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Caracterização e avaliação do papel do gene *wcbE* de *Burkholderia seminalis* linhagem TC3.4.2R3 na interação microbiana.

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Characterization and evaluation of the role of *wcbE* gene from *Burkholderia seminalis* strain TC3.4.2R3 in microbial interaction.

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RESUMO

GONÇALVES, P. J. R. O. **Caracterização e avaliação do papel do gene *wcbE* de *Burkholderia seminalis* linhagem TC3.4.2R3 na interação microbiana.** 2017. 149 f. Tese (Doutorado em Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2017.

Burkholderia seminalis tem sido encontrada como um micro-organismo não patogênico, promotor de crescimento vegetal, nodulador de raízes, biorremediador, agente de biocontrole e, também, como organismo patogênico em algumas plantas e pacientes com fibrose cística. O gene *wcbE* codifica uma glicosiltransferase e pertence ao cluster *wcb*, que está relacionado à síntese de cápsula e interações ambientais. O objetivo deste trabalho foi investigar o papel do gene *wcbE* e da temperatura nas interações microbianas de *B. seminalis* TC3.4.2R3. A produção de biofilme, EPS e compostos antifúngicos foi maior a 28 ºC. Por outro lado, a motilidade relacionada a flagelos, bem como a virulência no modelo de infecção de *Galleria mellonella* foram maiores a 37 ºC. As análises transcriptômicas sugeriram que ocorre uma aceleração do metabolismo e da fase de crescimento a 37 ºC, induzindo respostas de estarvação e estresse, onde a célula direciona sua energia para conseguir substrato para sintetizar proteínas necessárias à sobrevivência. A linhagem Δ*wcbE* produziu menos biofilme que a linhagem selvagem e foi atenuada em *G. mellonella* a 37 ºC, destacando a importância da glicosiltransferase de uma maneira temperatura-dependente na patogênese. A inativação do gene *wcbE* resultou na repressão dos genes *wcbC* e *wcbR*, os quais estão envolvidos com a biossíntese de polissacarídeo capsular e metabólitos secundários, respectivamente. Além disso, Δ*wcbE* perdeu a habilidade de inibir fungos fitopatogênicos, mostrando que a glicosiltransferase está também envolvida com a produção de antimicrobianos. A mineração do genoma revelou um domínio PKS no gene *wcbR*. Comparações entre WT e mutantes revelaram uma 10-deoximetimicina, cuja organização gênica se mostrou muito diferente do cluster *wcb*, e um composto com *m/z* 344.0129 ainda não identificado, o qual poderia representar um novo produto relacionado ao cluster *wcb*. Embora *B. seminalis* seja um membro do Bcc, produz compostos antifúngicos eficientes contra patógenos clínicos e ambientais, indicando que esta linhagem pode ter interações múltiplas no ambiente. A temperatura e o gene de glicosiltransferase desempenharam um papel crucial nas interações ambientais de *B. seminalis* TC3.4.2R3.

Palavras-chave: *Burkholderia seminalis*. Interações microbianas. Temperatura. Glicosiltransferase. Cluster *wcb*.

ABSTRACT

GONÇALVES, P. J. R. O. **Characterization and evaluation of the role of *wcbE* gene from *Burkholderia seminalis* strain TC3.4.2R3 in microbial interaction.** 2017. 149 p. Ph.D. thesis (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2017.

Burkholderia seminalis has been found as a nonpathogenic microorganism, plant-growth promoter, root-nodulating, bioremediators, biocontrol agent, and as a pathogenic agent, in some plants and cystic fibrosis patients. The *wcbE* gene, codify a glycosyltransferase, belongs to the *wcb* cluster that is related to synthesis of capsule and environmental interactions. The aim of this work was to investigate the role of *wcbE* gene and the temperature on microbial interactions of *B. seminalis* TC3.4.2R3. Production of biofilm, EPS and antifungal compounds are greater at 28 ºC. On the other hand, motility related to flagella and virulence in the infection model of *Galleria mellonella* were higher at 37 ºC. Transcriptomic analyses suggested an acceleration of metabolism and growth phase at 37 ºC, setting starvation and stress responses where cell directed its energy to get substrate to synthetize proteins needed to survival. The Δ*wcbE* produced less biofilm than WT and was attenuated in *G. mellonella* at 37 ºC, highlighting the importance of glycosyltransferase in the pathogenesis in a temperature-dependent manner. The inactivation of *wcbE* gene resulted in *wcbC* and *wcbR* repression, which are involved in capsular polysaccharide biosynthesis and secondary metabolites production, respectively. Furthermore, Δ*wcbE* lost the ability to inhibit phytopathogenic fungi, demonstrating that glycosyltransferase is also involved in antimicrobial production. The genome mining revealed a PKS domain in the *wcbR* gene. Comparisons between WT and mutants revealed a 10-deoxymethymycin, which showed a genic organization largely different from *wcb* cluster, and a *m/z* 344.0129 not identified compound that could represent a new product related to *wcb* cluster. Even though *B. seminalis* is a Bcc-member, it produces antifungal compounds efficient against plant and clinical pathogens indicating this strain may have multiple interactions in the environment. Temperature and glycosyltransferase played a crucial role on *B. seminalis* TC3.4.2R3 environmental interactions.

Keywords: *Burkholderia seminalis*. Microbial interactions. Temperature. Glycosyltransferase. *wcb* cluster.

1 CHAPTER 1 - Introduction

Agricultural productivity can be limited by different types of diseases caused by microorganisms, such as fungi and bacteria, which result in decreased quality and production. Therefore, the need arises to look for alternatives of low environmental impact and cost. Thus, the secondary antimicrobial metabolites produced by microorganisms could be an excellent alternative. Molecular biology tools, such as gene mining, may help elucidate the mechanisms involved in antimicrobial production and therefore, the study of these compounds at the genomic and metabolomic level is of great importance. Also, accurate study of the biology and adaptive strategies of the microorganism with biocontrol potential is very important.

A large variety of antimicrobial compounds produced by *Burkholderia* has been described. Pyochelin and a rhamnolipid were found conferring antimicrobial activity for *Burkholderia seminalis* strain TC3.4.2R3. This strain was isolated from sugarcane rhizosphere and had genes associated to the biosynthesis of antimicrobial compounds, which were identified through a Tn5 mutant library. Among the identified genes, a gene encoding a glycosyltransferase was found (*wcbE* – ref. *Bsem*_02955) in the *wcb* cluster associated to *B. seminalis* ability in inhibiting phytopathogens. The *wcb* cluster is related to lipopolysaccharide (LPS), exopolysaccharide (EPS) and capsular polysaccharide and, its possible link with antimicrobial production has only recently been suggested. The role of the glycosyltransferase gene has been investigated here for not only the production of antimicrobial compounds, but also for pathogenicity, growth production and adaptive strategies.

Studies have shown that temperature is an abiotic factor that affects the expression of many genes associated with virulence and adaptation of microorganisms in the host, including biofilm production, EPS, motility, seed germination of different species, virulence in different hosts and inhibition of different phytopathogenic fungi. In this way, we aimed to study the role of this glycosyltransferase and temperature in biocontrol potential and microbial interaction processes of *B. seminalis* TC3.4.2R3.

1.1 AIMS

Determine the role of the <u>*wcbE*</u> gene and temperature in *Burkholderia seminalis* TC3.4.2R3 microbial interactions.

1.1.1 Specific Aims

Chapter 1: To review pertinent literature.

Chapter 2: To evaluate the influence of temperature on the microbial interactions and biocontrol potential of *B. seminalis* TC3.4.2R3.

