The *Bradyrhizobium* sp. LmicA16 type VI secretion system is required for efficient nodulation of *Lupinus* spp.

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6 In memoriam

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Abstract

Many bacteria of the genus *Bradyrhizobium* are capable of inducing nodules in legumes. In this work, the importance of a type VI secretion system (T6SS) in a symbiotic strain of the genus *Bradyrhizobium* is described. T6SS of *Bradyrhizobium* sp. LmicA16 (A16) is necessary for efficient nodulation with *Lupinus micranthus* and *L. angustifolius*. A mutant in the gene *vgrG,* coding for a component of the T6SS nanostructure, induced less nodules and smaller plants than the wild type strain (wt) and was less competitive when co-inoculated with the wt strain. A16 T6SS genes are organized in a 26 kb DNA region in two divergent gene clusters of nine genes each. One of these genes codes for a protein (Tsb1) of unknown function but containing a methyltransferase domain. A *tsb1* mutant showed an intermediate symbiotic phenotype regarding *vgrG* mutant and higher mucoidy and motility than the wt strain in free living conditions. T6SS promoter fusions to the *lacZ* reporter indicate expression in nodules but not in free living cells grown in different media and conditions. The analysis of nodule structure revealed that the level of nodule colonization was significantly reduced in the mutants with respect to the wt strain.

Keywords Type VI secretion; *Rhizobium*–legume symbiosis; *Bradyrhizobium*; *Lupinus*; effector; Methyltransferase;

Introduction

The so-called bacterial type VI secretion system (T6SS) is proving to be of great importance in adaptive activities of bacteria such as competitiveness and interaction with eukaryotes [1,2]. The T6SS is a nanosyringe similar to inverted phage tails generally made up of 13 conserved components called TssA to TssM and able to secrete proteins (effectors) to target cells or to the extracellular milieu [3,4]. It was first described in two human pathogens, *Vibrio cholerae* and *Pseudomonas aeruginosa* [5,6] although some of what are now known as *tss* genes were previously ascribed to protein secretion in a symbiotic *Rhizobium leguminosarum* strain [7] and in the fish pathogen *Edwardsiella tarda* [8]. Three main elements form a T6SS: a cytoplasmic baseplate (TssEFGK), a trans-membrane complex formed by TssJLM and a tail. The tail contains an internal tube of hexameric Hcp (also named TssD) rings topped with a spike structure formed by a VgrG (TssI) trimer base and a conical tip PAAR monomer [9] and is wrapped by a contractile sheath made of TssB-C protomers. TssA is required to assemble the Hcp tube and the TssB-C sheath [10]. Components of T6SS are recycled by the ATPase TssH (ClpV) after tail contraction [4]. In addition to Tss core components, T6SS gene clusters encode effectors and accessory proteins refereed as Tag (Type six associated gene) required for assembly of the T6SS subunits and regulation of their expression [4,11]. Most of the effectors described so far are enzymes with antibacterial activity and can degrade the cell wall, DNA or RNA, the cytoplasmic membrane, etc [1]. Often, antibacterial genes are adjacent to cognate immunity ones preventing self-toxicity or toxicity from other bacteria. These genes are known as effector-immunity pairs (E/I pairs) [12] . Other effectors target eukaryots, or act in stress responses or in the acquisition of metals or nutrients from the extracellular milieu and do not require cognate immunity proteins [2,13].

T6SSs are present in ~25% of Gram-negative bacteria mainly in the Proteobacteria and Bacteroidetes phyla [3,14], among them are plant-associated bacteria including legume symbionts known as rhizobia [15,16]. However, there are very few studies about the role of rhizobial T6SSs in symbiosis with legumes and in the rhizosphere. It can be highlighted the aforementioned work by Bladergroen et al in 2003 [7], which showed that the presence of a T6SS impairs the symbiosis with peas of a *Rhizobium leguminosarum* strain. The main aim of this work was to study the function of the T6SS of a *Bradyrhizobium* strain that nodulates *Lupinus micranthus* and *L. angustifolius*. Lupines belong to the legume tribe *Genisteae* and are capable of establishing symbioses mainly with *Bradyhizobium* strains [17]. *Bradyrhizobium* sp. LmicA16 (A16) was isolated from *L. micranthus* in Northern Algeria. A16 was able to induce effective nodules with its original host and also with *L. angustifolius* [18]. This led us to obtain a draft of its genome in which a T6SS was identified. In this work it has been shown that the A16 T6SS is required for efficient nodulation with *L. micranthus* and *L. angustifolius*. It has also been determined that the T6SS is expressed in symbiosis and that one of the potential effector (Tsb1) encoded in the T6SS cluster, presenting a methyltransferase domain, could have a positive role in symbiosis.

Materials and methods

Strains and culture conditions used in this work

Strains are listed in Table S1. *Escherichia coli* was grown in Luria-Bertani (LB) broth at 37º C [19]. *Rhizobium* strains were cultured at 28ºC in Yeast Mannitol Broth (YMB) [20] or Tryptone Yeast (TY) [21], and selection of transconjugants was carried out in *Rhizobium* minimal broth (Rmin) [21]. Antibiotic concentrations were (μg x mL^{-1}): 100 Amp (ampicillin), 50 Km (kanamycin), 50 Spc (spectinomycin), 5 Tc (tetracycline), 20 (nalidixic acid), and 20 (cefotaxime).

Construction of vgrG and tsb1 mutants

The *vgrG* mutant was done by single recombination with plasmid pK18*mobsacB* (Table S1) harbouring an internal DNA sequence of *vgrG* gene (479 bp). The internal region was amplified by PCR (Table S2), cloned first into pCR2.1®-TOPO® and then in pK18*mobSacB* after digestion with *Eco*RI. *E. coli* S17.1 cells containing pK18mob-*vgrG* were used to conjugate the plasmid to A16.
The *tsb1* mutant was obtained also by single recombination with plasmid pVO-npt2-cefo-npt2-GFP containing the central region of the gene *tsb*2 (388 bp) (pVO-*tsb1*) (Tables S1 and S2). This region was amplified by PCR, then digested with *Xho*I/*Xba*I and cloned in the plasmid open with *SalI/XbaI*. Conjugation was made as before.

The mutants were selected and purified in Rmin supplemented with antibiotics and checked by PCR.

Complementation of the vgrG mutant

In order to complement the *vgrG* mutant, a DNA fragment of 2,445 bp containing *vgrG* and its promoter (Fig. 1) was amplified by PCR (Table S2). The amplicon was cloned in the plasmid pMP220 and conjugated to A16 *vgrG*-deficient strain using S17.1 (Table S1).

Plant assays and nitrogen fixation activity

Lupinus micranthus seeds were surface-sterilized in 96% sulfuric acid for 2 h, washed 10 times with sterile distilled water and kept submerged one day and then placed on water-agar (1%) plates at 20ºC in the dark to germinate. The seeds of *L. angustifolius* were sterilised by immersion in 96° ethanol for 1 min, in bleach (12%) for 5 min and washed 10 times. Seedlings were transferred into sterilized Leonard jars containing vermiculite and Jensen's solution [20] and covered with a 1 cm thick layer of sterile gravel. Each seedling was inoculated with 1 mL of a rhizobial suspension (10^8–10^9cells mL^{-1}). Plants were grown at 23–25ºC in a greenhouse (16/8 h day/night) for 5 weeks. At least three different replicates with four plants per replicate were used. Nitrogen fixation activity of nodules estimated by the acetylene reduction test with a Shimadzu GC-8 apparatus have been previously described [22].

