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Symbiotic Burkholderia Species Show Diverse Arrangements of nif/fix and nod Genes and Lack Typical High-Affinity Cytochrome cbb3 Oxidase Genes

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Genome analysis of fourteen mimosoid and four papilionoid beta-rhizobia together with fourteen reference alpha-rhizobia for both nodulation (nod) and nitrogen-fixing (nif/fix) genes has shown phylogenetic congruence between 16S rRNA/MLSA (combined 16S rRNA gene sequencing and multilocus sequence analysis) and nif/fix genes, indicating a free-living diazotrophic ancestry of the beta-rhizobia. However, deeper genomic analysis revealed a complex symbiosis acquisition history in the beta-rhizobia. Nonetheless, papilionoid-nodulating groups. Mimosoid-nodulating beta-rhizobia have nod genes tightly clustered in the nodBCIJHNASU operon, whereas papilionoid-nodulating Burkholderia have nodUSDABC and nodIJ genes, although their arrangement is not canonical because the nod genes are subdivided by the insertion of nif and other genes. Furthermore, the papilionoid Burkholderia spp. contain duplications of several nod and nif genes. The Burkholderia nifHDKNE and fixABC genes are very closely related to those found in free-living diazotrophs. In contrast, nifA is highly divergent between both groups, but the papilionoid species nifA is more similar to alpha-rhizobia nifA than to other groups. Surprisingly, for all Burkholderia, the fixNOQP and fixGHIS genes required for cbb3 cytochrome oxidase production and assembly are missing. In contrast, symbiotic Cupriavidus strains have fixNOOPGHIS genes, revealing a divergence in the evolution of two distinct electron transport chains required for nitrogen fixation within the beta-rhizobia.

Biological nitrogen fixation (BNF) by rhizobia has been studied intensively during the past century because this process supplies utilizable nitrogen (N) for agriculture at little cost to the environment. For most of this period, rhizobia were classified as closely related species currently placed in the Alphaproteobacteria genera Azorhizobium, Bradyrhizobium, Ensifer, Mesorhizobium, Neorhizobium, and Rhizobium. This view changed more recently with the identification of Betaproteobacteria (Burkholderia tuberculosis STM678T, Burkholderia phymatum STM815T, and Cupriavidus taiwanensis LMG 19424T) as nodulators of legumes (Chen et al. 2003a, 2005; Moulin et al. 2001). At first, the scientific community found the idea of Betaproteobacteria (beta-rhizobia) functioning as nitrogen-fixing symbionts in legume root nodules to be controversial, but Burkholderia and Cupriavidus species have been confirmed numerous times as the main microsymbionts for many mimosoid legumes (Gyaneshwar et al. 2011; Liu et al. 2012). Since then, research on nodulating Burkholderia species has proliferated (Gyaneshwar et al. 2011; Howieson et al. 2013; Lemaire et al. 2015b). Moreover, the research on the Brazilian mimosoid-nodulating beta-rhizobia has shown a strong correlation between Burkholderia nodulation and the host legume’s geographic distribution. B. caribensis (Chen et al. 2003b), B. diazotrophica (Sheu et al. 2013), B. mimosarum (Chen et al. 2006), B. nodosa (Chen et al. 2007), B. phymatum (Elliott et al. 2007b; Vandamme et al. 2002), B. subiae (Chen et al. 2008), B. symbiotica (Sheu et al. 2012), and C. taiwanensis (Chen et al. 2001, 2003a) are reported to nodulate Mimoso species. Also, Bournaud et al. (2013) provide additional evidence of a growing diversity of mimosoid-nodulating Burkholderia species, including B. phenoliruptrix. However, Bontemps et al. (2010; 2016) reported that the native Mimoso species of Mexico, which are distinct from the Brazilian species, are more likely to be nodulated by alpha-rhizobia than beta-rhizobia, although certain Burkholderia are known to nodulate M. occidentalis (Ormeño-Orrillo et al. 2012) and the widespread M. somnians and M. skinneri species in Mexico (Bontemps et al. 2016).

In contrast, papilionoid legume–nodulating Burkholderia species from the Cape Floristic Region (CFR) are not as well studied as the mimosoid legume–nodulating bacteria, although progress has been made (Garau et al. 2009; Howieson et al. 2013; Lemaire et al. 2015b), especially for those species associated with the CFR-endemic papilionoids, namely members of the Crotalearia, Hypocalyptea, Phaseoleae, and Podalyrieae tribes (Beukes et al. 2013; Lemaire et al. 2015b, 2016). Many of these isolates also nodulate cowpea (Vigna unguiculata L.) and siratro (Macroptilium atropurpureum L.) (Angus et al. 2013; Elliott et al. 2007a). Several are closely related to B. tuberum, based on 16S rRNA analysis (Elliott et al. 2007a), and a number of these beta-rhizobia have been described as new species, including B. dilworthii (De Meyer et al. 2014), B. dipogonis (Sheu et al. 2015), B. rhynchostae (De Meyer et al. 2013a), and B. spreitae (De Meyer et al. 2013b). Of the South African strains, only B. tuberum and B. dipogonis have been investigated.
for their ability to form a symbiosis with mimosoid plants, which they failed to nodulate (Elliott et al. 2007a; Liu et al. 2014).

