

# Commonalities and Differences in Regulation of *N*-Acyl Homoserine Lactone Quorum Sensing in the Beneficial Plant-Associated *Burkholderia* Species Cluster<sup>∇†</sup>

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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<sub>XEN</sub> and BraI/R<sub>UNA</sub> systems, respectively. Several phenotypes were analyzed, and it was determined that exopolysaccharide was positively regulated by the BraI/R-like system in the species *B. kururiensis*, *B. unamae*, and *B. xenovorans*, highlighting commonality in targets. However, the three BraI/R-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<sup>T</sup>. 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

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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. kururienensis* designated BraI/R<sub>KUR</sub>, which produces and responds to 3-oxo- $C_{12}$ -HSL. It was determined that the BraI/R<sub>KUR</sub> 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<sup>T</sup> possessed a BraI/R-like system (designated BraI/R<sub>XEN</sub>) 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 kururienensis*, *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 concentra-

tions: ampicillin, 100  $\mu\text{g ml}^{-1}$ ; tetracycline, 15  $\mu\text{g ml}^{-1}$  (*E. coli*) or 40  $\mu\text{g ml}^{-1}$  (*Burkholderia*); gentamicin, 10  $\mu\text{g ml}^{-1}$  (*E. coli*), 30  $\mu\text{g ml}^{-1}$  (*Agrobacterium*), or 40  $\mu\text{g ml}^{-1}$  (*Burkholderia*); kanamycin, 50  $\mu\text{g ml}^{-1}$  (*E. coli*) or 100  $\mu\text{g ml}^{-1}$  (*Burkholderia*); and nitrofurantoin, 50  $\mu\text{g ml}^{-1}$ . Conjugations in *B. kururienensis*, *B. unamae*, and *B. xenovorans* were performed by triparental mating using *E. coli* DH5 $\alpha$  (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.

$\beta$ -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<sup>T</sup>.** To identify the QS system of *B. unamae* MTL-641<sup>T</sup>, 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  $\lambda$  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*<sub>UNA</sub> and *braR*<sub>UNA</sub> loci were located within a 8-kb EcoRV fragment and cloned in pMOSBlue to generate pMOS-pLZ-1, which was sequenced.

To generate genomic *braI*<sub>UNA</sub>- and *braI*<sub>UNA</sub>-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<sup>T</sup> to generate *B. unamae* UNABRAI and *B. unamae* UNABRAR.

To identify the BraR<sub>UNA</sub> cognate AHL, *braR*<sub>UNA</sub> was PCR amplified from genomic DNA and cloned into pQE30 to generate pQEUNAR. The *braI*<sub>UNA</sub> 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  $\mu\text{M}$  concentrations of each synthetic AHL. The  $\beta$ -galactosidase activity was determined after 4 h at 37°C.

