## Title

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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 that clearly separates the mimosoid and papilionoid nodulating groups. Mimosoid-nodulating beta-rhizobia have *nod* genes tightly clustered in the *nodBCIJHASU* 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 nifHDKEN* 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 *fixNOQPGHIS* 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 tuberum* STM678$^T$, *Burkholderia phymatum* STM815$^T$, and *Cupriavidus taiwanensis* LMG 19424$^T$) 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. sabiae* (Chen et al. 2008), *B. symbiotica* (Sheu et al. 2012), and *C. taiwanenis* (Chen et al. 2001, 2003a) are reported to nodulate *Mimosa* 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 *Mimosa* 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 Crotalariae, Hypocalypteae, 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. rhynchosiae* (De Meyer et al. 2013a), and *B. sprentiae* (De Meyer et al. 2013b). Of the South African strains, only *B. tuberum* and *B. dipogonis* have been investigated

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*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure and two supplementary tables are published online.

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 *fdx*) 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 *nifHDK*, 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 $N_2$-fixation (Juty et al. 1997; Kaminski et al. 1996; Kelly et al. 1990). *Azorhizobium caulinodans*, the *Sesbania rostrata* symbiont, utilizes both *cytcbb3* and *cytbd* oxidases for $N_2$-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 alpha-rhizobia, 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

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

| Genome | IMG[a] genome no. | ANI clique no. | gANI/alignment fraction | Scaffold count | Size (Mbp) |
|---|---|---|---|---|---|
| *Burkholderia phenoliruptrix* BR3459 | 2518645580 | 1630 | 98.77/0.80 | 3 | 7.6 |
| *Burkholderia* sp. strain CCGE1001 | 649633021 | 1630 | | 2 | 6.8 |
| *Burkholderia mimosarum* LMG 23256$^T$ | 2513237083 | 35 | 99.48/0.88 | 268 | 8.4 |
| *Burkholderia mimosarum* STM3621 | 2513237082 | 35 | | 268 | 8.6 |
| *Burkholderia* sp. strain WSM4176 | 2516653074 | 431 | 97.14/0.68 | 13 | 9 |
| *Burkholderia tuberum* STM678$^T$ | 2501025500 | 431 | | 643 | 8.2 |
| *Burkholderia* sp. strain CCGE1002 | 646564515 | 1516 | 98.04/0.80 | 4 | 7.8 |
| *Burkholderia* sp. strain JPY251 | 2515154122 | 1516 | | 122 | 8.6 |
| *Burkholderia silvatlantica* PVA5 | 2501025501 | 1786 | 98.84/0.85 | 491 | 7.7 |
| *Burkholderia silvatlantica* SRMrh-20$^T$ | 2501025504 | 1786 | | 519 | 8 |
| *Cupriavidus taiwanensis* LMG 19424$^T$ | 644736347 | 1442 | 99.03/0.91 | 3 | 6.4 |
| *Cupriavidus taiwanensis* STM6018 | 2513237150 | 1442 | | 80 | 6.5 |
| *Burkholderia dilworthii* WSM3556$^T$ | 2508501124 | Singleton | N/A | 140 | 7.6 |
| *Burkholderia nodosa* DSM 21604 | 2515154189 | Singleton | N/A | 114 | 9.6 |
| *Burkholderia phymatum* STM815$^T$ | 642555112 | Singleton | N/A | 4 | 8.6 |
| *Burkholderia* sp. strain UYPR1.413 | 2513237166 | Singleton | N/A | 336 | 10.3 |
| *Burkholderia sprentiae* WSM5005$^T$ | 2510065045 | Singleton | N/A | 8 | 7.7 |
| *Burkholderia* sp. strain CCGE1003 | 648028011 | Singleton | N/A | 2 | 7 |
| *Burkholderia* sp. strain Ch1-1 | 2508501040 | Singleton | N/A | 4 | 8.7 |
| *Burkholderia* sp. strain H160 | 642979355 | Singleton | N/A | 310 | 7.8 |
| *Burkholderia* sp. strain JPY347 | 2515154123 | Singleton | N/A | 57 | 6.3 |
| *Burkholderia* sp. strain JPY366 | 2526164713 | Singleton | N/A | 69 | 6.7 |
| *Burkholderia* sp. strain WSM2230 | 2513237151 | Singleton | N/A | 33 | 6.3 |
| *Burkholderia* sp. strain WSM2232 | 2508501125 | Singleton | N/A | 72 | 7.2 |
| *Burkholderia unamae* MTI-641$^T$ | 2501025502 | Singleton | N/A | 960 | 9.6 |
| *Burkholderia xenovorans* LB400$^T$ | 637000053 | Singleton | N/A | 3 | 9.7 |
| *Cupriavidus* sp. strain AMP6 | 2524023212 | Singleton | N/A | 260 | 7.5 |
| *Cupriavidus* sp. strain UYPR2.512 | 2513237163 | Singleton | N/A | 365 | 7.8 |
| *Cupriavidus taiwanensis* STM6070 | 2513237165 | Singleton | N/A | 107 | 6.7 |

[a] 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 LMG 23256$^T$ and STM3621. Clique 3 provides evidence that *B. tuberum* STM678$^T$ 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$^T$ as their closest neighbor, with 98.55 and 99.04% 16S rRNA sequence similarity, respectively. Indeed, Mishra et al. (2012) described strain CCGE1002 as *B. tuberum* biovar *mimosae* and strain STM678 as *B. tuberum* biovar *papilionoideae*. However, the gANI value (<90) indicates they might not belong to the same species. Clique 5 includes the *B. silvatlantica* strains (Angus et al. 2013; Perin et al. 2006), and clique 6 comprises the *C. taiwanensis* strains STM6018 and LMG 19424$^T$. Strain STM6070 was originally described as *C. taiwanensis* (Klonowska et al. 2012), but gANI analysis places this strain in a singleton and 16S rRNA sequence similarity shows *C. necator* N-1$^T$ as its closest neighbor, with 99.13% similarity.

**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 (Ormeño-Orrillo 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 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 *nodDABCIJ* 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* (*nodC1*) is located adjacent to the *nodIJ* region, whereas the smaller copy, *nodC2*, is next to *nodBA*. This smaller sequence is very similar to the last 700 bp of the larger *nodC1* 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$^T$ and *Burkholderia* sp. strain WSM4176 and was absent in *B. dilworthii* WSM3556$^T$ (Fig. 1), *B. tuberum* STM678$^T$, 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 MultAlin-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*.

