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Regulon Studies and *In Planta* Role of the BraI/R Quorum-Sensing System in the Plant-Beneficial *Burkholderia* Cluster

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The genus *Burkholderia* is composed of functionally diverse species, and it can be divided into several clusters. One of these, designated the plant-beneficial-environmental (PBE) *Burkholderia* cluster, is formed by nonpathogenic species, which in most cases have been found to be associated with plants. It was previously established that members of the PBE group share an *N*-acyl-homoserine lactone (AHL) quorum-sensing (QS) system, designated BraI/R, that produces and responds to 3-oxo-C_{14}-HSL (OC14-HSL). Moreover, some of them also possess a second AHL QS system, designated XenI2/R2, producing and responding to 3-hydroxy-C_8-HSL (OHC8-HSL). In the present study, we performed liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis to determine which AHL molecules are produced by each QS system of this group of bacteria. The results showed that XenI2/R2 is mainly responsible for the production of OHC8-HSL and that the BraI/R system is involved in the production of several different AHLs. This analysis also revealed that *Burkholderia phymatum* STM815 produces greater amounts of AHLs than the other species tested. Further studies showed that the BraR protein of *B. phymatum* is more promiscuous than other BraR proteins, responding equally well to several different AHL molecules, even at low concentrations. Transcriptome studies with *Burkholderia xenovorans* LB400 and *B. phymatum* STM815 revealed that the BraI/R regulon is species specific, with exopolysaccharide production being the only common phenotype regulated by this system in the PBE cluster. In addition, BraI/R was shown not to be important for plant nodulation by *B. phymatum* strains or for endophytic colonization and growth promotion of maize by *B. phytofirmans* PsJN.

In the last decade, much attention has been given to betaproteobacteria in the genus *Burkholderia* mainly because of their great metabolic versatility, their capacity for colonizing very distinct niches, and their ability to cause serious infections in humans (1).

Currently the *Burkholderia* genus comprises over 60 validly described species; phylogenetic trees generated from diverse gene sequence analyses (e.g., 16S rRNA) have shown divisions within the genus, and consequently it has been separated into several clades. One clade, called the *Burkholderia cepacia* complex (BCC), is comprised of human pathogens, most abundantly isolated from the respiratory tracts of cystic fibrosis patients with chronic infections (2). Another *Burkholderia* species cluster which has been recently discovered comprises nonpathogenic *Burkholderia* spp., mostly described after the year 2000, which have been isolated from plants or from environmental samples (3). The latter group, also known as the plant-beneficial-environmental (PBE) *Burkholderia* group, had a strong impact on the ecological perception of *Burkholderia* spp., as it possesses several interesting features with potential biotechnological applications (3), including (i) degradation of different aromatic compounds and toxic molecules (for example, *Burkholderia xenovorans* LB400, which is also able to degrade polychlorinated biphenyls [4]); (ii) rhizospheric and endophytic colonization of plants combined with an ability to promote plant growth via various mechanisms, such as nitrogen fixation, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and/or indole-3-acetic acid (IAA) production (for example, *Burkholderia phytofirmans* PsJN, which is able to endophytically colonize and promote plant growth of several plant varieties [5]); and (iii) the ability to form N_2-fixing symbioses with mosses and higher plants, providing the first confirmed examples of rhizobia belonging to the betaproteobacteria (for example, *Burkholderia phymatum*, which is able to form symbiotic nodules and fix nitrogen in association with legumes [6, 7]).

Quorum sensing (QS) is a regulatory process that allows bacterial cells to communicate among themselves and to monitor their population density. This is possible through the production of signaling molecules which increase in accordance with population density, and, once a certain threshold is reached, bacteria sense and respond to them, modulating target gene expression. The most common and studied QS system of Gram-negative bacteria thus far uses *N*-acylhomoserine lactones (AHLs) as signal molecules and is based on two genes/proteins: an AHL synthase belonging to the LuxI protein family and a transcriptional regulator belonging to the LuxR family which detects the signal and regulates gene expression (8).

The first studies of QS in the PBE *Burkholderia* spp. have shown that this clade shares a highly conserved AHL QS system, called BraI/R, which is unrelated to the CepI/R present in BCC species.

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TABLE 1 *Burkholderia* and *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)[a]	Reference or source
Strains		
B. xenovorans LB400	Wild-type strain	4
LB400XENI2	*XenI2*::Km of *B. xenovorans* LB400	10
LB400BRAI	*braI*::Km of *B. xenovorans* LB400	10
LB400BRAR	*braR*::Km of *B. xenovorans* LB400	10
LB400BRAR(pBBRbraR$_{XEN}$)	*braR*::Km of *B. xenovorans* LB400 harboring pBBRbraR$_{XEN}$	10
B. phytofirmans PsJN	Wild-type strain	5
PsJNBRAI	*braI*::Km of *B. phytofirmans* PsJN	This study
PsJNBRAI(pBBRbraI$_{PsJN}$)	*braI*::Km of *B. phytofirmans* PsJN harboring pBBRbraI$_{PsJN}$	This study
B. phymatum GR01	Wild-type strain	7
GR01BRAI	*braI*::Km of *B. phymatum* GR01	This study
GR01BRAI(pBBRbraI$_{phym}$)	*braI*::Km of *B. phymatum* GR01 harboring pBBRbraI$_{phym}$	This study
B. phymatum STM815	Wild-type strain	57
STM815BRAI	*braI*::Km of *B. phymatum* STM815	This study
STM815BRAI(pBBRbraI$_{phym}$)	*braI*::Km of *B. phymatum* STM815 harboring pBBRbraI$_{phym}$	This study
STM815BRAI(pMPbraI$_{p}$)	*braI*::Km of *B. phymatum* STM815 harboring pMPbraI$_{p}$	This study
STM815BRAI(pMP220)	*braI*::Km of *B. phymatum* STM815 harboring pMP220	This study
STM815(pMPeps$_{p}$)	*B. phymatum* STM815 harboring pMPeps$_{p}$	This study
STM815BRAI(pMPeps$_{p}$)	*braI*::Km of *B. phymatum* STM815 harboring pMPeps$_{p}$	This study
B. tuberum DSM17489	Wild-type strain	57
B. tuberum pME6863	*B. tuberum* DSM17489 harboring pME6863	This study
B. tropica Ppe8	Wild-type strain	58
B. tropica pME6863	*B. tropica* Ppe8 harboring pME6863	This study
B. terrae DSM17804	Wild-type strain	59
B. terrae pME6863	*B. terrae* DSM17804 harboring pME6863	This study
B. phenazinium DSM10684	Wild-type strain	60
B. phenazinium pME6863	*B. phenazinium* DSM10684 harboring pME6863	This study
B. graminis DSM17151	Wild-type strain	60
B. graminis pME6863	*B. graminis* DSM17151 harboring pME6863	This study
E. coli DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	14
Plasmids		
pGEM-T Easy	Cloning vector; Amp^r	Promega
pRK2013	Tra$^+$ Mob$^+$, ColE1 replicon; Km^r	13
pMP220	Promoter probe vector, IncP, LacZ; Tc^r	28
pMPbraI$_{p}$	Promoter region of *braI* from *B. phymatum* STM815 cloned into pMP220; Tc^r	This study
pMPeps$_{p}$	Promoter region of the EPS operon of *B. phymatum* STM815 cloned into pMP220; Tc^r	This study
pKNOCK-Km	Conjugative suicide vector; Km^r	16
pKNOCK-BRAI$_{PsJN}$	Internal PCR *braI* fragment of *B. phytofirmans* PsJN cloned in pKNOCK-Km	This study
pKNOCK-BRAI$_{phym}$	Internal PCR *braI* fragment of *B. phymatum* STM815 cloned in pKNOCK-Km	This study
pME6863	*aiiA* gene under constitutive P*lac* control; Tc^r	22
pBBR1MCS-3	Broad-host-range vector; Tc^r	17
pBBRbraI$_{PsJN}$	*braI*$_{PsJN}$ cloned into pBBR1MCS-3; Tc^r	This study
pBBRbraI$_{phym}$	*braI*$_{STM815}$ cloned into pBBR1MCS-3, Tc^r	This study
pBBRbraR$_{XEN}$	*braR*$_{XEN}$ cloned into pBBR1MCS-3, Tc^r	10

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

Besides BraI/R, another AHL QS system, called XenI2/R2, is found in only a few of the PBE *Burkholderia* spp. (e.g., *B. xenovorans*). Studies with the BraI/R QS system of *B. kururiensis* M130, *B. unamae* MTI-641, and *B. xenovorans* LB400 have revealed that it responds preferentially to 3-oxo-C_{14}-HSL (OC14-HSL) and that the XenI2/R2 of *B. xenovorans* LB400 responds to 3-hydroxy-C_8-HSL (OHC8-HSL) (9, 10).

