Nitrogen

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Review

Nitrogen-Fixing Symbiotic *Paraburkholderia* Species: Current Knowledge and Future Perspectives

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Abstract: A century after the discovery of rhizobia, the first *Beta-proteobacteria* species (beta-rhizobia) were isolated from legume nodules in South Africa and South America. Since then, numerous species belonging to the *Burkholderiaceae* family have been isolated. The presence of a highly branching lineage of nodulation genes in beta-rhizobia suggests a long symbiotic history. In this review, we focus on the beta-rhizobial genus *Paraburkholderia*, which includes two main groups: the South American mimosoid-nodulating *Paraburkholderia* and the South African predominantly papilionoid-nodulating *Paraburkholderia*. Here, we discuss the latest knowledge on *Paraburkholderia* nitrogen-fixing symbionts in each step of the symbiosis, from their survival in the soil, through the first contact with the legumes until the formation of an efficient nitrogen-fixing symbiosis in root nodules. Special attention is given to the strain *P. phymatum* STM815^T that exhibits extraordinary features, such as the ability to: (i) enter into symbiosis with more than 50 legume species, including the agriculturally important common bean, (ii) outcompete other rhizobial species for nodulation of several legumes, and (iii) endure stressful soil conditions (e.g., high salt concentration and low pH) and high temperatures.

Keywords: beta-rhizobia; competition; symbiosis; legume interaction; root nodule; nitrogen fixation

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1. Introduction

Nitrogen is one of the five chemical elements essential to any living organism. Organic compounds, such as nucleic and amino acids, all contain nitrogen. The largest nitrogen reservoir on Earth is atmospheric dinitrogen gas (N_2) [1,2]. Although it might seem contradictory, N_2 cannot be directly used by most organisms, including plants, which makes it a limiting factor in crop productivity [3]. The triple bond between the two nitrogen atoms can only be broken by certain groups of microorganisms (prokaryotes and archaea), which transform the N_2 molecule into ammonia that is then ionized into ammonium, a nitrogenous form that plants can readily metabolize [4]. The term "biological nitrogen fixation" (BNF) refers to this process, which can be performed with the nitrogenase enzyme by both symbiotic and free-living bacteria [5]. Indeed, leguminous plants can host microsymbionts that fix N_2, called "rhizobia", in root (and sometimes stem) nodules [6–9]. To date, only the *Alpha-* and *Beta*-classes of *Proteobacteria* have been recognized as containing genuine rhizobia [10].

In this review, we summarize the most recent findings on N_2-fixing symbionts (Beta-rhizobia) belonging to the Beta-proteobacterial genus *Paraburkholderia*, detailing the information, to date, on their geographical distribution and phylogenetic relatedness, since their discovery 20 years ago up to the present day. We draw attention to key processes occurring in different environments where rhizobia are located prior to and during the symbiosis (i.e., soil, rhizosphere, plant root and nodules) and discuss the mechanisms employed to survive abiotic and biotic stresses, their interaction with host plants until the allocation and differentiation of the free-living rhizobia into N_2-fixing bacteroids. In particular, we focus on the strain *Paraburkholderia phymatum* STM815^T, which possesses exceptional features that allow it to outperform other rhizobia during the different stages of the symbiosis.

2. Two Decades since the Discovery of Nitrogen-Fixing Symbiotic *Beta-proteobacteria*

The rhizobium–legume symbiosis is not a strict symbiotic relationship as both partners may develop and persist in soils on their own [6–9]. Nonetheless, the evolutionary success of legumes in the plant kingdom can be attributed to their ability to establish this symbiosis with rhizobia [11,12]. It is believed that this interaction arose approximately 60 million years ago [13] and is probably derived from the same ancestor in the Rosid Clade as the slightly earlier evolved nodulating symbiosis between actinorhizal plants and *Frankia* [14,15]. By the time the major legume lineages diversified, nodules inhabited by N_2-fixing rhizobia had already evolved to form on their roots [16,17]. Of the six leguminous subfamilies, only *Papilionoideae* and *Caesalpinioideae* (which now contains the former Mimosoideae subfamily, informally referred to as the "mimosoid clade") are known to be capable of nodulation [13,18–21].

Rhizobial species have been isolated and characterized with the main focus of understanding their symbiotic association with legumes related to human and animal consumption, such as soybean (*Glycine max*), cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), or mungbean (*Vigna radiata*), among others [22]. For instance, the estimated global soybean production in 2021 was over 300 million tonnes [23]. Two decades ago, rhizobia were still presumed to be exclusive members of the class *Alpha-proteobacteria* [18,22,24]. This long-held belief unraveled in 2001, when three species belonging to the *Beta-proteobacteria* class were isolated from nodules. Indeed, two strains previously belonging to the *Burkholderia* genus, *Burkholderia* sp. STM815^T (named, at present, *Paraburkholderia phymatum* STM815^T = LMG 21445^T [25,26] (Table 1)) and *Burkholderia* sp. STM678^T (named, at present, *Paraburkholderia tuberum* STM678^T = LMG 21444^T [25,26]) were isolated in French Guiana and South Africa, respectively [10]. In parallel, Chen and associates identified *Cupriavidus taiwanensis*, originally known as *Ralstonia taiwanensis*, from root nodules of two species of *Mimosa* (*Caesalpinioideae*-mimosoid clade), *M. pudica* and *M. diplotricha*, in Taiwan and from the sputum of a cystic fibrosis patient [27,28]. Subsequently, rhizobia that belonged to *Alpha-* or *Beta-proteobacteria* were referred to as alpha- and beta-rhizobia, respectively [10]. This discovery was a paradigm shift, not only in the taxonomic diversity of legume symbionts but also in our understanding of the ecological versatility of *Burkholderia* [29]. Nonetheless, part of the scientific community received these findings with a certain skepticism [18] since both strains isolated by Moulin and coworkers were unable to re-infect the papilionoid plant-host from which they were purportedly isolated (*Machaerium lunatum*), and they only induced ineffective nodules on the promiscuous legume *Macroptilium atropurpureum* (commonly known as siratro) [10]. Therefore, even though the symbiotic ability of *C. taiwanensis* was confirmed by Chen et al., 2003, the same could not be said for the putative legume-nodulating *Burkholderia* strains [18,30]. Confirmation only came some years later when subsequent studies described several other nodulating *Burkholderia* strains that were isolated from *Mimosa* species [31,32], including strains that were later described at a species level, such as Br3461 (presently belonging to *Paraburkholderia nodosa* [33]) and MAP3-5 and PAS44^T (both belonging to *Paraburkholderia mimosarum* [34]). Even more importantly, the aforementioned studies confirmed the nodulating ability of these new *Mimosa* nodule isolates with GFP-tagged variants and high-resolution microscopy [18,31,32]. These same methods were then used to confirm the nodulating ability of the strains described by Moulin et al. (2001), by then renamed *P. phymatum* STM815^T and *P. tuberum* STM678^T [25], however not on either of the hosts from which they were originally isolated. Instead, STM815^T was shown to nodulate several species of *Mimosa* (Table 1) [35], whereas STM678^T could nodulate species of *Cyclopia*, a genus endemic to the South African Core Cape Subregion (CCR) [36]. In the same study by Elliott et al., 2007a, where *P. phymatum* STM815^T was proven to be a *Mimosa* symbiont, its ability to fix N_2 *ex planta* was also demonstrated; the same ability was demonstrated for *P. tuberum* STM678^T [35]. This work was performed in comparison with several newly identified non-symbiotic diazotrophic *Burkholderia* strains, since renamed *Paraburkholderia tropica* Ppe8^T [26,37] and *Paraburkholderia unamae* MTI-641^T [26,38]. Interestingly, the two

beta-rhizobia strains fixed less N_2 than the non-symbionts, suggesting that they had become progressively adapted to a non-free-living, symbiotic lifestyle more typical of rhizobia in general [18].

