Graz University of Technology

Doctoral Thesis

Biopolymer producing bacteria and analysis of ecosystem function

Bakterien als Produzenten von Biopolymeren und Untersuchung deren Funktion im Ökosystem

Dissertation zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften (Dr. rer. nat.) an der Fakultät für Technische Chemie, Verfahrenstechnik und Biotechnologie
der Technischen Universität Graz

Ilona Bernadette Gasser
geb. am 29.10.1983
in Innsbruck

Graz, 31. Juli. 2010

I. Abstract

Polyhydroxyalkanoates (PHA) are storage substances produced by bacteria to survive periods of starvation. Little is known about the ecology of PHA producing bacteria. Environments with fluctuating nutrient availability such as rhizosphere of crop plants or lichens are supposed to be a promising source of biotechnologically interesting PHA producing bacteria which cope with industrial demands. The rhizosphere of plants provides a continuous changing microhabitat with fluctuation in the concentrations of nutrients exuded by plant roots. In lichen thalli, bacteria also find changing conditions such as strong oscillation in water availability. Indigenous bacteria developed their own strategies to overcome these changing conditions, which lead to a competitive advantage for PHA producing bacteria. Antagonistic bacteria and microbial community DNA were isolated from different lichen thalli and subsequently analysed for PHA production *in vitro* by plate assay and lipophilic staining or with PCR strategy using specific primers for *phaC* gene encoding the key enzyme of PHA synthase. Lichen thalli were colonised by numerous bacteria which were potentially able to accumulate PHA and keep promise for biotechnological applications. To analyse the occurrence of PHA producing bacteria in the rhizosphere of crop plants, microbial fingerprint techniques such as single strand conformation polymorphism analyses were used in addition to culture dependent methods. These experiments revealed a significantly higher amount of potentially PHA producing bacteria in the rhizosphere of sugar beet compared to bulk soil. Results were confirmed by qPCR. Cluster analysis revealed plant species specific communities for sugar beet, wheat and oilseed rape. Also new PHA producing bacteria such as *Burkholderia terricola* were found, which produces up to 70% w/w of cell dry mass PHA. For understanding the ecological role of PHA, this strain was tested in different green house experiments in comparison to a PHA negative mutant. Separately tested, both strains were able to promote plant growth with extremely high colonization abilities. When tested in a competitive assay, the wild type of *B. terricola* occupied the ecto- and endosphere of roots and outcompeted the mutant strain, verified by confocal laser scanning microscopy. Summarized results confirm that changing environments such as rhizosphere or lichens inhabit PHA producing bacteria of biotechnological relevance. One of these strains, *B. terricola*, exhibited plant growth promoting properties and studies with its PHA negative derivate emphasized the importance of PHA production for successful colonisation competence of the rhizosphere.

II. Zusammenfassung

Polyhydroxyalkanoate (PHA) sind bakterielle Speicherstoffe, welche produziert werden, um nährstoffarme Zeiten zu überdauern. Bisher ist wenig über die ökologische Rolle PHA-produzierender Bakterien bekannt. Als viel versprechende Ressource zum Auffinden biotechnologisch interessanter PHA Produzenten gelten Habitate mit fluktuierender Nährstoffverfügbarkeit. Die Rhizosphäre von Pflanzen stellt aufgrund der Produktion von Wurzelexsudaten ein fluktuierendes Mikrohabitat dar. Auch im Flechtenthallus liegen sich ständig ändernde Bedingungen, wie Schwankungen in der Wasserverfügbarkeit, vor. Beide Habitate erfordern spezielle Anpassungen der indigenen Mikroorganismen. Von verschiedenen Flechten wurden antagonistische Bakterien und Gesamt-DNA isoliert und anschließend mit Plattentests und lipophilen Färbemethoden auf PHA Produktion bzw. molekularbiologisch mittels spezifischer Primer für das *phaC* Gen, welches für das Schlüsselenzym der PHA Synthase kodiert, auf die potentielle Fähigkeit hin, PHAs zu produzieren, untersucht. Eine Vielzahl biotechnologisch interessanter PHA Produzenten wurde nachgewiesen. Um das Vorkommen von PHA Produzenten in der Rhizosphäre von Kulturpflanzen zu untersuchen, wurden neben kultivierungsabhängigen auch molekulare Fingerprint-Methoden durchgeführt. Letztere ergaben eine hohe Pflanzenspezifität mikrobieller Gemeinschaften für Zuckerrübe, Raps und Weizen. Die Rhizosphäre von Zuckerrüben enthielt eine signifikant höhere Anzahl an PHA Produzenten als der undurchwurzelte Erdboden, was mittels qPCR bestätigt werden konnte. Als bisher unbekannter PHA Produzent konnte *Burkholderia terricola* aus der Rhizosphäre der Zuckerrübe isoliert und ein PHA Gehalt von bis zu 70% W/W der Zelltrockenmasse nachgewiesen werden. Um die ökologische Rolle von PHA zu untersuchen, wurden mit *B. terricola* und einer PHA negativen Mutante mehrere Pflanzenexperimente durchgeführt. Beide Stämme zeigten Pflanzenwachstums fördernde Wirkung, jedoch wenn Wildtyp und Mutante in einem Konkurrenzversuch an der Zuckerrübe getestet wurden, konnte der Wildtyp die PHB negative Mutante vollständig aus der Endo- und Ektosphäre der Wurzel verdrängen. Zusammengefasst bestätigen die Ergebnisse, dass fluktuierende Habitate, wie Rhizosphäre oder Flechten, große Mengen an biotechnologisch relevanten PHA Produzenten beherbergen. So zum Beispiel *B. terricola*, der neben der PHA Produktion auch die Fähigkeit zur Pflanzenwachstumsförderung besitzt. Bei kompetitiven Experimenten mit der PHB negativen Mutante konnte die Notwendigkeit von PHA für erfolgreiche Kolonisierungsskompetenz an der Rhizosphäre gezeigt werden.

III. Abbreviations

A/O	anaerobic/aerobic
ACC	1-aminocyclopropane-1-carboxylate
AHL	N-acyl homoserine lactone
bp	base pairs
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
CoA	3-hydroxyacyl-coenzymeA
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DSMZ	Deutsche Stammsammlung für Mikroorganismen & Zellkulturen
EMBL	European molecular biology laboratory
EMS	ethylmethansulfonat
fw	fresh weight
GC	gas chromatography
HPLC	high pressure liquid chromatography
IAA	indole 3-acetic acid
IR	infrared
MCL	medium chain length
MS	mass spectrometry
NADH	nicotinamidadenindinucleotide
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
PGPB	plant growth promoting bacteria
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PP	polypropylene
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
SCAM	strain collection of antagonistic bacteria
SCL	short chain length
SSCP	single-strand conformation polymorphism
TGGE	termperature gel gradient electrophoresis
TLC	thin layer chromatography
tn	transposon
UPGMA	unweighted-pair group methods using averages
UV	ultraviolet

4. Table of contents

5. Introduction

5.1. General aspects of Polyhydroxyalkanoates

Among biological polyesters polyhydroxyalkanoates (PHAs) provide a good alternative to petrochemical based plastics regarding their biodegradability and biocompatibility. PHAs exhibit material properties similar to those of polypropylene (Byrom 1987; Steinbüchel and Füchtenbusch 1998). Beside biodegradability and biocompatibility some other interesting material properties such as thermoplastic and elastomeric features can be achieved. Therefore PHAs are considered for several applications in industry (particularly packaging), agriculture, pharmacy and medicine (Steinbüchel *et al.* 1996) (Fig. 5.1.1.). PHAs are macromolecules accumulated in cells up to levels of 90% of cell dry weight, when a carbon source is provided in excess and another nutrient like nitrogen, phosphate, sulfur, magnesium or even oxygen is limited. Within the cell PHA exists as cytoplasmic granules surrounded by a membrane (Sudesh *et al.* 2000). These granules are coated with a monolayer of proteins and phospholipids (Fig. 5.1.1.). A common PHA type found in bacteria is a copolymer consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) units whereat the polymer composition depends on the nature of the carbon source (Lemos *et al.* 1998). Over hundred different types of such hydroxyl acids are possible. The most common type of PHA, polyhydroxybutyrate (PHB) was first described by Lemoigne in 1926 in *Bacillus megaterium*, consequently followed by a wide variety of prokaryotes producing this biopolymer. Depending on the number of carbon atoms in the monomers, PHA synthases of bacteria are classified in short chain length (SCL, three to five carbon atoms) or medium chain length (MCL, six to

14 carbon atoms) hydroxyalkanoic acids (Rehm and Steinbüchel 1999): If SCL or MCL are produced, depends on different conditions such as carbon source or the type of PHA synthase.

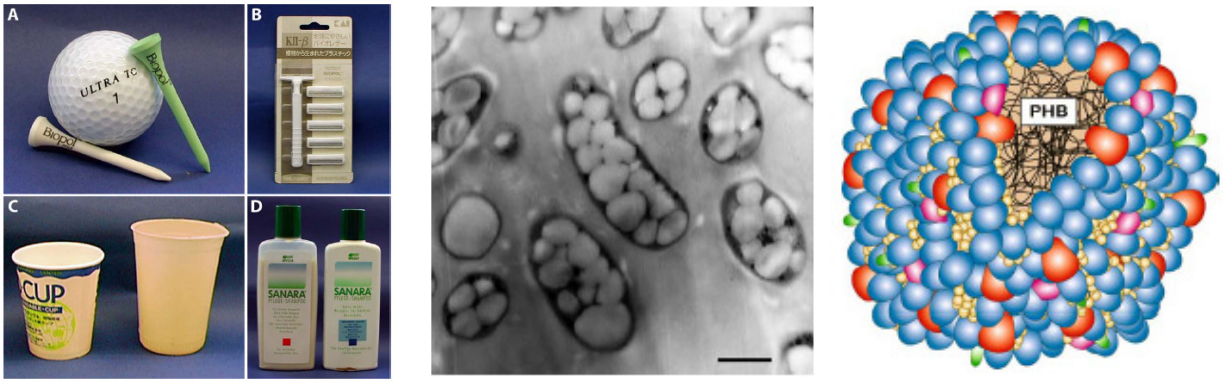

Fig. 5.1.1. Left picture shows commercial products made out of PHA (Biopol), for example golf pins (A), razor (B), cups (C) and shampoo bottles (D); Central picture presents an electron micrograph of an ultrathin section of Ralstonia eutropha H16 cells (scalebar 1 µm); Picture on the right side illustrates a model of the PHA granule, in which PHA synthase is red, phasin blue, PHA depolymerase magenta, transcriptional repressor of phasin green and phospholipids are demonstrated in yellow. Adapted from Pötter and Steinbüchel 2006.

