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INTER-CELLULAR COMMUNICATION (QUORUM SENSING) IN PLANT ENDOPHYTIC *BURKHOLDERIA* SP.

DISSERTATION

ZULMA ROCÍO SUÁREZ-MORENO

MENTOR: VITTORIO VENTURI PhD.

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All truths are easy to understand once they are discovered; the point is to discover them

Galileo Galilei

ABSTRACT

The genus *Burkholderia* includes over 60 species isolated from a wide range of environmental niches and can be tentatively divided into two major species cluster. The first cluster includes human and plant pathogens as the species of the *Burkholderia cepacia* complex (BCC). The other established cluster comprises at least 30 non-pathogenic species, which in most cases have been found to be associated with plants. In spite of the versatility of the *Burkholderia* genus, most of the regulation studies thus far have been performed in pathogenic species. In this study the existence of an *N*-acyl homoserine lactone (AHL) quorum sensing (QS) system in three beneficial *Burkholderia* species is investigated. Beneficial species studied are: the rice endophyte *B. kururiensis*, *B. unamae* isolated from maize rhizosphere and *B. xenovorans* LB400^{T}, a powerful degrader of recalcitrant compounds. Results indicate that the three species share an *N*-acyl homoserine lactone (AHL) quorum sensing (QS) system designated BraI/R, which is also present in other 21 species of the plant-associated *Burkholderia* cluster. Phenotypic analysis of BraI/R deficient mutants of the three species evidenced commonalities and differences in the regulation of several phenotypes, as biofilm formation, exopolysaccharide production and plant colonization. In addition, a second AHL QS system designated XenI2/R2 and an unpaired LuxR solo protein designated BxeR were also identified and characterized in *B. xenovorans* LB400^{T}. The transcriptional regulation between both *B. xenovorans* QS systems and the BxeR solo protein was evaluated. This study represents an extensive analysis of AHL QS in the *Burkholderia* plant associated cluster demonstrating commonalities as well as differences probably reflecting environmental adaptations of the various species.

POVZETEK

Rod *Burkholderia* vključuje preko 60 vrst, izoliranih iz širokega spektra ekoloških niš, ki jih lahko okvirno razdelimo v dve skupini. Prva skupina zajema človeške in rastlinske patogene, kot je na primer skupek vrst *Burkholderia cepacia* (*Burkholderia cepacia* complex; BCC), druga pa vključuje vsaj 30 nepatogenih vrst, ki so v večini primerov povezane z rastlinami. Kljub veliki raznovrstnosti rodu *Burkholderia* je bila do sedaj večina raziskav narejenih na patogenih vrstah. V okviru naše raziskave smo proučevali sistem medcelične komunikacije (quorum sensing; QS) na osnovi N-acil-homoserin-laktona (AHL) pri treh koristnih vrstah bakterij *Burkholderia*. V raziskavo smo vključili: *B. kururiensis*, endofitsko bakterijo riža, *B. unamae*, izolirano iz rizosfere koruze, ter *B. xenovorans* LB400, ki učinkovito razgrajuje nekatere zelo obstojne substance. Rezultati kažejo, da pri vseh treh vrstah obstaja sistem medcelične komunikacije QS na osnovi N-acil-homoserin-laktona (AHL) z oznako BraI/R, ki ga najdemo tudi pri drugih 21-tih vrstah iz rodu *Burkholderia*, povezanih z rastlinami. Fenotipske analize, pri katerih smo uporabili mutante BraI/R omenjenih treh vrst, so pokazale, da obstajajo med njimi tako podobnosti kot tudi razlike v regulaciji številnih fenotipskih lastnostih, kot so nastanek biofilmov, produkcija eksopolisaharidov in kolonizacija rastlin. Poleg tega smo v pri bakteriji *B. xenovorans* LB400 na nivoju medcelične komunikacije odkrili in karakterizirali še dodatni sistem AHL QS z oznako XenI2/R2 ter dodatni neparni protein LuxR z oznako BxeR. V nadaljevanju smo pri *B. xenovorans* natančneje proučili transkripcijsko regulacijo med obema sistemoma QS in neparnim proteinom BxeR. Raziskava predstavlja obsežno analizo medcelične komunikacije AHL QS v skupini bakterij *Burkholderia*, ki so povezane z rastlinami, pri čemer smo ugotovili, da obstajajo tako podobnosti kot tudi razlike med različnimi vrstami, kar je najverjetneje posledica različnih okoljskih adaptacij.

PUBLICATIONS

This thesis is based in these publications:

Suárez-Moreno ZR , Devescovi G, Myers, M, Hallack L, Mendonça-Previato L, Caballero-Mellado J and V. Venturi. (2010). Commonalities and differences in N-acyl homoserine lactone quorum sensing regulation in the novel species cluster of beneficial *Burkholderia*.*Appl Environ Microbiol.* 76, (13). 4302-4317.

Suarez-Moreno ZR, J. Caballero-Mellado, and V. Venturi. **(2008).** The new group of non-pathogenic plant-associated nitrogen-fixing *Burkholderia* spp. shares a conserved quorum-sensing system, which is tightly regulated by the RsaL repressor. *Microbiology*; 154, 2048 - 2059.

Suarez-Moreno ZR, Mendonça-Previato L, Caballero-Mellado J and V. Venturi. Common features in the novel group of plant associated *Burkholderia.* (Submitted to Archives in Microbiology).

Other publications:

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Kerényi A, **Suárez-Moreno ZR,** Venturi V and S Pongor.(2010) Multispecies microbial communities. Part II: Principles of molecular communications. Mikologia Lekarska, 17(2):113-116.

Subramoni S, Suarez-Moreno ZR, Venturi, V.2010. Lipases as Pathogenicity Factors in Plant Pathogens. *In* Handbook of Hydrocarbon and Lipid Microbiology.K. N. Timmis (ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Verlag GmbH, Heidelberg

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ABBREVIATIONS

2,4,5-T	2,4,5 trichlorophenoxyacetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AHL	Acyl-homoserine lactone
AI-2	Autoinducer 2
Approx.	Approximately
ARA	Acetylene reduction assay
ANOVA	Analysis of variance
BCC	Burkholderia cepacia complex
BNF	Biological Nitrogen Fixation
CAS	Chrome azurol S
CS	Cepacia syndrome
CDC	Center for disease control
CFU	Colony forming units
DNA	Desoxyrribonucleic Acid
DPD	4,5-hydroxy-2,3-pentanedione
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
e-DNA	Extracellular DNA
Gfp	Green fluorescent protein
HPLC	High performance liquid chromatography
HTH	Helix-Turn-Helix
HSL	Homoserine Lactone
ISR	Induced systemic resistance

KB	King'S B medium
LMG	Laboratorium voor Microbiologie, Universiteit Gent
MLST	Multilocus Sequence Typing
ORF	Open Reading Frames
PCB	Polychlorobiphenyls
PCR	Polymerase Chain Reaction
IAA	Indoleacetic Acid
PGPR	Plant growth promoting rhizobacteria
PGPB	Plant growth promoting bacteria
PCB	Polychlorobiphenyls
PAH	Polycyclic aromatic hydrocarbons
rRNA	ribosomal ribonucleic acid
Rf	Retention Factor
QS	Quorum sensing
SAM	S-adenosylmethionine
SDS-PAGE	Sodium dodecylsulphate- Polyacrylamide Gel Electrophoresis
TE	Tris EDTA
TLC	Thin Layer Chromatography
TCE	Trichloroethylene
SEM	scanning electron microscopy

1 INTRODUCTION

Bacteria may regulate the expression of their behaviours in a cell-density manner by using a sophisticated mechanism based on the production and sensing of signals, known as quorum sensing. This thesis investigates quorum sensing in the recent cluster of *Burkholderia* spp., which are most often associated to plants and also present in many different environments. In order to present the topic of this thesis, the introduction is divided in two major parts. The first part details the characteristics of the beneficial plant-associated *Burkholderia* cluster, while the second, outlines the current knowledge on quorum sensing mainly via *N*-acyl homoserine lactone signal molecules.

1.1 *BURKHOLDERIA*, A MULTIFARIOUS GENUS

The *Burkholderia* genus comprises at least 61 recognized species of Gram negative, non-spore-forming bacteria within the class of the β-proteobacteria, characterized by their remarkable diversity and ubiquity. *Burkholderia* species have been found in ground water and soil, and are able to colonize a wide range of hosts including plants, animals and fungi, with an interaction that may be pathogenic, symbiotic or both. In fact, some species have become known as opportunistic pathogens in humans, whereas others are considered powerful plant-growth promoters or environmentally beneficial (Figure 1-1). These characteristics have encouraged the study of the genus at clinical, ecological and biotechnological level. Particular emphasis has been given to understand their pathogenicity as well as traits and mechanisms underlying their multifaceted biotechnological potential (Coenye & Vandamme, 2003; Compant *et al.*, 2008b; Vial *et al.*, 2007; Woods & Sokol, 2006).

1.1.1 Taxonomy and evolution of the genus *Burkholderia*

Burkholderia was proposed as genus in 1992 by Yabuuchi *et al.*, but its former members were originally classified as *Pseudomonas* and *Bacilli* (Yabuuchi *et al.*, 1992). *Bacillus mallei* was the first species described in 1885 (Zopf, 1885), followed by *Pseudomonas andropogonis, P. gladioli* and *P. pseudomallei* (Severini, 1913; Smith & Hedges, 1905; Whitmore, 1913).

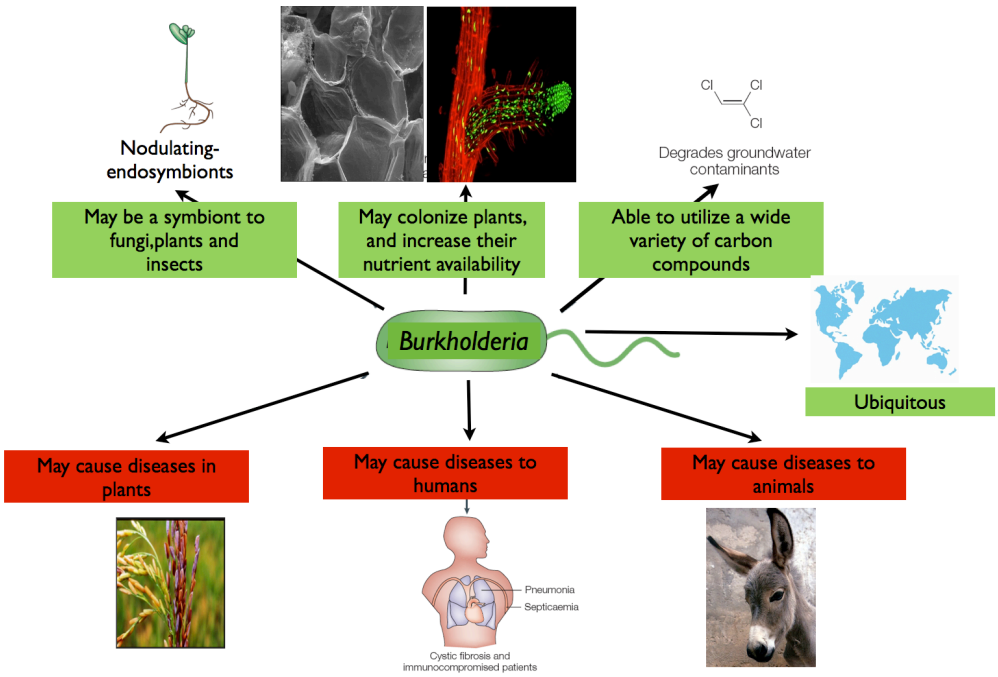


Figure 1-1.Relevant characteristics from the *Burkholderia* genus

Red squares illustrate pathogenic interactions, while green describe some beneficial properties. (Modified from Mahenthiralingham *et al.*, 2005)

In 1942, the american phytopathologist, Walter Burkholder described *Phytomonas caryophilli* and *Phytomonas alliicola* as pathogens of carnation and onion, respectively (Burkholder, 1942). In 1950, Burkholder reported the causing agent of the sour skin in onion as *Pseudomonas cepacia* (Burkholder, 1950). This species would then become the type species of the current genus, acquiring several names through the years i.e. eugonic oxidizers group 1 (Morris & Roberts, 1959), *P. kingii* (Jonsson, 1970), and *P. multivorans* (Stanier *et al.*, 1966), but the *P. cepacia* name was revived in honour of Walter Burkholder and prevailed from 1970 (Ballard *et al.*, 1970).

For a number of years, these bacteria continued to be recognized as members of the non-fluorescent *Pseudomonads*. In 1966 comprehensive studies of the *Pseudomonads* were performed highlighting the overall similarity of the so-called *mallei-pseudomallei* group by comparison of several phenotypic traits (Redfearn *et al.*, 1966; Stanier *et al.*, 1966). Palleroni and coworkers recognized the taxonomic heterogeneity among the *pseudomonads,* and delineated five species homology groups based on rRNA-DNA hybridization experiments (Palleroni *et al.*, 1973). *Pseudomonas* therefore became subdivided into five well-defined rRNA homology groups, which deserved at least five

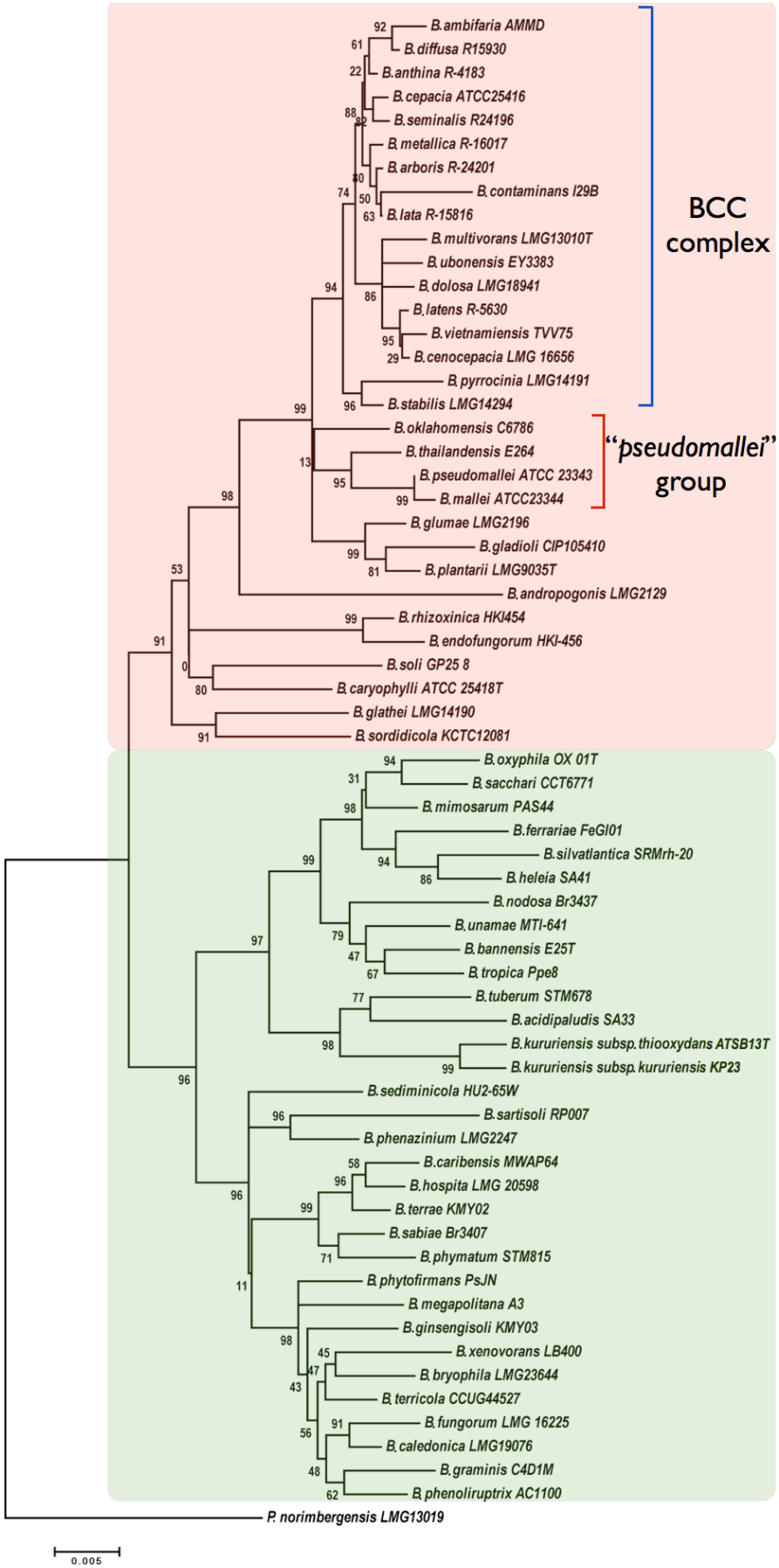
independent generic designations. Later, polyphasic taxonomy analyses including 16SrRNA sequence data, DNA-DNA hybridization and fatty acid analysis provided sufficient grounds for the creation of the *Burkholderia* genus to accommodate the seven species of the rRNA group II (*P. cepacia, P. caryophylli, P. gladioli, P. mallei, P pseudomallei, P. solanacearum* and *Pseudomonas picketti)* (Gillis *et al.*, 1995; Yabuuchi *et al.*, 1992).

Today the *Burkholderia* genus comprises 62 described species and their taxonomy has been continuously reviewed. Phylogenetic trees inferred from independent gene sequence analysis (*16SrRNA,recA, gyrB, rpoB, acdS*), have reiteratively evidenced divisions inside the *Burkholderia* genus with significant bootstrap values (>90) (Godoy *et al.*, 2003; Onofre-Lemus *et al.*, 2009; Payne *et al.*, 2005; Payne *et al.*, 2006; Tayeb *et al.*, 2008). All together, these analyses suggest that inside the *Burkholderia* cluster two main clades may be distinguished (Figure 1-2). One clade comprises the *B. cepacia* complex (BCC), the "*pseudomallei*" group, and plant-pathogens, as well as endosymbiotic species from phytopatogenic fungi. In contrast, the second clade contains a pool of non-pathogenic *Burkholderia* species associated to plants or to the environment, several of them being able to fix nitrogen and/or to degrade recalcitrant compounds.

This clustering is consistent with results from MLST (Multilocus Sequence Typing) and whole genome analysis, where species from plant-associated-nitrogen fixing clade (i.e *B. xenovorans*) locate distant from the BCC complex species and *pseudomallei* group species (Spilker *et al.*, 2008; Ussery *et al.*, 2009; Vanlaere *et al.*, 2009). Internal divisions seem to be reflective of the organisms environments, since most species included in the BCC-clade have shown pathogenic interactions with their hosts, while most species from the "plant-associated-nitrogen fixing" clade are reported to be beneficial. Details of the relationship of genome contents, virulence, and specific niche adaptation will become clearer as more *Burkholderia* genomes of diverse life style will be sequenced and available for comparative genomic analyses(Bontemps *et al.*, 2009).

Figure 1-2 Updated phylogenetic tree based on 16SrRNA sequences of the recognized species of the *Burkholderia* genus.

Sequences from type strains were aligned and the sequence of *Pandoraea norimbergensis* LMG 13019 was used as outgroup. (Bar = 0·005 nucleotide substitutions per nucleotide position). The evolutionary distances were computed using the Maximum Likelihood method and the evolutionary history was inferred using the Neighbor-Joining method. Numbers at branch nodes are bootstrap values. RED, the pathogenic *Burkholderia* clade; GREEN, Plant-associated group.


B. ambifaria AMMD
B. diffusa R15930
B. anthina R-4183
B. cepacia ATCC25416
B. seminalis R24196
B. metallica R-16017
B. arboris R-24201
B. contaminans I29B
B. lata R-15816
B. multivorans LMG13010T
B. ubonensis EY3383
B. dolosa LMG18941
B. latens R-5630
B. vietnamiensis TVV75
B. cenocepacia LMG 16656
B. pyrrocinia LMG14191
B. stabilis LMG14294
B. oklahomensis C6786
B. thailandensis E264
B. pseudomallei ATCC 23343
B. mallei ATCC23344
B. glumae LMG2196
B. gladioli CIP105410
B. plantarii LMG9035T
B. andropogonis LMG2129
B. rhizoxinica HKI454
B. endofungorum HKI-456
B. soli GP25 8
B. caryophylli ATCC 25418T
B. glathei LMG14190
B. sordidicola KCTC12081
B. oxyphila OX 01T
B. sacchari CCT6771
B. mimosarum PAS44
B. ferrariae FeGl01
B. silvatlantica SRMrh-20
B. heleia SA41
B. nodosa Br3437
B. unamae MTI-641
B. bannensis E25T
B. tropica Ppe8
B. tuberum STM678
B. acidipaludis SA33
B. kururiensis subsp. thiooxydans ATSB13T
B. kururiensis subsp. kururiensis KP23
B. sediminicola HU2-65W
B. sartisoli RP007
B. phenazinium LMG2247
B. caribensis MWAP64
B. hospita LMG 20598
B. terrae KMY02
B. sabiae Br3407
B. phymatum STM815
B. phytofirmans PsJN
B. megapolitana A3
B. ginsengisoli KMY03
B. xenovorans LB400
B. bryophila LMG23644
B. terricola CCUG44527
B. fungorum LMG 16225
B. caledonica LMG19076
B. graminis C4D1M
B. phenoliruptrix AC1100
P. norimbergensis LMG13019
0.005
BCC complex
"pseudomallei" group

1.1.2 An overview of the "pathogenic *Burkholderia*" group

In the early 1980s, *B. cepacia* became increasingly isolated in cultures of respiratory tract specimens from immunocompromised cystic fibrosis patients (Isles *et al.*, 1984). Further studies revealed that some patients might remain colonized with *B. cepacia* for prolonged periods, while others used to succumb to a rapidly progressive necrotizing pneumonia and sepsis, denominated cepacia syndrome (CS).

Polyphasic taxonomy studies revealed that *B. cepacia* was actually made up of five closely related, but distinct genomic species referred to as the *Burkholderia cepacia* complex (BCC). Each species was initially designated as a *genomovar,* which later acquired binomial species names (Vandamme *et al.*, 1997). Currently, the BCC complex is comprised of 17 species that share 98-100% similarity in the 16S rRNA, and 94-95% in the *recA* gene (See table 1-1) (Vanlaere *et al.*, 2008a; Vanlaere *et al.*, 2009).

It must be highlighted that BCC species, such as *B. vietnamiensis* and *B. ambifaria*, have also been isolated from natural environments as the rhizosphere of several plants. It is known that they may provide growth promotion to the plant and protection from attack by fungal and nematode pathogens, while others have been reported to have biodegradation properties. However, since clonally identical BCC strains may be found both in natural environments and as disease causing agents, their use in biocontrol is considered controversial, and thus rigorous protocols have been imposed to clearly establish if isolated specimens are pathogenic (Parke & Gurian-Sherman, 2001; Spilker *et al.*, 2009).

B.mallei, B. pseudomallei, B. thailandensis and *B. oklahomensis* compose the *"**pseudomallei-group**"*. *B. mallei* is the etiologic agent of glanders in horses, mules, donkeys and humans (Srinivasan *et al.*, 2001; Whitlock *et al.*, 2007), whereas *B. pseudomallei* is the causative agent of the human disease melioidosis, a potentially fatal septicemic infection frequent in the Southern Asia and Northern Australia (Inglis & Sagripanti, 2006; Levy *et al.*, 2008). *B. thailandensis* was isolated from the environment as a *B. pseudomallei*-like strain and has recently been associated to pneumonia outbreaks (Brett *et al.*, 1998; Glass *et al.*, 2006a). Similarly, *B. oklahomensis* was identified from patients accidentally exposed to ground soils in Oklahoma (Glass *et al.*, 2006b). Although all four species have been reported as animal and human pathogens, *B. thailandiensis* and *B. oklahomensis* are known to be less virulent than *B. mallei* and *B. pseudomallei* (DeShazer, 2007). Both *B. mallei* and *B. pseudomallei* are currently listed in the CDC list as bioterrorism/biothreat agents.

Table 1-1. Species comprised in the *Burkholderia* pathogenic clade

***Burkholderia* Group**	**Interaction**	**Habitat/Host**
Cepacia complex		
B. cepacia	Pathogen/Beneficial	Human, animals, plants, soil
B. cenocepacia	Pathogen	Human,
B. anthina	Pathogen	Human,
B. pyrrocinia	Pathogen	Human,
B. lata	Pathogen/environmental	Human,
B. contaminans	Pathogen/Beneficial	Human, soil
B. dolosa	Pathogen	Human,
B. difussa	Pathogen/environmental	Human,
B. ambifaria	Beneficial/Pathogen	Human,soil
B. stabilis	Pathogen	Human,
B, multivorans	Pathogen	Human,
B. latens	Pathogen	Human,
B. metallica	Pathogen	Human,
B. seminalis	Pathogen	Human,
B. arboris	Pathogen	Human,plants
B. vietnamiensis	Pathogen/beneficial	Human,soil, Rice roots
B. ubonensis	Pathogen	Human,
***Pseudomallei* group**		
B. mallei	Obligate pathogen	Animals,human,
B. pseudomallei	Oportunistic pathogen	Soil,human
B. thailandensis	Saprophyte	Soil
B. oklahomensis	Oportunistic pathogen	Soil
Phytopathogens		
B. gladioli	Pathogen	Gladiolus, Iris
B. glumae	Pathogen	Rice
B. caryophilli	Pathogen	Plants
B. andropogonis	Pathogen	Sorghum, clover, Orchids
B. plantarii	Pathogen	Rice
Endosymbionts of pathogenic fungi		
B. sordidicola	Endosymbiont	*Phanerochate sordida*
B. rhizoxinica	Endosymbiont	*Rhizopus microsporus*
B. endofungorum	Endosymbiont	*Rhizopus microsporus*

Some *Burkholderia* may also be pathogens of plants. *B. caryophylii* was described as pathogen of carnation (*Dyanthus caryophyllus*) but also causes onion rot and wilt in Russel prairie gentian (*Eustoma grandiflorum*)(Burkholder, 1942; Furuya *et al.*, 2000). *B. plantarii* causes seedling blight of rice (*Oryza sativa*) by producing tropolone, a phytotoxin responsible of root growth inhibition and wilting of the seeds (Azegami *et al.*, 1988; Urakami *et al.*, 1994). Analogously, *B. glumae* causes panicle blight in rice and synthesizes toxoflavin, considered essential for the disease in rice (Jeong *et al.*, 2003; Urakami *et al.*, 1994). *B. andropogonis*, is responsible of the stripe disease in sorghum (*Andropogon* sp.), leaf spot of clover (*Trifolium* sp.) and wilt in other 52 plant species (Coenye *et al.*, 2001a; Smith & Hedges, 1905). *B. gladioli* is known to induce bacterial soft rot in onions, leaf

sheath browning and grain rot in rice and leaf and corm diseases in *gladiolus* and iris species (Severini, 1913).

Other *Burkholderia* species are reported to be endosymbionts of phytopathogenic fungi (Partida-Martinez & Hertweck, 2005). *B. rhizoxinica* and *B. endofungorum* live within the fungal cells of *Rhizopus microsporus*, and are the true producers of the mycotoxins causing the rice seedling blight (Partida-Martinez & Hertweck, 2005; Partida-Martinez *et al.*, 2007). Similarly, *B. sordidicola* was found to be associated to *Phanerochate sordida*, which inhabits fallen branches of hardwood trees (Lim *et al.*, 2003). This diversity in the hosts range evidences the high versatility of the *Burkholderia* species.

1.1.3 The "Beneficial plant-associated *Burkholderia*" cluster

Two findings had a strong impact in the ecological perception of the *Burkholderia* species: (1) the identification of nitrogen fixation in *Burkholderia* species other than *B. vietnamiensis*, which belongs to the BCC, as for example in "*B. brasilensis*" and "*B. kururiensis*" (Baldani *et al.*, 1997b); and (2) the description of legume-nodulating *Burkholderia* and their subsequent characterization as real endosymbionts (Moulin *et al.*, 2001). These two insights, together with the increased exploration of plant growth promoting rhizobacteria (PGPR), led to the characterization of a considerable number of *Burkholderia* species, both from rhizosphere and/or endosphere, many of them diazotrophs and /or legume nodulating (Bontemps *et al.*, 2009).

As noted previously, these non-pathogenic plant-associated species constitute a single clade phylogenetically distant from the BCC-group. The main commonality among these *Burkholderia* is that their interaction with their host or with the environment has been reported to be beneficial, or neutral but not detrimental. Also, *Burkholderia* from this group are mostly associated to plants, although some species may also survive in sediments and bulk soil (Lim *et al.*, 2008). Remarkably, several species from this group may convert nitrogen in ammonia via biological nitrogen fixation (BNF). In addition, most of them are catabolically versatile enabling them to degrade recalcitrant compounds and tolerating environments with limited nutrient availability. As it will be described below, some species are able to promote the plant growth, while others are proposed for biotechnological uses as the phytoremediation. This species group will be denoted here as the "Beneficial plant-associated *Burkholderia* cluster", and their main features are summarized in Table 1-2.

Table 1-2. Main characteristics of the plant–associated beneficial *Burkholderia* group

SPECIES	ISOLATED FROM	HOST	RELEVANT CHARACTERISTICS	REFERENCE
B. kururiensis	ENDOPHYTE RIZOSPHERE TCE[3]-SOIL	*Oryza sativa* *Manihot sculenta* *Mussa acuminata* *Nicotiana tabacum*	***nif+acdS.*** Plant-growth promotion. aromatic compounds degradation.	(Anandham *et al.*, 2009; Baldani *et al.*, 1997b)
B. unamae	ENDOPHYTE ROOTS RHIZOSPHERE	*Zea mays* *S. officinarum* *Coffea Arabica*	***nif+.acdS.*** Phenol and benzene degradation. Plant growth promotion.	(Caballero-Mellado *et al.* , 2004)
B. xenovorans	RHIZOSPHERE PCB-polluted SOIL	*S. lycopersicum* *Coffea arabica*	***nif***+, ***acdS.***PCB degradation. Associated to plants.	(Bopp, 1986; Goris *et al.*, 2004)
B. beleia	RHIZOSPHERE	*Eleocharis dulcis*	***nif+.*** Grow in acidic environments.	(Aizawa *et al.* , 2009a)
B. silvatlantica	RHIZOSPHERE ENDOSPHERE	*Zea mays* *S. officinarum* *A. comosus*	***nif+.acdS.*** Plant growth promotion	(Perin *et al.* , 2006)
B. tropica	RHIZOSPHERE ENDOPHYTE	*S. officinarum* *L. esculentum* *Zea mays* *A. comosus*	***nif+.acdS.*** Production of PHAs and EPS. Plant growth promotion	(Reis *et al.* , 2004)
B. acidipaludis	ENDOPHYTE RHIZOSPHERE	*Eleocharis dulcis*	Alluminium tolerant	(Aizawa *et al.* , 2009b)
B. ginsengisoli	RHIZOSPHERE	*Panax ginseng*	ß-galactosidase activity	(Kim *et al.* , 2006)
B. phytofirmans	ENDOPHYTE, RHIZOSPHERE	*Allium cepa* *Solanum spp.* *Oryza sativa*	***acdS.***Plant growth promotion, antifungal activity.	(Frommel *et al.*, 1991; Sessitsch *et al.*, 2005)
B. sartisoli	RHIZOPHERE PAH-SOIL COMPOST	*Zea mays*	Aromatic compound degradation	(Vanlaere *et al.* , 2008)
B. graminis	RHIZOSPHERE	*Zea mays L.* *deliciosus- P.pinea* *S. lycopersicum*	***acdS.*** May induce systemic resistance and tolerance to salt and drought.	(Viallard *et al.* , 1998) (Barriuso *et al.*, 2005)
B. terrae	SOIL RHIZOSPHERE	Broad-leaved soil forest *Lycopersicum esculentum*	***nif+, nod+.***	(Yang *et al.* , 2006) (Wong-Villarreal & Caballero-Mellado, 2010)
B. caledonica	RHIZOSPHERE SANDY SOIL	*Vitis vinifera*	***acdS.***	(Coenye *et al.* , 2001)
B. phenoliruptrix	Isolated from a chemostat with 2,4,5 T	*Unknown*	***acdS.*** Degrades halogen-phenol substituted compounds	(Coenye et al. , 2004; Kellogg et al. , 1981; Kilbane et al. , 1982)
B. bryophila	Associated to mosses	*A. palustre,* *S. palustre,* *S. rubellum*	Growth Promotion in Lettuce. Antifungal activity	(Vandamme *et al.* , 2007)
B. megapolitana	Associated to mosses	*A. palustre*	Plant Growth Promotion Antifungal activity	(Vandamme *et al.* , 2007)
B. mimosarum	NODULES	*Mimosa pigra* *Mimosa pudica* *Mimosa scrabella*	***nod+, nif+.*** Main endosymbiont *Mimosa* spp.	(Chen *et al.* , 2006)
B. nodosa	NODULES	*M. bimucronata* *M. scrabella*	***nif+, nod+.*** Nodulates *Mimosa* roots	(Chen *et al.* , 2007)
B. tuberum	NODULES	*Asphatlatus carnosa* *Cyclopia* spp.	***nif+, nod+,acdS.*** Nodulates several plants	(Moulin *et al.* , 2001; Vandamme *et al.* , 2002)
B. sabiae	NODULES	*Mimosa caesalpiniifolia*	***nif+,nod+.*** PHA production	(Chen *et al.* , 2008)

SPECIES	ISOLATED FROM	HOST	RELEVANT CHARACTERISTICS	REFERENCE
B. phymatum	NODULES	*Machaerium lunatum* *Mimosa spp.* *Phaseolus vulgaris*	***nif+, nod+, acdS.*** N-fixation *in vivo*, and *ex planta.*	(Chen *et al.*, 2003; Talbi *et al.*, 2010; Vandamme *et al.*, 2002)
B. caribensis	NODULES, VERTISOIL	*Mimosa diplotricha*	***nif+, nod, acdS.*** High EPS production	(Achouak *et al.* , 1999)
B. saccharii	SOIL	*Saccharum officinarum*	PHA production	(Bramer *et al.* , 2001)
B. ferrariae	SOIL	Iron ore	***nif+*** Phosphate solubilizer	(Valverde *et al.* , 2006)
B. hospita	SOIL	A/B horizon agricultural soil	Acceptor of plasmids pJP4. pEMT1	(Goris *et al.* , 2002)
B. terricola	SOIL		***acdS.*** Acceptor of plasmids pJP4. pEMT1	(Goris *et al.* , 2002)
B. phenazinium	SOIL Associated to mosses	Unknown *S. rubellum*	Produces ionidin Acidophilic	(Izumi *et al.*, 2010; Viallard *et al.*, 1998)
B. oxyphila	ACIDIC SOIL		Catabolizes (+)-catechin into taxifolin.	(Otsuka *et al.*, 2010)
B. bannensis	RHIZOSPHERE	*Panicum repens*	***nif+,***	(Aizawa *et al.*, 2010)
B. sediminicola	WATER SEDIMENTS		PHA production	(Lim *et al.* , 2008)
B. fungorum	FUNGAL Endosymbiont	*P. chrysosporium*	***acdS.*** Aromatic compound degrader	(Coenye et al. , 2001)

Gray rows highlight the species studied in this thesis. ***nif+*** verified presence of nifH; ***nod*** plant endosymbionts, ***acdS*** verified presence of ACC deaminase gene; **PCB** polycholobiphenyl **PHA**, polyhydroxyalcanoates; **EPS**, exopolysaccharide production; **TCE** Trichloroethylene; **PAH** polyaromatic hydrocarbons.

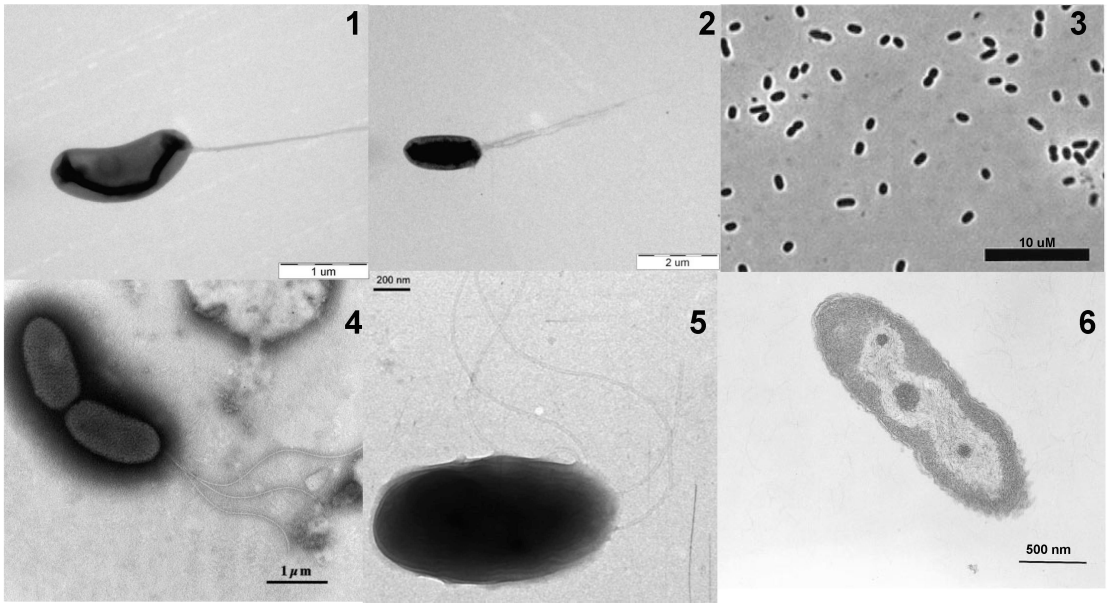


Figure 1-3. Micrographs of some beneficial plant-associated *Burkholderia* species

1. B.terrae ,2. *B. gisengisoli* ,3. *B. kururiensis*, 4. *B. tropica*, 5.*B. phymatum*, 6. *B. kururiensis.* Taken from (Kim *et al.*, 2006; Reis *et al.*, 2004; Vandamme *et al.*, 2002; Yang *et al.*, 2006; Zhang *et al.*, 2000)

1.1.3.1 Characteristics of the beneficial plant associated group

1.1.3.1.1 Microbiological features

Members of this cluster share the main microbiological characteristics of other *Burkholderia* species. Cells are single or in pairs, straight or curved rods, but not helical. Size may vary between 0.3 to 1 μm x 1.5 to 4 μm (Figure 1-3). Their optimal growth temperature is 30 °C, although some species may also grow at 37°C, such as *B. kururiensis* (Zhang *et al.*, 2000). The optimal pH is between 3 and 7, although in *B. heleia* and *B. oxyphila* tolerance to acidic conditions has been reported (Aizawa *et al.*, 2009a; Otsuka *et al.*, 2010). Usually these species are motile by means of one or several polar flagella, with exception of *B. kururiensis, B. heleia, B. bryophila and B. megapolitana* (Aizawa *et al.*, 2009a; Vandamme *et al.*, 2007; Zhang *et al.*, 2000). Poly-B-hydroxybutyrate (PHB) may be accumulated as carbon reserve material, as observed in *B.sabiae, B. sacchari, B. sediminicola* and *B.tropica.* (Bramer *et al.*, 2001; Chen *et al.*, 2008; Lim *et al.*, 2008; Reis *et al.*, 2004). In most species pigment production is not present, with the exception of *B. phenazinium,* which produces ionidine in the presence of L-threonine (Byng & Turner, 1976; Messenger & Turner, 1983; Viallard *et al.*, 1998).

Most *Burkholderia* members of this group have a strictly respiratory type of metabolism with oxygen as terminal electron acceptor, and with the exception of *B. sartisoli,* most species are also able to reduce nitrate to nitrite, but do not denitrify (Vanlaere *et al.*, 2008b). Similarly, with the exception of a recently proposed strain of *B. kururiensis* subsp. *thiooxydans,* none of the *Burkholderia* species from this group has exhibited chemolithoautotrophic growth with thiosulfate, tetrathionate or sulfur (Anandham *et al.*, 2009). The typical cellular fatty acids in plant beneficial *Burkholderia* contain 14, 16, 17 and 18 carbon atoms ($C_{16:0}$, $C_{17:0}$ cyclo, and $C_{18:0}$ ω7c), with $C_{16:0\ 3OH}$ as the most characteristic. Two different ornithine lipids may be present in some species and ubiquinone Q-8 has been found to be the predominant quinone system (Gillis *et al.*, 1995; Palleroni, 2005; Yabuuchi *et al.*, 1992). Catalase is produced, although oxidase activity may vary between species. *Burkholderia* species are chemorganotrophs. All species may use glucose, glycerol, inositol, galactose, sorbitol and mannitol as carbon source. The use of other carbon sources may vary between species, and thus has been used as discrimination trait (Palleroni, 2005).

1.1.3.1.2 Exopolysaccharide production

Bacteria physically interact with surfaces to form complex multicellular and often multispecies assemblies, known as biofilms. The main "cements" for all these cells are extracellular polymeric substances composed of mixtures of microbial biopolymers. Such mixtures are mainly composed by water, exopolysaccharides, glycoproteins, glycolipis and extracellular DNA (e-DNA). (Reviewed by (Danhorn & Fuqua, 2007; Karatan & Watnick, 2009)).

Exopolysaccharides (EPS) are sugar polymers that are excreted from the cells, and their production is important for the environmental adaptation and pathogenicity of bacteria. *Burkholderia* species such as *B. cepacia* (Cerantola *et al.*, 1996; Cunha *et al.*, 2004a) and *B. gladioli* (Park *et al.*, 2008) may produce several types of EPS polymers. In the beneficial plant-associated *Burkholderia* group, *B. caribensis, B. kururiensis and B. tropica* have been reported to have a remarkable synthesis of EPS (Achouak *et al.*, 1999; Mattos *et al.*, 2001; Serrato *et al.*, 2006). The structure of EPS for *B. caribensis, B. phytofirmans*, and *B. kururiensis* has been recently determined (Figure 1-4) (Mattos *et al.*, 2005; Silipo *et al.*, 2008; Vanhaverbeke *et al.*, 2003). Complementary studies have revealed similarities between *cepacian*, one of the heptasaccharide polymers produced by *B. cepacia*, and the EPS produced by *B. graminis, B. phytofirmans, B. phymatum* and *B. xenovorans*(Ferreira *et al.*, 2010). In contrast, structural analyses have indicated that *B. kururiensi*s EPS is composed of two types of polymers, a pentasaccharide named EPS A, and EPS B which is a mixture of two polymers composed by an hepta or octasaccharide repeating units (Hallack *et al.*, 2009; Mattos *et al.*, 2005).

In the *B*CC the genes involved in the EPS synthesis are organized in the *bce* cluster, which was first identified by Moreira *et al.* (Moreira *et al.*, 2003). Recently, it was determined that a similar cluster is also present in beneficial plant associated group. However, in the latter group, the *bce* cluster is not separated as in the BCC species, and the presence of an additional gene *bceV* has been determined by analysis of *Burkholderia* sequenced genomes (Figure 1-4)(Ferreira *et al.*, 2010). The same study also proposed a role for the EPS, since *B. xenovorans* exhibited increased tolerance to iron stress and desiccation in presence of EPS.

The EPS produced by *B. caribensis* was also found to be involved in the aggregation of soils (Vanhaverbeke *et al.*, 2003). However it is believed that the main function of the

EPS in plant-associated *Burkholderia* could be the attatchment to the root surfaces. Since EPS has been shown to be involved in plant colonization by symbiotic bacteria such as *Sinorhizobum melliloti* (Pellock *et al.*, 2000), it is expected that it may have a similar role in plant-*Burkholderia* interaction.

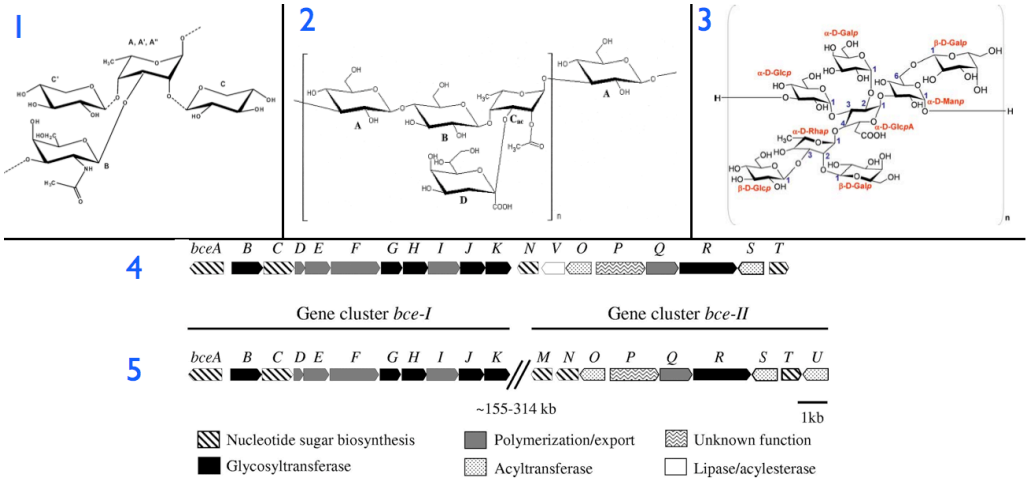


Figure 1-4. Exopolysaccharide structures in the beneficial plant-associated *Burkholderia* cluster

Exopolysaccharide structures from (1) *B. phytofirmans* PsJN1 (2) *B. caribensis* MWAP71 (3) EPS type B from *B. kururiensis* M130. (4-5) Genetic organization of the *bce* gene cluster directing the biosynthesis of cepacian by (4) species from the beneficial plant associated *Burkholderia* group (*B. graminis, B. xenovorans, B. phymatum* and *B. phytofirmans).* (5) *bce* cluster organization in members from the pathogenic clade (*B. mallei, B. pseudomallei, B.cepacia*) Structures were taken from (Ferreira *et al.*, 2010; Hallack *et al.*, 2009; Silipo *et al.*, 2008; Vanhaverbeke *et al.*, 2003).

1.1.3.1.3 Genome structure

Currently available *Burkholderia* genome sequences suggest that species of this genus owe their niche versatility to (i) their large genomes comprised of several large replicons and (ii) to lateral gene transfer events and plasmid acquisition (Chain *et al.*, 2006; Lim *et al.*, 2009; Lin *et al.*, 2008; Martinez-Aguilar *et al.*, 2008). The GC content in these *Burkholderia* species ranges from 61.2 to 64%. Four genome projects have been completed, *B. xenovorans* LB400^{T}(Chain *et al.*, 2006), *B. phytofirmans* PsJN^{T}, *B. phymatum* STM815^{T}, and *B. graminis* C4D1M ^{T} while the genomes of *B. unamae* MTI-641^{T} and *B. silvatlantica* SRMrh-20^{T}are currenlty being sequenced. Chromosomal analysis in several species suggested that genome sizes in this *Burkholderia* group range from 6.5 Mbp to 9.7 Mbp, represented in several replicons and megaplasmids, which are conserved within the species. An important exception is *B. xenovorans*, in which the type strain (LB400^{T}) hosts a 1.47 megaplasmid absent in most strains, although 0.8 Mbp plasmids have also been detected in strains LMG16224 and LMG22943. The megaplasmid of LB400 has been related to the acquisition of genetic material via horizontal gene transfer (Martinez-

Aguilar *et al.*, 2008). Although the selective advantage conferred by multireplicons is not clear, it has been proposed as essential and advantageous for the fitness in particular environments (Mahenthiralingam *et al.*, 2005).

1.1.3.2 Biogeography and distribution

Burkholderia species are considered ubiquitous, because they can survive in several environments without showing prevalent affinity for any of them (Tamames *et al.*, 2010). Beneficial plant-associated *Burkholderia* have been isolated from all continents. In fact, strains from the same species have been recovered from distant locations, and from completely different niches (Table 1-2 and Figure 1-5). For instance, *B. caribensis* strains were first isolated from vertisoils in Martinique, and were later found in nodules of *Mimosa* sp., in China (Achouak *et al.*, 1999; Liu *et al.*, 2010); *B. graminis* strains were isolated from rhizosphere soils in France and Australia, and strains of *B. tropica* have been identified in rhizosphere of several plants in Mexico, Brazil and South Africa (Reis *et al.*, 2004; Viallard *et al.*, 1998).

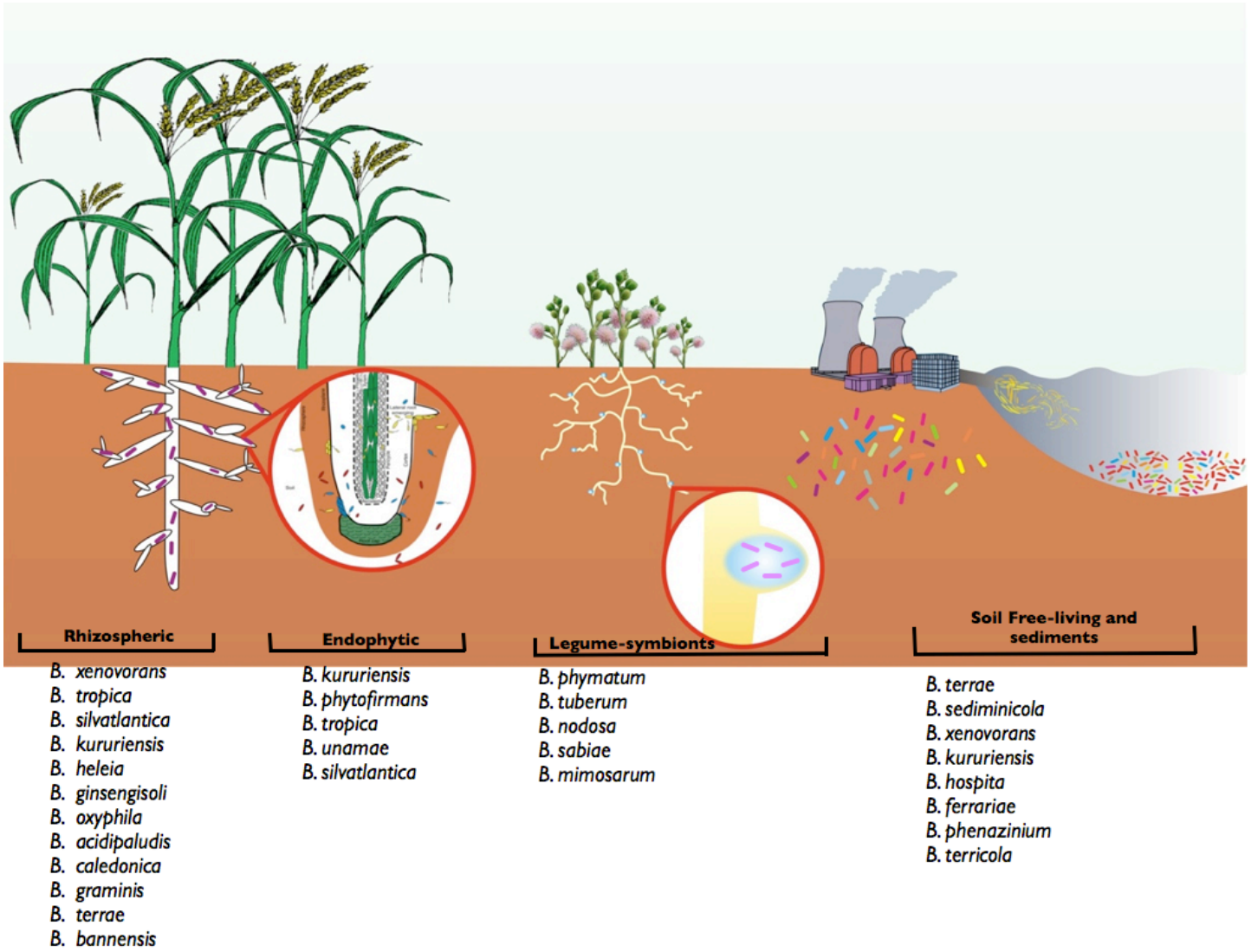
Species from this group of *Burkholderia* may be found free-living in the soil, associated to fungi and insects, although most of them have been recovered in association with plants (Figure 1-5). Beneficial plant associated *Burkholderia* may establish epiphytic, endophytic and endosymbiotic interactions with plants. So far, species of this group have not been reported to cause a detrimental effect in plant hosts, and some are currently under evaluation to assess their potential as biofertilizers due to their plant-growth promoting effects.

1.1.3.2.1 Beneficial *Burkholderia* in the rhizosphere

The rhizosphere is a biologically active zone of the soil around plant roots that contains soil-borne microbes including bacteria and fungi. In general, plant–microbe interactions in the rhizosphere can be beneficial to the plant, to the microbes or to neither of them (Singh *et al.*, 2004). Bacteria normally reach root surfaces by active motility facilitated by flagella and are guided by chemotactic responses (Badri *et al.*, 2009).

Figure 1-5. Occurrence of beneficial *Burkholderia* species in several niches: rhizosphere, endosphere, plant nodules, and soil.

Bacterial cells are coloured rods. Several sites of isolation are depicted, in the rhizosphere of rice, nodules of mimosa or free soil and sediments.



The rhizosphere is the most frequent niche for beneficial plant-associated *Burkholderia* (Table 1-2 and Figure 1-5). It has been speculated that such preference may be associated to their catabolic versatility that enables them to degrade root compounds and thus persist on the root surface (Chain *et al.*, 2006).

A considerable number of *B. unamae, B. tropica* and *B. silvatlantica* strains have been recovered from the rhizosphere, which strongly suggest that this is their main niche (Caballero-Mellado *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Estrada-de Los Santos *et al.*, 2001; Perin *et al.*, 2006a). *B. heleia, B. acidipaludis, B. oxyphila* and *B. ginsengisoli*, also have been recovered from the rhizosphere, but have their species descriptions are based in few isolates and therefore conclusions about their occurrence and diversity will require further analysis (Aizawa *et al.*, 2009a; Aizawa *et al.*, 2009b; Lim *et al.*, 2008).

It may be the case that plants have preference for a particular *Burkholderia* species. For instance, *B. unamae*, which occurs in the rhizosphere of maize, coffee, sugarcane and tomato, has been found significantly prevalent in sugarcane rhizosphere in Brazil in comparison to other species isolated under the same conditions (Caballero-Mellado *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Perin *et al.*, 2006a). Similarly, *B. tropica*, was found to be prevalently present in sorghum and maize rhizosphere in Mexico and Brazil (Perin *et al.*, 2006a; Wong-Villarreal & Caballero-Mellado, 2010).

Some species may not only occur in the rhizosphere, but also in other niches as the bulk soil. For example, B. *xenovorans* LB400^{T} was recovered from a polychlorobiphenyl (PCB) polluted soil, but other three strains have been also identified from the rhizosphere of coffee and tomato (Bopp, 1986; Caballero-Mellado *et al.*, 2007; Goris *et al.*, 2004). In a similar fashion, *B. sartisoli* originally isolated from polycyclic aromatic polluted (PAH) soils, was recently recovered from maize rhizosphere and compost heaps (Vanlaere *et al.*, 2008b). *B. caledonica,* which exists in sandy soils, was recovered from the rhizosphere of *Vitis vinifera* in Scotland (Coenye *et al.*, 2001b). Other species, as the legume-nodulating *B. phymatum* or the soil habitant *B. terrae*, may also exist in associated to the tomato rhizosphere (Wong-Villarreal & Caballero-Mellado, 2010).

1.1.3.2.2 Endophytic *Burkholderia*

Endophytes are defined as those bacteria able to colonize the internal tissue of the plant without causing external signs of infection or negative effects on their host. (Azevedo *et al.*, 2000; Bacon & Hinton, 2006; Compant *et al.*, 2010; Hallmann, 1997; Rosenblueth &

Martinez-Romero, 2006). The rhizosphere is the major source of bacterial endophytes from which they can spread inside the plant and colonize endosphere tissues. Although many species are able to enter into the endosphere, only those bacteria able to persist are considered *competent* endophytes (Hardoim *et al.*, 2008).

Compant *et al.*, have described the kinetics of the colonization as follows: (i) once attached to the root surfaces, bacteria may start endophytic colonization by penetrating at cracks, root emergence sites, and root tips in a mechanism that may be influenced by exopolysaccharides, motility traits as flagella and pili, or chemotaxis towards plant exudates, (ii) in the rhizoplane, bacteria translocate to reach the root cortical zone, and should then pass through the endodermis, and (iii) once in the endodermis, bacteria may penetrate the pericycle to reach the root xylem of the host (Compant *et al.*, 2010; Rosenblueth & Martinez-Romero, 2006). (Figure 1-6)

B. cepacia and *B. vietnamiensis* strains are often recovered as endophytes of plants as rice and maize, however their potential pathogenicity has impaired further biotechnological applications (Estrada-de Los Santos *et al.*, 2001). Among the beneficial *Burkholderia* group *B. tropica, B. kururiensis, B. unamae, B. silvatlantica, B. phytofirmans* and *B. acidipaludis* have also been isolated from surface-sterilized plants, and therefore are considered endophytes (Table 1-2).

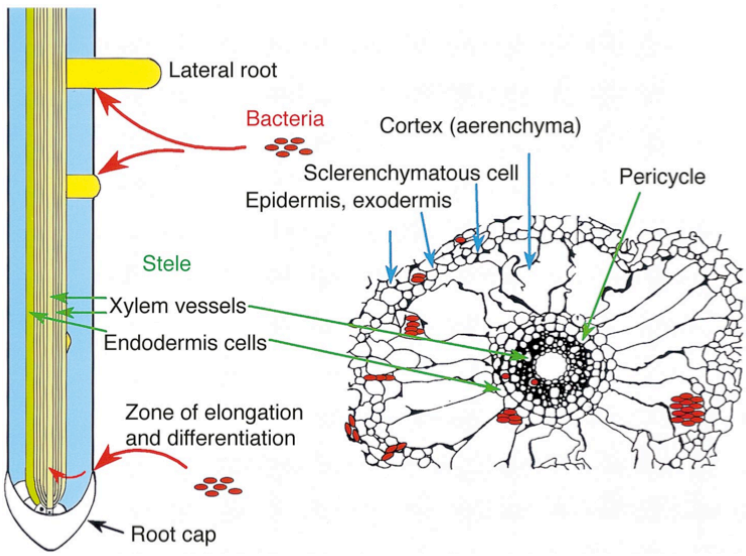


Figure 1-6. Classical scheme of the endophytic colonization

Bacterial cells are the red ovals. Possible sites of infection and colonization of roots shown on a longitudinal section of a young rice root and on a transverse section of a mature rice root (Taken from Hurek &Hurek, 1996)

B. phytofirmans is one of the most studied endophytes and has greatly contributed to the the understanding of plant-endophyte interaction. *B. phytofirmans* PsJN, was isolated in 1991 as a contaminant from onion roots infected with the mycorrhizal fungus *Glomus vesiculiferum* (Frommel *et al.*, 1991). Colonization experiments in potato with strain PsJN have shown enhancement in the root system, increased leaves, more adventitious secondary roots and higher lignin deposits, as well as a better water management in bacterized plants (Frommel *et al.*, 1991). *B. phytofirmans* PsJN has also been shown to colonize the endosphere and rhizosphere of tomato, grapevine and several potato cultivars promoting plant growth in all cases (Barka *et al.*, 2000; Nowak *et al.*, 1995; Nowak & Shulaev, 2003; Pillay & Nowak, 1997).

B. phytofirmans PsJN can also induce resistance of grapevine to *Botrytis cinerea*, the causal agent of gray mould, and was able to increase the growth of grapevine at low temperature (Barka *et al.*, 2000; Barka *et al.*, 2002). Other *B. phytofirmans* strains have been recovered from agricultural soils in Netherlands, rice endosphere in Korea, and sugarcane in Brazil (Luvizzotto *et al.*, 2010; Mukhopadhyay *et al.*, 1996; Salles *et al.*, 2006). Several isolates have shown to produce siderophores and indolacetic acid *in planta,* and in some cases plant growth promotion has also been verified(Sun *et al.*, 2009b; Trognitz *et al.*, 2009).

As will be discussed below, the primary focus of the study of endophytic *Burkholderia* is their potential for plant-growth promotion and bioremediation. Although the presence of bacterial endophytes in plants is variable, they are often capable of enhance the development of the plant (Cocking, 2003; Compant *et al.*, 2010).

1.1.3.2.3 Legume Nodulation by Beneficial *Burkholderia*

Legume nodulating bacteria collectively called *rhizobia*, live as saprophytes in the soil and in facultative symbiosis with plants. They induce the formation of root nodules, where they fix atmospheric nitrogen and provide it to the plant in exchange for carbon compounds [reviewed by (Masson-Boivin *et al.*, 2009)]. These bacteria were restricted to the α–proteobacteria class, however nodulation of *Mimosa* spp. by *Burkholderia*, *Cupriavidus* and *Herbaspirillum* sp. revealed that there were also β-rhizobia (Chen *et al.*, 2003; Moulin *et al.*, 2001). So far, most of the known β-rhizobia are taxonomically located in the group of beneficial *Burkholderia*, with the exception of one strain of *B. cepacia* isolated in Madagascar (Rasolomampianina *et al.*, 2005).

The legume-nodulating *Burkholderia* are: *B. phymatum, B. tuberum, B.nodosa, B.sabiae, B caribensis* and *B. mimosarum,* and they have been isolated from *Asphalantus carnosa, Mimosa spp., Machaerium lunatum, Rhynchosia ferufolia and Cyclopia spp.* It is worth noting that *B. caribensis* may also occur in soil, while *B. phymatum* may be associated to the tomato rhizosphere, being able to fix-nitrogen *ex-planta* (Chen *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2008; Chen *et al.*, 2003; Chen *et al.*, 2005; Elliott *et al.*, 2007a; Elliott *et al.*, 2007b; Garau *et al.*, 2009; Moulin *et al.*, 2001)

Plant-bacteria symbiosis is mainly mediated by bacterial chemotaxis towards flavonoid exudates in the root. It is also mediated by *Nod* factors (encoded by *nodAB* genes), which are lipo-oligosaccharides that act as signalling molecules for nodulating specific legume hosts (Spaink *et al.*, 1991). The presence of *nodAB* genes and the ability to re-induce nodulation and the presence of *nifHDK* genes have been verified in all legume nodulating *Burkholderia.*

Although plant-growth promotion in nodulating *Burkholderia* has not been explored yet, these species have been extremely useful to understand the phylogeny of the symbiosis. For example, the intense screening of legume–nodulating *Burkholderia* endosymbionts has indicated recently that these species are ancient symbionts in *Mimosa* sp. plants. (Bontemps *et al.*, 2009; Lloret & Martinez-Romero, 2005).

1.1.4 Plant growth promoting effects in the group of beneficial *Burkholderia*

When bacteria are competent to colonize plants and promote plant growth, they are referred to as plant growth promoting bacteria (PGPB) (Kloepper & Schrot, 1978). PGPB can affect plant growth directly by providing plants with a compound synthesized by the bacterium or facilitating the uptake of certain nutrients in the environment. Indirect plant growth promotion may be provided by preventing the deleterious effects of phytopathogens (Compant *et al.*, 2010; Glick, 1995).

1.1.4.1 Nitrogen fixation, a main feature of this *Burkholderia* group

Bacteria are the major source of plant growth promotion associated with nitrogen fixation in plants. The biological nitrogen fixation (BNF) is the conversion of atmospheric nitrogen into ammonia by symbiotic, free-living and associative bacteria (diazotrophs). This process is catalyzed by the oxygen-sensitive enzyme nitrogenase,

encoded by the *nifHDK* genes (Dixon & Kahn, 2004). The identification of diazotrophs is primarily based on the measurement of nitrogenase activity by using the acetylene reduction assay (ARA)(Dilworth, 1966), as well as the verification of the presence of the genes *nifHDK* (Menard *et al.*, 2007).

Among the *Burkholderia* species, the ability to fix nitrogen is prevalent in species of the beneficial group, although it was also described in the BCC member *B. vietnamiensis* (Gillis *et al.*, 1995; Menard *et al.*, 2007). Interestingly, the observed congruence between phylogenetic trees of 16SrRNA and *nifH* genes has indicated that most likely the common ancestor of all *Burkholderia* was a diazotroph, and that this function has been inherited in most species and lost in others (Bontemps *et al.*, 2009). In fact, genome comparison analyses have recently shown syntheny between the *nifHDK-nifENX* from *B. vietnamiensis* and *B. xenovorans* LB400^{T}, with a single indel related to the presence of a putative *iscS* gene in the *B. xenovorans nif* cluster (Menard *et al.*, 2007).

B. unamae, B.xenovorans, B. kururiensis, B. silvatlantica, B. tropica , B heleia, B. terrae, and *B. gisengisoli* are diazotrophs associated to plants, while *B. ferrariae* has been isolated from the soil (free-living) (Caballero-Mellado *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Chen *et al.*, 2003; Estrada-de Los Santos *et al.*, 2001; Moulin *et al.*, 2001; Perin *et al.*, 2006a; Reis *et al.*, 2004). As previously noted, species such as *B. sabiae, B.tuberum, B. phymatum, B. mimosarum* and *B. nodosa* may fix nitrogen in symbiosis with legumes.

Several diazotrophic *Burkholderia* species have been inoculated on plants and their contributions to the nitrogen uptake have been studied. The most remarkable examples are *B. kururiensis* in rice, *B. unamae* in tomato and maize and *B. tropica* in sugarcane. The use of *B. tropica* together with a bacterial consortium has been recommended in Brazil in order to increase the sugarcane yield (Ribeiro *et al.*, 2010). Similarly the rice endophyte, *B. kururiensis* M130 has been shown to increase the total nitrogen content of rice as much as the 30%, which, was also reflected in the grain yield (Baldani *et al.*, 1997a; Baldani & Baldani, 2005; Baldani *et al.*, 2000). Importantly, the positive effects exerted by diazotrophic bacteria are not only confined to the fixation of atmospheric N_2, but to a combination of other mechanisms that increase the nutrient availability, promote the solubilization of phosphates and the iron uptake via siderophores.

1.1.4.2 ACC deaminase and IAA production

One of the major mechanisms that plant growth promoting bacteria use to facilitate plant growth is the lowering of plant ethylene levels through the action of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. ACC is one of the precursors of the ethylene synthesis in plants, and the ACC deaminase degrades it to ammonia and α-ketobutyrate (Glick, 1995). Bacteria that express ACC deaminase normally bind to the surface of roots or seeds acting as a sink for ACC produced when the plant is stressed (Glick, 1995). A role of the ACC deaminase in promotion of plant-growth has been evidenced in the endophytes *B. phytofirmans* and *B. unamae,* as *acdS* defective mutans were impared in the growth-promotion in canola and tomato (Onofre-Lemus *et al.*, 2009; Sun *et al.*, 2009a). Moreover the ACC encoding gene, *acdS*, was found to be widely distributed in 20 tested species of the *Burkholderia* genus, 14 of them belonging to the beneficial plant-associated *Burkholderia* cluster (Table 1-2). The widespread ACC deaminase activity, and their common association to plants suggest that this group of *Burkholderia* could be a major contributor to plant growth under natural conditions (Blaha *et al.*, 2006; Onofre-Lemus *et al.*, 2009).

Bacteria that inhabit the rhizosphere may also influence plant growth by contributing to a host plant's endogenous pool of phytohormones, such as auxins. Beneficial bacteria synthesize the auxin indoleacetic acid (IAA) predominantly by an alternate tryptophan-dependant pathway, through indolepyruvic acid, although the role of bacterial IAA in plant growth promotion is currently being determined (Patten & Glick, 1996).Although IAA has been detected in *B. kururiensis, B. phytofirmans* and *B. unamae,* the biosyntesis pathway and the mechanisms involved in the plant enhancement remain to be understood (Caballero-Mellado *et al.*, 2007; Padua *et al.*, 2002; Sun *et al.*, 2009b).

1.1.4.3 Protective effects against biotic and abiotic factors

In some cases, PGPB may also trigger disease resistance in the host plant in a phenomenon called induced systemic resistance (ISR), as is the case of *B. phytofirmans* which is able to increase resistance against potato, tomato and grapevine pathogens (Barka *et al.*, 2000; Nowak *et al.*, 1995).

Several authors have correlated the expression of some genes in the defensive response with genes expressed in salt and drough stress situations (Bloemberg & Lugtenberg, 2001; Lugtenberg & Kamilova, 2009). *B. graminis* has been identified in rhizosphere of

corn, pasture and wheat and recently was reported to induce tolerance to abiotic factors in tomato plants. *In vivo* experiments with *B. graminis* isolates, resulted in enhancement in the shoot height and neck diameter of tomato plants as well as a protective response to salt stress and drought (Barriuso *et al.*, 2005; Barriuso *et al.*, 2008).