At this time, symbiotic beta-rhizobial strains were still classified as *Burkholderia*, alongside human, animal, and plant pathogens, such as members of the *Burkholderia cepacia* complex (BCC) or the *B. pseudomallei* group [18]. There have been several efforts to highlight the differences between the pathogenic *Burkholderia* and environmental strains. For instance, phylogenetic studies based on 16S ribosomal RNA (rRNA) and multilocus sequence analysis (MLSA) of housekeeping genes revealed the separation of these two lineages [18,39–41]. The difference in GC content between the two groups was viewed as further confirmation of this separation, as plant-associated *Burkholderia* have a lower GC content than the pathogenic BCC, while they also lack major virulence factors as well as several secretion systems [40]. However, it was only in 2014 that Sawana and colleagues performed a phylogenetic analysis based on 21 conserved protein sequences of 45 sequenced *Burkholderia* species and comparisons of conserved sequence indels, which they then used as the basis for their proposal to reclassify the environmental species (including the symbionts) into a new genus: *Paraburkholderia* [26]. Subsequently, additional environmental *Burkholderia sensu lato* species/strains have been accommodated into new genera delineated within the *Burkholderiaceae* family, such as *Caballeronia, Trinickia, Mycetohabitans, Robbsia*, and *Pararobbsia* [42–45]. Of these genera, only strains from *Paraburkholderia* and *Trinickia* contain proven N_2-fixing symbiotic strains [44]. Since the discovery of beta-rhizobia, there has been a global effort to isolate, sequence, and characterize new rhizobial strains in order to not only trace the evolutionary history of this polyphyletic group but also to comprehend the molecular mechanisms driving this symbiosis [18,46]. A clear example is *P. phymatum*, for which more than a hundred strains have been isolated from all over the world and from various plant hosts since the discovery of the first strain (STM815^T). At present, however, of these, only the strain STM815^T has a genome sequence available (Table 1) [47].

Table 1. List of *P. phymatum* isolated strains reported, to date, including the source and plant host.

***P. phymatum* Strains**	**Sources (Country)**	**Plant Host**	**Publication**
STM815^T	French Guiana	*Machaerium lunatum*/ *Mimosa pudica* *	Moulin et al., 2001 [10]/ Mishra et al., 2012 [48]
GR01, GR03, GR05, GR06 [1,2]	Morocco	*Phaseolus vulgaris*	Talbi et al., 2010 [49]
NGR114 [1,3]	Papua New Guinea	*M. pudica*	Elliott et al., 2007a [35]
NGR195A [1,3]	Papua New Guinea	*M. invisa*	Elliott et al., 2007a [35]
STM3619, STM3622, STM3623, STM3631, STM3665, STM3666, STM3667, STM3668, STM3669, STM3674, STM3675, STM3676, STM4205, STM4207, STM4208, STM4211, STM4212, STM4214, STM4216, STM4217, STM4219, STM4221, STM4223, STM4225, STM4302, STM4303, STM4305, STM4308, STM4312, STM4313, STM4316, STM4317, STM4318, STM4319, STM4320, STM4322, STM4323, STM4324, STM4325, STM4326, STM4327, STM4328, STM4332, STM4333, STM4334, STM4335, STM4337, STM4338, STM4339, STM4342, STM4343, STM4344, STM4345, STM4346, STM4337, STM6016, STM6019, STM6023, STM6028, STM6025, STM6027, STM6031, STM6017, STM6022 [1,4]	French Guiana	*M. pudica*	Mishra et al., 2012 [48]

Table 1. *Cont.*

P. phymatum Strains	Sources (Country)	Plant Host	Publication
SWF66029, SWF66286 [1,4]	China	*M. pudica*	Liu et al., 2011 [50]
SWF67297 [1,4]	China	*M. pudica*	Liu et al., 2012 [51]
MP20, MPJ1 [1]	India	*M. pudica*	Gehlot et al., 2013 [52]
CVRDII_2 [1,5]	Brazil	*Parapiptadenia pterosperma*	Bournaud et al., 2013 [53]
STM3714 [1,4]	Guinea	*M. pudica*	Melkonian et al., 2014 [7]
HBU52006, HBU52001, HBU35004, and 52 other strains [1,4]	China	*M. pudica*	Liu et al., 2020 [54]
HBU67642, HBU67643 [1,4]	China	*M. diplotricha*	Liu et al., 2020 [54]

* Contradictory information: it was reported to be isolated from *Machaerium lunatum*, although it was never proven to nodulate it; at present, *M. pudica* is considered the plant host. [1] Identified as being of the same genotype as *P. phymatum* STM815^T on the basis of 16S rRNA. [2] Additionally identified by repetitive extragenic palindromic (REP)-PCR fingerprinting. [3] Additionally identified by whole protein profile. [4] Additionally identified by PCR-RFLP. [5] Additionally identified by *recA* PCR amplification.

3. Geographical Distribution and Phylogeny of *Paraburkholderia* Species

Tracking the distribution of *Paraburkholderia* symbionts can be useful to determine the extent to which they can act as nodulating symbionts of legumes as well as to gain a better understanding of the evolution of alpha- and beta-rhizobia. *Paraburkholderia* species have been isolated globally. However, more specifically, with regard to symbiotic species, they have been isolated from nodules of native *Mimosa* species in South (Brazil, Uruguay, French Guiana, and Venezuela) and Central (Costa Rica and Panama) America [31,32,35,55,56], while in South Africa, they have been found mostly in association with papilionoid legumes [36,57–63]. Although a recent study has found the southern African indigenous mimosoid *Vachellia karroo* to be capable of forming nodules in association with *Paraburkholderia* strains [64], several of these strains are recognized, at present, as members of *P. tuberum sensu stricto* [65]. Additionally, *Paraburkholderia* have also been isolated from invasive legume nodules in China, Taiwan, India, Australia, and New Zealand [32,50,52,60,66,67]. However, the only confirmed centers of symbiotic diversification of native legume-nodulating *Paraburkholderia* species are in South and Central America (e.g., the Cerrado and Caatinga biomes in Brazil) and in the southern tip of South Africa (the CCR) [13,18,20,29,59,61–63,68–72]. *Paraburkholderia–Mimosa* symbioses exhibit some level of specificity; yet, competition studies between *Paraburkholderia*, *C. taiwanensis*, and *Rhizobium* species using different plant hosts and nitrogen-free conditions revealed that *Paraburkholderia* are the most dominant species, not due to the plant preference, but rather due to physical environmental factors [29,68]. One of the most important factors appears to be soil pH, as acidic soils are often associated with low nutrient availability, whereas neutral- and higher-pH soils are often linked to higher fertility. In low-pH Brazilian soils, *Paraburkholderia* are the predominant symbionts of the *Mimosa* species [69,73,74], as well as species in some other mimosoid genera, such as the Piptadenia group and *Calliandra* [48,75,76]. This contrasts with other centers of diversity of these legumes, such as Mexico, in which the native *Mimosa* and *Calliandra* species preferentially nodulate with *Rhizobium*/*Sinorhizobium* in the largely alkaline soils [76,77], and in India where the native *Mimosa* species (*M. hamata*, *M. himalyana*) nodulate with *Sinorhizobium* (*Ensifer*) and do not interact with the beta-rhizobial symbionts of the invasive *M. pudica* [52]. *Paraburkholderia* are similarly absent as symbionts of endemic *Mimosa* spp. in the high-pH soils of the metal-rich highland region of Minas in Uruguay; however, in this instance, they are nodulated with diverse species of *Cupriavidus* rather than with alpha-rhizobia [78]. Geology and altitude, particularly in relation to humidity and temperature, have also been shown to be ecological drivers determining the distribution of nodulating *Paraburkholderia* species in both of their major centers of diversity [29,58,61]. In fact, in several studies, it has been shown that the

diversity of *Paraburkholderia* species associated with *Mimosa* species in central Brazil and CCR papilionoid legumes in South Africa changes with elevation and is, therefore, associated with environmental conditions rather than the ability of *Paraburkholderia* symbionts to adapt to infertile acidic soils [29,57,59,61].