Chapter 3: To compare the transcriptomic analysis of *B. seminalis* TC3.4.2R3 in response to different growth temperatures.

Chapter 4: To examine the role of the glycosyltransferase *wcbE* gene in the pathogenicity of *B. seminalis* TC3.4.2R3.

Chapter 5: To purify secondary metabolites of *B. seminalis* TC3.4.2R3 in comparison with glycosyltransferase *wcbE* mutants.

1.2 LITERATURE REVIEW

1.2.1 General characteristics of Burkholderia

The genus *Burkholderia* was first isolated by Walter H. Burkholder in 1942, and was initially called as *Pseudomonas caryophylli* (BURKHOLDER, 1942). In 1950, Burkholder isolated a Gram-negative mobile bacillus and plant pathogen that causes onion rot, which was termed *Pseudomonas cepacia*, cepacia means derived from onion (BURKHOLDER, 1950). However, DNA-DNA hybridization studies indicated considerable genetic diversity among members of this genus (COMPANT et al., 2008). And later, Yabuuchi et al. (1992) included seven species of rRNA group II of *Pseudomonas* in the new genus *Burkholderia*. Since then, many species have been added to this genus, and 113 species are currently accepted, which are validated according to the Approved Bacterial Names List (DSMZ, 2017). However, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN, 2017) there are 103 species cited in this genus.

Members of the genus *Burkholderia* belong to the β-proteobacteria subdivision and are Gram-negative, aerobic, non-spore forming, non-fermenting, catalase positive and most species are mobile with single or multiple polar flagellum (SHEU et al., 2013). *Burkholderia seminalis* was only recently described by Vanlaere et al. (2008a) as Gram-negative, aerobic, , non-spore forming, which colonies are mucoid and yellow pigmented. Lineages grow in MacConkey and in selective medium for *Burkholderia cepacia* (BCSA), forming acids. The growth was observed at 30 and 37 ºC, nevertheless there was a strain that grew at 42 ºC (VANLAERE et al., 2008a).

The genus *Burkholderia* can be divided in three main clades. The first clade includes the *Burkholderia cepacia* complex (Bcc) and is composed of human pathogens, plant pathogens *(Burkholderia gladioli, Burkholderia plantarii* and *Burkholderia glumae*) and some environmental strains (*Burkholderia vietnamiensis* and *Burkholderia ambifaria*), among other species. The second clade is composed of environmental strains only, several of which have been isolated from polluted soils, and has been designated as *Paraburkholderia.* This clade also has endophytic strains of plants and insects and does not present human or animal pathogens. The third clade includes more than 40 environmental and plant-associated species, many of which are diazotrophic and beneficial to the host. *Burkholderia fungorum* is an exception in this clade, since it was found in human and animal samples. In addition to these three main clades, there are other species that do not cluster to other species of *Burkholderia*, as for example, *Burkholderia rhizoxinica*, *Burkholderia endofungorum*, *Burkholderia caryophylli*, etc (DEPOORTER et al., 2016; ESTRADA-DE LOS SANTOS et al., 2015; SAWANA; ADEOLU; GUPTA, 2014; SUÁREZ-MORENO et al., 2012).

The *Burkholderia cepacia* complex (Bcc) contains at least 20 closely related species (genomovars) (Table 1.1) that share a high similarity (> 97.5%) of 16S rRNA sequences and moderate DNA-DNA (30-60%) hybridization values. They have large genomes (7.5-8.5 Mb) with G + C composition of approximately 67 mol% in multiple replicons distributed in 2 or 4 chromosomes. The classification of this group of bacteria is well delineated, since they present genotypic, phenotypic and very particular metabolic characteristics (VANDAMME; DAWYNDT, 2011). In addition, 10% of its genome consists of genomic islands, since most species of *Burkholderia* present DNA of exogenous origin in the genome (plasmids, bacteriophages and insertion sequences), which guarantees them rapid evolution, plasticity, genotypic diversity and extensive genetic and physiological variability (LESSIE et al., 1996). The *Burkholderia seminalis* TC3.4.2R3 genome contains 7,674,286 bp and G + C composition of 67.22%. The annotated genome shows 6917 predicted coding sequences (ARAÚJO et al., 2016). Phylogenetic analyzes of Araújo et al. (2016) demonstrated that TC3.4.2R3 strain belongs to the Bcc group.

Table 1.1 – List of *Burkholderia cepacia* complex (Bcc) species.

Species	Isolate (origin)	Reference
B. ambifaria	Clinical and environmental	Coenye et al., 2001a .

B. anthina	Clinical and environmental	Vandamme et al., 2002.
B. arboris	Clinical, environmental and industrial contaminant	Vanlaere et al., 2008a.
B. cenocepacia	Clinical, environmental and industrial contaminant	Vandamme et al., 1997, 2003.
B. cepacia	Clinical and environmental	Palleroni; Holmes, 1981; Vandamme et al., 1997.
B. contaminans	Clinical and environmental	Vanlaere et al., 2009.
B. difusa	Clinical and environmental	Vanlaere et al., 2008a.
B. dolosa	Clinical and environmental	Coenye et al., 2001b; Vermis et al., 2004.
B. latens	Clinical	Vanlaere et al., 2008a.
B. lata	Clinical, environmental and industrial contaminant	Vanlaere et al., 2009.
B. metallica	Clinical	Vanlaere et al., 2008a.
B. multivorans	Clinical and environmental	Vandamme et al., 1997.
B. pseudomultivorans	Clinical and environmental	Peeters et al., 2013.
B. pyrrocinia	Clinical and environmental	Storms et al., 2004; Vandamme et al., 2000, 2002.
B. seminalis	Clinical and environmental	Vanlaere et al., 2008a.
B. stabilis	Clinical and environmental	Vandamme et al., 1997, 2000.
B. stagnalis	Clinical and environmental	De Smet et al., 2015.
B. territorii	Clinical and environmental	De Smet et al., 2015.
B. ubonensis	Clinical and environmental	Vandamme et al., 2003; Vanlaere et al., 2008a; Yabuuchi et al., 2000.

B. vietnamiensis	Clinical, environmental and industrial contaminant	Gillis et al., 1995; Vandamme et al., 1997.

Adapted from Vandamme; Dawyndt, 2011.

Bacteria of the genus *Burkholderia* present wide nutritional versatility thanks to the plasticity of their genomes and their ability to adapt to different environments (COMPANT et al., 2008). This characteristic gives them the advantage of inhabiting various ecological niches, and, for this reason, these bacteria can be found in the soil (YOO et al., 2007) or contaminated soil (VANLAERE et al., 2008b); in fresh or salt water (LIM; BAEK; LEE, 2008); in all parts of plants (AIZAWA et al., 2011), as symbiont (SINGH et al., 2006) or pathogen (LI et al., 2010); in several animal species (ELSCHNER et al., 2014); in humans and in hospital settings (KHOSRAVI et al., 2014). Among the plants colonized by *Burkholderia* are sugarcane, eucalyptus, rice, corn, coffee, onion, pineapple, banana, *Mimosa* sp., among others (AIZAWA et al., 2011; COENYE; VANDAMME, 2003). *B. seminalis* was found in non-pathogenic association with rice (PANHWAR et al., 2014), on the other hand, was the pathogenic agent responsible for rotting apricots (LI et al., 2010), in addition, this species was also found in water (FANG et al., 2011), in patients with cystic fibrosis and in nosocomial infections (VANLAERE et al., 2008a). *Burkholderia seminalis* TC3.4.2R3 studied in this work was isolated from sugarcane rhizosphere as a symbiont present in the sugarcane microbiome, able to control necrosis of orchids (ARAÚJO et al., 2016; MANO, 2016).

