Plant Soil (2011) 347:125–136
DOI 10.1007/s11104-011-0833-8

REGULAR ARTICLE

Analysis of the endophytic lifestyle and plant growth promotion of *Burkholderia terricola* ZR2-12

Ilona Gasser · Massimiliano Cardinale · Henry Müller · Stefanie Heller · Leo Eberl · Nicole Lindenkamp · Chlud Kaddor · Alexander Steinbüchel · Gabriele Berg

Received: 18 March 2011 / Accepted: 17 May 2011 / Published online: 10 June 2011

Abstract Members of the genus *Burkholderia* are highly versatile bacteria that can be beneficial as well as pathogenic for their eukaryotic hosts. Furthermore, many strains exhibit a remarkable biotechnological potential. To study the ecosystem function and lifestyle of *B. terricola*, we analysed the interactions with plants and survival in soil as well as the mechanisms behind it. We used a combination of in vitro and ad planta assays to study *Burkholderia*-plant interaction and assess the role of poly-β-hydroxybutyrate (PHB). Additionally, DsRed-labelled bacteria were analysed by confocal laser scanning microscopy (CLSM) to study root colonisation. *B. terricola* ZR2-12 treatment resulted in enhanced growth of sugar beet plants with a more than doubled biomass relative to the non-treated control. The strain was a remarkable good root coloniser, which was found in rhizosphere as well as endorhiza of sugar beet up to 10 $\log_{10}$ CFU g^{-1}. Using CLSM, we observed that ZR2-12 cells form large micro-colonies along the apoplastic spaces of the root. Xylem vessels were colonised by smaller aggregates and single cells, whereas in root tips mainly single cells were present. The colonisation patterns differed strongly between older and younger parts of the roots. PHB production of ZR2-12 (up to 70% (w/w) of cell dry mass) provided a competitive advantage for rhizosphere colonisation. *B. terricola* ZR2-12 belongs to the plant-associated *Burkholderia* cluster with biotechnological potential due to its excellent root colonisation and plant growth promotion.

Keywords *Burkholderia terricola* · PHB · CLSM · Plant growth promotion · Rhizosphere competence

Responsible Editor: Peter A.H. Bakker.

I. Gasser · M. Cardinale · H. Müller · G. Berg (✉)
Institute of Environmental Biotechnology,
Graz University of Technology,
Graz, Austria
e-mail: gabriele.berg@tugraz.at

S. Heller · L. Eberl
Institute of Plant Biology, University of Zurich,
Zurich, Switzerland

N. Lindenkamp · C. Kaddor · A. Steinbüchel
Institut für Molekulare Mikrobiologie und Biotechnologie,
Westfälische Wilhelms-Universität Münster,
Münster, Germany

Introduction

The genus *Burkholderia*, which was described by Yabuuchi et al. in 1992, comprises a diverse group of Betaproteobacteria with currently more than 60 validly described species. Members of the genus *Burkholderia* are known for their interaction with plants, animals and humans (Coenye and Vandamme 2003). Although some *Burkholderia* species are known for their human pathogenic potential (Mahenthiralingam et al. 2005), some other *Burkholderia* species exhibit beneficial effects for their host plants. Recently, it has

been suggested that the plant-associated *Burkholderia* form a cluster that is comprised of at least 29 non-pathogenic species (Caballero-Mellado et al. 2007; Suárez-Moreno et al. 2010). Examples are nitrogen-fixing *Burkholderia* strains like *B. tropica* (Reis et al. 2004) and *B. silvatlantica* (Perin et al. 2006), strains capable of nodulating leaves and roots of different plants (van Oevelen et al. 2002; Vandamme et al. 2002), species with an intimate association with bryophytes (Vandamme et al. 2007), and endophytic *Burkholderia* strains showing high 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Sessitsch et al. 2005). ACC deaminase activity, which is a widespread feature within the plant-associated cluster of *Burkholderia*, reduces the level of the plant hormone ethylene and protects plants against stress (Hontzeas et al. 2004; Onofre-Lemus et al. 2009). In addition, many *Burkholderia* species exhibit antifungal antibiotics, and protected plants against diseases caused by pathogenic fungi (McLoughlin et al. 1992; Hebbar et al. 1998). While several species have been characterised in great detail and the genomes of many species have been sequenced (Lin et al. 2008), less is known for other *Burkholderia* species. For instance, the lifestyle as well as ecosystem function of *Burkholderia terricola,* a species described by Goris et al. (2002), are largely unknown (Coenye and Vandamme 2003). While the type strain was isolated from agricultural soil degrading a wide range of carbon sources, we isolated another strain *B. terricola* ZR2-12 of this species from the rhizosphere of sugar beet, which has been shown to produce large amounts of poly-β-hydroxybutyrate (PHB) (Gasser et al. 2009). However, the ecological role of this organism in the rhizosphere is yet unexplored.

The rhizosphere is characterised by an enhanced abundance and activity of microorganisms relative to bulk soil as a result of root exudation (Sørensen 1997; Bais et al. 2006). Bacteria with antagonistic activity against plant pathogens have been found to be selectively enriched in the rhizosphere (Berg et al. 2002, rev. in Berg and Smalla 2009), including beneficial *Burkholderia* species (Caballero-Mellado et al. 2007) as well as PHB-producing bacteria (Gasser et al. 2009). Synthesis of storage compounds such as PHB is a strategy of bacteria to increase survival in changing environments (Steinbüchel and Valentin 2006). Only a few plant-associated *Burkholderia* strains are known to produce PHB (Brämer et al. 2002; Gasser et al. 2009), and little is known about the function of PHB production in *Burkholderia* species. Interestingly, for free-living nitrogen-fixing bacteria of the genera *Azospirillum, Herbaspirillum* and *Rhizobium,* a relationship between PHB accumulation and rhizosphere colonisation has been described (Castro-Sowinski et al. 2010). It has been suggested that PHB can also play a role in agricultural applications like microbial inoculants for biological control (Kadouri et al. 2005).

To understand the ecological role of *B. terricola* in terrestrial ecosystems, the interaction of *B. terricola* ZR2-12 (Gasser et al. 2009) with sugar beet plants and its persistence in soil were investigated. The ability to colonise the root—the rhizosphere as well as the endorhiza (root tissues below the epidermis)—was compared with *Pseudomonas fluorescens* WCS365, which is known to be an excellent root coloniser (Dekkers et al. 1998; Kamilova et al. 2005). Furthermore, using DsRed labelled *Burkholderia* cells in combination with confocal laser scanning microscopy (CLSM), plant colonisation patterns were studied in detail. The role of PHB in root colonisation was analysed using a PHB-negative mutant.