As for the alpha-rhizobia, the symbiosis between beta-rhizobia and their associated legumes also requires a specific communication process that includes the expression of nodulation (nod) and nitrogen fixation (nif, fix, and idx) genes located in the genome of the microsymbiont. The nod genes are responsible for the synthesis of the Nod factor (NF), which triggers the initial plant responses for nodule development. The core nod genes (nodABC) encode enzymes for synthesizing the lipo-chitin backbone of the NF, whereas expression of additional nod genes results in a NF decorated with chemical substitutions, which are important for host specificity (Wang et al. 2012). However, little is known about the NF-encoding genes in the beta-rhizobia or of the structure of the Nod factors.

The essential BNF genes in beta-rhizobia are the same as those in alpha-rhizobia, but the exact mechanism of how BNF functions in the symbiotic Burkholderia spp. has not been elucidated. BNF is an ATP-dependent and highly energy-consuming process executed by the nitrogenase enzyme complex, which is composed of two main functional subunits, dinitrogenase reductase and dinitrogenase (Kneip et al. 2007). In the alpha-rhizobia, low nitrogen availability in the nodule environment leads to the activation of the transcriptional regulator nifA, which triggers additional nif gene expression needed for converting nitrogen gas into ammonium. Under microaerobic conditions, certain fix genes are also essential for nitrogen fixation. FixL, an oxygen-sensing membrane-bound protein, autophosphorylates and transfers a phosphoryl group to the two-component signal transduction regulator FixJ (Foussard et al. 1997). Genes directly regulated by FixJ include nifA, which regulates ndHDK, and the Crp/Fnr regulator fixK, which, when expressed, induces the transcription of fixNOQP and fixGHIS. The fixNOQP and fixGHIS genes are required for the production and assembly of cbb3 cytochrome oxidase (Pitcher and Watmough 2004), an enzyme that is essential for many anaerobic biological processes, including anoxygenic photosynthesis and nitrogen fixation (Ekici et al. 2012). The cbb3-encoded enzyme is thought to be an essential oxidase for symbiotic bacteria because it has a high oxygen affinity, and oxygen is present in low concentrations in the nodule environment. However, studies on Azorhizobium, Azotobacter, and Klebsiella spp. have revealed that an alternate oxidase, encoded by cytbd, is also important for free-living N2-fixation (Juty et al. 1997; Kaminski et al. 1996; Kelly et al. 1990). Azorhizobium caulinodans, the Sesbania rostrata symbiont, utilizes both cytbb3 and cytbd oxidases for N2-fixation (Kaminski et al. 1996). All currently known legume microsymbionts possess the cbb3 cytochrome oxidase for nitrogen fixation. In addition to the cbb3 genes, electron flow through the electron transport chain is mediated by the FixABC flavoproteins.

In this work, we investigated 29 betaproteobacterial genomes from both symbiotic and free-living diazotrophic bacteria as well as from representative alphaproteobacterial symbionts to understand i) the possible origin of symbiosis genes, ii) the structural organization of the symbiosis genes in the genome, iii) the amount and possible direction of horizontal gene transfer (HGT) of symbiotic genes, and iv) symbiotic specificity in the papilionoid-nodulating group of beta-rhizobia. We also show that the symbiotic and free-living diazotrophic Burkholderia spp., in contrast to Cupriavidus spp. and alpharhizobia, lack the genes required for cbb3 cytochrome oxidase.

### RESULTS

#### Genome information and average nucleotide identity (ANI) analysis.

In total, 29 Betaproteobacteria genomes were investigated for their symbiotic (nod) and nitrogen fixation (nif, fix) genes (Supplementary Table S1). The 14 mimosoid-nodulating strains originated from Brazil, China, French Guiana, Mexico, New Caledonia, Taiwan, Uruguay, and the United States, with the

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**Table 1. Genome-wide average nucleotide identity (gANI) values for the investigated Betaproteobacteria**

