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Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do título de Doutora em Microbiologia.

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DECLARAÇÃO

Em adendo ao Certificado de Isenção **CEP-ICB nº 523/2012**, de 13/06/2012, e por solicitação do Prof. Dr. *Welington Luiz de Araújo*, do departamento de Microbiologia, informo que o título do projeto de doutorado da aluna *Priscila Jane Romano de Oliveira Gonçalves* foi alterado para ***"Caracterização e avaliação do papel do gene wcbE de Burkholderia seminalis linhagem TC3.4.2R3 na interação microbiana*** " visto que o projeto inicial teve algumas modificações, sendo que somente o estudo do gene da glicosiltransferase (*wcbE*) foi selecionado para estudo da tese.

São Paulo, 31 de março de 2017.

Profa. Dra. **Camila Squarzoni Dale**
Coordenadora 3 CEPSH-ICB/USP

Profa. Dra. **Luciane Valéria Sita**
Vice-coordenadora CEUA-ICB/USP

Ao meu bem mais precioso sobre a Terra,
minha família,

Dedico.

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“Suba o primeiro degrau com fé.
Não é necessário que você veja toda a escada,
apenas dê o primeiro passo. ”

Martin Luther King

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.

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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 *wcbE* 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).

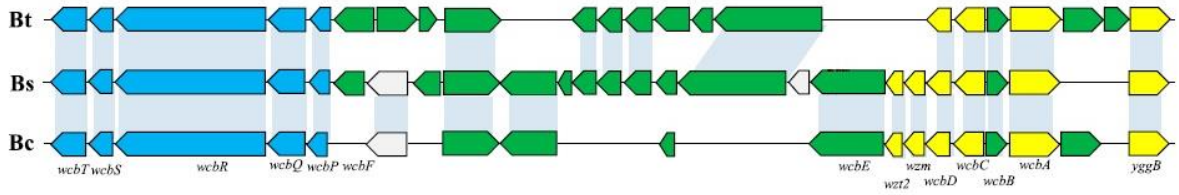
Araujo et al. (2016) selected 12 mutants of *B. seminalis* TC3.4.2R3 that lost biocontrol capacity, allowing the mapping of 8 genes essential for the biocontrol, all located on chromosome 1 of the bacterial genome. One of these genes was a glycosyltransferase (*wcbE* – *Bsem*_02955) present in the *wcb*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

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2 CHAPTER 2 - Microbial interactions and biocontrol potential under different temperatures in *Burkholderia seminalis* TC3.4.2R3.

2.1 ABSTRACT

Burkholderia sp. are considered very effective for biological control of phytopathogens, since they are able to produce many antimicrobial compounds. *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. Temperature may influence pathogenicity of many microorganisms; thus the aim of this work was to investigate microbial interactions and biocontrol potential of *B. seminalis* TC3.4.2R3 under different temperatures. We found that the behavior and pathogenicity of this strain are strongly influenced by temperature suggesting that this bacterium present different mechanisms to compete and survival in hosts. At environmental temperature (28 ^{o}C), this bacterium produced greater amounts of biofilm, EPS and higher inhibitory activity against phytopathogenic fungi. In contrast, at clinical temperature (37 ^{o}C), this TC3.4.2R3 strain present more motility and virulence against *Galleria mellonella* (Insecta) larvae. Seed germination was not influenced by *B. seminalis* regardless temperature. It is necessary to test *B. seminalis* in mammal infection model to confirm the strong environmental profile of this strain and encourage its use for biotechnological applications.

2.2 INTRODUCTION

The *Burkholderia* was first isolated by W. Burkholder, who reported that the causal agent of sour skin in onion was *Pseudomonas cepacia* (BURKHOLDER, 1950). Later, seven species of *Pseudomonas* were reclassified in *Burkholderia* genus based on phylogenetic analysis (YABUUCHI et al., 1992). Since then, many species have been added to this genus, with 113 currently accepted species, which are validated according to the Approved List of Bacterial Names (DSMZ, 2017). *Burkholderia* can inhabit various ecological niches and for this reason, these bacteria may be found in natural (YOO et al., 2007) or contaminated soil (VANLAERE et al., 2008b), water (LIM; BAEK; LEE, 2008), into all parts of plants (AIZAWA et al., 2011), in several animal species (ELSCHNER et al., 2014), in humans and in hospital settings (KHOSRAVI et al., 2014). Among *Burkholderia* species, *Burkholderia seminalis* was recently described by Vanlaere et al. (2008b) and was included in the Bcc group. This species is Gram-negative, aerobic, non-sporulating rods, yellow-pigmented colonies with mucoid aspect. *Burkholderia seminalis* has been found associated to rice as a nonpathogenic

microorganism and plant-growth promoter (PANHWAR et al., 2014), as root-nodulating bioremediator bacterium which produce great EPS amounts (HUANG et al., 2012), as a pathogenic agent of bacterial fruit rot in apricot (LI et al., 2010; LOU et al., 2011), in water (FANG et al., 2011), in sputum of cystic fibrosis patients and nosocomial infections (VANLAERE et al., 2008b). *B. seminalis* TC3.4.2R3 was isolated endophytically from sugarcane roots and was effective in biocontrol of orchid necrosis, caused by *B. gladioli* (ARAÚJO et al., 2016). Furthermore, produced antimicrobial compounds against *Fusarium oxysporum, F. verticillioides, C. paradoxa,* and *Phytophthora parasitic* (NEVES, 2011). In another study, it was observed that *B. seminalis* produced piochelin and a ramnolipid n the presence of against *F. oxysporum* (ARAÚJO; ARAÚJO; EBERLIN, 2017), suggesting that these compounds could be related to the interaction of this strain with fungi.

Indeed, the *Burkholderia* sp. are considered very effective for biological control of pathogens, since they exhibit the ability to produce antimicrobial agents against both fungi and bacteria (PARKE; GURIAN-SHERMAN, 2001). Several metabolites, such as cepacins, pyrrolnitrins, cepaciamides, cepacidines, quinolones, phenazines, siderophores, lipopeptides, among others (HILL et al., 1994; HUANG; WONG, 1998; MAO et al., 2006; PARKER et al., 1984) with antimicrobial activity have been reported for *Burkholderia*, and most of them present antifungal activity. However, few studies have evaluated the role of temperature in the synthesis of this compounds and in the interaction with different hosts.

Temperature is a very important parameter for microorganisms. Virulence factors, such as toxins production, transport and regulation are affected by temperature in *Pseudomonas syringae* (HOCKETT; BURCH; LINDOW, 2013) and *Yersinia entomophaga* (HURST et al., 2015). Motility, flagellar expression and biofilm production were found as thermo-regulated as well in *Pseudomonas syringae* (HOCKETT; BURCH; LINDOW, 2013) and *Campylobacter jejuni* (TEH; LEE; DYKES, 2016). Furthermore, surfactant and EPS production seems occur in a temperature-dependent manner in *Pseudomonas putida* (DUBERN; BLOEMBERG, 2006) and *Erwinia amylovora* (WEI; SNEATH; BEER, 1992), respectively. In the present work, we studied the *Burkholderia seminalis* strain TC3.4.2R3 and investigated the effects of different growth temperatures (28 ºC and 37 ºC) on microbial interactions with the host and other microorganisms.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strain and growth conditions

B. seminalis strain TC3.4.2R3 studied in this work was isolated from inner sugarcane roots tissues (LUVIZOTTO et al., 2010) as endophytic bacteria. Whole genome of the strain was sequenced and described by Araujo et al. (2016). *B. seminalis* was maintained at -80 ºC in Luria-Bertani (LB) broth (Difco Laboratories, Sparks, USA) and 20% glycerol, and was recovered on Luria-Bertani agar (Difco Laboratories, Sparks, USA) or broth with incubation for 24 h at 28 ºC or 37 ºC.

2.3.2 Swimming motility assay

Overnight bacterial culture was harvested and resuspended in PBS to an OD_{595} of 1.0. Bacterial suspensions were inoculated on LB with 0.3% w/v agar with a central tip and incubated at 28 ºC and 37 ºC. Motility was evaluated by appearance of growth rings around the inoculum area and the halo diameter (mm) was measured after 24, 48 and 120 h (DENMAN et al., 2014; FLANNAGAN; LINN; VALVANO, 2008). Experiment was delineated in triplicate.

2.3.3 Biofilm assays

Biofilm formation was assessed using the 96-well plate and accompanying peg-lid of the MBEC (Minimum Biofilm Eradication Concentration) Assay device (Innovatech Inc.). Wells received 150 µL bacterial suspension of *B. seminalis* standardized to 10^7 c.f.u. mL^{-1} in Tryptone Soya Broth (TSB) (Difco Laboratories, Sparks, USA). Eight wells were inoculated in at least three independent experiments. The peg-lid was placed on the plate and incubated at 28 ºC or 37 ºC for 24 h. The peg-lid was transferred to a fresh 96-well plate containing pre-warmed TSB and incubated for more 24 h. Then the peg-lid was submitted to rinses with Phosphate Buffered Saline (PBS) (200 µL per well) at room temperature for 2 min. Peg-lid was baked at 60 ºC for 20 min and stained with crystal violet 0.1% (w/v) (200 µL per well) and incubated for 30 min at room temperature. Three wash plates containing PBS (200 µL per well) rinsed the pegs following staining, subsequently the crystal violet was solubilized with 95% ethanol prior to measuring in OD_{595} (DENMAN; BROWN, 2013).

2.3.4 Scanning electron microscopy of biofilm

Scanning electron microscopy (SEM) of biofilms was conducted at Instituto Butantan, São Paulo, Brazil. *B. seminalis* cells were grown overnight at 28 ºC or 37 ºC and then absorbance was adjusted to OD_{595} 1.0. Cells were grown on membrane discs (13 mm) by seeding 2 mL of bacterial suspension in 12-well culture plates and incubating them for 24 h at 28 ºC or 37 ºC at 150 rpm. After, the medium was removed and samples were fixed following

Karnovsky (1965) protocol with modifications. Cells were fixed in Karnovsy fixative solution (paraformaldehyde 4%; glutaraldehyde 2.5%; 0.1 M cacodylate buffer pH 7.2) during 4 h. The membrane discs were washed 3 times for 15 min with cacodylate buffer. Cells were then treated with a 1% osmium tetroxide solution diluted in 0.2 M cacodylate buffer for 30 min. After fixation, the samples were washed 3 times for 15 min with 0.1 M cacodylate buffer again to remove traces of fixatives. For dehydration, membranes were placed in increasing ethanol solutions starting with 30, 50, 70, 90 and 100% for 15 min twice per step. Then the samples were prepared by critical point drying in Leica CPD 030. Sample sputtering was performed with gold–palladium in Leica SCD 050. The images were taken using a FEI QUANTA 250 LEO scanning electron microscopy (HEILMANN et al., 2005).

2.3.5 Measurement of exopolysaccharide weight

Exopolysaccharides were purified from cell culture media according to Kim et al. (2014) with some modifications. Briefly, *B. seminalis* was grown for 5 days at 28 ºC or 37 ºC in 10 ml of mannitol medium (0.2% yeast extract and 2% mannitol). The bacterial cultures were vortexed and centrifuged at 2,300 X *g* for 10 min. Supernatant was transferred to new tubes and phenol was added at a 10% final concentration and then incubated at 4°C for 5 h and further centrifuged at 9,100 X *g* for 15 min. The water phase was collected, added 4 volumes of isopropanol and incubated at –20 ºC for 18 h. The precipitated exopolysaccharide was centrifuged at 9,100 X *g* for 15 min. and suspended in 150 µL of distilled water. The extracted was lyophilizated and the dry-weight was measured.

2.3.6. Galleria mellonella infection model

Larvae were obtained from LiveFoods UK and stored in woodchips at 10 ºC prior to use. *B. seminalis* TC3.4.2R3 was inoculated in the hindmost larvae proleg using a 25 µL 22s gauge gas-tight Hamilton syringe with 10 µL of 10^8 c.f.u. mL^{-1} and incubated at 28 ºC or 37 ºC. PBS was used as a control. For each strain and treatment, ten larvae were inoculated per experiment in two independent experiments. The larvae survival was monitored up to 96 hours (SEED; DENNIS, 2008).

2.3.7 Effects of Burkholderia on seed germination

B. seminalis was grown in LB broth at 28 ºC or 37 ºC and 180 rpm for 48 h. Bacteria were harvest and absorbance adjusted to OD_{595} 1.0 with sterile distilled water. Seeds of maize (*Zea mays*) and cotton (*Gossypium hirsutum*) were surface sterilized in 70% alcohol for 1 min,

3% sodium hypochlorite for 1 min and rinsed twice in sterile water. Subsequently, they were immersed in the *B. seminalis* suspension. Control seeds were treated with sterile water (untreated control). A replicate consisted of 50 seeds were placed on moist filter paper and incubated in a germination chamber at 25 °C or 37 ºC and 12 h photoperiod for 5 days. During the experiment, filter paper was maintained humid adding a suitable volume of sterile water daily and wrapping containers in transparent plastic bags. Experiment was carried out in triplicate. The mean germination percentage was calculated at the end of the experiment (CASSÁN et al., 2009; KUNOVA et al., 2016).

2.3.8 Antagonism against plant pathogenic fungi

Overnight *B. seminalis* culture was adjusted to 10^8 c.f.u. mL^{-1} and 10 µL were cultivated for 48 h at 28 ºC or 37 ºC on Potato Dextrose Agar (Difco Laboratories, Sparks, USA). After bacterial growth, phytopathogenic fungi (6 mm in diameter disks) were transferred to the center of PDA medium and incubated at 28 ºC up to 10 days. After fungi growth, the inhibition zone was measured and compared in relation to the control, which consisted in the phytopathogenic fungi plates without *B. seminalis* TC3.4.2R3. The antagonism was evaluated against *Ceratocystis fimbriata, Ceratocystis paradoxa, Colletotrichum falcatum* and *Fusarium oxysporum*. The experiment was performed in triplicate (FÁVARO; SEBASTIANES; ARAÚJO, 2012).

2.3.9 Statistical analysis

All *in vitro* assays were performed at least in triplicate, with subsequent statistical analysis by one-way ANOVA with Tukey test (GraphPad Prism 6) and multiple comparisons. $P < 0.05$ was deemed statistically significant. A Kaplan-Meier survival plot with log-rank (Mantel-Cox) test and Bonferroni correction (GraphPad Prism 6) was used to compare larval survival in *Galleria mellonella* infection model.

2.4 RESULTS AND DISCUSSION

2.4.1 Swimming motility is stimulated at elevated incubation temperature in B. seminalis

Motility provides beneficial effects for bacteria, which include increased efficiency in nutrient acquisition, ability to avoid toxic substances, ability to translocate to specific hosts and access to optimal colonization sites and to disperse in the environment (RASHID; KORNBERG, 2000). Bacteria motility using different modes of translocation is classified in at

least three distinct forms as swimming, swarming and twitching. Swimming-type motility is characterized by mobile cells having a single polar (monotrichous) flagellum. These bacteria revert the flagellar rotation to swim in a new direction on semi-solid agar (0.3%) (HOCKETT; BURCH; LINDOW, 2013; RASHID; KORNBERG, 2000).

Swimming motility of *B. seminalis* on the surface of soft agar plates was compared under different temperatures. The rate of motility was significantly ($p < 0.0001$) higher at 37 ºC than 28 ºC (Fig. 2.1), suggesting that flagellum synthesis or function was stimulated at the warmer temperature (Fig. 2.2). It can be a strategy to achieve specific hosts. 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).

Figure 2.1 - Swimming motility in *B. seminalis* TC3.4.2R3 under different temperatures

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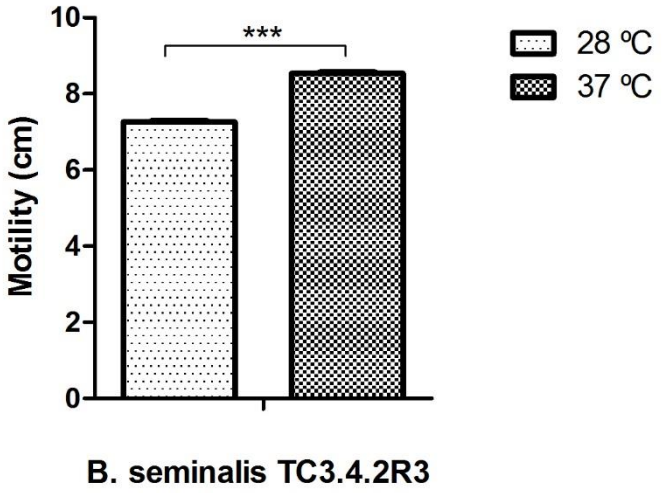
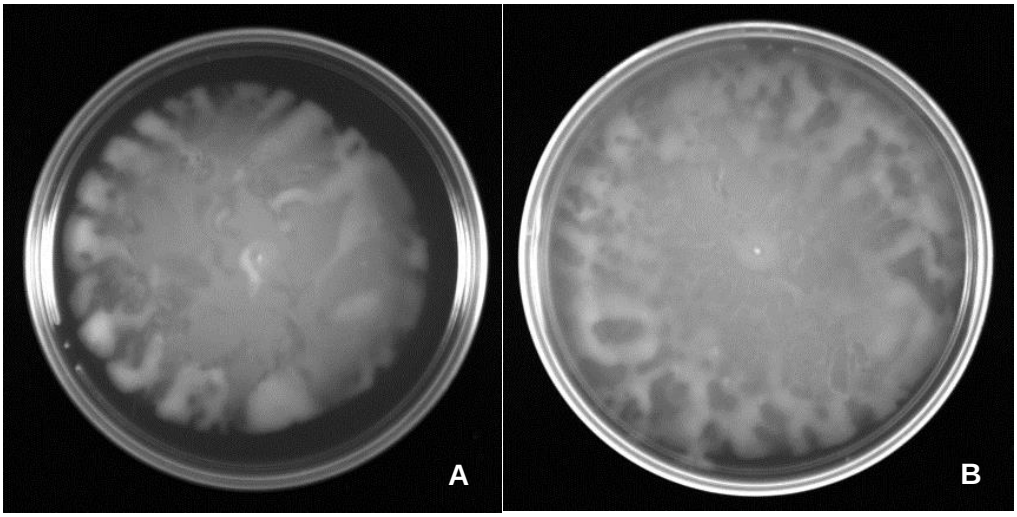


The error bars represent standard deviation; ***$p < 0.0001$

Figure 2.2 – Motility plates on soft agar (0.3%). A - *B. seminalis* at 28 ºC. B - *B. seminalis* at 37 ºC.



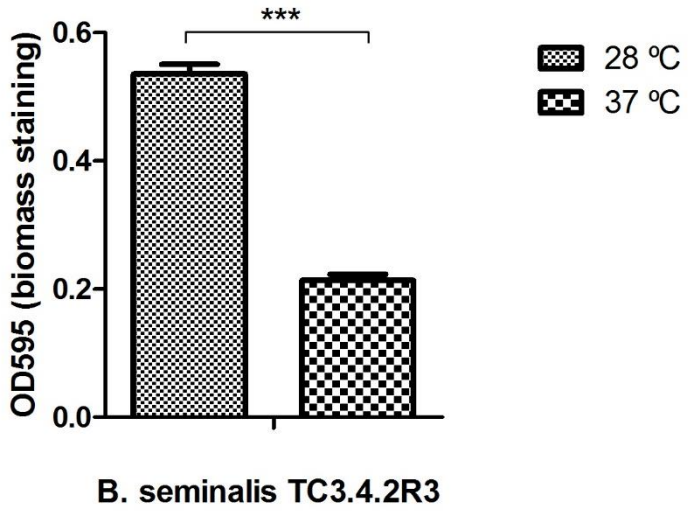
2.4.2 Biofilm formation under different conditions

Biofilms are complex, multicellular bacterial communities that can protect bacteria from antibiotics and the host immune system (LOUTET; VALVANO, 2010). We accessed the ability of *B. seminalis* TC3.4.2R3 to form biofilm using the MBEC biofilm device (Innovatech). Biofilm formation at 28 ºC was 2.5-fold higher than 37 ºC (Fig. 2.3). Lower temperature facilitated better biofilm production for *B. seminalis*. These results agree with motility results, since TC3.4.2R3 presented higher motility at 37 ºC. The growth rate was evaluated and the highest growth occurred at 37 ºC (Fig. 2.4) at $p < 0.0001$, even though less biofilm was produced at this temperature. These data indicate that altered biofilm phenotype cannot be attributed to differences in growth rate and biofilm production is not directly related to bacterial growth. Quantitative data of biofilm were confirmed by scanning electron microscopy (SEM), which showed higher cell aggregates at 28 °C than 37 °C (Fig. 2.5).

Comparisons of biofilm formation at 37 ºC among members of the Bcc group showed great variation between clinical and water isolates. The clinical isolates produced a much higher amount of biofilm than the environmental ones (IBRAHIM et al., 2012). *B. seminalis* TC3.4.2R3 is an environmental isolate and, therefore, produces higher amounts of biofilm at 28 ºC. However, biofilm formation seems to vary among *Burkholderia* spp. For instance, an increase of temperature for *B. pseudomallei* resulted in a significant decrease in biofilm formation. Biofilm production was higher at 30 ºC than 37 ºC (RAMLI et al., 2012). Boddey et al. (2006) found the similar result with *B. pseudomallei* 08 e *B. pseudomallei* K96243. On the other hand, *B. pseudomallei* strain 1026b showed biofilm increased two-fold at 37 ºC when compared to 30 ºC. Nevertheless, at 40 ºC biofilm amounts decreased six-fold (PLUMLEY et al., 2016). Similarly, *B. thailandensis* formed more biofilm and presented less flagelar

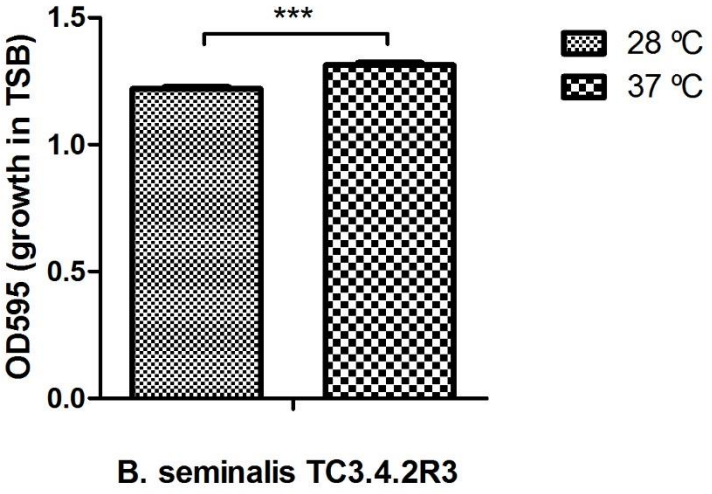
production and motility at 37 ºC. Although *B. thailandensis* is considered avirulent and an environmental isolate, this bacterium can be an occasional agent of pneumonia and melioidosis in susceptible humans (PEANO et al., 2014).

Figure 2.3 – Biofilm production of *B. seminalis* TC3.4.2R3 at different temperatures.



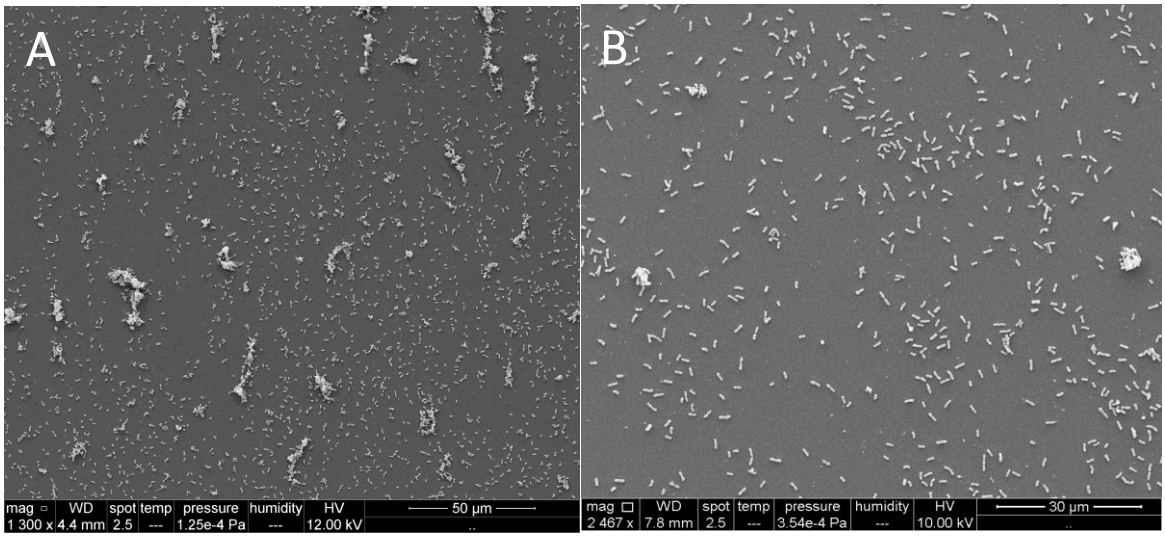
The error bars represent standard deviation of three technical replicates and eight biological replicates each; ****p* < 0.0005.

Figure 2.4 – Growth of *B. seminalis* TC3.4.2R3 in TSB at different temperatures.



The error bars represent standard deviation of three technical replicates and eight biological replicates each; ****p* < 0.0005.

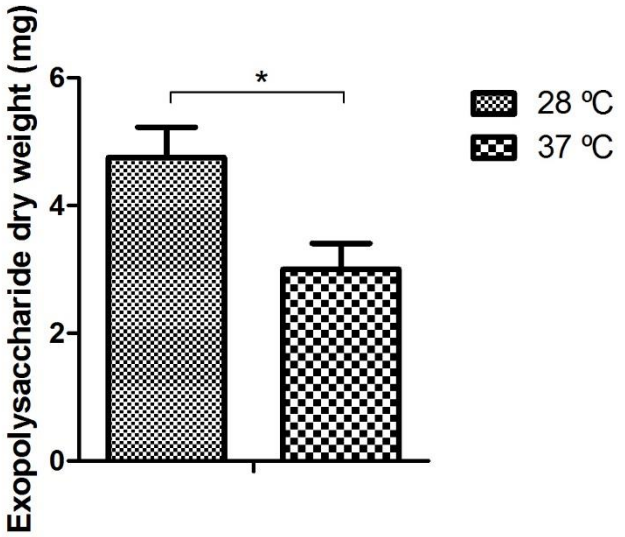
Figure 2.5 – Biofilm SEM images from *B. seminalis* TC3.4.2R3 at 28 ºC (A) and 37 ºC (B).



2.4.3 Exopolysaccharide production is influenced by temperature

Exopolysaccharide (EPS) production by *B. seminalis* TC3.4.2R3 occurred in a temperature-dependent manner (Fig. 2.6). Not only flagella, but also EPS are related to biofilm production, since is important in formation of the biofilm matrix (WANG et al., 2014). In *P. aeruginosa*, the EPS was the most important biofilm matrix components (FAZLI et al., 2014). In the present study, the production of EPS by strain TC3.4.2R3 was greater at 28 ºC than 37 ºC, similarly to biofilm. EPS production by *B. kururiensis* is regulated in response to growth and external conditions (HALLACK et al., 2010), as also observed for *B. tropica*, which biofilm formation is affected in a temperature dependent-way up to 24 h bacterial growth (SERRATO et al., 2006). *Pseudoalteromonas*, a marine bacterium from Antarctic Sea, also produces EPS in a temperature-dependent manner, since at -2 °C and 10 °C, the yield of EPS was 30 times higher than at 20 °C (NICHOLS; BOWMAN; GUEZENNEC, 2005). Members of the Bcc group have a high variability in the EPS production, which is strongly influenced by the bacterial environmental (CUZZI et al., 2014).

Figure 2.6 – EPS production by *B. seminalis* TC3.4.2R3 in dry weight at different temperatures.