Burkholderia has been used for many purposes, such as in agricultural management (SALLES; VAN ELSAS; VAN VEEN, 2006), biocontrol of rice foliar pathogens (YANG et al., 2008), biodegradation of thiocyanate, a contaminant in effluent from gold mine tailings (VU; MU; MOREAU, 2013), bioremediation of PAHs-contaminated soils (ANDREOLLI et al., 2011), growth promotion of plants (PAUNGFOO-LONHIENNE et al., 2014), through biological nitrogen fixation (MARTÍNEZ-AGUILAR et al., 2013) or phytohormone production (PERIN et al., 2006a) and in the industry for biopolymers production (LOPES et al., 2014). *B. cepacia*, for example, presents the metabolic capacity to degrade aromatic compounds like phenol and toluene (LANDA et al., 1994; SCHMIDBERGER et al., 2005) and halogenated compounds such as trichlorethylene, a toxic and carcinogenic substance, present in soil and polluted waters. Still, there are species of *Burkholderia* that cause disease in animals and are found in immunodepressive patients with cystic fibrosis (PERIN et al., 2006a). Specifically, *B. seminalis* has already been described as rice growth promoter (PANHWAR et al., 2014), bioremediator of diesel oil (HUANG et al., 2012), in the biocontrol of orchid necrosis

(ARAÚJO et al., 2016; LI et al., 2014) and as pathogen of apricot (LOU et al., 2011).

1.2.2 Microbial interactions involving Burkholderia spp.

Microbial interactions are very advantageous, since through them microorganisms can establish themselves as pathogens or symbionts in a host. In addition, various strategies such as competition and mutualism among species in a community can be regulated through the release of molecules involved in signaling. In animals and plants the associated microbial community may confer protection against pathogens, increase nutrient uptake and interfere with different physiological aspects of the host. Knowledge of the mechanisms involved in these microbial interactions is very important for the development of agents and / or conditions that can prevent or stimulate the formation of microbial communities in a given host (BRAGA; DOURADO; ARAÚJO, 2016).

Burkholderia species can colonize different environments, occupying various niches. *B. pseudomallei* is a free-living saprophyte that can infect humans and cause melioidosis, with pneumonia as the main clinical manifestation. In addition to causing disease in humans, this bacterium can also colonize many animals, such as cattle, goats, swine, monkeys, rabbits, dolphins, iguanas, among others (ELSCHNER et al., 2014). *B. mallei* is an obligatory animal pathogen and etiological agent of glanders, which causes debilitation and death in horses. Both *B. pseudomallei* and *B. mallei* are potentially virulent and belong to the biosafety level 3 (BERNHARDS et al., 2016). The Bcc members (Table 1) can cause infections in about 5% of the patients with cystic fibrosis in the world, which after colonization suffer a rapid decline. The acquisition is usually by the environment and *B. multivorans* is the most acquired bacterium (MCCLEAN et al., 2016). *B. cepacia* is an opportunistic pathogen that causes disease in immunocompromised patients and has been associated with infections in the bloodstream, respiratory and urinary tract. It is very found in nosocomial infections (SRINIVASAN; ARORA; SAHAI, 2016).

In addition to vertebrates, *Burkholderia* spp. also interact with invertebrates, since a *Burkholderia* was found as a symbiont in *Riptortus pedestris* (Hemiptera), an insect associated with common bean. The insect acquires the bacterium orally from the environment and then the bacterium colonizes the posterior region of the midgut (KIKUCHI; HOSOKAWA; FUKATSU, 2007), conferring greater survival to *R. pedestris* through the stimulation of humoral immunity. Kim; Lee (2015) observed in colonized insects a greater expression of antimicrobial peptides and an increase in antimicrobial activity in the hemolymph of the animal. Among the genes associated to this symbiosis are biosynthetic genes of peptideoglycan, purines,

polyhydroxyalkanoate (PHA) granules, among others (KIM; LEE, 2015). The O-antigen from *Burkholderia* lipopolysaccharide (LPS), although not essential for bacterial persistence in the late stages of symbiosis, plays an essential role in the early stages of association (KIM; PARK; LEE, 2016). *Burkholderia* was also found in symbiosis in the gut of *R. clavatus* and *Leptocorisa chinensis*, insects that are considered as pests of soybeans and rice, respectively, in Japan (KIKUCHI; MENG; FUKATSU, 2005). In *Gossyparia spuria* and *Acanthococcus aceris*, hemiptera of the superfamily Coccoidea, *Burkholderia* was found in body fat cells. In these insects, the bacterium is transmitted vertically from the mother to the offspring. *Burkholderia* leaves the fat cells and invades the follicular epithelium in the ovary, entering the perivitelinal space, where infests the oocytes (MICHALIK et al., 2016). Analyzes of 16S rRNA genes revealed that the midgut crypts of the eastern bug *Cavelerius saccharivorus*, pest of sugarcane, are dominated by symbionts of the genus *Burkholderia*, whose transmission is also vertical in these insects (ITOH et al., 2014). Nevertheless, in *Physopelta gutta*, popularly known as stinkbug, *Burkholderia* transmission does not occur vertically, but seems to be acquired by each host generation. Elimination of the symbiotic bacterium resulted in growth retardation, reduced size, high mortality, reduced fecundity and abnormal staining of the insect body. It is suggested that the abilities to fix nitrogen and produce antimicrobials against phytopathogenic fungi may be involved in the symbiotic association with these insects (TAKESHITA et al., 2015). *Blissus insularis*, an insect that feeds on the phloem and is considered as plague of grass, also acquires *Burkholderia* via environment, that is, through plants and soil (XU; BUSS; BOUCIAS, 2016). *Burkholderia* was also found in the gut of ants of the species *Tetraponera binghami* (BORM et al., 2002). *B. cenocepacia* in ants *Atta sexdens rubripilosa* secretes a potent antifungal agent that inhibits the germination of the entomopathogenic fungi conidia from *Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii* and *Escovopsis weberi*, but does not affect the symbiont fungus *Leucoagaricus gongylophorus* (SANTOS et al., 2004).

Furthermore, *Burkholderia* has been found in association with plants, either as endosymbionts or phytopathogens, both in the rhizosphere and in the aerial parts, as epiphytic or endophytic (COENYE; VANDAMME, 2003). The great majority of plant-associated *Burkholderia* species are not pathogenic and may be both neutral and beneficial to their hosts (DE COSTA; ERABADUPITIYA, 2005). *Burkholderia* can inhabit the rhizosphere of the plant, establishing themselves in the soil or in the surface of the root (COMPANT et al., 2008). The preference of the plant beneficial *Burkholderia* to the rhizosphere is probably due to its catabolic versatility, which allows the root exudates and other compounds derived from the root to be degraded, allowing their proliferation on the root surface (CHAIN et al., 2006). Bcc

species were isolated from several different plants, such as rice, cotton, wheat and corn in the United States (RAMETTE; LIPUMA; TIEDJE, 2005). *Burkholderia silvatlantica* Inhabits the rhizosphere of maize and sugarcane in Brazil (PERIN et al., 2006b). Luvizotto et al. (2010) Found 39 *Burkholderia* isolates in sugarcane roots, of which 19 belonged to the rhizosphere. 95% of these isolates were positive for nitrogen fixation, 84% inhibited *Xanthomonas albilineans* and 47% produced siderophores. The authors suggest that although most of the *Burkholderia* species found in the sugarcane rhizosphere are from Bcc, they can be used as plant growth promoters and biocontrol agents.