Figures 1 and 2 highlight the relationships between the different genome sequences, by showing that the mimosoid-nodulating beta-rhizobia are clearly separated from the papilionoid-nodulating beta-rhizobia. Although the *nodU* genes in all beta-rhizobia appear to be well-conserved in terms of gene identity (≥74%) and orthology (Fig. 3), in contrast, the gene neighborhoods are not conserved (Fig. 1, Supplementary Fig. S1). The *nolO* gene (sometimes annotated as *nodU*) and *nodU* both encode beta-1,4-*N*-acetylglucosamine oligosaccharide 3-*O*-carbamoyltransferase. Based on the low sequence identity between the papilionoid-nodulating *Burkholderia* spp. and the nonorthologous sequences in the other beta-rhizobia, *nolO* is likely to be a host-specific gene for the papilionoid-nodulating group. Although highly conserved among papilionoid group members, the genes from other beta-rhizobia probed with *nolO* are only ≤40% identical to the comparable genes in the papilionoid-nodulating *Burkholderia* species. Because NodU and NolO are both in the NodU gene family, the pBLAST search picked up the *nodU* sequence. Moreover, the queried mimosoid-nodulating genes are not orthologous to *nolO* in the papilionoid group. Interestingly, *nolO* of the papilionoid-nodulating group exhibits approximately 80% identity and

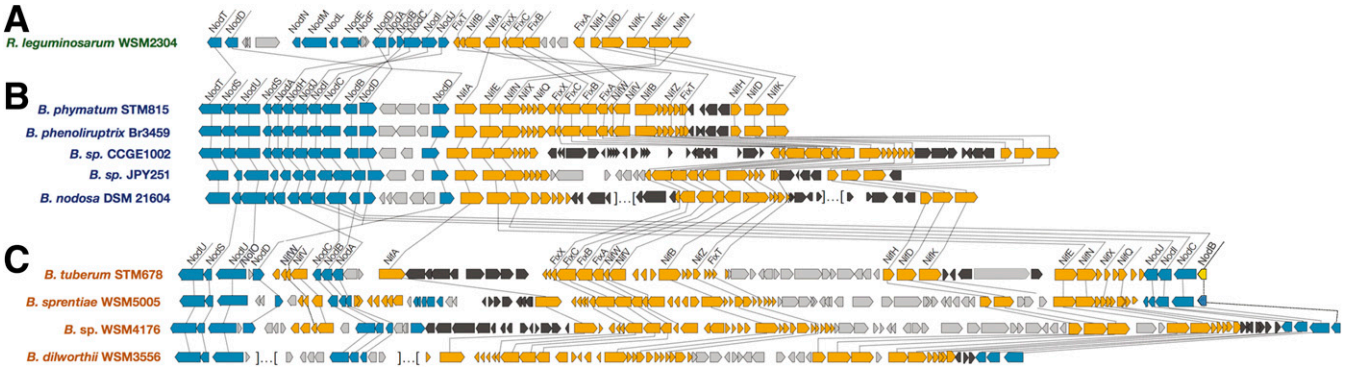


**Fig. 1.** Chromosomal arrangement of symbiotic genes. **A,** Example of an alpha-rhizobial species. Many alpha-rhizobia exhibit this organization of *nod* and *nif* genes. **B,** Mimosoid *Burkholderia* strains, and **C,** papilionoid *Burkholderia* strains. Blue indicates *nod* genes, orange indicates *nif* and *fix* genes, black are transposases, recombinases, and insertion elements as well as unknown genes. Gray represents nonsymbiosis-related genes.

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* spp. 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 *nifV1* 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% *nifV1* gene identity and orthology to the genes of the papilionoid beta-rhizobia. We could find a second *nifV2* (Fig. 5) 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

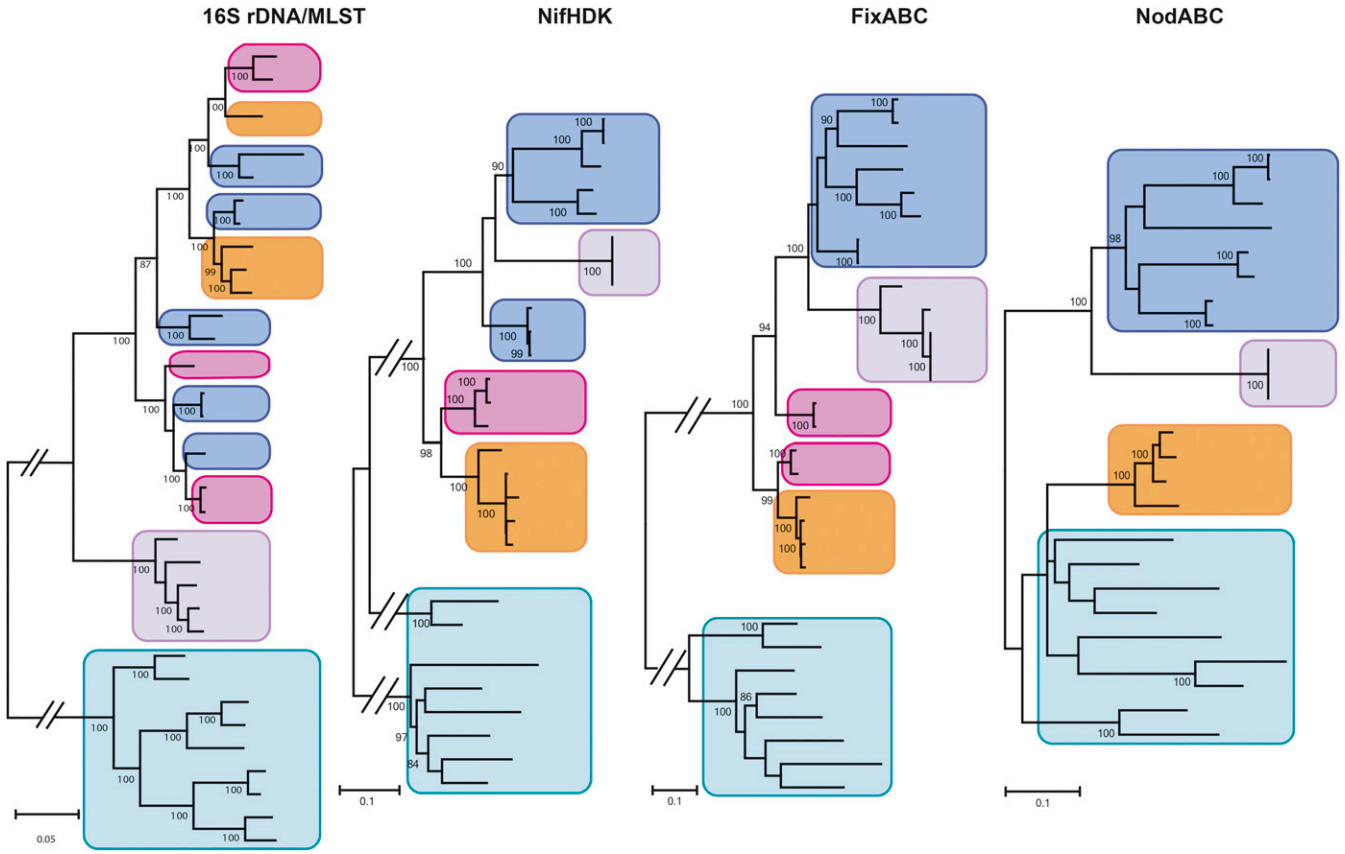


**Fig. 2.** Comparative maximum likelihood phylogenetic analysis using four different gene clusters. Colors indicate the different groups: blue-green: alpha-rhizobia, orange: papilionoid-nodulating *Burkholderia* strains; pink: diazotrophic *Burkholderia* strains; blue: mimosoid nodulating *Burkholderia* 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.

