Nodulation and effective nitrogen fixation of macroptilium atropurpureum (siratro) by burkholderia tuberum, a nodulating and plant growth promoting beta-proteobacterium, are influenced by environmental factors

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REGULAR ARTICLE

Nodulation and effective nitrogen fixation of *Macroptilium atropurpureum* (siratro) by *Burkholderia tuberum*, a nodulating and plant growth promoting beta-proteobacterium, are influenced by environmental factors

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Abstract
Background and aims *Burkholderia tuberum* STM678^T was isolated from a South African legume, but did not renodulate this plant. Until a reliable host is found, studies on this and other interesting beta-rhizobia cannot advance. We investigated *B. tuberum* STM678^T's ability to induce Fix$^+$ nodules on a small-seeded, easy-to-propagate legume (*Macroptilium atropurpureum*). Previous studies demonstrated that *B. tuberum* elicited either Fix$^-$ or Fix$^+$ nodules on siratro, but the reasons for this difference were unexplored.
Methods Experiments to promote effective siratro nodule formation under different environmental conditions were performed. *B. tuberum* STM678^T's ability to withstand high temperatures and desiccation was checked as well as its potential for promoting plant growth via mechanisms in addition to nitrogen fixation, e.g., phosphate solubilization and siderophore

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production. Potential genes for these activities were found in the sequenced genomes.
Results Higher temperatures and reduced watering resulted in reliable, effective nodulation on siratro. *Burkholderia* spp. solubilize phosphate and produce siderophores. Genes encoding proteins potentially involved in these growth-promoting activities were detected and are described.
Conclusions Siratro is an excellent model plant for *B. tuberum* STM678^T. We identified genes that might be involved in the ability of diazotrophic *Burkholderia* species to survive harsh conditions, solubilize phosphate, and produce siderophores.

Keywords Siratro · *Burkholderia* · Abiotic stress · Phosphate solubilization · Siderophore

Abbreviations

hpi	hours post-inoculation
dpi	days post-inoculation
CAS	chrome azurol S

Introduction

Burkholderia tuberum STM678^T, the major focus of this study, and other nitrogen-fixing members of the *Burkholderiaceae* have been isolated not only from a broad range of plant species, but also from various parts of the world—especially South Africa, Brazil, and Australia, three major centers of biological diversity (Gyaneshwar et al. 2011). The three other *Burkholderia* strains included in this study are *B. unamae* MTl-641^T, *B. silvatlantica* SRMrh20^T, and *B. silvatlantica* PVA5. They were isolated from both legume and non-legume plants, and all fix atmospheric nitrogen into ammonia (Table 1). Of these four, only *B. tuberum* nodulates legumes, but so far a model plant for the detailed study of this nitrogen-fixing, beta-proteobacterial species has not been developed. Other legume-nodulating *Burkholderia* species include *B. phymatum* STM815^T (Vandamme et al. 2002), *B. nodosa* Br3437^T, Br3641, Br2470 (Chen et al. 2007), and a number of *B. mimosarum* strains (Chen et al. 2006). The latter three species nodulate *Mimosa* spp. effectively and have closely related nodulation (*nodA*) and nitrogenase-encoding (*nifH*) genes. In contrast, the *B. tuberum* STM678^T *nodA* (<75 %) and *nifH* genes are distantly related (90 % gene identity) to the comparable genes from the *Mimosa*-nodulating strain *B. phymatum* STM815^T (Gyaneshwar et al. 2011).

Vandamme et al. (2002) were the first to name and describe the taxonomic position of *B. tuberum* STM678^T, which was isolated from the South African legume *Aspalathus carnosa* (tribe: Crotalarieae). However, this species has not yet been shown to nodulate its original host (see history in Elliott et al. 2007a and Gyaneshwar et al. 2011). Thus, Moulin et al. (2001) used siratro (*Macroptilium atropurpureum*, Phaseoleae), which is nodulated by a large number of alpha-rhizobial strains (Vincent 1970; Pueppke and Broughton 1999) to demonstrate that *B. tuberum* STM678^T could nodulate legumes. However, the siratro nodules that formed were reported as Fix$^-$ (ineffective in nitrogen fixation) and only partially infected. At the same time, *B. tuberum* STM678^T was shown to have the nodulation gene *nodA*, as well as two copies of *nodC* (Moulin et al. 2001), indicating that this strain had the potential to nodulate legumes. Later, Elliott et al. (2007a) reported that *B. tuberum* STM678^T developed Fix$^+$ (capable of nitrogen fixation) nodules not only on four species of *Cyclopia* (Podalyrieae), another genus of South African legumes that grows in the acidic soils of the fynbos, but also on siratro plants grown in glass tubes at 26 °C. These higher temperatures may be closer to the conditions encountered in the wild by the South African hosts and this legume. Siratro normally grows in moist, subtropical to tropical regions, but numerous studies have demonstrated that it is much more tolerant of dryness than other

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Table 1 Strains and plasmids used in this study

Strains	Relevant characteristics	Source or reference
Rhizobium tropici UMR1899 (CIAT899)	Acid tolerant.	Graham et al. 1982, 1994
Burkholderia tuberum STM678^T	Wild-type. Isolated from *Aspalathus carnosa* nodules in South Africa; nodulates *Cyclopia* spp. effectively.	Moulin et al. 2001; Vandamme et al. 2002; Chen et al. 2003.
Burkholderia tuberum TnGFP	GFP$^+$, Tet^R derivative of STM678.	Elliott et al. 2007a
B. unamae MTI-641^T	Wild-type. Isolated from maize and sugarcane in Mexico.	Caballero-Mellado et al. 2004
B. silvatlantica PVA5	Wild-type. Isolated from roots of *Gleditisia tricanthos* in Brazil.	de Faria et al. 1999.
B. silvatlantica SRMrh20^T	Wild-type. Isolated from maize and sugarcane in Brazil.	Perin et al. 2006.
Plasmids		
pHC60	GFP-plasmid, Tet^R	Cheng and Walker 1998.

legumes or various grass species (Ahmed and Quilt 1980; Sheriff and Ludlow 1984; Ohashi et al. 2000). Siratro survives drought because of its long taproot, which reaches distant water sources (Sheriff and Ludlow 1984). It also tolerates both acid (pH4.5) and alkaline soils (pH 8.5) (http://www.tropicalforages.info/key/Forages/Media/Html/Macroptilium_atropurpureum.htm). Although *Rhizobium*-induced siratro nodulation and growth was improved at higher temperatures (26 °C or greater), these traits were nonetheless still dependent on the inoculum employed (Herridge and Roughley 1976).

Because we observed that effective nodulation of siratro by *B. tuberum* STM678^T occurred sporadically at 21/22 °C, our objective was to establish whether temperature and other environmental factors could influence the ability of this bacterial species to establish Fix$^+$ nodules in a predictable way, with the ultimate goal of using this legume as a model system for studying host responses and for future mutant screening experiments. Lima et al. (2009) in a survey of nitrogen-fixing bacteria from various soils under different land uses in the Western Amazon region examined edaphic factors such as Ca^{2+}, Mg^{2+}, Cu^{2+}, base saturation, exchangeable bases, as well as pH on siratro nodulation. Similarly, soil pH, phosphate, and $CaCO_3$ content and granulation were found to influence rhizobial nodulation of siratro and *Mimosa* (Mishra et al. 2012). Both studies concluded that this small-seeded legume is a valuable plant for trapping both alpha- and beta-rhizobia.

We compared siratro nodulation by *B. tuberum* with that induced by *Rhizobium tropici* CIAT899, which is reported to be tolerant of acid soils and high concentrations of aluminum (Graham et al. 1982). In this study, we investigated temperature, desiccation, and artificial substrate type, each of which potentially could dictate effective nodulation of siratro by *B. tuberum* STM678^T under laboratory conditions. The fact that Elliott et al. (2007a) grew seedlings in 30 mL glass tubes suggests that siratro root nodulation was not negatively affected by light as has been observed for some legumes, e.g., *Pisum sativum* (van Brussel et al. 1982) and *Lotus japonicus* (Suzuki et al. 2011), so we did not examine this parameter in our studies.

In addition to agar-based cultures, we employed mixtures of perlite, vermiculite, and sand in both open containers and in closed Magenta jars, the latter with small holes drilled into the tops because closed containers often accumulate ethylene, which inhibits nodulation (Lee and LaRue 1992). We also utilized *Vigna unguiculata* (cowpea) as a host because it is reported to be nodulated by both *B. tuberum* (F.D. Dakora, personal communication) and *R. tropici* (Hernandez-Lucas et al. 1995). Also, because many bacteria promote growth via mechanisms other than nitrogen fixation, we examined whether *B. tuberum* STM678^T and three additional non-nodulating, but nitrogen-fixing *Burkholderia* species were able to secrete siderophores and to solubilize phosphate, two common mechanisms of plant growth promotion. Although *B. unamae* has already been shown to have many plant growth-promoting activities (Caballero-Mellado et al. 2007; Castro-González et al. 2011), we included it in our analysis for comparison.