Material and methods

Bacterial strains

Burkholderia terricola ZR2-12 (EMBL no. FN313521.1) was isolated from the rhizosphere of sugar beet (*Beta vulgaris* L.) in Deggendorf, Germany (Gasser et al. 2009). For soil and plant experiments, a spontaneous rifampicin-resistant mutant of *B. terricola* ZR2-12 was used. This mutant was characterised by the same growth parameters and traits as the wild-type. For colonisation assays, *Pseudomonas fluorescens* WCS365 was used for comparison because this is a well-investigated model strain for an excellent root coloniser (Dekkers et al. 1998; Kamilova et al. 2005).

Generation of the PHB-negative mutant *B. terricola* ZR2-12_PHB$^-$

In order to investigate the role of PHB biosynthesis in the root colonisation ability of *B. terricola*, we constructed a PHB-negative mutant of *B. terricola* ZR2-12. Mutants were obtained by chemical mutagenesis with ethyl methanesulfonate (EMS, Merck,

Darmstadt, Germany) according to Schlegel et al. (1970). Cell material of a 1 ml culture (grown for 24 h) was pelleted by centrifugation (2 min, 8000 rpm) and washed with phosphate buffered saline (PBS, pH 7.2). The pellet was re-suspended in 1 ml PBS containing 30 μg of EMS and incubated for 30 min. Afterwards the pellet was washed twice with PBS, re-suspended in mineral salts medium (MM) medium and plated onto MM agar plates (Schlegel et al. 1961). After 24 h incubation at 30°C, colonies were washed from plates with 1 ml MM and subjected to saccharose-density-centrifugation. (12500×*g*, 2 h). The lower fraction was taken for further processing according to Steinbüchel (2001). After chemical mutagenesis, a phenotypic screen of over 3000 colonies resulted in six strains, which were subjected to GC analysis, Nile red staining and microscopic analysis (Spiekermann et al. 1999; Timm and Steinbüchel 1990). Five of these strains produced lowered amounts of PHB (10.3% w/w of cell dry weight (CDW) when compared to the wild-type); one strain (ZR2-12_PHB$^-$) without PHB production was selected. Nile red staining and microscopic affirmation were done continuously. No difference in growth rate in nutrient broth (Sifin, Berlin, Germany) at 30°C between the wild-type and the PHB-negative mutant was observed. Mutant ZR2-12_PHB$^-$ was also indistinguishable from the wild-type in all other phenotypic features investigated (Table 1).

Seed priming and plant growth conditions

Seeds of sugar beet (cv. Calida, KWS SAAT AG, Einbeck, Germany) were surface disinfected by washing in water for 5 min, incubating in 70% ethanol for 5 min and by an additional washing step in sterile water. To inoculate seeds with bacteria, 1 g of seeds was incubated for 6 h in liquid culture of the respective strains grown in mineral salts medium (MM) (Schlegel et al. 1961) for 24 h at 30°C. For root competition assay, sugar beet seeds were co-inoculated with *B. terricola* ZR2-12 and *P. fluorescens* WCS365 (Dekkers et al. 1998). For control treatment, 1 g of seeds was incubated with sterile MM medium. After priming, inoculated seeds were dried back under laminar flow over night and sown in 400 ml polypropylene containers or in culture tubes type 'De Wit' (Duchefa, Haarlem, The Netherlands) containing standardised soil substrate consisting of a mixture from

Table 1 Phenotypic and biochemical characterisation of *B. terricola* ZR2-12 and its PHB$^-$ mutant. Different in vitro and ad planta tests in order to compare properties related to plant-microbe interaction and ecological function are listed

Strains	Antifungal activity and biocontrol						Plant-microbe-interaction				Storage compounds[8]			PHB production	
	Antifungal activity[1]	Biological control[2]	Growth on 3% NaCl w/v	Siderophore production[3]	AHL[4]	Proteolytic activity[3]	Plant growth promotion[5]	Nitrogen fixation[3]	ACC[6]	Auxin[7]	Wax ester	Triacyl-glyceride	PHB	growth condition	storage condition
ZR2-12	–	–	–	+	–	–	+	–	+	–	–	–	+	32% PHB w/w	70% PHB w/w
ZR2-12_PHB-	–	–	–	+	–	–	+	–	+	–	n.d.	n.d.	–	–	–

[1] Dual culture plate assay to assess antifungal in vitro activity against *Rhizoctonia solani* and *Verticillium dahliae*

[2] Biological control of *Rhizoctonia solani* AG4 on seedling of sugar beet 'Calida' according to Keijer et al. (2003)

[3] Plate assay (+ is >two millimetre clearance halos)

[4] Strains were cross-streaked against the reporter strain *Chromobacter violaceum* CV026 according to McClean et al. (1997)

[5] Increase of plant biomass of sugar beet 'Calida' compared to a non treated control under greenhouse conditions

[6] Plate assay on medium containing ACC as a sole nitrogen source according to Brown and Dilworth (1975)

[7] Colorimetric assay of liquid culture supernatants according to Sarwar and Kremer (1995)

[8] Determined by thin layer chromatography

slightly to fully decomposed raised bog peat and perlite at a pH-value of 5.6 (Gramoflor Vechta, Germany). Prior planting, the substrate was autoclaved at 121°C for 20 min. For microscopically observations, plants were grown in seed germination pouches (Mega International, Minneapolis, USA) moistened with 10 ml of sterile water. Plants were generally grown under greenhouse conditions with 14 h of light at 23°C.

Plant growth promotion and root colonization

To determine plant growth promotion ability of *B. terricola* ZR2-12, sugar beet plants were grown in polypropylene pots under greenhouse conditions with 14 h of light at 23°C. After 1, 2, and 3 weeks, plantlets were harvested and fresh weight of all plant parts was determined gravimetrically. The experiment was done in three replicates each with six seeds per pot and repeated three times.