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* 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*, *fixN*, *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 symbionts 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 diazotrophic *Burkholderia* strains, we searched the genomes for alternative cytochrome oxidases. All *Burkholderia* genomes harbored the genes for the high aeration cytochromes *aa3* and *bo* and the low aeration cytochrome *bd* (data not shown). For *B. tuberum*, cytochrome *bo* genes were nested within the *nif* and *fix* gene clusters, and two cytochrome d gene operons, consisting of the genes *cydA* and *cydB*, were found outside of the symbiotic region (data not shown).

Another set of *fix* genes required for $N_2$-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*.

## DISCUSSION

Biological nitrogen fixation has been studied intensely, using two very different systems, the symbiotic $N_2$-fixers and the free-living diazotrophic $N_2$-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 $N_2$-fixing symbiosis within a nodule requires the activation of a large number of *nod*, *nif*, *fix*, and $N_2$ 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

| IMG Gene ID | | 2512348632 | 2512348633 | 2512348639 | 2512348650 | 2512348651 | 2512348652 | 2512348653 | 2512348654 | 2512348655 | 2512348656 | 2512348662 | 2512348663 | 2512348665 | 2512348666 | 2512348668 | 2512348683 | 2512348684 | 2512348685 | 2512348691 | 2512348692 | 2512348693 | 2512348697 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gene | *nifW1* | *nifV1* | *nifA* | *fixX* | *fixC* | *fixB* | *fixA* | *nifW2* | *nifV2* | *nifB* | *fdx* | *hesB/yadR/yfhF* | *nifZ* domain | *nifZ* domain | *nifT/fixU* | *nifH* | *nifD* | *nifK* | *nifE* | *nifN* | *nifX* | *nifQ* |
| Papilionoid-legume nodulating strains | *Bukholderia tuberum* STM678 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | *Burkholderia sprentiae* WSM5005 | 94 | 97 | 92 | 99 | 99 | 98 | 98 | 98 | 87 | 98 | 97 | 99 | 98 | 99 | 96 | 100 | 98 | 99 | 98 | 97 | 96 | 97 |
| | *Burkholderia* sp. WSM4176 | 94 | 97 | 92 | 100 | 98 | 98 | 98 | 96 | 85 | 98 | 98 | 98 | 96 | 97 | 100 | 100 | 99 | 99 | 98 | 98 | 97 | 95 |
| | *Burkholderia dilworthii* WSM3556 | 63 | 96 | 88 | 96 | 96 | 96 | 98 | 91 | n.d. | 93 | 92 | 95 | 93 | 95 | 96 | 99 | 98 | 98 | 94 | 95 | 94 | 91 |
| Mimosoid-legume nodulating strains | *Burkholderia* sp. CCGE1002 | 85 | 84 | 44 | 72 | 86 | 80 | 84 | 66 | n.d. | 70 | 87 | 54 | 68 | n.d. | 70 | 91 | 88 | 87 | 79 | 77 | 76 | 57 |
| | *Burkholderia* sp. JPY251 | 80 | 87 | 43 | 73 | 87 | 80 | 82 | 63 | n.d. | 73 | 90 | 32 | 64 | n.d. | 70 | 91 | 88 | 87 | 79 | 77 | 75 | 58 |
| | *Burkholderia mimosarum* LMG 23256 | 64 | 82 | 43 | 76 | 83 | 79 | 80 | 57 | n.d. | 73 | 84 | 53 | 70 | n.d. | n.d. | 91 | 88 | 87 | 80 | 76 | 64 | 52 |
| | *Burkholderia nodosa* DSM 21604 | 60 | 81 | 43 | 76 | 84 | 77 | 79 | 58 | n.d. | 72 | 82 | 56 | 70 | n.d. | 65 | 90 | 87 | 86 | 80 | 76 | 66 | 53 |
| | *Burkholderia phenoliruptrix* BR3459 | 84 | 86 | 44 | 80 | 86 | 81 | 87 | 60 | n.d. | 75 | 84 | 69 | 66 | 56 | 75 | 90 | 89 | 87 | 84 | 79 | 71 | 61 |
| | *Burkholderia phymatum* STM815 | 85 | 86 | 45 | 80 | 86 | 81 | 87 | 61 | n.d. | 74 | 84 | 63 | 64 | 56 | 75 | 90 | 89 | 87 | 84 | 79 | 73 | 61 |
| Diazotrophic *Burkholderia* spp. | *Burkholderia xenovorans* LB400 | 66 | 74 | 42 | 96 | 95 | 94 | 95 | 92 | n.d. | 92 | 61 | 91 | 94 | 87 | 100 | 98 | 97 | 95 | 94 | 91 | 94 | 82 |
| | *Burkholderia unamae* MTI-641 | 72 | 78 | 43 | 86 | 88 | 84 | 91 | 78 | n.d. | 82 | 92 | 75 | 86 | 75 | 88 | 97 | 94 | 92 | 89 | 84 | 89 | 69 |
| | *Burkholderia silvatlantica* SRMrh-20 | 72 | 79 | 43 | 87 | 88 | 84 | 90 | 79 | n.d. | 82 | 92 | 73 | 85 | 75 | 88 | 95 | 94 | 92 | 89 | 85 | 88 | 68 |
| | *Burkholderia silvatlantica* PVA5 | 71 | 79 | 43 | 87 | 88 | 84 | 91 | 79 | n.d. | 82 | 90 | 73 | 85 | 75 | 88 | 95 | 94 | 92 | 89 | 84 | 89 | 67 |
| | *Burkholderia vietnamiensis* G4 | 62 | 75 | 42 | 86 | 88 | 82 | 86 | 69 | n.d. | 79 | 94 | 74 | 72 | 70 | 83 | 95 | 93 | 92 | 91 | 82 | 82 | 60 |
| *Cupriavidus* strains | *Cupriavidus taiwanensis* STM6070 | 75 | 41 | 43 | 75 | 82 | 76 | 82 | n.d. | n.d. | 36 | 76 | 34 | n.d. | n.d. | n.d. | 89 | 87 | 84 | 79 | 73 | 63 | n.d. |
| | *Cupriavidus* sp. AMP6 | 64 | 36 | 44 | 76 | 82 | 77 | 83 | 59 | n.d. | 36 | 77 | 34 | 71 | n.d. | n.d. | 89 | 87 | 84 | 79 | 71 | 62 | n.d. |
| | *Cupriavidus* sp. UYPR2.512 | 71 | 62 | 41 | 74 | 82 | 76 | 85 | 60 | n.d. | 56 | 79 | 34 | 69 | n.d. | n.d. | 90 | 88 | 85 | 80 | 76 | 65 | n.d. |
| *Rhizobiaceae* | *Rhizobium tropici* CIAT899 | 42 | 36 | 50 | 46 | 57 | 65 | 65 | 42 | n.d. | 36 | 65 | 38 | 54 | n.d. | 63 | 81 | 73 | 59 | 66 | 50 | 51 | 51 |
| | *Rhizobium etli* CFN 42, DSM 11541 | 38 | n.d. | 54 | 53 | 58 | 62 | 64 | 42 | n.d. | 31 | 60 | 37 | 65 | n.d. | 67 | 82 | 73 | 58 | 67 | 50 | 46 | 39 |
| | *Bradyrhizobium elkanii* WSM2783 | 45 | 47 | 81 | 56 | 63 | 64 | 64 | 44 | n.d. | 50 | 85 | 31 | 64 | 56 | 61 | 93 | 84 | 68 | 73 | 56 | 68 | 46 |
| | *Bradyrhizobium japonicum* USDA 110 | 42 | 33 | 70 | 48 | 59 | 64 | 60 | 45 | n.d. | 35 | 70 | 36 | 62 | 31 | 55 | 87 | 82 | 67 | 72 | 57 | 68 | 37 |
| | *Rhizobium phaseoli* Ch24-10 | 38 | 30 | 54 | 53 | 58 | 62 | 64 | 42 | n.d. | 31 | 60 | 41 | 65 | n.d. | 67 | 82 | 77 | 58 | 67 | 50 | 45 | 39 |
| | *Methylobacterium nodulans* ORS 2060 | 40 | 47 | 52 | 47 | 59 | 61 | 62 | 43 | n.d. | 49 | 70 | 40 | 54 | n.d. | 60 | 85 | 72 | 61 | 64 | 51 | 45 | 40 |
| | *Microvirga* sp. BSC39 | n.d. | 35 | 48 | n.d. | n.d. | 33 | 31 | n.d. | n.d. | 37 | n.d. | 32 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