**Isolation of the *braI*<sub>XEN</sub> and *braR*<sub>XEN</sub> and the *xenI2* and *xenR2* AHL QS systems and of the *bxeR* solo gene of *Burkholderia xenovorans* LB400<sup>T</sup> and construction of derivative genomic mutants.** *braI*<sub>XEN</sub>, *tsaL*<sub>XEN</sub>, and *braR*<sub>XEN</sub> were identified by *in silico* comparison of the DNA and protein sequences with the genome of *B. xenovorans* LB400<sup>T</sup>(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*<sub>XEN</sub>, *braI*<sub>XEN</sub>, *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<sup>T</sup> 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*<sub>XEN</sub> gene and its promoter region were PCR amplified and cloned into pMOS-Blue. A 623-bp fragment containing *braR*<sub>XEN</sub> 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 <sup>a</sup>	Characteristics <sup>b</sup>	Reference or source
<b>Strains</b>		
<i>B. kururiensis</i> M130	Amp <sup>r</sup> Rif <sup>r</sup> , isolated from surface-sterilized roots of rice	5
M130BRAI	<i>braI</i> ::Km of <i>B. kururiensis</i> M130	73
M130BRAR	<i>braR</i> ::Km of <i>B. kururiensis</i> M130	73
M130RSAL	<i>rsaL</i> ::Km of <i>B. kururiensis</i> M130	73
<i>B. xenovorans</i> LB400 <sup>T</sup>	Type strain	31
LB400BRAI	<i>braI</i> ::Km of <i>B. xenovorans</i> LB400 <sup>T</sup>	This study
LB400BRAR	<i>braR</i> ::Km of <i>B. xenovorans</i> LB400 <sup>T</sup>	This study
LB400XENI2	<i>xenI2</i> ::Km of <i>B. xenovorans</i> LB400 <sup>T</sup>	This study
LB400XENR2	<i>xenR2</i> ::Km of <i>B. xenovorans</i> LB400 <sup>T</sup>	This study
LB400BXER	<i>bxeR</i> ::Km of <i>B. xenovorans</i> LB400 <sup>T</sup>	This study
<i>B. unamae</i> MTI-641 <sup>T</sup>	Maize rhizosphere isolate	10
UNABRAI	<i>braI</i> ::Km of <i>B. unamae</i> MTI-641	This study
UNABRAR	<i>braR</i> ::Km of <i>B. unamae</i> MTI-641	This study
<i>P. putida</i> F117	AHL-negative derivative of <i>P. putida</i> IsoF; PpuI <sup>-</sup>	63
<i>E. coli</i> M15	NaI Str Rif Thi Lac <sup>-</sup> Ara <sup>+</sup> Gal <sup>+</sup> Mtl <sup>-</sup> F <sup>-</sup> RecA <sup>+</sup> Uvr <sup>+</sup> Lon <sup>+</sup>	Qiagen
<b>Plasmids</b>		
pRK2013	Tra <sup>+</sup> Mob <sup>+</sup> ColE1 replicon; Km <sup>r</sup>	27
pMOSBlue	Cloning vector; Amp <sup>r</sup>	Amersham-Pharmacia
pGEM-T Easy	Cloning vector; Amp <sup>r</sup>	Promega
pMP220	Promoter probe vector, IncP, LacZ; Tet <sup>r</sup>	70
pLAFR3	Broad-host-range cloning vector, IncP1; Tet <sup>r</sup>	72
pKNOCK-Km	Conjugative suicide vector; Km <sup>r</sup>	2
pLZ1	pLAFR3 containing <i>B. unamae</i> DNA	This study
pBBRMCS-5	Broad-host-range vector Gm <sup>r</sup>	42
pKRC-12	pBBR1MCS-5 carrying <i>P<sub>lasB</sub>-gfp</i> (ASV) <i>P<sub>lac</sub>-lasR</i> ; Gm <sup>r</sup>	63
pMOS-LZ-1	pMOSBlue carrying an 8-kb fragment containing partial <i>B. kururiensis</i> QS genes; Amp <sup>r</sup>	This study
pKNOCK-UNAI	Internal PCR <i>braI<sub>UNA</sub></i> fragment of <i>B. unamae</i> cloned in pKNOCK-Km	This study
pKNOCK-UNAR	Internal PCR <i>braR<sub>UNA</sub></i> fragment of <i>B. unamae</i> cloned in pKNOCK-Km	This study
pKNOCKXENR	Internal PCR <i>braR<sub>XEN</sub></i> fragment of <i>B. xenovorans</i> cloned in pKNOCK-Km	This study
pKNOCKXENI	Internal PCR <i>braI<sub>XEN</sub></i> fragment of <i>B. xenovorans</i> cloned in pKNOCK-Km	This study
pKNOCKXENI2	Internal PCR <i>xenI2</i> fragment of <i>B. xenovorans</i> cloned in pKNOCK-Km	This study
pKNOCKXENR2	Internal PCR <i>xenR2</i> fragment of <i>B. xenovorans</i> cloned in pKNOCK-Km	This study
pKNOCKBXE	Internal PCR <i>bxeR</i> fragment of <i>B. xenovorans</i> cloned in pKNOCK-Km	This study
MPXENI1	promoter of gene <i>braI<sub>XEN</sub></i> cloned in pMP220 vector	This study
pMPX2I	Promoter of gene <i>xenI2</i> cloned in pMP220 vector	This study
pMPBXE	Promoter of gene <i>bxeR</i> cloned in pMP220 vector	This study
pMP2786	Promoter of gene <i>Bxe_B2786</i> cloned in pMP220 vector	This study
pMP0016	Promoter of gene <i>Bxe_B0016</i> cloned in pMP220 vector	This study
pBBRXENI	<i>braI<sub>XEN</sub></i> cloned into pBBR-MCS5 Gm <sup>r</sup>	This study
pMPUNAI	promoter of gene <i>braI<sub>UNA</sub></i> cloned in pMP220 vector	This study
pGEMXENR1	<i>braR<sub>XEN</sub></i> cloned into pGEM; Amp <sup>r</sup>	This study
pGEMX2R	<i>xenR2</i> cloned into pGEM; Amp <sup>r</sup>	This study
pQEXENR1	<i>braR<sub>XEN</sub></i> cloned into pQE30 expression vector; Amp <sup>r</sup>	This study
pQEXENR2	<i>xenR2</i> cloned into pQE30 expression vector; Amp <sup>r</sup>	This study
pQEBXER	<i>bxeR</i> cloned into pQE30 expression vector; Amp <sup>r</sup>	This study
pQEUNAR	<i>braR<sub>UNA</sub></i> cloned into pQE30 expression vector; Amp <sup>r</sup>	This study
pQE30	Expression vector, ColE1 replication origin, T5 promoter, His epitope; Amp <sup>r</sup>	Qiagen

<sup>a</sup> A superscript "T" indicates a type strain.

<sup>b</sup> Cm<sup>r</sup>, chloramphenicol resistance; Gm<sup>r</sup>, gentamicin resistance; Tet<sup>r</sup>, tetracycline resistance; Amp<sup>r</sup>, ampicillin resistance; Rif<sup>r</sup>, rifampin resistance; Km<sup>r</sup>, 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<sub>XEN</sub>* and *XenI2/R2* AHL QS systems and cross-talk regulation between *BraI/R<sub>XEN</sub>*, *XenI2/R2*, and the unpaired *BxeR* solo.** The *braR<sub>XEN</sub>* 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<sub>XEN</sub>* and *xenI2* were amplified and cloned in the promoter probe vector pMP220 to generate pMPXEN1 and pMPX21 as described in Table S1 in the supplemental material.

*E. coli* M15(pQEXENR1)(pMPXEN1) and *E. coli* M15(pQEXENR2)(pMPX21) 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  $\mu$ M concentrations of each synthetic AHL to be evaluated. The  $\beta$ -galactosidase activity was then determined after 4 h at 37°C. Possible regulation by *BraR<sub>XEN</sub>* of the *xenI2* promoter and of the *braI<sub>XEN</sub>* promoter by *XenR2* was evaluated by transforming the plasmid gene promoter fusion into *E. coli* M15(pQEXENR1) and *E. coli* M15(pQEXENR2), respectively. The  $\beta$ -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 pMPXEN1 or pMPX21, as well as with the *bxeR* promoter transcriptional fusion pMPBXE. *E. coli* M15(pQEBXER)(pMPX21), *E. coli* M15 (pQEBXER)(pMPXEN1), and *E. coli* M15 (pQEBXER)(pMPBXE) were then grown in the presence of three independent AHL mixtures. One contained 1  $\mu$ M each of nine different unsubstituted AHLs at position 3, the second mixture contained 1  $\mu$ M concentrations each of seven different 3-oxo-substituted AHLs, and a third cocktail contained 1  $\mu$ M each of seven different 3-OH-substituted AHLs. In these three growth conditions, the  $\beta$ -galactosidase activities were determined.

