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QUORUM SENSING CONTROLS THE PRODUCTION OF THE SIDEROPHORE MALLEOBACTIN IN *BURKHOLDERIA THAILANDENSIS* E264

Maîtrise en microbiologie appliquée

Jury d'évaluation

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ABSTRACT

Siderophores are chelating molecules with a very high affinity for $Fe3^{+}$, an essential element for the survival of most living organisms. These molecules are produced and secreted by bacteria under iron-limiting conditions. Malleobactin and pyochelin are siderophores produced by the bacterium *Burkholderia thailandensis*, a closely related bacterium to *B. pseudomallei* and *B. mallei* that is not pathogenic to humans. Several species of Gram-negative bacteria communicate by synthesizing, secreting, and responding to small diffusible signal molecules, *N*-acyl homoserine lactones (AHLs), through a mechanism called quorum sensing (QS). The AHLs-mediated cell-to-cell signaling allows these bacteria to coordinate gene expression and regulate different functions such as biofilm formation and virulence factors. The signal molecules are produced by an AHL synthase gene *luxI* at low concentration and are diffusing around the cells. Transcription of QS-regulated target genes appears by LuxR homologue proteins only when high AHL concentration is present, which required a threshold bacterial cell density. Like *B. pseudomallei* has the bpsIR quorum sensing system, in *B. thailandensis* there are three complete quorum sensing systems (each including a synthase for a corresponding signal molecule), BtaIR*1,* BtaIR*2 and* BtaIR*3*; and two orphan regulators, Bta*R4* and BtaR*5*. Our hypothesis is that QS regulates the production of the siderophore malleobactin in *B. thailandensis* E264. First we measured siderophore production with a colorimetric method using CAS dye (chrome azurol S) in different mutants of pyochelin and maleobactin synthesis and determined that malleobactin acts as the main siderophore under iron depleted conditions. Then, the different quorum sensing mutants available in our laboratory (*btaI1*, *btaI2*, *btaI3*, *btaI1,2,3, btaR1*, *btaR2*, *btaR3*, *btaR4* and *btaR5*) were investigated. This allowed us to identify positive and negative regulation of siderophore production. Finally, we quantified the expression of the *mbaA* gene (the first gene for the operon of malleobactin synthesis) in these mutants using the qRT-PCR method. This study provides insights into the role of intercellular communication in the production of the main siderophore produced by *B. thailandensis.*

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LIST OF ABBREVIATIONS

AHL	N-acyl homoserine lactone
Bcc	*Burkholderia cepacia complex*
Bptm	*Burkholderia pseudomallei-thailandensis-mallei*
C_8-HSL	*N*-(Octanoyl)-L-homoserine lactone
C_{10}-HSL	*N*-(Decanoyl)-L-homoserine lactone
$3OHC_8$-HSL	*N*-(3-Hydroxy-octanoyl)-L-homoserine lactone
HPLC-MS	« High performance liquid chromatography coupled to tandem mass spectrometry »
INRS	Institut National de la Recherche Scientifique
OD_{600}	Optical density at 600 nm.
PCR	« Polymerase chain reaction »
QS	*Quorum sensing*
RSU	Relative siderophore units
qRT-PCR	« Quantitative reverse transcription-PCR »

INTRODUCTION

Bacteria are unicellular prokaryotic organisms that were among the first life forms to appear on Earth and they are present in most of its habitats. Adapted to a wide range of habitats and conditions, from extreme pH to extreme temperatures, bacteria also live in symbiotic and parasitic relationships with higher organisms (plants and animals).

Burkholderia is a genus of β-*Proteobacteriaceae*, Gram-negative, a rod-shaped bacteria. Previously, they were members of the genus *Pseudomonas*. The first species of this genus was described by Walter Hagemeyer Burkholder, and it was named as *Pseudomonas cepacia* (Burkholder, 1950). Ecologically they are saprophytes that intervene in the recycling of organic matter, but there are also pathogenic species such as members of the *Burkholderia cepacia* complex or *Burkholderia pseudomallei*, which is considered as a potential biological warfare agent (along with *B. mallei)*, due to their antibiotic resistance and high mortality rates of their associated diseases (Godoy *et al.*, 2003). For this project, we chose to work with *B. thailandensis* because it is not a pathogen while being closely related to *B. pseudomallei* and *B. mallei*, as they originate from a common ancestor (Brett *et al.*, 1998).

Iron is an essential element for most organisms, because it is involved in several metabolic pathways. It has two oxidation states, ferrous (II) and ferric (III), but only form (II) is used by the cell. Because most of the iron in nature is in its ferric form, bacteria have several mechanisms for iron uptake. The reversible Fe(II)/Fe(III) redox pair is best suited to catalyze a broad spectrum of redox reactions and to mediate electron chain transfer in most microbial habitats. Fe(II) is oxidized to Fe(III) either spontaneously by reacting with molecular oxygen or enzymatically during assimilation and circulation in host organisms. In the environment, Fe(III) forms ferric oxide hydrate complexes in the presence of oxygen and water at neutral to basic pH. These complexes are very stable, leading to a free Fe(III) concentration of only 10^{-9} to 10^{-18} M. In mammalian hosts, the assimilated iron is tightly bound to various proteins.

Hemoproteins such as hemoglobin contain about two-thirds of the body iron in the heme-bound state. Ferritin, the intracellular iron storage protein, is able to store up to 4,500 Fe(III) ions per oligomer and contains about 30% of the iron pool (Miethke *et al.*, 2007). Because of this, iron is poorly available to invading pathogenic bacteria.

Siderophores are small molecules, usually peptides, acting as iron chelators. Iron is necessary for life, since it is part of the metabolism of essential molecules, such as DNA or ATP (Caza *et al.*, 2013). In nature, most iron is in its insoluble form (III) and is not assimilable by bacteria. The soluble form (II) is scarce. Therefore, bacteria produce siderophores to capture the iron in its form (III) and uptake it into the cells, where it is reduced into form (II), soluble and assimilable by the cell (Ilbert *et al.*, 2013). The affinity for iron of siderophores is among the highest known in nature. In fact, in the case of pathogenic bacteria, they can compete and capture iron from proteins such as lactoferrin or hemoglobin, to ensure their survival within the host (Miethke *et al.*, 2007). Therefore, they are considered as virulence factors. In the case of *B. thailandensis*, *B. pseudomallei* and *B. mallei* it is known that they produce two siderophores: malleobactin (the most important, with higher affinity to iron) and pyochelin (smaller (324 Da) and with low affinity to iron, it acts like a secondary siderophore) (Franke *et al.*, 2013),(Kvitko *et al.*, 2012).

Since they are unicellular organisms, bacteria are considered independent and clonal organisms. However, bacteria can interact with each other, and "communicate" to coordinate diverse functions (production of virulence factors, emulsifying agents, biofilms formation, siderophore production, toxins, swarming motility, etc.). This phenomenon is known as *quorum sensing*. This communication depending on cell density was first described in *Vibrio fischeri* (Nealson *et al.*, 1979); it is considered the paradigm of *quorum sensing* in most Gram-negative bacteria. At lower cell densities, the signal molecules (inducers) are constitutively expressed at a low, basal level and thus they get accumulated in the surrounding. After reaching a certain threshold in cell density, these inducers will bind to the transcriptional regulator and will modify the transcription of certain target genes. The fact of being able to regulate diverse genes

and functions entails a great benefit for the bacterial population. It supposes an adaptive advantage, optimal management of resources and promotes survival.

In this project, the regulation of siderophore malleobactin by *quorum sensing* in *Burkholderia thailandensis* was studied. This siderophore is analogous to ornibactin produced by *Burkholderia cepacia* and similar genes are responsible for its production (Sokol *et al.*, 2000). We used *B. thailandensis* because it is a non-pathogenic species and genetically very close to *B. pseudomallei* and it also easier to work with. This work will provide a more detailed and extensive view of how *quorum sensing* controls certain genes in bacterial development.

1 LITERATURE REVIEW AND BACKGROUND

1.1 THE GENUS *BURKHOLDERIA*

Burkholderia is a genus of β-proteobacteria, Gram-negative bacilli, that they can live in a wide diversity of environments. The first species of this genus was first named *Pseudomonas cepacia* by W. H. Burkholder. He discovered this bacterium while he was studying the disease sour skin in onions (Burkholder, 1950). It was years after, when the genus *Burkholderia* was created to accommodate a subgroup of *Pseudomonas* species (Yabuuchi *et al.*, 1992). Currently, according to the LPSN (*List of prokaryotic names with standing in nomenclature)* this genus has 122 species (www.bacterio.net.)

The genus *Burkholderia* contains organisms that can live in very different environments (from saprophytes to strict pathogens). Some of the species of this genus are pathogens for animals and plants but there are also organisms that are not pathogenic, and some strains are beneficial for plant growth or bioremediation (Donald E Woods *et al.*, 2006). For instance, *Burkholderia thailandensis*, a non-pathogenic bacterium that is saprophytic from tropical soils, is closely related to two other pathogenic species, *Burkholderia pseudomallei* and *Burkholderia mallei*. These three species form a group known as the *Bptm* group.

The *Burkholderia* group has experienced several changes. New bioinformatic tools and new genomic information have provided more data to better understand the relationship between all the *Burkholderia* species. Thus, this group is currently composed of six genera: *Burkholderia, Caballeronia, Paraburkholderia, Robbsia, Mycetohabitans* and *Trinickia.* These genera were established after several studies based on 16S rRNA sequences (Mannaa *et al.*, 2018).

1.1.1 *B. pseudomallei* complex

Inside the genus *Burkholderia,* we can find this well-defined group of five species (*B pseudomallei, B. mallei, B. thailandensis, B. oklahomensis*, *B. humptydooensis* sp.) (Tuanyok *et al.*, 2017). The three first species share more than 95% genomic (Donald E Woods *et al.*, 2006).

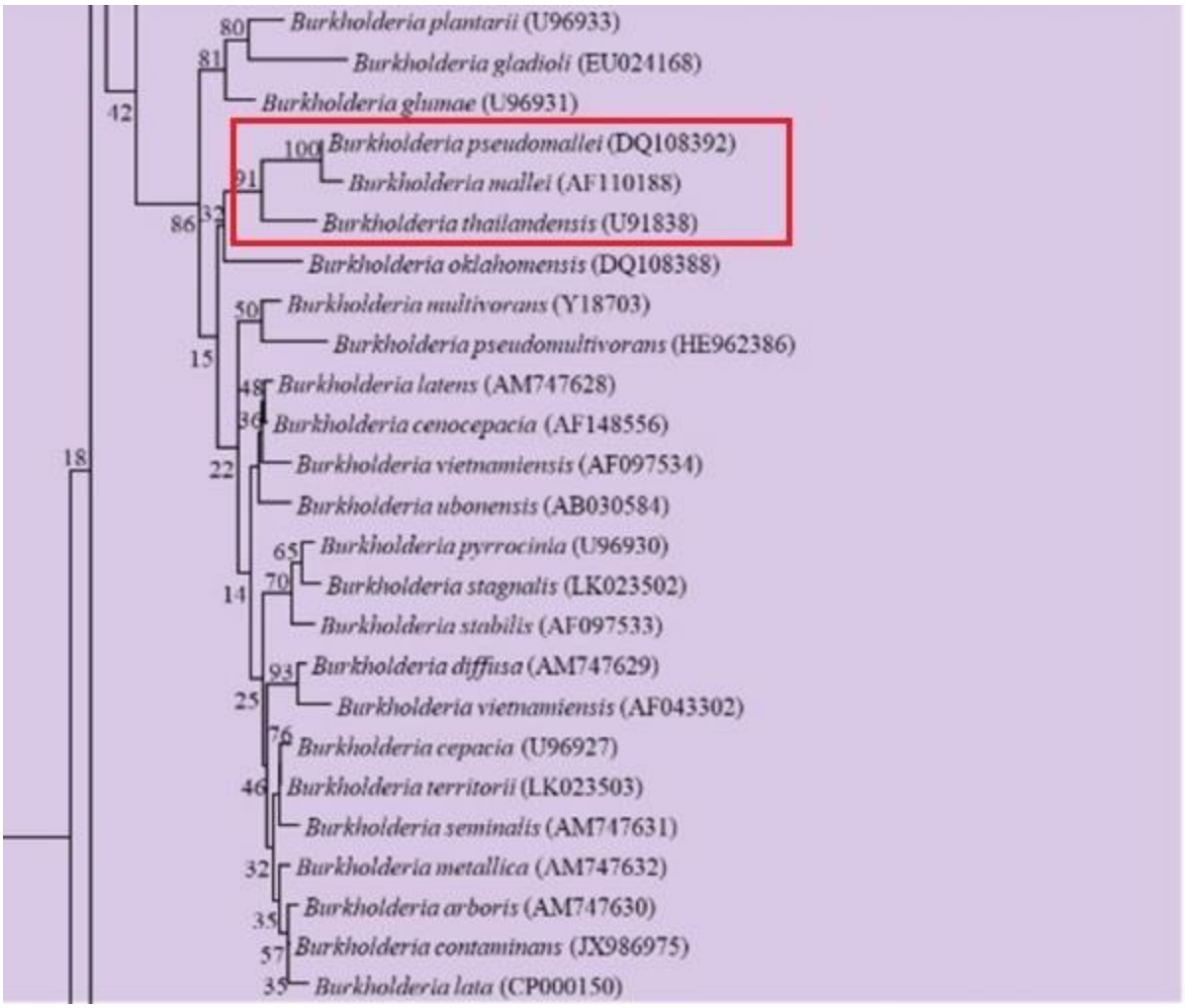

Figure 1. Proposed taxonomy of the *Burkholderia* genus. This scheme only shows the 25 species classified in the *Burkholderia* stricto group. Based on biochemical, immunological and genetic data, *B. pseudomallei, B. mallei and B. thailandensis* are closely related (adapted from Mannaa *et al.* 2018).

Burkholderia pseudomallei, a soil organism especially found in Southeast Asia and Northern Australia (Woods *et al.*, 2006) is a pathogen that causes melioidosis in humans, especially in those individuals with underlaying conditions, such a diabetes or renal disease (Woods *et al.*, 1993). Individuals who contract the disease can be asymptomatic or develop various clinical manifestations, such as chronic pneumonia, abscesses and even reach a septicemia that can be lethal. The treatment of meliodosis with antibiotics is complex and can be very long (up to 9 months). Also, this microorganism has a very long latency period, which can vary from 2 to 26 years (Smith

et al., 1987). In addition, this pathogen is considered as a potential biological weapon that may present a risk of bioterrorism (Jansen *et al.*, 2014). For these reasons, this bacterium needs to be handled with care and requires level 3 confinement (Rotz *et al.*, 2002).