1.1.4.4 Phosphate solubilization, siderophores and nematode control

Low levels of soluble phosphate can limit the growth of plants. Some PGPB are able to solubilize phosphate form either organic or inorganic bound phosphates thereby facilitating plant growth (Igual *et al.*, 2001 ; Lugtenberg & Kamilova, 2009). Phosphate solubilization has been observed in *B. tropica* isolated from tomato in Mexico, as well as in *B. ferrariae* obtained from an iron ore. Although, applications of this trait in both species remains to be evaluated, both species are diazothophic and exist in soil and rhizosphere, which makes them potential candidates for bioinoculation (Caballero-Mellado *et al.*, 2007; Valverde *et al.*, 2006) .

Siderophores are low molecular weight Fe (III)-specific ligands produced by microorganisms as scavenging agents in order to counteract low iron levels (Neilands, 1993). Production of siderophores by PGPB is considered to be important in the suppression of deleterious microorganisms and soilborne plant pathogens (Lugtenberg & Kamilova, 2009). The presence of siderophores was reported in *B. bryophila* and *B. megapolitana* associated to the mosses *Aulacomnium palustre, Sphagnum rubellum and Sphangnum pallustre* in Germany. Importantly, strains of both species also exhibited antifungal and antibacterial activity, as well as plant growth promotion in lettuce which makes them promising candidates for biological control (Vandamme *et al.*, 2007). The production of siderophores has also been verified in *B. phytofirmans, B. unamae, B. kururiensis, B. xenovorans, B. tropica,* and *B. silvatlantica*, which suggest that these species may also have biocontrol properties (Caballero-Mellado *et al.*, 2007; Sessitsch *et al.*, 2005).

Recently, it was hyphotesized that *B. tropica* could have a role in the nematode control in sugarcane. Statistical analysis of the occurrence of this species and *Xhipinema elongatum* indicated a negative correlation between the presence of the nematode and the bacteria. This suggests that *B. tropica* could be used to promote a reduction in the pathogenicity of the nematode community and thus reduce the sugarcane damage (Omarjee *et al.*, 2008).

1.1.5 Aromatic compound degradation in the beneficial plant-associated *Burkholderia*

In addition to their beneficial effects on plant growth, species of this *Burkholderia* cluster have a considerable biotechnological potential to reduce the concentration and toxicity of chemical pollutants from the environment. Strains of *B. xenovorans, B. kururiensis, B. unamae*, *B. sartisoli and B. phenoliruptrix* have been recovered from polluted soils or from plant rhizosphere and are able to tolerate and metabolize recalcitrant compounds (Bopp, 1986; Caballero-Mellado *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Coenye *et al.*, 2004; Vanlaere *et al.*, 2008b; Zhang *et al.*, 2000).

Two special cases are *B. sartisoli* and *B. phenoliruptrix*. *B. sartisoli RP007*^{T}, isolated from a polycyclic aromatic hydrocarbon (PAH) contaminated soil in New Zealand, is a versatile degrader of low molecular mass PAHs, and can utilize naphthalene, phenanthrene and anthracene as sole sources of carbon and energy. Recently, it was proposed as a bacterial biosensor for the phenanthrene detection (Laurie & Lloyd-Jones, 1999a; Laurie & Lloyd-Jones, 1999b; Tecon *et al.*, 2006).

B. phenoliruptrix AC1100 was isolated after successive plating of a mixed culture from a chemostat grown with 2,4,5 trichlorophenoxyacetic acid (2,4,5-T), a potent herbicide and component of the agent orange (Kellogg *et al.*, 1981). Subsequent experiments have shown that strain AC1100 is capable of removing up to the 97% of 2,4,5-T from heavily contaminated soils and is able to degrade other halogenated compounds (Kilbane *et al.*, 1982).

In recent years, microbial degradation of hazardous compounds in the rhizosphere (rhizoremediation) and the use of plants to extract and degrade harmful substances (phytoremediation) are considered as alternatives for decontamination of soils. These are *in situ*, solar powered remediation technologies, which require minimal site disturbance and maintenance resulting in a low cost technology. Furthermore, it has been demonstrated that plants grown in polluted soils naturally recruit endophytes with the necessary contaminat-degrading genes, which enhance the plant degrading ability (Siciliano *et al.*, 2001). The examples described here clearly show the enormous potential that beneficial plant-associated *Burkholderia* have for phytoremediation. Since remediation options are frequently expensive and invasive, phytoremediation turns out

to be a valuable alternative, especially for the treatment of large contaminated areas (Weyens *et al.*, 2009a; Weyens *et al.*, 2009b).

1.1.6 Three *Burkholderia* species with outstanding biotechnological potential

So far, the biotechnological potential of this group of *Burkholderia* species has been outlined. It is clear that species from the beneficial plant associated group are potential candidates for bioinoculation and bioremediation as they couple PGPB properties and increased aromatic compound degradation. Three representative species showing these features are *B. kururiensis, B. unamae and B. xenovorans.*

1.1.6.1 *B. kururiensis* M130; a rice endophyte

B. kururiensis M130, formerly named "*Burkholderia brasilensis,"* was isolated from surface sterilized rice roots in Brazil, although strains from the same species were recovered from cassava, banana, pineapple, tobacco as well as from sugarcane (Baldani *et al.*, 1997a; Magalhaes Cruz *et al.*, 2001; Weber *et al.*, 1999; Weber *et al.*, 2000). Subsequent taxonomical analysis including 16SrRNA, *nifH, glnB* as well as other markers determined that "*B. brasilensis*" was actually a *B. kururiensis* strain (Caballero-Mellado *et al.*, 2007; Marin *et al.*, 2003). *B. kururiensis* strains are diazotrophs and therefore they may increase the nitrogen availability to the plant, promoting enhancement in growth and yield (Baldani *et al.*, 1997a; Baldani *et al.*, 2000). Inoculation of rice seeds with *B. kururiensis* M130, under gnotobiotic conditions showed that 30 % of the total N accumulated by the plant had been fixed by *B. kururiensis* (Baldani *et al.*, 2000). Greenhouse experiments also indicated a prevalence of B. *kururiensis* inside the roots in comparison with aerial parts (Baldani *et al.*, 2000). This and other experiments also demonstrated that increases of 38% and 54% in the rice yield could be obtained upon inoculation of several rice cultivars with *B. kururiensis* M130, although the enhancement in growth appeared to be dependant on the rice cultivar, as other rice varieties did not show significant increases (Baldani *et al.*, 2000).

B. kururiensis KP23 on the other hand, was isolated from an acquifier sample collected from a TCE tricholoethylene-(TCE) polluted site in Kururi, Japan, and is able to degrade phenol and toluene, flurobenzene and p-cresol (Caballero-Mellado *et al.*, 2007; Zhang *et al.*, 2000). Moreover, *B. kururiensis* was able to induce degradation of TCE by

enriching with phenol as sole carbon source, and was recently reported to be able to grow in consortia that degrades 2,4,6 trichlorophenol, using phenol as a primary intermediate (Gomez-De Jesus *et al.*, 2009).

Plant growth promoting effects by *B. kururiensis* strain KP23^T were confirmed by rice colonization experiments (Baldani *et al.*, 2000). Significant increases were observed in fresh weight of roots, aerial parts, flowering and seed yields. Bacterial indolacetic acid (IAA) production was also verified both chemically as well as *in vivo*, and its potential role in the growth was hypothesized. (Mattos *et al.*, 2008). Also, the same study indicated that endophytic colonization by *B. kururiensis* could be significantly reduced upon the presence of nitrogen sources as $(NH_4)_2SO_4$ (Mattos *et al.*, 2008).

The infection route of *B. kururiensis* in rice was analyzed by scanning electron microscopy (SEM) and is presented in Figure 1-7 (Mattos *et al.*, 2008). Results indicated that colonization starts with attachment of bacteria to epidermal cells of the root surfaces. At this stage, filamentous structures (bacterial strings) were observed to promote bacteria-bacteria and plant bacteria cross-linking. Proliferation of *B. kururiensis* then occurs through basal air tissues and surface cracks probably mediated by cell wall degrading enzymes, similar to what was previously observed during plant colonization by *B. phytofirmans* PsJN (Compant *et al.*, 2005; Compant *et al.*, 2008a). Finally, intercellular invasion of the internal tissues and subsequent entrance into the root vascular system was observed, suggesting the entrance to the xylem as the diffusion corridor for *B. kururiensis* (Mattos *et al.*, 2008). A similar endophytic colonization pattern was observed for *B. tropica* Ppe8^T in sugarcane, *Azoarcus* (Hurek *et al.*, 1994), and *Serratia marcesens* in rice (Gyaneshwar *et al.*, 2001).

It has been hypothesized that exopolysaccharide production may be a competence factor in the attachment steps during endophytic colonization (Compant *et al.*, 2010). Structural analyses have indicated that *B. kururiensi*s EPS is composed by two types of polymers, a pentasaccharide named EPS A, and EPS B which is a mixture of two polymers composed by hepta or octasaccharide repeating units (Hallack *et al.*, 2009; Mattos *et al.*, 2005). Although the role of EPS in plant colonization has not been determined for *B. kururiensis*, it is believed that it may be as pivotal as in other plant associated bacteria. A close example is the legume nodulating *Sinorhizobium melliloti,* which also produces two types of EPS that are determinant for plant-bacteria interaction (Rinaudi & Gonzalez, 2009).

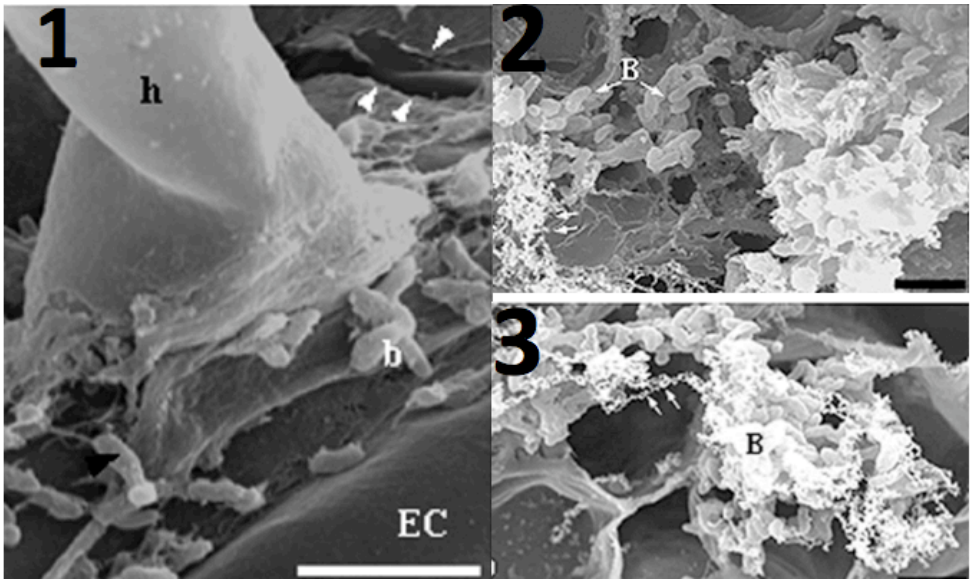


Figure 1-7. Rice endophytic colonization by *B. kururiensis* KP23^T

(**1**). Bacterial attachment to the root hair zone forming microaggregates on disrupted areas of the epidermal surface is shown by arrows (b) bacteria (h) root hair. (**2**) After invading the inner tissues through the hair base *Burkholderia* cross the epidermis eventually migrating to the cortex cell.3. Vascular cylinder showing bacteria arranged on strings (B) bacteria. Taken from Mattos *et al.* 2008.

1.1.6.2 *B.unamae*: rhizopshere and endosphere

B. unamae, is a nitrogen-fixing species which occurs in the rhizosphere of maize, coffee, sugarcane and tomato (Caballero-Mellado *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Perin *et al.*, 2006a). Rhizosphere colonization experiments performed in tomato have evidenced that *B. unamae* is able to promote plant growth, with significant increases in the plant weight, chlorophyll content and root length (Onofre-Lemus *et al.*, 2009). The same study also demonstrated that the plant-growth promotion effect is associated to the production of ACC deaminase. An additional plant growth promoting mechanism is the IAA production, which has been detected recently in *B. unamae* strains (Onofre-Lemus *et al.*, 2009).

Interestingly, *B. unamae* strains isolated from tomato rhizosphere have also shown abilities to degrade phenol and benzene (Caballero-Mellado *et al.*, 2007). Although the biochemical pathways involved in the aromatic compound degradation are currently unknown, the adaptability and versatility of *B. unamae* strains to survive both in the endosphere as in the rhizosphere of different plants, makes of them interesting candidates for phytoremediation and plant growth promotion(Caballero-Mellado *et al.*, 2004).

1.1.6.3 *B. xenovorans* LB400^T, a powerful PCB degrader

To date, among the plant-associated beneficial *Burkholderia, B. xenovorans* LB400^T is probably the most studied. Strain LB400 was isolated from a PCB-containing landfill in New York, as part of a screening to identify strains able to degrade polychlorobiphenyls (PCB) (Bedard *et al.*, 1986; Bopp, 1986). PCBs are extremely toxic compounds used as fungicides for citrus in agriculture, as well as for industrial and commercial purposes. These compounds are highly absorbed by the soil due to their high hydrofobicity, which makes their removal particularly difficult. *B. xenovorans* LB400^T degrades the widest range of PCB congeners, although may also degrade isoflavonoids, abietan diterpenoids and sulfonates (Seeger *et al.*, 2003; Smith *et al.*, 2004; Smith *et al.*, 2007a; Smith *et al.*, 2008a).

In *B. xenovorans* LB400^T, the *bph* cluster encodes for the enzymes involved in the biphenyl upper pathway responsible of the PCB degradation (Erickson & Mondello, 1993; Gibson *et al.*, 1993; Haddock *et al.*, 1993; Hofer *et al.*, 1993; Mondello, 1989). The *bph* operon is located in a genomic island within a 1.47 Mbp megaplasmid. Importantly, although other five *B. xenovorans* strains have been isolated from coffee and tomato rhizosphere, none of them can degrade PCB as they lack of the *bph* genes (Chain *et al.*, 2006; Martinez-Aguilar *et al.*, 2008). In addition to the *bph* loci other genes involved in transcription regulation, carbohydrate and aminoacid transport have also shown to be determinant for the upregulation of the biphenyl degradation(Denef *et al.*, 2004).

In comparison to the other *B. xenovorans* strains, LB400^T is also enriched in aromatic catabolic pathways contained in genomic islands, possessing biphenyl, 3-chlorocatechol and 2-aminophenol catabolic capacities, as suggested from genome analysis. *In silico* analyses have also evidenced eleven "central aromatic" catabolic pathways and twenty "peripheral aromatic" catabolic pathways, encoded by more than 170 genes, mostly organized in operons. Such abundance in catabolic pathways has led to hypothesize the existence of regulatory and signal transduction networks (Chain *et al.*, 2006; Denef *et al.*, 2006; Smith *et al.*, 2007b; Smith *et al.*, 2008b).

Apart from the extensively studied aromatic compound degradation characteristic of *B. xenovorans* LB400^T, most of the mechanisms regulating its adaptability and versatility remain to be understood. Determining factors involved in the adaptability of this

species is pivotal, considering that other *B. xenovorans* strains have been identified in close association to plants.

In spite of the increasing attention given to these species, the molecular mechanisms regulating the interaction with the plant or with the environment are poorly understood.

1.2 QUORUM SENSING

Quorum sensing (QS) is a cell-cell communication process in which bacteria use the production and detection of signals or autoinducers in order to monitor cell population density and make a coordinated response. When the autoinducer reaches a critical level, the population responds through the coordinated expression of specific target genes (Fuqua *et al.*, 1994). This synchronous response of bacterial populations confers them a form of multicellularity and enables them to adapt and survive continually to changing environments (Ryan & Dow, 2008).

1.2.1 Autoinduction and Quorum sensing: Evolution of a concept

The term "quorum sensing" was proposed in mid 90s (Fuqua *et al.*, 1994), however, the notion of "autoinduction" emerged from late 60s with the publication of pioneer studies on fruiting body formation in *Myxococcus xanthus* (McVittie *et al.*, 1962), and the work of Tomasz, who found that in *Streptococcus pneumoniae* a hormone-like extracellular product might help to regulate the competence. The molecule was later identified as a peptide, now recognized as a common signal molecule in gram-positive bacteria (Tomasz, 1965).

Pioneering work was also performed on the bioluminescent bacteria *Vibrio fisheri,* symbiont of marine fishes and squids. In *V. fisheri,* the light emission starts only during the middle of the logarithmic phase of growth. However, in 1970 Nealson, Platt & Hastings noted that by adding spent supernatants from stationary-phase cultures, they could induce the production of light in early log-phase cultures (Nealson *et al.*, 1970). This observation suggested that bacteria were able to produce a diffusible compound or "autoinducer", which was able to induce the luciferase synthesis when its concentration, hence cell-density, reached a critical level (Nealson *et al.*, 1970). Moreover, the autoinducer seemed to be species-specific, as supernatants from other *Vibrio* species were not able to induce the luciferase synthesis in *V. fisheri* (Eberhard, 1972).

The structure of the compound was then identified as N-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981), but the regulatory circuit controlling the bioluminescence in *V. fisheri* was found few years later (Engebrecht *et al.*, 1983; Engebrecht & Silverman, 1984). Engebrecht & Silverman identified seven luminescence genes (*lux)* organized in two transcriptional units. One unit comprises the *luxR* gene, while the other is an operon containing *luxI* followed by five genes required for light production *luxCDABEG* (Figure 1-8). In this system, *luxI* and *luxR* work as regulatory components (Engebrecht *et al.*, 1983). The role of the LuxI protein is synthesizing the AHL, while LuxR binds the AHL at high cell-density allowing it to then activate transcription of the *luxICDABE* operon (Engebrecht *et al.*, 1983; Engebrecht & Silverman, 1984).

These insights were relevant in the ecological context of *V. fisheri.* This marine species is capable of free-living lifestyle, but its preferred niche is the light organ of marine fishes and squids. While bacteria produces light, the host provides a rich and safe environment and uses light to attract both preys and mates, as well as avoiding predators (Ruby & McFall-Ngai, 1992). These animals may acquire *V. fisheri* from the surrounding seawater, but once the bacteria enter the confined space of the light organs, it synthesizes AHLs that move freely across the bacterial membranes to be accumulated as a function of population density. Upon reaching a certain concentration, the AHLs will bind the LuxR protein inside the cell, and the complex will activate the transcription the *lux* operon, and produce light (Fuqua *et al.*, 1996). (Figure 1-8)

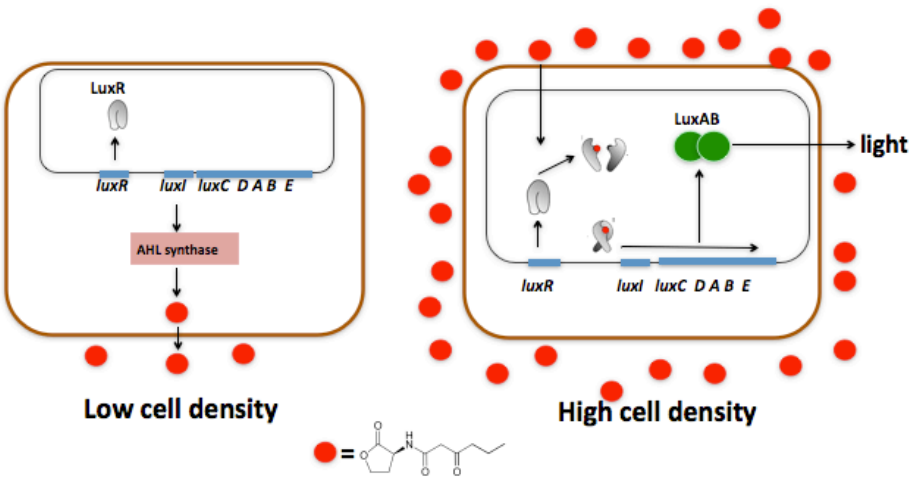


Figure 1-8.Elucidated QS system for *Vibrio fisheri*.

For many years it was thought that this regulatory mechanism was unique for certain marine species of *Vibrio*. However in the 90s, other bacteria were reported to produce AHLs, and new phenotypes were described as regulated in a cell density manner, which led to the proposal of the quorum-sensing concept (Fuqua *et al.*, 1996; Fuqua *et al.*, 1994). Species found to produce AHLs included *Erwinia carotovora* (Bainton *et al.*, 1992a; Bainton *et al.*, 1992b), *Pantoea stewarti* (Beck von Bodman & Farrand, 1995), *Agrobacterium tumefaciens (Piper et al., 1993)* and *Pseudomonas aeruginosa* (Gambello & Iglewski, 1991). Also, several species were identified to harbour more than one system, and in several cases these circuits revealed to be interconnected, with *P. aeruginosa* as the most studied example (Latifi *et al.*, 1995). The existence of quorum sensing was also reported in Gram-positive bacteria such as *Lactococcus lactis* (van der Meer *et al.*, 1993), *Staphylococcus aureus* (Ji *et al.*, 1995) and *Bacillus subtillis* (Ansaldi *et al.*, 2002), although in these cases different autoinducers were identified.

In recent years, more efforts have been made to explore the role of quorum sensing in multispecies communities and cross-kingdom communication (Ferluga & Venturi, 2008). Concepts as diffusion and efficiency sensing have been proposed to refine the repercussions of signalling in terms of space and distribution (Hense *et al.*, 2007; Redfield, 2002). Other studies have focused on identifying possible mechanisms to interfere QS to control pathogenicity and virulence, also known as quorum quenching. All together, these trends in research of bacterial socio-biology are beginning to create a more complete picture of the impact of bacterial communication in nature.

1.2.2 Canonical quorum sensing circuit and autoinducer types

The fundamental steps involved in responding to fluctuations in cell number are comparable in all QS systems. In a canonical system, the autoinducer molecules must be synthesized inside the cell and passively released or actively secreted. As the number of cells in a population increases, the extracellular concentration likewise increases. When autoinducers accumulate above the minimal threshold level required for detection, cognate receptors bind the autoinducers and trigger signal transduction cascades that result in population-wide changes in the gene expression (Figure 1-9).

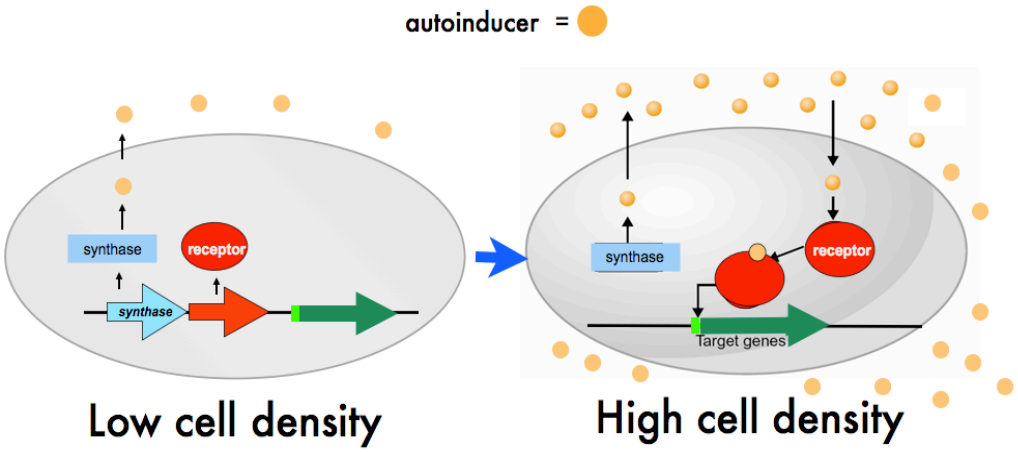


Figure 1-9. Canonical Scheme of Quorum Sensing

Although many chemical metabolites have signalling roles, not all are considered quorum-sensing autoinducers. To be considered a QS signal, a molecule should match several criteria as proposed by Winzer and colleagues(Winzer *et al.*, 2002):

(i) The production of the molecule should occur during specific stages of growth, under physicological conditions or in response to changes in the environment.

(ii) The molecule should be secreted and accumulated in an extracellular environment.

(iii) The signal should be able to generate a concerted response after reaching a threshold via recognition by a specific receptor (located on the cell surface or in the cytoplasm)

(iv) The response should not be related to synthesis or uptake of the autoinducer molecules

Apart from AHLs, other examples of compounds involved in bacterial quorum sensing are: oligopeptides, Autoinducer type-2 (AI-2), and quinolones (Figure 1-10).

Gram positive bacterial oligopeptides autoinducers range from 5 to 34 aminoacids in length, and are often postranslationally modified by the incorporation of lactone and thiolactone rings, lanthionines and isoprenyl groups. Due to their hydrophobicity, oligopeptide release requires specialized transporters. Two-component signalling proteins located in the membrane then mediate its perception with signal transduction

occuring by a phosporylation cascade (Camilli & Bassler, 2006). Different oligopeptide autoinducers contain subtle variations, which confer signalling specificity because of the discriminatory properties of their cognate receptors. Some examples are ComX and CSF of *Bacillus subtillis*, CSP from *S. pneumoniae*, and the four types of AIP (Autoinducer Peptide) from *S. aureus* (Ansaldi *et al.*, 2002; Ji *et al.*, 1995; Pestova *et al.*, 1996).

The Autoinducer 2 (AI-2) was first found in *V. harveyi,* although it is also found both in other Gram negative and Gram-positive bacteria and is proposed to enable interspecies communication (Bassler *et al.*, 1993; Schauder *et al.*, 2001). The AI-2 precursor, DPD (4,5-hydroxy-2,3-pentanedione), is first synthesized by LuxM and then cyclizes spontaneously to give rise to several related furanones that are thought to be in equilibrium. Importantly, in *V. harveyi* AI-2 contains boron, while the AI-2 active form in *Salmonella* does not. Different species of bacteria may recognize distinctly arranged DPD moieties, which allows bacteria to respond to their own DPD and also to that produced by other bacteria (Miller *et al.*, 2004). In *V. harveyi*, the AI-2 circuit also integrates with other autoinducers and is involved in the regulation of bioluminescence (Ng & Bassler, 2009). In other species it may control functions as the virulence factor production, motility and biofilm formation (Sperandio *et al.*, 1999; Stevenson & Babb, 2002; Xavier & Bassler, 2003).

The diffusible signal factor (DSF) was first identified in *Xanthomonas campestris*, but is also produced by other species including *X. oryzae, Xylella fastidiosa* and *Stenotrophomonas maltophilia.* Its structure corresponds to an unsaturated fatty acid (cis-11-methyl-2-dodecenoic acid), and a two-component system is implicated in DSF perception. The main components of this system are encoded by the *rpf* cluster (Barber *et al.*, 1997; Dow *et al.*, 2003). The *rpf*/DSF system controls diverse functions in these bacteria including virulence factor systems, aggregative behaviour and biofilm formation. A similar molecule was also recently reported in *Burkholderia cenocepacia* (BDSF) and its role in interspecies communication has been recently determined (Boon *et al.*, 2008; Ryan *et al.*, 2008).

One of the *Pseudomonas* autoinducers is known as *Pseudomonas* Quinolone Signal (PQS) which is a 2-heptyl-3hydroxy-4 quinolone (Pesci *et al.*, 1999). This molecule is synthesized from anthranilate via the action of *pqsA-D* and *pqsH* gene products. PQS regulates its own production by controlling the expression of the 4-quinolone biosynthetic genes and its own package into membrane vesicles that deliver

antimicrobials and toxins (Wade *et al.*, 2005). Together with other QS circuits in *P. aeruginosa*, PQS functions to control a battery of genes required for virulence and biofilm formation (Diggle *et al.*, 2003; Diggle *et al.*, 2007). Recently analog compounds have been reported in *Burkholderia* species, such as *B. ambifaria, B. thailandiensis* and *B. pseudomallei*. These PQS-like molecules typically bear a methyl group, and hence they have been designated as 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs)(Vial *et al.*, 2008).

Other autoinducers are the CAI-1 of *V. cholera* and *V. harveyi*, bradyoxetin from *Bradirhizobium japonicum* and the diketopiperazines from *Pseudomonas* spp. and *Proteus mirabilis*, among others (Reviewed by Ryan &Dow, 2008).

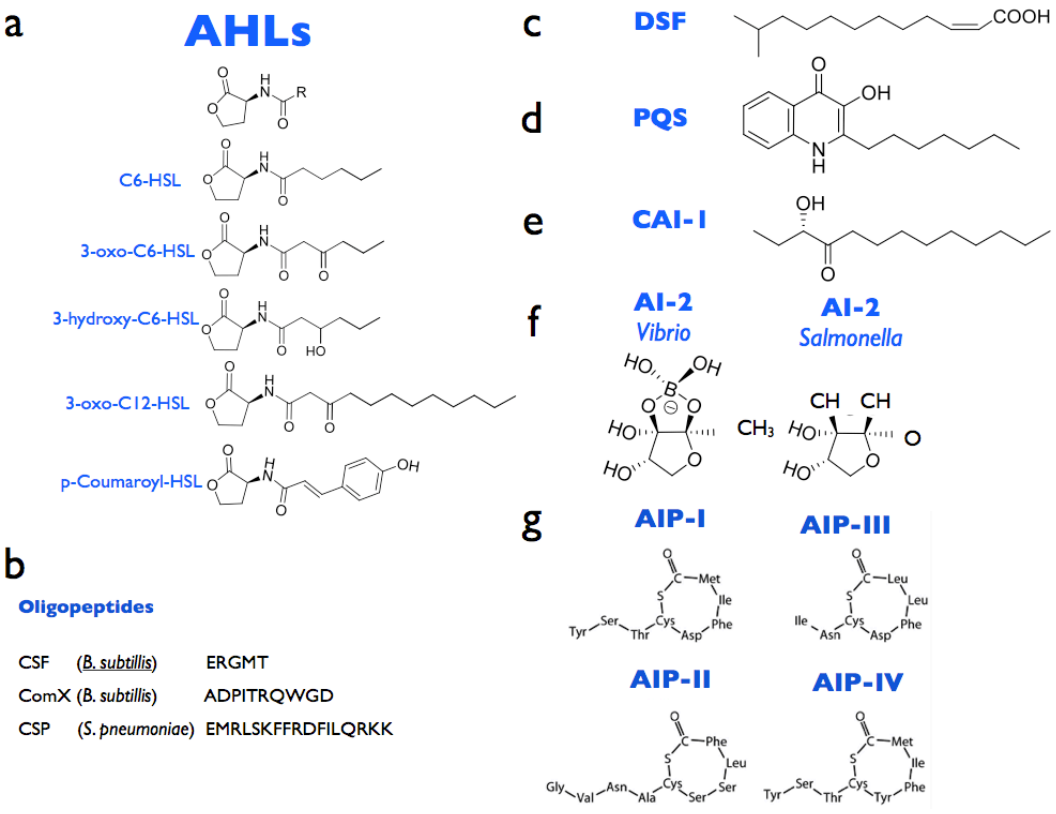


Figure 1-10. Structure of bacterial autoinducers

(a)AHLs Homoserine lactone produced by several Gram-negative bacteria. **(b)** Aminoacid sequences of three oligopeptides produced by Gram-positive bacteria **(c). DSF** produced by *Xanthomonas* spp.**(d)** Structure of the PQS autoinducer of *Pseudomonas aeruginosa.* **e.** Structure of *V. cholerae* CAI-1 **(f)** Autoinducer 2 (AI-2), in the presence of boron, it exists as S-THMF borate, whereas in absence of boron it exists as R-THMF (g) Cyclic peptides from *Staphylococcus aureus.*

1.2.3 AHL-based quorum sensing

Acyl homoserine lactones (AHLs) are the major class of autoinducer signals used by Gram-negative bacteria. These molecules have a conserved homoserine lactone ring with an acyl side chain, which may vary from 3 to 18 carbons (Figure 1-10a). The length and saturation level of the acyl chain coupled to the presence or absence of oxo or hydroxyl substitutions at the C3 position of the acyl chain provide variation and specificity. The homoserine lactone ring imparts hydrophilic character to the molecules, while the length, substitution and saturation modulate their hydrophobicity (Fuqua & Parsek, 2002). All AHLs are believed to be able to freely diffuse across the cell membrane; however efflux pumps may actively export some longer chain AHLs (Kohler *et al.*, 2001).

In an AHL-QS circuit, AHLs are synthesized by a LuxI-type protein, and diffuse in and out of the cell (Figures 1-11 and 1-12a). As the cell density increases, the concentration of the autoinducer increases both inside as outside of the cell until reaching a critical point or threshold. At this stage, the AHL binds the LuxR protein, which gets activated by exposing a DNA binding domain that recognizes a palindromic *lux* box *cis*-element in the promoter region of the target genes (Egland & Greenberg, 1999). The transcription of the genes is then driven, via interaction of the LuxR-AHL complex with the RNA polymerase (Figure 1-11) (Zhu & Winans, 2001). Some LuxR-type proteins may also bind promoters in the absence of AHLs, and upon the presence of the autoinducer, they unbind the *lux*-box allowing the transcription, in such cases these proteins are considered repressors (Von Bodman *et al.*, 1998). Importantly, often the *luxI* and *luxR* genes are under a positive induction feedback loop forming a regulatory circuit, which generates rapid amplification of the signal (Fuqua *et al.*, 1996; Fuqua *et al.*, 1994).

1.2.3.1 AHL synthesis : LuxI-type Proteins

AHL biosynthesis is primarily dependent on members of the LuxI family of synthases. These enzymes direct the formation of an amide bond between the S-adenosylmethionine (SAM) and the acyl moiety of a cognate acyl carrier protein. The homoserine lactone ring is derived from the SAM, while the acyl portion of the AHL is derived from fatty-acid precursors conjugated to the acyl carrier protein (acyl-ACP) (More *et al.*, 1996; Parsek *et al.*, 1999). Recently, one exception was reported in

Rhodopseudomonas palustris, which synthesizes the signal p-coumaroyl-HSL from p-coumaric acid obtained from extracellular environment (Schaefer *et al.*, 2008). The choice of fatty acyl substrates is partly due to substrate preference of the enzyme and to substrate availability. AHL synthases can accept several acyl-ACPs leading to the synthesis of a set of different AHLs, as demonstrated for YtbI from *Yersinia pseudotuberculosis*, which can synthesize up to 24 different AHLs (Ortori *et al.*, 2007).

LuxI-type proteins are normally from 190 to 226 aminoacids in size and share 4 blocks of conserved sequence domains. Within these blocks 10 residues are conserved in the amino terminal portion of the protein, which was verified since their mutation impaired AHL production by the synthases LuxI and RhlI (Figure 1-11) (Hanzelka *et al.*, 1997; Pappas *et al.*, 2004; Parsek *et al.*, 1997). Other structural features important for the catalytic activity of the LuxI proteins are the acyl-chain binding pockets, and the presence of threonin at the position 143. When acyl chain binding pockets are narrow, the synthase can only accommodate substrates with relatively short-acyl chains, as EsaI from *P. stewarti*, which synthezises 3-oxo-C6 HSL. In contrast, if the pocket is an elongated tunnel, the synthase will not have steric restriction to accommodate a long acyl chain, as happens with LasI, which synthesizes 3-oxo-C12-HSL (Gould *et al.*, 2004; Watson *et al.*, 2002b). Similarly, proteins that synthesize 3-oxo or 3-hydroxy AHLs generally have threonine or serine residues at position 143, while proteins that synthesize 3-unsubstituted AHLs generally have small non-polar residues at that position (Fuqua & Greenberg, 2002; Watson *et al.*, 2002b).

LuxM proteins are another class of AHL synthases with distinct aminoacid composition from LuxI homologues, but are analogous in function (Hanzelka *et al.*, 1999). AinS for *V. fisheri*, VanM for *V. anguillarum* and LuxM for *Vibrio harveyi* share 34% similarity at the amino terminal domain and are able to synthesize AHLs. Members of this family use the same substrates as LuxI–type proteins, although acyl-CoA may substitute the acyl-carrier protein (Bassler *et al.*, 1993; Hanzelka *et al.*, 1999; Milton *et al.*, 2001).

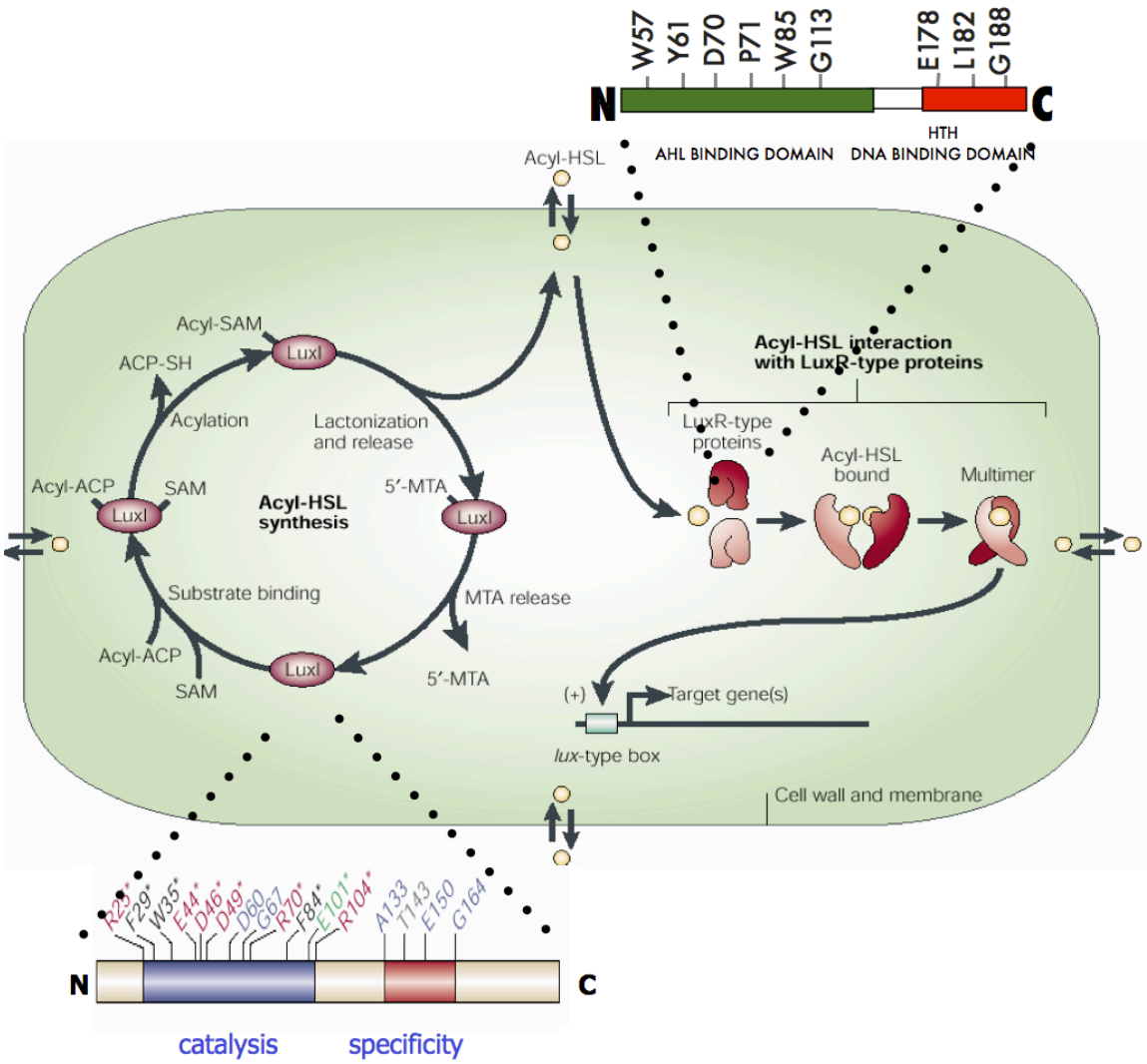


Figure 1-11. AHL-BASED QUORUM SENSING CIRCUIT

Model of AHL quorum sensing in a single generalized bacterial cell. On the left side, the steps for the AHL synthesis mechanism are shown, and the conserved residues of the LuxI-type synthase proteins are highlighted. Double arrows represent the AHL transit in and out of the cell. On the right side, the conformational changes of LuxR-type proteins upon binding the AHL cognate are depicted. The recognition of the *lux*-boxes in target gene promoters is shown. (Adapted from Fuqua& Greenberg, 2002).

1.2.3.2 AHL sensing: LuxR transcriptional regulators

AHL sensing is mediated by a transcriptional regulator of the LuxR family, referring to the protein regulating the bioluminescence process initially described in *V. fisheri*. The LuxR-type proteins are about 250 aminoacids long and comprise two functional domains: the amino-terminal region AHL-binding domain and the carboxy-terminal region containing a Helix-Turn-Helix (HTH) DNA-binding domain (Fuqua *et al.*, 2001). Protein sequence comparisons have revealed that LuxR type proteins share 18-25% identity, although their functional domains share much higher sequence conservation. Importantly, nine residues are identical in at least 95% of the LuxR proteins, six of them are located in the N terminal AHL-binding domain, while the other three are in the DNA-binding motif (Figure 1-11) (Fuqua *et al.*, 2001; Nasser & Reverchon, 2007). Overall structure-function analysis suggest that the DNA binding domain is highly conserved while the AHL binding domain tends to vary in several LuxR type proteins, most likely to accommodate the variety of activating signals (Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Vannini *et al.*, 2002).

LuxR-proteins may work as transcriptional activators or as repressors. Transcriptional activation occurs when the AHL-LuxR complex recognizes a palindromic *lux* box *cis*-element localized in the promoter region of the target genes, and recruits RNA polymerase through direct contact. A *lux*-box is an 18-22 bp inverted repeat, centered between -40 and -80 relative to the transcriptional site. Although many target genes of the LuxR type proteins contain *lux*-boxes within their promoters, target genes lacking discernible *lux*-boxes in their promoters have also been described (Egland & Greenberg, 2001; Zhu & Winans, 1999). LuxR-type protein folding normally requires the presence of the AHL ligand (Zhu & Winans, 2001). Once bound to the AHL cognate, LuxR-family proteins suffer conformational changes, dimerize and, then the N-terminal domain of each monomer binds the AHL ligand, while each DNA binding domain recognizes half of the *lux*-box (Schuster *et al.*, 2004; Vannini *et al.*, 2002).

In the absence of AHL, some LuxR-type proteins may act as repressors by binding promoters of target genes where they prevent transcription by blocking access to the RNA polymerase. Binding to the AHL causes conformation changes that release the repressor from DNA and relieve repression, as described for such as EsaR of *P. stewartii* and ExpR of *Erwinia* (Minogue *et al.*, 2002; Von Bodman *et al.*, 1998).

1.2.3.3 Multiple AHL QS systems

Some species harbour two or more circuits to coordinate their population-based responses. When several LuxI/R systems are present, they are often organized in hierarchical networks (Figure 1-12b).

The most studied example is the QS network of the opportunistic pathogen *P. aeruginosa*, which hosts two LuxI/R circuits: LasI/LasR and RhlI/RhlR, producing and responding to 3-oxo-C-12-HSL and C4-HSL, respectively. The two systems are arranged in a hierarchical fashion, since the LasI/R system activates transcription of *rhlI* and *rhlR* (Latifi *et al.*, 1996). Transcriptome analyses of *P. aeruginosa* revealed that approximately 300 QS regulated genes are widespread in the genome, suggesting that the QS circuitry constitutes a global regulatory system that affects many different cellular functions including many virulence factors (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

It is worth noting that further levels of complexity are involved in the regulatory circuitry of the LasR/RhlR systems; for example the LuxR homologues QscR and VqsR have been shown to interfere with the LasR protein (Chugani *et al.*, 2001; Lee *et al.*, 2006). Similarly the PQS autoinducer has been postulated to act as a link between the LasI/R and the RhlI/R system (Diggle *et al.*, 2003; Diggle *et al.*, 2007). A variation to this scheme was recently reported in an environmental *P. aeruginosa* strain PuPA3, in which the LasI/R and RhlI/R QS systems are not hierachically arranged, but act independently and often autonomously to regulate the same functions (Steindler *et al.*, 2009).

1.2.3.4 LuxR solo proteins

It has recently been observed that the number of LuxI and LuxR-type proteins is not always equal since some species may also harbour unpaired QS-like LuxR-type proteins (Case *et al.*, 2008). These proteins were initially denoted as orphans (Fuqua, 2006), and the name "LuxR-*solo*" proteins was recently proposed to more suitably describe their unpaired character (Subramoni & Venturi, 2009b). LuxR-solo elements also have an AHL-binding domain in the amino terminal and a DNA binding domain in the carboxy-terminal. In some cases, differences have been observed in the protein length, as well as in the typically conserved residues of the LuxR-type proteins (Patankar & Gonzalez, 2009a).

LuxR-solo proteins have been found and characterized in species holding one or several AHL-QS circuits, as well as in species lacking of LuxI synthases (Figure 1-12c) (Case *et al.*, 2008). Recent studies showed that when present in bacteria holding one or several AHL-QS systems, LuxR *solo* proteins may be integrated in the AHL-QS regulatory network (Figure 1-12d). Examples are QscR from *P. aeruginosa*, BisR from *Rhizobium leguminosarum*, CarR from *Erwinia* spp., and ExpR from *Sinorhizobium melliloti* (Cox *et al.*, 1998; Fuqua, 2006; Hoang *et al.*, 2008; Wilkinson *et al.*, 2002). In these cases, LuxR solo proteins recognize AHLs produced by the resident QS system (s), and play a role in the regulation of specific targets or in the homeostasis of the QS network. One particular example is PpoR from *Pseudomonas putida,* which has been found present both in AHL producers and non-producers. PpoR is able to bind AHLs and to regulate several loci (Subramoni & Venturi, 2009a).

It has been suggested that the presence of LuxR-solo proteins in non-AHL producing bacterial species may allow the bacteria to sense and respond to foreign AHLs, as well as to switch to a competitive behaviour towards neighbours (Subramoni & Venturi, 2009b). For instance, *E. coli*, *Salmonella*, and *Klebsiella* do not produce AHLs, but harbour the LuxR *solo* SdiA. These proteins have shown response to exogenous AHLs, and a role in regulation of accessory virulence factors in *Salmonella* has been proposed (Ahmer, 2004).

OryR and its ortholog Xcc, are LuxR solo proteins identified in the non-AHL producers *Xanthomonas oryzae* and *Xantomonas campestris* (Ferluga *et al.*, 2007; Zhang *et al.*, 2007). These two proteins present two substitutions in the conserved residues of the AHL binding domain. A role in interkingdom communication has been proposed for these two LuxR-solo proteins, as they recognize an unknown plant factor and regulate the proline iminopeptidase (*pip*) virulence genes (Ferluga & Venturi, 2008; Zhang *et al.*, 2007).

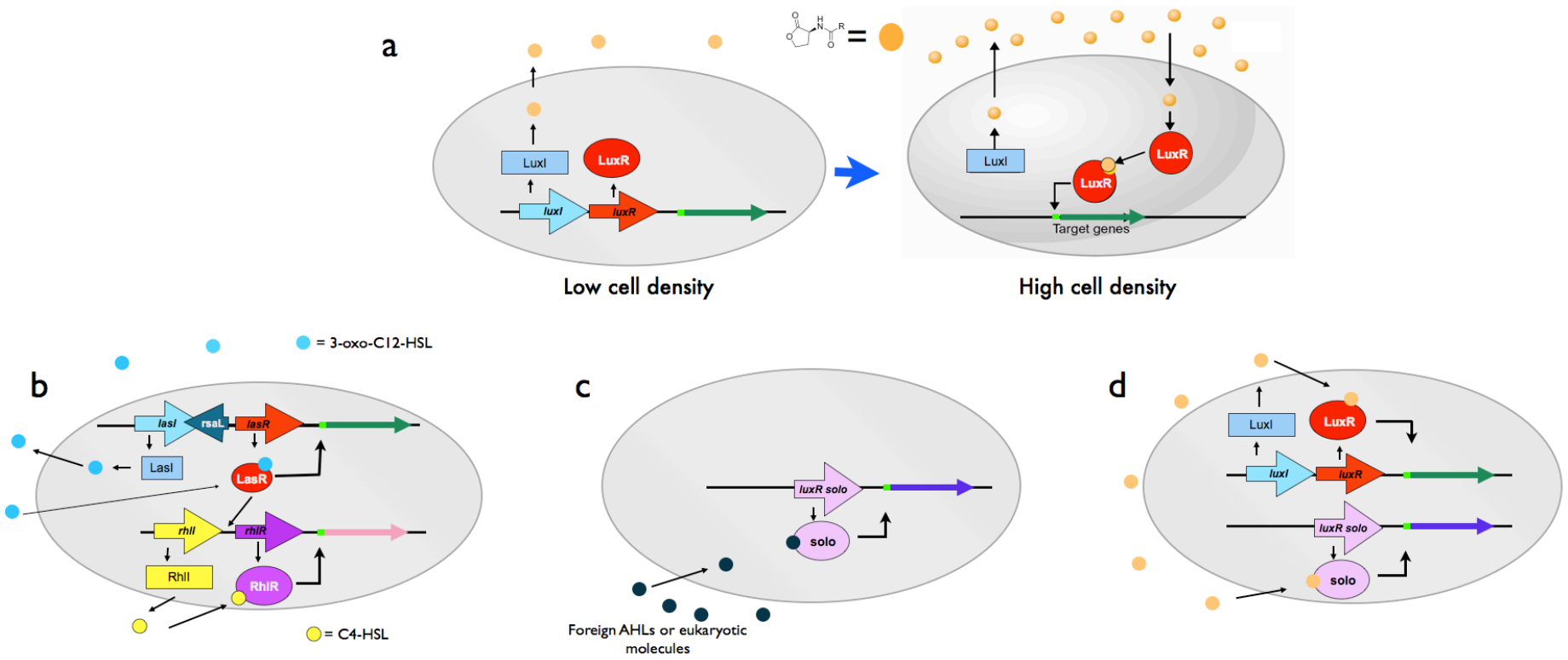


Figure 1-12. AHL-QS scenarios

(a). Cannonical AHL-QS systems composed by a LuxI-type and a LuxR –type proteins. (b) Multiple AHL-QS systems. (c) Unpaired Lux-R solo proteins in non-AHL producers which responds to foreing AHL or to eukaryotic signals (d) Unpaired-LuxR solo proteins in AHL-producing bacteria

1.2.4 Approaches to the study of AHL-QS: Bacterial biosensors

The first step in the study of AHL-QS consists in the identification of the AHL molecules. The most common strategy consists in the use of bacterial biosensors. These biosensors do not produce AHLs and contain a LuxR family protein cloned together with a cognate target promoter (usually the promoter of the cognate *luxI* synthase), which positively regulates the transcription of a reporter gene (e.g. *luxCDABE, lacZ*, *gfp* and pigment production). The biosensors respond sensitively to AHLs and do not require sophisticated instrumentation. Importantly the sensitivity of each biosensor is dependent on the affinity of the LuxR protein for AHLs. These assays provide information on the most likely chemical identity and concentration of the signal molecules present in bacterial cultures supernatants. [Figure 1-13; reviewed in (Steindler & Venturi, 2007)]. Unequivocal determination of the structure of AHLs is then detemined via HPLC coupled with mass-spectrometry.

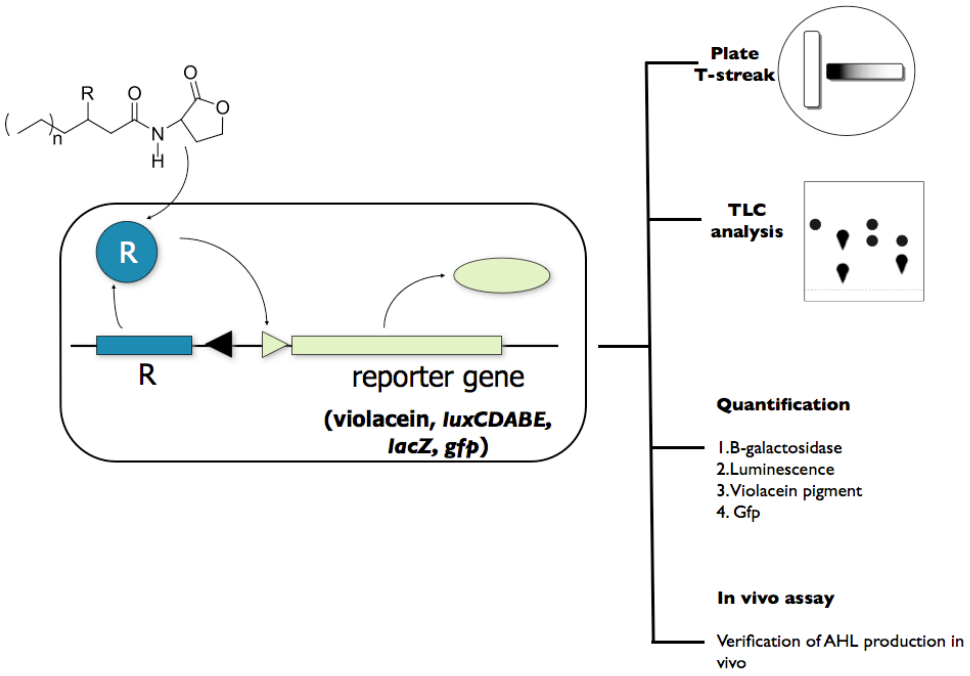


Figure 1-13. Construction and use of a QS-bacterial biosensors.

The exogenous AHL interacts with a LuxR family protein inside the bacterial biosensor (non-AHL producer), which results in the transcription of a reporter gene(s) from a LuxR family-AHL regulated promoter as shown by the open triangle. The LuxR family gene is usually expressed from a constitutive promoter as shown with a filled triangle. (Modified from Steindler and Venturi, 2007)

1.2.5 AHL-Quorum sensing in the genus *Burkholderia*

Several AHL-based QS systems have been identified in *Burkholderia*, and in some cases more than one circuit per species has been detected. The study of QS in human and plant pathogenic *Burkholderia* has been focused in determining the possible roles of cell-cell communication in virulence and pathogenicity. Characterized QS systems in *Burkholderia* species are presented in Table 1-3.

1.2.5.1 AHL-Quorum sensing in the BCC complex

The existence of AHL-QS in BCC species was first proposed in 1995, when it was reported that spent supernatants from *P. aeruginosa* were able to activate the production of siderophores, protease and lipase in a *B. cepacia* strain (McKenney *et al.*, 1995). Further studies determined the presence of an AHL-based QS system in a clinical isolate of *B. cenocepacia* strain (K56-2), which was designated *cepI*R (Lewenza *et al.*, 1999). The *cepI*R system produces and responds to C8-HSL, although minor amounts of C6-HSL have also been detected. CepR positively controls the transcription of *cepI* via binding to a 20 bp *lux*-box in the putative *cepI* promoter region, and negatively regulates its own transcription(Lewenza & Sokol, 2001).

These results were later verified by characterizing *cepI*R in *B. cenocepacia* strain H111 and in *B. cepacia* strain ATCC25416^{T}(Aguilar *et al.*, 2003a; Huber *et al.*, 2001). Evaluation of *cepI*R ortholog systems in other species of the BCC complex suggested that the *cepI*R system is conserved in species of the BCC complex (Table 1-3) (Gotschlich *et al.*, 2001; Lutter *et al.*, 2001). Many different BCC virulence phenotypes have been shown to be positively regulated by the *cepI*R system, including extracellular enzymes as protease, polygalacturonase, swarming motility, and biofilm formation, while negative regulation was observed in the production of the siderophore ornibactine [Reviewed by (Eberl, 2006; Venturi *et al.*, 2004)]. In addition, the *cepI*R system is required for full virulence in both rodent and worm models of infection (Kothe *et al.*, 2003; Sokol *et al.*, 2003).

B. vietnamiensis and *B. cenocepacia* possess more than one QS circuit. In *B. vietnamiensis* a second QS circuit*, bviI*R*,* produces and responds to C10-HSL, and is present only in *B. vietnamiensis* among BCC complex (Conway & Greenberg, 2002; Gotschlich *et al.*, 2001). The AHL production by this system is strain dependent with regard to type and quantity of AHL produced. Furthermore, cross-talk regulation has been observed between both

QS systems, as transcription of *bviI* requires both BviR and CepR, although BviR does not regulate the *cepIR* system (Malott & Sokol, 2007).

In epidemic strains of *B. cenocepacia* that possess the *B. cenocepacia* pathogenicity island (*cci*), CepR is required for the expression of an additional quorum-sensing system, the *cciIR* system (Malott *et al.*, 2005). The predominant AHL produced by the CciI synthase is C6-HSL, with minor amounts of C8-HSL. *cciI* and *cciR* are cotranscribed, and CciR negatively regulates the expression of the *cciIR* operon and *cepI*. CciIR also regulates extracellular protease production, swarming motility and is important for virulence in murine hosts (Baldwin *et al.*, 2004). In summary, the two B. *cenocepacia* QS systems form a global regulatory system, in which CepR is positioned hierarchically upstream of CciIR (O'Grady *et al.*, 2009).

Table 1-3. Known QS systems in *Burkholderia* species

SPECIES	QS CIRCUITS	MAIN AHL COGNATE	REFERENCE
BCC complex			
B. cepacia	*cepIR,*	C6-HSL;C8-HSL	(Aguilar *et al.*, 2003a)
B. cenocepacia	*cepIR, cciIR*	C6-HSL;C8-HSL	(Lewenza *et al.*, 1999)
	cepR2§		(Malott *et al.*, 2009b)
B. anthina	NI	C6-HSL;C8-HSL	
B. pyrrocinia	NI	C6-HSL;C8-HSL	(Gotschlich *et al.*, 2001)
B. lata	*cepIR*	C8-HSL	(Schmidt *et al.*, 2009)
B. ambifaria	*baflR*	C8-HSL	(Zhou *et al.*, 2003)
B. stabilis	*cepIR*	C6-HSL;C8-HSL	(Gotschlich *et al.*, 2001)
B, multivorans	*cepIR*	C6-HSL;C8-HSL	(Gotschlich *et al.*, 2001)
B. vietnamiensis	*cepIR;bviIR*	C6-HSL;C8-HSL; C10-HSL	(Malott *et al.*, 2005)
B. dolosa	NI	C6-HSL;C8-HSL	
Pseudomallei **group**			
B. mallei	*bmaIR1,*	C8-HSL	(Duerkop *et al.*, 2007)
	bmaIR3	3-OH-C8-HSL	(Duerkop *et al.*, 2008)
	bmaR4,bmaR5		
B. pseudomallei	*bpsIR1;*	C8-HSL,OHC8	(Song *et al.*, 2005)
	bpsIR2;	C8-HSL;3-OH-C8-HSL1;3-	(Kiratisin & Sanmee, 2008)
	bpsIR3,	OH-C10-HSL	
	bpsR4,bpsR5		
B. thailandensis	*btaIR1,*	C8-HSL	(Chandler *et al.*, 2009)
	btaIR2,	3-OH-C10-HSL,3-OH-C8-	(Duerkop *et al.*, 2009)
	btaIR3	HSL	(Chandler *et al.*, 2009)
	btaR4,btaR5	3-OH-C8-HSL	
Phytopathogens			
B. glumae	*tofIR*	C6-HSL;C8-HSL	(Kim *et al.*, 2004)
B. plantari	*plaIR*	C6-HSL;C8-HSL	(Solis *et al.*, 2006)

§ (LuxR-*solo* proteins are hightlighted in RED)

Recently, a LuxR-*solo* protein was characterized in *B. cenocepacia* and denoted as CepR2 (Malott *et al.*, 2009b). The CepR2 regulon includes targets of the CepIR-CciIR QS systems, which suggest that the role of CepR2 could be to balance the timing and level

of gene expression. Importantly CepR2 does not require AHL for solubility or activity (Malott *et al.*, 2009b).

1.2.5.2 AHL-QS in the "*pseudomallei*" group

Quorum sensing in the pathogens *B. pseudomallei*, *B. mallei* and *B. thailandensis* is more complex than in other *Burkholderia* species, as it consists of multiple *luxIR* homologs and LuxR-solo proteins (Table 1-3).

Studies in *B. pseudomallei* strains used the designations *bps*, *pml* and *bpm* when referring to the three *luxIR* pairs and two LuxR-*solo* proteins found in their genomes. The reason for these different names stems from the usage of different strains included in each study, which reported variable AHL profiles. Mutants in *bpsI* genes no longer produced C8-HSL and 3-oxo-C8-HSL. Also deficient mutants were less virulent and exhibited decreased patterns of colonization in murine hosts and *C. elegans* (Chan & Chua, 2005; Song *et al.*, 2005; Ulrich *et al.*, 2004; Valade *et al.*, 2004). QS negatively regulates the production of siderophores, as well as the response to oxidative stress (Lumjiaktase *et al.*, 2006; Song *et al.*, 2005). Recently, it has been shown that the LuxR *solo* proteins BpsR4 and BpsR5, negatively regulate *bpsI3*, while BpsR5 positively regulates *bpsI1* (Kiratisin & Sanmee, 2008) .

B. thailandensis also hosts three *luxIR* pairs and two LuxR-*solo* proteins. The characterization of *btaIR1*, *braIR2* and *btaIR3*, revealed a determinant role of *btaIR2* in antibiotic production (bactobolin), while complete deficiency in the AHL production affected bacterial aggregation, with no relevant effects in virulence (Chandler *et al.*, 2009; Duerkop *et al.*, 2009). In *B. mallei* the QS circuitry consists of two *luxIR* pairs and two LuxR-*solo* proteins, but in contrast to *B. pseudomallei*, it lacks the BpmIR2 (Table 1-5)(Duerkop *et al.*, 2007; Duerkop *et al.*, 2008).

The QS role was also studied in the rice pathogens, *B. glumae* and *B. plantarii*, and the *tofIR* and *plaIR* systems have been characterized (Devescovi *et al.*, 2007; Solis *et al.*, 2006). These two systems share 99% and 75 % identity with the *cepIR* system and their mutation reduces significantly their pathogenicity in rice (Devescovi *et al.*, 2007; Solis *et al.*, 2006).

1.2.5.3 AHL-QS in the plant beneficial *Burkholderia*

AHL-QS in this group of bacteria remains at large unexplored. The production of AHLs was reported in the description of *B. phytofirmans, B. megapolitana* and *B. bryophila* species (Sessitsch *et al.*, 2005; Vandamme *et al.*, 2007).

Recently, a study of two *B. graminis* isolates reported that both strains were able to produce AHLs (Barriuso *et al.*, 2008). Moreover, the biological effects on plant growth promotion and protection from salinity and stress were altered by the presence of AHLs produced by transgenic plants, although this effect was strain dependant (Barriuso *et al.*, 2008). Experiments with *gfp*-labelled biosensors suggested that these *B. graminis* strains are able to secrete AHL during the rhizosphere colonization, however no genetic analyses have been performed to unravel the genetic system responsible of the production of the autoinducers (Barriuso *et al.*, 2008).

In 2009, two strains of *B. phytofirmans* strains were reported to synthesize 3-oxo-C14-HSL, 3-hydroxy-C14-HSL and 3-hydroxy-C12 HSL. In strain PsJN the production of 3-hydroxy-C8-HSL was also detected (Trognitz *et al.*, 2009). In the same study a lactonase–expressing vector was mobilized into *B. phytofirmans* PsJN to reduce the AHL production and plant experiments in two potato cultivars were performed. The potato-plant response was evaluated by transcriptomic analysis and the results suggested that although the inoculation effects may depend on the strain, AHL plays a determinant role in the plant-endophyte interaction (Trognitz *et al.*, 2009). In spite of the novelty provided by these studies, the genetic elements involved in the AHL production and their targets have not been determined. Further studies focused both in the detection of AHL, the genetic mechanisms and their regulons need to be carried out in order to understand their role in the adaptability and versatility of this group of *Burkholderia*.

1.3 Aim of this thesis

The group of plant-associated beneficial *Burkholderia* represents an interesting subject of study as they share both ubiquity and catabolic versatility with other well-studied pathogenic *Burkholderia*, but unlike them, these species interact beneficially with the environment or with their plant hosts.

B. kururiensis, B. unamae and *B. xenovorans* may occur in the rhizosphere or endosphere of plants. In addition they have two important biotechnological features: the plant growth promotion and the degradation of recalcitrant pollutants. However, despite the increasing attention given to these species, the molecular mechanisms regulating most of their phenotypes are still not addressed or poorly understood.

The scope of this thesis was to determine the possible presence and role of *N*-acyl homoserine lactone quorum sensing (QS) in species of this cluster of *Burkholderia* spp. In order to perform these studies, *B. kururiensis*, *B. unamae* and *B. xenovorans* will be used, as they are representative species of this group of *Burkholderia*.