The error bars represent standard deviation; *$p < 0.05$

2.4.4 Pathogenicity of B. seminalis TC3.4.2R3 infection is thermo-regulated and host-dependent

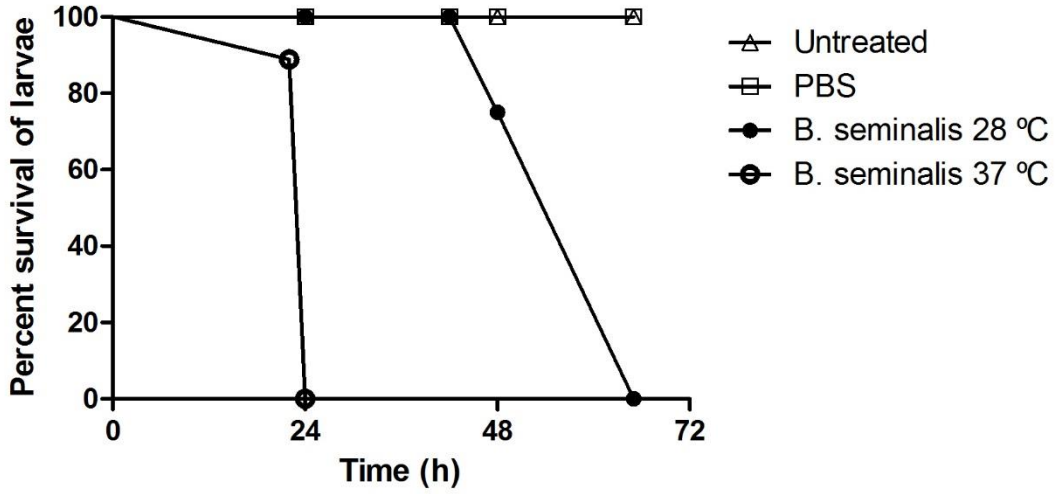
Non-mammalian infection models have become particularly attractive because they are fast, cheap, can survive at 37 ºC, allow high throughput screenings and are not limited by ethical concerns. In this context, *Galleria mellonella* larvae have been used as a model organism to study many bacterial pathogenicity because of the similarities of the innate immune systems of insects and mammals (PEREIRA et al., 2015). The wax moth larvae are a pest organism in beehives once they feed on honeycombs, pollen and bee eggs and larvae. The larvae grow up to three centimeters in length and achieve 300 to 500 mg (KAVANAGH; REEVES, 2004).

G. mellonella has been used recently as a model organism for investigating the pathogenicity of members of the Bcc (DENMAN et al., 2014; DENMAN; BROWN, 2013; IBRAHIM et al., 2012; SEED; DENNIS, 2008; UEHLINGER et al., 2009; WAND et al., 2011). The overall pathogenicity of *B. seminalis* isolates towards *G. mellonella* is shown in Fig. 2.7. The larvae killing occurred in a significantly ($p < 0.0001$) temperature-dependent manner. 100% of the larvae were killed following inoculation with *B. seminalis* after 24 h at 37 ºC. In contrast, at 28 ºC was observed less virulence in *B. seminalis* with extended time to larval killing. 100% mortality was observed after 65 h. No larval death was observed for untreated or larvae inoculated with PBS. At the final of experiment, all the larvae were sacrificed at -80 ºC.

IBRAHIM et al. (2012) evaluated the pathogenicity of three *B. seminalis* strains isolated from water (strain S9), lesion of apricot fruit (O901) and from rice rhizosphere (R456), to *G. mellonella*. The authors observed that the strains S9 and O901were less virulent than the strain R456, but in comparison with other clinical and water isolates, the evaluated strains may be

considered weak pathogen or none virulent. However, even though *B. seminalis* TC3.4.2R3 is a sugarcane rhizosphere isolate, it showed high virulence in wax moth larvae infection, mainly at 37 ºC showing that evaluation should be also conducted at a mammal model.

Figure 2.7 – *Galleria mellonella* pathogenicity assay.



Untreated: the larvae were not inoculated; PBS: control of larvae inoculated with PBS; *B. seminalis* 28 ºC: larvae inoculated with *B. seminalis* and incubated at 28 ºC; *B. seminalis* 37 ºC: larvae inoculated with *B. seminalis* and incubated at 37 ºC. Significance was determined using the log-rank (Mantel-Cox) test and Bonferroni correction; $p < 0.0001$.

Thus, we found that *B. seminalis* TC3.4.2R3 is virulent in a temperature-dependent way for the larvae. Many factors may be implicated in virulence, depending on the infection model. For Bcc strains, AHL-mediated quorum sensing is one of the most important factors in control of universal pathogenesis, and in turn this system may be regulate by host sign. Siderophores and lipopolysaccharide (LPS) were found as important virulence factors for *B. cenocepacia* in mammals and *G. mellonella*, but several virulence factors were host specific for these bacteria (SCHWAGER et al., 2013; UEHLINGER et al., 2009). It is likely that in *Galleria* flagella are involved in disease and is associated to host-pathogen interaction. It is necessary to investigated if *B. seminalis* causes disease in murine model, since it is within Bcc group (ARAUJO et al., 2016) and was indicated for biocontrol purposes against pathogens to the crops (ARAÚJO; ARAÚJO; EBERLIN, 2017).

2.4.5 Effects of Burkholderia on seed germination

*Burkholderi*a have features that confer ability for biological control and natural resources for crop protection (PARKE; GURIAN-SHERMAN, 2001). Considering the changes

in *B. seminalis* behavior under different temperatures described previously in this work, we investigate the effects of *B. seminalis* inoculation on seed germination at 25 ºC and 37 ºC (Table 2.1). Seed germination showed no significant differences among treatments. Maize inoculated with *B. seminalis* TC3.4.2R3 promoted an increase on seed germination percentage than control at 25 ºC, whereas at 37 ºC was observed the opposite. For cotton, *B. seminalis* caused slight decrease on seed germination at 25 ºC when compared to the control, nevertheless it was observed the opposite at 37 ºC.

Kunova et al. (2016) evaluated 3 species of *Streptomyces* for seed germination ability. They showed inhibition of lamb lettuce and tomato germination *in vivo* after the treatment with *S. albidoflavus* VT111I and *S. cyaneus* ZEA17I and species-specific effects. The authors suggested the production of some secondary metabolites or complex interactions among the *Streptomyces*, the natural microbiome and the host plant as possible causes of the negative effect observed. However, those negative effects became less severe during the growth of plant once they did not find reduction in the biomass dry weight after inoculation. A gibberellin-producing *Burkholderia* sp. strain KCTC 11096, which is a plant growth promoting rhizobacteria (PGPR), significantly increased the seed germination and growth of lettuce and Chinese cabbage. They suggested the use of *Burkholderia* sp. KCTC 11096 for early seed germination and plant growth could increase crop productivity (KANG et al., 2012). Even though other *Burkholderia* present the ability to promote seed germination, *B. seminalis* does not seem to influence on germination in both mono and dicotyledonous regardless of temperature, demonstrating that the interaction between *Burkholderia* and the host plant may be a complex interaction regulated by plant genotype and environmental conditions as observed for other endophytic species.

Table 2.1 – Percentages of seed germination at different temperatures.

	25 ºC		37 ºC	
	TC3.4.2.R3	Control	TC3.4.2.R3	Control
Maize	90.7 ± 4.16	86.7 ± 3.06	79.3 ± 12.06	81.3 ± 12.06
Cotton	76.0 ± 9.17	78.0 ± 10.39	88.7 ± 7.57	86.7 ± 4.62

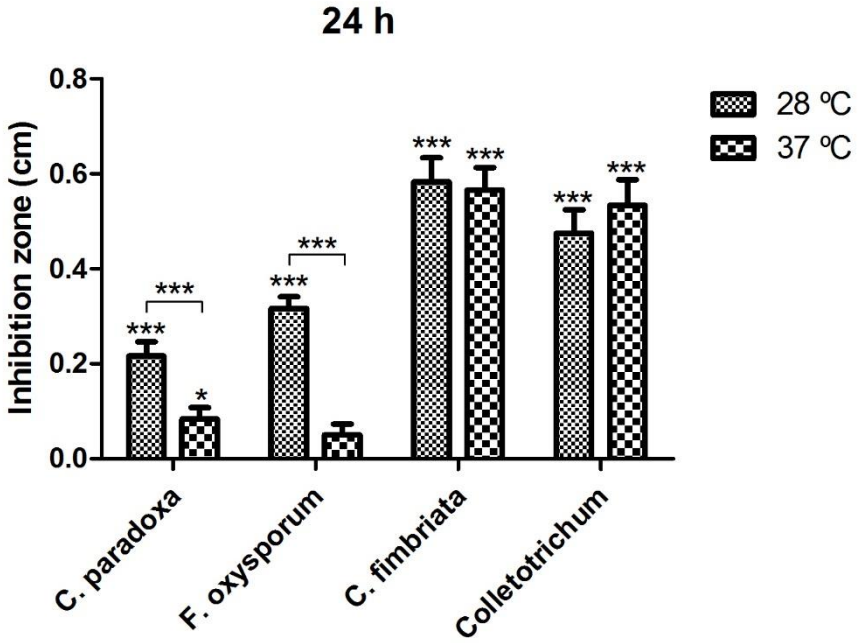
Values correspond to averages of triplicates following by standard deviation.

2.4.6 Antagonism against plant pathogenic fungi

Antagonism tests of *B. seminalis* against the fungi *Fusarium oxysporum, Ceratocystis paradoxa, Ceratocystis fimbriata* and *Colletotrichum* sp. revealed greater inhibition of fungi

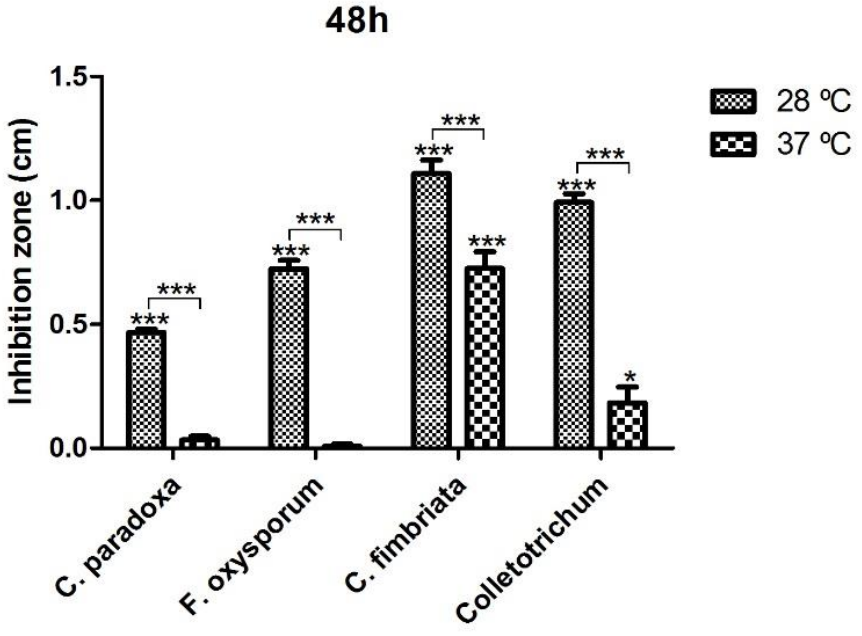
when bacteria were grown for 48 h on plate. Although there was fungal inhibition by *B. seminalis* grown for 24 h (Fig. 2.8), the best levels of biocontrol were obtained with *B. seminalis* after 48 h of growth (Fig. 2.9). In addition, it was possible to observe that at 37 ºC, *B. seminalis* produced less yellow pigment (Fig. 2.10). Biological control occurred in a temperature-dependent way as well. For all fungi tested, inhibition was significantly higher ($p < 0.0001$) when bacteria were grown at 28 ºC (Fig. 2.11 and 2.12). These results were expected once *B. seminalis* is an agricultural isolate found as endophytic in sugarcane, which tends to be more competitive in enviromental conditions and is able to control pathogenic fungi of plants efficiently (ARAÚJO; ARAÚJO; EBERLIN, 2017; ARAÚJO et al., 2016). A change in growth temperature of *B. cenocepacia* from 20 ºC to 37 ºC increased transcript levels of various chaperones and heat-shock proteins. Growth at 20 ºC induced cold-shock genes and transcription/translation regulators required at lower temperature. At 37 ºC, a largest number of virulence factor genes, such as T3SS and T6SS secretion system; extracellular protease *zmpA*; genes involved in adhesion as fimbriae, *flp*-type pili, lectin, *bapA*/*adhA*; surface polysaccharides; the quorum sensing *cepI*; a catalase involved in the reactive oxygen species detoxification were induced. The authors suggested that temperature adaptation during host infection might have one of the main roles in *B. cenocepacia* pathogenesis (SASS et al., 2013). Thus, it seems that temperature triggers expression of genes in a host-specific manner in *B. seminalis* TC3.4.2R3, not only in *G. mellonella* model, but also in the host plant and during interaction with fungi in the environment.

Figure 2.8 – Antagonism of *B. seminalis* against phytopatogenic fungi after previously growth of 24 h.



Inhibition zone was measured in centimeters (cm).The error bars represent standard deviation; *$p < 0.01$, ***$p < 0.0001$.

Figure 2.9 – Antagonism of *B. seminalis* against phytopatogenic fungi after previously growth of 48 h.



Inhibition zone was measured in centimeters (cm).The error bars represent standard deviation; *$p < 0.01$, ***$p < 0.0001$.

Figure 2.10 – Antagonism of *B. seminalis* TC3.4.2R3 previously grown for 24 h at 28 ºC (A) and 37 ºC (B) against *C. paradoxa*. Less yellow pigment is produced at elevated temperature.

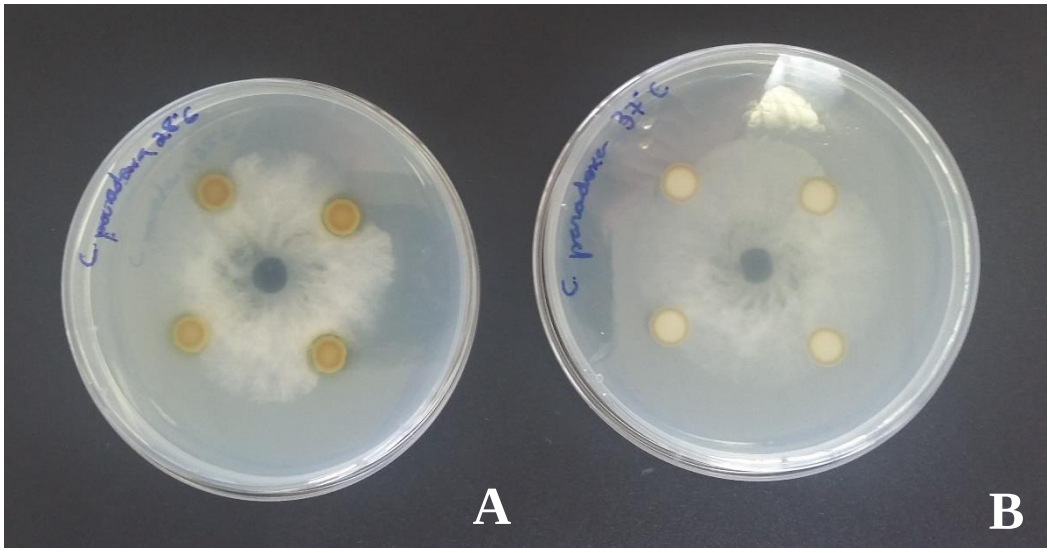


Figure 2.11 – Antagonism of *B. seminalis* previously grown for 48 h at 28 ºC (A) and 37 ºC (B) against *C. fimbriata*. (C) control.

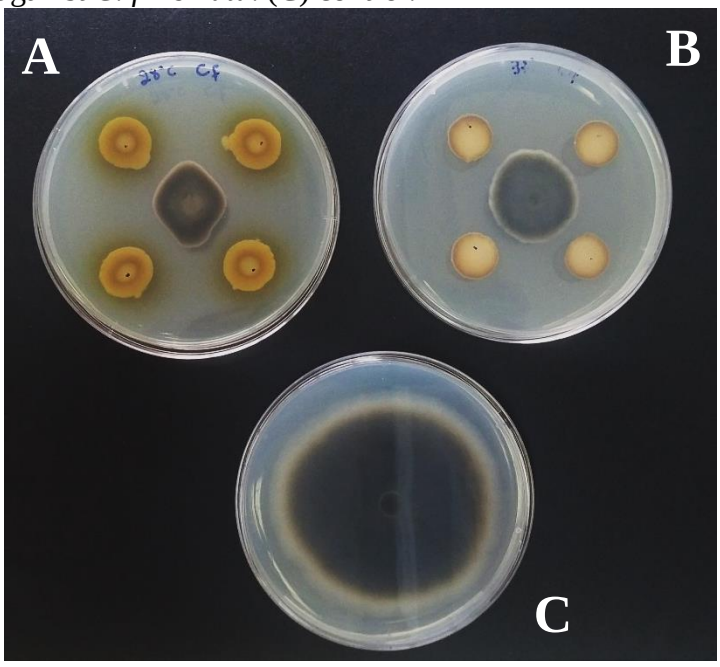
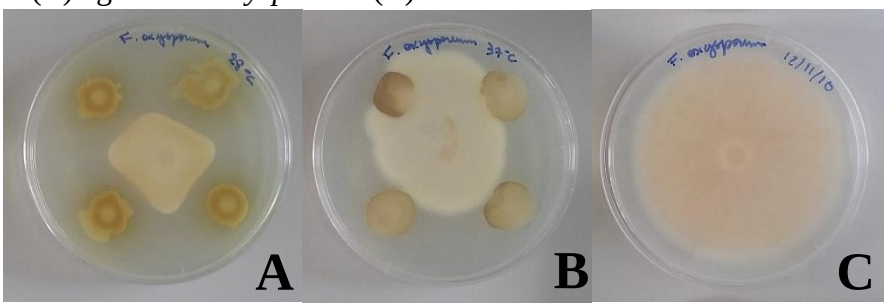


Figure 2.12 – Antagonism of *B. seminalis* previously grown for 48 h at 28 ºC (A) and 37 ºC (B) against *F. oxysporum*. (C) control.



2.5 CONCLUSION

B. seminalis TC3.4.2R3 behavior and pathogenicity are strongly influenced by temperature. At environmental temperature, these bacteria produced greater amounts of biofilm, EPS and antifungal compounds, suggesting that the control of phytopathogenic fungi is more effective at 28 ºC than 37 ºC. On the other hand, at clinical temperature, more motility and virulence against *G. mellonella* were observed. *B. seminalis* does not seem to influence on seed germination regardless on temperature. However, temperature seems to trigger expression of genes in a host-specific manner in *B. seminalis* TC3.4.2R3, ensuring different virulence mechanisms to compete for survival. Considering that *B. seminalis* is a Bcc member, more studies are necessary to confirm its avirulence in mammals, however the results obtained in this work point to the biocontrol potential of this strain.

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3 CHAPTER 3 - Comparative transcriptomic analysis of *Burkholderia seminalis* TC3.4.2R3 in response to clinical and environmental temperatures.

3.1 ABSTRACT

Temperature has a very important role on regulation of genes that control the biological activities including virulence and survival strategies for microbes. Changes in temperature can trigger responses in gene expression of many microorganisms. *B. seminalis* TC3.4.2R3 was isolated from inner sugarcane roots and able to control orchid necrosis caused by *B. gladioli* and kill *Galleria mellonella* larvae a temperature dependent way. In order to evaluate the effect of temperature on *B. seminalis* global expression, we performed a comparative transcriptome profile at 28 ºC and 37 ºC. The analyses led to identification of 600 genes whose expression was significantly changed. Among the 79 genes with decreased expression at 37 ºC were found genes related to transcriptional regulation, transport, energetic metabolism, hydrolases, flagella, pilus and few others, besides unknown function proteins. Induced gene encoded proteins related to stress response; carbon, lipid and energy metabolism; transcription and translation regulation; transport; flagella and pilus; EPS, PHB, toxin, ribosome, amino acid and vitamins biosynthesis; proteins modification; cell division and chromosome partitioning; unknown proteins; among many others. At 37 ºC we observed that genes related to primary metabolism in *B. seminalis* was induced suggesting a strategy to survival in stationary phase. Entericidin, bacteriocin and terpenes biosynthesis were induced at 37 ºC. Motility seemed to play a complex regulation of in *B. seminalis*, presenting genes up- and down-regulated. Analysis of genome sequence combined with phenotypic results did not provide clear hints to *B. seminalis* pathogenic potential, which encourages its uses in agriculture for biocontrol purposes in the future.

3.2 INTRODUCTION

Burkholderia seminalis is a Gram-negative, aerobic, not sporulated and yellow-pigmented bacteria, which belongs to *B. cepacia* complex *cepacia* (Bcc) group (VANLAERE et al., 2008). *B. seminalis* was isolated as endophytic from rice (PANHWAR et al., 2014) and sugarcane (LUVIZOTTO et al., 2010), but also causing rot in apricot (LI et al., 2010; LOU et al., 2011) or presented in nosocomial infections (VANLAERE et al., 2008). *B. seminalis* have been used to different purposes, such as plant-growth promoter (PANHWAR et al., 2014), bioremediators (HUANG et al., 2012) and biocontrol agent (ARAÚJO; ARAÚJO; EBERLIN, 2017; ARAÚJO et al., 2016).

Changes in temperature can trigger responses in gene expression of many microorganisms (PEANO et al., 2014). Virulence factors such as toxins production, transport and regulation are affected by temperature (HOCKETT; BURCH; LINDOW, 2013; HURST et al., 2015), as well as motility, flagellar genes and biofilm production (HOCKETT; BURCH; LINDOW, 2013; TEH; LEE; DYKES, 2016), which is highly influenced by temperature and it seems to occur in a lineage-dependent manner (TEH; LEE; DYKES, 2016). *B. thailandensis*, has flagella production and motility inhibited at 37 ºC and has been considered an avirulent environmental isolate, although, occasionally cause pneumonia in susceptible humans (PEANO et al., 2014). *Yersinia enterocolitica* can establish saprophytic or parasitic interactions with the host. It is regulated in a coordinated manner. Temperature affects gene expression promoting alterations in cell morphology, outer membrane and LPS synthesis, urease production, motility and others. Motility was repressed at 25 ºC and induced at 37 ºC (ROHDE; FOX; MINNICH, 1994). Furthermore, surfactant and EPS production seems occur in a temperature-dependent manner in *Pseudomonas putida*, (DUBERN; BLOEMBERG, 2006) and *Erwinia amylovora* (WEI; SNEATH; BEER, 1992), respectively, which indicates the importance of temperature for the functioning and competition in environment. Many genes involved in plant-microbe interactions, insect-microbe interactions and secondary metabolites production are also thermo-regulated. *P. syringae* PG4180 produces the phytotoxin coronatine and its precursor, coronafacic acid, which prevents pathogen recognition, at 18 °C whereas no detectable levels are produced at 28 °C. Coronatine mutants of *P. syringae* showed smaller lesions and exhibited lower levels of bacterial multiplication in plants than wild-type. Synthesis of these of compounds is also important for the initial steps in the plant-microbe interaction (BUDDE; ULLRICH, 2000). RNA-seq of the *P. syringae* revealed 1440 genes with temperature sensitive expression. Among the repressed genes at 30 ºC were genes involved in polysaccharide synthesis, phage and IS elements, type VI secretion, chemosensing and chemotaxis, translation, flagellar synthesis and motility, and phytotoxin synthesis and transport. Among the induced genes at 30 ºC were genes involved in transcriptional regulation, quaternary ammonium compound, metabolism and transport, and chaperone/heat shock proteins. Highlighting that many genes encoding traits important for plant-microbe interactions were thermo-regulated (HOCKETT; BURCH; LINDOW, 2013). *Yersinia entomophaga* was virulent in *Galleria mellonella* model when held at 25 °C but was avirulent when maintained at 37 °C. Once inside the hemocoelic cavity, the bacterium rapidly multiplies, which occurs due the production of unknown temperature-independent factors that suppress the host immune system (HURST et al., 2015). The rhizobacteria *Pseudomonas fluorescens*, *Bacillus megaterium* and *Paenibacillus*

macerans can suppress fusarium wilt of chickpea, but the extent of disease suppression is modulated by soil temperature. The fusarium wilt of chickpea was suppressed by these rhizobacteria at 20 or 30 °C, but not at 25 °C, the temperature at which disease developed most severely. However, the greatest levels of disease suppression were obtained at 30 °C, when rhizosphere colonization and production of inhibitory metabolites by the rhizobacteria were very high and, chickpea root dry weight and rate of seedling emergence increased. The production of antifungal metabolites, colonization, and growth promotion of plant were significantly affected by incubation temperature (LANDA; NAVAS-CORTÉS; JIMÉNEZ-DÍAZ, 2004).

Thus, temperature has a very important role on regulation of genes that control the biological activities during interaction with the host plant and animals. Considering that *B. seminalis* is a Bcc member and pointed to biocontrol purposes, the understanding of temperature influence on the regulation of gene expression is extremely necessary. Hence, we investigate the response of *B. seminalis* strain TC3.4.2R3, an environmental isolate, to different growth temperatures through comparative transcriptional analysis.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strain and growth conditions

B. seminalis strain TC3.4.2R3 studied in this work was isolated from sugarcane roots (LUVIZOTTO et al., 2010) as endophytic bacteria. Whole genome of the strain was sequenced and described by Araujo et al. (2016). *B. seminalis* was maintained at -80 ºC in Tryptone Soya Broth (TSB) (Difco Laboratories, Sparks, USA) and 20% glycerol, and was recovered on Tryptone Soya Agar (TSA) (Difco Laboratories, Sparks, USA) with incubation for 24 h at 28 ºC or 37 ºC.

3.3.2 RNA extraction and sequencing

B. seminalis was grown for 48 h under shaking (180 rpm) at 28 ºC or 37 ºC in TSB in triplicate. Samples were frozen with liquid nitrogen and macerated. The total RNA of each sample was extracted using Illustra RNAspin Mini Kit (GE Healthcare) according to the manufacturer's instructions. Samples were tested for quality in agarose gel and 60 ng/mL of each sample were dissected with RNAstable (Biomatrica). The dried RNA samples were sent to DUKE Center for Genomic and Computational Biology (GCB), Durham, United States. RNA quality was evaluated by Qubit and Bioanalyzer and, only samples with intact RNA were

processed. Removal of rRNA was done using Ribozero Bacteria (Gram-Negative) kit. The sequencing library was prepared according to Illumina Truseq Stranded RNAseq protocol, following the manufacturer's instructions. Sequencing was performed on Illumina Hiseq 2000/2500 platform (DENMAN et al., 2014) to generate single-end reads with 50 bp. Reads were mapped on *Burkholderia seminalis* strain FL-5-4-10-S1-D7 chromosomes (GenBank access number 3432768).

3.3.3 Mapping and analysis of Illumina reads

Sequence reads from each sample had rRNA and tRNA subtracted with Bowtie (LANGMEAD et al., 2009). The reference sequences used were *E. coli* and *Burkholderia.* The resulting sequences were mapped on the *B. seminalis* genome (GenBank Access number 3432768) with BWA software (http://bio-bwa.sourceforge.net/). The Samtools package was used to convert sam in bam format files, sorted and indexed. To determine differential expression of known transcripts, the resulting aligned reads were analyzed by Cuffdiff package (http://cole-trapnell-lab.github.io/cufflinks/). Transcripts with a q-value of ≤ 0.05 and $\log_2$ fold-change above 1 were considered as differentially expressed transcripts. Relative expression graphs and tables were assembled using the R program and the CummeRbund library. Annotation of genome was performed with Blast2GO software (https://www.blast2go.com/) (CONESA et al., 2005). Furthermore, transcripts were analyzed for Carbohydrate-active enzyme Annotation (http://csbl.bmb.uga.edu/dbCAN/index.php), presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP/), prediction of bacterial protein secretion (http://effectivedb.org/) and bacterial protein subcellular localization prediction (http://www.psort.org/psortb/).

3.4 RESULTS AND DISCUSSION

3.4.1 Transcriptome analysis and regulation of gene expression by temperature

We used RNA sequencing and comparative transcriptome analysis to identify genes and their respective expression levels at 28 ºC and 37 ºC of *B. seminalis* TC3.4.2R3, in order to identify genes that potentially contribute towards the temperature and phenotype changes in *B. seminalis* TC3.4.2R3. Through generation of more than 35 million reads per sample, we were able to map about 2 Gb of TC3.4.2R3 genomic sequence to the corresponding sequences in the *B. seminalis* FL-5-4-10-S1-D7 genome (GenBank access number 3432768), which comprise three genetic elements: chromosome 1 (3.50 Mb); chromosome 2 (3.05 Mb) and a mega-

plasmid (1.09 Mb). Table 3.1 has details of yield and quality of Illumina sequencing per sample. The average for depth was 235X-coverage for 28 ºC samples and 233X-coverage for 37 ºC samples.