Bacterial cell numbers were determined after 7, 14 and 21 days post-inoculation. According to the sampling time 0.4, 3.5 or 13.0 g of root material was sampled into sterile plastic bags and homogenised with mortar after adding 3 ml, 50 ml and 100 ml, respectively, of NaCl solution (0.9%). Suspensions were serially diluted and plated on nutrient agar II (Sifin, Berlin, Germany). After 3 days of incubation at 30°C colony forming units (CFU) were determined and calculated to CFU per gram root fresh weight. In experiments with the spontaneous rifampicin-resistant mutant of *B. terricola* ZR2-12, medium was supplemented with 80 μg ml^{-1} rifampicin (Fluka, St. Louis, USA).

Confocal Laser Scanning Microscopy (CLSM)

For microscopic analyses of ZR2-12 and its PHB-negative mutant (ZR2-12_PHB^-) associated with sugar beet roots (cv. Calida), DsRed-labelled strains were employed. To this end, plasmid pIN69 (Vergunst et al. 2010) was transferred into the wild-type and the mutant by triparental mating as described by De Lorenzo and Timmis (1994). Transconjugants were selected on *Pseudomonas* isolation agar (PIA, Becton Dickinson, Sparks, USA) supplemented with 100 μg ml^{-1} trimethoprim. In the absence of antibiotic selection, the plasmids remained stable for more than 2 weeks (data not shown). Resulting transconjugants ZR2-12 [pIN69] and ZR2-12_PHB^- [pIN69], respectively, were applied to seeds prior growing in seed germination pouches. This was done in three replicates (six seeds per pouch). After 1, 2 and 3 weeks sugar beet roots were observed with a TCS SPE confocal microscope (Leica Microsystems, Germany) using the following laser lines (nm)/detection wavelengths (nm): DsRed, 532/570−620; plant tissues (autofluorescence), 405/425−490. Confocal stacks were acquired with Z-step of 0.2–0.5 μm and sequential activation of laser lines/detection windows. Maximum projections, volume renderings and 3D models were created with the software IMARIS 7.0 (Bitplane, Zurich, Switzerland). Final figures were assembled and labelled with Adobe Photoshop CS3, version 10.0.1 (Adobe Systems Inc., USA).

Phenotypic and biochemical characterisation of *B. terricola* ZR2-12

ACC (1-aminocyclopropane 1-carboxylate) deaminase activity was tested on Brown and Dilworth (BD) minimal medium (Brown and Dilworth 1975) containing 0.7 gL^{-1} ACC as a sole nitrogen source. BD plates containing 0.7 gL^{-1} NH_4Cl served as positive controls and BD plates containing no nitrogen source were used as negative controls. ACC deaminase production was monitored after 7 days of incubation at 30°C. Indole-3-acetic acid synthesis was tested by a colorimetric assay in liquid culture according to Sarwar and Kremer (1995). Growth medium consisting of glucose (5.0 g), yeast extract (25.0 mg), L-tryptophane (0.2 g) and distilled water (to 1.0 l) was inoculated with cell material from the pre-culture (0.5x TSA, 30°C, 24 h) to an OD600 of 0.5. After cultivation at 20°C and 150 rpm for 72 h, cell free supernatant was mixed with Salkowski reagent (50.0 mM $FeCl_3$, 35.0% [v/v] perchloric acid) at the ratio of 3:2 and incubated for 30 min, due its sensitive reaction to light, in the dark. IAA concentration was measured photospectrometrically using the microplate-reader Spectramax-250 (Molecular devices, Union city, USA) at 530 nm and quantified using standard curve. Production of C4-C6 AHLs was checked by on-plate assay according to McClean et al. (1997). Strains to be tested were cross-streaked against the reporter strain *Chromobacter violaceum* CV026 on nutrient agar II. Development of purple

color due to violacein production of *C. violaceum* was evaluated after 24 h incubation at 30°C. For assessment of production of extracellular proteolytic enzymes, ZR2-12 was screened by plating on nutrient agar II containing 10% skim milk powder. Clearance halos indicating protease activity were measured after an incubation time of 3 days at 30°C. Detection of siderophores was performed under iron-limited conditions using universal siderophore assay developed by Schwyn and Neilands (1987). Iron (III) ions form a complex 10 with chrome-azurol-S dye. For testing, cell material was transferred onto chrome azurol-S medium and incubated for 3 to 5 day at 30°C. The diameter of the discoloured zone, which is indicative of siderophore activity, was measured. Thin layer chromatography (TLC) of lipid extracts from whole cells was performed as described previously (Uthoff et al. 2005). Triolein or oleate and oleyl oleate were applied as reference substances for triacylglycerides and wax ester, respectively, using hexane/diethyl ether/acetic acid (90:7.5:1 or 80:20:1, v/v/v) as solvent system for the separation of wax esters and triacylglycerides, respectively.

Antifungal activity in vitro was determined by a dual-culture assay on Waksman agar (WA). Fungi applied in this bioassay were the plant pathogens *Rhizoctonia solani* AG 4 (RHI Ben 4 from sugar beet) and *Verticillium dahliae* V25 (strain collection Graz University of Technology). Zone of inhibition was measured after 5 days of incubation at 20°C by the method of Berg et al. (2002). Additionally, *R. solani* AG4 was used for an in vitro biocontrol experiment with 3 days old seedling of sugar beet 'Calida' according to Keijer et al. (2003).

Starvation and drought stress experiment

Sterilised soil substrate (15 g; Gramoflor Vechta, Germany) was placed in a Petri dish and inoculated with 420 µl of a 24 h-culture of ZR2-12 or ZR2-12_PHB$^-$ in three replicates. After incubation for 60 days at room temperature, inoculated substrate was homogenised by suspending in 0.9% NaCl solution to a final volume of 100 ml and stirring for 30 min. Substrate was separated from the liquid by filtration through a folded filter type 591 (Whatman, Dassel, Germany) and dried for 24 h under laminar flow. The capacity to colonise sugar beet seedlings after the starvation period and subsequent drought stress event was tested by germinating surface sterilised, non-inoculated seeds in culture tubes type 'De Wit'. Prior transferring the soil substrate into the culture tubes, cell number was determined by re-suspending 0.5 g of dried soil substrate in 3 ml 0.9% NaCl solution and plating onto nutrient agar II. Two seeds were placed in each of the three culture tubes per strain. After 10 days bacterial cell counts associated with 1 g of sugar beet roots were determined.