<table>
<thead>
<tr>
<th>Genome</th>
<th>IMG genome no.</th>
<th>ANI clique no.</th>
<th>gANI/alignment fraction</th>
<th>Scaffold count</th>
<th>Size (Mbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia phenoliruptrix</em> BR3459</td>
<td>2518645580</td>
<td>1630</td>
<td>98.77/0.80</td>
<td>3</td>
<td>7.6</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain CCGE1001</td>
<td>649633021</td>
<td>1630</td>
<td>98.63/0.80</td>
<td>2</td>
<td>6.8</td>
</tr>
<tr>
<td><em>Burkholderia mimosarum</em> LMG 23256T</td>
<td>251327083</td>
<td>35</td>
<td>99.48/0.88</td>
<td>268</td>
<td>8.4</td>
</tr>
<tr>
<td><em>Burkholderia mimosarum</em> STM3621</td>
<td>251327082</td>
<td>35</td>
<td>99.48/0.88</td>
<td>268</td>
<td>8.6</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain WSM4176</td>
<td>2516653074</td>
<td>431</td>
<td>97.14/0.68</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain STM678</td>
<td>2501025500</td>
<td>431</td>
<td>97.14/0.68</td>
<td>645</td>
<td>8.2</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain CCGE1002</td>
<td>646564515</td>
<td>1516</td>
<td>98.04/0.80</td>
<td>4</td>
<td>7.8</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain JPY251</td>
<td>251514122</td>
<td>1516</td>
<td>98.04/0.80</td>
<td>122</td>
<td>8.6</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain PVA5</td>
<td>2501025501</td>
<td>1786</td>
<td>98.84/0.85</td>
<td>491</td>
<td>7.7</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain STM6018</td>
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<td>1786</td>
<td>98.84/0.85</td>
<td>519</td>
<td>8</td>
</tr>
<tr>
<td><em>Cupriavidus taiwanensis</em> LMG 19424T</td>
<td>644736347</td>
<td>1442</td>
<td>99.03/0.91</td>
<td>3</td>
<td>6.4</td>
</tr>
<tr>
<td><em>Cupriavidus</em> sp. strain STM6018</td>
<td>2513273175</td>
<td>1442</td>
<td>99.03/0.91</td>
<td>80</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain CH1-1</td>
<td>2508501040</td>
<td>Singleton</td>
<td>N/A</td>
<td>4</td>
<td>8.7</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain H160</td>
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<td>310</td>
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<td>57</td>
<td>6.3</td>
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<tr>
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<td>69</td>
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<td>Singleton</td>
<td>N/A</td>
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<td>6.3</td>
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<tr>
<td><em>Burkholderia</em> sp. strain STM6012</td>
<td>2508501125</td>
<td>Singleton</td>
<td>N/A</td>
<td>72</td>
<td>7.2</td>
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<td><em>Burkholderia</em> sp. strain STM641</td>
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<td>Singleton</td>
<td>N/A</td>
<td>960</td>
<td>9.6</td>
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<tr>
<td><em>Burkholderia</em> sp. strain STM600T</td>
<td>637000053</td>
<td>Singleton</td>
<td>N/A</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td><em>Cupriavidus</em> sp. strain AMP6</td>
<td>2524023212</td>
<td>Singleton</td>
<td>N/A</td>
<td>260</td>
<td>7.5</td>
</tr>
<tr>
<td><em>Cupriavidus</em> sp. strain UYPR2.512</td>
<td>251327163</td>
<td>Singleton</td>
<td>N/A</td>
<td>365</td>
<td>7.8</td>
</tr>
<tr>
<td><em>Cupriavidus</em> sp. strain STM6070</td>
<td>251327165</td>
<td>Singleton</td>
<td>N/A</td>
<td>107</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*IMG = Integrated Microbial Genomes database.*
majority isolated from Latin America. The four papilionoid-nodulating strains originated from the Fynbos region on the west coast of South Africa, primarily out of a program searching for climate change-adapted legumes (Howieson et al. 2008).

Genome-wide (g)ANI was obtained for all Betaproteobacteria genomes, which identified six cliques and 16 singletons (Table 1). Clique 1 contains B. phenoliruptrix BR3459 and CCGE1001, which also show high (99.52%) 16S rRNA sequence similarity with each other. Clique 2 comprises B. mimosarum strains LGM 23256 and STM3621. Clique 3 provides evidence that B. tuberum STM678 and WSM4176 could belong to the same species, because their gANI value is 97.14 and they also share 99.79% 16S rRNA sequence similarity. Clique 4 contains CCGE1002 and JPY251, both of which have B. tuberum STM678 as their closest neighbor, with 98.55 and 99.04% 16S rRNA sequence similarity, respectively. Indeed, Mishra et al. (2012) described strain CCGE1002 as its closest neighbor, with 99.13% similarity.

In total, 10 nod genes were investigated: nodA, nodB, nodC, nodD, nodH, nodI, nodJ, nodS, nodU, and nolO (Figs. 1, 2, and 3). All genomes of the microsymbionts contained one copy of nodA. However, phylogenetic analysis revealed, with regard to symbiotic gene arrangement, that two distinct groups of beta-rhizobia exist. They correspond to the mimosoid-nodulating Burkholderia species and the papilionoid-nodulating group (Figs. 1 and 2). The mimosoid-nodulating species exhibit an almost canonical arrangement of nod and nif genes when compared with the organization of the symbiotic genes in alpha-rhizobia (R. leguminosarum WSM2304 is the example used in Figure 1), albeit with several transposases, recombinases, insertion elements, and unknown genes within the nif/fix gene operons. In contrast, the nod genes of the papilionoid-nodulating group are interrupted by not only transposases and other sequences but, also, by nif/fix genes, resulting in a split in the canonical nodDABC1J organization. We base this conclusion on the observation that both nodB and nodC each appear to exist as two copies of differently sized genes that occur a considerable distance apart on the chromosome. The larger copy of nodC (nolC1) is located adjacent to the nodIJ region, whereas the smaller copy, nolC2, is next to nodBA. This smaller sequence is very similar to the last 700 bp of the larger nolC1 copy (Fig. 1). Further supporting the idea of a genomic rearrangement is the fact that, although a second nodB was detected in B. sprentiae WSM5005 and Burkholderia sp. strain WSM4176 and was absent in B. dilworthii WSM3556 (Fig. 1), B. tuberum STM678, the comparable nodB, is likely to be a pseudogene, based on the presence of numerous stop codons (not shown). A nodB-like sequence (Fig. 1, yellow gene) was detected only by querying the intergenic space adjacent to nodC1. Also, the nodB similarity with the one found adjacent to nodAC is very low, based on MulAlign-based sequence analysis (Corpet 1988), and no highly similar hits were found using a National Center for Biotechnology Information (NCBI) BLAST search, although this nodB sequence is longer than the one found next to nodAC.