A16 T6SS gene expression

In order to analyse the expression of the T6SS genes of A16, a 690 bp region (P6) comprising 160 bp of the *tssA* gene,175 bp of *vgrG* and 355 bp of the intergenic region between both genes which likely includes the promoter region for the genes, was amplified with primers shown in Table S2. P6 was fused in front of the promoter *lacZ* gene of plasmid pMP220 (Table S1). Plasmids containing fusions in the two possible orientations were named P6TssA and P6VgrG, depending on which gene they are oriented towards. P6 derivatives were conjugated to A16 strain. The orientation of the promoter region with respect to the *lacZ* gene was determined by PCR (Table S2).
P6TssA and P6VgrG expression was studied under different free-living conditions considering different pHs (5.5, 7, 8.5), culture medium (Rmin, YMB, TY), temperature (20°C, 28°), presence of root exudates and in bacteroids.
For analysis under free-living conditions, a preinoculum was grown for 5 days at 28°C. Then, ODs were adjusted to 0.6 and cultures grew for 24 hours. After that, *β-galactosidase* activity (OD_{540} x 1000 x t (min) / OD_{540} x vol (mL)) was measured following the protocol described by Miller (1972) [23].

Preparation of root exudates and growth condition

Germinated seeds of *Lupinus angustifolius, L. micranthus*, *Phaseolus vulgaris*, *Leucaena leucocephala*, *Glycine max*, and *Triticum aestivum* were placed on a 0.8 mm diameter hollow cylinder so the roots were in contact with Jensen´s solution. After four days of submergence, the solution was collected and filtered (0.2 µm). A16 grew in YMB until OD_{600}=0.6, and then 0.7 mL of the culture were mixed with 0.3 mL Jensen´s solution containing the different root exudates. Sterilized Jensen´s solution was used as negative control. Cultures plus exudates were incubated 3h at 28ºC and then cells were collected by centrifugation for analysis.

Bacteroids extraction

Extraction of bacteroids was done following the protocol of Ruiz-Argüeso 1978 [22] after five weeks post-inoculation with A16 (P6TssA), A16 (P6VgrG) and A16 (pMP220).

Rhizosphere competitive colonization assays

Tests on the competitive colonisation of the rhizosphere between the wt A16 strain and *vgrG* mutant was determined by inoculating *Lupinus angustifolius* with different ratio 1:10; 1:1; 10:1. In order to differentiate the strains, A16 expressed GFP constitutively by harbouring vector pHC60 (Table S1). After 5 weeks, the nodules were counted differently through fluorescence visualisation with NightOWL II LB 983 In Vivo

Imaging System that allowed differentiation of both strains. In addition, cells from nodules were picked with a sterile loop and streaked on YMB plates to confirm growth with the appropriate antibiotics (tetracyclin for A16 and kanamycin for the *vgrG* mutant).

Colony morphology

In order to compare the appearance of the T6SS mutants the strains were grown in the TY medium up to OD_{600}= 0.6. Then 10 µL of each culture was deposited as a drop in the center of TY and YMB plates. Growth was visualized five days after inoculation. This assay was repeated 3 times with 3 replicates. Viable cells from cultures were counted in Petri dishes and all strains have similar cfu/OD_{600}.

Motility assay

The motility assays is based on the protocol of Liu et al (2012) [24]. A cell suspension (5 µl) in TY medium at OD_{600} = 0.6 was added to the centre of a TY 0.7% agar plate, creating an initial droplet of 5 mm diameter. After 15 days of incubation, the diameter of the colonies was measured. This assay was repeated 2 times independently with 5 replicates.

Bioinformatics analyses and accession numbers

A16 T6SS was identified from a genomic sequence draft obtained in our group using Illumina HiSeq 2000, 500 bp paired-end libraries, 100 bp reads and 7 million reads. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 7. The NCBI submission number of the A16 T6SS is 2474916.

Confocal laser scanning microscopy and image analysis

Nodules were washed in PBS and 200 µm sections were obtained with a Vibratome 1000 Plus. Microscopy was performed with a laser scanning microscope (Leica SP8). Nodules were observed by monitoring the GFP fluorescence expressed from pHC60 and analyzed with ImageJ.

Results

T6SS gene cluster organization of Bradyrhizobium sp. LmicA16

A T6SS was identified in the A16 genome. Twelve of the thirteen *tss* structural genes (*tssA-M*), generally conserved in T6SS, were distributed in two divergent clusters. No *tssJ* gene was identified (Fig. 1). The T6SS region also comprises four accessory genes (*tagE, pppA, tagF* and *tagH*) presumably involved in regulation as shown for homologous proteins in other bacteria where the systems are post-translationally regulated, positively by *tagE* (Ser-Thr kinase) and negatively by the cognate phosphatase PppA [25]. In A16, PppA and TagF are two distinct proteins while in *Rhizobium etli* Mim1 they are two domains of the same protein (TagF) (Fig. 1) [26]. In addition to *tag* accessory genes, two other genes, *tsb1* and *tsb2*, encode proteins of unknown function which could be T6SS-dependent effectors (Fig. 1 and Table 1). It is noteworthy that these genes are not accompanied by other of unknown function which could indicate the presence of a possible E/I pair. Whereas no functional domain has been identified in Tsb2, Tsb1 has a methyltransferase domain (Table 1) identified in silico at Phyre2 web [27]. The analysis identified related structures (100 % confidence) of two protein-lysine methyltransferases (PKMT1 and PKMT2) from *Rickettsia typhi* [28], with which Tsb1 shares 28 % of sequence identity (coverage 95%).

A comparison of T6SS from various rhizobia are presented in Fig. 1 and Table 1. The chosen rhizobia were *Bradyhizobium diazoefficiens* USDA110, the first sequenced strain of this genus [29] and *Azorhizobium caulinodans* ORS571 and *Rhizobium etli* Mim1 for being strains where the role of T6SS in the symbiosis has been studied [26,30]. It was observed that genes *tss* and *tag* are present in all strains, but differently organized. T6SS organization and protein similarity of the two *Bradyrhizobium* are highly conserved. Genes *tsb1* and *tsb2* of unknown function are not conserved (Table 1).

Bernal et al described in 2018 [16] five T6SS phylogenetic groups based on TssB protein. TssB of A16 belongs to group 3 as TssB from other *Bradryrhizobium* strains (Fig. 2a). Tsb1 homologous were found mostly associated to T6SS of *Bradyrhizobium*, *Microvirga* and *Bosea* strains and, more distantly from *Rickettsia* spp.

(Fig. 2b). Tsb2 is phylogenetically related to T6SS-related proteins from *Bradyrhizobium, Bosea* and *Mesorhizobium* (Fig. 2c).

Expression of A16 T6SS

The DNA region between *vgrG* and *tssA* (P6), likely corresponding to the promoter region for the two divergent clusters in T6SS of A16 (Fig. 1) was used to determine the expression of the T6SS A16 in different conditions. P6 was fused in the two possible orientations to the *lacZ* reporter gene of pMP220 vector generating P6TssA and P6VgrG (Table S1). Their expression in free-living conditions (See Material and Methods) was not detected (data not shown). In bacteroids extracted from *L. angustifolius* nodules, weak activities (Miller units) of the P6TssA (280±28) and P6VgrG promoters (270±14) were observed, pMP220 (6±0.4). qPCR analysis was performed and expression of *hcp, vgrG, tsb1* and *tsb2* from *L. angustifolius* bacteroids was negligible compared to *rpoD* gene (data not shown).

Relevance of A16 T6SS in symbiosis

In order to evaluate the relevance of the T6SS of A16 in symbiosis, a mutant deficient in *vgrG* was generated. VgrG protein is essential for functional T6SS [31]. The growth of *vgrG* mutant in YMB and TY media showed no difference regarding the wt strain. In order to assess the potential effect of T6SS on the symbiosis, *L. micranthus* and *L. angustifolius* plants were inoculated with wt and *vgrG* strains and their symbiotic phenotype were examined. In contrast to the wt strain, no advantage on plant growth could be observed with the *vgrG* mutant, i.e. the plants had an aerial dry weight similar to that of the non-inoculated control (Figs. 3 and 4). In addition, the nodules from the mutant were 45-55 % bigger than those induced by the wt strain, the number and weight of nodules per plant were significantly lower in the *vgrG* mutant compared to the wt strain (Figs 3c and 4d).