Burkholderia mallei is the causative agent of glanders, a disease which mainly affects mules, horses and donkeys. It is an intracellular, facultative pathogen. Although it is not common for this disease to occur in humans, direct contact with this organism can cause localized skin infection, while inhalation can lead to septicemia and severe lung infections (Sanford, 1990).

B. thailandensis E264 (an avirulent environmental isolate from Central and North-East Thailand (Smith *et al.*, 1995) (Wuthiekanun *et al.*, 1995). It is very similar to *B. pseudomallei* but it is not pathogenic for animals nor plants. Several studies (genotypic, phenotypic and biochemical) have shown that it is a new species within the genus *Burkholderia* (Brett *et al.*, 1997, Brett *et al.*, 1998). Since then, it has been used for an easier study of their pathogenic relatives and the diseases they cause. Even if *B. pseudomallei* and *B. thailandensis* are very similar genetically speaking, they are considered and studied as a different species (Brett *et al.*, 1998).

1.2 QUORUM SENSING

Quorum sensing (QS) is the regulation of gene expression in response to cell density. Bacteria produce and secrete chemical signals, called autoinducers, that increase their concentration as cell density also increases. The reaching of a certain threshold in this concentration is what leads to the modification of gene expression (W. C. Fuqua *et al.*, 1994). QS is involved in the regulation of several cellular processes: symbiosis, virulence, competition, conjugation, antibiotic production, motility, sporulation and biofilm formation. Gram-negative bacteria use acylated homoserine lactones (AHL) as autoinducers (C. Fuqua *et al.*, 1998), while Gram-positive bacteria use oligo-peptides to communicate (Dunny *et al.*, 1997). This type of communication allows the bacteria to

coordinate gene expression, allowing them to function as multicellular organisms (Miller *et al.*, 2001).

Each QS system has been optimized to promote the survival of the bacteria in different specific ecological niches. Each of these system are specifically organized to solve the communication needs bacteria may have depending on the ecological niche they occupy. Because of this, the types of signals, receptors, and signal transduction mechanisms are specific to each bacterial species (Waters *et al.*, 2005).

1.2.1 Quorum sensing in Gram-negative bacteria (acyl-homoserine lactone signalling)

In Gram-negative bacteria, the regulation of gene expression by QS has homologies with the LuxI/LuxR system described in *Vibrio ficheri* (Waters et al., 2005). Specifically, this *quorum sensing* model consists in one synthase protein (LuxI) and a transcriptional regulator (LuxR) (**Figure 2**). LuxI protein is responsible for the biosynthesis of specific acylated homoserine lactones (AHL) that act as signaling molecules, called autoinducers. Consequently, a low level of autoinducer is produced. The autoinducer diffuses freely through the membrane, therefore, with the minimal basal expression, the extracellular concentration of autoindicer is equal to its intracellular concentration. As cell density increases, the amount of autoinducers outside the cell also increases, until they reach a concentration threshold which is sufficient for detection and subsequent binding to the LuxR protein (C. Fuqua *et al.*, 2002). After that, autoinducers interact with LuxR protein to form the LuxR-AHL complexes that control the expression of the QS target genes and the gene *luxI*, coding the LuxI synthase. This creates a positive feedback loop, and all the bacterial population switch into "*quorum sensing"* mode (Waters *et al.*, 2005). The LuxR-AHL complex acts as a negative feedback in the regulation of *luxR* expression. This negative regulation is a compensatory mechanism that decreases the expression of the *luxI* gene in response to the positive feedback circuit (Miller *et al.*, 2001). Using this mechanism, Gram-negative bacteria can efficiently couple gene expression to fluctuations in population density.

These systems are mostly used in intraspecies communication due to the extreme specificity that exists between the LuxR proteins and their AHL signal molecule. Several studies (Vannini *et al.*, 2002),(Zhang *et al.*, 2002) point out that in cultures where several species are mixed, with multiple AHL present in the environment, each species can distinguish, measure and respond only to its own signal. It should be noted that bacteria rarely use exclusively a single QS LuxIR system. They prefer to use one or several LuxIR systems, usually in conjunction with other types of QS circuits. Because of that, it is also necessary to have mechanisms to prevent the premature activation of the LuxIR circuit, since the signal molecule and the detector are synthesized and interact in the cytoplasm (Waters *et al.*, 2005).

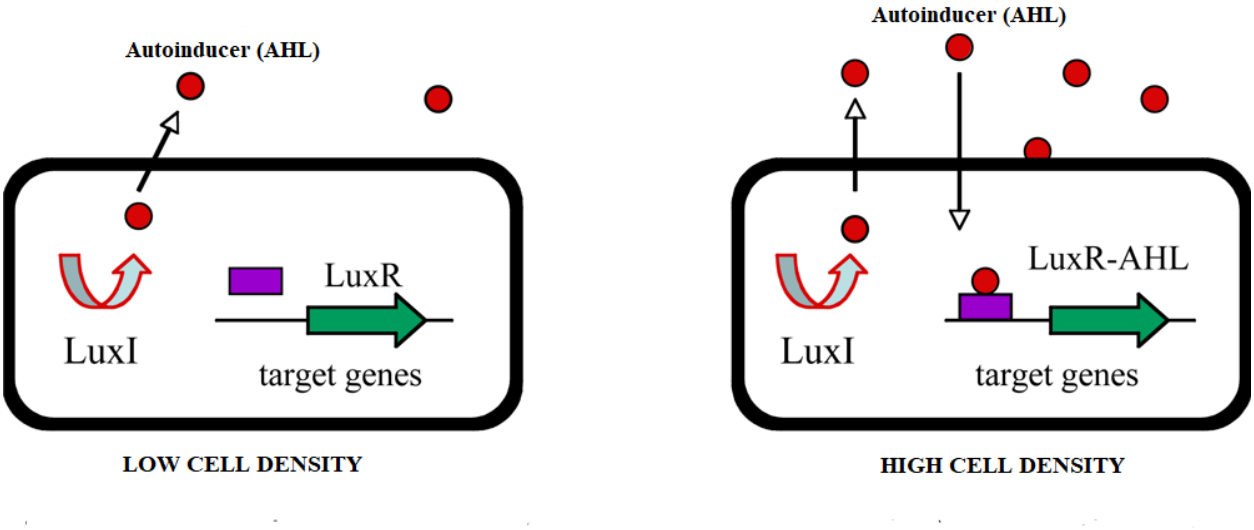

Figure 2. *Quorum sensing* in Gram-negative bacteria. A LuxI/LuxR-type signalling circuit. The AHL freely diffuses through the cell membrane at high cell density. LuxR is a transcriptional regulator that binds to the AHL and activates the transcription of QS-controlled genes.

1.2.2 Quorum sensing in *Burkholderia thailandensis*

It is common in many *Burkholderia* species to use AHL in their QS systems. Members of that group have homologous QS systems. *B thailandensis* and *B. pseudomallei* possess three complete QS LuxIR type systems: system 1 (QS-1), system 2 (QS-2) and system 3 (QS-3). Alternatively, *B. mallei* has retained system 1

and 3 but lacks QS-2 (Ulrich *et al.*, 2004); it seems it has been deleted during evolution (Ong *et al.*, 2004). Specifically, *B. thailandensis* has three complete LuxIR systems (named BtaIR1, BtaIR2 and BtaIR3) and two orphans systems that do not have a cognate autoinducer synthase (BtaR4 and BtaR5) (Ulrich *et al.*, 2004). The QS-1 circuit of *B. thailandensis* consists of the pair *btaI1* and *btaR1* genes that code for BtaI1 (synthase) and BtaR1 (transcriptional regulator), and its corresponding signal molecule is the N-octanoyl-homoserine lactone (C_8-HSL). The system 2, is composed of *btaI2* and *btaR2* genes that code for the BtaI2 synthase and the BtaR2 transcriptional regulator, respectively. BtaR2 activates expression of *btaI2* involved in both *N-(*3-hydroxy-decanoyl)-L-homoserine lactone (3OH-C_{10}-HSL) and *N*-(3-hydroxy-octanoyl)-L-homoserine lactone (3OH-C_8-HSL) biosynthesis (Duerkop *et al.*, 2009).

Finally, QS-3 encoded by the *btaI3* gene and the *btaR3* gene, encoding for BtaI3 synthase and BtaR3 transcriptional regulator. The AHL working in this system is 3OHC$_8$-HSL (Chandler *et al.*, 2009). As mentioned before, the three species of the *Bptm* group are very similar and are closely related, and they have conserved QS systems. However, *B. mallei* is a strict pathogen and *B. pseudomallei* is a soil bacterium but also highly infectious opportunistic pathogen. Working with both requires level 3 biosecurity facilities. As *B. thailandensis* is not a pathogen for humans, it serves as a model for the study of QS and other aspects of *Bptm* group biology, which can be carried out without strict level 3 biosafety contstrains (Majerczyk *et al.*, 2014).

By studying the QS systems in these three species of bacteria, it can be elucidated not only which genes, or groups of genes, are controlled and regulated by QS, but it also shows how different QS networks can benefit different ways of life. As mentioned before, *B. mallei* does not have the QS-2 system, it may be due to its adaptation to a strict pathogenic way of life (Ong *et al.*, 2004).

Previous investigations in the Déziel Lab described how the three QS systems in *B. thailandensis* interact between each other and suggested a model of how these QS systems are regulated by feedback loops, resulting from transcriptional and posttranscriptional interactions, allowing controlled coordination of the expression of genes (Le Guillouzer *et al.*, 2017) (**Figure 3**). In addition, the results of a RNA-seq study (Le Guillouzer, 2018) (all the genes that are controlled by QS in *B. thalandensis*) and

the screening Sok Gheck Tan made in 2014 (all the genes in *B. thailandensis)* using the CAS essay were compared to find out which other genes are involved in siderophore production regulation which are also controlled by QS.

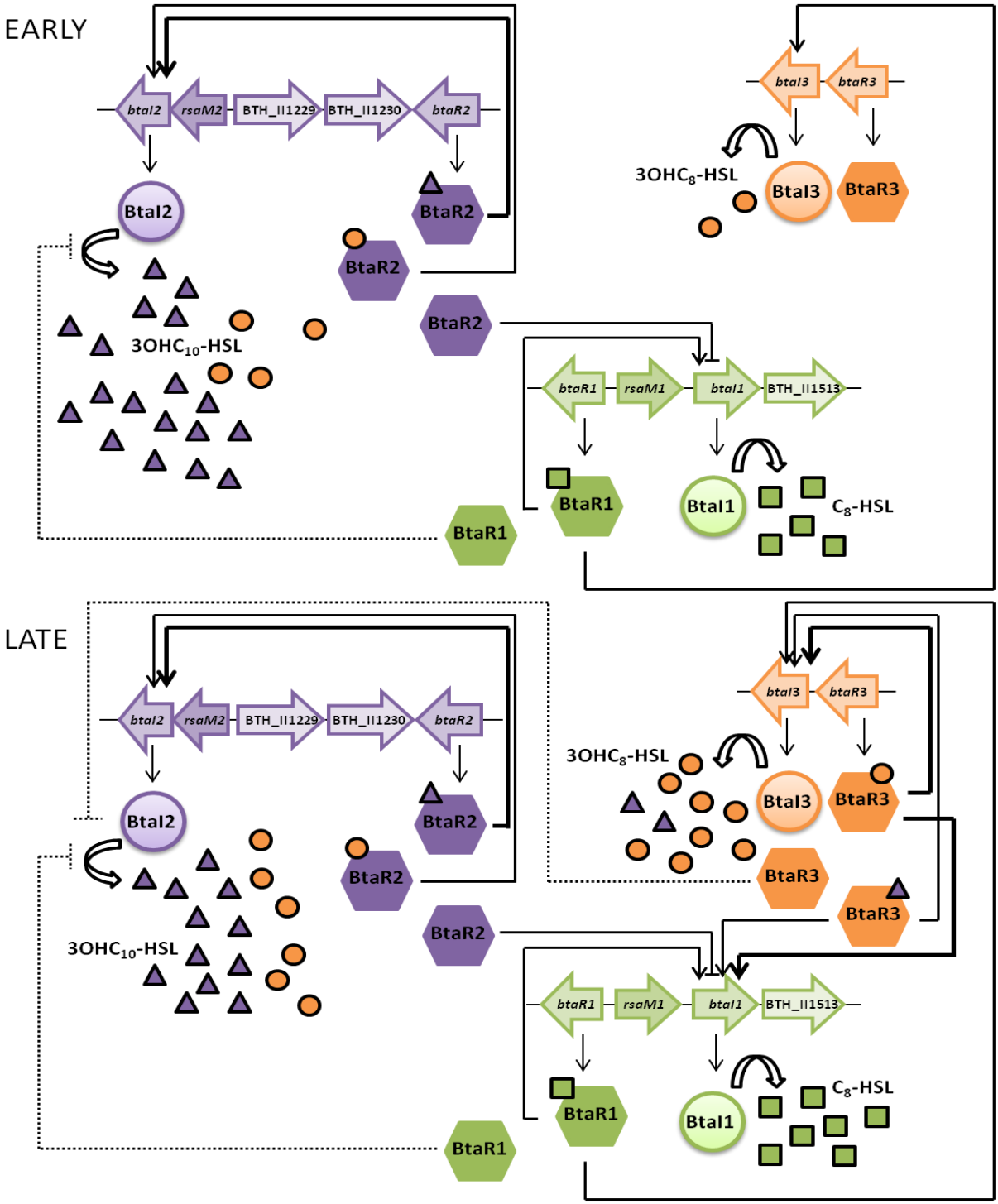

Figure 3. *Quorum sensing* in *Burkholderia thailandensis* E264. The three *quorum sensing* systems that are present in *B. thailandensis* and how they interact with each other. We can see an interdependance between the QS-1 and QS-2 systems. Also, it is shown that the QS-3 system is controlled by BtaR1, showing that these two systems are connected. Even that the QS-2 and QS-3 systems share common AHLs seem not to be transcriptionally linked (Le Guillouzer *et al.*, 2017)

1.3 THE IMPORTANCE OF IRON

Iron is a transition metal that is very abundant on Earth. Under physiological conditions, it exists mainly in one of two redox states, and may vary from one to the other: the ferrous form (Fe^{+2}) and the ferric form (Fe^{+3}). These properties make iron a tremendously versatile element to incorporate into proteins or as an electron carrier. Iron is absolutely indispensable for all forms of life, as it participates in many essential biological processes, such as photosynthesis, methanogenesis, production and consumption of hydrogen, respiration, oxygen transport, gene regulation and DNA biosynthesis. (Andrews *et al.*, 2003)

Despite being required in very small amounts so that organisms can develop their biological functions, iron is poorly bioavailable, since it is extremely insoluble in water in its ferric form, which is the most abundant in the planet. This is due to the amount of oxygen in the atmosphere, which makes the ferrous form, very soluble in water, to change into ferric form. That makes it a key and limiting factor for growth in many ecological niches (Andrews *et al.*, 2003).