90-100 80-89 70-79 60-69 50-59

underline represents orthology (bidirectional hit) Highest E-value 7.00E-07

**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 tuberum* STM678$^T$ 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. 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.

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 (*nodDABCIJ*) (Fig. 1A). Some alpha-rhizobia may lack *nodU* or *nodH* or may have a large number of additional *nod*, *nol*, 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)

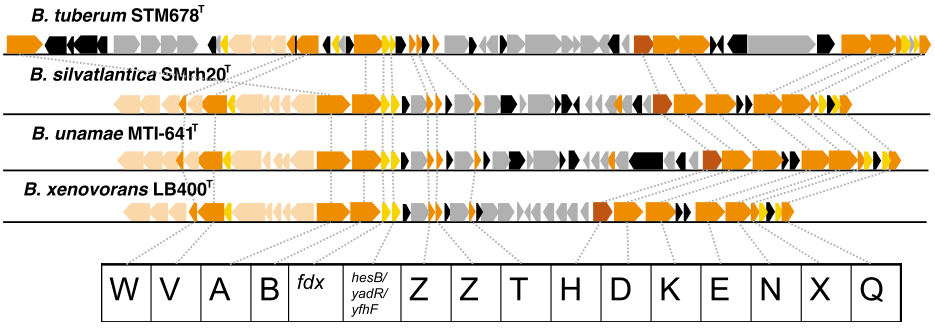


**Fig. 4.** Detailed *nif*/*fix* region in papilionoid and free-living diazotrophic *Burkholderia* strains. Light orange indicates *fix* genes; dark orange indicates *nif* genes; yellow indicates nitrogen fixation–related genes; black are transposases, recombinases, and insertion elements as well as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

| Category | | Host specific *nod* genes | | | Regul. | Common *nod* genes | | | | | | CCGE1002 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IMG Gene ID | | 2512348625 | 2512348626 | 2512348627 | 2512348629 | 2512348634 | 2512348635 | 2512348636 | 2512348700 | 2512348701 | 2512348702 | 646771261 |
| Gene | | *nodU* | *nodS* | *nolO/nodU* | *nodD* | *nodC2* | *nodB* | *nodA* | *nodI* | *nodJ* | *nodC1* | *nodH* |
| Papilionoid-legume nodulating strains | *Burkholderia tuberum* STM678 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | n.d. |
| | *Burkholderia sp.* WSM4176 | 99 | 98 | 98 | 94 | 90 | 94 | 98 | 98 | 96 | 87 | n.d. |
| | *Burkholderia sprentiae* WSM5005 | 99 | 97 | 98 | 93 | 89 | 95 | 99 | 96 | 97 | 88 | n.d. |
| | *Burkholderia dilworthii* WSM3556 | 98 | 94 | 96 | 99 | 79 | 94 | 98 | 96 | 94 | 89 | n.d. |
| Mimosoid-legume nodulating strains | *Burkholderia phenoliruptrix* BR3459 | 81 | 67 | 40 | 70 | 76 | 60 | 73 | 71 | 77 | 70 | 72 |
| | *Burkholderia phymatum* STM815 | 80 | 66 | 39 | 69 | 76 | 60 | 73 | 70 | 77 | 70 | 69 |
| | *Burkholderia sp.* CCGE1002 | 76 | 65 | 40 | 71 | 63 | 57 | 75 | 72 | 79 | 67 | 100 |
| | *Burkholderia sp* . JPY251 | 76 | 69 | 40 | 72 | 63 | 56 | 75 | 74 | 79 | 66 | 94 |
| | *Burkholderia sp.* JPY366 | 75 | 71 | 40 | 68 | 71 | 72 | 70 | 71 | 76 | 71 | 66 |
| | *Burkholderia sp.* UYPR1.413 | 75 | 66 | 39 | 66 | 75 | 58 | 71 | 73 | 76 | 75 | 64 |
| | *Burkholderia nodosa* DSM 21604 | 75 | 64 | 29 | 68 | 72 | 60 | 72 | 73 | 79 | 72 | 67 |
| | *Burkholderia mimosarum* STM3621 | 74 | 69 | 40 | 68 | 74 | 62 | 71 | 71 | 77 | 73 | 69 |
| | *Burkholderia mimosarum* LMG 23256 | 74 | 69 | 39 | 68 | 74 | 62 | 71 | 71 | 77 | 73 | 69 |
| *Cupriavidus* strains | *Cupriavidus sp.* UYPR2.512 | 78 | 64 | 33 | 68 | 69 | 59 | 68 | 67 | 76 | 66 | 69 |
| | *Cupriavidus sp.* AMP6 | 76 | 62 | 29 | 69 | 63 | 57 | 69 | 66 | 75 | 67 | 65 |
| | *Cupriavidus taiwanensis* LMG 19424 | 76 | 61 | 40 | 69 | 68 | 59 | 68 | 66 | 75 | 67 | 65 |
| | *Cupriavidus taiwanensis* STM6018 | 76 | 61 | 40 | 69 | 68 | 59 | 68 | 66 | 75 | 67 | 65 |
| | *Cupriavidus taiwanensis* STM6070 | 76 | 61 | 40 | 69 | 68 | 59 | 68 | 66 | 75 | 67 | 65 |
| *Rhizobiaceae* | *Bradyrhizobium elkanii* USDA 76 | 74 | 61 | 81 | 78 | 74 | 71 | 67 | 68 | 77 | 77 | n.d. |
| | *Bradyrhizobium japonicum* USDA 6 | 74 | 59 | 79 | 75 | 71 | 70 | 72 | 67 | 76 | 71 | n.d. |
| | *Ensifer arboris* LMG 14919 | 75 | 69 | 39 | 72 | 78 | 71 | 73 | 77 | 80 | 72 | 60 |
| | *Ensifer meliloti* 1021 | n.d. | n.d. | 39 | 73 | 70 | 67 | 64 | 73 | 77 | 68 | 59 |
| | *Mesorhizobium australicum* WSM2073 | 69 | 47 | 80 | 71 | 77 | 66 | 70 | 72 | 76 | 71 | 58 |
| | *Mesorhizobium loti* R7A | n.d. | 65 | 81 | 76 | 72 | 70 | 73 | 73 | 78 | 71 | 24 |
| | *Rhizobium etli* CFN 42, DSM 11541 | 61 | 71 | 75 | 73 | 73 | 66 | 70 | 74 | 77 | 71 | n.d. |
| | *Rhizobium leguminosarum bv. trifolii* WSM1325 | n.d. | 46 | n.d. | 69 | 75 | 63 | 67 | 70 | 73 | 67 | n.d. |