Analysis of nitrogen fixation was carried out by the acetylene reduction assay (ARA). This analysis revealed that ARA values were significantly lower in the nodules induced by the vgrG mutant strain in *L. angustifolius* (Figs. 3 and 4). In Fig. 4c the colonization of the nodules by the different strains is shown and it can be seen that the colonized area is clearly smaller in the nodules induced by *vgrG* mutant. This is consistent with the nitrogen fixation values obtained since they are referred to the fresh weight of the nodules and in the mutant nodules, there are clearly fewer bacteroids capable of fixing nitrogen. In order to complement the *vgrG* mutant, a DNA fragment containing the gene *vgrG* and the upstream promoter region designed as P6 were cloned in pMP220 (pvgrG) and conjugated into the *vgrG* mutant. The complemented strain approximately reproduced the nodulation and dry weight values of the wt strain (Figs. 3 and 4).

Additionally, the symbiotic effect of the mutant on *tsb1* was studied (Figs. 3 and 4). The *tsb1* mutant behaved with an intermediate phenotype with respect to the wt and *vgrG* mutant, ie, the values regarding the number and weight of nodules and nodule colonization were lower than those of the wt strain but higher than those of the *vgrG* mutant. It should be noted that the tsb1 mutant presented a more mucoid appearance than the wt strain and the *vgrG* mutant in TY medium (Fig. 5). Studies on T6SS from other bacteria have shown crosstalk between this system and mucoidy and also to motility [32]. A motility analysis was carried out in TY medium with 0.7 % agar and a greater motility could be observed for tsb1 mutant with regarding the wt strain and the *vgrG* mutant (Fig. 5).

T6SSs have a wide variety of functions among which is to obtain a fitness advantage against possible bacterial competitors [1.] Therefore, a competitiveness study in symbiosis with *L. angustifolius* was carried out using wt and *vgrG* mutant inoculating at different ratios. Values of nodule occupancy by the two strains showed a lower competitiveness of the *vgrG* mutant, indicating that the T6SS would be important for this activity. Thus, when both strains were inoculated in the same proportion, the wt strain occupied 80% of the nodules and when 10 times more mutant cells were inoculated, the proportion of wt nodules was double that of the nodules occupied by the mutant (Fig. 6).

All these results indicate that T6SS is important for the A16 symbioses.

Discussion

Rhizobia are key partners for a sustainable agriculture thanks to their ability to fix nitrogen in symbiosis with legumes, which favors a moderate use of nitrogen fertilizers mitigating the negative effects of their excessive utilization [33]. The efficiency of symbiosis varies greatly depending upon various factors such as legume host,

soil, environment, and rhizobial strains. Several characteristics of rhizobia important in this interaction have been described such as Nod factors, extracellular polysaccharides and secretion systems [34]. There are numerous examples where rhizobial type III or IV secretion systems (T3SS/T4SS) have been found to have neutral, positive or negative effects on symbiosis depending on the species or accession of the host [35]. The role of rhizobial T6SS has been studied only in a few strains although the increasing number of available genomes makes it possible to identify T6SSs in many rhizobia [3,36]. The first report in 2003 indicated that T6SS genes from *R. leguminosarum* RBL5523 impaired nodulation with peas [7]. In two studies, it was shown that the presence of T6SS had no effect on nodulation but rather did on competitiveness, the cases of *Paraburkholderia phymatum/ Vigna unguiculata* [37] and *Azorhizobium caulinodans/Sesbania rostrata* [30]. A positive effect of T6SS on symbiosis was recently shown for *Rhizobium etli/Phaseolus vulgaris* [26].

In this work a positive effect of T6SS on the *Bradyrhizobium* sp. */Lupinus* symbiosis is also demonstrated. The *vgrG* mutant strain, lacking the essential structural element VgrG, induced less nodules and the plant aerial dry weight was similar to that of the non-inoculated plants (Figs. 3 and 4). These results are similar to those obtained with other structural mutants in T6SS of *R. etli*, an effective endosymbiont of beans [26]. It is to note that when comparing the T6SS gene clusters of *R. etli* and that of the A16 (Fig. 1) it can be observed that the structural genes are conserved (although the corresponding percentage of amino acid identity is below 50%, Table 1), but the genes of unknown function, among which the effectors would be found, are different in the two strains. It is unknown how the beneficial effect of these T6SS occurs and whether the mechanism would be similar. The presence of possible T6SS-dependent effectors encoded outside the studied clusters elsewhere in the genomes is also unknown.

So far no T6SS effector targeting legume host cells has been described but it is tempting to speculate that the beneficial role of T6SS in symbiosis is also related to an attenuation of the defense response of the compatible host plant as described by T3SS-dependent rhizobial effectors as effector-triggered susceptibility [38]. As examples, the effector Bel2-5 from *Bradyrhizobium elkanii* USDA61 is involved in nodule organogenesis and repression of defense genes that impair nodulation in some host genotypes [39], NopC effector from *Sinorhizobium fredii* HH103 has a positive effect on *Glycine max* and *Vigna unguiculata* nodulation [40], NopAB and NopT from *Bradyrhizobium vignae* ORS3257 are key determinant for nodulation of *Vigna* spp. [41] and the effector NopM from *S. fredii* NGR234 favours nodulation by reducing plant reactive oxygen species (ROS) during infection [42]. By opposite, negative effects of T3SS effectors in symbiosis can occur if effector-triggered immunity (ETI) of the plant is induced, stopping rhizobial infection [43,44].

Lupinus angustifolius nodule infected cells by the A16 strain and by the *vgrG* and *tsb1* mutants are shown in Fig. 4c. It can be seen that the infected surface by the wt strain is much larger than that of the *vgrG* strain, while the surface area of *tsb1* is intermediate. This correlates with the higher nitrogen fixation activity and higher plant size produced with the inoculation of the wt strain compared to *vgrG* mutant. The *tsb1* mutant showed an in-between phenotype. The fact that the *vgrG* mutant has fewer infected cells could be due to the fact that some T6SS-dependent effectors favor infection in a similar way to some T3SS-dependent effectors. In this regard, the effect of the *tsb1* mutation suggests that the Tsb1 protein could be a possible T6SS effector having a positive effect on symbiosis. Tsb1 is a protein of unknown function with a methyltransferase domain and whole protein is structurally similar to rickettsial PKMT1 and PKMT2 [28] (Fig. 2b). PKMTs catalyze methylation of the outer membrane protein OmpB at multiple sites. OmpB is present in all rickettsial species mediating host cell adhesion and invasion, and is also an important antigen. OmpBs from virulent strains have multiple trimethylated lysine residues, whereas the avirulent strains hold mainly monomethyllysine. PKMT1 catalyzes mainly monomethylation and PKMT2 trimethylation and their primary substrate is OmpB, methylating multiple lysyl residues with broad sequence specificity [28]. Recently Engstrom et al (2020) [45] showed that methylation of outer membrane proteins prevents host ubiquitylation and autophagy. This suggests that Tsb1 may methylate some outer membrane proteins favouring interaction with its host legume. However, this is highly speculative because the function of class I methyltransferases which contain a Rossmann fold for binding S-Adenosyl methionine (SAM), and to which PMKT and Tsb1 structurally belong, have very diverse substrate specificities (small molecules, lipids, nucleic acids, etc.) and different target atoms for methylation (nitrogen, oxygen, carbon, sulfur, etc [46]. It should be noted that methyltransferase motifs have been found in other proteins within T6SS clusters of other bacteria although they are structurally diverse and different from Tsb1. Some examples are PF00487 from *Rhizobium etli* Mim1 [26]; Azc_2606 and Azc_2704; from *A. caulinodans* [30] and Tfe7 homologous to the ribosomal RNA large subunit methyltransferase D required for the fully methylation of 23S ribosomal RNA [47]. Interestingly, no gene encoding an immunity pair was found linked to these genes, suggesting a distinct activity of the antibacterial effectors as is the case of antifungal

effectors described in the opportunistic pathogen *Serratia marcescens* [48]. On the other hand, the *tsb1* mutant has a more mucoid morphological appearance than the parental strain or the *vgrG* mutant and also higher motility. Although relationships between T6SS, motility and biofilm production have been described [32] the relationship of Tsb1 to these processes requires further investigation.

