The new group of non-pathogenic plant-associated nitrogen-fixing Burkholderia spp. shares a conserved quorum-sensing system, which is tightly regulated by the RsaL repressor

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

INTRODUCTION

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

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

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the 4938 bp XmnI fragment harbouring braI, rsaL and braR is AM940944.
mainly plant-associated, rhizospheric and/or endophytic bacteria, many of which are nitrogen-fixing (Caballero-Mellado et al., 2004; Goris et al., 2004; Perin et al., 2006b; Reis et al., 2004) and legume-nodulating species (Chen et al., 2006; Elliott et al., 2007; Vandamme et al., 2002). Initially only Burkholderia vietnamiensis, a member of the BCC and found to be closely associated with rice plants, was reported as being able to fix atmospheric nitrogen (Gillis et al., 1995). B. kururiensis, a trichloroethylene degrader (Zhang et al., 2000), was soon after identified as a diazotrophic species (Estrada-de los Santos et al., 2001). Subsequently, many nitrogen-fixing isolates were recovered from different plants (rice, maize, sugar cane, sorghum, coffee and tomato) or from their rhizospheres and further classified as Burkholderia unanum, Burkholderia xenovorans, Burkholderia silwalantica, Burkholderia tropica, Burkholderia tuberum, Burkholderia phymatum, Burkholderia mimosarum and Burkholderia nodosa (Caballero-Mellado et al., 2007 and references therein). ‘Burkholderia brasilensis’ strain M130 (Baldani et al., 1997b), a plant- associated diazotrophic species never described validly, has recently been reclassified as B. kururiensis (Caballero-Mellado et al., 2007). It is expected that many more novel non-pathogenic plant-associated Burkholderia species will be described in the near future.

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

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

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

**METHODS**

**Bacterial strains, plasmids and media.** Burkholderia strains and plasmids used in this study are listed in Table 1. Burkholderia ‘brasiliensis’ M130 was isolated from rice in Brazil (Baldani et al., 1997b) and kindly provided by Dr Lucia Mendoça-Prevato from the Universidade Federal do Rio de Janeiro, Brazil. This strain has recently been reclassified as B. kururiensis M130 (Caballero-Mellado et al., 2007). Bacterial strains were grown at 30 °C in M9 minimal medium supplemented with glycerol (Sambrook et al., 1989), in King’s medium (King et al., 1954) or in BSE liquid medium (Estrada-de los Santos et al., 2001). Six AHL bacterial biosensors were used for AHL detection: Chromobacterium violaceum strain CV026 (McClean et al., 1997), Agrobacterium tumefaciens NT14/pZLR4 (Shaw et al., 1997), Escherichia coli/pSB1075, E. coli JM110/pSB401, E. coli MT102/pJBA132 and P. putida F117/pKRC12 (Riedel et al., 2001). Chromobacterium, Agrobacterium and Pseudomonas AHL detector strains were grown at 28 °C, while E. coli were grown at 37 °C. Antibiotics were added when required at the following final concentrations: ampicillin, 100 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; tetracycline, 15 µg ml⁻¹ (E. coli) or 40 µg ml⁻¹ (Burkholderia); gentamicin, 10 µg ml⁻¹ (E. coli), 30 µg ml⁻¹ (Agrobacterium) and 40 µg ml⁻¹ (Pseudomonas); kanamycin, 50 µg ml⁻¹ (E. coli and C. violaceum) or 100 µg ml⁻¹ (Pseudomonas and
Table 1. *Burkholderia* strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>Burkholderia strains</strong></td>
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<tr>
<td><em>B. kururiensis</em> M130</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt; Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Baldani et al. (1997b)</td>
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<tr>
<td>M130BRAI</td>
<td>braI::Km of <em>B. kururiensis</em> M130</td>
<td>This work</td>
</tr>
<tr>
<td>M130BRAR</td>
<td>braR::Km of <em>B. kururiensis</em> M130</td>
<td>This work</td>
</tr>
<tr>
<td>M130RSAL</td>
<td>rsaL::Km of <em>B. kururiensis</em> M130</td>
<td>This work</td>
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<td>Coenye et al. (2001)</td>
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<td><em>B. caribensis</em> DSM13236</td>
<td>Type strain</td>
<td>Achouak et al. (1999)</td>
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<tr>
<td><em>B. fungorum</em> DSM17061</td>
<td>Type strain</td>
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<td><em>B. graminis</em> DSM17151</td>
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<td>Viallard et al. (1998)</td>
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<td><em>B. hospita</em> DSM17164</td>
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<td><em>B. kururiensis</em> DSM13646</td>
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<td>Zhang et al. (2000)</td>
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<td><em>B. mimosarum</em> PAS44</td>
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<td>Goris et al. (2002)</td>
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<td><em>B. tropica</em> Ppe8&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>B. unanue</em> MT-641&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>B. xenovorans</em> DSM17367</td>
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<td><strong>Plasmids</strong></td>
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<td>pRK2013</td>
<td>Tra&lt;sup&gt;T&lt;/sup&gt;Mob&lt;sup&gt;T&lt;/sup&gt;ColE1 replicon; Km&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMOSBlue</td>
<td>Cloning vector; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Amersham-Pharmacia</td>
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<td>Promoter probe vector, IncP; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pZS1</td>
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<td>pZS2</td>
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<td>pMOS-Xmn-12</td>
<td>pMOSBlue carrying 5 Kb fragment containing partial <em>B. kururiensis</em> QS genes, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMOSBRAR</td>
<td>pMOSBlue carrying 490 bp fragment containing partial <em>B. kururiensis</em> braR gene, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMOSRSAL</td>
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<td>pKNOCK-Km</td>
<td>Conjugative suicide vector; Km&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMPbra1prom</td>
<td>Promoter of gene bra1 cloned in pMP220 vector</td>
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<td>pMPrsaLprom</td>
<td>Promoter of gene rsaL cloned in pMP220 vector</td>
<td>This study</td>
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<td>pQEbraRfw</td>
<td>braR cloned into pQE30 expression vector</td>
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<td>pQE30</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>braR18FW</td>
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<td>braR509Rv</td>
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<td>braLRv</td>
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<td>pQEbraRfw</td>
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<td>pQEbraRrv</td>
<td>5′-GGGAAATTCAGCAGCCGGATCTAAAA-3′</td>
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Burkholderia); rifampicin, 100 µg ml⁻¹. Conjugations in Burkholderia kururiensis were counter-selected in JMV medium (Reis et al., 2004) with the appropriate antibiotics.