No molecular studies have yet been performed on the BraI/R gene targets. A previous study has shown that the production of exopolysaccharides (EPS) is regulated by this system in at least three species of the PBE clade (*B. xenovorans* LB400, *B. unamae* MTI-641, and *B. kururiensis* M130), which suggests the existence of a common BraI/R regulon. However, phenotypes such as biofilm formation, plant colonization, and degradation of aromatic compounds seem to be regulated in a species-specific manner (10). The present work aimed to identify and compare the BraI/R QS regulons of *B. phymatum* and *B. xenovorans*, two species of the PBE cluster that occupy different environmental niches. In addition, we intended to determine the importance of this system for the plant-symbiotic and -endophytic lifestyles of *B. phymatum* and *B. phytofirmans* PsJN, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *Burkholderia* spp. and *Escherichia coli* strains and plasmids used in the present study are listed in Table 1. The list of primers used and the construction of recombinant

plasmids are given in Table S1 in the supplemental material. All *Burkholderia* strains were grown in King's medium B (KB) (11) at 30°C. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. *Agrobacterium tumefaciens* NTL4/pZLR4 (12) was used for AHL detection, and it was grown in LB medium at 30°C. The plasmid pGEM-T Easy vector (Promega, Madison, WI) was used for cloning. Antibiotics were added when required at the following final concentrations: ampicillin, 100 μg ml^{-1}; tetracycline (Tc), 15 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Burkholderia* spp.); gentamicin, 30 μg ml^{-1} (*A. tumefaciens*), or kanamycin (Km), 50 μg ml^{-1} (*E. coli*) or 100 μg ml^{-1} (*Burkholderia* spp.); and nitrofurantoin, 100 μg ml^{-1}. Conjugation experiments with the *Burkholderia* spp. were performed by triparental mating using *E. coli* DH5α(pRK2013) as a helper (13) and incubated 18 h at 30°C. Transconjugants were counterselected in KB with the appropriate antibiotics.

Recombinant DNA techniques and β-galactosidase activity. All recombinant DNA techniques were performed as described previously (14). 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 (15). Generated plasmids were sequenced by Macrogen (Europe).

Construction of gene knockout mutants and complemented strains. Genomic null mutants of the *braI* gene (encoding the AHL synthase) were created as follows. Internal fragments from the *braI* genes were PCR amplified using the primers Phytof.luxI2.F/Phytof.luxI2.R (Phytof.luxI2.F/R) and Phymat.luxI.F/R (see Table S1 in the supplemental material) for *B. phytofirmans* PsJN and *B. phymatum* STM815, respectively, and cloned in pKNOCK-Km (16). The generated plasmids, pKNOCK-$BRAI_{PsJN}$ and pKNOCK-$BRAI_{phym}$, respectively, were used as suicide delivery systems in order to create *braI* knockout mutants as previously described (16), and the mutants thus generated were termed PsJNBRAI and STM815BRAI, respectively. The plasmid pKNOCK-$BRAI_{phym}$ was also used to construct a *braI* knockout mutant of *B. phymatum* GR01, which was named GR01BRAI. The fidelity of all marker exchange events was confirmed by Southern analysis or by DNA sequencing following PCR (data not shown).

Complementation of the *braI* mutants was performed using the pBBRbraI_{PsJN} and pBBRbraI_{phym} plasmids for *B. phytofirmans* PsJN and *B. phymatum* strains, respectively. These plasmids were constructed as follows: a fragment containing the *braI* gene of *B. phytofirmans* PsJN or *B. phymatum* STM815 ($braI_{phym}$) was amplified using the primers Phytof .braI.F/R or Phymat.braI.F/R and cloned into pGem-T-Easy. The fragments were subsequently cloned into the high-copy-number plasmid pBBR1MCS-3 (17), yielding pBBRbraI_{PsJN} and pBBRbraI_{phym}. All plasmids were verified by sequencing (Macrogen) and then mobilized into their respective mutant strains.

AHL extraction and characterization. Production of AHLs was detected by thin-layer chromatography (TLC) using AHL biosensors after extraction of AHL from cell-free spent supernatant (12) of an overnight culture in 50 ml KB for most *Burkholderia* strains and 20 ml of KB for *B. phymatum* strains. The TLC plate was then overlaid with a thin layer of AB top agar seeded with *A. tumefaciens* NTL4/pZLR4 in the presence of 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) ml^{-1} as described previously (12).

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was used for the analysis of AHLs extracted from the bacterial supernatants. The dried extracts were redissolved in 100 μl of MeOH (plus 0.1%, vol/vol, formic acid), and any remaining insoluble material was removed by centrifugation. Sample temperature was maintained at 4°C in the autosampler prior to analysis. The injection volume was 5.0 μl. The high-performance liquid chromatography (HPLC) system used was a Shimadzu SIL-HTc autosampler with two Shimadzu LC-10ADvp pumps. The MS system used was a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer equipped with a TurboIon source. Chromatographic separation was achieved using a Phenomenex Gemini C_{18} reversed-phase column (3.0-μm particle size; 100 by 3.0 mm) with an appropriate guard column, maintained at 50°C, using a mobile phase flow rate set at 450 μl/min. Mobile phases consisted of aqueous 0.1% (vol/vol) formic acid (A) and 0.1% (vol/vol) formic acid in MeOH (B). The binary gradient began initially at 10% B and ran isocratically for the first 1 min before increasing linearly to 99% B over 9 min. After a further 5 min at this composition, the gradient was returned to 10% B over the next 1 min and allowed to reequilibrate for 4 min. The total run time was 20 min for each sample. MS detection was operated in a multiple reaction monitoring (MRM) mode, screening for all unsubstituted AHLs, 3-oxo-AHLs, and 3-OH-AHLs with acyl chain lengths of 4, 6, 8, 10, 12, and 14 carbon atoms (18). All AHL standards used in this experiment were chemically synthesized, purified, and characterized in the School of Molecular Medical Science, Centre for Biomolecular Science, University of Nottingham.

Determination of the AHL specificity for the BraI/R QS system of *B. phymatum* STM815. To identify the cognate AHL(s) for BraR of *B. phymatum* STM815, the promoter region of the *braI* gene was amplified with the primers PhymbraI.promF/R and cloned in the promoter-probe vector pMP220 (EcoRI-KpnI), generating $pMPbraI_p$. This plasmid was then mobilized into STM815BRAI. STM815BRAI($pMPbraI_p$) was then inoculated into 10 ml of M9 minimal medium (14) supplemented with 0.2% glucose, 0.3% Casamino Acids, Km, and Tc, grown overnight, and then diluted to an optical density at 600 nm (OD_{600}) of 0.2 into 10 ml of prewarmed medium (without antibiotics) containing a 1 mM, 100 nM, or 10 nM concentration of the specific AHL to be evaluated. β-Galactosidase activity was determined after 6 h of growth at 30°C and 180 rpm as described by Miller (19) with the modifications of Stachel et al. (20); all experiments were performed in triplicate, and the mean value is given.