Quantitative and qualitative analysis of PHB

Cells of ZR2-12 and ZR2-12_PHB$^-$ grown on MM with different NH_4Cl concentrations (0.05% (w/v) for storage conditions; 0.1% (w/v) for growth conditions) were harvested by centrifugation (15 min, 6000*g*, 4°C), washed in 0.9% (w/v) NaCl, and then lyophilized for 24 h. Analysis of PHB content was performed by methanolysis of 5 to 10 mg lyophilized cells in presence of 2 ml (85:15, v/v) methanol/sulfuric acid and 2 ml chloroform. The resulting methyl esters were analysed by gas chromatography using an Agilent 6850 GC (Agilent Technologies, Waldbronn, Germany) as described previously (Timm and Steinbüchel 1990). For qualitative determination of methyl esters, a coupled GC/MS was performed using an HP6890 gas chromatograph equipped with a model 5973 EI MSD mass selective detector (Hewlett Packard). The mass spectra obtained were evaluated using the NIST Mass Spectral Search program (Stein et al. 1998). Isolation of the accumulated polymer from lyophilized cells was done by chloroform extraction (50 ml) followed by shaking for 24 h and precipitation with 10 × ice cold ethanol. Proton ^{1}H NMR was used to quantify the levels of 3-hydroxyproprionate and $3HA_{MCL}$ present in the isolated polymer. NMR experiments were performed employing the Varian Unity Plus 600 spectrometer.

Statistics

Plant and colonisation experiments were done in three replicates and independently repeated three times. Results shown represent mean values of all replicates. For all statistical analysis of data (analysis of variance - ANOVA, Mann Whitney test, Tukey test, Student's *t*-test) the statistical package SPSS (SPSS Inc., Chicago, USA), was used.

Results

Effect of *B. terricola* treatment on plant growth

The effect of bacterial treatment on growth of sugar beet plants was monitored in sterilised soil substrate. Fresh mass of plants grown from seeds inoculated with 3.87±0.27 $\log_{10}$ CFU seed^{-1} was determined gravimetrically, and compared to the non-treated control. As shown in Fig. 1, *B. terricola* ZR2-12 inoculants stimulated growth of sugar beet seedlings. Three weeks post-inoculation, *Burkholderia*-primed seeds resulted in a statistically significant higher weight of seedlings (P=0.0019) relative to the non-treated control (1.26 g and 0.53 g on average per plant, respectively). The plant weight correlated with leaf length: leaves of *B. terricola* treated plants were on average 1.4 cm longer than those of the non-treated control plants. Under non-sterile conditions a plant growth promoting effect of *B. terricola* ZR2-12 was also significant, although the effect was less pronounced (data not shown).

Rhizosphere competence of *B. terricola* ZR2-12

Rhizosphere competence, the ability to colonise and persist in the root system under a wide range of conditions (Sørensen 1997), was assessed by monitoring the abundance of *B. terricola* ZR2-12 in the sugar beet root system. This experiment was carried out in comparison to *Pseudomonas fluorescens* WCS365, which has been documented to be a good root coloniser (Dekkers et al. 1998). After 10 days, bacterial treatment resulted in comparable numbers for both strains. Sugar beet roots were colonised with 9.86±0.97 $\log_{10}$ CFU g^{-1} fresh weight (fw) root *P. fluorescens* WCS365 cells and with 9.59±0.92 $\log_{10}$ CFU g^{-1} fw root *B. terricola* ZR2-12. No statistically significant difference for the rhizosphere competence of both strains (P=0.30) was calculated.

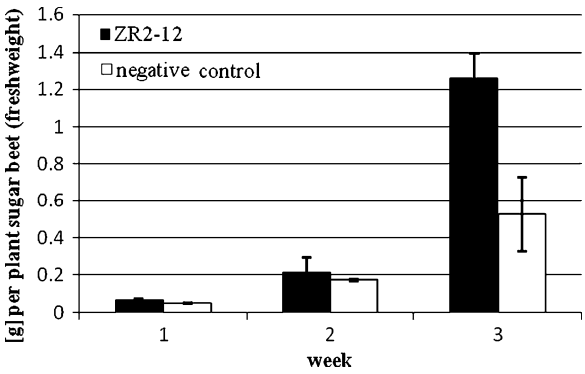

Fig. 1 Plant growth promoting effect of *B. terricola* ZR2-12. Seeds of sugar beet 'Calida' were primed with strain ZR2-12 before sowing, and fresh weight of seedling shoots was examined after 1, 2, and 3 weeks in comparison to the non-treated control. Error bars represent confidence intervals at P≤0.05. Significance of difference between ZR2-12 and negative control in week 3 was 0.0019

Microscopic analyses of root colonisation patterns

To analyse the colonisation pattern of *B. terricola* ZR2-12 in detail, CLSM (Confocal Laser Scanning Microscopy) was used in combination with seeds primed with a DsRed-tagged derivative of ZR2-12, ZR2-12 (pIN69). Inoculated plant roots of sugar beets were analysed after 3 weeks grown under gnotobiotic conditions. Surprisingly, cells of ZR2-12 [pIN69] were not only found on the root surface but also in the endorhiza. Indeed, a more detailed analysis using the Imaris software package revealed an extremely dense colonisation of the endorhiza (Fig. 2). Large microcolonies consisting of hundreds of *Burkholderia* cells were found along the apoplastic spaces of the root. In addition, xylem vessels were colonised by smaller aggregates and single cells, whereas in the root tips mainly single cells were present. Interestingly, *Burkholderia* cells were not found in the living plant parts (symplast) like phloem or inside cells.

Phenotypic characterisation of *B. terricola* ZR2-12

Several in vitro assays to investigate the physiological and biochemical potential of *B. terricola* ZR2-12 were performed (Table 1). We observed neither auxin production nor nitrogen fixation. No antagonistic activity against the soil-borne plant pathogenic fungi *Verticillium dahliae* and *Rhizoctonia solani* was observed (data not shown). *B. terricola* ZR2-12 was also not able to control *R. solani* in a biocontrol assay on sugar beet seedlings under greenhouse conditions, as described by Keijer et al. (2003). Furthermore, the strain did wether produce extracellular proteases nor it was resistant to high salinity. After 3 days of incubation on siderophore detection medium, decoloured halos of 1 mm in diameter were formed, suggesting production of siderophores. Strain ZR2-12 was able to grow on medium containing ACC as a sole nitrogen source, suggesting that the strain has ACC deaminase activity.