2 MATERIALS AND METHODS

2.1 GENERAL PROCEDURES

2.1.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

Bacterial strains used in this study are listed in Table 2-1. *Burkholderia* "*brasilensis*" M130 was isolated from rice in Brazil (Baldani *et al.*, 1997b) and kindly provided by Dr. Lucia Mendonça-Previato from the Universidad Federal do Rio de Janeiro, Brasil. This strain was recently re-classified as *B. kururiensis* M130 (Caballero-Mellado *et al.*, 2007). *B. unamae* MTI-641^{T} and SCCu-23, *B. ferrariae* FeGI0^{T} and *B. nodosa* BR3437^{T} were provided by Jesús Caballero-Mellado from the Universidad Nacional Autonoma de México. 21 *Burkholderia* type strains belonging to the plant-associated *Burkholderia* group were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

Table 2-1. Bacterial Strains used in this study

STRAINS	CHARACTERISTICS	REFERENCE OR SOURCE
Escherichia coli Strains	Genotype	
E. coli DH5α™	ϕ80d*lacZ* ΔM15,*end*A1, *rec*A1, *gyr*A96 (Nal^{R}),*thi*-1, *hsd*R17(r_K-,$m_K$$^{+}$), , *sup*E44,relA1,*deoR* Δ(lacZYA-argF)U169,*phoA*	Gibco BRL-Life Technologies
E. coli JM109	*end*A1, *rec*A1, *gyr*A96 (Nal^{R}),*thi*-1, *hsd*R17(r_K-,$m_K$$^{+}$), *rel*A1, *sup*E44,*e*14-(McrA^{-}), Δ(*lac-proAB*)/*F' pro A+B+ traD36lacI*^{Q} , Δ*lacZM*15	(Yanisch-Perron *et al.*, 1985)
E.coli HB101	*supE44, hsdS20* ($r_b$$^{-}$ $m_b$$^{-}$), *recA13, ara* -14, *leu*B6, *pro* A2, *lac*Y1, *gal* K2, *rpsL*20 (str^{R}), *xyl*-5, *mtl*-1	(Lacks & Greenberg, 1977)
E. coli M15	NaI^{S}, Str^{S}, Rif^{S}, Thi-, *ara*$^{+}$, *gal*$^{+}$, *mtl*$^{-}$, F^{-}, *recA*$^{+}$, *uvr*$^{+}$, *lon*$^{+}$,	Qiagen
E. coli CC118	*araD139*(Δ*ara-leu*)*7697*,Δ*lac X74, ΔphoA20, galE, galK,thi-1,rpsE*(Sp^{R}),*rpoB* (Rif^{R}),*argE (Am) recA.*	(Manoil & Beckwith, 1985)
E. coli S17-1 λ-pir	*recA*, *thi*, *pro*, *hsd*R^{-}M^{+}(RP4: 2-Tc::Mu-Km::Tn7) λpir (Tp^{R}, Sm^{R})	(Simon *et al.*, 1983)
E. coli DH5α (pRK2013)	Conjugation helper strain Tra^{+} Mob^{+} ColE1 replicon; Km^{R}	(Figurski & Helinski, 1979)
Biosensor Strains		
Pseudomonas putida F117	*ppuI*$^{-}$ derivative of *P. putida* IsoF;	(Steidle *et al.*, 2002)
Pseudomonas putida SM	*ppuI*$^{-}$, *rsaL*$^{-}$ derivative of *P.putida* WCS356	(Rampioni *et al.*, 2006)
Chromobacterium violaceum CVO26	*cviI*$^{-}$ Tn5 mutant derivative of ATCC31532	(McClean *et al.*, 1997)
Agrobacterium tumefaciens NTL4	Ti plasmidless derivative of C58 Tc^{S}	(Luo *et al.*, 2001)

***Burkholderia* Strains**		
Burkholderia kururiensis M130	Amp^{R} Rif ^{R}	(Baldani *et al.*, 1997a)
M130BRAI	*braI*::Km of *B. kururiensis* M130	This study
M130BRAR	*braR*::Km of *B. kururiensis* M130	This study
M130RSAL	*rsaL*::Km of *B. kururiensis* M130	This study
Burkholderia xenovorans LB400^{T}	Type Strain	(Goris *et al.*, 2004)
LB400BRAI	*braI*::Km of *B. xenovorans* LB400	This study
LB400BRAR	*braR*::Km of *B. xenovorans* LB400	This study
LB400XENI2	*xenI2*::Km of *B. xenovorans* LB400	This study
LB400XENR2	*xenR2*::Km of *B. xenovorans* LB400	This study
LB400BXER	*bxeR*::Km of of *B. xenovorans* LB400	This study
Burkholderia unamae MTl-641^{T}	Type Strain (Maize Rhizospere isolate)	(Caballero-Mellado *et al.*, 2004)
UNABRAI	*braI*::Km of *B. unamae* MTI-641^{T}	This study
UNABRAR	*braR*:Km of *B. unamae* MTI-641^{T}	This study
Burkholeria unamae SCCu-23	Sugarcane root isolate	
Burkholderia xenovorans CAC-124	Coffee plant rhizosphere isolate	(Goris *et al.*, 2004)
Burkholderia xenovorans LMG 16224	Human blood culture specimen isolate	(Goris *et al.*, 2004)
Burkholderia xenovorans TCo-382	Tomato Rhizosphere isolate	(Caballero-Mellado *et al.*, 2007)
Burkholderia xenovorans TCo-26	Tomato Rhizoplane isolate	(Caballero-Mellado *et al.*, 2007)
Burkholderia caledonica DSM17062^{T}	Type Strain	(Coenye *et al.*, 2001b)
Burkholderia caribensis DSM13236^{T}	Type Strain	(Achouak *et al.*, 1999)
Burkholderia fungorum DSM17061^{T}	Type Strain	(Coenye *et al.*, 2001b)
Burkholderia ferrariae FeGI0^{T}	Type Strain	(Valverde *et al.*, 2006)
Burkholderia graminis DSM17151^{T}	Type Strain	(Viallard *et al.*, 1998)
Burkholderia glathei LMG 14190^{T}	Type Strain	(Zolg & Ottow, 1975)
Burkholderia hospita DSM17164^{T}	Type Strain	(Goris *et al.*, 2002)
Burkholderia kururiensis DSM13646^{T}	Type Strain	(Zhang *et al.*, 2000)
Burkholderia mimosarum PAS44^{T}	Type Strain	(Chen *et al.*, 2006)
Burkholderia phenazinium DSM10684^{T}	Type Strain	(Viallard *et al.*, 1998)
Burkholderia nodosa BR3437^{T}	Type Strain	(Chen *et al.*, 2007)
Burkholderia phenoliruptrix DSM17773^{T}	Type Strain	(Coenye *et al.*, 2004)
Burkholderia phymatum DSM17167^{T}	Type Strain	(Vandamme *et al.*, 2002)
Burkholderia phytofirmans DSM17436^{T}	Type Strain	(Sessitsch *et al.*, 2005)
Burkholderia sacchari DSM17165^{T}	Type Strain	(Bramer *et al.*, 2001)
Burkholderia silvatlantica SRMrh-20^{T}	Type Strain	(Perin *et al.*, 2006b)
Burkholderia terrae DSM17804^{T}	Type Strain	(Yang *et al.*, 2006)
Burkholderia terricola DSM17221^{T}	Type Strain	(Goris *et al.*, 2002)
Burkholderia tuberum DSM18489^{T}	Type Strain	(Vandamme *et al.*, 2002)
Burkholderia tropica Ppe8^{T}	Type Strain	(Reis *et al.*, 2004)

Burkholderia strains were grown at 30°C in either M9 minimal medium supplemented with glycerol or glucose (Sambrook, 1989), King's medium (King *et al.*, 1954), or BSE liquid medium (Estrada-de Los Santos *et al.*, 2001). *Escherichia coli* and *Pseudomonas* spp. were grown in LB media. The preparation of all culture media, antibiotics and supplements is described in appendix 6.1.

Antibiotics were added when required at the following final concentrations: ampicillin 100 μg mL^1, streptomycin 100 μg mL^1, tetracycline 15 μg mL^{-1} (*E. coli*) or 40 μg mL^{-1} (*Burkholderia*), gentamicin 10 μg mL^{-1} (*E. coli*), 30 μg mL^{-1} (*Agrobacterium*) and 40 ug mL μg mL^{-1} (*Pseudomonas* and *Burkholderia*); kanamycin 50 μg mL^{-1} (*E. coli* and *C. violaceum*) or 100 μg mL^{-1} (*Pseudomonas* and *Burkholderia*); rifampicin 100 μg mL^{-1}, nitrofurantoin 50 μg mL^{-1}.

Growth curves and CFU/mL for wild type and mutants were determined in KB media. For long-term storage, single colonies were grown overnight in liquid media supplemented with antibiotics, when required. Subsequently 0.85 mL were transferred in a sterile vial containing 0.15 mL sterile glycerol and then frozen at -80 °C.

2.1.2 REAGENTS AND CHEMICALS

All chemicals used for the culture media preparation were purchased from Difco and Sigma. Molecular Biology reagents were acquired from New England Biolabs and Promega. The Centre for Biomolecular Sciences, University of Nottingham, supplied synthetic AHLs.

2.1.3 RECOMBINANT DNA TECHNIQUES

Recombinant DNA techniques, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, hybridization and radioactive labelling by random priming were performed as described (Sambrook, 1989). The composition and preparation of solutions used are provided in appendix 6.2.

2.1.3.1 Genomic DNA extraction

Total DNA from *Burkholderia* was isolated by Sarkosyl-pronase lysis (Better *et al.*, 1983). 5 mL from an overnight culture were harvested by centrifugation and suspended in 400 μL of TE (50mM Tris-HCl 20 mM EDTA, pH 8.0). Cells were then lysed by adding 500 μL of 2 % Sarkosyl (in TE) and 100 μL of pronase (5 mg/mL), followed by incubation at 37°C for 30 min. DNA was extracted by adding one volume of phenol: chloroform: isoamyl alcohol (25:24:1) with vigorous vortexing to mix. Each sample was then centrifuged at 14.000 rpm for 10 minutes and the upper aqueous phase was transferred to a new tube. An additional extraction with chloroform was performed, and

the DNA was precipitated from the aqueous phase by adding 40 µL of 5M NaCl and 2 volumes of ethanol absolute. The obtained pellet was washed with 70% ethanol, and air-dried. The DNA pellet was resuspended in 500 µL HPLC grade sterile water with 10 µL of 10 mg/mL RNAse. The quality of the genomic DNA was evaluated by agarose gel electrophoresis.

2.1.3.2 Plasmids

Plasmid DNA isolation from *E. coli* was performed by using EuroGold columns (EuroClone, Italy) according to the manufacturers instructions. Briefly, overnight cultures were submitted to alkaline lysis and neutralization. After removal of the cellular debris the lysates were loaded in a silica column and eluted with sterile water.

Cosmid DNA was extracted from *Pseudomonas*, from 10 mL overnight cultures. Cells were first harvested at 4000 rpm and washed with 1 mL TE. Washed cells were then resuspended in 200 µL of GTE solution (50 mM Glucose; 25mM Tris-Cl; 10 mM EDTA pH8.0), and lysed by adding 400 µL of solution II (0.2 N NaOH, 1 % SDS) during 5 minutes. 300 µL of 3 M KOAc were added to neutralize, followed by 10 min centrifugation at 14000 rpm. The supernatant was then transferred to a new tube and DNA was extracted by adding 1 volume of phenol: chloroform: isoamyl alcohol. The aqueous phase was recovered, and the DNA was precipitated with 1 volume of isopropanol. The DNA pellet was then washed with ethanol 70% and once air-dried, the cosmidic DNA was resuspended in 100 µL of sterile water.

2.1.3.3 DNA Agarose electrophoresis

DNA samples were analyzed routinely on agarose gels (0.8%-1.5 w/v) in TAE 1x (40 mM Trisma base, 1mM EDTA pH 8.0, 0.1142% glacial acetic acid). To visualize DNA, SYBR Green I nucleic acid gel stain (Invitrogen ®) was added 1:10000 into the gel solution just prior to pouring the gel. Electrophoresis was performed in Biorad mini sub™ cell chamber, at 5 V/cm, and TAE 1x was used as running buffer (Sambrook, 1989).

2.1.3.4 Cloning of PCR products

Cloning and expression vectors used are listed in Table 2-2. For cloning purposes, DNA fragments were PCR amplified from approx. 50 ng of chromosomal DNA. For general

PCR reactions, 0.6 U of GoTaq Flexi (Promega) were used, and the reaction mix was composed by 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.1μM of each primer. 0.05% DMSO was also added in order to increase reaction stringency. When required restriction sites were added to the primers in the 5' extremes (Table 3). PCR reactions were performed in an Applied Biosystems PCR GeneAmp 2400 thermocycler. The DNA template was initially denatured at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C, annealing at 55-65 °C for 30 seconds and extension at 72 °C for 1-3 minutes, according to the length of the fragment to be amplified. A last extension cycle at 72°C during 5 minutes was used to ensure completion of strands.

In all cases, fragments were separated by electrophoresis and extracted from the gel by using the EuroGOLD gel extraction kit (Euroclone, Italy). Blunt ended fragments were then cloned into the pMOS-Blue® (Amhersam Pharmacia), or protruding A- fragments were cloned into pGEM-T easy® (Promega), according to the instructions of the manufacturer. Subsequently, each fragment was excised with restriction enzymes, ligated into the final vector, and sequenced to verify the identity and orientation of the insert. A description of constructs generated in this work and the primers used is provided in Table 2-3.

Table 2-2. Cloning and expression vectors used in this study

Plasmids	Relevant characteristics	Reference
pMOS-Blue	Cloning vector; Amp^R	Amersham-Pharmacia
pBlueScript KS+	Cloning vector; Amp^R	Stratagene
pGEM-T easy	Cloning vector; Amp^R	Promega
pMP220	Promoter probe vector, IncP, LacZ, Tet^R	(Spaink *et al.*, 1987)
pLAFR3	Broad-host-range cloning vector, IncP1; Tet^R	(Staskawicz *et al.*, 1987)
pKNOCK-Km	Conjugative suicide vector; Km^R	(Alexeyev, 1999)
pQE30	Expression vector, ColE1 replication origin, T5 promoter, His epitope. Amp^R.	Qiagen
pBBRMCS-5	Broad-host-range vector Gm^R	(Kovach *et al.*, 1995)

New England Biolabs Restriction enzymes were used to cut both genomic as plasmidic DNA. Generally 0.5-1 μg of DNA were digested by using 10 U of enzyme in a final volume of 25 μL, with the addition of the buffer suggested by the provider.

Table 2-3. Plasmid constructs generated in this study.

PLASMIDS	DESCRIPTION	PRIMER NAME	PRIMER SEQUENCE	AMPLICON SIZE (bp)
pKnock plasmids				
pKNOCK-BRAI	Internal *Eco*RV *braI*$_{KUR}$ fragment of *B. kururiensis* cloned into pKNOCK-Km			
pKNOCK-BRAR	pKNOCK-Km containing an internal fragment of *braR*$_{KUR}$ fragment from *B. kururiensis*	braR18FW braR509Rv	5'-CATCCGACGAGACGCAAT-3' 5'-TTTGGAATGAGCGTTTGC-3'	490
pKNOCK-RSAL	pKNOCK-Km containing an internal fragment of *rsaL*$_{KUR}$ from *B. kururiensis*	braLFW braLRV	5'-TTGTTGAAATAAAGTCCCAG-3' 5'-CTGGAAAATCACTGGCA-3'	225
pKNOCK-UNAI	pKNOCK-Km containing an internal fragment of *braI* $_{UNA}$ from *B. unamae*	UNAIFW40 UNAIRV566	5'-GACAACGAGGACATCAACGA-3' 5'-TGTGGGTCTGCTGGCTCAT-3'	517
pKNOCK-UNAR	pKNOCK-Km containing an internal fragment of *braR*$_{UNA}$ from *B. unamae*	PKNUNARFW179 PKNUNARRV604	5'-AGTTCGTGGGGATTCAG-3' 5'-GTGGTCGGTCAGATGCC-3'	426
pKNOCKXENI	pKNOCK-Km containing an internal fragment of *braI*$_{XEN}$ from *B. xenovorans*	LUXIXENFW LUXIXENRV	5'-GGTTTTTCACGGGCGGCTC-3' 5'-CGATCGGCGTGGTGGTCA-3'	368
pKNOCKXENR	pKNOCK-Km containing an internal fragment of *braR*$_{XEN}$ from *B. xenovorans*	LUXRXENFW LUXRXENRV	5'-TACTTACGACGAGCAGGG-3 5'-GTTCAGGATGTGGGAGATTT-3	421
pKNOCKXENI2	pKNOCK-Km containing an internal fragment of *xenI2* from *B. xenovorans*	MUTXENI2FW MUTXENI2RV	5'-GCAGTACCGTCCATCTGGTT-3' 5'-TCGATCTCAGCAGACTGTCG-3'	482
pKNOCKXENR2	pKNOCK-Km containing an internal fragment of *xenR2* from *B. xenovorans*	MUTXENR2FW MUTXENR2RV	5'-CACCGATATCCCTTCTCTCG-3' 5'-GTGCGGCTCTTCTTGAATGT-3'	440
pKNOCKBXE	pKNOCK-Km containing an internal fragment of *bxeR* from *B. xenovorans*	XENSOLOFW XENSOLOREV	5'-GCTTTCTCACGCTCTTCACC-3' 5'-GGTGCGATGCCGATCACGCT-3'	595
pQE30 derivatives				
pQEBRAR	*braR*$_{KUR}$ from *B.kururiensis* cloned into pQE30 expression vector	pQEbraRfw pQEbraRrv	5'-GGGGATCCTCGCCGATACTGGCCGCA-3' 5'-GGGAAGCTTTCAGCCCGGATCTATAAGG-3'	714
pQEXENR1	*braR*$_{XEN}$ from *B. xenovorans* cloned into pQE30 expression vector	XENR1FW XENR1RV	5'-TCGGATCCGCACCGCTACTGAACGCC-3' 5'-TCAAGCTTTCAACCCGGGTCAATAAG-3	719
pQEXENR2	*xenR2* from *B. xenovorans* cloned into pQE30 expression vector	XENR2FW XENR2RV	5'-TCGGATCCACACCTATCTCTCGACGC-3' 5'-TCAAGCTTTCAGGGTTTTATGAGGCCC-3'	803
pQEBXER	*bxeR* from *B. xenovorans* cloned into pQE30 expression vector	BAMSOLOXENFW HINDSOLOXENRV	5'-TCGGATCCGAACACGAATGCGATACG-3' 5'-TCAAGCTTCTACGACACGTACGCCTG-3'	999
pQEXENI1	*braI*$_{XEN}$ from *B. xenovorans* cloned into pQE30 expression vector	XENI1_FW XENI1_RV	5'-TCGGATCCCAAACAGCAATCCGGATTGG -3' 5'-TCAAGCTTTTACGCGGCAGCGGCCATC-3'	603
PQEXENI2	*xenI2* from *B. xenovorans* cloned into pQE30 expression vector	pQEX2IFW pQEX2IRV	5'-GGGGATCCTCATTCGTCGTTGCCGGC-3' 5'-GGGAAGCTTTTAAATTGCCCGTTCCGT-3'	758
pQEUNAR	*braR*$_{UNA}$ from *B. unamae* cloned into pQE30 expression vector	PQEUNARFWBAM PQEUNARRVHIND	5'- GGGGATCCTCGCCCGTGCTCGACGC-3' 5'- GGGAAGCTTCCGATGGTGTTTAGCCC-3'	717

pMP220 derivatives				
pMPBRAI	pMP220 containing a 403 bp *Cla*I-*Eco*RV fragment with the *braI*$_{KUR}$ promoter region from *B.kururiensis*			
pMPXENI1	*braI*$_{XEN}$ promoter region from *B. xenovorans* cloned into pMP220	PXENIFWECO PXENIRVXBA	5'- CGGAATTCCCATTTTCGTGTCCTGT-3' 5'- AATCTAGATTCCTGACGCATTCCAA-3'	192
pMPX2I	*xenI2* promoter region from *B. xenovorans* cloned into pMP220	PC00415KPNFW PC00415XBARV	5'-AAGGTACCGCACTCAACATCTGGGG -3' 5'-AATCTAGAGGCAACGACGAATGACA-3'	267
pMPBXER	*bxe*R promoter region from *B. xenovorans* cloned into pMP220	PROMSOLOBAMFW PROMSOLOXBARV	5'-AGGATCCATTCACGCTCGAGACCAGTT-3 5'-TTCTAGATATCGCATTCGTGTTCCATT-3'	279
pMP2786	Promoter of gene Bxe2786 from *B. xenovorans* cloned in pMP220 vector	BXE2786FW BXE2786RV	5'-AAGGATCCATCACGAATCCACGACCG-3' 5'-AAAAAGCTTGCAACCGCAACGACGATT-3'	514
pMP1181	Promoter of gene Bxe1181 from *B. xenovorans* cloned in pMP220 vector	BXE_1181FW BXE_1181RV	5'-GGGGATCCGTCCAGTCGGTGAAGGT-3' 5'-CCCAAGCTTAGCGGTTTTCTTCAGCAT-3'	473
pMP0016	Promoter of gene Bxe0016 from *B. xenovorans* cloned in pMP220 vector	BXE0016FW BXE0016RV	5'-AAGGATCCCCACTGCTTGTCGGTCTC-3' 5'-CCCAAGCTTCGTTTCATACGCTCGACA-3'	385
pMPUNAI	promoter of gene *braI*$_{UNA}$from *B. unamae* cloned in pMP220 vector	PMPUNAIFWECO PMPUNARRVKPN	5'-CGGAATTCCGATGACCGAAGACAGA-3' 5'-GGGGTACCATGACAACTCTCCCGAT-3'	246
Other constructs				
pZS1	pLAFR3 containing the QS loci of *B. kururiensis*			
pZS2	pLAFR3 containing the QS loci of *B. kururiensis*			
pMOS-Xmn-1	pMOS-Blue containing a 5 Kb XmnI fragment of *B. kururiensis*			
pLZ1	pLAFR3 containing *B. unamae* QS loci			
pBBRXENI1	*braI*$_{XEN}$ cloned into pBBR-MCS5 Gm^R	XENIFWECORI XENI1RV	5'- CGGAATTCCCATTTTCGTGTCCTGT-3' 5'- TCAAGCTTTTACGCGGCAGCGGCCATC-3'	750
pBBRBXER	*bxe*R cloned into pBBR-MCS5 Gm^R	PROMSOLOBAMFW HINDSOLOXENRV	5'-AGGATCCATTCACGCTCGAGACCAGTT-3 5'-TCAAGCTTCTACGACACGTACGCCTG-3'	1279
pBBRXENR	*braR*$_{XEN}$ cloned into MCS5 Gm^R	PBBRXENR_FW PBBRXENR_RV_XBA	5'-ggGGTACCGATGTTTGCGAGCCTGA-3' 5'-aaTCTAGAACGCCGCCGCCTTGCTC-3'	773
pBBRRSAL	*rsaL*$_{KUR}$ cloned into pBBR-MCS5 Gm^R	RSALFWXBA RSALRVKPN	5'-CCTCTAGACAGCTGGGCCTTATAG-3' 5'-CCGGTACCAAGTGCGGAGAATCGG-3´	400
pGEMX2R	*xenR2* cloned into pGEM Amp^R	BXEC0416FW BXEC0416RV	5'-ATGACACCTATCTCTCG-3' 5'-CAGCTTCAGGGTTTTAT-3'	794
pREP4	p15A replicon, *lacI*$^+$, repressor of *E. coli* M15			

pKNOCK derived plasmids were Km^R, pQE30 and pMOS-Blue derived constructs were Amp^R , and pMP220 derived plasmids were Tc^R. *Bam*HI sites are highlighted in red, *Hind*III sites are highlighted in blue, *Xba*I sites are highlighted in green and *Kpn*I sites are highlighted in yellow, *Eco*RI in gray. Subindexes KUR, UNA and XEN correspond to *B. kururiensis* , *B. unamae* and *B. xenovorans* accordingly.

Reactions were incubated from 2 h to 10 h, and when required heat inactivation at 65 °C was performed to stop the reaction. For ligation reactions, a 1:2.5 ratio of vector to insert was used, and 1.5 U of T4 DNA ligase (Promega) were added in a final volume of 20 μL. Ligation reactions were incubated for 12 h at 16 °C.

2.1.3.5 Bacterial transformation and conjugation

Preparation of *E. coli* competent cells was done by using the protocol described in appendix 6.3 (Hanahan *et al.*, 1991). Transformation was performed by mixing 5 μL plasmidic DNA with 100 μL of competent cells. The mixture was incubated on ice for 30 minutes, and then heat shocked at 42°C for 90 seconds. The heat shock was stopped by incubation on ice for two minutes, and 1 mL of pre-warmed LB was added. Cells were then grown 1 hour in agitation at 37 °C, and transformants were selected on LB agar plates with the proper antibiotics.

Plasmids were mobilized from *E. coli* to *Pseudomonas* by tri-parental matings using the helper strain *E. coli* (pRK2013) (Figurski and Helinski, 1979). Overnight cultures from donor, helper and acceptor were diluted 1 to 25 in fresh LB media, and then grown during 6 hours. Each culture was then harvested and washed twice, and optical density was determined. Volumes equivalent to 5 $x10^8$ cells from donor and helper were mixed with the corresponding volume to 2 $x10^8$ cells from the acceptor. The mixture of cells was then centrifuged and resuspended in 100 μL of media, and cells were spotted on top of a 0.45 μM nitrocellulose filter, previously placed on a LB agar plate. The conjugation mixture was incubated 12 h at 30 °C. Thereafter, cells were then resuspended in 1 mL LB broth, serially diluted and plated on LB agar, with proper antibiotics. Mixtures of donor cells plus acceptor were used as negative control.

Plasmids were introduced in *B. kururiensis* by biparental conjugation using the *E. coli* S17-1 λ *pir* strain as donor (Simon *et al.*, 1983) and incubated for 22 hrs at 30ºC. Conjugations were counter-selected in JMV Media (Reis *et al.*, 2004) with the appropriate antibiotics. In *B. unamae* and *B. xenovorans* tri-parental conjugation was performed and transconjugants were counter-selected in KB with the appropriate antibiotics.

2.1.3.6 Southern Hybridization

Southern analyses were performed to verify the presence of genes, as well as for confirming the fidelity of all marker exchange events. For Southern analysis, approx. 1 μg of genomic DNA was digested overnight with 10 U of restriction enzyme (s) (New England Biolabs). Digested DNA was then electrophoresed in 0.8% agarose gels, visualized and photographed. Thereafter, the gel was depurinated by soaking it in a 0.125M HCl solution during 10 minutes, followed by denaturation during 30 minutes in denaturation solution (1.5M NaCl, 0.5M NaOH). The gel was then blotted to transfer the DNA to a nylon membrane (Hybond™-XL-Amersham, Biosciences), according to procedures described by the manufacturer. Subsequently, the membrane was dried and fixed by UV crosslinking.

Solutions for hybridization are described in appendix 6.2. Membranes were prehybridized in Denhardt's buffer for at least 1 hour before addition of the radiolabeled probe. Probes were prepared by using a Random Primed DNA labelling kit (Roche), with 25 ng of DNA-template and 0.05 mCi [α-^{32}P] dCTP, as stated in the provider's instruction handbook. For removal of unincorporated dNTPs, a G-25 sephadex column was used (Quick Spin Columns- Roche). Once purified, the probe was diluted to 20 mL with hybridization solution, and denatured by heating at 100°C for 10 minutes. At this stage the denatured probe was added to the membrane.

Hybridizations were performed overnight at 65°C. After probe removal, the membrane was washed once with wash solution 1 (2x SSC, 0.1%SDS) at room temperature followed by two washes of solution 2 (1X SSC, 0.1% SDS) at 65°C. Finally, the membrane was washed twice with (0.1x SSC 0.1% SDS) at 65°C. To identify the DNA fragments hybridized to the probe used, KODAK Biomax MS Films were exposed on the membrane, enclosed in a cassette and incubated at -80 °C, and developed after 6 hours.

2.1.3.7 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by electrophoresis under denaturing conditions by SDS-PAGE using the method described by Laemmli (Laemmli, 1970). Separating and stacking mixes were sequentially prepared as described in appendix 6.2. 10x12 cm glass plates and 0.5 mm spacers were used to assemble the gel mould. The separating acrylamide mix was

then poured into the gel mould, and 1 mL of isobutanol was added to prevent oxygen inhibition of polymerization. One hour later, isobutanol was removed, the stacking acrylamide mix was poured, and the comb was placed. After completion of the polymerization, the gel was fixed in an electrophoresis apparatus and 1x SDS-PAGE running buffer were added. Loading buffer 5x was added to each protein sample and then denatured by boling 10 minutes at 100 ºC. Denatured protein samples were then loaded in the gel wells and then run at 8 V/cm. Gels were stained by immersion during 30 minutes in a 0.25% Coomasie brilliant blue solution prepared in methanol:water: acetic acid (9:9:2). To destain, geles were soaked in a prewarmed destaining solution (30% methanol, 10% acetic acid) during 30 minutes.

2.2 DETECTION OF AHLs IN PLANT-ASSOCIATED BENEFICIAL *Burkholderia*

Acyl homoserine lactone detection in 27 strains of *Burkholderia* species from the plant-associated beneficial cluster was achieved by using six bacterial biosensors with different ranges of specificity. Detection range and references of the biosensors used are presented in table 2-4. *Chromobacterium*, *Agrobacterium* and *Pseudomonas* AHL detector strains were grown at 28 ºC, while *E. coli* were grown at 37ºC. Culture media used for the recovery and growth of the biosensors is detailed in appendix 6.1

P. putida F117 (pKRC12) and *C. violaceum* CV026 were used to detect the production of AHLs by T-streak analysis on plates. In such case, tester strains were streaked and grown on KB solid media close to the biosensor to form a "T" and the phenotypic change associated to the presence of exogenous AHLs was observed.

To tentatively identify the AHLs produced by the *Burkholderia* species, AHL-extraction from cell-free spent supernatants of late exponential phase cultures was carried out. TLC plates were then loaded with the sample extracts and with different AHL standards, and AHL identification was achieved by overlaying the TLC plate with bacterial biosensors. *Burkholderia* strains were grown overnight in 20 mL of M9 minimal medium supplemented with glucose and casaminoacids and the supernatants of the cultures were extracted with one volume of acidified ethyl acetate (Sigma). Organic phase was then air-dried and resuspended in 20 µL of ethyl acetate. Thereafter, C_{18} reverse-phase chromatography TLC plates (20x20 Merck, Darmsadt, Germany) were

loaded with the sample extracts and with different AHL standards. Chromatography was then performed by using methanol: water (60:40) as mobile phase (Shaw *et al.*, 1997). The plates were then overlaid with a thin layer of LB or AB top agar seeded with either, *E. coli* (pSB1075) or *A. tumefaciens* NTL4 (pZLR4), the latter in presence of 100 μg/ml X-gal, as described in Appendix 6.1 (Shaw *et al.*, 1997).

Table 2-4. AHL Biosensors used in this study.

BIOSENSOR	AHL RANGE DETECTION	REPORTER	REFERENCE
Agrobacterium tumefaciens NTL4 (pZLR4)	**3-oxo-C8-HSL*** All 3-oxo-HSL All 3-OH-HSL	β-galactosidase	(Shaw *et al.*, 1997)
Escherichia coli (pSB1075)	**3-oxo-C12-HSL** 3-oxo-C10-HSL 3-oxo-C14-HSL C12-HSL C10-HSL	*luxCDABE*	(Winson *et al.*, 1998)
E. coli JM109 (pSB401)	**3-oxo-C6-HSL** C6-HSL C8-3-oxo-HSL C8-HSL	*luxCDABE*	(Winson *et al.*, 1998)
P. putida F117 (pKRC12)	**3-oxo-C12-HSL** 3-oxo-C10-HSL	Gfp (Green Fluorescent Protein)	(Riedel *et al.*, 2001)
E. coli MT102 (pJBA132)	**3-oxo-C6-HSL** C6-HSL C8-3-oxo-HSL C8-HSL	Gfp	(Andersen *et al.*, 2001)
C. violaceum CV026	**C6-HSL** C8-HSL 3-oxo-C6-HSL 3-oxo-C8-HSL	violacein pigment	(McClean *et al.*, 1997)

* AHL in bold correspond to preferred cognate of each biosensor. (Steindler & Venturi, 2007)

2.3 IDENTIFICATION OF QS SYSTEMS IN THREE SPECIES OF THE PLANT-ASSOCIATED BENEFICIAL *Burkholderia* GROUP

The presence of *luxIR* pairs was studied in *B. kururiensis* M130, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T. Cosmid libraries were constructed for *B. kururiensis* M130 and *B. unamae* MTI-641^T, by using the cosmid pLAFR3 as vector (Staskawicz *et al.*, 1987). Insert DNA was prepared by partial *Eco*RI digestion of the genomic DNA and then ligated in the corresponding site in pLAFR3. The ligated DNA was then packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene) and the phage particles were transduced to *E. coli* HB101 as recommended by the supplier. In order to identify the cosmids containing the AHL QS genes, the *E. coli* HB101 harbouring each cosmid library were conjugated *en masse* into the AHL biosensor *P. putida* F117 (pKRC12) as acceptor (Riedel *et al.*, 2001). The transconjugants displaying *gfp* expression were further

studied. The identified QS loci for *B. kururiensis* M130 is designated here as *braIR*$_{KUR}$, whereas the QS loci for *B. unamae* MTI-641^{T} would be denoted as *braI/R*$_{UNA}$

In *B. xenovorans* LB400^{T} the identification of the QS elements was performed by *in silico* analysis of the available genome sequence (Chain *et al.*, 2006). Two gene sets with homology to autoinducer synthesis and response regulator systems had been previously detected during the genome sequencing. The first *luxIR*-like pair will be denoted here as *braIR*$_{XEN}$, and the second *luxIR* pair (BxeC0415, BxeC0416) will be denoted here as *xenI2/R2* (Chain *et al.*, 2006). The *B. xenovorans* LB400^{T} genome sequence was also examined for the presence of LuxR-*solo* proteins typically containing an N-terminal AHL-binding domain (PFAM03472) and a C-terminal Helix-Turn-Helix domain (PFAM 00196).

2.4 CONSERVATION OF BraIR AND XenI2/R2 IN OTHER *Burkholderia* SPECIES.

The presence of **BraIR-like** systems was determined by Southern analysis in 20 type strains from species within the group of plant-associated *Burkholderia*. Southern analysis was performed on *Eco*RI digested genomic DNAs, which were hybridized with an *Eco*RV 423 bp internal fragment of *braI*$_{KUR}$. PCR reactions were also performed in order to verify the presence of *bra*R-like and *rsaL*-like genes. The primers used in the PCR reaction for *bra*R were pQEbraRfw and pQEbraRrv, whereas braLFw and braLRv were used to detect *rsaL* (Table 2-3).

Similarly, the occurrence of highly identical systems to *xenI2/R2* in other 4 *B. xenovorans* strains, as well as in 21 *Burkholderia* species was determined by Southern analysis with a 794 bp probe comprising the *xenR2* gene. This analysis was performed on *Eco*RI or *Pst*I digested genomic DNAs. To generate the probe, *xenR2* was amplified by PCR from *B. xenovorans* genomic DNA and cloned into pGEM-T easy to generate pGEMX2R (Table 2-3). After verifying the sequence, a radiolabelled probe was generated using an excised *Spe*I-*Not*I fragment as template.

The presence of the unpaired LuxR-*solo* protein BxeR was also determined in the pool of plant associated *Burkholderia* species by Southern analysis, with a probe comprising a

999 fragment representing the entire *bxeR* gene. Such fragment was excised with *Bam*HI-*Hind*III from the plasmid pBBRBXER, after verification of the sequence.

2.5 GENERATION OF QS KNOCK OUT MUTANTS

2.5.1 pKNOCK Suicide constructs

Different genomic null mutants were created in the AHL QS system of *B. kururiensis, B. unamae* and *B. xenovorans,* by using pKNOCK-Km suicide constructs containing an internal fragment of each gene (Alexeyev, 1999). In each case, the pKNOCK construct was subsequently mobilized into the wild-type mutants by conjugation and transconjugants were selected in KB plates with antibiotics as previously described (100 μg/mL nitrofurantoin, 100 μg/mL kanamycin). The cloning details related to the pKNOCK constructs used in this thesis are described in table 2-3, whereas the names of the mutants corresponding to each suicide plasmid are presented in table 2-5. The fidelity of all marker exchange events in the selected transconjugants was then confirmed by Southern analysis.

In *B. kururiensis* M130*,* an internal 423 bp *Eco*RV fragment from the *braI* gene, as well as internal fragments to the genes *braR* and *rsaL* were cloned into pKNOCK-Km to yield PKNOCKBRAI, PKNOCKBRAR and PKNOCK-RSAL (Table 2-3). These plasmids were subsequently transferred to *B. kururiensis* M130 by biparental conjugation to generate the knock-out mutants M130BRAI, M130BRAR and M130RSAL, respectively.

In *B. unamae*, $braR_{UNA}$ and $braI_{UNA}$ null mutants were generated by cloning internal fragments from each gene, to yield the suicide vectors pKNOCK-UNAR and pKNOCK-UNAI. Each plasmid was then mobilized into strain MTl-641^{T} to generate *B. unamae* UNABRAI and *B. unamae* UNABRAR, respectively.

In *B. xenovorans* LB400^{T}*,* internal fragments from $braR_{XEN}$, $braI_{XEN}$, *xenI2*, *xenR2* and *bxeR* each gene were PCR amplified (Table 2-3), and cloned into pKNOCK-Km to yield the suicide plasmids pKNOCKXENR, pKNOCKXENI, pKNOCKXENI2, pKNOCKXENR2 and PKNOCKBXER. These plasmids were then conjugated into *B. xenovorans* LB400^{T} to generate genomic mutants LB400BRAR, LB400BRAI, LB400XENI2, LB400XENR2 and LB400BXER respectively.

Table 2-5. pKNOCK constructs used in this study for the generation of Knock-out mutants

GENE	SPECIES	pKnock plasmids	MUTANT NAME
braI$_{KUR}$[1]	*B. kururiensis*	**pKNOCK-BRAI**	M130BRAI
braR$_{KUR}$	*B. kururiensis*	**pKNOCK-BRAR**	M130BRAR
rsaL$_{KUR}$	*B.kururiensis*	**pKNOCK-RSAL**	M130RSAL
braI $_{UNA}$	*B. unamae*	**pKNOCK-UNAI**	UNABRAI
braR$_{UNA}$	*B. unamae*	**pKNOCK-UNAR**	UNABRAR
braI$_{XEN}$	*B. xenovorans*	**pKNOCKXENI**	LB400BRAI
braR$_{XEN}$	*B. xenovorans*	**pKNOCKXENR**	LB400BRAR
xenI2	*B. xenovorans*	**pKNOCKXENI2**	LB400XENI2
xenR2	*B. xenovorans*	**pKNOCKXENR2**	LB400XENR2
bxeR	*B. xenovorans*	**pKNOCKBXE**	LB400BXER

All pKNOCK plasmids are Km^R, and were mobilized into wild-type strains by conjugation. Subindexes KUR, UNA and XEN correspond to *B. kururiensis, B. unamae* and *B. xenovorans* respectively.

2.5.2 Complementation of knock-out mutants

B. kururiensis M130RSAL mutant was complemented *in trans* by conjugating the cosmid pZS1. Similarly, the *B. unamae* knock-out UNABRAR was complemented by conjugating the cosmid pLZ1.

To complement the *B. xenovorans braI*$_{XEN}$ and *bxeR* deficient mutants, both genes and their promoters were cloned into the pBBRMCS-5 vector (Kovach *et al.*, 1995). The *braI*$_{XEN}$ gene and its promoter region were PCR amplified and cloned into pMOS-Blue. A 623 bp fragment was then excised using *Xba*I-*Kpn*I and ligated into pBBR-MCS5 to generate pBBRXENI. Similarly the *bxeR* gene and its promoter region were PCR amplified and cloned into pGEM-T easy. A 1263 fragment was then excised using *Bam*HI-*Spe*I, and cloned into pBBRMCS-5 to generate pBBRBXER (Table 2-3). Both pBBRXENI and pBBRBXER were then mobilized by triparental conjugation into LB400BRAI and LB400BXER mutants, respectively.

2.6 DETERMINATION OF THE BIOLOGICALLY ACTIVE AHLs

A bioassay was designed to identify the biologically active AHL for each QS system. For this purpose the identified *luxR* genes were cloned in the expression vector pQE30 (Qiagen), while the promoter region of their paired LuxI synthase gene was cloned in the promoter probe vector pMP220 (Spaink *et al.*, 1987). Both plasmids were then transformed in *E. coli* M15, and the reporter β-galactosidase activity was measured upon the addition of individual AHLs. The details regarding the cloning of the pQE30 constructs, as well as the pMP220 derivatives are presented in table 2-3.

2.6.1 Over-expression of the LuxR-proteins and cloning of promoter-based transcriptional fusions

To determine the AHL cognate for $BraR_{KUR}$ from *B. kururiensis* M130, the gene $braR_{KUR}$ was PCR amplified from chromosomal DNA, and the PCR product was cloned as a *Bam*HI-*Hind*III fragment in pQE30, generating pQEBRAR. The gene promoter region of the $braI_{KUR}$ AHL synthase was first cloned in pBlueScript KSII+ as a 403 bp *Cla*I-*Eco*RI fragment. The fragment was then excised with *Xba*I-*Kpn*I, and cloned in the corresponding sites in the promoter probe vector pMP220, generating pMPbraI (Table 2-3). This construct was then transformed into an *E. coli* strain M15 containing an expression plasmid pQEBRAR harboring the $braR_{KUR}$ gene.

For identifying the $BraR_{UNA}$ cognate, $braR_{UNA}$ was PCR amplified from genomic DNA and cloned as a *Bam*HI/*Hind*III fragment into pQE30 to generate pQEUNAR. The $braI_{UNA}$ promoter region was PCR amplified and cloned as an *Eco*RI-*Kpn*I fragment into pMP220 promoter probe vector to generate pMPUNAI (Table 2-3).

The $braR_{XEN}$ and *xenR2* genes were PCR amplified using genomic DNA as template and amplimers were cloned into pGEM-T easy. Each gene was then excised with *Bam*HI/*Hind*III and ligated into the corresponding sites of pQE30 to generate pQEXENR1 and pQEXENR2 respectively (Table 2-3). The gene promoters of $braI_{XEN}$ and *xenI2* were amplified and cloned in the promoter probe vector pMP220 to generate pMPXENI1 and pMPX2I.

2.6.2 β-galactosidase activity measurements

E. coli M15 [pREP-4] was transformed with each pQE30-LuxR derivative and its paired promoter transcriptional fusion. In each case, a single colony was inoculated into 10 mL of LB-Amp-Km-Tet and grown overnight. Thereafter, each culture was diluted to OD_{600} 0.1 into 10 mL of prewarmed medium having 1 μM of a specific AHL to be evaluated. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6 and after 2 hrs at 37 °C β-galactosidase activity was determined.

β-galactosidase activities were determined essentially as described by Miller (Miller, 1972) with the modifications of Stachel *et al.* (Stachel *et al.*, 1985). The protocol used for the β-galactosidase activity is described in appendix 6.4.

2.7 ANALYSIS OF THE QS NETWORK IN *B. xenovorans* LB400^T

Since two *luxIR* pairs and one LuxR-*solo* protein were identified in *B. xenovorans* LB400^T, two types of experiments were designed to establish the existence of hierarchies in the QS network. Firstly, experiments were performed in *E.coli* M15 overexpressing either $BraR_{XEN}$, XenR2 or BxeR, in the presence of the each pMP220 derivatives upon the addition of AHL cognates. The second approach consisted in determining the transcription levels of each promoter in wild-type, $braI_{XEN}$, *xenI2* and *bxeR* deficient mutants.

2.7.1 Cross-talk between $BraIR_{XEN}$ and XenI2/R2

Possible regulation of the *xenI2 p*romoter by $BraR_{XEN}$, and of $braI_{XEN}$ promoter by XenR2, were evaluated by transforming the plasmid gene promoter fusion into *E. coli* M15 (pQEXENR) and *E. coli* M15 (pQEXENR2), respectively. β-galactosidase activity was measured in the presence of the cognate AHL after 4 hours growth.

2.7.2 BxeR and *bxeR* studies

Possible regulation of the *B.xenovorans* QS network by the LuxR-*solo* BxeR protein was assessed by over-expressing the BxeR protein and evaluating its interaction with its own promoter, as well as with $braI_{XEN}$ and *xenI2* promoters. To overexpress BxeR, the *bxeR* gene was PCR amplified and cloned into pGEM-T easy (see table 2-3). This fragment was then excised with *Bam*HI-*Hind*III and cloned into pQE30 to generate pQEBXER. The promoter of *bxeR* was PCR amplified and then excised and cloned as a *Bam*HI-*Xba*I into pMP220 previously digested with *Bgl*II-*Xba*I, to generate pMPBXER (Table 2-3).

Regulation of the AHL QS systems of *B. xenovorans* by BxeR was evaluated. *E. coli* overexpressing BxeR via the pQEBXER plasmid was transformed with either pMPXENI1 or pMPX2I as well as with the *bxeR* promoter transcriptional fusion pMPBXER. *E. coli* M15 (pQEBXER)(pMPX2I), *E. coli* M15 (PQEBXER)(pMPXENI1)

and *E. coli* M15 (PQEBXER)(pMPBXER) were then grown in the presence of three independent AHL mixtures; one contained 1 µM of each of 9 different unsubstituted AHLs at position 3, the second mixture contained 1 µM of each of 7 different 3-oxo substituted AHLs and third cocktail contained 1 µM of each of 7 different 3-OH substituted AHLs. In these three growth conditions ß-galactosidase activity was determined.

2.7.3 Transcription profiles in *B. xenovorans* LB400^T

To monitor the $braI_{XEN}$, *xenI2* and *bxeR* promoter activities, pMPXENI, pMPX2I, pMPBXER and pMP220 were conjugated in *B. xenovorans* wild type and mutants. Single colonies were grown overnight in KB-Tc, and the bacterial cultures were started with an initial inoculum of 5 x10^6 CFU in 20 mL of KB-Tc medium and ß-galactosidase activities were measured after 12 hours of growth.

2.8 STUDY OF *RsaL$_{KUR}$* AS REPRESSOR OF BraIR$_{KUR}$ SYSTEM

The *rsaL* gene from *B. kururiensis* was overexpressed in *E. coli* to determine the interaction between RsaL$_{KUR}$ and the $braI_{KUR}$ promoter. $rsaL_{KUR}$ was PCR amplified from genomic DNA and cloned as a *Xba*I-*Kpn*I fragment into pBBRMCS5, to generate pBBRRSAL.

A single colony of *E. coli* M15 (pQEBRAR)(pMPBRAI)(pBBRRSAL) was inoculated into 10 mL of LB-Amp-Km-Tet and grown overnight. Next day, the culture was diluted to OD_{600} 0.1 into 10 mL of prewarmed medium having 1 µM 3-oxo-C12-HSL. pQEBRAR expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6 and after to 2 hrs at 37 °C β-galactosidase activity was then determined.

To quantify AHL production in *B. kururiensis* and its *rsaL* deficient mutant, dried AHL extracts from *B. kururiensis* M130, M130RSAL and M130RSAL+pZS1 were obtained. The samples were then resuspended in an ethyl acetate volume, so that 1 µl of final extract corresponded to 10^9 cells of the original culture. The quantity of C12-3-oxo-AHL in the extracts was determined using 3-oxo-C12-AHL sensor *P. putida* SM17 (pRSAL220) as previously described (Rampioni *et al.*, 2006). *P. putida* SM17 is a double mutant of *ppuI* and *rsaL* genes consequently it does not produce either RsaL repressor, nor 3-oxo-C12-AHL. Adding exogenous 3-oxo-C12-AHL is quantified through β-

galactosidase activity by using strain SM17 harboring (prsal220); this plasmid contains the PpuR-3-oxo-C12-AHL regulated *rsaL* promoter fused to promoterless *lacZ* gene. Overnight cultures of SM17 (prsal220) were diluted in 10 ml of LB medium to an A_{660} of 0.1; the AHL extract to be quantified was then added and after 4 hours of growth β-galactosidase activity was determined. This 3-oxo-C12-AHL bacterial sensor has a linear dose response between 0.1 µM to 1 µM of 3-oxo-C12-AHL. Synthetic 3-oxo-C12-AHL was used as standard molecules.

2.9 PHENOTYPICAL ANALYSES OF QS DEFICIENT MUTANTS

QS deficient mutants were screened to determine the possible regulation of several phenotypes.

2.9.1 Protease and Lipase activities

Protease activity was evaluated by spotting 1 µL of a bacterial suspension having an optical density of 1 at OD_{600} into the surface of a KB plate supplemented with 2 % skim milk (Huber *et al.*, 2001). Lipolytic activity was determined in trybutyrin agar, and inoculation was performed as for the protease assay (Anderson, 1939). Preparation of the media for these and other tests is described in Appendix 6.1.

2.9.2 Bacterial motility assays

Swimming assays were performed on 0.25% KB agar plates and 0.25% Nutrient A Agar plates. Swarming assays were performed using 0.5% nutrient agar plates and M8 medium plates (M9 salts without NH_4Cl) (Kohler *et al.*, 2000) supplemented with 0.2% glucose and 0.05% glutamate and 0.5% agar (Murray & Kazmierczak, 2006). The inoculation was performed by spotting 1 µl of a bacterial suspension having an optical density of 1 at OD_{600}. The swimming and swarming zones were measured after 48 hours incubation at 30°C for all species WT and its QS mutant derivatives.

2.9.3 EPS production and quantification

EPS production was tested by cross-streaking single colonies in Yeast Extract Mannitol Media (Zlosnik *et al.*, 2008). To quantify the EPS production, single colonies were streaked and grown in YEM agar and allowed to grow for 3 to 5 days. Cells were harvested by adding 2 mL of phosphate buffer (pH 7.2) to the plates and gently scraping

with a sterile spreader. 0.5% (v/v) formaldehyde was added to the cell suspension and dilutions of bacterial cells were plated out on KB agar for estimation of cell numbers. EPS was stripped off from bacteria by gentle stirring and bacterial cells were removed by centrifuging at 16000 g. Four volumes of chilled acetone were added to the supernatant and the EPS was left to precipitate at 4°C for 12 hours. The precipitate was recovered by centrifuging at 3000 g and washed at least twice with acetone (Dharmapuri & Sonti, 1999). The pellet was allowed to dry at room temperature and dissolved in 1 ml of sterile water. EPS was estimated by the boiling phenol method (Dubois et al., 1956). 0.4 mL of the EPS sample was mixed with 0.2 mL of 5% (w/v) phenol followed by 1 mL of concentrated sulphuric acid. The mixture was incubated for 10 minutes at room temperature and then at 30°C for 20 minutes. Absorbance was measured at 490 nm against a reagent blank. A calibration curve was constructed using glucose concentration from 10μg to 100μg/mL.

2.9.4 Biofilm Formation

Biofilm formation was determined by surface attatchment experiments in microtiter plates, as described by Huber *et al.,* (2001). Single colonies were grown overnight in AB medium supplemented with 10 mM glucose. Grown cultures were then washed twice with fresh media and diluted to OD_{600} 0.01. Thereafter, 100 μL of bacterial dilution were then inoculated in the rounded bottom wells of the microtitre dishes and incubated at 30^0C. Biofilm formation was evaluated after 24, 48 h, 72h and 120 h. After the incubation time, OD_{550} was determined prior to medium removal. 100 μL of 1 % (w/v) aqueous solution of crystal violet were added and incubated at room temperature for 20 minutes, and then washed thoroughly with water. For quantification of the attached cells the crystal violet was solubilized in 120 μL of DMSO, and absorbance was determined at 570 nm.

2.9.5 Siderophore production.

The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol S (CAS) agar plates (Appendix 6.1)(Schwyn & Neilands, 1987). Single colonies of each strain to be tested were grown overnight in 5 mL of KB media. The cultures were harvested at 4000 rpm, and the pellets were washed and adjusted to an OD_{600} of 1 (Approx. 10^8 CFU). 2 μL of culture were spotted on the surface of the plate and then incubated for 48 h at 30ºC. Siderophore production was measured by the size of the orange halos formed around the colonies (Caballero-Mellado *et al.*, 2007).

2.9.6 Aromatic Compound Degradation

2.9.6.1 *B. kururiensis* M130

B. kururiensis M130 and its derivative mutants in $braI_{KUR}$, $braR_{KUR}$ and $rsaL_{KUR}$ genes were grown in BSE liquid medium (Estrada-de los Santos *et al.* 2001) for 18 h with reciprocal shaking. The cultures were harvested and the pellets washed with 10 mM $MgSO_4 \cdot 7H_2O$. The pellets were adjusted to a low (1×10^5 cfu/mL) or high (1×10^8 cfu/mL) cell density. Culture aliquots of M130 and mutant derivatives were inoculated with a multipoint replicator on SAAC medium plates (Caballero-Mellado *et al.*, 2007) containing 0.05% (w/v) phenol as a single carbon source. Benzene and toluene were also tested as carbon source, by adding 150 µL of these volatile compounds on filter paper placed in the lids of Petri dishes as described previously (Caballero-Mellado *et al.*, 2007). Presence or absence of growth was determined after incubation for 5 days at 29ºC.

In addition, SAAC liquid medium supplemented with phenol was inoculated with *B. kururiensis* wild type strain and mutants to low or high cell density as described above; optical density was determined after incubation with shaking (250 rpm) for 5 days at 29ºC. SAAC medium without a carbon source was used as a negative control for bacterial growth; succinic acid (0.2% w/v) as carbon source was a positive control.

2.9.6.2 *B. unamae* MTl-641^T

B. unamae MTl-641 ^{T}, UNABRAI, UNABRAR, and UNABRAR complemented with pLZ1 were grown in BSE liquid medium (Estrada-de Los Santos *et al.*, 2001) and the cultures were incubated at 29°C with reciprocal shaking (200 rpm) for 18 h. The cultures were adjusted to 10^4 CFU/mL, and 1.0 mL was then inoculated into 99 mL SAAC culture medium containing phenol 0.05% (Caballero-Mellado *et al.*, 2007). Growth was monitored at 24, 48 and 72 h serial dilution and CFU was determined. As control, wild type and derivative strains were grown with mannitol as carbon source.

2.9.6.3 *Burkholderia xenovorans* LB400^T

Single colonies of *B. xenovorans* wild-type and QS deficient mutants were grown in 5 mL of KB liquid media. Overnight grown cultures were then harvested at 4000 rpm and washed twice with PBS buffer. Each culture was then adjusted to OD_{600} 0.25 (Approx

2 x 10^8 cells/mL) and 1 µL was spotted in the surface of SAAC plates. To test the biphenyl degradation, 50 mg of biphenyl cristals were added on filter paper placed in the lids of Petri dishes and growth was monitored after 5 days of incubation at 30 °C (Caballero-Mellado *et al.*, 2007). SAAC plates were also supplemented with mannitol as positive control.

2.9.7 Analysis and identification of secreted proteins in *B. xenovorans*

Overnight cultures were washed and diluted to an OD_{600} of 0.05 in 40 mL of prewarmed LB. After 12 h of incubation, the cultures were centrifuged for 15 min at 8000 *g* at 4°C. Culture supernatants were filtered through a 0.45-µm filter (Millipore), and proteins were precipitated overnight at 4°C with 10% (v/v) of trichloroacetic acid (final concentration). The precipitates were separated by centrifugation at 15000 *g* for 20 min at 4°C and the pellets were washed twice with ice-cold acetone. Another centrifugation was performed at 15000 *g* for 20 min, and protein pellets were air dried, and resuspended in sample buffer. The suspension was then boiled for 10 min, and the proteins were separated by SDS-PAGE on gels containing 12% (w/v) polyacrylamide (Appendix 6.2). Proteins were identified by mass spectroscopy as described by Tomaic *et al.* (Tomaic *et al.*, 2009).

2.10 RICE ENDOPHYTIC COLONIZATION EXPERIMENTS AND PLANT GROWTH PROMOTING FEATURES OF *B. kururiensis*

Endophytic rice colonization was performed as previously described (Mattos *et al.*, 2008). Rice seeds (*Oryza sativa* cultivar Guarani) were dehusked surface sterilized, and transferred to water-based 0.5% agar for seed pre-germination, following incubation for three days at 28ºC, in absence of light. The pre-germinated rice seeds were aseptically transferred to glass tubes (4 cm in diameter, 29 cm in height) containing 20 mL of a nitrogen-free Hoagland's nutrient solution (Appendix 6.1) (Hoagland, 1975). Plantlets infection assays were carried out by inoculation of 500 µL of bacterial culture (approximately 10^9 CFU) into each glass tube. After incubation for 12 days with a 12 h photoperiod at 28ºC, plantlets were collected and cut (roots and aerial parts). The excised plant segments were subjected to surface sterilization with 1% sodium hypochlorite for 5 min followed by several washes with sterile water. Plant segments were then weighed and transferred to micro centrifuge tubes, containing 1 mL of sterile nutrient solution, and macerated with a pestle. From each of the obtained suspension, a

series of 10-fold dilutions were prepared using sterile saline, and aliquots of 100 μl were spread-plated onto LB medium and incubated for 4 days at 28ºC. Bacterial quantification was expressed as CFU/g of fresh weight plant tissue.

2.10.1 Nitrogen fixation assay

B. kururiensis M130 and its derivative mutants in $braI_{KUR}$, $braR_{KUR}$ and $rsaL_{KUR}$ genes were grown in BSE liquid medium (Estrada-de los Santos *et al.* 2001) for 18 h with reciprocal shaking. The cultures were harvested and the pellets washed with 10 mM $MgSO_4 \cdot 7H_2O$. The pellets were adjusted to a low (1 x 10^5 cfu/mL) or high (1 x 10^8 cfu/mL) cell density. 10 mL vials containing 5 mL of N-free semisolid BAz mineral medium (Estrada-de Los Santos *et al.*, 2001), in which azelaic acid was omitted and succinic acid added (5 g/L), were inoculated with *B. kururiensis* M130 and derivative mutants to a low or high cell density as described above. The cultures were incubated for 30 h at 29ºC, and then 10% (v/v) acetylene was injected to the vials and the cultures incubated for 15 h at 29ºC, and then assayed for nitrogenase activity (nitrogen fixation) by the acetylene reduction activity (ARA) method (Burris, 1972). Three replicate cultures were assayed for the wild type strain and each mutant.

2.10.2 Indole compounds biosynthesis.

B. kururiensis M130 and its derivative mutants were cultured in BSE medium described above; Succinate-fructose salts broth (Appendix 6.1) was inoculated to a low or high cell density and incubated for 24 h at 29ºC (Jain and Patriquin, 1984) . Indole compounds, including indoleacetic acid, were determined by using the Salkoswki´s colorimetric reaction (Tang & Bonner, 1948).

2.11 MAIZE COLONIZATION EXPERIMENTS WITH *B. unamae* MTI-641^T

B. unamae strains were grown in BSE liquid medium (Estrada-de Los Santos *et al.*, 2001) and the cultures were incubated at 29°C with reciprocal shaking (200 rpm) for 18 h. Thereafter the cultures were adjusted to an optical density (OD_{600}) of 0.15 (approximately 1.5 X 10^7 cfu/mL). Three germinated seeds of maize *(Zea mays)* were sown per pot containing sterile river sand, and each seed was inoculated with 1.0 mL of bacterial culture. The plants were grown for 9 days (7 days after plant emergence). 3 roots of each pot were cut in segments of approximately 1 cm and mixed. Two grams of the roots mixture were macerated with 10mM $MgSO_4 \cdot 7H_2O$ to obtain a dilution 10^{-1}

and further dilutions were prepared and streaked on BAc agar plates (Estrada-de Los Santos *et al.*, 2001) containing the appropriate antibiotic; three replicates were made from each dilution.

2.12 DNA SEQUENCING AND NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

All DNA sequences were performed either at the CRIBI center (University of Padova, Italy) or at Macrogen (www.macrogen.com). The nucleotide sequence of the 4938 bp *Xmn*I fragment harboring *braI*, *rsaL* and *braR* has been deposited in GenBank/EMBL/DDBJ under accession number AM940944. The BraI/R_{UNA} QS loci of *B. unamae* MTl-641^{T} is given as a 1977 bp fragment of pMOS-LZ1 under the accession number FN640548.

2.13 STATISTICAL ANALYSIS

All experiments were performed at least three times and means plus standard deviations are given. Statistical analysis included unpaired *t* tests, ANOVA and Dunnetts post-tests, and were performed with PRISM 4.0 software (GraphPad Software, San Diego California). A P value of < 0.05 was considered significant

3 RESULTS AND DISCUSSION

3.1 THE NEW CLUSTER OF PLANT ASSOCIATED BENEFICIAL *BURKHOLDERIA* SHARES A CONSERVED QS SYSTEM

3.1.1 Introduction and scope

Studies by different laboratories have highlighted that bacterial intercellular communication via the production and sensing of signal molecules, known as quorum sensing (QS), plays an important role in fitness and virulence of *Burkholderia* species (Eberl, 2006; Venturi *et al.*, 2004). In BCC species, like in many other Gram-negative bacteria, the most common signal molecules produced and detected are *N*-acyl homoserine lactones (AHLs), which allow bacteria to monitor their population density by responding to the concentration of AHLs. Various studies have established that in BCC the AHL QS system is highly conserved consisting of CepI, which synthesizes mainly *N*-octanoylhomoserinelactone (C8-AHL) and the CepR C8-AHL sensor-response regulator. The CepI/R AHL QS system was found to be involved in the regulation of similar phenotypes in many different species and strains of the BCC including virulence on several models (Gotschlich *et al.*, 2001). In addition to BCC members, also the rice pathogens *B. plantarii* and *B. glumae* were found to possess a CepI/R-like system producing and responding to C8-AHL and involved in the regulation of virulence associated factors (Devescovi *et al.*, 2007; Kim *et al.*, 2004; Solis *et al.*, 2006). QS is also relevant for *B. mallei, B. pseudomallei* and *B. thailandiensis,* although in these cases the QS regulation is exerted by multiple *luxIR* pairs, not related to the *cepIR* (Chandler *et al.*, 2009; Duerkop *et al.*, 2009).

As noted previously, the beneficial plant-associated *Burkholderia* species group is phylogenetically distant from the BCC-group. Their ecological importance mostly relies in the fact that most of the species have been isolated in association to plants, and furthermore, their interaction with their host or with the environment has been reported to be beneficial. In contrast to the well studied QS systems in other *Burkholderia* species, the existence of AHL based QS systems in this group has not been investigated. The purpose of this study was to determine if the new environmental group of plant-associated beneficial *Burkholderia* species possess AHL QS system(s) and establish their main characteristics in comparison with other *Burkholderia* species.

3.1.2 Results

3.1.2.1 AHL screening for the beneficial plant-associated *Burkholderia* species

It was of interest to determine the ability to produce AHLs in the novel group of beneficial *Burkholderia*. For this purpose, we analysed 27 strains representing 21 species by solid media T-streak tests with the AHL biosensor *P. putida* (pKR-C12), which is a LasR based sensor particularly sensitive to 3-oxo-C12-HSL and to C10-3-oxo-HSL (Riedel *et al.*, 2001), and with *C. violaceum* CV026 which is sensitive to short and medium acyl chains AHLs (McClean *et al.*, 1997). All strains gave a positive response with sensor *P. putida* (pKR-C12), while only *B. phenazinium* gave a response with *C. violaceum* (Table 3-1). These results suggested initially that most strains were able to produce AHLs, most likely with acyl chains longer than C10 since they activated the pKRC12 sensor (Table 3-1).

Table 3-1. AHL characterization for the beneficial plant-associated cluster of *Burkholderia* by T-STREAK and TLC analysis .

STRAINS	*P.putida* pKRC12	*C.violaceum* CV026	*A. tumefaciens* pNTL4
B. xenovorans LB400 ^{T}	+	-	OC8-OC10[§]
B. xenovorans CAC124	+	-	OC6-OC8-OC10
B. xenovorans LMG16224	+	-	OC6-OC8-OC10
B. xenovorans TCo-26	+	-	OC6-OC8-OC10
B. xenovorans-TCo-382	+	-	OC6-OC8-OC10
B.caledonica DSM17062	+	-	OC8
B.terricola DSM17221	+	-	OC8-OC10-OC12
B.graminis DSM17151	+	-	OC6-OC8-OC10
B.phytofirmans DSM17436	+	-	OC8
B.fungorum DSM17061	+	-	OC8-OC10
B.phenoliruptrix DSM17773	+	-	OC8-OC10-OC12
B.phenazinium DSM10684	+	+	OC6-OC8-OC10-OC12
B.tuberum DSM18489	+	-	OC6-OC8-OC10-OC12
B.silvatlantica SRMrh-20^{T}	+	-	OC8-OC10
B.kururiensis M130	+	-	OC8-OC10
B.kururiensis KP23^{T}	+	-	OC6-OC8
B.caribensis DSM13236^{T}	+	-	OC6-OC8-OC10
B.hospita DSM17164	+	-	OC6-OC8-OC10
B.mimosarum PAS44^{T}	+	-	OC8
B.phymatum DSM17167	+	-	OC6-OC8-OC10-OC12
B.sacchari DSM17165	+	-	OC8
B.terrae DSM17804	+	-	OC6-OC8-OC10-OC12
B. tropica Ppe8^{T}	+	-	OC8-OC10
B. unamae MTl-641^{T}	+	-	OC8-OC10
B. unamae SCCu-23	+	-	OC8-OC10
B. ferrariae FeGI0^{T}	+	-	OC6-OC8-OC10-OC12
B.nodosa BR3437^{T}	+	-	OC6-OC8-OC10-OC12

[§] OC6: 3-oxo-C6-HSL; OC8 : 3-oxo-C8-HSL; OC10: 3-oxo-C10-HSL;OC12: 3-oxo-C12-HSL. ^{T} Type strain.

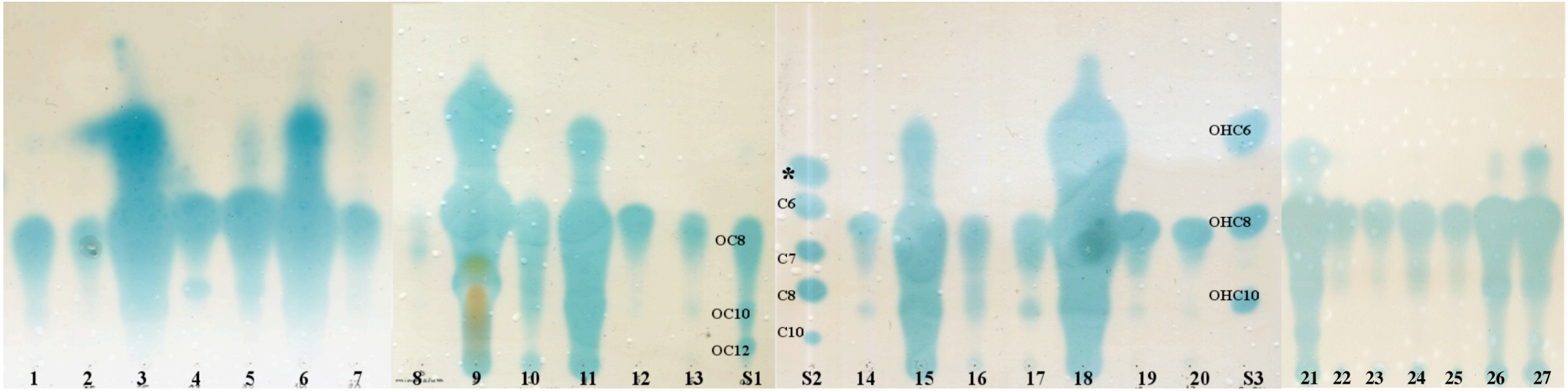


Figure 3-1. TLC Analysis of AHL produced by 27 strains from 21 *Burkholderia* species

Lane 1, *B. kururiensis* M130; lane 2, *B. caledonica* DSM 17062; lane 3, *B. caribensis* DSM 13236; lane 4, *B. fungorum* DSM 17061; lane 5, *B. graminis* DSM 17151; lane 6, *B. hospita* DSM 17164*;* lane 7, *B. kururiensis* DSM 13646*;* lane 8, *B. mimosarum* PAS44*;* lane 9, *B. phenazinium* DSM 10684; lane 10, *B. phenoliruptrix* DSM 17773; lane 11, *B. phymatum* DSM 17167; lane 12, *B. phytofirmans* DSM 17436; lane 13, *B. sacchari* DSM17165; lane 14, *B. silvatlantica* SRMrh-20; lane 15, *B. terrae* DSM 17804; lane 16, *B. terricola* DSM 17221; lane 17. *B. tropica* DSM 15359; Lane 18, *B. tuberum* DSM 18489; lane 19, *B. unamae* MTI-641^{T}; lane 20, *B. xenovorans* LB400^{T}; 21 *B. unamae* SCC-23; 22, *B. xenovorans* CAC124; 23, *B. xenovorans* LMG16224; 24 *B. xenovorans* TCo-382; 25, *B. xenovorans* TCo-26; 26, *B. ferrariae* FeGI0^{T};27, *B. nodosa* BR3437^{T}. Synthetic AHL compounds were used as reference. The * denoted a degradation production as indicated by Shaw *et al.*, 1997.

.

The AHL production was confirmed by TLC analysis of ethyl acetate extracts from spent supernatants using the biosensor *A. tumefaciens* NTL4 (pZLR4). This sensor was chosen based on its capability to respond to a wide range of AHL molecules (Shaw *et al.*, 1997). All strains were found to produce a very similar AHL profile, and the only difference was the amount of AHLs being produced by some strains (Figure 3-1). Using this AHL biosensor it was observed that all type strains most likely produced C8-3-oxo-HSL. However it is worth noting that the *A. tumefaciens* NTL4 (pZLR4) biosensor is most sensitive to this AHL and thus, very low amounts (~$5x10^{-4}$ pmol) are able to induce spots in TLC analysis (Shaw *et al.*, 1997). From these results, it was concluded that this AHL was prevalent in these species and therefore most probably they all possess at least one AHL QS system.

It was then decided to further study the AHL QS in three species with remarkable biotechnological properties, isolated from three different niches. The species were the rice endophyte *B. kururiensis* M130, the maize rhizosphere strain *B. unamae* MTI-641^T, and the PCB degrader *B. xenovorans* LB400^T.

3.1.2.2 AHL production in *B. kururiensis* M130, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T

From the TLC analysis performed above it was postulated that strains *B. kururiensis* M130, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T were able to produce AHLs, most likely 3-oxo-C8-HSL, 3-oxo-C10-HSL, and 3-oxo-C12 HSL (Figure 3-1). In order to identify the AHL molecules produced by these type strains, more analyses were performed using three other AHL biosensors. These were the LasR based biosensor *E. coli* (pSB1075), the LuxR-based *E. coli* (pSB401), (Winson *et al.*, 1998) and the LuxR-based *E. coli* pJBA132.

Ethyl acetate extracts of *B. kururiensis* M130 culture supernatants were separated by TLC and overlaid with AHL biosensor *A. tumefaciens* NTL4 (pZLR4) or *E. coli* pSB1075. These analyses showed that this strain produced four AHL molecules tentatively identified as 3-oxo-C12-, 3-oxo-C10-, 3-oxo-C8- and 3oxo-C6-HSL (Figure 3-2). TLC also confirmed the detection of the latter two AHL molecules when using *E. coli* pSB401. In addition these strains gave a positive response when using sensor *E.coli* pJBA132 in a plate T-streak analysis.(Table 3-2).

Importantly, identical results were also obtained for the strain *B. kururiensis* KP23^{T} (Figure 3-2). This means that the AHL production profile is the same for two strains from different origins.