Lastly, we looked for DNA sequences in the recently sequenced genomes of four plant-associated *Burkholderia* species (Table 1) that might be responsible for their performance under environmental stress. These include such traits as the ability to: 1) synthesize trehalose, a disaccharide with an unusual α,α 1-1

linkage between two glucose molecules, which is synthesized following desiccation or temperature stress by several alpha-rhizobia including *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (Streeter and Gomez 2006); 2) solubilize phosphate from forms that have limited solubility (Rodríguez et al. 2006); and 3) produce siderophores for obtaining iron from soil environments (Andrews et al. 2003). The presence of these traits as well as the ability to induce nitrogen-fixing nodules make the siratro-*B. tuberum* system an important new model system to study various facets of the interaction between plants and symbiotic microbes, particularly the beta-rhizobia.

Materials and methods

Culture of bacteria and plants

The bacteria used in this study are listed in Table 1. The plasmid pHC60 was mobilized into *R. tropici* CIAT899 using a triparental mating. For routine culture, the *Rhizobium* strains were grown either on TY or RDM (Vincent 1970) containing 10 μg/mL tetracycline (tet) to select for the plasmids, whereas the *Burkholderia* strains were grown on LB without salt or on YEM with or without 10 μg/mL tet. All bacteria were grown at 30 °C.

Seeds of *M. atropurpureum* were scarified for 1–2 min. using a scarifying cup (Brigham and Hoover 1956) prior to surface-sterilization. *V. unguiculata* subsp. *unguiculata* (PI339603) seeds were not scarified. Siratro seeds were first briefly washed with 95 % ethanol for 5 min. and then sterilized in a 50 mL conical centrifuge tube with full-strength commercial bleach on a rotating platform for 35–45 min. Five rinses of sterile water followed the bleaching step with the last rinse overnight. Cowpea seeds were placed in 10 % bleach for 10 min after the initial 95 % ethanol step, and also copiously rinsed with sterile water after sterilization. The sterilized seeds were placed on water agar (1 %) for 3 days in the dark to assess germination and sterility before planting. For seeds to be sown in Magenta jars, autoclaved perlite:vermiculite (1:1 by volume) or perlite:vermiculite:sand (1:1:1 by volume) watered with ¼ strength Hoagland's solution minus N was utilized. Magenta jar tops had holes drilled into them, and the holes were covered with sterile rayon adhesive film (AeraSeal, Excel Scientific, Wrightwood, CA) to permit airflow. The entire apparatus was autoclaved before seed planting. When plants touched the top of the Magenta jar, an autoclaved extender consisting of a Magenta jar unit with one end sawed off was added to the system to allow further shoot elongation.

Seeds were also sown directly after sterilization in autoclaved containers half-filled with either perlite: vermiculite (1:1 by volume) or a mixture of sand: perlite:vermiculite (1:1:1 by volume) watered with ¼ strength Hoagland's solution minus N. The moisture in the dishpans was monitored with a soil moisture meter (General Specialty Tools & Instruments; Model DSMM500) to give an initial reading of 10–15 %, the pans were weighed, and once a week, ¼ strength Hoagland's solution minus N was added to the dishpans to the same weight to restore the moisture levels.

For the studies in 30 mL culture tubes, either Jensen's or ¼ strength Hoagland's solution minus N media were used for agar slants (1 % Plant Agar, PhytoTechnology Laboratories, Shawnee Mission, KS), and the bottoms of the tubes were covered with aluminum foil. Two siratro seeds were planted after the sterilization procedure per tube and inoculated immediately with 1 mL of 10^6/mL bacteria resuspended in sterile water or phosphate-buffered saline after centrifugation. For the dishpans, 100–150 mL of inoculum were added, whereas 20 mL were used for the Magenta jars and the polypropylene pots.

Microscopy

For confocal microscopy, GFP-labeled bacteria in association with plant roots and nodules were visualized using a Zeiss LSM 5 Exciter. Images were obtained with the Zeiss ZEN acquisition/imaging software using a 5× or 10× objective and excitation at 488 nm for observation of GFP, and 545 nm for observation of root autofluorescence.

Light microscopic analysis using epifluorescence was performed as described in Fujishige et al. (2008).

Temperature influence on bacteria and desiccation effects on bacteria and siratro

For the temperature studies, 10 μL spots of lag/early log (13 h), log (17 h), and stationary (40 h) phase cultures of *B. tuberum* and *R. tropici* were diluted to a starting concentration of OD_{600}=0.1 and then

spotted in triplicate onto LB agar plates and incubated at temperatures of 30 °C, 37 °C, or 40 °C for 24 h.

For the desiccation studies, assays were set up according to the method described by Hugenholtz et al. (1995). Briefly, nitrocellulose filters (0.45 μm pore) were sterilized under UV light for 24 h before spotting the center in triplicate with 100 μL of each overnight bacterial culture (17 or 40 h) diluted in fresh LB to OD_{600}=0.1. Filters were placed inside standard-sized sterile petri dishes and were allowed to air-dry, then placed inside a desiccation chamber filled with fresh silica desiccant (Sigma) and sealed using a vacuum line. The filters remained undisturbed within the chamber for 24 h, after which they were transferred with a sterile forceps onto fresh LB agar plates and incubated at 30 °C for 4 days to recover desiccation-resistant strains.

For the in vivo studies on water-stress effects, siratro plants were grown in sterilized perlite:vermiculte (dishpans) or perlite:vermiculte:sand (pots and Magenta jars) and watered with ¼ strength Hoagland's solution minus N. After 2 weeks of watering twice weekly, one set of *B. tuberum*-inoculated siratro plants kept the initial regime whereas the second set was watered every other week with the moisture content restored to ca. 15 % at this time. Treatments representing the uninoculated as well as inoculated plants were observed for signs of desiccation stress after another 4–6 weeks.

Phosphate and siderophore assays

Phosphate solubilization by strains *B. tuberum* STM678^T, *B. unamae* MTl-641^T, *B. silvatlantica* strain PVA5, and *B. silvatlantica* strain SRMrh20^T was performed using both solid and liquid Pikovskaya (1948) (PVK) medium as modified by Xie et al. (2009). The liquid medium contained 36.7 mM $CaHPO_4$. The bacterial strains were grown in liquid TY until stationary phase (24–30 h) at which time the cells were harvested by centrifugation (8,000 × g, 10 min), and the pellets washed with sterile water three times to remove traces of the TY medium. The cell pellets were diluted to OD_{600}=0.2 in sterile water. For solid cultures, 10 μL droplets were spotted onto plates, which were incubated at 30 °C for up to 10 days. The size of the colony and the clearing zone around the colony were measured. Quantification of the phosphate solubilized in liquid cultures was performed by use of the QuantiChrom Phosphate Assay kit (BioAssay systems; cat # DIPI-500), recording the absorption at 620 nm.

Both liquid and solid PVK medium were set to an initial pH of 7.55, 6.53, and 5.28, and then inoculated in triplicate. For the inoculated cultures, a 1 mL aliquot of a single culture of each strain was selected at random at 24 and 48 h intervals, centrifuged for 3 min to pellet the cells, and the pH of the supernatant was recorded. As a control, tubes of uninoculated medium of the same starting pH as the inoculated tube were prepared and treated the same as the test samples.

All four strains were also tested on solid PVK medium supplemented with 0.035 % bromocresol purple, which has been shown to be an effective pH indicator ranging in color from purple at pH7.0 to yellow at pH5.0 (Yao and Byrne 2001). A triplicate set of the plates were inoculated with 10 μL of 48-h grown cultures and incubated at 30 °C. Colony size, zones of clearance, and zones of color diffusion were measured after 72 h, and the ratio of zone of clearance to colony size calculated. The ratio was used to account for any difference in growth rate that occurred on media of different pH levels.

Chrome azurol S (CAS) agar medium devoid of nutrients was used as an indicator of the presence of siderophores using 0.9 % (w/v) agar as a gelling agent. The medium for a liter of the overlay medium included: CAS, 60.5 mg; hexadecyltrimetyl ammonium bromide (HDTMA or CTAB), 72.9 mg; piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 30.24 g; and 10 mL of 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl (Schwyn and Neilands 1987). Bacteria were first grown in liquid TY until OD_{600}=0.1, at which time 10 μL droplets were spotted onto TY agar plates, allowed to dry, and incubated at 30 °C for 2 days. Molten CAS agar was poured over the bacterial spots grown on solid TY. The overlaid plates were observed for yellow to orange halos around the bacterial spots.

