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The tight-adhesion proteins TadGEF of *Bradyrhizobium diazoefficiens* USDA 110 are involved in cell adhesion and infectivity on soybean roots

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ABSTRACT

Adhesion of symbiotic bacteria to host plants is an essential early step of the infection process that leads to the beneficial interaction. In the *Bradyrhizobium diazoefficiens*-soybean symbiosis few molecular determinants of adhesion are known. Here we identified the tight-adhesion gene products TadGEF in the open-reading frames blr3941–blr3943 of the *B. diazoefficiens* USDA 110 complete genomic sequence. Predicted structure of TadG indicates a transmembrane domain and two extracytosolic domains, from which the C-terminal has an integrin fold. TadE and TadF are also predicted as bearing transmembrane segments. Mutants in *tadG* or the small cluster *tadGEF* were impaired in adhesion to soybean roots, and the root infection was delayed. However, nodule histology was not compromised by the mutations, indicating that these effects were restricted to the earliest contact of the *B. diazoefficiens* and root surfaces. Knowledge of preinfection determinants is important for development of inoculants that are applied to soybean crops worldwide.

1. Introduction

Bradyrhizobium diazoefficiens (previously, *Bradyrhizobium japonicum*, Delamuta et al., 2013) belongs to the important bacterial class of rhizobia, which fixes atmospheric N_2 in symbiosis with legume plants. This symbiosis is the main contributor to N-nutrient input into the biosphere, constitutes a key step in the N biogeochemical cycle, and is applied in sustainable agriculture worldwide. The symbiosis is achieved after a complex process of infection of legume roots, in which bacterial adhesion to host root surfaces is an essential early step (Oldroyd and Downie, 2008). Adhesion was carefully studied in the *B. diazoefficiens*-soybean symbiosis, where exopolysaccharide, lectins, and pili formed by polymerization of a 21-kDa MW subunit were recognized as determinants (Dardanelli et al., 2003; Ho et al., 1988, 1990a,b, 1994; Lodeiro and Favelukes, 1999; Lodeiro et al., 2000a; Loh et al., 1993; Oehrle et al., 2000; Pérez-Giménez et al., 2009; Pueppke, 1984; Smith and Wollum, 1991; Vesper and Bauer, 1985; Vesper et al., 1987). However, molecular details of this process are unknown, since none of the above-mentioned studies made use of site-directed mutants in adhesins that might be directly involved in the contact of bacteria and root surfaces.

Among bacterial pili, fimbrial low-molecular weight protein (Flp) pili constitute a special class. The structural Flp subunit is around 6–8 kDa in size (Inoue et al., 1998; Kachlany et al., 2000) and is generally encoded among tight adherence (*tad*) loci, which contain other structural and regulatory genes for Flp pilus biosynthesis. The tad genes are widely distributed in bacteria (Imam et al., 2011; Tomich et al., 2007), but evidence on their functions was reported only in *Aggregatibacter actinomycetemcomitans* (Kachlany et al., 2001; Rosan et al., 1988), *Caulobacter crescentus* (Skerker and Shapiro, 2000), *Micrococcus luteus* (Angelov et al., 2015), *Pectobacterium* sp. (Nykyri et al., 2013), and *Pseudomonas aeruginosa* (de Bentzmann et al., 2006). These works confirmed the function of *flp/tad* genes in cell adhesion, biofilm development, host colonization and virulence, and extended their roles to

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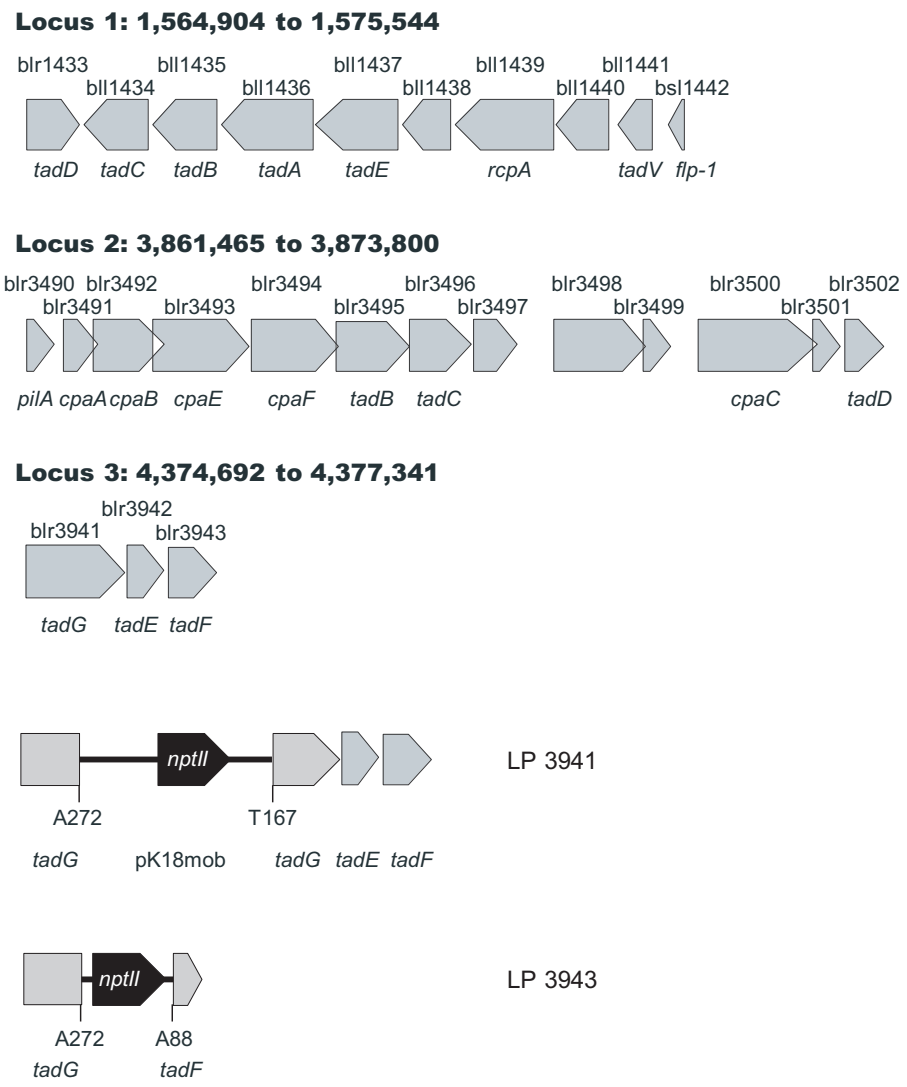