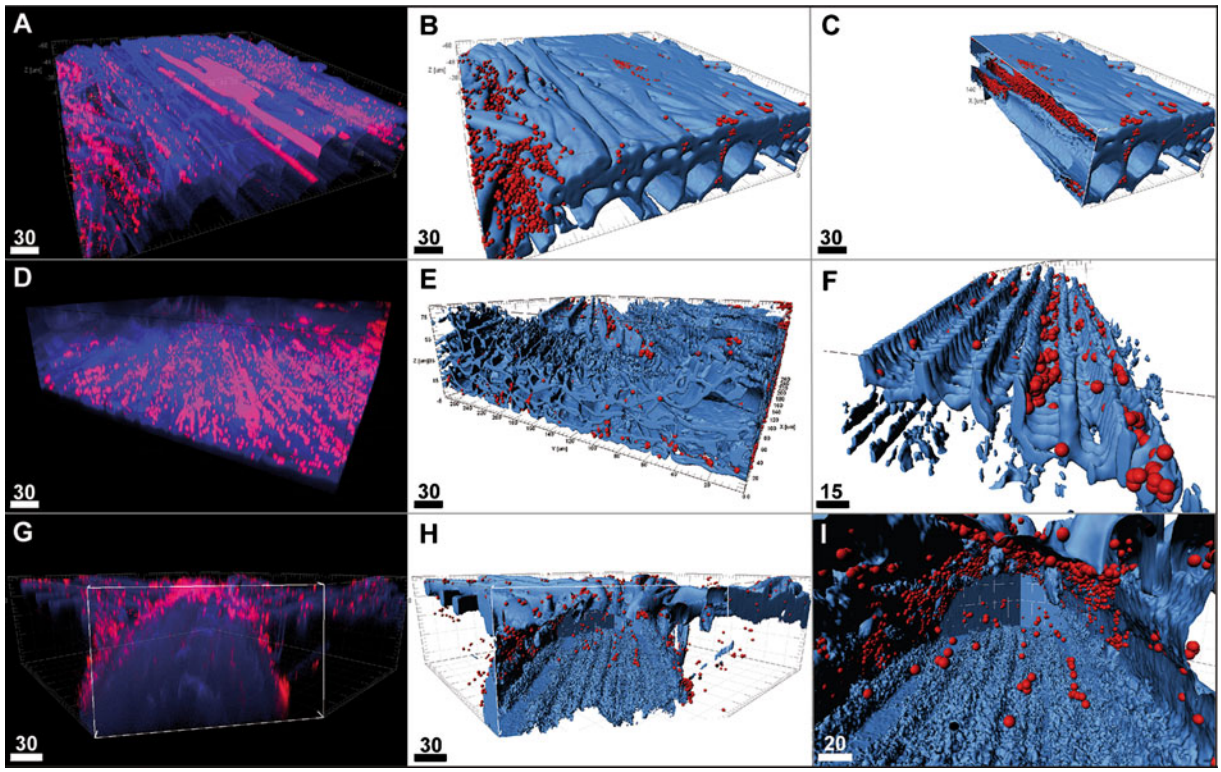

Fig. 2 Endophytic colonisation of sugar beet roots 'Calida' by *B. terricola* ZR2-12 [pIN69] observed by confocal laser scanning microscopy. **a–c**: dense colonies of *B. terricola* ZR2-12 in the apoplast of the older parts of the root, 3 weeks after plant germination; **d–f**: small colonies and single cells of *B. terricola* ZR2-12 colonising the inside of the xylem vessel in the middle part of the root, 2 weeks after plant germination; **g–i** endophytic cells of *B. terricola* ZR2-12 in the vicinity of the root tip, 3 weeks after plant germination. (a, d and g: volume rendering; b, c, e, f, h and i: 3D-reconstructions created with the software IMARIS 7.0 (Bitplane, Zurich, Switzerland))

B. terricola is capable of producing PHB up to 70% (w/w) of cell dry mass when grown under polymer storage conditions by feeding with 1% (w/v) glucose and 0.05% (w/v) NH_4Cl. Up to 32% PHB (w/w) of cell dry weight was synthesized by ZR2-12 cultivated in MM with 0.1% (w/v) NH_4Cl and 1% (w/v) glucose (growth conditions). Performing TLC, common fatty acids but neither wax esters nor triglycerides could be detected (data not shown).

Comparative studies of the wild-type and the PHB-negative mutant ZR2-12_PHB$^-$ on plant colonisation

To assess whether the ability of PHB-production influences the colonisation competence of *B. terricola,* sugar beet roots grown from primed seeds were inoculated by similar amounts of either the wild-type or the PHB-negative mutant strain. This treatment resulted into comparable bacterial abundances in the rhizosphere after 3 weeks (6.05±0.59 and 6.03±0.57 $\log_{10}$ CFU g^{-1} fw root) (Table 2, experiment A). To assess whether PHB-production may facilitate faster colonisation of roots, sterile seeds of sugar beets were planted into *B. terricola* containing soil (Table 2, experiment B). After 10 days, cell numbers of roots of germinated seedlings were determined. Both the wild-type and PHB$^-$-mutant colonised the plants well with 9.45 and 9.69 $\log_{10}$ CFU g^{-1} fw root, respectively. These data suggest that PHB production does not affect the strains ability to colonise plant roots.

As formation of storage compounds like PHB can be linked to response to fluctuating nutrient supply, we tested the survival rate of both strains under drought stress in plant free soil. *Burkholderia* treated sterilised soil (Table 2, experiment C) was long-term incubated and air dried for 24 h. After starvation and drought stress, survival was determined by plating,

Table 2 Comparison of colonisation competence between wild-type strain *B. terricola* ZR2-12 and its PHB-negative mutant ZR2-12_PHB$^-$. For experiments A and B abundances of *B. terricola* on roots and for C and D abundance in soil (log_{10} CFU g^{-1} soil±SD) were determined

Experimental design	Abundance of *B. terricola* log_{10} CFU g^{-1} root or soil	
	ZR2-12	ZR2-12_PHB$^-$
A: Rhizosphere competence (seed priming)[1)]	6.05±0.598	6.03±0.578
B: Rhizosphere competence (root inoculation)[2)]	9.45±0.927	9.69±0.967
C: Soil colonisation before drought stress[3)]	9.64±0.906	9.56±0.877
D: Soil colonisation after drought stress[3)]	5.49±0.507	5.40±0.510