Table 3.1 – Transcriptome yield and quality.

Sample Name	PF* Yield (bp)	Number of reads	Q30%	Average Quality Score	Barcode	Depth coverage
PR-S1	1,825,288,292	35,789,967	93.32	37.26	TTAGGC	233
PR-S2	1,898,429,381	37,224,106	93.73	37.38	GATCAG	242
PR-S3	1,791,337,311	35,124,261	93.53	37.32	GTCCGC	229
PR-S4	1,863,094,566	36,531,266	93.66	37.36	CGTACG	238
PR-S5	1,862,172,843	36,513,193	93.75	37.38	ACTGAT	238
PR-S6	1,757,953,017	34,469,667	93.79	37.39	ATTCCT	225

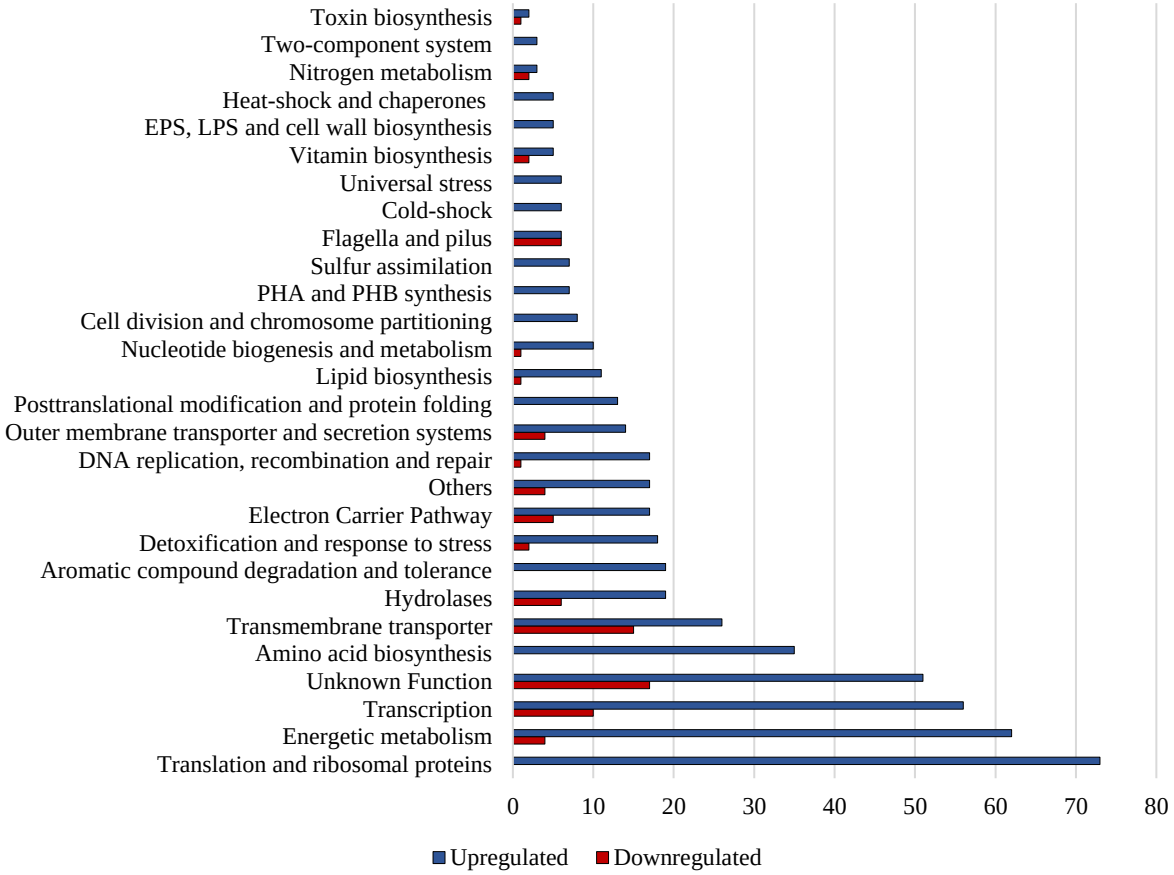
* PF: Passed Filter. PR-S1 to PR-S3: 28 ºC triplicate samples. PR-S4 to PR-S6: 37 ºC triplicate samples.

By adopting a q-value of ≤ 0.05 we found 1270 differentially expressed genes (1186 up-regulated genes and 84 down-regulated genes). However, when considering log2 fold-change above 1 to classify a transcript as being differentially expressed, transcriptional analysis revealed actually 600 differentially expressed genes (521 up-regulated genes and 79 down-regulated genes) in 37 ºC relative to 28 ºC (Appendix A). Functional classification of up- and down-regulated genes showed that the most of these genes encode core function such as energy metabolism, transport, regulatory proteins, cellular processes, aromatic compound degradation and detoxification, among others (Fig. 3.1). Many genes encoding proteins with unknown function or hypothetical proteins were also modulated by temperature.

The higher effect of growth at 37 ºC *versus* 28 ºC was on translation and ribosomal proteins, once 73 genes appeared to be up-regulated at 37 ºC. Energetic metabolism and transcription regulation had 62 and 56 up-regulated genes, but 4 and 10 down-regulated genes, respectively. Genes with unknown function and hypothetical proteins presented 51 up-regulated and 17 down-regulated genes, indicating that these unknown genes could be related to adaptation to different environmental conditions. Following, 35 genes involved in amino acid biosynthesis were highly expressed at 37 ºC. The transmembrane transport had 26 genes up-regulated and 15 down-regulated at 37 ºC. Nineteen genes encoding hydrolases were up-regulated, whereas 6 genes were down-regulated at 37 ºC. Interestingly, the aromatic compound degradation and tolerance showed thermo-regulation as well, with 19 genes up-regulated at 37 ºC. Changes in temperature triggered responses to stress and detoxification processes in *B. seminalis*, where 18 genes were up-regulated, only 2 were down-regulated. Part of the energetic

metabolism, but specifically, from electron chain pathway, 17 genes were expressed at higher levels, while 5 were down-regulated. Genes involved in DNA replication, recombination and repair were highly expressed (17 genes), only one gene showed lower expression level. Outer membrane transporter and secretion systems had 14 genes up-regulated and 4 down-regulated. Growth at 37 ºC also resulted in up-regulation and down-regulation of genes involved in lipid biosynthesis (11 genes-up; 1-down), nucleotide biogenesis and metabolism (10-up; 1-down), flagella and pilus (6-up; 6-down), vitamin biosynthesis (5-up; 2-down), nitrogen metabolism (3-up; 2-down) and toxin biosynthesis (2-up; 1-down). The following categories were all up-regulated in *B. seminalis* TC3.4.2R3, posttranslational modification and protein folding (13 genes); cell division and chromosome partitioning (8 genes); polyhydroxyalkanoate (PHA) and polyhydroxybutyrate (PHB) synthesis (7 genes); sulfur assimilation (7 genes); EPS, LPS and cell wall biosynthesis (5 genes); two-component system (3 genes); universal stress proteins (6 genes); cold-shock (6 genes) and, finally, heat-shock and chaperones (5 genes) (Fig. 3.1). Furthermore, there were other genes unclassified in the categories above, among these up-regulated genes we found the quorum sensing gene, acyl-homoserine-lactone synthase, diguanylate cyclase and signal peptides. Among those others down-regulated genes were an aminoglycoside resistance gene and a signal transduction gene involved in chemotaxis (Appendix A).

Figure 3.1 – Number of differentially expressed genes under temperature changes in *B. seminalis*.



It was demonstrated in the Chapter 2 that, at 37 ºC, *B. seminalis* showed more swimming motility. Here, the transcriptome analysis showed that 3 genes involved to flagella (WJ13_RS21675, WJ13_RS27715 and WJ13_RS27810) and 3 genes of pilus assembly were up-regulated. However, 4 genes of flagella biosynthesis (WJ13_RS21060, WJ13_RS21065, WJ13_RS21070 and WJ13_RS29715) and 2 genes of pilus assembly were down-regulated. Peano et al. (2014) found 12 down-regulated genes involved in flagellar motility of *B. thailandensis* at 37 ºC, however 3 other flagellar genes were up-regulated in the same temperature. Authors explained this modulation due to the complex regulation of flagellar apparatus, and the same might be highlighted here. Flagellar motility may be related to increased virulence in *B. pseudomallei* (CHUA; CHAN; GAN, 2003), nevertheless, *B. mallei*, the only obligate pathogen in the *Burkholderia* genus, is non-motile (GALYOV; BRETT; DESHAZER, 2010). Thus, pathogenic *Burkholderia* species appear to have adopted very different strategies in terms to regulation flagellar motility during host infection (PEANO et al., 2014), and more studies are necessary to consider flagella up-regulation, indeed, a virulence factor for *B. seminalis*.

It was possible to note an increased expression of genes involved in primary metabolism, such as ribosomal proteins, RNA polymerases, nucleotide biosynthesis, protein processing and energy-requiring cellular processes. It can be attributed to the growth phase, which is accelerated at 37 ºC. Transcripts were extracted from *B. seminalis* in the stationary phase, thus cells present more metabolic requirements. It was expected to find genes involved in transcription, translation, hydrolases, proteins folding, amino acid, lipids and vitamins biosynthesis up-regulated. In addition, it was not surprising that genes involved in cell division and chromosome partitioning, as well as DNA replication, recombination and repair appeared highly expressed. Stationary phase is a starvation period when cells are less active and more resistant. Starved cells of *E. coli* are more resistant to heat shock, oxidative stress, osmotic challenge and exposure to toxic chemicals than exponential-phase cells (KOLTER; SIEGELE; TORMO, 1993; NYSTRÖM, 2004; PLETNEV et al., 2015). During starvation, certain proteins are required for reinitiating exponential growth, thus many activators of transcription of rRNAs are expressed to replace the ribosomes degraded during stationary phase and synthetize new ones needed for growth (SIEGELE; KOLTER, 1993). Many sigma factors control expression of genes encoding proteins involved in maintaining viability during prolonged starvation. Starved microorganisms slow their growth rate dramatically and reduce protein synthesis, while protein turnover increases, once the proteins synthesized in the early stages of starvation are hydrolases, mainly proteases and peptidases. The protein turnover facilitates *de novo* protein synthesis as an amino acid source in the absence of an exogenous carbon source (LLORENS; TORMO; MARTÍNEZ-GARCÍA, 2010; SHAIKH et al., 2010). The proteins synthesized during the first few hours of starvation are critical for survival. However, proteins synthesis is still required over the starvation period to play roles on nutrient scavenging, heat-shock response and recovery from starvation (WATSON; CLEMENTS; FOSTER, 1998). *E. coli* was able to produce new proteins in the stationary phase, and among the amino acid biosynthetic pathways, with significant activity during the stationary phase, were serine, alanine, glutamate/glutamine, and aspartate/asparagine biosynthesis (SHAIKH et al., 2010). However, it was possible to find not only glutamine and serine, but also glycine, histidine, isoleucine, arginine, threonine and lysine biosynthesis pathways up-regulated in *B. seminalis* at 37 ºC.

Respiratory chain produces reactive oxygen species in starvation and there is a repression of aerobic metabolism to save endogenous reserves. Stress-induced oxidation may affect peptide chain elongation, protein folding, DNA organization, gene expression, carbon catabolism, and general stress protection. Thus, chaperones, histone-like proteins H-NS, the universal protein UspA, elongation factors EF-Tu and EF-G, glutamine synthetase, glutamate

synthase, aconitase, malate dehydrogenase, and pyruvate kinase could be induced (NYSTRÖM, 2004). For *B. seminalis* TC3.4.2R3 grown at 37 ºC genes that encode chaperons and heat-shock proteins (WJ13_RS00345, WJ13_RS02915, WJ13_RS08560, WJ13_RS11020, WJ13_RS17410 and WJ13_RS24470), universal stress proteins (WJ13_RS12750, WJ13_RS27520, WJ13_RS27525, WJ13_RS27535, WJ13_RS27665 and WJ13_RS28740), and several genes involved in cellular oxidant detoxification (Appendix A) were up-regulated. Moreover, ferritins, which sequester ferrous iron and reduce the formation of oxidative radicals formed within the cell (LLORENS; TORMO; MARTÍNEZ-GARCÍA, 2010) appeared to be up-regulated. The oxidative stress resistance is not only important in starvation survival, but also in pathogenesis, by protecting the cells from oxygen radical-mediated host defenses. Superoxide free radicals are neutralized by superoxide dismutase, generating hydrogen peroxide, which is broken down by catalase (WATSON; CLEMENTS; FOSTER, 1998). Heat shock causes damage to the cells, unfolding or denaturation of proteins (PHADTARE, 2004). Thus, we found induced heat-shock proteins and chaperones, which assist in protein folding. Also, cold-shock genes were found up-regulated at 37 ºC. Cold-shock response is exhibited upon temperature downshift (PHADTARE, 2004), however, they might be induced also by other types of stress and could be the most abundant proteins in cells at later stages of growth. The cold-shock transcripts are no stable and the translation occurs in a cold-induced manner, preferentially (GUALERZI; GIULIODORI; PON, 2003). Since there was no decreasing in the tested temperature, it is likely that even cold-shock genes appeared induced due stress conditions, they are not been translated in *B. seminalis*.

The quick response to the availability of nutrients requires the starved cells have an efficient expressed transport system for nutrient uptake (KOLTER; SIEGELE; TORMO, 1993), which is in agreement with our results of many transmembrane and outer membrane transporters have been highly expressed at 37 ºC.

It was found an up-regulated gene encoding a ppGpp (WJ13_RS08595) in *B. seminalis*. The small signaling molecule guanosine tetraphosphate plays a role in the stringent response, binding to RNA polymerase and altering gene expression, in particular the expression of genes involved in ribosome biogenesis, inducing starvation responses (POTRYKUS; CASHEL, 2008). Furthermore, production of cell envelope structures, such as peptidoglycan, lipopolysaccharides and lipoproteins are expected to have expression increased, once they confer protection to the cell during stationary phase adaptation (LLORENS; TORMO; MARTÍNEZ-GARCÍA, 2010; PLETNEV et al., 2015). Here, we observed that genes involved in exopolysaccharide (WJ13_RS02090, WJ13_RS09605, WJ13_RS09700, WJ13_RS09705,

WJ13_RS09710 and WJ13_RS26575) and lipopolysaccharide (WJ13_RS31700) biosynthesis were up-regulated.

Polyhydroxyalkanoates (PHA) and polyhydroxybutyrates (PHB) are energy reserve polymers produced by bacteria to survive periods of starvation in natural habitats (GASSER; MÜLLER; BERG, 2009). PHA synthesis, for example, is triggered by exhaustion of an essential nutrient in the presence of excess carbon. PHB is the best characterized PHA (PEANO et al., 2014). The genes WJ13_RS08070, WJ13_RS10330, WJ13_RS11665, WJ13_RS11670, WJ13_RS17885, WJ13_RS23790 and WJ13_RS29825 that encode proteins related to PHB biosynthesis and transport were induced in *B. seminalis* TC3.4.2R3 at 37 ºC.

During stationary phase several secondary metabolites, antibiotics and toxins, are synthesized (LLORENS; TORMO; MARTÍNEZ-GARCÍA, 2010). Two toxins were induced entericidin (WJ13_RS04525) and bacteriocin (WJ13_RS16305). Enterecidins have a lipoprotein nature and their genes are activated in stationary phase with bacteriolytic functions (BISHOP et al., 1998). Bacteriocins are an abundant class of antimicrobial molecules (RILEY; GORDON, 1999). Bacteriocins are largely found in *Burkholderia*, such as pyocins and cepaciacins (GOVAN; HARRIS, 1985). Furthermore, we found up-regulated genes in the biosynthesis of terpenes and terpenoids. The terpenoid metabolites present medically or agriculturally useful activity, the vast majority have been isolated from plants or fungi, with only a relative handful having been obtained from bacterial sources (YAMADA et al., 2015). By genome mining is possible to identify a cluster encoding terpenes biosynthetic genes in *B. seminalis* TC3.4.2R3 (Chapter 5).

Interestingly, several genes involved in the aromatic compounds biodegradation were induced at 37 ºC. Many *Burkholderia* are able to biodegrade aromatic compounds in the environment (SEO; KEUM; LI, 2009), however, enzymes for the degradation of aromatic compounds are only synthesized when the respective substrates are present (GERISCHER, 2002). Aromatic intermediates of bacterial metabolism seems to induce enzymes involved in cellular aromatic compounds, such as the aromatic amino acid phenylalanine, which was able to induce phenylacetic acid degradation in *B. cenocepacia* K56-2 (HAMLIN; BLOODWORTH; CARDONA, 2009).

Most clinical isolates of *B. cenocepacia* produce siderophores, such as ornibactin and pyochelin under iron-limiting conditions, and production of both siderophores has been correlated with morbidity and mortality in cystic fibrosis patients, besides contributing to pathology in animal models of respiratory infection (AGNOLI et al., 2006; OOI et al., 2013). Although siderophores biosynthesis pathways were not found up-regulated in *B. seminalis* at

37 ºC, genes encoding terpenes, toxins and heat-shock proteins, even in a small amount, were up-regulated. Those genes together with the ability of *Burkholderia* to withstand stress conditions, suggests that *B. seminalis* could be pathogenic at warmer temperatures, however it was not verified *in vivo* in *Galleria mellonella* infection model (Chapter 2). Thus, more experiments with different hosts should be conduct in order to confirm the virulence factors found up-regulated at 37 ºC.

3.5 CONCLUSION

In the present analysis, we observed that many genes were regulated at temperature dependent way. At 37 ºC genes involved in primary metabolism, such as energy metabolism, transcription and translation regulation, transport, proteins modification and cellular processes were up-regulated in *B. seminalis* TC3.4.2R3. Also, aromatic compound degradation, terpenes, toxins biosynthesis and detoxification genes were up-regulated, beyond hypothetical proteins. Only few genes were down-regulated. The obtained results showed that at 37 ºC the metabolism seem to be higher than at 28ºC and, hence, the growth phase, setting starvation conditions rapidly. Cell directed its energy to get substrate to synthetize proteins needed to survival. Very few genes involved in secondary metabolism and virulence factors were up-regulated at 37 ºC in comparison with 28 ºC.

We focused our investigation on the effects of 28 ºC and 37 ºC growth temperatures, an environmental signal associated to colonization of the human host, on the gene expression, once Bcc members could represent intriguing examples of transition state between environmental bacteria and human pathogen. However, analysis of *B. seminalis* TC3.4.2R3 genome sequence combined with phenotypic results (Chapter 2) did not provide clear hints to its pathogenic potential, which encourages its use in agriculture for biocontrol purposes in the future.

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4 CHAPTER 4 - The role of *wcbE* gene of *Burkholderia seminalis* TC3.4.2R3 in environmental interactions.

4.1 ABSTRACT

The *wcbE* gene, codify a glycosyltransferase enzyme, belongs to the *wcb* cluster that is related to synthesis of capsule and environmental interactions. The strain TC3.4.2R3 of *Burkholderria seminalis* was isolated from inner root tissues of sugarcane and further studies demonstrated that can control orchid necrosis, caused by *B. gladioli* and inhibit *in vitro* phytopathogenic fungi. However, the *wcbE* mutant lost the ability to *in vitro* inhibit fungi and control this orchid disease. Therefore, in the present study, a *wcbE* mutant of the bacterium *Burkholderia seminalis* TC3.4.2R3 was evaluated and the role of this gene in microbial in interactions described. The results showed that in *wcbE* mutant, genes involved in capsular polysaccharide biosynthesis and export and secondary metabolites production were repressed in *wcb* cluster, suggesting that the TN5 mutation affected not only the *wcbE* gene, but also other genes in *wcb* cluster in *B. seminalis*. Indeed, even though motility, EPS production, hydrogen peroxide resistance, seed germination and plant growth were not affected, the biofilm formation and virulence in *Galleria mellonella* infection model were highly affected by the inactivation of glycosyltransferase in a temperature-dependent manner. Thus, glycosyltransferase gene has a crucial role on *B. seminalis* environmental interactions.

4.2 INTRODUCTION

Burkholderia seminalis, is a β-*proteobacteria*, Gram-negative, aerobic, non-sporulating rods, yellow-pigmented, mucoid and belongs to the Bcc group (VANLAERE et al., 2008). This species has been isolated from different environments, including water (FANG et al., 2011), soil (HALL et al., 2015), plants as endophyte (ARAÚJO et al., 2016) and even humans (VANLAERE et al., 2008). *B. seminalis* might establish pathogenic and nonpathogenic interactions with hosts. Because of this ambiguity, decision on the industrial or biotechnological use of a *Burkholderia* strain must be taken based on its pathogenic potential and only after careful molecular and phenotypic characterization (EBERL; VANDAMME, 2016).

B. seminalis strain TC3.4.2R3 was isolated from sugarcane rhizosphere (LUVIZOTTO et al., 2010). Further, a TN5 mutants library was screened and which that lost the ability to inhibit phytopathogenic fungi and control orchid necrosis was identified, revealing that a glycosyltransferase gene (*wcbE* – ref. *Bsem*_02955), that belongs to *wcb* cluster, is related to these control (ARAÚJO et al., 2016). This *wcb* cluster is associated to capsule biosynthesis (CUCCUI et al., 2012) and environmental interaction (KIM et al., 2005). Mutants with

inactivated glycosyltransferase gene were named M3, M4 and M7, and did not produce the siderophore pyochelin and the ramnnolipid Rha-Rha-C15-C14 when challenged against *Fusarium oxysporum,* while wild-type strain produced and inhibit the fungus. Authors suggested the involvement of *wcb* cluster in antimicrobial production (ARAÚJO; ARAÚJO; EBERLIN, 2017).

Glycosyltransferases catalyse the transfer of sugar molecules (KAPITONOV; YU, 1999; LAIRSON et al., 2008) between compounds and are present in several antibiotic biosynthesis pathways (GARRIDO et al., 2006; STINGELE; NEWELL; NEESER, 1999; WALSH; FREEL MEYERS; LOSEY, 2003). Also, glycosyltransferases can be present in virulence factors biosynthesis, as in lipopolysaccharides (LPS) (LEIPOLD; VINOGRADOV; WHITFIELD, 2007), exopolysaccharides (EPS) (BARTHOLDSON et al., 2008) and flagellin (HANUSZKIEWICZ et al., 2014). Therefore, it is possible to note the glycosyltransferase importance on survival and adaption strategies for bacteria. Thus, the aim of this work was to investigate the role of *wcbE* gene of *B. seminalis* TC3.4.2R3 in mechanisms related to environmental interactions, including in plant and animal host.

4.3 MATERIAL AND METHODS

4.3.1 Bacterial strain and growth conditions

B. seminalis strain TC3.4.2R3 studied in this work was isolated from sugarcane roots (LUVIZOTTO et al., 2010) as endophytic bacteria. Whole genome of the strain was sequenced and described by Araujo et al. (2016). The TC3.4.2R3 mutants presented a TN5 transposon inserted in the *wcbE* gene, which encodes a glycosyltransferase, which were called M3, M4 and M7 (ARAÚJO; ARAÚJO; EBERLIN, 2017). The complemented M3, namely cM3, presented pBBR1-MCS vector. *B. seminalis* strains were maintained at -80 ºC in Luria-Bertani (LB) broth (Difco Laboratories, Sparks, USA) and 20% glycerol, and was recovered on Luria-Bertani agar (Difco Laboratories, Sparks, USA) or broth with incubation for 24 h at 28 ºC.

4.3.2 Mutant complementation

The mutant M3, generated by random mutation with TN5 (Km^+), was complemented with the replicative plasmid pBBR1-MCS (Cm^+). For this, *wcbE* gene (ref. *Bsem_02955*) of glycosyltransferase was totally amplified from *Burkholderia seminalis* TC3.4.2R3 genome using the following primers forward 5'ccgcgAAGCTTcgtagagctggtttccttgc 3' and reverse 5'cggccCTCGAGagcacttcccggagtgaac 3'. The ends of insert and vector were digested with the enzymes XhoI and HindIII. Then, the fragments were ligated by T4 ligase in the 3:1 ratio of

insert and vector, respectively. The cloned glycosyltransferase gene had expression controlled by T3 promoter of the vector. The pBBR1-MCS::*wcbE* plasmid was transformed into electrocompetents *E. coli* S17λ cells. Plasmid was inserted into mutant *B. seminalis* M3 towards conjugation with *E. coli* S17 in 1:1, 2:1, 3:1 (M3:S17) ratios at 28 ºC or 37 ºC. The complements were confirmed by colony PCR and screening on LB selective plaque containing chloramphenicol (Cm^+) 50 µg.mL^{-1} and kanamycin (Km^+) 200 µg.mL^{-1} (LIM, 2015).

4.3.3 Motility assay

Overnight bacterial cultures of wild-type (WT), mutant (M3) and complemented (cM3) were harvested and resuspended in PBS to an OD_{595} of 1.0. Bacterial suspensions were inoculated on LB with 0.3%, 0.5% and 1% w/v agar with a central tip and incubated at 28 ºC or 37 ºC. Motility was evaluated by appearance of growth rings outside the inoculum area and the diameter in millimeters of these halos was measured after 24, 48 and 120 h of growth (DENMAN et al., 2014; FLANNAGAN; LINN; VALVANO, 2008). Experiment was delineated in triplicate.

4.3.4 Biofilm assays

Biofilm formation was assessed using the 96-well plate and accompanying peg-lid of the MBEC (Minimum Biofilm Eradication Concentration) Assay device (Innovatech Inc.). Wells received 150 µL bacterial suspensions of *B. seminalis* WT, M3 and cM3 standardized to 10^7 c.f.u. mL^{-1} in Tryptone Soya Broth (TSB) (Difco Laboratories, Sparks, USA). Eight wells were inoculated in at least three independent experiments. The peg-lid was placed on the plate and incubated at 28 ºC or 37 ºC for 24 h. The peg-lid was transferred to a fresh 96-well plate containing pre-warmed TSB and incubated for more 24 h. Then the peg-lid was submitted to rinses with Phosphate Buffered Saline (PBS) (200 µL per well) at room temperature for 2 min. Peg lid was baked at 60 ºC for 20 min and stained with crystal violet 0.1% (w/v) (200 µL per well) and incubated for 30 min at room temperature. Three wash plates containing PBS (200 µL per well) rinsed the pegs following staining, subsequently the crystal violet was solubilized with 95% ethanol prior to measuring OD_{595} (DENMAN; BROWN, 2013).

4.3.5 Scanning electron microscopy of biofilm

Scanning electron microscopy (SEM) of biofilms was conducted at Instituto Butantan, São Paulo, Brazil. *B. seminalis* cells (WT, M3 and cM3) were grown overnight at 28 ºC and then absorbance was adjusted to OD_{595} 1.0. Cells were grown on membrane discs (13 mm) by

seeding 2 mL of bacterial suspension in 12-well culture plates and incubating them for 24 h at 28 ºC or 37 ºC and 150 rpm. After, the medium was removed and samples were fixed following Karnovsky (1965) protocol with same modifications. Cells were fixed in Karnovsy fixative solution (paraformaldehyde 4%; glutaraldehyde 2.5%; 0.1 M cacodylate buffer pH 7.2) during 4 h. The membrane discs were washed 3 times for 15 min with cacodylate buffer. Cells were then treated with a 1% osmium tetroxid solution diluted in 0.2 M cacodylate buffer for 30 min. After fixation, the samples were washed 3 times for 15 min with 0.1 M cacodylate buffer again to remove traces of fixatives. For dehydration procedure, membranes were placed in increasing ethanol solutions starting with 30, 50, 70, 90 and 100% for 15 min twice per step. Then the samples were prepared by critical point drying in Leica CPD 030. Sputtering of samples was performed with gold–palladium in Leica SCD 050. The images were taken using a FEI QUANTA 250 LEO scanning electron microscopy (HEILMANN et al., 2005).