In addition, iron can be extremely toxic under aerobic conditions, because it is a very strong oxidizing agent. Because of that, organisms have mechanisms to obtain the small amount of iron available in the medium and to be able to maintain adequate levels of intracellular free iron ions so they do not become toxic (Andrews *et al.*, 2003).

1.4 SIDEROPHORES

Siderophores are small, high-affinity peptides secreted by bacteria and fungi and they serve to transport iron across cell membranes (Krewulak *et al.*, 2008). Siderophores are amongst the strongest soluble Fe^{3+} binding agents known. They normally only are produced under iron stress conditions (when there is not enough free iron available in the medium).

Bacteria produce siderophores to solubilize the iron before transporting it. Gram-negative bacteria take iron-siderophore complexes through the outer membrane

receptors, which lead the process that continues through the cytosolic membrane and all mediated by the energy produced by the TonB-ExbB-ExbD system (Krewulak *et al.*, 2008). Periplasmic binding proteins take the iron-siderophore complex from the outer membrane to the ATP-binding cassette (ABC) proteins of the cytosolic membrane, which will release the complex to the cytosol of the cell (Fig. 4). Inside the cytosol, the iron, is released and the siderophore is recycled and sent back outside the cell to search for more iron. (Andrews *et al.*, 2003)

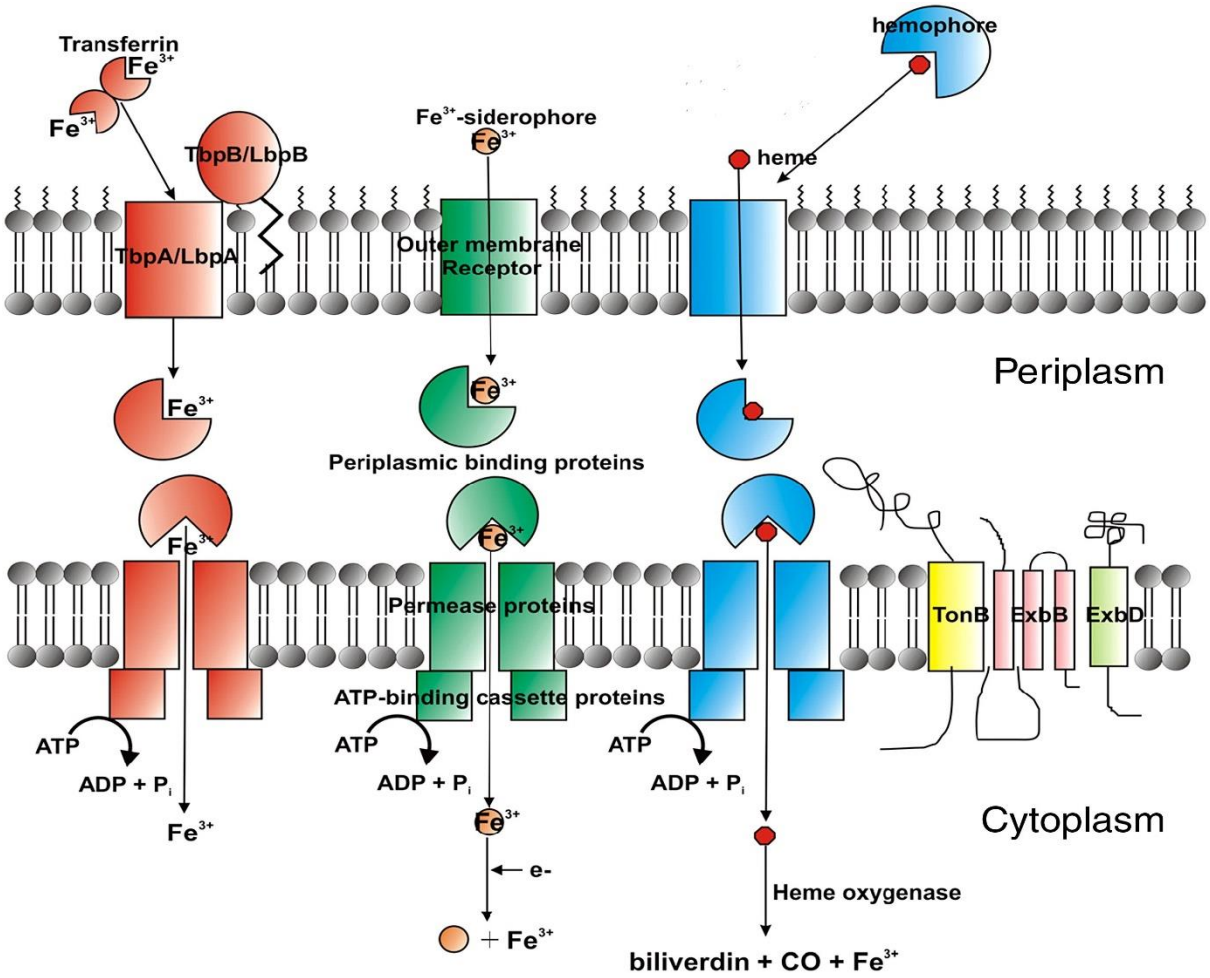

Figure 4. Iron uptake in Gram-negative bacteria. Represented in green, the siderophore iron uptake. (Krewulak, K. D., & Vogel, H. J. (2008). Structural biology of bacterial iron uptake. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(9), 1781-1804.)

The synthesis of siderophores is a special case of peptide synthesis. To begin with, siderophores are non-ribosomal peptides and synthesis is carried out by nonribosomal peptide synthetases (NRPSs). These are huge multienzyme complexes that are in charge of that synthesis (Schwarzer *et al.*, 2003). Normally, bacteria use various NRPSs to synthesize peptides and are usually encoded by genes organized in an operon. The NRPSs have a modular organization. These modules can be divided into domains, which would represent each enzymatic unit that catalyzes each synthesis process (Winn *et al.*, 2016). This reflects the enormous complexity that surrounds this type of structure (**Figure 5**).

1.4.1 Siderophores in *Burkholderia thailandensis*

B. thailandensis produces the same siderophores as *B. pseudomallei*. These siderophores are pyochelin (**Figure 6**) and another siderophore very similar to ornibactin (**Figure 7**) called malleobactin (**Figure 8 and 9**) (Butt *et al.*, 2017). In iron-deficient conditions, the bacteria produce these two siderophores to supply this deficiency.

Malleobactin is considered as the main siderophore because of its greater chelating power of iron in comparison with pyochelin, which is a weaker siderophore (Kvitko *et al.*, 2012). In fact, malleobactin is not a single molecule exclusively (**Figure 8**). They are a family of molecules, which despite having a virtually identical structure, are represented by several congeners that vary their tridimensional structure minimally and make them have totally different functions, most of them still unknown. (Franke *et al.*, 2015). In fact, only Malleobactin E (**Figure 9**) can be considered as a true siderophore, comparing its quelating power with ornibactin (Franke *et al.*, 2015)

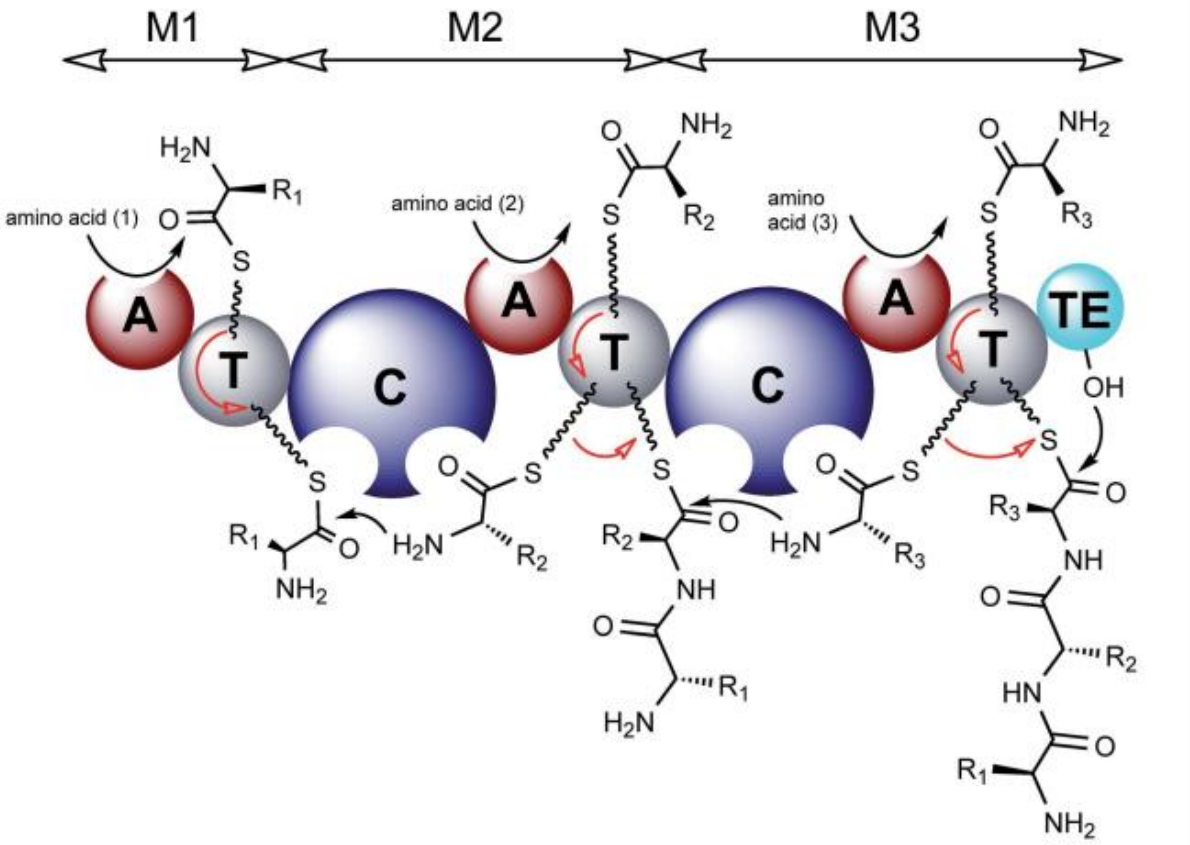

Figure 5. Model of NRPS biosynthesis. Amino acids are activated by adenylation domain (A). The thiolation domain (T) captures the aminoacyl-AMP intermediate and the condensation domain (C) catalyses peptide bond formation. M1 is the inititation module and subsequent modules are know as elongation modules. Each elongation module will incorporate a single amino acid. That means that they will be as many elongation modules as there are amino acids in the final peptide. The final module has a thioesterase domain (TE) which catalyse the release of the final peptide by hydrolysis or cyclisation. Adapted from (Winn *et al.*, 2016).

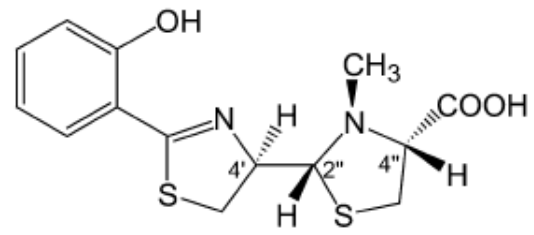

Figure 6. Pyochelin structure. Adapted from (Brandel *et al.*, 2012)

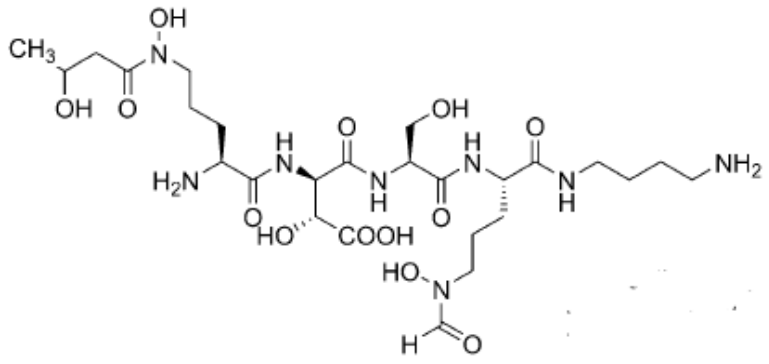

Figure 7. Ornibactin structure. Adapted from (Franke *et al.*, 2015)

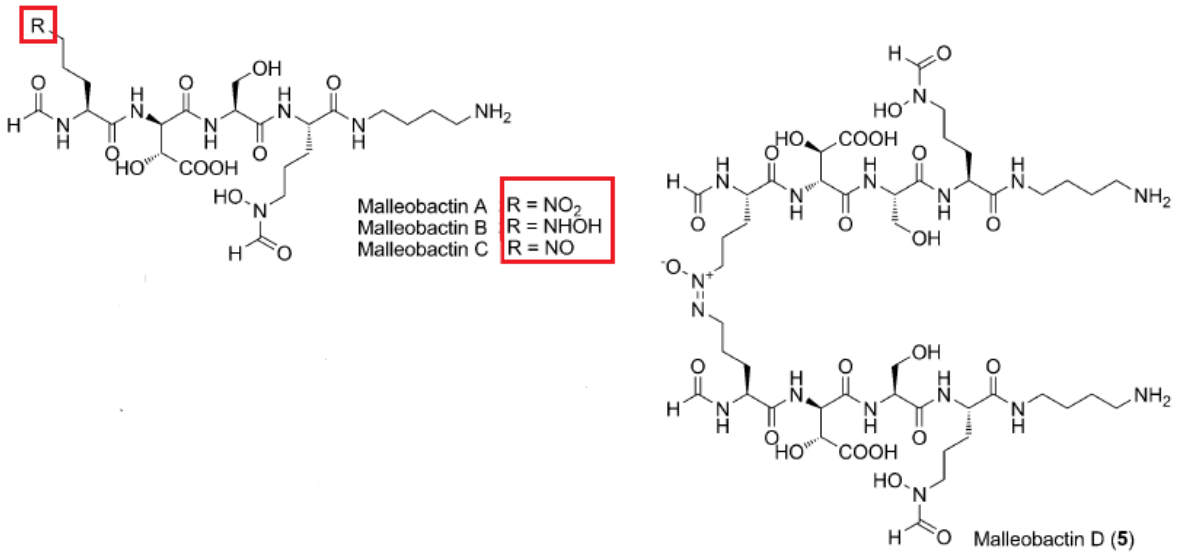

Figure 8. Structure of malleobactins. Malloebactin is not a single molecule but a family of them. Adapted from (Franke *et al.*, 2013)