**AHL extraction, visualization, and quantification.** AHLs were purified from spent supernatant and separated by using a C<sub>18</sub> 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  $\mu$ g of X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -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<sub>4</sub>Cl) (40) supplemented with 0.2% glucose, 0.05% glutamate, and 0.5% agar (52). The inoculation was performed by spotting 1  $\mu$ l of a bacterial suspension having an OD at 600 nm (OD<sub>600</sub>) 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<sub>600</sub> of 0.01. Then, 100  $\mu$ l 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<sub>550</sub> 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  $\mu$ l 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  $\mu$ l 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<sub>600</sub> of 1. Then, 2- $\mu$ l portions of culture were spotted on the surface of the plate, followed by incu-

bation 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<sub>600</sub> of 0.05 in 40 ml of prewarmed LB. After 12 h of incubation, the cultures were centrifuged for 15 min at 8,000  $\times$  g at 4°C. Culture supernatants were filtered through a 0.45- $\mu$ m-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  $\times$  g for 20 min at 4°C, and the pellets were washed twice with ice-cold acetone. Another centrifugation was performed at 15,000  $\times$  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* MTI-641<sup>T</sup> and mutant derivatives using phenol as a carbon source.** *B. unamae* MTI-641<sup>T</sup>, 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  $\sim 10^4$  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  $\mu$ l of bacterial culture ( $\sim 10^9$  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  $\mu$ l 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<sub>600</sub> of 0.15 ( $\sim 1.5 \times 10^7$  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  $\sim 1$  cm and mixed. Two grams of the roots mixture was macerated with 10 mM MgSO<sub>4</sub>  $\cdot$  7H<sub>2</sub>O to get a dilution 10<sup>-1</sup>, 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/DBJ. The *BraI/R<sub>UNA</sub>* QS loci of *B. unamae* MTI-641<sup>T</sup> 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 MTI-641<sup>T</sup> was constructed and screened against the LasR-based *P. putida* F117(pRK12) 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<sub>UNA</sub>* and *braR<sub>UNA</sub>*) displayed homology to *luxI* and *luxR* family genes, while a third ORF, located in



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<sup>T</sup>. 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<sub>6</sub>-HSL, 3-oxo-C<sub>8</sub>-HSL, 3-oxo-C<sub>10</sub>-HSL, and 3-oxo-C<sub>12</sub>-HSL (Fig. 2A and C). In *B. xenovorans* the most likely production of C<sub>14</sub>-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<sub>UNA</sub> 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<sub>UNA</sub> positively regulates the *braI*<sub>UNA</sub> synthase through a positive autoinduction loop. The production of the putative AHLs synthesized by BraI/R<sub>UNA</sub> could be rescued by genetic complementation of the UNABRAR mutant by providing *braI*<sub>UNA</sub> in the cosmid pLZ1 (Fig. 2A and B).

*B. xenovorans* mutants LB400BRAI and LB400BRAR were both unable to produce 3-oxo-C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, and 3-oxo-C<sub>14</sub>-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<sub>8</sub>-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<sub>XEN</sub> mutant of *B. xenovorans* did not produce 3-oxo-C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, or 3-oxo-C<sub>14</sub>-HSL, suggesting a positive-feedback loop (Fig. 2D). The synthesis of these AHLs could be complemented in the LB400BRAI mutant by providing *braI*<sub>XEN</sub> in trans via plasmid pBBRXENI1 (Fig. 2D). TLC analysis of the AHL mol-

ecules produced by the *xenI2* and *xenR2* mutants revealed the putative production of 3-oxo-C<sub>6</sub>-HSL, 3-oxo-C<sub>8</sub>-HSL, 3-oxo-C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, and 3-oxo-C<sub>14</sub>-HSL, confirming that BraI<sub>XEN</sub> 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<sub>14</sub>-HSL.** In order to determine to which AHL BraR<sub>XEN</sub> and BraR<sub>UNA</sub> best responded, the proteins BraR<sub>XEN</sub> and BraR<sub>UNA</sub> were overexpressed in *E. coli* M15 in the presence of different AHL molecules, and the cognate *braI*<sub>XEN</sub> and *braI*<sub>UNA</sub> 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*<sub>XEN</sub> promoter increased 30-fold in the presence of 3-oxo-C<sub>14</sub>-HSL, 10-fold in the presence of 3-oxo-C<sub>16</sub>-HSL, and only 2-fold in the presence of 3-oxo-C<sub>12</sub>-HSL, demonstrating a specific preference of BraR<sub>XEN</sub> for 3-oxo-C<sub>14</sub>-HSL (Fig. 3A). An analog experiment performed in *E. coli* M15(pMPUNAI)(pQEUNAR1) suggested that *braI*<sub>UNA</sub> promoter activity increased 3.5-fold upon the presence of 3-oxo-C<sub>14</sub>-HSL and 2-fold when 3-oxo-C<sub>12</sub> HSL was present in the media, indicating that 3-oxo-C<sub>14</sub>-HSL was most likely the cognate AHL for BraR<sub>UNA</sub> (Fig. 3B). We had previously concluded that BraR<sub>KUR</sub> responded to 3-oxo-C<sub>12</sub>-HSL by using a similar approach (73). In the present study, we tested whether AHLs with acyl chains longer than C<sub>12</sub> could be recognized by BraR<sub>KUR</sub> and determined that it responded well to 3-oxo-C<sub>16</sub>-HSL and very well to 3-oxo-C<sub>14</sub>-HSL, (Fig. 3D). It was therefore concluded that 3-oxo-C<sub>14</sub>-HSL was the most likely cognate AHL for BraIR-like systems.