The T6SS region in A16 is probably post-translationally regulated by the Ser-Thr kinase *tagE* (positively) and negatively by the cognate phosphatase PppA [25]. In A16 PppA and TagF are two distinct proteins while in *Agrobacterium tumefaciens* and in *Rhizobium etli* they are two domains of the same protein (TagF). TagF and TagH are encoded in A16 T6SS cluster, the interaction of these two proteins has been demonstrated critical for T6SS repression in *A. tumefaciens* and *Pseudomonas aeruginosa* [25,49]. Bacteria deploy TagE-dependent activation and TagF-mediated repression mechanisms to control T6SS but the environmental signals to activate the system are not identified. In this work we have found low expression in nodules and no free-living activity under the conditions tested. This suggests an early role in symbiosis. The role of T6SS in free-living conditions is still unknown but it would be involved in interbacterial competition, as has been demonstrated in A16 strain and in those already mentioned of *A. caulinodans* [30] and *P. phymatum* [37]. The capacity of T6SS from phytobacteria to outcompete other bacteria has also been seen in *Pseudomonas* spp*., Pantoea ananatis, A. tumefaciens* etc [15,16].

To our knowledge, this is the first time that a T6SS of a symbiont of the genus *Bradyrhizobium* has been shown to be necessary for effective symbiosis with legumes. Although possible functions have been suggested for tsb1 gene, future work is needed to identify and characterize T6SS-dependent effectors and to understand the specific role they can play in symbiosis and/or in the rhizosphere. This information can be of great use in fields such as ecology, agriculture, and medicine.

Supplementary information

Tables S1 and S2

Author Contributions:

LR, FB and LT designed the study. The experiments are performed by LT. LT, BFSS and LR analysed the data. LT an LR wrote the initial draft and all authors contributed to manuscript revision and approved it to be published.

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Table 1. Core and accessory gene754sof A16 T6SS and putative functions. Comparison to*R. etli*Mim1,*B.diazoefficiens* USDA110 and to *A. caulinodans*ORS_571

***Bradyrhizobium* sp. LmicA16**		**Strains**	***B. diazoefficiens* USDA110**			***Rhizobium etli* Mim1**			***Azorhizobium caulinodans* ORS571**		
T6SS genes (aa)[1]		**Function or characteristic**	**ORF(aa)(%)**[2]			**ORF (aa)(%)**[2]			**ORF (aa) (%)**[2]		
tagE	(602)	Ser/Thr protein kinase	*tagE*	(602)	(86)	*tagE*	(303)	(31)			
pppA	(258)	Ser/Thr phosphatase type 2	*blr3603*	(259)	(85)	tagF	(455 Ct)	(24)			
tagF	(233)	DUF2094	*tagF*	(234)	(79)	*tagF*	(455 Nt)	(29)			
tssM	(1182)	Integral membrane	*tssM*	(1179)	(92)	*tssM*	(1158)	(31)	*tssM*	(1178)	(28)
tssL	(508)	Integral membrane	*tssL*	(509)	(89)	*tssL*	(512)	(34)	*tssL*	(464)	(44)
tssK	(445)	Baseplate complex	*tssK*	(450)	(94)	*tssK*	(446)	(35)	*tssK*	(444)	(38)
tagH	(458)	FHA domain	*tagH*	(461)	(77)	*tagH*	(394)	(32)			
tsb2[3]	(188)	Unknown	*blr3597*	(188)	(87)	-			-		
tssI/vgrG	(622)	Puncturing device	*tssI*	(623)	(95)	*tssI*	(754)	(34)	*tssI*	(680)	(35)
tssA	(437)	Baseplate complex	*tssA*	(447)	(81)	*tssA*	(354)	(36)	*tssA*	(381)	(51)
tssB	(175)	Cytoplasmic sheath	*tssB*	(184)	(94)	*tssB*	(181)	(47)	*tssB*	(170)	(41)
tssC	(500)	Cytoplasmic sheath	*tssC*	(503)	(98)	*tssC*	(493)	(48)	*tssC*	(494)	(48)
tssD/hcp	(161)	Secretiontube	*tssD*	(162)	(94)	*tssD*	(158)	(23)	*TssD*	(158)	(29)
tssE	(182)	Baseplate complex	*tssE*	(183)	(84)	*tssE*	(169)	(21)	*tssE*	(150)	(30)
tssF	(655)	Baseplate complex	*tssF*	(444)	(89)	*tssF*	(593)	(30)	*tssF*	(607)	(30)
tssG	(354)	Baseplate complex	*tssG*	(355)	(88)	*tssG*	(333)	(26)	*tssG*	(356)	(29)
tssH(clpV)	(878)	Disassembler ATPase	*tssH*	(880)	(91)	*tssH*	(919)	(49)	*tssH*	(875)	(50)
tsb1[3]	(528)	SAM-Methyltransferase	*bll3586*	(533)	(83)	*PF00487*	(84)	-	*Azc-2606*	(255)	(24)

[1]Gene names *tss* (type six secretion) and *tag* (T6SS-associated gene) were proposed by Shalom *et al.* 2007.[2]Amino acid identity to A16 strain.

[3] Names used in this work after Type six secretion *Bradyrhizobium* (*tsb*)

