha proteo	***Rhodobacter***	Rsph	CerI	CerR
	sphaeroides			
	Rhizobium meliloti	Rmel	TraI	
	Rhizobium loti	Rloti	TraI	
	***Rhizobium* sp**	Rhizobium	TraI	
	Agrobacterium	Atum	TraI	TraR
	tumefaciens			
	Agrobacterium	Arhi	TraI	TraR
	rhizogenes			
	Rhizobium	Rleg	RaiI	RaiR
	leguminosarum			
	Rhizobium etli	Retli	RaiI	RaiR
	***Ruegeria* sp**	Ruegeria	TraI	
Epsilon proteo	***Campylobacter***	Cjej	LuxS	-
	jejuni			
	Helicobacter pylori	Hpyl	LuxS	-
	Helicobacter	HpylJ99	LuxS	-
	***pylori* J99**			

Firmicutes	***Clostridium perfringens***	Cperf	LuxS	-
	Bacillus halodurans	Bhal	LuxS	-
	Bacillus anthracis	Bant	LuxS	-
	Bacillus cereus	Bcer	LuxS	-
	Bacillus subtilis	Bsub	LuxS	-
	Staphylococcus aureus	Saur	LuxS	-
	Listeria innocua	Linno	LuxS	-
	Listeria monocytogenes	Lmono	LuxS	-
	Lactococcus lactis	Llac	LuxS	-
	Lactobacillus plantarum	Lplan	LuxS	-
	Oceanobacillus iheyensis	Oihe	LuxS	-
	Staphylococcus epidermidis	Sepi	LuxS	-
	Streptococcus pyogenes	Spyo	LuxS	-
	Streptococcus pneumoniae	Spneu	LuxS	-
	Streptococcus gordonii	Sgord	LuxS	-
	Streptococcus mutans	Smut	LuxS	-
	Streptococcus agalactiae	Saga	LuxS	-
	Clostridium acetobutylicum	Cace	LuxS	-
Actinobacteria	***Bifidobacterium longum***	Blon	LuxS	-
Deinococcus	***Deinococcus radiodurans***	Drad	LuxS	-
Spirochaete	***Borrelia burgdorferi***	Bbur	LuxS	-

10. ANNEXE II: ESSAIS DE PRODUCTION DE HMAQS DANS DIFFÉRENTES CONDITIONS

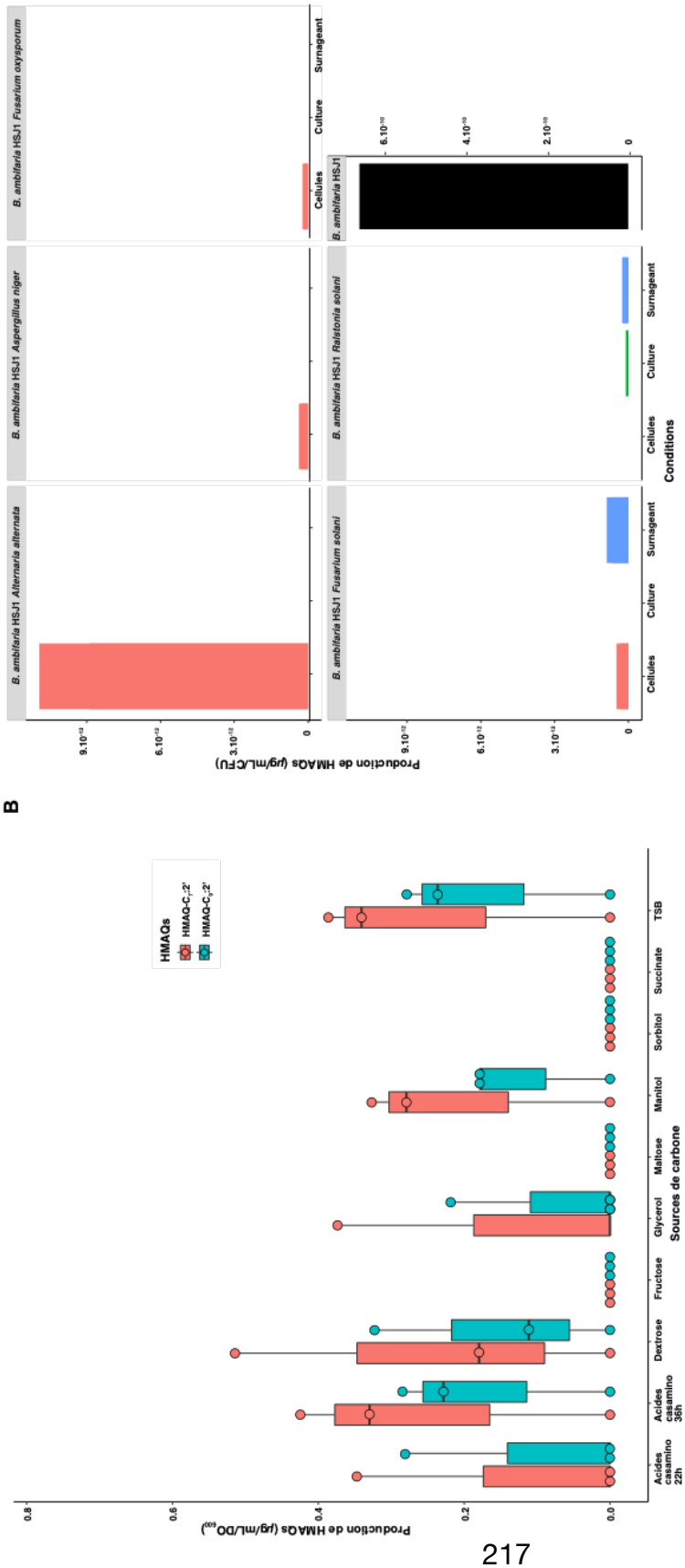

Figure annexe 10-I Pré-tests pour la production de HMAQs chez *B. ambifaria* HSJ1 en milieu M9 avec différentes sources de carbone et en milieu TSB en présence de différents champignons en culture liquide.

A) Recherche de source de carbone portentielle pour composer un milieu sur mesure pour la production de HMAQs. B) Stimulation de la production de HMAQs en presence de cellules, surnageant de culture ou culture de champignons. La quantification des HMAQs a été faite par LC-MS à partir d'une culture de 5mL dont la méthode est décrite dans les paragraphes matériel et méthodes des chapitres 4 et 6.