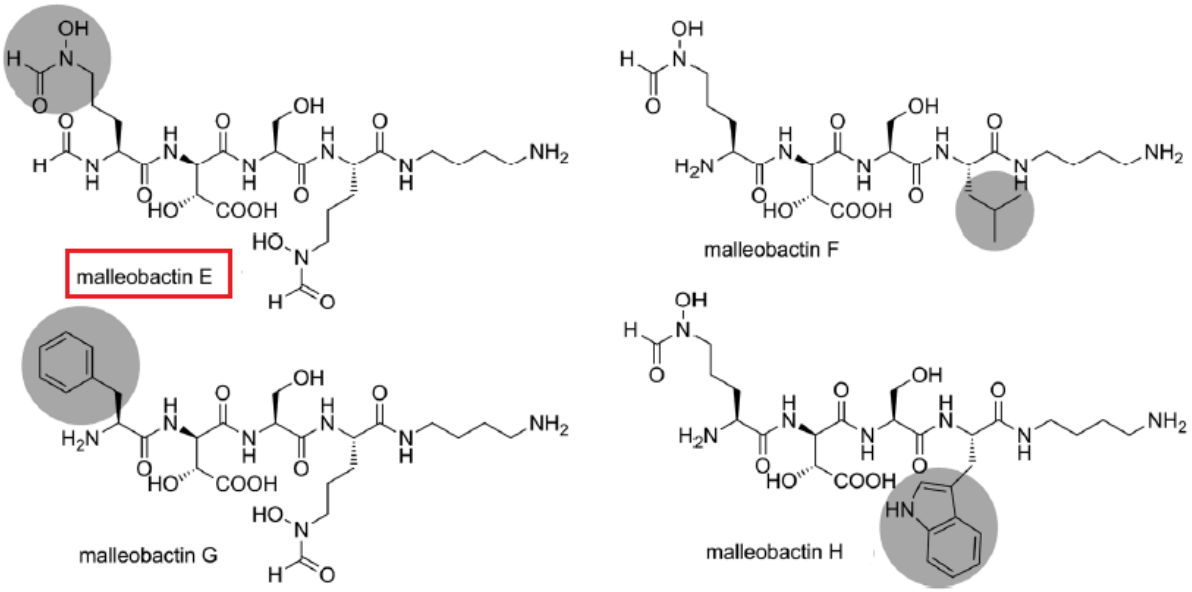

Figure 9. Malleobactin congeners. Only malleobactin E can be considered as a true siderophore. Adapted from (Franke *et al.*, 2015)

Malleobactin and pyochelin are products of very similar gene clusters and depend on NRPS to be synthesized (**Figure 5**). Pyochelin is synthesised from one molecule of salicylate and two molecules of L-cysteine, and requires the activity of two NRPSs, PchE and PchF (Quadri *et al.*, 1999), while malleobactin synthesis requires also two NRPS, which are MbaA and MbaB (Alice *et al.*, 2006, Franke *et al.*, 2013).

On the other hand, malleobactin is very similar to ornibactin, with an almost identical synthetic gene cluster (Franke *et al.*, 2013) (**Figure 10**). Ornibactin, a siderophore produced by the majority of bacteria belonging to the *Burkholderia cepacia* complex (Butt *et al.*, 2017) is also regulated by QS in *B. cepacia* (Lewenza *et al.*, 2001). In Figure 10 both gene clusters in different *Burkholderia* species are compared. The main genes (red ones) of each biosynthesis operon are almost identical.

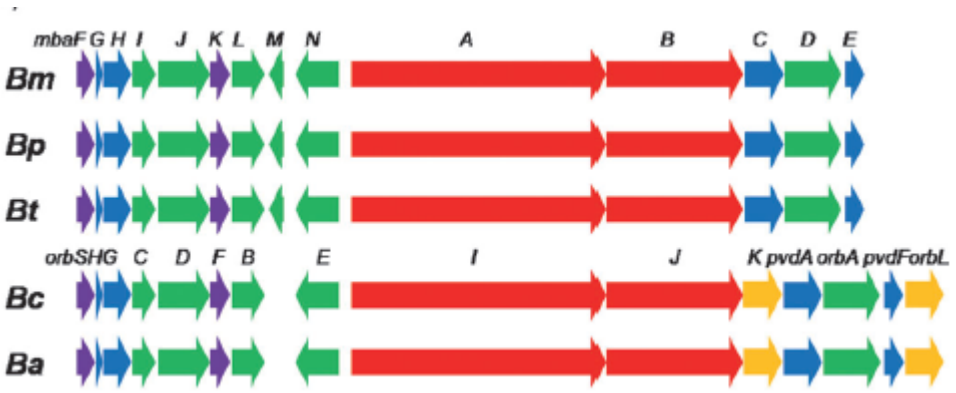

Figure 10. Malleobactin (*mba*) and ornibactin (*orb*) biosynthesis gene clusters in different *Burkholderia* species. *Bptm* group share the same gene cluster for malleobactin and that both biosynthesis gene clusters are very similar. (**Bm** = *Burkholderia mallei*; **Bp** = *Burkholderia pseudomallei*; **Bt** = *Burkholderia thailandensis*; **Bc** = *Burkholderia cenocepacia*; **Ba** = *Burkholderia ambifaria*). Adapted from (Franke *et al.*, 2013)

1.5 BACKGROUND

Previous studies and experiments in the Déziel laboratory showed that different QS mutants of *B. thailandensis* strain E264 produce different amounts of siderophores (**Figure 11**). By using the CAS agar method (described later), they measured the halo surrounding the colonies on plates, that represents siderophore production. It was clear that the QS triple mutant produced the highest amount of siderophore and thus, suggested QS exert a negative regulatory effect on siderophore production (Tan, Sok Gheck, 2014). Also, they discovered several genes related to malleobactin synthesis genes. In this project, strains with mutations in *mbaS (*sigma factor directly related to malleobactin biosynthesis) and *mbaF (*gene related to a siderophore-iron reductase) were also tested.

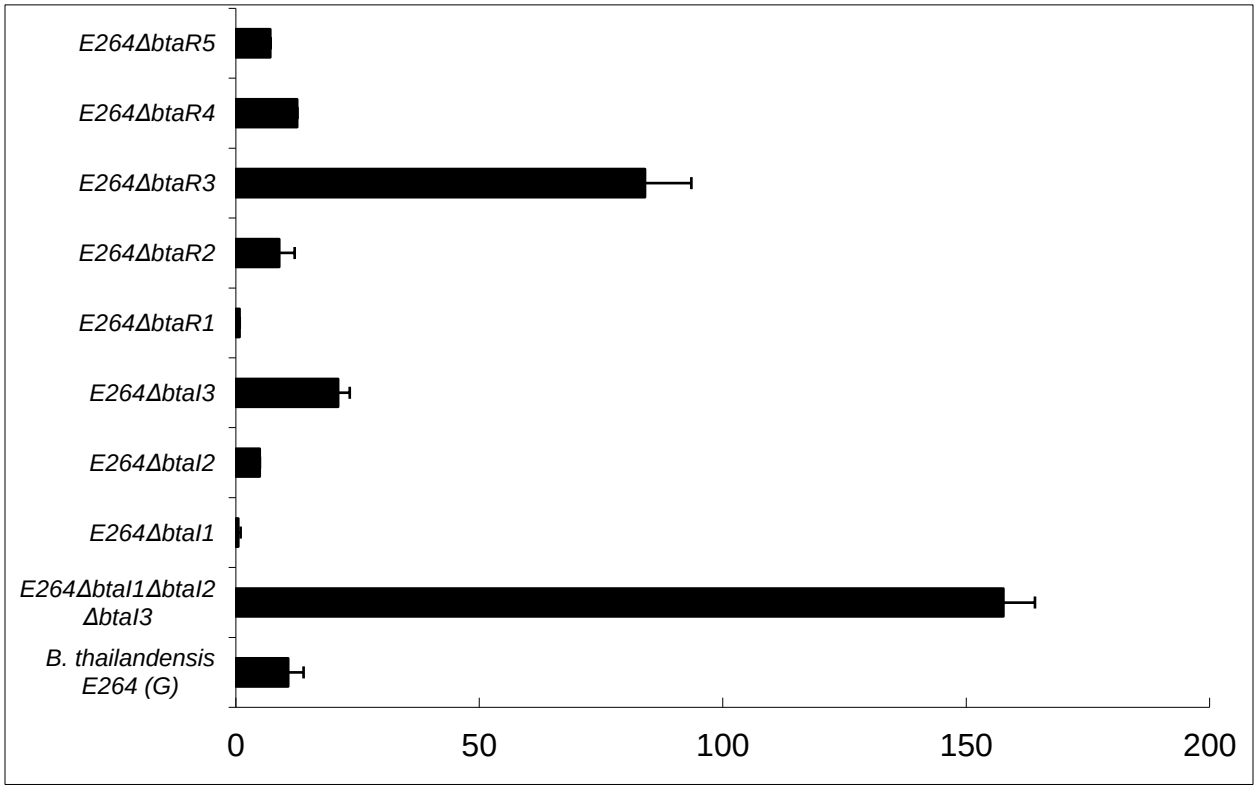

Figure 11. Siderophore production in different *quorum sensing* mutants. 24h culture. Area of halos in mm^2. Different mutants have different siderophore production (figure courtesy of Marie Christine Groleau).

These preliminary data suggested the triple QS mutant is a superproducer of siderophores in comparison with the wild type. This experiment was performed only once and it is not possible in this assay to differentiate between siderophores produced. Also, the QS system 3 seems to have the biggest impact in siderophore production**.**

1.7 PROJECT HYPOTHESIS

Considering that this triple mutant has no active QS and it is a superproducer, it is hypothesized that QS plays a repressor role in siderophore production. Based on preliminary results, this project was started to verify that QS is involved in malleobactin production regulation. And also, to elucidate how this regulation occurs and which system (or systems) of the QS are involved.

1.6 PROJECT OBJECTIVES

There were two main objectives on this project:

- Verify if QS is, in fact, involved in malleobactin production regulation. Production of malleobactin will be investigated in various QS mutants of E264
- Elucidate how *QS* controls the gene expression involved in malleobactin synthesis, the relative expression of the *mbaA* gene will be measured in all the QS mutants.

2. MATERIAL AND METHODS

2.1 SIDEROPHORE PRODUCTION AND GENE EXPRESSION

All bacterial strains shown in Table 1 were used to perform experiments described in this project.

B. thailandensis	**Description**	**Reference**
E264	Wild-type	(Brett *et al.*, 1998)
JBT101	E264 Δ*btaI1*	(Chandler *et al.*, 2009)
JBT102	E264 Δ*btaI2*	(Chandler *et al.*, 2009)
JBT103	E264 Δ*btaI3*	(Chandler *et al.*, 2009)
JBT112	E264 Δ*btaI1*Δ*btaI2*Δ*btaI3*	(Chandler *et al.*, 2009)
JBT107	E264 Δ*btaR1*	(Chandler *et al.*, 2009)
JBT108	E264 Δ*btaR2*	(Chandler *et al.*, 2009)
JBT109	E264 Δ*btaR3*	(Chandler *et al.*, 2009)
JBT110	E264 Δ*btaR4*	(Chandler *et al.*, 2009)
JBT111	E264 Δ*btaR5*	(Chandler *et al.*, 2009)
BTH_I2418-142 :: ISlacZ-PrhaBo-Tp (BT04142)	E264 Δ*mbaA*	(Gallagher *et al.* 2013)
BTH_II1828-251:: ISlacZ-PrhaBo-Tp (BT12751)	E264 Δ*pchE*	(Gallagher *et al.* 2013)

Table 1. List of strains used in the project.

It is necessary to have a minimal medium depleted in iron to make bacteria produce siderophores. The medium M9 was selected to use in all the cultures made in this project. This medium has 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0,5 g NaCl et 3 mg

$CaCl_2$ per litre of water (ddH_2O). Sucrose was used as a carbon source at 0.2% (w/v). Two grams of Casamino acids (Fisher Scientific)) were added to enhance siderophore production. Glassware was cleaned with 6M HCl prior to use. All the bacterial strains used in this project were taken from frozen stocks and routinely grown overnight in a rich medium (Tryptic Soy Broth (TSB) Difco Laboratories, Mississauga, Ontario, Canada) overnight and then washed and inoculated in M9 medium. The incubator used was the 1565 Sheldon manufacturing, USA, at 37ºC, 240 rpm agitation speed in a Rotor TC-7 roller drum (New Brunswick, Canada).

To remove iron traces from the M9 medium, 5 g/L of the resin Chelex-100 (Sigma-Aldrich) were used. The resin added to the medium stayed at 4ºC and 50 rpm agitation speed overnight. After that, the medium was filtered (0.2 µm pore size) to obtain iron-depleted M9 medium.

2.1.1 Siderophore production measurement

To measure siderophore production in QS mutants, the *mbaA* mutant (this gene is in charge of malleobactin synthesis, thus this mutant should not produce this siderophore), and the *pchE* mutant (it does not produce any pyochelin), CAS (Chrome Azurol S) medium was used. This is a universal siderophore assay containing chrome azurol S and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The CAS/HDTMA complexes tightly with ferric iron to produce a blue colour. When a strong iron chelator such as a siderophore removes iron from the dye complex, the colour changes from blue to orange (Louden *et al.*, 2011)

Two different types of CAS assay were performed: in liquid and solid medium. By doing this, whether the medium affects the siderophore production can be studied.

-Solid Medium (CAS plates): to prepare this medium, the preparation protocol described by (Louden *et al.*, 2011, Wen *et al.*, 2012) was followed. Using an overnight culture of test bacteria in Tryptic Soy Broth (TSB) (Difco Laboratories, Mississauga, Ontario, Canada), cells were washed twice with M9 medium (described before). Plates were inoculated with 20 µL of the cells washed and incubated at 37° overnight.