Fig. 1. Organization of *tad* and related genes in *B. diazoefficiens* USDA 110. The genes were identified with BLAST alignments and subsequently found clustered in three separate loci. This organization is in contrast to other species, where these genes are in a single operon. At the bottom the mutants obtained in this work are sketched. In LP 3941 the pK18mob suicide plasmid was inserted into *tadG* coding region between the aminoacid positions indicated (note that the recombination fragment was duplicated after the single crossing-over). In LP 3943 the fragment comprised between the aminoacid positions indicated was replaced by a Km^r cassette.

bacterial transformation. However, although in *Ensifer* (*Sinorhizobium*) *meliloti* the Flp pili are important for competitive nodulation (Zatakia et al., 2014), the tad genes were not studied yet in rhizobia–legume symbiosis. To advance in our knowledge on adhesion of *B. diazoefficiens*, here we studied the role of tadGEF, which are thought to encode components of the apparatus that anchors the Flp pilus to the cell surface (Tomich et al., 2007).

2. Materials and methods

2.1. Bioinformatics

Sequence similarity searches were done using BLAST and PSI-BLAST at NCBI site. Retrieved sequences were aligned using CLUSTALX and a phylogenetic estimation was performed using maximum likelihood calculations with the program PhyML (Guindon et al., 2009) and using the model JTT with gamma rate variation among sites. To validate the topologies obtained, 100 replicants in a non-parametric bootstrapping were used. To further characterize the ORF blr3941, predictions of putative transmembrane domains were performed with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/last access date: 8-26-15), DAS (Cserzo et al., 1997) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html last access date: 8-25-15). Fold assignment methods as FFAS03 (Jaroszewski et al., 2005), HHPred (Söding, 2005) and Phyre (Kelley et al., 2015) were also applied. Using the putative templates detected, structural models were built using the program MODELLER (Rosan et al., 1988). Structural models were also obtained using *ab initio* methods as I-TASSER (Zhang, 2008).

2.2. Strains and culture conditions

Insertion and deletion mutants of *B. diazoefficiens* USDA 110 were obtained as already described (Quelas et al., 2010) with the strains, plasmids and primers indicated in Table 1. Briefly, to construct the LP 3941 gene disruption mutant, a 317 bp fragment (fragment A) was amplified using primers A-fw and A-rv. This PCR fragment was cloned in pGEM T-Easy (pEJM01) and then the fragment A was transferred to the EcoRI site of pK18mob (pEJM02). Finally, pEJM02 was conjugated from *Escherichia coli* DH5α to *B. japonicum* USDA 110 by triparental mating using the helper plasmid pRK2013, and the candidate mutants were selected in YM agar

Table 1
Bacterial strains, plasmids, and primers used in this study.

Name Strains	Relevant characteristics Relevant genotype or phenotype	References or source
E. coli		
DH5α	*rec*A1, Δ*lac*U169, (Φ80d*lac*ZΔM15)	Bethesda Res. Lab.
B. diazoefficiens		
USDA 110	Wild type Nod$^+$ Fix$^+$ (Cm^r)	USDA culture collection
LP 3941	USDA 110 insertional mutant with the pK18mob vector inserted into the coding region of blr3941 at position 4,375,509 (Km^r Cm^r)	This study
LP 3943	USDA 110 deletional mutant blr3941–blr3943 with a Km^r cassette replacing bases 4,375,509 to 4,376,946 (Km^r Cm^r)	This study
Name Plasmids	Relevant characteristics Relevant genotype	References or source
pRK2013	Helper plasmid tra$^+$, *oriT*, ColE1 (Km^r)	Figurski and Helinski (1979)
pGEM T-Easy	Multicopy plasmid vector for cloning PCR products (Ap^r)	Promega corp.
pK18mob	Suicide plasmid in rhizobia, Mob$^+$ (Km^r)	Schäfer et al. (1994)
pG18mob2	Suicide plasmid in rhizobia, Mob$^+$ (Gm^r)	Schäfer et al. (1994)
pUC4K	Plasmid with *nptI* gene (source for Km-resistance cassette) (Ap^r Km^r)	Vieira and Messing (1982)
pEJM01	pGEM T-Easy carrying an internal fragment of blr3941 (A) from 4,375,192 to 4,375,509 USDA 110 genome nucleotides (Ap^r)	This study
pEJM02	pK18mob carrying the internal fragment A of blr3941 (Km^r)	This study
pEJM03	pGEM T-Easy carrying an internal fragment of blr3943 (B) from 4,376,946 to 4,377,272 USDA 110 genome nucleotides (Ap^r)	This study
pEJM04	pG18mob2 carrying the internal fragment A of blr3941 (Gm^r)	This study
pEJM05	pEJM04 carrying the internal fragment B of blr3943 (Gm^r)	This study
pEJM06	pEJM05 carrying the Km-resistance cassette between fragments A and B (Gm^r Km^r)	This study
Name Primers	Relevant characteristics Sequence	References or source
A-fw	5′ GACCGACATCAGCAATCTCA 3′	This study
A-rv	5′ AGTGCGTTCAAACCCAAATC 3′	This study
B-fw	5′ GTCAAGAGCGGCAGTACCTC 3′	This study
B-rv	5′ GTATGGGAAGCTCGAGTTGG 3′	This study
Aext-fw	5′ TTCTCGGCAAGCAGACCT 3′	This study
Aext-rv	5′ CGCCTCGTTCTCGTTGTAGC 3′	This study
Bext-rv	5′ CCCCATAAGCCACACGTT 3′	This study
Km-fw	5′ CATCGGGCTTCCCATACA 3′	This study
Km-rv	5′ TGCCATTCTCACCGGATT 3′	This study
M13-fw	5′ GTAAAACGACGGCCAGT 3′	Promega corp.
M13-rv	5′ GCGGATAACAATTTCACACAGG 3′	Promega corp.