underline represents orthologous (bidirectional hit)

| 90-100 | 80-89 | 70-79 | 60-69 | 50-59 |
|---|---|---|---|---|

**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* STM678 gene. 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

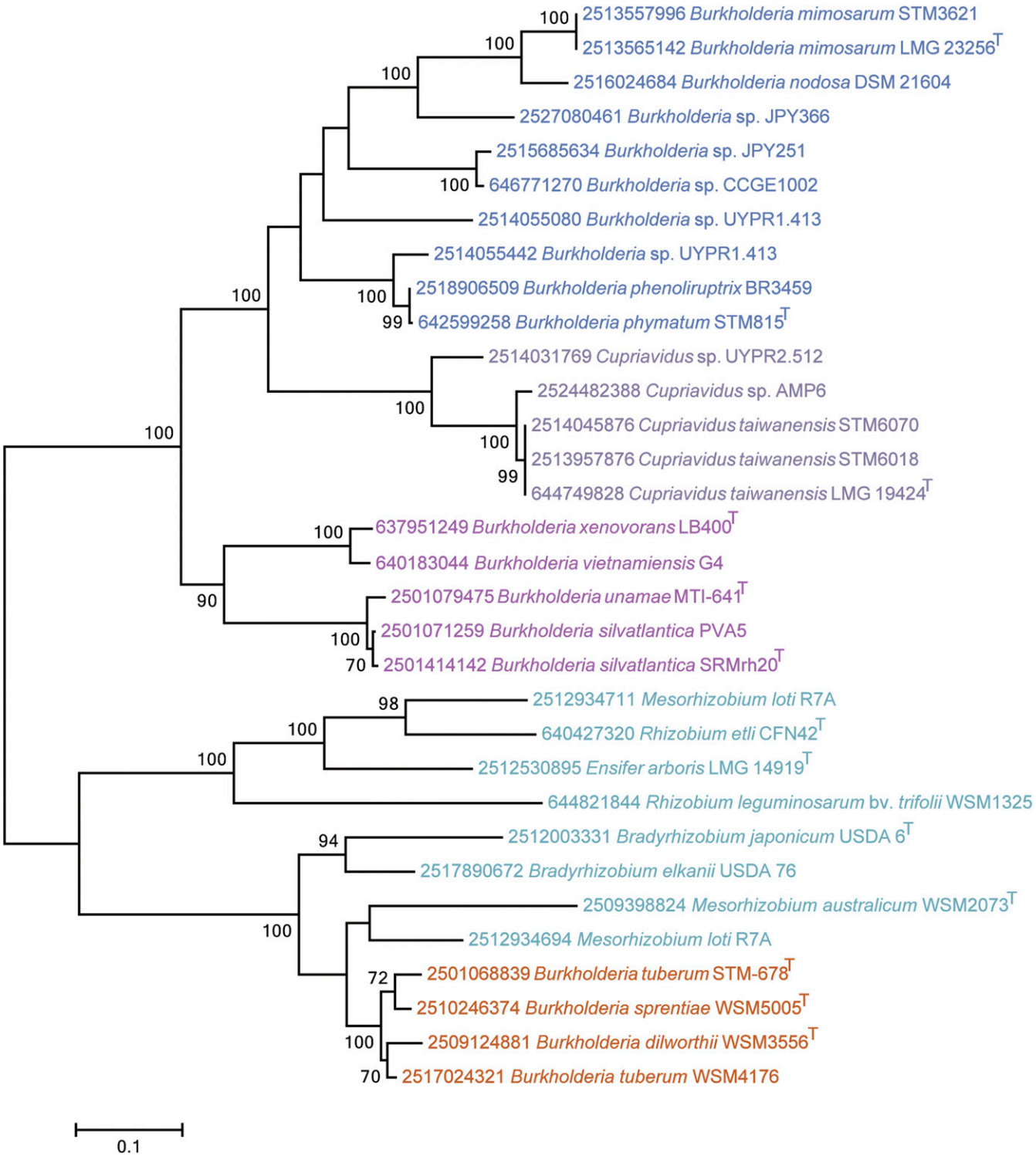


**Fig. 6.** Maximum likelihood *nifA* phylogenetic tree based on 1,888-bp gene alignment. Colors indicate the different groups: alpha-rhizobia, blue-green; papilionoid nodulating *Burkholderia* strains, orange; diazotrophic *Burkholderia* strains, pink; mimosoid nodulating *Burkholderia* strains, blue; 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, subsequent 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, *Mimosa*-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 alpha-rhizobia-like *nodA*, *nodB*, and *nodC* and beta-rhizobia-like *nodI* and *nodJ* genes. The *nodIJ* 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 *nodI* and *nodJ* 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 (*fixNOQPGHIS)* 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 inmembers 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 *cytcbb3* or *cytbd* showed that they were still able to fix nitrogen symbiotically, whereas *cytcbb3* and *cytdb* 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 $N_2$-fixation process or for protecting it from $O_2$ inhibition.

In addition to the *FixN*, *fixO*, *fixQ*, *fixP*, *fixG*, *fixH*, *fixI*, and *fixS* genes, another set of *fix* genes (*fixABCX*) is important for $N_2$-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. xenovorans*, whereas the mimosoid beta-rhizobia share similarity with *B. silvatlantica* and *B. unamae*.