4.3.6 Measurement of exopolysaccharide weight

Exopolysaccharides were purified from cell culture media according to Kim et al. (2014) with some modifications. Briefly, *B. seminalis* WT, M3 and cM3 were grown for 5 days at 28 ºC or 37 ºC in 10 ml of mannitol medium (0.2% yeast extract and 2% mannitol). The bacterial cultures were vortexed and centrifuged at 2,300 X *g* for 10 min. Supernatant was transferred to new tubes and phenol was added at a final concentration of 10%. Mixture was incubated at 4 °C for 5 h and centrifuged at 9,100 X *g* for 15 min. The water phase was collected and 4 volumes of isopropanol was added, incubations was at –20 ºC overnight. The precipitated exopolysaccharide was centrifuged under the same conditions as above and suspended in 150 µL of distilled water. Following lyophilization, dry-weight was measured. EPS samples were standardized to 1 mg.mL-1 in sterile water and tested against *Ceratocystis paradoxa.*

4.3.7 Hydrogen peroxide resistance assay

Oxidative stress was assessed via hydrogen peroxide resistance assays. Cells from overnight cultures of WT and mutant M3 were harvested and resuspended in PBS. *E. coli* CLM24 was used as a control. The density was standardized to 10^7 c.f.u. mL^{-1}. Hydrogen peroxide was added to a final concentration of 2, 5 and 10 mM followed by 30 min incubation at 25 ºC. Serial dilutions were plated on LB agar to quantify c.f.u.s and calculate percentage survival relative to controls (DENMAN; BROWN, 2013; LEFEBRE; VALVANO, 2001).

4.3.8 Galleria mellonella infection model

Larvae were obtained from LiveFoods UK and stored in woodchips at 10 ºC prior to use. Larvae were injected in the hindmost proleg using a 25 µL 22s gauge gas-tight Hamilton syringe with 10 µL of 10^8 c.f.u. mL^{-1} of *B. seminalis* WT and M3. PBS was used as a control. For each strain and treatment, ten larvae were inoculated per experiment in two independent experiments. Following injection, larvae were incubated at 28 ºC or 37 ºC, and their survival was monitored for up 96 hours (SEED; DENNIS, 2008).

4.3.9 Effects of Burkholderia on seed germination and plant growth

B. seminalis strains WT, M3 and cM3 were grown in LB broth at 28 ºC and 180 rpm for 48 h. Bacteria were harvest and absorbance adjusted to OD_{600} 1.0 with sterile distilled water. Seeds of maize (*Zea mays*) and cotton (*Gossypium hirsutum*) were surface sterilized in 70% alcohol for 1 min, 3% sodium hypochlorite for 1 min and rinsed twice in sterile water. Subsequently, they were immersed in a sufficient amount of *B. seminalis* suspension. Control seeds were treated with sterile water (untreated control). Fifty seeds were placed on moist filter paper and incubated in a germination chamber at 25 °C and 12 h photoperiod for 5 days. During the experiment, filter paper was maintained humid adding a suitable volume of sterile water daily and wrapping containers in transparent plastic bags. Experiment was repeated twice. The mean germination percentage was calculated at the end of the experiment. Maize seedlings followed for plant growth assay. Forty seedlings per treatment were planted in polystyrene seed trays (50 cm^3/cell) containing non-sterilized soil and vermiculite as substrate and watered with tap water. The plants were left to adapt for 2 days in the growth chamber at 25 °C and 12 h photoperiod and watered as necessary with tap water to keep the soil moist. After this, 1 mL of WT, M3 and cM3 OD_{595} 1.0 was added according to the treatment close to the roots. In the control treatment was added 1 mL of water. Plants growth were assessed after 12 days of inoculation. Plants were carefully removed from the boxes and the substrate was carefully removed from roots. The following parameters were measured, as indicators: (a) shoot and root fresh weight; (b) shoot and root length; (c) shoot and root dry weight. Dry weight was obtained after 5 days at 60 ºC (CASSÁN et al., 2009; KUNOVA et al., 2016).

4.3.10 Real Time qPCR

For the analysis of gene expression by real-time qPCR, the pellets of *B. seminalis* WT and mutants M3, M4 and M7, after 18, 24 and 48 h of growth under shaking at 28 ºC, were frozen with liquid nitrogen and macerated. RNA of each sample was extracted using GE Healthcare kit according to the manufacturer's instructions. Three independent biological replicates per

treatment were used, from which technical replicates were made, totaling 6 replicates for each sample. SuperScriptIII (Invitrogen, Foster city, CA) was used for the RT-PCR reactions, according to the manufacturer's instructions, by using 1 µg of total *B. seminalis* RNA. The thermocycling conditions for reverse transcription were comprised of an initial step at 25 °C for 10 min, followed by 50 ºC for 50 min and 85 °C for 5 min. SYBR Green PCR Reagent kits (Applied Biosystems, Foster city, CA) were used for the qPCR reactions using 50, 33 and 25 ng of the resulting cDNA. The increase in fluorescence emitted by SYBR Green Reagent was used to measure PCR products. The primers used, as well as monitored genes were listed in Table 4.1. The *recA* gene was chosen as endogenous control for experimental normalization, once it is a constitutively expressed gene. All the real-time qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The default thermocycler program was used for all genes. Nonspecific amplification and negative control reactions were checked by dissociation curves. Expression of each gene was figured out using $2^{-\Delta\Delta Ct}$ method and the control treatment as calibrator (DOURADO et al., 2015).

Table 4.1 - List of primers pairs used in real-time qPCR and the corresponding genes.

Primer	Primer sequence (5' → 3')	Putative Gene Function
BSem_02959_F	AAATATCGCACATGACCCGC	Biosynthesis protein/ export of capsular polysaccharide
BSem_02959_R	GCGTCACCTGAATCATGGTC	
BSem_02955_F	CGAATGTCTCTTTCCGCCTG	Glycosyltransferase group I
BSem_02955_R	TTCAAGCCAATTCCCTGCAC	
BSem_02954_F	TTTCTCGTCCAGGCGTTTTG	Hypothetical protein
BSem_02954_R	CGAGTCGTTCATTGTGGAGC	
BSem_02952_F	TCCTTTCTCGACGACAAGCT	Lipopolysaccharide biosynthesis protein
BSem_02952_R	TCGTTCATCTCCTGCTCCAG	
BSem_02940_F	CAGCAGTCGAAGCTGTTCC	Capsular polysaccharide biosynthesis fatty acid synthase - type I PKS
BSem_02940_R	GTGTTCGCGTACAGCGTGTC	

F: forward – R: reverse.

4.3.11 Statistical analysis

All *in vitro* assays were performed at least in triplicate, with subsequent statistical analysis by one-way ANOVA with Tukey test (GraphPad Prism 6) and multiple comparisons. $P < 0.05$ was deemed statistically significant. A Kaplan-Meier survival plot with log-rank

(Mantel-Cox) test and Bonferroni correction (GraphPad Prism 6) was used to compare larval survival within *Galleria mellonella* infection model.

4.4 RESULTS AND DISCUSSION

4.4.1 Mutant complementation

Conjugation of mutant *B. seminalis* M3 to *E. coli* S17 was efficient in all proportions and temperatures tested. It was possible to obtain several conjugative colonies of *B. seminalis* denominated cM3, which were confirmed by PCR, selection with antibiotics and phenotypic tests.

4.4.2 Motility assay

Motility confers many benefits to bacteria such as increased efficiency of nutrient acquisition, avoidance of toxic substances, translocation to preferred hosts and access to optimal colonization sites within them, and dispersal in the environment during the course of transmission. The bacterial motility with different modes of translocation is classified in at least three different forms, namely: swimming, swarming and twitching. Swimming and swarming depend on flagella, whereas twitching depends on type IV pili. Swimming-type motility is characterized by mobile cells having a single polar (monotrichous) flagellum. These bacteria revert the flagellar rotation to swim in a new direction on semi-solid agar (0.3%), whereas swarming and twitching can be verified on 0.5% and 1% agar, respectively (GIBIANSKY et al., 2010; RASHID; KORNBERG, 2000; TURNER et al., 2010). Comparisons between the three types of motility in *B. seminalis* showed that, regardless of temperature, the preferred type of motility is swimming (Table 4.2).

The swimming motility of *B. seminalis* was compared at different temperatures and it was observed that at 28 ºC the motility is significantly ($p < 0.0001$) lower than at 37 ºC for all strains (Fig. 4.1). At 28 ºC, WT and M3 were statistically equal ($p < 0.0001$). At 37 ºC, there were no differences among strains motilities (Fig. 4.1).

Table 4.2 - Differents types of motility in *B. seminalis* WT at 37 ºC.

B. seminalis	*Swimming*	*Swarming*	*Twitching*

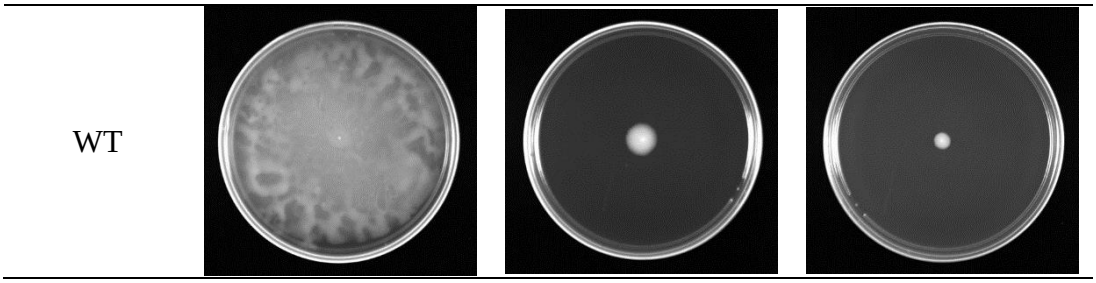
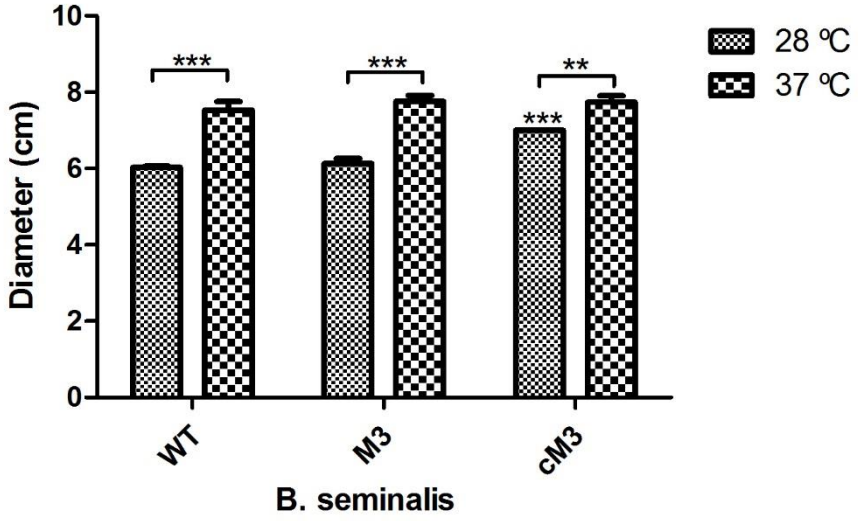


Figure 4.1 - Swimming motility in *B. seminalis* TC3.4.2R3 strains WT, M3 and cM3 under different temperatures.



The error bars represent standard deviation; $^{**}p < 0.001$; $^{***}p < 0.0001$

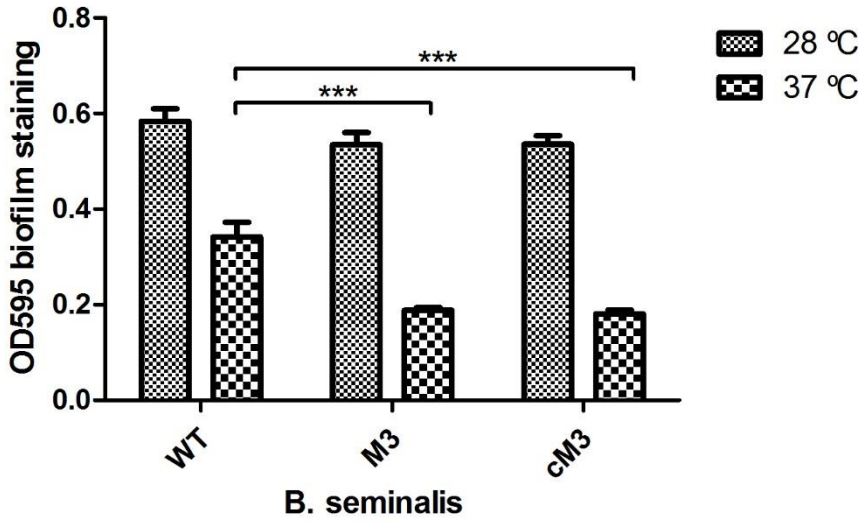
4.4.3 Biofilm assays

Biofilms are surface-associated and multicellular-structure communities of microorganisms. Biofilm formation is a complex process that involves many factors such as bacterial motility, production of extracellular polymeric substances (EPS) and quorum sensing signals (WANG et al., 2014). The growth rate was evaluated and the highest growth occurred at 37 ºC (data not shown), even though less biofilm was produced at this temperature (Fig. 4.2), indicating that altered biofilm phenotype cannot be attributed to differences in growth rate and biofilm production is not directly related to bacterial growth. At 28 ºC, there were no differences in biofilm production among strains ($p < 0.0001$). Although cM3 motility has been greater than others strains, the biofilm was statistically the same. Nevertheless, at 37 ºC, differences were observed. Less biofilm was produced, which is in agreement with the motility results once at 28 ºC, bacteria were less mobile. It is known that the ability to form biofilms is often dependent on motility, with flagella being required for initial surface attachment, thus the less mobile a

bacterium, the more biofilm it forms (ANDREAE; TITBALL; BUTLER, 2014; WANG et al., 2014).

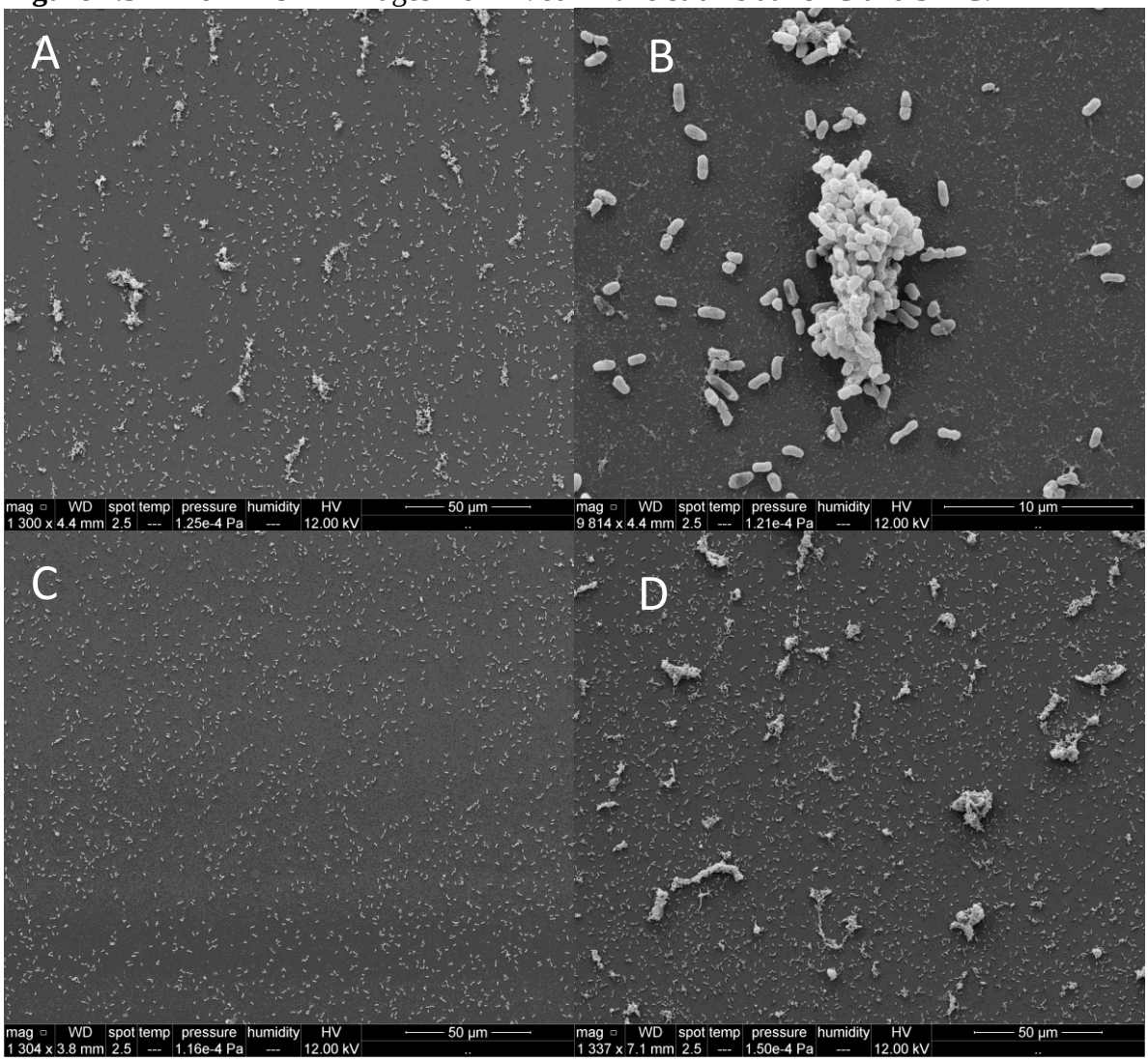
At 37 ºC, the lack of glycosyltransferase gene causes less biofilm formation (Fig. 4.2). WT produced more biofilm than M3 ($p < 0.0001$). In *Clostridium difficile*, was demonstrated the role of glycosyltransferases in the biosynthesis of the flagellin glycan chain. These glycosyltransferases were located upstream and downstream of the RT027 flagellin biosynthetic locus and reduced bacterial motility, promoted autoaggregation and biofilm formation and reduced adhesion of *C. difficile* to intestinal epithelial cell (VALIENTE et al., 2016). Glycosyltransferase was also critical for biofilm formation and virulence of *Enterococcus faecalis*. Deletion of the glycosyltransferase *bgsA*, presented in *bgs* locus in *E. faecalis*, leaded to impaired biofilm formation and reduced attachment to host cells (THEILACKER et al., 2011). Deletion of the glycosyltransferases genes located in *xag* cluster of *Xanthomonas campestris* resulted in decreased EPS production, abolished biofilm formation and attenuated the bacterial resistance to oxidative stress (TAO; SWARUP; ZHANG, 2010). An increase of temperature for *B. pseudomallei* resulted in a significant decrease in biofilm formation. Biofilm production was higher at 30 ºC than 37 ºC (BODDEY et al., 2006; RAMLI et al., 2012), such as results presented here for *B. seminalis*. Quantitative data of biofilm were confirmed by scanning electron microscopy (SEM). Images showed higher cell agglomerates at 28 °C than at 37 °C for all isolates. At 37 °C, the cell pellets were smaller than at 28 °C. The mutant produced a very small amount of biofilm and the visualization of cellular agglomerates was sporadic in the tested temperatures (Fig. 4.3). The higher production of biofilm at 28 ºC, suggests a preference for environmental hosts, such as plants, which should benefit the microorganism. Furthermore, the phenotypic changes of *wcbE* mutant showed to be temperature-dependent, highlighting the importance of glycosyltransferase in *B. seminalis* at 37 ºC

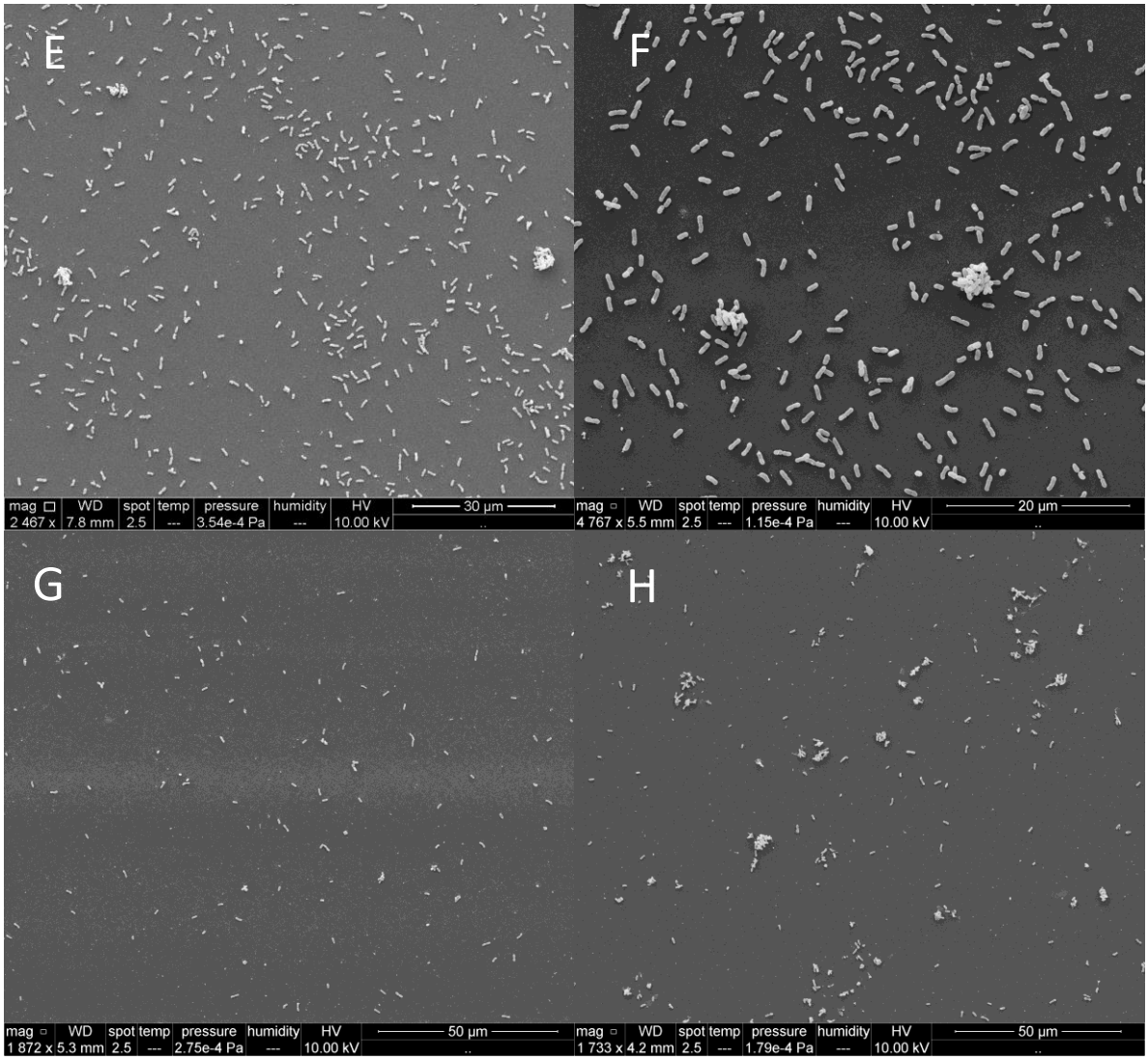
Figure 4.2 – Biofilm production of *B. seminalis* WT, M3 and cM3 at different temperatures.



The error bars represent standard deviation; ***$p < 0.0001$

Figure 4.3 – Biofilm SEM images from *B. seminalis* strains at 28 ºC and 37 ºC.



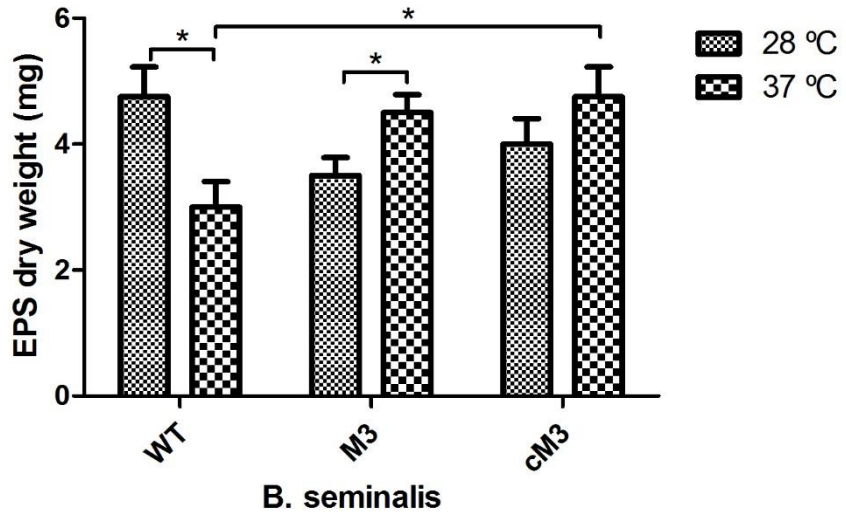


Biofilm of *B. seminalis* WT, M3 and cM3 in different magnifications. A- WT at 28 ºC; B- Detail of an agglomerate of WT at 28 ºC; C- M3 at 28 ºC; D – cM3 at 28 ºC; E- WT at 37 ºC; F- Detail of small agglomerates; G- M3 at 37 ºC and H- cM3 at 37 ºC.

4.4.4 Measurement of exopolysaccharide weight

EPS are usually related to biofilm production because its importance in biofilm matrix (FAZLI et al., 2014; WANG et al., 2014). For WT strain, there was a clear correlation between EPS and biofilm production, since at 28 ºC was observed the higher biofilm and EPS amounts compared to 37 ºC, the same was not observed in the mutant. Furthermore, no significant differences were observed among WT and M3. At 28 ºC, *B. seminalis* WT produced larger amounts of EPS than M3 and cM3. At 37 ºC, M3 and cM3 produced more EPS than WT, nevertheless they had produced less biofilm than WT at the same temperature. In addition, for M3 and cM3 the higher EPS production occurred at 37 ºC, whereas the best biofilm happened at 28 ºC (Fig. 4.4). It is known that species of the Bcc group have a high variability in the EPS production, which is strongly influenced by the bacteria environmental (CUZZI et al., 2014).

Figure 4.4 – EPS production of *B. seminalis* WT, M3 and cM3 in dry weight at different temperatures.

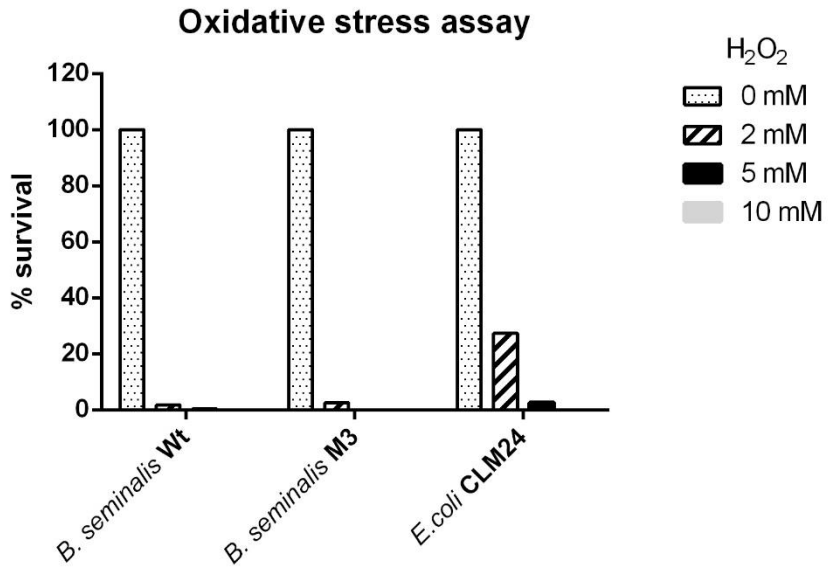


The error bars represent standard deviation, *$p < 0.05$.