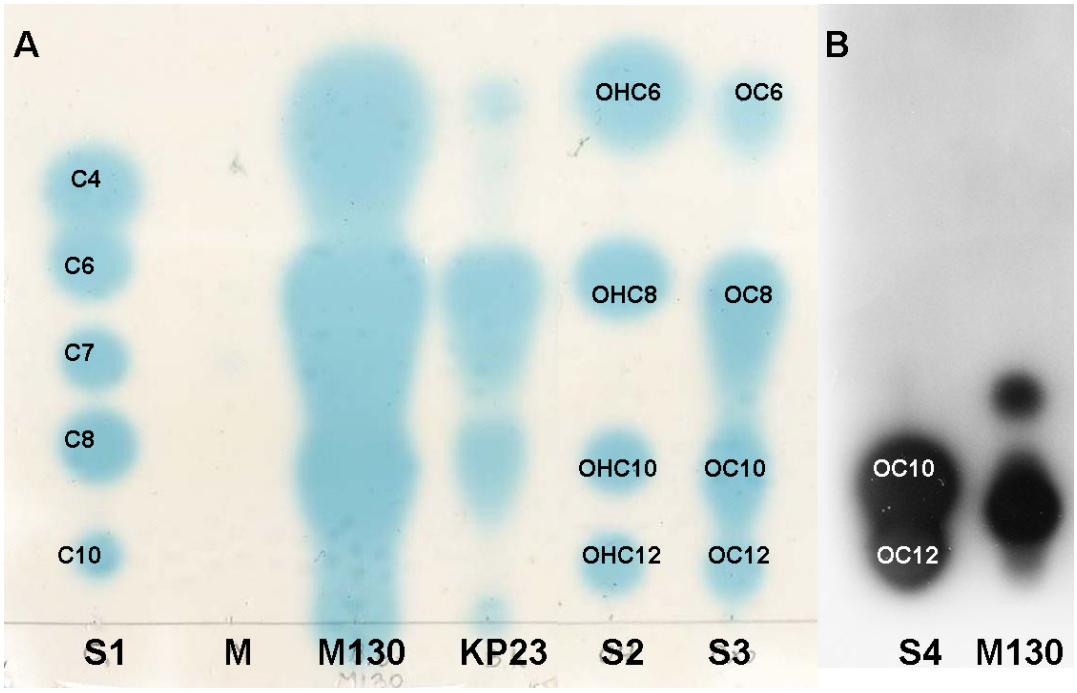


Figure 3-2. TLC analysis of AHL produced by *B. kururiensis* strains M130 and KP23^{T}.

AHL were extracted from 100 mL of M9C glucose media, corresponding to 1x10^{10} cells approx. Samples were run in TLC with methanol:water (60:40) as mobile phase, and overlaid with A). A. *tumefaciens* NTL4 (pZLR4) B. *E.coli* . Synthetic AHL compounds were used as reference. **M**: Uninoculated media, S1 unsubstituted, HSL standards. **S2** 3-hydroxy-HSL, **S3** 3-oxo-HSL standards.

Table 3-2. AHL identification in *B. kururiensis* M130, *B. xenovorans* LB400^{T} and *B. unamae* MTI-641^{T}

BIOSENSOR	*B. kururiensis* M130	*B. xenovorans* LB400 ^{T}	*B. unamae* MTL-641^{T}
A. tumefaciens NTL1 (pZLR4) **All 3-oxo-HSL**§	OC6-OC8-OC10-OC12	OC6-OC8-OC10-OC12-OC14	OC6-OC8-OC10-OC12
E.coli (pSB401) **OC6,OC8,C8,C6**	OC6-OC8	OC6-OC8	OC6-OC8
E.coli (pSB1075) **OC10,OC12**	OC10-OC12	OC10-OC12-OC14	OC10-OC12
E. coli (pJBA132) **OC6,OC8,C6,C8,C10**	+	+	+
TENTATIVE PROFILE	OC6-OC8-OC10-OC12	OC6-OC8-OC10-OC12-OC14	OC6-OC8-OC10-OC12

§ Green bold characters are the AHL to which each sensor responds to, as described in materials and methods. OC6: 3-oxo-C6-HSL; OC8 : 3-oxo-C8-HSL; OC10: 3-oxo-C10-HSL;OC12: 3-oxo-C12-HSL, OC14:3-oxo-C14-HSL.

A similar screening was performed in *B. unamae* MTI-641^{T}and *B.xenovorans* LB400^{T}. The results suggested that these two strains most likely produced 3-oxo-C6-HSL, 3-oxo- C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL (Figure 3-3). In *B. xenovorans* LB400^{T} the possible production of 3-oxo-C14-HSL was also detected. Remarkably, the profiles obtained for all three species using *A. tumefaciens* sensor were similar, as summarized in table 3-2. As in *B. kururiensis*, AHL production in these species was detected in more than one strain (Table 3-1), which could indicate that the QS systems are conserved within the species.

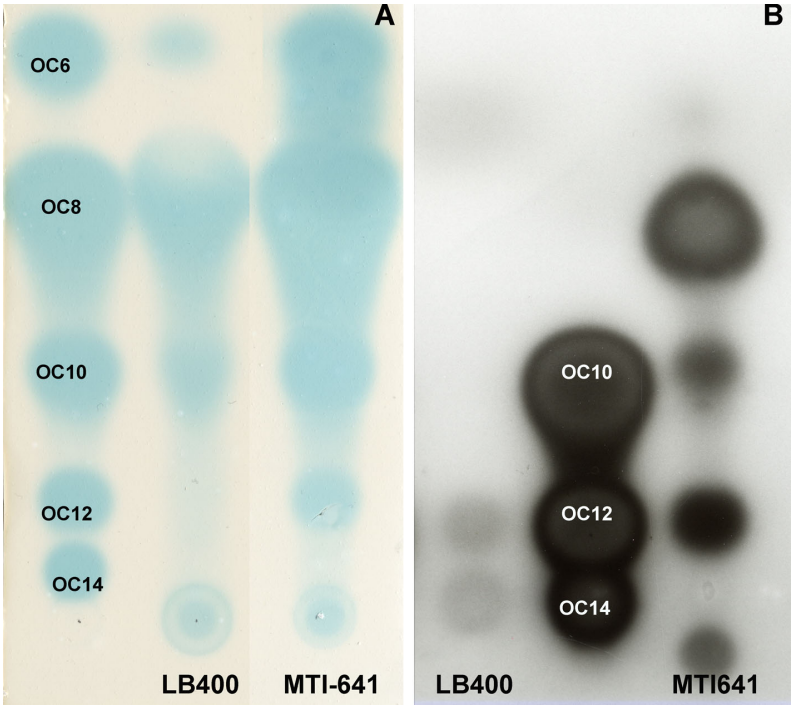


Figure 3-3. TLC analysis for *B.xenovorans* LB400^{T} and *B. unamae* MTI641^{T}

AHL were extracted from 100 mL of M9C glucose media, corresponding to 10^{10} cells approx. Samples were run in TLC with methanol:water (70:30) mobile phase, and overlaid with (A) *A. tumefaciens* pZTR4, (B) *E. coli* pSB1075, as described in materials and methods. Synthetic AHL compounds were used as reference.

3.1.2.3 Identification of the BraIR system in *B. kururiensis* M130

In order to identify the AHL QS system(s), a genomic library was constructed for the strain *B. kururiensis* M130. The AHL QS locus of strain M130 was identified via conjugation *en masse* of the cosmid library into the LasR-based biosensor F117 (pKRC12). Two cosmids, which shared several fragments of identical DNA, were identified as pZS1 and pZS2, which induced *gfp* expression were isolated and contained the AHL QS locus of *B. kururiensis* M130, designated with the *luxI*-type gene called *braI*

and the *lux*R-type gene *bra*R. The *braI*R locus was localized in a 5 kb *Xmn*I fragment which was cloned in pMOSBlue creating pMOSXmn12. This plasmid was sequenced and analysed for the prediction of Open Reading Frames (ORF) and *luxIR*-like genes. The 4938 bp *Xmn*I sequenced fragment was deposited in GenBank with the accession number for the nucleotide sequence AM940944 (Appendix 6.5). (Figure 3-4)

DNA sequence analysis of this locus revealed the presence of three ORFs, two of them (*braI* and *bra*R) displayed homology to *luxI*- and *lux*R-family genes and a third ORF, located in between *bra*R and *braI* divergently transcribed from *braI*, displayed high similarity to the *rsaL* negative regulatory gene from *P. aeruginosa* and *P. putida* (Figure 3-4). The BraR protein consisted of 235 amino acids displaying approximately 40% of identity to LasR and PpuR of *P. aeruginosa* and *P. putida* respectively. The BraI protein consisted of 196 amino acids displaying 50% identity to LasI and PpuI, and RsaL consisted of 105 amino acids having approximately 50% identity to RsaL proteins of *P. aeruginosa* and *P. putida*. It was therefore concluded that *B. kururiensis* possessed an AHL QS system related to the LasI/R and PpuI/R systems of *Pseudomonas*.

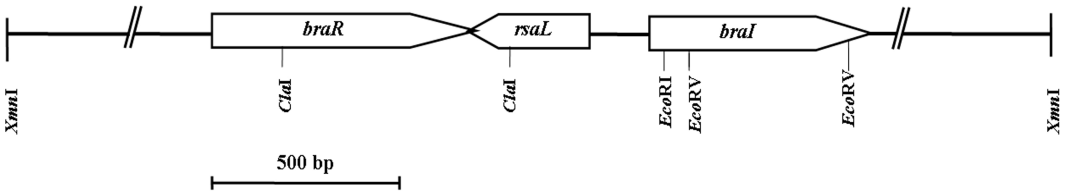


Figure 3-4. Map of the 5 Kbp DNA fragment from *B. kururiensis* M130 containing the 1.7 Kb *braIR* system described in this study.

ORFs were identified in the 4938 bp *Xmn*I fragment by using GENEMARK (Lukashin & Borodovsky, 1998). Each ORF was then analyzed by BLAST database analysis (Altschul *et al.*, 1990) and the identity at protein level is reported in the text.

Interestingly, the highest scores reported during similarity analysis, showed identities ranging from 62 to 82% at protein level, between the BraIR system and uncharacterized putative QS systems in *B. xenovorans* (ORFs Bxe_B0608, Bxe_B0609 and Bxe_B_0610), *B. phytofirmans* (ORFs Bphyt_4277, Bphyt_4276 and Bphyt_4275), *B. graminis* (ORFs BgramDRAFT_3087, BgramDRAFT_3088 and BgramDRAFT3089) and *B. phymatum* (Bphy_4439,Bphy_4438,Bphy4437). All these proteins were found in sequenced genomes from species from the beneficial plant associated *Burkholderia* cluster. In contrast, the BraIR system resulted to be only 31% similar to the CepIR system from *B.*

cepacia, BviIR of *B. vietnamiensis,* CciIR from *B. cenocepacia* and PmiIR from *B. pseudomallei.* From these results it was deduced that the BraIR-like systems are less related to the known QS systems studied in *Burkholderia*, while are closely related to QS systems from the *Pseudomonas* genus.

3.1.2.4 Identification of the *braIR*$_{UNA}$ system from *B.unamae* MTI-641^{T}

The genes encoding for AHL production in *B. unamae* MTI-641^{T} were also identified by screening a cosmid genomic library via conjugation *en masse* in *P. putida* F117 (pKRC12). One transconjugant carrying the cosmid pLZ1 displayed *gfp* expression and was further studied. The QS loci was located within an 8 Kbp *Eco*RV fragment and cloned in pMOSBlue to generate pMOS-pLZ-1, which was sequenced.

Analysis of the predicted ORFs revealed an organization of the QS loci identical to the one described for the *braI*R system *B. kururiensis* (Figure 3-5). The LuxR-type protein consists of 235 aminoacids 77% identical to BraR, while the acyl-homoserine lactone synthase comprises 197 aminoacids displaying 82% identity to BraI. RsaL from *B. unamae* is 103 aminoacids long with 72% identity to RsaL from *B. kururiensis.* This genetic resemblance, together to the similarity in the AHL profile, suggests that the AHL QS system of *B. unamae* MTI-641^{T} may be considered orthologous to the BraI/R system of *B. kururiensis*. The QS system of *B. unamae* MTI-641^{T} was then designated as *braI*/R$_{UNA}$, while the ortholog in *B. kururiensis* was consequentially designated as *braI*/R$_{KUR.}$ The fragment containing the identified QS loci of *B. unamae* were deposited in GenBank/EMBL/DDBJ under the accession number FN640548 (Appendix 6.5).

3.1.2.5 Identification of QS genes in the genome of *B. xenovorans* LB400^{T}

In silico analysis of the sequenced genome of *B. xenovorans* LB400^{T} revealed that it possessed two complete *luxI*/R systems, here designated as *braI*/R$_{XEN}$ and *xenI2*/*R2* (Chain *et al.*, 2006). The *braI*/R$_{XEN}$ system was located in chromosome two, while *xenI2*/*R2* was located in the 1.47 Mbp megaplasmid and will be described in Chapter 3.3. The *braI*/R$_{XEN}$ system displays a similar organization to *braI*/R$_{KUR}$ and *braI*/R$_{UNA}$ as described above (Figure 3-5). In fact, BraR$_{XEN}$, RsaL$_{XEN}$ and BraI$_{XEN}$ (234, 105 and 197 aminoacids, respectively) show high identity values (>75%) to BraI/R$_{KUR}$ and BraI/R$_{UNA}$. Alignments of the BraI and BraR orthologs evidencing the high overall

identity are showed in figure 3-6. BraI and BraR proteins also have the typical residues of the LuxI synthases and LuxR-type proteins.

By performing a nucleotide sequence analysis of the promoter regions from the six AHL synthase *braI*-like genes belonging to the beneficial *Burkholderia* cluster, it was observed that a consensus putative *lux*-box was located between -77 and -82 with respect to the ATG translational start codon (Figure 3-5). Interestingly this sequence is identical in *B. graminis* C4D1M, *B. phytofirmans* PsJN and *B. xenovorans* LB400^{T}.

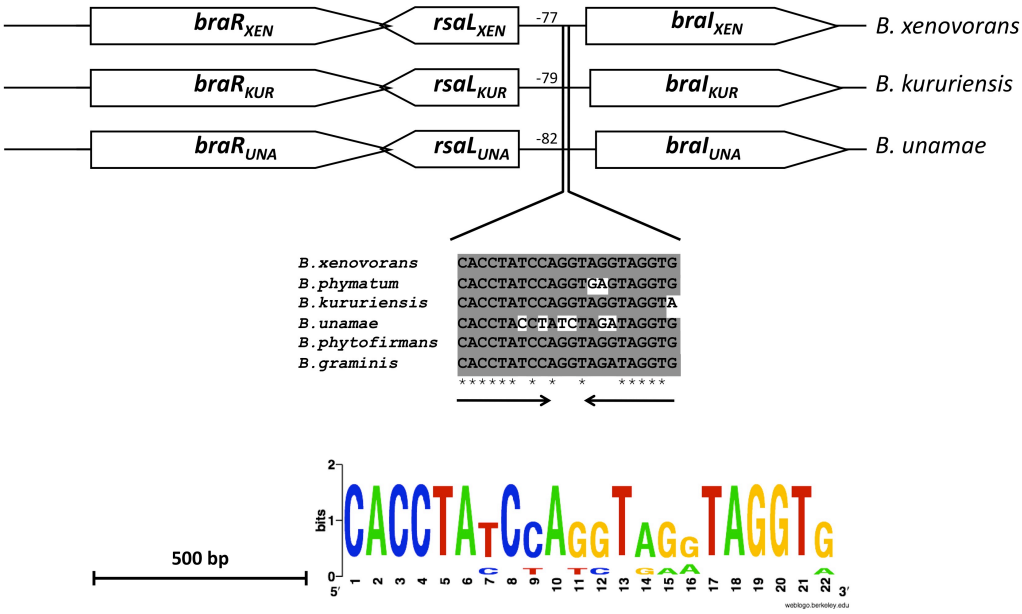


Figure 3-5. Genetic maps of the BraIR-like systems in *B. kururiensis* M130, *B. xenovorans* LB400^{T} and *B. unamae* MTI-641^{T}.

ORFs from *B. unamae* were identified by using GENEMARK (Lukashin & Borodovsky, 1998), and then analyzed by BLAST database analysis (Altschul *et al.*, 1990) in the UNIPROT database(Jain *et al.*, 2009). Identity at protein level is reported in the text. Promoter regions were aligned with ClustalW (Thompson *et al.*, 2002), and analyses of promoter regions were performed with the programs BPROM (Softberry, Inc.), virtual footprint promoter analysis (Munch *et al.*, 2005), and SCOPE (Carlson *et al.*, 2007). The asterisks represent the residues completely conserved within the analyzed sequences. The square contains the consensus sequence for the predicted *lux*-box obtained by WEBLOGO(Crooks *et al.*, 2004).

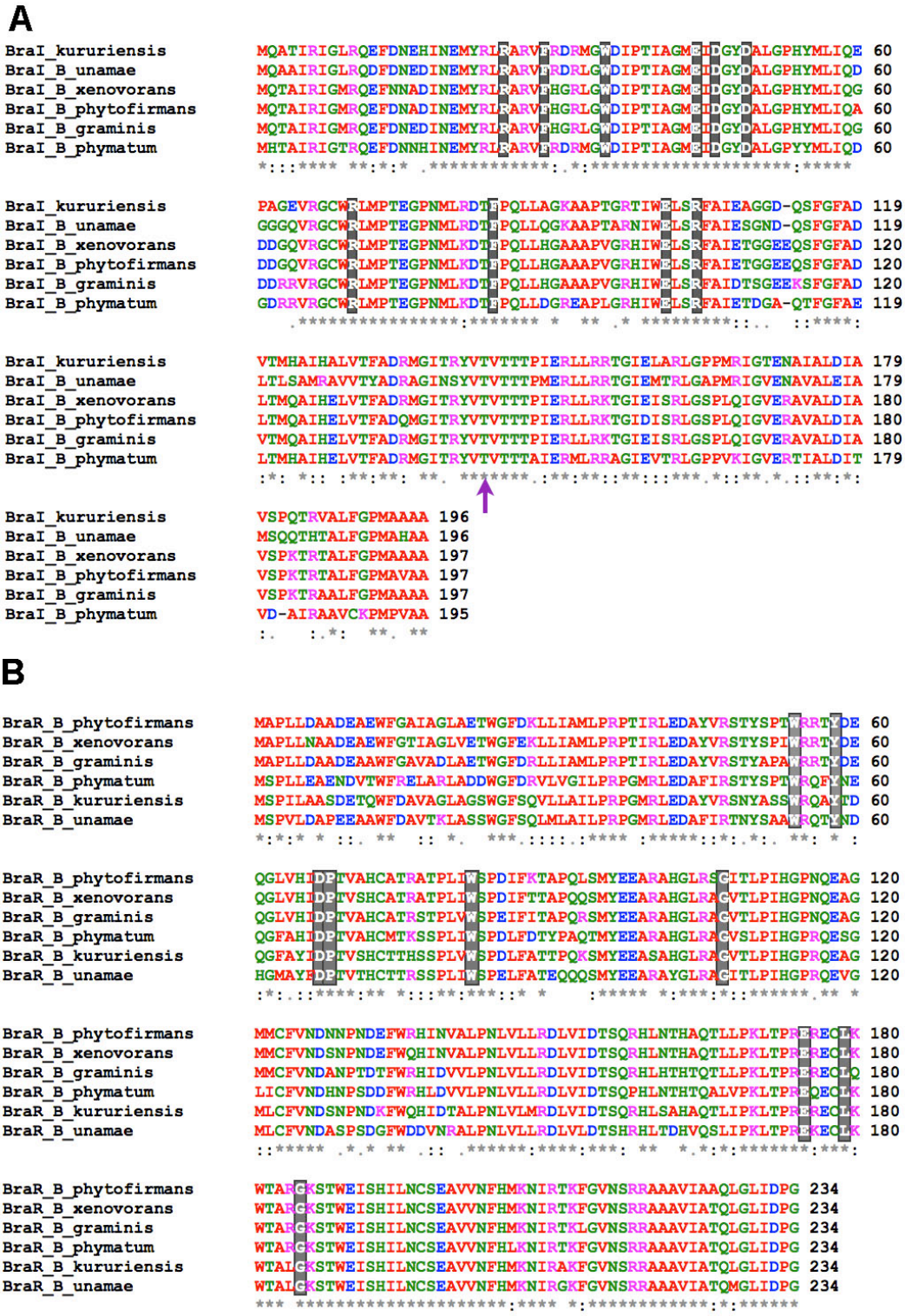


Figure 3-6. Protein alignment of BraI (A) and BraR (B) orthologs in the beneficial plant associated *Burkholderia* species.

BraIR-like proteins were retrieved from the genome projects of *B. graminis* C4D1M, *B. phymatum* STM815 and *B. phytofirmans* PsJN from NCBI/EMBL/DDJB. Proteins were aligned with ClustalW (Thompson *et al.*, 2002), The asterisks represent the residues completely conserved within the LuxI and LuxR-type proteins (Patankar & Gonzalez, 2009a; Watson *et al.*, 2002a). Shaded residues indicate the typical residues of each family of proteins. The purple arrow in A indicates the presence of T143 typical of AHL synthases producing oxo-HSL.

3.1.2.6 Generation of knock-out mutants of the BraIR-like systems

In order to create QS mutants, *braIR-like* genes were inactivated in strains *B. kururiensis* M130, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T as described in materials and methods. The *braI* mutants in the three species were designated as M130BRAI, UNABRAI and LB400BRAI, while *braR* mutants were designated as M130BRAR, UNABRAR and LB400BRAR, respectively.

TLC analysis of ethyl acetate extracts with two different biosensors for *B. kururiensis* M130BRAI and M130BRAR, and *B. unamae* UNABRAI and UNABRAR indicated that these mutants were unable to synthesize AHLs (Figure 3-7 A to D). This suggested that the BraIR system was responsible for synthesizing all AHLs identified in culture supernatants from both strains. In addition, it was concluded that no other AHL QS system was present in *B. kururiensis* or in *B. unamae*. The absence of AHLs in M130BRAR and UNABRAR also demonstrated that BraR-like proteins positively regulate their corresponding BraI synthases through a positive auto-induction loop typical of AHL QS systems. In *B. unamae*, the production of the putative AHLs synthesized by BraI/R_{UNA} could be rescued by genetic complementation of the UNABRAR mutant by providing *bra*R_{UNA} in the cosmid pLZ1 (Figure 3-7).

Similarly, *B. xenovorans* LB400^T mutants LB400BRAI and LB400BRAR were both unable to produce 3-oxo-C10 -HSL, 3-oxo-C12- HSL and 3-oxo-C14-HSL as shown by the AHL production profile observed using the LasR-based biosensor *E. coli* pSB1075 (Figure 3-7E-F). However, AHL production profile of the two mutants using *A. tumefaciens* NTL4(pZLR4) revealed the presence of one AHL spot, probably 3-OH-C8-HSL or 3-oxo-C8-HSL, evidencing that most likely the second AHL QS system present in *B. xenovorans* was responsible for the synthesis of this AHL (Figure 3-7E). The synthesis of 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL could be complemented in the LB400BRAI mutant by providing *braI*$_{XEN}$ in *trans* via plasmid pBBRXENI1. As in *B. unamae* and in *B. kururiensis,* the *bra*R_{XEN} mutant did not produce 3-oxo-C10-HSL, 3-oxo-C12- HSL nor 3-oxo-C14 HSL suggesting a positive feedback loop, which was rescued when the mutation was complemented *in trans* by conjugating the plasmid pBBRXENR (Figure 3-7E-F).

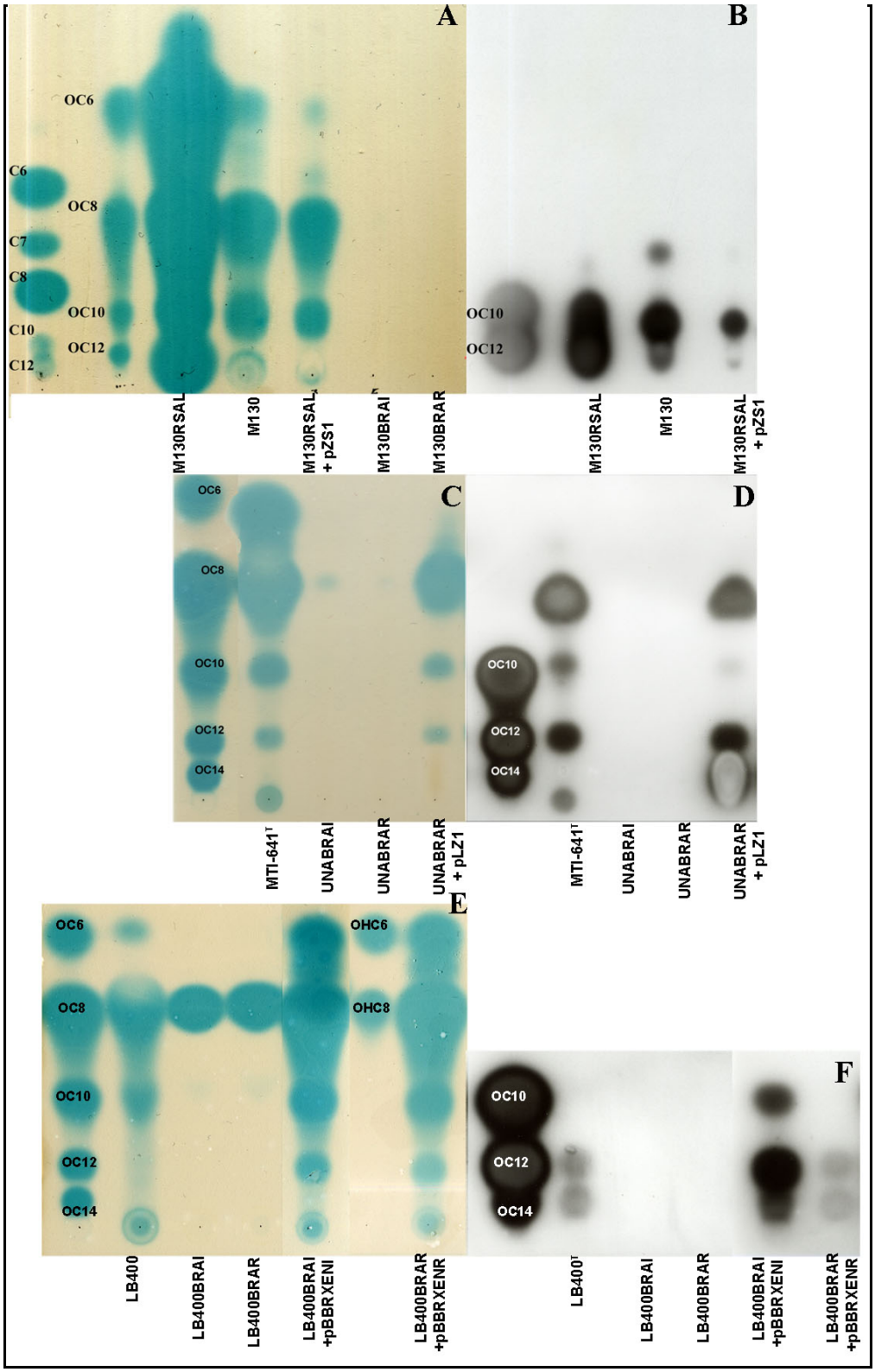


Figure 3-7. TLC Analysis of BraIR mutants of *B. kururiensis* M130 (A,B), *B. unamae* MTI-641^T (C,D) and *B. xenovorans* LB400^T(E,F).

(A-B) *B. kururiensis* extracts correspond to 5x10^{10} C.F.U, with the exception of M130RSAL, which corresponds to 10^9 CFU. *B. unamae* (C-D) and *B. xenovorans* (E-F) extracts correspond to 2.5 x10^{10} CFU. All AHLs were extracted from 100 mL cultures in M9C-Glucose, as described in materials and methods. *A. tumefaciens* pNTL4 was used for overlaying in A,C and E, while *E. coli* pSB1075 was used in B,D and F. TLC for *B. unamae* and *B. xenovorans* were run using a 70:30 methanol:water mobile phase.

3.1.2.7 BraIR-like systems recognize 3-oxo-C14-HSL

Although the AHL profiles identified for the three species studied were similar, it was of interest to determine which was the biologically active molecule among the AHLs produced. A bioassay was set-up to determine to which AHL $BraR_{KUR}$, $BraR_{XEN}$, and $BraR_{UNA}$ best responded to. The three *braR* genes were cloned in the pQE30 expression vector as indicated in material and methods (section 2.6), and the proteins were over-expressed in *E. coli* M15 harbouring a transcriptional fusion of the *lacZ* reporter gene to their cognate $braI_{KUR}$, $braI_{XEN}$ and $braI_{UNA}$ promoters, respectively. Promoter activities were then determined upon the addition of different AHL molecules (1 μM), and the cognate was recognized as the molecule able to generate the highest levels of ß-galactosidase activity.

Measurements of promoter activities in the presence of many different AHLs showed that the activity of the *braI*-like promoters displayed highest transcription in the presence of 3-oxo-C14-HSL. For instance, $braI_{KUR}$ promoter increased twenty two-fold in the presence of 3-oxo-C14-HSL, although increases of eighteen and fourteen fold were also observed when 3-oxo-C16-HSL and 3-oxo-C12-HSL were added to the media, respectively. The activities induced by these three AHLs were statistically different between them ($p<0.05$). These results suggested that $BraR_{KUR}$ has specific preference for 3-oxo-C14-HSL (Figure 3-8A), although it could eventually respond to 3-oxo-C16-HSL and 3-oxo-C12-HSL. In the analog experiment for $BraR_{UNA}$ performed in *E. coli* M15 (pMPUNAI)(pQEUNAR1), the $braI_{UNA}$ promoter activity increased 3.5 fold upon the presence of 3-oxo-C14-HSL and 2 fold when 3-oxo-C12 HSL was present in the media, indicating that most likely 3-oxo-C14-HSL was also the cognate AHL for $BraR_{UNA}$ (Figure 3-8A).

Similar studies were performed using $BraR_{XEN}$. Promoter activity in *E. coli* M15 (pMPXENI1)(pQEXENR1) upon the presence of many different AHLs showed that the activity of the $braI_{XEN}$ promoter increased thirty-fold in the presence of 3-oxo-C14-HSL, ten-fold in the presence of 3-oxo-C16-HSL and only two-fold in the presence of 3-oxo-C12-HSL, demonstrating a specific preference of $BraR_{XEN}$ for 3-oxo-C14-HSL (Figure 3-8C). It was therefore concluded that 3-oxo-C14-HSL was the most likely cognate AHL for BraIR-like systems.

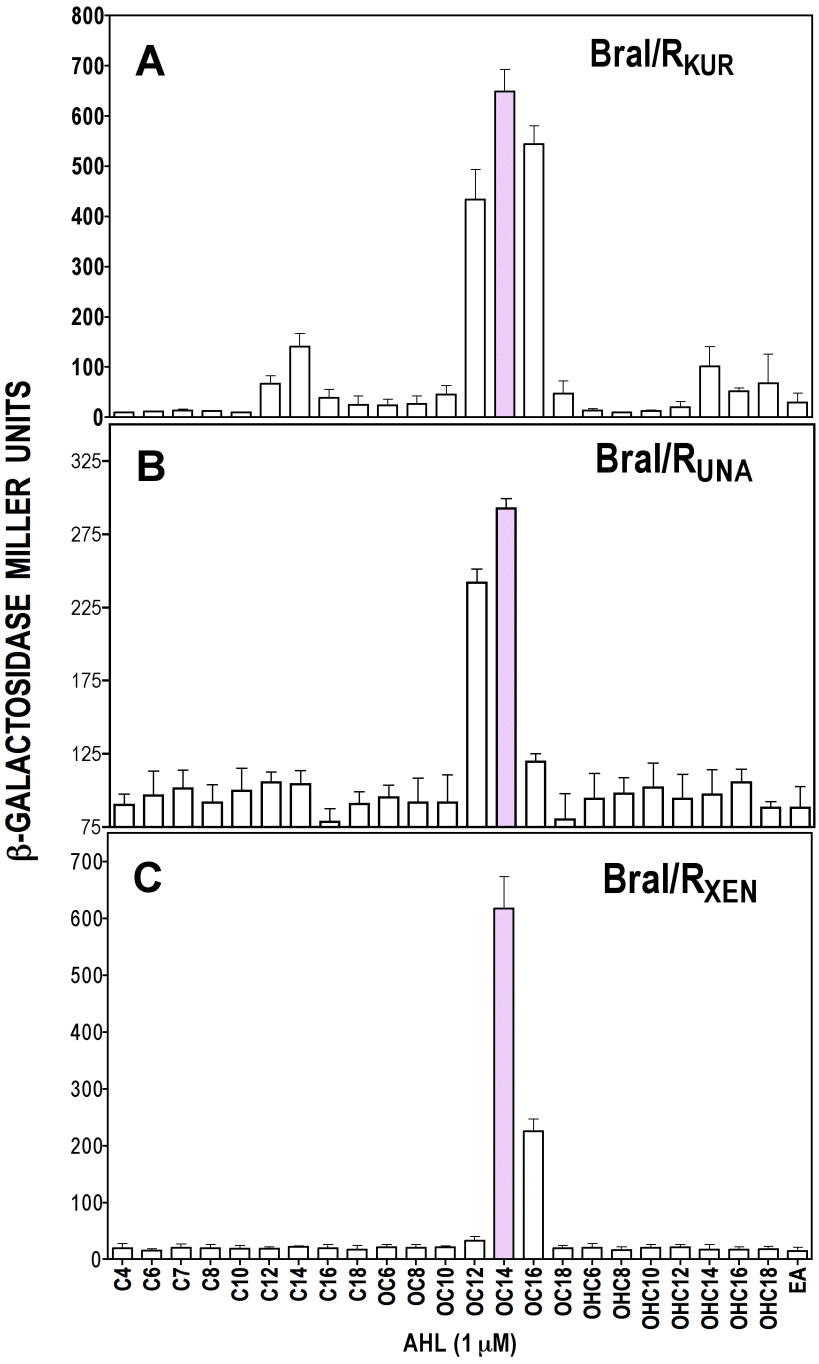


Figure 3-8. Identification of biologically active AHLs for the BraR-like proteins.

Bars correspond to ß-galactosidase activities determined for *E. coli* harboring each pQEBraR and its corresponding synthase promoter fused to *lacZ* in pMP220, upon the addition of various exogenous AHLs (1 μM), as described in materials and methods. (A) For BraIR$_{KUR}$, *E. coli* harbouring pQEBRAR+pMPbraI was used. (B) For BraIR$_{UNA}$, pQEUNAR+pMPUNAI was used. (C). For BraIR$_{XEN}$ pQEXENR+pMPXENI was used. Pink bars represent the preferred AHL molecule for each BraR-like protein. The results are means of three values ±SD of three independent biological replicates. Means were compared with ANOVA analysis in combination with Dunnett post test, means were considered statistically different when $p<0.01$. EA: Ethyl Acetate.

Importantly, the production of 3-oxo-C14-HSL was detected by TLC analysis in *B. xenovorans* LB400^T, but not in *B. kururiensis* and *B. unamae*. It is speculated that this molecule is produced by these strains, as $braI_{KUR}$ and $braI_{UNA}$ promoter activities were considerably activated upon the addition of this AHL. The detection of 3-oxo-C14-HSL by TLC could be limited by the sensitivity of the biosensors used and by the amounts of AHLs produced by the cell under the conditions tested.

3.1.2.8 BraIR system in *B. kururiensis* is strongly repressed by RsaL

The *rsaL* gene located intergenically between the $braIR_{KUR}$ genes in *B. kururiensis* M130, encodes for the $RsaL_{KUR}$ protein. Orthologs to this protein have been reported to negatively regulate the transcription of the *luxI*-family AHL synthase in *P. aeruginosa* and *P. putida* (Bertani *et al.*, 2007; Rampioni *et al.*, 2006). Since it was observed that strain M130 produced low amounts of AHLs (Figure 3-7A), it was of interest to determine whether RsaL was negatively regulating $braI_{KUR}$. The *rsaL* gene was inactivated in strain M130 generating a knock-out mutant designated M130RSAL. The AHLs produced by this mutant were extracted and analyzed by TLC and this showed that AHLs production dramatically increased in the *rsaL* mutant (Figure 3-7A-B). This mutant could be complemented since providing the *rsaL* gene in *trans* in a plasmid (cosmid pZS1) restored AHL production to wild-type levels (Figure 3-7A-B).

In order to estimate the levels of repression exerted by $RsaL_{KUR}$, the AHL production was quantified in *B. kururiensis* M130 and M130RSAL. Since it was determined that one of the functional AHLs for $BraR_{KUR}$ was 3-oxo-C12-HSL, levels of this AHL in extracts from M130 and M130RSAL were determined using the sensor *P. putida* SM17 (prsaL220) (Rampioni *et al.*, 2007b). This sensor is suitable for 3-oxo-C12-HSL quantification, as it is very specific for this AHL and its linear dose response was shown to be from 0.1 mM to 1 mM. Results of this experiment showed that C12-3-oxo-AHL levels produced by M130 wild type were very low and were enhanced dramatically (almost 2000 times) in the *B. kururiensis rsaL* mutant (Table 3-3). The estimated concentration in a stationary phase culture of *B. kururiensis* M130 was approximately 20 nM whereas it increased dramatically to 45 μM for the *rsaL* mutant. The production of 3-oxo-C12-AHL in the *rsaL* mutant was restored to wild-type levels when the *rsaL* gene was provided in *trans* via cosmid pZS1 (Table 3-3).

Table 3-3. Quantification of 3-oxo-C12-HSL (OC12) in *B. kuruviensis* M130 and M130RSAL

Strain	Volume Culture[1]	Optical Density	EA volume[2]	Volume for assay [3]	β-galactosidase (Miller Units)[4]	OC12 Estimated concentration (μM)[5]
WT	200	2.8	616	200	577.78 ± 59.2	0.020 ± 0.004
	200	3.23	710.6	200	514.81 ± 8.01	0.018 ± 0.001
	200	4.52	994.4	200	543.83 ± 6.55	0.028 ± 0.001
M130RSAL	50	4.37	240.35	1	2533.33 ± 38.19	49.76 ± 3.937
	50	4.535	249.42	1	2147.73 ± 177.50	42.725 ± 4.102
	50	4.535	249.42	1	2354.70 ± 125.82	47.508 ± 2.907
M130RSAL	50	2.91	145.5	50	651.09 ± 51.26	0.109 ± 0.014
(pZS1)	50	2.2	110	50	599.86 ±34.65	0.072 ±0.007
	50	2.74	137	50	694.56 ± 90.56	0.114 ± 0.023

(1)Single colonies were independently inoculated and grown overnight in KB medium and optical density was measured. AHL were extracted from spent supernatants and resuspended in an ethyl acetate volume (2) corresponding to an amount of 1×10^9 CFU. (3)AHL levels were measured with *P. putida* SM17 (prsaL220) since C12-3-oxo-AHL levels are proportional to β-galactosidase activity (Miller Units). Extracted AHL where diluted in order to obtain β-galactosidase values into the linear range of the sensor (0.1-1 μM).(4) Values represent the means of three independent experiments with three replicates ± SD (5) Estimated concentrations of C12-3-Oxo AHL in spent supernatants

3.1.2.8.1 RsaL directly represses the $braI_{KUR}$ transcription.

An additional experiment was performed in order to verify if the repression of the AHL production exerted by $RsaL_{KUR}$ was direct. For this purpose $BraR_{KUR.}$ and $RsaL_{KUR}$ were overexpressed in *E.coli* M15 cells, and the $braI_{KUR}$ promoter activity was measured in the presence of 3-oxo-C12-HSL (Figure 3-9). Results suggest that when both proteins were present in a heterologous host, the $braI_{KUR}$ promoter activities were reduced three times in comparison to the transcription levels achieved when only $BraR_{KUR}$ was present. This result suggests that $RsaL_{KUR}$ directly represses the transcription of $braI_{KUR}$ by binding the *rsaL-braI* bidirectional promoter and thus interfering in its recognition by $BraR_{KUR}$.

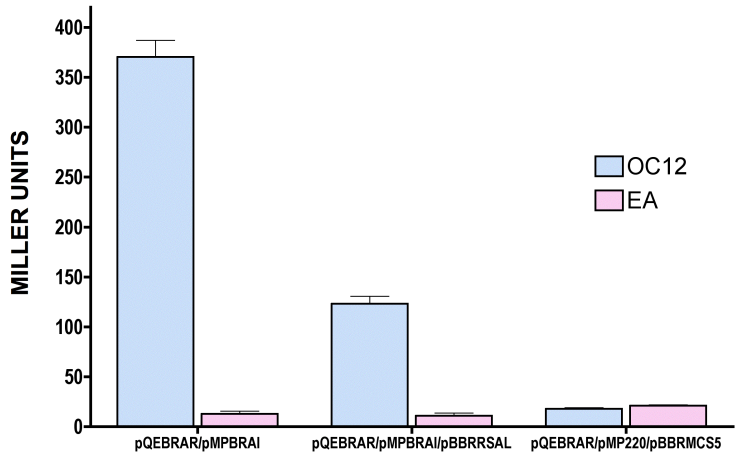


Figure 3-9. $braI_{KUR}$ promoter activity in presence of $RsaL_{KUR}$ and $BraR_{KUR}$.

Bars represent the $braI_{KUR}$ ß-galactosidase activities, in presence (blue bars) or absence (pink bars) of 3-oxo-C12-HSL (OC12). The plasmids harboured by *E.coli* M15 cells are pQEBRAR over-expressing $BraR_{KUR,}$ and pBBRRSAL which overexpresses $RsaL_{KUR}$. pMPBRAI is the transcriptional fusion of the $braI_{KUR}$ promoter. *E. coli* harbouring pQEBRAR/pMP220/pMPBBRMCS5 was used as negative control .

3.1.2.9 The BraIR-like system is conserved in 20 species of the beneficial plant associated *Burkholderia* cluster

As described above, the ability to produce AHLs with a similar profile is conserved within the species from the beneficial plant associated *Burkholderia* group; it was therefore of interest to determine if the genetic loci responsible of the AHL production were similar to *braI*R. Southern analysis at high stringency levels was performed to determine the presence of BraIR-like systems in other species from the group. A 423 bp *Eco*RV fragment excised from $braI_{KUR}$ was used as probe (Figure 3-10), against genomic DNA of the type strains. Hybridization results suggested that the BraIR-like AHL QS system was well conserved in 20 species of the novel cluster of *Burkholderia* species (Figure 3-10).

Southern results were also confirmed by PCR amplification of the *bra*R-like genes and the *rsaL*-like genes in 20 species by using two pair sets of oligonucleotide primers directed towards $braR_{KUR}$ and $rsaL_{KUR}$, which generated the expected amplimers of approx. 200 bp and 750 bp in most of the species tested (Figure 3-11). The amplimers for *B. graminis, B. phymatum* and *B. phytofirmans* were sequenced, and their sequences corresponded with the *bra*R and *rsaL* orthologs. Importantly, in *B. unamae* and *B. silvatlantica* the same result was obtained for two different strains within the same species, demonstrating that *braI*R is not strain-specific (lanes 13-16). In the case of *B. terrae* no amplification was obtained for the *bra*R-like gene, however positive amplification of the *rsaL*-like gene and hybridization with the $braI_{KUR}$ probe, confirmed the most likely presence of a BraIR-like system in this species. Importantly no amplification was observed using either set of primers in members of the BCC complex and with *P. aeruginosa*. These results suggest that PCR reactions by using these sets of primers may constitute an important tool for the identification of the BraIR systems in *Burkholderia* belonging to this novel cluster (Figure 3-11).

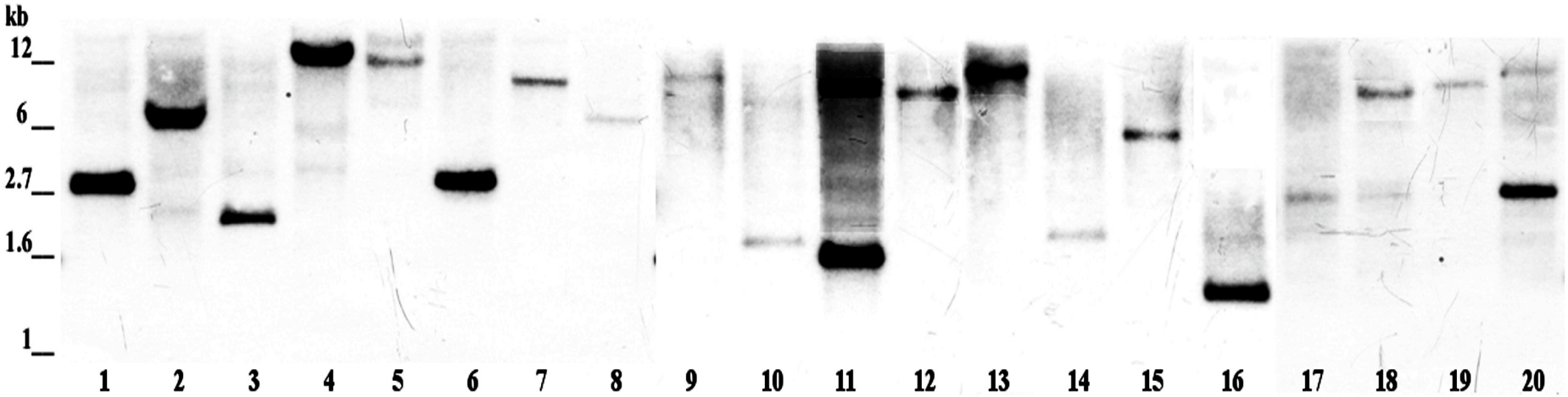


Figure 3-10. Autoradiogram of a Southern Blot analysis of total *Eco*RI-digested genomic DNA hybridized with the *braI* probe from *B.kururiensis* M130.

Lane 1, *B. kururiensis* M130; lane 2, *B. caledonica* DSM 17062; lane 3, *B. fungorum* DSM 17061; lane 4, *B. graminis* DSM 17151; lane 5, *B. hospita* DSM 17164; lane 6, *B. kururiensis* DSM 13646; lane 7, *B. mimosarum* PAS44; lane 8, *B. tropica* DSM 15359; lane 9, *B. caribensis* DSM 13236; lane 10, *B. phytofirmans* DSM 17436; lane 11, *B. sacchari* DSM17165; lane 12, *B. silvatlantica* SRMrh-20^{T}; lane 13, *B. terrae* DSM 17804; lane 14, *B. tuberum* DSM 18489; lane 15, *B. unamae* DSM 17197; lane 16, *B. xenovorans* DSM 17367; lane 17, *B. phenazinium* DSM 10684; lane 18, *B. phenoliruptrix* DSM 17773; lane 19, *B. phymatum* DSM 17167;lane 20, *B. terricola* DSM 17221.

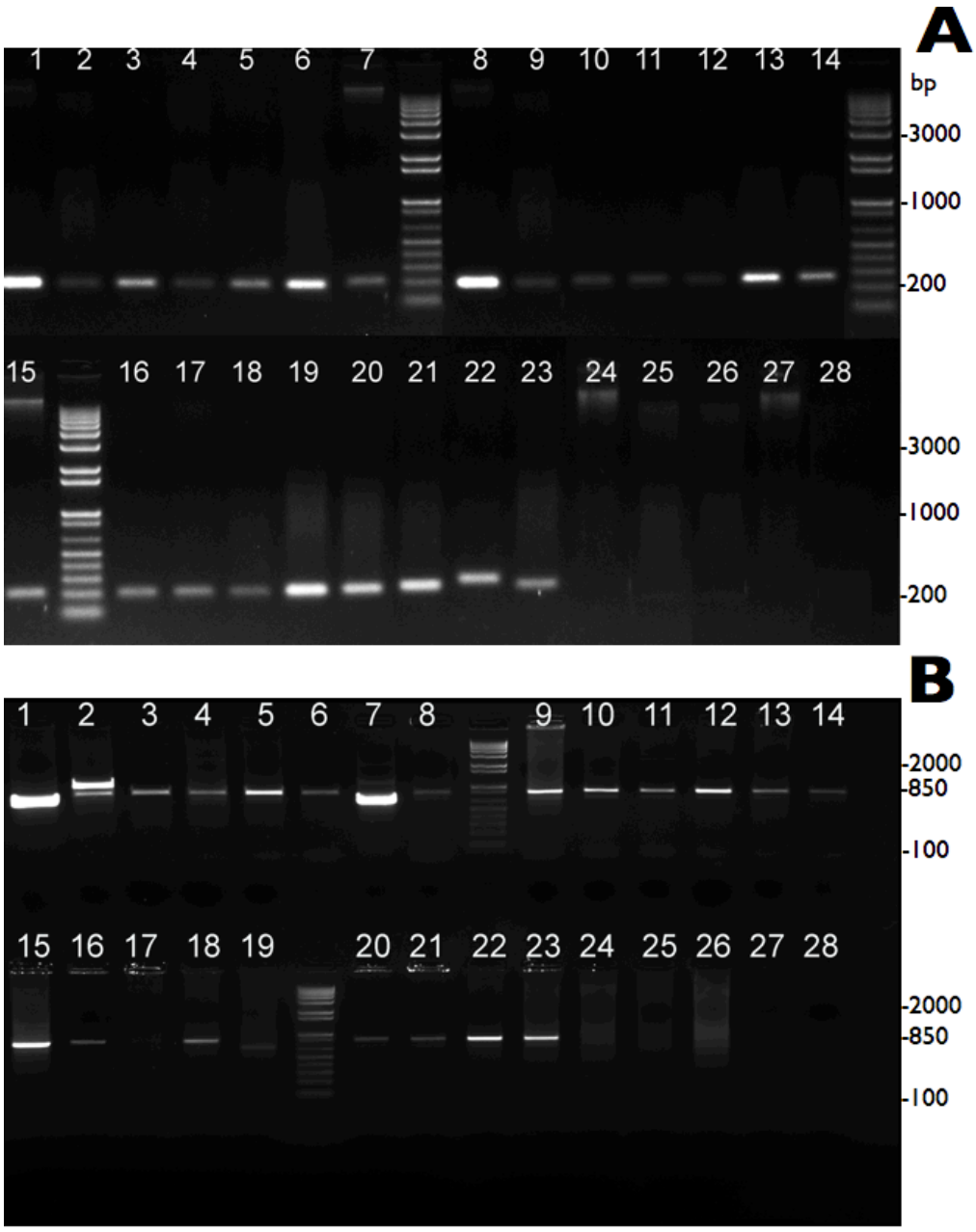


Figure 3-11. Conservation of BraIR demonstrated by PCR amplification of *braR* and *rsaL* genes in species of the beneficial plant–associated *Burkholderia* cluster.

A. Amplification of a 225 bp internal fragment of *rsaL* orthologs by using braLFW and braLRV oligonucleotides. B. Amplification of *braR*-like genes. (1) *B. kururiensis* M130, (2)*B. caledonica* DSM17062,(3) *B. caribensis* DSM13236, (4) *B. fungorum* DSM17061, (5) *B. graminis* DSM17151, (6) *B. hospita* DSM17164, (7) *B.kururiensis* KP23^{T}, (8) *B.mimosarum* PAS44^{T}, (9) *B. phenazinium* DSM10684^{T}, (10) *B. phenoliruptrix* DSM17773^{T} (11) *B. phymatum* DSM17167, (12) *B. phytofirmans* PsJN (13) *B. silvatlantica* SRMrh20^{T}(14) *B. silvatlantica* SRCL-318,(15) *B. unamae* MTL-641^{T}, (16)*B. unamae* SSCu-23 (17) *B. terrae* DSM17804 (18) *B. terricola* DSM17221, (19) *B. tropica* DSM15359 (20)*B. tropica* Ppe8^{T},(21) *B. tuberum* DSM 18489, (22) *B. sacchari* DSM 17165 (23) *B. xenovorans* LB400^{T}, (25) *P. aeruginosa* PAO1,(26) *B. glumae* AU6208 (27)*B. cepacia* ATCC 25416 (28) Negative control (No DNA).

3.1.3 Concluding discussion

From its formation in 1992, the genus *Burkholderia* has been extensively studied since its members are catabolically versatile, are found in many different environments and some are of medical importance (Yabuuchi *et al.*, 1992). Validly described species have been isolated from a wide range of niches including soil, water, wastes, plants, fungi, animals and humans. Importantly, several species have been reported to have either a beneficial or pathogenic interaction with plants, animals or humans (Reis *et al.*, 2004; Vandamme & Mahenthiralingam, 2003). Currently available *Burkholderia* genome sequences suggest that this genus owes its niche versatility to its large genomes comprised of several large replicons, as well as to lateral gene transfer events and plasmid acquisition (Chain *et al.*, 2006; Lim *et al.*, 2009; Lin *et al.*, 2008; Martinez-Aguilar *et al.*, 2008).

A new cluster composed of more than 30 beneficial *Burkholderia* species has emerged in recent years following the rapid increase in the number of described species identified in natural environments. This group is phylogenetically distant from the BCC complex, and phenotypes such as plant association, nitrogen fixation and aromatic compound degradation are present in several species from the group. Despite the metabolic versatility and agro-biotechnological potential of this new group, the regulatory mechanisms underlying their association with their hosts are at large unknown. This chapter investigated the AHL based QS systems in 21 species from this *Burkholderia* species group, and reports the identification and characterization of one system in strains from three representative species of the cluster, namely *B. kururiensis*, *B. xenovorans* and *B. unamae*.

Screening for AHL production in 27 strains from 21 species of the beneficial plant-associated *Burkholderia* group was performed by using three biosensors, which clearly showed the production of this type of autoinducers in all species tested. Although, these tests identified tentatively the molecules produced, a combination of three biosensors covering a wide range of the known AHLs evidenced the production of a common AHL pattern in all strains tested. These results suggested that AHL-QS is widespread in this *Burkholderia* species cluster.

These insights are in accordance with previous studies, which demonstrated that other members of the cluster were also able to produce AHLs, namely *B. bryophila*, *B.*

megapolitana, *B. phytofirmans* and *B. graminis* (Barriuso *et al.*, 2008; Trognitz *et al.*, 2009; Vandamme *et al.*, 2007).

The BraIR-like systems are conserved in the beneficial plant-associated cluster

AHL QS systems were identified in strains *B. kururiensis* M130^T, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T. These strains were chosen based on their reported ability to fix nitrogen, live in close association with the plant and to degrade aromatic compounds. An additional criterion was the diversity in their preferred niche: endosphere, rhizosphere and free-soil. *B. kururiensis* M130 has been reported to endophytically colonize rice roots in Brazil, and field experiments have reported increases in rice yield due to the contribution to total nitrogen (Baldani *et al.*, 2000). On the other hand, *B. unamae* MTI 641^T was identified in the rhizosphere of maize and has demonstrated ability to promote the growth of tomato plants (Onofre-Lemus *et al.*, 2009). *B. xenovorans* LB400^T was isolated from PCB polluted soil and has become a model system for the breakdown of highly persistent contaminants (Goris *et al.*, 2004; Seeger *et al.*, 2003; Smith *et al.*, 2007a).

These three strains possess a QS system composed by a *luxIR* pair designated here as the BraIR system. *In silico* analyses demonstrated that BraIR-like systems were also present in the sequenced genomes of cluster mates as *B. phytofirmans, B. graminis* and B. *phymatum*. Importantly these QS systems exhibit low overall similarity values when compared with other QS systems from the *Burkholderia* species (<31%), while are closer to the LasI/R and PpuI/R from the *Pseudomonas aeruginosa* and *putida*, respectively.

These three BraIR systems characterized are to date the first reported AHL QS systems in the beneficial plant-associated *Burkholderia* group. It was showed that the BraIR systems are highly conserved in the plant associated *Burkholderia* species cluster indicating that they are part of the core genome of this *Burkholderia* group and most likely were not acquired by recent gene transfer events. The existence of a conserved QS system could facilitate inter-species communication within this *Burkholderia* cluster, possibly providing advantages in multispecies niche adaptation. Furthermore, the relatedness between the BraI/R-like systems with the LasI/R and PpuI/R from

Pseudomonas spp., could suggest that their coding genes might have been involved in lateral gene transfer during early speciation events (Case *et al.*, 2008; Malott *et al.*, 2005).

Commonalities of the BraIR systems: a conserved *lux*-box, a common AHL cognate and positive feedback loop.

Several characteristics were identified as common in the three BraIR systems presented here. First, it was established that the BraI/R_{XEN}, BraI/R_{UNA} and BraI/R_{KUR} systems produce several 3-oxo-AHL derivatives, but respond preferentially to 3-oxo-C14-HSL. This is in accordance with previous studies, which demonstrated that other members of the cluster, namely *B. phytofirmans* (strains $PsJN^T$ and RG6-12) and *B. graminis* (strains M1 and M14), also produce 3-oxo-C14-HSL (Barriuso *et al.*, 2008; Trognitz *et al.*, 2009). Given the high similarity observed between BraR-like proteins it is expected that 3-oxo-C14-HSL is also the cognate AHL for the BraI/R-like systems in other species of this *Burkholderia* cluster.

The production of 3-oxo-C14-HSL was clearly detected by TLC analysis in *B. xenovorans* $LB400^T$. It is speculated that this molecule is most likely produced also by *B. kururiensis* and *B. unamae*, since $braI_{KUR}$ and $braI_{UNA}$ promoter activities in the presence of $BraR_{KUR}$ and $BraR_{UNA}$ and 3-oxo-C14-HSL was considerably activated. The limited detection of 3-oxo-C14-HSL by TLC in these strains may be due to the sensitivity of the biosensors used, and needs to be confirmed by futher experimentation including HPLC-Maldi-TOF analyses.

A common *lux*-box was located in the promoter region of the BraI-like synthases characterized here, which highlights the high degree of conservation exhibited by the *braIR* systems. The existence of a common system sharing features like *lux*-boxes could indicate a common function, and thus the presence of common targets for the BraIR-like systems, which might have coevolved together with the QS genes. Further identification of BraIR targets in different species substantiates this possibility.

Generation of knock-out mutants in the *braR* and *braI* genes suggested that BraR-like proteins positively regulate the *braI* synthase promoters, as deficient mutants in $braR_{KUR}$, $braR_{XEN}$ and $braR_{UNA}$, were also negative for the production of the AHLs encoded by their partner *braI* synthase. This commonality between the BraIR systems is frequent in AHL-QS systems in several species, and guarantees an immediate amplification of the signal (Fuqua & Greenberg, 2002; Fuqua & Parsek, 2002). Importantly, the BraIR

system showed to be the only AHL-QS system present in *B. unamae* and *B. kururiensis*, whereas *B. xenovorans* possessed at least another AHL QS system. This fact suggests that while some *luxIR* pairs, as the *braIR*, are conserved within species, others may be subject of horizontal transfer events (Lerat & Moran, 2004).

BraIR$_{KUR}$ systems is tightly regulated by RsaL

The three identified *braIR* systems possess a repressor gene called *rsaL* which is located in between the *braI* and *braR* genes as is also the case for the *lasI/R* and *ppuI/R* genes of *P. aeruginosa* and *P. putida,* respectively. RsaL of *P. aeruginosa* is a tetrahelical HTH protein, which directly represses *lasI*. In *P. aeruginosa, rsaL* deficient mutants produce approximately 10-fold more 3-oxo-C12-HSL in late logarithmic/stationary phase of growth when compared to the wild-type parent strain (Rampioni *et al.*, 2006; Rampioni *et al.*, 2007a). It has been proposed that the biological role of RsaL in *P. aeruginosa* is to maintain 3-oxo-C12-HSL homoeostasis and to then possibly change steady-state levels upon varying environmental conditions (Rampioni *et al.*, 2007a).

Considering the relevance of the RsaL repressor protein in the *P. aeruginosa* LasIR system, experiments were designed to understand the role of RsaL in *B. kururiensis* M130. Our results suggested that the scenario in *B. kururiensis* M130 is somewhat different since this strain is synthesizing very low levels of AHLs under laboratory conditions, with the situation dramatically changing when RsaL$_{KUR}$ is inactivated. In fact, the *rsaL$_{KUR}$* deficient mutant produces over 2000-fold more AHLs, as suggested by quantification analyses. The role of RsaL is hypothesized not to be one of homeostasis but rather a switch to turn on/off the AHL QS system. Most probably under certain environmental conditions the system is very efficiently switched on. This could possibly occur through a mechanism of inactivation of RsaL resulting in very fast increase in AHL production. The ability of BraI/R$_{KUR}$ to synthesize such high levels of AHLs is unique and also questions the role of this molecule in cell-density dependent regulation in *B. kururiensis*, as AHLs may also act as iron chelators or as anti-bacterial agents (Kaufmann *et al.*, 2005; Schertzer *et al.*, 2009).

Our experiments showed that *braI$_{KUR}$* repression by RsaL$_{KUR}$ is direct, and occurs by binding of the RsaL$_{KUR}$ to the bidirectional *rsaL-braI$_{KUR}$* promoter. These results are in agreement with studies in *P. aeruginosa,* which have suggested that when both proteins

are bound to DNA, the repressor activity of the RsaL is dominant over the LasR activation (Rampioni *et al.*, 2006; Rampioni *et al.*, 2007a). In fact, RsaL proteins from the BraIR-like systems share six conserved residues proposed for the RsaL-DNA interaction, and therefore most likely function in the same way (Rampioni *et al.*, 2006; Rampioni *et al.*, 2007a).

Measurements of 3-oxo-C12-HSL levels, one of the AHLs produced by strain M130, suggest that stationary phase *B. kururiensis rsaL* mutants accumulate almost 50 μM C12-3-oxo-AHL in their supernatant whereas only 20 nM for the parent strain. It is true that some AHL QS systems respond to nM amounts of AHLs; however the ability of *B. kururiensis* to produce high amounts of AHL such as 45 μM, indicates that strain M130 can most probably respond very quickly to a sudden need. The on/off very stringent regulation and the capacity to synthesize such high concentration of AHLs might also be an indication that the molecule could play another role in the life of this bacterium unrelated to quorum sensing. It is tempting to speculate that as this group of *Burkholderia* have been isolated from so varied and geographically distant environments, the BraIR-like system might provide these bacteria via RsaL a specific response to a particular stimulus regulating a particular set of genes. Importantly, our studies have shown that RsaL is very well conserved among this group of *Burkholderia* and that most strains produce very low quantity of AHLs indicating that stringent regulation of the BraI/R system is likely to be widespread.

The identification of the *braIR* systems described here has important ecological relevance considering the occurrence and distribution of the beneficial plant-associated group of *Burkholderia* species in nature. These species have been reported to colonize geographically distant environments including polluted soils, the rhizosphere and endosphere of several species of plants. This is an indication that species grouped in this cluster can respond and adapt to varying environmental conditions, and consequently possess a large set of regulatory systems allowing them to sense and respond to many different stimuli (Compant *et al.*, 2008b; Vial *et al.*, 2007). Since QS has shown to play an important role in survival and adaptation in other *Burkholderia* species, it is likely that a similar function could be hypothesized for this type of communication in members of the beneficial *Burkholderia* cluster.

3.1.4 Final Remarks

In the *Burkholderia* genus two major AHL-QS networks had been described so far: the CepI/R system producing and responding to C8-AHL found in members of the *Burkholderia cepacia* complex (Eberl, 2006; Venturi *et al.*, 2004), and the multiple LuxIR systems from the *B. mallei-pseudomallei* group (Chandler *et al.*, 2009; Duerkop *et al.*, 2009). In this study a new AHL-QS, the BraIR-like systems, has been found and characterized in members of the beneficial plant-associated *Burkholderia* species group.

This study describes the BraIR-like systems in strains from three different species. The three identified systems share the same gene organization, which together to the similarity in the AHL profile are sufficient grounds to consider them orthologs. The three BraR-like proteins respond best to 3-oxo-C14-HSL, and are tightly regulated by RsaL. Furthermore, the BraIR-like system is conserved along the members of the beneficial-plant associated *Burkholderia* species group. Further studies are required to decipher the role of this BraIR as regulatory system in the plant-*Burkholderia* interaction, which also could contribute to the understanding of the high levels of conservation within species from this *Burkholderia* clade.

3.2 IDENTIFICATION OF TARGETS FOR THE BraIR-LIKE QS SYSTEMS FROM BENEFICIAL PLANT-ASSOCIATED *BURKHOLDERIA* CLUSTER

3.2.1 Introduction

In the *Burkholderia* genus there are three major AHL QS networks: the CepI/R system producing and responding to C8-AHL found in members of the *Burkholderia cepacia* complex (Eberl, 2006; Venturi *et al.*, 2004), the multiple *luxIR* pairs systems present in strains from the *B. mallei-pseudomallei-thailandiensis* group (Chandler *et al.*, 2009; Duerkop *et al.*, 2009), and the BraIR-like systems present in the beneficial-plant associated cluster and described in the previous chapter (Suarez-Moreno *et al.*, 2008). The CepIR system is conserved along the species of the BCC complex and has shown to be pivotal in their adaptation and pathogenicity by regulating targets as motility, biofilm formation, and the exoenzyme and siderophore production, among others (Huber *et al.*, 2001; Sokol *et al.*, 2003; Sokol *et al.*, 2007). On the other hand, the complex QS networks from the *B. pseudomallei*, *B. thailandiensis* and *B. mallei* have shown relevant roles in pathogenicity and fitness by regulating phenotypes as aggregation or antibiotic production (Chandler *et al.*, 2009; Duerkop *et al.*, 2009).

In the previous chapter, the presence of an AHL-based QS system was identified in three species of the beneficial-plant associated *Burkholderia* group and designated as the BraIR-like system. This QS system was conserved in type strains of 20 species of the cluster exhibiting high overall similarity at protein sequence level. Furthermore, studies in three BraIR-like systems showed that the BraR proteins recognize long chains AHL, with preference for 3-oxo-C14-HSL. The BraIR system is repressed by RsaL, since it negatively regulates the expression of the *braI* synthase gene. No phenotypes are known to be regulated for the BraIR systems and thus their roles as regulatory systems are still poorly understood.

The purpose of this study was to determine some of the targets for the BraIR-like systems and their possible role in the environmental adaptation of these versatile species group. We established that BraIR was not involved in the regulation of nitrogen fixation and in several other important phenotypes. However, BraI/R regulated EPS production and the biofilm formation. In addition, the plant-*Burkholderia* interaction and

the aromatic compound degradation were also regulated by BraI/R. In conclusion, this study represents an extensive analysis of AHL QS regulation in the *Burkholderia* plant associated cluster demonstrating commonalities as well as differences probably reflecting environmental adaptations of the various species.

3.2.2 Results

To determine the possible targets of the BraIR-like systems, several phenotypes were analyzed in the wild type and QS mutant strains of *B. kururiensis* M130, *B. unamae* MTI-641^T and *B. xenovorans* LB400^T. The mutant strains tested are listed in table 3-4.

Table 3-4. *Burkholderia* strains and QS mutants studied in this chapter

STRAIN	DESCRIPTION
B. kururiensis M130	Isolate form surface sterilize roots of rice
M130BRAI	*B. kururiensis* deficient in *braI*$_{KUR}$
M130BRAR	*B. kururiensis* deficient in *braR*$_{KUR}$
M130RSAL	*B. kururiensis* deficient in *rsaL*$_{KUR}$
B. unamae MTI-641^T	Type strain solated from maize rhizosphere
UNABRAI	*B. unamae* MTI-641^T deficient in *braI*$_{UNA}$
UNABRAR	*B. unamae* MTI-641^T deficient in *braR*$_{UNA}$
UNABRAR +pLZ1	*B. unamae* MTI-641^T deficient in *braR*$_{UNA}$ harbouring the cosmid pLZ1
B. xenovorans LB400^T	Type strain, isolate from PCB-polluted soils
LB400BRAI	*B. xenovorans* LB400^T deficient in *braI*$_{XEN}$
LB400BRAR	*B. xenovorans* LB400^T deficient in *braR*$_{XEN}$

Mutants were generated as described in materials and methods.

3.2.2.1 Exopolysaccharide production is positively regulated by the *braIR*-like systems in *Burkholderia* species

The exopolysaccharide production was evaluated in wildtype strains and QS mutants derived from *B. xenovorans* LB400^T, *B. kururiensis* M130 and *B. unamae* MTI-641^T, by streaking single colonies of wild type strains and QS mutants in Yeast Extract Mannitol Agar (YEM). Our results suggest that in all three species, EPS production was positively regulated by the BraIR-like QS system, since the AHL-synthase mutants M130BRAI, LB400BRAI and UNABRAI mutants were significantly less mucoid than the corresponding wild type strains (Figure 3-12). In all three cases the EPS production was restored by chemical complementation when 1 μM of the cognate AHL (3-oxo-C14-HSL) was provided to the media.