-Liquid CAS Medium: to perform this analysis, the Blue Dye recipe described in Louden *et al.* (2011) was followed. Measure the OD at 600 nm of an overnight culture in TSB (3 ml), and wash the cells twice in M9 and let them grow in 4 ml of M9 medium overnight (with starting OD at 0.1). After that, centrifuge 5 minutes at 4500 rpm and take the supernatant. After that, mix 0.5 ml of *B. thailandensis* culture supernatant and 0.5 ml of blue dye (Chrome Azurol S solution) and leave at room temperature for 10 minutes. Then, the absorbance at 630 nm was measured (CAS absorbance) of each sample, and by using the following formula, the RSU (relative siderophore units) normalized to cell density was calculated (Wen *et al.*, 2012). Three replicates were measured for each strain.

$$RSU = \frac{A_{630}\ \text{M9 control} - A_{630}\ \text{supernatant}}{A_{600}\ \text{of cell culture}} \times 100$$

Équation 1. Equation used to calculate the RSU (Relative Siderophore Units) in CAS medium assay. Normalized by optical density of the cultures. (Wen *et al.*, 2012)

Before the RT-qPCR method to measure the expression of the *mbaA* gene was performed, another experiment, using a reporter gene method (Hoang *et al.*, 2000), using the plasmid Mini-CTX Lux as vector was tested to measure the expression in different QS mutants.

2.1.2 Relative gene expression measurement (RT-qPCR)

Transcription of the gene of interest *mbaA* was also determined. To do so, I used RT-qPCR. I performed this method in two steps: first, RNA extraction and RT-PCR (reverse transcription PCR) to generate the cDNA and qPCR (quantitative PCR) to measure the expression of the *mbaA* gene in all various backgrounds compared to a housekeeping gene *ndh* (Subsin *et al.*, 2007).

The RNA extraction was performed at OD_{600} of 0,5 when the production of siderophore was maximum.

2.1.2.1 RNA Extraction

The protocol with PureZOL RNA Isolation Reagent (Bio-Rad) was used. These are the steps that were followed:

1. Homogenizing samples: centrifuge the cell culture and resuspend the pellet in 1mL of PureZOL RNA Isolation Reagent.
2. Phase separation: add 200 µL of chloroform to separate the nucleic acids. Centrifuge at 4ºC. Keep the organic phase.
3. RNA precipitation: add 500 µL 100% Isopropanol to do this. Centrifuge at 4ºC. The small pellet is the RNA. Keep the pellet and remove the supernatant.
4. RNA Wash: resuspend the pellet in 1 mL of ethanol 100%. Centrifuge at 4ºC. Remove the supernatant and let the RNA dry inside the biological hood at least 15 min.
5. RNA Resuspension: resuspend the RNA in 50 µl of RNase-free water. Keep the RNA on ice or storage at -80ºC.

Once the RNA is isolated, a DNase treatment was performed to remove possible DNA traces that may remain in the RNA solution. *TURBO DNase* (Ambion) was used to do so. The enzyme was inactivated for 30 min at 37ºC. This step was performed twice. After that, inactivate the enzyme followeing the *RiboPure* (Ambion) protocol. Using the *DNase Inactivation Reagent* for 3 minutes and centrifuged (1 min 8000 rpm) after that. The purified RNA's were transferred in a clean new tube and kept them at -80ºC.

2.1.2.2 RT-PCR

To perform the RT-PCR, primers shown in Table 2 were used. Dilute the RNA samples were at adjusted to 50 ng/µL. The *iScript Reverse Transcription Supermix* (Bio-Rad) was used. These were the steps to perform this kind of PCR:

Hybridization at 25ºC for 5 minutes

Reverse transcription at 42ºC for 30 minutes

Inactivation at 85ºC for 5 minutes.

After that, we kept the cDNA we obtained at -20ºC. We used it to perform the qPCR.

Gene	Sequence
mbaA Forward	GGAATCAGTCGATCCAGCTC
mbaA Reverse	AATACCGGGTGATGGTTCAG
ndh Forward	TGGCATGGTTTCGAATTCCACCAG
ndh Reverse	GAAATAATGCGTCGTGCTGCCGAT

Table 2. Primers used to do all the PCRs performed. Sequences of the primers for our gene of interest (*mbaA*) and the housekeeping gene (*ndh*). Sequences of the primers were obtained in www.burkholderia.com and were made by Alpha DNA (Montreal, Quebec, Canada).

2.1.2.3 qPCR

To perform this type of PCR, the *SsoAdvanced universal SYBR Green supermix* was used, with the gene *ndh* as housekeeping, reference gene (Subsin *et al.*, 2007). The thermocycler used was a Rotor-Gene 6000 (Corbett Life Science). These were the conditions:

Hold: 95ºC 30 seconds

Cycle (40 times): 95ºC 15 seconds

55ºC 30 seconds

72ºC 15 seconds

Melt: ramp from 72ºC to 95ºC, rising by 1ºC each step.

The results were analysied using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001)

2.2 HPLC/MS

Analysis with HPLC-MS were performed with the HPLC separation mode Waters Alliance HT *2795* coupled to a mass spectrometer (Micromass Quattro Premier XE; Waters Corporation Milford, Massachusetts, USA). The column Kinetex-C8 (Phenomenex) was used for the chromatography. Initially, the mobile phase was 100% water. The program gradually changed the concentration between water and acetonitrile until it reached 100% acetonitrile after 7 minutes. This concentration was maintained until minute 11, and after that it was returned gradually to 100% water until the end of the program: 15 minutes total. All the molecules that exit the HPLC are injected into the

mass spectrometer. The analyses were performed by electrospray ionization in negative mode. To detect the siderophores, a scanning program between *m/z* 200 and *m/z* 800 mass interval was used.

2.3 BIOSCREEN

To study the growth of the cells, the BioScreen apparatus (OY Growth Curves AB, Ltd, Helsinki, Finlande) was used. This instrument allows the incubation with agitation, coupled to a spectrometer to measure the bacterial growth. The study was performed during 24h hours, in a M9 minimal medium with an inoculation of an overnight culture in TSB with an initial OD_{600} 0.05. The incubation temperature was 37ºC and three replicates of each strains were studied. The OD_{600} was measured every 15 minutes during 24 hours.

DATA ANALYSES

Statistical analyses (Dunnet's multiple comparisons test) were performed with GraphPad Prism v7.0.

3. RESULTS

Experiments were performed to characterize siderophore production and the expression of the *mbaA* gene in different QS mutants

3.1 Growth with different levels of iron availability

These are the results observed while testing three different iron conditions: medium with iron added, medium treated with chelex-100 resin and medium treated with the chelator 2,2-dipyridyl (100µM). These results show the effect of iron bioavailability on *B. thailandensis* growth, when siderophore production is defective

Figure 12 shows that all strains grow well in an iron rich medium, despite their differences in levels of siderophore production. Also, the triple QS mutant grows faster but reaches the stationary phase earlier than the other strains.

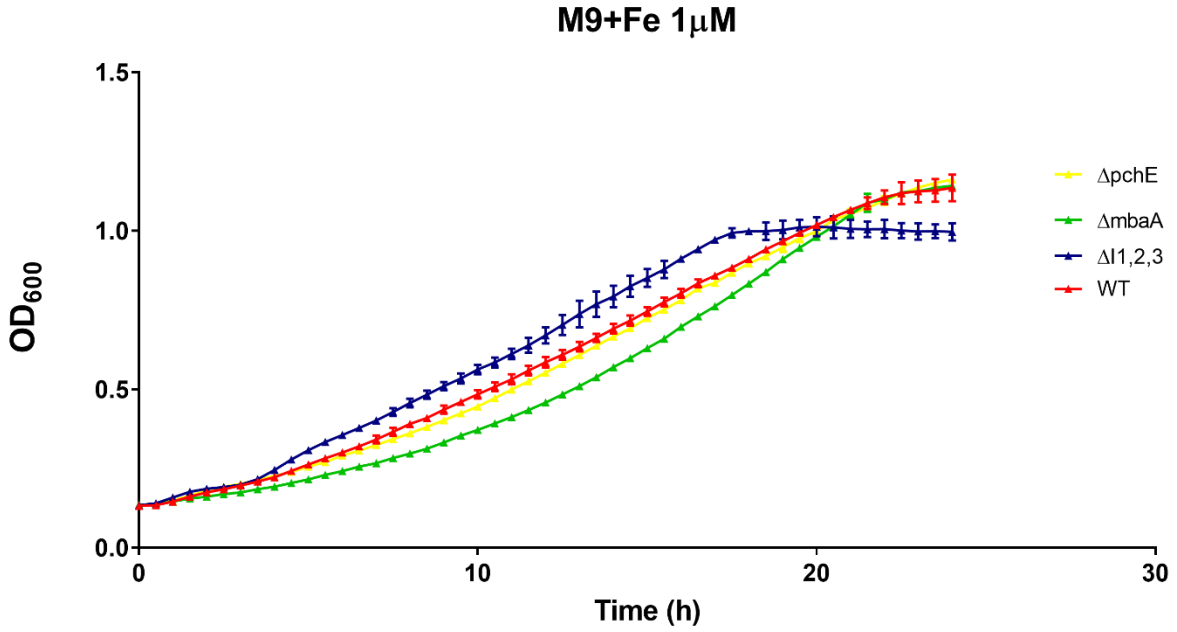

Figure 12. Growth curve of *B. thailandensis* E264 during 24h at 37°C with iron added in a M9 minimal medium. Measured with Bioscreen. $FeCl_3$ (1 µM) used as iron source. We compared the pyochelin-negative mutant (Δ*pchE*), the malleobactin-negative mutant (*ΔmbaA*), the triple mutant in all three luxI homologues of the *quorum sensing* circuitry (Δbta*I1,btaI2,btaI3)* and the wild type (WT).

Figure 13 presents the growth curves in the same culture medium but without iron added and also treated with Chelex-100 resin to remove transition metal ions, including iron. We can see how the bacteria grow less rapidly, and how the mutants that do not produce any of the two siderophores (Δ*mbaA* and Δ*pchE*) grow a bit less than the others that produce siderophores.

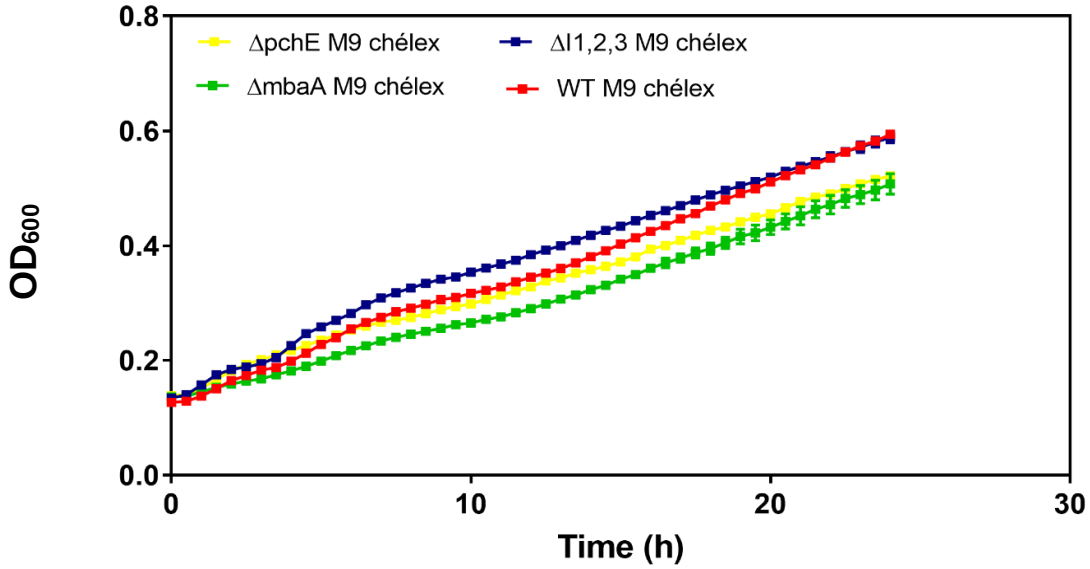

Figure 13. Growth curve during 24h at 37º in a M9 minimal medium pretreated with Chelex-100 resin. Measured with Bioscreen. The Chelex-100 resin is a heavy metals chelator, with very high affinity for iron. This was an iron-depleted medium. We compared the pyochelin mutant (Δ*pchE*), the malleobactin mutants (Δ*mbaA*), the triple mutant in all three *btaI quorum sensing* AHL synthases (Δ*I1,2,3)* and the wild type (WT).

2,2,-Dipyridyl is a very strong chelator agent, with a very high affinity for transition metals, such as Fe ,Co, Ni, Cu, Zn. It will chelate iron, which is why it cannot be used to treat the medium normally, but it can be used it to show the difference in chelator power between malleobactin and pyochelin. Here, the Δ*mbaA* mutant does not grow, because it lacks of malleobactin, the strongest siderophore in *B. thilandensis.* The *ΔpchE* mutant

can grow better because pyochelin is not a very strong siderophore, and malleobactin seems to be enough to assure the growth of the cells (Fig. 14).

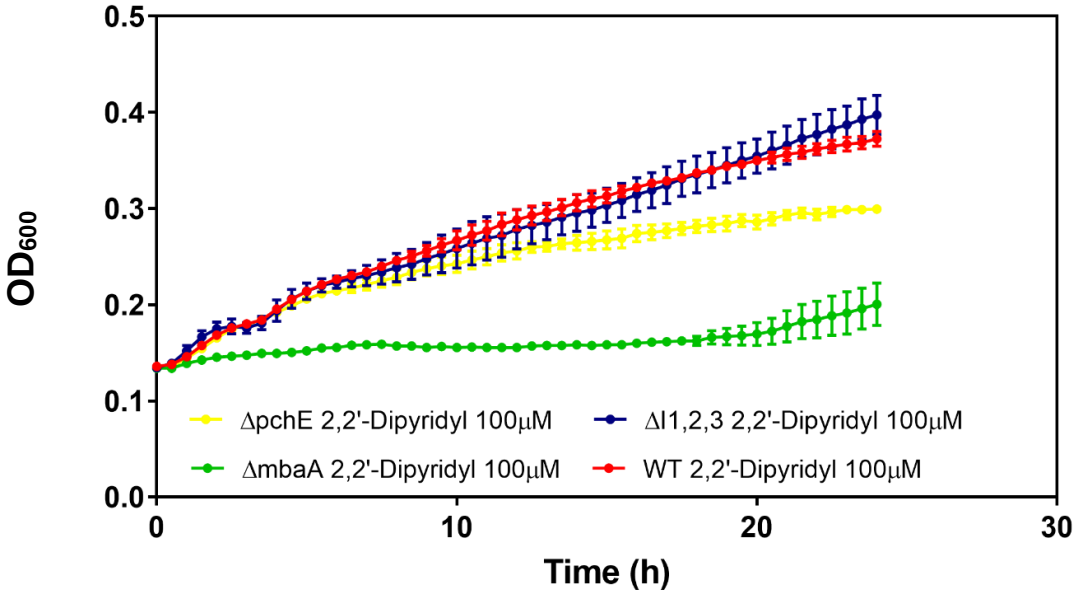

Figure 14. Growth curve during 24h at 37º in a M9 minimal medium treated with 2,2-Dipyridyl. Measured with Bioscreen. We compared the pyochelin mutant (*ΔpchE*), the malleobactin mutants (*ΔmbaA*), the triple mutant in all three I *quorum sensing* systems (*ΔI1,2,3)* and the wild type (WT).