Once rhizobia are isolated and identified, phylogenetic analyses are routinely performed to categorize these strains into distinct lineages (i.e., "delineation"). The genes encoding the 16S rRNA subunit and housekeeping locus, *recA* (encoding recombinase A), are most commonly used for resolving species relationships among *Paraburkholderia* isolates, while the symbiotic potential of the strains is characterized by investigating the genes necessary for nodulation (especially the *nod* genes) and nitrogen fixation (the *nif* and *fix* genes) [48,58]. The *nodA* gene, whose product is essential for the synthesis of the backbone of the rhizobial signaling molecule—the nod factor (NF) [79,80]—shows the clearest evolutionary divergence among symbiotic *Paraburkholderia* strains [31,81]. The phylogenetic analysis of *nodA* gene sequences from South African *Paraburkholderia* strains/species together with those from South and Central America have repeatedly shown distinct origins [29,35,57,58], with non-South African sequences (including *P. phymatum* and *P. mimosarum*, for instance) grouping with other beta-rhizobial sequences (*Cupriavidus* and *Trinickia symbiotica*) [44,65], while South African species, such as *P. tuberum*, form part of a different lineage all together and show greater relatedness to sequences from the alpha-rhizobial genera *Methylobacterium*, *Bradyrhizobium*, and *Microvirga* [29,35,57,65]. At a more local scale, a recent detailed investigation of the evolutionary relationship of CCR rhizobia (including *nodA* sequences of both alpha- and beta-rhizobia from this region) found that the horizontal gene transfer (HGT) of the nodulation loci occurred between the genera *Mesorhizobium*, *Rhizobium*, and *Paraburkholderia* from this region and again recovered two distinct *Paraburkholderia* lineages, one based in the CCR and the other consisting of global strains [71]. Based upon species phylogenies, nodulating *Paraburkholderia* species have been delineated and two major groups are recognized [44,48,51,82]: the South American mimosoid-nodulating *Paraburkholderia,* which include *P. caribensis* [81], *P. diazotrophica* [83], *P. mimosarum* [34], *P. nodosa* [33], *P. phenoliruptrix* [84], *P. phymatum* [10,25,35], *P. piptadeniae* [85], *P. ribeironis* [85], *P. sabiae* [86], *P. atlantica* [87], *P. franconis* [87], *P. guartelaensis* [88], and *P. youngii* [89], and the papilionoid legume-nodulating *Paraburkholderia* from South Africa that include *P. dilworthii* [90], *P. dipogonis* [67], *P. kirstenboschensis* [91], *P. podalyriae* [65], *P. steynii* [92], *P. strydomiana* [92], *P. rhynchosiae* [93], *P. sprentiae* [94,95], and *P. tuberum* [10,25,36,65].

Several *Mimosa*-associated strains were originally tentatively placed within *P. tuberum* on the basis of 16S rRNA and *recA* phylogenies; these strains still separated according to host/geography on the basis of the symbiotic loci, which led to the definition of two symbiovars, sv. papilionideae and sv. mimosae [48]. A symbiovar is characterized by a specific subset of symbiotic loci that enables a set of strains (that could be members of multiple rhizobial species) to effectively interact with a specific host [96]. Recent investigations of these *Mimosa*-nodulating *P. tuberum* strains have, however, delineated these into the new species *P. youngii* and added several strains to the species *P. atlantica* [89], with the strains recognized, at present, as part of *P. tuberum sensu stricto*, all originating from South Africa only [65,89].

The presence of a deeply branched lineage of nodulating genes in *Beta-proteobacteria* suggests that beta-rhizobial nodulation genes have a long and independent evolutionary history [29,71]. In the case of the *nif* and *fix* genes, better congruence is found between their phylogenies and that of the 16S rRNA and housekeeping genes [18,29,68,81]. It is believed that the set of *nif* and *fix* genes in both mimosoid and papilionoid-nodulating *Paraburkholderia* have been acquired from diazotrophic *Burkholderia*, which they, in turn, might have received from alpha-rhizobia [18,58,68,81]. However, the gene encoding the transcriptional regulator NifA, appears to have a different origin, implying that multiple HGT events may have occurred [82]. *Paraburkholderia* rhizobia have evolved primarily due to the frequent lateral transfer of symbiosis genes within the Cape Fynbos biome, which

gives the South African species a polyphyletic origin [71]. In contrast, the monophyletic character of South American *Paraburkholderia* indicates that their symbiotic genes might have been acquired simultaneously and from a different origin than those acquired by South African species [97], and probably less recently. Different biogeographic patterns and legume subfamilies on the two once-connected continents may have aided the divergence of mimosoid and papilionoid-nodulating *Paraburkholderia*. The origin of Fynbos *Paraburkholderia* in South Africa and the origin of South American mimosoid-nodulating *Paraburkholderia* in Cerrado and Caatinga overlapped during the Eocene, about 30 million years ago [62,98,99]. *Paraburkholderia* populations in Asia and Oceania are closely related to South American strains and were most probably brought over with their invasive *Mimosa* species (*M. pudica*, *M. pigra*, and *M. diplotricha*) [32,51]. The exceptions are the isolates from the South African native legume, *Dipogon lignosus*, which is invasive in New Zealand and Australia; strains isolated from invasive *D. lignosus* have been placed within the species *P. dipogonis* [67] and are related to the South African papilionoid-nodulating *Paraburkholderia* species, *P. rhynchosiae* and *P. dilworthii* [67]. According to Bontemps et al., the nodulation genes of the South American *Paraburkholderia* symbionts were acquired around the time when legumes began to diversify 65–50 million years ago, implying that the evolution of symbiotic *Paraburkholderia* in their South American center has an old and stable genetic history [18,29,100].

4. High Competitiveness of *Paraburkholderia* in the Rhizosphere

Rhizobia persist for a long time in three separate soil environments: bulk soil, the rhizosphere, and the plant root. Rhizobial tolerance to the harsh surroundings and rivalry with other species for survival are examples of the numerous pressures that soil bacteria must endure in these environments [101,102]. In addition, as plants restrict the number of nodules developed in their roots by a system called autoregulation of nodulation (AON), compatible rhizobia need to compete to form these nodules. Hence, competitiveness is commonly observed as a feature directly influencing the strain's relative fitness [7]. As previously mentioned, beta-rhizobia have consistently outcompeted alpha-rhizobia in nodulating *Mimosa* species. This dominance, which is also influenced by the host species and geography, could possibly be explained by the inherent higher competitiveness of beta-rhizobia [7,68]. In this section, both abiotic and biotic challenges that rhizobia encounter in the rhizosphere prior to root colonization are described, as well as the strategies and traits that *Paraburkholderia* employ to overcome them.

4.1. Abiotic Factors and Stress Responses

Environmental stresses, such as temperature, salinity, drought, pH, or the presence of toxic compounds, have a significant impact on rhizobial survival. In situations where these stresses are encountered, bacteria rely on stress tolerance mechanisms and the production of specific enzymes to remove damaging components [103,104]. Stress tolerance strategies include the influence of physical–chemical soil properties, the regulation of cell envelope composition, solute accumulation, and biofilm and exopolysaccharide (EPS) production [103].