Loss of symbiosis genes.

Nodulation and nitrogen fixation genes were not detected in strains CCGE1001, CCGE1003, H160, JPY347, WSM2230, and WSM2232, although they were initially reported to nodulate and fix nitrogen with their original host (Ormen-Roillo et al. 2012; Walker et al. 2014a and b) (J. M. Tiedje personal communication). Therefore, these genomes were omitted from further analyses.

nod genes.

In total, 10 nod genes were investigated: nodA, nodB, nodC, nodD, nodH, nodI, nodJ, nodS, nodU, and nolO (Figs. 1, 2, and 3). All genomes of the microsymbionts contained one copy of nodA. However, phylogenetic analysis revealed, with regard to symbiotic gene arrangement, that two distinct groups of beta-rhizobia exist. They correspond to the mimosoid-nodulating Burkholderia species and the papilionoid-nodulating group (Figs. 1 and 2). The mimosoid-nodulating species exhibit an almost canonical arrangement of nod and nif genes when compared with the organization of the symbiotic genes in alpha-rhizobia (R. leguminosarum WSM2304 is the example

![Fig. 1. Chromosomal arrangement of symbiotic genes. A, Example of an alpha-rhizobial species. Many alpha-rhizobia exhibit this organization of nod and nif genes. B, Mimosoid Burkholderia strains, and C, papilionoid Burkholderia strains. Blue indicates nod genes, orange indicates nif and fix genes, black are transposases, recombinases, and insertion elements as well as unknown genes. Gray represents nonsymbiosis-related genes.](image-url)
orthology to the genes of several alpha-rhizobia, namely *Bradyrhizobium* and *Mesorhizobium* spp. and *Rhizobium etli* (Fig. 3, shades of orange). A pBLAST against the NCBI database revealed 26 *Bradyrhizobium* sequences, 28 from *Mesorhizobium*, and a few *Microvirga* and *Rhizobium* strains with *nolO* DNA identities of ≥80% (data not shown). In addition, a second copy of *nolO* is present in the *B. tuberum* genome (with 99% identity) along with *nodS*, but the two genes are interrupted and flanked by transposases and recombinases (data not shown). This *nolO* gene is also 90 bp shorter than the *nolO* gene in the *nod* operon (Fig. 1), due to the presence of insertional elements.

On the other hand, the host-specificity gene *nodH*, which adds a sulfate group to the NF, is absent in the papilionoid-nodulating beta-rhizobia but present in the other beta-rhizobia and several of the alpha-rhizobia analyzed (Fig. 3). This *nodH* gene is likely to be a host-specificity gene, along with *nodU*, for the mimosoid-nodulating legumes. Finally, the *nodIJ* genes, which are part of the core *nod* genes and function as lipo-oligosaccharide transport system ATP-binding proteins, are highly conserved within the beta- and alpha-rhizobial strains (Fig. 3).

*nif* genes.

A broad spectrum of *nif* genes was investigated in this study (Figs. 1, 2, 4, 5, and 6). A *nifH*, *nifD*, and *nifK* phylogenetic analysis using 768-, 1,484-, and 1,411-bp gene sequences, respectively, positioned the free-living diazotrophic *Burkholderia* spp. closest to the papilionoid-nodulating *Burkholderia* strains, and clearly separated from the mimosoid-nodulating beta-rhizobia (Fig. 2). The alpha-rhizobia clustered as the outgroup, with the bradyrhizobia being closest to the beta-rhizobia.

In the free-living diazotrophic *Burkholderia* species, *nifA* and *nifB* are adjacent to each other (Fig. 4). However, in both the papilionoid- and mimosoid-nodulating *Burkholderia* strains, *nifA* and *nifB* are interrupted by other *nif* and *fix* genes. Among these interrupting genes, the *nifZ* gene sequence is duplicated in both the papilionoid-nodulating beta-rhizobia and the diazotrophic *Burkholderia* species, as well as in some of the mimosoid *Burkholderia* strains. Moreover, both *Bradyrhizobium elkanii* WSM2783 and *Bradyrhizobium japonicum* USDA 110 have a second copy of *nifZ*. Also, *nifW* but not always *nifV* genes appear to be duplicated in the papilionoid-nodulating beta-rhizobia and the free-living nitrogen fixers (Fig. 5). These clusters are not adjacent to each other but, rather, on opposite sides of *nifA* in the papilionoid strains (Fig. 4). The *nifVI* gene is homologous in all *Burkholderia* species but shows low gene identity with the alpha-rhizobial strains and the *Cupriavidus* strains, with the exception of *Cupriavidus* sp. strain UYPR2.512, which has 62% *nifVI* gene identity and orthology to the genes of the papilionoid beta-rhizobia. We could find a second *nifVII* in only three of the four papilionoid-nodulating *Burkholderia* genomes; none of the other bacteria have an additional copy of *nifV*.