11. ANNEXE III: SECONDARY METABOLITES FROM THE BURKHOLDERIA PSEUDOMALLEI COMPLEX: STRUCTURE, ECOLOGY, AND EVOLUTION

Métabolites secondaires du complex de *Burkholderia pseudomallei* : structure, ecologie et évolution

Auteurs :

Jennifer R. Klaus[a], Pauline M. L. Coulon[b], Pratik Koirala[a], Mohammad R. Seyedsayamdost[c], Eric Déziel[b], and Josephine R. Chandler[a]#

[a]Department of Molecular Biosciences, University of Kansas, Lawrence KS 66045

[b]Centre Armand Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique (INRS), Laval (Québec) H7V 1B7, Canada;

[c]Department of Chemistry, Princeton University, Princeton NJ 08544

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11.1. Abstract

Bacterial secondary metabolites play important roles in promoting survival, though few have been carefully studied in their natural context. Numerous gene clusters code for secondary metabolites in the genomes of members of the Bptm group, made up of three closely related species with distinctly different lifestyles: the opportunistic pathogen *Burkholderia pseudomallei*, the non-pathogenic saprophyte *Burkholderia thailandensis,* and the host-adapted pathogen *Burkholderia mallei*. Several biosynthetic gene clusters are conserved across two or all three species, and this provides an opportunity to understand how the corresponding secondary metabolites contribute to survival in different contexts in nature. In this review, we discuss three secondary metabolites from the Bptm group: bactobolin, malleilactone (and malleicyprol), and the 4-hydroxy-3-methyl-2-alkylquinolines, providing an overview of each of their biosynthetic pathways and insight into their potential ecological roles. Results of studies on these secondary metabolites provide a window into how secondary metabolites contribute to bacterial survival in different environments, from host infections to polymicrobial soil communities.

11.2. Introduction

The Bptm group is comprised of three closely related species in the *Burkholderia* genus: *Burkholderia pseudomallei,* an opportunistic pathogen that causes the disease melioidosis; *Burkholderia thailandensis,* a non-pathogenic saprophyte; and *Burkholderia mallei,* a host-restricted animal pathogen. These three species have distinctly different lifestyles despite their close sequence relatedness, providing a unique opportunity to address questions regarding adaptations that have evolved to benefit bacteria in different environments. Of particular interest, there are many gene clusters coding for the biosynthesis of secondary metabolites in these three species. In all, at least 24 unique biosynthetic gene clusters have been identified (summarized in Biggins *et al.*, [2014]; Kunakom & Eustaquio, [2019]). The products of 12 of those gene clusters have been characterized (Gupta *et al.*, 2017; Liu & Cheng, 2014; Thapa & Grove, 2019). While 18 are unique to just one or two members of the Bptm group, five are conserved across all three species (Table 1).

The Bptm group is a useful set of model bacterial species with which to further deepen our understanding of the biology and ecology of secondary metabolites. This is because of the genetic tractability of all three species, their ease of growth in standard laboratory conditions, and the abundance and diversity of encoded secondary metabolite biosynthetic gene clusters. Understanding more about the functions of the small molecule products could give insight into the conditions under which they are most helpful to the producing bacteria (or conditions when they are harmful) and uncover important new biology about the Bptm group. Additionally, these studies could expand our existing knowledge of small-molecule biochemistry. Metabolites produced by the Bptm

group could serve as lead compounds or scaffolds to be modified for alternative medicinal or therapeutic purposes (Kunakom & Eustáquio, 2019).

In this review, we will focus on three of the secondary metabolites produced by members of the Bptm group: the bactobolin antibiotics, malleilactone, and the hydroxyalkylquinolines. We focus on these three because results of recent studies have provided insight into their importance in natural environments. For each, we will briefly describe what is known about their structure, function, and biosynthesis. Then we will discuss the ecological importance of these metabolites and how they might benefit populations during infections or surviving within complex soil communities.

Table 11.1 Secondary metabolite biosynthetic gene clusters conserved in *B. thailandensis* (*Bt*) and *B. pseudomallei* (*Bp*) or in all three strains within the Bptm group.[a,b]

Cluster	*Bt* Locus[a]	Other	Natural Product	Reference
1	BTH_I1952	*Bp, Bm*	Unknown	
2	**BTH_I2418**	*Bp, Bm*	**Malleobactin**	(Alice *et al.*, 2006)
3	**BTH_II0204**	*Bp*	**Terphenyl**	(Biggins *et al.*, 2011)
4	**BTH_II0229**	*Bp, Bm*	**Isonitrile**	(Brady *et al.*, 2007)
5	BTH_II0562	*Bp*	Unknown	
6	BTH_II1209	*Bp*	Unknown	
7	**BTH_II1233**	*Bp*	**Bactobolin**	(Seyedsayamdost *et al.*, 2010)
8	BTH_II1828	*Bp*	Pyochelin	(Franke *et al.*, 2013)
9	**BTH_II1930**	*Bp*	**HMAQ**	(Vial *et al.*, 2008)
10	**BTH_II2088**	*Bp, Bm*	**Malleilactone**	(Biggins *et al.*, 2012; Franke *et al.*, 2012)
11	**BTH_II2349**	*Bp, Bm*	**Thailandene**	(Park *et al.*, 2020)

[a]The gene ID for the first gene within the gene cluster is given. Biosynthetic gene clusters for which the product has been identified are shown in bold type.

[b]Note that *Bt* and *Bm* do not share any secondary metabolite biosynthetic gene clusters that are absent in *Bp*. Three biosynthetic gene clusters shared by *Bp* and *Bm* are not listed

11.3. Bactobolin

11.3.1. Structure, function, and biosynthesis

The bactobolins are a family of polar, hybrid polyketide-non-ribosomal peptide antibiotics first discovered in the 1970s in spent cultures of a "*Pseudomonas*" species (Kondo *et al.*, 1979). Recent studies initiated by the Greenberg and Clardy labs provided more insight into the structural diversity of this family, the biosynthetic genes and biosynthesis, and its mechanism of action (for a review of their discovery, see ref. Greenberg *et al.*, [2020]). Bactobolins are produced by both *B. thailandensis* and *B. pseudomallei.* They consist of a bicyclic enol lactone core that is formed in part with an unusual amino acid building block, 3-hydroxy-4,4-dichloro-L-valine (3-OH-4,4-Cl_2-Val, **Fig. 1A**). Nine bactobolin derivatives have been characterized to date, as well as a family of related molecules, acybolins, which are produced by the same gene cluster (**Fig. 1B**) (Carr *et al.*, 2011; Okada *et al.*, 2016; Seyedsayamdost *et al.*, 2010). Bactobolin A, the most abundant variant, inhibits growth of a variety of bacterial species with minimal inhibitory concentrations (MICs) in the range of 0.1-10 μg/mL. It targets both Gram-positive bacteria, e.g. *Staphyococcus aureus* with an MIC of ~0.3 μg/mL, and Gram-negative bacteria, e.g. *Escherichia coli* with an MIC of ~1.5 μg/mL (Chandler *et al.*, 2012b; Seyedsayamdost *et al.*, 2010). Bactobolin A binds to the 50S ribosome, where it interacts directly with the tRNA in the P-site. Both the C-5 OH and the dichloride groups are important for ribosome interaction and activity (Amunts *et al.*, 2015; Carr *et al.*, 2011; Seyedsayamdost *et al.*, 2010). Binding to the ribosome is thought to block the peptidyl transfer step during protein synthesis. This activity is similar to that of another antibiotic, blasticidin S, although the precise binding site of blasticidin S differs from that of bactobolin (Amunts *et al.*, 2015). The bactobolin target site is also unique from other antibiotics known to interact with or interfere with the peptidyl transferase center (Chandler *et al.*, 2012b). Thus, bactobolin blocks translation through a unique mode of action.