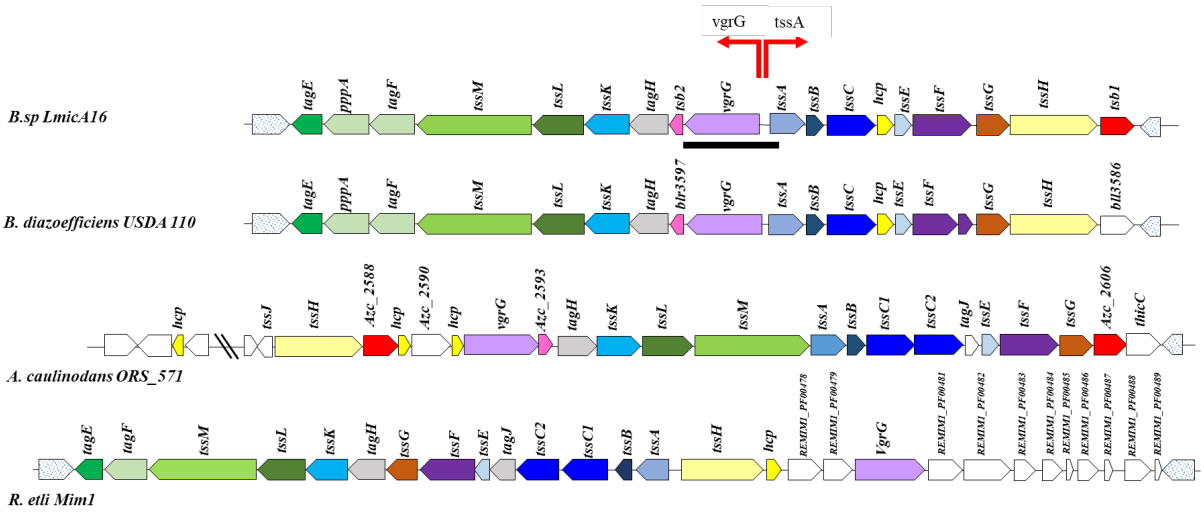
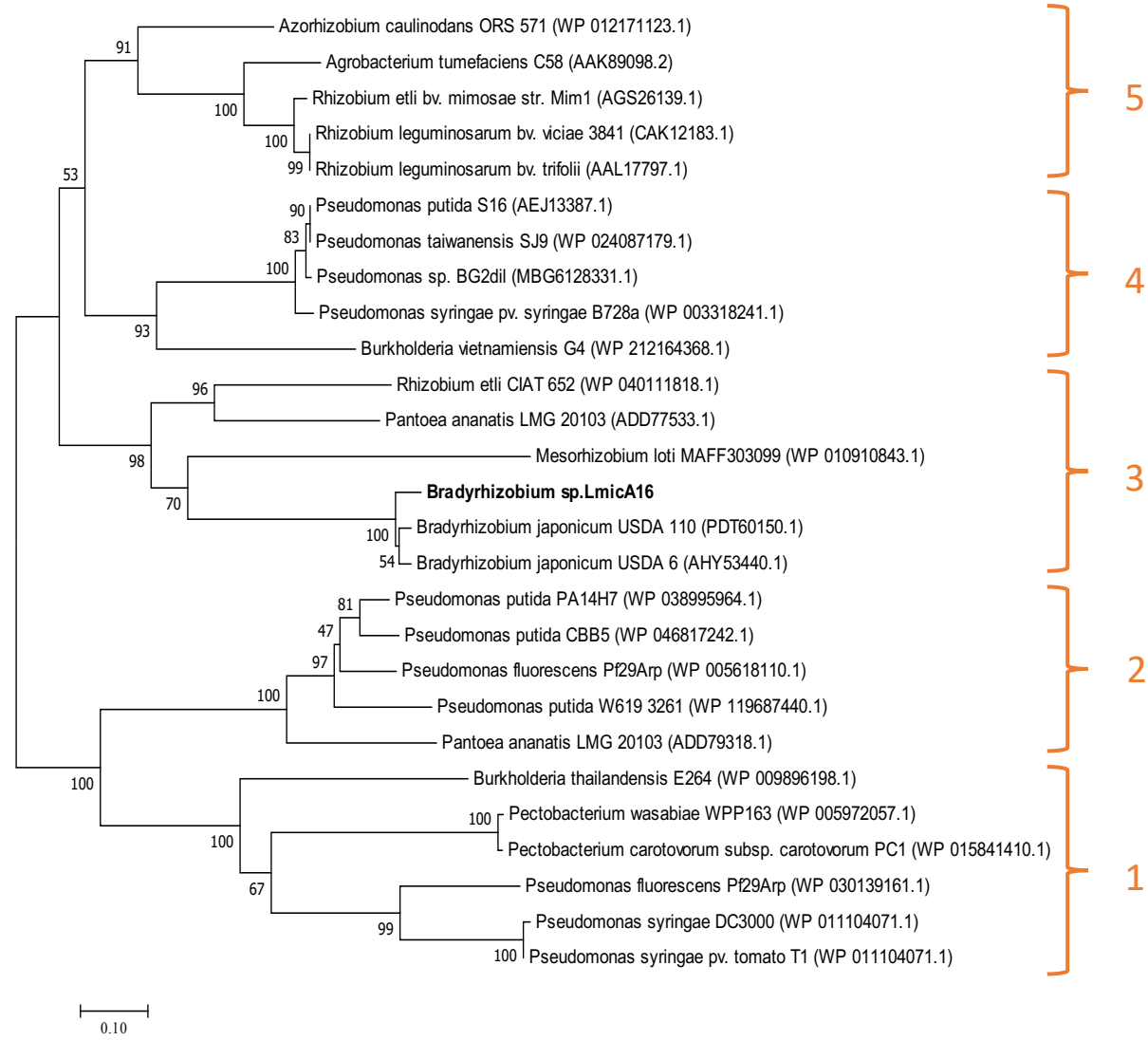


Fig. 1 Genetic organization of *Bradyrhizobium* sp. LmicA16 T6SS cluster and homologous region of *B. diazoefficiens* USDA110, *A. caulinodans* ORS571 and *R. etli* Mim1. Two divergent clusters named vgrG and tssA are indicated by red arrows. Orthologous genes show the same color; white genes correspond to non-conserved genes. The black bar under *vgrG* shows the amplified region to complement vgrG mutant.