5.2. Biosynthesis of Polyhydroxybutyrate

The synthesis of Polyhydroxybutyrate (PHB) requires different enzymes (Fig. 5.2.1.). The enzyme PHA synthase is encoded by *phaC* and uses 3-hydroxyacyl-coenzymeA (CoA) substrates for polymerization. In turn for production of CoA substrates the enzymes ß-ketothiolase (encoded by *phaA*) and acetoacetyl-CoA reductase (encoded by *phaB*) are needed (Kranz *et al* 1997) (Fig. 5.2.1.). Generally an alphabetical order is used for genes coding for proteins which are involved in biosynthesis of PHA (*phaA* (ß-ketothiolase), *phaB* (acetoacetyl-CoA reductase) *phaC* (PHA synthase) etc.).

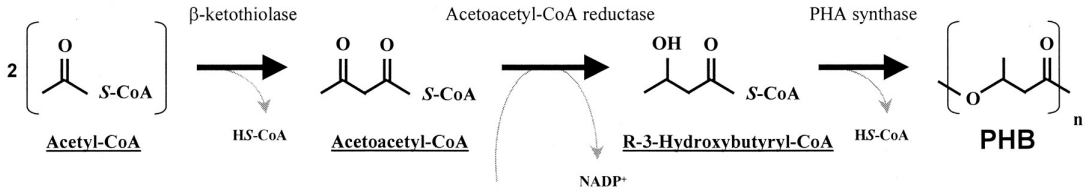

Fig. 5.2.1. PHB biosynthetic pathway, whereat substrates and products are underlined and cofactors and side products are boldfaced. Adapted from Taroncher-Oldenburg *et al*. 2000.

Nomenclature of genes required for degradation is referred in reverse alphabetical order, incipient with *phaZ* for PHA depolymerases (Rehm and Steinbüchel 1999). Furthermore PHA synthase genes are divided into four classes, differentiated by the organization of the gene locus and structure (Solaiman *et al.* 2005) (Fig. 5.2.2.). A good overview about PHA synthases and different metabolic interconnections between the different pathways is given by Pötter and Steinbüchel (2006) and Luengo *et al*. (2003).

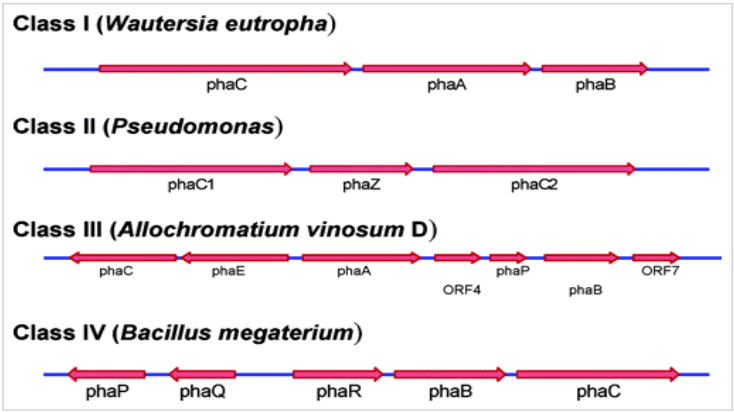

Fig. 5.2.2. Genetic organization of the four classes of *pha* loci (*phaA*: ß-ketothiolase; *phaB* acetoacetyl reductase; *phaC*, *phaC1* and *phaC2*: PHA synthase; *phaE* and *phaR*: subunit of class III and class IV PHA synthase, respectively; *phaP* phasin; phaQ homologous to helix turn- helix multiple antibiotic resistance protein; *phaZ*: PHA depolymerase; ORF putative open reading frame with unknown function). Adapted from Solaiman *et al.* 2005.

5.3. Environmental habitats and ecology of PHA producing bacteria

In environments of fluctuating availability and limitation of nutrients microorganisms have evolved different strategies to overcome times of adverse growing conditions. One possibility is the production of the storage polymer PHA. Occurrence of PHA producing bacteria is to be expected in ecosystems, which exhibit the mentioned properties – fluctuation of carbon availability or transient limitation of essential nutrients. As it seems to be a common phenomenon that PHA amounts are increased by perturbation of environments, it has been established that occurrence of PHA is an indication for an unbalanced growth of microorganisms in disturbed environments (Nickels *et al*. 1979).

Early studies have shown high diversity of PHAs in marine environments with strong fluctuations of available nutrients (Findlay and White 1983). From estuarine microbial mats PHA producing strains of *Halomonas* and *Labrenzia* have been isolated (Villanueva *et al*. 2010). From freshwater sludge of the Weende Quelle in Göttingen, Germany, the well-investigated model strain *Ralstonia eutropha* H16 was isolated (Schlegel *et al*. 1961). Abundance of PHA synthase genes also has been detected in river water of the Rio de la Plata, Buenos Aires in Argentina (Lopez *et al*. 1997). PHB accumulation is shown for an isolate from groundwater sediment, where availability of nutrients is fluctuating with the quality of percolating water (Bengtsson 1991). In activated sludge from wastewater treatment PHB producing microorganisms fulfil important roles in removal of unwanted substances. From activated sludge a mixture of different PHA types, ranging from C4 to C7 monomer chain lengths, have been isolated using chloroform extraction (Lowell *et al*. 1974).

Oshiki *et al.* (2008) have found PHA producing representatives of the genera *Dechloromonas, Accumulibacter, Thauera, Zoogloea, Comamonas, Competibacter* and a novel cluster of Betaproteobacteria in activated sludge.

For marine sediments it is shown that presence of PHA is an indicator for unbalanced growth (Nickels *et al*. 1979). Evidences, which support this finding to be true also for terrestrial ecosystems, come from reports of presence of PHA producing bacteria in somehow disturbed soils. PHA producing *Bacillus* strains have been isolated from gas field soil (Tajima *et al*. 2003). Kung *et al*. (2007) have found PHA producing bacteria in oil contaminated soils, industrial waste drainage sites and agricultural soils. Contrary reports about PHA producing bacteria and PHA amounts associated with the rhizosphere are found in literature. The rhizosphere is defined as the volume of soil adjacent to and influenced by plant root and as a habitat of great microbial diversity (Sorensen 1997; Berg and Smalla 2009). On the one hand, several studies show a high abundance of PHA producing bacteria or high amounts of PHA in the rhizosphere, sometimes in comparison to bulk soil. In the rhizosphere of rice plants a higher amount of PHA has been found in comparison to bulk soil (Reichardt *et al*. 1997). Rhizosphere of sugar beet, oilseed rape and wheat harbour high amounts of PHA producing microorganisms as determined by screening of isolates, community DNA fingerprints and qPCR (Gasser *et al*. 2009). On the other hand, a study with rhizospheric strains on roots of oilseed rapes has shown a lower PHA content at the rhizosphere in comparison to sand (Tunlid *et al*. 1985). However presence of PHA producing bacteria in rhizosphere soil can be explained by the fact that roots on the one hand limit nutrient access for rhizospheric bacteria by consumption of essential inorganic nutrients, and on the other hand enrich rhizospheric soil with fluctuating root exudates mainly consisting of organic compounds. It is remarkable that especially carbohydrate and oil producing crop

plants display frequent and intense association with PHA producing bacteria (de Lima *et al*. 1999; Romo *et al*. 2007; Gasser *et al*. 2009). Since decades it is known that PHA is produced by bacteroids of root nodules of legumes and also of free living nitrogen fixing bacteria (Wong and Evans 1971; Stam *et al*. 1986). Data suggest that accumulated PHA may prolong the period of nitrogen fixation in the dark (Bergersen *et al*. 1991) or may help the bacterial cell in maintaining its redox potential by energy production and NADH consumption (Dunn 1998; Encarnación *et al*. 2002). Recently PHA producing microorganisms have been detected in the microbiota of lichens (Gasser *et al*. 2010).

5.4. Studies with PHA negative mutants

The ecological and environmental role of PHA synthase genes or their deficiency remains to be determined. For *Ralstonia eutropha* and *Bacillus megaterium* it could be shown that there was a clear decrease in the ability of the PHB negative mutant strain to survive starvation in comparison to the wild type strains (López *et al.* 1995). *PhaC*$^-$ strains of *Rhodobacter capsulatus* were even unable to grow on solid media containing higher amount of certain fatty acids like acetone or caproate. Whereas *phaA*$^-$ and *phaAB*$^-$ stains are able to grow on this media. Kranz *et al.* (1997 b) presumed that PHA production can function as a detoxification mechanism when bacteria grow on fatty acids. When potentially toxic substances are polymerized or used for derivates (for example for CoA) it can result in resistance to that substance. The question remains how far fatty acid detoxification properties are ecologically beneficial in certain environments but it is conceivable bacteria getting more competitive thereby. Different groups worked with *Azospirillum brasilense*, a

bacterium which is able to accumulate PHB up to 75% of its dry weight (Tal and Okon 1985; Tal *et al.* 1990; Itzigsohn *et al.* 1995). Kadouri *et al.* (2002; 2003) experienced with *A. brasilense* mutants defective in their capability to produce PHA (*phaC*$^-$) or to degrade PHA (*phaZ*$^-$) that this mutant strains survive not as long as wild type strains. The term "*stress alleviation*" was established. When exclusively PHA is used as energy source it could be demonstrated to support physiological features (nitrogenase activity and aerotaxis) which are eminently energy consuming (Tal and Okon 1985). Continuative it could be shown that bacteria, which are able to produce PHA, survived the stress of starvation better than their PHA negative mutants (López *et al*. 1995; Ruiz *et al.* 1999).

But there are also open discussions because non PHA producing bacteria were isolated from soils which apparently endure stress situations in natural habitats as well as PHA producer (Wang and Bakken 1998). Therefore it seems PHAs are not the only way out to endure stresses in environment (Willis and Walker 1998), but it may help bacteria to be more competitive. For *Burkholderia terricola* this could be demonstrated in a competitive assay. The PHA producing wild type strain was able to occupy root of sugar beet and completely outcompete the PHA negative mutant derivate (Gasser *et al.* 2010). Rhizosphere competence and root colonization could be enhanced by high growth rates and energy recovery of available compounds (Simons *et al.* 1996; Jjemba and Alexander 1999). *Azospirillum brasilense* was also used for plant root colonization and plant growth promoting experiments under sterile and not sterile conditions in soil, resulting in no difference under tested conditions between wild type and *phaC* negative mutant. Roots were colonized by both strains to the same extent (Kadouri *et al.* 2003). However for *Pseudomonas fluorescens* a correlation between growth rate and rhizosphere competence was detected by Simons *et al.* (1996).