4.4.5 Hydrogen peroxide resistance assay

B. seminalis demonstrated to be very sensible to oxidative stress. Survival of WT and mutant decreased dramatically in 2mM of hydrogen peroxide and presented no significant difference. *B. seminalis* did not survive from 5 mM of H_2O_2. *E. coli* CLM24 survived at least ten times more than *B. seminalis*, but did not survived in 10 mM (Fig. 4.5). Yuen et al. (2012) also found no significant difference between *B. pseudomallei* WT and Δ*wzm*, gene responsible by capsular polysaccharide production located in *wcb* cluster, strains at concentrations ranging from 2.5% to 25% H_2O_2. However, the mutant strain was slightly more sensitive to 30% H_2O_2 than the wild-type strain. Since H_2O_2 diffuses through the bacterial membrane leading to lethality, authors suggested that Δ*wzm* was more susceptible due its compromised membrane structure. After entrance in a human host, environmental bacteria experience initially an aerobic niche, such as skin wounds, and then, in the host tissues or bloodstream, enter an anoxic environment (PEANO et al., 2014). Also, phagocytic cells generate superoxide and other reactive oxygen species, which presented antibacterial activity and, hence, resistance and adaptation to oxidative stress are critical aspects of bacterial pathogenesis during host colonization (HASSETT; COHEN, 1989).

Figure 4.5 - Percentage of WT and M3 survival in different concentrations of hydrogen peroxide.



There are no error bars because survival were calculated based on means, each mean was plotted as a single data bar.

4.4.6 Galleria mellonella infection model

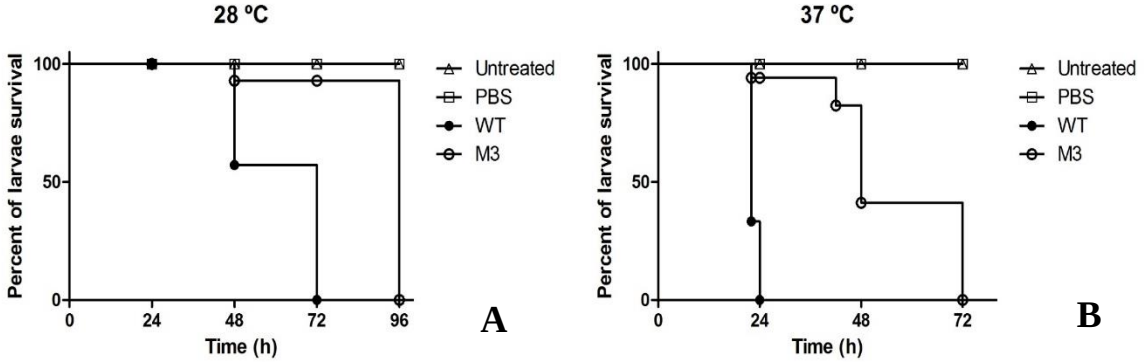
Non-mammalian infection models have become particularly attractive because they are fast, cheap, can survive at 37 ºC, allow high throughput screenings and are not limited by ethical concerns (PEREIRA et al., 2015). In this context, *Galleria mellonella* larvae have been used as a model organism to study the pathogenicity of members of the Bcc (DENMAN et al., 2014; DENMAN; BROWN, 2013; IBRAHIM et al., 2012; SEED; DENNIS, 2008; UEHLINGER et al., 2009; WAND et al., 2011).

The overall pathogenicity of *B. seminalis* WT and M3 towards *G. mellonella* (Fig. 4.6) was statistically different between WT and mutant infection and the larvae killing occurred in a manner that was temperature-dependent. At 28 ºC was observed less virulence in *B. seminalis* with extended time to larval killing. 100% mortality was observed in 72 h for WT infection and 96 h for M3 mutant one (Fig. 4.6). 100% of the larvae were killed following inoculation with *B. seminalis* WT after 24 h at 37 ºC (Fig. 4.6). In contrast, the rate of killing of *G. mellonella* was significantly lower with the glycosyltransferase mutant in the same conditions (log-rank test and Bonferroni correction; $P < 0.0001$) and 100% mortality was observed after 72 h only. No killing was observed for the control larvae. At the final of experiment, all the larvae were sacrificed at -80 ºC.

The results showed inactivation of *wcbE* did result in altered virulence relative to the wild-type in both temperatures. Biofilms results showed that WT produced more agglomerates than M3, and usually biofilm is linked to the pathogenicity. Thus, less biofilm could reduce

mutant virulence in comparison with WT. In addition, it is known that glycoproteins have been identified important as virulence factors in pathogenic bacteria and localized on the bacterial cell surface, where they may be involved in interactions with the host. There are studies recorded various O-linked glycosylation modifications in *Burkholderia* with potential virulence functions (HANUSZKIEWICZ et al., 2014; SCOTT et al., 2011). Thus, the mutant could be synthetizing a compromised capsular polysaccharide involved in virulence. It appears the *wcbE* plays a role in *in vitro* virulence. Cuccui et al. (2012) inactivated genes of *wcb* cluster, specifically *gmhA, wcbJ* and *wcbN*, which are required for sugar biosynthesis in *B. pseudomallei* K96243. The result was attenuation in the BALB/c mice highlighting the role of *wcb* cluster, and specifically glycosyltransferase gene, in virulence as well. Furthermore, at 37 ºC, it was observed more motility related to flagella, which could be linked to higher virulence, since flagella are virulence factors that support the pathogen in achieving specific sites in the host (CHUA; CHAN; GAN, 2003).

Figure 4.6 - *G. mellonella* pathogenicity assay 96 h after infection at 28 ºC (A) and 37 ºC (B).

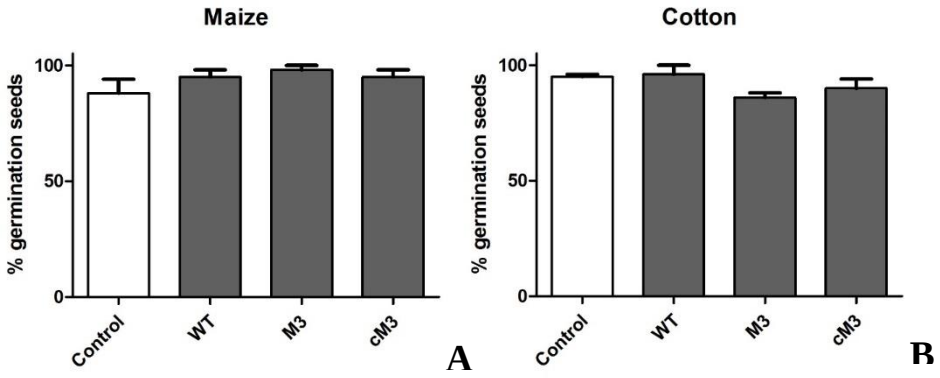


The significance of the results was determined using the log-rank (Mantel-Cox) test and Bonferroni correction; $p < 0.0001$.

4.4.7 Effects of Burkholderia on seed germination and plant growth

Once *Burkholderi*a have features that confer ability for biological control and natural resources for crop protection (PARKE; GURIAN-SHERMAN, 2001), we tested the potential of *B. seminalis* on a monocotyledon (maize) (Fig. 4.7) seeds and a dicotyledon (cotton) (Fig. 4.7) seeds and if glycosyltransferase influences it. No statistical differences between uninoculated and inoculated seeds were observed, indicating that the glycosyltransferase lack did not play a role in the early growth stages of the plants.

Figure 4.7 – % of germination of maize (A) and cotton (B) seeds inoculated with *B. seminalis* strains.



The error bars represent standard deviation.

The ability of *Burkholderia* promote plant-growth is well known. Establishment of epiphytic and endophytic associations with plants can lead to the stimulation of plant growth, nitrogen fixation, induction of plant nodulation and enhancement of the host's resistance to stresses (COMPANT et al., 2008). *B. seminalis* was able to improve rice growth by producing indoleacetic acid (IAA) and organic acids (PANHWAR et al., 2014). Here we tested the potential of TC3.4.2R3 in promoting growth of maize seeds in comparison with mutant. For the shoot length, WT and M3 had similar values to the control. Only cM3 had different mean, which was lower than the other treatments (Table 4.3). For root length, no statistical differences were found among treatments. In fresh weight treatment, it was possible to see that WT was significantly heavier than M3 and cM3 for shoot. However for roots, cM3 showed the highest fresh weight. Dry weight was the same for all strains in both shoot and root. (Table 4.3). It is possible to see that cM3 behavior oscillates between wild-type and mutant phenotypes. No evidences that glycosyltransferase could influence on bacteria-plant interaction were seen in the tested parameters. According to our results, *B. seminalis* did not influence on seed germination and plant growth, nor did glycosyltransferase play a role on this interaction.

Table 4.3- Effects of *B. seminalis* strains inoculation on the growth of maize seedlings.

Treatments	Length (cm)		Fresh weight (mg)		Dry weight (mg)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	40.87 ± 2.11	27.21 ± 3.23	1.04 ± 0.18	1.42 ± 0.24	0.10 ± 0.02	0.14 ± 0.03
WT	40.81 ± 2.49	26.93 ± 4.50	1.08 ± 0.26	0.96 ± 0.27*	0.10 ± 0.02	0.12 ± 0.03
M3	39.52 ± 2.48	27.68 ± 3.83	0.91 ± 0.21*	0.87 ± 0.23*	0.10 ± 0.02	0.10 ± 0.03
cM3	38.54 ± 2.45*	25.78 ± 4.95	0.89 ± 0.36*	1.18 ± 0.25	0.10 ± 0.02	0.14 ± 0.03

Data values are means of replicates followed by standard deviation. Means within the same column followed by * were significantly different from control at $p < 0.01$.

4.4.8 Real time qPCR

The *wcb* is located in the chromossome 1 of *B. seminalis* TC3.4.2R3 and has 43,395 bp, with 24 putative genes, distributed in 9 units of transcription and 6 operons. The genes of the *B. seminalis* TC3.4.2R3 cluster *wcb* are schematized according to their functions in Fig. 4.8. Not all species that present *wcb* cluster, present *wcbE* gene. The *wcb* cluster is similar among *Burkholderia* sp., with conserved terminal portions and a very variable central part, which could be attributed to the different lifestyles of strains (Fig. 4.9) (ARAÚJO; ARAÚJO; EBERLIN, 2017; KIM et al., 2005; SIM et al., 2010).

Figure 4.8 – Genetic context of *wcb* cluster in *B. seminalis* (*Bsem*_02938 to *Bsem*_02961).

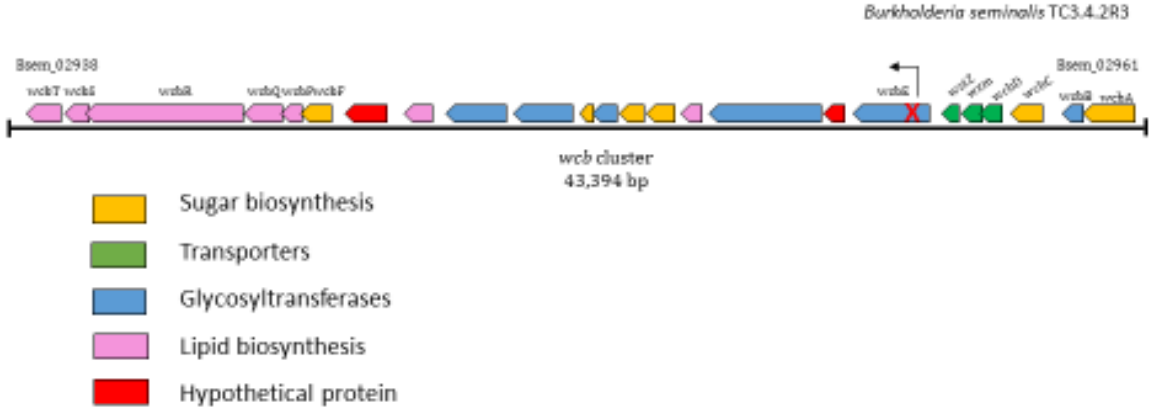
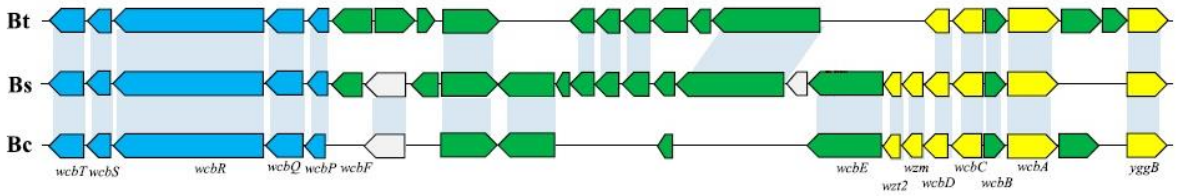


Figure 4.9 - Comparison of the *wcb* capsule synthesis region in *B. thailandensis* (Bt), *B. seminalis* (Bs), and *B. cenocepacea* (Bc).

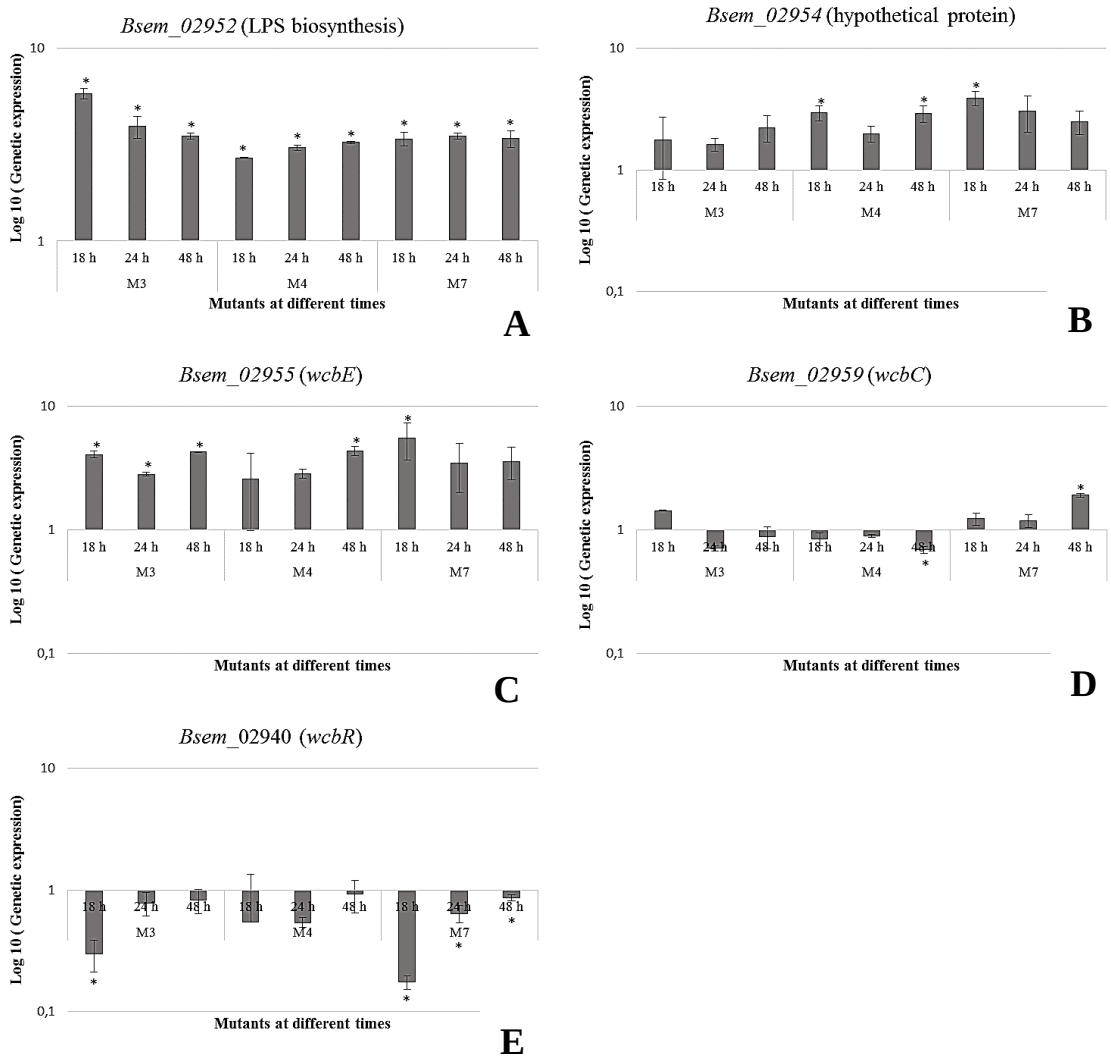


The orthologous genes in the three species are denoted with connecting lines. The putative function of the genes are color coded as lipid biosynthesis (blue), sugar biosynthesis (green), transport (yellow) and hypothetical proteins (white). Adapted from Araujo et al. (2016).

Expression of *Bsem*_02959, *Bsem*_02955, *Bsem*_02954, *Bsem*_02952 and *Bsem*_02940 genes (Table 4.1), in the mutants M3, M4 and M7 in comparison to WT was analyzed by Real Time qPCR in three different times. These times were chosen according growth curve of *B. seminalis*, at 18 h, bacteria are in the exponential phase (log), at 24 h, the stationary phase starts and at 48 h, bacteria are at the end of stationary phase. Excepting *Bsem*_02955 (*wcbE*) gene, the other genes were chosen randomly in three different operons within *wcb* cluster.

*Bsem*_02954 gene, which corresponds to a hypothetical protein and the *Bsem*_02952 gene, which encodes a lipopolysaccharide biosynthetic protein, were overexpressed in all mutants at all times tested (Fig. 4.10A). However, expression of *Bsem*_02952 gene was, at least, two times higher than that of hypothetical protein (Fig. 4.10A-B) and, there was statistical difference for all mutants at all times tested. Expression of *Bsem*_02954 gene was statistically significant only for mutants M4, at 18 h and 48 h, and M7, at 18 h (Fig. 4.10B). *Bsem*_02955 (*wcbE*) gene, which presentes TN5 insertion, was induced as well (Fig. 4.10C). It was possible to note that the amplified region did not corresponded to the insertion region and, thus, it is likely that part of glycosyltransferase gene was transcribed, which does not necessarily mean that it is being translated and processed into a functional protein. *Bsem*_02959 (*wcbC*) was repressed at all times, except at 18 h for M3 and 48 h for M7 (Fig. 4.10D). Statistically significant repression occurred in M3 at 24 h and in M4 at 48 h (Fig. 4.10D). *Bsem*_02959 gene, located in a capsular transport operon in *B. seminalis* TC3.4.2R3, had 97% similarity to a capsular polysaccharide biosynthesis / export protein of *B. cepacea*. This gene, *wcbC,* also encodes an outer membrane transporter in *B. pseudomallei* K96243, responsible for capsular polysaccharide export to the cell surface (CUCCUI et al., 2012). The *wcbR* expression was also repressed in all mutants (Fig. 4.10E). The *wcbR* gene encodes a capsular polysaccharide biosynthesis fatty acid synthase - type I PKS. Type I PKS genes are involved with secondary metabolites production, which is in agreement to mutant phenotype, once mutants lost the ability to inhibit phytopatogenic fungi (ARAÚJO; ARAÚJO; EBERLIN, 2017). Probably, the process of biosynthesis and capsular transport occur in a coordinated manner in *B. seminalis* and, therefore, the expression of the transporter *wcbC* would only occur through the presence of capsular polysaccharide, which is not possible with glycosyltransferase inactivation. Repression of *wcbC* could lead to the non-formation of capsular polysaccharide, and, hence, should be expected less biofilm and EPS production, as well as decreased virulence. Indeed, it was observed for *wcbE* mutant. Repression of *wcbR* could lead to the non-formation or decrease in the secondary metabolites production, which would result in loss of the ability in inhibiting phytopathogens, and it was surely observed (chapter 5).

Figure 4.10 – Gene expression of selected *wcb* genes in M3, M4 and M7 at different times.



The error bars represent standard deviation;* $p < 0.05$. (A) Gene expression of *Bsem*_02952, (B) Gene expression of *Bsem*_02954, (C) Gene expression of *Bsem*_02955 (*wcbE*), (D)Gene expression of *Bsem*_02959 (*wcbC*) and (E) Gene expression of *Bsem*_02940 (*wcbR*), at 18, 24 and 48 h.

In general, different times did not influence the differential expression in *B. seminalis*. A consensus was not observed in the expression results at the different times, and therefore, time was not a good variable to differentiate the genes in *wcb* cluster. There was also no pattern profile in gene expression among the mutants, probably due to the different location of the TN5 insert in each mutant, although even M4 and M7, which have insertion at a very close site, were different. Other genes of this cluster should be analyzed for expression to understand the machinery of *wcb* cluster as a whole and which genes are related to glycosyltransferase *(wcbE).*

4.5 CONCLUSION

The glycosyltransferase gene (*wcbE*) is linked not only with capsule production in *B. seminalis* TC3.4.2R3, but also other important mechanisms in the interaction in the

environment. Apparently, motility, EPS production and hydrogen peroxide resistance were not affected by the glycosyltransferase inactivation. However, motility and EPS production were affected by temperature changes. Furthermore, it seems that glycosyltransferase did not influence on seed germination nor plant-growth. At 37 ºC, mutant showed less biofilm than wild-type, highlighting the importance of glycosyltransferase in a temperature-dependent manner. In addition, at 37 ºC virulence was more severe than 28 ºC in *Galleria* infection model for both strains, the motility was also higher. Nevertheless, glycosyltransferase mutants were more attenuated than wild-type at both temperatures. Thus, biofilm and flagella could be involved in the observed virulence. 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. Thus, we can conclude that glycosyltransferase gene has a crucial role on *B. seminalis* environmental interactions.

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5 CHAPTER 5 - Biosynthesis of antifungal compounds is related to the glycosyltransferase gene (*wcbE*) in *Burkholderia seminalis* TC3.4.2R3.

5.1 ABSTRACT

The *Burkholderia* can produce several antimicrobial compounds and interact with different plant species, suggesting that could be used to development a strategy for biocontrol of phytopathogens. The *wcbE* gene codify a glycosyltransferase and belongs to *wcb* cluster, which is related to the synthesis of capsule in many *Burkholderia* species. In *B. seminalis* TC3.4.2R3 the inactivation of the *wcbE* gene caused loss of ability to inhibit *in vitro* pathogens and control orchid necrosis, caused by *B. gladioli*. Since the *wcb* cluster is related to the synthesis of capsule, the role of *wcb*E in antimicrobial synthesis is still unknow. A genome mining revealed a PKS gene in *wcb* cluster suggesting that this gene in association with *wcbE* could be related to the synthesis of a secondary metabolite. Comparisons between wild-type strain and mutant chromatograms showed two peaks missing in the mutant suggesting that these peaks could be related to glycosyltransferase gene. LC-ESI-IT-TOF analyses of those peaks detected the polyketide 10-deoxymethymycin for one peak, which has glycosyltransferases involved in the biosynthesis. The other peak had no matches in the databases and could represent a new product, which could have a biotechnological application. Even though *B. seminalis* TC3.4.2R3 is a Bcc-member, it produces antifungal compounds efficient against plant and clinical pathogens indicating that this strain may have multiple interactions in the environment.

5.2 INTRODUCTION

The *Burkholderia* are considered very effective for biological control of pathogens, since they exhibit the ability to produce antimicrobial agents against both fungi and bacteria (PARKE; GURIAN-SHERMAN, 2001; VIAL et al., 2007). Several metabolites, such as cepacins, pyrrolnitrins, cepaciamides, cepacidines, quinolones, phenazines, siderophores, lipopeptides, enacyloxins, among others (EL-BANNA; WINKELMANN, 1998; HILL et al., 1994; HUANG; WONG, 1998; MAHENTHIRALINGAM et al., 2011; MAO et al., 2006; PARKER et al., 1984; ROSS et al., 2014) with antimicrobial activity have been reported for *Burkholderia*, and most of them present antifungal activity.

Burkholderia seminalis is Gram-negative, aerobic, non-sporulated, yellow-pigmented, which belongs to the Bcc group (VANLAERE et al., 2008). *B. seminalis* strain TC3.4.2R3 was isolated from sugarcane rhizosphere and was found to control the phytopathogens *Fusarium verticiloides* and *Xanthomonas albilineans* (LUVIZOTTO et al., 2010) and orchid necrosis,

caused by *B. gladioli* (ARAÚJO et al., 2006). *Rhizoctonia solani*, the agent of rice sheath blight, was strongly inhibited by *B. seminalis* (LI et al., 2011; ZHU et al., 2016). This biocontrol ability could be associated to the synthesis pyrrolnitrin, ramnolipid, siderophores ornibactin and pyoquelin, ACC deaminase and indole-acetic acid (IAA) (ARAÚJO et al., 2016). The glycosyltransferase gene, *wcbE*, was found associated with ramnolipid and pyoquelin production, which ensured the control of *Fusarium oxysporum* (ARAÚJO; ARAÚJO; EBERLIN, 2017).

Indeed, glycosyltransferases have been found in antimicrobial biosynthesis pathways (WALSH; FREEL MEYERS; LOSEY, 2003). Garrido et al. (2006) described two glycosyltransferases genes in *Streptomyces olindensis* involved in cosmomycin biosynthesis. Two glycosyltransferase genes involved in the synthesis of naphthoxanthene were detected in *Streptomyces* spp. (KUDO et al., 2011). The antibiotic teicoplanin had two glycosyltransferases characterized in its biosynthetic gene cluster (LI et al., 2004). The glicosyltransferase, GtfB, was found to transfer glucose residues in the biosynthesis of vancomycin group antibiotics (MULICHAK et al., 2001). *Streptomyces* produces macrolide polyketide antibiotics. Its production is based on glycosyltransferase self-resistance mechanism, where a glycosylation ensures protection of microorganism against its own antimicrobial agent (BOLAM et al., 2007). The glycosyltransferase genes (*lanGT1*, *lanGT2* and *lanGT4*) are involved in landomycin biosynthesis (TAM et al., 2015; TREFZER et al., 2001). The *ocfC* gene encodes a glycosyltransferase in the *ocf* gene cluster for production of the antifungal occidiofungin in *Burkholderia contaminans* MS14 (CHEN et al., 2013). However, in none of these examples, the glycosyltransferase gene was in a capsule cluster.

Many agricultural crops have limited production due to different types of diseases caused by microorganisms such as phytopathogenic fungi. To avoid decreased quality and economic losses, several pesticides and agricultural practices have been used, which leads to environmental and health problems (RAAIJMAKERS; MAZZOLA, 2012).

The genome-guided discovery technologies combine two complementary strategies, the analysis of genome sequences and experiments to support the predictions obtained *in silico*. This approach has resulted in the discovery of several natural products from *Burkholderia* sp., making the search for these compounds faster and more punctual (ESMAEEL et al., 2016; LIU; CHENG, 2014). However, many products remain unknown (CALTEAU et al., 2014). Considering the taxonomic position of *B. seminalis*, the understanding of the molecular mechanisms and genes involved in antifungal activities could provide important information on its biocontrol potential and eliminate health risks. Herein we demonstrated the relationship

between the glycosyltransferase gene (*wcbE*) in the antimicrobial compounds biosynthesis of *B. seminalis* TC3.4.2R3 against some phytopathogenic fungi.

5.3 MATERIALS AND METHODS

5.3.1 Strains and growth conditions

B. seminalis strain TC3.4.2R3 studied in this work was obtained from sugarcane rhizosphere (LUVIZOTTO et al., 2010) and had the genome sequenced further (ARAÚJO et al. 2016). The TC3.4.2R3 mutants presented a Tn5 transposon inserted in the *wcbE* gene, which encodes a glycosyltransferase, which were called M3, M4 and M7 (ARAÚJO; ARAÚJO; EBERLIN, 2017). The complemented M3, namely cM3, presented pBBR1-MCS vector. *B. seminalis* strains were maintained at -80 ºC in Luria-Bertani (LB) broth (Difco Laboratories, Sparks, USA) and 20% glycerol, and was recovered on Luria-Bertani agar (Difco Laboratories, Sparks, USA) or broth with incubation for 24 h at 28 ºC. When required, chloramphenicol (Sigma Aldrich) was added at a concentration of 50 µg mL^{-1} for the complemented strain cM3 and kanamycin (Sigma Aldrich) at 200 µg mL^{-1} for cM3 and mutants.

The phytopathogenic fungi *Fusarium oxysporum*, *Ceratocystis paradoxa*, *Ceratocystis fimbriata* and *Colletotrichum falcatum* were grown on PDA (Potato Dextrose Agar) (Difco Laboratories, Sparks, USA) at 28 ºC. Yeasts *Candida albicans* and *Candida glabrata* were grown on Müller-Hinton (Difco Laboratories, Sparks, USA) agar at 37 ºC.