Temperature is one of the factors that can affect the survival of rhizobia in soil, the molecular communication between host and symbiont, as well as the development of the nodule [103]. The ideal growth temperature for symbiotic *Paraburkholderia* species is 28 °C, while some species, namely, *P. atlantica*, *P. franconis*, and *P. phymatum*, tolerate temperatures as high as 37 °C [87,105]. This trait already gives them a distinct advantage over most alpha-rhizobia, whose optimal temperature ranges from 25 °C to 30 °C [103]. It is well-known that alpha-rhizobia exposed to a high-temperature stress respond by activating their survival program, which involves the production of EPS, chaperones, and heat-shock proteins [103,106].

Drought and desiccation hinder the uptake of water, removing most of the water from the cells and causing osmotic stress [103]. Compared to non-symbiotic bacteria, rhizobia are

often more susceptible to salt and osmotic stress [107]. *Paraburkholderia* have, however, been shown in several studies to better tolerate this type of stress compared to alpha-rhizobia. In comparison to the highly stress-resistant alpha-rhizobium *Rhizobium tropici* CIAT899 [108], *P. phymatum* GR01, which was isolated from common bean in semi-arid soils in Morocco [49] (Table 1), not only survives better in free-living conditions at higher salt concentrations and in a hyperosmotic medium, but also exhibits higher nodule competitiveness under these conditions. The higher survival rate of the *Paraburkholderia* strain could be attributed to the accumulation of the disaccharide trehalose, the sugar alcohol mannitol, and the amino acid alanine, which are known as osmoprotectants [109]. The most well-known response to desiccation by species of *Paraburkholderia* is the production of EPS, which includes the production of cepacian (CEP), the major EPS produced by the *Burkholderiaceae* family. It is synthesized by both symbiotic species, such as *P. phymatum* and *P. caribensis*, as well as endophytic species, such as *P. phytofirmans* or *P. xenovorans* [110,111]. In *P. xenovorans* LB400^{T}, CEP has been related to desiccation tolerance [110,112]. Other examples include *Paraburkholderia* isolates from the South African legume *D. lignosus*, which presented greater tolerance to water stress induced by different concentrations of polyethylene glycol (PEG) than *Bradyrhizobium* or *Rhizobium* species [60].

Another abiotic factor that negatively impacts rhizobial persistence and survival in the rhizosphere is acidity [48,69,75,107,113]. Interestingly, *Paraburkholderia* and *Burkholderia* species are found enriched in acidic soils [48,69,75,114]. Organic acids secreted by plant roots, such as oxalate or citrate, contribute to the acidification of the environment and, at the same time, can be utilized by bacteria as carbon sources. Therefore, a link between their pH tolerance and their ability to grow using oxalate as a carbon source has been suggested [114–116].

Heavy metals are released into the environment with the use of pesticides, fertilizers, herbicides, insecticides, or fungicides, along with other human practices that can harm beneficial rhizobial communities [107]. Copper, nickel, cadmium, zinc, chromium, and lead are the most common contaminants [117]. Rhizobia tolerate these in a variety of ways, e.g., by producing EPS and biopolymers, but also by changing the properties of these compounds, thereby converting them into less toxic forms [103]. Carbonyl, carboxyl, and hydroxyl groups in the EPS matrix of *P. xenovorans* have been shown to scavenge and complex metals, such as iron and zinc [110]. Recently, Soares Neto et al., 2022, showed that *P. atlantica* is the dominant symbiont in nodules of several *Mimosa* species grown in heavy metal-contaminated soils, suggesting that symbiosis with *Paraburkholderia* might play a significant role in healing soils containing excesses of toxic metals [74].

4.2. Competition between Soil Bacteria for Survival and Root Nodulation

Bacterial diversity is lower in the rhizosphere than in bulk soil; however, the rhizospheric population is nonetheless more active due to competition between its constituents and the consumption of plant root exudates (REs) [118]. On a local scale, competition reduces biodiversity while increasing ecological stability. Soil bacteria have developed a variety of competitive mechanisms in order to prevent rival strains from colonizing a common niche. These strategies can be categorized into two types: the first is exploitative or passive competition, which involves rapid growth to consume the available resources resulting in metabolic alterations that profit the original strain and that can be exploited against competitors, while the second is interference or direct competition that involves direct antagonism to exclude competitors [119–122].

4.2.1. Exploitative or Passive Competition

Basic cellular functions, such as the assimilation of nutrients and energy sources, surface structure formation, signaling, and proliferation, can influence rhizobial nodulation competitiveness [123]. Bacteria with high metabolic adaptability are more likely to survive as they can better metabolize a wider range of nutrients and plant-secreted compounds [122]. REs provide a source of carbon and energy in the rhizosphere, and

bacteria able to assimilate these compounds can change the environment through a variety of metabolic processes [119,124]. Members of the *Burkholderiaceae* have been shown to be metabolically versatile, which allows them to persist in a wide range of environments and confers a competitive advantage [7,68,125]. Oxalotrophy, a trait found in several symbiotic *Paraburkholderia* species, such as *P. phymatum* STM815^T, *P. phenoliruptrix* LMG 22037^T, *P. caribensis* LMG 18531^T, and *P. tuberum* STM678^T, has been recognized as an example of metabolic diversity in the *Paraburkholderia* group. It contributes to the effective colonization of lupin and maize by *P. phytofirmans* LMG 22487^T, for instance, by allowing the bacterium to utilize oxalate, one of the main RE components in soil-grown plants that can be toxic to strains that cannot metabolize it [116]. The production of secondary metabolites that improve direct nutrient consumption also contributes to exploitative competition. An example is the production of siderophores, which has been shown in *Sinorhizobium meliloti* Rm5000 to inhibit competitor strains through the siderophore sequestering of environmental iron [121,126,127]. Previous transcriptome studies looking at the responses of two beta-rhizobia (*P. phymatum* STM815^T and *C. taiwanensis* LMG 19424^T) and one alpha-rhizobium (*Rhizobium mesoamericanum* STM3625) to *M. pudica* REs showed that only *P. phymatum* up-regulates the expression of a putative siderophore (Bphy_4034-4047), hinting at an important role of this siderophore in the first steps of the symbiotic interaction [128].

4.2.2. Interference or Active Competition

Interference competition, also referred to as active competition, employs antagonistic mechanisms to inhibit competitors [119]. It includes increasing access to space through enhancing motility, disrupting the competitiveness of other bacteria by inhibiting their quorum sensing (QS) systems, and eliminating competitors through the production of antibiotics or secreted toxins [120].

As demonstrated by the *pilA1* integral pilin subunit mutant strains in *S. meliloti* Sm1021 or the chemotaxis receptors *mcpB* and *mcpC* mutant in *Rhizobium leguminosarum* bv. *viciae* VF39SM, motility and chemotaxis can affect the nodulation efficiency in alpha-rhizobia and lead to a significant decrease in nodulation competitiveness [129,130]. Within the *Paraburkholderia* genus, it has been demonstrated that enhanced motility and EPS formation may be features that give *P. phymatum* STM815^T a competitive advantage in nodulation [7,68,125]. In this species, both flagella biosynthesis and chemotaxis have been shown to be positively controlled by the alternative RNA polymerase sigma factor RpoN or σ^{54}, which regulates nitrogen utilization systems under nitrogen-deplete conditions [131]. During inter-bacterial competition experiments between beta-rhizobial strains, being one of the fastest-growing strains may have contributed towards the higher competitiveness of *P. phymatum* STM815^T in nodulating common bean, siratro, and cowpea when compared to four other *Paraburkholderia* strains (*P. tuberum* STM678^T, *P. mimosarum* LMG 23256^T, *P. diazotrophica* LMG 26031^T, and *P. sabiae* LMG 24235^T) and one *Trinickia* strain (*T. symbiotica* LMG 26032^T) [125]. Bacterial cell motility is also regulated by QS, which is usually based on *N*-acylhomoserine lactone (AHL) molecules and contributes to balancing the density of rhizobial relocation into the nodule in relation to proliferation in the root zone [123,132]. The *P. phymatum* strains, STM815^T and GR01, possess the BraI/R QS system, which, although not essential for the nodulation of common bean or *M. pudica*, may provide a competitive advantage by utilizing AHLs produced by neighboring bacteria [133]. The BraI/R system regulates EPS production, which may provide a competitive advantage to *P. phymatum*. Indeed, the *P. phymatum* EPS CEP has been proposed to contribute to nodule competitiveness *in planta*, as a CEP mutant was impaired in attaching to common bean roots and formed less nodules compared to the wild-type and complemented strains [111].