3.2 Siderophore production

At first, for the CAS essay a solid medium was tested. With this method, an orange halo can be seen when siderophore production occurs. After trying it several times, it was clear that it is not a very sensitive nor reliable method. Mostly because it can only measure an accumulation of siderophores, and it is not possible to know when exactly that production happens, or if it changes over time. Nevertheless, as shown in **Figure 15** it is possible to appreciate that different QS mutants produce different amounts of siderophore. The triple I mutant, and also the I3 mutant are the ones with the highest production. In addition, Figure 14 shows that Δ*mbaA* does not produce malleobactin, and it has a very small halo. Decreased siderophore production can also be observed in

the halos of the other two mutants, Δ*mbaS (*sigma factor directly related to malleobactin biosynthesis) and Δ*mbaF (*gene related to a siderophore-iron reductase).

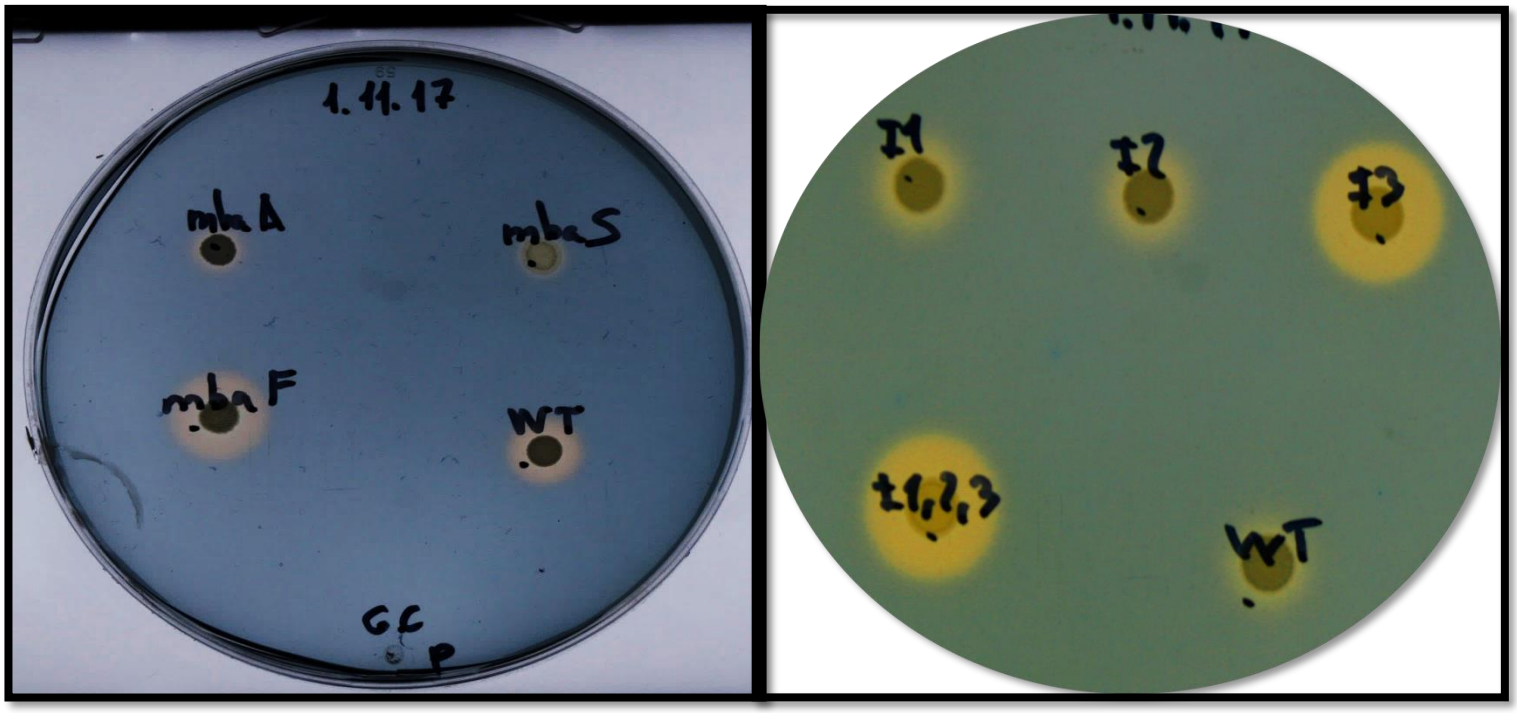

Figure 15. Siderophore halos production on CAS agar plates. Colonies shown on the plates. Different *quorum sensing* mutants show different halo diameter. Plates were incubated at **37ºC for 24h.** .

As a more reliable alternative, the CAS assay was performed as a liquid assay. Cultures were grown in a M9 minimal medium pretreated with Chelex-100 resin, as described before, and samples were taken periodically to assess the concentration of total siderophore present in the cultures. The data reveal when the siderophore production starts and how QS impacts that production (**Figure 16**).

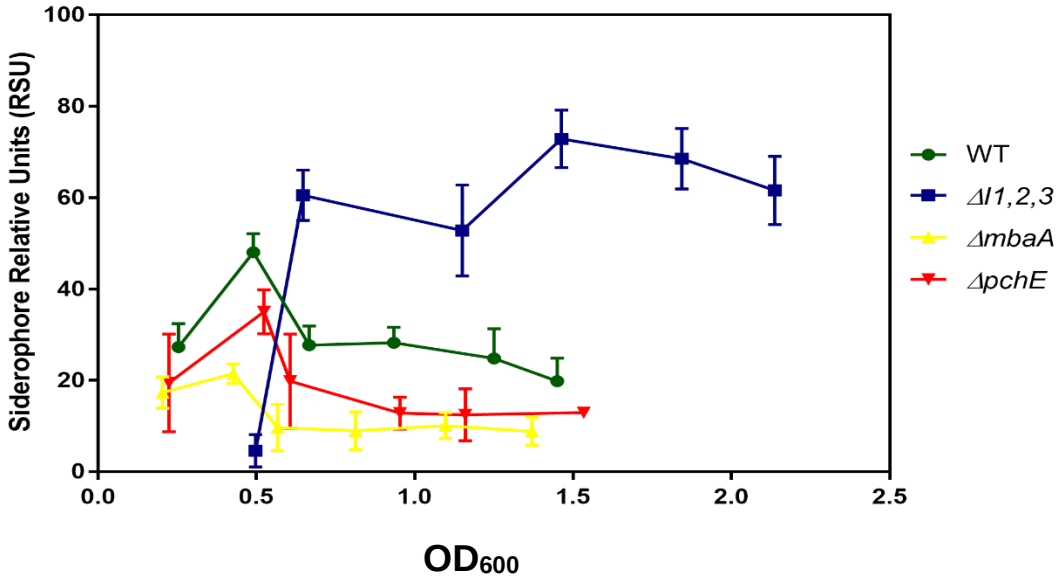

Figure 16. Siderophore production kinetic curve during 12h at 37ºC in a M9 minimal medium treated with Chelex-100 resin. Measured with CAS assay. We compared the pyochelin mutant (*ΔpchE*), the malleobactin mutants (*ΔmbaA*) and the triple mutant in all three I *quorum sensing* systems (*ΔI1,2,3)* and the wild type (WT). Measured in RSU (Relative Siderophore Units) as shown in **Equation 1**.

Here, it is shown that the triple QS mutant acts as a siderophore superproducer compared to the WT and also, it grows faster than the others. In addition, it is possible to see the difference in chelating power between pyochelin and malleobactin by looking at Δ*mbaA* and Δ*pchE* mutants.

The next graphic (**Figure 17**) shows again how the triple mutant is a superproducer of siderophores compared to the WT, and also, it seems to be that the system *btaR3/btaI3* has the strongest negative influence on siderophore production.

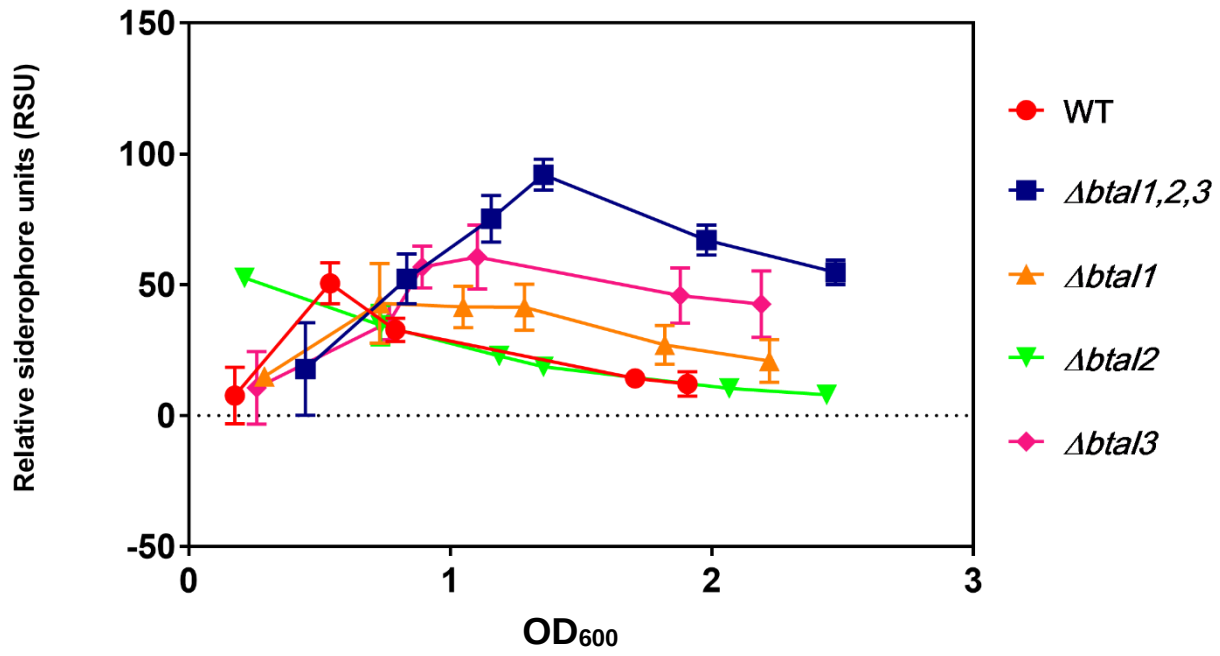

Figure 17. Siderophore production kinetics during 12h at 37ºC in a M9 minimal medium treated with Chelex-100 resin. Measured with CAS assay. The production in all the QS mutants in the synthase of the three systems (Δ*btaI1*, Δ*btaI2, ΔbtaI3)* and also the triple mutant (Δ*btaI1,2,3)* was compared with the wild type. Measured in RSU (Relative Siderophore Units) as shown in **Equation 1.**

Use of mutants in the *luxI* homologues only indirectly informs us on which QS systems are involved in regulation of siderophore production because the various AHLs produced are shared between the various *btaR* homologues of *B. thailandensis* (Le Guillouzer et al. 2017; see figure 3). Thus total siderophore production was assessed in the indivual *btaR*- mutants. Figure 18 shows that the system 3 has the strongest influence on siderophore production once again. In addition, BtaR5 and BtaR2 appear to positively affect the production.

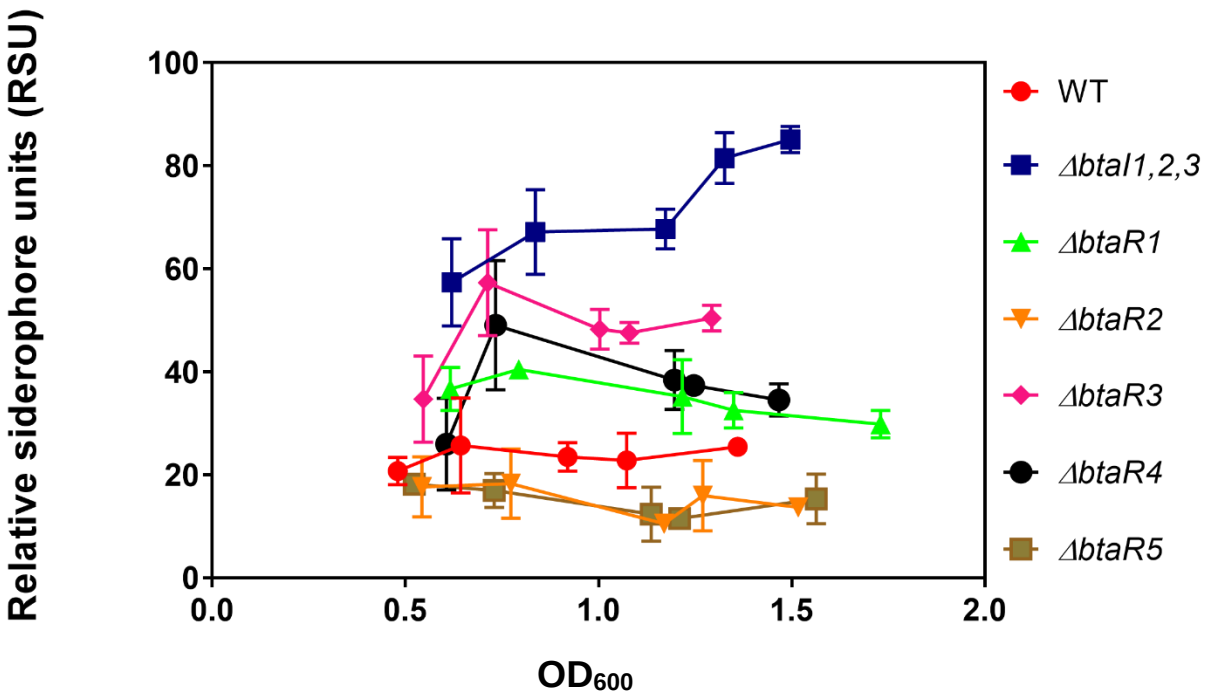

Figure 18. Siderophore production kinetics during 12h at 37ºC in a M9 minimal medium treated with Chelex-100 resin. Measured with CAS assay. The production in all the QS mutants in the transcriptional regulator (Δ*btaR1*, Δ*btaR2, ΔbtaR3, ΔbtaR4, ΔbtaR5)* and also the triple mutant in the synthases(Δ*btaI1,2,3)* was compared with the wild type. Measured in RSU (Relative Siderophore Units) as shown in **Equation 1.**

Since with the CAS essay, it is not possible to distinguish between all the siderophores that are produced, the idea was to try another method to measure siderophore production. Indeed, figure 16 shows that malleobactin is the strongest siderophore but pyochelin also contributes to the total CAS assay RSU data. The goal was to measure only the malleobactin produced.