5.3.2 In silico detection of biosynthetic genes

The whole genome of *B. seminalis* strain TC3.4.2R3 was sequenced and described by Araujo et al. (2016). Detection of putative secondary metabolism and antibiotic genes in chromosome 1 of *B. seminalis* was done by the antiSMASH tool (http://antismash.secondarymetabolites.org) (WEBER et al., 2015).

5.3.3 Antifungal activity assay

Overnight *B. seminalis* strains (WT, M3, M4, M7 and cM3) were inoculated on PDA and plant pathogenic fungi mycelia disks (6 mm in diameter) were transferred to the center of the plate. Fungi tested in antagonism were *Ceratocystis fimbriata*, *Ceratocystis paradoxa*, *Colletotrichum falcatum* and *Fusarium oxysporum*. As a control, the pathogens were inoculated without *Burkholderia.* After five to ten days of incubation at 28 ºC, the inhibition zone of the pathogen was measured in relation to the control. The tests were performed in triplicate.

Bioassays with organics extracts were conducted in the same manner. Statistical analyses was carried out by one-way ANOVA with Tukey test (GraphPad Prism 6) and multiple comparisons. $P < 0.05$ was deemed statistically significant (DE FÁVARO; DE SEBASTIANES; ARAÚJO, 2012).

5.3.4 Secondary metabolites purification

B. seminalis overnight grown were inoculated in 500 mL of LB and allowed to grow for 24, 48 and 120 h at 28 ºC and 180 rpm (EL-BANNA; WINKELMANN, 1998). After this, cultures were centrifuged for 25 min, 4 ºC and 6000 rpm. Pellets were discarded and the secondary compounds in supernatant of wild-type (WT) and mutants were obtained by consecutive extractions with organic solvents of different polarities: ethyl acetate, chloroform, hexane and butanol. Solvent and supernatant were mixed at a ratio of 1:1 (v/v), and after agitation, the aqueous and organic phases were separated in a separation funnel. The organic extracts were then concentrated under vacuum at 40 °C (DE FÁVARO; DE SEBASTIANES; ARAÚJO, 2012). Extracts were dissolved in the same solvent that were extracted. Following, they were tested against molds and yeasts by adding 125, 500 or 1250 µg on filter discs (6 mm) on PDA with fungi. Controls consisted of only solvent, only fungi and hygromycin discs (25 $\mu g.ml^{-1}$). Only extracts from 120 h treatment were tested against the yeasts *Candida albicans* and *C. glabrata* through disc diffusion method (KHAN et al., 2009).

5.3.5 Secondary metabolites detection

Firstly, the metabolic profile of the crude extracts produced by the strains was analyzed by reversed phase high performance liquid chromatography (HPLC, AKTA Purifier 10, GE Healthcare) at the Department of Biochemistry and Biophysics, Instituto Butantan, São Paulo, Brazil. Crude extracts were dissolved in methanol and analyzed by a linear gradient of 0–100% of solvent B in 35 min in a C18 column (ACE® C18, 10 µm; 100 Å, 250 mm × 4.6 mm) in a two solvent system: (A) water/ trifluoroacetic acid (TFA) (99,9/0,1 %) and (B) methanol/water/TFA (90/9,9/0,1 %), at a constant flow 1.0 $mL.min^{-1}$ and absorbance at 214, 254 and 280 nm. For fractionation by solid phase extraction (SPE) using a C-18 cartridge (55 µm, 70A, Strata C18-E Phenomenex), crude extracts were dissolved in water, applied to the cartridge and extracted with methanol at 25, 40, 65 and 100%. After a bioassay against *C. paradoxa*, a new SPE was performed with methanol at 70, 90 and 100%. Then, the SPE fractions obtained and coded as SPE70, SPE90 and SPE100 were analyzed by HPLC through a linear gradient of 0–100% of solvent B in 35 min in the same condition as above, and also

used in the bioassay on *C. paradoxa*. Bioassay-guided fractionation with *C. paradoxa* was performed.

Chromatograms of the WT, M3 and cM3 SPE fractions were compared and those peaks that were present only in the WT were collected and analyzed by liquid chromatography mass spectrometry (LCMS-*IT-TOF,* Shimadzu Co., Japan).

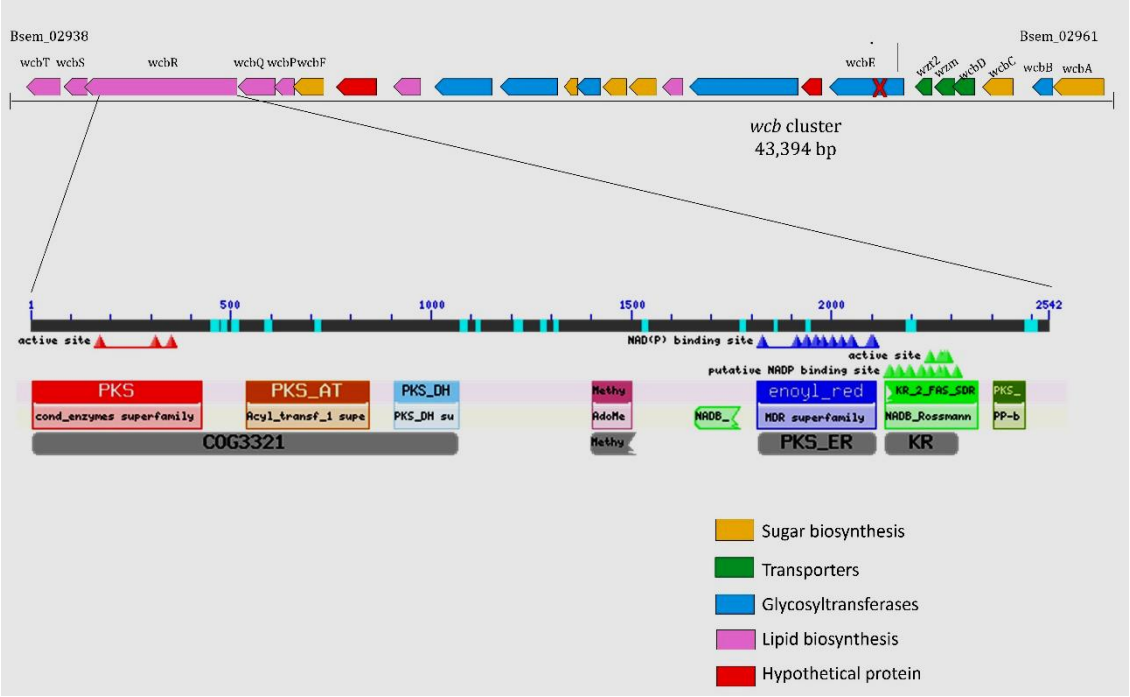
Briefly, samples were diluted in 50% methanol and manually injected into the mass spectrometer, at a constant flow of 50 µL.min^{-1} and solvent 50% methanol in water. Simultaneous positive and negative ionization was performed, by using ESI ion source. Ions were automatically fragmented by 50% collision induced dissociation (CID) to generate MS^2 spectra. Both MS and MS^2 were collected in the range of 50 to 2000 *m/z* and fragmentation patterns were compared with those reported in databases, such as MassBank (CAVALCANTE et al., 2017).

5.4 RESULTS AND DISCUSSION

5.4.1 In silico detection of biosynthetic genes

An antiSMASH homology search of genes encoding known protein motifs involved in secondary metabolism and antibiotic production revealed 4 putative clusters in the chromosome 1 of *B. seminalis* TC3.4.2R3. It was possible to predict an NRPS and a type I PKS (T1PKS) cluster, which are related to siderophore and antifungal metabolite production, respectively (SINGH; CHAUDHARY; SAREEN, 2017). Many species within *Burkholderia* genera present a high number of NRPS and PKS clusters (ESMAEEL et al., 2016). Moreover, the chromosome contained clusters for the production of arylpolyene and terpene. The T1PKS was located on *wcb* cluster (Fig. 5.1), specifically the *wcbR* gene, which encodes a capsular polysaccharide biosynthesis fatty acid synthase in *B. seminalis* TC3.4.2R3. According to antiSMASH tool, that T1PKS cluster found in *B. seminalis* has similarity with a galactoglucan biosynthetic gene cluster of *Sinorhizobium meliloti* and with O-antigen biosynthetic cluster of *B. pseudomallei* and *B. mallei.* Although the gene organization in *wcb* cluster is more similar to those described by *B. cepacia* and *B. cenocepacia*.

Figure 5.1 – Genetic context of *wcb* cluster in *B. seminalis* (*Bsem*_02938 to *Bsem*_02961).



In detail, the T1PKS in *wcbR* gene. The red X represents the transposon insertion in the glycosyltransferase gene *wcbE* presented in M3, M4 and M7 mutants.

5.4.2 Antifungal activity assay

All the mutants lost the ability to inhibit the plant pathogenic fungi *F. oxysporum*, *C. paradoxa*, *C. fimbriata* and *C. falcatum* (Table 5.1 and Fig. 5.2). The complemented strain cM3 recovered the inhibition potential. The inhibition ability seemed to be conditioned to glycosyltransferase expression.

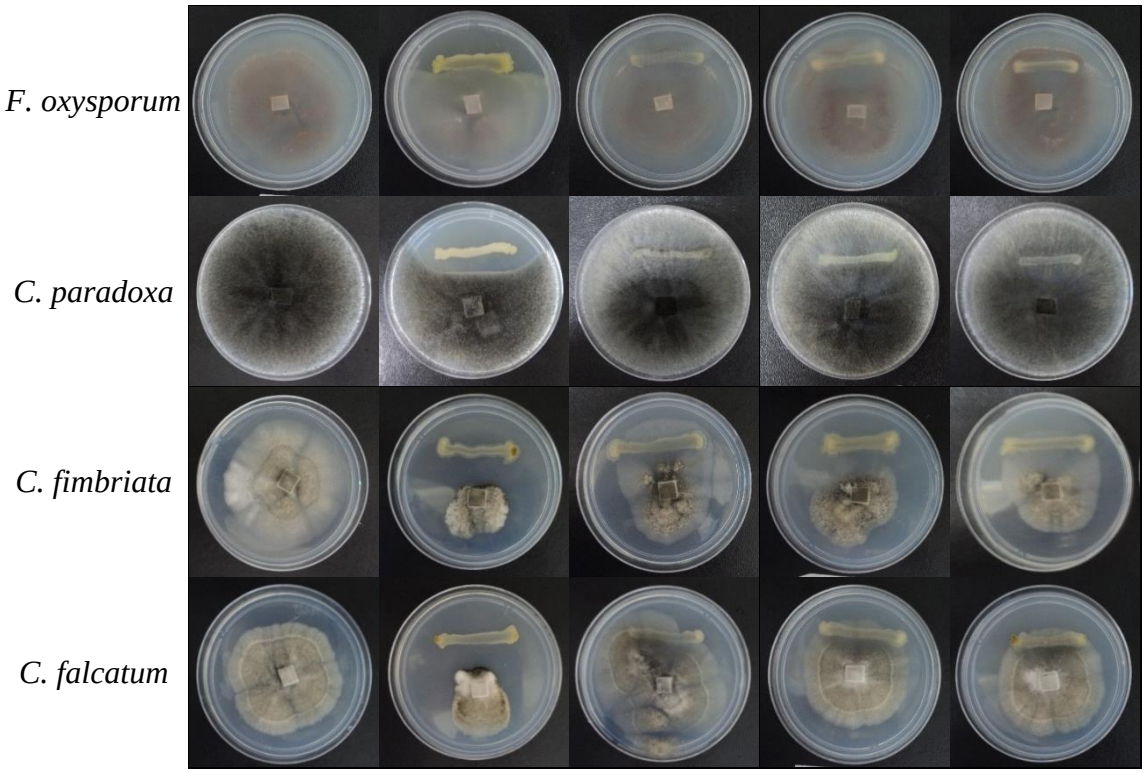
Table 5.1 – Colony diameter (cm) of plant pathogenic fungi against *B. seminalis* strains.

Treatments	**Phytopathogenic fungi**			
	F. oxysporum	*C. paradoxa*	*C. fimbriata*	*C. falcatum*
Control	8.47 ± 0.06	8.47 ± 0.06	7.67 ± 0.15	6.90 ± 0.10
WT	6.03 ± 0.06*	4.97 ± 0.15*	3.83 ± 0.15*	3.60 ± 0.10*
M3	8.43 ± 0.12	8.40 ± 0.10	7.63 ± 0.15	6.67 ± 0.15
M4	8.40 ± 0.10	8.30 ± 0.10	7.40 ± 0.17	6.57 ± 0.21
M7	8.33 ± 0.21	8.30 ± 0.20	7.50 ± 0.10	6.60 ± 0.10

Values represent the mean of triplicates following by standard variation. *Statistically significant $p < 0.05$.

Figure 5.2 – Antagonism test. Fungi *versus* *B.seminalis* Wild-Type (WT) and glycosyltransferase mutants (M3,M4 and M7).

Control **WT** **M3** **M4** **M7**



5.4.3 Secondary metabolites purification

The antagonism tests with 125 µg of organic crude extracts did not cause inhibition of fungi growth. The higher inhibition rate was observed with 1250 µg of crude extracts. Hygromycin reduced significantly the fungi growth from 25 µg. Results of antagonism tests of extracts (1250 µg) against *Fusarium oxysporum*, *Ceratocystis paradoxa*, *Colletotrichum* and *Ceratocystis fimbriata* at different times are summarized in Tables 5.2-5.5. Data are according to ANOVA and Tukey test ($p \leq 0.05$).

Table 5.2 - Biological activity of *B. seminalis* crude extracts obtained with ethyl acetate against plant pathogenic fungi.

	Ethyl acetate											
B. seminalis	***F. oxysporum***			***C. paradoxa***			***C. falcatum***			***C. fimbriata***		
	24 h	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**
M3	+	+	+	-	-	-	-	-	-	-	-	-
M4	-	+	+	-	-	-	-	+	-	-	-	-
M7	-	+	+	-	-	-	-	-	-	-	-	-
WT	+	-	+	+	+	+	+	+	+	-	-	+

The + indicates that means were statistically significant for Tukey test at $p < 0.05$. The –indicates no difference in comparison to controls.

Table 5.3 - Biological activity of *B. seminalis* crude extracts obtained with chloroform against plant pathogenic fungi.

B. seminalis	Chloroform											
	F. oxysporum			***C. paradoxa***			***C. falcatum***			***C. fimbriata***		
	24 h	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**
M3	-	-	-	-	-	-	-	-	-	-	-	-
M4	-	-	-	+	-	-	-	-	-	-	-	-
M7	-	-	-	-	+	-	+	-	-	-	+	-
WT	-	-	+	+	+	-	+	+	-	-	+	-

The + indicates that means were statistically significant for Tukey test at $p < 0.05$. The –indicates no difference in comparison to controls.

Table 5.4 - Biological activity of *B. seminalis* crude extracts obtained with hexane against plant pathogenic fungi.

B. seminalis	Hexane											
	F. oxysporum			***C. paradoxa***			***C. falcatum***			***C. fimbriata***		
	24 h	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**
M3	-	-	-	-	-	-	-	-	-	-	-	-
M4	-	-	-	-	-	-	-	-	-	-	-	-
M7	-	-	-	-	-	-	-	-	-	-	-	-
WT	-	-	-	-	-	+	-	-	+	-	-	-

The + indicates that means were statistically significant for Tukey test at $p < 0.05$. The –indicates no difference in comparison to controls.

Table 5.5 - Biological activity of *B. seminalis* crude extracts obtained with butanol against plant pathogenic fungi.

B. seminalis	Butanol											
	F. oxysporum			***C. paradoxa***			***C. falcatum***			***C. fimbriata***		
	24 h	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**
M3	-	-	-	-	-	-	-	-	-	+	+	-
M4	-	-	-	-	-	-	-	-	-	+	+	-
M7	-	-	-	-	-	-	-	-	-	+	+	-
WT	-	-	-	-	-	-	-	-	-	+	+	-

The + indicates that means were statistically significant for Tukey test at $p < 0.05$. The –indicates no difference in comparison to controls.

It was possible to observe that the extracts obtained with ethyl acetate (Table 5.2) were more effective in controlling fungi. At 120 h, WT extract inhibited the growth of all fungi. The inhibition profile for each fungus was variable when tested with the same extract, showing that the action of *B. seminalis* extracts was species-specific. Some extracts from mutants presented activity as well, which could be attributed to the different molecules extracted in the process. The inhibition profile of extracts was the same as the observed in tests with bacteria on plate, i.e., only WT had activity. Conjugations of sugars generally result in higher solubility, polarity

and chemical stability of secondary metabolites (LIANG; QIAO, 2007), thus it was expected to find the molecule(s) with a polar solvent.

Extracts obtained with Chloroform showed activity very variable through the time (Table 5.3). No activity for WT extracts against *F. oxysporum* up to 120 h, however no activity was detected at 120 h when facing other fungi. These results were inconsistent, which did not support the chloroform to extraction of *B. seminalis* compounds. For hexane extracts, only that obtained after 120 h of culture presented activity, and only against *C. paradoxa* and *Colletotrichum* (Table 5.4). The butanol extracts were effective only against *C. fimbriata*, at 24 h and 48 h (Table 5.5). From these tests, it is possible to hypothesize when a molecule is produced and consumed by the fungus. In general, extracts obtained after 120 h of bacteria growth presented more antifungal activity.

Extracts from 120 h treatment were tested against *Candida albicans* and *C. glabrata* (Table 5.6).

Table 5.6 - Biological activity of *B. seminalis* crude extracts obtained with different solvents against clinical fungi.

B. seminalis	***C. albicans***				***C. glabrata***			
	Ethyl acetate	**Chloroform**	**Hexane**	**Butanol**	**Ethyl acetate**	**Chloroform**	**Hexane**	**Butanol**
M3	-	-	-	-	+	-	-	+
M4	-	-	-	-	+	+	-	+
M7	-	-	-	-	+	-	-	+
WT	+	+	-	-	+	+	-	+

The + indicates that means were statistically significant for Tukey test at $p < 0.05$. The – indicates no difference in comparison to controls.

Only WT extracts inhibited *C. albicans*. No activity was observed with hexane and butanol extracts. For *C. glabrata*, a multidrug resistant pathogen, extracts of all strains, obtained in ethyl acetate and butanol were effective. Furthermore, chloroform extracts of M4 and WT were also able to control that yeast.

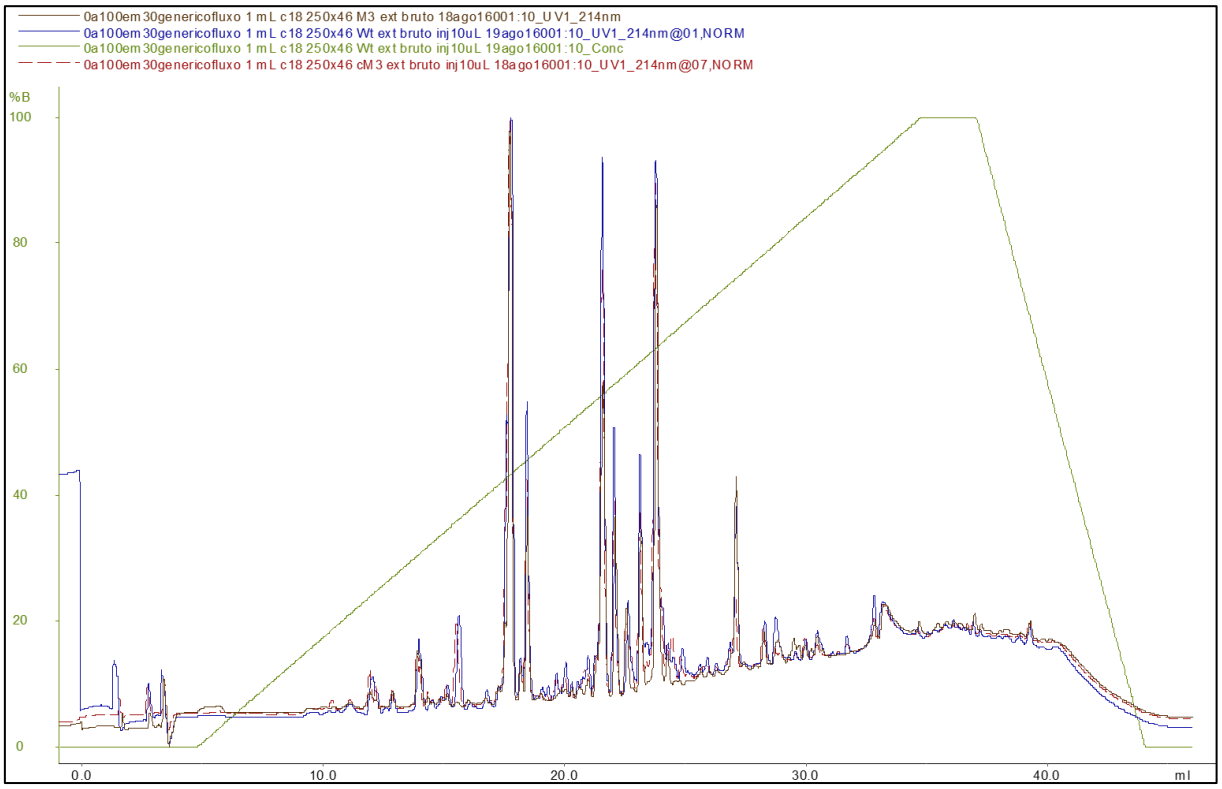
These assays demonstrated very variable activity among mutants and WT extracts. Crude extracts usually have antimicrobial property very concentrated, which might be associated to mutants extracts be able to inhibit fungi. Furthermore, it is likely that different molecules with different activities had been extracted. In general, WT extracts had more activity when obtained in ethyl acetate. Thus, the determination of secondary metabolites was done from ethyl acetate extracts.

The crude extract was fractionated using SPE. The first bioassay-guided fractionation showed activity in the SPE100 fraction and no activity was observed for SPE25, SPE40 and SPE65 fractions. In the second assay, activity was found in SPE70 and SPE90 fractions. Thus, was possible to locate a region responsible for antimicrobial activity.

5.4.4 Secondary metabolites detection

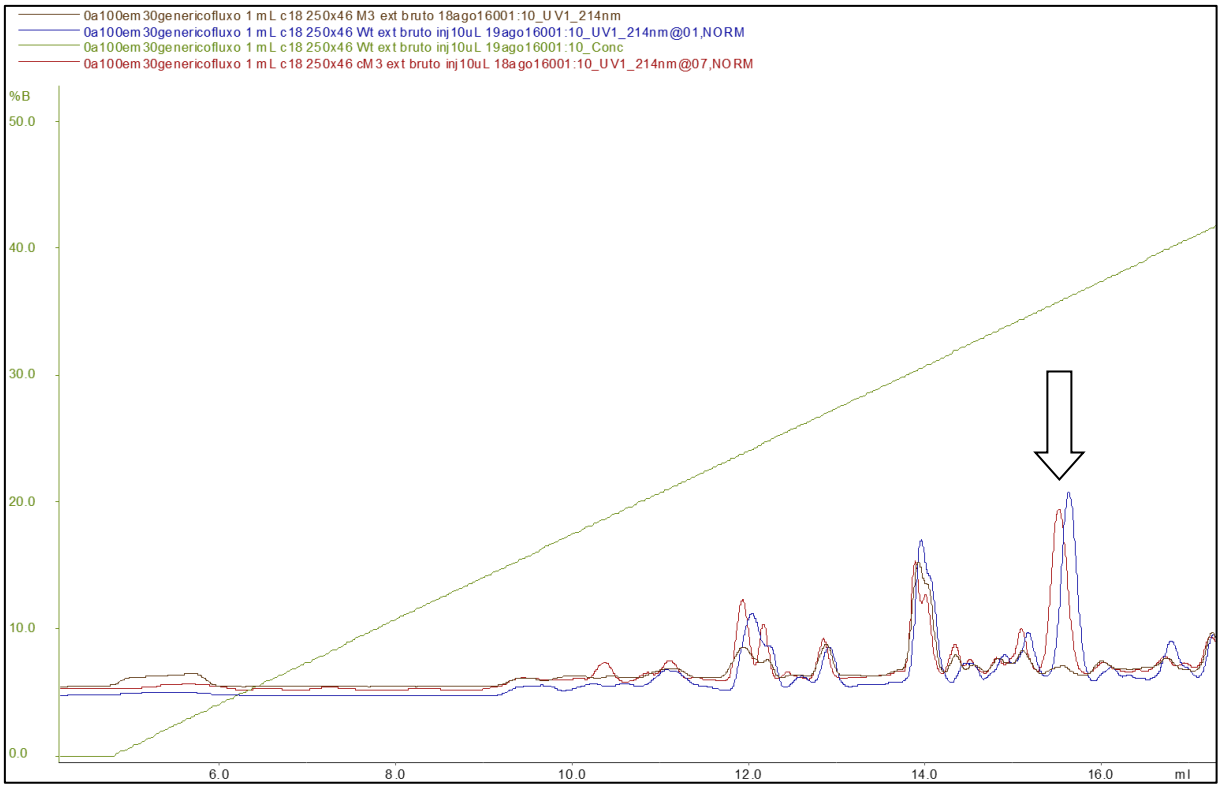
Crude extracts were analyzed by HPLC and peaks from WT, cM3 and M3 were compared (Fig. 5.3). All peaks showed absorbance at 214 nm. The chromatographic metabolic profiles of the three strains are very similar. However, two peaks were detected in WT and cM3 and missing in M3. The first peak presented retention time of 15.6 min (with 36% of B) (Fig. 5.4), and the second peak in 31.7 min (89.8% of B) (Fig. 5.5), which could be responsible for the biological activity

Figure 5.3 - Comparison of chromatographic profile of extracts from *B. seminalis* WT, M3 and cM3 at 214 nm.



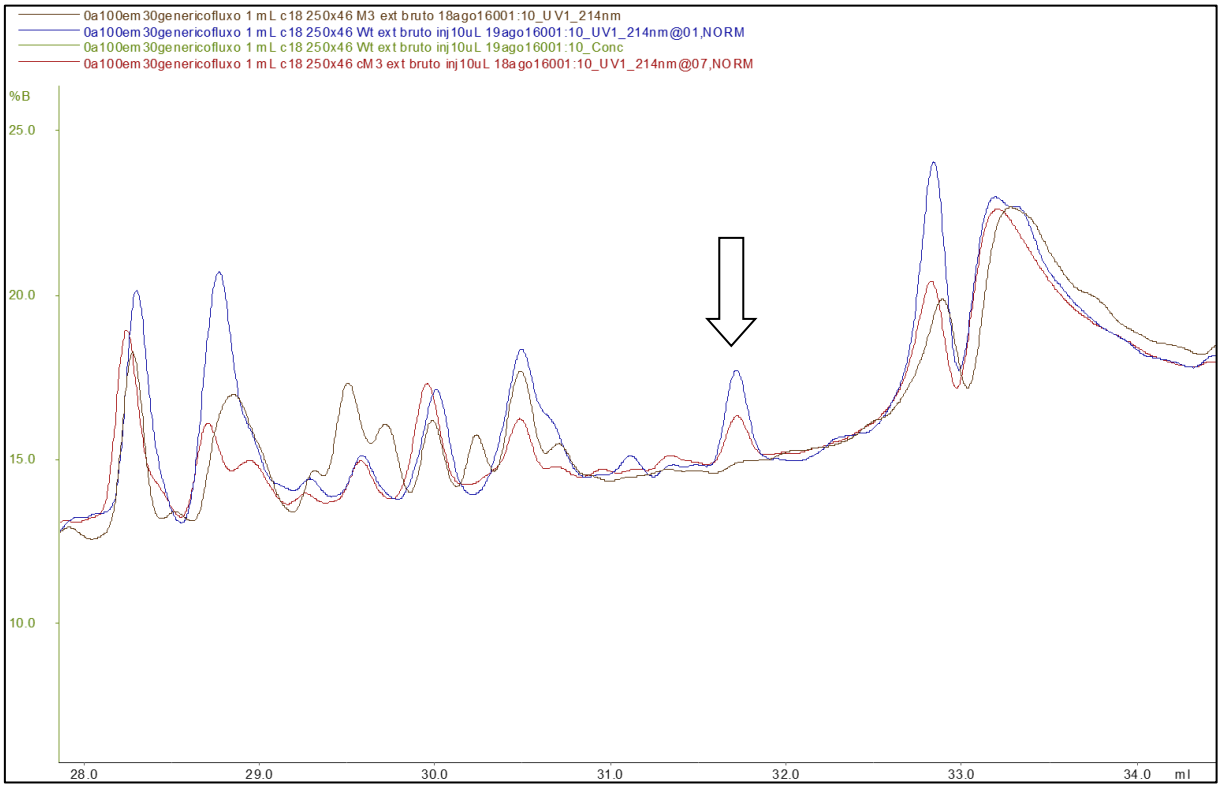
Lines: Green: concentration of B; blue: WT curve; red: cM3 curve and brown: M3 curve. Linear gradient of 0–100% of solvent B in 35 min in a C18 column in a two solvent system A and B, at a constant flow 1.0 mL.min^{-1} and absorbance at 214, 254 and 280 nm.