The eradication of competitors can also occur through the production of antimicrobial compounds that can either be released into the environment (contact-independent systems) or directly injected into competitors by contact between cells (contact-dependent systems). Indeed, rhizobia can produce and secrete secondary metabolites, such as antimicrobial

peptides or antibiotics, which can inhibit growth or actively kill other bacteria [119]. While bacteriocins are peptidic toxins, antibiotics are secondary metabolites that are byproducts of metabolic pathways with a non-ribosomal origin [134]. Bacteriocins have a narrow spectrum of activity; therefore, they usually target closely related species without harming the producing cell [135–137]. In the *Paraburkholderia* genus, genes potentially coding for bacteriocins have been found in the newly identified nitrogen-fixing *P. lycopersici* Tne-862^T and in several endophytic species, such as *P. phytofirmans* and *P. xenovorans* [138,139]. Conversely, the *Burkholderiaceae* family is known to produce secondary metabolites with antimicrobial activity. The most highly investigated species and strains in this regard belong to *Burkholderia sensu stricto* (that includes the BCC and *B. pseudomallei* groups), which suggest that newly derived genera, such as *Paraburkholderia*, may have an undiscovered potential for novel antimicrobial production [140]. To date, bioinformatic analyses have found putative genes coding for antibiotics; however, only the production of sulfazecin (a monobactam antibiotic) in *Paraburkholderia acidophila* has been demonstrated [141,142]. Contact-dependent mechanisms usually employ secretion systems to deliver toxins by cell-to-cell contact into the target cell. One of the best-known contact-dependent mechanisms employs the type 5b or two-partner secretion system (T5SSb or TPSS), also known as the contact-dependent growth inhibition (CDI) system [143–146]. In *Burkholderia dolosa*, a new class of CDI was described that involves a gene cluster called *bcpAIOB*, where BcpA is an adhesin with the toxin encoded in the C-terminal domain, BcpI is the immunity protein that can form a tight complex with BcpA, BcpB is a beta-barrel outer-membrane protein that secretes the toxin, while the protein BcpO still has an unknown function [143,144,146–148]. This gene cluster is present in several *Paraburkholderia* species, including *P. xenovorans* and *P. rhizoxinica*, and in the symbiotic *P. phymatum* [144]; however, nothing is known about its role in symbiotic N_2-fixing rhizobia. The other secretion system that plays a role in inter- and intra-bacterial competition is the type 6 secretion system (T6SS). The T6SS possesses some elements that are similar to a phage tail spike. The genes encoding the core structure are annotated as "*tss*" (from type-six secretion) and code for the baseplate (TssAEFGK), the membrane complex (TssJLM), a tail tube (TssD or Hcp), a tail tip (TssI or VgrG), and a contractile sheath (TssBC). Similar to the phage spikes, T6SS penetrates the target cells delivering multiple effector proteins [149]. The T6SS is frequently present (in nearly 25%) in Gram-negative bacteria and was originally discovered in the alpha-rhizobial symbiont *R. leguminosarum* bv. *trifolii*, where it prevented the nodulation of the non-host legume pea [150–152]. Two complete T6SS clusters have been identified in the genome of the symbiotic *P. phymatum*, and both have been shown to be partially responsible for the high competitiveness of *P. phymatum* when nodulating cowpea roots in the presence of other beta-rhizobia [153]. The so-called T6SS-3 is more akin to that present in pathogenic *Burkholderia* species and can be activated at 37 °C [105]. The second T6SS cluster in *P. phymatum*, T6SS-b, is mainly present in soil bacteria and is positively controlled by the sigma factor RpoN under N-limiting conditions [105,131]. The expression of T6SS-b is induced by citrate (a metabolite found in common bean RE), temperatures of 20/28 °C, and is in contact with germinated common bean seeds, suggesting a possible function during early symbiosis [105].

5. Interaction with the Plant Host

A successful rhizobium–legume symbiosis requires the initiation of the interaction between free-living bacteria and a plant host before the rhizobia can nodulate the legume and perform BNF inside the root nodules. Through specific signaling molecules, compatible symbionts and host legumes detect and recognize each other. This interaction then triggers nodule organogenesis, the entry of the rhizobia into the plant, and the eventual accommodation of the rhizobia within the nodule, which require changes in gene expression and metabolism in both partners [154–158]. As beta-rhizobia have only been discovered relatively recently, there is still little information about the molecular mechanisms underlying beta-rhizobial symbiosis. The following section covers recent discoveries in this area,

unraveling the genetic network used by nitrogen-fixing *Paraburkholderia* to interact with a plant host.

5.1. "Opening the Gate": Rhizobium–Legume Recognition

Rhizobia have two different routes by which they infect plants. The less common route is crack entry, where bacteria occupy the intercellular spaces between epidermal and cortical cells [13,159]. The second route that is more frequently encountered involves the infection of root hairs and is mediated by rhizobial nodulation factors (NFs) [160]. The transcriptional regulator, NodD, a member of the LysR family of transcriptional activators, is activated by specific plant flavonoids and controls the expression of *nod* genes, the structural genes that code for the NFs [156,161]. On legume hosts, they function as specialized morphogenic signal molecules that induce early responses, such as root hair deformation, calcium-spiking, and the division of cortical cells, eventually resulting in effective nodules [79,161]. NFs are composed of a chitin oligomer backbone of two to six units of β-(1,4)-linked N-acetyl-D-glucosaminyl residues that are N-acetylated on their non-reducing ends [162]. The *nod* genes are divided into two sets: "common" genes (*nodA, nodB, nodC, nodI*, and *nodJ*) present in all rhizobial species that use the NF for host communication, and "species-specific" genes. The "common" genes synthesize the core structure of the NFs: *nodA* encodes an acyl transferase, *nodB* codes for a deacetylase, and *nodC* encodes a *N*-acetylglucosaminyl transferase (Figure 1, Table 2) [163]. Additionally, present in all rhizobia, the *nodIJ* genes encode an ABC transporter involved in the secretion of NFs. The set of "species-specific" genes are *noe*, *nol*, and additional *nod* genes. Their products are responsible for producing a wide range of modifications to the NF core by introducing different residues, for example, N-methyl by NodS, sulfate groups by NodH, or a O-carbamoyl by NodU (Figure 1, Table 2) [161,164,165]. As a result of these changes, rhizobia can increase the diversity of NFs, a trait that has been considered a sign of rhizobia adjusting to different host plants [166]. Alpha- and beta-rhizobia share the *nod* core genes; however, they differ in the presence of the species-specific genes. The beta-rhizobial *nod* genes are induced by RE, as has been shown for *P. phymatum* and *C. taiwanensis* with *M. pudica* REs [128]. In this study, *P. phymatum*, which is able to nodulate over 50 legume species, showed a higher induction of *nod* genes expression than *C. taiwanensis* and *R. mesoamericanum* [47,49,125,128]. The *Paraburkholderia* species nodulating mimosoids often have a single copy of each *nod* gene arranged in the operon *nodBCIJHASU* [82,167] (Figure 1). The *nodUSDABC* genes in papilionoid legume-nodulating *Paraburkholderia* species are separated from *nodIJ* by the insertion of *nif* genes and transposases, and some *nod* genes are duplicated, such as *nodB* and *nodC* [82,167] (Figure 1). They also seem to lack *nodH* and possess *nolO,* suggesting that their NFs are not sulfated, but have carbamoyl groups. Common to both *Paraburkholderia* groups is the presence of *nodSU,* which indicates that methyl and carbamoyl groups are potentially added to the NF core [82,167]. In addition, the gene *noeM* (coding for a fatty acid hydrolase) has been proposed as a nodulation gene specific for rhizobia nodulating *M. pudica*, as it is present in all strains nodulating *M. pudica* (*Ensifer/Sinorhizobium*, *Mesorhizobium*, *Neorhizobium*, and *Rhizobium* in alpha-rhizobia and *Paraburkholderia*, *Trinickia*, and *Cupriavidus* in beta-rhizobia) [168].