Generally conditions in green house experiments with optimal light, temperature and ideal soil moisture are often not comparable with those in outdoor rhizosphere environments which could make it difficult to compare the relevance of PHAs for ecology in both conditions. Kadouri *et al.* (2005) provided with his review an insight into the ecological and agricultural significance of bacterial polyhydroxyalkanoates.

6. Essence of the doctoral thesis

First hypothesis of this work was related to the rhizosphere, which was expected to harbour an interesting reservoir for PHA producing bacteria. The rhizosphere of plants provides a continuous changing environment with fluctuations in the concentrations of nutrients exuded by plant roots. Indigenous microorganisms must be adapted to these changing conditions. In Publication I, in contrast to bulk soil, rhizospheres of sugar beet, wheat and oilseed rape plants were shown to have a promising potential to harbour PHA producing bacteria. The highest number of positive tested strains was found in the rhizosphere of sugar beet. The relative copy number of *phaC* was statistically significantly enhanced in all tested rhizospheres. Also new PHA producing bacterial species such as *Burkholderia terricola* were detected.

Hypothesis number two claimed lichens to be an emerging bioresource for PHA producing bacteria among terrestrial ecosystems. In lichen thalli bacteria also find dramatically changing abiotic conditions such as strong oscillations in water availability. Production and storage of PHA might be a competitive advantage in this changing environment. In Publication II by applying both cultivation dependent and independent methods results clearly demonstrate that lichen thalli are colonised by numerous bacteria, which were potentially able to accumulate PHA and keep promise for biotechnological applications.

Investigated habitats were predicted to harbour new PHA producing bacterial strains with a high biotechnological potential. According to results in Manuscript I, one example for a biotechnological useful strain is *Burkholderia terricola*, isolated from

the rhizosphere of sugar beet, a microhabitat with changing conditions. *B. terricola* is able to produce up to 70% w/w of cell dry mass PHA. Furthermore this strain is a promising plant growth promoting agent, has extremely high colonization abilities and can be found in rhizosphere as well as endorhiza of sugar beet plants.

PHA plays a crucial role in competitive root colonisation. Inclusions of storage substances (e.g. PHA) is a bacterial strategy to increase survival and store energy in changing surroundings such as in the rhizosphere of plants or in lichens. Understanding the role of PHA in root colonization of *B. terricola* is an important quest in microbial ecology, which was investigated in Manuscript I by experiments conducted with a PHA negative mutant of *B. terricola*. The ability to produce PHA enhanced the strength of competition for space. When mutant and wild type strain were tested in a competitive assay *B. terricola* was able to occupy root and endosphere and completely outcompete the mutant strain.

Combination of chemical, molecular and microbiological methods can elucidate the ecological function of PHA in environmental habitats. Manuscript II reviews current knowledge about occurrence of PHA producing microorganisms in different environments. In aquatic as well as in terrestric habitats PHA producing microorganisms fulfil functions of ecological and biotechnological importance. The review also discusses well established and recently used cultivation independent methods for detecting PHA and PHA producing bacteria in environmental samples.

7. References

Bengtsson G (1991) Bacterial exopolymer and PHB production in fluctuating ground-water habitats. *FEMS Microbiol Ecol* 86: 15-24

Berg G and 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

Bergersen FJ, Peoples MB and Turner GL (1991) A role for poly-β-hydroxybutyrate in bacteroids of soybean root nodules. *Proc R Soc London* 245: 59–64

Byrom D (1987) Polymer synthesis by microorganisms: Technology and economics. *Trends Biotechnol* 5: 246–250

De Lima TCS, Grisi BM and Bonato MCM (1999) Bacteria isolated from a sugarcane agroecosystem: their potential production of polyhydroxyalcanoates and resistance to antibiotics. *Rev Microbiol* 30: 214-224

Dunn MF (1998) Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS Microbiol Rev* 22: 105–123

Encarnación S, Vargas MC, Dunn MF, Dávalos A, Mendoza G, Mora Y and Mora J (2002) AniA Regulates Reserve Polymer Accumulation and Global Protein Expression in *Rhizobium etli*. *J Bacteriol* 184: 2287–2295

Findlay RH and White DC (1983) Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Appl Environ Microbiol* 45: 71–78

Gasser I, de Castro Junior JV, Müller H and Berg G (2010) Lichen-associated bacteria antagonistic to phytopathogens and their potential to accumulate polyhydroxyalkanoates. *IOBC/wprs bulletin, Biological Control of Fungal and Bacterial Plant Pathogens* „Climate change: Challenge or Threat to Biocontrol?" (in press)

Gasser I, Müller H and Berg G (2009) Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants. *FEMS Microbiol Ecol* 70: 142-50

Itzigsohn R, Yarden O and Okon Y (1995) Polyhydroxyalkanoate analysis in Azospirillum brasilense. *Can J Microbiol* 41: 73-76

Jjemba PK and Alexander M (1999) Possible determinants of rhizosphere competence of bacteria. *Soil Biol Biochem* 31: 623-632

Kadouri D, Burdman S, Jurkevitch E and Okon Y (2002) Identification and Isolation of Genes Involved in Poly(ß-Hydroxybutyrate) Biosynthesis in Azospirillum brasilense and Characterization of a phbC Mutant. *Appl Environ Microb* 68: 2943-2949

Kadouri D, Jurkevitch E and Okon Y (2003) Involvement of the Reserve Material Poly-ß-Hydroxybutyrate in Azospirillum brasilense Stress Endurance and Root Colonization. *Appl Environ Microb* 69: 3244-3250

Kadouri D, Jurkevitch E, Okon Y and Castro-Sowinski S (2005) Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates. *Crit Rev Microbiol* 31: 55-67

Kranz RG, Gabbert KK and Madigan MT (1997 b) Positive selection systems for discovery of novel polyester biosynthesis genes based on fatty acid detoxification *Appl Environ Microbiol* 63: 3010-3013

Kranz RG, Gabbert KK, Locke TA and Madigan MT (1997) Polyhydroxyalkanoate production in Rhodobacter capsulatus: genes, mutants, expression, and physiology *Appl Environ Microbiol* 63: 3003-3009

Kung SS, Chuang YC, Chen CH and Chien CC (2007) Isolation of polyhydroxyalkanoates-producing bacteria using a combination of phenotypic and genotypic approach. *Lett Appl Microbiol* 44: 364–371

Lemoigne M (1926) Produits de deshydration et de polymerisation de lacide β-oxybutyrique. *Bull Soc Chim Biol* 8: 770-782

Lemos PC, Viana C, Salgueiro EN, Ramos AM, Crespo JPSG and Reis MAM (1998) Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. *Enzyme Microb Tech* 22: 662-671

López NI, Floccari ME, García AF, Steinbüchel A and Méndez BS (1995) Effect of poly3-hydroxybutyrate content on the starvation survival of bacteria in natural waters. *FEMS Microbiol Ecol* 16:95-102

López NI, Pettinari MJ and Méndez BS (1997) Detection of reserve polymer synthesis genes in natural bacterial populations. *FEMS Microbiol Ecol* 22: 129-136

Luengo JM, García B, Sandoval A, Naharro G and Olivera ER (2003) Bioplastics from microorganisms. *Curr Opin Microbiol* 6: 251-260

Nickels JS, King JD and White DC (1979) Poly-,3-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. *Appl Environ Microbiol* 37: 459-465

Oshiki M, Onuki M, Satoh H and Mino T (2008) PHA-accumulating microorganisms in full-scale wastewater treatment plants. *Water Sci Technol* 58: 13-20

Pötter M and Steinbüchel A (2006) Biogenesis and Structure of hydroxyalkanoate Granules. Microbiol Monogr Vol 1 J.M. Shively: Inclusions in Prokaryotes Springer Berlin / Heidelberg

Rehm BHA and Steinbüchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int J Biol Macromol* 25: 3-19

Reichardt W, Mascarin G, Padre B and Doll J (1997) Microbial Communities of Continuously Cropped, Irrigated Rice Fields. *Appl Environ Microb* 63: 233–238

Romo DMR, Grosso MV, Solano NCM and Castaño DM (2007) A most effective method for selecting a broad range of short and mediumchain-length polyhidroxyalcanoate producing microorganisms. *Electronic Journal of Biotechnology* 10 (3)

Ruiz JAN, López I and Méndez BS (1999) Polyhydroxyalkanoates degradation affects survival of Pseudomonas oleovorans in river water microcosms. *Rev Argent Microbiol* 31: 201-204

Schlegel HG, Gottschalk G and Von Bartha R (1961) Formation and Utilization of Poly-β-Hydroxybutyric Acid by Knallgas Bacteria (*Hydrogenomonas*). *Nature* 191: 463-465

Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA and Lugtenberg BJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting Pseudomonas bacteria. *Mol Plant Microbe Interact* 9 :600-7

Solaiman DKY and Ashby RD (2005) Rapid Genetic Characterization of Poly(hydroxyalkanoate) Synthase and Its Applications. *Biomacromolecules* 6: 532–537

Sørensen J (1997) The rhizosphere as a habitat for soil microorganisms. In J. D. Van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc, New York, N.Y. 21-45

Stam H, Devries W, Stouthamer AH and Vanverseveld HW (1986) Utilization of poly-b-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571. *FEMS Microbiol Lett* 35: 215–220

Steinbüchel A (1996) PHB and other polyhydroxyalkanoic acids. In: H. J. Rehm, G. Reed, A. Pühler and P. Stadler (eds.) Biotechnology, 2nd Edition, 6: 403-464

Steinbüchel A and Füchtenbusch B (1998) Bacterial and other biological systems for polyester production. *Trends Biotechnol* 16: 419–27

Sudesh K, Abe H, Doi Y (2000) "Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters" *Prog Polym Sci* 25: 1503-1555

Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y and Munekata M (2003) Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil. *J Biosci Bioeng* 95: 77-81

Tal S and Okon Y (1985) Production of the reserve material poly-β-hydroxybutyrate and its function in Azospirillum brasilense Cd. *Can. J. Microbiol*. 31: 608–613