Unlike *nifB*, the phylogeny of *nifA* does not follow the other *nif* gene phylogenies. The phylogenetic tree shows that the papilionoid-nodulating beta-rhizobia were nested within the alpha-rhizobia clade, and the free-living diazotrophic *Burkholderia* group were separate, but they are phylogenetically closer to the mimosoid-nodulating beta-rhizobia, which formed a tight well-supported clade (Fig. 6). The *nifA* gene identity of the papilionoid-nodulating strains compared with all other investigated strains

![Fig. 2. Comparative maximum likelihood phylogenetic analysis using four different gene clusters. Colors indicate the different groups: blue-green: alpha-rhizobia, orange: papilionoid-nodulating *Burkholderia* strains; pink: diazotrophic *Burkholderia* strains; blue: mimosoid nodulating *Burkholderia* strains; and purple: *Cupriavidus* strains. Bootstrap values after 500 replicates are expressed as percentages; values less than 50% are not shown. The scale bar indicates the fraction of substitutions per site.](image-url)
was below or close to the 50% cut-off value except for *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* in the alpha-rhizobia, which showed 81 and 70% gene identity, respectively (Fig. 5).

Phylogenetic analysis of *nifE* and *nifN* revealed the presence of a single copy in each of the genomes (Fig. 5) and they showed the same clustering as identified for the *nifH, nifD,* and *nifK* genes (Fig. 2). However, in the mimosoid-nodulating beta-rhizobia, *nifE, nifN, nifX,* and *nifQ* are in an operon together with *nifA,* whereas in the papilionoid-nodulating beta-rhizobia, *nifEN* is positioned close to *nifHDK* (Figs. 1 and 4), albeit with transposase genes and other such elements in between them. However, for the free-living diazotrophic *Burkholderia* species, the *nifHDK* gene is almost adjacent to *nifENXQ* (Fig. 4).

Each of the *nifB, nifZ, nifX* and *nifQ* genes performs different functions in the nitrogen fixation process (Curatti et al. 2007). However, their phylogeny was identical to the *nifHDK* gene sequence phylogeny, such that the papilionoid-nodulating beta-rhizobia were closest to the free-living diazotrophic *Burkholderia* spp., the mimosoid-nodulating beta-rhizobia were a second branch, and the alpha-rhizobia made up the outgroup (data not shown).

**fix genes.**

In total, 13 *fix* genes were investigated: *fixA, fixB, fixC, fixG, fixH, fixI, fixX, fixO, fixP, fixQ, fixS, fixT,* and *fixX* (Supplementary Table S2). Genome analysis revealed the absence of both the *fixNOQP* and *fixGHIS* gene clusters in all *Burkholderia* strains, including both symbiotic and free-living diazotrophs. Furthermore, several alpha-rhizobia contained multiple copies of these genes, whereas, for the *Cupriavidus* strains, only a single copy was present. Because an alternative high oxygen affinity cytochrome needs to be present in symbiotic and free-living *Burkholderia* species, the *nifHDK* gene is almost adjacent to *nifENXQ* (Fig. 4).

**DISCUSSION**

Biological nitrogen fixation has been studied intensely, using two very different systems, the symbiotic *N₂*-fixers and the free-living diazotrophic *N₂*-fixers. In the current study, we investigated 37 symbiosis and nitrogen-fixation genes in 43 bacterial genomes that included 14 mimosoid- and four papilionoid-nodulating beta-bacteria as well as five free-living diazotrophic beta-bacteria, 14 alpha-rhizobia, and six nonsymbiotic, nonfixing beta-bacteria.

The development of a mature *N₂*-fixing symbiosis within a node requires the activation of a large number of *nod, nif, fix,* and *N₂* fixation–related genes at specific stages in the developmental process. The formation of a legume root nodule, in the majority of cases, is dependent on the presence of several microsymbiont *nod* genes, which encode proteins for Nod Factor (NF) production, transport, and regulation. All known genes *cydA* and *cydB,* were found outside of the symbiotic region (data not shown).

Another set of *fix* genes required for *N₂*-fixation is *fixABCX.* Genome analysis revealed the presence of this gene cluster in all the genomes analyzed. Additionally, these genes exhibited similar phylogenies as the *nifHDK* gene cluster, with the alpha-rhizobia as the outgroup, the mimosoid *Burkholderia* and *Cupriavidus* strains together and quite distant from the papilionoid *Burkholderia* and free-living diazotrophic *Burkholderia* strains (Fig. 2).

**Other nitrogen fixation–related genes.**

Several genes embedded within the *nif* region, e.g., two subunits of a 4Fe-4S ferredoxin iron-sulfur binding domain–containing protein that were originally annotated as ferredoxin (*fdx*) and *hesB/yadR/yhf* were positioned next to *nifB* (Fig. 4). The position of these genes is well conserved among the diazotrophic strains and all symbiotic strains. A *nif*-specific ferredoxin III was also detected next to *nifQ* in all the strains examined as well as a second gene annotated as a probable nitrogen fixation gene (Fig. 4, highlighted in yellow), which was adjacent to *nifX.*