The *B. thailandensis* bactobolin biosynthetic gene cluster has 21 genes that are highly conserved in *B. pseudomallei.* The entire gene cluster is notably absent from *B. mallei,* suggesting that it is important specifically in non-host environments. Within the gene cluster, *btaLMO* code for large modular type I polyketide synthases, while *btaKN* code for non-ribosomal peptide synthetases. A small operon, *btaABCDE*, is responsible for synthesizing and incorporating the non-canonical valine analog (3-OH-4,4-Cl_2-Val), with *btaE* coding for the adenylation domain, and *btaA* and *btaC* coding for an iron-dependent hydroxylase and an iron-dependent chlorinase, respectively. A third iron-dependent enzyme, BtaU, is responsible for adding the OH group at the C-5 position. Two other genes embedded in the bactobolin gene cluster are involved in quorum sensing, a population density-dependent type of cell-cell signaling that relies on acyl-homoserine lactone (AHL) signals. The genes are *btaI2 and btaR2*, which code for an AHL synthase and an AHL receptor, respectively. AHLs can vary in structure, in which differences in the length or substitutions

of the acyl group determine the specificity of the particular system. In this case, BtaI2 synthesizes the AHLs *N*-(3-hydroxyoctanoyl)-L-homoserine lactone ($3OHC_8$-HSL) and *N*-(3-hydroxydecanoyl)-L-homoserine lactone ($3OHC_{10}$-HSL) (Duerkop *et al.*, 2009). BtaR2 specifically recognizes the AHLs synthesized by BtaI2 and upon binding these AHLs, activates expression of the bactobolin biosynthetic genes (Duerkop *et al.*, 2009). Thus, bactobolin biosynthesis is induced at high cell density by quorum sensing.

Interestingly, the bactobolin gene cluster is also activated by antibiotics, notably trimethoprim. Trimethoprim-mediated induction leads to divergent production of molecules related to bactobolins called the acybolins (Okada *et al.*, 2016). Acybolins share the bicyclic core and dichloride substituents of bactobolin, but they contain a much longer peptide linker, acylated at the N-terminus with a 3-hydroxydecanoyl group, the same acyl group found in the $3OHC_{10}$-HSL signal. Acybolins may be the prodrug form of the bactobolins. However, they are by themselves active against bactobolin-resistant *Bacillus subtilis* (Greenberg *et al.*, 2020), suggesting that they may also serve as antibiotics with a different target than the bactobolins. Much remains to be learned about the biology of acybolins and their relationship to quorum sensing.

11.3.2. Ecological role

Production of antibiotics might be important for competition with other microbes in soil. Interactions within natural microbial communities are quite complex, which can present many barriers to their study. However, reductionist binary co-culture approaches provide an alternative for studying microbial interactions without the added complexity of natural communities. Bactobolin production by *B. thailandensis* provided an opportunity to develop such a model that could be used for studying the link between quorum sensing and interspecies competition. A laboratory co-culture model was developed with *B. thailandensis* and another saprophytic soil bacterium, *Chromobacterium subtsugae* (formerly *Chromobacterium violaceum*) (Chandler *et al.*, 2012a). The model was also used to test the importance of the BtaR2-BtaI2 quorum sensing circuit that controls bactobolin production. Specifically, these results showed that genetic disruptions of *btaR2, btaI2* or the bactobolin biosynthesis gene *btaK* reduce the ability of *B. thailandensis* to compete with *C. substugae* (Chandler *et al.*, 2012a). These results support the view that bactobolin and the BtaR2-BtaI2 quorum-sensing system are important for interspecies competition.

Quorum sensing commonly controls production of antibiotics, and there is growing evidence that quorum sensing-controlled antibiotics are important for interspecies competition in other bacteria (An *et al.*, 2006; Mazzola *et al.*, 1992; Moons *et al.*, 2006; Moons *et al.*, 2005; Smalley *et al.*, 2015). Several hypotheses have been proposed to explain how quorum control of antibiotic production might benefit microbes during competition (**Fig. 1C**). Quorum sensing might provide a cost-savings strategy to ensure that metabolically costly antibiotics are produced only when the population density is sufficient to produce a killing dose (Hibbing *et al.*, 2010). An alternative

hypothesis is that delaying antibiotic production deprives a competitor of the ability to mount defenses to sublethal antibiotic and survive accumulated concentrations that are lethal (Hibbing *et al.*, 2010). These two strategies do not need to be mutually exclusive.

To explore the potential benefits of using quorum sensing to control antibiotic production, results of the *B. thailandensis-C. substugae* laboratory co-culture model were used to develop an *in silico* model where antibiotic production costs and timing could be manipulated through mathematical equations. The *in silico* models showed that early antibiotic production delayed growth during a time when too little antibiotic was produced to be effective, thereby reducing competitiveness (Chandler *et al.*, 2012a). The *in silico* results are consistent with the hypothesis that quorum sensing provides a metabolic cost-savings to antibiotic-producing bacteria that is important for competition with other species. The idea that quorum sensing mitigates costs of producing metabolically expensive goods is supported by other studies outside of the context of competition (Heilmann *et al.*, 2015; Nickzad & Déziel, 2016; Xavier *et al.*, 2011). These studies provide new insight into how quorum sensing might promote survival of bacterial populations in natural communities.

The *B. thailandensis-C. subtsugae* co-culture model was also used to explore the role of antibiotic defense mechanisms during competition (Benomar *et al.*, 2019). *C. subtsugae* has two antibiotic efflux pumps that can recognize bactobolin as a substrate. Cells treated with sublethal concentrations of bactobolin activate production of one of these efflux pumps, CseAB-OprN. This activation increases resistance to subsequent higher bactobolin doses. In co-cultures, treatment with sublethal bactobolin concentrations increases *C. subtsugae* survival during higher-dose treatment by 1,000-fold compared with cultures that did not receive the sublethal treatment. These results show that the activation of antibiotic resistance mechanisms following exposure to sublethal antibiotic concentrations is an important survival strategy during competition. Quorum sensing could be useful to coordinate the delivery of a sudden killing dose of antibiotic during competition in order to avert the induction of antibiotic resistance.