Tal S, Smirnoff P and Okon Y (1990) The regulation of poly-β-hydroxybutyrate metabolism in Azospirillum brasilense during balanced growth and starvation. *J Gen Microbiol* 136: 1191-1196

Taroncher-Oldenburg G, Nishina K and Stephanopoulos G (2000) Identification and Analysis of the Polyhydroxyalkanoate-Specific beta -Ketothiolase and Acetoacetyl Coenzyme A Reductase Genes in the Cyanobacterium Synechocystis sp. Strain PCC6803. *Appl Environ Microb* 66: 4440-4448

Tunlid A, Baird BH, Olson S, Findlay RH, Odham G and White DC (1985) Determination of phospholipid ester-linked fatty acids for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* L. *Can. J. Microbiol* 31: 1113–1119

Villanueva L, Del Campo J and Guerrero R (2010) Diversity and physiology of polyhydroxyalkanoate-producing and -degrading strains in microbial mats. *FEMS Microbiol Ecol*. Epub ahead of print

Wallen LL and Rohwedder WK (1974) Poly-beta-hydroxyalkanoate from activated sludge. *Environ Sci Technol* 8: 576–579

Wang JG and Bakken LR (1998) Screening of Soil Bacteria for Poly-β-Hydroxybutyric Acid Production and Its Role in the Survival of Starvation. *Microbial Ecol* 35 (1)

Willis LB and Walker GC (1998) The phbC (poly-b-hydroxybutyrate synthase) gene of Rhizobium (Sinorhizobium) meliloti and characterization of phbC mutants. *Can J Microbiol* 44: 554–564

Wong PP and Evans HJ (1971) Poly-3-hydroxybutyrate Utilization by Soybean (*Glycine max* Merr.) Nodules and Assessment of Its Role in Maintenance of Nitrogenase Activity. *Plant Physiol* 47: 750-755

8. Appendix

Compendium of Publications and Manuscripts

Publication I

Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants

Publication II

Lichen-associated bacteria antagonistic to phytopathogens and their potential to accumulate polyhydroxyalkanoates

Manuscript I

Analysis of ecosystem function and endophytic life style of the polyhydroxybutyrate-producing *Burkholderia terricola* ZR2-12

Manuscript II

Review: Environmental habitats and ecology of PHA producing bacteria

Publication I

Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants

FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants

Ilona Gasser, Henry Müller & Gabriele Berg

Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

Correspondence: Gabriele Berg, Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria. Tel.: +43 316 8738310; fax: +43 316 8738819; e-mail: gabriele.berg@tugraz.at

Received 5 February 2009; revised 15 April 2009; accepted 22 May 2009.
Final version published online 27 July 2009.

DOI:10.1111/j.1574-6941.2009.00734.x

Editor: Philippe Lemanceau

Keywords
polyhydroxyalkanoates; rhizosphere; SSCP; *phaC*.

Abstract

Polyhydroxyalkanoates are energy reserve polymers produced by bacteria to survive periods of starvation in natural habitats. Little is known about the ecology of polyhydroxyalkanoate-producing bacteria. To analyse the occurrence of this specific group on/in seven different plant species, a combined strategy containing culture-dependent and -independent methods was applied. Using microbial fingerprint techniques (single-strand conformation polymorphism analysis with specific primers for *phaC* gene encoding the key enzyme of the polyhydroxyalkanoate synthesis), a high number of bands were especially found for the rhizosphere. Furthermore, cluster analysis revealed plant species-specific communities. Isolation of bacteria, recognition of brightly refractile cytoplasmatic inclusions, lipophilic stainings and a PCR strategy targeted on the *phaC* gene were used as a culture-dependent strategy for the detection of polyhydroxyalkanoate-producing bacteria. Results again represent a high degree of plant specificity: the rhizosphere of sugar beet contained the highest number of positive strains. This was confirmed by quantitative PCR: the relative copy number of *phaC* was statistically and significantly enhanced in all rhizospheres in comparison with bulk soil. New polyhydroxyalkanoate-producing bacterial species were detected: for example, *Burkholderia terricola*, *Lysobacter gummosus*, *Pseudomonas extremaustralis*, *Pseudomonas brassicacearum* and *Pseudomonas orientalis*. Our results confirm the hypothesis that the rhizosphere is an interesting hidden reservoir for polyhydroxyalkanoate producers.

Introduction

Plants constitute microbial ecosystems, which harbour diverse and specific bacterial communities (Smalla *et al.*, 2001; reviewed by Garbeva *et al.*, 2004; Berg & Smalla, 2009). A long list of microorganisms are known for positive interactions with their host plant and fulfil important functions for them (Whipps, 2001; Lugtenberg *et al.*, 2002; Raaijmakers *et al.*, 2008). One of these functions is plant growth promotion by bacterial synthesis of phytohormones, the breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate deaminase and/or increased mineral and N availability in soil (Glick *et al.*, 2004). Another important function of plant-associated bacteria is pathogen defence (Cook *et al.*, 1995; Weller *et al.*, 2002). However, so far not all plant–microorganism interactions and their effects on each other are understood.

Plants themselves can be divided into different microenvironments, which are characterized by specific biotic and abiotic conditions. Especially in the rhizosphere, according to root exudations, the conditions and nutrient contents are dynamically changing (Bais *et al.*, 2006). Indigenous microorganisms must be adapted to the changing conditions of their environment and fluctuations in the concentration of nutrients exuded by plant roots. Prokaryotes have evolved multitudinous mechanisms of resistance towards stress conditions such as those that have an inherent ability to form cysts and spores to survive desiccated environments (Potts, 1994). Additionally, bacteria are able to exhibit a metabolic versatility in order to overcome fluctuations in the chemical conditions of their environment. Inclusion of storage substances is another bacterial strategy that increases survival in changing surroundings (Müller *et al.*, 1999). Polyhydroxyalkanoates are one such group that bacteria produce to store

energy (Steinbüchel *et al.*, 2002). A long list of bacterial species such as *Ralstonia eutropha*, *Bacillus megaterium* and *Pseudomonas oleovorans* are able to accumulate polyhydroxyalkanoates (Füchtenbusch *et al.*, 2000; Rehm *et al.*, 2002; McCool *et al.*, 2006). The synthetic pathway was already analysed in detail (Rehm & Steinbüchel, 1999; Steinbüchel *et al.*, 2006), and on this basis, biotechnological applications for the biodegradable polymers as bioplastics were developed (Zinn *et al.*, 2001; Chen & Wu, 2005; Koller *et al.*, 2007). The majority of polyhydroxyalkanoate-producing bacteria were isolated from soil and activated sludge (Wang & Bakken, 1998; Khardenavis *et al.*, 2005). Recently, new bioresources such as marine environments were analysed regarding their potential to harbour new polyhydroxyalkanoate producers (Arun *et al.*, 2009). In these studies, culture-dependent strategies such as microscopic methods or chemical extractions of polyhydroxyalkanoates were applied to isolate and characterize the polyhydroxyalkanoate production of bacteria. Although fast-growing *Rhizobium* species, which are naturally occurring inhabitants of plants, are well known as producers of polyhydroxyalkanoates in sludge and industrial wastewater (Kadouri *et al.*, 2005; Ben Rebah *et al.*, 2009; Ratcliff & Denison, 2009), little is known about plants as a reservoir for polyhydroxyalkanoate-producing bacteria. The hypothesis of this study was that especially the rhizosphere should provide conditions under which diverse polyhydroxyalkanoate-producing bacteria are enriched.

The objective of this work was to analyse the diversity and distribution of polyhydroxyalkanoate-producing bacteria in the rhizosphere and other microenvironments of seven different plant species by a hierarchical combination of cultivation-dependent and -independent methods. By the cultivation-dependent approach, bacterial isolates were screened for their ability to form polyhydroxyalkanoates *in vitro* as well as for the presence of polyhydroxyalkanoate-synthase genes using a PCR strategy. On the other hand, a cultivation-independent method [single-strand conformation polymorphism (SSCP) analysis with specific primers for *phaC*, targeted the key enzyme of the polyhydroxyalkanoate synthesis] was used to determine the occurrence of genes within the microbial community of plant habitats. Both strategies resulted in the conclusion that the rhizosphere is an interesting reservoir for polyhydroxyalkanoate producers and harbours as yet undiscovered potential of these biotechnologically relevant bacteria.

Materials and methods

Experimental design and sampling

For a preliminary screening, we analysed the genetic potential of single strains and total bacterial communities to synthesize polyhydroxyalkanoates. Therefore, we used isolates from the culture collection Strain Collection of Antagonistic Microorganisms (SCAM), Graz University of Technology, Austria and samples from other research projects of our working group. The latter included samples from the rhizosphere of oilseed rape (*Brassica napus* L.), polygonum (*Polygonum bistorta* L.), olive (*Olea europaea* L.), sugar beet (*Beta vulgaris* L.) and from the phyllosphere and carposphere of strawberry (*Fragaria* × *ananassa* [Duchesne] Decaisne & Naudin) and grapevine (*Vitis vinifera* L.) (Zachow *et al.*, 2008; Schmid & Berg, 2009). In addition, bacterial communities isolated from peat mosses (*Sphagnum fallax* Klinggr.) were tested (Opelt *et al.*, 2007).

For a systematic study, bacterial communities of two different field trials were analysed. In Field trial 1, in Deggendorf (Germany), bacteria were isolated from rhizosphere of sugar beet (*B. vulgaris* L.), oilseed rape (*B. napus* L.) and wheat (*Triticum aestivum* L.). In Field trial 2, strawberry (*Fragaria* × *ananassa* [Duchesne] Decaisne & Naudin cv. Elsanta) and oilseed rape (*B. napus* L.) rhizosphere-associated bacteria were investigated at three different growth stages of host plants (young, flowering and senescent plants) at different locations in Germany (Braunschweig, Rostock). The different crop plants were grown in a randomized block design with a minimum of four replicates per crop plant. In general, plants were analysed at three different developmental stages: (1) young plants, (2) flowering plants and (3) early senescent plants. Detailed information about the field trials are described in Zachow *et al.*, 2008 (Field trial 1) and Berg *et al.*, 2006 (Field trial 2).

For all analyses, plant roots with adhering soil, leafs, stems and fruits of investigated plants were sampled into sterile plastic bags. Samples taken from six plants were considered as one composite sample. For the isolation of plant-associated microorganisms 5 g each of roots and other organs were pooled and transferred into a new Stomacher bag. Samples were extracted in a Stomacher laboratory blender (BagMixer, Interscience, St Nom, France). After addition of 50 mL 0.85% NaCl solution samples were shaken for 3 min; and then the supernatants were decanted into 50-mL tubes. The supernatants decanted into 50-mL tubes were used for cultivation as well as for cultivation-independent investigation procedures.