Total RNA isolation. RNA isolations were carried out from three independent cultures of *B. phymatum* STM815 and *B. xenovorans* LB400 and their respective QS mutants STM815BRAI and LB400BRAR. The cultures were grown in KB incubated at 30°C and 180 rpm until they reached an optical density at 600 nm (OD_{600}) of 5.0 (end of logarithmic phase) (data not shown). RNA isolation was carried out from 2×10^9 cells using a Ribopure bacterial RNA isolation kit (Ambion Inc., Austin, TX, USA) following the manufacturer's instructions. Isolated RNA was treated with DNase at 37°C for 1 h and purified. The purity of RNA was assessed by PCR on total RNA (250 ng) with GoTaq polymerase (Promega) using specific primers for each species. RNA quality and concentration were assessed by Nanodrop (Thermo Scientific, Wilmington, DE, USA).

Microarray experiment and analysis. Custom microarrays for *B. phymatum* STM815 and *B. xenovorans* LB400 wild-type and mutant strains were designed and manufactured by MYcroarray, Inc. (Ann Arbor, MI, USA) in a 40,000-spot chip based on the genome sequences of the wild-type strains (PRJNA17409 and PRJNA57823 which correspond to strains STM815 and LB400, respectively). Each microarray slide had one array composed of 40,960 potential addresses for probes, of which 37,300 or 34,604 spots contained 45- to 47-mer probes for *B. phymatum* or *B. xenovorans* genes, respectively. The empty spaces were filled with random control probes. Each specific gene was surveyed by one unique probe sequence. There were four identical replicates of each *B. xenovorans* probe sequence, such that a total of 8,651 genes were surveyed by each array. For *B. phymatum* there were five identical replicates of each probe sequence, yielding a total of 7,460 genes being surveyed by each array. Microarray analysis was performed on three biological replicates of RNA samples collected for each strain, as described above. Labeling, hybridization, and scanning were performed by MYcroarray, Inc. Briefly, slides were hybridized with Cy3-labeled samples and scanned with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Data were extracted from the scanned images using GenePix Pro software (version 6.1.0.4). Gene functions were annotated from the National Center for Biotechnology Information (NCBI) database. Data analysis aimed at finding the differentially expressed genes (wild type × QS mutant) was carried out using Microsoft

Excel 2010. The cutoff *P* value used was 0.05 with a minimum 1.5-fold change.

Semiquantitative reverse transcription-PCR (SQ RT-PCR) and analysis. Reverse transcription was performed in a 20-μl reaction mixture containing 2.5 μg of total RNA, 200 ng of random primers/μg of RNA (Promega), and 30 U of avian myeloblastosis virus (AMV) reverse transcriptase, following the manufacturer's instructions. Conditions used for RT were 65°C for 3 min, 25°C for 10 min, 42°C for 90 min, and 70°C for 10 min. The primers 16s_BXE_RV/FW and Bphy_R0017-F/R were used to measure the transcription of the 16S rRNA gene. Second-strand synthesis was performed using GoTaq Flexi polymerase (Promega) with 1 μl of undiluted (any test gene) or 1:100 diluted (16S rRNA) cDNA reaction mixture as the template. The number of PCR cycles for each gene (the primers used are listed in Table S2 in the supplemental material) was standardized so that the product amplification was in the linear range; 10 to 20 μl of the PCR product was analyzed by agarose gel electrophoresis. The intensities of the bands were measured and normalized to the intensity of the 16S rRNA gene using Kodak 1D software (21) to obtain the fold difference. The validation of each gene was performed with samples from three independent isolations.

Mobilization of the *aiiA* lactonase gene into *Burkholderia* spp. to generate AHL-depleted strains. The pME6863 plasmid was mobilized into some PBE *Burkholderia* spp. to generate their transconjugants. The pME6863 plasmid carries the *aiiA* gene from the soil bacterium *Bacillus* sp. strain A24, which encodes a lactonase enzyme able to degrade AHLs (22).

EPS production and EPS promoter activity. EPS production was measured by streaking single colonies in yeast extract-mannitol (YEM) or nutrient-sucrose agar (NSA) medium, as described previously (23). EPS was extracted as follows. The bacterial strain was grown in 50 ml of YEM medium for 3 days at 30°C and 180 rpm; the supernatant was then separated from the bacteria through centrifugation, followed by the addition of 3 volumes of cold ethanol. The mixture was left overnight at 4°C under agitation before being centrifuged at 10,000 rpm for 20 min; after centrifugation, the precipitate was left to dry and then resuspended in 1 ml of sterile deionized water. The EPS was then quantified by the boiling-phenol method, as described previously (24).

The gene promoter of *bceI* of *B. phymatum* STM815 was amplified with the primers PhymEPS.promF/R and cloned in the promoter-probe vector pMP220 (EcoRI-PstI), generating pMPeps$_p$. This plasmid was then mobilized into *B. phymatum* STM815 and its QS mutant, STM815BRAI. STM815(pMPeps$_p$) and STM815BRAI(pMPeps$_p$) were inoculated into 10 ml of KB supplemented with Km and Tc, grown overnight, and then diluted to an OD_{600} of 0.2 into 20 ml of prewarmed medium (without antibiotics). β-Galactosidase activity was determined throughout different points of the growth curve, as described above. Complementation was done chemically by the addition of 10 to 20 μM concentrations of AHLs (mixture of 3-oxo-C_{14}-HSL, 3-oxo-C_{12}-HSL, and 3-oxo-C_{10}-HSL).

Legume nodulation assays. *Mimosa pudica* cultivation and nodulation tests were carried out as described previously (25). Briefly, seeds were surface sterilized with concentrated sulfuric acid for 10 min, followed by 3% sodium hypochlorite for 10 min, and then washed with sterile water. Seeds were germinated on 1% water agar plates at 28°C in darkness. Nodulation experiments were carried out in tubes containing modified liquid Jensen's N-free plant nutrient medium (26). The seedlings were inoculated 7 days after germination, with a bacterial concentration of 1×10^4 bacterial cells ml^{-1}. Plants were harvested 60 days after inoculation; the nodules were counted, and the dry weight of the plants was measured. Ten to 13 plants were tested for each bacterial strain; the experiment was performed in duplicate.

Common bean (*Phaseolus vulgaris* cvs. Flamingo and Negro Jamapa) cultivation and nodulation tests were carried out as described previously (7). Briefly, seeds were surface sterilized with 96% ethanol for 30 s followed by immersion in 5% sodium hypochlorite for 5 min, washed with sterile water, soaked in water for 2 h, and germinated in darkness at 30°C. Seedlings (2 per jar) were planted in autoclaved Leonard-type jars containing vermiculite and N-free nutrient solution (27). Plants were inoculated at sowing with approximately 10^9 cells ml^{-1} of bacteria. Plants were grown for 35 days in a greenhouse under the following conditions: 16/8-h day/night cycle and day/night temperatures of 28/20°C. Twelve plants were tested for each strain, and the experiment was done in duplicate (Flamingo) or triplicate (Negro Jamapa).

Plant endophytic colonization assays. Endophytic colonization and plant growth promotion were tested on two different maize cultivars (cultivars Kaloe and Mazurka; DOW AgroSciences Vertrebsgesellschaft mbH, Austria). Seeds were surface sterilized for 5 min in 70% ethanol and for 5 min in 5% sodium hypochlorite solution. They were then washed five times for 1 min in sterile water, and aliquots of the washing water were spread on 10% tryptic soy broth (TSB) agar to check for sterility. *B. phytofirmans* PsJN, PsJNBRAI, and PsJNBRAI(pBBRbraI$_{PsJN}$) were grown overnight in LB medium or in LB medium supplemented with the appropriate antibiotics for the mutant strains. Five milliliters of the overnight culture was used to inoculate 50 ml of fresh LB medium, and cultures were incubated at 28°C in a shaker until an OD_{600} of 1. Cells were washed and resuspended in fresh LB medium. Surface-sterilized seeds were soaked in bacterial suspensions for 30 min with soft shaking. Control seeds were soaked in sterile LB medium only. Seeds were placed on water agar (15%) and incubated in the dark at 27°C. Emerging sprouts were analyzed after 4 days of incubation. The number of germinated seeds, number of roots, and length of sprout and roots were then determined.