(Vincent, 1970) supplemented with 20 μg ml^{-1} cloramphenicol (Cm) and 150 μg ml^{-1} kanamycin (Km). The insertion was confirmed by PCR using combinations of primers M13-fw and M13-rv with primers external to fragment A (Aext-fw and Aext-rv). To construct the deletion mutant LP 3943, the fragment A was moved to the EcoRI site of pG18mob2 (pEJM04). The fragment B (376 bp) was amplified with B-fw and B-rv primers, cloned in pGEM T-Easy as before (pEJM03), and moved to pEJM04 between PstI and SphI sites (pEJM05), thus leaving a BamHI site between fragments A and B. Hence, the Km^r cassette containing *nptII* was moved from pUC-4K to this site with BamHI to generate pEJM06. Finally, pEJM06 was conjugated to USDA 110 as described above and the double crossing-over was selected by resistance to 150 μg ml^{-1} Km and sensitivity to 100 μg ml^{-1} gentamicin (Gm). Furthermore, the deletion was confirmed using a combination of primers specific for the Km^r cassette (Km-fw and Km-rv) with primers external to fragments A and B (Aext-fw and Bext-rv).

For all experiments of adhesion, biofilm formation and nodulation, the bacteria were grown in YM as described (Lodeiro and Favelukes, 1999).

2.3. Adhesion

Adhesion to soybean roots was measured as described (Mongiardini et al., 2008). In brief, 10 aseptically grown seedlings per treatment were incubated for 4 h in a rhizobial suspension of approximately 10^3 cells ml^{-1} in N-free modified Fåhraeus solution (MFS) (Lodeiro et al., 2000b) at 28 °C with rotary shaking at 50 rev min^{-1}. Rootlets with adhered rhizobia were washed four times, each by shaking with fresh MFS for 1 min at 120 rev min^{-1}. After washing, two different methods were employed to quantify rhizobial adhesion.

In method I, the rootlets were distributed on the bottom of petri dishes, and overlaid with molten (45 °C) YM agar supplemented with cycloheximide and the appropriate antibiotic concentration for selection of the assayed strain. After plate incubation at 28 °C, rhizobia remaining adhered on the embedded primary root surfaces developed microcolonies, which were counted along the visible surface of each primary root under a stereomicroscope at 25 × magnification. The total number of rhizobial colony-forming units (CFU) on the whole root surface was estimated as described (Mongiardini et al., 2008).

In method II, primary roots were vortexed for 2 min in the presence of 0.5 mm diameter glass beads and the supernatants were diluted and plated in YM agar with the corresponding antibiotic for the rhizobial strain used. After colony development, CFU were counted and corrected for the dilution factor to estimate the total CFU released from the primary roots.

To measure rhizobial adhesion to polypropylene or glass beads, 2.5 g beads per flask were used. Rhizobia were incubated with these materials for 4 h at 28 °C and 50 rev min^{-1} and washed as described before. After washing, adsorbed rhizobia were counted according to method II, except that glass beads were not included at the vortexing step.