![Fig. 3. Nodulation (nod) genes investigated, including host specific, regulatory, and common nod genes. The third rows names nod genes identified in each strain (left column) following comparison with the *Burkholderia tajunus* STM6782* gene. Each gene family member is depicted by a color family: papilionoid-nodulating *Burkholderia* strains, orange; mimosoid-nodulating *Burkholderia* strains, blue; *Cupriavidus* strains, purple; and alpha-rhizobia, blue-green. Any gene that is ≥50% identical to the *B. tajunus* query gene is indicated by an orange color; the darker the color, the greater the identity. Genes with an identity <50% are marked in the color of the family they belong to. Genes not detected (d.n.d.) are colored gray.](image-url)
NF share the same chitin-like N-acetyl glucosamine oligosaccharide backbone but differ in backbone length and additional NF modifications, many of which mediate host specificity (Perret et al. 2000; Wang et al. 2012). *Burkholderia* NFs are so far unknown, but the genome analysis presented here provides insights into the potential NF structures of mimosoid and papilionoid beta-rhizobia. The mimosoid beta-rhizobia have a single copy of *nod* genes arranged in the operon *nodBCIJHASU* (Fig. 1), which differs from the canonical arrangement of the typical alpha-rhizobial *nod* genes (*nodDABC*J) (Fig. 1A). Some alpha-rhizobia may lack *nodU* or *nodH* or may have a large number of additional *nod*, *not*, and *noe* genes for modifying host specificity. In contrast, the papilionoid beta-rhizobia *nod* genes seem simpler; the genes, like *Bradyrhizobium* and *Mesorhizobium* spp. seem to be chromosomal, show evidence for duplicate *nodB* and *nodC* genes, and have neither *nodH* (Fig. 3) nor * noeE* (data not shown). The presence of more than one *nodB* and *nodC* in these genomes is related to the insertion of *nif* genes within the *nod* operon, and the lack of *nodH* (and * noeE*) indicates that papilionoid beta-rhizobial NFs are not sulfated. On the other hand, the presence of *nodSU* and *nolO* indicates that methyl and carbamoyl groups are added to the core NF. Both *NodU* and *NolO* add carbomyl groups on the glucosamine residue at the nonreducing end to alpha-rhizobial NF (Broughton et al. 2000).

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**Fig. 4.** Detailed *nif*/*fix* region in papilionoid and free-living diazotrophic *Burkholderia* strains. Light orange indicates *fix* genes; dark orange indicates *nif* genes; yellow indicates nitrogen fixation–related genes; black are transposases, recombinases, and insertion elements as well as unknown genes, and gray represents nonsymbiosis-related genes. The map was aligned to *nifB*. The *nif* is colored bronze to indicate the start of the nitrogenase operon.

<table>
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*Fig. 5.* Nitrogen fixation genes investigated, including *nif*, *fix*, and nitrogen fixation–related genes. The genes named were identified in each strain (left column) following a comparison with a *Burkholderia* *tuberum* strain. Each gene family member is depicted by a color family: papilionoid-nodulating *Burkholderia* strains, orange; mimosoid-nodulating *Burkholderia* strains, blue; free-living diazotrophic *Burkholderia* strains, pink; *Cupriavidus* strains, purple; and alpha-rhizobia, blue-green. Any gene that is ≥50% identical to the *B. tuberum* query gene is indicated by an orange color; the darker the color, the greater the identity. Genes with an identity <50% are marked in the color of the family they belong to. Genes not detected (n.d.) are colored gray.
and, based on our sequence analysis, these same substitutions are likely to be present on the NF in *B. tuberum*. Furthermore, apart from *nodU*, *nolO*, and *nodS*, no other host-specific *nod* genes occur in the papilionoid beta-rhizobia, which suggests that the NFs of this group of bacteria may be similar to those reported for *Burkholderia tuberum* (previously named “*Bradyrhizobium aspalathi*”), which was isolated from *Aspalathus linearis* (Boone et al. 1999; Elliott et al. 2007a).

The *nodABC* gene phylogenetic analysis shows a clear distinction between the mimosoid and papilionoid beta-rhizobia, as suggested in previous studies (Bontemps et al. 2010; Gyaneshwar et al. 2011). Within the mimosoid beta-rhizobial branch, the *nodA*, *nodB*, and *nodC* genes seem to have been acquired all at once, as indicated by their monophyletic origin and their presence on a symbiotic plasmid (Fig. 2). This is supported by previous work, which indicated the acquisition of

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**Fig. 6.** Maximum likelihood *nifA* phylogenetic tree based on 1,888-bp gene alignment. Colors indicate the different groups: alpha-rhizobia, blue-green; papilionoid nodulating *Burkholderia* strains, orange; diazotrophic *Burkholderia* strains, pink; mimosoid nodulating *Burkholderia* strains, blue; and *Cupriavidus* strains, purple. Bootstrap values after 500 replicates are expressed as percentages and values less than 50% are not shown. The scale bar indicates the fraction of substitutions per site. The IMG (Integrated Microbial Genomes database) gene identification number is mentioned before the species name.
nod genes by the mimosoid 
Burkholderia spp. and, then, subse-
quently transfer to Cupriavidus spp. (Bontemps et al. 2010; Parker 2015). By contrast, the papilionoid beta-rhizobia seem to have acquired these genes from a different source, because they form a subcluster within the alpha-rhizobial clade, indicating a close relationship between these two groups (Fig. 2). This finding might indicate an old acquisition event. Moreover, numerous transposase genes, integrase genes, and insertion elements reside within the symbiotic gene cluster in the papilionoid beta-rhizobia, resulting in the nodB and nodC genes being interrupted and also in various gene duplications. Lessie and colleagues (1996) showed that insertion elements promoted genomic rearrangement in B. cepacia, enabling this species, which like the environmental species studied here, has to adapt rapidly in terms of physiology and biochemistry to changes in the environment. Moreover, like B. cepacia, the symbiotic and environmental Burkholderia species have large genomes consisting of multiple replicons (Chen et al. 2003b; Martínez-Aguilar et al. 2008) and insertion elements (this study). Genomic replacement and the movement of various elements may promote the expression of symbiotic genes in the papilionoid beta-rhizobia in a similar manner to that observed for B. cepacia with regard to expression of genes for the degradation of xenobiotics (Lessie et al. 1996).