The analysis using our triple QS mutant in presence of the three different AHL was performed in liquid CAS assay (**Figure 19**) to see how they affect siderophore production, and also it was studied which inducer molecule has the biggest impact on the expression of *mbaA* (**Figure 20**) Siderophore production and gene expression are compared in discussion section.

Attempts to purify or specifically detect malleobactin by LC/MS were done as described in the thesis of Sok Gheck Tan (2014). However, we were unable to achieve this, and the procedure may require multiple columns and steps in order to succesfully

detect or isolate these molecules. (Franke *et al.*, 2013) described how they purified malleobactin.

In addition, another siderophore production analysis using CAS method was performed. But this time, AHL were added to the triple I mutant, and they all were compared to the WT (Figure 19). Addition of AHL, clearly caused a significant reduction in the level of siderophores produced, although there was no significant change in reduction between any of the different AHL molecules that were tested.

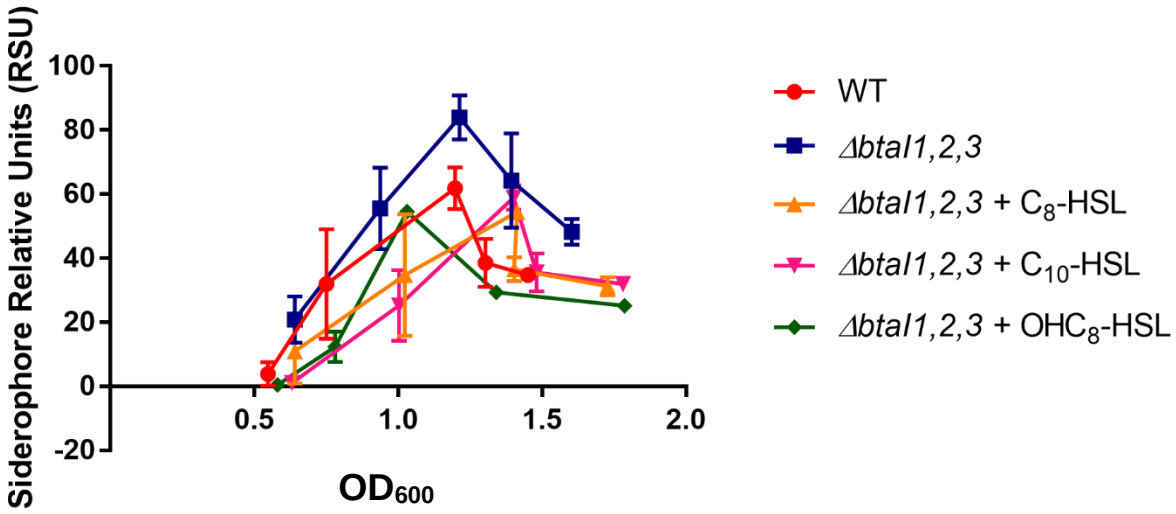

Figure 19. Siderophore production kinetics during 12h at 37ºC in a M9 minimal medium treated with Chelex-100 resin. Measured with CAS assay. AHL were added to the triple btaI mutant to see their effect in QS regulation. Three repetitions each point.

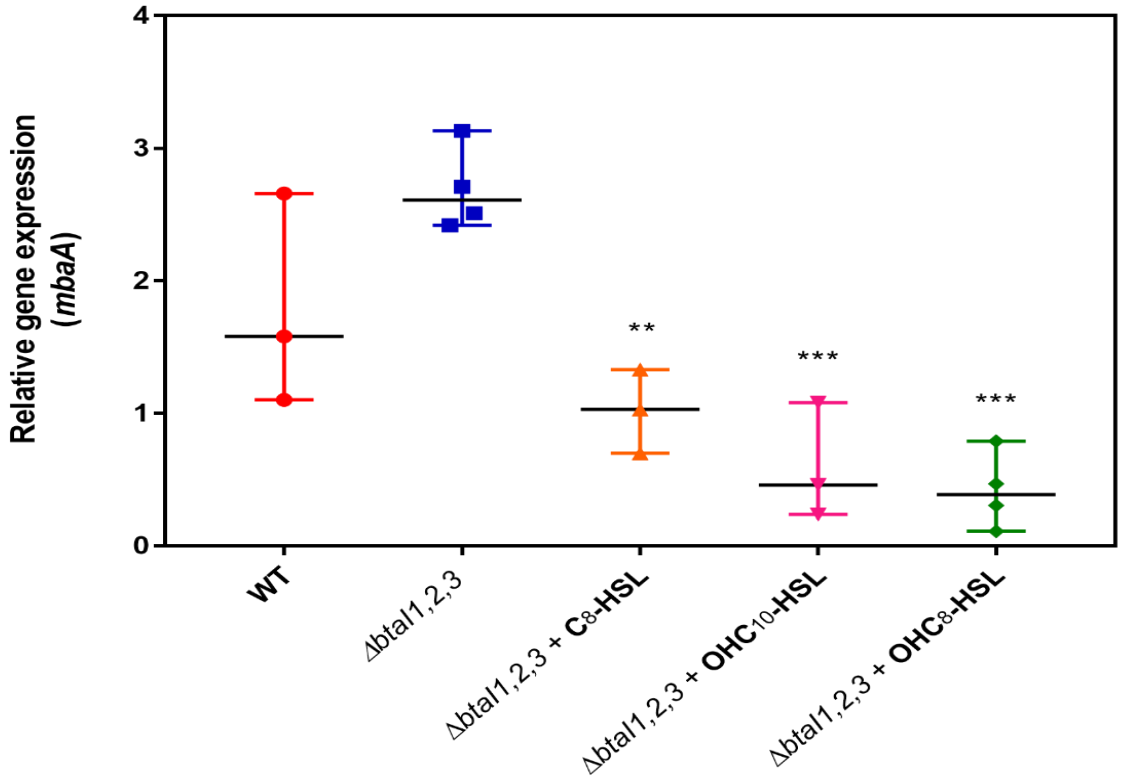

Figure 20. Relative gene expression of gene *mbaA* in the triple *btaI*- mutant with AHLs added. The effect in expression of the three different autoinducers is shown. The gene *ndh* was used as a housekeeping gene. Three replicates each strain. Samples taken at 0.5 OD. (**, p<0,01, ***, p<0,001, compared with the triple mutant without AHL, Dunnet's multiple comparisons test.)

The expression of all the QS mutants and the WT using a reporter gene was checked. They all had the same expression, with no significative difference (data not shown). The plasmid construction and the primers used were revised, the insertion could be inverted and showing the expression of something else, but that was not the case. A new reporter gene was built, with new primers and new insert. This time, it was showing no luminescence at all. The construction was sent to sequencing, and it was alright. The insert was there, in the right place, in the right sense. Because all this, the qRT-PCR method was chosen to study gene expression.

3.3 Relative gene expression (RT-qPCR)

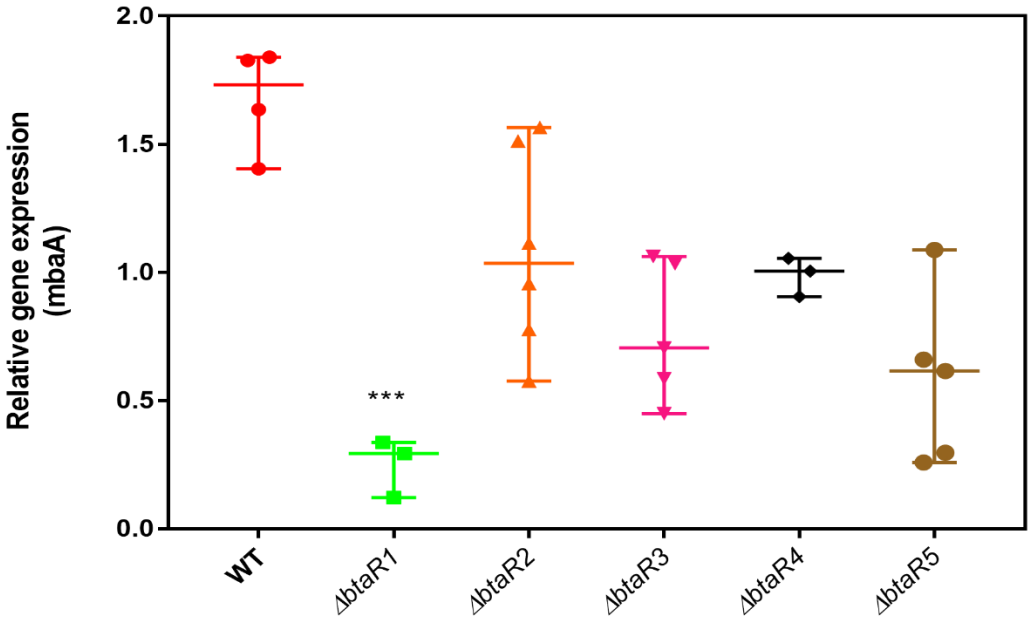

Figure 21. Relative gene expression of gene *mbaA* in all the QS mutants in the transcriptional regulator. The gene *ndh* was used as a housekeeping gene. Six replicates each strain (some points were lost during the qPCR). Samples taken at 0.5 OD. Cells were grown in M9 medium. (***, *p*<0,001, all R mutants compared between them, Dunnet's multiple comparisons test.)

As shown in figure 21, only the Δ*btaR1* mutant seems to be significantly different from the other mutants vs WT.

To try to better understand whether the timing of sampling would explain these unexpected resultats, when exactly the peak of expression of *mbaA* is occurring was studied. Three different ODs were tested and there was no difference between the three of them (**Figure 22**). It seems that expressions happens very early during the bacterial growth and remains constant. In addition, by looking at the results of the RNA-Seq performed in (Le Guillouzer, 2018) in a TSB medium, which compares identifies the genes controlled by QS in *B.* thailandensis,the gene *mbaA* is not among them. Another gene expression study was repeated under the same conditions of the RNA-Seq (**Figure 22**). It was possible to see gene expression.

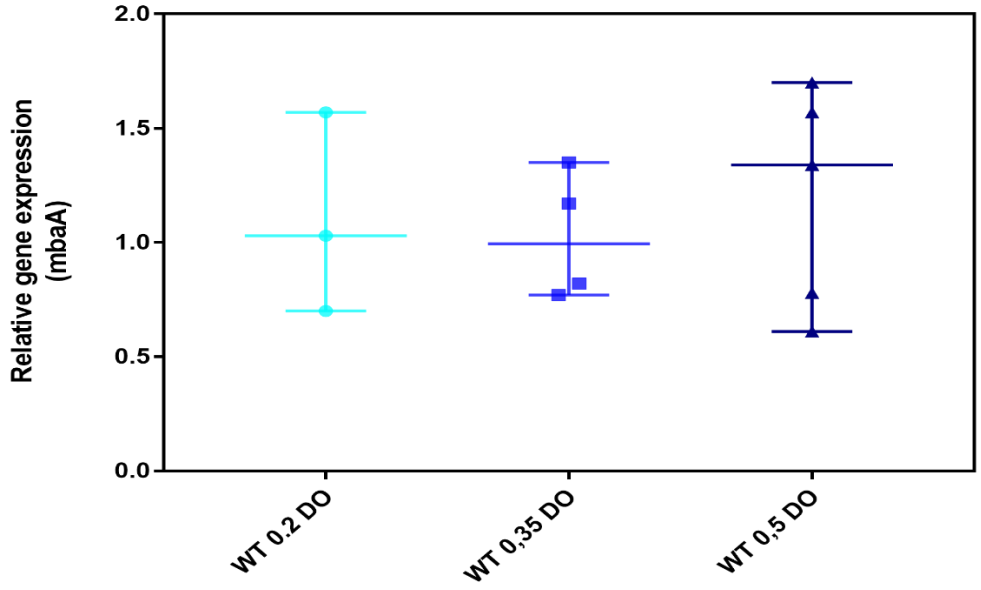

Figure 22. WT different *mbaA* gene expression at different ODs in TSB medium. Even if it is a rich medium, gene expression is mesurable and the genes are expressed. Different OD points were tested to identify when the pick of expression of the *mbaA* gente starts. There is no difference between them.

4. DISCUSSION AND CONCLUSION

Our preliminary data indicated that there is a regulation of siderophore production by QS in *B. thailandensis*. This regulation affects the main siderophore of this bacterium, malleobactin. Due to all this, our goal in this project was to elucidate how that regulation occurs and which genes are involved. To do so, two main objectives were established: to study malleobactin production in all the QS available in the lab and also, to study malleobactin gene expression and see how QS is controlling it.

The results obtained when measuring the siderophore production with the colorimetric test (CAS) show how the triple *btaI* QS mutant produced more siderophores than the WT, so it is a clear siderophore superproducer in all cases (Figure 18). Furthermore, it was observed in all the btaI mutants and btaR mutants studied that the system QS-3 seems to have the most important role in the downregulation of production, since both mutants (btaR3 and btaI3) produce more siderophores than the WT.

These results are not supported by the results obtained in the study of the *mbaA* gene expression. First of all, the *mbaA* gene in the WT in a iron-depleted medium is well expressed and, surprisingly, in all the different btaR QS mutants, the *mbaA* gene is less expressed than the WT. There is no significant difference between them, except for the mutant R1, which seems to be the one that expresses the least. On the other hand, the same study repeated with the triple btaI QS mutant with different AHLs added to observe the effect of each inducer, shows a diminution in the expression of *mbaA*, in comparison with the triple *btaI* mutant without AHL. There are no significant differences between the effects of the three AHL, but there are significant differences between the WT and the AHL of the system 3. These results show that the QS system in *B. thailandensis* is very complex, as we can see in Figure 3, and there are systems that can share AHL, and all of them have a repressor role in the expression of the *mbaA* gene.

It was expected that the triple *btaI* mutant to be a siderophore superproducer according to our background experiments, and this was confirmed in our study measuring siderophore production. Also, and in line with previous results, QS system number 3 had the biggest impact on siderophore production regulation. Following these

observations, levels of *mbaA* gene expression should match the siderophore production results, but this was not the case.