Results

Nodulation tests Earlier, we inoculated siratro plants that had been grown in dishpans containing perlite and vermiculite and watered twice weekly with nitrogen-free medium with *B. tuberum* STM678^T, and found that at a day/night temperature of 22 °C/18 °C only ineffective nodules formed on the roots (data not shown). Because Elliott et al. (2007a) reported the presence of

Fix$^+$ nodules on siratro when the temperature was increased to 26 °C day/22 °C night, we tested siratro nodulation not only at a higher temperature, but also under restricted water availability and in various substrates to see whether reliable nodulation and nitrogen fixation could be achieved with *B. tuberum*.

When dry weights and shoot lengths were measured after 6 weeks of growth in dishpans at higher temperatures and restricted water availability, a significant increase in the *B. tuberum*-inoculated plants compared to the uninoculated controls was observed using a one-way ANOVA with Tukey's post-hoc test (Fig. 1a). Nevertheless, under these conditions, plants were slow to show evidence of nitrogen fixation. It took approximately 4–6 weeks before the foliage turned a dark green. The shoot lengths of the *B. tuberum*-inoculated plants (Fig. 1b) in particular were highly variable because not all plants developed the elongated internodes seen in Fig. 2a at the same time. Surprisingly, siratro plants inoculated with *R. tropici* and grown under the same conditions were small, had yellow leaves, short internodes, and did not develop nitrogen-fixing nodules (Figs. 1b and 2b). Their average dry weight and stem length were also lower than those of the *B. tuberum*-inoculated siratro plants, but not statistically different than the values obtained for the uninoculated controls (Fig. 1).

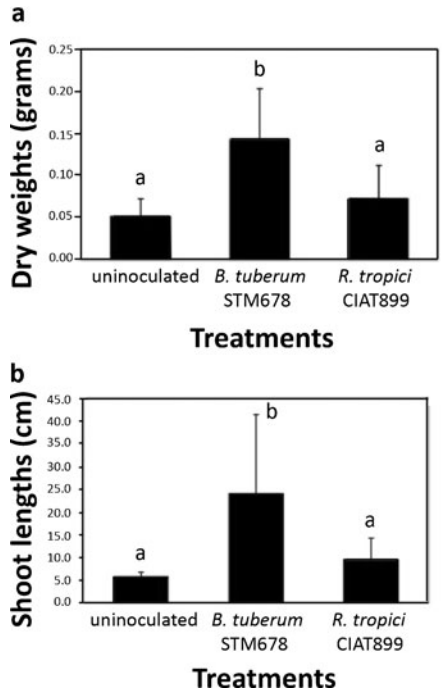

Fig. 1 *Burkholderia tuberum* STM678^T enhances plant biomass and siratro shoot length better than *R. tropici* CIAT899. The plants were harvested 6 weeks post inoculation, their shoot lengths measured, and then the plants were dried in a 65 °C oven for 3 days to obtain biomass measurements. *B. tuberum* STM678^T- or *R. tropici* CIAT899-inoculated plants showed an increase in dry weight (**a**) and shoot lengths (**b**) compared to uninoculated plants. Overall, *B. tuberum*-inoculated plants were more robust with respect to height, weight, and greenness compared to *R. tropici*-inoculated plants. One-way ANOVA with Tukey's post hoc test was done for comparison of the means. Different letters represent values that differ significantly, $P<0.01$

Siratro nodules are determinate in that they lack a persistent nodule meristem (Hirsch 1992). A comparison of nodule development between GFP-labeled *R. tropici* CIAT899 and *B. tuberum* STM678^T demonstrated very little difference in the early nodulation stages. Both rhizobia elicited root hair deformation and entered the root hairs via infection threads (Fig. 2d and e). *B. tuberum*-elicited nitrogen-fixing nodules were established on a well-developed siratro root system (Fig. 2c), and the shoots also expanded, producing climbing stems at about 6–8 weeks (Fig. 2a). Examination of the internal structure of siratro nodules showed that the nodule cells consisted of two types: interstitial cells devoid of bacteria and cells filled with green-fluorescing bacteria (Fig. 2f). This type of cell arrangement is characteristic of nodules that develop in response to infection thread penetration (Sprent 2007).

Plants in Magenta jars were nodulated within 3 weeks, and nitrogen fixation was also delayed. Under the same conditions, *R. tropici* CIAT899 again induced only ineffective nodules. We repeated the experiment in glass tubes and found that although the *B. tuberum*-inoculated plants formed Fix$^+$ nodules, the *R. tropici*-inoculated plants did not (data not shown). However, the *B. tuberum*-inoculated plants became water-stressed after 8 weeks in the glass tubes due to the drying of the agar. They also occasionally formed bacteria-free callus-like structures on the roots, even on the uninoculated plants, indicating that an agar-based screen in glass tubes might not be suitable for making meaningful conclusions about nodulation and nitrogen fixation ability.

Temperature and desiccation stress Because siratro is tolerant of dry conditions and nodulation by *B. tuberum* is enhanced at higher temperatures, we reasoned that the bacteria used to inoculate siratro might tolerate these abiotic stresses. *B. tuberum* and *R. tropici* were thus compared for their potential to tolerate

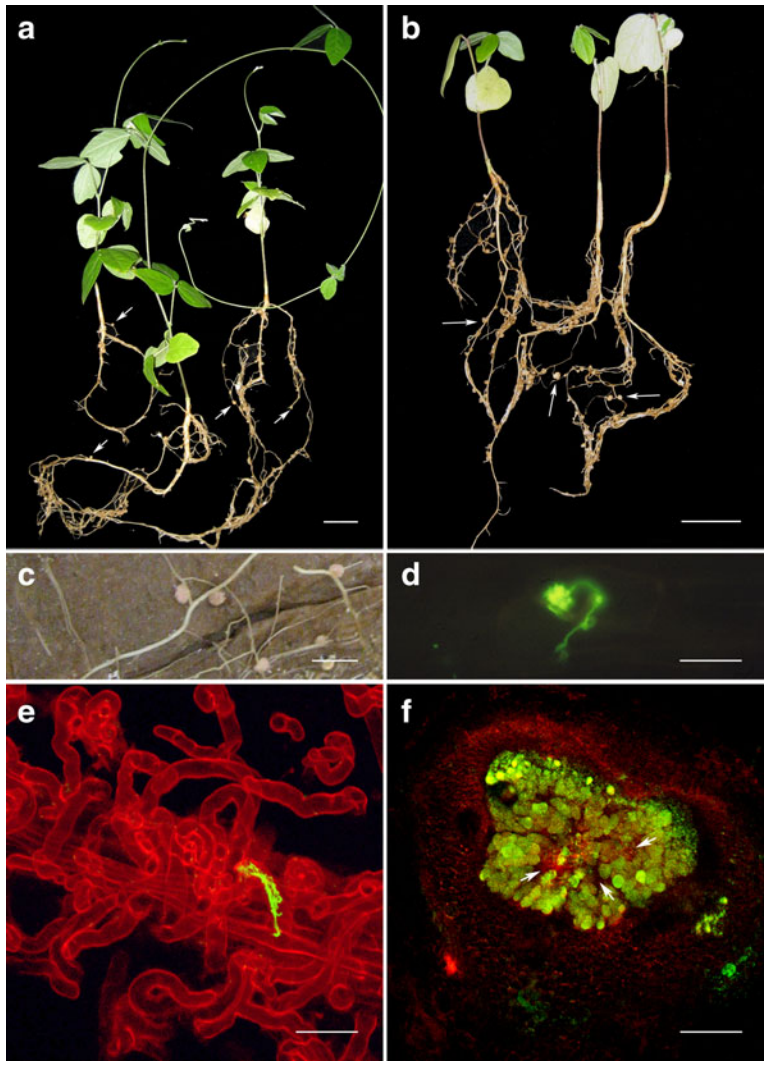

Fig. 2 Effects of *B. tuberum* STM678^T and *R. tropici* CIAT899 on siratro nodulation. **a** Healthy, siratro plant with elongated internodes and root nodules (*arrows*) had been inoculated with *B. tuberum* STM678^T and harvested 7.5 weeks post-inoculation. Bar=2 cm. **b** Stunted, yellow siratro plant with numerous nodules (*arrows*) that had been inoculated with *R. tropici* CIAT899 and harvested 7.5 weeks post-inoculation. Bar=2 cm. **c** Pink nodules from a *B. tuberum* STM678^T-inoculated siratro 6 weeks post-inoculation. Bar=5 mm. **d** Infection thread formed within a siratro root hair by GFP-labeled *R. tropici* CIAT899. Bar=50 μm. **e** Infection thread formed within a siratro root hair by GFP-labeled *B. tuberum* STM678^T. Bar=50 μm. **f** Young siratro nodule filled with GFP-labeled *B. tuberum* STM678^T. Interstitial cells (*arrows*) are devoid of STM678^T. Bar=1 mm

temperature and desiccation stress in culture. *R. tropici* had been shown earlier to acquire thermo-tolerance in response to high temperature exposure, although under such conditions, the bacteria did not fix nitrogen symbiotically (Michiels et al. 1994). We hypothesized that tolerance to severe stress could be one mechanism whereby *B. tuberum* could effectively nodulate siratro under adverse conditions. A number of possible mechanisms, such as trehalose synthesis, involvement of heat shock proteins, or glycine betaine/proline accumulation as well as exopolysaccharide production, may confer temperature and/or desiccation tolerance to *Burkholderia*.