Burkholderia australis was isolated from the rhizosphere of sugarcane and showed nitrogen fixation (N_2) and plant growth promotion abilities (PAUNGFOO-LONHIENNE et al., 2014). *Burkholderia* strain AU4i from pea rhizosphere, promoted root and shoot growth in pea, wheat and grass, conferred phosphate solubilization, indole-acetic acid (AIA) production, N_2 fixation, ammonia, siderophore and hydrogen cyanide (HCN) production. In addition, it inhibited pathogenic fungi *Fusarium* sp., *Rhizoctonia* sp., *Alternaria* sp. and *Rosellinia* sp. in both *in vitro* and *in vivo* experiments (DEVI et al., 2015). *B. seminalis* strain R456 has been isolated from the rice rhizosphere and, besides being non-pathogenic, protects rice seedlings from *Rhizoctonia solani* (LI et al., 2011, 2014). *Burkholderia* spp. have been isolated from *Fabaceae* roots nodules and *Rubiaceae* leaves (COMPANT et al., 2008). *Burkholderia* spp. exhibit affinity for the colonization of leguminous plants of the genus *Mimosa*, suggesting a coevolutionary process between these species (COMPANT et al., 2008). *B. phymatum* was isolated from *Mimosa* spp. in Taiwan, Venezuela and Brazil (CHEN et al., 2005a, 2005b); *B. nodosa* was isolated from *M. scrabella* nodules in Brazil (CHEN et al., 2007). In the same way, Bontemps et al. (2010) isolated symbionts of 50 *Mimosa* spp. from Brazilian Cerrado and Caatinga, all nodulating species belonged to the genus *Burkholderia.*

Soil and crop management practices, as well as land use patterns, affect the diversity of *Burkholderia* spp. in the rhizosphere of plants. The diversity of *Burkholderia* spp. colonizing plants in different geographic locations may be related to human activity. It is also possible that some plants exhibit preferences for rhizospheric species of *Burkholderia* in particular (SUÁREZ-MORENO et al., 2012). And, although most populations of *Burkholderia* are non-pathogenic to the plants, intensive agriculture activities can accelerate the proliferation of human pathogens (SALLES; VAN ELSAS; VAN VEEN, 2006).

Some bacteria of the rhizosphere can colonize the internal tissue of the plant, cross the cortex of the root reaching the vascular system, which can occur with the aid of cell wall degradation enzymes, and thus establish an endophytic population in various organs of the plant

(COMPANT et al., 2008; SUÁREZ-MORENO et al., 2012). These bacteria can benefit the plant in many ways, such as hormone production, phosphate solubilization, siderophores production, inhibition of phytopathogens, among others (COMPANT; CLÉMENT; SESSITSCH, 2010).

B. cepacia was isolated from *Citrus* sp. cultivated in Brazil (ARAÚJO et al., 2002). *B. silvatlantica* from sugarcane leaves (PERIN et al., 2006b). *B. unamae* is associated to maize, sugarcane and coffee (CABALLERO-MELLADO et al., 2004). *B. tropica* was isolated from diferente parts of sugarcane, maize and teosinte grown in different climatic and geographic regions of Brazil, Mexico and South Africa (REIS et al., 2004). *B. gladioli* was found in leaves and pseudobulb of orchids, causing necrosis (MANO et al., 2015). *B. seminalis* strain TC3.4.2R3 was isolated as endophyte from sugarcane roots (ARAÚJO et al., 2016). *B. brasiliensis*, currently called *B. kururiensis* strain M130, was isolated from rice, bananas, cassava, pineapple and sugarcane (COUTINHO et al., 2013; MATTOS et al., 2001), while *B. vietnamiensis* was also isolated from rice and promoted a significant increase in the production of this crop in Vietnam (VAN et al., 2000). *B. phytofirmans* strain PsJN can express high levels of ACC (1-aminocyclopropane-1-carboxylate) deaminase. This enzyme hydrolyzes the ACC, the precursor of the ethylene hormone, in ammonia and α-ketobutyrate, reducing the levels of ACC and, consequently, ethylene in the plant (GLICK et al., 2007; SESSITSCH et al., 2005). Interactions between potato and *B. phytofirmans* strain PsJN increased tolerance of plant against heat stress (NOWAK et al., 2007) and the cold tolerance of the vine (AIT BARKA; NOWAK; CLÉMENT, 2006), demonstrating that the association of *Burkholderia* with plants can also increase resistance against abiotic stresses.

Many species of *Burkholderia* are found in association with mycorrhizae, since some bacteria of the rhizosphere adhere strongly to the hyphae and can penetrate them, while others associate directly to the surface of the root (COMPANT et al., 2008). *B. terrae* was isolated from the *Laccaria proxima* mycosphere and is able to use 15 of the 18 exudates produced by this fungus. In addition, it is capable of migrating and translocating other plant growth promoting rhizobacteria through fungal hyphae (WARMINK et al., 2011; WARMINK; VAN ELSAS, 2008). *Burkholderia* spp. form associations with a large number of fungal taxa. *Burkholderia glathei, B. terrae, B. fungorum* and *B. phytofirmans* can interact and disperse with *A. alternata, F. solani* and *R. solani* (STOPNISEK et al., 2015). Bacteria can capture volatile organic compounds, for example terpenes, emitted by certain fungi and respond with changes in their motility. The release of these compounds by fungi is influenced by nutritional conditions and stage of growth (SCHMIDT et al., 2016). *B. fungorum* and *B. sordidicola* were

directly isolated from the phytopathogenic fungi *Phanerochaete chrysosporium* and *Phanaerochaete sordida*, respectively (COENYE et al., 2001c; LIM et al., 2003), whereas *B. endofungorum* and *B. rhizoxinica* are endosymbionts of *Rhizopus microsporus* (PARTIDA-MARTINEZ et al., 2007). *Burkholderia* sp., endosymbiotic of *Rhizopus microsporus*, produced a mycotoxin, rhizoxin, which caused rice seedling patches, which was attributed to the fungus. Rhizoxin inhibits the mitosis of rice cells and weakens or even kills the plant, thus both the host and the symbiont benefit from the nutrients of the decomposing plant (PARTIDA-MARTINEZ; HERTWECK, 2005). *B. phenazinium* and *B. sordidicola* were found in association with ectomycorrhizae of *Pinus* (NGUYEN; BRUNS, 2015). This association is advantageous for both because the bacteria could supply carbon to the fungus, while this fungus can provide nitrogen sources to the bacteria. Bacterial communities differ among fungal species, but when it is a single fungal species, the associated bacterial community appears to be stable over the years (NGUYEN; BRUNS, 2015). Moreover mutualistic interactions, *Burkholderia* can also form antagonistic interactions with fungi, mainly phytopathogenic fungi and oomycetes, and also bacteria. This antagonism is usually due to the production of secondary metabolites. Many compounds with activity have already been described for *Burkholderia* spp., such as pyrrolnitrin, phenazines, cepacidins, lipopeptides, rhamnolipids, siderophores, among others (ARAÚJO; ARAÚJO; EBERLIN, 2017).

1.2.3 Antimicrobial compounds in Burkholderia

Bacteria belonging to the genus *Burkholderia* sp., mainly those from the Bcc group, are considered to be quite efficient for the biological control of pathogens of plant species of interest, since they have the ability to produce antimicrobial agents, derived from their secondary metabolism, against fungi and bacteria (PARKE; GURIAN-SHERMAN, 2001).