[1)] Sugar beet seeds 'Calida' were primed with both *Burkholderia* strains (3.87±0.27 log_{10} CFU $seed^{-1}$) and germinated in sterile soil. Bacterial cell numbers per g root fresh weight were determined after 3 weeks

[2)] Sugar beet seeds 'Calida' were set into soil inoculated with the *Burkholderia* strains. Bacterial cell numbers per g root fresh weight were determined after 10 days

[3)] Sterilised soil substrate was placed in a Petri dish and inoculated with a 24 h-culture of the *Burkholderia* strains. After incubation for 60 day at room temperature, bacterial cell numbers were determined before and after air drying (24 h under laminar flow)

resulting in a reduction of cell numbers ranging from 9.64 to 5.49 log_{10} CFU g^{-1} fw soil for wild-type and from 9.56 to 5.40 log_{10} CFU g^{-1} fw soil for the mutant (Table 2, experiment C and D).

In a next step, competitive assays were used to determine the effect of PHB production on plant colonisation competence. In two independent and three times repeated experiments, seeds were primed with ZR2-12 [pIN69] and ZR2-12_PHB$^-$ or with ZR2-12_PHB$^-$ [pIN69] and ZR2-12 and grown in seed germination pouches under gnotobiotic conditions for up to 3 weeks. When the DsRed tagged PHB-negative mutant was co-inoculated with the wild-type strain, the former was out-competed and no fluorescent cells were detected after 3 weeks. When the DsRed tagged wild-type was used in the competition assays, fluorescent cells were found to colonise the plant, albeit the colonisation patterns differed between older and younger parts of the root (Fig. 3). While dense colonies were present in the apoplastic spaces of the older root parts (Fig. 3), small colonies and single cells were found in the middle part of the roots. In the vicinity of the root tip, only single cells were present. Colonisation density increased with plant age. Older, middle aged and younger parts of roots from 3 weeks old plants were stronger colonised than those from 2 weeks or 1 week old plants, indicating a succession of colonisation in dependence of plant age and root part. Analysis with Imaris software showed a dense colonisation of the root surface and the endorhiza by *B. terricola*. By serially diluting crushed roots and plating on nutrient agar an average cell number of 9.86 log_{10} CFU g^{-1} fw root was determined.

Discussion

In addition to plant and human pathogens, the genus *Burkholderia* harbours various species, which are able to enhance plant growth and health. This study showed that the so far poorly analysed species *B. terricola* belongs to the recently established *Burkholderia* cluster of plant beneficial bacteria (Suárez-Moreno et al. 2008). Their members are characterised by (i) their ability to colonise the rhizosphere or the internal intercellular spaces in several plants and promote plant growth, (ii) the potential to increase plant nutrient availability via nitrogen fixation and/or phosphate solubilisation, (iii) the capacity to degrade aromatic compounds, or (iv) the ability to form symbiotic interactions with plants (van Oevelen et al. 2002; Caballero-Mellado et al. 2007; Hontzeas et al. 2004; Reis et al. 2004; Sessitsch et al. 2005; Perin et al. 2006). *B. terricola* ZR2-12 belongs to the first group; the strain was able to promote plant growth and exhibited an endophytic lifestyle with enormous ability in colonisation of endophytic habitats. Such endophytes, which are defined as microorganisms living inside of plant organs and tissues without causing disease symptoms, have become highly interesting models to study plant-microbe interactions (Mei and Flinn 2010).