Experimental analysis Incubation at temperatures of 30 °C, 37 °C, and 40 °C, demonstrated that *B. tuberum* inoculated onto plates during the early growth phases, both lag and log, exhibited limited growth at 30 °C and no growth at the higher temperatures. In contrast, *R. tropici* at the same stages of growth grew at both 37 °C and 40 °C, confirming this species' tolerance to high temperatures. On the other hand, for cultures incubated at stationary phase, *B. tuberum* grew at all temperatures whereas *R. tropici* stationary phase cells survived only at 30 °C.

Because the *B. tuberum*-inoculated plants formed effective nodules at higher temperatures, we reasoned that inoculated host plants might be more desiccation-tolerant than the uninoculated plants. *R. tropici* was not included in these experiments because it induced a Fix$^-$ nodule phenotype on siratro at 26 °C. Following an additional 6–8 weeks of desiccation-simulating

conditions (see Materials and Methods), the *B. tuberum*-inoculated siratro plants were smaller than the unstressed *B. tuberum*-inoculated plants, but nonetheless were green and developed pink Fix^+ nodules, strongly suggesting that the bacteria were fixing nitrogen (Supplementary Information Fig. 1). Although many of the well-watered, inoculated plants produced vining internodes, fewer of the water-stressed inoculated plants did. By contrast, the uninoculated plants in both treatments were stunted, yellow, and lacked nodules.

In vitro studies investigating the desiccation tolerance of *B. tuberum* and *R. tropici* showed that both species were viable following simulated dry conditions (data not shown). Although *R. tropici* tolerated desiccation stress in early (exponential) growth phase and late (stationary) phase cultures, *B. tuberum* exhibited the most robust recovery from desiccation stress when in the stationary growth phase. One explanation for both the thermal and desiccation tolerance results reported here may be the overall slower growth of *B. tuberum* $STM678^T$ compared to *R. tropici* CIAT899 and hence longer recovery time.

Genome analysis Because only a draft genome of *R. tropici* CIAT899 exists (Pinto et al. 2009), we could not make comparisons between it and the *Burkholderia* species with regard to specific gene sequences. In some cases, we utilized other *Rhizobium* genomes to make the comparison between alpha- and beta-rhizobia.

We hypothesized that trehalose, two glucose molecules held together by a glycosidic α-(1, 1) bond and important in temperature and desiccation tolerance in alpha-rhizobia, may also be significant for stress adaptation in *B. tuberum*. *Bradyrhizobium japonicum* possesses three different trehalose biosynthetic pathways: 1) from UDP-glucose and glucose-6-phosphate via the enzyme trehalose 6-phosphate synthase (TPS; EC:2.4.1.15; *otsA*); 2) from the conversion of maltooligosaccharides to maltooligosyl trehalose via the enzyme maltooligosyl trehalose synthase (MOTS; EC:5.4.99.15; *treY*); and 3) from the direct catalysis of maltose to trehalose by way of trehalose synthase (TS; EC:5.4.99.16; *treS*) (Streeter and Gomez 2006).

The four beta-rhizobia in Table 1 have genes for the same three pathways as *Br. japonicum* as well as multiple copies of them. For the first pathway, the *B. tuberum* genome contains one operon consisting of *otsA* (TPS) and *otsB* (trehalose 6-phosphatase; EC:3.1.3.1.2; Fig. 3) as well as three additional copies of *otsA*, two of which are paralogs (data not shown). A similar situation exists for the other three *Burkholderia* strains (Fig. 3), except that none of the *otsA* genes have paralogs as seen in the *B. tuberum* genome.

Genes encoding MOTS and maltooligosyl trehalose hydrolase (EC: 3.2.1.14; *treZ*) were also found in the genomes of all four *Burkholderia* species. The gene neighborhoods are also very well conserved among the four *Burkholderia* species, and are similar in terms of gene organization to that of *Rhizobium* and *Bradyrhizobium* species, especially *Br. japonicum* USDA110 (Fig. 4). The gene sequences in the TS operon—glycogen branching enzyme (*glgX*; also known as *treX*), and alpha-1,4-glucan:alpha-1,4-glucan 6-glycosyltransferase (*glgB*)—were also detected in both the beta- and alpha-rhizobial genomes.

Lastly, the genomes of the plant-associated *Burkholderia* strains, except for *B. unamae*, possess gene sequences encoding a neutral trehalase (EC:3.2.1.28; *treF*). This DNA sequence was not detected in the alpha-rhizobial genomes used in our analysis (data not shown).

Phosphate solubilization Available phosphate (P) is generally in short supply in soils. Most of it is tied up in insoluble forms in either organic or inorganic complexes, which are unavailable to plants. Plant growth-promoting bacteria have the ability to hydrolyze organic P via a variety of mechanisms including: 1) nonspecific acid phosphatases (NSAPs), 2) phytases, and 3) phosphonatases and C-P lyases (Rodríguez et al. 2006). By contrast, inorganic phosphate is usually solubilized via the production of organic acids (Rodríguez and Fraga 1999).

Experimental analysis Inorganic P-solubilizing activity was observed for each of the four plant-associated *Burkholderia* species when PVK agar medium was supplemented with glucose as a carbon source, but *B. tuberum* was not as effective at solubilizing P compared to non-nodulating *Burkholderia* spp. (Fig. 5a). *R. tropici* CIAT899 solubilized P at a level similar to *B. tuberum* but statistically lower compared to *B. unamae* (Fig. 5a). Additionally, only PVA5 and $SRMrh20^T$ were positive for solubilization of P when sucrose instead of glucose was added as a carbon source (Supplementary Information Fig. 2). Results from the QuantiChrom™ assay kit, which detects insoluble P confirm that the four *Burkholderia* spp. solubilize P after 24 h in liquid PVK medium (Fig. 5b). *R. tropici* CIAT899 was not tested in this assay.

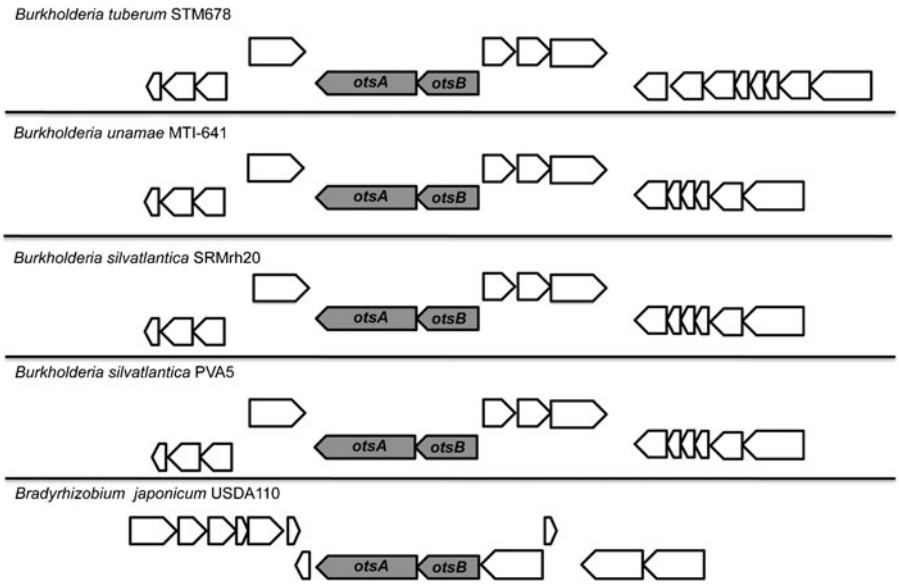

Fig. 3 Gene maps of the *otsA*/*otsB* operon. The gene neighborhoods (shaded) of the four *Burkholderia* spp. are conserved with that of *Bradyrhizobium japonicum* USDA110

Because organic acid secretion is one of the principle ways that bacteria accomplish inorganic P solubilization (Rodríguez and Fraga 1999), we examined whether the ability of the four plant-associated *Burkholderia* strains to solubilize phosphate was correlated with an alteration in pH. With the exception of *B. tuberum* STM678^T, all the media in which the *Burkholderia* strains grew showed a pH drop close to 3.30 after 20 h of incubation regardless of the initial pH of the medium (Table 2). By 44 hpi, *B. tuberum* also elicited a significant pH drop in the medium. All four *Burkholderia* spp. solubilized $CaHPO_4$ to varying degrees on solid PVK plates by 72 hpi (Supplementary information Fig. 2).