Figure 5.4 – Detail of peak 1 in chromatographic profile of extracts from *B. seminalis* WT, M3 and cM3 at 214 nm.



The arrow points to the peak 1 (15.6 min and 36% of B) missing in mutant. Lines: Green: concentration of B; blue: WT curve; red: cM3 curve and brown: M3 curve. Linear gradient of 0–100% of solvent B in 35 min in a C18 column in a two solvent system A and B, at a constant flow 1.0 mL.min^{-1} and absorbance at 214, 254 and 280 nm.

Figure 5.5 - Detail of peak 2 in chromatographic profile of extracts from *B. seminalis* WT, M3 and cM3 at 214 nm.



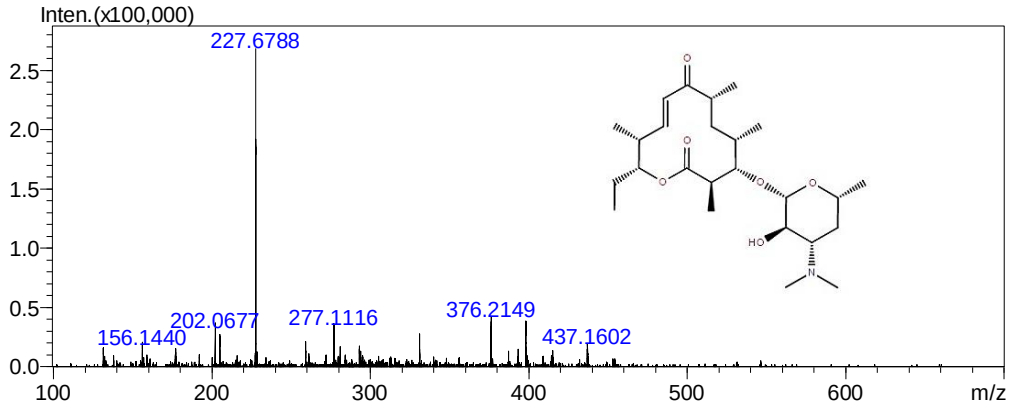
The arrow points to the peak 2 (31.7 min and 89.8% of B) missing in mutant. Lines: Green: concentration of B; blue: WT curve; red: cM3 curve and brown: M3 curve. Linear gradient of 0–100% of solvent B in 35 min in a

C18 column in a two solvent system A and B, at a constant flow 1.0 mL.min^{-1} and absorbance at 214, 254 and 280 nm.

Peaks 1 and 2 were analyzed in ESI-IT-TOF-MS. The diffuse metabolite detected for peak 1 from wild-type *B. seminalis* was *m/z* 227.6788 (Fig. 5.6). For peak 2 was detected *m/z* 344.0129 (Fig. 5.7). The *m/z* 227.6788 and its dissociation pattern of its doubly protonated molecule [M + 2H] 2+ pointed to 10-deoxymethymycin. The dissociation pattern of *m/z* 344.0129 protonated molecule [M + H] + showed no matches to the known substances.

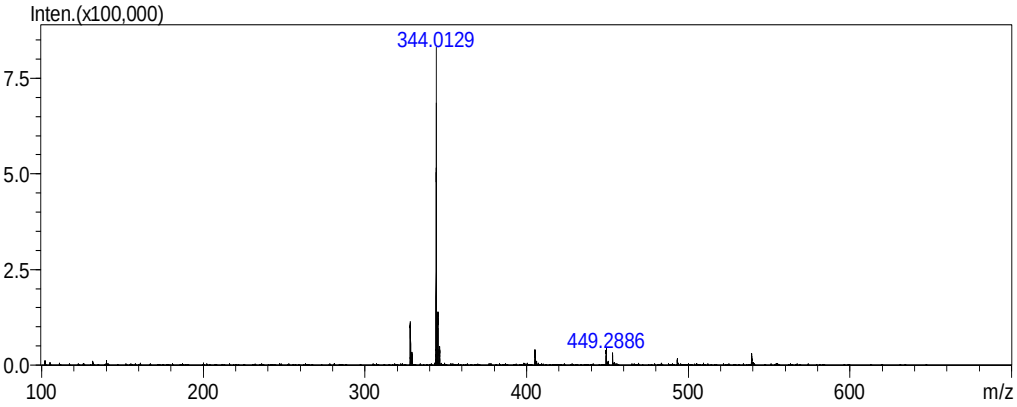
The 10-deoxymethymycin is a polyketide belonging to the macrolides class. This substance is derived from one acetate and five propionates followed by glycosylation with desosamine through the action of polyketide synthases (PKSs). That methymycin was isolated from *Streptomyces venezuelae* and presented biological activity against *Bacillus subtilis* (ZHANG; SHERMAN, 2001). The deoxysugar moiety of macrolide antibiotics is essential for the biological activity and glycosyltransferases are involved in the process by attaching sugar to specific macrolactones (HONG et al., 2004). Indeed, some *Burkholderia* might produce macrolides. *B. rhizoxina*, for example, produces rhizoxin, an antimitotic macrolide (PARTIDA-MARTINEZ; HERTWECK, 2007). Considering the role of glycosyltransferases DesVII/VIII in the biosynthesis of 10-deoxymethymycin by transferring of desosamine onto the polyketide aglycones (XUE et al., 1998), it is likely that the *wcbE* inactivation resulted in the absence of this compound or a very similar compound in the mutant. Nevertheless, the structure of *m/z* 227.6788 remains to be confirmed, since the 10-deoxymethymycin was only described for *Streptomyces venezuelae* so far (BRZOZOWSKI; WUEST, 2016). Furthermore, the deoxymethymycin biosynthetic cluster has a different organization (Fig. 5.8) from *wcb* cluster, suggesting that other gene cluster is involved. The methymycin cluster has the *pikC* gene, which encodes the only P450 hydroxylase identified in the *pik* cluster that modifies the intermediates and produces the methymycin (BRZOZOWSKI; WUEST, 2016; XUE et al., 1998). No similar hydroxylase was found in *wcb* cluster. It might be that the ion *m/z* 344.0129 represents a new molecule and is involved in the antifungal activity related to the *wcb* cluster, however further studies at structural level are necessary to confirm this hypothesis.

Figure 5.6 – LC-ESI-IT-TOF spectra of peak 1 of *B. seminalis* WT ethyl acetate extracts.



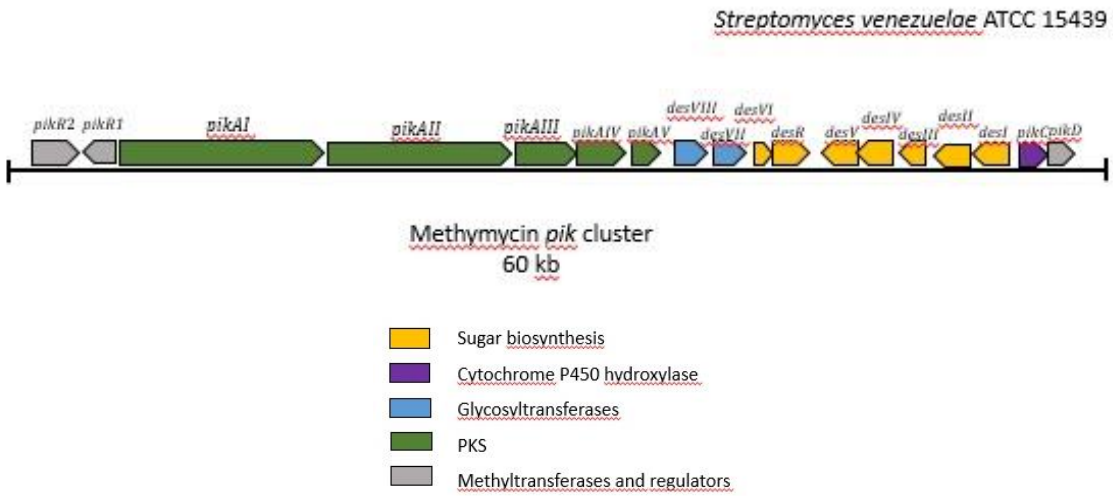
The predominance of the ion *m/z* 227.6788 detected in the extract of *B. seminalis*, and the proposed structure of a 10-deoxymethymycin.

Figure 5.7 – LC-ESI-IT-TOF spectra of peak 2 of *B. seminalis* WT ethyl acetate extracts.



The predominance of the ion *m/z* 344.0129 detected in the extract of *B. seminalis.*

Figure 5.8 – Genetic context of 10-deoxymethymycin cluster in *S. venezuelae.*



Adapted from Brzozowski; Wuest, 2016; Xue et al., 1998.

5.5 CONCLUSION

It was demonstrated that mutants deficient in the glycosyltransferase gene lost the ability to inhibit phytopathogenic fungi, highlighting the role of *wcbE* gene in the antimicrobial synthesis. The compound(s) were better extracted in ethyl acetate, probably due its polarity. 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. The other peak had no matches in the databases used, which could represent a new product to be studied structurally. *B. seminalis*, despite being a Bcc-member, was efficient in control plant and clinical pathogens, and could represent an alternative for biocontrol in crops in the future.

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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.

APPENDIX A– Transcriptome data details.

UPREGULATED GENES			
Gene	**log2(FC)***	**q_value**	**Description**
Transcription			
WJ13_RS00125	23.916	0.000466097	XRE family transcriptional regulator
WJ13_RS00630	100.006	0.000466097	family transcriptional regulator
WJ13_RS00810	122.556	0.000466097	family transcriptional regulator
WJ13_RS01205	11.698	0.000466097	Fis family transcriptional regulator
WJ13_RS02100	154.505	0.000466097	Crp Fnr family transcriptional regulator
WJ13_RS02405	106.605	0.000466097	family transcriptional regulator
WJ13_RS02455	100.479	0.000466097	family transcriptional regulator
WJ13_RS03100	141.593	0.000466097	family transcriptional regulator
WJ13_RS04290	177.877	0.000466097	family transcriptional regulator
WJ13_RS07405	209.825	0.000466097	Antitermination
WJ13_RS07430	292.053	0.000466097	DNA-directed RNA polymerase subunit beta
WJ13_RS07435	260.468	0.000466097	DNA-directed RNA polymerase subunit beta
WJ13_RS07600	204.507	0.000466097	DNA-directed RNA polymerase subunit alpha
WJ13_RS08575	108.962	0.000466097	transcription elongation fator
WJ13_RS08600	183.957	0.000466097	DNA-directed RNA polymerase subunit ômega
WJ13_RS08860	112.576	0.00907023	N utilization substance B
WJ13_RS10720	120.041	0.000466097	family transcriptional regulator
WJ13_RS11345	125.674	0.000466097	transcription elongation fator
WJ13_RS12700	110.751	0.00202726	family transcriptional regulator
nusA	173.622	0.000466097	transcription elongation fator
WJ13_RS13260	102.329	0.0197474	pseudouridine synthase
WJ13_RS14045	102.942	0.000466097	RNA polymerase subunit sigma-24
WJ13_RS14180	106.742	0.000466097	Fur family transcriptional regulator
WJ13_RS14860	106.285	0.0053698	polyribonucleotide nucleotidyltransferase
WJ13_RS14955	13.689	0.000877212	family transcriptional regulator
WJ13_RS16245	2.007	0.000466097	DNA-binding
WJ13_RS16770	183.309	0.000466097	RNA polymerase subunit sigma
WJ13_RS18100	196.598	0.00202726	RNA polymerase factor sigma-32
WJ13_RS20005	136.211	0.000466097	DNA-binding
WJ13_RS20335	198.567	0.000466097	family transcriptional regulator
WJ13_RS20340	114.819	0.00127646	family transcriptional regulator
WJ13_RS20860	201.001	0.000466097	H-NS histone
WJ13_RS21010	215.729	0.000466097	transcriptional regulator
WJ13_RS21015	2.194	0.000466097	transcriptional regulator
WJ13_RS25020	105.568	0.000466097	transcriptional regulator
WJ13_RS25115	107.606	0.000466097	family transcriptional regulator
WJ13_RS25760	143.857	0.000466097	transcription elongation fator
WJ13_RS26210	165.676	0.000466097	Rrf2 family transcriptional regulator
WJ13_RS26580	164.147	0.000466097	anti-sigma fator
WJ13_RS26585	192.682	0.000466097	RNA polymerase sigma fator
WJ13_RS27500	235.559	0.000466097	Crp Fnr family transcriptional regulator
WJ13_RS27710	22.612	0.000466097	family transcriptional regulator

WJ13_RS28995	175.666	0.00993522	family transcriptional regulator
WJ13_RS29120	115.417	0.00786374	family transcriptional regulator
WJ13_RS29330	122.203	0.000466097	RNA polymerase-binding
WJ13_RS29905	176.172	0.000466097	RNA-binding Hfq
WJ13_RS29970	111.726	0.000466097	RNA polymerase sigma fator
WJ13_RS30025	186.754	0.000466097	transcription termination factor Rho
WJ13_RS31790	124.169	0.000466097	Rrf2 family transcriptional regulator
WJ13_RS32130	163.283	0.000466097	family transcriptional regulator
WJ13_RS32225	158.118	0.000466097	family transcriptional regulator
WJ13_RS32270	106.537	0.000466097	family transcriptional regulator
WJ13_RS32475	135.501	0.000466097	family transcriptional regulator
WJ13_RS32825	157.649	0.000466097	family transcriptional regulator
WJ13_RS33185	230.141	0.000466097	DNA-binding
WJ13_RS33690	103.885	0.000466097	family transcriptional regulator
Unknown function			
WJ13_RS00820	121.512	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS01200	168.821	0.000466097	Gp49-like PF05973 family
WJ13_RS02080	104.059	0.000466097	Uncharacterized conserved DUF488 family
WJ13_RS02085	135.131	0.000466097	PF07369 family
WJ13_RS02205	248.814	0.000466097	hypothetical protein
WJ13_RS02870	112.231	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS03395	238.084	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS03490	123.362	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS03885	16.622	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS03890	125.057	0.000466097	yfdX Family
WJ13_RS05365	114.276	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS06120	111.969	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS07240	177.715	0.000466097	PF08786 domain
WJ13_RS07245	104.752	0.000466097	hypothetical protein
WJ13_RS08565	187.535	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS09250	120.664	0.003748	MULTISPECIES: DUF179 domain-containing
WJ13_RS11060	175.692	0.000466097	PF07411 domain
WJ13_RS11190	136.625	0.00933904	MULTISPECIES: hypothetical protein
WJ13_RS11200	108.509	0.000466097	hypothetical protein
WJ13_RS11255	166.967	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS11375	103.493	0.000466097	hypothetical protein
WJ13_RS11675	115.038	0.000466097	hypothetical protein
WJ13_RS13180	122.739	0.000466097	Uncharacterized conserved DUF2147 family
WJ13_RS14050	102.214	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS14485	118.755	0.000466097	CNP1-like Family
WJ13_RS15870	114.895	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS16130	103.044	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS17405	101.439	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS18605	465.716	0.000466097	PF12091 family
WJ13_RS18625	151.962	0.000466097	PF08895 domain
WJ13_RS19470	140.331	0.000466097	PF11065 family

WJ13_RS21510	169.814	0.000466097	PF12512 family
WJ13_RS21935	130.576	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS22895	227.662	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS23430	117.961	0.000466097	Sel1 repeat-containing
WJ13_RS24460	196.399	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS25135	101.223	0.000466097	PF04248 domain
WJ13_RS25300	129.096	0.000466097	PF11136 family
WJ13_RS26125	117.635	0.000466097	pentapeptide MXKDX repeat
WJ13_RS27475	260.483	0.000466097	hypothetical protein
WJ13_RS27905	159.268	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS28880	1.129	0.000466097	hypothetical protein WJ13_00945
WJ13_RS28885	106.974	0.000466097	hypothetical protein
WJ13_RS29405	151.999	0.000466097	Uncharacterised
WJ13_RS30725	127.892	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS30915	112.151	0.000466097	hypothetical protein
WJ13_RS32210	104.154	0.000466097	hypothetical protein
WJ13_RS32215	177.135	0.000466097	hypothetical protein
WJ13_RS32220	115.965	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS32830	108.325	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS33715	109.951	0.000466097	hypothetical protein
Energetic metabolism			
WJ13_RS00815	110.252	0.00166302	FAD-binding
WJ13_RS02735	171.949	0.000466097	alcohol dehydrogenase
WJ13_RS02775	167.158	0.000466097	phosphoenolpyruvate carboxykinase
WJ13_RS02785	185.464	0.00273644	isocitrate lyase
WJ13_RS06010	183.566	0.000466097	aldehyde dismutase
WJ13_RS06640	125.853	0.000466097	UTP--glucose-1-phosphate uridylyltransferase [Burkholderia stagnalis]
WJ13_RS08605	119.589	0.000466097	guanylate kinase
WJ13_RS10825	108.029	0.000466097	enoyl- hydratase
WJ13_RS10830	187.612	0.000877212	aldehyde dehydrogenase
WJ13_RS10835	143.732	0.000466097	beta-ketoadipyl thiolase
WJ13_RS11260	222.895	0.000466097	pyridine nucleotide-disulfide oxidoreductase
WJ13_RS13195	110.584	0.000877212	dihydrolipoamide dehydrogenase
WJ13_RS13200	105.657	0.000466097	dihydrolipoamide succinyltransferase
WJ13_RS13205	137.993	0.00598751	2-oxoglutarate dehydrogenase subunit E1
WJ13_RS13210	128.463	0.000466097	GTP-binding
WJ13_RS13415	143.928	0.000466097	succinyl- --3-ketoacid- transferase
WJ13_RS13420	144.263	0.000466097	succinyl- --3-ketoacid- transferase
WJ13_RS14095	111.194	0.000466097	adenylosuccinate lyase
WJ13_RS14230	214.441	0.000466097	glyceraldehyde-3-phosphate dehydrogenase
WJ13_RS14235	149.773	0.0030723	transketolase
WJ13_RS14420	111.955	0.000466097	sugar kinase
WJ13_RS15270	136.558	0.000466097	transaldolase
WJ13_RS16465	188.763	0.000466097	type II citrate synthase
WJ13_RS16470	174.239	0.000466097	MULTISPECIES: succinate dehydrogenase assembly factor 2

WJ13_RS16475	158.685	0.000466097	succinate dehydrogenase
WJ13_RS16490	165.099	0.000466097	succinate dehydrogenase
WJ13_RS16500	129.746	0.000466097	malate dehydrogenase
WJ13_RS18060	112.077	0.000466097	phosphopantetheine adenylyltransferase
WJ13_RS18330	121.381	0.000466097	phosphocarrier HPr
WJ13_RS18450	114.339	0.0189239	lipoyl synthase
WJ13_RS21225	109.249	0.00964309	aldehyde dehydrogenase
WJ13_RS21365	18.401	0.000466097	ATP synthase F0F1 subunit epsilon
WJ13_RS21370	249.625	0.000466097	ATP synthase subunit beta
WJ13_RS21375	232.301	0.000466097	ATP F0F1 synthase subunit gamma
WJ13_RS21380	211.891	0.000466097	ATP synthase subunit alpha
WJ13_RS21385	121.342	0.000466097	ATP synthase F0F1 subunit delta
WJ13_RS21390	160.691	0.000466097	ATP synthase subunit B
WJ13_RS21395	217.422	0.000466097	ATP synthase F0F1 subunit C
WJ13_RS21400	188.481	0.000466097	ATP synthase F0F1 subunit A
WJ13_RS21405	180.891	0.000466097	ATP synthase subunit I
WJ13_RS24450	128.725	0.0030723	isocitrate dehydrogenase
WJ13_RS24455	115.443	0.0105442	isocitrate dehydrogenase
WJ13_RS24480	210.206	0.000877212	ATP-dependent Clp protease ATP-binding subunit
WJ13_RS24700	194.194	0.0120222	oxidoreductase
WJ13_RS25195	141.568	0.000466097	fructose-1,6-bisphosphate aldolase
WJ13_RS25290	228.263	0.000466097	succinate-- ligase
WJ13_RS25295	243.956	0.000466097	succinyl- synthetase subunit beta
WJ13_RS27495	163.229	0.000466097	alcohol dehydrogenase
WJ13_RS29390	114.025	0.000877212	L-glyceraldehyde 3-phosphate reductase
WJ13_RS29875	112.169	0.0111359	adenylosuccinate synthetase
WJ13_RS29900	115.029	0.000466097	GTPase
WJ13_RS30070	189.363	0.000466097	pyruvate dehydrogenase
WJ13_RS30075	17.255	0.000466097	ABC transporter substrate-binding
WJ13_RS30080	140.099	0.000466097	acetoin dehydrogenase
WJ13_RS30085	136.534	0.000466097	ATP-NAD kinase
WJ13_RS30190	148.771	0.00754549	3-beta hydroxysteroid dehydrogenase isomerase family
WJ13_RS30920	179.496	0.000466097	IMP dehydrogenase
WJ13_RS30980	209.397	0.000466097	phosphoenolpyruvate synthase
WJ13_RS31205	18.833	0.000877212	isocitrate lyase
WJ13_RS31225	117.671	0.000877212	malate synthase A
WJ13_RS31695	19.263	0.000466097	enolase
WJ13_RS31980	167.261	0.00933904	acetyl- acetyltransferase
Electron Carrier Pathway			
WJ13_RS05915	129.993	0.000466097	hybrid-cluster NAD(P)-dependent oxidoreductase
WJ13_RS05920	190.487	0.000466097	(Fe-S)-binding
WJ13_RS07630	126.387	0.000466097	cytochrome C
WJ13_RS07935	116.999	0.0266157	ubiquinol-cytochrome c reductase iron-sulfur subunit
WJ13_RS07940	102.927	0.0133681	cytochrome B
WJ13_RS07945	135.602	0.000466097	cytochrome C

WJ13_RS08250	125.607	0.000466097	electron transfer flavo subunit beta [Burkholderia stagnalis]
WJ13_RS08255	130.781	0.000466097	electron transporter
WJ13_RS11000	189.347	0.000466097	glutaredoxin
WJ13_RS14820	120.697	0.000466097	NADH dehydrogenase
WJ13_RS14835	208.053	0.000466097	NADH-quinone oxidoreductase subunit A
WJ13_RS18255	102.837	0.000466097	cytochrome oxidase subunit I
WJ13_RS23805	183.748	0.000466097	(2Fe-2S)-binding
WJ13_RS24695	256.269	0.000466097	sulfite reductase
WJ13_RS26720	165.702	0.000466097	ferredoxin
WJ13_RS27485	103.647	0.000466097	cytochrome C [Burkholderia territorii]
WJ13_RS29505	141.054	0.000466097	ferredoxin--NADP(+) reductase
Translation and ribosomal proteins			
WJ13_RS07390	23.317	0.000466097	elongation factor Tu
WJ13_RS07410	276.113	0.000466097	50S ribosomal L11
WJ13_RS07415	273.445	0.000466097	50S ribosomal L1
WJ13_RS07420	306.407	0.000466097	50S ribosomal L10
WJ13_RS07425	257.066	0.000466097	50S ribosomal L7 L12
WJ13_RS07445	233.318	0.000466097	30S ribosomal S12
WJ13_RS07450	209.747	0.000466097	30S ribosomal S7
WJ13_RS07455	260.004	0.000466097	elongation factor G
WJ13_RS07460	232.918	0.000466097	elongation factor Tu
WJ13_RS07465	172.986	0.000466097	30S ribosomal S10
WJ13_RS07470	257.994	0.00166302	50S ribosomal L3
WJ13_RS07475	266.126	0.00273644	50S ribosomal L4
WJ13_RS07480	266.405	0.0474884	50S ribosomal L23
WJ13_RS07485	228.849	0.000466097	50S ribosomal L2
WJ13_RS07490	251.581	0.000466097	30S ribosomal S19
WJ13_RS07495	282.212	0.000466097	50S ribosomal L22
WJ13_RS07500	219.791	0.000466097	30S ribosomal S3
WJ13_RS07505	218.167	0.000466097	50S ribosomal L16
WJ13_RS07510	236.551	0.000466097	50S ribosomal L29
WJ13_RS07515	243.876	0.000466097	30S ribosomal S17
WJ13_RS07520	259.832	0.000466097	50S ribosomal L14
WJ13_RS07525	240.072	0.000466097	50S ribosomal L24
WJ13_RS07530	239.399	0.000466097	50S ribosomal L5
WJ13_RS07535	228.767	0.000466097	30S ribosomal S14
WJ13_RS07540	214.171	0.000466097	30S ribosomal S8
WJ13_RS07545	223.502	0.000466097	50S ribosomal L6
WJ13_RS07550	200.576	0.000466097	50S ribosomal L18
WJ13_RS07555	190.811	0.000466097	30S ribosomal S5
WJ13_RS07560	146.977	0.000466097	50S ribosomal L30
WJ13_RS07565	18.808	0.000466097	50S ribosomal L15
WJ13_RS07575	229.469	0.000466097	translation initiation factor IF-1
WJ13_RS07580	233.807	0.000466097	50S ribosomal L36
WJ13_RS07585	22.435	0.000466097	30S ribosomal S13
WJ13_RS07590	176.483	0.000466097	30S ribosomal S11

WJ13_RS07605	199.873	0.000466097	50S ribosomal L17
WJ13_RS08325	23.655	0.000466097	integration host factor subunit beta
WJ13_RS08330	237.396	0.000466097	30S ribosomal S1
WJ13_RS10170	247.613	0.000466097	30S ribosomal S9
WJ13_RS10175	23.607	0.000466097	50S ribosomal L13
WJ13_RS10925	115.936	0.000466097	GTP-binding
WJ13_RS13245	12.117	0.0139504	translation initiation factor IF-2
WJ13_RS13255	219.713	0.0237101	ribosome maturation
WJ13_RS13355	308.799	0.000466097	50S ribosomal L20
WJ13_RS13360	235.353	0.000466097	50S ribosomal L35
WJ13_RS13365	230.097	0.000466097	translation initiation factor IF-3
WJ13_RS14250	102.059	0.000466097	16S rRNA methyltransferase
WJ13_RS14500	258.538	0.000466097	50S ribosomal L27
WJ13_RS14505	258.774	0.000466097	50S ribosomal L21
WJ13_RS14865	169.836	0.000466097	30S ribosomal S15
WJ13_RS16790	173.216	0.000466097	30S ribosomal S21
WJ13_RS17980	2.017	0.000466097	ribosome hibernation promoting factor
WJ13_RS18040	188.519	0.000466097	50S ribosomal L25
WJ13_RS21055	201.712	0.000466097	30S ribosomal S21
WJ13_RS24320	24.706	0.000466097	30S ribosomal S20
WJ13_RS24435	148.536	0.000877212	elongation factor G
WJ13_RS24555	215.159	0.000466097	50S ribosomal L33
WJ13_RS24560	210.234	0.000466097	50S ribosomal L28
WJ13_RS26515	231.644	0.000466097	elongation factor P
WJ13_RS26625	2.633	0.000466097	50S ribosomal L32
WJ13_RS26630	111.131	0.000466097	MULTISPECIES: ribosomal L32p
WJ13_RS26860	176.576	0.000466097	50S ribosomal L19
WJ13_RS26875	153.736	0.000466097	30S ribosomal S16
WJ13_RS28935	180.252	0.000466097	50S ribosomal L34
WJ13_RS29140	148.492	0.000466097	peptide deformylase
WJ13_RS29340	326.365	0.000466097	23S rRNA methyltransferase
WJ13_RS30045	210.913	0.000466097	50S ribosomal L31 type B
WJ13_RS30230	240.654	0.000466097	50S ribosomal L9
WJ13_RS30235	264.664	0.000466097	30S ribosomal S18
WJ13_RS30245	223.713	0.000466097	30S ribosomal S6
WJ13_RS30965	100.194	0.000466097	#NOME?
WJ13_RS31050	113.079	0.000466097	ribosome recycling factor
WJ13_RS31060	122.257	0.000466097	elongation factor Ts
WJ13_RS31065	200.822	0.000466097	30S ribosomal S2
Amino acid biosynthesis			
WJ13_RS05615	124.411	0.000466097	branched chain amino acid ABC transporter substrate-binding
WJ13_RS05910	131.937	0.000466097	glycine betaine ABC transporter substrate-binding
glyA	130.537	0.000466097	serine hydroxymethyltransferase
WJ13_RS06460	115.781	0.000466097	glycine betaine ABC transporter substrate-binding
WJ13_RS06470	159.988	0.0053698	choline ABC transporter permease subunit