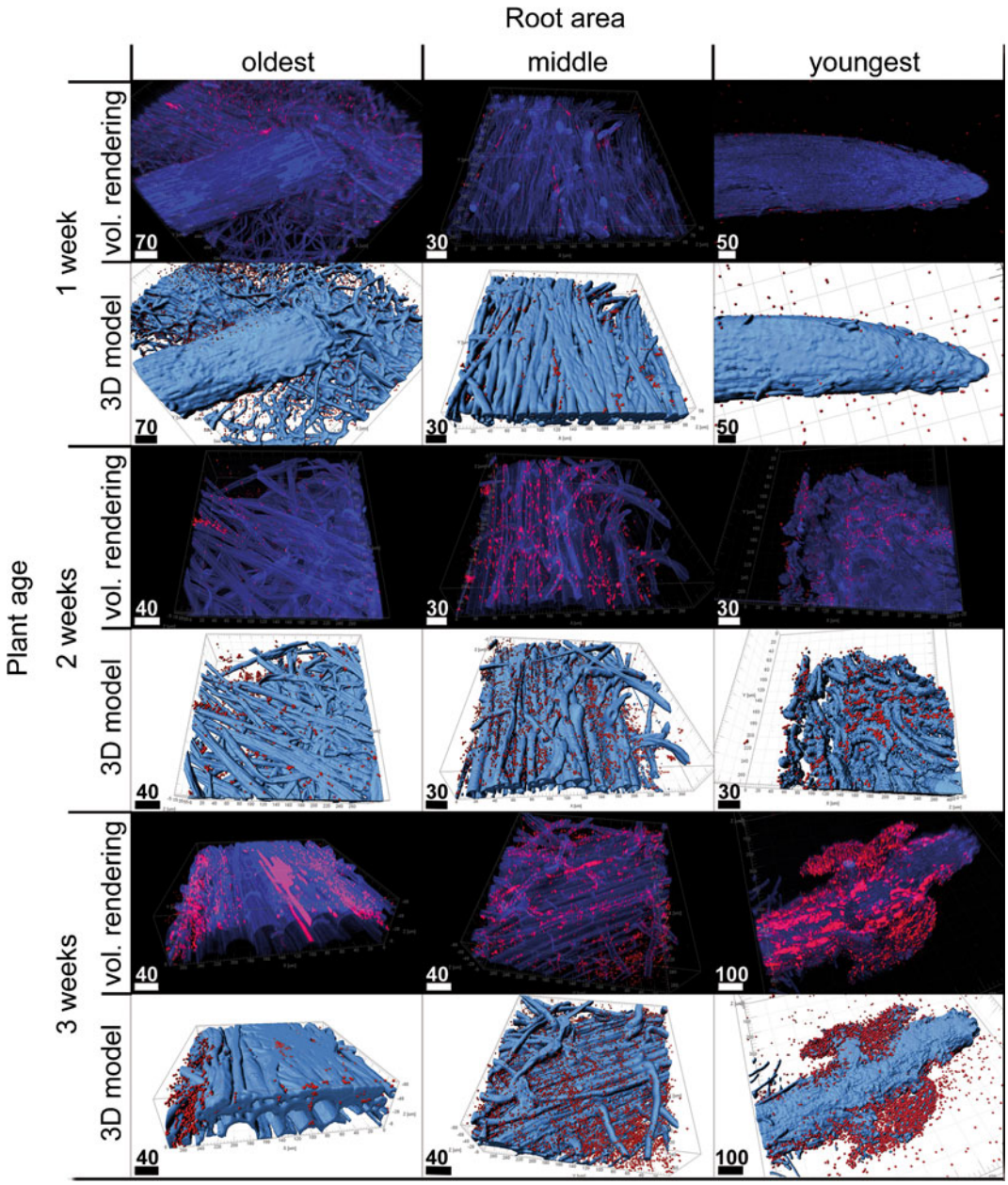

Fig. 3 Time course of colonisation of different parts of sugar beet roots 'Calida' by *B. terricola* ZR2-12 [pIN69] investigated by confocal laser scanning microscopy over a time period of 1 to 3 weeks. 3D models and volume renderings were created with the software IMARIS 7.0 (Bitplane, Zurich, Switzerland), which show cross sections of different areas of the root (oldest, middle, and youngest) densely colonised by *B. terricola* ZR2-12 [pIN69]

B. terricola ZR2-12 was able to significantly enhance plant growth. The observed ACC deaminase activity may, at least for a part, explain this plant growth promotion effect. Our investigations improve no evidence that *B. terricola* ZR2-12 could provide nutrient uptake of the plant like nitrogen and/or phosphate or to production of phytohormones like auxin. However, additional work will be required to determine factors involved in plant growth promotion. No antifungal activity against plant pathogens was detected, which has been described for other *Burkholderia* species (Vandamme et al. 2007; Schmidt et

al. 2009). In addition, we found no AHL activity in our applied test systems. In contrast to the pathogenic *Burkholderia* cluster, little is known about the role of AHLs in members of the non-pathogenic, plant-associated *Burkholderia* cluster, which is characterised by conserved AHL-dependent regulatory system (Sokol et al. 2007; Suárez-Moreno et al. 2008).

The most remarkable feature of ZR2-1 is the production of up to 70% (w/w) of cell dry mass of PHB. Therefore, we decided to study the role of PHB production for rhizosphere competence, the ability to colonize roots under a wide range of conditions, in more detail. When tested in separate systems, no statistically significant differences between wild-type strain and a PHB-negative mutant were observed for rhizosphere competence, tolerance of drought stress in soil, ability to survive in soil and time curve of root colonisation. However, when both strains were tested in a competitive assay, *B. terricola* ZR2-12 wild-type was able to out-compete the PHB-negative mutant. These findings are in accordance with results obtained with nitrogen-fixing bacteria of the genera *Azospirillum, Azotobacter, Herbaspirillum* and *Rhizobium,* for which a positive correlation between PHB accumulation and root colonisation has been demonstrated (rev. in Castro-Sowinski et al. 2010). Furthermore, we could show that a high competition for colonisation was correlated with plant growth promotion, which has been predicted previously (Dekkers et al. 1998; Lugtenberg and Kamilova 2009).

The mutant employed in this study was generated by chemical mutagenesis and the genetic defect(s) of the mutant has not been identified. Intriguingly, using Tn5 mutagenesis we were not able to generate a PHB-deficient mutant. We therefore speculate that the strain may contain more than one set of PHB biosynthesis genes, which may also explain the enormous amounts of PHB produced.

Microbial inoculants on the basis of plant growth promoting or biocontrol agents have a great potential for sustainable and environmentally friendly agriculture (rev. in Berg 2009). In addition, microorganisms can act as stress-protecting agents, indicating that plant associated bacteria are an important factor for the response of plants to climate change (rev. in Compant et al. 2010). *B. terricola* ZR2-12 is a promising candidate to develop a strategy for plant growth promotion because i) it is a potent root coloniser, ii) exhibits an endophytic lifestyle, iii) can well survive in plant-associated microhabitats with changing conditions, iv) produces no antibiotics or toxins, v) is harmless for humans and the environment, and vi) is easy to cultivate. The interaction between plant-associated microorganisms themselves as well as with their host plants is highly complex (Sørensen 1997). Here we showed that this research may not only be important to understand the ecological function of the bacterium but also for biotechnological applications.

Acknowledgment This research was funded in frame of the fForte-Wissenschafterinnenkolleg "FreChe Materie", by the Austrian Ministries BMVIT, BMWFJ and BM.W_f as well as from the Land Steiermark (Styria, Austria) and by a grant of the Austrian Science Foundation (FWF) to G.B. We would like to thank Martin Koller (Graz) for the fruitful discussions about PHB and Annette C. Vergunst (Leiden) for providing us plasmid pIN69.

References

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Ann Rev Plant Biol 57:234–266

Berg G (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84:11–18

Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68:1–13

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl Environ Microbiol 68:3328–3338

Brämer CO, Silva LF, Gomez JG, Priefert H, Steinbüchel A (2002) Identification of the 2-methylcitrate pathway involved in the catabolism of propionate in the polyhydroxyalkanoate-producing strain *Burkholderia sacchari* IPT101(T) and analysis of a mutant accumulating a copolyester with higher 3-hydroxyvalerate content. Appl Environ Microbiol 68:271–279

Brown CM, Dilworth MJ (1975) Ammonia assimilation by *Rhizobium* cultures and bacteroids. J Gen Microbiol 86:39–48

Caballero-Mellado J, Onofre-Lemus J, Estrada-de los Santos P, Martínez-Aguilar L (2007) The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Appl Environ Microbiol 73:5308–5319

Castro-Sowinski S, Burdman S, Matan O, Okon Y (2010) Natural functions of bacterial polyhydroxyalkanoates. Microbiol Monogr 14:39–61

Coenye T, Vandamme P (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol 5:719–729

Compant S, van der Heijden MGA, Sessitsch A (2010) Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiol Ecol 73:197–214

De Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in Gram-negative bacteria with TN5- and TN10-derived mini transposons. Methods Enzymol 235:386–405