When grown in PVK medium containing bromocresol purple at pH7.55, 6.53, and 5.28, the two *B. silvatlantica* strains demonstrated the greatest amount of phosphate solubilization at all the pH values tested whereas *B. tuberum* exhibited the least (Table 3). The sizes of the clearance zones and of the diameters of the color diffusion zones, related to the change from neutral to acidic pH, are presented in Table 3. A correlation between the sizes of the diffusion zone and the zone of P solubilization is observed.

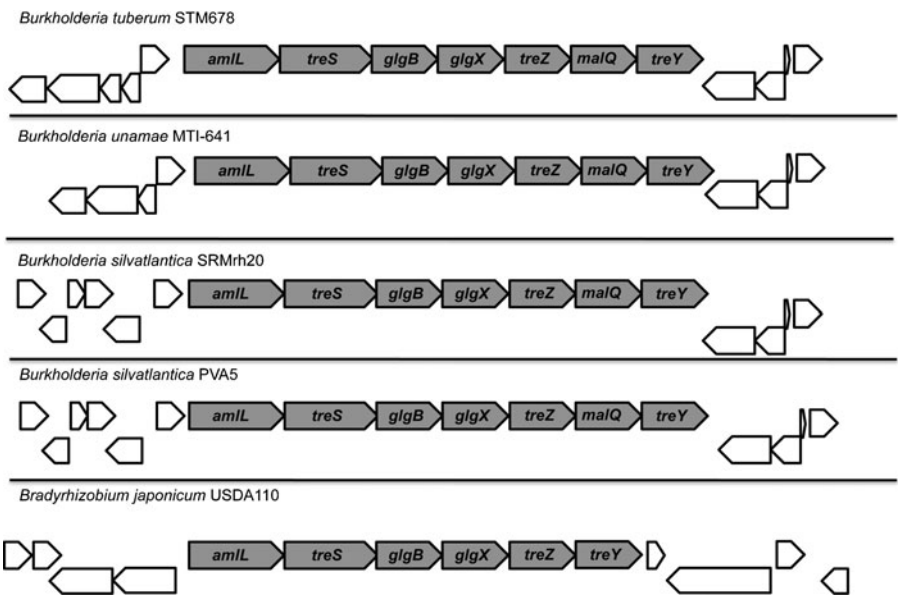

Fig. 4 MOTS and maltooligosyl trehalose hydrolase gene maps. The gene neighborhoods (shaded) of the four *Burkholderia* spp. are conserved with that of *Bradyrhizobium japonicum* USDA110

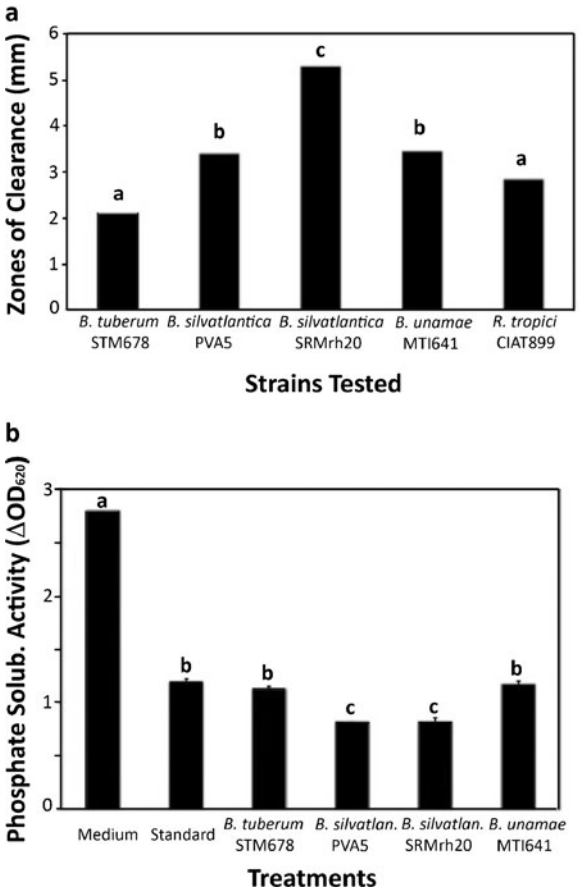

Fig. 5 *Burkholderia* species solubilize inorganic phosphate. **a** P-solubilizing activity for the four plant-associated *Burkholderia* species and *R. tropici* CIAT899 under the same conditions with glucose as a carbon source. Measurements indicate the zone of clearance (in mm), calculated by measuring the surrounding halo after 48 h after equal OD readings of bacteria had been inoculated onto plates. **b** Quantification of P-solubilization activity in broth culture (the medium alone contains 36 μM P) using the QuantiChrom™ assay kit, which detects insolubilized phosphate. *B. tuberum* and *B. unamae* solubilize P at levels close to that of the standard (30 μM) after 24 h, whereas B. silvatlantica PVA5 and SRMrh20 solubilize significantly more inorganic P. One-way ANOVA with Tukey's post hoc test was done for comparison of the means. Different letters represent values that differ significantly, $P<0.01$

Genome analysis Inorganic phosphate solubilization activity that is related to the production of organic acids (Rodríguez and Fraga 1999) is a property of several enzymes, particularly glucose dehydrogenase and the cofactor pyrroloquinoline quinone (PQQ). We searched for amino acid sequences of similar proteins in the *Burkholderia* genomes by querying with a *Sinorhizobium meliloti* sequence annotated as a probable glucose dehydrogenase (pyrroloquinoline-quinone) protein predicted to be involved in 6-phosphogluconate synthesis via gluconate (Fig. 6a). Each *Burkholderia* species has at least one coding sequence, and *B. silvatlantica* SRMrh20^T and PVA5 each have two copies (Table 4). Overall, the *B. tuberum* genome has the fewest number of gene sequences that could be involved in phosphate solubilization, which may explain the difference in activity of this strain on the PVK plates versus the other three.

Although we did not test the four *Burkholderia* strains for their ability to solubilize organic phosphate experimentally, we found gene sequences for NSAPs and similar enzymes, but not genes encoding phytases or phosphonatases and C-P lyases (Table 4). The genomes of all four *Burkholderia* spp. examined possess DNA sequences that encode a purple acid phosphatase-like protein (Table 4), which in *B. cenocepacia* has a pH optimum of 8.5 (Yeung et al. 2009). Purple acid phosphatase (PAP) generally breaks down phosphoric acid esters and phosphoric acid anhydrides, but the *B. cenocepacia* PAP was reported to be active towards phosphotyrosine, phosphoserine, and phosphoenolpyruvate (Yeung et al. 2009). The gene neighborhoods of the diazotrophic *Burkholderia* strains are almost identical while that of *B. tuberum* STM678^T differs.

A gene for a *Burkholderia*-type acid phosphatase (AcpA), generally used for the breakdown of organic P complexes, is also present in the genomes of all four of the species studied herein and in some alpha-rhizobia (Fig. 6b). Because *B. tuberum* exhibited lower phosphate solubilization compared to the non-nodulating strains, we also compared the genomes of two of the sequenced nodulating strains with the genomes of *B. unamae*, *B. silvatlantica* PVA5, and *B. silvatlantica* SRMrh20^T. The *acpA* gene neighborhoods of both *B. tuberum* and *B. phymatum* STM815^T differed not only from each other, but also from the comparable neighborhoods in *B. unamae* and in the two *B. silvatlantica* species. Interestingly, for the genomes of two sequenced *B. mimosarum* strains, the *acpA* neighborhoods matched those of the non-nodulating strains, suggesting that the arrangement of genes putatively involved in organic phosphate solubilization is not correlated with nodulation ability per se. Also, two

Table 2 pH of PVK medium 20 and 44 h post-inoculation (hpi)

Strain	pH7.55[a]		pH6.53[a]		pH5.28[a]	
	20 hpi	44 hpi	20 hpi	44 hpi	20 hpi	44 hpi
Uninoculated[b]	6.01	6.21	5.38	5.54	5.03	4.86
B. tuberum STM678^T	7.35±0.04	3.93±0.02	6.45±0.03	3.71±0.16	4.33±0.14	3.65±0.08
B. unamae MTI-641^T	3.33±0.05	3.17±0.01	3.38±0.06	3.09±0.03	3.28±0.09	3.14±0.02
B. silvatlantica PVA5	3.41±0.04	3.18±0.01	3.28±0.05	3.20±0.12	3.27±0.06	3.07±0.01
B. silvatlantica SRMrh20^T	3.35±0.01	3.16±0.03	3.32±0.02	3.02±0.02	3.27±0.07	3.07±0.01

[a] Original pH of medium and subsequent measurements in triplicate are displayed

[b] The uninoculated control was incubated under the same conditions as the inoculated cultures. The values indicate only a single reading recorded per time point

AcpA-encoding genes were detected in the genomes of the non-nodulating strains, with no orthologs in *B. tuberum* STM678^T, which had only one *acpA* (Table 4).