a


Azorhizobium caulinodans ORS 571 (WP 012171123.1)
Agrobacterium tumefaciens C58 (AAK89098.2)
Rhizobium etli bv. mimosae str. Mim1 (AGS26139.1)
Rhizobium leguminosarum bv. viciae 3841 (CAK12183.1)
Rhizobium leguminosarum bv. trifolii (AAL17797.1)
Pseudomonas putida S16 (AEJ13387.1)
Pseudomonas taiwanensis SJ9 (WP 024087179.1)
Pseudomonas sp. BG2dil (MBG6128331.1)
Pseudomonas syringae pv. syringae B728a (WP 003318241.1)
Burkholderia vietnamiensis G4 (WP 212164368.1)
Rhizobium etli CIAT 652 (WP 040111818.1)
Pantoea ananatis LMG 20103 (ADD77533.1)
Mesorhizobium loti MAFF303099 (WP 010910843.1)
Bradyrhizobium sp.LmicA16
Bradyrhizobium japonicum USDA 110 (PDT60150.1)
Bradyrhizobium japonicum USDA 6 (AHY53440.1)
Pseudomonas putida PA14H7 (WP 038995964.1)
Pseudomonas putida CBB5 (WP 046817242.1)
Pseudomonas fluorescens Pf29Arp (WP 005618110.1)
Pseudomonas putida W619 3261 (WP 119687440.1)
Pantoea ananatis LMG 20103 (ADD79318.1)
Burkholderia thailandensis E264 (WP 009896198.1)
Pectobacterium wasabiae WPP163 (WP 005972057.1)
Pectobacterium carotovorum subsp. carotovorum PC1 (WP 015841410.1)
Pseudomonas fluorescens Pf29Arp (WP 030139161.1)
Pseudomonas syringae DC3000 (WP 011104071.1)
Pseudomonas syringae pv. tomato T1 (WP 011104071.1)
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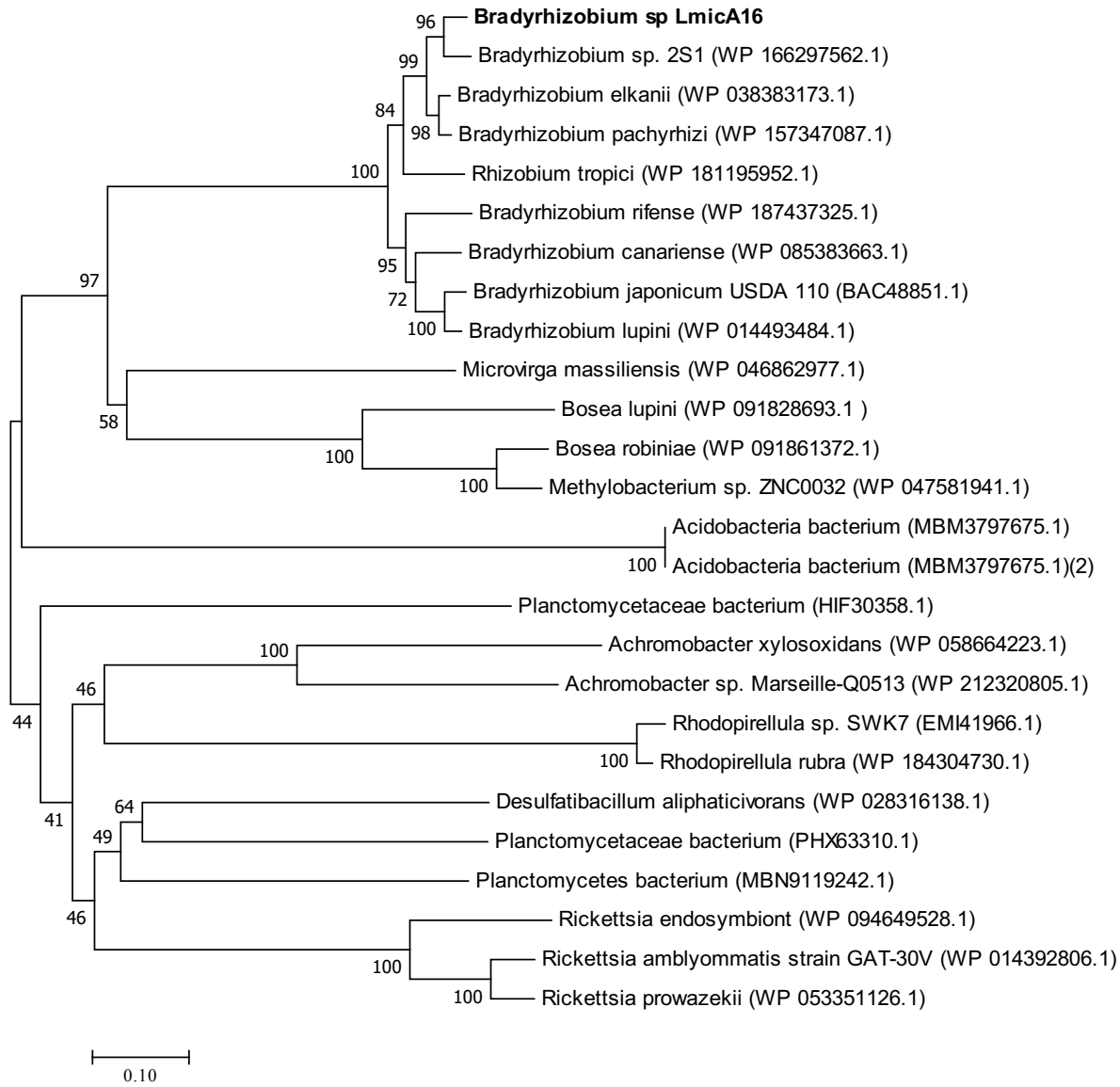
Bradyrhizobium sp LmicA16
Bradyrhizobium sp. 2S1 (WP 166297562.1)
Bradyrhizobium elkanii (WP 038383173.1)
Bradyrhizobium pachyrhizi (WP 157347087.1)
Rhizobium tropici (WP 181195952.1)
Bradyrhizobium rifense (WP 187437325.1)
Bradyrhizobium canariense (WP 085383663.1)
Bradyrhizobium japonicum USDA 110 (BAC48851.1)
Bradyrhizobium lupini (WP 014493484.1)
Microvirga massiliensis (WP 046862977.1)
Bosea lupini (WP 091828693.1)
Bosea robiniae (WP 091861372.1)
Methylobacterium sp. ZNC0032 (WP 047581941.1)
Acidobacteria bacterium (MBM3797675.1)
Acidobacteria bacterium (MBM3797675.1)(2)
Planctomycetaceae bacterium (HIF30358.1)
Achromobacter xylosoxidans (WP 058664223.1)
Achromobacter sp. Marseille-Q0513 (WP 212320805.1)
Rhodopirellula sp. SWK7 (EMI41966.1)
Rhodopirellula rubra (WP 184304730.1)
Desulfatibacillum aliphaticivorans (WP 028316138.1)
Planctomycetaceae bacterium (PHX63310.1)
Planctomycetes bacterium (MBN9119242.1)
Rickettsia endosymbiont (WP 094649528.1)
Rickettsia amblyommatis strain GAT-30V (WP 014392806.1)
Rickettsia prowazekii (WP 053351126.1)
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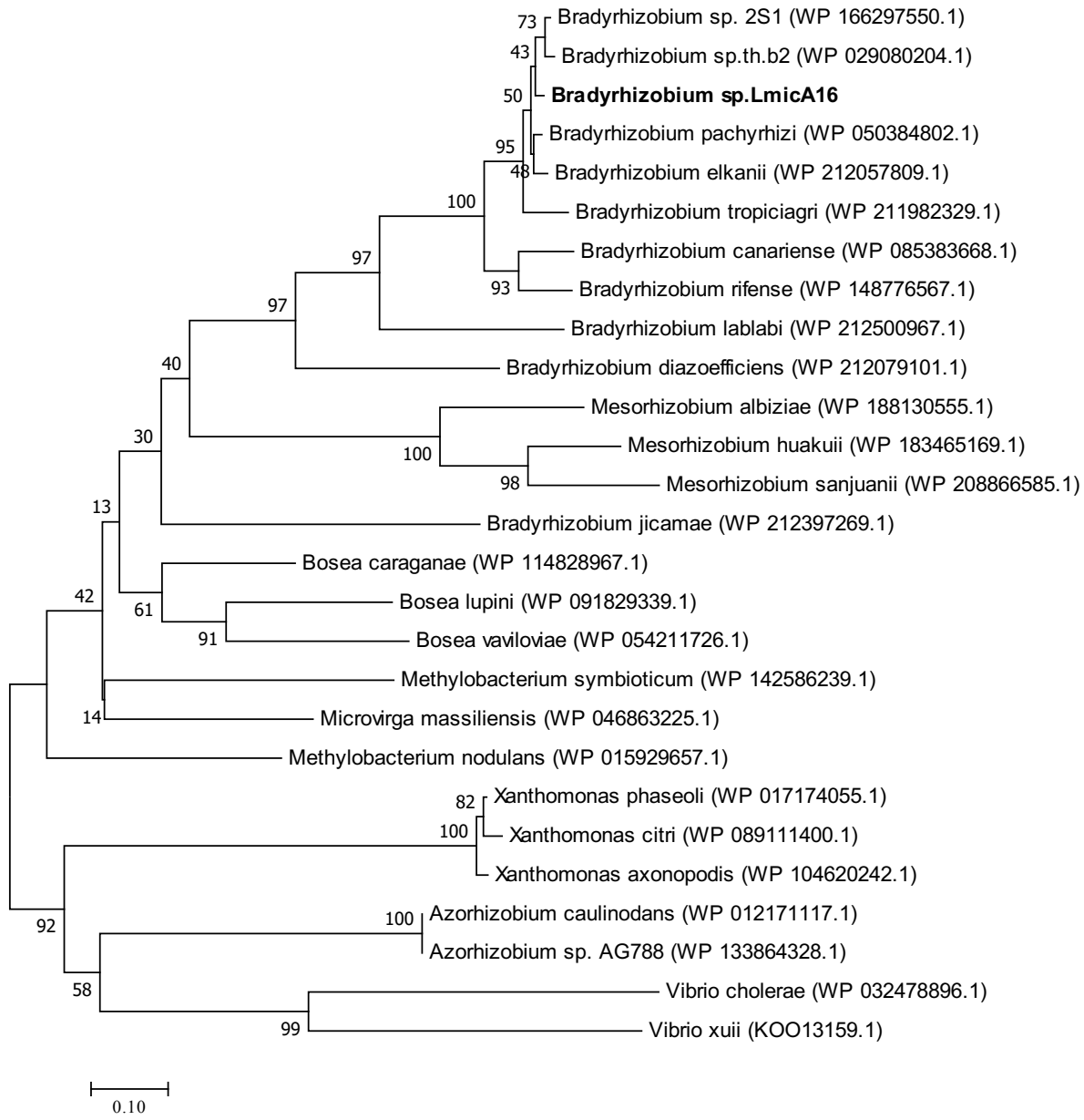
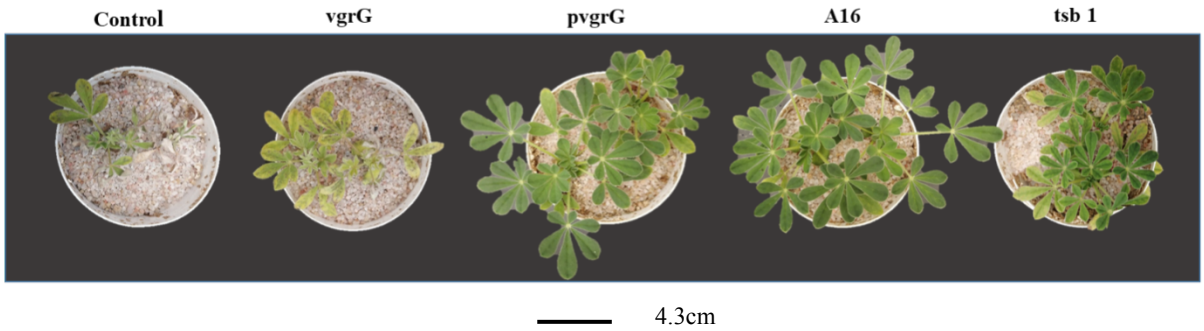
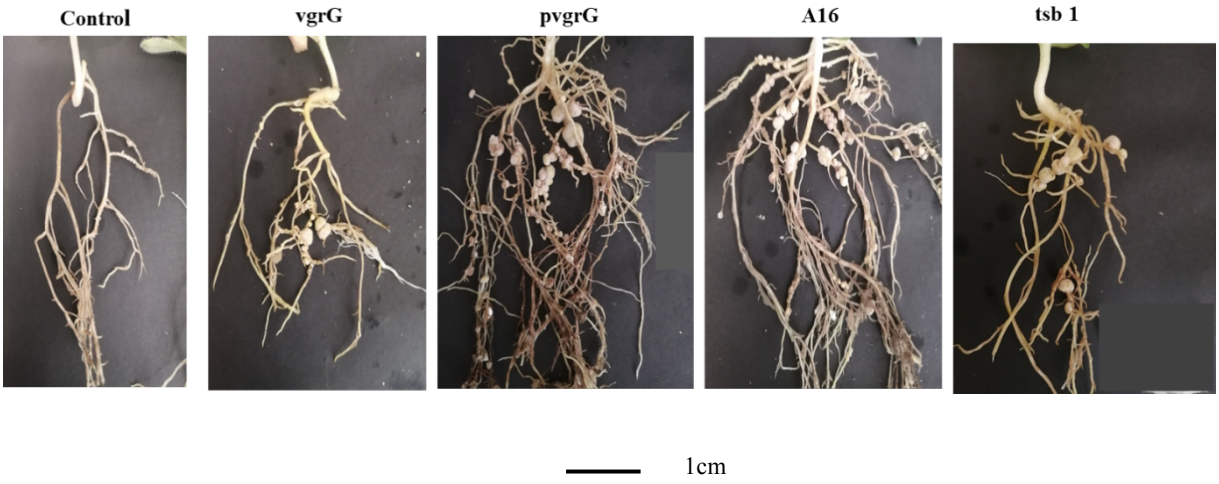