For the analysis of endophytic colonization, three emerged sprouts per treatment were surface sterilized and cut into small pieces. Plant material was placed into a sterile plastic bag and overlaid with sterile 0.9% sodium chloride solution, and then bacterial cells were dislodged from the plant material by oscillation using a Pulsifier (Microgen Bioproducts Ltd.). A dilution series of the supernatant was spread on LB agar supplemented with the appropriate antibiotics and was incubated overnight at 28°C to calculate the number of CFU per gram of plant material.

In order to study the effect of *B. phytofirmans* PsJN and its mutants on the development of young plants, seeds of two maize varieties (Mazurka and Kaleo) were surface sterilized and inoculated with bacteria as described above. Inoculated seeds were planted in sterile magenta boxes filled with sterilized soil treated by three cycles of freezing at −80°C and thawing. This treatment was performed to eliminate insects and eggs of insects. The plants were grown for 3 weeks in the greenhouse, after which the number of emerging plants and the length and the number of leaves were counted, and the fresh weights of the roots and the above-ground plant parts were determined.

RESULTS

Characterization of the AHLs produced by the QS systems of three different PBE *Burkholderia* spp. In order to perform a complete analysis of which AHL molecules are produced by each QS system of the PBE *Burkholderia* spp., *braI* knockout mutants of *B. phymatum* STM815 and *B. phytofirmans* PsJN were constructed, yielding STM815BRAI and PSJNBRAI, respectively. As expected, TLC analysis using bacterial biosensors revealed that STM815BRAI was unable to produce any detectable AHL molecules as the wild-type strain possesses only a single AHL QS system (BraI/R), whereas PSJNBRAI produced a putative OHC8-HSL molecule, which was most likely produced by the second AHL QS system, XenI2/R2, present in wild-type *B. phytofirmans* (see Fig. S1 in the supplemental material). Extractions of AHLs from these mutants and wild-type strains of *B. phymatum* and *B. phytofirmans* were performed, and these extracts were subjected to LC-ESI-MS/MS analysis, together with those from *B. xenovorans* LB400 and its QS mutants, LB400BRAI and LB400XENI2. These analyses showed that XenI2/R2 is mainly responsible for the production of OHC8-HSL and that the BraI/R system is involved in

TABLE 2 Identification of the different AHLs produced by the BraI/R system of *B. phytofirmans* STM815, *B. phytofirmans* PsJN, and *B. xenovorans* LB400 and by the XenI2/R2 system of *B. xenovorans* LB400

	AHL production by type and chain length[c]																	
	Unsubstituted						3-Oxo-HSL						3-Hydroxy-HSL					
Strain	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}
STM815[a]	−	+	++	+++	++	++	−	++	+++	+++	+++	++	−	+	+++	+++	+++	++
STM815BRAI[a]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
PsJN[b]	−	−	+	−	+	+	−	−	−	+	+	++	−	−	++	+	+	+++
PsJNBRAI[b]	−	−	+	−	−	−	−	−	−	−	−	−	−	−	++	+	−	−
LB400[b]	−	−	+	−	+	++	−	−	+	+	++	+++	−	−	++	+	++	+++
LB400BRAI[b]	−	−	+	−	−	−	−	−	−	−	−	−	−	−	++	+	−	−
LB400XENI2[b]	−	−	+	−	+	++	−	−	+	+	++	+++	−	−	+	+	++	+++

[a] AHL extraction from cell-free spent supernatant from a culture grown overnight in 20 ml of KB.
[b] AHL extraction from cell-free spent supernatant from a culture grown overnight in 50 ml of KB.
[c] −, no production; +, relative peak intensity <100,000; ++, relative peak intensity between 100,000 and 1,000,000; +++, relative peak intensity >1,000,000. See Table S3 in the supplemental material for further details.

the production of several different AHLs, varying in the length of their acyl chains (from 6 to 14 carbons) and in the substitution (or not) of a ketone or hydroxyl at position C-3 (Table 2; see also Table S3 in the supplemental material). From this analysis it was also evident that *B. phymatum* STM815 produces greater amounts of AHLs than the other species under these growth conditions (Table 2; see also Table S3).

The BraI/R QS of *B. phymatum* STM815 responds to several different AHL molecules. It has been established previously that the BraR proteins of *B. unamae* MTI-641, *B. kururiensis* M130, and *B. xenovorans* LB400 respond well to OC14-HSL (9, 10). However, as we established here that the BraI/R of *B. phymatum* is responsible for the production of significant amounts of several different AHLs in addition to OC14-HSL, it was of interest to determine which is the cognate AHL for the $BraR_{phym}$. In order to determine which AHL(s) $BraR_{phym}$ best responded to, we cloned the promoter region of $braI_{phym}$ into the pMP220 reporter plasmid (28), yielding $pMPbraI_p$, which was then introduced into the STM815 derivative *braI* mutant, STM815BRAI. The $braI_{phym}$ promoter activity was determined in the presence of exogenously added AHL molecules, which were found to be most abundantly produced by strain STM815 (Table 2; see also Table S3 in the supplemental material). Testing promoter activity in STM815BRAI($pMPbraI_p$) upon addition of different AHLs at three different concentrations (1 μM, 100 nM, and 10 nM) showed that the activity of the $braI_{phym}$ promoter was induced with all the AHLs tested, with the exception of C8-HSL (Fig. 1). This result suggests that the $BraR_{phym}$ is more promiscuous than the other BraR proteins tested so far, as it is able to respond to several different AHL molecules, even at low concentrations.

Determination of the BraI/R regulon by transcriptome analysis. The BraI/R AHL QS system is very well conserved among all members of the PBE *Burkholderia* group; thus, it was of interest to study its regulon in two species. Microarray profiling was performed with two PBE *Burkholderia* spp. originating from different environmental niches, namely, the legume symbiont *B. phymatum* STM815 and the soil isolate *B. xenovorans* LB400. RNA was extracted from the wild-type strains and their respective QS mutants, STM815BRAI and LB400BRAR, at the end of logarithmic phase from three biological replicates. A set of eight differentially expressed genes from each microarray experiment was then chosen for validation with SQ RT-PCR, which displayed comparable results with the microarray/transcriptomic data (see Table S4 in the supplemental material).

The BraI/R regulon of *B. xenovorans* LB400. The BraI/R QS system of LB400 influenced the transcriptional levels of 347 genes distributed among its three chromosomes by 1.5-fold or more, representing approximately 4% of the protein-encoding genes in the strain. The system positively regulated 296 of these genes, whereas 61 were downregulated (Fig. 2; see also Table S5 in the supplemental material).

The highest percentage of the regulated genes corresponded to cell process and metabolism genes (42.4%), and the second most represented set of genes encode hypothetical proteins (27.1%) (Fig. 2). A closer analysis of these hypothetical proteins with the STRING tool (29) showed that most were conserved among bacteria, also having homologues in such distant groups as eukaryotes and archaea. However, some of them were shared only with other *Burkholderia* spp., as for example loci Bxe_A2661, Bxe_B0810, Bxe_B2781, and others were unique to *B. xenovorans*, such as Bxe_B0044, Bxe_B0045, and Bxe_B0046 (see Table S5).

A significant finding was the overrepresentation of genes encoding components of ABC transporters, representing 12.1% of all differentially expressed genes (Fig. 2). This finding suggests that QS is important for the interaction of LB400 with the environment, as ABC transporters are essential for the utilization of environmental nutrients. Moreover, 5.7% of the genes shown to be regulated by QS were involved in the degradation of aromatic compounds, a well-known characteristic of *B. xenovorans*, and several of them were related to benzoate degradation (see Table S5 in the supplemental material).

The BraI/R regulon of *B. phymatum* STM815. The BraI/R QS system of *B. phymatum* STM815 acted directly or indirectly on the transcription of 176 genes by 1.5-fold or more, which represents approximately 2.3% of the protein-coding genes in this strain. The system positively regulated 54 of these genes, whereas 122 were downregulated (Fig. 2; see also Table S6).