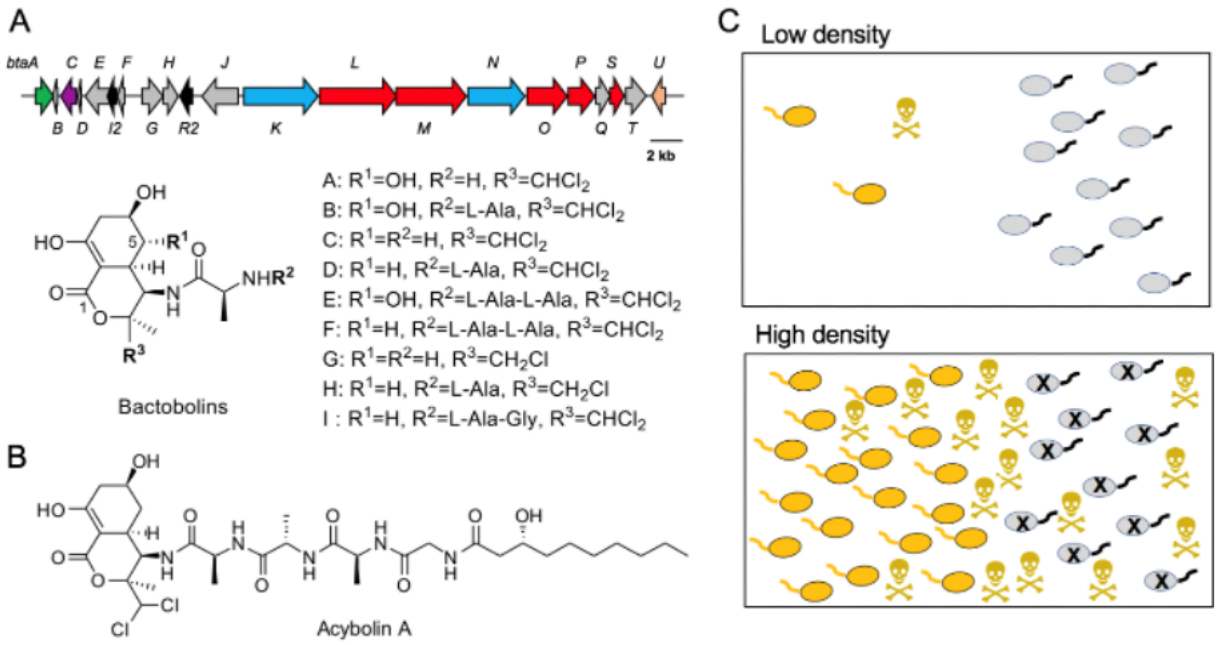

Figure 11.1 Bactobolins

(A) The *bta* gene cluster and structure of bactobolin variants A-H. The *bta* genes are indicated here by color: *btaKLMNOPS* encode nonribosomal peptide synthetases (blue) and polyketide synthases (red), *btaC* encodes the enzyme that adds chloride substituents (purple), *btaA* encodes the enzyme that adds the hydroxy group onto the valine (green), *btaU* encodes the hydroxylase that adds the OH onto C5 (salmon) and the quorum-sensing genes are coding by *btaI2* and *btaR2* (black). All other genes encode predicted transporters, bactobolin biosynthetic genes, or are of unknown function. (B) Structure of acybolin A with the bactobolin warhead. (C) Density-dependent effects of antibiotic production. Top panel: at a low density, antibiotic-producing bacteria (yellow) produce little antibiotic and are unlikely to kill the competitor. This production might slow growth of the producing bacteria or enable the competitor (grey) to mount a defense response. Bottom panel: at high cell density, enough antibiotics are produced to kill the competitor (black X). Quorum sensing delays antibiotic production until a sufficient killing dose can be produced.

11.4. Malleilactone and malleicyprol

11.4.1 Structure, function, and biosynthesis

The malleilactone and malleicyprol (*mal* or *bur*) biosynthetic genes are encoded in a ~35 kb region that is conserved in all three species of the Bptm group. This region includes the genes *malA-M* encoding polyketide synthases and non-ribosomal peptide synthetases as well as a divergently-encoded transcriptional regular gene *malR* (**Fig. 2A**). Two other genes are found only in *B. pseudomallei* and *B. mallei; malA*$_a$ and *malB*$_b$, encoding hypothetical proteins. Otherwise, the gene organization is highly conserved with the encoded proteins sharing ~80-90% amino acid identity in all three species. Early studies identified malleilactone and its tautomer burkholderic acid as the products of the *mal* genes (Biggins *et al.*, 2012; Franke *et al.*, 2012). However, recent studies by Hertweck and colleagues have shown that malleicyprol is the immediate and toxic bioactive

product of this pathway in *B. thailandensis* (Trottmann *et al.*, 2019). Malleicyprol contains an unusual and unstable cyclopropanol warhead, which converts to malleilactone/burkholderic acid under typical work-up conditions. These products have been detected in both *B. thailandensis* and *B. pseudomallei*. It is safe to assume, therefore, that the pathway in *B. mallei* also gives rise to malleicyprol, though the *mal* genes and gene product from this strain remain to be studied (Biggins *et al.*, 2012; Franke *et al.*, 2012; Klaus *et al.*, 2018) (**Fig. 2B**).

To date, the steps of malleilactone and malleicyprol biosynthesis have only been studied in *B. thailandensis* (Biggins *et al.*, 2012; Franke *et al.*, 2012; Trottmann *et al.*, 2020). A key piece to the puzzle has recently been reported regarding the initial reactions in the biosynthetic pathway and the installation of the unusual cyclopropanol group. The three-carbon unit of the warhead is derived from methionine. In the current proposed pathway, a set of four enzymes (BurBCDE or MalBCDE) convert methionine to dimethylsulfoniopropionate (DMSP) (Trottmann *et al.*, 2020). The adenylation domain of MalA then charges an adjacent thiolation domain with DMSP. This intermediate condenses with malonyl-CoA to form a thioesterified gonyol intermediate. Lending credence to this model is the detection of gonyol in *B. thailandensis* overexpressing the *mal* biosynthetic genes and when *malA* is heterologously expressed in *E. coli* (Trottmann *et al.*, 2020). Additional steps in the biosynthetic pathway have not yet been examined experimentally, but the long linear arm of malleicyprol is likely constructed independently on MalF, and the two polyketides are then joined to deliver the final product (Biggins *et al.*, 2012). Specifically, bioinformatic analyses suggest that MalF elongates a caprylic acid starter unit via two condensation reactions, the first with methylmalonyl-CoA, followed by reduction and dehydration to form a branched olefin, and the second with malonyl-CoA. MalF could subsequently join the two polyketides, one derived from elongated caprylic acid and the second from DMSP (Biggins *et al.*, 2012). Formation of the substituted furan lactone may also occur on MalF, though this proposal awaits experimental verification. Studies with the malleilactone gene cluster have brought new and interesting biosynthetic reactions to the fore and identified the cytotoxic product that is important for host infection.