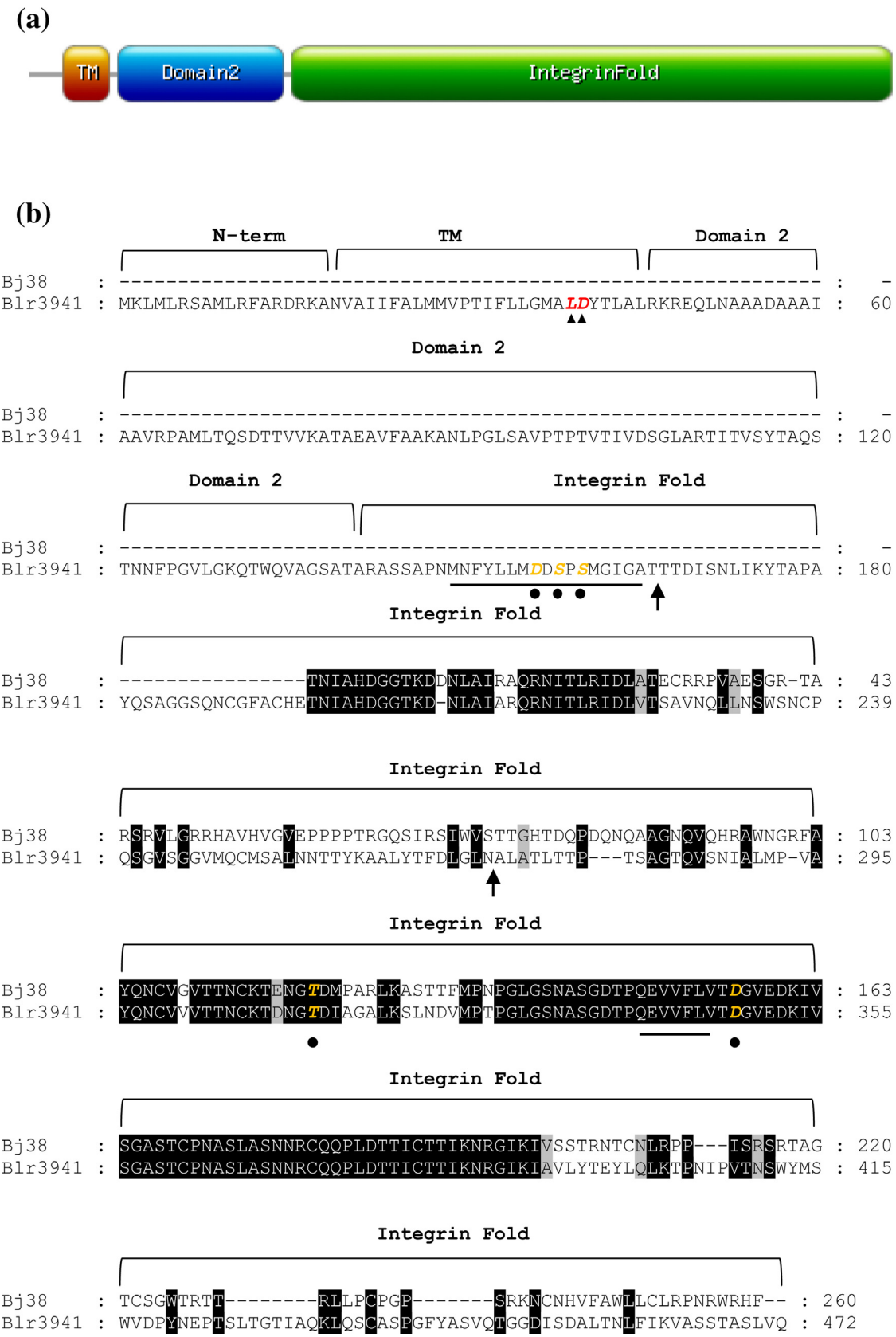

Fig. 2. Primary structure of TadG and its alignment with BJ38 (Ho et al., 1999). (a) Domains organization deduced with bioinformatic methods. (b) Aminoacids sequence alignment indicating the different domains of TadG. The possible cleavage site by a membrane protease is shown by triangles. The integrin fold contains the von Willebrand sequences (underlined) and is probably the extracellular portion of the protein. Residues marked with dots within the integrin fold are the MIDAS motif. Identical aminoacids between TadG and BJ38 are in black background while conserved aminoacids are in grey background. The Thr167 and Ala272 targeted by the pK18mob insertion in LP 3941 or start of deletion in LP 3943 are indicated by arrows.