As in *B. xenovorans* LB400, the highest percentage of BraI/R-regulated genes (38.6%) corresponded to various cellular processes and metabolism genes. Again, hypothetical proteins were highly represented, with 17.6% of the total genes regulated above 1.5-fold (Fig. 2). Of these, Bphy_1208 and Bphy_1217 were found only in *Burkholderia* spp., and Bphy_1241 was unique to STM815 (see Table S6 in the supplemental material).

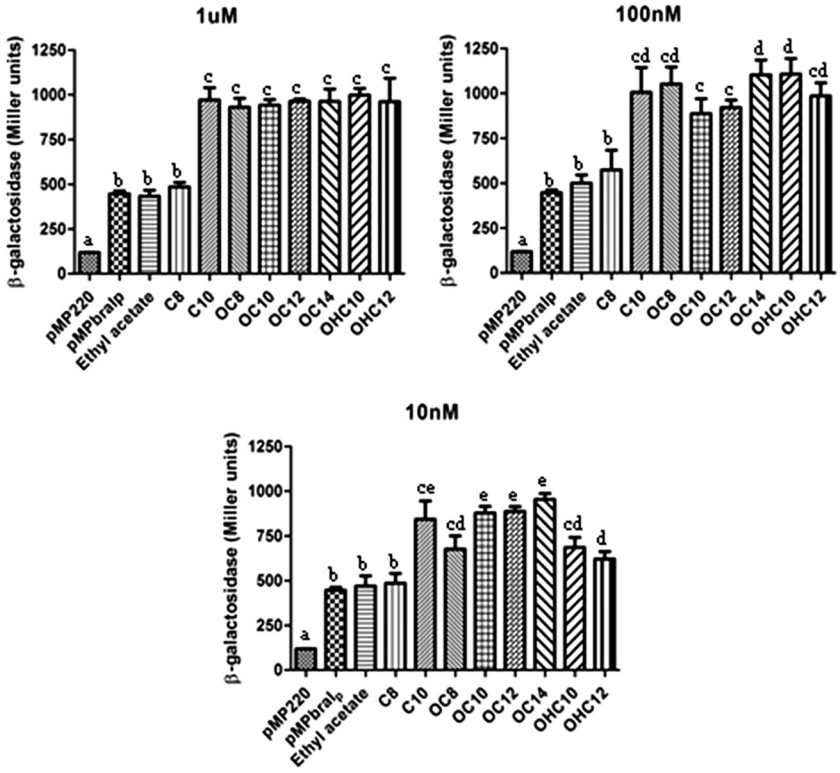

FIG 1 Determination of the biologically active AHL for the BraR AHL sensor/regulator of *B. phymatum* STM815. Bars correspond to β-galactosidase activities determined for STM815BRAI harboring pMPbraI$_p$ or pMP220. Various exogenous AHLs in different concentrations (1 μM, 100 nM, and 10 nM) were added as indicated, and the β-galactosidase activities were determined. The results are mean values ± the standard deviations of three independent biological replicates. Means indicated by different letters are significantly different at a *P* value of ≤0.05. Carbon chain lengths are indicated (OC, oxo-C; OHC, hydroxy-C).

A significant number (14.8%) of the regulated genes was shown to be involved in DNA/RNA modifications, especially in recombination and DNA repair; in addition, 11.9% were involved in the synthesis and composition of the bacterial membrane and cell wall, with a good representation of genes related to the production of lipopolysaccharides (LPS). No genes for nodulation or nitrogen fixation were found to be regulated by BraI/R.

Comparison of the BraI/R regulons of *B. xenovorans* LB400 and *B. phymatum* STM815. It was interesting to discover that the BraI/R system of STM815, which possesses one AHL QS system, was responsible for the regulation of a smaller number of genes (i.e., 50% fewer) than the BraI/R regulon of LB400, which also possesses an additional AHL QS system. Another important difference was the major type of regulation in each strain; in LB400,

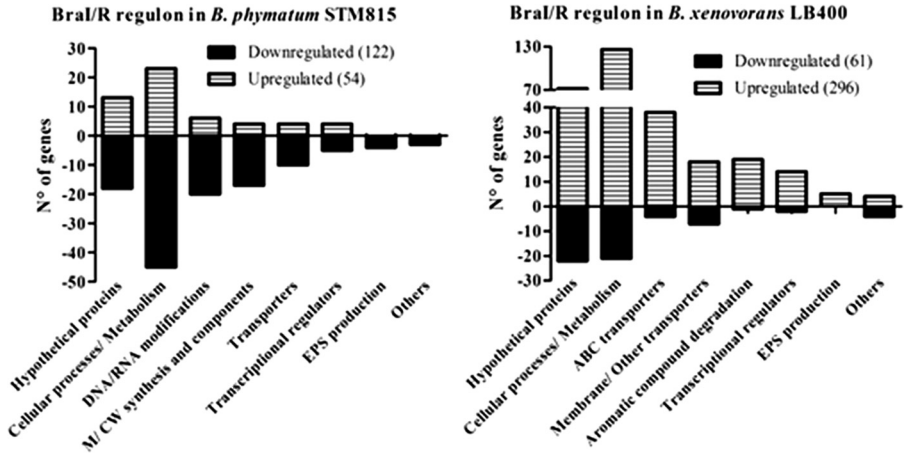

FIG 2 Functional classification of differentially expressed genes found in transcriptome analysis between *B. phymatum* STM815 wild type and its *braI* mutant and *B. xenovorans* LB400 wild type and its *braR* mutant. Only genes with a fold difference greater than 1.5 are included. M, membrane; CW, cell wall; EPS, exopolysaccharide.

TABLE 3 Homologous genes regulated by the BraI/R QS system in both *B. xenovorans* LB400 and *B. phymatum* STM815

Locus[a]	Gene product	% identity[b]	Fold change
Bphy_0422	Conserved hypothetical protein	89	1.61
Bxe_A0642	Conserved hypothetical protein		1.64
Bphy_0313	Peptidyl-tRNA hydrolase	92	−3.01
Bxe_A4135	Peptidyl-tRNA hydrolase		−1.56
Bphy_1058	Nucleotide sugar dehydrogenase	86	−4.10
Bxe_A2245	UDP-glucose 6-dehydrogenase		2.48
Bphy_1064	Putative transmembrane protein	80	−2.26
Bxe_A2239	Putative transmembrane protein		1.64

[a] Bphy, *B. phymatum* STM815; Bxe, *B. xenovorans* LB400.
[b] Identity between genes of *B. xenovorans* LB400 and *B. phymatum* STM815.

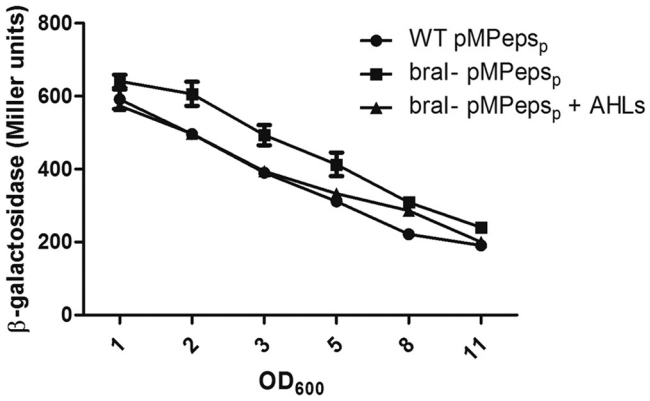

FIG 4 Transcription of the EPS operon is negatively regulated by the BraI/R QS system of *B. phymatum* STM815. β-Galactosidase activities of a transcriptional fusion of the EPS operon promoter region to *lacZ* were assayed in the *B. phymatum* STM815 wild type (WT pMPeps$_p$), its QS mutant (braI− pMPeps$_p$), and the QS mutant complemented by the addition of 2 μM AHL to the growth medium (braI− pMPeps$_p$ + AHLs). Strains were grown in KB. The results are mean values ± the standard deviations of three independent biological replicates.