Screening Burkholderia isolates for the production of AHLs. B. kururiensis M130 was first tested for the production of AHLs in a T-streak analysis on solid medium as described by Piper et al. (1993) using the AHL biosensors CV026, MT102/pBHA32 and FI17/pKRC12 on KB agar plates. In the same way, type strains of 19 other valid Burkholderia species were tested for AHL production (Table 1). Production of AHLs was further characterized by TLC after AHL extraction from cell-free spent supernatants as follows. Burkholderia strains were grown overnight in 20 ml M9 minimal medium supplemented with glucose and Casamino acids and the supernatants of the cultures were extracted and analysed on C₁₈ reverse-phase TLC plates as previously described (Shaw et al., 1997). The plate was then overlaid with a thin layer of LB or AB top agar seeded with either E. coli/pSB1075 or A. tumefaciens NTL4/pZLR4, the latter in the presence of 100 µg X-Gal ml⁻¹, as described previously (Shaw et al., 1997).

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

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

Different genomic null mutants were created in the AHL QS system of B. kururiensis as follows. First, an internal 423 bp EcoRV fragment from the braI gene was cloned in pNOCK-Km (Alexeyev, 1999), generating pNOCK-braI. This latter plasmid was then used as a suicide delivery system in order to create a braI knockout mutant of strain M130 as previously described (Alexeyev, 1999), generating M130BRAI. Similarly, an internal (490 bp) fragment of braR was PCR amplified by using braR18Fw and braR509Rv primers (Table 1) and cloned into pMOSBlue, yielding pMOSBRAI. A XbaI–KpnI insert was then cloned into pNOCK-Km and used as suicide vector for generating M130BRAI. Using a similar approach, an rsaL mutant was then generated by amplifying a 225 bp fragment by using braLRfw and braLRv primers (Table 1) and cloned into pMOSBlue, generating pMOSRSAI, and subsequently cloned as a XbaI–KpnI fragment in the corresponding sites in pNOCK-Km, yielding pNOCK-rsal. This latter plasmid was used to generate the rsaL knockout mutant designated M130RSAI. The fidelity of all marker-exchange events was confirmed by Southern analysis (data not shown).