Isolation of community DNA for SSCP and quantitative real time (qRT)-PCR analysis

Total genomic DNA from soil microbial communities were extracted by mechanical disruption and homogenization of up to 500 mg soil in a FastPrep® Instrument (MP Biomedicals, Carlsbad) for 40 s at speed 6.0. Afterwards, DNA was purified by the FastDNA® Spin Kit for soil (MP Biomedicals). DNA was additionally purified by the GeneClean Turbo Kit (MP Biomedicals) containing the special binding buffer

guanidine thiocyanate. The procedure of SSCP analysis was carried out according to Schwieger & Tebbe (1998) using the *phaC*-specific primers I-179L (5′-ACAGATCAACAAGTTC TACATCTTCGAC-3′) and 5′phosphorylated I-179R (5′-GG TGTTGTCGTTGTTCCAGTAGAGGATGTC-3′) (Solaiman *et al.*, 2000) in a reaction mixture of 30 μL Taq&Go PCR Mastermix (MP Biomedicals) supplemented with 0.5 μM of each primer and about 60–80 ng of template DNA. The thermal cycle programme consisted of one cycle of 94 °C for 5 min, 53 °C for 1 min, 72 °C for 2 min; 36 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min; and a final incubation step at 72 °C for 10 min. The PCR products were purified by the GeneClean Turbo Kit (MP Biomedicals) and subjected to a λ-exonuclease (New England Biolabs, Ipswich) digestion and DNA single-strand folding. The polyacrylamide gel electrophoresis was performed on a TGGE apparatus (Biometra, Göttingen, Germany) at 26 °C and 400 V for 24 h using 8% (w/v) acrylamide gels. Afterwards, the gels were silver stained according to the procedure described by Bassam *et al.* (1991).

To determine the relative abundance of the gene *phaC*, a qRT-PCR using the RotorGene 6000 apparatus (Corbett Research, Sydney, Australia) was performed. The same PCR conditions and primer set as described above, except for the addition of SybrGreen I to a final concentration of 0.4 × (Invitrogen, Carlsbad) and exactly 40 ng template DNA, were applied. Following the PCR reactions, the amplicons were verified by melting-point analysis and agarose gel electrophoresis. Four samples of each habitat were analysed in two independent PCR experiments as duplicates. Additionally, a standard curve was generated by quantifying *phaC* in aliquots of a dilution series of a total community DNA sample. Abundances of the amplified gene in relation to the soil samples were calculated by applying the equation $E^{\Delta C_t}$, where E is the PCR efficiency according to the standard curve. For statistical analyses, calculated values were subjected to *post hoc* Tukey's test.

Isolation of bacteria

An aliquot was taken from the supernatant as described above in the sampling procedure, serially diluted and plated onto R2A, a nutrient-poor medium suitable for the growth of diverse plant-associated bacteria (Difco, Detroit). Plates were incubated for 3 days at 20 °C. Colonies that developed on agar were differentiated by colour, elevation, shape and edge appearance. From each sample, 16 colonies were picked up with a sterilized toothpick and plated onto Luria–Bertani (LB) plates for further screening.

Screening of polyhydroxyalkanoate-producing bacteria

Bacterial isolates were screened for their genetic potential for polyhydroxyalkanoate production and their *in vitro* ability to produce polyhydroxyalkanoates. The screening was based upon both molecular and microscopic approaches. Colony PCR techniques were used for screening bacterial polyhydroxyalkanoate producers isolated from the environment using a *phaC*-specific primer set (Sheu *et al.*, 2000) in a PCR reaction mixture with a total volume of 30 μL consisting of 5 × Taq&Go PCR Mastermix (MP Biomedicals), 0.5 μM of each primer [phaCF1: 5′-ATCAACAA(GGG/A)T(TT/A) CTAC(AA/G)TC(CC/T)T(CC/G)GACCT-3′ and phaCR1: 5′-AGGTAGTTGT(TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)(GGG/A)TAG(TTT/G)TCCA-3′], 3% dimethyl sulphoxide and 1 M betaine (Sigma). The thermal cycle programme consisted of one cycle of 94 °C for 10 min, 51 °C for 2 min, 72 °C for 2 min; 35 cycles of 94 °C for 20 s, 57 °C for 45 s, 72 °C for 1 min; and a final incubation step at 72 °C for 5 min. One microlitre of a DNA preparation obtained by suspending cell material from an overnight culture in 50 μL demineralized water and incubating at 96 °C for 10 min served as the template. PCR-amplified DNA fragments were observed by gel electrophoresis using 1.0% agarose gels. Five microlitres of each amplification mixture were subjected to agarose gel electrophoresis and ethidium bromide staining. The amplified DNA fragments were visualized by UV illumination. Exemplarily, DNA fragments were sequenced to prove their identity. To detect the formation of polyhydroxyalkanoate-containing granules microscopically, bacteria were plated onto a solid minimal medium containing high amount of glucose (1.0 g L^{-1}) and low amount of nitrogen (0.5 g L^{-1} NH_4Cl), which is optimized for polyhydroxybutyrate production. Plates were incubated for at least 5 days at 30 °C before the cells were stained by Sudan black B (Steinbüchel & Oppermann-Sanio, 2003).

Identification and characterization of polyhydroxyalkanoate-producing bacteria

A single colony on LB medium was picked with a sterile toothpick into 50-μL demineralized water in a 1.5-mL Eppendorf tube. The cell suspension was heated to 96 °C for 10 min. The 30-μL reaction mixture contained 6 μL 5 × Taq&Go (MP Biomedicals), 1.5 μL of primer pair mix EubI-forward (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492r-reverse (5′-TACGGYTACCTTGTTACGACTT-3′) both at a concentration of 10 pmol μL^{-1} and 20–30 ng template. The PCR products were purified with GeneClean Turbo Kit as recommended by the manufacturer. The fragments were sequenced using the reverse primer 1492r. For identification of related sequences, a database alignment using BLAST algorithm was performed.

Altogether, 58 representative strains were characterized by their BOX fingerprint using the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (Rademaker & De

Bruijn, 1997). BOX groups were defined according to the similarity of BOX patterns and cluster analysis at 80%. To estimate this borderline, we used a higher proportion than evaluated for variability by analysing three independent replicates of the same strain (< 89%).

Statistics

Computer-assisted evaluation of polyhydroxyalkanoate community profiles obtained by SSCP and BOX-PCR was performed using the GELCOMPAR® program, version 4.1 (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed with the unweighted pair group method with arithmetic average algorithm.

Nucleotide sequence accession numbers

Bacterial sequence accession numbers for sequences submitted to the EMBL nucleotide sequence database are FN313510–FN313537.

Results

Preliminary screening for polyhydroxyalkanoate-producing bacteria in plant-associated communities

To get an overview of the occurrence and diversity of polyhydroxyalkanoate-producing bacteria in plant-associated microenvironments, total community DNA and single-strain DNA derived from different plant species (oilseed rape, polygonum, olive, sugar beet, strawberry and grapevine) as well as from different microenvironments (phyllosphere, carposphere, rhizosphere and endosphere) were analysed for the occurrence of *phaC* genes. While no positive results were obtained for all aboveground microenvironments (phyllosphere and carposphere), in endo- and rhizosphere samples, the occurrence of *phaC* could be demonstrated. This was confirmed for all plant species. For example, total community DNA of 10 olive endospheres and 11 olive rhizospheres were tested on *phaC* genes. Interestingly, only in samples from rhizospheres (nine out of 10), but not in endosphere samples, *phaC* genes were detected.

Polyhydroxyalkanoate-producing bacteria in the rhizosphere of sugar beet, wheat and oilseed rape (Field trial 1)

In a comparative study, 180 bacteria were isolated from the rhizosphere of sugar beet, oilseed rape and wheat. We applied two methods to detect polyhydroxyalkanoate-producing organisms: microscopic analysis and PCR detection of the *phaC* gene. On comparing results obtained by PCR, sugar beet was found to be associated with a higher number (90%) of polyhydroxyalkanoate-producing bacteria than oilseed rape (32%) and wheat (31%) (Fig. 1a). Using the microscopic technique, less positive strains were found. However, also with this technique, the highest proportion was found in the rhizosphere of sugar beet. In general, a low correlation between results of both techniques was observed; only 25 isolates showed a correlation between intracellular granules and a positive PCR signal. Furthermore, 66 isolates carried the *phaC* gene but showed no visible polyhydroxyalkanoate accumulation during cultivation.

In addition, for profiling and quantifying *phaC* in plant habitats, two cultivation-independent techniques were applied. The qualitative approach using qRT-PCR resulted in statistically significant differences between the samples from the analysed rhizospheres and those from bulk soil (Fig. 1b), whereas in the rhizosphere of wheat and oilseed rape, the abundance was approximately twofold, the copy number in the sugar beet rhizosphere was > 8.5-fold enhanced. Using the qualitative approach, all SSCP profiles of polyhydroxyalkanoate synthesis gene *phaC* of the rhizosphere community showed a high diversity. The highest diversity was found in the rhizosphere of wheat and the lowest for the oilseed rape rhizosphere. Furthermore, these fingerprints were specific for each plant species. This is shown in Fig. 2 for the SSCP pattern of bacterial communities of oilseed

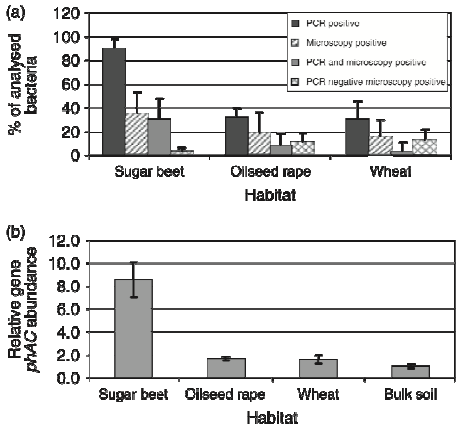

Fig. 1. (a) Proportion of polyhydroxyalkanoate-producing bacteria isolated from the rhizosphere of sugar beet, oilseed rape and wheat (Field trial 1). They were screened for their capacity to form polyhydroxyalkanoates using PCR and microscopic methods. Error bars represent confidence intervals at $P \leq 0.05$. (b) Relative number of *phaC* gene copies in the rhizosphere of sugar beet, oilseed rape and wheat in comparison with bulk soil (relative copy number = 1) from Field trial 1 determined by qRT-PCR from total genomic DNA. Error bars represent confidence intervals at $P \leq 0.05$.