85.3% of the genes were activated by the system, whereas in STM815, 69.3% were repressed by it.

This comparison surprisingly revealed that only four genes were regulated in both strains (Table 3); two of these coded for a hypothetical protein and a peptidyl-tRNA hydrolase, while the other two were related to the production of EPS. Little can be predicted about the hypothetical protein as it does not have any known conserved domains, but a BLAST search revealed that it is a very common protein among *Burkholderia* spp. (data not shown). Peptidyl-tRNA hydrolases are important for protein translation, as they release tRNA from peptidyl-tRNA by cleaving the ester bond between the peptide and the tRNA, and they have been shown to be essential for the survival of *E. coli* (30, 31). The two EPS-related genes are part of an EPS production cluster, known as *bce-1*, composed of 11 genes homologous to the ones responsible for the synthesis of cepacian, the major EPS produced by a large percentage of clinical isolates of the BCC (32). In addition to these two EPS genes that are regulated in both strains, more genes from the same cluster were regulated by the BraI/R system in each bacterium, with two other genes regulated in STM815 and three regulated in LB400 (Fig. 3). However, the type of regulation of these EPS genes is different in each strain, as they are repressed by the QS system in STM815 and activated by it in LB400.

Confirmation of the regulation of EPS production by promoter studies and EPS quantification. Careful analysis of the promoter region of the *bce-1* genes of LB400 and STM815 did not reveal any evident BraR putative regulatory region via a *lux*-box-like element. We therefore cloned the promoter region of one of the *eps* genes (i.e., Bphy_1057, undecaprenyl-phosphate glucose phosphotransferase) of *B. phymatum* STM815 into the pMP220 reporter plasmid (28), yielding the plasmid pMPeps$_p$, which was then introduced into the STM815 wild-type strain and its derivative *braI* mutant. β-Galactosidase activity was determined at different growth stages, and it confirmed the negative regulation of the EPS promoter by BraI/R (Fig. 4). Promoter activity increased approximately 1.5-fold in the *braI* mutant throughout the growth phase and was restored to statistically similar levels by complementation with exogenous addition of AHLs.

We also quantified EPS from the wild-type and QS mutant strains of STM815 and LB400. Moreover, we also performed the same experiment with the strains *B. phytofirmans* PsJN and *B. phymatum* GR01 and with their respective *braI* mutants in order to elucidate if BraI/R was involved in the regulation of EPS production also in these strains. *B. phymatum* STM815 and GR01 showed that their QS mutants produced substantially larger amounts of EPS (9.5- and 240-fold increase, respectively) than the wild-type strains (Fig. 5). In contrast, the QS mutants of *B. xenovorans* LB400 and *B. phytofirmans* PsJN produced less EPS than the wild-type strains, with a difference of 1.5- and 2.9-fold, respectively (Fig. 5). In all four cases, EPS production was completely or partially restored to the wild-type levels when mutants were complemented with plasmids harboring their corresponding *braI* or *braR* genes, as appropriate. These results were in agreement with the microarray and gene promoter data and further confirmed the role of the BraI/R QS system in the regulation of EPS production in different PBE *Burkholderia* spp.

The regulation of EPS production by the BraI/R QS system is a common feature among the PBE *Burkholderia* group. Our re-

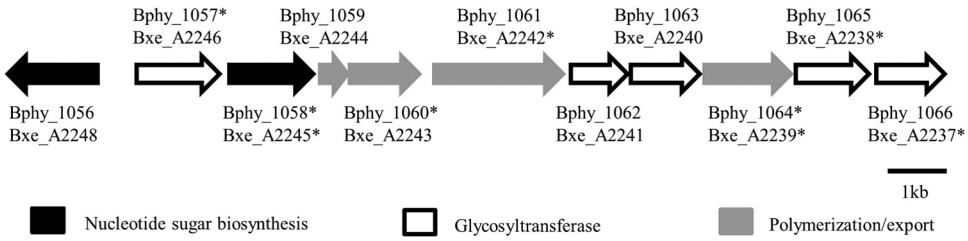

FIG 3 The cepacian production cluster *bceI* from *B. xenovorans* LB400 and *B. phymatum* STM815. *, genes found to be regulated by the BraI/R QS system of LB400 (Bxe) or STM815 (Bphy) by microarray studies.

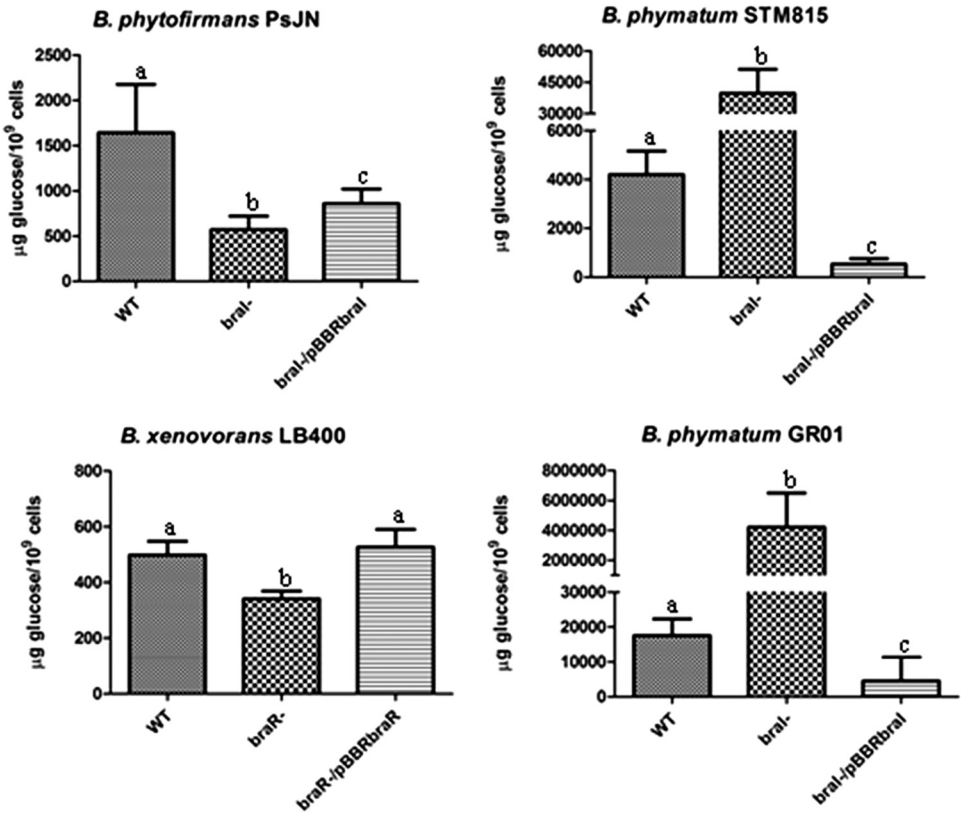

FIG 5 EPS quantification of *B. phytofirmans* PsJN, *B. phymatum* STM815, *B. xenovorans* LB400, and *B. phymatum* GR01 wild type (WT), QS mutants (braI− or braR−), and complemented mutants (braI−/pBBRbraI or braR−/pBBRbraR) was performed by the boiling-phenol method (described in Materials and Methods). Experiments were performed in triplicate, and means ± standard deviations are plotted. Means indicated by different letters are significantly different at a *P* value of ≤0.05.

sults have shown the involvement of BraI/R in the regulation of EPS production in *B. phymatum*, *B. xenovorans*, *B. phytofirmans*, *B. kururiensis*, and *B. unamae* (see above and reference 10). These observations suggest a possible common role for the BraI/R system among the PBE *Burkholderia* group. To test this, we mobilized the plasmid pME6863 (22), harboring the gene coding for the AiiA lactonase enzyme, into five other PBE species, namely, *B. tuberum* DSM17489, *B. tropica* Ppe8, *B. terrae* DSM17804, *B. phenazinium* DSM10684, and *B. graminis* DSM17151. We initially confirmed that the presence of pME6863 significantly reduced AHL production by AHL extraction and analysis with a biosensor strain (see Fig. S2 in the supplemental material). EPS production was then tested on solid growth medium, and all five *Burkholderia* strains carrying the pME6863 plasmid displayed different profiles of EPS production than the wild-type strains (data not shown). These results demonstrated that the conserved BraI/R AHL QS system is involved in the regulation of EPS production in the PBE *Burkholderia* group.