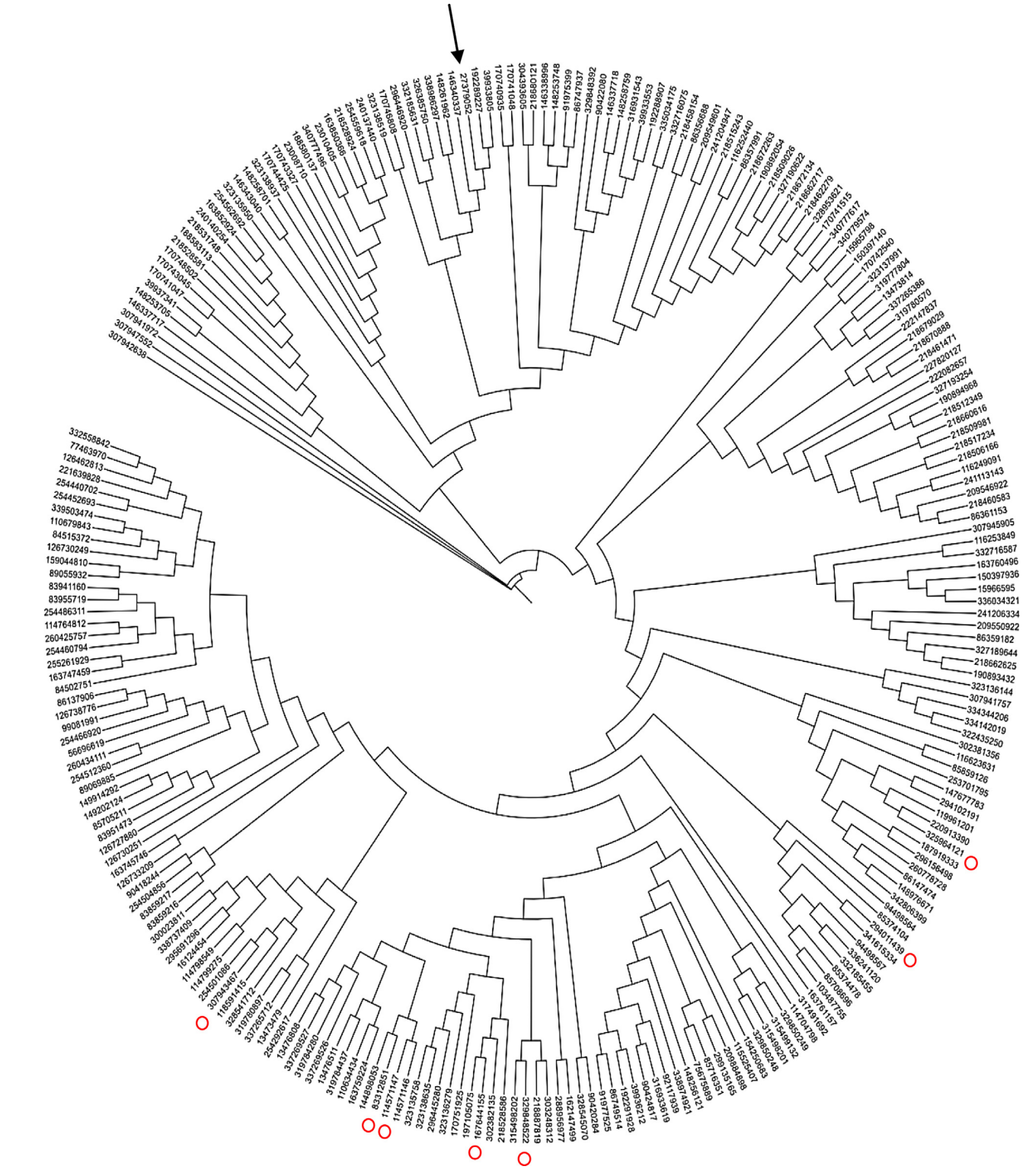

Fig. 3. Phylogenetic estimation using the program PhyML (Guindon et al., 2009) by maximum likelihood of blr3941 and close homologous proteins. The GI of each protein is shown at the tip of the corresponding tree branch. The black arrow indicates blr3941. The closest functionally characterized proteins to blr3941 are TadG (circles). All the other proteins are unknown.

Total counts of microcolonies on (or CFU released from) all primary roots or beads, expressed as the percent of the total number of CFU present in the original inoculum, represented the adhesion index. Confidence intervals ($p < 0.05$) were calculated taking into account the binomial distribution of data (Mongiardini et al., 2008).

2.4. Biofilms in multiwell plates

Rhizobia were grown in YM medium at 28 °C and 180 rev min^{-1} rotary shaking to an OD_{500} of 1.0. Then, rhizobia were diluted in MFS to an OD_{500} of 0.1. The microtiter plate assay for biofilm

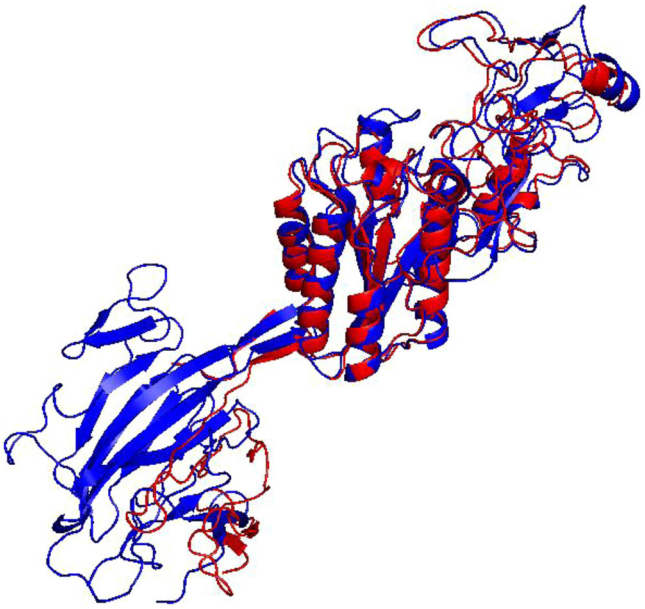

Fig. 4. Proposed 3D model for carboxyl-terminal end domains from blr3941 (red) aligned with the protein Tip pilin GBS104 from Group B *Streptococcus agalactiae* (blue). The model was obtained using the program I-TASSER, but similar ones were obtained using MODELLER. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

quantification was used as described (Fujishige et al., 2006; Pérez-Giménez et al., 2009). Briefly, 150 μl of cells or MFS were added to individual wells of a 96-well polystyrene plate. The plates were sealed with sterile parafilm "M" and incubated at 28 °C. At different times the medium was removed and the OD_{500} was measured to verify that there was no difference in growth rate among the wells. Then, the biofilms were stained with 0.1% crystal violet for 20 min. and read at 570 nm.

2.5. Nodulation assays

Infectivity of soybean plants was evaluated as described (Bhuvaneswari et al., 1980). Nodulation profiles were obtained by inoculating 30 plants with the indicated concentrations of rhizobia ml^{-1} in plastic growth pouches watered with MFS. At the time of inoculation, the positions of the root tip (RT) were marked for each plant on the surface of the plastic growth pouches. The total number of nodules and the number of nodules above the RT marks were recorded on primary roots after 22 days of growth in the greenhouse at 26 °C/18 °C day/night temperature.