The congruence between the 16S rRNA/MLST and *nif*/*fix* 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 orthologous 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). Additional gene sequences for the trees were sourced from the GenBank database and added to the genome alignments. The sequence identity was verified by comparative alignment analysis using the BLASTP program from NCBI. Coverage and identity cut-off values were set at 75 and 30%, respectively, for reliable homolog identification.

**Homologous gene identification for gene identity comparisons.**

To find gene homologs for the *nod* and *nif/fix* genes of interest, the BLASTP algorithm in the JGI IMG Expert Review database was used to compare amino acid sequences of genes across the selected genomes. Moreover, a Python script was developed to extract the percentage of gene identity, the associated e-value, and the orthology notation from the IMG BLASTP results. The initial BLASTP searches were constrained to e-values $\leq 10^{-2}$ and sequence identities ≥30%, with the exception of a few genes that showed meaningful homology at lower percentages (Maymon et al. 2015). The final results were filtered to an e-value of $<10^{-7}$, and multiple genes from the same genome were frequently identified as homologous 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* STM678$^T$ 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 *fdx* gene and the *hesB/yadR/yfhF* 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.

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## LITERATURE CITED

Angus, A. A., Lee, A., Lum, M. R., Shehayeb, M., Hessabi, R., Fujishige, N. A., Yerrapragada, S., Kano, S., Song, N., Yang, P., Estrada de los Santos, P., de Faria, S. M., Dakora, F. D., Weinstock, G., and Hirsch, A. M. 2013. Nodulation and effective nitrogen fixation of *Macroptilium atropurpureum* (siratro) by *Burkholderia tuberum*, a nodulating and plant growth promoting beta-proteobacterium, are influenced by environmental factors. Plant Soil 369:543-562.

Aoki, S., Ito, M., and Iwasaki, W. 2013. From β- to α-proteobacteria: The origin and evolution of rhizobial nodulation genes *nodIJ*. Mol. Biol. Evol. 30:2494-2508.

Arigoni, F., Kaminski, P. A., Hennecke, H., and Elmerich, C. 1991. Nucleotide sequence of the *fixABC* region of *Azorhizobium caulinodans* ORS571: Similarity of the *fixB* product with eukaryotic flavoproteins, characterization of *fixX*, and identification of *nifW*. Mol. Gen. Genet. 225:514-520.

Beukes, C. W., Venter, S. N., Law, I. J., Phalane, F. L., and Steenkamp, E. T. 2013. South African papilionoid legumes are nodulated by diverse *Burkholderia* with unique nodulation and nitrogen-fixation loci. PLoS One 8:e68406.

Bontemps, C., Elliott, G. N., Simon, M. F., Dos Reis Júnior, F. B., Gross, E., Lawton, R. C., Neto, N. E., de Fátima Loureiro, M., De Faria, S. M., Sprent, J. I., James, E. K., and Young, J. P. W. 2010. *Burkholderia* species are ancient symbionts of legumes. Mol. Ecol. 19:44-52.

Bontemps, C., Rogel, M. A., Wiechmann, A., Mussabekova, A., Moody, S., Simon, M. F., Moulin, L., Elliott, G. N., Lacercat-Didier, L., Dasilva, C., Grether, R., Camargo-Ricalde, S. L., Chen, W., Sprent, J. I., Martínez-Romero, E., Young, J. P. W., and James, E. K. 2016. Endemic *Mimosa* species from Mexico prefer alphaproteobacterial rhizobial symbionts. New Phytol. 209:319-333.

Boone, C. M., Olsthoorn, M. M., Dakora, F. D., Spaink, H. P., and Thomas-Oates, J. E. 1999. Structural characterisation of lipo-chitin oligosaccharides isolated from *Bradyrhizobium aspalati*, microsymbionts of commercially important South African legumes. Carbohydr. Res. 317: 155-163.

Bournaud, C., de Faria, S. M., dos Santos, J. M. F., Tisseyre, P., Silva, M., Chaintreuil, C., Gross, E., James, E. K., Prin, Y., and Moulin, L. 2013. *Burkholderia* species are the most common and preferred nodulating symbionts of the Piptadenia group (tribe Mimoseae). PLoS One 8: e63478.

Broughton, W. J., Jabbouri, S., and Perret, X. 2000. Keys to symbiotic harmony. J. Bacteriol. 182:5641-5652.

Chen, W. M., de Faria, S. M., Chou, J. H., James, E. K., Elliott, G. N., Sprent, J. I., Bontemps, C., Young, J. P. W., and Vandamme, P. 2008. *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. Int. J. Syst. Evol. Microbiol. 58:2174-2179.

Chen, W. M., de Faria, S. M., James, E. K., Elliott, G. N., Lin, K. Y., Chou, J. H., Sheu, S. Y., Cnockaert, M., Sprent, J. I., and Vandamme, P. 2007. *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. Int. J. Syst. Evol. Microbiol. 57:1055-1059.

Chen, W. M., de Faria, S. M., Straliotto, R., Pitard, R. M., Simões-Araùjo, J. L., Chou, J. H., Chou, Y. J., Barrios, E., Prescott, A. R., Elliott, G. N., Sprent, J. I., Young, J. P. W., and James, E. K. 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: A study of novel *Mimosa*-nodulating strains from South America. Appl. Environ. Microbiol. 71:7461-7471.

Chen, W. M., James, E. K., Coenye, T., Chou, J. H., Barrios, E., de Faria, S. M., Elliott, G. N., Sheu, S. Y., Sprent, J. I., and Vandamme, P. 2006. *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. Int. J. Syst. Evol. Microbiol. 56: 1847-1851.

Chen, W.-M., James, E. K., Prescott, A. R., Kierans, M., and Sprent, J. I. 2003a. Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. Mol. Plant-Microbe Interact 16:1051-1061.

Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., De Vos, P., Mergeay, M., and Vandamme, P. 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. Int. J. Syst. Evol. Microbiol. 51:1729-1735.

Chen, W. M., Moulin, L., Bontemps, C., Vandamme, P., Béna, G., and Boivin-Masson, C. 2003b. Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J. Bacteriol. 185:7266-7272.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16:10881-10890.

Cosseau, C., and Batut, J. 2004. Genomics of the *ccoNOQP*-encoded *cbb3* oxidase complex in bacteria. Arch. Microbiol. 181:89-96.

Curatti, L., Hernandez, J. A., Igarashi, R. Y., Soboh, B., Zhao, D., and Rubio, L. M. 2007. In vitro synthesis of the iron-molybdenum cofactor of nitrogenase from iron, sulfur, molybdenum, and homocitrate using purified proteins. Proc. Natl. Acad. Sci. U.S.A. 104:17626-17631.

De Meyer, S. E., Cnockaert, M., Ardley, J. K., Maker, G., Yates, R., Howieson, J. G., and Vandamme, P. 2013b. *Burkholderia sprentiae* sp. nov., isolated from *Lebeckia ambigua* root nodules. Int. J. Syst. Evol. Microbiol. 63:3950-3957.