Siderophore secretion

Alpha-rhizobia are known to bind iron through the secretion of siderophores, and several plant-associated *Burkholderia* species have already been shown to be capable of this activity (Caballero-Mellado et al. 2007; Suárez-Moreno et al. 2012; Weisskofp et al. 2011). Similar to phosphate solubilization, these activities are beneficial because they support the growth and development of plants, especially in marginalized soils, by making unattainable mineral nutrients available.

Experimental analysis Iron acquisition via siderophore secretion was detected using the CAS overlay agar plate assay on all four *Burkholderia* species. The presence of yellow/orange halos around the bacterial colonies indicates the presence of iron-binding siderophores. *B. tuberum* consistently formed the smallest halos whereas *B. unamae* established the largest (Supplementary information Fig. 3). To determine whether a connection could be made between halo size and genes involved in siderophore production, we analyzed the sequenced genomes of the four *Burkholderia* species.

Genome analysis Genome analysis revealed that several genes described as siderophore receptors, siderophore transport system components, and siderophore-interacting proteins are found in the genomes of the four plant-associated species. Interestingly, the *B. unamae*

Table 3 Zones of clearance and color change on PVK agar plates containing bromocresol purple 72 hpi

Strain	pH7.55[a]		pH6.53[a]		pH5.28[a]	
	Halo:colony average[b]	Diameter of color diffusion[c] (mm)	Halo:colony average[b]	Diameter of color diffusion[c] (mm)	Halo:colony average[b]	Diameter of color diffusion[c] (mm)
B.tuberum STM678^T	1.33	11	1.29	11	1.43	n.r.
B.unamae MTI-641^T	1.53	26	1.67	21	1.63	n.r.
B. silvatlantica PVA5	2.00	31	1.79	28	1.81	n.r.
B.silvatlantica SRMrh20^T	1.92	29	1.80	33	1.69	n.r.

[a] Original pH of medium and subsequent measurements in triplicate are displayed

[b] The ratio of the zone of clearance to the colony size is reported. A value >1 indicates solubilization of $CaHPO_4$

[c] The average diameter of the diffusion of color is shown. A change in color from purple to yellow indicates a decrease in the pH of the medium

n.r., not recorded. Measurements for pH5.28 are not shown due to a complete diffusion of yellow throughout the plates

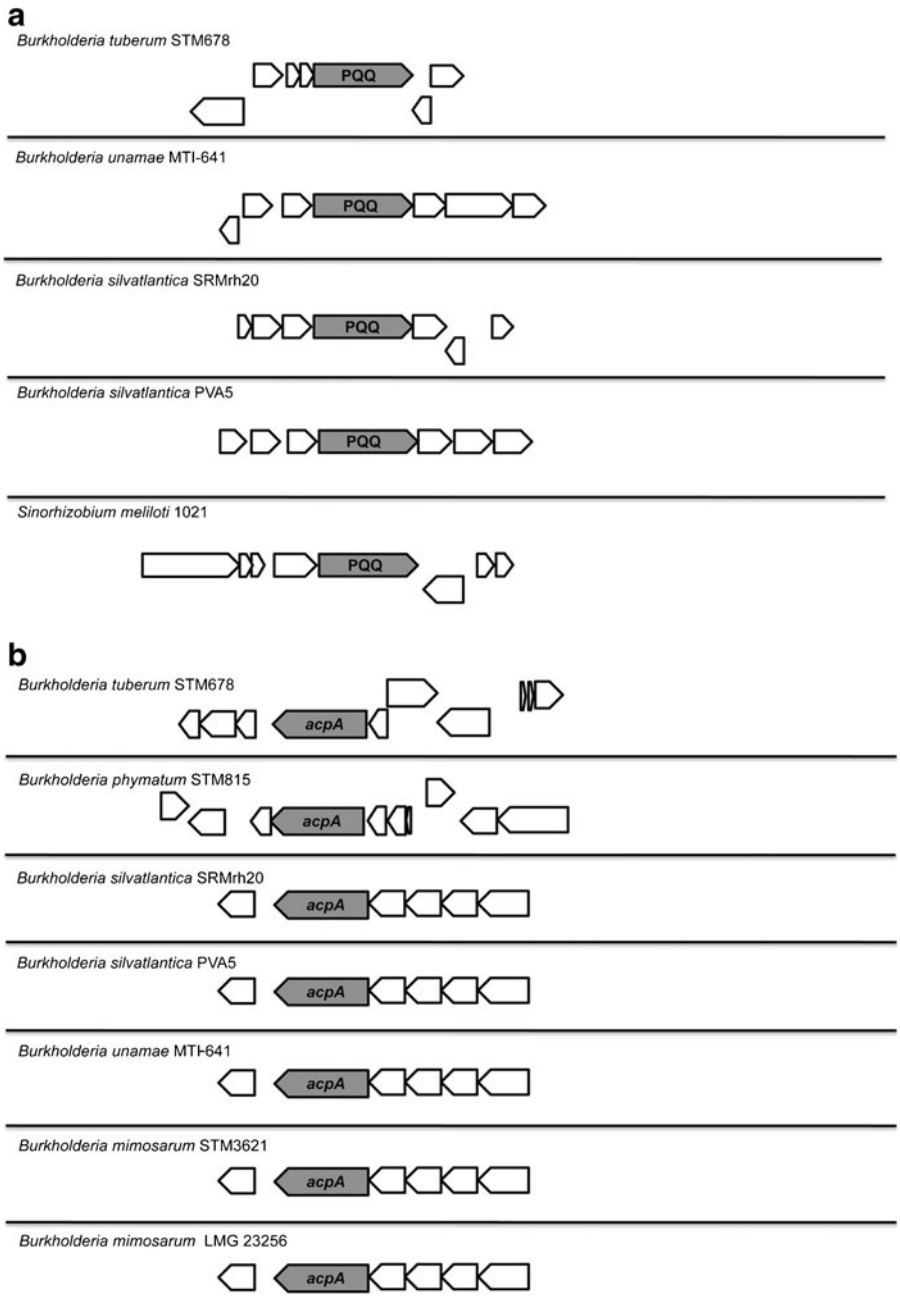

Fig. 6 P solubilization gene maps. **a** The PQQ gene neighborhoods of four *Burkholderia* spp. compared to that of *S. meliloti*. **b** The orientation and surrounding neighborhoods for the *acpA* gene in the nodulating species *B. tuberum* and *B. phymatum* are different from the non-nodulating *Burkholderia* species. However, the nodulating *B. mimosarum* strain gene neighborhoods are identical to those of *B. unamae* and the two *B. silvatlantica* strains

genome contains 16 genes potentially encoding TonB-dependent siderophore receptors whereas each *B. silvatlantica* strain has four (Table 5). *B. tuberum* has three genes annotated as TonB siderophore receptors (Table 5), one of which may be non-functional (see below). Some of the TonB-dependent siderophore receptors listed in Table 5 are in an operon (see consecutive gene numbers and similar superscripted letters) with 1) an ABC-type cobalamin/Fe^{3+}-siderophore transport system, 2) an ATPase component (EC:3.6.3.34), 3) an ABC-type cobalamin/Fe^{3+}-siderophore transport system, permease component, and 4) an ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component genes (Table 5). In addition, several ABC-type Fe^{+3}-siderophore transport system, and ATPase and permease component genes were found in the genomes, but were not associated with siderophore protein-encoding genes. Genes encoding a potential periplasmic component of a

Table 4 List of locus tags for gene sequences encoding proteins that are likely to be involved in phosphate solubilization

Gene function and name	Locus tag			
	B. tuberum STM678^T	*B. unamae* MTI641^T	*B. silvatlantica* PVA5	*B. silvatlantica* SRMrh20^T
Inorganic Phosphate Solubilization				
Membrane-bound PQQ-dependent dehydrogenase glucose/quinate/shikimate family				
	GCWU001488_06170	GCWU001489_05348	GCWU001490_06856	GCWU001491_04527
	-	-	GCWU001490_04976	GCWU001491_06776
Organic Phosphate Solubilization				
Putative purple alkaline phosphatase				
	GCWU001488_04845	GCWU001489_02415	GCWU001490_05774	GCWU001491_07093
Acid phosphatase AcpA				
	GCWU001488_05111	GCWU001489_03077	GCWU001490_05252	GCWU001491_06457
	-	GCWU001489_01734	GCWU001490_01837	GCWU001491_02862

hydroxamate transport system were also present (Table 5). Such a transport system had been detected earlier in *B. unamae* (Caballero-Mellado et al. 2007).