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

Determination of the biologically active AHL of the BraI/R AHL QS system. To test which is the cognate AHL for BraR of B. kururiensis M130, we amplified the gene promoter regions of the braI and the rsaL regulatory genes. The braIR promoter regions were cloned in pMP220, generating pMPbraI and pMPrsaL, respectively. These two latter plasmids were transformed into E. coli M15/pREP-4 containing the expression plasmid pQE30 harbouring the braI promoter, generating pQEbraI, as follows: braI was PCR amplified by using oligonucleotides pQEbraIRfw and pQEbraIRrv using chromosomal DNA as template, and the PCR product was cloned as a BamHI–KpnI fragment in pQE30. The BraI protein was expressed according to the manufacturer’s instructions (Qiagen) and was verified by SDS-PAGE.

E. coli M15/pQEbraIR/pMPbraI and E. coli M15/pQEbraI/pMPrsaI were inoculated into 10 ml of LB-Amp-Km-Tet and grown overnight, and then diluted to OD₆₆₀ 0.1 into 10 ml prewarmed medium containing 1 µM of a specific AHL to be evaluated. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD₆₆₀ of 0.6, and after 2 h at 37 °C, β-galactosidase activity was determined.

Quantification of C12-3-oxo-AHL. Dried AHL extracts were prepared as described above and resuspended in ethyl acetate with an amount which corresponded to 1 µl final extract corresponding to 10⁶ cells of the original culture. The quantity of C12-3-oxo-AHL in the extracts was determined using C12-3-oxo-AHL sensor P. putida SM17/pPral220 as previously described (Rampioni et al., 2006). Briefly, P. putida SM17 is a double ppul rsaL mutant; consequently, it does not produce the RsaL repressor and C12-3-oxo-AHL. Exogenous C12-3-oxo-AHL is quantified through β-galactosidase activity by using strain SM17 harbouring pral220; this plasmid contains the Ppul-C12-3-oxo-AHL-regulated rsaL promoter fused to a promoterless lacZ gene. Overnight cultures of SM17/pral220 were diluted in 10 ml LB medium to an OD₆₆₀ of 0.1; the AHL extract to be quantified was then added and after 4 h growth, β-galactosidase activity was determined. This C12-3-oxo-AHL bacterial sensor has a linear dose response between 0.1 and 1 µM of C12-3-oxo-AHL. Synthetic C12-3-oxo-AHL was used as standard molecules (obtained from P. Williams, University of Nottingham, UK). The experimental set-up for the quantification is detailed in Table 2.

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

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

**Bacterial motility assays.** Swimming assays were performed on 0.3% KB agar plates; swarming assays were performed using M8 medium plates (M9 salts without NH₄Cl) (Kohler et al., 2000) supplemented with 0.2% glucose and 0.05% glutamate and containing 0.5% agar (Murray & Kazmierczak, 2006). The inoculation was performed by spotting 1 μl of a bacterial suspension having an OD₆₀₀ of 1. The swimming and swarming zones were measured after 48 h incubation at 30 °C for the wild-type and its QS mutant derivatives.

**Assays for nitrogen-fixation assays and other phenotypic features**

Media and culture conditions. *B. kururiensis* M130 and its derivative mutants in the braI, braR and rsaL genes were grown in BSE liquid medium (Estrada-de Los Santos et al., 2001) for 18 h with reciprocal shaking. The cultures were harvested and the pellets washed and adjusted to a low (1 × 10⁸ c.f.u. ml⁻¹) or high (1 × 10⁹ c.f.u. ml⁻¹) cell density. All the assays described below were assessed with *B. kururiensis* M130 and its derivative mutants to a low and high cell density, and the cultures were incubated at 29 °C.

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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture volume</th>
<th>Optical density</th>
<th>Ethyl acetate volume</th>
<th>Volume for assay</th>
<th>β-Galactosidase activity (Miller units)</th>
<th>Estimated concentration (μM) of C12-3-oxo-AHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>200</td>
<td>2.8</td>
<td>616</td>
<td>200</td>
<td>577.7 ± 59.2</td>
<td>0.020 ± 0.004</td>
</tr>
<tr>
<td>M130RSAL</td>
<td>50</td>
<td>4.52</td>
<td>994.4</td>
<td>200</td>
<td>348.3 ± 6.35</td>
<td>0.018 ± 0.001</td>
</tr>
<tr>
<td>M130RSAL/pZS1</td>
<td>50</td>
<td>5.33</td>
<td>249.42</td>
<td>1</td>
<td>2174.7 ± 177.5</td>
<td>42.725 ± 41.02</td>
</tr>
<tr>
<td></td>
<td>2.74</td>
<td>110</td>
<td>145.5</td>
<td>50</td>
<td>651.09 ± 51.24</td>
<td>0.109 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>2.74</td>
<td>137</td>
<td>599.86 ± 34.65</td>
<td>50</td>
<td>694.56 ± 90.56</td>
<td>0.072 ± 0.007</td>
</tr>
</tbody>
</table>

*Single colonies were independently inoculated and grown overnight in KB medium and optical density was measured. AHLs were extracted from spent supernatants and resuspended in an ethyl acetate volume (†) corresponding to an amount of 1 × 10⁸ c.f.u.