De Meyer, S. E., Cnockaert, M., Ardley, J. K., Trengove, R. D., Garau, G., Howieson, J. G., and Vandamme, P. 2013a. *Burkholderia rhynchosiae* sp. nov., isolated from *Rhynchosia ferulifolia* root nodules. Int. J. Syst. Evol. Microbiol. 63:3944-3949.

De Meyer, S. E., Cnockaert, M., Ardley, J. K., Van Wyk, B.-E., Vandamme, P. A., and Howieson, J. G. 2014. *Burkholderia dilworthii* sp. nov., isolated from *Lebeckia ambigua* root nodules. Int. J. Syst. Evol. Microbiol. 64: 1090-1095.

Edgren, T., and Nordlund, S. 2004. The *fixABCX* genes in *Rhodospirillum rubrum* encode a putative membrane complex participating in electron transfer to nitrogenase. J. Bacteriol. 186:2052-2060.

Ekici, S., Pawlik, G., Lohmeyer, E., Koch, H.-G., and Daldal, F. 2012. Biogenesis of *cbb3*-type cytochrome c oxidase in *Rhodobacter capsulatus*. BBA-Bioenergetics 1817:898-910.

Elliott, G. N., Chen, W. M., Bontemps, C., Chou, J. H., Young, J. P. W., Sprent, J. I., and James, E. K. 2007a. Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. Ann. Bot. (Lond.) 100:1403-1411.

Elliott, G. N., Chen, W. M., Chou, J. H., Wang, H. C., Sheu, S. Y., Perin, L., Reis, V. M., Moulin, L., Simon, M. F., Bontemps, C., Sutherland, J. M., Bessi, R., de Faria, S. M., Trinick, M. J., Prescott, A. R., Sprent, J. I., and James, E. K. 2007b. *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa* spp. and fixes nitrogen *ex planta*. New Phytol. 173:168-180.

Farris, J. S., Källersjö, M., Kluge, A. G., and Bult, C. 1994. Testing significance of incongruence. Cladistics 10:315-319.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17:368-376.

Foussard, M., Garnerone, A.-M., Ni, F., Soupène, E., Boistard, P., and Batut, J. 1997. Negative autoregulation of the *Rhizobium meliloti fixK* gene is indirect and requires a newly identified regulator, FixT. Mol. Microbiol. 25:27-37.

Garau, G., Yates, R. J., Deiana, P., and Howieson, J. G. 2009. Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with papilionoid herbaceous legumes adapted to acid, infertile soils. Soil Biol. Biochem. 41:125-134.

Gyaneshwar, P., Hirsch, A. M., Moulin, L., Chen, W. M., Elliott, G. N., Bontemps, C., Estrada-de Los Santos, P., Gross, E., Dos Reis, F. B., Sprent, J. I., Young, J. P. W., and James, E. K. 2011. Legume-nodulating Betaproteobacteria: diversity, host range, and future prospects. Mol. Plant-Microbe Interact 24:1276-1288.

Howieson, J. G., De Meyer, S. E., Vivas-Marfisi, A., Ratnayake, S., Ardley, J. K., and Yates, R. J. 2013. Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua*—A perennial suffrutescent legume of the fynbos. Soil Biol. Biochem. 60:55-64.

Howieson, J. G., Yates, R. J., Foster, K., Real, D., and Besier, B. 2008. Prospects for the future use of legumes. Pages 363-394 in: Leguminous Nitrogen-Fixing Symbioses. M. J. Dilworth, E. K. James, J. I. Sprent, and W. E. Newton, eds. Elsevier, London, U.K.

Juty, N. S., Moshiri, F., Merrick, M., Anthony, C., and Hill, S. 1997. The *Klebsiella pneumoniae* cytochrome *bd'* terminal oxidase complex and its role in microaerobic nitrogen fixation. Microbiology 143:2673-2683.

Kaminski, P. A., Kitts, C. L., Zimmerman, Z., and Ludwig, R. A. 1996. Azorhizobium caulinodans uses both cytochrome bd (quinol) and cytochrome cbb3 (cytochrome c) terminal oxidases for symbiotic N2 fixation. J. Bacteriol. 178:5989-5994.

Kelly, M. J. S., Poole, R. K., Yates, M. G., and Kennedy, C. 1990. Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii*: Mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air. J. Bacteriol. 172:6010-6019.

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C., Jeon, Y. S., Lee, J.-H., Yi, H., Won, S., and Chun, J. 2012. Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol. 62:716-721.

Klonowska, A., Chaintreuil, C., Tisseyre, P., Miché, L., Melkonian, R., Ducousso, M., Laguerre, G., Brunel, B., and Moulin, L. 2012. Biodiversity of *Mimosa pudica* rhizobial symbionts (*Cupriavidus taiwanensis*, *Rhizobium mesoamericanum*) in New Caledonia and their adaptation to heavy metal-rich soils. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 81:618-635.

Kneip, C., Lockhart, P., Voss, C., and Maier, U. G. 2007. Nitrogen fixation in eukaryotes—New models for symbiosis. BMC Evol. Biol. 7:55.

Lemaire, B., Dlodlo, O., Chimphango, S., Stirton, C., Schrire, B., Boatwright, J. S., Honnay, O., Smets, E., Sprent, J., James, E. K., and Muasya, A. M. 2015b. Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa). FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 91:1-17.

Lemaire, B., Van Cauwenberghe, J., Chimphango, S., Stirton, C., Honnay, O., Smets, E., and Muasya, A. M. 2015a. Recombination and horizontal transfer of nodulation and ACC deaminase (*acdS*) genes within *Alpha-* and *Betaproteobacteria* nodulating legumes of the Cape Fynbos biome. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 91:fiv118.

Lemaire, B., Van Cauwenberghe, J., Verstraete, B., Chimphango, S., Stirton, C., Honnay, O., Smets, E., Sprent, J., James, E. K., and Muasya, A. M. 2016. Characterization of the papilionoid-*Burkholderia* interaction in the Fynbos biome: The diversity and distribution of beta-rhizobia nodulating *Podalyria calyptrata* (Fabaceae, Podalyrieae). Syst. Appl. Microbiol. 39:41-48.

Lessie, T. G., Hendrickson, W., Manning, B. D., and Devereux, R. 1996. Genomic complexity and plasticity of *Burkholderia cepacia*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 144:117-128.

Liu, W. Y., Ridgway, H. J., James, T. K., James, E. K., Chen, W.-M., Sprent, J. I., Young, J. P., and Andrews, M. 2014. *Burkholderia* sp. induces functional nodules on the South African invasive legume *Dipogon lignosus* (Phaseoleae) in New Zealand soils. Microb. Ecol. 68:542-555.

Liu, X., Wei, S., Wang, F., James, E. K., Guo, X., Zagar, C., Xia, L. G., Dong, X., and Wang, Y. P. 2012. *Burkholderia* and *Cupriavidus* spp. are the preferred symbionts of *Mimosa* spp. in southern China. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 80:417-426.