There are two fundamental facts that may be hindering this study and so, it is not possible to relate expression with production in a direct way. As discussed above, malleobactin is a NRPS peptide. This means that the expression of the *mbaA* gene does not directly correspond to the siderophore itself, but to a proteic macrostructure, the NRPS, which is responsible for producing the small peptides which are the siderophores. If the entire siderophore production process were a chain, we are studying just the first link (gene expression) and the last one (measuring the amount of siderophores in the supernatant of a bacterial culture at a certain OD). But the links in between these two have not been investigated, and could have a greater impact than originally expected.

The second noteworthy fact that may be affecting the ability to relate expression to production, is that maleobactin is not a single molecule exclusively. They are a family of molecules, which despite having a virtually identical structure, is represented by several congeners that vary their tridimensional structure minimally and make them have potentially totally different functions, which are still unknown. Actually, only maleobactin E can be considered as a true siderophore. When comparing the iron chelation power of all maleobactins with the chelating power of a true siderophore such as ornibactin (a well-known and studied siderophore) it happened that only one malleobactin had a certain chelating power, but rather weak compared to ornibactin (Franke *et al.*, 2015). The fact that malleobactin is a weak siderophore and that there are also many different ones with unknown function, complicates analysis and comparison of the expression of the gene with siderophore production.

There are data suggesting that there may be siderophores whose production is not regulated by QS. In *Burkholderia cepacia* for example, which produces four different siderophores (salicylic acid, ornibactin, pyochelin, and cepabactin), not all siderophores are regulated by QS. Ornibactin depends on QS, but not pyochelin, showing that its production is not affected in QS mutants (S. Lewenza *et al.*, 1999).

It is possible that the first gene of the biosynthesis operon of malleobactin (*mbaA*) is not regulated by QS. It may be that it is another gene/operon that receives such regulation. As it was shown, there are differences in the concentrations of siderophores detected in the supernatants. It may be that the gene set to be responsible for the siderophore transport outside the cell and recycling are those regulated by QS. Previous studies in our lab (Tan, Sok Gheck, 2014), revealed that there are several mutations that affects siderophore production in the siderophore superproducer, the triple *btaI* mutant (Δ*btaI1,2,3*). It is possible that there is another gene that is controlled by QS and is implicated in the difference we see in siderophore production.

By comparing the results of the screening performed by (Tan, Sok Gheck, 2014) and the RNA-Seq analysis by (Le Guillouzer, thesis 2018) our goal was to find genes that appear in both of them (Table 2). Those are genes that may change siderophore production in the triple I QS mutant and they are also regulated by QS. These are now genes of interest to continue the project.

Table 3. List of genes of interest.

Locus tag	Gene	Operon	Description
BTH_I1403	*scmR*	BTH_I1401-BTH_I1403	LysR family transcriptional regulator
BTH_II1929	*hmqG*	BTH_II1929-BTH_II1935	hypothetical protein
BTH_II1933	*hmqC*	BTH_II1929-BTH_II1935	hypothetical protein
BTH_II2025	*TonB*	BTH_II2024-BTH_II2026	MotA/TolQ/ExbB proton channel family protein
BTH_II2035		BTH_II2032-BTH_II2035	antigen
BTH_II2188	*prpC*	BTH_II2186-BTH_II2189	methylcitrate synthase
BTH_II2189	*prpB*	BTH_II2186-BTH_II2189	2-methylisocitrate lyase
BTH_II2341			hypothetical protein
BTH_II2342		BTH_II2342-BTH_II2348	hypothetical protein
BTH_I3190	*aqpZ*		aquaporin Z
BTH_I1879	*hscA*	BTH_I1878-BTH_I1879	chaperone protein HscA

Genes are controlled by *quorum sensing* and they have an impact in the siderophore production. In yellow, the mutation that produces less siderophores than the WT. In grey, the superproducers. Data extracted from (Tan, Sok Gheck, 2014) and (Le Guillouzer, thesis 2018).

It would be interesting to test these genes to see how they can impact malleobactin production and *mbaA* gene expression. By looking at table 3 the most interesting gene to study now is the *tonB* gene homologue, which could be involved in

siderophore transport through the membrane (**Figure 3**). It may have an impact in siderophore recycling or accumulation outside the cell, and that can explain the different production seen in the CAS assay between all the QS mutants.

Another interesting fact is that in the RNAseq performed in (Le Guillouzer, 2018) there are more than 1900 genes controlled by QS in *B. thailandensis.* The gene *mbaA* is not among them. We already tried to measure siderophore production in a rich medium (TSB) and working in the same conditions. We saw siderophore production (**Figure 21**, less than the production we saw in the iron-limited condition M9 minimal medium, but still, it is measurable, and that means the genes are expressed. So maybe, the reason they are not in the RNAseq list, is not because the experiment was performed in a rich medium with no iron limited conditions, maybe it is because QS does not directly control *mbaA* gene, and that would support our gene expression results, where there were not any significant difference between mutants.

4.1 CONCLUSION AND PERSPECTIVES

While our data confirm that QS controls siderophore production (it was possible to see the differences in siderophore production in the CAS experiments performed in all the QS mutants), *mbaA* gene expression data indicate this QS regulation is very complex and it could have a repressor role in gene expression.

It is certain that malleobactin production is affected by QS, due to the liquid CAS experiments (**Figure 15**) and the different size of halos in the CAS plates (**Figure 14**) Since malleobactin is the main siderophore in *B. thailandensis,* the effect in the halos has to be due to it. On the other hand, it was not possible to confirm which genes are responsible for the increase amount of siderophore produced. There could be another unknown siderophore involved.

As further perspective, it is proposed to check pyochelin production and *pcheE* gene expression (first gene in pyochelin biosynthesis operon) as well and also, to measure malleobactin production using HPLC/MS, trying different columns and configuration to obtain optimal conditions for detection of siderophore molecules.

It will be interesting also to study the list of genes shown in Table 3 and determine how they are regulated and see how those genes affect malleobactin production and *mbaA* gene expression. Specially, *TonB* gene (if is down, less amount of siderophore could get back into the cell and would increase its accumulation in the medium) and *Fur* gene (it would be interesting to know how is regulated and the combined effects of QS and loss of this gene).

RESUMÉ (ANNEXE)

Les bactéries sont des organismes procaryotes unicellulaires. Ces organismes sont parmi les premières formes de vie apparues sur Terre et sont présents dans la plupart de ses habitats. Différentes espèces de bactéries se sont adaptées à une large variété d'habitats et de conditions environnementales extrêmes de Ph et température. Certaines espèces bactériennes vivent également dans des relations symbiotiques et parasitaires avec des organismes supérieurs (plantes et animaux).

Burkholderia est un genre de bactérie à Gram négatif, appartenant à la classe phylogénétique β-Proteobactea. Autrefois, le genre *Burkholderia* faisait partie du genre *Pseudomonas*. La première espèce du genre *Burkholderia* a été décrite par Walter Hagemeyer Burkholder et a été nommée *Pseudomonas cepacia* (Burkholder, 1950). Le genre *Burkholderia* regroupe un ensemble de bactéries saprophytes qui interviennent dans le recyclage de la matière organique. Il existe également des espèces pathogènes telles que les membres du complexe *Burkholderia cepacia* ou *Burkholderia pseudomallei*, considérées comme un agent de guerre biologique potentiel (avec *B. mallei*) en raison de leur la résistance aux antibiotiques et les taux de mortalité élevés de leurs maladies associées (Godoy et al., 2003).

Dans ce projet, nous avons choisi de travailler avec *B. thailandensis*, car cette espèce n'est pas pathogène et il est très similaire aux espèces *B. pseudomallei* et *B. mallei*, car elles proviennent d'un ancêtre commun (Brett et al., 1998).

Le fer est un élément essentiel de la vie cellulaire, car il intervient dans plusieurs voies métaboliques. Cet élément a deux états d'oxydation, ferreux (II) et ferrique (III), mais seule la forme (II) est assimilable par la cellule. Comme la majeure partie du fer dans la nature se trouve sous sa forme ferrique, les bactéries ont développé plusieurs mécanismes d'absorption du fer. Le couple redox Fe (II)/Fe (III) réversible convient le mieux pour catalyser un large spectre de réactions rédox et pour assurer le transfert de la chaîne d'électrons dans la plupart des habitats microbiens. Le Fe (II) est oxydé en Fe (III) spontanément par réaction avec l'oxygène moléculaire ou

par voie enzymatique lors de l'assimilation et de la circulation dans les organismes hôtes. Dans l'environnement, Fe (III) forme des complexes d'oxyde ferrique en présence d'oxygène et de l'eau à un pH neutre à basique. Ces complexes sont très stables, conduisant à une concentration en Fe (III) libre de seulement 10-9 à 10-18 M. Chez les hôtes mammifères, le fer assimilé est étroitement lié à diverses protéines. Les hémoprotéines telles que l'hémoglobine contiennent environ les deux tiers du fer contenu dans l'état lié à l'hème. La ferritine, la protéine de stockage du fer intracellulaire, est capable de stocker jusqu'à 4 500 ions Fe (III) par oligomérisation et contient environ 30% du contenu total de fer (Miethke et al., 2007). De cette façon, le fer est moins disponible pour les bactéries.

Les sidérophores sont de petites molécules, généralement des peptides, qui agissent comme étant des chélateurs du fer. Le fer est nécessaire pour la vie, car il fait partie du métabolisme de molécules essentielles, telles que l'ADN ou l'ATP (Caza et al., 2013). Dans la nature, la plupart du fer se trouve sous sa forme insoluble (III) et n'est pas assimilable par les bactéries. La forme soluble (II) est rare. Par conséquent, les bactéries produisent des sidérophores qui capturent le fer sous sa forme (III) et l'absorbent dans les cellules, où il est réduit à la forme (II), soluble et assimilable par la cellule (Ilbert et al., 2013). L'affinité des sidérophores pour le fer est l'une des plus élevées connues dans la nature. La production de sidérophores chez les bactéries pathogènes leur confère la capacité de participer dans la compétition pour le faire et le capturer à partir des protéines telles que la lactoferrine ou l'hémoglobine. Ceci afin d'assurer leur survie dans l'hôte (Miethke et al., 2007). Par conséquent, les sidérophores sont considérés comme des facteurs de virulence.

B. thailandensis, *B. pseudomallei* et *B. mallei*, produisent deux sidérophores : la malléobactine (la plus importante, avec une plus grande affinité pour le fer) et la pyochéline (plus petite et avec une faible affinité pour le fer agit comme sidérophore secondaire) (Franke et al., 2013), (Kvitko et al., 2012).

Par ailleurs, les bactéries sont considérées comme des organismes indépendants et clonaux. Cependant, les bactéries peuvent interagir les unes avec les autres et « communiquer» pour coordonner diverses fonctions (production de facteurs de virulence, d'agents émulsifiants, la formation de biofilms, la production de sidérophores, la production de toxines, et la motilité, entre autres). Ce phénomène est connu sous le nom de *quorum sensing* (QS). Cette communication dépendant de la densité cellulaire a été décrite pour la première fois chez la bactérie *Vibrio fischeri* (Nealson et al., 1979). Les bactéries produisent des molécules de signalisation (autoinducteurs) et après avoir atteint une certaine densité cellulaire, ces autoinducteurs se lient un régulateur de transcription provocant la modulant ainsi la transcription de certains gènes cibles. *B. thailandensis*, *B. mallei* et *B. pseudomallei* ont un système LuxIR semblable à celui de *V. fischeri*. Plus précisément, *B. thailandensis* dispose de trois systèmes LuxIR complets (nommés BtaIR1, BtaIR2 et BtaIR3) et de deux systèmes orphelins dépourvus d'autoinducteur synthase apparenté (BtaR4 et BtaR5) (Depoorter et al., 2016). Le fait de pouvoir réguler divers gènes et fonctions présente un grand avantage pour la population bactérienne. Cela suppose un avantage adaptatif, une gestion optimale des ressources et favorise la survie.

Dans ce projet, la régulation de la production de malléobactine par le QS chez *Burkholderia thailandensis* a été étudiée. Ce sidérophore est analogue à l'ornibactine produite par *Burkholderia cepacia* et des gènes similaires dans les deux bactéries sont responsables de leur production (Sokol et al., 2000). Nous avons utilisé *B. thailandensis* car il s'agit d'une espèce non pathogène et génétiquement très proche de *B. pseudomallei*, donc cela représente une meilleure facilité pour l'étude au laboratoire en éliminant les risques associés à l'utilisation de bactéries pathogènes. Tout d'abord nous avons mesuré la production de sidérophores avec une méthode colorimétrique utilisant le colorant CAS (chrome azurol S) chez différents mutants de la synthèse de pyochéline et malléobactine et nous avons déterminé que ce dernier est le principal siderophore produit dans les conditions testées. Ensuite, les différents mutants du QS disponibles dans notre laboratoire (*btaI1*, *btaI2*, *btaI3*, *btaI1,2,3, btaR1*, *btaR2*, *btaR3*, *btaR4* et *btaR5*) ont été investigués. Cela a permis d'identifier des régulations positives et

négatives. Finalement, nous avons quantifié l'expression du gène *mbaA* (le premier gène de l'opéron de la synthèse de la malleobactin) chez ces mutants en utilisant la méthode qRT-PCR.

Les données que nous avons obtenues montrent que différents mutants du QS ont une production différente de sidérophores. De plus, le mutant qui n'a pas de régulation du QS, le triple mutant I, est celui qui produit la plus grande quantité de sidérophores. Les autres mutants dans les différents systèmes QS montrent que le système trois (BtaIR3) joue le rôle le plus important dans cette variation. Cependant, en ce qui concerne à l'expression des gènes, aucune différence significative n'est observée entre les mutants, ils ont tous montré une expression similaire. Cela pourrait indiquer que le système de contrôle de la production de malleobactin ne régule pas directement l'expression génique de la malleobactine (*mbaA*).

Étant donné que notre méthode utilisée (CAS) mesure la production totale de siderophores, il n'est donc pas possible de distinguer les différentes molécules. Aussi, cette méthode bien que rapide n'est pas très sensible. En perspective, il serait pertinent d'utiliser une méthode plus fiable pour mesurer la production de malléobactine, telle que la HPLC/MS, en utilisant la malleobactine pure comme standard interne. D'autre part, il serait intéressant de vérifier si la pyochéline a également ce comportement, elle n'est pas régulée directement par QS, ce qui obligerait à étudier sa production et son expression.

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