Nodule sectioning, staining, and microscopy were carried out at the Central Microscopy Service of the Faculty of Veterinary Sciences, National University of La Plata (UNLP), Argentina as described (Quelas et al., 2010). Briefly, nodules were excised from plants, and then transversally cut in halves and placed in 2% (v/v) glutaraldehyde. Then, nodules were postfixed in 1% (w/v) OsO_4 in phosphate buffer for 1 h at room temperature, washed, dehydrated, infiltrated with epoxy-resin, and sectioned. For light microscopy, 2 μm thick sections were dried onto glass slides and stained with saturated solution of Toluidine blue. For electron microscopy, 70 nm ultrathin sections were placed on 200-mesh copper grids that had been coated with collodion. The sections were stained with 0.5–1% (w/v) uranyl acetate for 10 min and 1% (w/v) lead citrate for 5 min, washed in distilled water, and air dried. The grids were viewed in a JEM 1200 EX II (Jeol) transmission electron microscope at 80 kV, and photographs were taken on Kodak electron image film.

3. Results and discussion

3.1. Bioinformatic characterization of tad genes

We searched in the *B. diazoefficiens* USDA 110 genome (Kaneko et al., 2002) for homology to known tight-adhesion genes, and found homologous to *tadABCD* as well as *flp-1* (encoding Flp pilin) in two clusters, and homologous to *tadGEF* in a separate cluster (Fig. 1). Moreover, we observed a putative paralogous to *tadE* in bll1437. In this work we focused on the blr3941-blr3943 cluster, since its products might stabilize the basal structure of the Flp pili (Tomich et al., 2007) and in particular, blr3941 has some sequence similarity to the previously described *B. diazoefficiens* lectin BJ38 (Fig. 2), which is required for adhesion and infection (Ho et al., 1990b, 1994, 1999).

To better characterize blr3941, we performed an evolutionary analysis of 262 close homologous proteins retrieved with PSIBLAST after three runs using an *E*-value of $1\ 10^{-4}$ as cutoff, which indicated that blr3941 is related with the Flp pili proteins family (Kachlany et al., 2001; Pérez et al., 2006; Schreiner et al., 2003; Skerker and Shapiro, 2000; Tomich et al., 2007) (Fig. 3). The phylogenetic

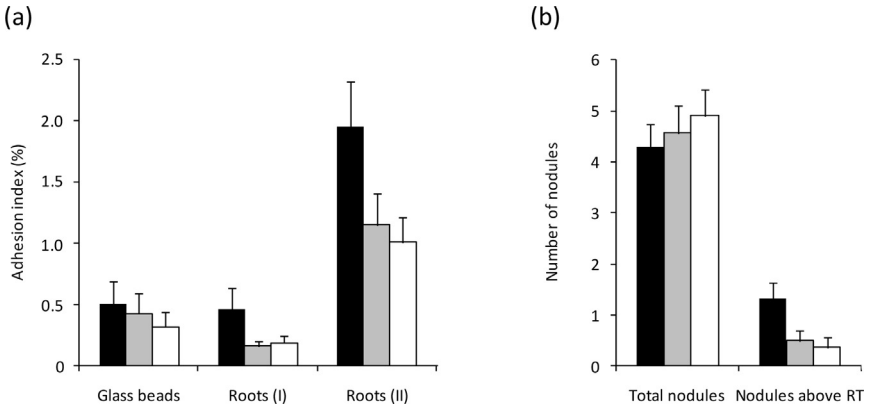

Fig. 5. Adhesion and infection of *B. diazoefficiens*. Black bars: wild-type; grey bars: insertional *tadG* mutant LP 3941; white bars: deletional *tadGEF* mutant LP 3943. (a) Adhesion to glass beads or soybean roots. Results are expressed as adhesion index (±confidence interval, $p < 0.05$), which is the amount of CFU that remained adhered to (or released from) the surface, expressed as percentage of the initially inoculated. Initial inoculum size was ca. 10^3 CFU ml^{-1}. Method I: microcolonies developed onto soybean root surfaces were counted directly with a dissecting microscope. Method II: colonies were counted after release of bacteria from the surfaces by vortexing for 3 min in the presence of 0.5 mm glass beads. (b) Infectivity of *B. diazoefficiens* in soybean roots, measured as the number of nodules developed in the primary roots above the position of the root tip (RT) marked at the moment of inoculation in comparison with the total number of nodules developed in these same roots (±confidence interval, $p < 0.05$). Nodules developed above the RT mark represent infections produced within 6 h after inoculation (Bhuvaneswari et al., 1980). Plants were inoculated with each strain at a density of ca. 10^4 rhizobia per root.