**XenR2 responds to 3-OH-C<sub>8</sub>-HSL and is not transcriptionally regulated by BraR<sub>XEN</sub> in *B. xenovorans* LB400<sup>T</sup>.** 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<sub>8</sub>-HSL, 50-fold in the presence of 3OH-C<sub>6</sub>-HSL, and 25-fold in the presence of 3-oxo-C<sub>8</sub>-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<sub>8</sub>-HSL was detected as being produced by XenI2 (see above). The response to 3-oxo-C<sub>8</sub>-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*<sub>XEN</sub> promoter, and the cognate AHL of the BraI/R<sub>XEN</sub> system did not activate the *xenI2* promoter (data not shown). In addition, the AHL production profiles, as observed in TLC analysis, *braI*<sub>XEN</sub><sup>-</sup> and *braR*<sub>XEN</sub><sup>-</sup> deficient mutants continue to produce *xenI2* and *xenR2* AHLs, and vice versa, suggesting the systems do not regulate each other.



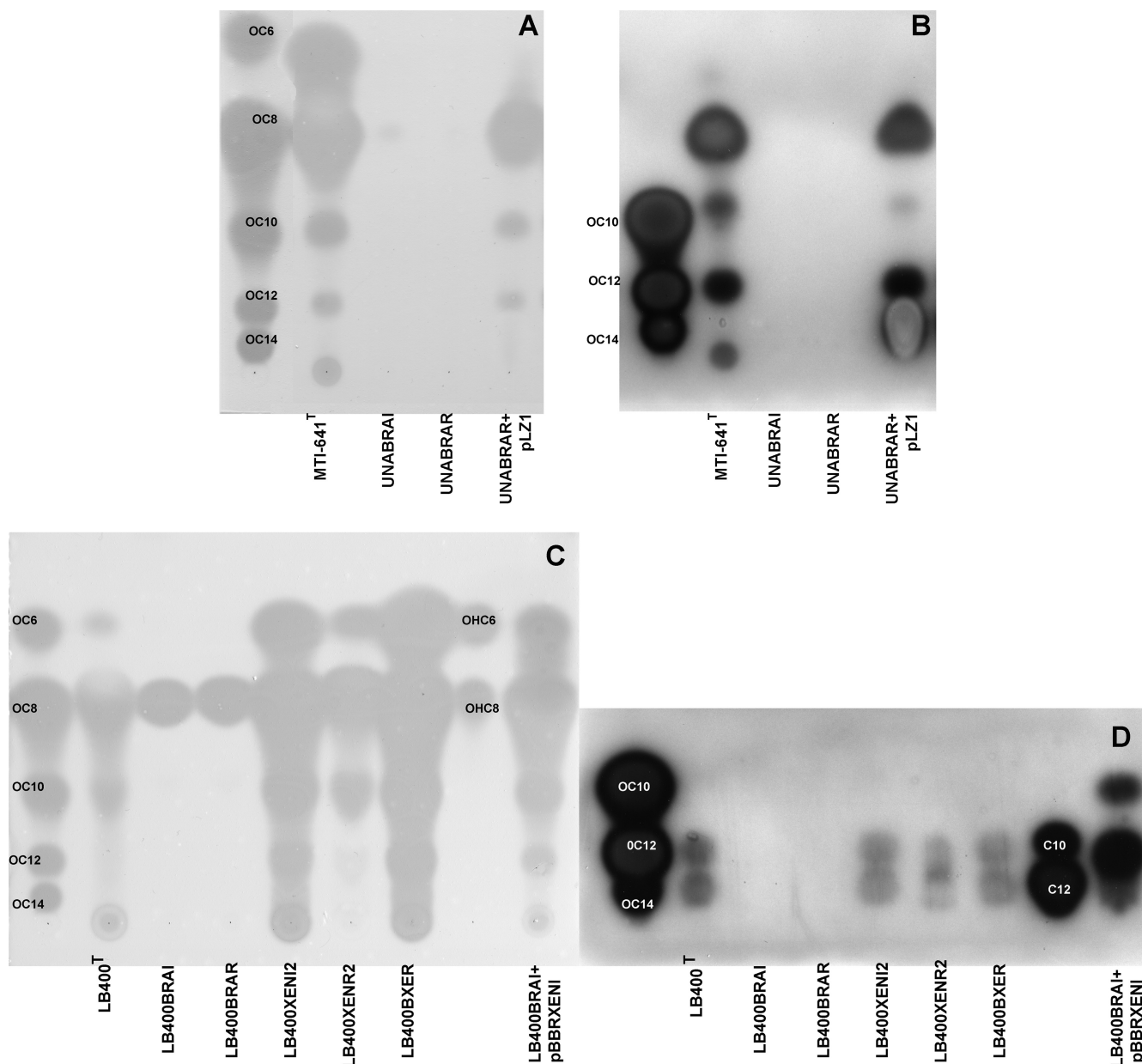


FIG. 2. TLC analysis of the AHLs produced by the *Burkholderia* and mutant derivatives. (A and B) *B. unamae* profiles. (C and D) *B. xenovorans* LB400<sup>T</sup> profiles. AHL extraction was performed as described in Materials and Methods, and TLCs were performed in 70% methanol for 6 h; for each strain, the equivalent of  $2.5 \times 10^{10}$  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<sub>6</sub>-HSL; OC8, 3-oxo-C<sub>8</sub>-HSL; OC10, 3-oxo-C<sub>10</sub>-HSL; OC12, 3-oxo-C<sub>12</sub>-HSL; OC14, 3-oxo-C<sub>14</sub>-HSL; OHC6, 3-OH-C<sub>6</sub>-HSL; OHC8, 3-OH-C<sub>8</sub>-HSL; C10, C<sub>10</sub>-HSL, C12; C<sub>12</sub>-HSL.