The BraI/R QS system is not essential for legume nodulation by *B. phymatum* strains. It was of interest to determine the role of the BraI/R of *B. phymatum* in symbiotic nodulation. We therefore tested the ability of *braI* mutants of *B. phymatum* STM815 and GR01 to produce effective nodules on mimosa (*Mimosa pudica*) and common bean (*Phaseolus vulgaris*), respectively.

Results showed that *M. pudica* was nodulated by the wild-type strain of STM815 and by the QS mutant strain to the same extent, as no difference in the numbers of nodules was observed (Fig. 6A). In both cases the nodules were effective, as the dry weights of the inoculated plants were greater than those of the uninoculated (and therefore nonnodulated) controls (Fig. 6B). Similar results were obtained when the *B. phymatum* GR01 strain was used in nodulation experiments with common bean. The *braI* mutant GR01BRAI was as competent as the wild-type in producing symbiotic nodules on *P. vulgaris* var. Flamingo and *P. vulgaris* var. Negro Jamapa (see Table S7 in the supplemental material). Moreover, the dry weights of plants inoculated with the QS mutant were similar to those of plants inoculated with the wild-type strain (see Table S8). These results clearly indicate that the BraI/R QS system of *B. phymatum* does not play a major role in the symbiotic lifestyle of this species.

The BraI/R QS system is not important for the endophytic colonization and plant growth promotion of maize by *B. phytofirmans* PsJN. *B. phytofirmans* PsJN colonizes endophytically and promotes the growth of several plant species, including maize (*Zea mays*), and so this plant was used as a model to study the role of BraI/R in endophytic colonization. Although PsJN colonizes and interacts with a wide variety of genetically unrelated plants, the intensity of its effects may vary considerably between different genotypes of a plant species (33, 34). For this reason, we performed experiments using two different maize cultivars (cultivars Kaleo and Mazurka).

PsJN colonized both maize varieties equally well, as we recov-

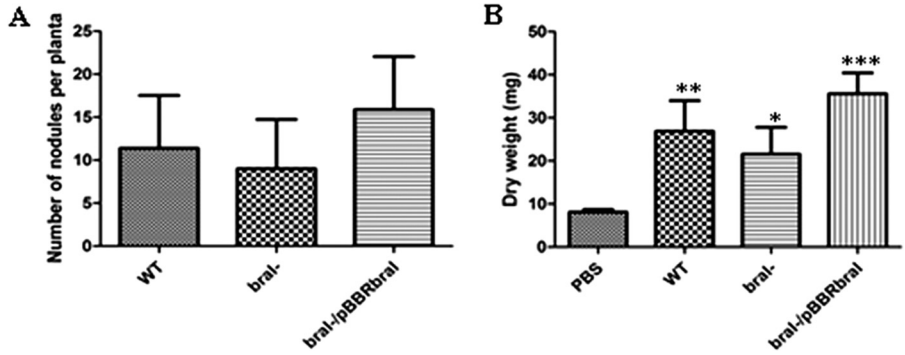

FIG 6 Nodulation of *M. pudica* by *B. phymatum* STM815 (WT), its QS mutant (braI−), or the complemented QS mutant (braI−/pBBRbraI) measured 60 days after infection. (A) Mean number of nodules per plant. (B) Mean dry weight of plants inoculated with the three different strains or left uninoculated (PBS, phosphate-buffered saline). Nodulation tests were repeated twice, with 10 to 13 replicate plants. Bars indicate means ± standard deviations. Statistical analyses (Student's *t* test) were performed to compare the mutant to the wild-type strain (A) or inoculated plants to the uninoculated ones (B). Significant differences are indicated as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

ered similar numbers of viable cells from 4-day-old seedlings (1.3E+08 CFU g^{-1} fresh weight [FW] in cultivar Mazurka and 1.4E+08 CFU g^{-1} FW in cultivar Kaleo). The PSJNBRAI mutant strain was not affected in its ability to colonize either maize variety, as the number of viable cells recovered was comparable to that of the wild-type strain (1.2E+08 CFU g^{-1} FW in cultivar Mazurka and 7.6E+07 CFU g^{-1} FW in cultivar Kaleo).

Germination was not significantly affected by the PsJN wild-type strain in either maize variety. By 4 days after seed inoculation, no significant differences were observed in the numbers of germinated seeds and roots. Seedling root and shoot lengths were increased but not significantly with respect to uninoculated seeds treated with broth only. Inoculation with the PSJNBRAI mutant strain gave similar results to those with the wild-type strain and the uninoculated controls. In addition, PsJN did not significantly affect the growth of young plants of cultivar Mazurka. Three weeks after seed inoculation and growth in soil in the greenhouse, no significant differences were observed in the numbers of leaves and lengths of sprouts and roots or sprout fresh weight with respect to uninoculated control plants. No effect was observed in the plants inoculated with the PSJNBRAI mutant strain. In contrast to cultivar Mazurka, PsJN did show significant effects on the development of young plants of cultivar Kaleo. Sprout length was increased from an average value of 18 cm in the control to 43 cm in PsJN-inoculated plants, while the average root fresh weight was increased from 0.3 to 1.0 g $plant^{-1}$, and the average sprout weight increased from 0.7 to 1.7 g $plant^{-1}$. The PSJNBRAI mutant strain had similar effects on young plants of cultivar Kaleo, thus showing that the *braI* knockout mutation did not affect the plant growth-stimulating effect of the endophyte. Taken together, these results demonstrate that the BraI/R QS system of *B. phytofirmans* PsJN is not involved in either endophytic colonization or plant growth promotion of maize.

DISCUSSION

The recently described PBE *Burkholderia* group is attracting attention from the scientific community, as it is composed of metabolically versatile bacteria with great agrobiotechnological potential. This group shares a highly conserved AHL QS system, known as BraI/R, which in *B. kururiensis*, *B. unamae*, and *B. xenovorans* was shown to produce and respond to OC14-HSL (10). In this study, we present LC-ESI-MS/MS analyses of AHL extracts from members of this group (i.e., *B. phymatum* STM815, *B. phytofirmans* PsJN, *B. xenovorans* LB400, and their AHL QS mutants). We have identified and compared the regulons of BraI/R QS of *B. phymatum* STM815 and *B. xenovorans* LB400, which revealed that the regulons of this QS system are species specific, which may be a result of niche adaptation. Moreover, this QS system was shown not to be important for legume nodulation by *B. phymatum* strains or for endophytic colonization and growth promotion of maize by *B. phytofirmans* PsJN.

The BraI/R system is responsible for the production of AHL molecules varying from 6 to 14 carbons with or without oxo or hydroxyl substitutions at the C-3 position (Table 2; see also Table S3 in the supplemental material). Some other LuxI-type proteins were already reported to produce several different types of AHL molecules. For instance, the AHL autoinducer synthases of some *Rhizobium* spp. are known to produce many different AHLs, as well as AfeI from *Acidithiobacillus ferrooxidans* (35, 36). LuxI-type proteins catalyze AHL synthesis from *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (acyl-ACP). It has been proposed that the production of different types of AHLs is not only a function of the enzyme acyl chain specificity but may also be influenced by the available cellular pool of acyl-ACP substrates in each bacterium (37). Consequently, growth conditions could also affect acyl-ACP availability (38). This could explain why we observed that some AHL molecules were produced only in M9-glucose medium and not in KB or vice versa; e.g., *B. xenovorans* LB400 and *B. phytofirmans* PsJN were able to produce C6-HSL only when grown in M9-glucose medium (see Table S3).