The first of three *B. tuberum* siderophore receptors, GCWU001488_01779, is adjacent to a gene putatively encoding a protein involved in ABC-type cobalamin/Fe^{3+}-siderophore transport (GCWU001488_01778) (Table 5). GCWU001488_01779 is orthologous and 69–70 % identical to genes encoding putative siderophore receptors in *B. glumae* (bglu_2g14800) and *B. gladioli* (bgla_2g10490). The immediate gene neighborhoods of *B. glumae* and *B. gladioli* are almost identical to that of *B. tuberum*, but a putative transposon adjacent to GCWU001488_01778 suggests that this insertion in the *B. tuberum* operon could result in the truncation of a putative ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component (GCWU001488_01777). On the other hand, homologs to either the ATPase or periplasmic component genes observed in *B. unamae* and *B. tuberum* were not detected in the two strains of nitrogen-fixing *B. silvatlantica* examined in this study (Table 5).

The second putative TonB siderophore receptor (GCWU001488_04562) in *B. tuberum* is orthologous to a gene of the same name in several *Burkholderia* species and also in *B. unamae* (64 % identity; GCWU001489_04829), but not to genes in either of the two *B. silvatlantica* strains (Table 5). Like the *B. unamae* siderophore receptor, this gene is not adjacent to other iron transporter-encoding genes. The *B. tuberum* siderophore receptor is predicted to be in COG1629 (Fe receptor), whereas the *B. unamae* ortholog is annotated as a protein in COG4774 (catecholate receptor). However, so far no experimental evidence exists for the latter type of receptor in *B. unamae*.

The third gene annotated as a *B. tuberum* TonB siderophore receptor (GCWU001488_05039) is orthologous to a comparable sequence in a number of *Burkholderia* species, including the ones studied here (from 74 to 76.3 % sequence identity), and also to genes in the *B. xenovorans* (84.2 %) and *B. phytofirmans* (84 %) genomes. A gene that putatively encodes 2OG-Fe(II) oxygenase (GCWU001488_05038) is adjacent to the TonB siderophore receptor as well as to its ortholog siderophore receptors in the other three nitrogen-fixing *Burkholderia* spp. (Table 5).

In *B. unamae*, a gene annotated as lysine/ornithine N-monooxygenase (GCWU001489_07200) shows 59–60 % identity to a *Bordetella pertussis* gene encoding a biosynthesis enzyme (*alcA*) for alcaligin, a dihydroxamate siderophore. Although similarities existed between the *B. unamae* sequence and a *B. tuberum* gene, only 31 % identity on the protein level was observed between them. Moreover, no other *alc*-like genes were found in *B. tuberum* or in the two *B. silvatlantica* strains. Adjacent to the *B. unamae alcA*-like gene lies a sequence encoding an arabinose efflux protein and adjacent to that is an *alcB-like* gene with 70.9 % identity to a protein annotated in *Burkholderia* sp. CCGE1002 as a siderophore biosynthesis protein (YP_003610151.1), and with ca. 58 % identity to an alcaligin biosynthesis protein in two strains of *Achromobacter xylosoxidans* (EGP43454.1, YP_003979104.1), and 55 % identity to an *alcB* gene product in *Bordetella pertussis* Tohama I

Table 5 Number (and locus tags) of genes in *Burkholderia* spp. genomes that are likely to encode proteins for siderophore-mediated iron uptake

	B. tuberum STM678T	*B. unamae* MTI-641 T	*B. silvatlantica* PVA5	*B. silvatlantica* SRMrh20T
TonB-dependent siderophore receptor	3 GCWU001488_01779[z,a] GCWU001488_04562[z'] GCWU001488_05039[x,k]	16 GCWU001489_01847[x] GCWU001489_02174[x,l] GCWU001489_02646[y] GCWU001489_03097[b,y] GCWU001489_04829[x] GCWU001489_05097 [y] GCWU001489_05762[c,y] GCWU001489_05953[d,y] GCWU001489_05997[e,z] GCWU001489_06000[z] GCWU001489_06507[f,y] GCWU001489_07702[g,y] GCWU001489_08020[x] GCWU001489_08176[h,z] GCWU001489_08634[i,y] GCWU001489_08731[y]	4 GCWU001490_04037[x,m] GCWU001490_05013[x] GCWU001490_05887[y] GCWU001490_05954[y]	4 GCWU001491_03470[y] GCWU001491_06738 [x] GCWU001491_06980[y] GCWU001491_07451[x, n]
ABC-type cobalamin/Fe^{3+}-siderophore transport systems, ATPase components (EC:3.6.3.34)	1 GCWU001488_01778[a] GCWU001488_03141[o]	5 GCWU001489_03782[p] GCWU001489_05763,[c] GCWU001489_05949[d] GCWU001489_06508[f] GCWU001489_07448	1 GCWU001490_02093[q]	1 GCWU001491_00020[r]
ABC-type Fe^{3+}-siderophore transport system, permease component	2 GCWU001488_01777[a*] GCWU001488_03140[o]	5 GCWU001489_03783 [p] GCWU001489_05765[c] GCWU001489_05951[d] GCWU001489_06510[f] GCWU001489_07447	1 GCWU001490_02094[q]	1 GCWU001491_00021[r]
ABC-type siderophore export system, fused ATPase and permease components	0	4 GCWU001489_05766,[c] GCWU001489_05950[d]	0	0

Table 5 (continued)

	B. tuberum STM678T	*B. unamae* MTI-641 T	*B. silvatlantica* PVA5	*B. silvatlantica* SRMrh20T
		GCWU001489_06511 [f], GCWU001489_08630		
ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component	1 GCWU001488_01777[a*] GCWU001488_03145	5 GCWU001489_03778 GCWU001489_05764[c] GCWU001489_05952[d] GCWU001489_06509[e] GCWU001489_07446	1 GCWU001490_02089	1 GCWU001491_00016
2OG-Fe(II) oxygenase	1 GCWU001488_05038[k]	1 GCWU001489_02175[l]	1 GCWU001490_04038[m]	1 GCWU001491_07450[n]
Ferric dicitrate sensor; FecR family	0	4 GCWU001489_05998[d] GCWU001489_07701[f] GCWU001489_08177[h] GCWU001489_08636	0	0
Lysine/ornithine N-monooxygenase (*alcA*-like)	0	GCWU001489_07200[o]	0	0
Siderophore biosynthesis protein (*alcB*-like)	0	GCWU001489_07202 [o]	0	0
Siderophore synthetase component (*alcC*-like)	0	1 GCWU001489_07203[o]	0	0
Siderophore ferric iron reductase (*alcD*-like)	0	1 GCWU001489_07204[o]	0	0
Uncharacterized protein related to arylsulfate sulfotransferase involved in siderophore biosynthesis	1 GCWU001488_05679	1 GCWU001489_06523	0	0
FAD-binding 9, siderophore-interacting domain protein	1 GCWU001488_05828	1 GCWU001489_02672	1 GCWU001490_04846	1 GCWU001491_06907

[a-r] Genes with the same superscripted letter are within the same operon; [x] COG4774, outer membrane receptor for monomeric catechol; [y] COG4773, outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid; [z] COG1629, outer membrane receptor proteins, mostly Fe transport; [zz] COG4206, outer membrane cobalamin receptor protein; [*] truncated

(NP_881084.1). Immediately adjacent to the *alcB*-like sequence is a gene coding for a siderophore synthetase component (GCWU001489_07205), with 63 % amino acid sequence identity to *alcC* in *A. xylosoxidans* (EFV887439.1), 61 % identity to the *alcC* gene in the various *Bordetella* species (NP_881085.1, CAA3891.1, and NP_885606.1), and 59 % to *alcC* in *Pseudomonas stutzeri* (YP_004716031.1). Lastly, a siderophore ferric iron reductase for alcaligin synthesis (*alcD*), (GCWU001489_07204), which is annotated in *Burkholderia* spp. CCGE1002 as a hypothetical protein (YP_003610153.1; 61 % identity), is found in *B. unamae* (Table 5, Fig. 7). The gene call was made on 42 % identity to the *alcD* gene in *Bordetella pertussis*, *B. bronchiseptica*, and *B. parapertussis* (NP_881086.1, CAA3891.1, and NP_885607.1). These and other proteins, such as arylsulfate sulfotransferase (Mathew et al. 2001), may be involved in siderophore synthesis and are listed in Table 5.