§AHL levels were measured with *P. putida* SM17prsaL220 since C12-3-oxo-AHL levels are proportional to β-galactosidase activity (Miller units). Extracted AHL volumes were diluted in order to obtain β-galactosidase values in the linear range of the sensor (0.1–1 μM).

§Values represent the means ± S.E.M. of three independent experiments with three replicates.

||Estimated concentrations of C12-3-oxo AHL in spent supernatants.

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

**Siderophore production.** The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol S (CAS) agar plates (Schwyn & Neillands, 1987), as described previously (Caballero-Mellado et al., 2007). *B. kururiensis* wild-type strain M130 and the derivative mutants were grown for 18 h at 29 °C in the CAA liquid medium supplemented with 0.3% (w/v) succinic acid. The cultures were harvested and the pellets washed and adjusted to a low or high cell density as described above. Aliquots of the cultures (10⁴ or 10⁸ c.f.u.) were inoculated with a multipoint replicator on CAS-CAA medium (Caballero-Mellado et al., 2007), and then incubated for 48 h at 29 °C. Siderophore production, as indicated by orange haloes formed around the colonies, was determined as described previously (Caballero-Mellado et al., 2007).

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

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

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

| Strain          | Culture volume | Optical density | Ethyl acetate volume | Volume for assay | β-Galactosidase activity (Miller units)§ | Estimated concentration (μM) of C12-3-oxo-AHL||
|-----------------|----------------|-----------------|----------------------|------------------|----------------------------------------|---------------------------------------------|
| Wild-type       | 200            | 2.8             | 616                  | 200              | 577.7 ± 59.2                           | 0.020 ± 0.004                               |
| M130RSAL        | 50             | 4.52            | 994.4                | 200              | 348.3 ± 6.35                           | 0.018 ± 0.001                               |
| M130RSAL/pZS1   | 50             | 2.91            | 145.5                | 50               | 651.09 ± 51.24                         | 0.109 ± 0.014                               |
|                 | 50             | 2.2             | 110                  | 50               | 599.86 ± 34.65                         | 0.072 ± 0.007                               |
|                 | 50             | 2.74            | 137                  | 50               | 694.56 ± 90.56                         | 0.114 ± 0.023                               |

*Single colonies were independently inoculated and grown overnight in KB medium and optical density was measured. AHLs were extracted from spent supernatants and resuspended in an ethyl acetate volume (†) corresponding to an amount of 1 × 10⁸ c.f.u.

§AHL levels were measured with *P. putida* SM17prsaL220 since C12-3-oxo-AHL levels are proportional to β-galactosidase activity (Miller units). Extracted AHL volumes were diluted in order to obtain β-galactosidase values in the linear range of the sensor (0.1–1 μM).

§Values represent the means ± S.E.M. of three independent experiments with three replicates.

||Estimated concentrations of C12-3-oxo AHL in spent supernatants.
The AHL QS system of B. kururiensis M130

To investigate the production of AHLs in the B. kururiensis M130 rice endophytic bacterial strain, we used four different AHL biosensors as described in Methods. Strain M130 was found to give a positive result when tested with a plate T-streak assay with LasR-based biosensor M130 was found to give a positive result when tested with a plate T-streak assay with LasR-based biosensor NTL4/pZLR4 or E. coli pSB1075. The results showed that this strain produced four AHL molecules tentatively identified as C12-3-oxo-, C10-3-oxo-, C8-3-oxo- and C6-3oxo-AHL (Fig. 1). It was therefore concluded that B. kururiensis M130 produces AHLs and therefore most probably possesses at least one AHL QS system.