Little is known about the biological activities of malleilactone and malleicyprol. The *mal* biosynthetic genes contribute to *B. thailandensis* virulence in the social amoeba *Dictyostelium discoidium* (Biggins *et al.*, 2012) and to virulence of *B. thailandensis* and *B. pseudomallei* in *Caenorhabditis elegans* nematodes (Biggins *et al.*, 2012; Klaus *et al.*, 2018). Results of two independent studies in a mouse model of melioidosis also suggest these genes are important for infection in mammals. The mouse studies used pools of transposon mutants to infect mice, and the effects of a particular gene on virulence was determined by tracking the frequency of mutants with particular gene disruptions in the pooled infection population (input population) vs. that recovered from the infection (recovered population). For the studies, mice were infected with pools of ~100 (Moule *et al.*, 2015) or ~20,000 (Gutierrez *et al.*, 2015) *B. pseudomallei* transposon mutants, and *mal*-disrupted mutants were found to be 3-7-fold reduced in the lung (both studies) and 40-fold

reduced in the spleen (Moule *et al.*, 2015) in the recovered population relative to the input population. These results suggest that the *mal* genes might play a role in *B. pseudomallei* dissemination to different organs in a mouse. The mechanism of virulence may be through the toxic effects of malleicyprol, which have been demonstrated *in vitro* to be in the 0.5 μg/mL range (Trottmann *et al.*, 2019). Malleicyprol toxicity has been proposed to be through its biologically active warhead (Trottmann *et al.*, 2019), although its specific mode of action has not been tested. Malleilactone has also been shown to coordinate iron (Biggins *et al.*, 2012); thus it is also possible malleilactone contributes to virulence by sequestering essentially needed iron in the host where its abundance is limited.

11.4.2. Ecological role

The conservation of the *mal* genes in all three species of the Bptm group suggests that the *mal* gene products are important for survival in both host- and non-host environments. However, few studies have directly tested the importance of this gene cluster in or outside of the host. The toxic effects of malleicyprol might contribute to infections *in vivo* or be important for evading eukaryotic predators in soil or killing microbial competitors in polymicrobial communities. The molecules might also have other, as-yet unknown functions in promoting survival in each of these environments. While many questions remain about the biological importance of malleilactone and/or malleicyprol, studies into the regulation of the *mal* genes do provide some insight into where and when this gene cluster might be important.

Like other secondary metabolites, expression of the *mal* genes is very low or absent in standard laboratory conditions (Biggins *et al.*, 2012; Franke *et al.*, 2012; Seyedsayamdost, 2014; Truong *et al.*, 2015). For early characterization of malleilactone, the promoter of the native *mal* cluster was replaced with a synthetic inducible promoter to enable production and isolation from lab-grown cultures (Biggins *et al.*, 2012; Franke *et al.*, 2012). In a later study (Seyedsayamdost, 2014), a small-molecule screen was used to search for elicitors of this gene cluster. The results revealed several antibiotics that can promote *mal* gene transcription (Seyedsayamdost, 2014), which has since been corroborated by other studies (Okada *et al.*, 2016; Truong *et al.*, 2015). Both trimethoprim and another folate biosynthesis pathway inhibitor, sulfamethoxazole, induce the *mal* genes. In addition, piperacillin and cephalosporins are strong inducers while the fluoroquinolone antibiotics and mitomycin C (a DNA crosslinker) cause moderate *mal* induction (Seyedsayamdost, 2014). Of note, trimethoprim and sulfamethoxazole are widely used to treat melioidosis caused by *B. pseudomallei* infections in humans, though it remains unclear what, if any, biological significance this finding has *in vivo*.

The discovery that antibiotics induce *mal* gene expression led to the hypothesis that this regulation might be through the MalR regulator encoded upstream of the *mal* genes. MalR belongs to the LuxR family of quorum sensing signal-responsive transcriptional regulators. LuxR family

receptors typically activate gene expression upon binding to AHL quorum-sensing signals, which are produced by LuxI-family signal synthases (for reviews, see Abisado *et al.*, [2018]; Waters & Bassler, [2005]). Although many cognate LuxR-LuxI pairs are genetically linked, MalR is not encoded near any LuxI genes, and for this reason, MalR is termed an orphan LuxR. MalR is also not responsive to any of the three native AHL signals produced by *B. thailandensis* and *B. pseudomallei* or a range of other AHLs (Klaus *et al.*, 2018; Truong *et al.*, 2015). However, MalR is important for trimethoprim induction of the *mal* genes in both *B. thailandensis* and *B. pseudomallei* (Klaus *et al.*, 2018; Truong *et al.*, 2015). Trimethoprim activates MalR by driving expression of *malR*, which appears to occur in response to an accumulation of folate and methionine biosynthesis intermediates (Li *et al.*, 2020). The finding that *mal* gene expression is triggered in response to certain antibiotics supports the idea that the products of the *mal* genes might enhance *Burkholderia* survival in polymicrobial soil communities.

The importance of the *mal* genes during host infections suggests that there are likely specific regulatory systems driving their expression in the host. While direct links have yet to be experimentally demonstrated *in vivo*, at least four possibilities have emerged. First, the MarR-family transcriptional repressor MftR de-represses *malR* to drive up malleilactone production in response to its inducer urate (Grove, 2010; Gupta *et al.*, 2017; Gupta & Grove, 2014). Urate is commonly produced by host-supplied xanthine oxidoreductase as part of the host's antimicrobial response (for a review, see Martin *et al.*, [2004]). Thus, MftR could be driving expression of the *mal* genes by responding to host-produced urate. Second, expression of the AraC-family transcriptional regulator HrpB increases *malR* transcription (Lipscomb & Schell, 2011). HrpB activates other genes, specifically those encoding a type 3 secretion system (T3SS) in response to plant cell contact in the plant pathogen *Ralstonia solanacearum* (Marenda *et al.*, 1998). However, it is inconclusive whether HrpB functions similarly in *Burkholderia* in response to host cues (Lipscomb & Schell, 2011). Third, microarray (Tuanyok *et al.*, 2005) analyses revealed that *malR* is activated during growth in iron-limited conditions relative to iron-replete conditions. These latter results support the idea that malleilactone might be important for sequestering iron during conditions of iron starvation, a notion that is consistent with the ability of malleilactone to bind iron (Biggins *et al.*, 2012). Finally, the *mal* genes are also controlled by ScmR, a LysR-type transcriptional regulator that largely suppresses secondary metabolism and virulence in a quorum sensing-regulated fashion in *B. thailandensis* (Klaus *et al.*, 2018; Le Guillouzer *et al.*, 2020; Mao *et al.*, 2017). Therefore, interference with the function of ScmR provides yet another route for activating the *mal* genes.