Quantification of the EPS produced by *B. kururiensis* showed that M130BRAI presented a threefold decrease in the EPS produced when compared to the wild type ($P<0.05$)

(Figure 3-12B), whereas in *B. unamae* UNABRAI the reduction was 10-fold (Figure 3-12D). Experiments performed to quantify the EPS in *B. xenovorans* LB400^{T} were unfruitful as the LB400BRAI cells remained adhered to the agar, and thus the EPS extraction and cell counting could not be carried out.

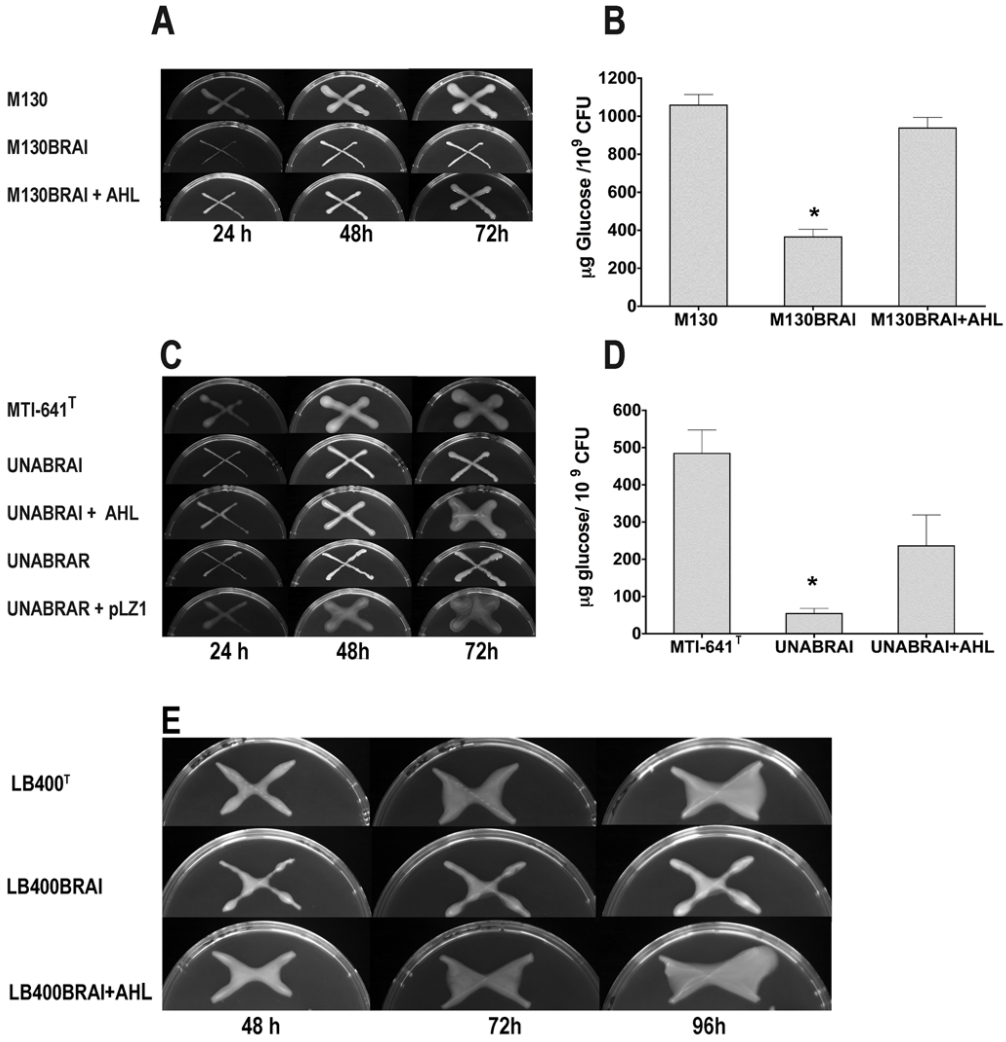


Figure 3-12. EPS production is positively regulated by BraIR in *B. kururiensis* M130 (A-B), *B. unamae* MTI-641^{T} (C-D) and *B. xenovorans* LB400^{T} (E)

Single colonies were cross-streaked in YEM media, and EPS production was visually monitored overtime. After incubation time, cells were recovered from the plate and the EPS was extracted and precipitated as described in materials and methods. Complementation was achieved by adding 1 µM AHLs 3-oxo-C14-HSL. Cells were counted by serial dilution plating and quantification was performed by the boiling phenol method (DuBois *et al.*, 1956).

3.2.2.2 Regulation of the biofilm production in beneficial *Burkholderia* species

The biofilm formation was evaluated in wild type and QS mutants by using a microtiter plate assay as described in materials and methods. The cells were grown in minimal media and after incubation time (72 h) the culture media was removed and the formed biofilm was stained with crystal violet. After removal of the dye, wells were washed thoroughly and the biofilm was measured indirectly by determining the amount of remaining crystal violet attached to the biofilm.

Our results suggest that biofilm formation was negatively regulated by QS in *B. unamae* MTI-641^{T}, since UNABRAR mutants accumulated approximately three times more biofilm than the wild type. Importantly, biofilm accumulation was restored to wild type levels in the UNABRAR mutant by providing *in trans* the *bra*R_{UNA} gene via cosmid pLZ1 (Figure 3-13A). Interestingly, the *B. unamae* UNABRAI mutant produced the same amount of biofilm like the wild-type.

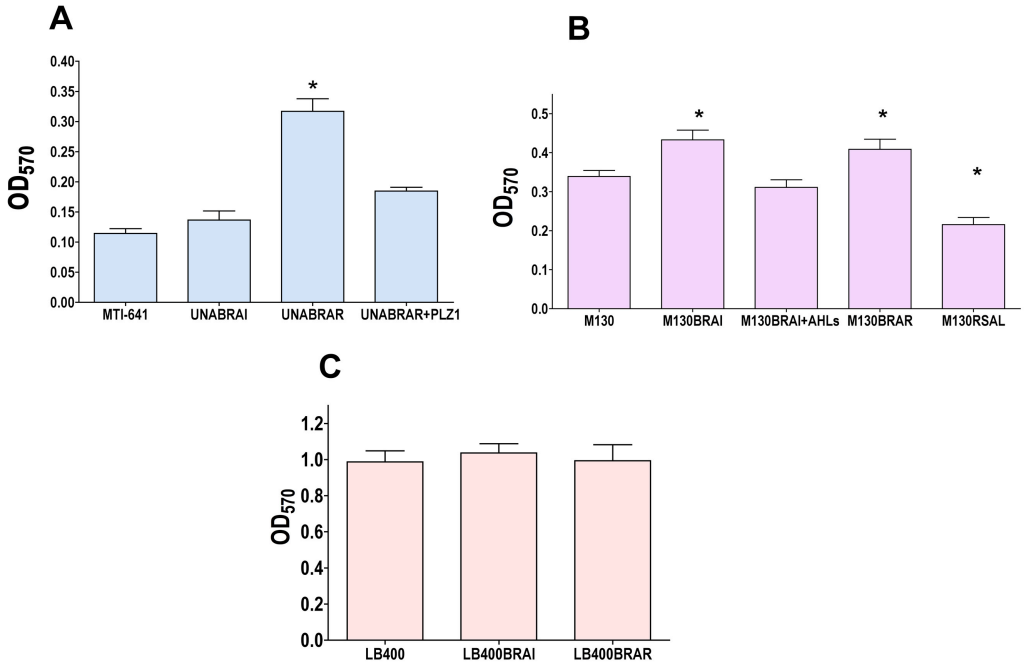


Figure 3-13. Biofilm accumulation in *Burkholderia* species analyzed in this study

Biofilm production in (A) *B. unamae*, (B) *B. kururiensis* and (C) *B. xenovorans* wild-type and QS mutants after 72 h incubation. Complementation was achieved by adding 1 μM AHLs 3-oxo-C14-HSL. Experiments were performed in triplicate and means ± SD were plotted. Means were compared with ANOVA analysis in combination with Dunnetts post-test performed with PRISM 4.0 software (GraphPad Software, San Diego California). A P value of < 0.05 was considered significant in comparison to wild type (*).

In contrast, *B. kururiensis* AHL deficient mutants M130BRAI and M130BRAR displayed only a slight increase in biofilm formation when compared to the wild-type ($P<0.01$); this increase was restored to wildtype levels, when 3-oxo-C14-HSL was provided to the media containing the M130BRAI (Figure 3-13B). Remarkably a corresponding reduction was observed in the overproducing mutant M130RSAL ($P<0.01$). Biofilm formation was clearly observed in *B. xenovorans* strains, but no major differences were observed in any of the *B. xenovorans* QS mutants under the conditions tested (Figure 3-13 C). It was concluded that biofilm formation was regulated by QS in *B. unamae* and in *B. kururiensis* but was not in *B. xenovorans*.

3.2.2.3 QS regulation of the plant-*Burkholderia* interaction

3.2.2.3.1 Rice endophytic colonization by *B. kururiensis* M130

Since *B. kururiensis* M130 was isolated as a rice endophyte, we tested the role of QS in rice colonization and growth. A significant decrease in colonization of the QS mutants was observed in comparison to that obtained for the wild type, both in the roots as well as in the aerial parts of the plant ($P<0.05$)(Figure 3-14 A and B). In addition, roots of plantlets colonized with the wild type exhibited an increase in length and branching when compared to roots colonized with the QS mutants (Figure 3-14C). This data suggested that under the conditions tested QS positively regulates endophytic rice colonization by *B. kururiensis* M130.

3.2.2.3.2 The $BraIR_{KUR}$ system is not involved in regulating either nitrogenase activity or indolacetic acid production (IAA)

An important feature of *B. kururiensis* M130 is its ability to fix nitrogen by converting N_2 into NH_3 through the synthesis of a nitrogenase complex. Since this is a very high energy demanding reaction, bacteria stringently regulate this process (Dixon & Kahn, 2004). It was therefore of interest to determine whether the $BraIR_{KUR}$ system was involved in the regulation of nitrogenase activity in *B. kururiensis* M130, and if therefore the root enhancement effect observed above, was associated to an increase in the nitrogen fixation. The acetylene reduction assay (ARA) was used to detect expression of the nitrogenase complex under nitrogen-free conditions in parent strain M130, in the AHL hyper-producing $rsaL_{KUR}$ mutant and in the non-AHL producing M130BRAI and M130BRAR mutants.

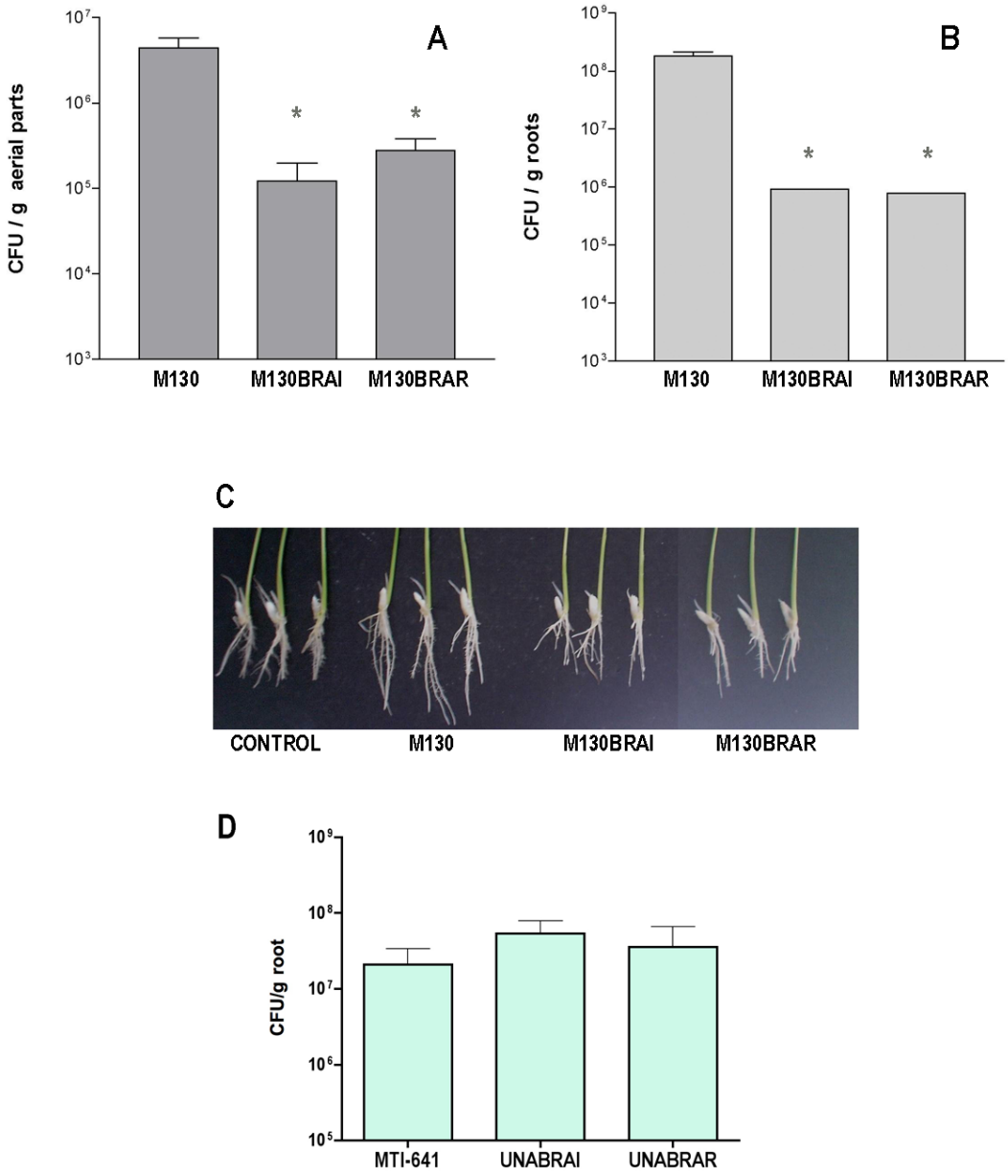


Figure 3-14. Plant colonization assays performed with *B. kururiensis* M130 and *B. unamae* MTI641^T

(A-C)Rice endophytic colonization by *B. kururiensis* wild-type and impaired QS mutants M130BRAI and M130BRAR Rice seeds were surface sterilized and inoculated as described in materials and methods. Bacterial colonization was measured by grinding and plating surface sterilized plantlets, after 12 days post inoculation, and CFU/g levels were plotted. Mean values were compared by using ANOVA and a p value<0.05 was considered significant (*).(A) *B. kururiensis* rice endophytic colonization of aerial parts (B) *B. kururiensis* root endophytic colonization levels. (C) Enhanced root development promoted by *B. kururiensis* M130 wildtype inoculation in comparison to QS mutants and non-inoculated plants.(D) Maize rhizosphere colonization by *B. unamae* MTI-641 wildtype and QS mutants.

Nitrogenase activity was comparable among the wild-type strain and the three QS mutant derivatives ($braI_{KUR}$, $braR_{KUR}$ and $rsaL_{KUR}$) both at low and high cell density. It was

therefore concluded that the AHL-QS does not regulate nitrogenase-complex formation in *B. kururiensis* M130 (Table 3-5). Similarly, the biosynthesis of indolic compounds, including indoleacetic acid (IAA), was determined. Results suggested that no dramatic difference was observed between the IAA production in wildtype and QS mutants (Table 3-5).

3.2.2.3.3 Maize Rhizosphere colonization by *B. unamae* MTI-641^T

Since *B. unamae* MTI-641T was isolated from the maize rhizosphere (Caballero-Mellado *et al.*, 2004), we determined the role of QS in maize rhizosphere colonization. Experiments revealed that the wild-type strain and the UNABRAI and UNABRAR genomic mutants colonize the maize roots at similar levels, indicating that in this strain AHL QS does not play a major role in root colonization under the conditions we tested, (Figure 3-14 D).

Table 3-5. Nitrogen Fixation, aromatic compound degradation and IAA acid production in *B. kururiensis* M130 and QS mutants

ACTIVITY	Low cell density (10^5 UFC)[1]				High cell density (10^8 UFC)			
	M130	M130BRAI	M130BRAR	M130RSAL	M130	M130BRAI	M130BRAR	M130RSAL
C_2H_2 Reduction[2]	13.5	13.3	13.6	13.9	15.8	15.5	15.8	16.0
Indolic compounds[3]	6.1	9.5	6.8	6.6	13.4	18.0	17.2	14.1
Growth in plates with:								
Succinic acid	+	+	+	+	+	+	+	+
Toluene	+	+	+	+	+	+	+	+
Benzene	±	±	±	±	±	±	±	±
Phenol	±	±	±	±	±	±	±	±
Growth in liquid [4]								
Succinic Acid	0.476	0.430	0.435	0.369	0.451	0.400	0.423	0.366
Phenol compounds	0.010	0.020	0.042	0.009	0.058	0.066	0.105	0.026

(1) Cell number inoculated per mL of liquid culture media or on agar culture media. (2) nmol C_2H_4/h/ culture (average of 3 replicate cultures) (3) μg/ml culture medium. Indolic compounds including IAA (average of 2 replicates), (4) Optical density (average of 2 replicates). +, Good growth; ±, poor growth.

3.2.2.4 Siderophores, motility, lipase and protease studies

The strains were tested for the siderophore production in CAS plates as described in materials and methods. Although the three wild-type strains showed remarkable siderophore production, none of their corresponding QS mutants showed significant differences.

Burkholderia wild type strains and their QS mutants were also tested for motility in several semisolid media. Testing swarming in KB, M8 and Nutrient agar media

supplemented with 0.5% agar, did not evidence any swarming motility in any of the tested strains. Swimming motility was examined by using KB and nutrient agar supplemented with 0.25% agar. Although swimming motility was observed in the three strains studied, no significant difference was observed between the wild type strains and their corresponding QS mutants.

Proteolytic and lypolitic enzyme activities were tested in media supplemented with skim milk and trybutyrin, respectively. No proteolytic activity was observed in any of the tested strains, under the conditions tested. In contrast, lypolitic activity was observed in the all three species tested, but none of their QS mutants exhibited appreciable differences in comparison with their wild-type strains.

3.2.2.5 Two secreted proteins are regulated in a cell density manner in *B. xenovorans* LB400^T

Very often AHL QS in bacteria regulates the expression of secreted proteins, thus it was of interest to determine whether the levels of any of the secreted proteins were altered in the mutants in *Burkholderia*. For this experiment, *B. xenovorans* LB400^T was used since among the three species of the cluster studied here, it is the only one of which the genome has been sequenced and annotated.

We analyzed the profile of secreted proteins of the wild type strain LB400 ^{T} vs. the profile of the *braI*$_{XEN}$ mutant LB400BRAI. Several protein bands were identified which displayed different levels and the corresponding gene promoters were cloned and studied. As depicted in Figure 3-15A, results revealed the presence of two proteins with an apparent molecular mass of 40 kDa and 21 kDa in the LB400BRAI mutant that were absent or present in very low amounts in the wild type. It was postulated that these proteins were negatively regulated by the BraI/R$_{XEN}$ system. Mass spectrometry analysis indicated that the 40kDa protein corresponds to a putative 377 a.a. outer membrane porin (OmpC family), encoded by the locus Bxe_B2786. This corresponding gene encoded for a porin belonging to the OmpC family (pFAM00267), and a putative *lux* box centered at -96 was identified in its promoter region(Figure 3-15C) .

In order to confirm whether this ORF was regulated by BraIR$_{XEN}$, the Bxe_B2786 gene promoter region was cloned into the promoterless probe vector pMP220 to generate pMP2786. This construct was then conjugated into the *B. xenovorans* LB400 ^{T} wild type

and LB400BRAI mutant. Determination of β-galactosidase activity showed a 50-fold increase in the activity values obtained in the LB400BRAI mutant compared to the wild-type confirming that this ORF was negatively regulated by the BraI/R_{XEN} system. Promoter activity was significantly reduced when *braI*$_{XEN}$ was provided in *trans* by conjugating the plasmid pBBRXENI1 into LB400BRAI (Figure 3-15B).

The 21 kDa protein which was also abundantly present in the LB400BRAI mutant corresponded to a putative ABC-type transporter periplasmic ligand binding protein postulated to be involved in toluene resistance, encoded by the locus Bxe_B0016. By measuring gene promoter activities in wild type versus mutants we were able to confirm the negative regulation by the BraI/R_{XEN} system since promoter activity increased 5 fold in the LB400BRAI mutants (Figure 3-15B). In this case, however, promoter activity was not restored to wild type levels when we provided the *braI*$_{XEN}$ gene *in trans* via pBBRXENI1; the reason for this is currently not known.

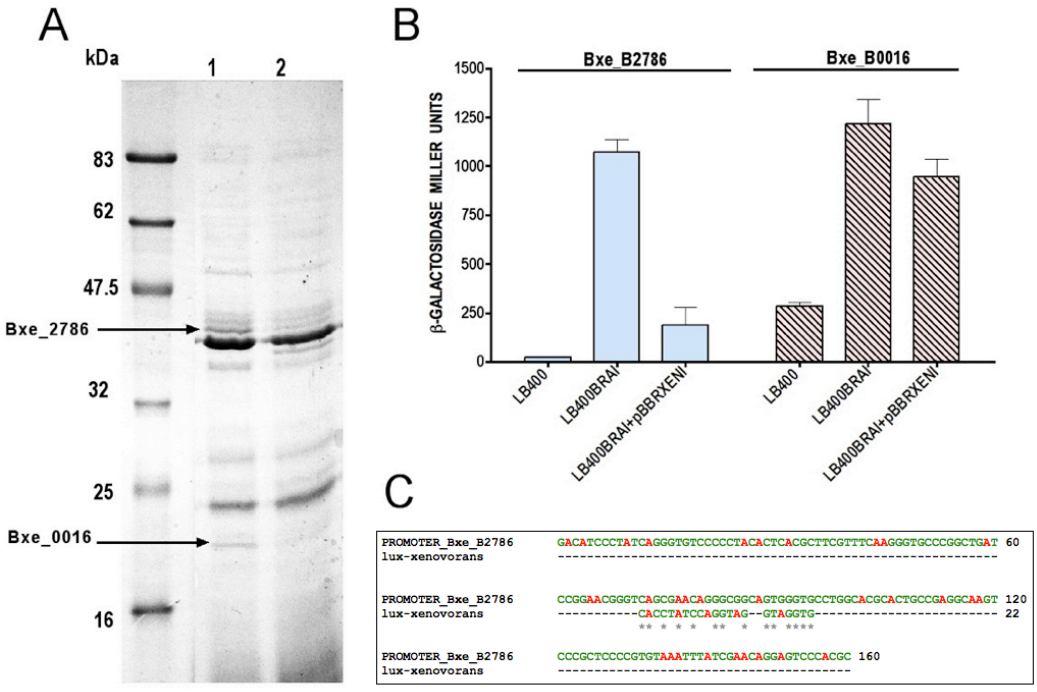


Figure 3-15. Analysis of secreted proteins for *B. xenovorans* LB400^T and LB400BRAI.

(A). Secreted protein profile for *B. xenovorans* LB400 ^{T} (2) and LB400BRAI mutant (1). (B). Bxe_B2786 and Bxe_B0016 promoter activities in wild-type, LB400BRAI mutant and LB400BRAI mutant complemented with pBBRXENI. Bacterial cultures were started with an initial inoculum of 5 x10^6 CFU in 10 mL of KB-Tc, and KB-Tc-Gm medium and ß-galactosidase activities were measured after 12 hours of growth when cells had reached stationary phase. All experiments were performed in triplicate and means values ± SD are plotted (C) Putative *lux*-box identified in the promoter region of the locus Bxe_B2786.

3.2.2.6 The degradation of aromatic compounds is regulated by QS in *B. unamae* and *B. xenovorans* but not in *B. kururiensis*

One of the main commonalities between the three *Burkholderia* species studied is their recognized ability to degrade aromatic compounds, although each species has been proved to degrade a different profile of compounds (Caballero-Mellado *et al.*, 2007). The catabolic pathways involved in the aromatic compound degradation are important for the fitness of these species to their niches.

3.2.2.6.1 Phenol degradation is QS regulated in *Burkholderia unamae*

The ability of *B. unamae* MTI-641^T to utilize phenol as carbon source was previously reported (Caballero-Mellado *et al.*, 2007). AHL QS deficient mutants (UNABRAR and UNABRAI) were found to have a decreased ability to utilize phenol compared to the wild type (Figure 3-16). The ability to grow in the presence of phenol was partially restored in UNABRAR mutant when complemented *in trans* with the cosmid pLZ1. These results suggest that the phenol degradation is QS regulated in *B. unamae*. As a control experiment we replaced phenol with mannitol as carbon source and determined that the mutants grew in a similar fashion as the wild-type.

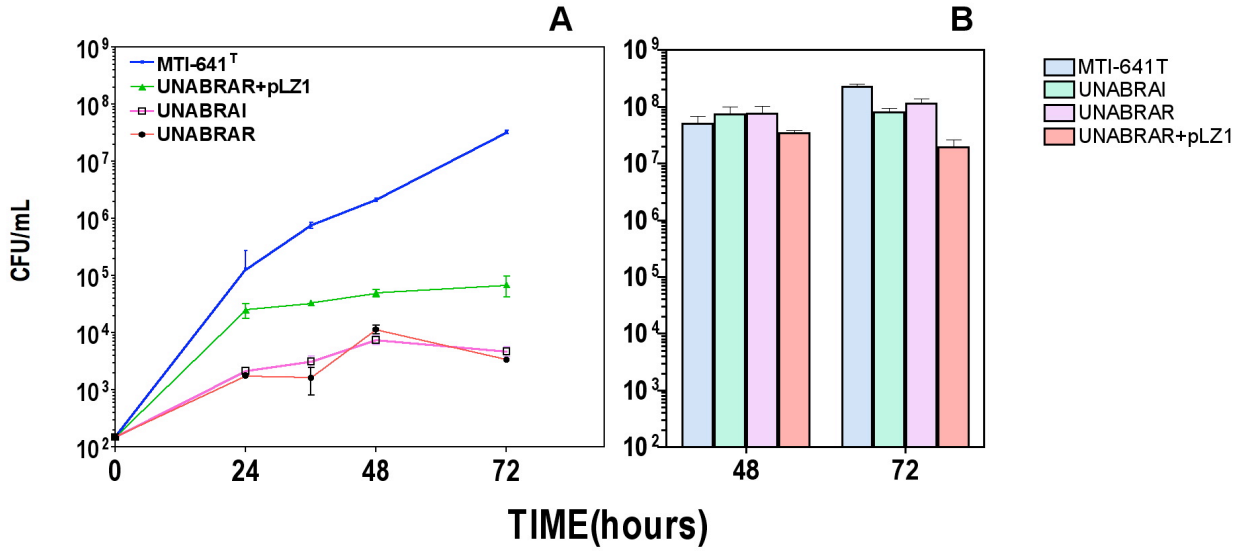


Figure 3-16.Phenol degradation profile in *B.unamae* wild-type and QS mutants.

(A). Growth profile was obtained with a 1x 10^3 CFU as starter inoculums, and CFU was determined after incubation time by serial dilution. (B) As control, phenol was replaced by mannitol as carbon source, and no important differences were observed in CFU at 48h and 72h, demonstrating that cells were viable and grew in a similar fashion.

3.2.2.6.2 Biphenyl degradation is positively regulated by QS in *B. xenovorans* LB400^T

Given the known ability of *B. xenovorans* LB400^T to degrade biphenyl compounds, a plate assay experiment was set up to determine the possible regulation of this phenotype by BraIR$_{XEN}$. Results suggested that the *braI*$_{XEN}$ deficient mutant was unable to metabolize the biphenyl as carbon source, and thus no growth was observed. Biphenyl degradation was partially recovered when *braI*$_{XEN}$ was provided in *trans* by conjugating the plasmid pBBRXENI1 into LB400BRAI. Interestingly, no effect was observed in the biphenyl degradation in the *braR*$_{XEN}$ deficient mutant. As a control experiment, when the same media was supplemented with mannitol as carbon source, all the mutants exhibited the same growth (Figure 3-17). These results suggest that the degradation of biphenyl is regulated in a cell density manner.

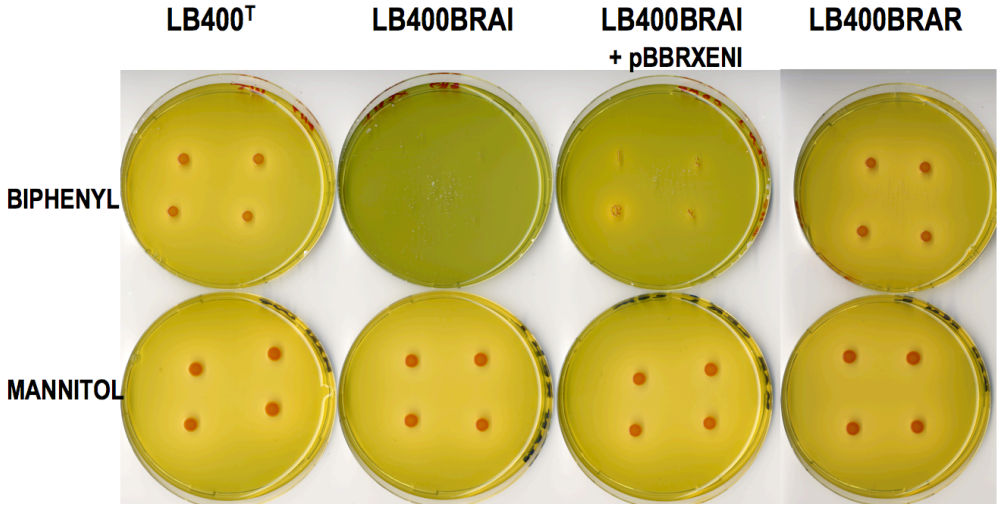


Figure 3-17. Biphenyl degradation in *B. xenovorans* LB400^T and *braIR*$_{XEN}$ defficient mutants

Overnight cultures of each strain were spun down and washed twice with PBS buffer, and then adjusted to OD 0.25. 1 µL of each suspension was then spot in SAAC plates. 50 mg of biphenyl crystals were provided on filter paper circles placed on the lids of the Petri dishes, which were sealed with polyethylene tape and incubated upside down at 30 °for 5 days. As positive control, plates containing 5 mM mannitol were inoculated exhibiting growth in the same fashion.

3.2.2.6.3 The degradation of phenol, benzene or toluene is not regulated by BraIR$_{KUR}$ in *B. kururiensis*

B. kururiensis M130 was reported to degrade benzene, toluene and phenol. To determine the role of QS in the regulation of the degradation of these compounds, *B. kururiensis* wild-type and QS mutants were grown in SAAC plates and each compound was

supplied as carbon source. No difference was observed in the degradation of these compounds, which suggest that the pathways used for their catabolism are not regulated in a cell density manner (Table 3-5). Importantly, all *B. kururiensis* strains grew in a similar fashion when inoculated in media supplemented with succinate (Table 3-5).

3.2.3 Concluding discussion

AHL-QS in the genus *Burkholderia* is involved in the regulation of many different phenotypes, including the production of extracellular proteases (Gingues *et al.*, 2005), lipases (Devescovi *et al.*, 2007), chitinases, polygalacturonases, siderophores (Lewenza & Sokol, 2001), motility, biofilm formation and virulence (Huber *et al.*, 2001).

Which genes/phenotypes are regulated by the highly conserved BraIR system in beneficial plant-associated *Burkholderia* species? In this study we have analyzed whether several important phenotypes, often associated with AHL QS in other bacterial species, are regulated in a cell density manner by the BraIR-like systems in three species from the plant associated beneficial *Burkholderia* clade.

The exopolysaccharide production is a common target of the BraIR-like systems

BraI/R-like systems were not involved in the regulation of motility, siderophore production, nor lipolytic activity. In contrast, EPS production was found to be positively regulated by the BraI/R-like systems in *B. kururiensis, B xenovorans* and *B. unamae*. It has been reported that EPS production is also subject to QS regulation in other plant associated bacteria like *Pantoea stewarti* (Von Bodman *et al.*, 1998), *Ralstonia solanacearum* (Flavier *et al.*, 1997), *Pseudomonas syringae* (Quiñones *et al.*, 2005) and *Sinorhizobium melliloti* (Marketon *et al.*, 2003). Significantly, in these species EPS production affected host invasion and the pathogenic or symbiotic interaction with the plant were diminished via alteration in surface attachment (biofilm formation) as described for *P. stewarti* (Koutsoudis *et al.*, 2006) and *S. meliloti* (Hoang *et al.*, 2008; Rinaudi & Gonzalez, 2009). Importantly, in *B. kururiensis* M130 both rice colonization and EPS production were reduced in the QS mutants, which could indicate that EPS production may be a determinant for the endophytic colonization.

It has been reported that one of the EPS polymers produced by *B. kururiensis* (EPS B) has structural similarity to the EPS produced by *B. cepacia* (cepacian) (Hallack *et al.*, 2009), which is considered a virulence factor required to the formation of thick and

mature biofilms (Cunha *et al.*, 2004b). A similar polymer has been recently detected in members of the plant associated *Burkholderia* cluster as *B. graminis, B. phytofirmans, B. phymatum* and *B. xenovorans,* and is believed that it may be involved in resistance to desiccation and iron stress (Ferreira *et al.*, 2010). Future understanding of the genes involved in the EPS synthesis in these species may contribute to identify the gene targets of BraIR in this biosynthetic pathway.

BraIR regulation of the biofilm formation varies according to the species.

Although EPS production was a common QS regulated trait in the three species, biofilm formation was found to be negatively regulated by *bra*R_{UNA} in *B. unamae*, while only slighty modulated in *B. kururiensis* M130, and no QS regulation of biofilm formation was observed in *B. xenovorans* LB400^T. The reasons for this differential regulation among the three species are currently unknown, but evidence that the EPS and the biofilm formation are not always correlated.

It is worth noting that the two strains in which biofilm was QS regulated are normally associated to plants. Although more strains should be tested, from this result it could be hypothesized that plant-associated bacteria could have included the adherence properties into the QS regulon as a form of niche adaptation, which is not required by soil-free living bacteria as *B. xenovorans* LB400^T.

The BraIR system and the plant colonization

Maize-rhizosphere colonization experiments indicated that BraI/R_{UNA} QS system does not regulate the ability to colonize this host under the conditions used. This also suggests that the rhizospheric colonization of *B. unamae* was not affected by the reduced EPS production, nor by the increased biofilm formation exhibited by the *bra*R_{UNA} deficient mutant. This might imply that these two factors are not important for the rhizospere colonization by this strain.

In contrast, rice endophytic colonization by *B kururiensis* M130 was positively regulated by QS. As noted previously the impaired QS mutants were also able to increase their biofilm formation, while they were deficient in the EPS production. All together this data supports the notion that EPS could have a role in the entrance and spreading of this bacterial species inside the plant, while more compact biofilms would affect the mobility of the endophyte. Similar behaviours have been observed in other plant-

beneficial species as *S. melliloti,* in which impairment in the production of EPS led to reduced nodulation and root enhancement in alfalfa and pea (Glenn *et al.*, 2007; Marketon *et al.*, 2003; Rinaudi & Gonzalez, 2009).

Furthermore, a root enhancement effect was evidenced upon inoculation of rice seeds with *B. kururiensis* M130, which was considerably attenuated when plants were inoculated with QS mutants. Our results suggest that this effect was not associated with increases in the nitrogen fixation, nor in the IAA production, as these two phenotypes were not affected in vitro by mutation in the QS genes; however, it cannot be excluded that these two features might be regulated *in planta.*

In summary, these results suggest that plant-bacteria interaction may be subject to regulation in a cell-density manner in plant associated *Burkholderia.* However, more experiments need to be performed in order to determine if plant colonization by these species may be dependant on the plant genotype, both at endophytic as at rhizospheric level. In fact, *B. phytofirmans* PsJN has been shown to have variable colonization levels in different cultivars in several plant species (Pillay & Nowak, 1997; Trognitz *et al.*, 2009). Exploring QS regulation of colonization in several plant species could contribute to the assessment of *B. kururiensis* and *B. unamae* for their use as bioinoculants.

The BraIR system and the degradation of aromatic compounds

Several species from the new plant-associated *Burkholderia* cluster have extraordinary ability to degrade aromatic compounds, and this potential has been extensively reviewed (Iwaki *et al.*, 2007; O'Sullivan & Mahenthiralingam, 2005; Parnell *et al.*, 2006). Among the strains studied here, *B. unamae* MTI-641^{T} has been shown to be able to degrade phenol and benzene, while *B. kururiensis* M130 may use these two compounds and trichloroethylene or toluene as carbon source (Caballero-Mellado *et al.*, 2007). In contrast, *B. xenovorans* LB400^{T} degrades exclusively biphenyl compounds or its halogenated derivatives (Bopp, 1986).

Here it was demonstrated that the phenol degrading ability is regulated by the BraI/R_{UNA} QS system in *B. unamae* MTI-641^{T}. Although the catabolic pathway involved in the degradation of this substrate has not been elucidated yet, the presence of a phenol monooxygenase has been previously demonstrated in several strains of *B. unamae,* including MTI-641^{T} (Caballero-Mellado *et al.*, 2007). Further studies are required to

determine if this enzyme is the specific target of the QS regulation in the phenol metabolism.

The importance of QS in the phenol degradation was previously reported (Valle *et al.*, 2004). Phenol consumption ability was reported to be associated with AHL production by members of microbial communities in activated sludges, and supplementation with AHLs was able to sustain phenol-degrading activity beyond the point of starvation. It was hypothesized that this effect was associated with surfactant production and composition rearrangement within the community (Valle *et al.*, 2004).

Analysis of the benzene, phenol and toluene degradation by *B. kururiensis* M130 and its corresponding QS mutants suggested that the use of these carbon sources is not subject to quorum regulation. Interestingly, *B. kururiensis* was recently reported to be able to grow in consortia that degrades 2,4,6 trichlorophenol, using phenol as a primary intermediate (Gomez-De Jesus *et al.*, 2009). It cannot be excluded that QS might have a role in the consortial behaviour of *B. kururiensis* M130 *in vivo,* which may lead to increased degradation of aromatic compounds.

As noted previously, *B. xenovorans* LB400^{T} has gained notoriety in the scientific community due to its ability to degrade biphenyl derivatives (Bedard *et al.*, 1986). In this study, experiments in plate containing biphenyl as unique carbon source suggested that the BraIR$_{XEN}$ system has a role in the degradation of biphenyl compounds since the *braI*$_{XEN}$ deficient mutant was unable to degrade this compound. This is the first report that QS is involved in the biphenyl degradation by *B. xenovorans* LB400^{T}. These results may be in agreement with previous studies in which the biphenyl degradation was found to be dependent on the phase of growth, being higher at the late-exponential phase (Denef *et al.*, 2005; Parnell *et al.*, 2009). Further experiments that evaluate the growth kinetics of QS mutants in presence of several biphenyl congeners would contribute to understand the effects of QS in the metabolic pathway.

QS regulates the expression of secreted proteins in *B. xenovorans* LB400^{T}

Secreted proteins profiling for *B. xenovorans* evidenced several bands present in the AHL synthase mutant LB400BRAI profile and absent in wild-type profile. However transcriptional analysis of their promoters indicated that only two of them were transcriptionally regulated by BraI/R$_{XEN}$, and only one of them could be fully complemented by supplying the *braI*$_{XEN}$ gene *in trans*. This latter gene encoded for a

porin belonging to the OmpC family (pFAM00267). A similar protein was identified in *B. cenocepacia* and it was suggested that it might function as a pore for small molecules involved in osmoregulatory control (Baldwin *et al.*, 2004). Several porins have been reported to be regulated by QS in several species including *B. cepacia* (Aguilar *et al.*, 2003b), *Azospirillum lipoferum* (Boyer *et al.*, 2008) and *P. aeruginosa* (Arevalo-Ferro *et al.*, 2003). However the implications of such regulation remains to be explored.

3.2.4 Final Remarks

In this study we describe the identification of several targets for the BraIR systems in *B. xenovorans* LB400^T, *B. unamae* MTI-641^T and *B. kururiensis* M130. EPS production was found to be the only phenotype to be regulated by the BraI/R system in all three strains. A summary of all phenotypes tested and role of BraI/R is depicted in figure 3-18. These results demonstrate that species-specific targets may have also been acquired as response to niche adaptation. It was determined that the biofilm production is regulated by BraIR in *B. unamae*, and in *B. kururiensis,* while no regulation was observed in *B. xenovorans*. Importantly the aromatic compound degradation is QS regulated in *B. unamae* MTI-641^T and *B. xenovorans* LB400^T while no effect was observed in *B. kururiensis* M130.

Importantly QS displayed a pivotal role positively regulating the endophytic colonization of rice plants by *B. kururiensis* M130. In conclusion, this study represents an extensive analysis of AHL QS regulated phenotypes in the *Burkholderia* plant associated cluster, demonstrating commonalities as well as differences probably reflecting environmental adaptations of the various species.

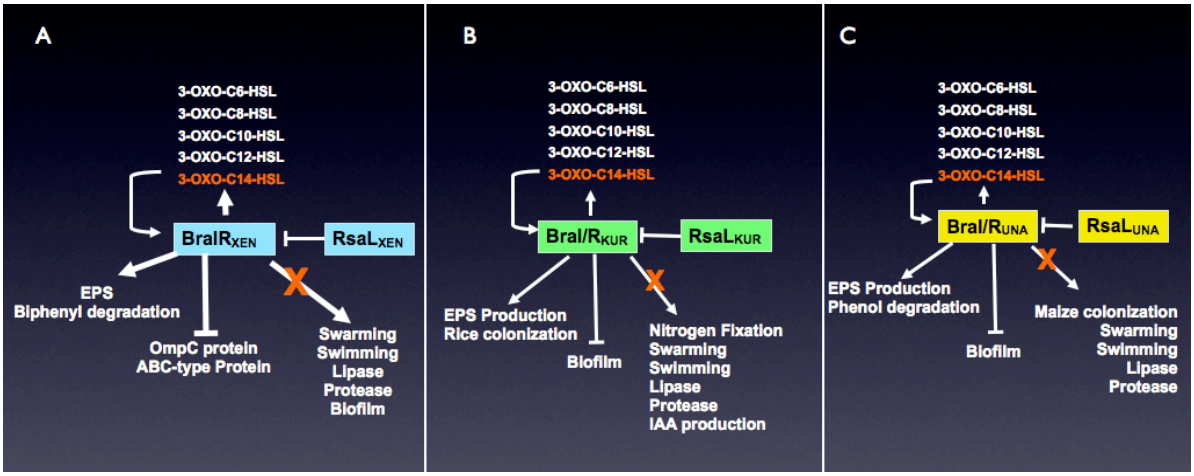


Figure 3-18. Summarizing scheme of the identified targets for the BraIR system in (A) *B. xenovorans,* (B) *B. kururiensis,* and (C) *B. unamae*

3.3 EXISTENCE OF OTHER QS ELEMENTS IN BENEFICIAL PLANT ASSOCIATED *Burkholderia* SPECIES

3.3.1 Introduction and scope

Quorum sensing is important for the fitness and versatility in *Burkholderia* species and AHL QS systems have been identified and characterized in several *Burkholderia* species (Chandler *et al.*, 2009; Eberl, 2006; Lewenza *et al.*, 1999). The BCC complex species share the CepIR system (Gotschlich *et al.*, 2001), while the clade composed by *B. mallei, B. pseudomallei* and *B. thailandiensis* harbours several *luxIR* pairs, two of them orthologs present in all three species (Duerkop *et al.*, 2008; Duerkop *et al.*, 2009; Kiratisin & Sanmee, 2008). On the other hand, species from the beneficial plant-associated *Burkholeria* group share the BraIR system, which is determinant for the regulation of the EPS production, and other essential traits (Suarez-Moreno *et al.*, 2008).

Some bacteria contain multiple *luxIR* circuits and many possess unpaired LuxR-*solo* proteins. These latter elements are LuxR-like proteins, which lack a cognate LuxI-AHL synthase and may bind exogenous AHLs or eukaryotic molecules to expand the existing regulatory network of the bacterium (Subramoni & Venturi, 2009a; Subramoni & Venturi, 2009b). Several LuxR-solo proteins in *Burkholderia* species have been identified, for example CepR2 from *B. cenocepacia,* and BpsR4 and BpsR5 from *B. pseudomallei,* and its orthologs in *B. mallei* and *B. thailandiensis* (Kiratisin & Sanmee, 2008; Malott *et al.*, 2009a).

In addition to the $BraIR_{XEN}$ system present in *B. xenovorans* $LB400^T$, an additional *luxIR* pair is present in the 1.47 Mb megaplasmid (Chain *et al.*, 2006). No genetic studies have thus far been performed to characterize this QS system. This chapter describes the characterization of the second AHL-QS system identified in the genome of *B. xenovorans* $LB400^T$. This system was found to be present in several but not all the species belonging to the cluster of plant-associated *Bukholderia*. Furthermore, we also report the presence of a LuxR *solo* protein in *B. xenovorans* $LB400^T$ as well as in several other species of this cluster. We performed experiments aimed at elucidating if the two complete AHL QS systems in *B. xenovorans* $LB400^T$ are hierarchically organized or whether they act independently. The results presented here evidence that AHL QS contribute to the ecologic versatility of the beneficial plant–associated *Burkholderia* cluster.

3.3.2 Results

3.3.2.1 The XenI2/R2 system of *B. xenovorans* LB400^T

In *silico* analysis of the sequenced genome of *B. xenovorans* LB400^T revealed that this strain possessed two complete *luxIR* systems, the *braI*/R_{XEN} and *xenI2*/*R2* (Chain *et al.*, 2006). The *xenI2*/*R2* system is located in the loci Bxe_C0415 and Bxe_C0416 of the 1.47 Mb megaplasmid (Figure 3-19).

The AHL synthase XenI2 is composed of 249 aa, while the sensor/response regulator XenR2 is 262 aa long. This LuxIR pair displays approximately 70% identity to two uncharacterized putative LuxIR-family members of the species cluster, namely *B. graminis* (locus BgramDRAFT_4129 and Bgram_DRAFT 4128) and *B. phytofirmans* (locus Bphyt_0126 and Bphyt_0127). It was therefore concluded that like *B. xenovorans* LB400^T, also the genomes of these two other species of the cluster possess two AHL QS systems. XenI2 displays 49% identity to BtaI3 of *Burkholderia thailandiensis*, BpsI3 of *Burkholderia pseudomallei* and BmaI3 of *Burkholderia mallei.* These three *Burkholderias* do not belong to this newly described plant-associated species cluster and are more related phylogenetically to the *Burkholderia* cepacia complex (Coenye & Vandamme, 2003). Similarly, XenR2 displays high identity (approximately 42%) to BtaR3 and BpsR3 of *B. thailandiensis* and *B pseudomallei,* respectively. The *xenI2*/*R2* and *braI*R_{XEN} systems of strain LB400^T display a low level of relatedness since they have less than 25% similarity at protein level.

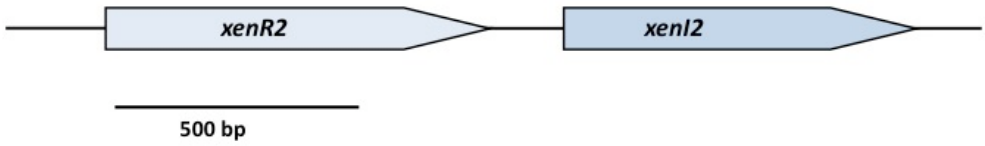


Figure 3-19. Map of the *xenI2/R2* system of *B. xenovorans* LB400^T

Loci Bxe_C0415 and Bxe_C0416 correspond to *xenI2* and *xenR2* respectively and were retrieved from the genome sequence of *B. xenovorans* LB400^T (Chain *et al.*, 2006).

A phylogenetic analysis based on concatenated LuxR-LuxI sequences was obtained in order to estimate the relationships between the XenI2/R2 system and other QS systems present in *Burkholderia* species (Figure 3-20). This approach was used as it has been reported that LuxI and LuxR proteins have coevolved, thus concatening the protein

sequence may provide more evolutionary information (Lerat & Moran, 2004; Malott *et al.*, 2005). From the topology of the tree it can be inferred that the XenI2/R2 system is closely related to the CciIR system and the BpsIR3, BmaIR3 and BpsIR3, which most likely share a common ancestor. Importantly XenI2/R2 is distant from the BraIR-like and the CepIR-like systems.

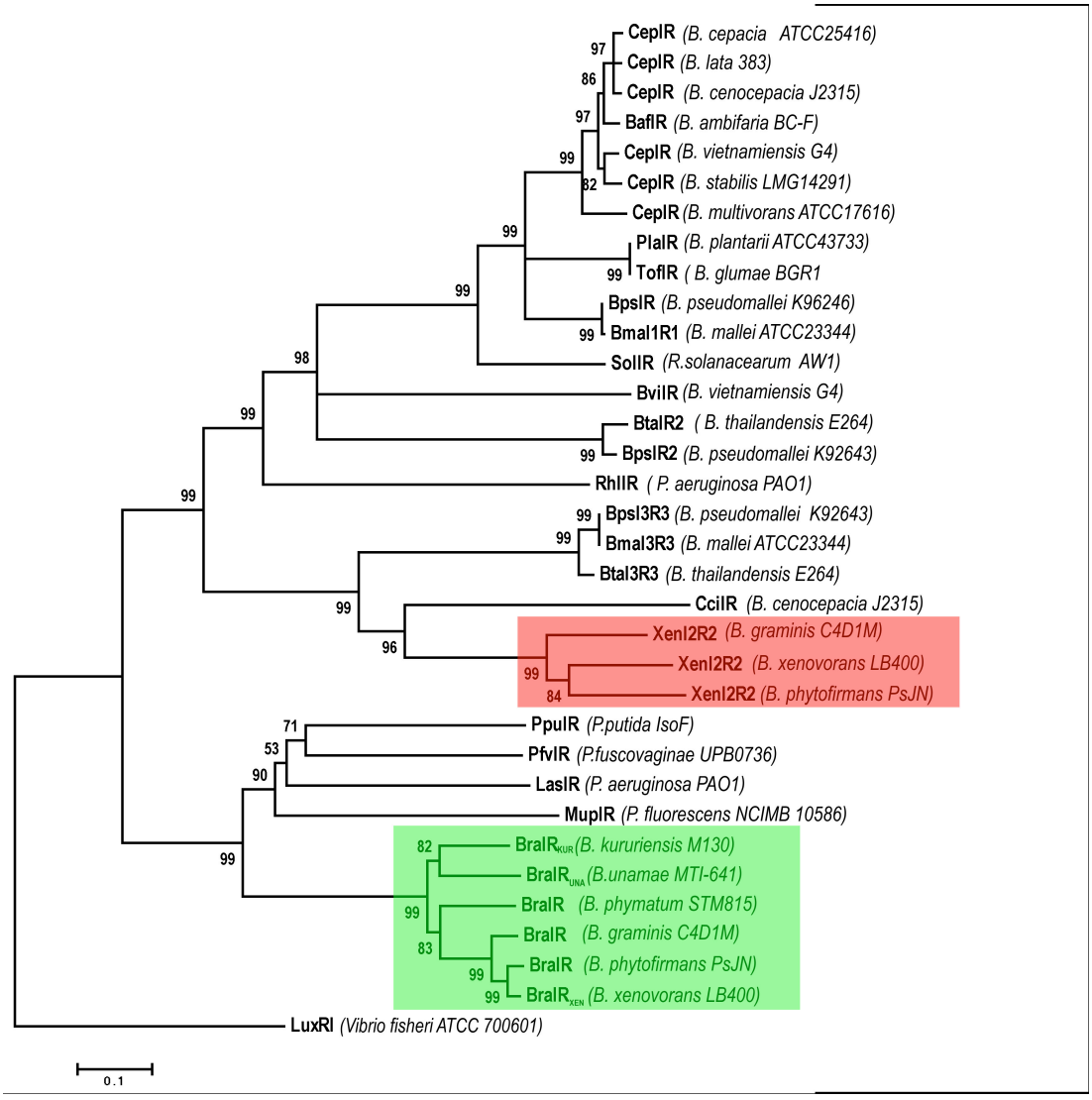


Figure 3-20. Phylogenetic analysis of the LuxIR pairs present in *Burkholderia* species.

34 LuxI and LuxR sequences from different *Burkholderia* and other species were retrieved from the NCBI database, concatenated and aligned with ClustalW (Thompson *et al.*, 1994). RED square shows the XenI2/R2-like systems and GREEN squares highlights the BraIR-like systems presented in chapter 3.1.The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Numbers at branch nodes are bootstrap values. Phylogenetic analyses were conducted in MEGA4. Concatenated LuxIR sequences from *V. fisheri* (LuxIR) were used as outgroup. The acronyms XEN: *B. xenovorans*, UNA: *B. unamae*, KUR, *B. kururiensis.*

3.3.2.2 XenI2/R2 produces and responds to 3-OH-C8-HSL

From TLC analysis performed with *B. xenovorans* LB400^T spent supernatants, it was previously shown that this strain produces several AHLs, and that its BraIR$_{XEN}$ system preferentially responds to 3-oxo-C14-HSL and 3-oxo-C16-HSL (See chapter 3.1). In order to identify the molecules produced by XenI2, single mutants in *xenI2* and *xenR2* loci were generated to yield LB400XENI2 and LB400XENR2, as described in materials and methods.

TLC analysis of the *xenI2/R2* deficient mutants with the biosensors *A. tumefaciens* pNTL4 and *E. coli* pSB1075, revealed that these two mutants most probably produce 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C-10-HSL, 3-oxo-C-12-HSL and 3-oxo-C14-HSL as the wild type, confirming that BraI$_{XEN}$ is responsible for the production of this group of AHLs. The *braI$_{XEN}$* mutant on the other hand evidenced the presence of only one spot with the same retention factor (Rf) of 3-hydroxy-C8-HSL standard, which suggested that this molecule was most likely produced by XenI2 (Figure 3-21A-B).

An experiment was then set up to identify the AHL cognate for XenR2. The *xenR2* gene was cloned in the pQE30 vector generating pQEXENR2, and the protein was expressed in *E. coli* M15 cells in the presence of their cognate *xenI2* promoter transcriptional fusion, pMPX2I. β galactosidase activity was then determined upon the addition of individual AHLs. The results indicated that the activity of *xenI2* promoter increased 100-fold upon the presence of 3-OH-C8-HSL, 50-fold in the presence of 3OH-C6-HSL and 25-fold in the presence of 3-oxo-C8-HSL ($P<0.001$) (Figure 3-22). These results are in accordance with the AHL production profile described before, which indicated that XenI2 most likely produced 3-OH-C8-HSL. The response to 3-oxo-C8-HSL suggested that XenR2 when exposed to high concentrations of AHLs, displayed a relaxed specificity allowing it to respond to structurally related AHLs. From these experiments it was concluded that XenI2/R2 system produces and responds to 3-hydroxy-C8-HSL.

In order to visualize the AHLs produced only by XenI2 without the background from the AHLs produced by BraI$_{XEN}$, AHLs extracts from wildtype and QS mutants were separated by TLC and overlaid with *E.coli* M15 (pQEXENR2)(pMPX2I) as biosensor.

The results obtained suggested that with the exception of *xenI2* deficient mutant, all the QS mutants and the wildtype produced 3-hydroxy-C8-HSL.

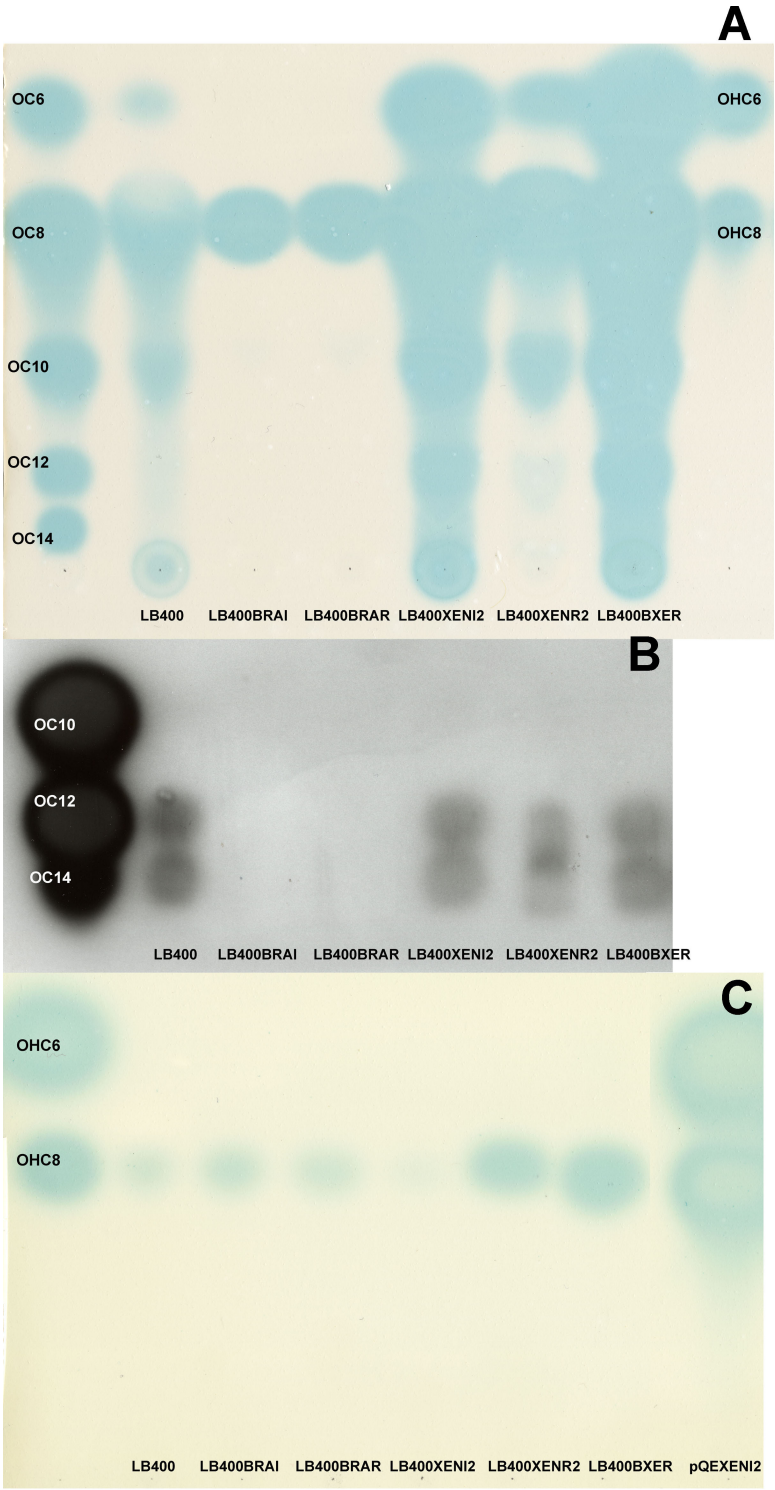


Figure 3-21. TLC Profile for *B. xenovorans* LB400^T QS mutants.

AHLs were extracted from spent supernatants and separated by TLC as described in materials and methods. Overlaid with (A) *A. tumefaciens* pNTL4, (B) *E. coli* pSB1075 (C) *E. coli* (pQEXENR2)(pMPXENI2). For each strain the volume of extract equivalent to 2.5 x 10^{10} cells was loaded, and synthetic AHL compounds were used as reference.

This analysis also revealed that the LB400XENR2 mutant, deficient in *xenR2,* continues to produce 3-hydroxy-C8-HSL, indicating that this gene is not required for the AHL production by XenI2 (Figure 3-21C). Moreover, the ethyl acetate extract from an *E. coli* strain overexpressing XenI2 (pQEXENI2) indicated that this synthase might also produce 3-hydroxy-C6-HSL (Figure 3-21B-C). It is worth noting that the use of *E.coli* M15 (pQEXENR2)(pMPXENI2) as sensor seemed to detect specifically the AHLs produced by XenI2/R2, with no background from the AHL produced by $BraI_{XEN.}$

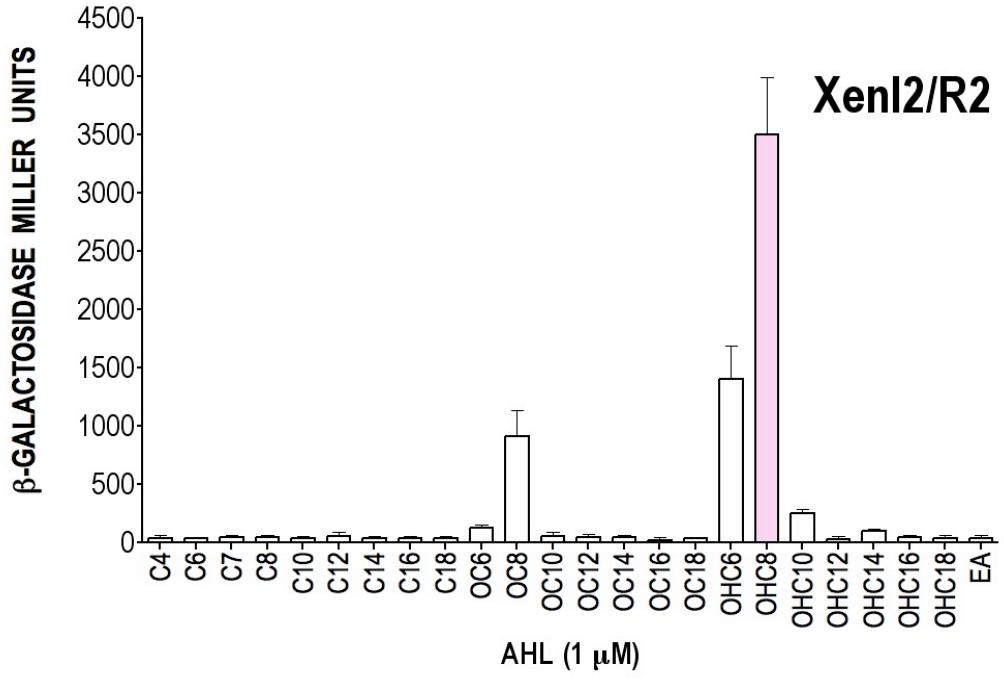


Figure 3-22. Determination of the biologically active AHL for XenR2

Bars correspond to ß-galactosidase activities determined for *E. coli* harbouring each pQEXENR2 and the *xenI2* promoter fused to *lacZ* in pMP220, upon the addition of various exogenous AHLs (1 μM). The pink bar denotes the identified cognate for XenR2. The results are means of three values ±SD of three independent biological replicates. Means were compared with ANOVA analysis in combination with Dunnett post test, means were considered statistically different when $p<0.01$. EA: Ethyl Acetate. Activity values for OC8, and OHC6 and OHC8 were statistically different than the control with EA.

3.3.2.3 *B. xenovorans* LB400^T possesses an unpaired LuxR solo

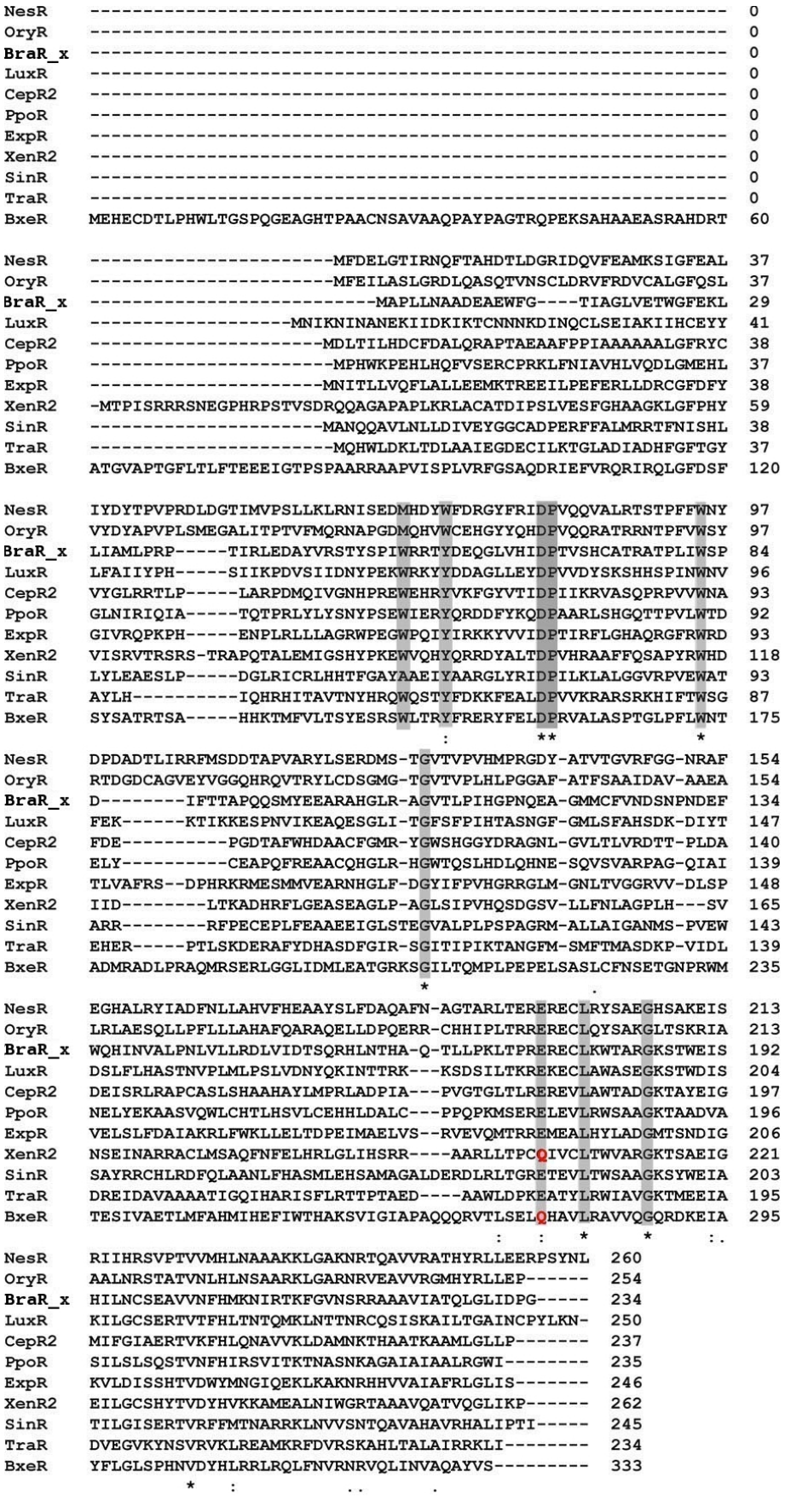
Analysis of the genome sequence of *B. xenovorans* LB400^T revealed the presence of an unpaired LuxR-*solo* protein having the typical modular structure of QS LuxR family proteins (Patankar & Gonzalez, 2009a; Subramoni & Venturi, 2009b). The protein encoded by this ORF, which is designated here as BxeR (Bxe_B2275), encodes for a 333 aa protein having the autoinducer binding domain (Pfam03472) from position 100 to 254, and the HTH domain (Pfam PF00196) from position 271 to 328.

Protein alignment of several LuxR proteins showed that BxeR possesses the six conserved amino acids of the AHL-binding domain. Interestingly, one of the three conserved residues in the HTH motif presents an E178Q substitution, which in fact was also substituted in the DNA binding domain of XenR2 protein (Figure 3-23). BxeR is 95% and 90% similar to uncharacterized orthologs of *B. phytofirmans* (Bphyt_6042) and *B. graminis* (BgramDRAFT_2595).

In order to visualize the possible effect of BxeR in the QS network of *B. xenovorans* LB400^T, a *bxeR* deficient mutant was generated as described in materials and methods, and designated as LB400BXER. TLC analyses of spent supernatants of this mutant did not reveal important changes in the AHL profile observed in comparison to the wild type AHL profile (Figure 3-21).

Figure 3-23. Protein alignment of the LuxR family proteins, including BraI$_{XEN}$, XenR2 and BxeR from *B. xenovorans* LB400^T

The sequences of *Sinorhizobium melliloti* NesR (SMc0432), ExpR(NP_387385), SinR (NP_385944), *Xanthomonas oryzae* pv. *oryzae* OryR(Q5H3E9),*Vibrio fischeri* LuxR (AAA27542), *Burkholderia cenocepacia* CepR2 (BCAM0188), *Pseudomonas putida* WCS358 PpoR (FM992077), *Agrobacterium tumefaciens* TraR (AAA64793). were aligned with those from XenR2, BxeR and BraI$_{XEN}$ from *B. xenovorans* LB400^T. Typical LuxR-family conserved residues are shaded in grey, identical residues are marked with an * . Multiple alignment was performed using ClustalW2 program (Larkin *et al.*, 2007; Thompson *et al.*, 1994).The red Q residues represent the E178Q substitution common between XenR2 and BxeR.