Dekkers LC, Bloemendaal CJ, De Weger LA, Wijffelman CA, Spaink HP, Lugtenberg B (1998) A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. Mol Plant Microb Interact 11:45–56

Gasser I, Müller H, Berg G (2009) Ecology and characterisation of polyhydroxyalkanoate-producing microorganisms on and in plants. FEMS Microbiol Ecol 70:142–150

Goris J, Dejonghe W, Falsen E, De Clerck E, Geeraerts B, Willems A (2002) Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. Syst Appl Microbiol 25:340–352

Hebbar PK, Martel MH, Heulin T (1998) Suppression of pre- and postemergence damping-off in corn by *Burkholderia cepacia*. Eur J Plant Pathol 104:29–36

Hontzeas N, Saleh SS, Glick RB (2004) Changes in gene expression in canola roots induced by ACC-deaminase-containing plant-growth-promoting bacteria. Mol Plant Microb Interact 17:865–871

Kadouri D, Jurkevitch E, Okon Y, Castro-Sowinski S (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Crit Rev Microbiol 31:55–67

Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg B (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environ Microbiol 7:1809–1817

Keijer J, Korsman MG, Dullemans AM, Houterman PM, De Bree J, Van Silfhout CH (2003) In vitro analysis of host plant specificity in *Rhizoctonia solani*. Plant Pathol 46:659–669

Lin CH, Bourque G, Tan P (2008) A comparative synteny map of 861 *Burkholderia* species links large-scale genome rearrangements to fine-scale nucleotide 862 variation in prokaryotes. Mol Biol Evol 25:549–558

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

Mahenthiralingam E, Urban T, Goldberg J (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. Nat Rev Microbiol 3:144–156

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GSAB, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143:3703–3711

McLoughlin TJ, Quinn JP, Bettermann A, Bookland R (1992) *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. Appl Environ Microbiol 58:1760–1763

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent Pat Biotech 4:81–95

Onofre-Lemus J, Hernández-Lucas I, Girard L, Caballero-Mellado J (2009) ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. Appl Environ Microbiol 70:6581–6590

Perin L, Martínez-Aguilar L, Paredes-Valdez G, Baldani JI, Estrada-de los Santos P, Reis VM, Caballero-Mellado J (2006) *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. Int J Syst Evol Microbiol 56:1931–1937

Reis VM, Estrada-de los Santos P, Tenorio-Salgado S, Vogel J, Stoffels M, Guyon S, Mavingui P, Baldani VL, Schmid M, Baldani JI, Balandreau J, Hartmann A, Caballero-Mellado J (2004) *Burkholderia tropica* sp nov., a novel nitrogen-fixing, plant-associated bacterium. Int J Syst Evol Microbiol 54:2155–2162

Sarwar M, Kremer RJ (1995) Determination of bacterially derived auxins using a microplate method. Lett Appl Microbiol 20:282–285

Schlegel HG, Kaltwasser H, Gottschalk G (1961) A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. Arch Microbiol 38:209

Schlegel HG, Lafferty R, Krauss I (1970) The isolation of mutants not accumulating poly-beta-hydroxybutyric acid. Arch Microbiol 71:283–294

Schmidt S, Blom JF, Pernthaler J, Berg G, Baldwin A, Mahenthiralingam E, Eberl L (2009) Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex. Environ Microbiol 11:1422–1437

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Sessitsch A, Coenye T, Sturz AV, Vandamme P, Ait Barka E, Faure D, Reiter B, Glick BR, Wang-Pruski G, Nowak J (2005) *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant beneficial properties. Int J Syst Evol Microbiol 55:1187–1192

Sokol PA, Malott RJ, Riedel K, Eberl L (2007) Communication systems in the genus *Burkholderia*: global regulators and targets for novel antipathogenic drugs. Future Microbiol 2:555–563

Sørensen J (1997) The rhizosphere as a habitat for soil microorganisms. In: Van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Marcel Dekker, Inc., New York, pp 21–45

Spiekermann P, Rehm BHA, Kalscheuer R, Baumeister D, Steinbüchel A (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 171:73–80

Stein S, Levitsky A, Fateev O, Mallard G (1998) The NIST mass spectral search program. Windows-Software Version 1.6d

Steinbüchel A (2001) Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. Macromol Biosci 1:1–24

Steinbüchel A, Valentin HE (2006) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228

Suárez-Moreno ZR, Caballero-Mellado J, Venturi V (2008) The new group of non-pathogenic plant-associated nitrogen-fixing *Burkholderia* spp. shares a conserved quorum-sensing system, which is tightly regulated by the RsaL repressor. Microbiology 154:2048–2059

Suárez-Moreno ZR, Devescovi G, Myers M, Hallack L, Mendonça-Previato L, Caballero-Mellado J, Venturi V (2010) Commonalities and differences in N-acyl homoserine lactone quorum sensing regulation in the novel species cluster of beneficial *Burkholderia*. Appl Environ Microbiol 76:4302–4317

Timm A, Steinbüchel A (1990) Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl Environ Microbiol 56:3360–3367

Uthoff S, Stöveken T, Weber N, Vosmann K, Klein E, Kalscheuer R, Steinbüchel A (2005) Thio wax ester biosynthesis utilizing the unspecific bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase of *Acinetobacter calcoaceticus* ADP1. Appl Environ Microbiol 71:790–796

Van Oevelen S, De Wachter R, Vandamme P, Robbrecht E, Prinsen E (2002) Identification of the bacterial endosymbionts in leaf galls of *Psychotria* (Rubiaceae, angiosperms) and proposal of *'Candidatus Burkholderia kirkii'* sp. nov. Int J Syst Evol Microbiol 52:2023–2027

Vandamme P, Goris J, Chen WM, de Vos P, Willems A (2002) *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov. nodulate the roots of tropical legumes. Syst Appl Microbiol 25:507–512

Vandamme P, Opelt K, Knochel N, Berg C, Schonmann S, DeBrandt E, Eberl L, Falsen E, Berg G (2007) *Burkholderia bryophila* sp. nov. and *Burkholderia megapolitana* sp. nov., moss-associated species with antifungal and plant-growth-promoting properties. Int J Syst Evol Microbiol 57:2228–2235

Vergunst AC, Meijer AH, Renshaw SA, O'Callaghan D (2010) *Burkholderia cenocepacia* creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. Infect Immun 78:1495–1508

Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T, Arakawa M (1992) Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group I1 to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol 36:1251–1275