Together, the regulatory pathways controlling expression of the *mal* genes are illustrated in Fig. 2c. Results from studies of these pathways suggest that products of the *mal* genes are important for promoting survival under stress, which may be growth-inhibiting antibiotics, host immune responses, and/or nutrient (iron) limitation.

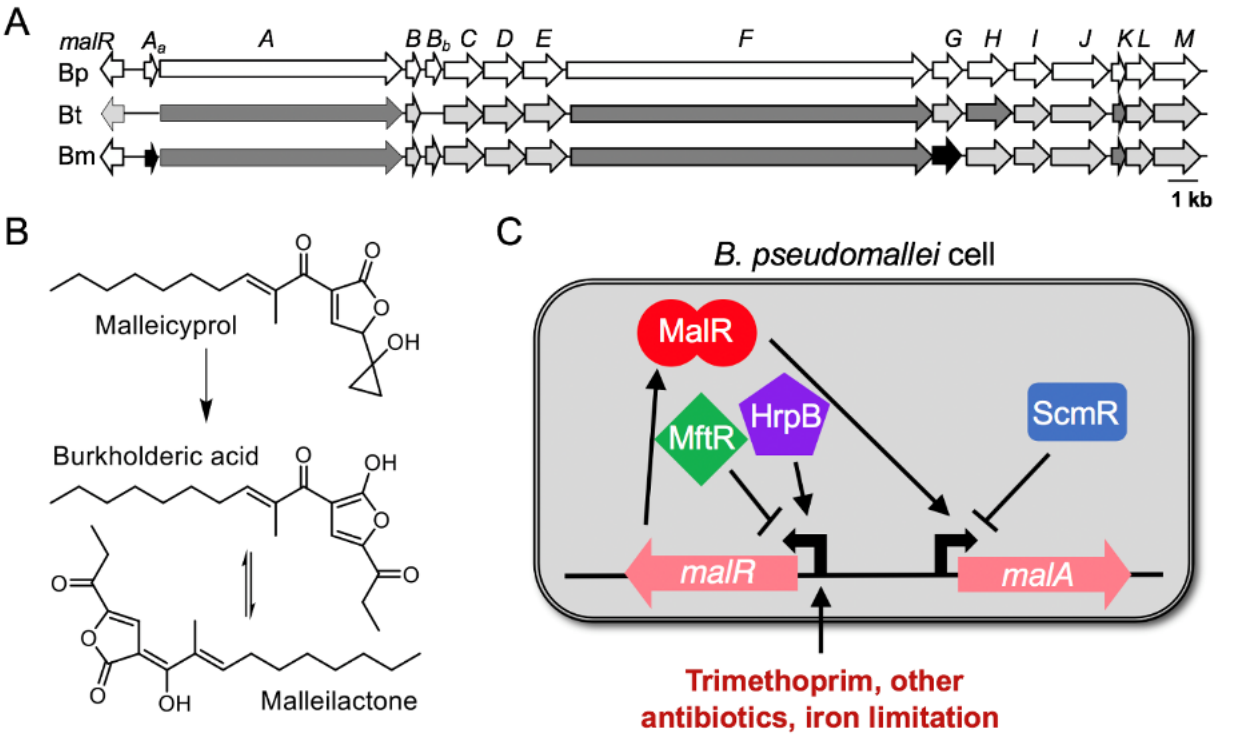

Figure 11.2 Malleilactone and malleicyprol

(A) The *mal* gene cluster from *B. pseudomallei* (Bp), *B. thailandensis* (Bt) and *B. mallei* (Bm). Shading indicates % identity to the *B. pseudomallei* protein sequences. White, 100%; light gray, 90-99%; dark gray, 80-89%; black <80%. Lowercase letters indicate genes that are present in *B. pseudomallei* and *B. mallei* but missing in *B. thailandensis.* (B) Structures of malleicyprol, malleilactone, and burkholderic acid. It is thought that malleicyprol degrades to form malleilactone and its tautomer burkholderic acid. (C) Model of *mal* gene regulation. Regulation occurs at the promoter of the first *mal* gene *(malA*) by the transcriptional activator MalR, encoded upstream from *malA*. Expression of *malR* is influenced by the urate-responsive repressor MftR, the LysR-type repressor ScmR, the host-responsive activator HrpB, trimethoprim, and conditions of iron limitation.

11.5. 4-Hydroxy-3-methyl-2-alkylquinolines

11.5.1. Structure, function and synthesis

A few bacteria produce 2-alkyl-4(1*H*)-quinolones, also known as 4-hydroxy-2-alkylquinolines (HAQs), as secondary metabolites. These molecules are involved in interbacterial competition (acting as antibiotics), virulence, iron acquisition, and cell-cell signaling, among other processes (Déziel *et al.*, 2004; Diggle *et al.*, 2007; Kilani-Feki *et al.*, 2011; Kilani-Feki *et al.*, 2012; Li *et al.*, 2018; Mahenthiralingam *et al.*, 2011; Mori *et al.*, 2007; Reen *et al.*, 2018). The HAQs mostly produced by *B. thailandensis* and *B. pseudomallei* are 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs), which are biosynthesized from products of the *hmqABCDEFG* genes (**Fig. 3A & B**). Based on bioinformatics analyses, these genes are present in 98% of *B. pseudomallei* and 79% of

B. thailandensis sequenced strains (Coulon *et al.*, 2019). Interestingly, the cluster is only present in 36% of sequenced *Burkholderia cepacia* complex (Bcc) strains (Coulon *et al.*, 2019), and thus is more highly prevalent in *B. pseudomallei* and *B. thailandensis* than the Bcc group. It is noteworthy that the *hmq* genes are entirely absent in host-adapted *B. mallei*, supporting the idea that they may have a role in the saprophytic lifestyle of the other species.

The *hmqABCDE* genes share high similarity to the *pqsABCDE* genes involved in biosynthesis of the *Pseudomonas aeruginosa* HAQs (Coulon *et al.*, 2019; Vial *et al.*, 2008). Among the *P. aeruginosa* HAQs are the *Pseudomonas* quinolone signal (PQS) 3,4-dihydroxy-2-heptylquinoline and its precursor 4-hydroxy-2-heptylquinoline (HHQ) (Coulon *et al.*, 2019; Vial *et al.*, 2008). Both PQS and HHQ are quorum-sensing signals that bind to the transcriptional regulator MvfR (also called PqsR) (Xiao *et al.*, 2006), which activates transcription of >100 genes in a population density-dependent manner (Déziel *et al.*, 2005; Diggle *et al.*, 2003). The *P. aeruginosa pqs* genes differ from the *Burkholderia hmq* genes in several important ways. First, *Burkholderia* species uniquely code for HmqF, which adds a double bond to the alkyl chain (Agarwal *et al.*, 2012), and HmqG, which adds the methyl group on the quinoline cycle (Vial *et al.*, 2008). Second, the *Burkholderia* genomes are missing a *pqsH* homolog, which is responsible for the conversion of HHQ into PQS. There is also no homolog of the MvfR/PqsR receptor in the vicinity. Although there seems to be no *Pseudomonas*-type PQS-dependent quorum-sensing system in the *Burkholderias,* HMAQs do modulate transcription of the *hmq* genes through an unknown mechanism (Chapalain *et al.*, 2017b; Le Guillouzer *et al.*, 2017; Piochon *et al.*, 2020). There are also complex regulatory interactions between the *hmq* system and the AHL quorum-sensing systems (Chapalain *et al.*, 2017a; Le Guillouzer *et al.*, 2017; Majerczyk *et al.*, 2014a; Majerczyk *et al.*, 2014b).