NesR ---------- 0
OryR ---------- 0
BraR_x ---------- 0
LuxR ---------- 0
CepR2 ---------- 0
PpoR ---------- 0
ExpR ---------- 0
XenR2 ---------- 0
SinR ---------- 0
TraR ---------- 0
BxeR MEHECDTLPHWLTGSPQGEAGHTPAACNSAVAAQPAYPAGTRQPEKSAHAAEASRAHDRT 60
NesR ----------MFDELGTIRNQFTAHDTLDGRIDQVFEAMKSIGFEAL 37
OryR ----------MFEILASLGRDLQASQTVNSCLDRVFRDVCALGFQSL 37
BraR_x ----------MAPLLNAADEAEWFG----TIAGLVETWGFEKL 29
LuxR ----------MNIKNINANEKIIDKIKTCNNNKDINQCLSEIAKIIHCEYY 41
CepR2 ----------MDLTILHDCFDALQRAPTAEAAFPPIAAAAAALGFRYC 38
PpoR ----------MPHWKPEHLHQFVSERCPRKLFNIAVHLVQDLGMEHL 37
ExpR ----------MNITLLVQFLALLEEMKTREEILPEFERLLDRCGFDFY 38
XenR2 -MTPISRRRSNEGPHRPSTVSDRQQAGAPAPLKRLACATDIPSLVESFGHAAGKLGFPHY 59
SinR ----------MANQQAVLNLLDIVEYGGCADPERFFALMRRTFNISHL 38
TraR ----------MQHWLDKLTDLAAIEGDECILKTGLADIADHFGFTGY 37
BxeR ATGVAPTGFLTLFTEEEIGTPSPAARRAAPVISPLVRFGSAQDRIEFVRQRIRQLGFDSF 120
NesR IYDYTPVPRDLDGTIMVPSLLKLRNISEDMHDYWFDRGYFRIDPVQQVALRTSTPFFWNY 97
OryR VYDYAPVPLSMEGALITPTVFMQRNAPGDMQHVWCEHGYYQHDPVQQRATRRNTPFVWSY 97
BraR_x LIAMLPRP-----TIRLEDAYVRSTYSPIWRRTYDEQGLVHIDPTVSHCATRATPLIWSP 84
LuxR LFAIIYPH-----SIIKPDVSIIDNYPEKWRKYYDDAGLLEYDPVVDYSKSHHSPINWNV 96
CepR2 VYGLRRTLP-----LARPDMQIVGNHPREWEHRYVKFGYVTIDPIIKRVASQPRPVVWNA 93
PpoR GLNIRIQIA-----TQTPRLYLYSNYPSEWIERYQRDDFYKQDPAARLSHGQTTPVLWTD 92
ExpR GIVRQPKPH-----ENPLRLLLAGRWPEGWPQIYIRKKYVVIDPTIRFLGHAQRGFRWRD 93
XenR2 VISRVTRSRS-TRAPQTALEMIGSHYPKEWVQHYQRRDYALTDPVHRAAFFQSAPYRWHD 118
SinR LYLEAESLP-----DGLRICRLHHTFGAYAAEIYAARGLYRIDPILKLALGGVRPVEWAT 93
TraR AYLH----------IQHRHITAVTNYHRQWQSTYFDKKFEALDPVVKRARSRKHIFTWSG 87
BxeR SYSATRTSA-----HHKTMFVLTSYESRSWLTRYFRERYFELDPRVALASPTGLPFLWNT 175
NesR DPDADTLIRRFMSDDTAPVARYLSERDMS-TGVTVPVHMPRGDY-ATVTGVRFGG-NRAF 154
OryR RTDGDCAGVEYVGGQHRQVTRYLCDSGMG-TGVTVPLHLPGGAF-ATFSAAIDAV-AAEA 154
BraR_x D--------IFTTAPQQSMYEEARAHGLR-AGVTLPIHGPNQEA-GMMCFVNDSNPNDEF 134
LuxR FEK------KTIKKESPNVIKEAQESGLI-TGFSFPIHTASNGF-GMLSFAHSDK-DIYT 147
CepR2 FDE----------PGDTAFWHDAACFGMR-YGWSHGGYDRAGNL-GVLTLVRDTT-PLDA 140
PpoR ELY----------CEAPQFREAACQHGLR-HGWTQSLHDLQHNE-SQVSVARPAG-QIAI 139
ExpR TLVAFRS--DPHRKRMESMMVEARNHGLF-DGYIFPVHGRRGLM-GNLTVGGRVV-DLSP 148
XenR2 IID--------LTKADHRFLGEASEAGLP-AGLSIPVHQSDGSV-LLFNLAGPLH---SV 165
SinR ARR--------RFPECEPLFEAAEEIGLSTEGVALPLPSPAGRM-ALLAIGANMS-PVEW 143
TraR EHER-----PTLSKDERAFYDHASDFGIR-SGITIPIKTANGFM-SMFTMASDKP-VIDL 139
BxeR ADMRADLPRAQMRSERLGGLIDMLEATGRKSGILTQMPLPEPELSASLCFNSETGNPRWM 235
NesR EGHALRYIADFNLLAHVFHEAAYSLFDAQAFN-AGTARLTERERECLRYSAEGHSAKEIS 213
OryR LRLAESQLLPFLLLAHAFQARAQELLDPQERR-CHHIPLTRRERECLQYSAKGLTSKRIA 213
BraR_x WQHINVALPNLVLLRDLVIDTSQRHLNTHA-Q-TLLPKLTPRERECLKWTARGKSTWEIS 192
LuxR DSLFLHASTNVPLMLPSLVDNYQKINTTRK---KSDSILTKREKECLAWASEGKSTWDIS 204
CepR2 DEISRLRAPCASLSHAAHAYLMPRLADPIA---PVGTGLTLREREVLAWTADGKTAYEIG 197
PpoR NELYEKAASVQWLCHTLHSVLCEHHLDALC---PPQPKMSERELEVLRWSAAGKTAADVA 196
ExpR VELSLFDAIAKRLFWKLLELTDPEIMAELVS--RVEVQMTRREMEALHYLADGMTSNDIG 206
XenR2 NSEINARRACLMSAQFNFELHRLGLIHSRR----AARLLTPCQIVCLTWVARGKTSAEIG 221
SinR SAYRRCHLRDFQLAANLFHASMLEHSAMAGALDERDLRLTGRETEVLTWSAAGKSYWEIA 203
TraR DREIDAVAAAATIGQIHARISFLRTTPTAED----AAWLDPKEATYLRWIAVGKTMEEIA 195
BxeR TESIVAETLMFAHMIHEFIWTHAKSVIGIAPAQQQRVTLSELQHAVLRAVVQGQRDKEIA 295
NesR RIIHRSVPTVVMHLNAAAKKLGAKNRTQAVVRATHYRLLEERPSYNL 260
OryR AALNRSTATVNLHLNSAARKLGARNRVEAVVRGMHYRLLEP------ 254
BraR_x HILNCSEAVVNFHMKNIRTKFGVNSRRAAAVIATQLGLIDPG----- 234
LuxR KILGCSERTVTFHLTNTQMKLNTTNRCQSISKAILTGAINCPYLKN- 250
CepR2 MIFGIAERTVKFHLQNAVVKLDAMNKTHAATKAAMLGLLP------- 237
PpoR SILSLSQSTVNFHIRSVITKTNASNKAGAIAIAALRGWI-------- 235
ExpR KVLDISSHTVDWYMNGIQEKLKAKNRHHVVAIAFRLGLIS------- 246
XenR2 EILGCSHYTVDYHVKKAMEALNIWGRTAAAVQATVQGLIKP------ 262
SinR TILGISERTVRFFMTNARRKLNVVSNTQAVAHAVRHALIPTI----- 245
TraR DVEGVKYNSVRVKLREAMKRFDVRSKAHLTALAIRRKLI-------- 234
BxeR YFLGLSPHNVDYHLRRLRQLFNVRNRVQLINVAQAYVS--------- 333

3.3.2.4 Analysis of hierarchies in the QS network of *B. xenovorans* LB400^T

When a bacterium harbours more than one LuxIR system, they could be organized in a hierarchical network. Two types of experiments were designed aimed at establishing whether there is any hierarchical transcriptional organization between the $BraIR_{XEN}$ and XenI2/R2 AHL QS systems of *B. xenovorans* and the LuxR-solo protein. For this purpose the $braI_{XEN}$, *xenI2* and *bxeR* promoters were cloned into the promoterless probe vector pMP220 as described in materials and methods, to generate pMPXENI, pMPX2I and pMPBXER, respectively.

For the first set of experiments, the *bxeR*, $braI_{XEN}$ and *xenI2* promoter transcriptional fusions were mobilized into *B. xenovorans* wild-type and mutants LB400BRAI, LB400XENI2 and LB400BXER, and β-galactosidase was determined in the stationary phase of growth. Results demonstrated that the $braI_{XEN}$ promoter displayed low activity in the wild type as well as in all mutants tested. The *xenI2* promoter also displayed low activities in the $braI_{XEN}$ and *xenI2* genomic knock-out mutants, but resulted in a threefold increase when harboured by the *bxeR* mutant, which suggested that the *xenI2* synthase promoter was negatively regulated by the BxeR solo protein. Although no difference in the AHL production profile for the *bxeR* deficient mutant was observed, *xenI2* promoter activity was reduced when *bxeR* was provided *in trans* (Figure 3-24).

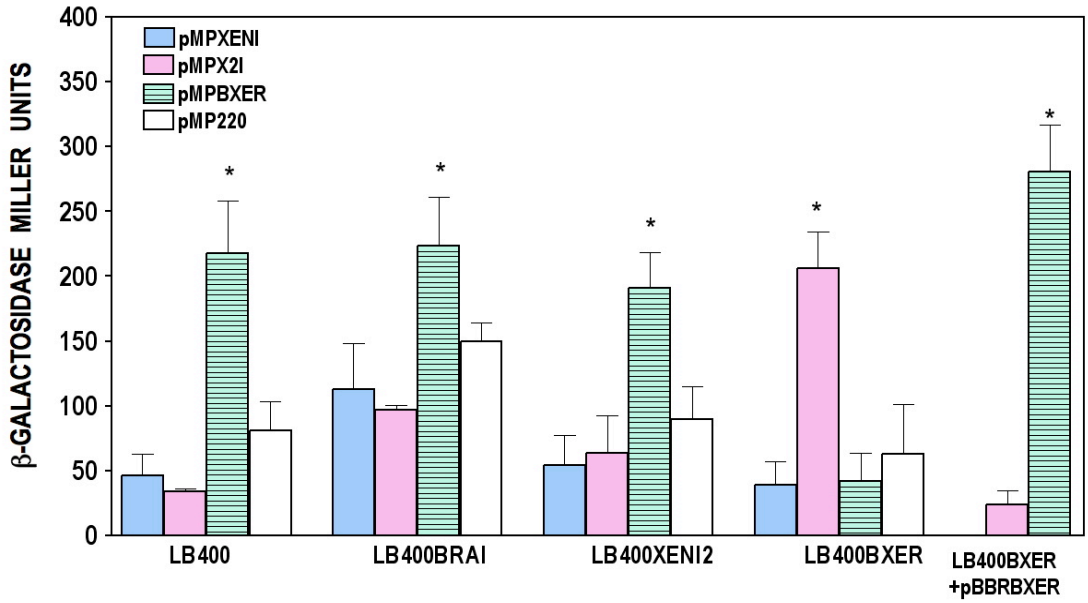


Figure 3-24. *$braI_{XEN}$*, *xenI2* and *bxeR* promoter activities in wild type and QS mutant strains of *Burkholderia xenovorans* LB400 ^{T}

Bacterial cultures were started with an initial inoculum of 5 x10^6 CFU in 20 mL of KB-Tc medium and ß-galactosidase activities were measured after 12 hours of growth. All experiments were performed in triplicate and means values with standard deviation are shown in the graph. ANOVA analyses in combination with Dunnetts post-test were performed with PRISM 4.0 software (GraphPad Software, San Diego California). A P value of < 0.05 was considered significant (*) for each strain in comparison with the values obtained with the pMP220 empty vector.

Analysis of the β-galactosidase activities in the LB400BXER mutant indicated the *bxeR* transcriptional promoter fusion decreased three-fold in activity when harboured in the *bxeR* mutant hence it is autoregulated. This activity was recovered when *bxeR* was provided *in trans* (Figure 3-24).

A second set of experiments was performed in order to determine if the LuxR-type proteins BxeR, XenR2 and $BraR_{XEN}$ were able to activate the $braI_{XEN}$, *xenI2* and *bxeR* promoters, as an evidence of possible cross-talk regulation. For this purpose the *bxeR*, $braI_{XEN}$ and *xenI2* promoter transcriptional fusions were transformed in *E. coli* M15 cells harbouring pQEBXER, pQEXENR2, or pQEXENR1, and β-galactosidase was determined in the presence of the AHL cognates for each LuxR-type protein. To determine if BxeR was able to activate any of these promoters in the presence of AHL, three independent AHL mixtures were used, representing 23 different AHLs (See Figure 3-25).

The experiments suggested that XenR2 protein did not activate the $braI_{XEN}$ promoter in the presence of its cognate, 3-hydroxy–C8-HSL (Figure 3-25A). Similarly, $BraR_{XEN}$ was not able to activate the *xenI2* promoter in the presence of cognate 3-oxo-C14-HSL (Figure 3-25B). These results are in agreement with the AHL production profiles, in which $braI/R_{XEN}$ deficient mutants continue to produce *xenI2/R2* AHLs, and viceversa, suggesting the systems do not regulate each other. From the results obtained, it was concluded that the $BraIR_{XEN}$ and XenI2/R2 systems were not transcriptionaly regulating each other (Figure 3-25A-B).

The role of the BxeR solo in regulating the $braI_{XEN}$ and *xenI2* promoters was also determined. Results indicated that the BxeR solo protein did not drive the transcription of any of AHL synthase promoters of *B. xenovorans* in response to the AHLs tested, as no increase in the promoter activity was observed under any of the conditions tested (Figure 3-25C). Similarly, no activation of the pMPBXER promoter was observed under the presence of the BxeR protein and any of the AHLs mixtures tested. This last result

is contrasting with the behaviour of the same promoter in the *bxeR* deficient mutant, which indicated that this protein regulates its own transcription (Figure 3-24). However, it could suggest that self-regulation of BxeR could be dependent on other molecule(s) or protein produced by *B. xenovorans*.

From studies of the synthase promoters in the LB400BXER mutant it was concluded that BxeR negatively regulates the *xenI2* promoter. In order to determine if this repression was due to competition for the *xenI2* promoter between BxeR and XenR2 protein, an experiment was set-up in *E. coli* to overexpress both BxeR and XenR2 in the presence of its cognate 3-hydroxy-C8-HSL and *xenI2* promoter transcriptional fusion. From the experimental results it was concluded that the repression exerted by BxeR is not direct, as no difference was observed in the promoter activities when BxeR was overexpressed (Figure 3-25D).

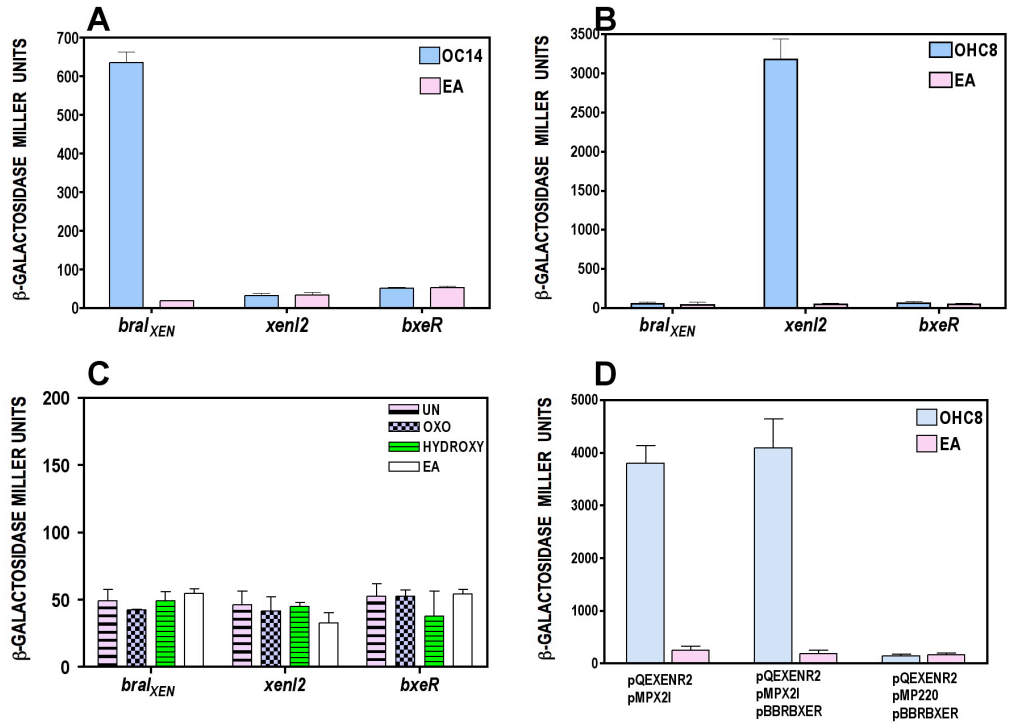


Figure 3-25. Cross-talk experiments between the LuxR type proteins of *B. xenovorans* and the promoters *braI*$_{XEN}$, *xenI2* and *bxeR*.

The bars represent the β-galactosidase activities for each promoter when the LuxR-like proteins were overexpressed **(A)** BraR$_{XEN}$ **(B)**XenR2 **(C)** BxeR. In A and B, 1 μM of the AHL cognate was used. In C, three mixtures were used. UN mix contained 1 μM of each of 9 different unsubstituted AHLs at position 3, OXO mixture contained 1 μM of each of 7 different 3-oxo substituted AHLs and HYDROXY cocktail contained 1 μM of each of 7 different 3-OH substituted AHLs, EA ethyl acetate, used as negative control. **(D)** *xenI2* promoter activity in the presence of XenR2 and BxeR. Activity values were analyzed with ANOVA in combination with Dunnetts post-test using PRISM 4.0 software (GraphPad Software, San Diego California). A P value of < 0.05 was considered significant.

3.3.2.5 Phenotypical analysis of the XenI2/R2 and BxeR deficient mutants

In order to determine the role of the XenI2/R2 and the BxeR systems, several phenotypes were tested in wild-type and deficient mutants LB400XENI2, LB400XENR2 and LB400BXER.

Protease production, swarming, swimming motility, EPS production, biofilm formation and siderophore production were tested. The experiments performed did not evidence any significant difference in the behaviour of the LB400XENI2, LB400XENR2 and LB400BXER in comparison with the wild type in any of the mentioned tests ($P>0.05$). Growth curves were also generated for all strains tested and no differences were observed between mutants and wild-type (Figure 3-26A). Similarly, the use of biphenyl as carbon source was tested in all strains, and no difference was observed between the wild type and *xenI2/R2* mutants suggesting that the degradation of this compound is not regulated by XenI2/R2 nor by BxeR. The fact that XenI2/R2 was not found to regulate any of the phenotypes tested may be an indication that this system is not a global regulatory system in *B. xenovorans* LB400^{T}.

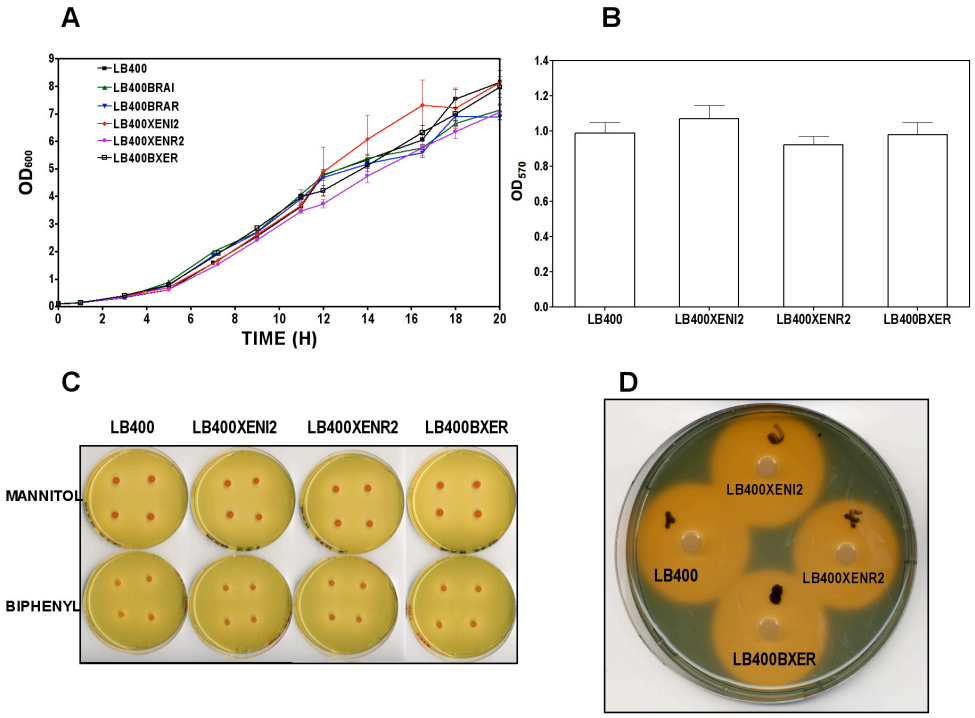


Figure 3-26. Phenotypic analysis of *xenI2*, *xenR2* and *bxeR* defficient mutants

(A) Growth profile of QS deficient mutants in KB media. (B) Biofilm production monitored after 72 hours of incubation (C) Biphenyl degradation (D) Siderophore production in CAS media. Experiments were performed as described in materials and methods.

3.3.2.6 XenI2/R2 and BxeR are not conserved along the members of the beneficial plant associated *Burkholderia* cluster

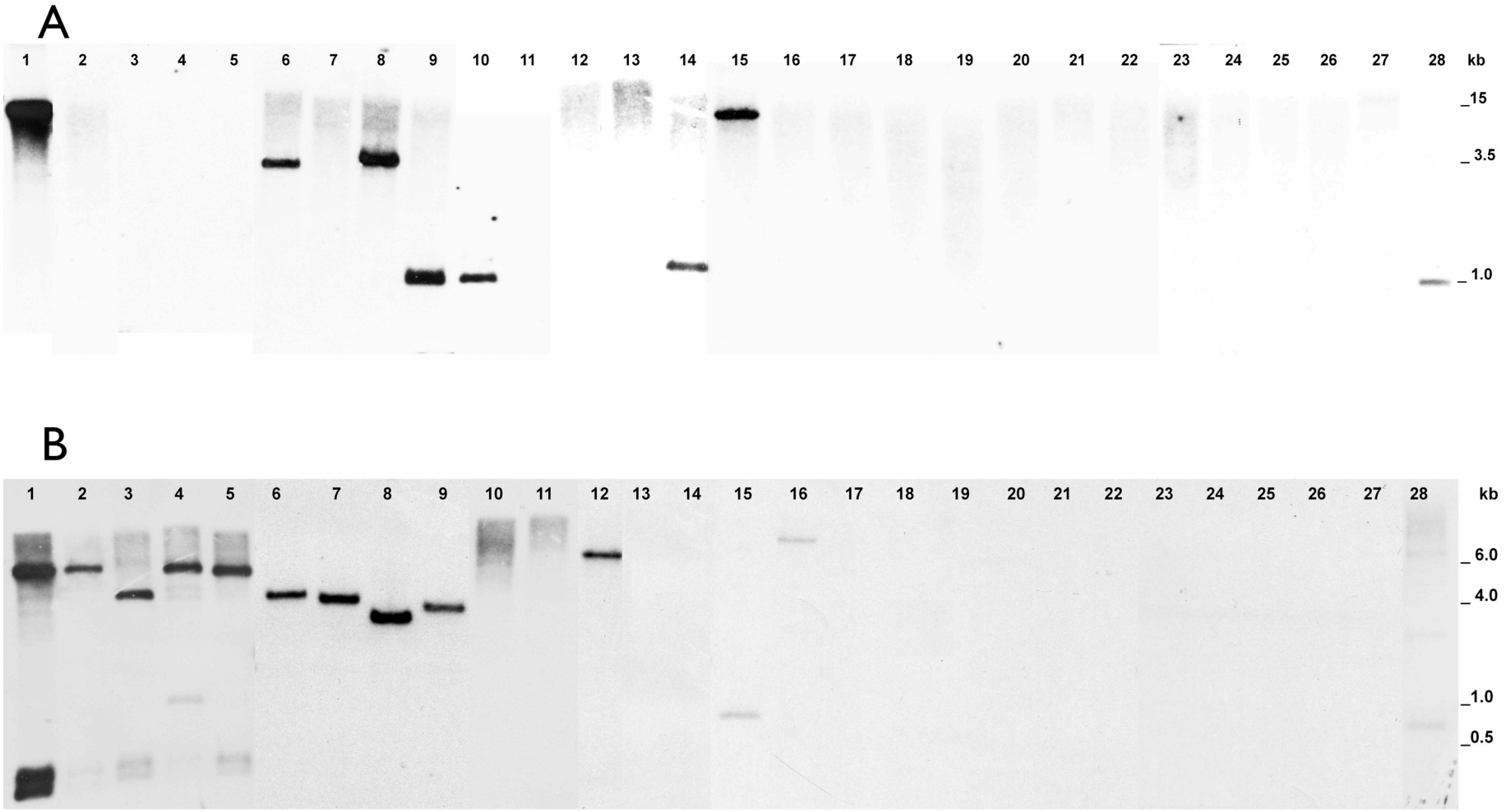
Similarity scores obtained during BLAST analysis of the XenI2/R2 and BxeR proteins evidenced the presence of highly similar proteins in *B. graminis* and *B. phytofirmans*. Thus, it was of interest to determine the possible conservation of these elements in other species from the group, as well as in *B. xenovorans* strains other than LB400^T.

The distribution of the *xenI2/R2* system and of the *bxeR* solo in other *B. xenovorans* strains and in the remaining members of the beneficial *Burkholderia* cluster was determined by Southern hybridization at high stringency conditions with 27 strains representing 22 species using *xenR2* and *bxeR* as probes. The *xenR2* probe hybridized to six different species (*B. graminis, B. phytofirmans, B. fungorum, B. terricola, B. ferrariae,* and *B. silvatlantica*). Interestingly however, no hybridization was observed in any *B. xenovorans* strain other than LB400^T.

In contrast, the *bxeR* solo was found to be present in all *B. xenovorans* strains, as well as in seven members of the cluster, four of which also possessed the *xenI2/R2* system (Figure 3-27). Figure 3-28 summarizes the hybridization results obtained with *xenR2* and *bxeR*, and suggests a possible correlation between both elements, as they are prevalent in members of the *B. graminis* clade. It is worth mentioning that the strain *B. glathei* LMG 14190^T was also tested for the presence of *bxeR* and *xenR2* genes. This species has been beneficially associated to plants but is not included in the beneficial plant-associated group at taxonomical level (Viallard *et al.*, 1998). Interestingly, positive hybridization was obtained with both probes, indicating that those genes are also present in other members of the *Burkholderia* genus. In both experiments negative hybridization was obtained with *E. coli* DH5α genomic DNA, which was used as negative control.

Figure 3-27. Conservation analysis of the *xenR2* and *bxeR* along members from the beneficial plant-associated *Burkholderia* group

Autoradiogram of a Southern Blot analysis of total genomic DNA digested with restriction enzymes *Eco*RI (E),*Pst*I (P),*Sal*I(S) hybridized with the *xenR2* probe **(A)** and the *bxeR* probe **(B)** from B. *xenovorans* LB400. Lanes:1, *B xenovorans* LB400/E*; 2, B. xenovorans* CAC124/E; 3, *B. xenovorans* LMG16224/E; *4, B. xenovorans* TCo382/E*; 5, B. xenovorans TCo26*/E*; 6, B graminis* DSM17151/P; 7, *B. caledonica DSM17062*/P*;8, B phytofirmans* PsJN/P*;9, B. fungorum* DSM17061/P;10, *B.ferrariae* FeGI01^T /P*;11, B. nodosa* BR3437^T */P;*12, *B. phenoliruptrix* DSM17773/E ;13,*B. sacchari* DSM17165/E;14, *B. silvatlantica* SMRrh-20^T/E;15,*B. terricola* DSM17221/E*;*16,*B. tuberum* DSM18489/E;17,*B. caribensis* DSM13236/P;18, *B.mimosarum* PAS44/P;19, *B. phenazinium* DSM10684/P; 20, *B. terrae* DSM17804/P; 21, *B. tropica* DSM15359/P; 22, *B. phymatum* DSM17167/P; 23,*B.unamae* MTI-641^T/S;24,*B.hospita* DSM17164/P;25, *B.kururiensis* M130/P;26, *B.kururiensis* KP23/P;27,*E. coli* DH5/E; 28, *B. glathei* LMG 14190^T/P.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 kb
15
3.5
1.0
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 kb
6.0
4.0
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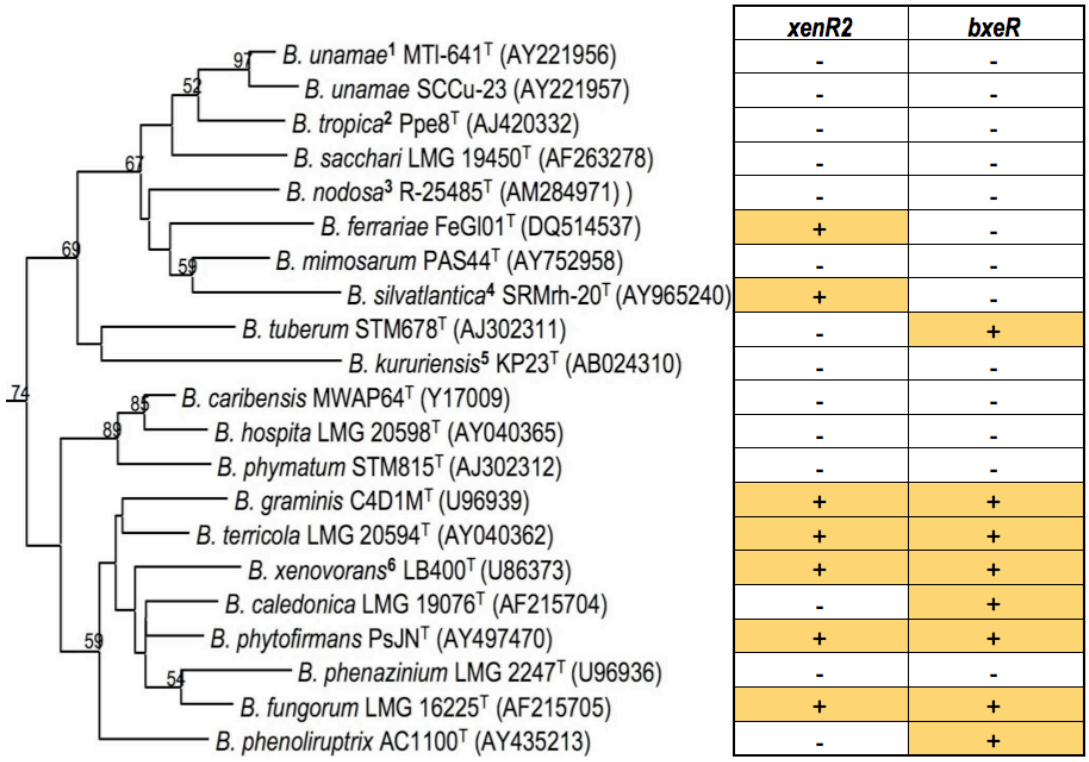

xenR2	bxeR
-	-
-	-
-	-
-	-
-	-
+	-
-	-
+	-
-	+
-	-
-	-
-	-
-	-
+	+
+	+
+	+
-	+
+	+
-	-
+	+
-	+

Figure 3-28. Conservation analysis of *bxeR* and *xenR2* along members of the beneficial plant-associated *Burkholderia* species in relation to their taxonomical distribution

The phylogenetic tree shows the relations between 21 species belonging to the beneficial plant-associated *Burkholderia* species group based on the 16SrRNA sequence analysis inferred using the Neighbor-Joining method. Numbers in brackets correspond to the gene accession number, and nodal numbers correspond to the bootstrap values after 1000 repetitions. The table highlights the positive or negative hybridization with the probes *xenR2* and *bxeR*. An additional reference strain tested was *B. terrae* DSM17804, in such case none of the probes gave positive hybridization.

3.3.3 Concluding discussion

The diversity of the *Burkholderia* genus is exemplified by the high diversity of ecological niches occupied by the different species ranging from soil to aqueous environments (Compant *et al.*, 2008b). *B. xenovorans* strain LB400^T is one of the most important aerobic PCB degraders yet discovered (Bopp, 1986). Mostly research involving this strain was focused in the study of the biphenyl degrading pathways, however the recent genome sequencing has evidenced the existence of many more catabolic pathways (Chain *et al.*, 2006).

Annotation of the *B. xenovorans* LB400^T genome revealed that it harbours two LuxIR pairs. BraIR$_{XEN}$, produces and responds to 3-oxo-C14-HSL and has been shown to regulate the EPS production and other important traits, as described in the previous chapter. This chapter describes the characterization of the second QS system in *B. xenovorans,* which has been named here as XenI2/R2.

XenI2/R2 produces and responds to 3-OH-C8-HSL and is not widely distributed along the beneficial plant-associated *Burkholderia* species group.

XenI2/R2 produces and responds to 3-OH-C8-HSL. Interestingly, also *B. phytofirmans* strain PsJN was reported to produce C8-3OH-HSL; these AHLs are however not produced by strain RG6-12, indicating that *xenI2/R2* may also not be present in all *B. phytofirmans* strains (Trognitz *et al.*, 2009). A similar scenario occurs for the CciIR system, which is found only in some *B. cenocepacia* strains and has been shown to be associated with a pathogenicity island (Malott *et al.*, 2005). Homology and phylogenetic studies demonstrated that XenI2/R2 is closely related to the CciIR system and to the BtaIR3, BmaIR3 and BpsIR3 systems of *B.mallei, B. pseudomallei* and *B. thailandiensis,* respectively (Case *et al.*, 2008; Malott *et al.*, 2005). In fact these systems produce and respond to the same AHL supporting the hypothesis of a probable common ancestor for these systems (Chandler *et al.*, 2009; Duerkop *et al.*, 2009).

The *cciIR* system has recently been characterized as a global regulator exerting an important regulatory control on *cepI/R* (O'Grady *et al.*, 2009). This system is located in a pathogenicity island (Baldwin *et al.*, 2004), which is not present in *B. xenovorans* LB400. However, it is worth noting that components of the aminoacid metabolism gene cluster within the *B. cenocepacia* pathogenicity island show remarkable syntheny with segments in the megaplasmid of strain LB400^T. In fact, six of the eight branched chain aminoacid metabolism genes are highly conserved between both species. These regions in the LB400 megaplasmid do not possess the hallmarks of genomic islands, suggesting that the megaplasmid may represent an ancestral replicon from which species as *B. cenocepacia* and *B. thailandiensis* have laterally acquired the pathogenicity islands. This would also explain the conservation observed of XenI2/R2 with the CciI/R system(Chain *et al.*, 2006).

B. xenovorans xenI2/R2 is found in only one third of the members of the *Burkholderia* plant associated cluster. Surprisingly, *xenI2/R2* was not present in four other *B.*

xenovorans strains, which implies that the occurrence of XenI2/R2 is strain-dependant rather than species-dependant. In fact, in strain LB400^T *xenI2/R2* is located in a megaplasmid indicating that it is not part of the core chromosome of *B. xenovorans*.

Phenotypical analysis of single mutants in either *xenI2* or *xenR2*, did not evidence significant differences in comparison to the wild-type strains. The fact that XenI2/R2 was not found to regulate any of the phenotypes tested may be an indication that this system is not a global regulatory system in *B. xenovorans* LB400^T. A similar scenario has been found in the BtaIR3 from *B. thailandiensis*, in which deletion in the *btaI3* gene did not cause significant differences in the carbon source metabolism, nor in the growth rate (Chandler *et al.*, 2009). In contrast, a role as global regulatory system has been recently proposed for *cciI*R as deduced from transcriptomic studies (O'Grady *et al.*, 2009). Global transcriptome and/or proteome analyses with *B. xenovorans xenI2/R2* deficient mutants could contribute to determine the regulon of this system.

BraI/R$_{XEN}$ and XenI2/R2 are not transcriptionaly regulating each other

Our studies showed that under the conditions we tested, no transcriptional regulatory hierarchies are present between BraI/R$_{XEN}$ and XenI2/R2. In addition, transcriptional studies of *braI$_{XEN}$* and *xenI2* gene promoters in *B. xenovorans* showed that both genes are expressed at very low levels indicating that they are most likely under transcriptional regulation. The *braI/R$_{XEN}$* system is most probably regulated by the intergenically located *rsaL* repressor, as previously described for the *braI/R$_{KUR}$* system (Suarez-Moreno *et al.*, 2008), and it is expected that the *xenI2/R2* system may also be coupled to other regulatory mechanisms.

The lack of interaction between QS systems is peculiar in *B. xenovorans* LB400^T, when compared to other *Burkholderia* species harbouring several LuxIR pairs. For instance, in *B. cenocepacia*, the CepR positively regulates the expression of the *cciI* and *cci*R genes, which are co-transcribed, and CciR is also involved in the negative regulation of *cepI* (Malott *et al.*, 2005). In *B. pseudomallei* a complex hierarchical organization has been proposed as consequence of the presence of five LuxR-like proteins (Kiratisin & Sanmee, 2008). However, the absence of cross-talk between systems has also been reported recently in the environmental strain *P. aeruginosa* PuPA3 isolated from the rhizosphere (Steindler *et al.*, 2009).

B. xenovorans LB400 harbours a LuxR-solo protein

The genome of *B. xenovorans* contains an unpaired LuxR family solo, designated BxeR, having the typical *N*-terminal AHL-binding domain and the *C*-terminus helix-turn-helix motif. Protein alignment showed that BxeR possesses the six conserved amino acids of the AHL-binding domain, while one of the three conserved residues in the HTH motif presents an E178Q substitution. Importantly such variation is also present in the DNA binding domain of XenR2 protein, meaning that probably both proteins may recognize similar features when binding DNA.

Promoter analysis suggested that BxeR positively autoregulated its own transcription and negatively regulated the expression of *xenI2*. BxeR was found to be present in all *B. xenovorans* strains, as well as in most of the type strains of the *B. graminis* clade, and in the species *B. tuberum*.

The role of LuxR-type solos in AHL producers has been reviewed recently (Patankar & Gonzalez, 2009a; Subramoni & Venturi, 2009b); several examples demonstrate that they are most often integrated with the resident AHL QS regulatory networks, as for example are ExpR and NesR from *S. melliloti* (Hoang *et al.*, 2008; Patankar & Gonzalez, 2009b), PpoR from *P. putida* (Subramoni & Venturi, 2009a), and QscR from *P. aeruginosa* (Lequette *et al.*, 2006). Our results suggest that such integration might exist for BxeR since the negative regulation of *xenI2* promoter was observed in the *bxeR* deficient background.

Although the experiments performed did not evidence binding to AHLs, the conservation of the residues in the AHL binding domain suggests that BxeR might bind AHL molecules. Generation of double and triple mutants in the LuxR-type proteins biochemical studies could contribute to determine the possible cognate AHL of this protein, as well as the role of this QS element in the regulatory network.

3.3.4 Final Remarks

In this study we describe the characterization of the XenI2/R2 system and BxeR solo in *B. xenovorans* LB400^{T}. The XenI2/R2 QS system and a LuxR solo were found to be present in few members of the cluster being independent of the *braIR*-like system. Importantly, the BxeR solo was found to negatively regulate the expression of the XenI2 synthase. The fact that no phenotype was found to be regulated, by neither BxeR

nor XenI2/R2 so far, suggests that these two elements are not global regulators, and that they could respond to a specific environmental condition. Future work will focus on determining the complete regulons of the two systems and of the LuxR-type *solo* BxeR in this important recently described cluster of *Burkholderia* species. The information presented in this chapter contributes to begin to assemble the picture on AHL-QS regulation in the environmental strain LB400^T as summarized in Figure 3-29.

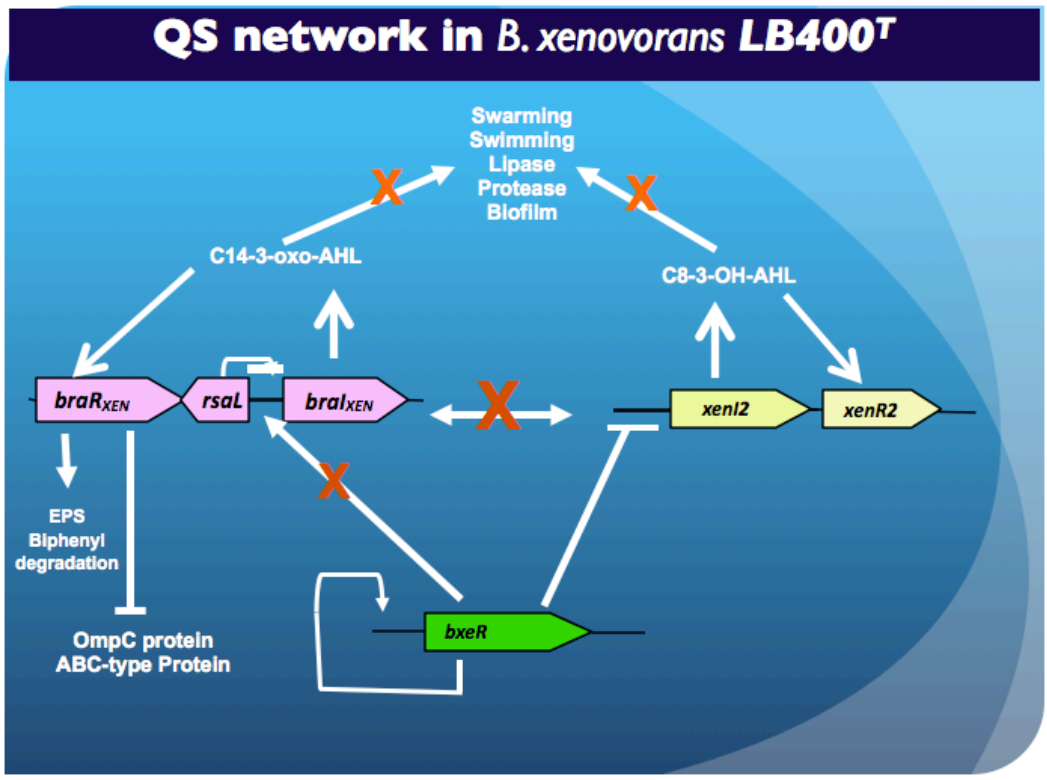

Figure 3-29. Quorum sensing network in *B. xenovorans* LB400^T elucidated in this work

4 SUMMARIZING DISCUSSION

4.1 MAIN SCOPE OF THIS WORK

The ubiquity of *Burkholderia* species, suggest that they possess versatile metabolic pathways based on complex regulatory mechanisms, which support their fitness in challenging environments. Although most studies thus far have involved the plant and human pathogenic *Burkholderia* species, in the last 15 years we witnessed the emergence of a new group of beneficial plant-associated *Burkholderia* species.

The main scope of this work was to identify and characterize the AHL-quorum sensing system(s) in the beneficial plant-associated *Burkholderia* species. Three representative species were chosen to perform these studies: *B. kururiensis* , *B. xenovorans* and *B. unamae*. This discussion aims to summarize the main insights of this work.

4.2 AHL-QS IS PRESENT IN THE BENEFICIAL PLANT ASSOCIATED *BURKHOLDERIA* SPECIES GROUP

At the beginning of this study, the production of AHL molecules had only been reported in *B. phytofirmans* PsJN (Sessitsch *et al.*, 2005). From that finding, we decided to analyze a type-strain collection from the beneficial *Burkholderia* clade. Our results not only revealed AHL production in all species, but also demonstrated overall similarity in their AHL profiles. Such AHLs profiles are different from those observed in BCC and in other *Burkholderia* species. This was the first report that AHL production was conserved in the novel group of *Burkholderia*.

4.2.1 BraIR as a common system in the beneficial *Burkholderia* species-group: ecological implications

All species were shown to possess a conserved AHL QS system, which we designated as BraI/R. Several ecological and phylogenetic implications arise from this finding:

1. **A common ancestor**: the conservation of the BraIR system along the beneficial plant associated *Burkholderia* clade suggests that this system belongs to their core genome and that most likely it was present in the common ancestor of these species. A common ancestor could have acquired *braI/R* in early events during evolution, in a point where the pathogenic *Burkholderia* group might have diverged from the plant beneficial clade (Case *et al.*, 2008; Lerat & Moran, 2004). This hypothesis is also

supported by the syntheny in the neighborhood of the BraIR genes, comprising for instance gluconolactonase and ABC-transporter genes in the four species sequenced. Further analysis based on genomic comparison from more species will contribute to understand if BraIR belongs to a genomic island or if it is ancestral in *Burkholderia* species (Chain *et al.*, 2006).

2. **Ecological Implications**: Conservation of the BraIR system may be associated to the common niches among beneficial plant-associated *Burkholderia* species, such as the plant rhizosphere or soil. In the rhizosphere, behaviours that are influenced by QS usually facilitate nutrient acquisition, modulate collective defense against competitors and permit community escape in the face of population destruction (Badri *et al.*, 2009). The BraIR-like systems could regulate such traits, and thus maintaining BraIR could constitute an advantage at niche level. BraIR could also allow sensing or interacting with other species from the same niche responding to similar signals (i.e. *Pseudomonas, Rhizobium* and *Sinorhizobium*)(Case *et al.*, 2008).

Selection pressure exerted by the environment could have forced this *Burkholderia* species-group to maintain the BraIR-like system. Our results suggested that BraIR-like systems regulate important traits, which confer to cells advantages for living in the soil or in association to plants. Examples are EPS production and aromatic compound degradation. EPS has been shown to be important for plant colonization and the degradation of aromatic compounds may be determinant to metabolize root exudates (Chain *et al.*, 2006; Marketon *et al.*, 2003). Such processes require important investments of energy, which most probably are worthwhile at high population densities. In addition, endophytes like *B. kururiensis,* and legume-symbionts, such as *B. phymatum,* could control the expression of genes at certain time-frames in order to avoid triggering early response reactions from the plant that could affect their survival during colonization or nodulation (Hartmann *et al.*, 2009). Soil free living *Burkholderia,* such as *B. xenovorans* or *B. caribensis* could have kept a BraIR-like system to control targets like the EPS production, which represents an advantage in agglutination and proliferation in soils (Vanhaverbeke *et al.*, 2003).

3. **RsaL as a major regulator of the BraIR-like systems**: Surprisingly BraIR systems are more closely related to the LasIR and PpuIR system from the *Pseudomonas* genus, than to the CepIR system from the BCC. The *braI/R* systems, just like LasI/R-like-systems, also harbour the *rsaL* regulator gene.

RsaL has been shown to be a major direct negative regulator of the *lasIR* system and is hypothesized that it contributes to AHL homeostasis in *P. aeruginosa* (Rampioni *et al.*, 2009). RsaL was shown here to be a stringent negative regulator of the $braIR_{KUR}$ system. In fact, *rsaL* mutants result in significant overexpression of the $braI_{KUR}$ synthase, which then results in the production of very large amounts of AHLs. It is therefore possible that RsaL allows response to a sudden environmental or physiological signal that could activate the system. In addition, as *rsaL* mutants produce > 45 μM of AHLs, this could indicate an additional role of AHLs in this group, such as iron chelation or anti-bacterial activity, as recently proposed (Kaufmann *et al.*, 2005; Schertzer *et al.*, 2009).

Transcriptomic analyses have suggested that RsaL is a global regulator in *P. aeruginosa* (Rampioni *et al.*, 2007c). A similar approach could be followed here to determine a probable large regulon for RsaL in *Burkholderia* species.

4.3 EXPLORING THE BraI/R REGULON: COMMON AND SPECIES- SPECIFIC TARGETS

Conservation along the beneficial *Burkholderia* group and high identity among BraIR systems led us to hypothesize about a common function for the BraIR system among the species. In fact, sequence analysis of the promoter regions from several *braI* genes highlighted the presence of a conserved *lux*-box in six BraIR systems. This was an indication of the possible existence of common DNA targets for the BraIR system in the different species.

Many phenotypes were tested for BraI/R control, but very few were found to be regulated, which could suggest that unlike the LasIR of *Pseudomonas* spp., BraIR is not a global regulatory system. However more work needs to be performed in order to reach this conclusion. Some alternatives could be the performance of transcriptomic, proteomic and promoter trapping/search analyses, which would decipher the BraI/R regulon.

BraIR was found to negatively regulate exopolysaccharide production in all three species studied. This confirmed that there are common targets for BraIR-like systems, and suggested that most likely other genes could be commonly regulated by BraIR. However, phenotypes like the degradation of aromatic compounds or the biofilm formation were regulated by BraI/R in a species-specific manner.

Unlike EPS production, BraI/R negatively regulates biofilm formation in plant associated *B. unamae* and *B. kururiensis*. These two traits are often linked in plant colonizing bacteria, in which EPS decrease could be accompanied by decrease in biofilm formation (Russo *et al.*, 2006). EPS also has a role in the protection against desiccation and iron stress in other *Burkholderia* species (Ferreira *et al.*, 2010). It could also contribute to maintain a suitable water reservoir that allows cell proliferation. EPS deficient cells could therefore aggregate and form more compact biofilms as suggested recently in *Sinorhizobium* (Rinaudi *et al.*, 2010). Experiments aimed to analyze the architecture of biofilms, as well as the structural analysis of EPS and its biosynthesis pathways will probably clarify how these two features are correlated.

One of the main questions addressed in this work was the possible role of QS in plant colonization by *Burkholderia*. Our results suggested that rice colonization was regulated by quorum in the case of *B. kururiensis* M130, whereas maize colonization by *B. unamae* was not. This result could indicate that the factors involved in the competence for colonization used by both species are unrelated and therefore the relevance of QS in each case may be different. Also it should be considered that the effects of colonization might also depend on the plant species.

These results gain a particular significance considering the biotechnological potential of *B. kururiensis* M130 as a biofertilizer. There is evidence about the benefits from rice inoculation with *B. kururiensis* M130 obtained under laboratory and field conditions (Guimarães *et al.*, 2002; Mattos *et al.*, 2008)(Guimarães *et al.*, 2002; Mattos *et al.*, 2008). Reducing the use of fertilizers, particularly nitrogen, by inoculation with this strain could provide economic benefits to the farmers. Our results indicated that QS is determinant for successful rice colonization and further plant-growth promotion. Thus, designing bioinoculants based on this strain should guarantee that the bacterial cell communication is functional to obtain the expected plant enhancement effect.

4.4 BraI/R REGULATES AROMATIC COMPOUND DEGRADATION IN *B. unamae* AND *B. xenovorans*

A commonality between the three *Burkholderia* strains studied was their ability to use recalcitrant compounds as carbon sources. BraIR system positively regulates phenol degradation in *B. unamae* and byphenyl degradation in *B. xenovorans*. Although the role of AHL-QS had already been hypothesized in the degradation of organic compounds in

bioreactors containing microbial communities (Valle *et al.*, 2004)(Valle *et al.*, 2004), the evidence presented here constitutes the first direct evidence of regulation of aromatic compound degradation in a cell density manner in *Burkholderia*. Future work needs to determine the precise targets of BraI/R, which are involved in biodegradation of these compounds.

B. xenovorans LB400^{T} has become a model for the study of the catabolism of biphenyl congeners and the role of quorum regulation presented here explains previous results, in which higher rates of biphenyl degradation were obtained during stationary phase of growth (Parnell *et al.*, 2009).

In both *B. unamae* and *B. xenovorans* manipulation of the BraIR system could provide a way to increase the rates of aromatic compound degradation; for example, an AHL-overproducing mutant could provide higher rates of compound turnover.

4.5 XenI2/R2 AND BxeR: TWO ADDITIONAL FACTORS IN THE *Burkholderia* QS EQUATION

Some members of this *Burkholderia* group contain a second AHL QS system designated XenI2/R2, as presented in chapter 3.3.

In contrast to the BraIR system, XenI2/R2 is more similar to LuxIR pairs present in other *Burkholderia* species (i.e. CciI/R, BpsI3/R3 among others) and is present in only 6 species of the cluster, but not among all *B. xenovorans* strains. These facts indicate that XenI2/R2 could have been acquired by lateral gene transfer events at strain level, although the reasons that could have motivated this event remain to be studied.

XenI2/R2 and BraI/R are not hierarchically organized and no phenotypes regulated by XenI2/R2 were identified. It is currently unknown what is the advantage of harboring a second system, and if the XenI2/R2 regulon overlaps with the BraIR$_{XEN}$ regulon. Generation of double mutants and global transcriptome analysis could contribute to answer these questions.

An unpaired LuxR solo designated BxeR, was identified in the sequenced genomes of *B. xenovorans* LB400^{T}, *B. graminis* and *B. phytofirmans* PsJN^{T}. No AHL cognate was identified for BxeR and it is not known if it could respond to any environmental signal. This protein positively regulates its own transcription and represses the *xenI2* promoter in *B.*

xenovorans. Importantly, BxeR is conserved among all *B. xenovorans* strains tested, which is an indication that belongs to the core genome of this species. BxeR, just like XenI2/R2, is prevalent among members of the *graminis-xenovorans* subclade.

4.6 FORECOMING WORK-FUTURE DIRECTIONS:

All these results are important since our previous knowledge of QS in the *Burkholderia* genus was mostly dominated by studies on the CepIR system and the complex networks from the *mallei-pseudomallei* group. The identification and characterization of BraIR-like systems, XenI2/R2 and BxeR presented here highlights the importance of QS in the beneficial plant associated *Burkholderia* group.

Future directions of QS research in beneficial plant-associated *Burholderia* could include: (i) global transcriptomic assays to determine the regulon of BraIR, XenI2/R2 and BxeR in *B. xenovorans* LB400^{T}, (ii) unequivocal determination of the structure of all AHLs via reverse HPLC and mass spectroscopy, (iii) the role of RsaL in AHL QS regulation via possible perception of environmental signals (iv) the possible presence of other signal molecules and (v) the analysis of the QS-regulated plant-growth promotion mechanisms in *B. kururiensis* M130.

The diversity and versatility of this group of species is largely unexplored; understanding the regulatory mechanisms underlying the striking features of this group of species will contribute to develop their biotechnological applications.

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6 APPENDIX

6.1 CULTURE MEDIA, SUPPLEMENTS AND ANTIBIOTICS

All media were prepared in distilled water, and pH was adjusted. Media were sterilized at 121°C for 15 minutes.

1. LB-LURIA BROTH (Sambrook, 1989)

	g/L
Tryptone	10
Yeast Extract	5
NaCl	10
Agar	15

pH 6.7 ± 0.1.

2. JMV (Reis *et al.*, 2004)

	g/L
Mannitol	5
$KH_2 PO_4$	1.8
$K_2 HPO_4$	0.6
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCl_2 .2H_2O$	0.02
Yeast Extract	0.05
Agar	13

Completed with distilled water. pH 5.7 Adjusted with KOH . When required, glutamate, was added to 1 g/L.

3. KING's B (King *et al.*, 1954)

	g/L
Proteose Peptone No. 3	20
$MgSO_4$	1.5
$KH_2 PO_4$	1.2
Glycerol	10
Agar	13

Completed with distilled water. pH 7 ±0.1.

4. M9C GLUCOSE (Sambrook, 1989)

	g/L
Na_2HPO_4	6
KH_2PO_4	3
NaCl	0.5
NH_4Cl	1
Casaminoacids	2

Completed with distilled water, and adjusted to pH 7.4. Autoclaved and added:

$MgSO_4$ 1 M	2 mL
Glucose 20%	10 mL
$CaCl_2$ 1M	0.1mL

When required glucose was replaced by 2 mL glycerol.

5. BSE (Estrada-de Los Santos *et al.*, 2001)

	g/L
Succinate	5
KH_2PO_4	0.4
K_2HPO_4	0.4
$MgSO_4.7H_2O$	0.2
Yeast extract	0.5
Agar	13

Completed with distilled water. pH 6.5

6. NUTRIENT AGAR

	g/L
Peptone	5
Meat Extract	3
Agar	15g

7. BAz (Caballero-Mellado *et al.*, 2007)

	g/L
Azelaic acid	2
KH_2PO_4	0.4
K_2HPO_4	0.4
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$Na_2MoO_4.H_2O$	0.002
$FeCl_3$	0.01
Bromothymol blue	0.075
Agar	18

pH 5.7 Adjusted with KOH

8. YEM (Yeast Extract Mannitol) (Zlosnik *et al.*, 2008)

	g/L
Yeast Extract	0.5
Mannitol	4
Agar	15

9. SAAC (Caballero-Mellado *et al.*, 2007)

	g/L
KH_2PO_4	0.4
K_2HPO_4	0.4
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$Na_2MoO_4.H_2O$	0.002
$(NH_4)_2SO_4$	0.5
$FeCl_3$	0.01
Bromothymol blue	0.075
Agar	18

10. AB MINIMAL MEDIUM FOR *A. tumefaciens* (Chilton *et al.*, 1974)

	g/L
K_2HPO_4	3
NaH_2PO_4	1
NH_4Cl	1
$MgSO_4.7H_2O$	0.3
KCl	0.15
$CaCl_2.2H_2O$	0.01
$FeSO_4.7H_2O$	0.0025
Glucose	5

When required top agar, 7.5 g/L agar were added prior autoclaving.

11. AB BIOFILM MEDIA Modified from (Huber *et al.*, 2001). For preparing this media 100 mL of component A were added to 900 mL of component B.This mix was supplemented with 10 mL of 1 M glucose as carbon source.

Component A

	g/L
$(NH_4)_2SO_4$	20
Na_2HPO_4	60
KH_2PO_4	30
NaCl	30

Component B

	0.9 L
$MgCl_2.6H_2O$ 1M	2 mL
$CaCl_2.2H_2O$	0.2 mL
$FeCl_3$ 0.01 M	0.17 mL

12. TRIBUTYRIN AGAR

	g/L
Peptone	5
Yeast Extract	3
Tributyrin	10
Agar	12

13. M8 SWARMING MEDIA

	g/L
Na_2HPO_4	6
KH_2PO_4	3
NaCl	0.5
Casaminoacids	2
Glucose	2
Glutamate	0.5
Agar	5

14. CAS MEDIA FOR SIDEROPHORE TESTING

To prepare the CAS media, 100 mL of autoclaved CAS-iron solution were mixed with 850 mL of MM9-Pipes Solution. The mix was complemented with 30 mL of 10 % casaminoacids solution , 2 mL 1 M $MgSO_4$ and 1 mL of 0.1 M $CaCl_2$.

CAS-Iron Solution

	100 mL
Chrome azurol Blue S (CAS)	60.5
1mM FeCl3 in 10 mM HCl	10 mL
Hexadecyltrimethylammonium bromide (HDTMA)	72.9 g

MM9-PIPES

	900 mL
Glycerol	2 mL
NaOH 50 % w/v	12 g
PIPES	30.24 g
Agar	15 g
MM9 Salts 10x	100 mL
Water	750 mL

MM9 SALTS 10X

	g/L
Na_2HPO_4	60
KH_2PO_4	30
NaCl	5
NH_4Cl	10
pH 7.4	

15. N-FREE HOAGLAND MEDIA

	L
KH_2PO_4 1M	1 mL
K_2HPO_4 1 M	1mL
$MgSO_4$. $7H_2O$ 1M	2 mL
$CaSO_4$. $2H_2O$	0.172 g
Mineral Stock Solution	1 mL
Iron Solution	1 mL

pH 6,5 – 7,0

Iron Solution

	100 mL
Na_2H_2EDTA	1,21 g
$FeCl_3.6H_2O$	0.6 g

Mineral stock solution

	g/L
H_3BO_3	2.86
$MnCl_2.2H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$Na_2MoO_4.2H_2O$	0.02

16. ANTIBIOTIC STOCKS

Antibiotic	Stock (mg/mL)	Solvent	Concentration *Burkholderia* (µg/mL)	Concentration *E.coli* (µg/mL)
Ampicillin	100	Water	100	50
Streptomycin	100	Water	100	50
Tetracycline	40	Ethanol 50%	40	10
Gentamicin	40	Water	40	10
Kanamycin	100	Water	100	50
Rifampicin	100	N,N dimethylformamide	100	-
Nitrofurantoin	100	N,N dimethylformamide	100	-

6.2 MOLECULAR BIOLOGY SOLUTIONS:

Solutions were prepared with destilated deionized water. Solutions were sterilized by filtration through 0.22uM.

1. TAE 50X

	g/L
Trizma Base	242
EDTA	18.6
Acetic Acid Glacial	57

2. SOUTHERN SOLUTIONS

Depurination Solution

	g/L
HCl 11 N	11 mL
Water	898 mL

Denaturation Buffer

	g/L
NaCl	87.66
NaOH	20 g

Saline Solution Citrate (SSC 20 X)

	g/L
Tri-sodium citrate	88.23
NaCl	175.32

Denharts 100 X

	100 mL
Bovine Serum Albumin	2g
Ficoll 400	2g
Polyvinyl pyrrolidone	2g

Hybridization solution

	400 mL
20x SSC	100 mL
100x Denhardts	20 mL
SDS 10%	20 mL

3. SDS- POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) SOLUTIONS (Sambrook, 1989)

SDS PAGE GEL 12 % SEPARATING GEL

	15 mL
H2O	4.9
30% ACRYLAMIDE MIX	6.0
1.5 M TRIS (pH 8.8)	3.8
10 % SDS	0.15
10% ammonium persulfate	0.15
TEMED	0.006

SDS PAGE GEL 4 % STACKING

	5 mL
H2O	3.4 mL
30% ACRYLAMIDE MIX	0.83 mL
1 M TRIS (pH6.8)	0.63
10% SDS	0.05
10 % ammonium persulfate	0.05
TEMED	0.005

Tris Glycine Electrophoresis Buffer

	L
Tris	25 mM
Glycine	250 mM
SDS 10%	10 mL

SDS PAGE Loading Buffer 5X

250 mM Tris. HCl (pH6.8)
0.5 M Dithiothreitol
10% SDS
0.5 % Bromophenol Blue
50% Glycerol

6.3 COMPETENT CELL PREPARATION

Based on methodology from Hanahan (Hanahan, Jessee et al., 1991).

1. Take the frozen stock of cell type and streak out on an LB media plate. Incubate overnight at 37 C.
2. Pick a colony off of the fresh streak plate and inoculate 10 mL of LB media. Grow overnight
3. Inoculate 1 mL of the overnight culture into 100 mL of prewarmed LB media and grow at 37 C in a shaker until the OD_{600} reaches 0.6
4. Transfer the cells to prechilled falcon tubes and pellet the cells at 4,000 rpm at 4 ºC for 10 -15 minutes. Drain thoroughly by inverting and tapping on paper towels to remove all traces of media.
5. Resuspend cells by pipetting in 1/3 the original culture volume in $CaCl_2$ 0.1 M, and incubate on ice for at least 3 hours.
6. Pellet the cells by centrifuging at 4000 rpm at 4. Resuspend cells by pipetting in 8 mL of RF2. Incubate on ice for 15 minutes.
7. Pipet cells into 100 µL of cells into sterile prechilled tubes .
8. Snap freeze cells in dry ice or liquid nitrogen and transfer to -80 ºC.

RF2 Solution

	L	Final Concentration
0.5M MOPS (pH 6.8)	20 mL	10 mM
$RbCl_2$	1.2	10 mM
$CaCl_2$ $2H_2O$	11	75 mM
Glycerol	118 mL	15 %

Sterilize by filtration through 22 µm

6.4 ß-GALACTOSIDASE ACTIVITY MEASUREMENT.

1. Pellet 1 mL of overnight grown culture, and resuspend it in the same volume of prechilled Z buffer.
2. Resuspend 100 µL of cells in 900 µL of Z buffer and determine the OD_{600}
3. Permeabilize 100 µL of cells by adding 20 µL of SDS 0.05%, 20 µL of chloroform and 500 µL of Z buffer. Vortex vigorously for 20 seconds. Incubate at 30 ºC for 20 minutes
4. Add 100 µL of 0.4 % ONPG (*o*-nitrophenyl-b-D-galactoside), and allow the reaction to take place.
5. Stop the reaction after sufficient yellow color has developed by adding 250 µL 1M Na_2CO_3.
6. Spin each sample for 10 min. at maximum speed to remove debris and chloroform and record the optical density of the supernatant at 420 nm
7. Calculate the Miller units of activity :

MILLER UNITS= $(OD_{420} \times 1000) / (OD_{600} \times t)$

Z Buffer Composition

Na_2HPO_4	0.06M
NaH_2PO_4	0.04M
KCl	0.01M
$MgSO_4$	0.001M
ß-mercaptoethanol	0.05M

6.5 GENE SEQUENCES

GenBank: AM940944.1

Burkholderia kururiensis braR gene, rsaL gene and braI gene (quorum sensing system), strain M130

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REFERENCE   1
  AUTHORS   Suarez-Moreno,Z.R., Caballero-Mellado,J. and Venturi,V.
  TITLE     The new group of non-pathogenic plant-associated nitrogen-fixing
            Burkholderia spp. shares a conserved quorum-sensing system, which
            is tightly regulated by the RsaL repressor
  JOURNAL   Microbiology (Reading, Engl.) 154 (PT 7), 2048-2059 (2008)
   PUBMED   18599833
REFERENCE   2  (bases 1 to 4938)
  AUTHORS   Venturi,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-FEB-2008) Venturi V., Bacteriology, International
            Centre for Genetic Engineering and Biotechnology, ICGEB Trieste
            Component, Padriciano 99, 34012 Trieste, ITALY
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Burkholderia unamae unaR, rsaL and unaI genes, strain MTI-641^{T}

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REFERENCE   1
  AUTHORS   Suarez-Moreno,Z.R., Devescovi,G., Myers,M., Mendoca Previato,L.,
            Caballero-Mellado,J. and Venturi,V.
  TITLE     N-acyl homoserine lactone quorum sensing in the novel species
            cluster of beneficial Burkholderia
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1977)
  AUTHORS   Venturi,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-DEC-2009) Venturi V., Bacteriology, I.C.G.E.B,
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The new group of non-pathogenic plant-associated nitrogen-fixing *Burkholderia* spp. shares a conserved quorum-sensing system, which is tightly regulated by the RsaL repressor

Zulma Rocí Suárez-Moreno,[1] Jesús Caballero-Mellado[2] and Vittorio Venturi[1]

Correspondence
Vittorio Venturi
venturi@icgeb.org

[1]Bacteriology Group, International Centre for Genetic Engineering & Biotechnology, Trieste, Italy

[2]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Ap. Postal 565-A, Cuernavaca, Morelos, Mexico

A novel group of nitrogen-fixing plant-associated *Burkholderia* species has emerged in the last few years. The purpose of this investigation was to determine if these species possess an *N*-acylhomoserine lactone (AHL) quorum-sensing (QS) cell–cell signalling system, and whether it is important for nitrogen fixation and other phenotypic features in *Burkholderia kururiensis*. It was determined that *B. kururiensis*, and other members of this *Burkholderia* species cluster, contain at least one highly conserved system, designated BraI/R, which produces and responds to *N*-dodecanoyl-3-oxo-homoserine lactone (C12-3-oxo-AHL). The BraI/R AHL QS is not involved in the regulation of nitrogen fixation or in several other important phenotypes, indicating that it may not be a global regulatory system. The BraI/R system is similar to LasI/R of *Pseudomonas aeruginosa* and, as with *lasI/R*, there is a repressor gene, *rsaL*, between the *braI/R* genes. *B. kururiensis* normally synthesizes very low levels of C12-3-oxo-AHL, but the situation dramatically changes when RsaL is missing since an *rsaL* mutant displays a marked increase in AHL production. This unique stringent regulation indicates that RsaL could be an on/off switch for AHL QS in *B. kururiensis* and the ability to produce very high levels of AHL also questions the role of this molecule in the novel group of *Burkholderia*. The presence of a well-conserved and distinct AHL QS system among all the diazotrophic *Burkholderia* is a further indication that they are closely related, and that this system might play an important and conserved role in the lifestyle of this novel group of bacterial species.