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

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

It was of interest to determine how conserved was the ability to produce AHLs via the BraI/RsaL/BraR QS system in the novel group of nitrogen-fixing Burkholderia. We analysed the production of AHLs by TLC analysis of extracts from spent supernatants of 20 different Burkholderia species using the A. tumefaciens NTL4/pZLR4 biosensor. As depicted in Fig. 3(A), all 20 species were found to produce a very similar AHL profile to that of B. kururiensis M130; the only difference was the amount of AHLs produced by some strains. Using this AHL biosensor, it was observed that all strains produced C8-3-oxo-AHL; however, it must be noted that the A. tumefaciens NTL4/pZLR4 biosensor is most sensitive to this AHL. In addition, it was observed that all strains produced C8-3-oxo-AHL; however, it must be noted that the A. tumefaciens NTL4/pZLR4 biosensor is most sensitive to this AHL. In addition, all strains reacted very strongly in a plate T-streak assay with sensor P. putida/pKR-C12, which is a LasR-based sensor particularly sensitive to C12-3-oxo-AHL (data not shown). By Southern analysis it was then established that the braI/R AHL QS system was well conserved in all 20 species of the novel cluster of Burkholderia species (see Methods and Fig. 3B). Using two pair sets of oligonucleotide primers directed towards braI and rsaL (Table 1 and Methods), it was possible to PCR amplify the braI and rsaL
genes in all 20 species (data not shown), making these PCRs a potentially important tool for the identification of *Burkholderia* isolates belonging to this novel cluster. No amplification using either sets of primers was observed with members of the BCC complex and with *P. aeruginosa* (data not shown).

The *BraIR* system is tightly regulated by *RsaL* and *BraR* responds to C12-3-oxo-AHL

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

---

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

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

The Rsal protein located between the _braI/R_ genes in _B. kururiensis_ M130 was reported to negatively regulate the transcription of the _luxI_ family AHL synthase in _P. aeruginosa_ and _P. putida_ (Bertani & Venturi, 2004; Rampioni _et al._, 2006, 2007b). As it was observed that strain M130 produced low amounts of AHLs (Fig. 1), we were interested to determine whether Rsal was negatively regulating _braI_. The _rsaL_ gene was inactivated in strain M130, generating a knockout mutant designated M130RSAI. The AHLs produced by this mutant were extracted and analysed by TLC and this showed that AHLs were produced by this mutant, generating knockout mutants designated M130RSAL. The AHLs produced by this mutant, especially the biologically active C12-3-oxo-AHL (Fig. 1B). This mutant could be complemented by providing the _rsaL_ gene in _E. coli_ expressing the BraR protein, and various exogenous AHLs (1 μM) were provided as indicated and β-galactosidase activities were determined. The results are mean values ± SEM of three independent experiments.

![Fig. 4. Determination of the biologically active AHL of the BraI/R AHL QS system. White bars correspond to β-galactosidase activities determined for the pMPbraI promoter; black bars correspond to pMPrsaI measurements. Both transcriptional fusions were harbouring independently in _E. coli_ expressing the BraR protein, and various exogenous AHLs (1 μM) were provided as indicated and β-galactosidase activities were determined. The results are mean values ± SEM of three independent experiments.](http://mic.sgmjournals.org)

from M130 and M130RSAL were precisely determined using the sensor _P. putida_ SM17/prsaI220 (Rampioni _et al._, 2007a). This sensor is suitable for C12-3-oxo-AHL quantification as it is very specific for this AHL and its dose response was shown to be linear from 0.1 to 1 μM. Results of this experiment showed that C12-3-oxo-AHL levels produced by M130 wild-type were very low and were enhanced dramatically (almost 2000 fold) in the _B. kururiensis rsaL_ mutant (Table 2). The estimated concentration in a stationary-phase culture of _B. kururiensis_ M130 was approximately 20 nM whereas it increased dramatically to 45 μM for the _rsaL_ mutant. The production of C12-3-oxo-AHL in the _rsaL_ mutant was restored to wild-type levels when the _rsaL_ gene was provided in _trans_ via cosmid pZS1 (Table 2).