Fig. 2 Neighbor-joining phylogenetic trees based on proteins TssB, Tsb1 and Tsb2. **(a)**, Neighbor-joining phylogenetic tree based on the TssB protein. The five groups were determined according to the phylogenetic distribution of T6SS clusters in plant-associated bacteria by Bernal et al. (2018) [17]. **(b)** phylogenetic tree based on Tsb1 **(c)** Phylogenetic tree based on Tsb2. Bootstrap values using 1000 replicates are indicated at branching points. The accession numbers are shown in brackets. Bars: 0.1 estimated substitutions.

a



b



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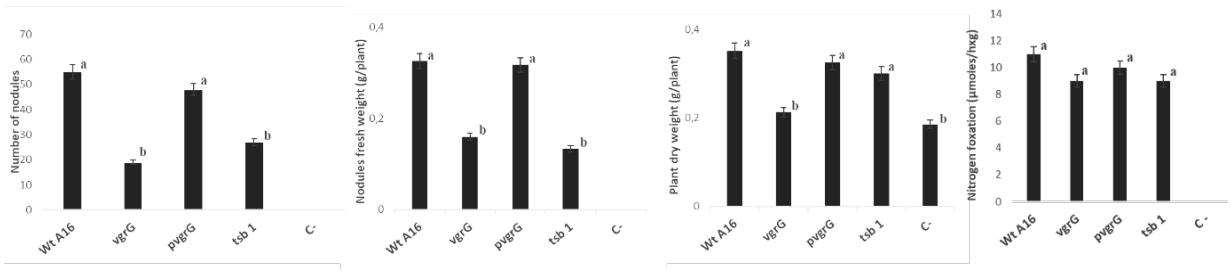

Fig. 3 Symbiotic phenotype of A16 and derivatives with *L. micranthus* 5 weeks after inoculation. C- (control) correspond to uninoculated plant. **(a)** Aerial part. **(b)** Roots and nodules. **(c)** Quantitative data from nodules and dry weight of plants. Values are the average of at least 12 plants from 3 replicates (4 plants/replicate). Values with the same letter are not significantly different using a Student´s test (p=0.05).

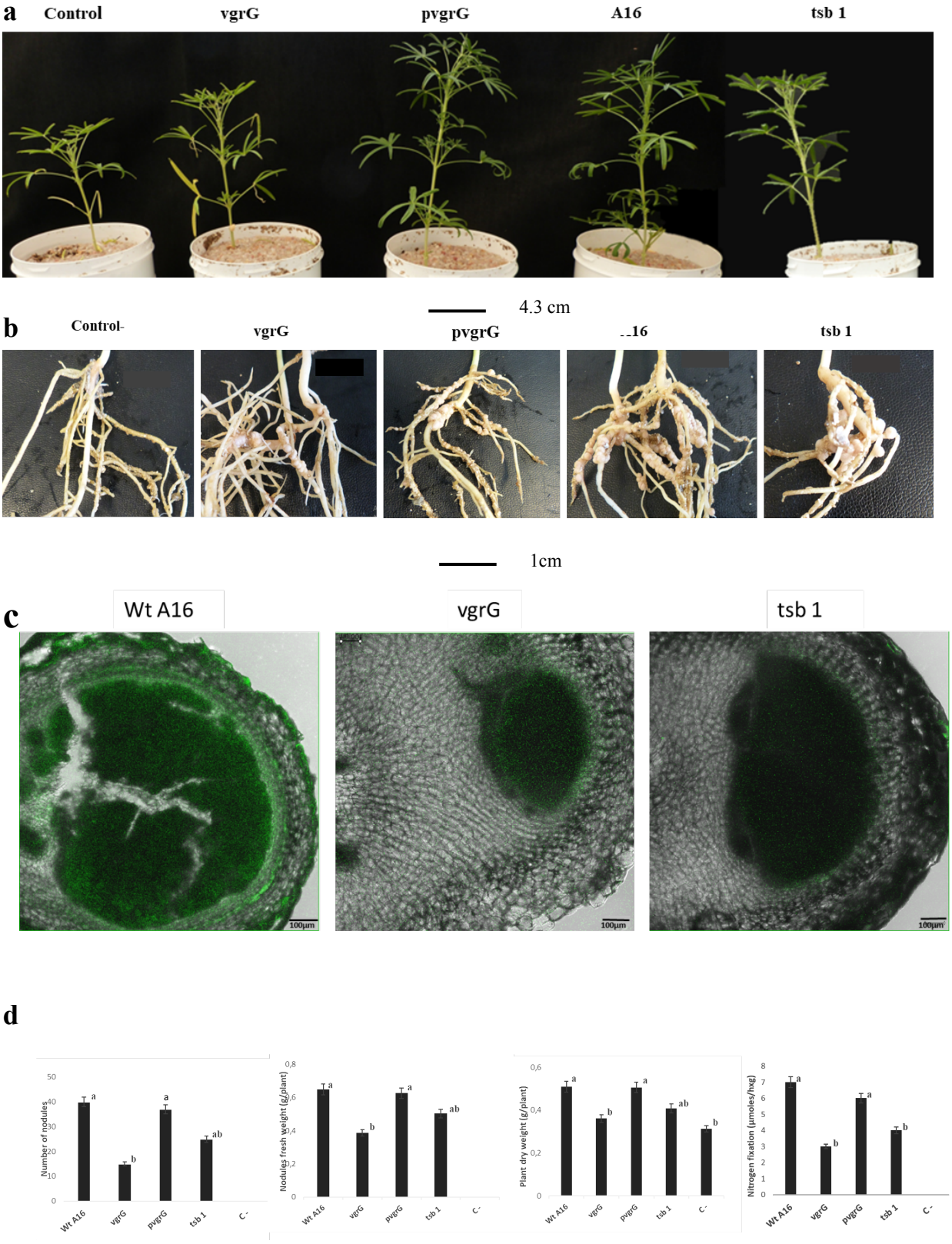

Fig. 4 Symbiotic phenotype of A16 and derivatives with *L. angustifolius* 5 weeks after inoculation. C- (control) correspond to uninoculated plant. **(a)** Aerial part. **(b)** Roots and nodules. **(c)** Nodule structure induced by A16, *vgrG* and *tsb1* strains harboring plasmid pHC60 expressing GFP protein constitutively. **(d)** Quantitative data from nodules and dry weight of plants. Values are the average of at least 12 plants from 3 replicates (4 plants/replicate). Values with the same letter are not significantly different using a Student´s test (p=0.05).