**Regulation of the AHL systems in *B. xenovorans* by BxeR solo.** The role of BxeR solo in regulating the *braI*<sub>XEN</sub> 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*<sub>XEN</sub> and *xenI2* gene promoters were also determined in *B. xenovorans* wild-type and in the mutants LB400BRAI, LB400XENI2, and LB400BXER. The results dem-

onstrated that the *braI*<sub>XEN</sub> 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*<sub>XEN</sub> 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

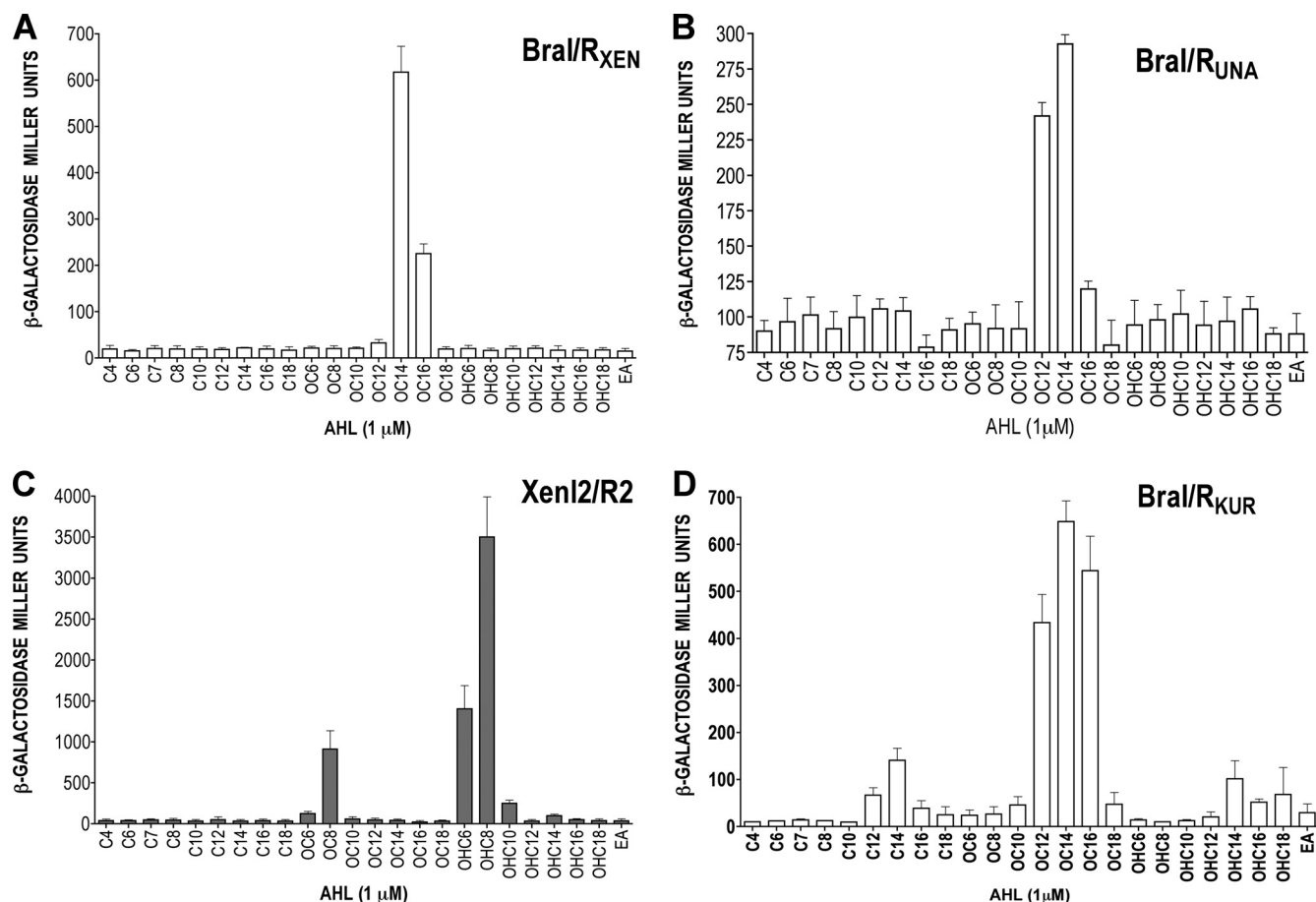


FIG. 3. Determination of the biologically active AHL for BraR<sub>XEN</sub>, XenR2, BraR<sub>UNA</sub>, and BraR<sub>KUR</sub> AHL sensor/regulators. (A) Determination of the cognate AHL for the BraI/R<sub>XEN</sub> system of *B. xenovorans*. Bars correspond to  $\beta$ -galactosidase activities determined for *E. coli* harboring pQEXENR1 and pMPXENI1. (B) Determination of the cognate AHL for the BraI/R<sub>UNA</sub> system of *B. unamae*. Bars correspond to  $\beta$ -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  $\beta$ -galactosidase activities determined for *E. coli* harboring pQEXENR2 and pMPX2I. (D) Determination of the cognate AHL for the BraI/R<sub>KUR</sub> system of *B. kururiensis*. Bars correspond to  $\beta$ -galactosidase activities determined for *E. coli* harboring pQEBRAR and PBRAI (73). Transcriptional fusions were harbored independently in *E. coli* expressing either BraR<sub>XEN</sub> or XenR2 proteins; various exogenous AHLs (1  $\mu$ M) were provided as indicated, and the  $\beta$ -galactosidase activities were determined. The results are mean values  $\pm$  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  $\mu$ M concentration of the cognate AHL (3-oxo-C<sub>14</sub>-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