Despite these key differences, other aspects of HMAQ and HAQ biosynthesis are largely similar (Fig. 3c, [Coulon *et al.*, 2019; Vial *et al.*, 2008]). Anthranilic acid is the precursor of both HMAQ and HAQ biosynthesis (Déziel *et al.*, 2004; Vial *et al.*, 2008). In both *Burkholderia* and *Pseudomonas*, anthranilic acid is synthesized via tryptophan degradation by the *kynBUA*-encoded kynurenine pathway, and/or from chorismic acid via the TrpEG anthranilate synthase (Farrow & Pesci, 2007). A second anthranilate synthase encoded by *phnAB* is unique *to P. aeruginosa*. From anthranilic acid, HmqA/PqsA synthesizes anthraniloyl-CoA. HmqD/PqsD then transfers malonyl-CoA to anthraniloyl-CoA to form 2-aminobenzoylacetate-CoA (Drees *et al.*, 2018). Next, the putative 2-aminobenzoylacetyl-CoA thioesterase HmqE/PqsE can remove the CoA from 2-aminobenzoylacetyl-CoA to yield 2-aminobenzoylacetate (2-ABA) (Drees *et al.*, 2018). In *Burkholderia,* the HmqG methylase is thought to add a methyl group onto 2-ABA to make methyl-2-ABA (Vial *et al.*, 2008). In both *Burkholderia* and *P. aeruginosa,* (methyl)-2-ABA is modified by PqsBC/HmqBC by incorporating fatty acid-derived alkyl groups to make the hydroxy (methyl) alkylquinolone molecules. In *P. aeruginosa,* PqsL also converts 2-ABA to 2-hydroxyl-ABA (2-HABA) (Drees *et al.*, 2018; Lépine *et al.*, 2004). 2-HABA is then alkylated by PqsBC to make *N*-oxide

derivatives of HAQs. In *Burkholderia* HmqL is thought to play a similar role as PqsL in *N*-oxide HMAQ biosynthesis (Klaus *et al.*, 2020).

11.5.2. Ecological role

The ecological role and biological function of HMAQs remains poorly understood despite their prevalence across many different *Burkholderia* species. Several studies support the idea that HMAQs could be important for competing with other microbes in mixed soil communities. These molecules have antibacterial (Li *et al.*, 2018; Piochon *et al.*, 2020) and antifungal activities (Kilani-Feki *et al.*, 2011; Kilani-Feki *et al.*, 2012; Mahenthiralingam *et al.*, 2011). In addition, in a recent study (Klaus *et al.*, 2020), HMAQs were shown to be important for *B. thailandensis* to kill *B. subtilis* in co-cultures. Genetic disruption of the *B. thailandensis hmqL* abolished production of the *N*-oxide derivatives of HMAQs, confirming the role of HmqL in biosynthesis of the *N*-oxides. Further, the most abundant *N*-oxide congener, HMNQ-NO (4-hydroxy-3-methyl-2-nonenyl-quinoline *N*-oxide, see **Fig. 3B**), has potent killing effects against *B. subtilis* (Klaus *et al.*, 2020). These results support the role of HmqL and HMAQs, in particular HMAQ-NO such as HMNQ-NO, in competition at least against Gram-positive bacteria (Piochon *et al.*, 2020). Interestingly, the *hmqL* gene needed for biosynthesis of *N*-oxide derivatives is missing from the genome of the Bcc (Vial *et al.*, 2008). It is possible HMAQs are sufficient to kill competitors in these species (Piochon *et al.*, 2020). The finding that some of the *Burkholderia* species lack an *hmqL* gene suggests another interesting possibility: that HMAQs have other important functions in soil bacteria that remain as-yet undiscovered.

B. thailandensis and *B. pseudomallei* predominantly produce C_9 congeners, while the Bcc species predominantly produce C_7 congeners (Diggle *et al.*, 2006; Vial *et al.*, 2008). The C_9 congeners are synthesized from decanoic acid while C_7 congeners are synthesized from octanoic acid, as they are in *P. aeruginosa* and probably also in other *Burkholderia* (Agarwal *et al.*, 2012; Dulcey *et al.*, 2013). It is unclear what evolutionary factors drive alkyl chain length and substitution in different species of *Burkholderia*, although it is tempting to speculate that the particular HAQs produced by each species promotes survival in that bacterium's particular environment. For example, differences in alkyl chain length or quinolone substitution could alter the killing activity against different target species. A recently developed procedure for HMAQ chemical synthesis (Piochon *et al.*, 2020) provided an avenue to explore this idea. Studies with these synthetic molecules showed that differences in the alkyl chain length and substitution do alter antimicrobial activities against different species (Klaus *et al.*, 2020; Piochon *et al.*, 2020). Although the basis for these differences are currently unknown, it is possible that changes in the alkyl group length or substitution change the specific target site or ability to penetrate different types of bacterial cells. These results suggest the possibility that the antimicrobial activity against different types of competitors might drive the evolution of the structure of the HMAQ.

There is also a diversity of *hmq* biosynthesis products within each organism. It has been proposed that synthesizing diverse secondary metabolites from the same pathway could provide certain advantages, such as synergistic activities (Challis & Hopwood, 2003). In support of this idea, two different products of the *B. thailandensis* HMAQ biosynthesis pathway were shown to have the synergistic ability to kill other bacteria (Wu & Seyedsayamdost, 2017). One of these was the major *B. thailandensis* product HMNQ. The other was HQNO (2-heptyl-4(1H)-quinoline N-oxide (see **Fig. 3B**), which is a minor product of *B. thailandensis* HMAQ

biosynthesis and which is readily produced by *P. aeruginosa*. It was shown that HMNQ and HQNO have distinctly different targets; HMNQ disrupts the proton motive force (Wu & Seyedsayamdost, 2017), while HQNO blocks the cytochrome bc1 complex (Hacker *et al.*, 1993; Hazan *et al.*, 2016; Van Ark & Berden, 1977). Thus, by blocking energy production through two different mechanisms, these two molecules act synergistically to inhibit bacterial growth (Wu & Seyedsayamdost, 2017). Divergent molecule synthesis could be important for competing with other bacteria because it could enhance the effects of the antimicrobials produced from the pathway. Divergent molecule synthesis could also avert the development of antibiotic resistance in competitors, as it would require mutations in several pathways occurring simultaneously to overcome the dual-killing mode of action.