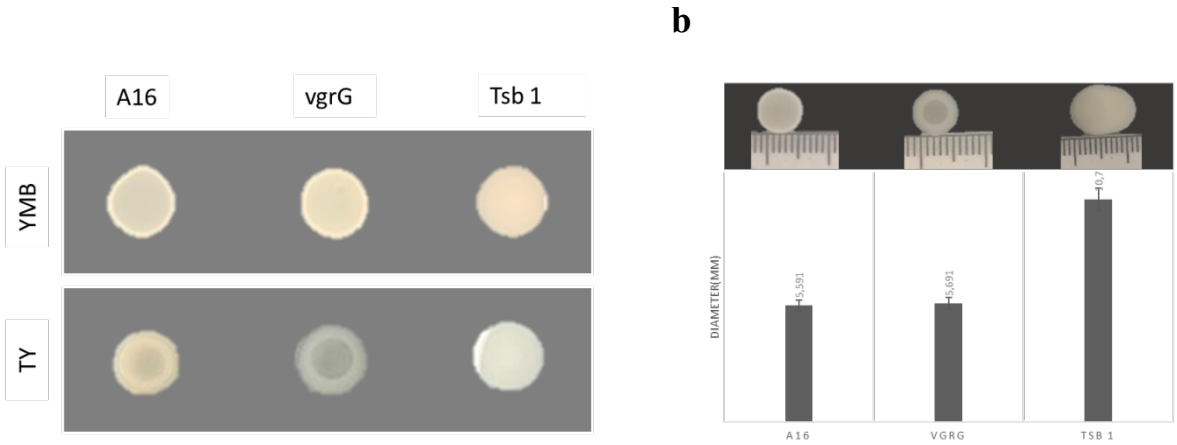

Fig. 5 Colony morphology and motility of A16 mutants vgrG and tsb1 mutants in different media (YMB, TY). (a) 10 µl culture of $1{,}6.10^{8}\pm 0.1$ CFU·mL−1 was spotted on the plates. (b) 5 µl of the same culture was spotted on 0.7% TY agar during 20 days. Values are the average of 2 assays (5 replicates/assay).

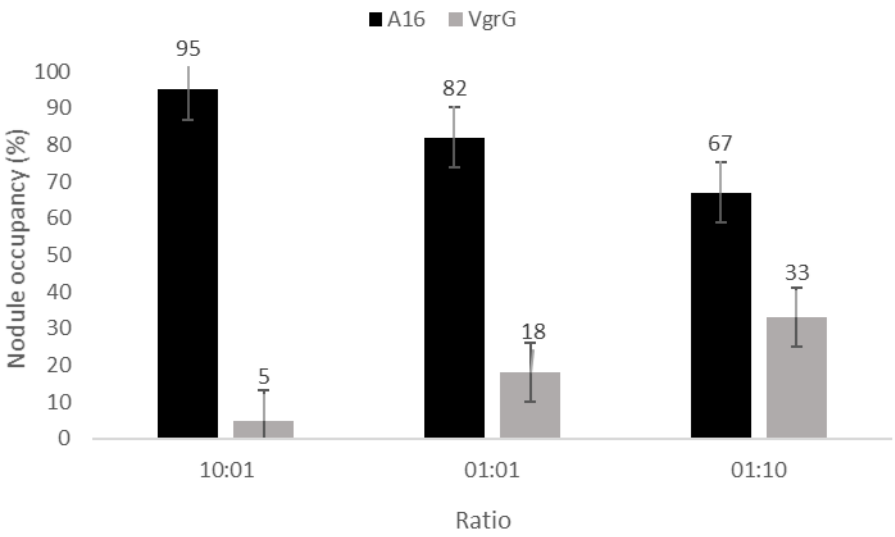

Fig. 6 Study of the competitiveness of the wild type A16 strain with the vgrG mutant at different ratios inoculated with *L. angustifolius*. Values are the average of at least 10 plants from 2 replicates (5 plants/replicate).

694 **Suppplementary information**

695 Table S1. Bacterial strains and plasmids used in this work

Strain or plasmids	Relevant characteristics	Reference of source
***E. coli* strains**		
DH5α	Host for DNA cloning	Hanahan1983
S17.1	Host for conjugation	Simon , Priefer and Puhler1983
***Bradyrhizobium* strains**		
LmicA16 (A16)	Wild-type strain isolated from *Lupinus micranthus*	Bourebaba et al., 2016
vgrG	A16 *vgrG* mutant, Km^r	This work
vgrG(pvgrG)	*vgrG* strain complemented by pvgrG	This work
tsb1	A16 *tsb1* mutant, Km^r	This work
Plasmids		
PCR2.1 TOPO	Vector for cloning PCR products, Amp^r, Km^r	Invitrogen
pK18*mobsacB*	Suicide vector; *sacB*,Km^r	Schäfer et al. 1994
pK18*vgrG*	pK18*mobsacB* with a 388bp internal fragment of *vgrG* from BA16 strain	This work
pVO-npt2- cefo-npt2-GFP (pVO)	Suicide vector; $Cefo^r$,Km^r	Okazaki et al., 2016
pVOtsb1	pVO with a 284 bp internal fragment of *tsb1*from BA16 strain	This work
pMP220	Broad-host-range plasmid, Tc^r	Spaink et al. 1987
P6VgrG	pMP220 derivative containing the P6*vgrG* in front of reporter gene *lacZ*	This work
P6TssA	pMP220 derivative containing the P6*tssA* in front of reporter gene *lacZ*	This work
pvgrG	pMP220 derivative containing the *vgrG* under P6 promoter	This work
pHC60	Broad-host range vector carrying GFP under control of a constitutive lac promoter, Tc^r	Cheng and Walker, 1998

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713

714 **Table S2**. Primers used in this work

715

Primer	Sequence	Amplicon	Use
MutvgrG.A16 Fw	GCTGCCGAAATAGACGTGTT	496pb	Obtaining vgrG mutant
MutvgrG.A16 Rv	ACTCCAAATCGTCGCATAGC		
P6VgrG Fw	**CCCAAGCTT**CCGTTTGCGTTGACGACC	2.445pb	*vgrG* mutant complementation
P6VgrG Rv	**CCCAAGCTT**GTAGCGTGCCAGCGGATA		
P6.Fw	GTCTTGAGCTCGCAAAGGCG	690pb	Obtaining P6. tssA and P6.vgrG
P6.Rv	TCGAGATCGCGGTTCAGGAT		
pMP220.Fw	AGCTCCTGAAAATCTCGTCG		Determination of P6 orientation
pMP220.Rv	AACGGCCTCACCCCAAAAAT		
Mutmethyl.A16 Fw	CGGACT**CTCGAG**CCCGAACCACTTGGCGGCAATC	388 pb	Obtaining tsb1 Mutant
Mutmethyl.A16 Rv	GCTGAC**TCTAGA**GAGTCCCTGGGGTGCCAAATTC		

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