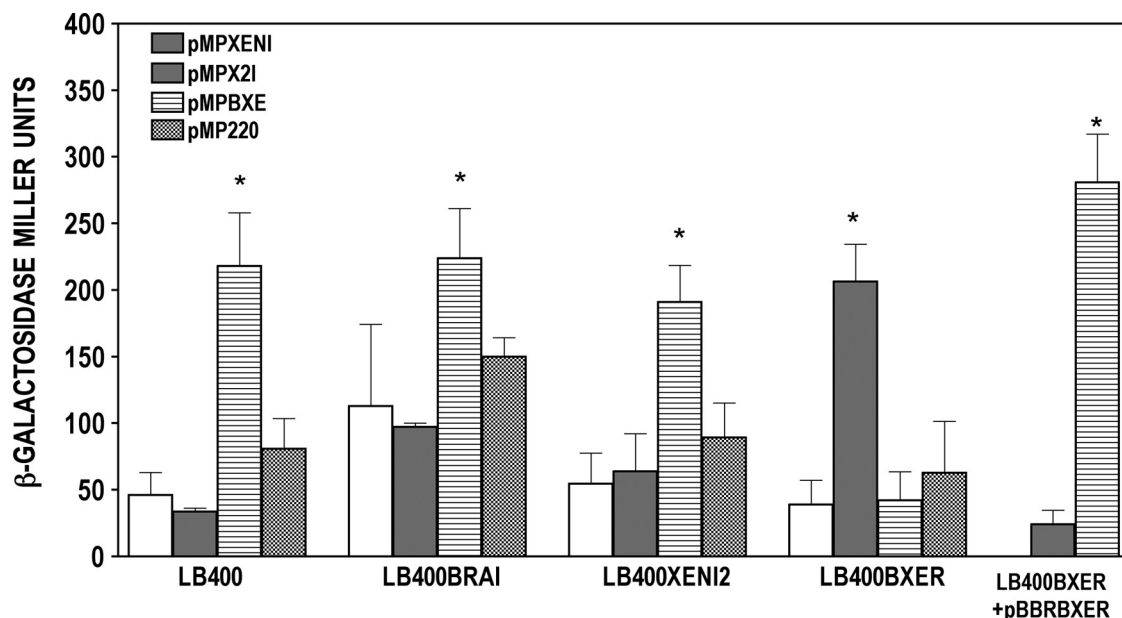


FIG. 4. *braI<sub>XEN</sub>*, *xenI2*, and *bxeR* promoter activities in wild-type and QS mutant strains of *B. xenovorans* LB400<sup>T</sup>. Bacterial cultures were started with an initial inoculum of  $5 \times 10^6$  CFU in 20 ml of KB-Tc medium, and the  $\beta$ -galactosidase activities were measured 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<sub>XEN</sub>* and *braR<sub>XEN</sub>* 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<sub>UNA</sub>* 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<sub>14</sub>-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<sub>XEN</sub> negatively regulates production of a 40-kDa Porin-1 family protein in *B. xenovorans* LB400<sup>T</sup>.** 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<sup>T</sup> versus the profile of the *braI<sub>XEN</sub>* 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<sub>XEN</sub> 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<sub>XEN</sub>, 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<sup>T</sup> wild-type and LB400BRAI mutant strains. Determination of the  $\beta$ -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<sub>XEN</sub> system. Promoter activity was significantly reduced when *braI<sub>XEN</sub>* 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