**The BraI/R system is not involved in regulating nitrogenase activity in _B. kururiensis_**

_B. kururiensis_, like many other closely related _Burkholderia_ species, is able to fix nitrogen, converting N₂ into NH₃ through the synthesis of a nitrogenase complex. Since this is a very high-energy-demanding reaction, bacteria stringently regulate this process (Dixon & Kahn, 2004). It was therefore of interest to determine whether the BraI/R QS system was involved in the regulation of nitrogenase activity in _B. kururiensis_ M130. In order to create QS mutants, _braI_ and _braR_ were inactivated in strain M130, generating knockout mutants designated M130BRAR and M130BRAI, respectively. Both these mutants were unable to synthesize AHLs (Fig. 1), indicating that the BraI/R system was responsible for synthesizing all four AHLs identified in culture supernatants and that most probably no other AHL QS system was present in _B. kururiensis_. In addition, this demonstrated that BraR positively regulates the _braI_ synthase through a positive auto-induction loop typical of AHL QS systems. The acetylene reduction assay was used to detect expression of the nitrogenase complex under nitrogen-free conditions in parental strain M130 and in the AHL-hyperproducing _rsaL_ mutant and the non-AHL-producing _braI_ and _braR_ mutants. Nitrogenase activity was comparable among the wild-type and the three QS mutant derivatives (_braI_, _braR_ and _rsaL_) at both low and high cell density (data not shown). It was therefore concluded that nitrogenase complex formation is not regulated by AHL QS in _B. kururiensis_.

**Analysis of the role of BraI/R in regulating several other phenotypes in _B. kururiensis_**

It was of interest to determine whether other important phenotypes were regulated by AHL QS in rice-associated _B. kururiensis_ M130. We examined growth in the presence of three aromatic compounds, namely toluene, benzene and phenol, and in the presence of the tricarboxylic acid cycle intermediate succinic acid as sole carbon sources. Strain M130 grows poorly on all three aromatic compounds at both low and high cell density whereas it can utilize...
succinic acid very efficiently; in all growth tests no significant differences were observed between the parent strain and the three QS mutant derivatives (data not shown). Similarly, we tested whether AHL QS was involved in siderophore production, lipase activity, and motility by swimming and swarming. *B. kururiensis* M130 displayed all these phenotypes; however, we detected no significant differences between the wild-type and all three AHL QS mutant derivatives under the conditions tested (data not shown). Interestingly, we also determined that strain M130 produces indoleacetic acid (IAA) and similarly we observed that this production was not regulated by AHL QS (data not shown).

**DISCUSSION**

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

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

Between the *braI/R* genes of *B. kururiensis*, as with the *lasI/R* and *ppulI/R* genes of *P. aeruginosa* and *P. putida*, respectively, there is repressor gene, *rsal*. (Fig. 2). Rsal of *P. aeruginosa* directly represses *lasI*, and *rsal* mutants produce approximately 10-fold more C12-3-oxo-AHL in the late-exponential/stationary phase of growth when compared to the wild-type parent strain (Rampioni et al., 2006, 2007b). It has been proposed that the biological role of *RsaL* in *P. aeruginosa* is to maintain C12-3-oxo-AHL homeostasis and to then possibly change steady-state levels upon varying environmental conditions (Rampioni et al., 2007b). The scenario in *B. kururiensis*, however, is somewhat different since the parent strain under laboratory conditions synthesizes very low levels of C12-3-oxo-AHL with the situation dramatically changing when Rsal is missing: the *rsal* mutant produces over 2000-fold more AHLs. The role of Rsal is hypothesized not to be one of homeostasis but rather a switch to turn on/off the AHL QS system; most probably under certain environmental conditions the system is very efficiently switched on. This could possibly occur through a mechanism of inactivation of Rsal, resulting in a very fast increase in AHL production. The ability of BraI/R to synthesize such high levels of AHLs is unique and also questions the role of this molecule in *B. kururiensis* in cell-density-dependent regulation. Stationary-phase *B. kururiensis* *rsal* mutants accumulate almost 50 μM C12-3-oxo-AHL in their supernatant versus only 20 nM in the parent strain. It is true that some AHL QS systems respond to nanomolar amounts of AHLs; however, the ability of *B. kururiensis* to produce such high amounts of AHL as 45 μM indicates that strain M130 can most probably respond very quickly to a sudden need. The very stringent on/off regulation and the capacity to synthesize such high concentration of AHLs might also be an indication that the molecule may play another role in the life of this bacterium unrelated to QS. Future work needs to determine the gene targets, if any, of BraI/R as this
might provide a clue to the precise role of this system and which environmental signal(s) it responds to. The fact that several phenotypes that we have tested are not regulated by Bral/R is an indication that, unlike the LasI/R system of P. aeruginosa, the Bral/R system of B. kururiensis is not a global regulatory system. It is tempting to speculate that as this group of Burkholderia have been isolated from such varied and geographically distant environments, the Bral/R system might provide these bacteria, via RsaL, a specific response to a particular stimulus regulating a particular set of genes. Importantly, our studies have shown that RsaL is very well conserved among this group of Burkholderia and that most strains produce very low quantities of AHLs, indicating that stringent regulation of the Bral/R system is likely to be widespread.

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

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