The question of which species was the donor of nod and nif genes and which was the recipient in this process is complicated by the presence of numerous mobile elements within the symbiotic islands of the papilionoid beta-rhizobia and the varied relationships between the symbiotic and diazotrophic Burkholderia species. The Burkholderia species that we investigated were grouped according to habitat (symbiotic versus nonsymbiotic) and host (mimosoid versus papilionoid). These groups are spread across the phylogeny in the 16S rRNA/MLST (combined 16S rRNA gene sequencing and multilocus sequence typing) tree, indicating that HGT of symbiosis genes would have occurred after divergence of the different phylogenetic lineages (Fig. 2). Additionally, these beta-rhizobia seem to have a complex acquisition history of their symbiotic genes, as supported by a recent study (Lemaire et al. 2015a). However, Mimoso-nodulating beta-rhizobia have distinct nod, nif, and fix genes, indicating these might have been obtained from an unknown ancestral source and diverged separately from the other groups (Fig. 2). In contrast, the papilionoid-nodulating Burkholderia spp. have both alpharhizobia-like nodA, nodB, and nodC and beta-rhizobia-like nodI and nodJ genes. The nodI1 gene phylogeny analysis indicates a single acquisition event in the beta-rhizobia, demonstrating two independent nod gene acquisitions in the papilionoid Burkholderia. A similar analysis led Aoki et al. (2013) to state that the nodI1 and nodI1 gene sequence originated from gene duplication in the Betaproteobacteria, followed by a transfer to the Alphaproteobacteria and not the other way around. However, Lemaire et al. (2015a) did not observe HGT between the alpha- and beta-rhizobia investigated in their study. Our results support HGT between alpha- and beta-rhizobia, but additional research is necessary to understand the direction and frequency. On the other hand, some of the papilionoid- and mimosoid-nodulating Burkholderia nif genes exhibit strong homology based on gene identity with free-living diazotrophic nif genes (nifH, nifD, nifK, nifE, nifN), whereas others, nifA and nifVW, do not. Our results, thus, highlight the complex origin of symbiosis genes in the beta-rhizobia and their organization within the genome. In addition, the complete loss of symbiosis genes has been discovered in a number of the beta-rhizobial genomes investigated (López-Guerrero et al. 2012; Ormeño-Orrillo et al. 2012). The mechanisms that drive the gain or loss of the symbiotic genes are not known to date.

Lastly, we discovered the complete absence of the essential cbb3 cytochrome oxidase mechanism (fixNOQPHGIS) in all Burkholderia genomes investigated but found the alternative bd cytochrome in Burkholderia symbionts and diazotrophs. FixN, fixO, fixQ, fixP, fixG, fixH, fixI, and fixS genes are responsible for the production and assembly of the cbb3 cytochrome oxidase (Pitcher and Watmough 2004). Homologous cbb3 cytochrome oxidase genes are present in members of genera Brucella, Caulobacter, Campylobacter, Helicobacter, Neisseria, Pseudomonas, Ralstonia, and Vibrio, suggesting this oxidase is required for the successful colonization of one or both anoxic or micro-oxic tissues (Cosseau and Batut 2004; Parkhill et al. 2000; Pitcher and Watmough 2004). However, studies on Azorhizobium caulinodans single mutants in cytcb3 or cytbd showed that they were still able to fix nitrogen symbiotically, whereas cytcb3 and cytbd double mutants lacked symbiotic fixation ability (Kaminski et al. 1996). Similar findings were discovered in the diazotrophs Azotobacter vinelandii and Klebsiella pneumonia, emphasizing the importance of the cytbd system in nitrogen fixation (Juty et al. 1997; Kaminski et al. 1996; Kelly et al. 1990). Our genome analysis revealed the presence of the cytbd system in all investigated genomes. Their gene products could, therefore, be important as an alternative energy source for the N2-fixation process or for protecting it from O2 inhibition.

In addition to the FixN, fixO, fixQ, fixP, fixG, fixH, fixI, and fixS genes, another set of fix genes (fixABCX) is important for N2-fixation. According to previous studies, FixAB shows similarity to an electron transfer flavoprotein and FixCX to a ubiquinone oxidoreductase involved in electron transfer (Arigoni et al. 1991; Tsai and Saier 1995). More recently, it has been confirmed that FixA, FixB, FixC, and FixX proteins are involved in the electron transfer pathway dedicated to the generation of reductant for nitrogenase (Edgren and Nordlund 2004). Our fixABC gene phylogenetic analysis suggests that both the papilionoid and mimosoid beta-rhizobia have acquired these genes from a free-living diazotrophic ancestor (Fig. 2). Moreover, the papilionoid beta-rhizobia’s closest relative seems to be B. xenorovans, whereas the mimosoid beta-rhizobia share similarity with B. silvatonatica and B. unamae.