To colonize the root, rhizobia form microcolonies or biofilms on the roots. Within the curled root hairs, rhizobial cells migrate through infection threads (ITs) toward the nodule primordia, where the rhizobia are released and differentiate into N_2-fixing bacteroids [118,154,155,169–171]. The main components of the root biofilm are EPS [172,173]. These molecules bind to the host EPS protein receptor 3 (EPR3), thereby inactivating the defense signaling pathway [174,175]. In beta-rhizobia, the role of EPS in the induction of root hair curling and the formation of ITs has not yet been studied. Recently, in *P. phymatum* STM815^T, the *bceN* gene that encodes a protein with GDP-D-mannose 4,6-dehydratase enzyme activity was shown to be involved in the synthesis of the polysaccharide CEP, which has been shown to play a role in the first steps of establishing a symbiosis with common bean (see above) [111]. It differs from the typical EPS found in alpha-rhizobia

that is encoded by the *exo*/*exs* and *wge*/*wga* gene clusters, both of which are absent in the genomes of *Paraburkholderia* strains [111].

Table 2. Function of *nod* gene products in the biosynthesis of the Nod factors (NFs) produced by rhizobia, found in *Paraburkholderia* genomes from Figure 1.

Gene	Protein Encoded	Functions
nodA	Acyl transferase	Transfer of fatty acyl chain to the NFs' backbone
nodB	Deacetylase	Removal of acetyl moiety from nitrogen attached to monomer at non-reducing end
nodC	NAG transferase	Catenation of monomeric NAG
nodD	LysR family transcriptional regulator	Activation of *nod* genes transcription
nodH	Sulphotransferase	Transfers PAPS to reducing end of NFs
nodU, *nolO*	Carbamoyl transferase	Carbamoylation
nodI	NF export ATP-binding protein I	Secretion of NFs
nodJ	Transport permease protein NodJ	Secretion of NFs
nodQ	Subunits of ATP sulfurylase and kinase	Produces PAPS and activates sulphate compounds
nodS	Methyltransferase	Addition of methyl group
nodT	Outer-membrane lipoprotein	Interacting with NodI and NodJ

NAG, N-acetyl-glucosaminyl; PAPS, 3′-phosphoadenosine-5′-phosphosulphate.

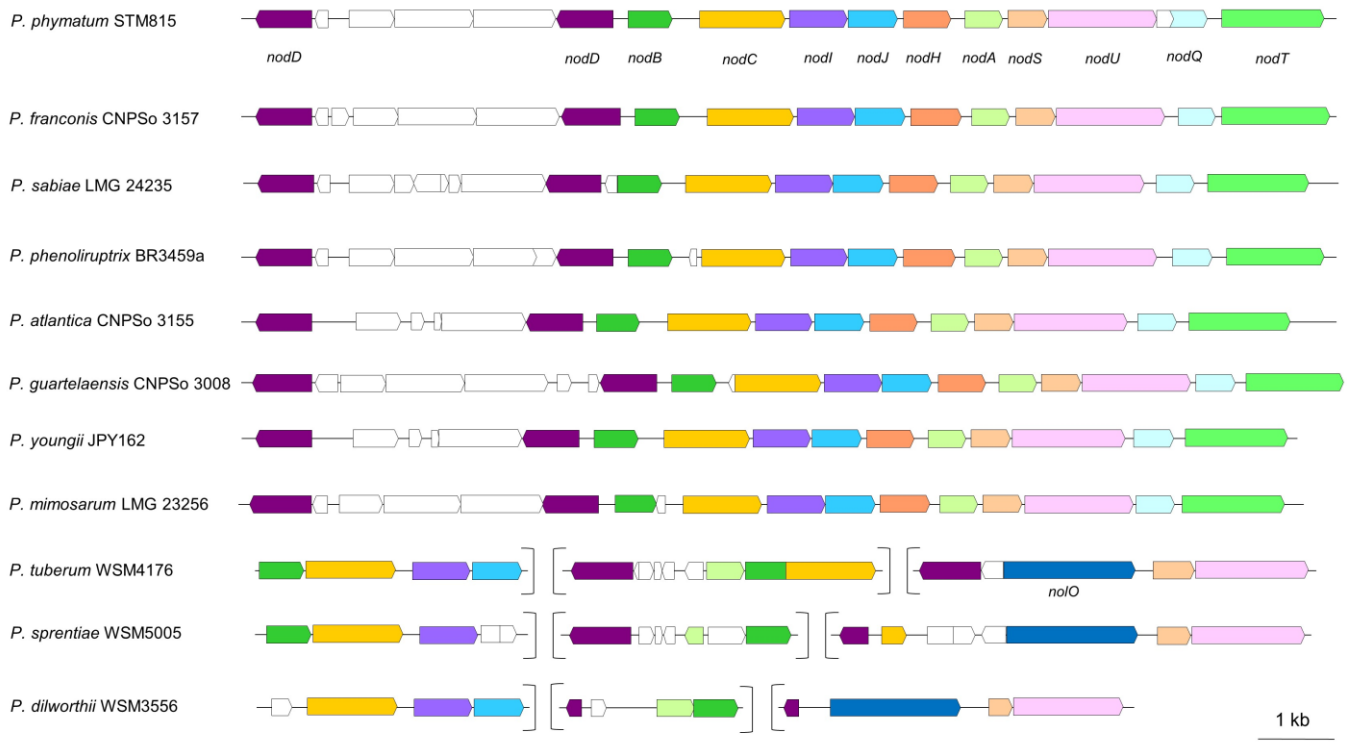

Figure 1. Organization of *nod* genes present in the genomes of mimosoid and papilionoid legume-nodulating *Paraburkholderia*-type strains. Homolog genes are similarly colored. White ORFs indicate genes coding for proteins with unknown functions. Figure created with data obtained from MicroScope Genome Browser version 3.16.0 and Synteny map function [176]. *P. phymatum* STM815 and *P. tuberum* WSM4176 genomes are used as query sequences.

5.2. The Steps towards Nodule Development

The recognition of NF-NF receptors (NFRs) (a transmembrane LysM-type serine/threonine receptor kinase) activates calcium oscillation in the plant cell nucleus, which activates gene expression that allows the initiation of the bacterial infection at the epidermis and promotes plant cell division in the cortex [155,177]. Nodule organogenesis requires

hormonal signaling, particularly auxin and cytokinin, which are important for plant growth, development, embryogenesis, and tropism [155,178–180].