WJ13_RS06475	116.651	0.0181096	glycine betaine L-proline ABC transporter ATP-binding
WJ13_RS06815	139.459	0.000466097	glutamine--tRNA ligase
WJ13_RS07865	10.133	0.000466097	Imidazoleglycerol-phosphate dehydratase
WJ13_RS07880	12.473	0.000466097	1-(5-phosphoribosyl)-imidazole-4-carboxamide isomerase
WJ13_RS08355	123.527	0.000466097	3-phosphoserine phosphohydroxythreonine aminotransferase
WJ13_RS08595	106.147	0.00662066	guanosine-3,5-bis(diphosphate)-3-pyrophospho-hydrolase
WJ13_RS09020	108.422	0.000466097	L-isoaspartate O-methyltransferase
WJ13_RS10450	224.644	0.000466097	proline hydroxylase
WJ13_RS11995	148.298	0.000466097	saccharopine dehydrogenase
WJ13_RS13580	137.225	0.0382547	tryptophan halogenase
WJ13_RS13585	126.425	0.000466097	lysine transporter
WJ13_RS14080	122.988	0.0161888	glutamate dehydrogenase
WJ13_RS14475	100.363	0.00598751	proline--tRNA ligase
WJ13_RS14890	108.741	0.000877212	2-isopropylmalate synthase
WJ13_RS16355	122.256	0.000466097	aspartate-semialdehyde dehydrogenase [Burkholderia stagnalis]
WJ13_RS17570	131.332	0.000466097	argininosuccinate synthase
WJ13_RS20875	188.538	0.000466097	branched chain amino acid ABC transporter substrate-binding
WJ13_RS21175	112.536	0.000466097	glycine cleavage system H
WJ13_RS21450	153.299	0.000466097	branched-chain amino acid ABC transporter substrate-binding
WJ13_RS23740	100.406	0.0040761	aromatic amino acid aminotransferase
WJ13_RS24310	133.075	0.000466097	ornithine carbamoyltransferase
WJ13_RS27675	13.261	0.000466097	aminocyclopropane-1-carboxylate deaminase D-cysteine desulfhydrase family
WJ13_RS30690	109.007	0.00273644	acetylornithine deacetylase
WJ13_RS31070	138.065	0.000466097	methionine aminopeptidase
WJ13_RS31115	145.452	0.00127646	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
WJ13_RS31535	179.006	0.000466097	aspartate kinase
WJ13_RS31635	119.477	0.000466097	4-hydroxy-tetrahydrodipicolinate synthase
WJ13_RS31705	174.467	0.000466097	CTP synthetase
WJ13_RS31785	163.679	0.000466097	cysteine desulfurase
WJ13_RS32300	110.023	0.000466097	amino acid ABC transporter substrate-binding
Transmembrane transporter			
WJ13_RS03110	216.725	0.000466097	MFS transporter
WJ13_RS07400	206.613	0.000466097	pre translocase subunit
WJ13_RS07570	271.728	0.000466097	pre translocase subunit
WJ13_RS07900	138.087	0.000466097	membrane
WJ13_RS09525	160.709	0.000466097	Membrane
WJ13_RS09980	108.505	0.000466097	pre translocase subunit
WJ13_RS11340	20.076	0.000466097	Porin
WJ13_RS11350	10.491	0.000466097	Membrane
WJ13_RS14075	178.342	0.000466097	ABC transporter
WJ13_RS14565	107.172	0.00933904	pre translocase subunit

WJ13_RS17140	15.375	0.000466097	Membrane
WJ13_RS18265	110.249	0.000466097	Membrane
WJ13_RS19805	133.133	0.00964309	copper-transporting ATPase
WJ13_RS20455	10.941	0.000466097	ABC transporter
WJ13_RS21640	16.674	0.000466097	multidrug ABC transporter ATPase
WJ13_RS22905	104.029	0.000466097	copper-transporting ATPase
WJ13_RS23750	121.894	0.000466097	Membrane
WJ13_RS23755	115.599	0.000466097	periplasmic lipo involved in iron transport
WJ13_RS23860	147.876	0.000466097	MULTISPECIES: membrane
WJ13_RS24630	193.009	0.0053698	Membrane
WJ13_RS25695	195.945	0.000877212	Membrane
WJ13_RS27655	20.849	0.000466097	MULTISPECIES: membrane
WJ13_RS29395	151.947	0.000466097	Membrane
WJ13_RS29550	150.179	0.000466097	peptide ABC transporter substrate-binding
WJ13_RS29950	182.989	0.000466097	Transmembrane
WJ13_RS33720	111.908	0.000466097	alpha-ketoglutarate transporter
Outer membrane transporter and secretion systems			
WJ13_RS02095	117.302	0.000466097	metal ABC transporter ATPase
WJ13_RS02460	110.865	0.000466097	Outer membrane (porin)
WJ13_RS07255	114.085	0.0286264	type VI secretion Vgr
WJ13_RS07730	122.774	0.000466097	Membrane
WJ13_RS08370	206.186	0.000466097	Membrane
WJ13_RS08570	132.508	0.00598751	Porin
WJ13_RS11150	176.471	0.000466097	type VI secretion
WJ13_RS11155	165.238	0.00273644	type VI secretion system
WJ13_RS11160	12.928	0.00127646	family type VI secretion
WJ13_RS11165	11.688	0.000466097	type VI secretion
WJ13_RS11775	116.774	0.000466097	hemolysin secretion activation family
WJ13_RS14425	141.454	0.000466097	Membrane
WJ13_RS19450	19.852	0.000466097	ABC transporter substrate-binding
WJ13_RS31025	177.137	0.000466097	outer membrane assembly factor
Flagella and pilus			
WJ13_RS21675	11.775	0.030772	flagellar motor switch
WJ13_RS27715	208.125	0.000466097	twitching motility
WJ13_RS27810	111.851	0.000466097	ATP synthase subunit epsilon
WJ13_RS13130	116.985	0.00273644	pilus assembly
WJ13_RS13150	124.656	0.000466097	pilus assembly
WJ13_RS13160	248.613	0.000466097	pilus assembly
Hydrolases			
WJ13_RS00245	12.446	0.000466097	phosphatase
WJ13_RS05950	266.206	0.000466097	peptidase M19 [Burkholderia stagnalis]
WJ13_RS07235	12.193	0.000466097	RHS repeat-associated core domain
WJ13_RS10235	156.604	0.000466097	carboxymethylenebutenolidase
WJ13_RS12160	108.658	0.0497241	Thioesterase
WJ13_RS13155	157.025	0.000466097	prepilin peptidase
WJ13_RS13430	107.471	0.000466097	Thioesterase

WJ13_RS13990	126.945	0.00339654	Zn-dependent hydrolase
WJ13_RS14480	18.662	0.000466097	RNA pyrophosphohydrolase
WJ13_RS18315	132.459	0.000466097	peptidase S41
WJ13_RS22120	135.206	0.000466097	leucyl aminopeptidase
WJ13_RS25265	124.252	0.000466097	glycosyl hydrolase
WJ13_RS26555	13.514	0.000466097	S26 family signal peptidase
WJ13_RS26570	146.004	0.0030723	serine peptidase
WJ13_RS27605	131.284	0.000466097	glutamyl-tRNA amidotransferase
WJ13_RS29155	145.309	0.000466097	Protease
WJ13_RS29480	101.097	0.00127646	Peptidase
WJ13_RS30065	144.427	0.003748	acetoin dehydrogenase
WJ13_RS31430	101.642	0.000466097	peptidase P60
Lipid biosynthesis			
WJ13_RS10005	206.323	0.000466097	polyisoprenoid-binding
WJ13_RS10010	138.704	0.000466097	polyisoprenoid-binding
WJ13_RS11250	14.527	0.000466097	pentalenene synthase
WJ13_RS12090	122.126	0.0263965	acyl- dehydrogenase
WJ13_RS16850	106.319	0.000466097	decarboxylase
WJ13_RS18025	117.258	0.000466097	4-diphosphocytidyl-2C-methyl-D-erythritol kinase
WJ13_RS20620	118.818	0.00202726	patatin-like phospholipase family
WJ13_RS24535	209.563	0.000466097	aminotransferase
WJ13_RS24575	137.711	0.000466097	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
WJ13_RS26600	252.124	0.000466097	acyl carrier
WJ13_RS31195	125.435	0.000466097	acyl- -binding
Cell division and chromosome partitioning			
WJ13_RS13265	170.218	0.000466097	segregation and condensation B
WJ13_RS14545	113.736	0.000466097	cell division
WJ13_RS14655	100.843	0.000466097	division cell wall cluster transcriptional repressor
WJ13_RS20535	17.034	0.000466097	chromosome partitioning
WJ13_RS24790	133.594	0.000466097	cell division
WJ13_RS25740	209.429	0.000466097	cell division
WJ13_RS30575	168.848	0.000466097	trigger factor
WJ13_RS31690	110.537	0.000466097	cell division
Universal stress			
WJ13_RS12750	145.256	0.000466097	universal stress
WJ13_RS27520	115.267	0.0159381	universal stress
WJ13_RS27525	166.405	0.00166302	universal stress
WJ13_RS27535	174.316	0.000466097	universal stress
WJ13_RS27665	134.192	0.000466097	universal stress
WJ13_RS28740	107.787	0.000466097	universal stress
Cold-shock			
WJ13_RS00345	211.425	0.000466097	cold-shock
WJ13_RS02915	197.574	0.000466097	cold-shock
WJ13_RS08560	270.784	0.000466097	cold-shock
WJ13_RS11020	427.849	0.000466097	cold-shock
WJ13_RS17410	37.124	0.000466097	cold-shock

WJ13_RS24470	260.532	0.000466097	cold-shock
Heat-shock and chaperones			
WJ13_RS09835	189.711	0.0030723	molecular chaperone
WJ13_RS23605	14.899	0.000466097	heat-shock Hsp20
WJ13_RS27460	186.444	0.000466097	heat-shock Hsp20
WJ13_RS30115	212.443	0.000466097	ATP-dependent chaperone
WJ13_RS31680	105.577	0.000877212	Hsp33 chaperonin
Nitrogen metabolismo			
WJ13_RS23440	119.884	0.000466097	oxidoreductase
WJ13_RS27490	116.844	0.000466097	nitroreductase [Burkholderia territorii]
WJ13_RS31900	246.263	0.000466097	glutamine synthetase
Sulfur assimilation			
WJ13_RS10165	270.246	0.000466097	iron-sulfur cluster insertion
WJ13_RS20055	17.897	0.000466097	radical SAM
WJ13_RS24705	154.079	0.0255075	phosphoadenosine phosphosulfate reductase
WJ13_RS24710	182.915	0.000466097	sulfate adenylyltransferase
WJ13_RS24715	169.201	0.00127646	sulfate adenylyltransferase
WJ13_RS31775	149.057	0.000466097	iron-sulfur cluster assembly
WJ13_RS31780	136.429	0.000466097	scaffolding
Two-component system			
WJ13_RS02105	14.671	0.000466097	two-component system response regulator
WJ13_RS21515	162.422	0.000466097	two-component system response regulator
WJ13_RS30445	143.295	0.000466097	histidine kinase
DNA replication, recombination and repair			
WJ13_RS07735	110.883	0.00166302	transposase
WJ13_RS08365	108.161	0.0053698	DNA gyrase subunit A
WJ13_RS14435	143.572	0.000466097	ribonucleotide-diphosphate reductase subunit beta
WJ13_RS16820	13.703	0.0202423	exodeoxyribonuclease VII small subunit
WJ13_RS17905	100.158	0.000466097	single-stranded DNA-binding
WJ13_RS20545	113.145	0.000466097	initiator
WJ13_RS21560	18.354	0.000466097	DNA-binding
WJ13_RS25310	144.195	0.00273644	DNA recombination repair
WJ13_RS28920	105.522	0.000466097	DNA gyrase subunit B
WJ13_RS28925	138.082	0.000466097	DNA polymerase III subunit beta
WJ13_RS28940	108.052	0.000466097	ribonuclease P component
WJ13_RS29255	136.057	0.000466097	exodeoxyribonuclease III
WJ13_RS29850	113.908	0.000466097	DNA repair ATPase
WJ13_RS30000	122.562	0.000466097	nucleoid-associated
WJ13_RS30240	222.202	0.000466097	primosomal replication N
WJ13_RS31285	12.199	0.000466097	initiator
WJ13_RS33740	126.356	0.000466097	integrase
Nucleotide biogenesis and metabolismo			
WJ13_RS03085	298.975	0.000466097	purine-nucleoside phosphorylase
WJ13_RS05760	109.981	0.000466097	purine-nucleoside phosphorylase
WJ13_RS09790	119.713	0.000466097	phosphoribosylaminoimidazole synthetase
WJ13_RS14465	107.706	0.000466097	hypoxanthine phosphoribosyltransferase

WJ13_RS16300	138.573	0.000466097	amidophosphoribosyltransferase
WJ13_RS16870	106.021	0.00662066	AMP nucleosidase
WJ13_RS17280	150.354	0.0442946	pyridine nucleotide-disulfide oxidoreductase
WJ13_RS18035	14.444	0.000466097	ribose-phosphate pyrophosphokinase
WJ13_RS24340	122.806	0.000466097	adenylate kinase
WJ13_RS29945	194.114	0.000466097	Nucleoside-diphosphate kinase
EPS, LPS and cell wall biosynthesis			
WJ13_RS02090	145.371	0.000466097	UDP-glucose 4-epimerase
WJ13_RS09605	140.335	0.000466097	peptidoglycan-associated lipo
WJ13_RS09700	128.329	0.0348497	glycosyl transferase 2 family
WJ13_RS09705	209.274	0.000466097	glucose-1-phosphate thymidylyltransferase
WJ13_RS09710	160.126	0.0133681	dTDP-glucose 4,6-dehydratase
WJ13_RS26575	15.757	0.00127646	sugar dehydratase
WJ13_RS31700	132.536	0.000466097	2-dehydro-3-deoxyphosphooctonate aldolase [Burkholderia territorii]
Aromatic compound degradation and tolerance			
WJ13_RS03270	159.159	0.000466097	butanediol dehydrogenase
WJ13_RS03305	140.248	0.000466097	phenylacetate-- ligase
WJ13_RS07820	147.715	0.000466097	Toluene tolerance
WJ13_RS10840	156.856	0.000466097	enoyl- hydratase
WJ13_RS10845	160.654	0.000466097	phenylacetic acid degradation
WJ13_RS10850	219.371	0.000466097	phenylacetate-- ligase
WJ13_RS11010	142.336	0.000877212	dioxygenase
WJ13_RS17285	312.418	0.00339654	Rieske (2Fe-2S)
WJ13_RS17290	360.928	0.000466097	anthranilate 1,2-dioxygenase
WJ13_RS17295	328.221	0.000466097	Rieske (2Fe-2S)
WJ13_RS17310	128.836	0.000877212	muconate cycloisomerase
WJ13_RS17315	232.644	0.000466097	catechol 1,2-dioxygenase
WJ13_RS17320	289.356	0.000466097	muconolactone delta-isomerase
WJ13_RS20630	151.705	0.0050505	phenylacetate- oxygenase
WJ13_RS20635	141.205	0.000466097	phenylacetate- oxygenase subunit
WJ13_RS20640	147.051	0.000466097	phenylacetic acid degradation
WJ13_RS20645	11.206	0.000466097	phenylacetate- oxygenase subunit
WJ13_RS20650	150.641	0.000466097	phenylacetic acid degradation
WJ13_RS20675	221.258	0.000466097	4-hydroxyphenylpyruvate dioxygenase
Detoxification and response to stress			
WJ13_RS00830	113.521	0.000466097	acetoacetate decarboxylase
WJ13_RS03105	31.316	0.000466097	mannonate dehydratase
WJ13_RS07950	186.032	0.000466097	stringent starvation A
WJ13_RS07955	119.174	0.000466097	stringent starvation B [Burkholderia territorii]
WJ13_RS09520	254.881	0.000466097	peroxidase
WJ13_RS09955	194.156	0.000877212	hydroperoxidase
WJ13_RS14415	120.785	0.000466097	thiol peroxidase
WJ13_RS15665	379.546	0.000466097	alkyl hydroperoxide reductase
WJ13_RS16895	113.191	0.000466097	organic hydroperoxide resistance
WJ13_RS17110	146.584	0.000466097	rubrerythrin
WJ13_RS23895	101.226	0.000466097	chemical-damaging agent resistance C

WJ13_RS24220	106.809	0.000466097	peptide methionine sulfoxide reductase
WJ13_RS24365	14.095	0.000466097	Superoxide dismutase
WJ13_RS26565	127.125	0.000466097	glutaredoxin
WJ13_RS30020	309.709	0.000466097	Thioredoxin
WJ13_RS30220	101.628	0.000466097	phosphate transport regulator
WJ13_RS30290	136.817	0.000466097	phosphate starvation-inducible
WJ13_RS30665	323.311	0.000466097	alkyl hydroperoxide reductase
PHA and PHB synthesis			
WJ13_RS08070	123.808	0.000466097	beta-ketoacyl-ACP reductase
WJ13_RS10330	114.368	0.00202726	3-hydroxybutyrate dehydrogenase
WJ13_RS11665	203.407	0.000466097	beta-ketoacyl-ACP reductase
WJ13_RS11670	150.491	0.000466097	polyhydroxyalkanoate biosynthesis repressor
WJ13_RS17885	145.705	0.00691755	phasin
WJ13_RS23790	107.472	0.000466097	biopolymer transporter
WJ13_RS29825	111.248	0.00723284	poly-beta-hydroxybutyrate polymerase
Posttranslational modification and protein folding			
WJ13_RS09610	129.315	0.000466097	Tol system periplasmic component
WJ13_RS12670	165.023	0.003748	serine kinase
WJ13_RS17015	14.308	0.000466097	peptidylprolyl isomerase
WJ13_RS18295	119.659	0.000466097	pre translocase subunit
WJ13_RS18640	102.504	0.000466097	thiol:disulfide interchange
WJ13_RS24570	14.029	0.000466097	peptidylprolyl isomerase
WJ13_RS30485	110.318	0.000466097	methionine sulfoxide reductase B
WJ13_RS30560	157.473	0.0128314	DNA-binding
WJ13_RS30565	165.376	0.000877212	ATP-dependent Clp protease ATP-binding subunit
WJ13_RS30570	169.139	0.000466097	ATP-dependent Clp protease proteolytic subunit
WJ13_RS31565	111.485	0.000466097	peptidylprolyl isomerase
WJ13_RS31570	184.045	0.000466097	cyclophilin
WJ13_RS31600	142.557	0.000466097	isoprenylcysteine carboxyl methyltransferase
Vitamin biosynthesis			
WJ13_RS11355	133.549	0.000466097	inosine-5-monophosphate dehydrogenase
WJ13_RS17890	120.626	0.000466097	cobyric acid synthase (B12)
WJ13_RS23460	125.643	0.000466097	flavin reductase
WJ13_RS29335	120.824	0.000466097	cobalamin B12 biosynthesis
WJ13_RS29870	100.773	0.000466097	nicotinate phosphoribosyltransferase
Toxin biosynthesis			
WJ13_RS04525	115.401	0.000466097	entericidin
WJ13_RS16305	101.352	0.000466097	bacteriocin production
Others			
WJ13_RS00130	199.586	0.000466097	MULTISPECIES: phage
WJ13_RS03880	10.769	0.000466097	diguanylate cyclase
WJ13_RS05945	130.182	0.000466097	4-vinyl reductase
WJ13_RS06555	174.111	0.000466097	CBS domain-containing
WJ13_RS07305	1.138	0.00166302	acetyltransferase domain
WJ13_RS08360	170.851	0.000466097	signal peptide
WJ13_RS08840	108.563	0.00817149	methyltransferase

WJ13_RS11385	119.495	0.00472098	acyl-homoserine-lactone synthase
WJ13_RS13300	152.385	0.000466097	signal peptide
WJ13_RS19810	12.807	0.000466097	GCN5 family acetyltransferase
WJ13_RS22900	149.888	0.000466097	isoprenylcysteine carboxyl methyltransferase
WJ13_RS24300	135.472	0.000466097	nucleotide-binding
WJ13_RS24990	131.501	0.000466097	inorganic pyrophosphatase
WJ13_RS25455	121.671	0.000466097	MULTISPECIES: phage tail assembly chaperone
WJ13_RS28155	118.634	0.000466097	methyltransferase type 12
WJ13_RS30960	129.945	0.000466097	cyclase
WJ13_RS31675	157.398	0.000466097	gamma carbonic anhydrase family

***Log2(FC) = logarithm2 of fold change.**

DOWNREGULATED GENES			
Gene	**log2(FC)***	**q_value**	**Description**
Transcription			
WJ13_RS01685	-206.388	0.0281772	family transcriptional regulator
WJ13_RS01735	-330.668	0.000466097	family transcriptional regulator
WJ13_RS01925	-231.266	0.0111359	family transcriptional regulator [Burkholderia territorii]
WJ13_RS05245	-250.823	0.0318189	XRE family transcriptional regulator
WJ13_RS05315	-262.633	0.000877212	family transcriptional regulator
WJ13_RS05525	-22.478	0.0432201	family transcriptional regulator [Burkholderia territorii]
WJ13_RS05540	-203.136	0.0369331	family transcriptional regulator
WJ13_RS14205	-209.146	0.0298229	transcriptional regulator
WJ13_RS16020	-203.027	0.0373553	family transcriptional regulator
WJ13_RS30940	-18.762	0.0380149	DNA-binding
Unknown function			
WJ13_RS02140	-672.741	0.000466097	MULTISPECIES: hypothetical protein
WJ13_RS05360	-371.432	0.0199956	DUF2917 domain-containing
WJ13_RS11980	-298.486	0.0335017	PGDYG family
WJ13_RS12190	-448.151	0.0156405	PF13663 domain
WJ13_RS15595	-202.449	0.0470791	hypothetical protein
WJ13_RS16135	-415.578	0.000466097	hypothetical protein
WJ13_RS16140	-179.081	0.0417702	hypothetical protein
WJ13_RS16155	-161.723	0.0477112	conserved domain
WJ13_RS16620	-850.059	0.000466097	PF13663 domain
WJ13_RS18525	-209.279	0.0350966	tetratricopeptide repeat family
WJ13_RS20785	-289.876	0.00202726	MULTISPECIES: hypothetical protein
WJ13_RS21885	-20.415	0.0474884	DUF3348 domain-containing
WJ13_RS25970	-515.144	0.0139504	hypothetical protein
WJ13_RS26755	-260.092	0.00817149	DUF4088 domain-containing
WJ13_RS29740	-376.032	0.0485992	hypothetical protein
WJ13_RS31475	-609.187	0.0463686	PF13663 domain
WJ13_RS32995	-373.749	0.000877212	MULTISPECIES: hypothetical protein
Energetic metabolism			
WJ13_RS02695	-21.991	0.0259082	ABC transporter ATP-binding

WJ13_RS11885	-266.091	0.00166302	NAD(FAD)-dependent dehydrogenase
WJ13_RS12265	-209.735	0.0216331	glycerate dehydrogenase
WJ13_RS21040	-273.009	0.00273644	ATPase
Electron Carrier Pathway			
WJ13_RS01580	-199.064	0.0293568	cytochrome C
WJ13_RS05390	-47.283	0.0288622	ferredoxin
WJ13_RS05550	-225.147	0.0290971	dehydrogenase
WJ13_RS05755	-188.184	0.0473029	short-chain dehydrogenase
WJ13_RS24085	-17.353	0.0491447	carbohydrate kinase
Transmembrane transporter			
WJ13_RS01740	-312.382	0.0040761	lysine transporter
WJ13_RS01830	-225.174	0.0380149	lysine transporter
WJ13_RS01860	-212.463	0.00878843	MFS transporter
WJ13_RS03585	-230.334	0.0438026	amino acid transporter
WJ13_RS06785	-192.546	0.0229666	MFS transporter
WJ13_RS08125	-248.802	0.0197474	membrane
WJ13_RS11880	-270.864	0.00440179	anion permease
WJ13_RS12025	-214.715	0.0210708	biopolymer transporter
WJ13_RS12220	-185.426	0.0399881	MFS transporter
WJ13_RS12330	-201.642	0.0239631	MFS transporter
WJ13_RS12615	-245.087	0.0136598	amino acid ABC transporter permease
WJ13_RS19230	-246.291	0.00440179	hemolysin secretion D
WJ13_RS19500	-187.183	0.0427938	heme ABC transporter permease [Burkholderia territorii]
WJ13_RS20735	-188.876	0.036264	MFS transporter
WJ13_RS30930	-202.025	0.0489634	membrane
Outer membrane transporter and secretion systems			
WJ13_RS01645	-172.163	0.0499424	ABC transporter permease
WJ13_RS05270	-196.275	0.0229666	RND transporter
WJ13_RS11910	-183.048	0.0290971	RND transporter
WJ13_RS12020	-254.478	0.00878843	energy transducer
Flagella and pilus			
WJ13_RS21060	-697.371	0.000466097	flagellin
WJ13_RS21065	-262.527	0.0030723	flagellar hook
WJ13_RS21070	-808.695	0.000466097	flagellar
WJ13_RS29715	-228.248	0.00662066	flagellar biosynthesis
WJ13_RS02135	-203.362	0.036264	pilus assembly
WJ13_RS25280	-281.276	0.00993522	general secretion pathway
Hydrolases			
WJ13_RS03285	-20.496	0.036264	CAAX protease
WJ13_RS05770	-218.038	0.0169454	alpha beta hydrolase
WJ13_RS06190	-204.256	0.026176	prolyl aminopeptidase
WJ13_RS11890	-343.372	0.000877212	MBL fold metallo-hydrolase
WJ13_RS21155	-227.315	0.0305424	lipase
WJ13_RS27170	-217.151	0.0416046	phosphohydrolase
Lipid biosynthesis			

WJ13_RS27060	-460.249	0.0483777	tRNA delta(2)-isopentenylpyrophosphate transferase
Nitrogen metabolism			
WJ13_RS12215	-211.093	0.0197474	2-nitropropane dioxygenase
WJ13_RS20880	-220.468	0.0122862	nitronate monooxygenase
DNA replication			
WJ13_RS18365	-189.065	0.0279387	magnesium chelatase
Nucleotide biogenesis and metabolism			
WJ13_RS18275	-222.307	0.0256856	competence
Detoxification and response to stress			
WJ13_RS12250	-32.391	0.0458814	lactoylglutathione lyase
WJ13_RS12255	-206.841	0.0263965	hydroxymethylglutaryl- lyase
Vitamin biosynthesis			
WJ13_RS12335	-217.743	0.0358047	precorrin-4 C(11)-methyltransferase
WJ13_RS12465	-190.596	0.0339731	cobyrinic acid a,c-diamide synthase
Antibiotic and toxin biosynthesis			
WJ13_RS12430	-27.713	0.018636	uroporphyrin-III methyltransferase
Others			
WJ13_RS12085	-382.517	0.000466097	N-acetyltransferase
WJ13_RS12200	-21.152	0.0449768	GNAT family N-acetyltransferase
WJ13_RS16240	-207.169	0.0417702	aminoglycoside resistance
WJ13_RS20960	-37.172	0.0128314	chemotaxis

***Log2(FC) = logarithm2 of fold change.**