Microorganisms form communities in various environments. The abundance of each species in these communities is a result of the specific interaction between the microorganisms present. In this context, the secondary metabolites play a crucial role in this interaction, since they can act positively or negatively on other species. These secondary metabolites, also called natural products, are organic compounds with low molecular weight, which unlike primary metabolites, are not directly involved in the growth, development, or reproduction of the organism that produces it. The microorganisms are able to synthesize a large number of secondary metabolites, but the exact number is unknown, since there are fewer natural products described than gene clusters of secondary metabolism (NETZKER et al., 2015; SEYEDSAYAMDOST; CLARDY, 2014).

Secondary fungal metabolites may act on fungal sterols by altering the permeability of the cell membrane, may affect the fungal cell wall, inhibit nucleic acid synthesis or inactivate conidiosphores, resulting in reduced or complete inhibition of growth and sporulation (LI; QUAN; FAN, 2007). On the other hand, the effect on bacteria can be observed by inhibition of growth, alteration in permeability and cell wall synthesis, plasma membrane, inhibition of protein synthesis, inhibition of biofilm, inhibition or interference in quorum sensing, among others (FRANCOLINI et al., 2004; TEASDALE et al., 2009). In addition, secondary metabolites can confer competitive advantage against other bacteria, fungi, plants and animals; act as agents in the transportation of metals; in the symbiosis between microbes and plants, nematodes, insects and other animals; act as sex hormones or effectors of chemical and morphological differentiation, for example, by stimulating or inhibiting sporulation and spore germination (DEMAIN; FANG, 2000).

Secondary metabolites do not belong to a homogeneous chemical group, but can be synthesized by two pathways. The first pathway is ribosomal, which involves the production of peptides associated with the host defense system. The other route of synthesis is the non-ribosomal, which uses a large variety of non-protein substrates, such as hydroxy acids and polyketide substances in the synthesis of petids of interest. This pathway has been characterized by producing a wide variety of secondary metabolites, which have a wide structural variety. These substances are biosynthesized by multifunctional enzymes called non-ribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS). There are also the NRPS-PKS hybrids (SINGH; CHAUDHARY; SAREEN, 2017).

A large number of secondary metabolites with antimicrobial activity, such as cepacins, pyrrolnitrines, cepaciamides, cepacidines, alteridines, quinolones, phenazines, siderophores, lipopeptides, among many others compounds with antifungal activity have been reported for *Burkholderia* spp. (HILL et al., 1994; HUANG; WONG, 1998; MAO et al., 2006; PARKER et al., 1984). According to Parke; Gurian-Sherman (2001), the biological control using *Burkholderia* spp. could partially replace the use of common chemical pesticides, fumigants and fungicides or biocides of wide spectrum. Bacteria of the genus *Burkholderia*, in a field experiment, were able to colonize the rhizosphere of several crops like corn, wheat, rice, peas, sunflower and radish, and significantly increased host plant growth, as well as reduced the presence of pathogens (CHIARINI et al., 2006; COENYE; VANDAMME, 2003).

Bevivino et al. (2000) observed that the strain *B. cepacia* MCI 7, described as a plant growth promoting agent of corn plants, was able to control the fungal infection by *F. moniliforme* in the early stages of plant growth. *Burkholderia* sp. strain 2.2.N was able to inhibit

the growth of *Micrococcus luteus, Saccharomyces cerevisiae* and *Aspergillus niger*. A zone of inhibition was observed with fractions of the supernatant, revealing that the antimicrobial activity did not depend on the presence of living cells and, rather, on the secreted extracellular compounds (CAIN et al., 2000). The control of pea rot caused by fungi *Pythium aphanidermatum* and *Aphanomyces euteiches* was evaluated by Heungens; Parke (2001), in different stages of the fungus life cycle, post-infection. The wild-type strain of *B. cepacia* AMMDR1, when in large quantities, significantly reduced seed colonization by *P. aphanidermatum* and roots by *A. euteiches*. The authors suggest that the process of inhibition of pathogens is dependent on the bacterial density, the stage of development of the pathogen and also on the interaction with the host plant. Luvizotto et al. (2010), in an experiment with several strains of bacteria associated with sugarcane of the genus *Burkholderia*, found that they were able to inhibit *Fusarium verticillioides* and *Xanthomonas albilineans in vitro* and suggested its use as a biocontrol agent, mainly for sugarcane. Pandey; Kang; Maheshwari (2005) described a 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by *Burkholderia* sp. with antagonistic activity against *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. *Rhizoctonia solani*, such as *Fusarium oxysporum*, were also inhibited by *Burkholderia gladioli* pv. *agaricicola* volatile organic compounds (ELSHAFIE et al., 2012). *Burkholderia tropica* also demonstrated ability to inhibit the fungi *C. gloesporioides, S. rolffsi, F. culmorum* and *F. oxysporum*, protecting corn from phytopathogen attacks (TENORIO-SALGADO et al., 2013). *Burkholderia* sp. strains MSh1 and MSh2, isolated from tropical turf swamp soil in Malaysia were able to produce active antibacterial compounds against several multiresistant bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 700699 and vancomycin resistant enterococci (VRE) ATCC 700802(ONG et al., 2014).

Parker et al. (1984) found two acetylenic compounds, cepacin A and cepacin B, with antimicrobial activity in *Burkholderia cepacia* SC11,783 against *Staphylococcus* sp. and other Gram-negative bacteria. *B. mallei, B. pseudomallei* and *B. thailandensis* have produced burkholderic acid, which is related to the yellow pigmentation of these species. This acid had no antibacterial activity and cytotoxicity was considered weak to moderate (FRANKE; ISHIDA; HERTWECK, 2012). Occidiofungin, a glycopeptide antibiotic with antifungal activity, was found in *B. contaminans* (CHEN et al., 2013; LU et al., 2009) and *B. pyrrocinia* (WANG et al., 2016).

Pyrrolnitrine, a product of Burkholderia cepacia, which blocks the transfer of electrons between dehydrogenase and cytochrome, components of the respiratory chain, has been identified by El-Banna; Winkelmann (1998) and presented broad antimicrobial spectrum

against filamentous fungi, yeasts and Gram-positive bacteria. Pyrrolnitrine produced by *B. cepacia* B23 was able to control the anthracnose caused by *Colletotrichum gloeosporioides* in papaya (KADIR et al., 2008). The production of pyrrolnitrin by a strain of *Pseudomonas cepacia* B37w was associated with the inhibition of the symptoms of root dryness by *Fusarium sambucinum* (BURKHEAD; SCHISLER; SLININGER, 1994). *B. cepacia* strain 5.5B, pyrrolnitrine producer, was able to supply rod rot in poinsettia caused by *Rhizoctonia solani*, whereas the RR13-1 and UV 19-4 mutant strains, which produced 5,000 to 20,000 times less amounts of the metabolite, were less able In inhibiting the disease (HWANG; CHILTON; BENSON, 2002). Pyrrolnitrin was also produced by *Burkholderia ambifaria, Burkholderia pyrrocinia* all *Burkholderia lata*, all Bcc members, under quorum sensing control. A large amount of antibiotics produced by *Burkholderia* sp. are controlled by quorum sensing. The AHL receptor mutants did not produce pyrrolnitrine, and consequently failed to inhibit the growth of *Rhizoctonia solani* (SCHMIDT et al., 2009). Pyrrolnitrin was also detected in *B. cenocepacia, B. ubonensi, B. pseudomallei, B. thailandensis* and *B. oklahomensis* (DEPOORTER et al., 2016).