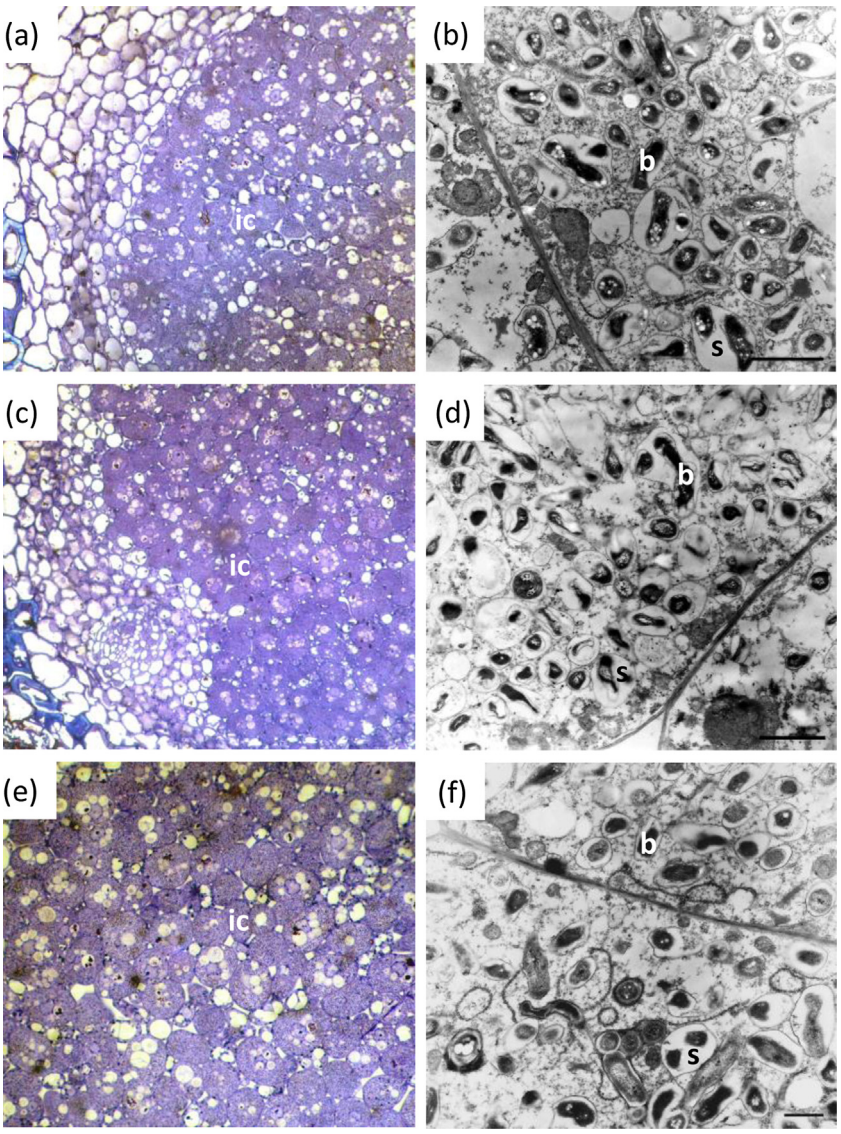

Fig. 6. Nodules developed in soybean roots by the wild-type USDA 110 (a, b) and the insertional *tadG* mutant LP 3941 (c, d) or the deletional *tadGEF* mutant LP 3943 (e, f). Nodules were cut and observed under the light microscope at 400 × where infected cells (ic) are clearly visible (a, c, e) or under the transmission electron microscope at 5000 × where symbiosomes (s) and bacteroids (b) are seen (b, d, f; bar: 1 μm).

estimation indicated that blr3941 shares common ancestors with TadG and TadE.

Using DAS, TMpred and TMHMM, we found that residues 20–45 of blr3941 are probably transmembrane. This defines a cytosolic N-terminal domain, comprising residues 1–20, and a C-terminal domain, comprising residues 46–472. The N-terminal domain possesses similarity with flagellins from *Archaeobacteria* (Inter Pro database IPR 002774) and with a group of proteins similar to TadE (PFam database PF 07811.4). In the conserved regions there is a sequence motif that could be involved in protein cleavage by a membrane protease (Fig. 2).

All the 3D predictions methods used, homology based as well as *ab initio* based, agree in detecting a Rossman fold domain in the C-terminal domain of blr3941,similar to the one found in von Willebrand factor and integrin I domains. All the methods found statistically significant scores with the protein Tip pilin GBS104 from Group B *Streptococcus agalactiae* (Krishnan et al., 2013) which corresponds in structure with PDB ID: 3TXA (FFas03 score = −31, HHpred *E*-value 2.27 10^{-22} and Phyre2 100% confidence). In this way, residues 145–463 in blr3941 could probably fold as the N3 domain of 3TXA while residues between 73 and 145 possibly form a second domain that resembles N1 and N2 domains of 3TXA; however in blr3941 this region is significantly shorter (Fig. 4).

Also, it has been characterized that 3TXA has a well conserved motif called MIDAS which is also found in von Willebrand factor and integrin domains playing a key role in protein–protein interactions and protein recognition (Loftus et al., 1994). MIDAS motif contains Asp229-X-Ser231-X-Ser233, Thr354 and Asp384 in 3TXA which importantly are also conserved in the blr3941 sequence and their close homologous as derived from the analysis of the phylogenetic inference mentioned above. MIDAS motif in blr3941 corresponds to Asp156-X-Ser158-X-Ser160, Thr312 and finally Asp348 (Fig. 2).

We obtained the same folding prediction for the TadG proteins already characterized in *Actinobacillus*. In addition, the presence of von Willebrand domain was already reported in TadG from *Actinobacillus actinomycetemcomitans* (Tomich et al., 2007). Taken together, these findings strongly indicate that blr3941 encodes the homologous of TadG in *B. diazoefficiens*, and henceforth we will refer to this locus as *tadG*. From these bioinformatic studies we can suggest that *B. diazoefficiens* TadG has four domains, one

cytosolic, one inserted into the membrane, and two as globular extracytosolic portion, where the larger domain located at the C-terminal end of the protein folds as integrin fold (Figs. 2 and 4). This larger domain contains the region targeted by the recombination fragment A between Thr167 and Ala272; therefore, the pK18mob insertion in strain LP 3941 disrupted this domain while the deletion in strain LP 3943 eliminated the portion comprised between Ala272 and the C-terminal end of TadG (Figs. 1 and 2). The predicted molecular mass of the whole extracytosolic portion after the predicted cleavage site (44 kDa) approaches that of the lectin BJ38 (Ho et al., 1990b, 1994, 1999; Loh et al., 1993).