López-Guerrero, M. G., Ormeño-Orrillo, E., Acosta, J. L., Mendoza-Vargas, A., Rogel, M. A., Ramírez, M. A., Rosenblueth, M., Martínez-Romero, J.,

and Martínez-Romero, E. 2012. Rhizobial extrachromosomal replicon variability, stability and expression in natural niches. Plasmid 68:149-158.
Martínez-Aguilar, L., Díaz, R., Peña-Cabriales, J. J., Estrada-de Los Santos, P., Dunn, M. F., and Caballero-Mellado, J. 2008. Multichromosomal genome structure and confirmation of diazotrophy in novel plant-associated *Burkholderia* species. Appl. Environ. Microbiol. 74:4574-4579.
Maymon, M., Martínez-Hidalgo, P., Tran, S. S., Ice, T., Craemer, K., Anbarchian, T., Sung, T., Hwang, L. H., Chou, M., Fujishige, N. A., Villella, W., Ventosa, J., Sikorski, J., Sanders, E. R., Faull, K. F., and Hirsch, A. M. 2015. Mining the phytomicrobiome to understand how bacterial coinoculations enhance plant growth. Front. Plant Sci. 6: 00784.
Mishra, R. P. N., Tisseyre, P., Melkonian, R., Chaintreuil, C., Miché, L., Klonowska, A., Gonzalez, S., Bena, G., Laguerre, G., and Moulin, L. 2012. Genetic diversity of *Mimosa pudica* rhizobial symbionts in soils of French Guiana: Investigating the origin and diversity of *Burkholderia phymatum* and other beta-rhizobia. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 79:487-503.
Moulin, L., Munive, A., Dreyfus, B., and Boivin-Masson, C. 2001. Nodulation of legumes by members of the beta-subclass of Proteobacteria. Nature 411:948-950.
Ormeño-Orrillo, E., Rogel, M. A., Chueire, L. M. O., Tiedje, J. M., Martínez-Romero, E., and Hungria, M. 2012. Genome sequences of *Burkholderia* sp. strains CCGE1002 and H160, isolated from legume nodules in Mexico and Brazil. J. Bacteriol. 194:6927.
Parker, M. A. 2015. A single sym plasmid type predominates across diverse chromosomal lineages of *Cupriavidus* nodule symbionts. Syst. Appl. Microbiol. 38:417-423.
Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S., and Barrell, B. G. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-668.
Perin, L., Martínez-Aguilar, L., Paredes-Valdez, G., Baldani, J. I., Estrada-de Los Santos, P., Reis, V. M., and Caballero-Mellado, J. 2006. *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. Int. J. Syst. Evol. Microbiol. 56:1931-1937.
Perret, X., Staehelin, C., and Broughton, W. J. 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64:180-201.
Pitcher, R. S., and Watmough, N. J. 2004. The bacterial cytochrome *cbb3* oxidases. Biochimica et Biophysica Acta (BBA) -. Bioenergetics 1655:388-399.
Reeve, W., Ardley, J., Tian, R., Eshragi, L., Yoon, J. W., Ngamwisetkun, P., Seshadri, R., Ivanova, N. N., and Kyrpides, N. C. 2015. A genomic encyclopedia of the root nodule bacteria: Assessing genetic diversity through a systematic biogeographic survey. Stand. Genomic Sci. 10:14.
Sheu, S.-Y., Chen, M.-H., Liu, W. Y. Y., Andrews, M., James, E. K., Ardley, J. K., De Meyer, S. E., James, T. K., Howieson, J. G., Coutinho, B. G., and Chen, W.-M. 2015. *Burkholderia dipogonis* sp. nov., isolated from root nodules of *Dipogon lignosus* in New Zealand and Western Australia. Int. J. Syst. Evol. Microbiol. 65:4716-4723.
Sheu, S. Y., Chou, J. H., Bontemps, C., Elliott, G. N., Gross, E., dos Reis Junior, F. B., Melkonian, R., Moulin, L., James, E. K., Sprent, J. I., Young, J. P., and Chen, W. M. 2013. *Burkholderia diazotrophica* sp. nov., isolated from root nodules of *Mimosa* spp. Int. J. Syst. Evol. Microbiol. 63:435-441.
Sheu, S.-Y., Chou, J.-H., Bontemps, C., Elliott, G. N., Gross, E., James, E. K., Sprent, J. I., Young, J. P. W., and Chen, W.-M. 2012. *Burkholderia symbiotica* sp. nov., isolated from root nodules of *Mimosa* spp. native to north-east Brazil. Int. J. Syst. Evol. Microbiol. 62:2272-2278.
Swofford, D. L. 1991. PAUP: Phylogenetic Analysis Using Parsimony, version 3.1. Smithsonian Institution, Washington, DC.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.
Tsai, M. H., and Saier, M. H., Jr. 1995. Phylogenetic characterization of the ubiquitous electron transfer flavoprotein families ETF-α and ETF-β. Res. Microbiol. 146:397-404.
Vandamme, P., Goris, J., Chen, W. M., de Vos, P., and Willems, A. 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. 25:507-512.
Varghese, N. J., Mukherjee, S., Ivanova, N., Konstantinidis, K. T., Mavrommatis, K., Kyrpides, N. C., and Pati, A. 2015. Microbial species delineation using whole genome sequences. Nucleic Acids Res. 43: 6761-6771.
Walker, R., Watkin, E., Tian, R., Bräu, L., O'Hara, G., Goodwin, L., Han, J., Lobos, E., Huntemann, M., Pati, A., Woyke, T., Mavromatis, K., Markowitz, V., Ivanova, N., Kyrpides, N., and Reeve, W. 2014a. Genome sequence of the acid-tolerant *Burkholderia* sp. strain WSM2230 from Karijini National Park, Australia. Stand. Genomic Sci. 9:551-561.
Walker, R., Watkin, E., Tian, R., Bräu, L., O'Hara, G., Goodwin, L., Han, J., Reddy, T., Huntemann, M., Pati, A., Woyke, T., Mavromatis, K., Markowitz, V., Ivanova, N., Kyrpides, N., and Reeve, W. 2014b. Genome sequence of the acid-tolerant *Burkholderia* sp. strain WSM2232 from Karijini National Park, Australia. Stand. Genomic Sci. 9:1168-1180.
Wang, D., Yang, S., Tang, F., and Zhu, H. 2012. Symbiosis specificity in the legume: Rhizobial mutualism. Cell. Microbiol. 14:334-342.

## AUTHOR-RECOMMENDED INTERNET RESOURCES

GenBank database: http://www.ncbi.nlm.nih.gov
Joint Genome Institute (JGI) website:
https://img.jgi.doe.gov/cgi-bin/mer/main.cgi
The JGI/IMG genomic database:
https://img.jgi.doe.gov/cgi-bin/m/main.cgi?section=FindGenesBlast&page=geneSearch%20Blast
NCBI BLAST database: https://blast.ncbi.nlm.nih.gov/Blast.cgi