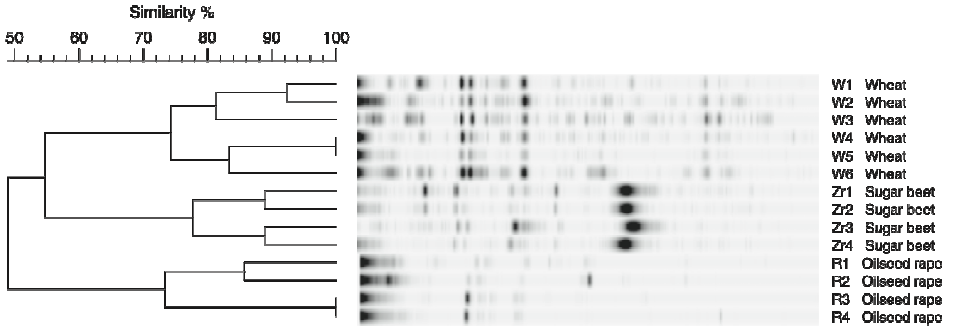

Fig. 2. Cluster analysis of community fingerprints of polyhydroxyalkanoate-synthesizing bacteria in the rhizosphere of wheat, sugar beet and oilseed rape using the hierarchical method, unweighted pair group method with arithmetic mean, after calculation of the band-based Dice similarity coefficient. Independently analysed replicates of the treatments were marked 1–4. W, wheat; Z, sugar beet; R, oilseed rape.

rape, wheat and sugar beet, which form distinct groups according to cluster analysis.

Polyhydroxyalkanoate-producing bacteria in the rhizosphere of strawberry and oilseed rape at different stages of plant development and in comparison with bulk soil (Field trial 2)

It is well known that the rhizosphere communities show a strong shift during their plant developmental stages (Smalla *et al.*, 2001). Therefore, in this field trial, the polyhydroxyalkanoate-producing bacterial community was analysed at three different growth stages of strawberry and oilseed rape plants (young, flowering and senescent plants). On comparing both host plants, no significant differences were found in the proportion of polyhydroxyalkanoate-producing bacteria regarding the plant development stage (data not shown). The proportion of positive tested bacteria ranged from 90% (young strawberry plants) to 36% (flowering oilseed rape plants). In this field trial, which was performed at two locations (Rostock and Braunschweig, Germany), the proportion of polyhydroxyalkanoate producers in the rhizosphere was also compared with those in bulk soil. Altogether, at both sites, the proportion of polyhydroxyalkanoate-producing bacteria was statistically and significantly enhanced in the rhizosphere in comparison with bulk soil (Fig. 3).

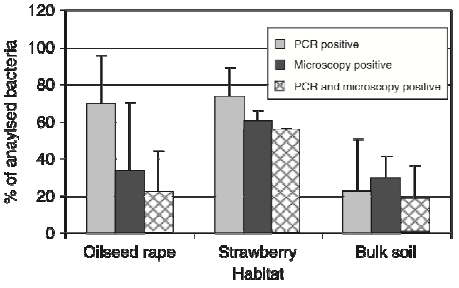

Fig. 3. Proportion of polyhydroxyalkanoate-producing bacteria isolated from the rhizosphere of oilseed rape and strawberry in comparison with bulk soil from Braunschweig and Rostock (Germany) (Field trial 2). They were screened for their capacity to form polyhydroxyalkanoates using PCR and microscopic methods. Error bars represent confidence intervals at $P \leq 0.05$.

Identification and characterization of polyhydroxyalkanoate-producing bacteria

To identify polyhydroxyalkanoate-producing bacteria, 58 of the strains, which were positive in microscopic as well as molecular evaluation, were first characterized on the genotypic level using BOX-PCR. GELCOMPAR was used for the comparison of BOX patterns. This is exemplarily shown in Fig. 4 for the isolates obtained from the rhizosphere of sugar beet, oilseed rape and wheat from the Deggendorf site (Field trial 1). Altogether, 23 different BOX profiles were obtained. According to the specific profile in BOX pattern, 30 bacterial isolates were selected for further partial sequencing of 16S rRNA genes. This selection and characterization of bacterial polyhydroxyalkanoate producers is shown in Table 1. Altogether, eight bacterial genera (*Pseudomonas*, *Sinorhizobium*, *Variovorax*, *Lysobacter*, *Ensifer*, *Pantoea*, *Erwininia* and *Burkholderia*) as well as several species could be identified.

Discussion

In our study, we show that plant habitats, especially the rhizosphere, are an interesting reservoir for

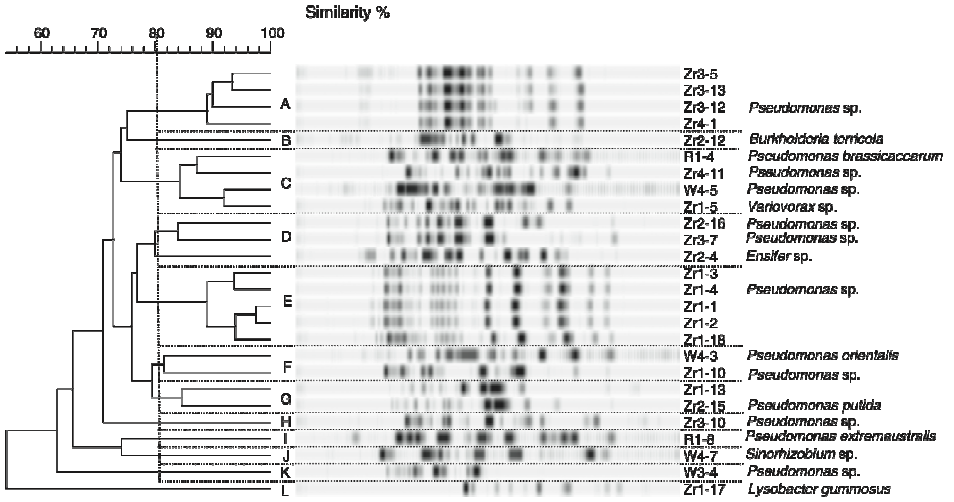

Fig. 4. Dendrogram showing the relationship of isolates obtained from the rhizosphere of sugar beet, oilseed rape and wheat from the site in Deggendorf (Field trial 1), based on BOX-PCR fingerprints using cluster analysis using the unweighted pair group method and arithmetic averages.

polyhydroxyalkanoate-producing bacteria. This was shown by two independent strategies in a hierarchical experimental design. Using the cultivation-independent method SSCP of bacterial *phaC* genes, a high number of different bands were observed in all rhizosphere samples. In addition, the characterization of isolates results in a high proportion (up to 90%) of polyhydroxybutyrate-producing strains on plants.

In the study, different ecological aspects of polyhydroxyalkanoate-producing bacteria were analysed. Altogether, seven different plant species were included in the investigation. Clear plant-specific effects were found for the rhizosphere community. Root exudates are a key factor for the enrichment of specific microbial populations in the rhizosphere; this was shown using stable isotope probing in combination with microbial fingerprints by Haichar *et al.* (2008). Exudates consist of ions, free oxygen and water, enzymes mucilage and a diverse array of carbon-containing primary and secondary metabolites (Uren, 2000). Ten to 44% of the photosynthetically fixed carbon is excreted by the root (Bais *et al.*, 2006). Organic acids, sugars, amino acids, lipids, coumarins, flavonoids, proteins, enzymes, aliphatics and aromatics are examples of the primary substances found at the soil–root interface. These substances are not continuously produced, but all of them can be used by bacteria to store the energy in polyhydroxyalkanoate granules.

In general, all applied techniques confirm our conclusions and showed the same tendencies. While the cultivation-independent methods to detect *phaC* genes in the environment worked well, discrepancies exist between the methods to determine polyhydroxyalkanoate production on the strain level. Here, microscopic techniques including staining and molecular techniques were applied. In general, molecular screening resulted in a higher proportion of positive strains. However, the occurrence of the *phaC* gene in the genome is not necessarily correlated with the *in vitro* production. This should be considered in the interpretation of the high percentages obtained. On the other side, the microscopic techniques also have their disadvantages. The medium developed for these investigations is not appropriate for all bacteria. In addition, despite modern microscopes, it is sometimes difficult to detect the granules in the single cells. Because of these problems, we decided to include only those microorganisms into further investigations, which were positive in both approaches. For the molecular approaches to detect polyhydroxyalkanoates in single strains and bacterial communities, different primers were applied. The primer set published by Sheu *et al.* (2000), which was used for PCR with single isolates, are supposed to target a broad range of *phaC* genes. However, because of the highly degenerated nature of the sequence, this primer caused unspecific amplicons, when total community DNA was used as template. For this reason, we decided to use a less universal and also less degenerated primer set published by Solaiman *et al.* (2000) to perform SSCP and qRT-PCR analyses.