The presence of siderophores was described in *B. bryophila* and *B. megapolitana* associated with the mosses *Aulacomnium palustre*, *Sphagnum rubellum* and *Sphagnum pallustre*. Both species exhibited antifungal and antibacterial activity, as well as promoted the growth of lettuce, making them candidates for biological control (VANDAMME et al., 2007). *Burkholderia contaminans* MS14 produced ornibactin, a siderophore with antibacterial activity (DENG et al., 2017). *B. paludis* produced pyochelin, a siderophore that was able to inhibit *S. aureus* and *E. faecalis* (ONG et al., 2016). *B. seminalis* strain TC3.4.2R3 also produced pyochelin and the rhamnolipid Rha-Rha-C15-C14 with fungicidal effect, when co-cultivated with *Fusarium oxysporum* (ARAÚJO; ARAÚJO; EBERLIN, 2017).

Burkholderia gladioli in co-culture with *Rhizopus microsporus* produces a potent antibiotic of the family of the enaciloxins, polyketides with antifungal and antibacterial capacity (ROSS et al., 2014). This same class of antibiotics is also produced by *B. ambifaria* against *C. albicans* and *S. aureus*, common in patients with cystic fibrosis (MAHENTHIRALINGAM et al., 2011). *B. gladioli*, necrosis of orchid leaves, was controlled by *B. seminalis* TC3.4.2R3 (ARAÚJO et al., 2016).

1.2.4 Genes associated with antimicrobial production

Secondary metabolites with antimicrobial action produced by microorganisms represent an interesting alternative to conventional chemicals. Genes associated with the production of

defense response activation molecules in plants may be targets to be exploited for biological control. Considering the progress in the field of genetic engineering of microorganisms and plants, the identification of genes involved in the synthesis of these molecules with antimicrobial activity, may allow the use of microorganisms and recombinant plants to control phytopathogens. Access to complete genomic sequences has facilitated the discovery of new antibiotics through genomic mining by bioinformatics tools. Thus, the search for genes involved in the biosynthesis of natural products has become extremely important (ZERIKLY; CHALLIS, 2009). Many gene clusters of antibiotic biosynthesis have been identified in *Burkholderia* sp., where biosynthetic clusters of NRPS (nonribosomal peptide synthesis) and PKS (polyketide synthetase) may contribute to the metabolic diversity of these bacteria. In addition, many regulatory genes may also be involved, directly or indirectly, in the process, since in *Burkholderia* spp. the synthesis of most antibiotics is regulated by quorum sensing (DEPOORTER et al., 2016).

Mano (2010) used *Burkholderia cenocepacia* strain TC3.4.2R3, later identified as *B. seminalis*, isolated endophytically from sugarcane roots, to control orchid soft rot. In that study, it was verified this biological control is associated with the genic expression of phospholipase similar to patatin, glycosyltransferase group 1, glutamate synthase, MFS (Principal Facilitating Superfamily) transporters, dehydrogenases, polyhydroxyalkanoates (PHAs), among others.

Neves (2011) tested a *Burkholderia* Tn5 mutant library with 600 clones, and found that 5.38% showed loss of ability to inhibit *F. oxysporum*, 17.14% *F. verticillioides* and *C. paradoxa*, and 5.71% inhibit *Phytophthora parasitic*. Using this strategy, it was observed that the thioredoxin, methyltransferase, ferredoxin and glycosyltransferase genes were associated with inhibition of pathogens, suggesting the synthesis of different molecules with antimicrobial properties in the process. Araujo et al. (2016) found clusters of pyrrolnitrine biosynthesis, rhamnolipid, ornibactin and pioquelin siderophores, ACC deaminase and indoleacetic acid (IAA), which were associated with the biocontrol capacity in *B. seminalis* TC3.4.2R3.

1.2.4.1 Glycosyltransferases

Glycosyltransferases catalyze the transfer of a mono or oligosaccharide residue to specific acceptor molecules, forming glycosidic linkages that initiate the elongation of a carbohydrate chain. Accepting molecules can be lipids, proteins, heterocyclic compounds, and other carbohydrate residues. These proteins represent one of the most abundant enzymes in biological systems, account for about 1% to 2% of the gene products of an organism and are responsible for catalyzing the synthesis of glycoconjugates. Glycoconjugates include

glycolipids, glycoproteins and polysaccharides. Glycosylation is a highly specific reaction with respect to the anomeric configuration of the sugar residue and the addition site (KAPITONOV; YU, 1999; LAIRSON et al., 2008). Glycosylation reactions catalyzed by glycosyltransferases are also essential for the biological activity of secondary metabolites, such as antibiotics, as well as being involved in the synthesis of numerous natural active compounds containing carbon, such as oligosaccharides. Conjugations of sugars generally result in improved solubility, increased polarity, and chemical stability of secondary metabolites (LIANG; QIAO, 2007; LUZHETSKYY; VENTE; BECHTHOLD, 2005).

Glycosyltransferases are classified into families based on similarities of amino acid sequences, catalytic specificity and consensus sequences (ROSS et al., 2001). The structural characterization of representatives of a large number of the 103 families of glycosyltransferases currently described (CAZy database, 2017) Revealed an extraordinary degree of diversity in the conformation of its folds. In contrast, several recent research on the structure of the glycosyltransferase revealed a very different situation, since only two global folds (GT-A and GT-B) were observed for all nucleotide-dependent glycosyltransferase structures. To date, the three-dimensional structures of glycosyltransferases have revealed these two large structural folds (LAIRSON et al., 2008).

Several types of glycosyltransferases are present in plants, which participate in sugar transfer reactions to a large group of receptor molecules (ROSS et al., 2001). For example, glycosyltransferases belonging to family 8 act on plant growth and the absence of this gene results in lower growth, diminished adhesion between foliar and root cells facilitating their dehydration (SCHEIBLE; PAULY, 2004). Glycosyltransferases are considered crucial for the modification of secondary plant metabolites, acting on hormone homeostasis, plant resistance to abiotic stresses, are involved in signal transduction, thus participating in the growth and development of plants (WANG; HOU, 2009).

Analyzing the sequence of the actinomycete cluster A40926 *Nonomuraea* sp., Sosio et al. (2003) found a glycosyltransferase gene involved in the biosynthesis of the antibiotic teicoplanin from the glycopeptide family and suggested that glycolysis occurs during secretion of the antibiotic in contrast to the mechanism of glycosyl transfer occurring in the compounds within the cell. Glycosyl transfer may have a cell protection function, promoting a temporary inactivation of the antibiotic newly produced by the microorganism. Only when the latent antibiotic is secreted, the hydrolytic glycosyltransferases reactivate the antibiotic by removing the hexose. Garrido et al. (2006) have described two glycosyltransferase enzymes in *Streptomyces olindensis*, the *cosG* and *cosK* that participate in the glycosylation in the

biosynthetic pathway of the antibiotic cosmomycin. Two glycosyltransferase genes involved in the synthesis of the antibiotic naftoxanthene FD-594 in *Streptomyces* sp. were detected by Kudo et al. (2011). Stingele; Newell; Neeser (1999) identified and analyzed 13 genes of *Streptococcus thermophilus* responsible for the production of exopolysaccharides and found glycosyltransferases composing the central part of these genes. The repeating unit is first added by transferring sugar residues to a lipophilic anchor by specific glycosyltransferases. Unlike the other glycosyltransferases, the first glycosyltransferase does not catalyze the glycosidic bond, but transfers a sugar-1P to a lipophilic anchor, subsequently, the complete repeating unit is exported and polymerized.