The congruence between the 16S rRNA/MLST and niffix genes suggests that the beta-rhizobia were free-living diazotrophs before acquiring nodulation ability (Bontemps et al. 2010; Chen et al. 2003b; Elliott et al. 2007a; Gyaneshwar et al. 2011). This hypothesis is also consistent with the ability of B. phymatum and B. tuberum to fix nitrogen ex planta (Elliott et al. 2007b). However, the nifA results suggest an additional exchange with the alpha-rhizobia, supporting previous reports suggesting the possible transfer of nif genes from beta- to alpha-rhizobia (Bontemps et al. 2010). Thus, two separate nif gene acquisition events seem to have taken place in the beta-rhizobia, one acquisition from the free-living diazotrophic Burkholderia spp., as indicated by nifHDKEN, and one from the alpha-rhizobia (nifA). Additionally, nifZ is duplicated in the papilionoid Burkholderia spp., the free-living diazotrophic Burkholderia, and in some of the mimosoid Burkholderia spp. and alpha-rhizobia but not in Cupriavidus spp. (Fig. 5). These inferences are based on a limited sample size and also on unfinished genome sequences, but with better sequencing technology and the sequencing of additional beta-rhizobial genomes, firmer conclusions about Burkholderia symbiotic genes will be forthcoming.

MATERIALS AND METHODS

Selection of strains.

Four groups of Betaproteobacteria were investigated: mimosoid-nodulating Burkholderia strains, papilionoid-nodulating Burkholderia strains, Cupriavidus strains, and free-living diazotrophic strains. In addition, 14 Alphaproteobacteria strains were also included as reference rhizobia for comparison.
ANI analysis.

ANI analysis was carried out by calculating the bidirectional gANI and alignment fraction (AF) between all genomes (Varghese et al. 2015). These computations included the identification of orthogonal genes with 70% or more identity as a filter, and the length of these genes was used to compute AF, while the percent identity was used to compute gANI. Furthermore, those genome pairs that have at least a gANI of 96.5 and an AF of 60 to each other were used as input pairs for clustering, in which maximal cliques were identified using the Bron–Kerbosch algorithm. For more information about the tool and its application, see Varghese et al. (2015) and the Joint Genome Institute (JGI) website.

Sequence search, phylogenetic analysis, and congruence tests.

BLASTP search (E = 1e⁻⁵) was performed, using the protein sequences of *nod, nif*, and *fix* genes from the Alphaproteobacteria reference strains to find putative homologs in the JGI IMG (Integrated Microbial Genomes) database, which includes the GEBA-RNB (Genomic Encyclopedia for Bacteria and Archaea–Root Nodule Bacteria) initiative that encompasses genome sequences of 107 rhizobial strains isolated from various locations around the world (Reeve et al. 2015). Moreover, the NCBI database was also queried the NCBI BLAST database.

Sequence alignment, alignment editing, and phylogenetic analyses were performed using MEGA 6.06 (Tamura et al. 2013). Phylogenetic trees were constructed, using the maximum likelihood reconstruction method (Felsenstein 1981) and applying the model resulting from the MEGA model test. The strength of each topology was verified using 500 bootstrap replications. The possibility of concatenating several gene sequences was investigated using the congruence tests (tree topology observation) and partition-homogeneity test with the PAUP software (Farris et al. 1994; Swofford 1991). Each concatenation was investigated for 1,000 replicates. If significant *P* values (*P < 0.01*) were obtained, the datasets are significantly different and were not combined for analysis. The EzTaxon-e server was used to obtain 16S rRNA sequence similarity values (Kim et al. 2012). When no gene match was discovered for a query gene, the lowest e-value were selected, even if other results had higher gene identity to the query gene. In those cases, the results with the lowest e-value were selected, even if other results had higher gene sequence identity. When no gene match was discovered for a genome, those absent genes were designated as n.d. (not detected). All of the query genes are from the *B. tuberum* STM678³ genome, except *nodH*, which belongs to *Burkholderia* sp. strain CCGE1002. Each of the 22 genes was searched against the investigated genomes selected for this analysis. The nitrogen fixation genes analyzed included a curated set of 14 *nif* genes, 13 *fix* genes, and two nitrogen fixation–related genes, i.e., a *fixD* gene and the *hesB*/*vdgR/*phf* gene, which encodes an iron-sulfur cluster assembly protein.

Each gene family member is depicted in Figures 1 through 6 by a color family. Any gene that is ≥50% identical to the *B. tuberum* query gene is indicated by different shades of orange; the darker the color, the greater the identity. Genes with an identity lower than 50% are marked in the color of the family to which they belong.

ACKNOWLEDGMENTS

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LITERATURE CITED


**AUTHOR-RECOMMENDED INTERNET RESOURCES**

Joint Genome Institute (JGI) website: https://img.jgi.doe.gov/cgi-bin/mcr/main.cgi
The JGI/IMG genomic database: https://img.jgi.doe.gov/cgi-bin/mcr/main.cgi?section=FindGenesBlast&page=genesearch%20blast