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INTRODUCTION

The genus *Burkholderia* in recent years has been phylogenetically well defined, consisting of species that are functionally remarkably diverse (Coenye & Vandamme, 2003). In fact, *Burkholderia* species have been isolated from many different environmental niches, including soil and water, and can form associations with plants, animals and humans. Phylogenetic trees, based on 16S rRNA sequence analysis, show clearly the separation of the genus *Burkholderia* into two major clusters (Caballero-Mellado *et al.*, 2007), one of them mainly represented by plant, animal and human pathogenic species, including the opportunistic human ones referred to as *Burkholderia cepacia* complex (BCC) (Coenye & Vandamme, 2003). The BCC, a group of at least nine species, has received most attention by the scientific community since, besides being isolated from diverse environmental sources, they have also been recovered from clinical specimens. Patients with cystic fibrosis (CF) are particularly susceptible to members of the BCC, which can cause serious chronic lung infections and, together with *Pseudomonas aeruginosa*, represent the biggest threat to CF individuals (Coenye & LiPuma, 2003; LiPuma, 2003). Other members of the genus *Burkholderia* can have beneficial or pathogenic interactions with plants; for example *Burkholderia glumae* and *Burkholderia caryophylli* are serious pathogens to rice and carnation, respectively (Gonzalez *et al.*, 2007). The second major cluster, phylogenetically distant from the BCC, is formed exclusively by novel environmental non-pathogenic species described later than the year 2000, which include

Abbreviations: AHL, *N*-acylhomoserine lactone; BCC, *Burkholderia cepacia* complex; C8-AHL, *N*-octanoylhomoserine lactone; C12-3-oxo-AHL, *N*-dodecanoyl-3-oxo-homoserine lactone; CF, cystic fibrosis; GFP, green fluorescent protein; QS, quorum sensing.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the 4938 bp *Xmn*I fragment harbouring *bral*, *rsaL* and *braR* is AM940944.

mainly plant-associated, rhizospheric and/or endophytic bacteria, many of which are nitrogen-fixing (Caballero-Mellado *et al.*, 2004; Goris *et al.*, 2004; Perin *et al.*, 2006b; Reis *et al.*, 2004) and legume-nodulating species (Chen *et al.*, 2006; Elliott *et al.*, 2007; Vandamme *et al.*, 2002). Initially only *Burkholderia vietnamiensis*, a member of the BCC and found to be closely associated with rice plants, was reported as being able to fix atmospheric nitrogen (Gillis *et al.*, 1995). *B. kururiensis*, a trichloroethylene degrader (Zhang *et al.*, 2000), was soon after identified as a diazotrophic species (Estrada-de los Santos *et al.*, 2001). Subsequently, many nitrogen-fixing isolates were recovered from different plants (rice, maize, sugar cane, sorghum, coffee and tomato) or from their rhizospheres and further classified as *Burkholderia unamae*, *Burkholderia xenovorans*, *Burkholderia silvatlantica*, *Burkholderia tropica*, *Burkholderia tuberum*, *Burkholderia phymatum*, *Burkholderia mimosarum* and *Burkholderia nodosa* (Caballero-Mellado *et al.*, 2007 and references therein). '*Burkholderia brasilensis*' strain M130 (Baldani *et al.*, 1997b), a plant-associated diazotrophic species never described validly, has recently been reclassified as *B. kururiensis* (Caballero-Mellado *et al.*, 2007). It is expected that many more novel non-pathogenic plant-associated *Burkholderia* species will be described in the near future.

Very large genomes, in most cases more than 7 Mb, and replicon multiplicity are characteristic features in the genus *Burkholderia*, in both pathogenic species and opportunistic pathogens (Mahenthiralingam *et al.*, 2005), as well as in diazotrophic environmental species (Martínez-Aguilar, 2008). Such genomic complexity in the novel diazotrophic *Burkholderia* species could account for their ability to colonize the rhizosphere and endophytic environments of a wide range of taxonomically unrelated host plants (Martínez-Aguilar, 2008). Additionally it provides them and other *Burkholderia* species with extraordinary nutritional versatility, in some cases having unique catabolic potential, being able to degrade recalcitrant xenobiotics, making them potentially useful for bioremediation purposes (O'Sullivan & Mahenthiralingam, 2005; Vial *et al.*, 2007). Very little, however, is known about the molecular and genetic aspects of plant interaction of this novel group of environmental *Burkholderia*.

In contrast, many molecular and genetic studies have been performed in members of the BCC complex. In addition, several models, including insect, plant, worm and animal, have been established for the study of virulence of BCC members (Mahenthiralingam *et al.*, 2005). A number of studies by different laboratories have highlighted that bacterial intercellular communication via the production and sensing of signal molecules, known as quorum sensing (QS), plays an important role in BCC fitness and virulence (reviewed by Eberl, 2006; Venturi *et al.*, 2004). In BCC species, as in many other Gram-negative bacteria, the signal molecules produced and detected are *N*-acylhomoserine lactones (AHLs), which allow bacteria to monitor their population density by responding to the extracellular concentration of AHLs. An AHL QS system is most commonly mediated by two proteins belonging to the LuxI-AHL synthase and to LuxR-AHL-response regulator protein families. AHLs interact directly, at quorum concentration, with the cognate LuxR-type protein, which then binds at specific gene promoter sequences affecting the expression of QS target genes (Fuqua *et al.*, 2001). Importantly, various studies have established that in BCC the AHL QS system is highly conserved, consisting of CepI, which synthesizes mainly *N*-octanoylhomoserine lactone (C8-AHL) and the CepR C8-AHL sensor-response regulator. Significantly, the CepI/R AHL QS system was found to be involved in the regulation of similar phenotypes in many different species and strains of the BCC, including virulence in several models (reviewed by Eberl, 2006; Venturi *et al.*, 2004). In addition to BCC members, also the plant-pathogenic *Burkholderia plantarii* and *Burkholderia glumae* were found to possess a CepI/R-like system producing and responding to C8-AHL and involved in the regulation of virulence-associated factors (Devescovi *et al.*, 2007; Kim *et al.*, 2004; Solis *et al.*, 2006).

The purpose of this study was to determine if the new environmental group of plant-associated diazotrophic *Burkholderia* species possesses an AHL QS system and whether it is important for nitrogen fixation and other important phenotypic features. We established that all the strains tested so far contain at least one highly conserved system, designated BraI/R, which is related to the LasI/R and PpuI/R AHL QS systems of *P. aeruginosa* and *Pseudomonas putida*, respectively, and is not similar to the CepI/R system. Importantly, the BraI/R system is under stringent negative regulation by the RsaL repressor, which could have the unique role of switching the system on/off. BraI/R is not involved in the regulation of nitrogen fixation or in several other important phenotypes.

METHODS

Bacterial strains, plasmids and media. *Burkholderia* strains and plasmids used in this study are listed in Table 1. *Burkholderia* '*brasilensis*' M130 was isolated from rice in Brazil (Baldani *et al.*, 1997b) and kindly provided by Dr Lucia Mendoça-Previato from the Universidad Federal do Rio de Janeiro, Brazil. This strain has recently been reclassified as *B. kururiensis* M130 (Caballero-Mellado *et al.*, 2007). Bacterial strains were grown at 30 °C in M9 minimal medium supplemented with glycerol (Sambrook *et al.*, 1989), in King's medium (King *et al.*, 1954) or in BSE liquid medium (Estrada-de los Santos *et al.*, 2001). Six AHL bacterial biosensors were used for AHL detection: *Chromobacterium violaceum* strain CVO26 (McClean *et al.*, 1997), *Agrobacterium tumefaciens* NTL4/pZLR4 (Shaw *et al.*, 1997), *Escherichia coli*/pSB1075, *E. coli* JM109/pSB401, *E. coli* MT102/pJBA132 and *P. putida* F117/pKRC12 (Riedel *et al.*, 2001). *Chromobacterium*, *Agrobacterium* and *Pseudomonas* AHL detector strains were grown at 28 °C, while *E. coli* were grown at 37 °C. Antibiotics were added when required at the following final concentrations: ampicillin, 100 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; tetracycline, 15 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Burkholderia*); gentamicin, 10 μg ml^{-1} (*E. coli*), 30 μg ml^{-1} (*Agrobacterium*) and 40 μg m^{-1} (*Pseudomonas*); kanamycin, 50 μg ml^{-1} (*E. coli* and *C. violaceum*) or 100 μg ml^{-1} (*Pseudomonas* and

Table 1. *Burkholderia* strains, plasmids and primers used in this study

Strain, plasmid orprimer	Relevant characteristics	Reference or source
***Burkholderia* strains**		
B. kururiensis M130	Amp^R Rif^R	Baldani *et al.* (1997b)
M130BRAI	*braI*::Km of *B. kururiensis* M130	This work
M130BRAR	*braR*::Km of *B. kururiensis* M130	This work
M130RSAL	*rsaL*::Km of *B. kururiensis* M130	This work
B. caledonica DSM17062	Type strain	Coenye *et al.* (2001)
B. caribensis DSM13236	Type strain	Achouak *et al.* (1999)
B. fungorum DSM17061	Type strain	Coenye *et al.* (2001)
B. graminis DSM17151	Type strain	Viallard *et al.* (1998)
B. hospita DSM17164	Type strain	Goris *et al.* (2002)
B. kururiensis DSM13646	Type strain	Zhang *et al.* (2000)
B. mimosarum PAS44	Type strain	Chen *et al.* (2006)
B. phenazinium DSM10684	Type strain	Viallard *et al.* (1998)
B. phenoliruptrix DSM17773	Type strain	Coenye *et al.* (2004)
B. phymatum DSM17167	Type strain	Vandamme *et al.* (2002)
B. phytoformans DSM17436	Type strain	Sessitsch *et al.* (2005)
B. sacchari DSM17165	Type strain	Bramer *et al.* (2001)
B. silvatlantica SRMrh-20	Type strain	Perin *et al.* (2006b)
B. terrae DSM17804	Type strain	Yang *et al.* (2006)
B. terricola DSM17221	Type strain	Goris *et al.* (2002)
B. tropica $Ppe8^T$	Type strain	Reis *et al.* (2004)
B. tuberum DSM18489	Type strain	Vandamme *et al.* (2002)
B. unamae $MTl\text{-}641^T$	Type strain	Caballero-Mellado *et al.* (2004)
B. xenovorans DSM17367	Type strain	Goris *et al.* (2004)
Plasmids		
pRK2013	Tra^+ Mob^+ ColE1 replicon; Km^R	Figurski & Helinski (1979)
pMOSBlue	Cloning vector; Amp^R	Amersham-Pharmacia
pBluescript KS	Cloning vector; Amp^R	Stratagene
pMP220	Promoter probe vector, IncP; Tet^R	
pMPbraI	*braI* promoter cloned into pMP220, Tet^R	This study
pMPrsaL	*rsaL* promoter cloned into pMP220, Tet^R	This study
pLAFR3	Broad-host-range cloning vector, IncP1; Tet^R	Staskawicz *et al.* (1987)
pZS1	pLAFR3 containing *B. kururiensis* DNA	This study
pZS2	pLAFR3 containing *B. kururiensis* DNA	This study
pMOS-Xmn-12	pMOSBlue carrying 5 Kb fragment containing partial *B. kururiensis* QS genes, Amp^R	This study
PMOSBRAR	pMOSBlue carrying 490 bp fragment containing partial *B. kururiensis braR* gene, Amp^R	This study
pMOSRSAL	pMOSBlue carrying 225 bp fragment containing partial *B. kururiensis rsaL* gene, Amp^R	This study
pKNOCK-Km	Conjugative suicide vector; Km^R	Alexeyev (1999)
pKNOCK-braI	Internal *Eco*RV *braI* fragment of *B. kururiensis* cloned in pKNOCK-Km	This study
pKNOCK-braR	Internal PCR *braR* fragment of *B. kururiensis* cloned in pKNOCK-Km	This study
pKNOCK-braL	Internal PCR *rsaL* fragment of *B. kururiensis* cloned in pKNOCK-Km	This study
pMPbraIprom	Promoter of gene *braI* cloned in pMP220 vector	This study
pMPrsaLprom	Promoter of gene *rsaL* cloned in pMP220 vector	This study
pQEBRAR	*braR* cloned into pQE30 expression vector	This study
pQE30	Amp^R	Qiagen
Primers		
braR18FW	5′-CATCCGACGAGACGCAAT-3′	This study
braR509Rv	5′-TTTGGAATGAGCGTTTGC-3′	This study
braLFw	5′-TTGTTGAAATAAAGTCCCAG-3′	This study
braLRv	5′-CTGGAAAATCACTGGCA-3′	This study
pQEbraRfw	5′-GGGGATCCTCGCCGATACTGGCCGCATC-3′	This study
pQEbraRrv	5′-GGGAAGCTTTCAGCCCGGATCTATAAGGCC-3′	This study

Burkholderia); rifampicin, 100 μg ml^{-1}. Conjugations in *Burkholderia kururiensis* were counter-selected in JMV medium (Reis *et al.*, 2004) with the appropriate antibiotics.

Screening *Burkholderia* isolates for the production of AHLs. *B. kururiensis* M130 was first tested for the production of AHLs in a T-streak analysis on solid medium as described by Piper *et al.* (1993) using the AHL biosensors CVO26, MT102/pJBA132 and F117/pKRC12 on KB agar plates. In the same way, type strains of 19 other valid *Burkholderia* species were tested for AHL production (Table 1). Production of AHLs was further characterized by TLC after AHL extraction from cell-free spent supernatants as follows. *Burkholderia* strains were grown overnight in 20 ml M9 minimal medium supplemented with glucose and Casamino acids and the supernatants of the cultures were extracted and analysed on C_{18} reverse-phase TLC plates as previously described (Shaw *et al.*, 1997). The plate was then overlaid with a thin layer of LB or AB top agar seeded with either *E. coli*/pSB1075 or *A. tumefaciens* NTL4/pZLR4, the latter in the presence of 100 μg X-Gal ml^{-1}, as described previously (Shaw *et al.*, 1997).

Recombinant DNA techniques. Recombinant DNA techniques, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, end filling with the Klenow enzyme, hybridization, radioactive labelling by random priming, and transformation of *E. coli* were performed as described by Sambrook *et al.* (1989). Southern hybridizations were performed using N^+ Hybond membranes (Amersham Biosciences); plasmids were purified using Jet star columns (Genomed); total DNA from *Burkholderia* was isolated by Sarkosyl-Pronase lysis as described previously (Better *et al.*, 1983). Triparental mating from *E. coli* to *Pseudomonas* was carried out by using the helper strain *E. coli*/pRK2013 (Figurski & Helinski, 1979), whereas plasmids were introduced into the *Burkholderia* by biparental conjugation using *E. coli* S17-1 as donor (Simon *et al.*, 1983), incubated for 22 h at 30 °C.

Isolation of the AHL QS system of *B. kururiensis* M130 and construction of gene knockout mutants. A cosmid library was constructed for *B. kururiensis* by using the cosmid pLAFR3 (Staskawicz *et al.*, 1987) as vector. Insert DNA was prepared by partial *Eco*RI digestion of the genomic DNA and then ligated into the corresponding site in pLAFR3. The ligated DNA was then packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene) and the phage particles were transduced to *E. coli* HB101 as recommended by the supplier. In order to identify the cosmid containing the AHL QS genes, *E. coli* HB101 harbouring the cosmid library was used as donor in a triparental conjugation with *P. putida* F117/pKRC12 as acceptor. The transconjugants that displayed green fluorescent protein (GFP) expression were further studied. Two cosmids, pZS1 and pZS2, contained the AHL QS locus of *B. kururiensis* M130, designated as the *luxI*-type gene *braI* and the *luxR*-type gene *braR*. The *braIR* locus was localized in a 5 kb *Xmn*I fragment, which was cloned in pMOSBlue creating pMOSXmn12 (see Fig. 2).

Different genomic null mutants were created in the AHL QS system of *B. kururiensis* as follows. First, an internal 423 bp *Eco*RV fragment from the *braI* gene was cloned in pKNOCK-Km (Alexeyev, 1999), generating pKNOCK-braI. This latter plasmid was then used as a suicide delivery system in order to create a *braI* knockout mutant of strain M130 as previously described (Alexeyev, 1999), generating M130BRAI. Similarly, an internal (490 bp) fragment of *braR* was PCR amplified by using braR18Fw and braR509Rv primers (Table 1) and cloned into pMOSBlue, yielding pMOSBRAR. A *Xba*I–*Kpn*I insert was then cloned into pKNOCK-Km and used as suicide vector for generating M130BRAR. Using a similar approach, an *rsaL* mutant was then generated by amplifying a 225 bp fragment by using braLFw and braLRv primers (Table 1) and cloned into pMOSBlue, generating pMOSRSAL, and subsequently cloned as a *Xba–Kpn*I fragment in the corresponding sites in pKNOCK-Km, yielding pKNOCK-rsaL. This latter plasmid was used to generate the *rsaL* knockout mutant designated M130RSAL. The fidelity of all marker-exchange events was confirmed by Southern analysis (data not shown).

Conservation of *braI/R* in 19 other *Burkholderia* species. The presence of *braI/R* in the other 19 species within the novel group of nitrogen-fixing *Burkholderia* was determined by Southern analysis and by PCR. Southern analysis was performed on *Eco*RI-digested genomic DNAs that were hybridized with an *Eco*RV 423 bp internal fragment of *braI* (see Fig. 2). PCRs were performed in order to amplify *braR* and *rsaL* in the 19 species. The primers used in the PCR for *braR* were pQEbraRfw and pQEbraRrv and for *rsaL* were braLFw and braLRv (Table 1). All amplified fragments were sequenced in order to confirm the presence *braR* and *rsaL* genes.

Determination of the biologically active AHL of the BraI/R AHL QS system. To test which is the cognate AHL for BraR of *B. kururiensis* M130, we amplified the gene promoter regions of the *braI* AHL synthase and of the *rsaL* regulator and cloned them using the promoter-probe vector pMP220 as follows. A 403 bp *Cla*I–*Eco*RI fragment containing the promoter regions of the *braI* synthase gene and putative regulator *rsaL* was cloned in pBlueScript KSII+. The fragment was then excised as a *Xba*I–*Kpn*I fragment or as a *Bam*HI–*Kpn*I fragment and cloned in the corresponding sites in the promoter-probe vector pMP220, generating pMPbraI and pMPrsaL, respectively. These two latter constructs were transformed into *E. coli* M15/pREP-4 containing the expression plasmid pQEBRAR harbouring the *braR* gene. The *braR* gene was cloned in expression vector pQE30, generating pQEBRAR, as follows: *braR* was PCR amplified by using oligonucleotides pQEbraRfw and pQEbraRrv using chromosomal DNA as template, and the PCR product was cloned as a *Bam*HI–*Kpn*I fragment in pQE30. The BraR protein was expressed according to the manufacturer's instructions (Qiagen) and was verified by SDS-PAGE.

E. coli M15/pQEBRAR/pMPbraI and *E. coli* M15/pQEBRAR/pMPrsaL were inoculated into 10 ml of LB-Amp-Km-Tet and grown overnight, and then diluted to OD_{600} 0.1 into 10 ml prewarmed medium containing 1 μM of a specific AHL to be evaluated. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 0.6, and after 2 h at 37 °C β-galactosidase activity was determined.

Quantification of C12-3-oxo-AHL. Dried AHL extracts were prepared as described above and resuspended in ethyl acetate with an amount which corresponded to 1 μl final extract corresponding to 10^9 cells of the original culture. The quantity of C12-3-oxo-AHL in the extracts was determined using C12-3-oxo-AHL sensor *P. putida* SM17/pRSAL220 as previously described (Rampioni *et al.*, 2006). Briefly, *P. putida* SM17 is a double *ppuI rsaL* mutant; consequently, it does not produce the RsaL repressor and C12-3-oxo-AHL. Exogenous C12-3-oxo-AHL is quantified through β-galactosidase activity by using strain SM17 harbouring prsal220; this plasmid contains the PpuR-C12-3-oxo-AHL-regulated *rsaL* promoter fused to a promoterless *lacZ* gene. Overnight cultures of SM17/prsal220 were diluted in 10 ml LB medium to an OD_{660} of 0.1; the AHL extract to be quantified was then added and after 4 h growth, β-galactosidase activity was determined. This C12-3-oxo-AHL bacterial sensor has a linear dose response between 0.1 and 1 μM of C12-3-oxo-AHL. Synthetic C12-3-oxo-AHL was used as standard molecules (obtained from P. Williams, University of Nottingham, UK). The experimental set-up for the quantification is detailed in Table 2.

Determination of enzyme activities. Proteolytic and lipolytic activities were determined in the appropriate indicator plates as

previously reported (Huber *et al.*, 2001). β-Galactosidase activities were determined essentially as described by Miller (1972) with the modifications of Stachel *et al.* (1985). All experiments were performed in triplicate and the mean value is given.

Bacterial motility assays. Swimming assays were performed on 0.3 % KB agar plates; swarming assays were performed using M8 medium plates (M9 salts without NH_4Cl) (Kohler *et al.*, 2000) supplemented with 0.2 % glucose and 0.05 % glutamate and containing 0.5 % agar (Murray & Kazmierczak, 2006). The inoculation was performed by spotting 1 μl of a bacterial suspension having an OD_{600} of 1. The swimming and swarming zones were measured after 48 h incubation at 30 °C for the wild-type and its QS mutant derivatives.

Assays for nitrogen-fixation assays and other phenotypic features

Media and culture conditions. *B. kururiensis* M130 and its derivative mutants in the *braI*, *braR* and *rsaL* genes were grown in BSE liquid medium (Estrada-de los Santos *et al.*, 2001) for 18 h with reciprocal shaking. The cultures were harvested and the pellets washed with 10 mM $MgSO_4.7H_2O$. The pellets were adjusted to a low (1×10^5 c.f.u. ml^{-1}) or high (1×10^8 c.f.u. ml^{-1}) cell density. All the assays described below were assessed with *B. kururiensis* M130 and its derivative mutants to a low and high cell density, and the cultures were incubated at 29 °C.

Nitrogen-fixation assay. Ten-millilitre vials containing 5 ml nitrogen-free semisolid BAz mineral medium (Estrada-de los Santos *et al.*, 2001), in which azelaic acid was omitted and succinic acid added (5 g l^{-1}), were inoculated with *B. kururiensis* M130 and derivative mutants to a low or high cell density as described above. The cultures were incubated for 30 h at 29 °C, then 10 % (v/v) acetylene was injected into the vials and the cultures incubated for 15 h at 29 °C, and then assayed for nitrogenase activity (nitrogen fixation) by the acetylene reduction activity method (Burris, 1972). Three replicate cultures were assayed for the wild-type strain and each mutant.

Growth on aromatic compounds. Aliquots of the cultures of M130 and mutant derivatives were inoculated with a multipoint replicator on SAAC medium plates (Caballero-Mellado *et al.*, 2007) containing 0.05 % (w/v) phenol as sole carbon source; when benzene or toluene was tested as carbon source, 150 μl of these volatile compounds was added on filter paper placed in the lids of Petri dishes as described previously (Caballero-Mellado *et al.*, 2007). Presence or absence of growth was determined after incubation for 5 days at 29 °C. In addition, SACC liquid medium supplemented with phenol was inoculated with *B. kururiensis* wild-type strain and mutants to low or high cell density as described above; optical density was determined after incubation with shaking (250 r.p.m.) for 5 days at 29 °C. SAAC medium without a carbon source was used as a negative control for bacterial growth; succinic acid (0.2 % w/v) as carbon source was a positive control.

Siderophore production. The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol S (CAS) agar plates (Schwyn & Neilands, 1987), as described previously (Caballero-Mellado *et al.*, 2007). *B. kururiensis* wild-type strain M130 and the derivative mutants were grown for 18 h at 29 °C in the CAA liquid medium supplemented with 0.3 % (w/v) succinic acid. The cultures were harvested and the pellets washed and adjusted to a low or high cell density as described above. Aliquots of the cultures (10^4 or 10^8 c.f.u.) were inoculated with a multipoint replicator on CAS-CAA medium (Caballero-Mellado *et al.*, 2007), and then incubated for 48 h at 29 °C. Siderophore production, as indicated by orange haloes formed around the colonies, was determined as described previously (Caballero-Mellado *et al.*, 2007).

Biosynthesis of indole compounds. *B. kururiensis* M130 and its derivative mutants were cultured in BSE medium described above; the mineral culture medium described previously (Jain & Patriquin, 1984) was inoculated to a low or high cell density and incubated for 24 h at 29 °C. Indole compounds, including indoleacetic acid, were determined by using Salkoswki's colorimetric reaction (Tang & Bonner, 1948).

DNA sequencing and nucleotide sequence accession numbers. All DNA sequencing was performed by Macrogen (http://www.macrogen.com). The nucleotide sequence of the 4938 bp *Xmn*I

Table 2. Quantification of C12-3-oxo-AHL in *B. kururiensis* M130 and M130RSAL

Strain	Culture volume*	Optical density*	Ethyl acetate volume†	Volume for assay‡	β-Galactosidase activity (Miller units)§	Estimated concentration (μM) of C12-3-oxo-AHL‖
Wild-type	200	2.8	616	200	577.78 ± 59.2	0.020 ± 0.004
	200	3.23	710.6	200	514.81 ± 8.01	0.018 ± 0.001
	200	4.52	994.4	200	543.83 ± 6.55	0.028 ± 0.001
M130RSAL	50	4.37	240.35	1	2533.33 ± 38.19	49.76 ± 3.937
	50	4.535	249.42	1	2147.73 ± 177.50	42.725 ± 4.102
	50	4.535	249.42	1	2354.70 ± 125.82	47.508 ± 2.907
M130RSAL/pZS1	50	2.91	145.5	50	651.09 ± 51.26	0.109 ± 0.014
	50	2.2	110	50	599.86 ± 34.65	0.072 ± 0.007
	50	2.74	137	50	694.56 ± 90.56	0.114 ± 0.023

*Single colonies were independently inoculated and grown overnight in KB medium and optical density was measured. AHLs were extracted from spent supernatants and resuspended in an ethyl acetate volume (†) corresponding to an amount of 1×10^9 c.f.u.
‡AHL levels were measured with *P. putida* SM17prsaL220 since C12-3-oxo-AHL levels are proportional to β-galactosidase activity (Miller units). Extracted AHL volumes were diluted in order to obtain β-galactosidase values in the linear range of the sensor (0.1–1 μM).
§Values represent the means ± SEM of three independent experiments with three replicates.
‖Estimated concentrations of C12-3-oxo AHL in spent supernatants.

fragment harbouring *braI*, *rsaL* and *braR* has been deposited in GenBank/EMBL/DDBJ under accession number AM940944.

RESULTS

The AHL QS system of *B. kururiensis* M130

To investigate the production of AHLs in the *B. kururiensis* M130 rice endophytic bacterial strain, we used four different AHL biosensors as described in Methods. Strain M130 was found to give a positive result when tested with a plate T-streak assay with LasR-based biosensor *P. putida* F117/pRKC12, which is particularly sensitive to C12-3-oxo-AHL and also to a lesser extent to C10-3-oxo-AHL (data not shown). Ethyl acetate extracts of M130 culture supernatants were then fractionated by TLC, overlaid with AHL biosensor *A. tumefaciens* NTL4/pZLR4 or *E. coli*/pSB1075. The results showed that this strain produced four AHL molecules tentatively identified as C12-3-oxo-, C10-3-oxo-, C8-3-oxo- and C6-3oxo-AHL (Fig. 1). It was therefore concluded that *B. kururiensis* M130 produces AHLs and therefore most probably possesses at least one AHL QS system.

In order to identify the gene(s) directing the synthesis of and response to AHL QS signal molecules, the AHL QS locus of strain M130 was cloned via complementation *in trans* of the LasR-based F117/pRKC12 biosensor with a cosmid gene bank of strain M130. Two cosmid clones were identified which directed GFP synthesis from the *lasI* gene promoter and inserts were found to contain the AHL QS system of strain M130. DNA sequence analysis of this locus revealed the presence of three ORFs; two of them (*braI* and *braR*) displayed homology to *luxI*- and *luxR*-family genes and a third ORF, located in between *braR* and *braI*, divergently transcribed from *braI*, displayed high similarity to the *rsaL* negative regulatory gene (Fig. 2). The BraR protein consisted of 235 amino acids displaying approximately 40 % identity to LasR and PpuR of *P. aeruginosa* and *P. putida*, respectively; the BraI protein consisted of 196 amino acids displaying 50 % identity to LasI and PpuI, and RsaL consisted of 105 amino acids having approximately 50 % identity to RsaL proteins of *P. aeruginosa* and *P. putida*. It was therefore concluded that *B. kururiensis* possesses an AHL QS system related to the LasI/R and PpuI/R systems of *Pseudomonas*.

The BraI/RsaL/BraR system is conserved among the novel group of nitrogen-fixing *Burkholderia*

It was of interest to determine how conserved was the ability to produce AHLs via the BraI/RsaL/BraR QS system in the novel group of nitrogen-fixing *Burkholderia*. We analysed the production of AHLs by TLC analysis of extracts from spent supernatants of 20 different *Burkholderia* species using the *A. tumefaciens* NTL4/pZLR4 biosensor. As depicted in Fig. 3(A), all 20 species were found to produce a very similar AHL profile to that of *B. kururiensis* M130; the only difference was the amount of AHLs produced by some strains. Using this AHL biosensor, it was observed that all strains produced C8-3-oxo-AHL; however, it must be noted that the *A. tumefaciens* NTL4/pZLR4 biosensor is most sensitive to this AHL. In addition, all strains reacted very strongly in a plate T-streak assay with sensor *P. putida*/pKR-C12, which is a LasR-based sensor particularly sensitive to C12-3-oxo-AHL (data not shown). By Southern analysis it was then established that the *braI/R* AHL QS system was well conserved in all 20 species of the novel cluster of *Burkholderia* species (see Methods and Fig. 3B). Using two pair sets of oligonucleotide primers directed towards *braR* and *rsaL* (Table 1 and Methods), it was possible to PCR amplify the *braR* and *rsaL*

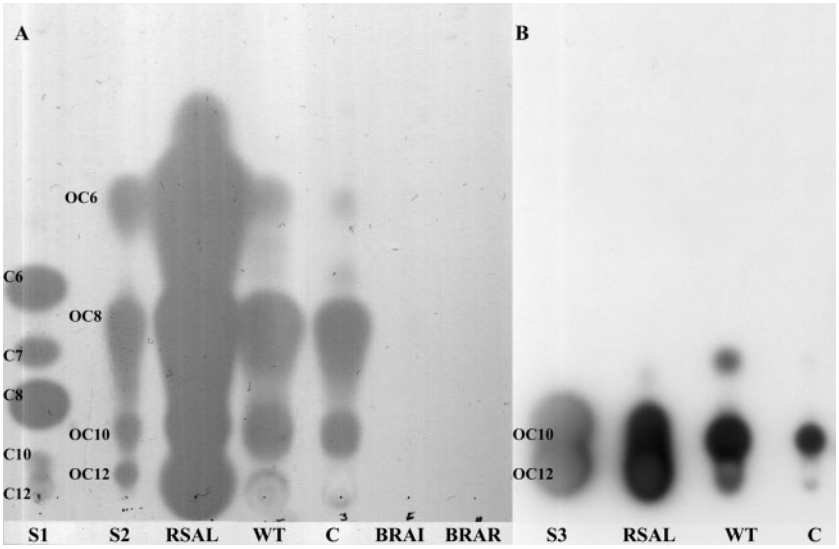

Fig. 1. (A) TLC analysis of AHLs produced by parent strain *B. kururiensis* M130 and mutant derivatives by using the *A. tumefaciens* pNTL4 biosensor. In the WT, BRAI and BRAR lanes, a volume corresponding to 5×10^{10} c.f.u. was loaded, whereas for the *rsaL* mutant (lane RSAL) and for the control (C, *rsaL* mutant harbouring pZS1), a volume corresponding to 10^9 c.f.u. was loaded. (B) TLC analysis of AHLs produced by parent strain *B. kururiensis* M130 and mutant derivatives by using the *E. coli* pSB1075 biosensor. In the WT lane, a volume corresponding to 5×10^{10} c.f.u. was loaded, whereas for the *rsaL* mutant (lane RSAL) and for the control (C, *rsaL* mutant harbouring pZS1) a volume corresponding to 10^9 c.f.u. was loaded. Synthetic AHL compounds were used as reference.

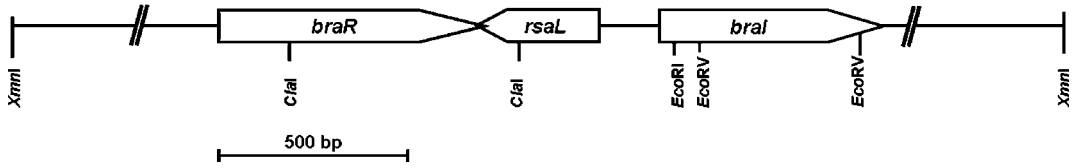

Fig. 2. Map of the 5 kbp DNA fragment from *B. kururiensis* M130 containing the 1.7 kb *braIR* system described in this study. Shown are several enzyme restriction sites and the location of the *braI*, *braR* and *rsaL* genes.

genes in all 20 species (data not shown), making these PCRs a potentially important tool for the identification of *Burkholderia* isolates belonging to this novel cluster. No amplification using either sets of primers was observed with members of the BCC complex and with *P. aeruginosa* (data not shown).

The BraI/R system is tightly regulated by RsaL and BraR responds to C12-3-oxo-AHL

In order to characterize BraR's AHL specificity, the protein was overexpressed in *E. coli* M15 in the presence of different AHL molecules and cognate *braI* and *rsaL*

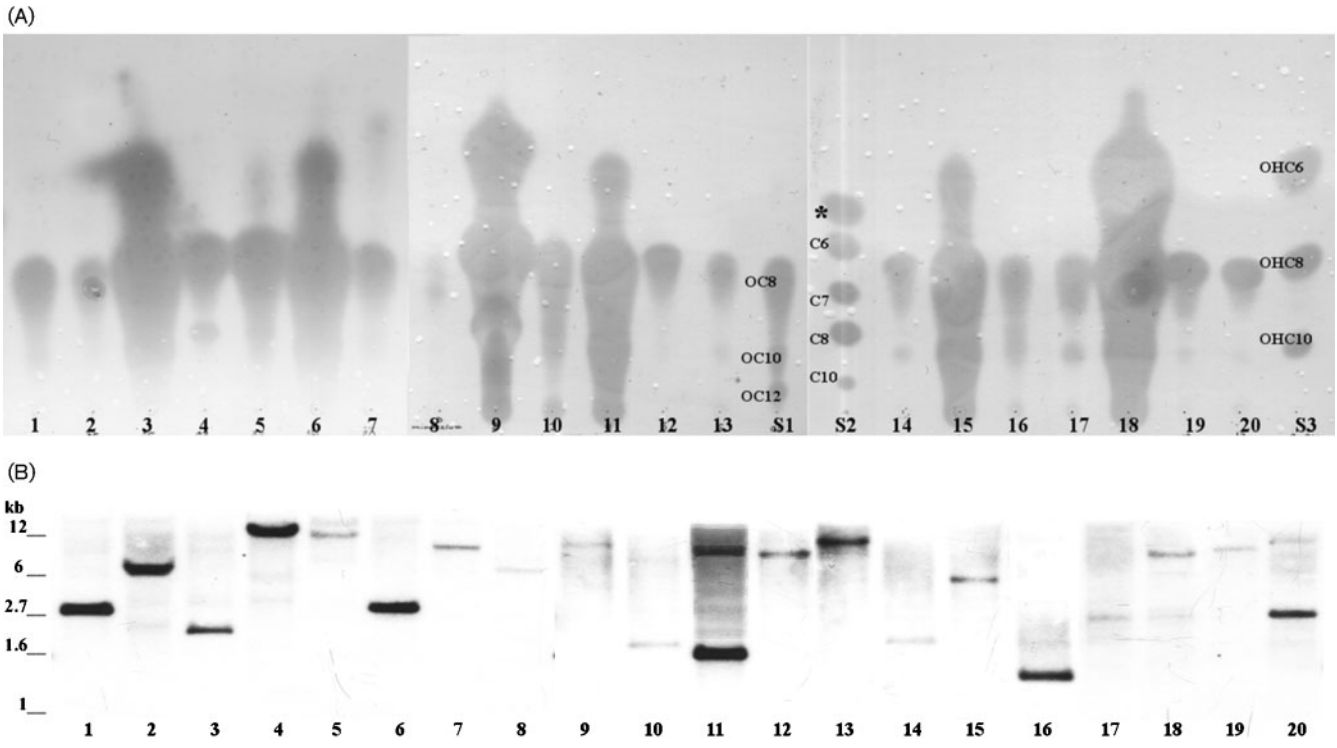

Fig. 3. (A) TLC analysis of AHLs produced by type strains of 20 *Burkholderia* species. Synthetic AHL compounds were used as reference. Lanes: 1, *B. kururiensis* M130; 2, *B. caledonica* DSM 17062; 3, *B. caribensis* DSM 13236; 4, *B. fungorum* DSM 17061; 5, *B. graminis* DSM 17151; 6, *B. hospita* DSM 17164; 7, *B. kururiensis* DSM 13646; 8, *B. mimosarum* PAS44; 9, *B. phenazinium* DSM 10684; 10, *B. phenoliruptrix* DSM 17773; 11, *B. phymatum* DSM 17167; 12, *B. phytofirmans* DSM 17436; 13, *B. sacchari* DSM 17165; 14, *B. silvatlantica* SRMrh-20; 15, *B. terrae* DSM 17804; 16, *B. terricola* DSM 17221; 17, *B. tropica* DSM 15359; 18, *B. tuberum* DSM 18489; 19, *B. unamae* DSM 17197; 20, *B. xenovorans* DSM 17367. The * denotes a degradation product as indicated by Shaw *et al.* (1997). (B) Autoradiogram of a Southern blot analysis of total *Eco*RI-digested genomic DNA hybridized with the *braI* probe from *B. kururiensis* M130. Lanes: 1, *B. kururiensis* M130; 2, *B. caledonica* DSM 17062; 3, *B. fungorum* DSM 17061; 4, *B. graminis* DSM 17151; 5, *B. hospita* DSM 17164; 6, *B. kururiensis* DSM 13646; 7, *B. mimosarum* PAS44; 8, *B. tropica* DSM 15359; 9, *B. caribensis* DSM 13236; 10, *B. phytofirmans* DSM 17436; 11, *B. sacchari* DSM 17165; 12, *B. silvatlantica* SRMrh-20^T; 13, *B. terrae* DSM 17804; 14, *B. tuberum* DSM 18489; 15, *B. unamae* DSM 17197; 16, *B. xenovorans* DSM 17367; 17, *B. phenazinium* DSM 10684; 18, *B. phenoliruptrix* DSM 17773; 19, *B. phymatum* DSM 17167; 20, *B. terricola* DSM 17221.

promoters activities were then determined. The *braI* and *rsaL* gene promoters were cloned in the broad-host-range low-copy-number β-galactosidase promoter probe vector pMP220, yielding pMPbraI and pMPrsaL, respectively. These two transcriptional fusions were transformed into *E. coli* containing pQEBRAR, which is a plasmid expressing the cognate BraR protein, generating *E. coli* M15/pMPbraI/pQEBRAR and *E. coli* M15/pMPrsaL/pQEBRAI. Testing promoter activities in the presence of many different AHLs showed that the activity of the *braI* promoter increased 20-fold in the presence of C12-3-oxo-AHL, demonstrating a specific preference for C12-3-oxo-AHL (Fig. 4). The same activity profile was observed for the *rsaL* promoter, further confirming that C12-3-oxo-AHL is the biologically active molecule for BraI/R system of *B. kururiensis*.

The RsaL protein located between the *braI/R* genes in *B. kururiensis* M130 was reported to negatively regulate the transcription of the *luxI* family AHL synthase in *P. aeruginosa* and *P. putida* (Bertani & Venturi, 2004; Rampioni *et al.*, 2006, 2007b). As it was observed that strain M130 produced low amounts of AHLs (Fig. 1), we were interested to determine whether RsaL was negatively regulating *braI*. The *rsaL* gene was inactivated in strain M130, generating a knockout mutant designated M130RSAL. The AHLs produced by this mutant were extracted and analysed by TLC and this showed that AHLs production dramatically increased in the *rsaL* mutant, especially the biologically active C12-3-oxo-AHL (Fig. 1B). This mutant could be complemented by providing the *rsaL* gene *in trans* in a plasmid (cosmid pZS1), which restored AHL production to wild-type levels (Fig. 1). Since it was determined that the functional AHL for BraR of strain M130 was C12-3-oxo-AHL, levels of this AHL in extracts from M130 and M130RSAL were precisely determined using the sensor *P. putida* SM17/prsaL220 (Rampioni *et al.*, 2007a). This sensor is suitable for C12-3-oxo-AHL quantification as it is very specific for this AHL and its dose response was shown to be linear from 0.1 to 1 μM. Results of this experiment showed that C12-3-oxo-AHL levels produced by M130 wild-type were very low and were enhanced dramatically (almost 2000 fold) in the *B. kururiensis rsaL* mutant (Table 2). The estimated concentration in a stationary-phase culture of *B. kururiensis* M130 was approximately 20 nM whereas it increased dramatically to 45 μM for the *rsaL* mutant. The production of C12-3-oxo-AHL in the *rsaL* mutant was restored to wild-type levels when the *rsaL* gene was provided *in trans* via cosmid pZS1 (Table 2).

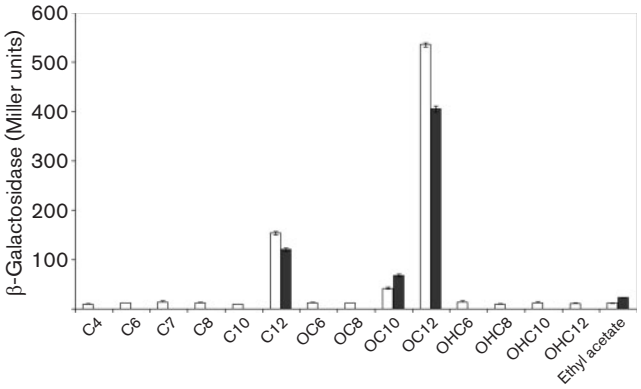

Fig. 4. Determination of the biologically active AHL of the BraI/R AHL QS system. White bars correspond to β-galactosidase activities determined for the pMPbraI promoter; black bars correspond to pMPrsaL measurements. Both transcriptional fusions were harboured independently in *E. coli* expressing the BraR protein, and various exogenous AHLs (1 μM) were provided as indicated and β-galactosidase activities were determined. The results are mean values ± SEM of three independent experiments.

The BraI/R system is not involved in regulating nitrogenase activity in *B. kururiensis*

B. kururiensis, like many other closely related *Burkholderia* species, is able to fix nitrogen, converting N_2 into NH_3 through the synthesis of a nitrogenase complex. Since this is a very high-energy-demanding reaction, bacteria stringently regulate this process (Dixon & Kahn, 2004). It was therefore of interest to determine whether the BraI/R QS system was involved in the regulation of nitrogenase activity in *B. kururiensis* M130. In order to create QS mutants, *braR* and *braI* were inactivated in strain M130, generating knockout mutants designated M130BRAR and M130BRAI, respectively. Both these mutants were unable to synthesize AHLs (Fig. 1), indicating that the BraI/R system was responsible for synthesizing all four AHLs identified in culture supernatants and that most probably no other AHL QS system was present in *B. kururiensis*. In addition, this demonstrated that BraR positively regulates the *braI* synthase through a positive auto-induction loop typical of AHL QS systems. The acetylene reduction assay was used to detect expression of the nitrogenase complex under nitrogen-free conditions in parental strain M130 and in the AHL-hyperproducing *rsaL* mutant and the non-AHL-producing *braI* and *braR* mutants. Nitrogenase activity was comparable among the wild-type and the three QS mutant derivatives (*braI*, *braR* and *rsaL*) at both low and high cell density (data not shown). It was therefore concluded that nitrogenase complex formation is not regulated by AHL QS in *B. kururiensis*.

Analysis of the role of BraI/R in regulating several other phenotypes in *B. kururiensis*

It was of interest to determine whether other important phenotypes were regulated by AHL QS in rice-associated *B. kururiensis* M130. We examined growth in the presence of three aromatic compounds, namely toluene, benzene and phenol, and in the presence of the tricarboxylic acid cycle intermediate succinic acid as sole carbon sources. Strain M130 grows poorly on all three aromatic compounds at both low and high cell density whereas it can utilize

succinic acid very efficiently; in all growth tests no significant differences were observed between the parent strain and the three QS mutant derivatives (data not shown). Similarly, we tested whether AHL QS was involved in siderophore production, lipase activity, and motility by swimming and swarming. *B. kururiensis* M130 displayed all these phenotypes; however, we detected no significant differences between the wild-type and all three AHL QS mutant derivatives under the conditions tested (data not shown). Interestingly, we also determined that strain M130 produces indoleacetic acid (IAA) and similarly we observed that this production was not regulated by AHL QS (data not shown).

DISCUSSION

B. kururiensis has been reported to colonize geographically distant and different environments including polluted aquifers in Japan (Zhang *et al.*, 2000) and in association with plant roots in Brazil (Weber *et al.*, 2000). This is an indication that *B. kururiensis*, and closely related species grouped in the cluster of nitrogen-fixing *Burkholderia*, can respond and adapt to varying environmental conditions. Consequently, this bacterial species is likely to possess a large set of regulatory systems, allowing it to sense and respond to many different stimuli in different niches. It is plausible that in the future more strains belonging to these species will be isolated from different habitats and also associated with different host plants. In this study it was decided to begin to investigate the regulatory mechanisms in this important new group of *Burkholderia*; we chose to investigate *B. kururiensis* M130, which has been isolated in rice and behaves as an endophyte (Baldani *et al.*, 1997a, 2000). Here it was demonstrated that strain M130 possesses an AHL QS system, which we designated BraI/R, which produces and responds to C12-3-oxo-AHL. The production of several 3-oxo-AHL derivatives by *B. kururiensis* was shown to be due only to BraI/R since mutants in both genes resulted in no production. The production of C12-3-oxo-AHL was better detected using a LasR-based biosensor, whereas when using the *A. tumefaciens* NTL4/pZLR4 biosensor C8-3-oxo-AHL appeared as the predominant AHL produced since this biosensor is most sensitive to this AHL. Since the most biologically active AHL is C12-3-oxo-AHL, at present it is not known whether the other 3-oxo-AHL derivatives produced by BraI have a biological function.

BraI/R to our knowledge is the first reported AHL QS system in the new group of *Burkholderia* and, importantly, we showed that it is highly conserved in this cluster, formed mainly for diazotrophic and legume-nodulating species. This is an indication that the system is part of the core genome of this group of bacteria rather than being acquired by a recent lateral gene transfer event. In fact, following our observation that parts of this AHL QS system could also be PCR amplified in all the strains tested, this locus might be useful in identifying strains belonging to this novel *Burkholderia* species cluster. Interestingly, BraI/R is closely related to the LasI/R system of *P. aeruginosa* and to the PpuI/R system of *P. putida*; all three systems produce and respond to C12-3-oxo-AHL. The LasI/R system of *P. aeruginosa* is interconnected with other regulatory systems and is a global regulator involved in the regulation of many genes playing a key role in virulence and colonization (Smith & Iglewski, 2003; Venturi, 2006). The PpuI/R systems of *P. putida*, however, have not been extensively studied and it is not known if AHL QS in *P. putida* behaves as a global regulatory response (Juhas *et al.*, 2005). In this study we analysed whether several important phenotypes, often associated with AHL QS in other bacterial species, are regulated by AHL QS. As most *Burkholderia* in this group are diazotrophic, it was important to establish if BraI/R was involved in this important trait. In *B. kururiensis* M130, however, atmospheric nitrogen fixation is not regulated by AHL QS. Similarly, motility, siderophore production, ability to grow on certain aromatic compounds, and production of exoenzymes were all not regulated by BraI/R.

Between the *braI/R* genes of *B. kururiensis*, as with the *lasI/R* and *ppuI/R* genes of *P. aeruginosa* and *P. putida*, respectively, there is repressor gene, *rsaL* (Fig. 2). RsaL of *P. aeruginosa* directly represses *lasI*, and *rsaL* mutants produce approximately 10-fold more C12-3-oxo-AHL in the late-exponential/stationary phase of growth when compared to the wild-type parent strain (Rampioni *et al.*, 2006, 2007b). It has been proposed that the biological role of RsaL in *P. aeruginosa* is to maintain C12-3-oxo-AHL homeostasis and to then possibly change steady-state levels upon varying environmental conditions (Rampioni *et al.*, 2007b). The scenario in *B. kururiensis*, however, is somewhat different since the parent strain under laboratory conditions synthesizes very low levels of C12-3-oxo-AHL with the situation dramatically changing when RsaL is missing: the *rsaL* mutant produces over 2000-fold more AHLs. The role of RsaL is hypothesized not to be one of homeostasis but rather a switch to turn on/off the AHL QS system; most probably under certain environmental conditions the system is very efficiently switched on. This could possibly occur thorough a mechanism of inactivation of RsaL, resulting in a very fast increase in AHL production. The ability of BraI/R to synthesize such high levels of AHLs is unique and also questions the role of this molecule in *B. kururiensis* in cell-density-dependent regulation. Stationary-phase *B. kururiensis rsaL* mutants accumulate almost 50 μM C12-3-oxo-AHL in their supernatant versus only 20 nM in the parent strain. It is true that some AHL QS systems respond to nanomolar amounts of AHLs; however, the ability of *B. kururiensis* to produce such high amounts of AHL as 45 μM indicates that strain M130 can most probably respond very quickly to a sudden need. The very stringent on/off regulation and the capacity to synthesize such high concentration of AHLs might also be an indication that the molecule may play another role in the life of this bacterium unrelated to QS. Future work needs to determine the gene targets, if any, of BraI/R as this

might provide a clue to the precise role of this system and which environmental signal(s) it responds to. The fact that several phenotypes that we have tested are not regulated by BraI/R is an indication that, unlike the LasI/R system of *P. aeruginosa*, the BraI/R system of *B. kururiensis* is not a global regulatory system. It is tempting to speculate that as this group of *Burkholderia* have been isolated from such varied and geographically distant environments, the BraI/R system might provide these bacteria, via RsaL, a specific response to a particular stimulus regulating a particular set of genes. Importantly, our studies have shown that RsaL is very well conserved among this group of *Burkholderia* and that most strains produce very low quantities of AHLs, indicating that stringent regulation of the BraI/R system is likely to be widespread.

In the genus *Burkholderia*, therefore, there are two major AHL QS systems: the CepI/R system producing and responding to C8-AHL found in members of the BCC (Eberl, 2006; Venturi *et al.*, 2004) and the BraI/R system reported here, producing and responding to C12-3-oxo-AHL found in many recently classified diazotrophic and plant-associated *Burkholderia* species (Caballero-Mellado *et al.*, 2004, 2007; Estrada-de los Santos *et al.*, 2001; Perin *et al.*, 2006a). The CepI/R system is a global regulatory system whereas the BraI/R system is stringently regulated by RsaL and most probably regulates a small set of genes in response to a particular environmental stimulus. Future work will focus on the precise regulatory role of BraI/R and its unique regulation by the RsaL repressor.

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Commonalities and Differences in Regulation of *N*-Acyl Homoserine Lactone Quorum Sensing in the Beneficial Plant-Associated *Burkholderia* Species Cluster▽†

Zulma Rocío Suárez-Moreno,[1] Giulia Devescovi,[1] Mike Myers,[2] Letícia Hallack,[4] Lucia Mendonça-Previato,[4] Jesús Caballero-Mellado,[3] and Vittorio Venturi[1]*

Bacteriology Group[1] and Protein Networks Group,[2] International Centre for Genetic Engineering and Biotechnology, Trieste, Italy; Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Ap. Postal 565-A, Cuernavaca, Morelos, México[3]; and Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Bloco G, Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, 21944-970 Rio de Janeiro, RJ, Brazil[4]

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The genus *Burkholderia* includes over 60 species isolated from a wide range of environmental niches and can be tentatively divided into two major species clusters. The first cluster includes pathogens such as *Burkholderia glumae*, *B. pseudomallei*, and *B. mallei* and 17 well-studied species of the *Burkholderia cepacia* complex. The other recently established cluster comprises at least 29 nonpathogenic species, which in most cases have been found to be associated with plants. It was previously established that *Burkholderia kururiensis*, a member of the latter cluster, possesses an *N*-acyl homoserine lactone (AHL) quorum-sensing (QS) system designated "BraI/R," which is found in all species of the plant-associated cluster. In the present study, two other BraI/R-like systems were characterized in *B. xenovorans* and *B. unamae* and were designated the BraI/R_{XEN} and BraI/R_{UNA} systems, respectively. Several phenotypes were analyzed, and it was determined that exopolysaccharide was positively regulated by the BraIR-like system in the species *B. kururiensis*, *B. unamae*, and *B. xenovorans*, highlighting commonality in targets. However, the three BraIR-like systems also revealed differences in targets since biofilm formation and plant colonization were differentially regulated. In addition, a second AHL QS system designated XenI2/R2 and an unpaired LuxR solo protein designated BxeR solo were also identified and characterized in *B. xenovorans* LB400^T. The two AHL QS systems of *B. xenovorans* are not transcriptionally regulating each other, whereas BxeR solo negatively regulated *xenI2*. The XenI2/R2 and BxeR solo proteins are not widespread in the *Burkholderia* species cluster. In conclusion, the present study represents an extensive analysis of AHL QS in the *Burkholderia* plant-associated cluster demonstrating both commonalities and differences, probably reflecting environmental adaptations of the various species.

From its establishment in 1992, the genus *Burkholderia* has been extensively studied since its members are catabolically versatile and are found in many different environments and some are of medical importance (87). Validly described species have been isolated from a wide range of niches, including soil, water, wastes, plants, fungi, animals, and humans. Importantly, several species have been reported to have either a beneficial or a pathogenic interaction with plants, animals, or humans (62, 81). Currently available *Burkholderia* genome sequences suggest that this genus owes its niche versatility to its large genomes comprised of several large replicons, as well as to lateral gene transfer events and plasmid acquisition (13, 44).

Taxonomic analysis of more than 60 species described to date shows an internal division of the genus that can be viewed in two major clusters (11, 49). The first cluster includes pathogens such as *Burkholderia glumae*, *B. pseudomallei*, and *B. mallei*, as well as the 17 well-studied species of the *Burkholderia cepacia* complex (BCC) (83). The second and more recently established cluster comprises more than 25 related environmental nonpathogenic species, which in most cases have been found to be associated with plants. Several interesting properties found in members of the latter cluster include (i) their ability to colonize the rhizosphere or the internal intercellular spaces in several plants and promote plant growth, as is the case for *B. kururiensis* and *B. phytofirmans* (35, 50); (ii) the potential to increase plant nutrient availability via nitrogen fixation and/or phosphate solubilization, as demonstrated for *B. unamae*, *B. tropica*, and *B. silvatlantica* (10, 59, 62); (iii) the strong catabolic potential to degrade aromatic compounds, with *B. xenovorans* being the best known example (31); and (iv) their ability to form symbiotic interactions with plants as occurs, for example, with *B. tuberum*, *B. phymatum*, *B. mimosarum*, *B. nodosa*, and *B. sabiae* (15–17, 24, 80), and with mosses, as reported for *B. megapolitana* and *B. bryophila* (82). However, despite the increasing attention given to these species, the molecular mechanisms regulating most of their properties are still not addressed or poorly understood.

Bacteria communicate with neighbors and monitor their population density by producing and sensing signaling molecules in a process called quorum sensing (QS) (29). The concentration of the signaling molecule increases alongside the

* Corresponding author. Mailing address: Bacteriology Group, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy. Phone: 390403757319. Fax: 39040226555. E-mail: venturi@icgeb.org.

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bacterial population density and, when it reaches a critical level, bacteria respond and modulate target gene expression. A typical QS system in Gram-negative bacteria involves the production and response to an acylated homoserine lactone (AHL) signal molecule produced by an AHL synthase which in most cases belongs to the LuxI protein family. A transcriptional regulator belonging to the LuxR family forms a complex with the cognate AHL at threshold ("quorum") concentration and affects the transcriptional status of target genes. Traits under QS control in bacteria are most beneficial to a bacterial community and include biofilm formation, virulence, plant-growth promoting activity, and antibiotic production.

QS has been extensively studied in some *Burkholderia* species. For example, all species of the BCC complex share a conserved QS system designated CepI/R that produces and responds to *N*-octanoyl homoserine lactone (C_8-AHL) regulating virulence and several important phenotypes such as biofilm formation and siderophore production (23, 36, 84). Several *Burkholderia* species have been shown to harbor more than one LuxI/R family pair and produce numerous AHL signal molecules, as reported for *B. cenocepacia*, *B. vietnamiensis*, *B. mallei*, *B. pseudomallei*, and *B. thailandensis* (21, 22, 39, 45, 47, 78). Furthermore, unpaired LuxR "solo" proteins that lack a cognate LuxI AHL synthase (74) have been characterized for *B. cenocepacia* (46). In contrast, little is known about the role of QS for members of the nonpathogenic plant-associated nitrogen-fixing *Burkholderia* cluster. In a previous study we reported the presence of an AHL-based QS system in the rice endophyte *B. kururiensis* designated BraI/R_{KUR}, which produces and responds to 3-oxo-C_{12}-HSL. It was determined that the BraI/R_{KUR} system is stringently regulated by RsaL, and it is not involved in the regulation of nitrogen fixation or in other several important phenotypes (73). Importantly, BraI/R was found to be highly conserved in 20 species of the recently described cluster (73).

In the present study, the AHL QS systems of *B. unamae* and *B. xenovorans*, were identified and characterized in order to obtain more insight into AHL QS in the cluster of plant-associated *Burkholderia* species. We demonstrate that the genome of *B. xenovorans* LB400^T possessed a BraI/R-like system (designated BraI/R_{XEN}) but evidenced the presence of a second AHL QS system, which we designated XenI2/R2. This system was found to be present in several but not all of the species belonging to the cluster of plant-associated *Burkholderia*. We performed experiments aimed at elucidating whether the two systems in *B. xenovorans* are hierarchically organized or whether they act independently. In addition, we report the presence of a LuxR solo protein in several species of this cluster. Finally, we also identified gene targets for the conserved BraI/R-like system and studied other aspects of the AHL QS systems in three *Burkholderia* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Burkholderia kururiensis*, *B. xenovorans*, *B. unamae*, *Pseudomonas* spp., and *E. coli* strains and plasmids used in the present study are listed in Table 1. The list of primers used and the construction of most recombinant plasmids is given in Table S1 in the supplemental material. All other *Burkholderia* strains used in the present study are listed in Table S2 in the supplemental material. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. All *Burkholderia* strains were grown in King's medium (KB) (38) or LB at 30°C. Antibiotics were added when required at the following final concentrations: ampicillin, 100 μg ml^{-1}; tetracycline, 15 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Burkholderia*); gentamicin, 10 μg ml^{-1} (*E. coli*), 30 μg ml^{-1} (*Agrobacterium*), or 40 μg ml^{-1} (*Burkholderia*); kanamycin, 50 μg ml^{-1} (*E. coli*) or 100 μg ml^{-1} (*Burkholderia*); and nitrofurantoin, 50 μg ml^{-1}. Conjugations in *B. kururiensis*, *B. unamae*, and *B. xenovorans* were performed by triparental mating using *E. coli* DH5α (pRK2013) as a helper (27) and incubated 22 h at 30°C. Transconjugants were counterselected in KB with the appropriate antibiotics.

Recombinant DNA techniques. Recombinant DNA techniques, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, end filling with the Klenow enzyme, hybridization, radioactive labeling by random priming, and transformation of *Escherichia coli* were performed as described previously (65). Southern hybridizations were performed using Amersham Hybond-XL membranes (Amersham, Biosciences); plasmids were purified by using EuroGold columns (EuroClone, Italy); total DNA from *Burkholderia* was isolated by Sarkosyl-pronase lysis as described previously (8). Generated plasmids were sequenced by Macrogen (South Korea). The fidelity of all marker exchange events was confirmed by Southern analysis.

β-Galactosidase activities were determined essentially as described by Miller (51) with the modifications of Stachel et al. (71); all experiments were performed in triplicate, and the mean value is given.

Identification, inactivation, and characterization of QS genes in *B. unamae* MTl-641^T. To identify the QS system of *B. unamae* MTl-641^T, a cosmid library was constructed by using the cosmid pLAFR3 (72) as a vector. Insert DNA was prepared by partial EcoRI digestion of the genomic DNA and then ligated in the corresponding site in pLAFR3. The ligated DNA was then packaged into λ phage heads by using Gigapack III Gold packaging extract (Stratagene), and the phage particles were transduced to *E. coli* HB101 as recommended by the supplier. In order to identify the cosmid containing the AHL QS genes, the *E. coli* HB101 harboring the cosmid library was conjugated *en masse* into the AHL biosensor *Pseudomonas putida* F117(pKRC12) as acceptor (63). One transconjugant carrying the cosmid pLZ1 displayed green fluorescent protein (GFP) expression and was further studied. The $braI_{UNA}$ and $braR_{UNA}$ loci were located within a 8-kb EcoRV fragment and cloned in pMOSBlue to generate pMOS-pLZ-1, which was sequenced.

To generate genomic $braR_{UNA}$- and $braI_{UNA}$-null mutants, internal fragments from each gene were PCR amplified with primers described in Table S1 in the supplemental material and then cloned into pKNOCK-Km(2) to yield the suicide vectors pKNOCK-UNAI and pKNOCK-UNAR. Each plasmid was then mobilized into strain MTl-641^T to generate *B. unamae* UNABRAI and *B. unamae* UNABRAR.

To identify the $BraR_{UNA}$ cognate AHL, $braR_{UNA}$ was PCR amplified from genomic DNA and cloned into pQE30 to generate pQEUNAR. The $braI_{UNA}$ promoter region was PCR amplified and cloned into pMP220 promoter probe vector to generate pMPUNAI (see the cloning details in Table S1 in the supplemental material). *E. coli* M15(pQEUNAR)(pMPUNAI) was then inoculated into 10 ml of LB-Amp-Km-Tet, grown overnight, and diluted to an optical density (OD) of 0.1 in 10 ml of prewarmed medium with 1 μM concentrations of each synthetic AHL. The β-galactosidase activity was determined after 4 h at 37°C.

Isolation of the $braI_{XEN}$ and $braR_{XEN}$ and the *xenI2* and *xenR2* AHL QS systems and of the *bxeR* solo gene of *Burkholderia xenovorans* LB400^T and construction of derivative genomic mutants. $braI_{XEN}$, $rsaL_{XEN}$, and $braR_{XEN}$ were identified by *in silico* comparison of the DNA and protein sequences with the genome of *B. xenovorans* LB400^T(13) as the three loci Bxe_B0610, Bxe_B0609, and Bxe_B0608. Similarly, *xenI2*, *xenR2*, and *bxeR* solo were identified as the genomic loci Bxe_C0416, Bxe_C0415, and Bxe_B2275, respectively. To generate knockout mutants of $braR_{XEN}$, $braI_{XEN}$, *xenI2*, *xenR2*, and *bxeR*, internal fragments from each gene were PCR amplified (see Table S1 in the supplemental material), and cloned into pKNOCK-Km to yield the suicide plasmids pKNOCKXENR, pKNOCKXENI, pKNOCKXENI2, pKNOCKXENR2, and PKNOCKBXER. These plasmids were then conjugated into *B. xenovorans* LB400^T to generate the genomic mutants LB400BRAR, LB400BRAI, LB400XENI2, LB400XENR2, and LB400BXER, respectively. Growth curves and CFU/ml for wild type and mutants were determined in KB media.

For complementation purposes, the $braI_{XEN}$ gene and its promoter region were PCR amplified and cloned into pMOS-Blue. A 623-bp fragment containing $braR_{XEN}$ was then excised using XbaI-KpnI and ligated into pBBR-MCS5 (42) to generate pBBRXENI. Similarly, the *bxeR* gene and its promoter region were PCR amplified and cloned into pGEM-T Easy. A 1,263-bp fragment was then excised using BamHI-SpeI and cloned into pBBRMCS-5 to generate pBBRBXER (see the primers in Table S1 in the supplemental material).

The presence of systems highly identical to that of *xenI2* and *xenR2* in four

TABLE 1. *B. kururiensis, B. xenovorans, B. unamae, P. putida*, and *E. coli* strains and plasmids used in this study

Strain or plasmid[a]	Characteristics[b]	Reference or source
Strains		
B. kururiensis M130	Amp^r Rif^r, isolated from surface-sterilized roots of rice	5
M130BRAI	*braI*::Km of *B. kururiensis* M130	73
M130BRAR	*braR*::Km of *B. kururiensis* M130	73
M130RSAL	*rsaL*::Km of *B. kururiensis* M130	73
B. xenovorans LB400^T	Type strain	31
LB400BRAI	*braI*::Km of *B. xenovorans* LB400^T	This study
LB400BRAR	*braI*::Km of *B. xenovorans* LB400^T	This study
LB400XENI2	*xenI2*::Km of *B. xenovorans* LB400^T	This study
LB400XENR2	*xenR2*::Km of *B. xenovorans* LB400^T	This study
LB400BXER	*bxeR:*:Km of of *B. xenovorans* LB400^T	This study
B. unamae MTl-641^T	Maize rhizosphere isolate	10
UNABRAI	*braI*::Km of *B. unamae* MTl-641	This study
UNABRAR	*braR:*:Km of *B unamae* MTl-641	This study
P. putida F117	AHL-negative derivative of *P. putida* IsoF; PpuI$^-$	63
E. coli M15	NaI Str Rif Thi Lac− Ara+ Gal+ Mtl− F− RecA+ Uvr+ Lon+	Qiagen
Plasmids		
pRK2013	Tra$^+$ Mob$^+$ ColE1 replicon; Km^r	27
pMOSBlue	Cloning vector; Amp^r	Amersham-Pharmacia
pGEM-T Easy	Cloning vector; Amp^r	Promega
pMP220	Promoter probe vector, IncP, LacZ; Tet^r	70
pLAFR3	Broad-host-range cloning vector, IncP1; Tet^r	72
pKNOCK-Km	Conjugative suicide vector; Km^r	2
pLZ1	pLAFR3 containing *B. unamae* DNA	This study
pBBRMCS-5	Broad-host-range vector Gm^r	42
pKRC-12	pBBR1MCS-5 carrying P_{lasB}-*gfp* (ASV) P_{lac}-*lasR*; Gm^r	63
pMOS-LZ-1	pMOSBlue carrying an 8-kb fragment containing partial *B. kururiensis* QS genes; Amp^r	This study
pKNOCK-UNAI	Internal PCR $braI_{UNA}$ fragment of *B. unamae* cloned in pKNOCK-Km	This study
pKNOCK-UNAR	Internal PCR $braR_{UNA}$ fragment of *B. unamae* cloned in pKNOCK-Km	This study
pKNOCKXENR	Internal PCR $braR_{XEN}$ fragment of *B. xenovorans* cloned in pKNOCK-Km	This study
pKNOCKXENI	Internal PCR $braI_{XEN}$ fragment of *B. xenovorans* cloned in pKNOCK-Km	This study
pKNOCKXENI2	Internal PCR *xenI2* fragment of *B. xenovorans* cloned in pKNOCK-Km	This study
pKNOCKXENR2	Internal PCR *xenR2* fragment of *B. xenovorans* cloned in pKNOCK-Km	This study
pKNOCKBXE	Internal PCR *bxeR* fragment of *B. xenovorans* cloned in pKNOCK-Km	This study
PMPXENI1	promoter of gene $braI_{XEN}$ cloned in pMP220 vector	This study
pMPX2I	Promoter of gene *xenI2* cloned in pMP220 vector	This study
pMPBXE	Promoter of gene *bxeR* cloned in pMP220 vector	This study
pMP2786	Promoter of gene Bxe_B2786 cloned in pMP220 vector	This study
pMP0016	Promoter of gene Bxe_B0016 cloned in pMP220 vector	This study
pBBRXENI	$braI_{XEN}$ cloned into pBBR-MCS5 Gm^r	This study
pMPUNAI	promoter of gene $braI_{UNA}$ cloned in pMP220 vector	This study
pGEMXENR1	$braR_{XEN}$ cloned into pGEM; Amp^r	This study
pGEMX2R	*xenR2* cloned into pGEM; Amp^r	This study
pQEXENR1	$braR_{XEN}$ cloned into pQE30 expression vector; Amp^r	This study
pQEXENR2	*xenR2* cloned into pQE30 expression vector; Amp^r	This study
pQEBXER	*bxeR* cloned into pQE30 expression vector; Amp^r	This study
pQEUNAR	$braR_{UNA}$ cloned into pQE30 expression vector; Amp^r	This study
pQE30	Expression vector, ColE1 replication origin, T5 promoter, His epitope; Amp^r	Qiagen

[a] A superscript "T" indicates a type strain.
[b] Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Rif^r, rifampin resistance; Km^r, kanamycin resistance.

other *B. xenovorans* strains, as well as in 21 *Burkholderia* species, was determined by Southern analysis. This analysis was performed on EcoRI- or PstI-digested genomic DNAs and hybridized with a 794-bp probe comprising the *xenR2* gene. This fragment was amplified by PCR from *B. xenovorans* genomic DNA and cloned into pGEM-T Easy to generate pGEMX2R (see Table S1 in the supplemental material). The plasmid was sequenced, and a probe was generated by random priming using the excised SpeI-NotI fragment as a template.