Glycosyltransferases are also present in the biosynthesis of lipopolysaccharides, cell membrane components (LEIPOLD; VINOGRADOV; WHITFIELD, 2007). While the function of glycosyltransferase in capsule synthesis is to catalyze the sequential transfer of sugar residues from nucleotide precursors, such as UDP and GTP, to a membrane acceptor (undecaprenol phosfate-P-Gl*cp*NAc) (RECKSEIDLER et al., 2001). Kim et al. (2007) investigated the glycosylation of flavonoids with an *E. coli* expressing glycosyltransferases of *Xanthomonas campestris*. The authors observed that there was production of various types of compounds with antibacterial activities, including flavonoids. Ko et al. (2008) found a flavonoid C-glycosyltransferase in rice using as substrate an O-methylated flavonoid. Glycosyltransferases are also involved in the synthesis of peptideoglycan, since they catalyze the glycosylation of a disaccharide peptide which is a basic unit of peptideoglycan (BARRETT et al., 2004) and in the synthesis of the polysalic acid present in the capsules of bacteria as *E. coli* (VIMR et al., 1992).

The glycosyltransferase gene was also found in *B. seminalis* TC3.4.2R3 associated to pyochelin and rhamnolipid production (ARAÚJO; ARAÚJO; EBERLIN, 2017). In *B. cenocepacia*, glycosyltransferase is present in the glycosylation of flagellin in ten different sites. This glycosylation reduces the recognition of flagellin by the host, providing an evasive strategy for bacterial infection (HANUSZKIEWICZ et al., 2014). A glycosyltransferase encoded by the *bceE* gene of *B. cenocepacia* is essential for the synthesis of exopolysaccharide, which plays a role in the colonization and persistence of this bacterium in the host (VIDEIRA; GARCIA; SÁ-CORREIA, 2005). Bartholdson et al. (2008) also inactivated the gene encoding a glycosyltransferase (*bceB*) and inhibited EPS biosynthesis by altering the mucoid phenotype of *B. ambifaria*. Thus, it can be seen that glycosyltransferases are involved in the synthesis of a number of compounds important for the adaptation and competition of the bacteria in their environment.

1.2.4.2 The *wcb* cluster

The *wcb* cluster is involved in the biosynthesis and transport of capsular polysaccharide in *Burkholderia*, dramatically altering its virulence (WARAWA et al., 2009). This cluster is responsible for exopolysaccharide (EPS) production in Bcc isolates (ZLOSNIK; SPEERT, 2010). The inactivated *wcbE* gene, located in the *wcb* cluster, had homology with a glycosyltransferase and in *B. pseudomallei* resulted in loss of lipopolysaccharide (O-PS type I) and attenuated virulence in animal model (RECKSEIDLER et al., 2001), besides the complete loss of capsular polysaccharide type I (CUCCUI et al., 2012; DANDO et al., 2016). The glycosyltransferase insertional inactivation in *B. pseudomallei* resulted in a mutant without capsule that had the virulence 10,000 times more attenuated when compared to the wild type, demonstrating the glycosyltransferase can be determinant in bacterium virulence (RECKSEIDLER-ZENTENO et al., 2010). Other study with *B. pseudomallei,* strain JW270, demonstrated that the loss of the *wcb* capsule operon causes a dramatic >4.46 log attenuation in a hamster intraperitoneal infection model (GUTIERREZ; WARAWA, 2016). Nevertheless, *wcb* cluster was not essential for the mucoid phenotype in *B. cenocepacia* H111 from cystic fibrosis lungs and, there could be strain specific factors involved in control of exopolysaccharide production (MILLER et al., 2015).

The *B. pseudomallei* K96243 CPS I coding region or *wcb* cluster is composed by a central sugar biosynthesis cassette with glycosyltransferase genes surrounded by genes encoding polysaccharide transport proteins to movement *B. pseudomallei* CPS I to the outer membrane. There is also a lipid anchor in *B. pseudomallei* CPS I (CUCCUI et al., 2012).

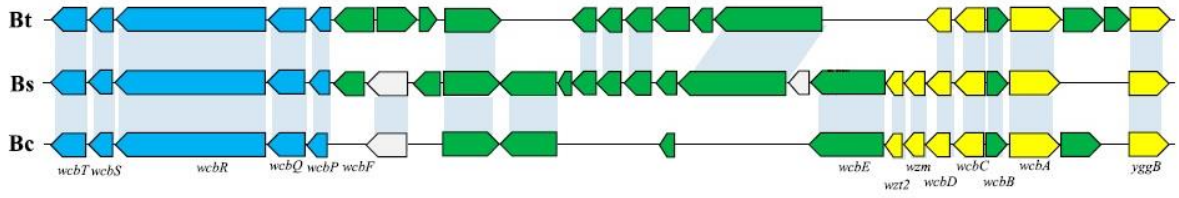
Araujo et al. (2016) selected 12 mutants of *B. seminalis* TC3.4.2R3 that lost biocontrol capacity, allowing the mapping of 8 genes essential for the biocontrol, all located on chromosome 1 of the bacterial genome. One of these genes was a glycosyltransferase (*wcbE* – *Bsem*_02955) present in the *wcb* cluster. The authors suggest the importance of the bacterial capsule in the interactions of *Burkholderia* with the host.

B. thailandensis exhibited *wcb* genes acquired, very similar to those from *B. pseudomallei,* corresponding to a capsular polysaccharide biosynthesis. Although the capsular polysaccharide is essential for virulence in mammals, *B. thailandensis* was not virulent in murine infections, suggesting that more factors are required to successfully colonize and infect mammals (SIM et al., 2010). The *wcb* genes in the capsule synthesis region confer virulence in both *B. mallei* and *B. pseudomallei*. The same region in *B. thailandensis* showed contents differences among the genes. These observations suggested a different capsule is produced by

B. thailandensis compared to *B. mallei* and *B. pseudomallei* (KIM et al., 2005).

Comparisons of the *wcb* cluster of *B. seminalis* TC3.4.2R3, *B. thailandensis* E264 and *B. cenocepacea* J2315 revealed a terminal portion very similar, but a central region variable (Fig.1.1), which could be explained by the pathogenic and saprophytic differences presented in those *Burkholderia* lifestyles (ARAÚJO et al., 2016; KIM et al., 2005; SIM et al., 2010).

Figure 1.1 - Comparison of the *wcb* capsule synthesis region in *B. thailandensis* (Bt), *B. seminalis* (Bs), and *B. cenocepacea* (Bc).



The orthologous genes in the three species are denoted with connecting lines. The putative function of the genes are color coded as lipid biosynthesis (blue), sugar biosynthesis (green), transport (yellow) and hypothetical proteins (white). Adapted from Araujo et al. (2016).

1.2.5 Temperature influence on gene expression regulation.

Changes in temperature can trigger responses in the gene expression of many microorganisms. *B. thailandensis*, for example, has flagellum production and cell motility inhibited when grown at 37 °C. Although an environmental isolate of *B. thailandensis* is considered avirulent, it has also been considered an occasional agent of pneumonia and melioidosis in susceptible humans. Comparisons of *B. thailandensis* and *B. pseudomallei* genomes, the causative agent of highly virulent melioidosis, revealed many differences between these species, such as the presence of a *Yersinia* fimbriae cluster and a specific capsular polysaccharide present only in *B. Pseudomallei* (PEANO et al., 2014). However, *B. thailandensis* shares a number of virulence factors with *B. pseudomallei*, and although it is avirulent in a model of infection with mice (SIM et al., 2010), is highly virulent in models of infection with nematodes and insects (WAND et al., 2011). In *B. thailandensis*, genes affected by temperature changes, whose significant differential expression was lower at 37 °C than at 28 °C, belong to the cell motility, secretion, and signal transduction (chemotaxis) classes. Genes encoding ATP synthases were also repressed at 37 ºC, in addition to genes involved in nitrogen metabolism and stress proteins. Among the few induced genes, secretory proteins involved in the flagellar system and chaperones have been observed, which is consistent with their role as heat protection proteins. Modulation of the flagella production at 37 °C may be a strategy of reducing the antigenic presentation to the host, without the complete loss of cell motility, which is required for infection. Reduced expression of energy generating genes, such as ATP

synthases, reflect cell economy, since at 28 °C, the cell needs more ATP due to the high flagellar activity (PEANO et al., 2014). In *Yersinia enterocolitica*, flagellar motility was expressed at 25 °C, but was repressed at 37 °C, whereas virulence factors were regulated in the opposite way (ROHDE; FOX; MINNICH, 1994).