Table 1. List of polyhydroxyalkanoate-producing (positive in PCR and microscopy) bacteria isolated from sugar beet, oilseed rape and wheat

BOX group	Isolate no.	Microenvironment	Closest database match (EMBL no.)	% Homology
C	R1-4	Rhizosphere oilseed rape	*Pseudomonas brassicacearum* (FN313510)	99
I	R1-8	Rhizosphere oilseed rape	*Pseudomonas extremaustralis* (FN313511)	99
K	W3-4	Rhizosphere wheat	*Pseudomonas* sp. (FN313512)	100
F	W4-3	Rhizosphere wheat	*Pseudomonas orientalis* (FN313513)	99
C	W4-5	Rhizosphere wheat	*Pseudomonas* sp. (FN313514)	100
J	W4-7	Rhizosphere wheat	*Sinorhizobium* sp. (FN313515)	100
E	ZR1-4	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313516)	99
C	ZR1-5	Rhizosphere sugar beet	*Variovorax* sp. (FN313517)	100
F	ZR 1-10	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313518)	100
L	ZR 1-17	Rhizosphere sugar beet	*Lysobacter gummosus* (FN313519)	99
D	ZR2-4	Rhizosphere sugar beet	*Ensifer* sp. (FN313520)	100
B	ZR2-12	Rhizosphere sugar beet	*Burkholderia terricola* (FN313521)	100
G	ZR2-15	Rhizosphere sugar beet	*Pseudomonas putida* (FN313522)	100
D	ZR2-16	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313523)	98
D	ZR3-7	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313524)	98
H	ZR3-10	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313525)	99
A	ZR3-12	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313526)	100
C	ZR4-11	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313527)	98
M	LV2-2-10	Rhizosphere sugar beet	*Pseudomonas fluorescens* (AJ969079)	87
N	R1-2-4	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313528)	100
O	Sel1-2-6	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313529)	99
P	At1-1-2	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313530)	100
Q	Phs2-1-6	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313531)	99
R	At2-2-11	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313532)	99
S	SDK1-2-10	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313533)	99
S	SDK1-2-16	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313534)	100
T	SDK1-2-18	Rhizosphere sugar beet	*Pantoea* sp. (FN313535)	99
U	SDK2-2-6	Rhizosphere sugar beet	*Pseudomonas* sp. (AJ969083)	100
V	SDK2-2-8	Rhizosphere sugar beet	*Pseudomonas* sp. (FN313536)	100
X	RE 1-3-41	Rhizosphere sugar beet	*Erwinia rhapontici* (FN313537)	99

Arabic numerals represent the plots (1–4) and the strain number (1–16).
Letters represent the location or microhabitat: R, oilseed rape (Deggendorf, Germany); W, wheat (Deggendorf, Germany); ZR, sugar beet (Deggendorf, Germany); Sel, Seligenstadt, Germany (ZR); At, Attily, France (ZR); Phs:, Hilprechtshausen, Germany (ZR); SDK Schoondijke, the Netherlands (ZR); LV, LaVeuve, France (ZR).

This study was carried out to understand the ecological behaviour and reservoirs of polyhydroxyalkanoate-producing bacteria. In addition, new polyhydroxyalkanoate-producing bacterial species were detected: for example, *Burkholderia terricola*, *Lysobacter gummosus*, *Pseudomonas extremaustralis*, *Pseudomonas brassicacearum* and *Pseudomonas orientalis*. Our results confirm the hypothesis that the rhizosphere is an interesting and yet unexploited reservoir for polyhydroxyalkanoate producers, and an interesting bioresource not only for plant growth-promoting and antagonistic bacteria.

Acknowledgements

We thank Martin Koller for helpful discussions and Johanna Klostermann (Graz) for mentoring. 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).

References

Arun A, Arthi R, Shanmugabalaji V & Eyini M (2009) Microbial production of poly-beta-hydroxybutyrate by marine microbes isolated from various marine environments. *Bioresource Technol* **100**: 2320–2323.

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

Bassam BJ, Caetano-Anolles G & Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* **80**: 81–84.

Ben Rebah F, Prévost D, Tyagi RD & Belbahri L (2009) Poly-beta-hydroxybutyrate production by fast-growing rhizobia

cultivated in sludge and in industrial wastewater. *Appl Biochem Biotech* **158**: 155–163.

Berg G & Smalla K (2009) Plant species versus soil type: which factors influence the structure and function of the microbial communities in the rhizosphere? *FEMS Microbiol Ecol* **68**: 1–13.

Berg G, Opelt K, Zachow C, Lottmann J, Götz M, Costa R & Smalla K (2006) The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol Ecol* **56**: 250–261.

Chen GQ & Wu Q (2005) The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* **26**: 6565–6578.

Cook RJ, Tomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G & Kim DS (1995) Molecular mechanisms of defense by rhizobacteria against root disease. *P Natl Acad Sci USA* **92**: 4197–4201.

Füchtenbusch B, Wullbrandt D & Steinbüchel A (2000) Production of polyhydroxyalkanoic acids by *Ralstonia eutropha* and *Pseudomonas oleovorans* from an oil remaining from biotechnological rhamnose production. *Appl Microbiol Biot* **53**: 167–172.

Garbeva P, van Veen JA & van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annu Rev Phytopathol* **42**: 243–270.

Glick BR, Penrose DM & Ma W (2004) Bacterial promotion of plant growth. *Biotechnol Adv* **9**: 135–138.

Haichar FE, Marol C, Berge O, Rangel-Castro JI, Prosser JI, Balesdent J, Heulin T & Achouak W (2008) Plant host habitat and root exudates shape soil bacterial community structure. *ISME J* **2**: 1221–1230.

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

Khardenavis A, Guha PK, Kumar MS, Mudliar SN & Chakrabarti T (2005) Activated sludge is a potential source for production of biodegradable plastics from wastewater. *Environ Technol* **26**: 545–552.

Koller M, Hesse P, Bona R, Kutschera C, Atlic A & Braunegg G (2007) Potential of various archae- and eubacterial strains as industrial polyhydroxyalkanoate producers from whey. *Macromol Biosci* **7**: 218–226.

Lugtenberg BJJ, Chin-A-Woeng TFC & Bloemberg GV (2002) Microbe–plant interactions: principles and mechanisms. *Antonie van Leewenhoek* **81**: 373–383.

McCool GJ, Fernandez T, Li N & Cannon MC (2006) Polyhydroxyalkanoate inclusion-body growth and proliferation in *Bacillus megaterium*. *FEMS Microbiol Lett* **138**: 41–48.

Müller S, Bley T & Babel W (1999) Adaptative responses of *Ralstonia eutropha* to feast and famine conditions analysed by flow cytometry. *J Biotechnol* **75**: 81–97.

Opelt K, Chobot V, Hadacek F, Schömann S, Eberl L & Berg G (2007) Investigations of the structure and function of bacterial communities associated with *Sphagnum* mosses. *Environ Microbiol* **9**: 2795–2809.

Potts M (1994) Desiccation tolerance of prokaryotes. *Microbiol Rev* **58**: 755–805.

Raaijmakers JM, Paulitz CT, Steinberg C, Alabouvette C & Moenne-Loccoz Y (2008) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* DOI 10.1007/s11104-008-9568-6.

Rademaker JLW & De Bruijn FJ (1997) Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. *DNA Markers: Protocols, Applications and Overviews* (Caetano-Anollés G & Gresshoff PM, eds), pp. 151–171. John Wiley & Sons Inc, New York.

Ratcliff BC & Denison RF (2009) Rhizobitoxine producers gain more poly-3-hydroxybutyrate in symbiosis than do competing rhizobia, but reduce plant growth. *ISME J* **3**: 870–872.

Rehm B & Steinbüchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int J Biol Macromol* **25**: 3–19.

Rehm B, Antonio RV, Spiekermann P, Amara AA & Steinbüchel A (2002) Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: *in vitro* evolution, site-specific mutagenesis and development of a PHB synthase protein model. *Biochim Biophys Acta* **1594**: 178–190.

Schmid F & Berg G (2009) Grapevine-associated microorganisms: antagonistic potential towards *Botrytis cinerea* varies between habitats, cultivation methods and grapevine species. *IOBC/WPRS Bull* **43**: 351–354.

Schwieger F & Tebbe CC (1998) A new approach to utilize PCR-single strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microb* **64**: 4870–4876.

Sheu DS, Wang YT & Lee CY (2000) Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. *Microbiology* **146**: 2019–2025.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Roskot N, Heuer H & Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant dependent enrichment and seasonal shifts. *Appl Environ Microb* **67**: 4742–4751.

Solaiman DKY, Ashby RD & Foglia TA (2000) Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Appl Microbiol Biot* **53**: 690–694.

Steinbüchel A & Oppermann-Sanio FB (2003) *Mikrobiologisches Praktikum*. Springer-Verlag, Berlin.

Steinbüchel A, DeBaets S & Vandamme EJ (2002) *Biopolymers*. Wiley-VCH, Weinheim.

Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A & Valentin H (2006) Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiol Lett* **103**: 217–230.

Uren NC (2000) Types, amounts and possible functions of compounds released into the rhizosphere by soil grown plants. *The Rhizosphere: Biochemistry, and Organic Substances at the Soil Interface* (Pinton R, Varani Z & Naniperi P, eds), pp. 19–40. Marcel Dekker, New York.

Wang JG & Bakken LR (1998) Screening of soil bacteria for poly-β-hydroxybutyric acid production and its role in the survival of starvation. *Microb Ecol* **35**: 94–101.

Weller DM, Raaijmakers JM, Gardener BB & Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* **40**: 309–348.

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot* **52**: 487–511.

Zachow C, Tilcher R & Berg G (2008) Sugar beet-associated bacterial and fungal communities show a high indigenous antagonistic potential against plant pathogens. *Microb Ecol* **55**: 119–129.

Zinn M, Witholt B & Egli T (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Adv Drug Deliver Rev* **53**: 5–21.

Publication II

Lichen-associated bacteria antagonistic to phytopathogens and their potential to accumulate polyhydroxyalkanoates

Ilona Gasser, João Vieira de Castro Junior, Henry Müller and Gabriele Berg

Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria

IOBC/wprs Bulletin, Biological Control of Fungal and Bacterial Plant Pathogens „Climate change: Challenge or Threat to Biocontrol?" (in press)

Abstract Bacteria are known to produce polyhydroxyalkanoates (PHA) as a storage substance, which has properties similar to those of petroleum-derived Polypropylene. Therefore, it is important to find PHA producing bacteria, which cope with industrial demands. Among terrestrial ecosystems, the highly active lichen-associated bacterial communities are expected to be a bio-resource for PHA-producers. Bacteria associated with the lichens *Peltigera canina, Lobaria pulmonata, L. immixta, L. virens, and Pseudocyphellaria aurata* collected from different European countries were isolated and tested for their ability to antagonize the phytopathogenic fungi *Rhizoctonia solani* and *Verticillium dahliae*. Antagonists were subsequently screened on their ability to produce PHAs by applying a multiphasic approach. In a first step, a cultivation independent method was developed to determine the occurrence of gene *phaC* within the microbial community of different lichens. Using cultivation-dependent techniques, bacterial isolates from different lichens were tested

on PHA accumulation *in vitro* employing a plate assay. In addition, the presence of the PHA synthase gene *phaC* was determined using PCR analysis. This study indicates that the thalli space of lichens contains a remarkable and very interesting population of PHA producing bacteria, and more studies should be conducted to understand much better the interaction and function of these bacteria in lichens as well as their potential for biocontrol strategies. In conclusion, lichen habitats are an excellent source for PHA-accumulating bacteria.