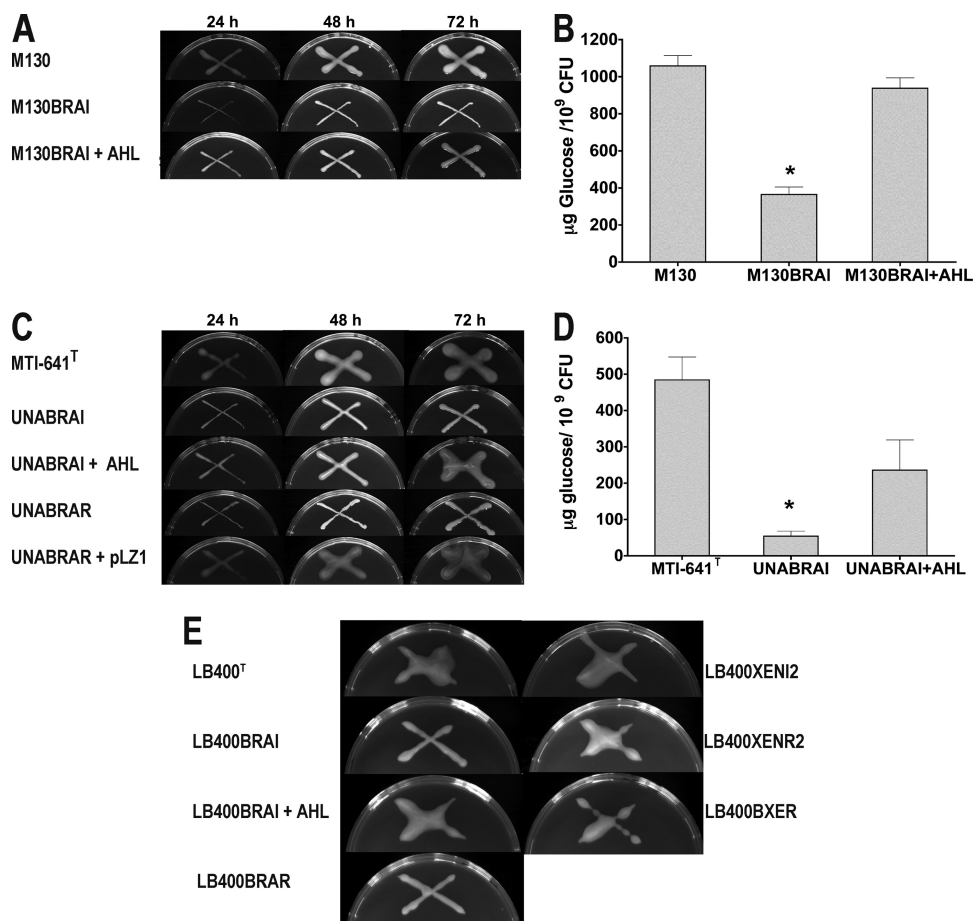


FIG. 5. EPS Production of *B. kururiensis* M130 (A and B), *B. unamae* MTI-641<sup>T</sup> (C and D), and *B. xenovorans* LB400<sup>T</sup> wild-type and QS mutants (E). Single colonies were streaked in YEM agar plates. Chemical complementation was achieved by adding 1  $\mu$ M AHL to the growth medium. 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  $\pm$  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<sub>XEN</sub>* 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<sub>XEN</sub>* 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* MTI-641<sup>T</sup>.** 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<sup>T</sup> 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<sup>T</sup> and the *B. xenovorans* LB400<sup>T</sup>, which has become a

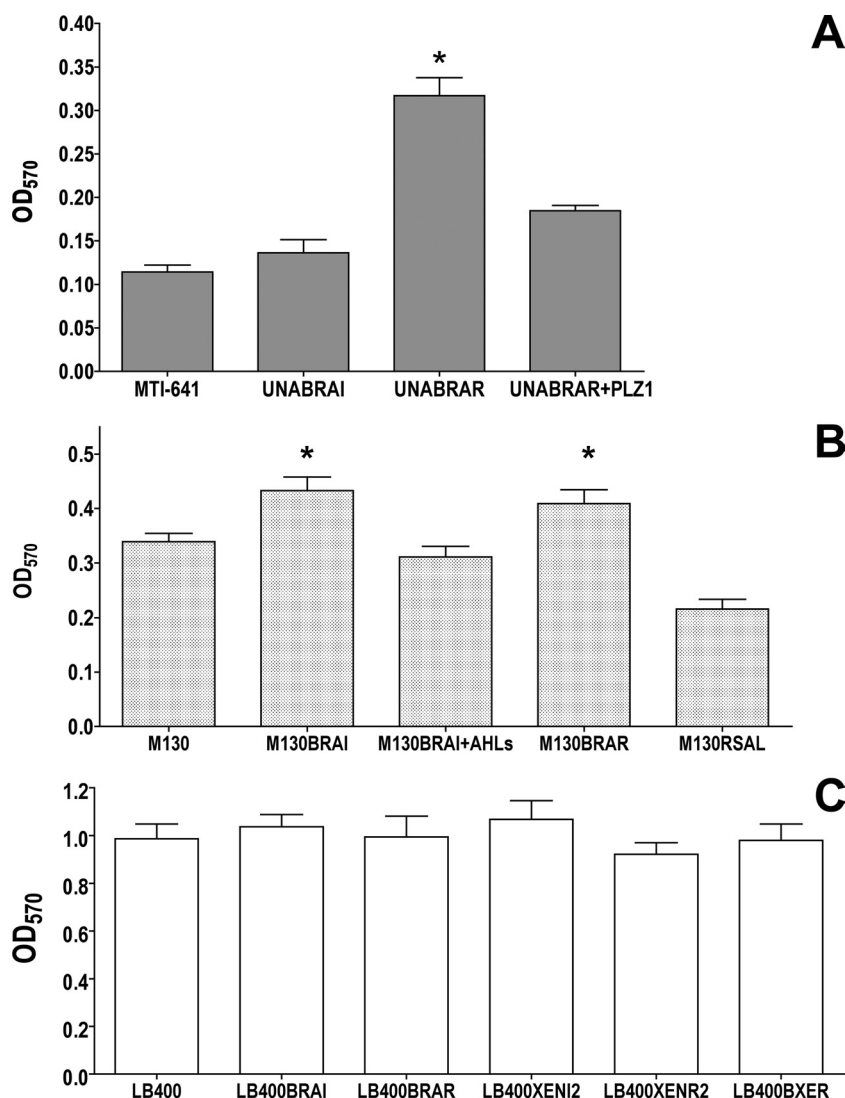


FIG. 6. Biofilm production in *B. unamae* (A), *B. kururiensis* (B), and *B. xenovorans* (C) wild-type and QS mutants after 72 h of incubation. “+AHLs” refers to complementation by adding 1  $\mu$ M 3-oxo- $C_{14}$ -HSL. Experiments were performed in triplicate, and means  $\pm$  the standard error of 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<sub>KUR</sub> of rice endophyte *B. kururiensis* M130 (73).