B. pseudomallei strain 1026b had biofilm production increased two-fold at 37 °C when compared to 30 °C. Already at 40 ºC and 42 ºC, there was a decrease of about six times in the formation of biofilm, compared to 37 ºC, demonstrating the important role of temperature in the biofilm regulation. Diguanylates cyclases (DGCs) act by increasing the concentrations of cyclic di-GMP, thereby decreasing motility. The molecular mechanism involved in the thermoregulation of biofilm formation is not yet fully understood, but the genes encoding proteins of cyclic di-GMP metabolism are involved in the process, since the enzymatic competition between DGCs and phosphodiesterases (PDEs) alters the intracellular levels of cyclic di-GMP (PLUMLEY et al., 2016). On the other hand, Ramli et al. (2012) tested the influence of temperature on the biofilm formation of 24 wild lines of *B. pseudomallei* obtained from different sites of infection, such as respiratory tract, biopsy of the spleen, wounds, urine, pus and blood. The biofilm production at 30 ºC was higher than at 37 ºC for all tested strains. Likewise, *B. pseudomallei* 08 and *B. pseudomallei* K96243 showed increased biofilm production when incubated at 27 °C with respect to 37 °C. In addition, temperature was a key regulatory signal for the formation of microcolonies and adhesion to eukaryotic cells for *B. pseudomallei* 08 (BODDEY et al., 2006).

Temperature also influences the formation of *Campylobacter jejuni* biofilm in a lineage-dependent process, suggesting that different strains of *C. jejuni* have different optimal growth temperatures, and different temperatures will induce or repress the expression of certain genes that may be involved in the biofilm formation (TEH; LEE; DYKES, 2016).

Many genes encoding important functions for plant-bacterial interaction are also thermoregulated. In *Pseudomonas syringae*, for example, the analysis of RNA-seq revealed 1440 genes whose expression was temperature-dependent. In general, genes regulating and synthesizing polysaccharides, IS and phage elements, type IV secretion systems, chemotaxis, flagellar synthesis, phytotoxin synthesis, and transport were repressed at 30 °C compared to 20 °C, whereas transcriptional regulatory genes, metabolism and transport of quaternary ammonium compounds, chaperones and hypothetical proteins were induced with increasing temperature. During *in plant* assays, cells that became mobile at lower temperatures accessed preferred colonization sites, such as glandular trichomes and intercellular epidermal cell grooves, to protect themselves from dissection and other stresses. In addition, increased motility

and EPS expression at lower temperatures appeared to contribute to the initial adhesion of cells to the surface, and hence stimulate biofilm formation in *P. syringae* (HOCKETT; BURCH; LINDOW, 2013). *P. syringae* pv. *glycinea* PG4148 produces a toxin called coronatine only when grown at lower temperatures (BUDDE; ULLRICH, 2000). This toxin increases the occurrence and severity of bacterial disease in plants, making the species more pathogenic at 18 ºC than at 28 ºC. On the other hand, incubation temperature significantly affected the activity of rhizobacteria important for the control of *F. oxysporum*, which causes fusarium wilt in chickpeas. *F. oxysporum* was inhibited by rhizobacteria belonging to the genera *Pseudomonas* and *Bacillus* at 20 ºC and 30 ºC, but not at 25 ºC, at which temperature the disease develops with greater severity. At 30 ºC, the highest production rate of metabolites with inhibitory activity by rhizobacteria was observed (LANDA; NAVAS-CORTÉS; JIMÉNEZ-DÍAZ, 2004).

The gene expression of the insect-active toxin complex in *Y. entomophaga* (Yen-TC) was great at 25 °C, but the toxin was not detected when the bacterium was incubated at 37 °C. However, sonicated *Y. entomophaga* culture filtrates, grown at 25 °C or 37 °C, were still active against *Galleria mellonella*, suggesting that Yen-TC release by the cell occurs in a temperature-dependent manner. The Yen-TC toxin mediates the entrance of the bacterium into the hemocellic cavity of the insect, through the dissolution of the midgut of the larvae. Once inside the hemocellic cavity, the bacterium multiplies rapidly and suppresses the immune system of the host, in addition to possibly limiting the growth of the endemic microbial community (HURST et al., 2015). Thus, it is possible to note the importance of temperature in the regulation of the gene expression, controlling biological activities, virulence and strategies of survival in microorganisms.

6 CONCLUDING REMARKS

Temperature and *wcbE* glycosyltransferase gene play a very important role on *B. seminalis* TC3.4.2R3 environmental interactions. Production of biofilm, EPS and antifungal compounds are greater at 28 ºC. On the other hand, motility related to flagella and virulence in the infection model of *Galleria mellonella* were higher at 37 ºC.

Transcriptomic analyses revealed only few genes downregulated at 37 ºC, while 87% of all differentially expressed genes were induced. There was an acceleration of metabolism and growth phase, setting starvation responses faster than 28 ºC. We found mainly upregulated genes involved in primary metabolism, such as energy metabolism, transcription and translation regulation, transport, proteins modification and cellular processes. Aromatic compound degradation, terpenes, toxins biosynthesis and detoxification genes were also found, beyond

hypothetical proteins. Motility present a complex regulation with genes being up and downregulated. Cell directed its energy to get substrate to synthetize proteins needed to survival.

The glycosyltransferase gene (*wcbE*), presented in the capsular cluster *wcb*, is linked not only with capsule production in *B. seminalis* TC3.4.2R3, but also other important mechanisms in the interaction in the environment. Motility, EPS production, hydrogen peroxide resistance, seed germination and plant-growth were not affected by the glycosyltransferase inactivation. However, biofilm production and virulence were strongly affected by *wcbE* gene. The Δ*wcbE* produced less biofilm than WT and was attenuated in *G. mellonella* at 37 ºC, highlighting the importance of glycosyltransferase in a temperature-dependent manner. When *wcbE* gene was inactivated, genes involved in capsular polysaccharide biosynthesis and export and secondary metabolites production (*wcbC* and *wcbR*) were repressed, proving that *wcbE* is responsible to synthetize capsule and, suggesting the capsule role on biofilm formation and hence, virulence.

Mutants deficient in the glycosyltransferase gene lost the ability to inhibit phytopathogenic fungi, highlighting the role of *wcbE* gene in the antimicrobial synthesis. The genome mining revealed a PKS domain in *wcb* cluster. The chromatograms of WT and M3 were compared and two peaks were detected lacking in the mutant. One of these peaks corresponded to a 10-deoxymethymycin, a polyketide macrolide, which presents glycosyltransferases in its biosynthesis. However, methymycin biosynthetic cluster is large different from *wcb* cluster. The other peak had no matches in the databases used, which could represent a new product related to *wcb* cluster to be studied structurally.

B. seminalis TC3.4.2R3 was efficient in control plant and clinical pathogens, and could represent an alternative for biocontrol in crops in the future. Nevertheless, it is a Bcc member with a high ability of adaptation and the possibility of a transition state between environmental bacteria and human pathogen should be studied very carefully.

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