Rhizobia can modulate the activity of these plant hormones either via production or degradation during the colonization process. Several phenomena related to the production of plant hormones by microorganisms have been reported within the alpha-rhizobia. Indole-3-acetic acid (IAA) is the form of auxin most studied [181]. Bacteria can synthesize IAA in a tryptophan-dependent or independent manner [182]. The beta-rhizobial symbiont *P. phymatum* STM815^T has recently been shown to produce IAA via the indole-acetamide (IAM) pathway [183]. The genes involved in this pathway (*iaaMH*) are up-regulated in the presence of *M. pudica* RE and common bean nodules compared to free-living conditions, suggesting a role of *iaaMH* in the early steps of symbiosis [128,184]. Rhizobia can also promote nodulation by decreasing ethylene levels in the plant root nodule with the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The ACC deaminase, encoded by the gene *acdS* in bacteria, catalyzes the degradation of ACC, an immediate precursor of ethylene, into ammonium and α-ketobutyrate [180]. In addition, rhizobitoxin (RTX) is a phytotoxin that acts as an inhibitor of the plant ACC synthase, involved in the biosynthesis of ethylene in host roots, leading to a reduction in ACC production and decreased plant ethylene levels. The dihydrorhizobiotoxin desaturase enzyme, encoded by the *rtxC* gene, is involved in the final step of RTX synthesis [185]. The *acdS* gene has been found in genomes of both alpha- and beta-rhizobia harboring *nodC*, being present in 100% of the *Paraburkholderia* and *Bradyrhizobium* genomes studied [186]. In contrast, *rtxC* was found less frequently in *Paraburkholderia* (33.3%) and *Bradyrhizobium* (45.1%) [186]. In *P. phymatum* STM815^T, *acdS* and the whole RTX coding operon were up-regulated in the presence of *M. pudica* RE [128]. Hence, possessing both mechanisms to reduce ethylene levels could potentiate the nodulation ability of a strain.

It would appear as if certain rhizobial enzymes involved in carbon metabolism play an important role during symbiosis. Mutants of *P. phymatum* for phosphoglycerate mutase (Bphy_0266), an enzyme important for glycolysis and gluconeogenesis, and fructose 1,6-biphosphatase (Bphy_0685), a regulatory enzyme in gluconeogenesis, are not able to induce ITs or nodules, although they still possess the ability to cause root hair deformation [187]. Some of the possible explanations for this observation are that either the accumulation of intermediates would interfere during nodulation, or that growth by gluconeogenesis is indispensable for *P. phymatum* during the early steps of nodulation [187].

5.3. "Achieving the Goal": Differentiation to BNF-Performing Bacteroids

Once rhizobia are released from the ITs inside the cortical cells, they grow and divide infecting the plant cells. Within the plant cell, bacterial cells are enclosed in a newly formed compartment surrounded by the plant-derived symbiosome membrane (also called the peribacteroid membrane, originating from the plasma membrane of the infected plant cell). The symbiosome membrane together with the enclosed bacteroids is referred to as the symbiosome [13,157,188,189]. In the nodule, bacteria differentiate into N_2-fixing bacteroids. At this stage of differentiation, the genes involved in N_2-fixation, the so-called *nif* genes, are found to be up-regulated. These genes are often clustered on symbiotic plasmids or in the chromosome as genomic islands [171]. The *nif* genes are involved in the synthesis of the nitrogenase enzyme that catalyzes, with a high energetic cost, the conversion of N_2 to biologically useful ammonia, following the reaction [157]:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

In most of the cases, BNF is conducted by molybdenum (Mo) nitrogenases; although alternative nitrogenases (vanadium or iron-only) exist in nature [190], they have never been found in rhizobia. The transcriptional regulator NifA activates the expression of the *nif* genes. NifA belongs to the enhancer binding protein (EBP) family and works together with the sigma factor RpoN. As proteins belonging to the EBP family, NifA has a central conserved AAA+ ATPase domain that interacts with RpoN, flanked by an amino-terminal

regulatory and a carboxy-terminal DNA-binding domains. The DNA-binding domain recognizes and binds to an upstream activator sequence (UAS) (TGT-N_{10}-ACA) roughly 100 bp upstream of the transcription start site (TSS). RpoN associates reversibly with the core RNA polymerase that recognizes promoter consensus sequences at −12 and −24 bp relative to the TSS and requires a DNA loop to interact with NifA [5,191,192]. *P. phymatum* mutants in *nifA* and *rpoN* are impaired in fixing N_2 [184]. During symbiosis with *P. vulgaris*, RpoN not only activates the expression of *nif* genes, but it also regulates the hydrogenase cluster, respiration-related genes (see below), and genes involved in ammonium transport and nitrogen metabolism [193]. In Bellés-Sancho et al., 2021, a combined metabolomics and dual RNA-sequencing (RNA-seq) approach on nodules was used to unravel the role of *P. phymatum* NifA during symbiosis with *P. vulgaris* [194]. In addition to the common response of nitrogen deprivation from non-fixing bacteroids, NifA was found to regulate other important symbiotic characteristics, such as stress response, transport of C_4-dicarboxylates, and auxin biosynthesis, all of which were validated by growing *P. phymatum* in vitro. By using dual RNA-seq and therefore looking at the transcript profile of both partners simultaneously, the *nifA* mutant was shown to also induce significant changes in common bean, such as an increase in brassinosteroids and the down-regulation of genes involved in AON. These results are in line with the previously reported higher number of nodules that clustered together in common beans inoculated by a *nifA* mutant [184,194]. In the follow-up study, the role of NifA as a repressor of bacterial auxin biosynthesis was demonstrated by mutant analysis and the construction of reporter constructs. The *P. phymatum NifA* represses the expression of the *iaaMH* genes in all stages of symbiosis. Moreover, in the absence of NifA, the overproduction of IAA and IAM induced the clustering phenotype and contributed to hypernodulation, suggesting a role of bacterial auxin in plant colonization. In *P. phymatum*, NifA also controls the *fixABCX* genes [194], which are present in both alpha- and beta-rhizobia and are involved in the transfer of electrons to nitrogenase. The gene products FixA and FixB are homologous to beta and alpha subunits of mammalian electron-transfer flavoproteins (ETFs), whereas FixC and FixX are homologous to ETF cognate acceptor ETF-quinone oxidase (ETF-QO), which usually transfers electrons to terminal electron acceptors [82,195].

Rhizobia are aerobic bacteria that require oxygen to respire; however, oxygen can permanently damage the nitrogenase enzyme if its active site becomes oxidized. N_2-fixation in nodules must, therefore, occur in a microaerophilic environment, and bacteria adopt several strategies to create this low-oxygen environment [196]. Under these circumstances, rhizobia have been shown to use a high-affinity cytochrome cbb_3 oxidase encoded by the *fixNOQP* and *fixGHIS* genes, which functions as a terminal oxidase at very low oxygen concentrations [9,167]. Interestingly, no genes coding for this cytochrome oxidase have been detected in beta-rhizobial genomes [82,184]. Instead, an RNA-seq analysis of *P. phymatum*–common bean nodules and from *P. phymatum* grown in microaerobic conditions showed a strong induction in the expression of genes coding for a potential cytochrome o ubiquinol oxidase (*cyo*). Indeed, their disruption led to impaired nitrogenase activity, consistent with the role of this oxidase in functional nodules [184]. The *nif* genes of *P. phymatum* are strongly up-regulated in microoxic conditions compared to aerobic conditions, but are not activated by nitrogen limitation [184]. Nonetheless, in free-living conditions, even between *Paraburkholderia* diazotrophic species there is a difference in N_2-fixing activity, as upon the presence of some fixed nitrogen in the growth media, symbiotic strains fix less than non-symbiotic species, such as *Burkholderia vietnamiensis* TVV75, *P. tropica*, and *P. unamae* [35]. These results suggest that there are differences in the nitrogen-fixation regulation between *Paraburkholderia* strains that are free-living and those that are symbiotic diazotrophs [18,35]. Yet, the ability of *Paraburkholderia* symbionts to fix nitrogen *ex planta* already marks a fundamental distinction from alpha-rhizobia. One of the possible reasons is the presence of the *nifV* gene in diazotrophic *Paraburkholderia*. Indeed, this gene is not present in the majority of alpha-rhizobial genomes, except for a few *Bradyrhizobium* species that possess the ability to fix N_2 *ex planta* [82,197,198]. The *nifV* gene encodes a