It was established that the BraI/R<sub>XEN</sub>, BraI/R<sub>UNA</sub>, and BraI/R<sub>KUR</sub> 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<sup>T</sup> 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<sub>UNA</sub> and BraI/R<sub>XEN</sub> 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-



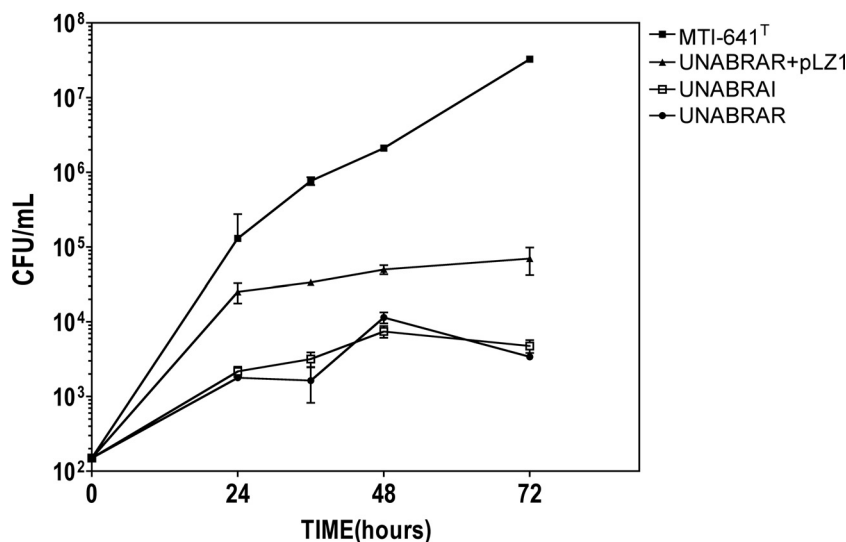


FIG. 7. Phenol degradation profile in *B. unamae* wild type and QS mutants. A growth profile was obtained with  $10^3$  CFU as starter the inoculum. As a control, phenol was replaced by mannitol as the carbon source, and all strains exhibited the same behavior.

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* (cepacia) (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<sub>UNA</sub> 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<sub>UNA</sub> 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<sub>UNA</sub> 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<sub>XEN</sub>, and only one of them could be fully complemented by supplying the *braI<sub>XEN</sub>* 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-

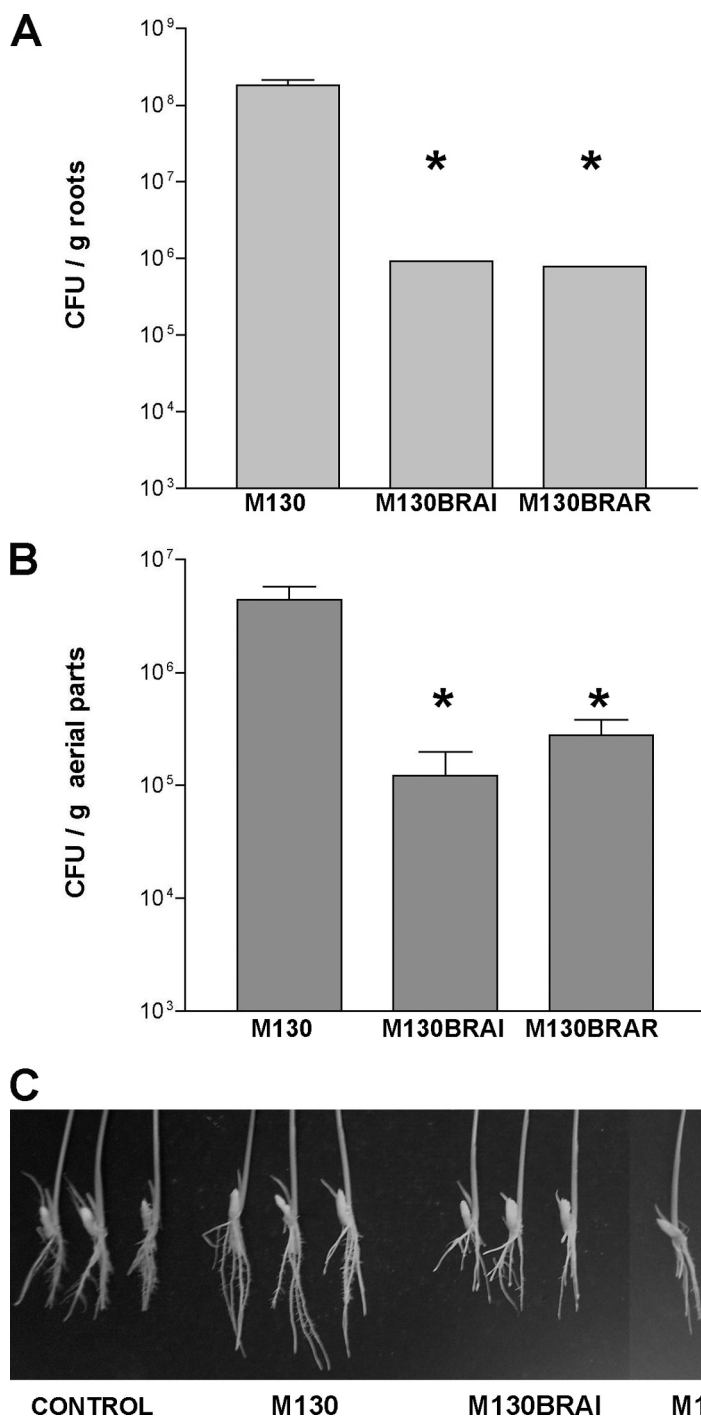


FIG. 8. Rice colonization assays performed with *B. kururiensis* M130 wild type and impaired QS mutants M130BRAI and M130BRAR. Plantlets were surface sterilized and inoculated as described in Materials and Methods. Bacterial colonization was measured by grinding and plating surface-sterilized plants 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<sup>T</sup> *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<sub>8</sub>-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<sub>XEN</sub> and XenI2/R2. In addition, transcriptional studies of *braI*<sub>XEN</sub> 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<sub>XEN</sub> system is most probably regulated by the intergenically located *rsaL* repressor, as previously described for the BraI/R<sub>KUR</sub> 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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