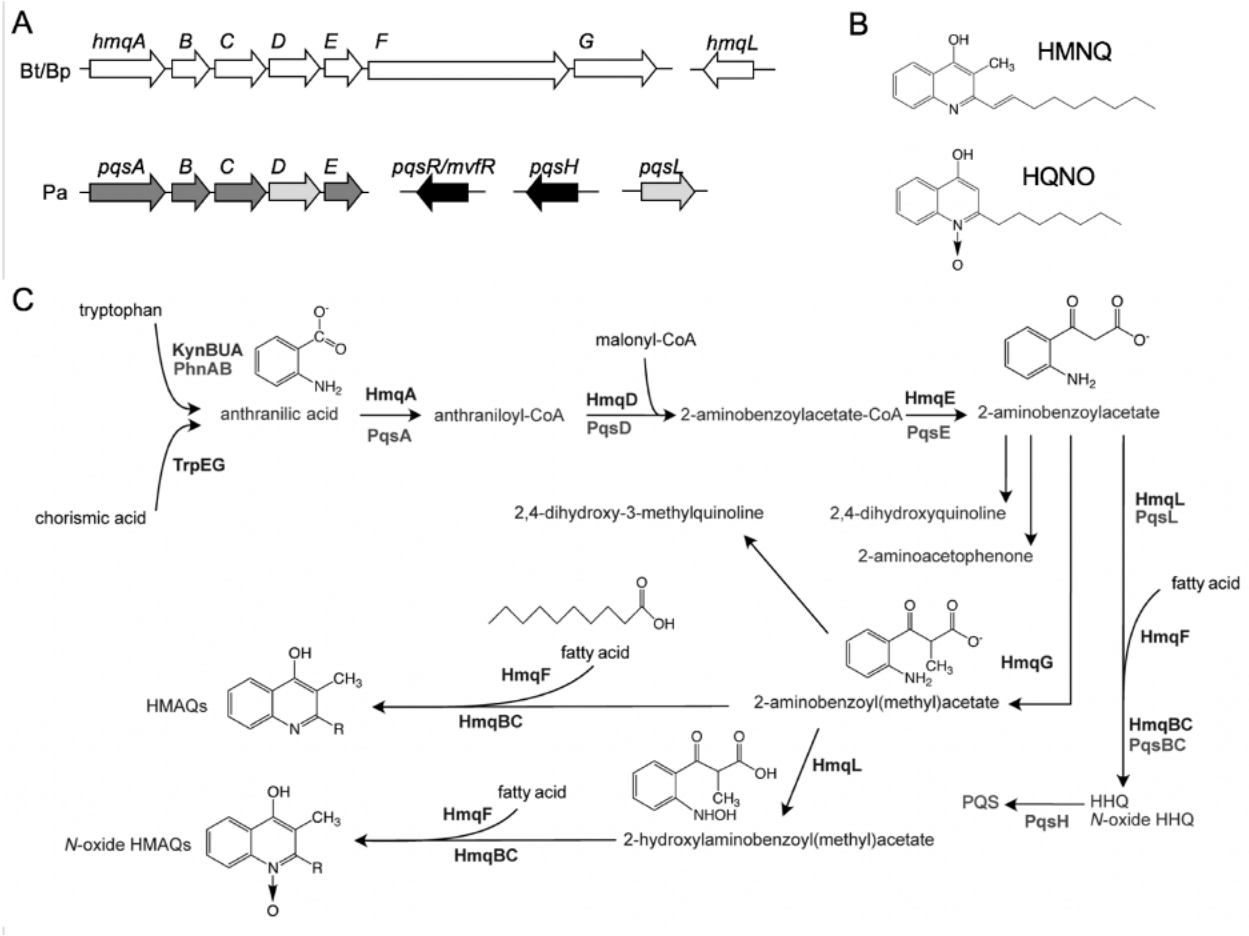

Figure 11.3 4-Hydroxy-2-alkylquinolines and substituted congeners

(A) The *hmq* gene cluster in *B. thailandensis* (Bt) and *B. pseudomallei* (Bp) and the homologous *pqs* gene cluster in *P. aeruginosa* (Pa). Shading of Pa proteins indicates % sequence identity to Bt sequences. Light grey, 50-60%; dark grey, 30-40%; black, no homology. Bt and Bp proteins are >85% identical. (B) Structures of HMNQ (4-hydroxy-3-methyl-2-nonenylquinoline) and HQNO (2-heptyl-4(1H)-quinoline *N*-oxide). (C) Biosynthesis of hydroxyl-alkylquinolines by *hmq* gene products in *B. thailandensis/B. pseudomallei* and by *pqs* gene products in *P. aeruginosa.* The specific steps are described in text.

11.6 Conclusions and future directions

Secondary metabolites can have important and often underappreciated roles in promoting survival in different host and non-host environments. Secondary metabolites might also have other useful purposes, such as expanding the existing knowledge of small-molecule biochemistry or in the development of new antimicrobial compounds to treat bacterial infections. Secondary metabolite studies are slowed down by technical challenges such as their low production under laboratory conditions or issues with purification and stability. It is also challenging to demonstrate their role in promoting survival of the producing microorganisms in their natural ecological niches. However, genetic and chemical tools to elicit secondary metabolite production as well as the development of laboratory co-culture models have helped to overcome some of these challenges. With these tools, we are now beginning to understand how some of these secondary metabolites are synthesized and regulated, and what their functions might be. Results of secondary metabolite studies provide useful knowledge both toward advancing research applications and toward appreciating how and when these molecules might become activated (or suppressed) in biologically-relevant conditions.

We believe that the Bptm group provides an excellent opportunity to further understand the biology of secondary metabolites. This is because of the abundance and diversity of encoded secondary metabolite gene clusters as well as the advancement of genetic, biochemical, and ecological systems for studying these species. There is a growing body of work describing the Bptm metabolites and in particular bactobolin, malleilactone, and HMAQs. The results generated provide a strong foundation with which to develop a better understanding of how secondary metabolites affect the ecology of these bacteria. Many questions remain to be answered, such as discovering targets and other activities of the metabolites, the regulatory interplay between the metabolite biosynthesis gene clusters, and uncovering the role of these metabolites in more complex natural communities. Addressing some of these questions will require already-existing genetic and chemical tools or altogether new approaches, such as synthetic ecology systems that expand on existing ones by introducing more species or other variables. Application of the tools and approaches developed in the Bptm group could also provide insights into roles of secondary metabolites in the ecology and physiology of other bacteria.

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