homocitrate synthase that catalyzes the condensation of acetyl-coenzyme A (acetyl-CoA) and α-ketoglutarate to form *R*-homocitrate that is needed for the FeMo-cofactor of the nitrogenase enzyme. When an alpha-rhizobium establishes a symbiosis with a host legume, the plant produces homocitrate using the FEN1 homocitrate synthase and supplies it to the bacteroid [9,197–199]. This dependency on the plant is a strategy used by plants to control the N_2-fixing activity of the bacteroids [171,200]. In *P. phymatum*, *nifV* is expressed in symbiosis with papilionoid and mimosoid legumes and also in free-living conditions and when homocitrate is abundant in all plant hosts inoculated with *P. phymatum*. However, NifV was not essential during symbiosis with its original host *M. pudica*, suggesting that the plant may assist in providing homocitrate. General transcriptome studies comparing *P. phymatum* symbiotic with free-living gene expression profiles have shown an over-representation of genes related to "inorganic ion transport" and "energy production and conversion" up-regulation during symbiosis with common bean [184]. Nonetheless, the opposite trend is observed in alpha-rhizobial bacteroids that down-regulate genes involved in these two functional categories [200]. Plants provide bacteroids with carbon and energy sources in the form of C_4-dicarboxylates, primarily malate and succinate. Malate is carried across the symbiosome membrane and is imported into the bacteroid by the dicarboxylic acid transporter, DctA, which is controlled by the DctB/C sensor-regulator system. The tricarboxylic (TCA) cycle is then used to metabolize malate, producing reduced electron carriers, such as NADH and $FADH_2$, required for ATP production during N_2-fixation [9,200,201]. Acetyl-CoA from the TCA cycle is metabolized to polyhydroxybutyrate (PHB) in low-oxygen conditions, where it is employed as carbon storage alongside glycogen and lipids, and to maintain redox balance [171,195]. In analogy to alpha-rhizobia, the PHB-associated protein and PHB synthesis genes are activated in *P. phymatum* during nitrogen shortage, whereas PHB depolymerase and genes that may be implicated in PHB stability are activated during symbiosis with *P. vulgaris* [184]. In alpha-rhizobia, the developmental program in bacteroids prevents rhizobia from assimilating ammonia fixed from the BNF, thereby giving all their reduced nitrogen to the plant [157]; however, it has not been proven in beta-rhizobial symbionts. Ammonia is transported through the symbiosome membrane from the bacteroids by simple diffusion, unspecific protein channels, channels for NH_3 or cations, or aquaporin-like channels [157]. Then, in plant cells, ammonia is assimilated as amino acids, mainly glutamine and asparagine, or ureides [9,157]. In alpha-rhizobia, additional pathways have been characterized and their importance in symbiosis has been proven. For instance, in the photosynthetic *Bradyrhizobium* sp. ORS278, the genes *ccbL1/cbbS1* encoding a ribulose 1,5 bis-phosphate carboxylase oxygenase (or RuBisCO) have been identified and their role in the carbon fixation pathway confirmed. Gourion and coworkers demonstrated that RuBisCO is necessary for an efficient symbiosis between *Bradyrhizobium* sp. ORS278 and *Aeschynomene indica* [202]. Interestingly, *P. phymatum* STM815^T also carries *ccbL1* (Bphy_6497, actually annotated as *rbcL*) on plasmid pBPHY01, and this gene is up-regulated during symbiosis compared to free-living conditions [184]. However, whether Bphy_6497 is important for a functional symbiosis is not known. The NiFe hydrogenase that recycles part of the energy used in the N_2-fixation process by oxidizing the H_2 released during BNF is up-regulated in common bean nodules induced by *P. phymatum* and has been shown to improve BNF [184,203]. An isocitrate lyase, involved in the glyoxylate shunt pathway, was also up-regulated in *P. phymatum* bacteroids [184]. Similar to alpha-rhizobial host legumes [204,205], specific metabolic requirements are needed depending on the plant hosts colonized by *P. phymatum*. Several host-specific intermediates belonging to distinct metabolic pathways accumulated in the *P. phymatum* symbioses with common bean, cowpea, siratro, and *M. pudica*; for example, intermediates from the biosynthesis of valine, leucine, and isoleucine were found in common bean nodules, metabolites involved in pyrimidine biosynthesis in cowpea nodules, three C_4-dicarboxylic acids (fumarate, malate, and tartaric acid) in siratro nodules, and lastly metabolites involved in flavonoid and isoflavonoid biosynthesis in *M. pudica* nodules [206].

6. Concluding Remarks

Since the identification of *Paraburkholderia* strains as N_2-fixing symbionts twenty-two years ago, the discovery of new rhizobial strains has not receded, as there is an ongoing international effort to isolate and characterize rhizobia from tropical soils and indigenous hosts, which might not have obvious commercial value. This practice stems not only from research groups attempting to understand the evolutionary path of rhizobia, but also from the industrial sector seeking to identify elite microorganisms capable of competing in the soil, surviving harsh environmental conditions, and fixing N_2, with the final aim of employing them as bioinoculants. Several studies have focused on achieving a better understanding of the molecular mechanisms involved in the interaction between host legumes and rhizobia, with a particular emphasis on the common and distinct mechanisms used by alpha- and beta-rhizobia. It is interesting to note that the *Paraburkholderia* beta-rhizobial strain with the most information available is *P. phymatum*, as it has been continuously used as the main model of beta-rhizobial interactions with plants. This strain is outstanding for its ability to outcompete rival strains for nodulation of several crops [7,68,125,153], to survive in harsh soil conditions [109], and for its exceptionally broad legume host range (over 50 legume species) [35,47,125], all qualities needed to compete with native soil rhizobia. Moreover, *P. phymatum* possesses mechanisms employed by plant growth-promoting rhizobia (PGPR) to promote plant growth, such as the production of auxin [183,194] and free-living N_2-fixation [183,184,194,206]; it also possesses the potential for ACC deaminase activity and iron uptake since putative genes related to both activities are present in its genome [128]. These additional features of the plant growth-promotion potential of *P. phymatum*, such as phosphate solubilization or siderophore production, could also support its application as a bioinoculant in agriculture. In this discovery process, functional genomic approaches have greatly contributed towards a better understanding of the molecular mechanisms underlying each step leading to the establishment of a successful beta-rhizobial symbiosis. However, even with this significant progress made at the genomic level, the importance of the identified genes remains to be characterized phenotypically. Creating mutant strains lacking or overexpressing these genes would be the preferred approach to demonstrate their role during symbiosis, or for the symbiotic condition being investigated. Since the ultimate goal is to apply these strains in the soil as agricultural inoculants, the following step would be to translate this rhizobial research to the field, where different environmental settings/stresses could be applied to discover to what extent each *Paraburkholderia* strain can contribute towards plant growth promotion. Indeed, in the case of *P. phymatum*, even if it is not used as a plant inoculant in the future, with a greater understanding of its competitiveness and N_2-fixing mechanisms, the genetic traits involved in these phenotypes could still be used to genetically modify and create synthetic inoculant strains aimed at improving crop yield.

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