B. phymatum STM815 was shown to produce much larger amounts of each AHL molecule than *B. xenovorans* LB400 and *B. phytofirmans* PsJN (Table 2; see also Table S3). Moreover, the $BraR_{phym}$ is able to respond to almost all of the AHLs that it produces, even in concentrations as low as 10 nM, indicating that $BraR_{phym}$ has a broader signal specificity than the other BraR molecules already tested (Fig. 1) (10). The high production and promiscuous response to different types of AHLs by BraR even at low concentrations could mean that *B. phymatum* is an efficient eavesdropper of AHLs produced by neighboring bacteria and possibly provides it with a competitive advantage (39).

The presence of the BraI/R QS system among all the species of

the PBE group tested so far possibly suggests that it was originally present in their common ancestor and is part of their core genome (10). Does BraI/R therefore share a regulon? For this purpose we performed transcriptome analysis of two PBE *Burkholderia* spp. from different environmental niches, namely, the legume symbiont *B. phymatum* STM815 and the soilborne, efficient biodegrader *B. xenovorans* LB400. The regulon of *B. xenovorans* is composed of several hypothetical proteins (27.1%), of which 10 are unique to the species and thus could be a topic of future research. Interestingly, 12.1% of all the genes being regulated encode ABC transporter components, which are important for the transport of a variety of substrates, such as metals, small ions, mono- and oligosaccharides, peptides, and amino acids essential for the utilization of environmentally available nutrients (40). Moreover, an important number of genes involved in the degradation of aromatic compounds, especially benzoate, were also regulated by BraI/R. It is possible that these two functions are linked, as ABC transporters are known to be involved in the uptake of aromatic compounds (40).

Aromatic compounds are widely distributed throughout the biosphere, predominantly in the form of recycled plant material, and comprise >25% of the earth's biomass (41). The natural turnover of these molecules is very slow because of the inherent thermodynamic stability of the aromatic ring. For this reason, microbial biodegradative pathways play a crucial role in the carbon cycle of aromatic compounds. Benzoate is a key aromatic intermediate in these pathways, being an end product of the degradation of many aromatic compounds, such as toluene, phenol, and even polychlorobiphenyl. *B. xenovorans* possesses three functional benzoate degradation pathways, of which two are paralogous copies of the benzoate oxidation (box) pathway (the chromosomal and megaplasmid box [box_C and box_M, respectively] clusters) (42, 43). Our studies revealed regulation of the box_M cluster by the BraI/R system, which is also in accordance with previous studies in which it was shown that the expression of this pathway is activated during the transition to the stationary phase of growth (44, 45).

In contrast to *B. xenovorans* LB400, the regulon of BraI/R of *B. phymatum* was overrepresented in loci involved in the regulation of DNA/RNA modification enzymes, in DNA repair and recombination, and in the regulation of components of the membrane/cell wall or of enzymes important for their synthesis. The negative regulation of genes related to LPS production is an interesting finding, as previous studies had already shown that the rhizobial LPS undergoes structural modifications during symbiosis with legumes (46–50). LPS is the primary component of the bacterial outer layer and is comprised of three structural regions: the lipid A that is anchored in the bacterial outer membrane, the core oligosaccharide, and the O-chain polysaccharide (or O antigen), which may or may not be present. Studies with alphaproteobacterial rhizobia (alpha-rhizobia) have shown that there are compositional differences between the bacterial and bacteroid LPS (46–50), and this has led to the hypothesis that the bacterial LPS structure inside legume root nodules is probably controlled, to a large extent, by the *in planta* microenvironment. From our studies, it cannot be excluded that AHL QS might play a role in LPS biosynthesis and modification during legume nodulation by *B. phymatum*; no studies thus far correlating LPS production/modification and nodule formation have been reported for betaproteobacterial rhizobia (beta-rhizobia).

The regulon of *B. phymatum* was determined using a *braI* mutant, whereas for *B. xenovorans* we used a *braR* mutant; this could have generated slight differences, as BraR could be involved in gene regulation independently of AHLs. Moreover, *B. xenovorans* contains a LuxR solo which could respond to BraI-produced AHLs expanding the QS regulon. As AHL QS systems strictly require both LuxI and LuxR-like proteins, we believe that our results using two different mutants of two species can be reliably compared. These transcriptome studies indicated a unique role for BraI/R in each species, rather than a common one, as only four common genes were regulated in two species of the PBE cluster. Interestingly, two of the four genes commonly regulated by both BraI/R systems are involved in the production of the EPS cepacian (Fig. 3). The regulation of EPS genes in *B. phymatum* and *B. xenovorans* was also confirmed by gene promoter and phenotypic studies. The regulation of EPS in several other members of the PBE *Burkholderia* group was also established by the introduction of an AHL-lactonase gene. The regulation of EPS production by AHL QS is, therefore, a common feature in PBE *Burkholderia* species. Exopolysaccharides are abundant extracellular products that accumulate on the bacterial cell surface and are secreted into its surroundings. The formation of a highly polymerized, hydrated, anionic matrix with a peripheral localization suggests that EPS protects bacteria against various environmental stresses, such as desiccation or toxic molecules, and it can also provide the first contact between bacteria and plant cell surfaces (51). These factors make the production of EPS an important phenotype for both plant and soil bacteria and might explain why it is regulated by BraI/R in the PBE *Burkholderia* spp. It would be interesting to perform further transcriptomic analyses with PBE species that occupy similar niches in order to provide further insights into the evolution of the role of the BraI/R system.

Although several studies with alpha-rhizobia have shown the importance of LPS and EPS in their symbiotic interactions with legumes (52), the *braI* mutants of *B. phymatum* were, surprisingly, not affected in their ability to form nodules on *M. pudica* and *P. vulgaris*. However, all of these alpha-rhizobial studies were performed with mutants impaired in the production of LPS or EPS, while the *braI* mutants of *B. phymatum* probably produce more of these molecules, as our transcriptome studies have shown that the BraI/R QS system is negatively regulating LPS and EPS genes. The AHL QS systems of several α-rhizobia have been shown to play important roles in legume nodulation and nitrogen fixation (36, 53). Our study represents the first analysis of the role of QS in nodulation by β-rhizobia, and it has revealed, somewhat surprisingly, that AHL QS does not play a major role in it; we are currently undertaking further research into this phenomenon.

A recent study demonstrated the importance of the XenI2/R2 QS system of *B. phytofirmans* PsJN for its endophytic colonization and growth promotion of *Arabidopsis thaliana*, but a PsJN knockout mutant for the BraI/R system showed only reduced endophytic colonization compared to the wild type (54). In the present study, however, we showed that the BraI/R QS system of PsJN does not play a major role in its endophytic colonization and plant growth promotion of maize. This contrasts with studies on rice, in which the BraI/R AHL QS system of *B. kururiensis* M130 has been shown to be important in its endophytic colonization (10). It is possible that different endophytes use different regulatory processes in order to colonize their hosts, with the plant also playing an active role in the colonization process (55).

It is important to highlight that these *Burkholderia* spp. were

found to possess another type of QS system, based on the production and recognition of a molecule known as the *Burkholderia* diffusible signal factor (BDSF) (56). This system was shown to regulate swarming motility, biofilm formation, and virulence in *B. cenocepacia* (56), and it could be involved in plant-bacterium interaction in the PBE *Burkholderia* spp.

In summary, this study highlighted that, regardless of how well conserved BraI/R is among the PBE *Burkholderia* species, the regulon is not conserved, possibly indicating that its role has evolved to be tailored to different models of growth in the various niches that this cluster of *Burkholderia* species occupies. Surprisingly, BraI/R does not play a major role in these *Burkholderia* species *in planta*, which means that other regulatory systems, possibly both global and specific, are regulating genes in response to the *in planta* growth of these bacteria. The production of EPS, however, is regulated by the BraI/R system throughout the PBE *Burkholderia* cluster, thus indicating a common role of EPS at high densities in this group of bacteria. Future studies will need to focus on the direct targets as well as the role played by the second AHL QS system that some species in this PBE *Burkholderia* cluster have evolved to possess.

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