Furthermore, DAS, TMPred and TMHMM also predicted transmembrane segments in blr3942 and blr3943. Both possess the N-terminal sequence GXXXXEF at the N-terminal portion of the first predicted transmembrane helix, suggesting that they are pseudopilins that might be processed by peptidases at this position (Tomich et al., 2007). However, secondary or tertiary structure prediction of these proteins was not possible given the lack of close homologs appropriate to be used as templates. Given the positive identification of TadG, the features identified in blr3942 and blr3943, and the positions of these two loci contiguous to *tadG*, we named them *tadE* and *tadF*. The deletion in strain LP 3943 eliminated TadE and the TadF N-terminal portion—which contains the GXXXXEF sequence—from N-terminal end to Ala88 (Fig. 1).

3.2. Adhesion and infection of tadG and tadGEF mutants.

Growth of both insertional *tadG* (LP 3941) and deletional *tadGEF* (LP 3943) mutants was similar to the wild type in YM broth. However, when we intended *trans*-complementation of these mutants with a copy of the wild-type *tadGEF* gene cluster under a constitutive promoter in the replicative plasmid pCB303 (Quelas et al., 2010), growth was compromised in the presence of selective antibiotics. In addition, the plasmid was too unstable in the absence of selection pressure during the adhesion experiments, indicating that uncontrolled expression of *tadGEF* produced deleterious effects at least in this process.

When cells from liquid culture were observed under light microscopy, polar cell–cell associations of 3–4 cells were occasionally found in the three strains. Both mutants were unaffected in their ability to form biofilms in polypropylene multiwell plates. We also measured adhesiveness of dilute bacterial suspensions to hydrophobic (polypropylene) or hydrophilic (glass) beads. Adhesion of both mutants to polypropylene beads was similar to the wild type. This result differs from previous observations on the role of other pili in adhesion of *B. diazoefficiens* to polypropylene plates (Vesper and Bauer, 1986). Similarly, adhesion to glass beads was only marginally affected in the mutants (Fig. 5a).

In addition to adhesion to inert materials, we studied adhesion to soybean roots. We used two methods for quantification of adhesion. On the one hand, we counted the CFU directly on the root surfaces, which allowed an estimation of the number of cell groups that adhered to the surfaces, independently of their sizes, since either an individual cell or a clump of aggregated cells would give rise to a single CFU on the root (method I). On the other hand, we released the bacteria from the roots by vigorous agitation, which allowed the estimation of the total number of cells adhered on the roots, independently of their previous degree of clumping on the root surface (method II). Hence, the comparison of CFU counted by methods I and II allowed the estimation of the mean association of cells on the roots (Mongiardini et al., 2008). With these methods we observed that, by contrast to the above observations with inert materials, adhesion to plant roots was significantly impaired in the mutants (Fig. 5a), indicating that at least TadG or some structure related with the Flp pili recognized some specific feature on the root surface early during the process of root adhesion, an activity that could not be substituted by the paralogous of *tadE* (bll1437; Fig. 1). By comparing the adhesion index obtained with methods I and II, we estimated that the mean aggregation of rhizobia was 4–7 cells per CFU on the roots, without differences among the mutants and the wild-type. This result indicates that the decrease in adhesiveness observed in the mutants is probably related to impairments of the bacteria-root surface contact and not to diminished bacteria–bacteria surface contact. The observed decrease in adhesion of the mutants is in agreement with earlier observations of *tadG* mutants of *A. actinomycetemcomitans* (Kachlany et al., 2000).

The rapid root recognition mediated by TadGEF might be significant for infection, which occurs a few hours after the initial contact of the bacteria with the roots (Bhuvaneswari et al., 1980). Therefore, we evaluated the infectivity of our mutants on soybean roots in plastic growth pouches. We observed that infectivity was significantly reduced in both mutants in relation to the wild-type. This was visualized as a shift in the nodule frequency peak from basal to apical root regions, which were developed later after inoculation. However, total number of nodules was similar among the different strains, indicating that the mutants had a slower infection rate but not a defect either in nodule induction or colonization (Fig. 5b). Likewise, a deletion of *flp* (*pilA1*) in *E. meliloti* impaired its competition for nodulation against the wild-type, a phenotype that presumably originates in a defect in early adhesion and infectivity (Zatakia et al., 2014).

In agreement with the above results, we observed that once nodules elicited by the LP 3941 or LP 3943 mutants initiated their development, it continued normally, reaching a morphology and histology indistinguishable to that of wild-type nodules (Fig. 6). This result is coincident with the lack of expression of *tadG*, *tadE* or *tadF* in the nodule bacteroids (Pessi et al., 2007).

3.3. Concluding remarks

The above results indicated that adhesion of *B. diazoefficiens* to soybean roots might require TadGEF, which are constitutive of Flp pili and, in the particular case of TadG, it has some properties similar to the BJ38 bradyrhizobial lectin. The requirement of these structures was at the level of bacteria-root surface contact and did not imply a difference in bacterial clumping, which is in agreement with the observed lack of effects on biofilm formation. Thus, impairment of adhesion in *tadG* and *tadGEF* mutants led to a delayed infection, while it did not affect nodule development.

Conflict of interest

None.

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