To generate pMP2786 and pMP0016, the promoter regions of Bxe_B2786 and Bxe_B0016 were PCR amplified and cloned as EcoRI-XbaI fragments in pMP220, respectively (primers are indicated in Table S1 in the supplemental material).

Determination of the cognate AHL for BraI/R$_{XEN}$ and XenI2/R2 AHL QS systems and cross-talk regulation between BraI/R$_{XEN}$, XenI2/R2, and the unpaired BxeR solo. The $braR_{XEN}$ and *xenR2* genes were PCR amplified using genomic DNA as a template, and amplimers were cloned into pGEM-T Easy.

Each gene was then excised with BamHI/HindIII and ligated into the corresponding sites of pQE30 to generate pQEXENR1 and pQEXENR2, respectively (for the primers, see Table S1 in the supplemental material). The gene promoters of $braI_{XEN}$ and *xenI2* were amplified and cloned in the promoter probe vector pMP220 to generate pMPXENI1 and pMPX2I as described in Table S1 in the supplemental material.

E. coli M15(pQXENR1)(pMPXENI1) and *E. coli* M15(pQEXENR2)(pMPX2I) were inoculated into 10 ml of LB-Amp-Km-Tet, grown overnight, and then diluted to an OD of 0.1 in 10 ml of prewarmed medium with 1 μM concentrations of each synthetic AHL to be evaluated. The β-galactosidase activity was then determined after 4 h at 37°C. Possible regulation by $BraR_{XEN}$ of the *xenI2 p*romoter and of the $braI_{XEN}$ promoter by XenR2 was evaluated by transforming the plasmid gene promoter fusion into *E. coli* M15(pQEXENR1) and *E. coli* M15(pQEXENR2), respectively. The β-galactosidase activity was measured in the presence of the cognate AHL after 4 h of growth.

BxeR and *bxeR* studies. To overexpress BxeR, *bxeR* was PCR amplified and cloned into pGEM-T Easy (see the primers in Table S1 in the supplemental material). This fragment was then excised with BamHI-HindIII and cloned into pQE30 to generate pQEBXER. When required, the same fragment was used as a template to generate the radioactive DNA probe representing *bxeR*. Plasmid pMPBXE, containing the promoter of *bxeR*, was PCR amplified and then excised and cloned, using BamHI-XbaI, into pMP220 to generate pMPBXE (see the primers in Table S1 in the supplemental material).

Regulation by BxeR solo of the AHL QS systems of *B. xenovorans* was determined as follows. *E. coli* overexpressing BxeR via the pQEBXER plasmid was transformed with either pMPXENI1 or pMPX2I, as well as with the *bxeR* promoter transcriptional fusion pMPBXE. *E. coli* M15(pQEBXER)(pMPX2I), *E. coli* M15 (PQEBXER)(pMPXENI1), and *E. coli* M15 (PQEBXER)(pMPBXE) were then grown in the presence of three independent AHL mixtures. One contained 1 μM each of nine different unsubstituted AHLs at position 3, the second mixture contained 1 μM concentrations each of seven different 3-oxo-substituted AHLs, and a third cocktail contained 1 μM each of seven different 3-OH-substituted AHLs. In these three growth conditions, the β-galactosidase activities were determined.

AHL extraction, visualization, and quantification. AHLs were purified from spent supernatant and separated by using a C_{18} reversed-phase thin-layer chromatography (TLC) plates as previously described (68). For visualization by TLC, the plate was overlaid with a thin layer of AB top agar seeded with *Agrobacterium tumefaciens* NTL4(pZLR4) in the presence of 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml, as described previously (68), or with LB top agar seeded with *E. coli* pSB1075 (86).

Motility assays, biofilm formation, EPS and siderophore production, and lipase and protease activities. The lipolytic activity was determined in trybutyrin agar, as previously described (3). The protease activity was determined in the KB plates supplemented with 2% skim milk (36).

Swimming assays were performed on 0.25% KB agar plates and 0.25% nutrient agar. Swarming assays were performed using 0.5% nutrient agar plates and M8 medium plates (M9 salts without NH_4Cl) (40) supplemented with 0.2% glucose, 0.05% glutamate, and 0.5% agar (52). The inoculation was performed by spotting 1 μl of a bacterial suspension having an OD at 600 nm (OD_{600}) of 1. The swimming and swarming zones were measured after 48 h of incubation at 30°C for all wild-type species and their QS mutant derivatives.

EPS production was tested by streaking single colonies in yeast extract mannitol medium, as described previously (88). EPS was extracted and quantified by the boiling phenol method, as described previously (20).

Biofilm formation was determined as previously described (36, 55). Briefly, single colonies were grown overnight in AB medium supplemented with 10 mM glucose, washed, and diluted to an OD_{600} of 0.01. Then, 100 μl of bacterial dilution was inoculated into the round-bottom wells of microtiter dishes, followed by incubation at 30°C. Biofilm formation was evaluated after 24, 48, 72, and 120 h. After the incubation time, the OD_{550} was determined prior to medium removal. Importantly, the mutants under these growth conditions did not display significant growth differences compared to the wild type (data not shown). Next, 100 μl of 1% (wt/vol) aqueous solution of crystal violet was added, followed by incubation at room temperature for 20 min, and the mixture was then washed thoroughly with water. For quantification of the attached cells, the crystal violet was solubilized in 120 μl of dimethyl sulfoxide, and the absorbance was determined at 570 nm.

The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol S (CAS) agar plates (66, 73). The cultures were harvested, and the pellets washed and adjusted to an OD_{600} of 1. Then, 2-μl portions of culture were spotted on the surface of the plate, followed by incubation for 48 h at 30°C. Siderophore production was measured by the size of the orange halos formed around the colonies.

Analysis and identification of secreted proteins. Overnight cultures were washed and diluted to an OD_{600} of 0.05 in 40 ml of prewarmed LB. After 12 h of incubation, the cultures were centrifuged for 15 min at 8,000 × *g* at 4°C. Culture supernatants were filtered through a 0.45-μm-pore-size filter (Millipore), and the proteins were precipitated overnight at 4°C with 10% (vol/vol) trichloroacetic acid (final concentration). The precipitates were separated by centrifugation at 15,000 × *g* for 20 min at 4°C, and the pellets were washed twice with ice-cold acetone. Another centrifugation was performed at 15,000 × *g* for 20 min, and protein pellets were air dried and resuspended in sample buffer. The suspension was then boiled for 10 min, and the proteins were separated by SDS-PAGE on gels containing 12% (wt/vol) polyacrylamide. The proteins were identified by mass spectroscopy as described by Tomaic et al. (76).

Growth of *B. unamae* MTl-641^T and mutant derivatives using phenol as a carbon source. *B. unamae* MTl-641^T, UNABRAI, UNABRAR, and UNABRAR complemented with pLZ1 were grown in BSE liquid medium (25), and the cultures were incubated at 29°C with reciprocal shaking (200 rpm) for 18 h. The cultures were adjusted to ~10^4 CFU/ml, and 1.0 ml was then inoculated into 99 ml of SAAC culture medium containing either phenol or mannitol 0.05% (11). Growth was tested at 24, 48, and 72 h.

Plant colonization assays. Plant colonization was conducted as previously described (50). Briefly, rice seeds were dehusked, surface sterilized, and transferred to water-based 0.5% agar for seed pregermination, following incubation for 3 days at 28°C, in the absence of light. The pregerminated rice seeds were aseptically transferred to glass tubes (4 cm in diameter, 29 cm in height) containing 20 ml of a nitrogen-free Hoagland's nutrient solution (33). Plantlets infection assays were carried out by inoculation of 500 μl of bacterial culture (~10^9 CFU) into each glass tube. After incubation for 12 days with a 12-h photoperiod at 28°C, plantlets were collected and cut (roots and aerial parts). The excised plant segments were subjected to surface sterilization with 1% sodium hypochlorite for 5 min, followed by several washes with sterile water. The plant segments were then weighed, transferred to microcentrifuge tubes containing 1 ml of sterile nutrient solution, and macerated with a pestle. From each of the obtained suspensions, a series of 10-fold dilutions were prepared using sterile saline, and aliquots of 100 μl were spread plated onto LB medium, followed by incubation for 4 days at 28°C. Bacterial quantification was expressed as CFU/g of fresh weight plant tissue.

B. unamae strains were grown in BSE liquid medium (25), and the cultures were incubated at 29°C with reciprocal shaking (200 rpm) for 18 h. Thereafter, the cultures were adjusted to an OD_{600} of 0.15 (~1.5×10^7 CFU/ml). Three germinated seeds of maize (*Zea mays*) were sown per pot containing sterile river sand, and each seed was inoculated with 1.0 ml of bacterial culture. The plants were grown for 9 days (7 days after plant emergence). The three roots of each pot were cut into segments of ~1 cm and mixed. Two grams of the roots mixture was macerated with 10 mM $MgSO_4 \cdot 7H_2O$ to get a dilution 10^{-1}, and further dilutions were prepared and streaked onto BAc agar plates (25) containing the appropriate antibiotic; three replicates were made from each dilution.

Statistical analysis. All experiments were performed at least three times, and mean values are given. Statistical analysis included unpaired *t* tests and analysis of variance (ANOVA) with Dunnett's post-test and were performed with Prism 4.0 software (GraphPad, San Diego, CA). A *P* value of <0.05 was considered significant.

DNA sequencing and nucleotide sequence accession numbers. All DNA sequencing was performed either at the CRIBI center (University of Padua, Padua, Italy) or at Macrogen, and the nucleotide sequences were deposited in GenBank/EMBL/DDBJ. The BraI/R_{UNA} QS loci of *B. unamae* MTl-641^T is listed as a 1,977-bp fragment of pMOS-LZ1 under accession number FN640548.

RESULTS

AHL QS systems in *B. xenovorans* and *B. unamae*. To identify the AHL QS system(s) of *B. unamae*, a cosmid gene library of strain MTl-641^T was constructed and screened against the LasR-based *P. putida* F117(pRKC12) AHL biosensor (63). One cosmid (pLZ1) was identified and possessed an AHL QS system that consisted of three open reading frames (ORFs); two of them ($braI_{UNA}$ and $braR_{UNA}$) displayed homology to *luxI* and *luxR* family genes, while a third ORF, located in

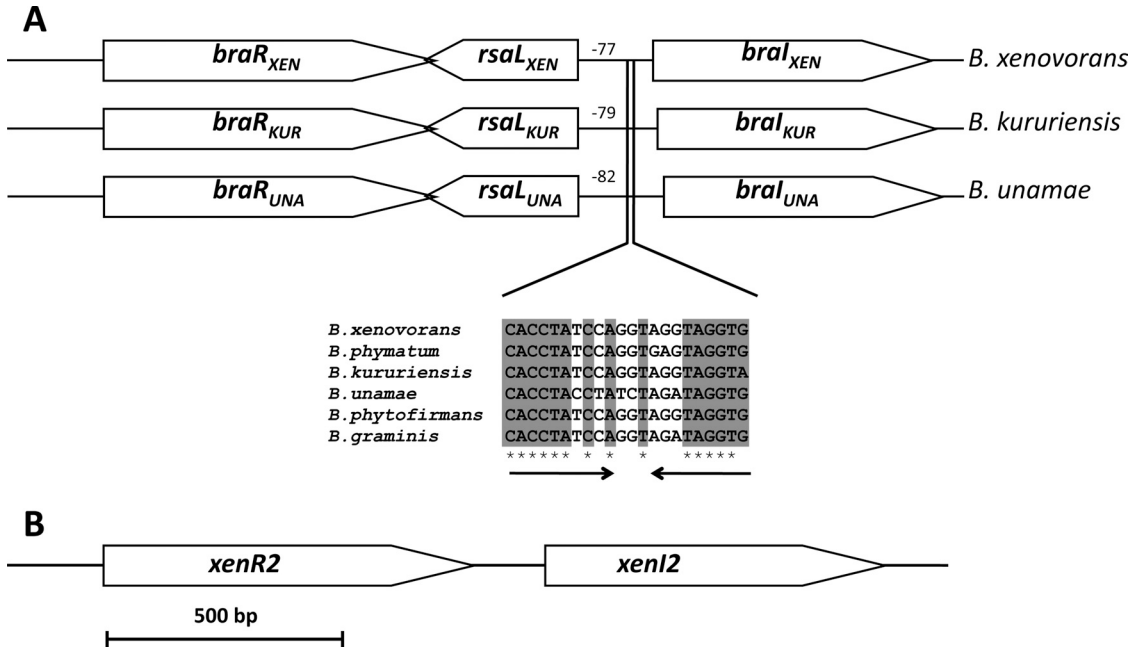

FIG. 1. (A) Genetic maps of the *B. xenovorans* LB400^T (*braI*$_{XEN}$ and *braR*$_{XEN}$), *B. kururiensis* M130 (*braI*$_{KUR}$ and *braR*$_{KUR}$), and *B. unamae* MTl-641^T (*braI*$_{UNA}$ and *braR*$_{UNA}$) QS systems. An alignment of putative *lux* boxes is shown in several members of the *Burkholderia* cluster; numbers indicate the positions upstream where the *lux* box is centered with the respect to the putative ATG start codon. (B) Map of the *xenI2* and *xenR2* system of *B. xenovorans* LB400^T.

between *braI*$_{UNA}$ and *braR*$_{UNA}$, and divergently transcribed from *braI*$_{UNA}$, displayed high similarity to the *rsaL* repressor. The BraR$_{UNA}$ protein consists of 235 amino acids 77% identical to BraR$_{KUR}$, while the acyl-homoserine lactone synthase BraI$_{UNA}$ comprises 197 amino acids displaying 82% identity to BraI$_{KUR}$. RsaL$_{UNA}$ from *B. unamae* is 103 amino acids long with 72% identity to RsaL$_{KUR}$ from *B. kururiensis* (Fig. 1A). It was therefore concluded that BraI/R$_{UNA}$ was an ortholog to the *B. kururiensis* BraI/R$_{KUR}$ system.

In silico analysis of the sequenced genome of *B. xenovorans* LB400^T revealed that it possessed two complete *luxI* and *luxR* systems, referred to here as the *braI*$_{XEN}$ and *braR*$_{XEN}$ system (BraI/R$_{XEN}$ system) and the *xenI2* and *xenR2* system (13). The BraI/R$_{XEN}$ system was located in chromosome two, whereas the *xenI2* and *xenR2* system was located in the 1.47-Mb megaplasmid. The first system, the BraI/R$_{XEN}$ system, displays an organization similar to that of the BraI/R$_{KUR}$ and BraI/R$_{UNA}$ systems described above. In fact, BraR$_{XEN}$, RsaL$_{XEN}$, and BraI$_{XEN}$ (234, 105, and 197 amino acids, respectively) show high identity values (>75%) to BraI/R$_{KUR}$ and BraI/R$_{UNA}$ and, since they have very high identities and produce and respond to the same AHLs, these three systems may be considered orthologous (see below). Highly similar systems with the same gene organization were also found in the sequenced genomes of three other members of this species cluster, namely, *B. phytofirmans* (ORFs Bphyt_4277, Bphyt_4276 and Bphyt_4275), *B. graminis* (ORFs BgramDRAFT_3087, BgramDRAFT_3088, and BgramDRAFT3089), and *B. phymatum* (Bphy_4439, Bphy_4438, and Bphy_4437). When a nucleotide sequence analysis in the promoter regions of the five AHL synthase *braI*-like genes was performed, it was observed that a consensus putative *lux*-box was located between −77 and −82 with respect to the ATG translational start codon (Fig. 1A).

The XenI2/R2 system of *B. xenovorans* LB400^T. As mentioned above, in the genome of *B. xenovorans* LB400^T a second putative AHL QS system was found and designated the *xenI2* and *xenR2* system. This system is located in the loci Bxe_C0415 and Bxe_C0416 of the 1.47-Mb megaplasmid (Fig. 1B). The AHL synthase XenI2 is composed of 249 amino acids, and the sensor/response regulator XenR2 is composed of 262 amino acids; both display ca. 70% identity to two uncharacterized putative LuxI/R-family members of the species cluster, namely, *B. graminis* (loci BgramDRAFT_4129 and BgramDRAFT 4128) and *B. phytofirmans* (loci Bphyt_0126 and Bphyt_0127). It was therefore concluded that, like *B. xenovorans* LB400^T, the genomes of these two other species of the cluster also possess two AHL QS systems. Interestingly, XenI2 displays 49% identity to BtaI3 of *Burkholderia thailandensis* and BpsI3 of *Burkholderia pseudomallei* and BmaI3 of *Burkholderia mallei*; these three *Burkholderia* spp. do not belong to this newly described plant-associated species cluster and are more related to the *Burkholderia cepacia* complex (18). Similarly, XenR2 displays high identity (ca. 42%) to BtaR3 and BpsR3 of *B. thailandensis* and *B pseudomallei*, respectively. The two AHL QS systems of strain LB400 display a low level of relatedness since they have less than 25% similarity at protein level.

***B. xenovorans* LB400^T possesses an unpaired LuxR solo protein.** Analysis of the genome sequence of *B. xenovorans* revealed also the presence of an unpaired LuxR solo protein having the typical modular structure of QS LuxR family proteins (58, 74). This ORF, which we designated BxeR (Bxe_B2275), contained the helix-turn-helix (HTH) DNA binding

motif, as well as AHL-binding domain typical of LuxR QS family of proteins. BxeR encodes a 333-amino-acid protein with an autoinducer binding domain (Pfam03472) from positions 100 to 254 and an HTH domain (Pfam PF00196) from positions 271 to 328. BxeR contains the six well-conserved amino acids in the AHL-binding domain typically shared by the QS LuxR-family members (data not shown). BxeR is 95 and 90% similar to uncharacterized orthologs of *B. phytofirmans* (Bphyt_6042) and *B. graminis* (BgramDRAFT_2595), respectively.

Presence of the *xenI2* and *xenR2* system and of the *bxeR* solo gene in the recently described *Burkholderia* cluster. In order to determine the distribution of the *xenI2* and *xenR2* system and of *bxeR* solo in other *B. xenovorans* strains, as well as in the remaining members of the beneficial *Burkholderia* cluster, Southern hybridization under high-stringency conditions was performed with 26 strains representing 22 species using *xenR2* and *bxeR* as probes (see Table S2 in the supplemental material). The *xenR2* probe hybridized to six different species (*B. graminis*, *B. phytofirmans*, *B. fungorum*, *B. terricola*, *B. ferrariae*, and *B. silvatlantica*). Interestingly, however, no hybridization was observed in any *B. xenovorans* strain other than LB400^T. In contrast, the *bxeR* solo gene was found to be present in all *B. xenovorans* strains, as well as in seven members of the cluster of plant-associated species, four of which also possessed the *xenI2* and *xenR2* system.

AHL production by *B. unamae* and *B. xenovorans*. It was of interest to determine which AHLs were produced by *B. unamae* and by *B. xenovorans*. TLC analysis suggested that both strains most likely produced 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL (Fig. 2A and C). In *B. xenovorans* the most likely production of C_{14}-3-oxo-HSL was also detected. In both species, the *braI* and *braR* orthologs were inactivated to generate knockout mutants *B. unamae* UNABRAI and UNABRAR and *B. xenovorans* LB400BRAI and LB400BRAR. It was established that both *B. unamae* UNABRAI and UNABRAR were unable to synthesize AHLs (Fig. 2A and B), indicating that the BraI/R_{UNA} system is responsible for synthesizing all of the AHLs identified, and most probably no other AHL QS system was present in *B. unamae*. In addition, the lack of AHL production in UNABRAR suggests that $BraR_{UNA}$ positively regulates the $braI_{UNA}$ synthase through a positive autoinduction loop. The production of the putative AHLs synthesized by BraI/R_{UNA} could be rescued by genetic complementation of the UNABRAR mutant by providing $braR_{UNA}$ in the cosmid pLZ1 (Fig. 2A and B).

B. xenovorans mutants LB400BRAI and LB400BRAR were both unable to produce 3-oxo-C_{10}-HSL, 3-oxo-C_{12}-HSL, and 3-oxo-C_{14}-HSL as shown by the AHL production profile observed using the LasR-based biosensor *E. coli* pSB1075 (Fig. 2D). AHL production profile of the two mutants using *A. tumefaciens* NTL4(pZLR4) evidenced the putative production of 3-OH-C_8-HSL, indicating that the XenI2/R2 was most probably responsible for the synthesis of this AHL (Fig. 2C; see also below). As in *B. unamae*, the $BraR_{XEN}$ mutant of *B. xenovorans* did not produce 3-oxo-C_{10}-HSL, 3-oxo-C_{12}-HSL, or 3-oxo-C_{14}-HSL, suggesting a positive-feedback loop (Fig. 2D). The synthesis of these AHLs could be complemented in the LB400BRAI mutant by providing $braI_{XEN}$ in *trans* via plasmid pBBRXENI1 (Fig. 2D). TLC analysis of the AHL molecules produced by the *xenI2* and *xenR2* mutants revealed the putative production of 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, 3-oxo-C_{12}-HSL, and 3-oxo-C_{14}-HSL, confirming that $BraI_{XEN}$ is responsible for the production of this later group of AHLs. Finally, TLC analysis of the AHL molecules produced by the *bxeR* knockout solo mutant LB400BXER did not show significant differences between their AHL profile in comparison to the wild type. All of the generated knockout mutants did not exhibit any growth differences in the media that we have used.

BraIR-like systems respond to 3-oxo-C_{14}-HSL. In order to determine to which AHL $BraR_{XEN}$ and $BraR_{UNA}$ best responded, the proteins $BraR_{XEN}$ and $BraR_{UNA}$ were overexpressed in *E. coli* M15 in the presence of different AHL molecules, and the cognate $braI_{XEN}$ and $braI_{UNA}$ promoter activities were determined. Testing the promoter activity in *E. coli* M15(pMPXENI1)(pQEXENR1) upon the presence of many different AHLs showed that the activity of the $braI_{XEN}$ promoter increased 30-fold in the presence of 3-oxo-C_{14}-HSL, 10-fold in the presence of 3-oxo-C_{16}-HSL, and only 2-fold in the presence of 3-oxo-C_{12}-HSL, demonstrating a specific preference of $BraR_{XEN}$ for 3-oxo-C_{14}-HSL (Fig. 3A). An analog experiment performed in *E. coli* M15(pMPUNAI)(pQEUNAR1) suggested that $braI_{UNA}$ promoter activity increased 3.5-fold upon the presence of 3-oxo-C_{14}-HSL and 2-fold when 3-oxo-C12 HSL was present in the media, indicating that 3-oxo-C_{14}-HSL was most likely the cognate AHL for $BraR_{UNA}$ (Fig. 3B). We had previously concluded that $BraR_{KUR}$ responded to 3-oxo-C_{12}-HSL by using a similar approach (73). In the present study, we tested whether AHLs with acyl chains longer than C_{12} could be recognized by $BraR_{KUR}$ and determined that it responded well to 3-oxo-C_{16}-HSL and very well to 3-oxo-C_{14}-HSL, (Fig. 3D). It was therefore concluded that 3-oxo-C_{14}-HSL was the most likely cognate AHL for BraIR-like systems.

XenR2 responds to 3-OH-C_8-HSL and is not transcriptionally regulated by $BraR_{XEN}$ in *B. xenovorans* LB400^T. Similarly for XenR2, we tested the response of many different AHLs using *E. coli* M15(pMPX2I)(pQEXENR2). The activity of *xenI2* promoter increased 100-fold upon the presence of 3-OH-C_8-HSL, 50-fold in the presence of 3OH-C_6-HSL, and 25-fold in the presence of 3-oxo-C_8-HSL (Fig. 3C). This result is in part in accordance with the AHL production profile detected for the *xenI2* genomic mutant LB400XENI2, in which 3-OH-C_8-HSL was detected as being produced by XenI2 (see above). The response to 3-oxo-C_8-HSL suggested that XenR2, when exposed to high concentrations of AHLs, displayed a relaxed specificity, allowing it to respond to structurally related AHLs.

Experiments aimed at establishing whether there is any hierarchical transcriptional organization between the two AHL QS systems of *B. xenovorans* strongly suggested that the systems were not transcriptionally regulating each other (data not shown). The cognate AHL for XenI2/R2 system did not activate the $braI_{XEN}$ promoter, and the cognate AHL of the BraI/R_{XEN} system did not activate the *xenI2* promoter (data not shown). In addition, the AHL production profiles, as observed in TLC analysis, $braI_{XEN}$- and $braR_{XEN}$-deficient mutants continue to produce *xenI2* and *xenR2* AHLs, and vice versa, suggesting the systems do not regulate each other.

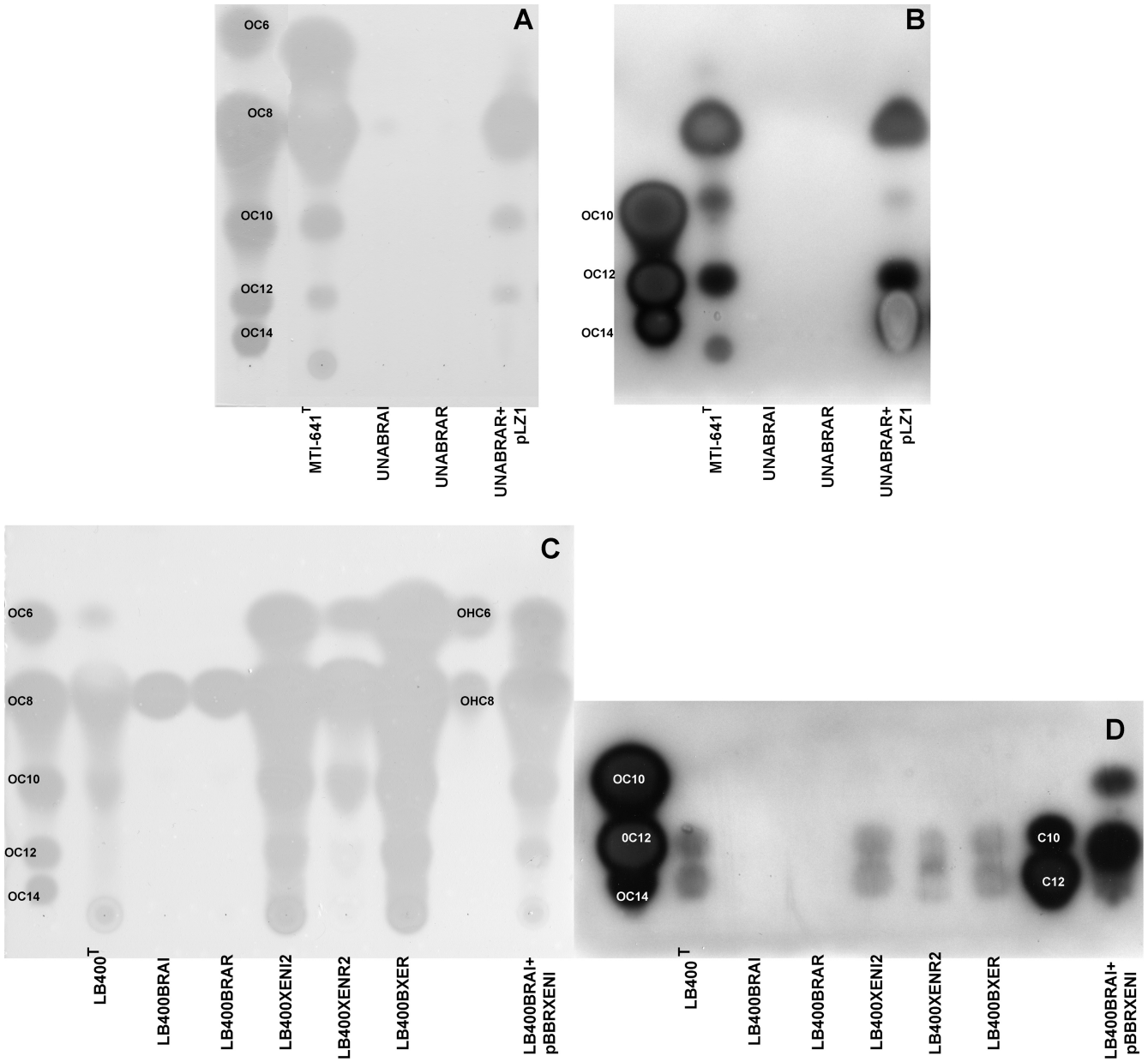

FIG. 2. TLC analysis of the AHLs produced by the *Burkholderia* and mutant derivatives. (A and B) *B. unamae* profiles. (C and D) *B. xenovorans* LB400 ^{T} profiles. AHL extraction was performed as described in Materials and Methods, and TLCs were performed in 70% methanol for 6 h; for each strain, the equivalent of 2.5×10^{10} cells was loaded. In panels A and C, *A. tumefaciens* (pNTL4) was used to detect the AHL signals, and in panels B and D, *E. coli* pSB1075 was used. Synthetic AHL compounds were used as a reference. OC6, 3-oxo-C_6-HSL; OC8, 3-oxo-C_8-HSL; OC10, 3-oxo-C_{10}-HSL; OC12, 3-oxo-C_{12}-HSL; OC14, 3-oxo-C_{14}-HSL; OHC6, 3-OH-C_6-HSL; OHC8, 3-OH-C_8-HSL; C10, C_{10}-HSL, C12; C_{12}-HSL.

Regulation of the AHL systems in *B. xenovorans* by BxeR solo. The role of BxeR solo in regulating the $braI_{XEN}$ and *xenI2* promoters, when tested both in *E. coli* and in *B. xenovorans*, was determined as described in Materials and Methods. In *E. coli*, results indicated that BxeR solo did not drive the transcription of any of AHL synthase promoters of *B. xenovorans* in response to the AHLs tested (data not shown).

The activities of the $braI_{XEN}$ and *xenI2* gene promoters were also determined in *B. xenovorans* wild-type and in the mutants LB400BRAI, LB400XENI2, and LB400BXER. The results demonstrated that the $braI_{XEN}$ promoter displayed low activity in the wild type, as well as in all mutants tested. The *xenI2* promoter also displayed low activities in the $braI_{XEN}$ and *xenI2* genomic knockout mutants but resulted in a 3-fold increase when harbored by the *bxeR* mutant, which suggested that the *xenI2* synthase promoter was negatively regulated by the BxeR solo protein. Although no difference in the AHL production profile for the *bxeR*-deficient mutant was observed, *xenI2* promoter activity was reduced when *bxeR* was provided in *trans* (Fig. 4). Finally, expression of *bxeR* was not regulated by the AHL systems but was found

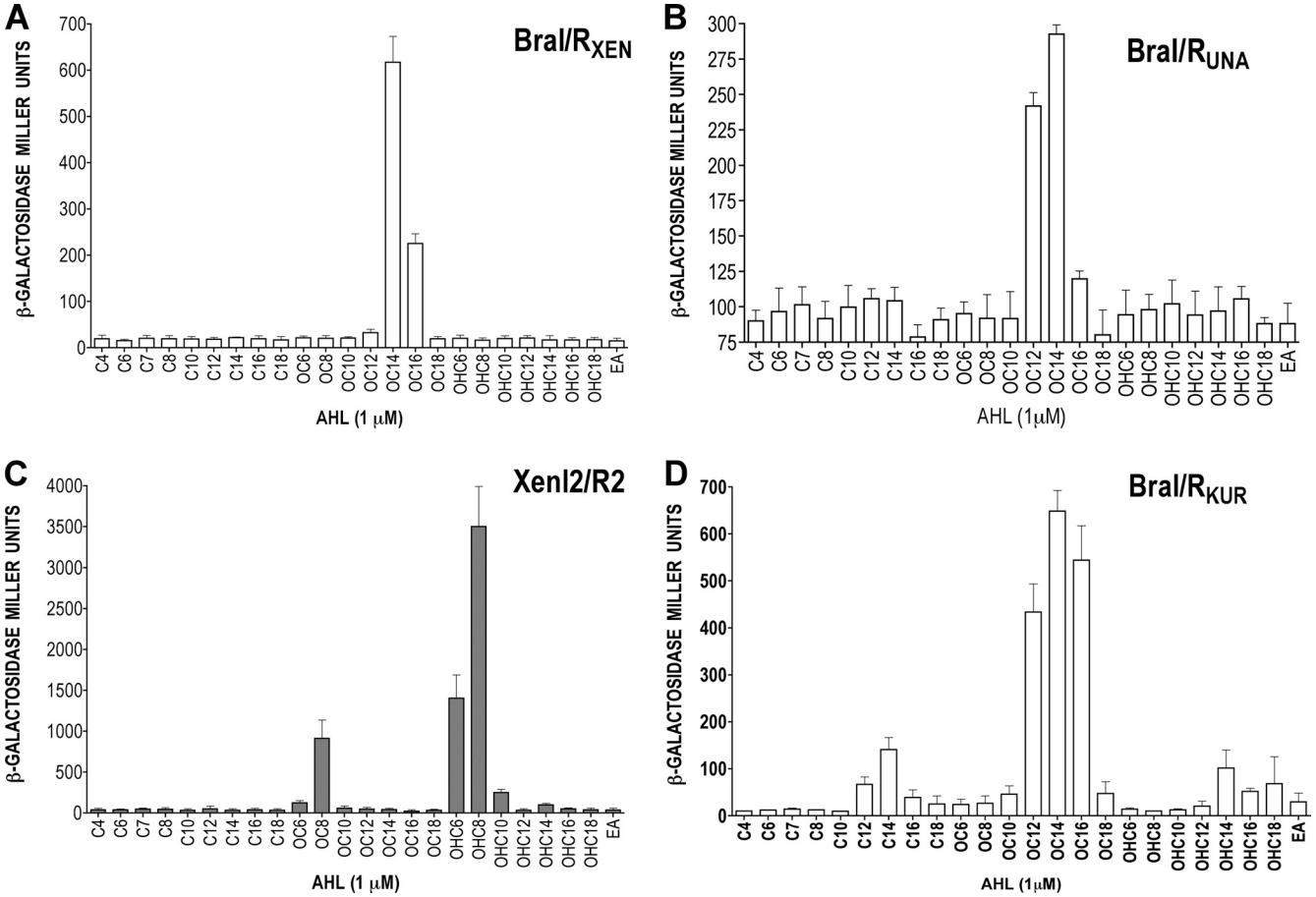

FIG. 3. Determination of the biologically active AHL for BraR$_{XEN}$, XenR2, BraR$_{UNA}$, and BraR$_{KUR}$ AHL sensor/regulators. (A) Determination of the cognate AHL for the BraI/R$_{XEN}$ system of *B. xenovorans*. Bars correspond to β-galactosidase activities determined for *E. coli* harboring pQEXENR1 and pMPXENI1. (B) Determination of the cognate AHL for the BraI/R$_{UNA}$ system of *B. unamae*. Bars correspond to β-galactosidase activities determined for *E. coli* harboring pQEUNAR and pMPUNAI combination. (C) Determination of the cognate AHL for the XenI2/R2 system of *B. xenovorans*. Bars correspond to β-galactosidase activities determined for *E. coli* harboring pQEXENR2 and pMPX2I. (D) Determination of the cognate AHL for the BraI/R$_{KUR}$ system of *B. kururiensis*. Bars correspond to β-galactosidase activities determined for *E. coli* harboring pQEBRAR and PBRAI (73). Transcriptional fusions were harbored independently in *E. coli* expressing either BraR$_{XEN}$ or XenR2 proteins; various exogenous AHLs (1 μM) were provided as indicated, and the β-galactosidase activities were determined. The results are mean values ± the standard deviations of three independent biological replicates. EA, ethyl acetate.

to be positively autoregulated, since the *bxeR* transcriptional promoter fusion decreased 3-fold in activity when harbored in the *bxeR* mutant LB400BXER, and was recovered when *bxeR* was provided in *trans* (Fig. 4). All measurements shown in Fig. 4 were also performed in four other different growth stages; the results have shown trends similar to the growth point depicted in Fig. 4 (data not shown).

EPS production is regulated by QS in *B. kururiensis*, *B. unamae*, and *B. xenovorans*, whereas biofilm formation is QS regulated only in *B. unamae*. Swimming, swarming, protease activity, lipase activity, siderophore production, exopolysaccharide (EPS) production, and biofilm formation were tested in the wild-type strains and compared to QS derivative mutants of *B. xenovorans*, *B unamae*, and *B. kururiensis* to determine whether these phenotypes were regulated by QS. Growth curves were determined for QS mutants, and no significant differences were observed with any of the mutants in their growth rates or in the CFU/ml compared to the wild type (data not shown).

No differences between the *Burkholderia* wild-type strains and their QS mutants were observed with regard to motility, secreted proteolytic and lipolytic enzyme activities, or siderophore production, suggesting that these phenotypes were not QS regulated under the conditions tested (data not shown). However, in all three species, EPS production was found to be positively regulated by the BraI/R-like QS system, since the AHL-synthase mutants M130BRAI, LB400BRAI, and UNABRAI mutants were significantly less mucoid than the corresponding wild-type strains (Fig. 5). In all three cases, EPS production was restored by chemical complementation when a 1 μM concentration of the cognate AHL (3-oxo-C_{14}-HSL) for each system was added to the medium. Quantification of the EPS produced for *B. kururiensis* showed that M130BRAI showed a 3-fold decrease in EPS produced compared to the wild type (Fig. 5B), whereas in *B. unamae* UNABRAI the reduction was 10-fold (Fig. 5D). In *B. xenovorans*, EPS production was reduced only in LB400BRAI and LB400BRAR mutants, while no difference was observed in

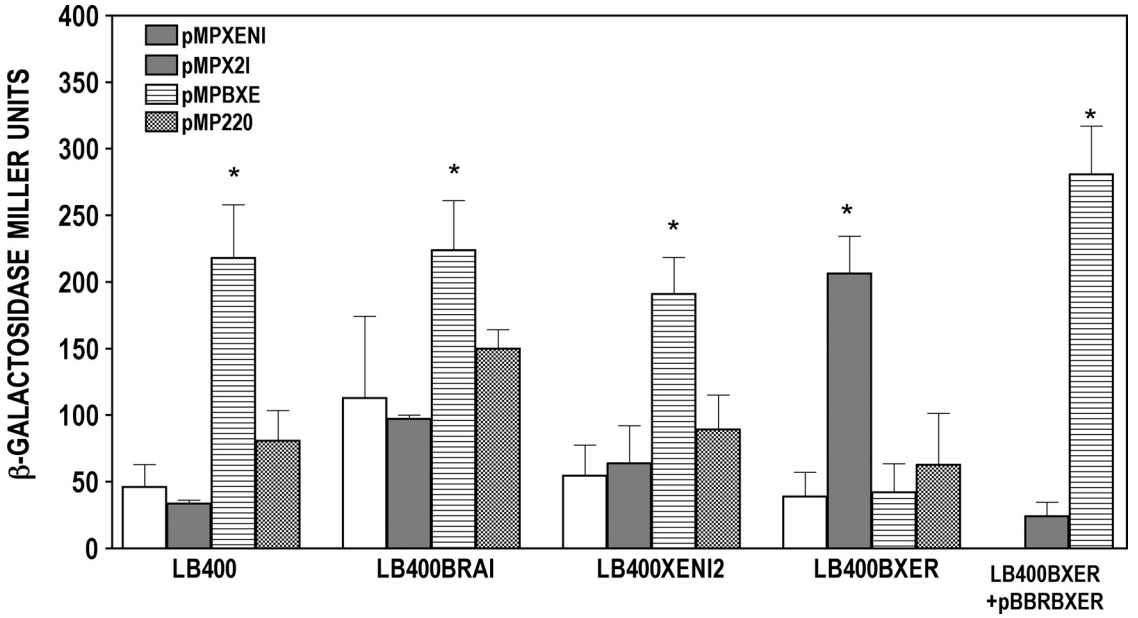

FIG. 4. $braI_{XEN}$, *xenI2*, and *bxeR* promoter activities in wild-type and QS mutant strains of *B. xenovorans* LB400^T. Bacterial cultures were started with an initial inoculum of 5×10^6 CFU in 20 ml of KB-Tc medium, and the β-galactosidase activities were measured over 12 h of growth. All experiments were performed in triplicate, and means values with standard deviations are indicated in the graph. ANOVA in combination with Dunnett's post-test and were performed with Prism 4.0 software (GraphPad). A P value of <0.05 was considered significant (*).

LB400BXER and in the *xenI2* and *xenR2* mutants. These results suggested that EPS production was controlled only by $braI_{XEN}$ and $braR_{XEN}$ in *B. xenovorans* (Fig. 5E).

Biofilm formation was found to be negatively regulated by QS in *B. unamae*, since UNABRAR mutants accumulated approximately three times more biofilm than the wild type. Biofilm accumulation was restored to wild-type levels in the UNABRAR mutant by providing in *trans* the $braR_{UNA}$ gene via cosmid pLZ1 (Fig. 6A). Interestingly, the *B. unamae* UNABRAI mutant produced the same amount of biofilm as the wild type. In contrast, the *B. kururiensis* AHL-deficient mutants M130BRAI and M130BRAR displayed only slight increases in biofilm formation compared to the wild-type ($P < 0.01$); this increase was restored when 3-oxo-C_{14}-HSL was provided to the medium containing M130BRAI (Fig. 6B). No major differences in biofilm formation were observed in any of the *B. xenovorans* QS mutants under the conditions tested (Fig. 6C). It was concluded that biofilm formation was regulated by QS in *B. unamae* and in *B. kururiensis* but was not QS dependent in *B. xenovorans*.

Phenol degradation is QS regulated in *B. unamae*. The ability of *B. unamae* to utilize phenol as carbon source was previously reported as a unique feature of this species among other N-fixing plant-associated *Burkholderia* spp. (11). AHL QS-deficient mutants (UNABRAR and UNABRAI) were found to have a decreased ability to utilize phenol compared to the wild type (Fig. 7). The ability to grow in the presence of phenol was partially restored in UNABRAR mutant when complemented in *trans* with the cosmid pLZ1. These results suggest that the phenol degradation is QS regulated in *B. unamae*. Importantly, using the same media and replacing phenol with mannitol as a carbon source showed that the mutants grew in a fashion similar to that of the wild type (data not shown).

BraI/R_{XEN} negatively regulates production of a 40-kDa Porin-1 family protein in *B. xenovorans* LB400^T. It was of interest to find gene targets of the well-conserved BraI/R-like system among the recently described *Burkholderia* species cluster. It was decided to determine whether the levels of any of the secreted proteins were altered in the mutant compared to the wild type. For this experiment, *B. xenovorans* was used since, of the three species of the cluster studied here, it is the only one of which the genome has been sequenced and annotated. We analyzed the profile of secreted proteins of the wild-type strain LB400^T versus the profile of the $braI_{XEN}$ mutant LB400BRAI. As depicted in Fig. S1 in the supplemental material, analysis revealed the presence of two proteins with apparent molecular masses of 40 and 21 kDa in the LB400BRAI mutant that were absent or present in very low amounts in the wild type. It was postulated that these proteins were negatively regulated by the BraI/R_{XEN} system. Mass spectrometry analysis indicated that the 40-kDa protein corresponds to a putative 377-amino-acid outer membrane porin (OmpC family), encoded by the locus Bxe_B2786. In order to confirm whether this ORF was regulated by BraI/R_{XEN}, the Bxe_B2786 gene promoter region was cloned into the promoterless probe vector pMP220 to generate pMP2786. This construct was then conjugated into the *B. xenovorans* LB400^T wild-type and LB400BRAI mutant strains. Determination of the β-galactosidase activity showed a 50-fold increase in activity values obtained in the LB400BRAI mutant compared to the wild type, confirming that this ORF was negatively regulated by the BraI/R_{XEN} system. Promoter activity was significantly reduced when $braI_{XEN}$ was provided in *trans* by conjugating the plasmid pBBRXENI1 into LB400BRAI (Fig. S1). The 21-kDa protein that was also abundantly present in the LB400BRAI mutant corresponded to a putative ABC-type

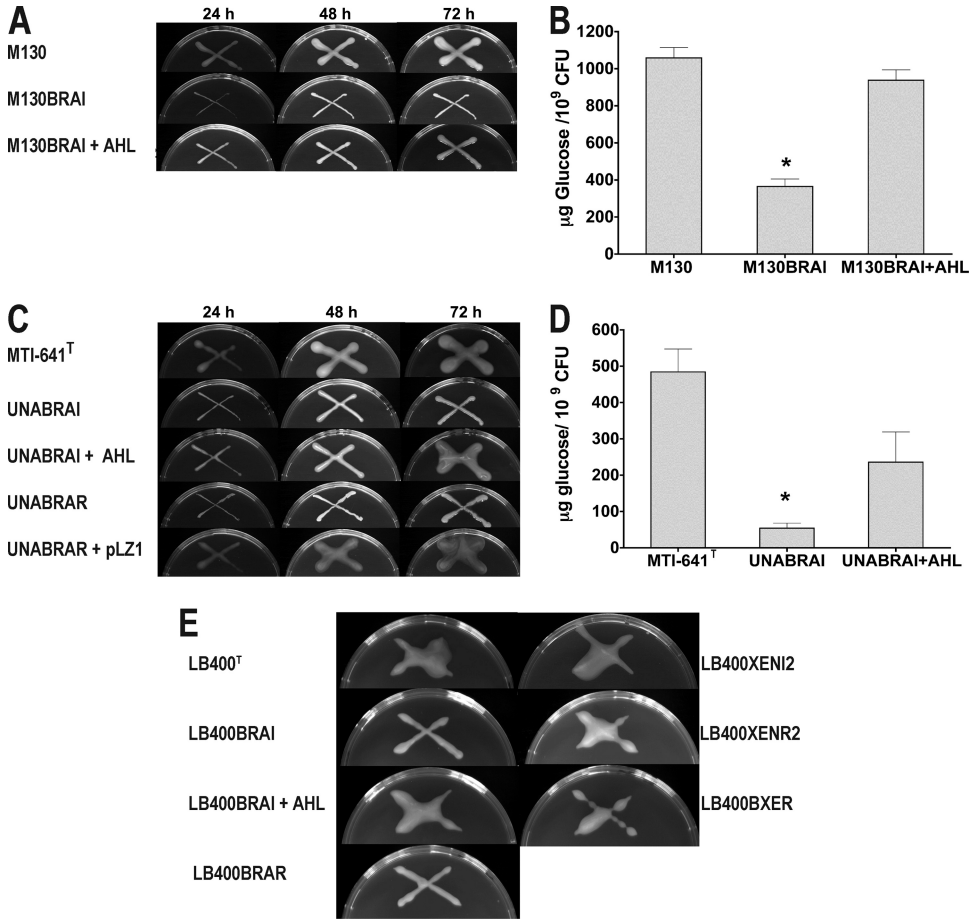

FIG. 5. EPS Production of *B. kururiensis* M130 (A and B), *B. unamae* MTl-641^T (C and D), and *B. xenovorans* LB400^T wild-type and QS mutants (E). Single colonies were streaked in YEM agar plates. Chemical complementation was achieved by adding 1 μM AHL to the growth medium. Bar graphs show EPS quantification for *B. kururiensis* and *B. unamae* by using the boiling phenol method (described in Materials and Methods). Experiments were performed in triplicate, and means ± the standard deviations are plotted. ANOVA in combination with Dunnett's post-test was performed using Prism 4.0 software (GraphPad). A P value of <0.05 was considered significant.

transporter periplasmic ligand binding protein postulated to be involved in toluene resistance, encoded by the locus Bxe_B0016. By promoter activity analysis in the wild type versus the mutants, we were able to confirm the negative regulation by the BraI/R_{XEN} system since the promoter activity increased 5-fold in the LB400BRAI mutants (see Fig. S1 in the supplemental material). In this case, however, the promoter activity was not restored to wild-type levels when we provided the *braI*$_{XEN}$ gene in *trans* via pBBRXENI1; the reason for this is currently not known.

QS regulates *in planta* growth in *B. kururiensis* M130 but does not affect rhizosphere colonization in *B. unamae* MTl-641^T. Since members of this recently described *Burkholderia* cluster are very often associated with plants, it was of interest to study the role of QS *in planta*. Since *B. kururiensis* M130 was isolated as a rice endophyte, we tested the role of QS in rice colonization and growth. A significant decrease in colonization of the QS mutants was observed in comparison to that obtained for the wild type, both in the roots and in the aerial parts of the plant (Fig. 8A and B). In addition, the roots of plantlets colonized with the wild type exhibited an increase in length and branching compared to roots colonized with the QS mutants (Fig. 8C). These data suggested that, under the conditions tested, QS positively regulates endophytic rice colonization by *B. kururiensis* M130.

Since *B. unamae* MTI-641^T was isolated from the maize rhizosphere (12), we determined the role of QS in maize rhizosphere colonization and growth. Experiments revealed that the wild-type strain and the UNABRAI and UNABRAR genomic mutants colonize the maize roots at similar levels, indicating that in this strain AHL QS does not play a major role in root colonization (data not shown).

DISCUSSION

A new cluster composed of beneficial *Burkholderia* species has been consolidated in the last years following the rapid increase in the number of described species. Despite the metabolic versatility and agrobiotechnological potential of this new group, the regulatory mechanisms underlying their interaction with the environment are largely unknown. In the present study, we investigated the AHL QS systems of two species of the cluster: the maize rhizosphere isolate *B. unamae* MTI-641^T and the *B. xenovorans* LB400 ^{T}, which has become a

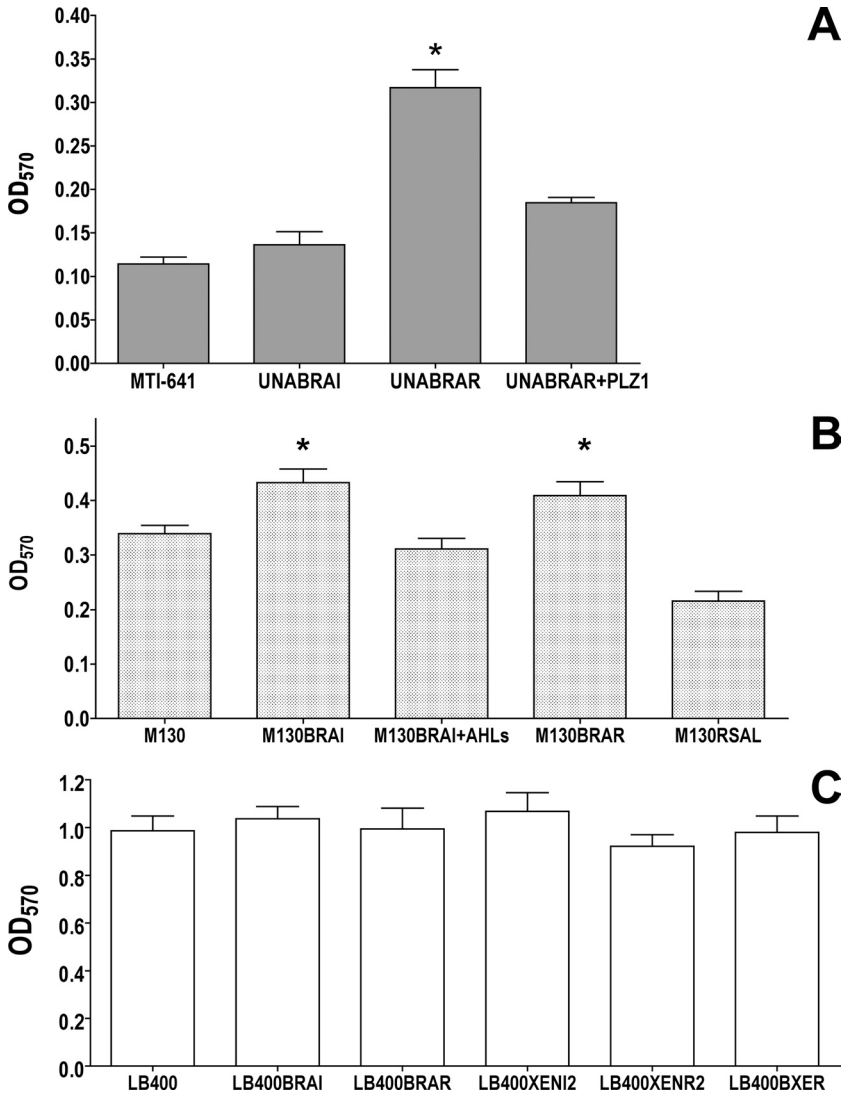

FIG. 6. Biofilm production in *B. unamae* (A), *B. kururiensis* (B), and *B. xenovorans* (C) wild-type and QS mutants after 72 h of incubation. "+AHLs" refers to complementation by adding 1 μM 3-oxo-C_{14}-HSL. Experiments were performed in triplicate, and means ± the standard error of the mean are plotted. ANOVA in combination with Dunnett's post-test were performed using Prism 4.0 software (GraphPad). A P value of <0.05 was considered significant compared to the wild type (*).

model system for the bacterial breakdown of highly persistent contaminants (31, 67, 69). In addition, we extended our studies on the role of BraI/R_{KUR} of rice endophyte *B. kururiensis* M130 (73).

It was established that the BraI/R_{XEN}, BraI/R_{UNA}, and BraI/R_{KUR} systems respond to C_{14}-3-oxo-HSL. This is in accordance with previous studies which demonstrated that other members of the cluster, namely, *B. phytofirmans* (strains $PsJN^T$ and RG6-12) and *B. graminis* (strains M1 and M14), produce C_{14}-3-oxo-HSL (7, 77). In summary, these results suggest that 3-oxo-C_{14}-HSL is the most probable cognate AHL for the BraI/R-like systems in this *Burkholderia* cluster. As expected, BraI/R ortholog systems are also present in the sequenced genomes of cluster mates *B. phytofirmans*, *B. graminis*, and *B. phymatum*. The existence of a conserved QS system could facilitate interspecies communication within this *Burkholderia* cluster, possibly providing advantages in multispecies niche adaptation. The relatedness between the BraI/R-like systems to the LasI/R and PpuI/R from *Pseudomonas* spp. could suggest that their coding genes might have been involved in lateral gene transfer (12, 45).

The BraI/R_{UNA} and BraI/R_{XEN} systems were not involved in the regulation of motility, siderophore production, or lipolytic or proteolytic activity under the conditions tested. EPS production, however, is positively regulated by the BraI/R-like systems in *B. kururiensis*, *B xenovorans*, and *B. unamae*. It has been reported that EPS production is also subject to QS regulation in other plant-associated bacteria such as *Pantoea stewartii* (85), *Ralstonia solanacearum* (28), *Pseudomonas syringae* (61) and *Sinorhizobium meliloti* (48). Significantly, in these species EPS production affected host invasion and the pathogenic or symbiotic interaction with the plant was diminished via alteration in surface attachment (biofilm formation) as described for *P. stewartii* (41) and *S. meliloti* (34, 64). Impor-

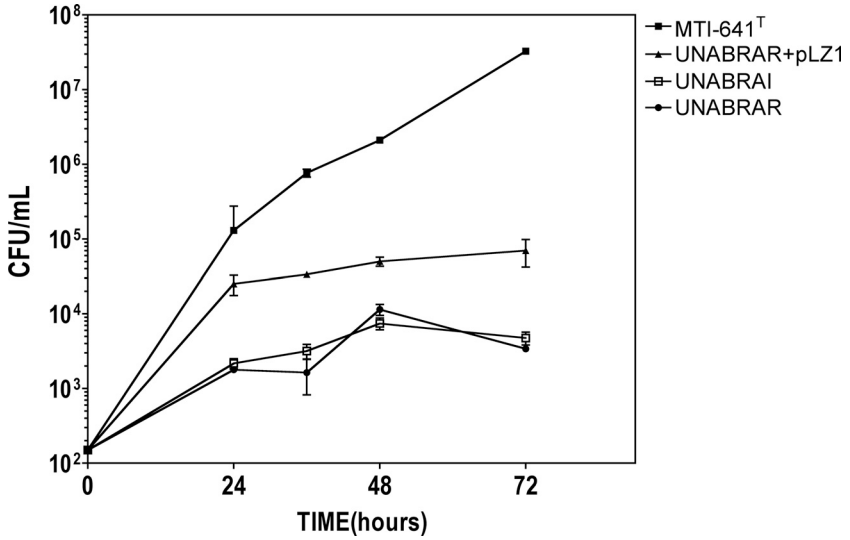

FIG. 7. Phenol degradation profile in *B. unamae* wild type and QS mutants. A growth profile was obtained with 10^3 CFU as starter the inoculum. As a control, phenol was replaced by mannitol as the carbon source, and all strains exhibited the same behavior.

tantly, in *B. kururiensis* M130 both rice colonization and EPS production were reduced in the QS mutants, which could indicate that EPS production is involved in endophytic colonization. It has been reported that one of the EPS polymers produced by *B. kururiensis* (EPS B) has structural similarity to the EPS produced by *B. cepacia* (cepacian) (32), which is considered a virulence factor required to the formation of thick and mature biofilms (19). A similar polymer has been recently detected in members of the plant-associated *Burkholderia* cluster, such as *B. graminis*, *B. phytofirmans*, *B. phymatum*, and *B. xenovorans*, and is believed to be involved in the plant-bacterium interaction (26). Future understanding of the genes involved in the EPS synthesis in these species may help in identifying the gene targets of QS in this pathway.

Although EPS production was a common QS regulated trait in the three species studied, biofilm formation was found to be negatively regulated by BraI/R_{UNA} in *B. unamae*, whereas it was only slightly modulated in *B. kururiensis*, and no QS regulation of biofilm formation was observed for *B. xenovorans*. The reasons for this differential regulation among the three species are currently unknown, but it is worth noting that the maize rhizospheric colonization of *B. unamae* was not affected by the reduced EPS production in QS mutants, which suggests that EPS does not affect plant colonization in this strain, whereas it does in *B. kururiensis* M130.

Several species from the new plant-associated *Burkholderia* cluster have shown extraordinary ability to degrade phenolic compounds, and this potential has been extensively reviewed (37, 54, 56). The presence of a phenol monooxygenase and the ability to degrade phenol have been previously demonstrated in *B. unamae* (11). Here, it is demonstrated that this trait is regulated by the BraI/R_{UNA} QS system in *B. unamae*. The ability to degrade phenol was reported to be associated with AHL production by members of the microbial community in activated sludges, and supplementation with AHL was able to sustain phenol-degrading activity beyond the point of starvation. It was hypothesized that this event was associated to surfactant production and composition rearrangement within the community (79). Interestingly, *B. kururiensis* was recently reported to be able to grow in consortia that degrade 2,4,6-trichlorophenol, using phenol as a primary intermediate (30). From these findings, it cannot be excluded that QS might have a role in the consortial behavior of *Burkholderia*, leading to increased degradation of aromatic compounds.

Maize colonization experiments performed with *B. unamae* indicated that the BraI/R_{UNA} QS system does not regulate ability to colonize this host under the conditions used, whereas rice colonization by *B. kururiensis* was positively regulated by QS. These results suggest that plant-bacterium interaction may be subject to regulation in a cell density manner in plant-associated *Burkholderia*. However, more experiments need to be performed in order to determine whether plant colonization (at both the endophytic and the rhizospheric levels) may be dependent on the plant genotype. In fact, *B. phytofirmans* PsJN has been shown to have variable colonization levels in different cultivars in several plant species (60, 77).

Secreted proteins profiling for *B. xenovorans* evidenced several bands present in the AHL synthase mutant LB400BRAI profile and absent in wild-type profile. However, transcriptional analysis of their promoters indicated that only two of them were transcriptionally regulated by BraI/R_{XEN}, and only one of them could be fully complemented by supplying the $braI_{XEN}$ gene in *trans*. This latter gene encoded for a porin belonging to the OmpC family (pFAM00267), and a putative *lux* box centered at −96 was identified in its promoter region. A similar protein was identified in *B. cenocepacia*, and it was suggested that it may function as a pore for small molecules involved in osmoregulatory control (6). Several porins have been reported to be regulated by QS in several species, including *B. cepacia* (1), *Azospirillum lipoferum* (9) and *P. aeruginosa* (4), but the implications of such regulation remains to be explored.

We report that *B. xenovorans xenI2* and *xenR2* are found in only one-third of the members of the *Burkholderia* plant-asso-

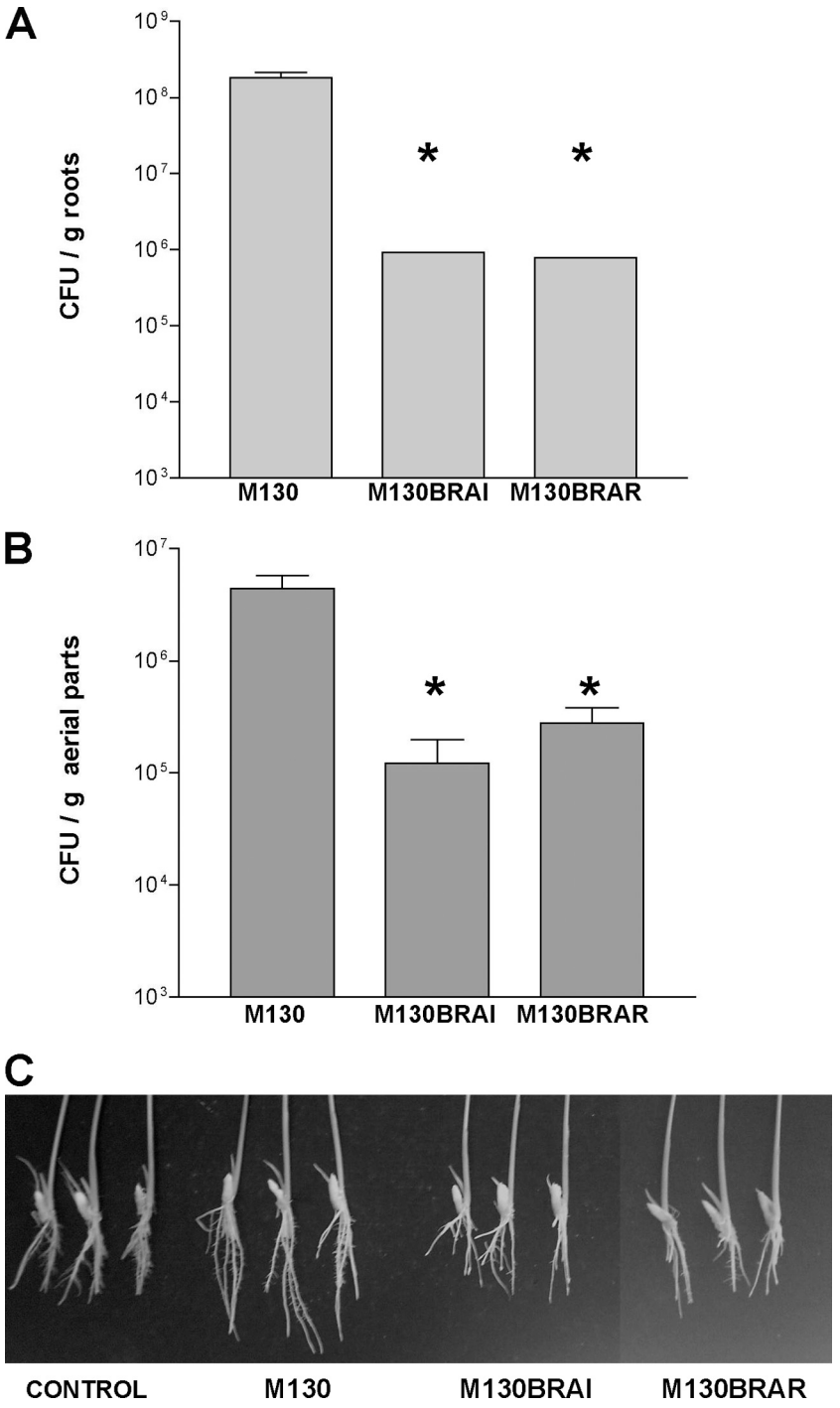

FIG. 8. Rice colonization assays performed with *B. kururiensis* M130 wild type and impaired QS mutants M130BRAI and M130BRAR. Plantlets were surface sterilized and inoculated as described in Materials and Methods. Bacterial colonization was measured by grinding and plating surface-sterilized plants after 12 days, and CFU/g levels are plotted. A P value of <0.05 was considered significant compared to the wild type (*). (A) Endophytic root colonization levels. (B) Endophytic aerial colonization. (C) Enhanced root development promoted by *B. kururiensis* M130 wild-type inoculation compared to QS mutants and noninoculated plants.

ciated cluster. Surprisingly, *xenI2* and *xenR2* are not present in four other *B. xenovorans* strains, which implies that the occurrence of XenI2/R2 is strain dependent rather than species dependent. In fact, in strain LB400^T *xenI2* and *xenR2* are located in a megaplasmid, indicating that it is not part of the core chromosome of *B. xenovorans*. Interestingly, *B. phytofirmans* strain PsJN was also reported to produce C_8-3OH-HSL, but these AHLs are not produced by strain RG6-12, indicating that *xenI2* and *xenR2* may not be present in all *B. phytofirmans* strains (77). A similar scenario occurs for the CciIR system,

which is found only in some BCC *B. cenocepacia* strains and has been shown to be associated with a pathogenicity island (45). Homology studies demonstrated that XenI2/R2 is closely related to the CciIR system and to the BtaIR3, BmaIR3, and BpsIR3 systems of *B. mallei*, *B. pseudomallei*, and *B. thailandensis*, respectively (12, 45), possibly indicating a probable common ancestor for these systems (14, 22). The *cciI* and *cciR* system has recently been characterized as a global regulator exerting an important regulatory control of the *cepI* and *cepR*, the other AHL QS present in *B. cenocepacia* K-52 (53).

Our studies showed that under the conditions we tested, no transcriptional regulatory hierarchies are present between BraI/R_{XEN} and XenI2/R2. In addition, transcriptional studies of $braI_{XEN}$ and *xenI2* gene promoters in *B. xenovorans* showed that both genes are expressed at very low levels, indicating that they are most likely under transcriptional regulation. The BraI/R_{XEN} system is most probably regulated by the intergenically located *rsaL* repressor, as previously described for the BraI/R_{KUR} system (73).

The genome of *B. xenovorans* contains an unpaired LuxR family solo, designated BxeR, with the typical *N*-terminal AHL-binding domain and the C-terminus HTH motif. Protein alignment showed that BxeR possesses the six conserved amino acids of the AHL-binding domain, while one of the three conserved residues in the HTH motif presents an E178Q substitution, which in fact was also substituted in the DNA-binding domain of XenR2 protein. BxeR positively autoregulated its own transcription and negatively regulated the expression of *xenI2*. BxeR was present in all *B. xenovorans* strains, as well as in most of the type strains of the *B. graminis* clade, and in the species *B. tuberum*. It is not known whether BxeR binds to AHLs, but the conservation of the residues in the AHL binding domain suggests that BxeR might bind AHL molecules. The role of LuxR-type solos in AHL producers has been reviewed recently (58, 74); several examples demonstrate that LuxR-type solos present in AHL producing bacteria are most often integrated with the resident AHL QS regulatory networks, as are the cases of ExpR and NesR from *S. meliloti* (34, 57), PpoR from *P. putida* (75), and QscR from *P. aeruginosa* (43). Our results suggest that such integration might exist for BxeR, since the negative regulation of *xenI2* promoter was observed in the *bxeR*-deficient background.

In the present study we describe the characterization of the QS systems in species from the new plant-associated *Burkholderia* cluster isolated from three different environmental niches. It was confirmed that the cluster shares a highly conserved BraI/R-like QS system, and the existence of a common core of targets is proposed, although species-specific targets may have been acquired as a response to niche adaptation. A second QS system and a LuxR solo protein were found to be present in a few members of the cluster that are independent of the BraI/R-like system. Future work will focus on determining the complete regulons of the two systems and of the LuxR type solo BxeR in this important recently described cluster of *Burkholderia* species.

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