Discussion

B. tuberum STM678^T was originally identified as a *Bradyrhizobium* species (Muofhe and Dakora 1998) because of its slow growth on plates upon isolation from nodules, and was named *Bradyrhizobium aspalati* because it was isolated from *Aspalathus carnosa* (see Elliott et al. 2007a and Gyaneshwar et al. 2011). However, it induced effective nodulation on siratro roots (Elliott et al. 2007a), although Moulin et al. (2001) had reported the phenotype as Fix$^-$.

We wanted to obtain a deeper understanding of the conditions that modulated whether Fix$^+$ or Fix$^-$ nodules are produced on siratro so that effective nodulation could be obtained on a reliable and predictable basis. We found that a porous soil mixture with infrequent watering resulted in *B. tuberum* STM678^T-induced Fix$^+$ nodules on siratro. However, when *R. tropici* CIAT899, a strain that is also tolerant of acid soils and thus expected to be a good reference strain, was used as an inoculum under the conditions tested, Fix$^-$ nodules developed on siratro. In contrast, cowpea plants included in the same dishpans as the siratro plants nodulated and fixed nitrogen (data not shown).

An analysis of the literature concerning *R. tropici* CIAT899 does not provide definitive answers. Hernandez-Lucas et al. (1995) reported that this strain induced two types of nodules on siratro—those that had leghemoglobin and those that lacked it and contained senescent, darkened cells. It was not clear from this report whether the nodules that contained leghemoglobin were actually fixing nitrogen. Collavino et al. (2005) tested the *guaA* mutant of *R. tropici* CIAT899 on siratro and found that it elicited ineffective nodules, but a mature, wild-type, Fix$^+$ nodule was not illustrated in this study. However, unpublished data from one of the authors (O.M. Aquilar, pers. com.) indicates that Fix$^+$ nodules were elicited by this strain. The difference in phenotypes described in our results and the previously published work cannot be explained at this time, in part due to the differences in growth conditions. Changes in temperature and water availability could make a significant difference in nodule outcome. Moreover, it is also possible that *R. tropici* CIAT899 only marginally nodulates siratro as suggested by the results of Hernandez-Lucas et al. (1995).

R. tropici CIAT899 was originally described as *R. leguminosarum* bv. *phaseoli* and nodulates bean and *Leucaena* sp. effectively (Martínez-Romero et al. 1991; Riccillo et al. 2000) at high temperatures although acetylene reduction activity was reduced in bean (Michiels et al. 1994). This strain's ability to nodulate a diverse number of legumes and under different environmental conditions is most likely conditioned by the large variety of Nod factors produced by CIAT899. *R. tropici* Nod factors have typical backbones consisting of four or five β-1,4 linked *N*-acetylglucosamine residues, but the number and types of substitutions on the lipo-chitooligosaccharide varies significantly depending on the environmental conditions. For example, under low pH, 59 Nod factors were produced whereas 29 different structures were synthesized at neutral pH (Móron et al. 2005). Additional and different Nod factors were also detected following salt stress (Estévez et al. 2009). In contrast, Boone et al. (1999) identified only two major Nod factors of *B. tuberum* STM678^T, then known as *Bradyrhizobium aspalati*. These factors have either a tetrameric or pentameric *N*-acetylglucosamine backbone, but instead of having substitutions on the reducing end as observed in alpha-rhizobia, these Nod factors are substituted only on the non-reducing end of the molecule. In any case, whether or not different Nod factors arise following abiotic stress has not been tested for the beta-rhizobia. Other than for *B. tuberum* STM678^T, no other Nod factor structures have been determined for the nodulating *Burkholderia* species.

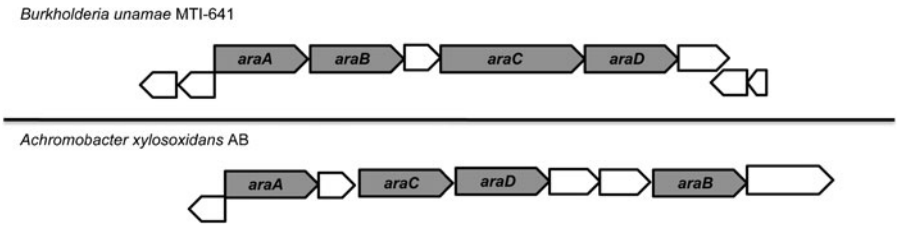

Fig. 7 Genes potentially encoding a hydroxamate siderophore. Of the four *Burkholderia* genomes surveyed, only the genome of *B. unamae* has genes coding for alcaligin, a hydroxamate siderophore. The *B. unamae* gene neighborhood is compared to the orthologous operon in *Achromobacter xylosoxidans* AB

B. tuberum STM678^T-inoculated siratro plants remained green and developed pink, Fix$^+$ nodules even under desiccating conditions, which differs from *Rhizobium* strains in general (Michiels et al. 1994). Both *B. phymatum* STM815^T and *B. tuberum* STM678^T have been shown to fix nitrogen *ex planta*, although not to the levels of *B. vietnamiensis* TVV75 (Elliott et al. 2007b). This ability may be the reason that *B. tuberum* is able to fix nitrogen symbiotically under stressful conditions.

Besides nitrogen fixation, rhizosphere bacteria enhance plant growth in a number of diverse ways, among them protecting plants from abiotic stress, and improving phosphate or iron nutrition. Various environmental stresses such as reduced water availability and increased temperature also significantly influence nodulation effectiveness. We found that siratro nodulation was improved by higher temperatures and was effective even under conditions of reduced water availability. In contrast, *R. tropici* elicited Fix$^-$ nodules on siratro when grown under these conditions. Trehalose is synthesized by a broad range of organisms including plants, bacteria, archaea, and insects (Iturriaga et al. 2009) as a means of dealing with either desiccation or thermal stress. The beta-rhizobial genomes, except for *B. unamae*, possessed an additional trehalose biosynthetic pathway compared to alpha-rhizobial genomes. A partial *otsA* gene sequence (AFH35528.1) from *R. tropici* exhibited only 46 % amino acid sequence identity to the *otsA* sequence in the *Burkholderia* spp. However, we cannot make a more direct comparison with *R. tropici* because only a draft genome analysis of this species has been published (Pinto et al. 2009). Information about the number of trehalose biosynthetic pathways in this species is thus not available at this time.

Of the four *Burkholderia* spp. investigated, *B. silvatlantica* PVA5 and SRMrh20^T exhibited the largest amount of phosphate solubilization activity and also possessed the most gene sequences potentially involved in inorganic and organic phosphate breakdown based on analysis of the sequenced genomes. The drop in pH upon mineralizing $CaHPO_4$ in the PVK plates strongly suggests that production of glucuronic or another organic acid may be involved in inorganic P solubilization, and that the plant-associated *Burkholderia* species are likely inhabitants of soils that are low in soluble phosphate. *Burkholderia* species have been described previously as having a preference for acidic soils (Garau et al. 2009; Dos Reis et al. 2010). A recent study of *Burkholderia* in French Guinea suggests that beta-rhizobia exclusive of *Cupriavidus taiwanensis*, which prefers alkaline soils, were dominant in low pH soils (Mishra et al. 2012). In a similar vein, Estrada-de los Santos et al. (2011) isolated only a few *Burkholderia* species associated with agricultural plants growing in the alkaline soils of northern Mexico in contrast to *Cupriavidus* species, which were isolated more frequently. Isolation of *Burkholderia* species from alkaline soils in Australia has been reported (see Gyaneshwar et al. 2011), but more studies are needed.

Siderophores, which make iron available, are important for host colonization by bacteria (Mietzner and Morse 1994). Of the four nitrogen-fixing *Burkholderia* strains, *B. unamae* produced the largest halos using the CAS-overlay medium whereas *B. tuberum* exhibited the least activity. Genome analysis showed that *B. unamae* contains more gene sequences for TonB-dependent associated siderophore receptors and their associated genes than did the other three species, which may explain the increased activity. Although *B. unamae* does not nodulate plants, it was originally isolated from the roots of maize and sugarcane (Table 1) and thus can be classified as a plant-associated diazotroph. The genome of *B. tuberum*, which contains *nod* genes, has three siderophore receptors and the genomes of the other

two *B. silvatlantica* strains each have four. Both *B. silvatlantica* SRMrh20^T (Perin et al. 2006) and PVA5 (de Faria et al. 1999) were isolated from the interstices of plant roots and fix nitrogen, but are not capable of nodulating roots.

Earlier reports indicated that siratro's small size, ability to grow under controlled conditions, prolonged seed viability, and promiscuity with respect to nodulation (Vincent 1970; Pueppke and Broughton 1999) supported its usefulness as a plant for studying responses to rhizobia. Recently, Lima et al. (2009) and Mishra et al. (2012) endorsed its value as a trap plant for both alpha- and beta-rhizobia. Based on our studies, we believe that this small-seeded legume is also an excellent model system for analyzing plant responses to *B. tuberum* inoculation under a wide range of environmental conditions.

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