Keywords: PHA, rhizosphere, lichens, bacteria, biotechnology, antagonism

Introduction

Biopolymers are an alternative to petroleum-based polymers with a wide range of environmental advantages. Bacteria are able to produce polyhydroxyalkanoates (PHA) as storage substances, of which some (e.g. polyhydroxybutyrate) have properties similar to those of polypropylen (PP). Therefore it is necessary to find PHA producing bacteria which cope with industrial demands. The microbial world is found in all kinds of ecological niches: in yet unexplored habitats with its microbial diversity, bacterial strains with great promise for biotechnological applications could be found. Lichens represent fungal symbioses with an algae and/or cyanobacteria as partner. Bacterial communities were found as further and functionally relevant associates. Among the highly diverse microbial community, Alphaproteobacteria belong often to the dominant bacterial groups in lichens (Gonzáles *et al*. 2005; Cardinale *et al*. 2006; Liba *et al*. 2006; Cardinale *et al*. 2008; Grube *et al*. 2009). The involvement of associated bacteria in nutrient cycling is indicated by characterization of cultivable

strains and by the presence of functional genes in the total fraction (Grube *et al*. 2009). The symbiosis of the three partners (fungi, algae and bacteria) enables lichens to tolerate environments, which for one individual partner would be too extreme for long-term surviving (Nash 2008). Beside secondary metabolites and bioactive properties of lichens (Boustie and Grube 2005; Boustie *et al*. 2010), bacteria find a long-existing and specific habitat with dramatically changing abiotic conditions. Inclusion of storage substances may be a competitive advantage. The objective of this work was to analyze the PHA producing potential of antagonistic bacteria associated with lichens.

Material and methods

Isolation and cultivation of bacteria and community DNA

Bacteria were isolated from the lichen species *Peltigera canina, Lobaria pulmonata, L. immixta, L. virens and Pseudocyphellaria aurata* which were sampled in Austria (Styria), Norway (Bergen), Switzerland (St. Gallen) and Portugal (Madeira). An amount of 2-4 g fresh material was suspended in 0.85% NaCl and homogenized for 3 min by agitation. The samples were plated onto R2A agar medium (Difco, Detroit, USA) and were incubated for 5 days at 20 °C. The bacteria were selected according to their morphological characteristics and purified by cultivation in Nutrient Agar medium (NA, Sifin, Berlin, Germany) for 48 hours at 20 °C. Microbial community DNA from lichens was extracted by mechanical disruption and homogenization of 300 mg material in a FastPrep Instrument (BIO 101 Systems: Qbiogene, Carlsbad, USA) for 30 s at speed 5.0 (Grube *et al*. 2009).

Analysis of antagonistic activity

A dual culture method was used to determine the ability of the isolated strains to inhibit the growth of fungal pathogens. Representative isolates of all lichens were tested for their antagonistic activity against *Rhizoctonia solani* RHI SO W4 and *Verticillium dahliae* V25**.** These assays were carried out on petri dishes containing Waksman agar (Berg *et al*. 2002). Only positive antagonistic strains were used for the screening of PHA producing bacteria.

Screening of PHA producing bacteria

In the present study, different lichen-associated bacteria were tested on their ability to produce PHBs by applying a multiphasic approach. Using cultivation-dependent techniques, bacterial isolates originating from different lichen species were screened on their ability to form PHAs *in vitro* by microscopic approach as well as on the presence of PHA synthase genes using polymerase chain reaction (PCR) with the specific primers phaCF1 and phaCR4 (Sheu *et al*. 2000). On the other hand, a cultivation independent method was developed to determine the occurrence of genes for the key enzyme of the PHA synthesis within the microbial community of lichens. Briefly, for PCR reaction the thermal cycle programme consisted of one cycle of 94 °C for 10 min, 51 °C for 2 min, 72 °C for 2 min, and 35 cycles of 94 °C for 20 s, 57 °C for 45 s, 72 °C for 1 min, a final incubation step at 72 °C for 5 min. As template for PCR from single bacteria isolates, 1 µl of a DNA preparation was used obtained by suspending cell material from an over-night culture in 50 µl de-mineralized water and incubating at 98 °C for 10 min. As template for PCR from community DNA, 1 µl of total DNA extract was applied. PCR-amplified DNA fragments were observed by gel electrophoresis using 1% agarose gels. Exemplarily, DNA fragments were sequenced to prove their identity. For microscopy, to detect formation of PHA

granules in single isolates, bacteria were plated onto a solid minimal medium containing high amount of glucose (1.0 g/L) and low amount of nitrogen (0.5 g/L NH4Cl) which is optimised for PHB-production. Plates were incubated for at least five days at 30 °C.

Results and discussion

Screening of PHA producing bacteria

By applying both, cultivation-independent as well as cultivation-dependent methods, the results clearly demonstrate that lichens are colonised by numerous bacteria which are potentially able to accumulate polyhydroxybutyrates as energy and carbon source. The use of total community DNA extracted from the selected lichens in this study (*Peltigera canina, Lobaria pulmonata, L. immixta and Pseudocyphellaria aurata*) was used for an initial screening (Fig. 1.), which revealed the presence of *phaC* genes in all tested lichen species. For further studies, 122 strains were randomly selected from five different lichens (*Peltigera canina, Lobaria pulmonata, Lobaria immixta, Pseudocyphellaria aurata and Lobaria virens*) to analyze the ability to produce PHAs.

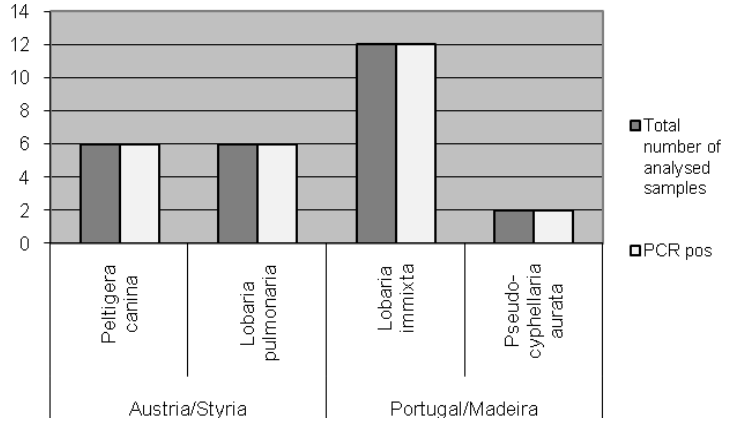

Fig 1. Presence of *phbC* gene in total bacterial community DNA in samples isolated from *Peltigera canina* and *Lobaria pulmonaria* (Austria/Styria) and *L. immixta* and *Pseudocyphellaria aurata* (Portugal/Madeira).

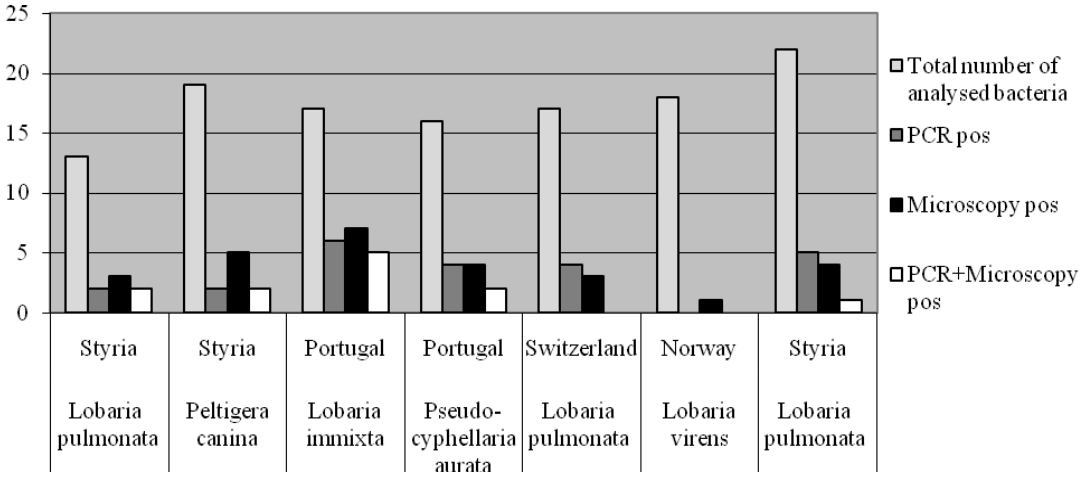

Fig 2. Bacteria isolated from the lichen species *Peltigera canina, Lobaria pulmonata, L. immixta, L. virens and Pseudocyphellaria aurata*, tested for their potential to produce PHA by PCR and microscopy. The lichens specimens were sampled in Austria (Styria), Norway (Bergen), Switzerland (St. Gallen) and Portugal (Madeira).

As shown in Fig. 2., for 23 out of 122 (28%) analysed bacterial isolates a positive PCR signal was obtained. To confirm PCR results by *in vitro* production of PHA, bacterial isolates were grown on minimal medium, and were examined under the microscope for granules. For 12 (14.6%) isolates, a correlation between intracellular granules and a positive PCR signal was shown. However, 99 isolates carry the *phbC* gene but show no visible PHA accumulation during cultivation. The reason could be different demands on conditions for producing PHA. According to our results, lichens represent a promising reservoir for polyhydroxyalkanoate producing bacteria. The ecological background behind could be storage strategies in a habitat of strong oscillation in water availability, as present in lichen thalli. It seems possible that bacteria benefit from PHA production because of the particular bacterial diet in the lichen thalli, mainly consisting of carbohydrate rich intercellular material.

Acknowledgments

This research was funded by a grant of the Austrian Science Foundation (FWF) to G.B. and 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).

References

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 Microb* 68: 3328-3338.

Boustie, J. & Grube, M. 2005: Lichens-a promising source of bioactive secondary metabolites. *Plant Gen. Resourc* 3: 273–278.

Boustie, J., Tomasi, S. & Grube, M. Bioactive lichen metabolites: a focus on some alpine species. *Phytochem. Rev* (under review).

Cardinale, M., Castro, Jr. J.V., Müller, H., Berg, G. & Grube, M. 2008: In situ analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of Alphaproteobacteria. *FEMS Microb Ecol* 66: 63-71.

Cardinale, M., Puglia, A.M. & Grube, M. 2006: Molecular analysis of lichen-associated bacterial communities. *FEMS Microb Ecol* 57: 484-495.

Gonzalez, I., Ayuso-Sacido, A., Anderson, A. & Genilloud, O. 2005: Actinomycetes isolated from lichens: Evaluation of their diversity and detection of biosynthetic gene sequences. *FEMS Microb Ecol* 54:401–415.

Grube, M. & Berg, G. 2009: Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biology Rev* 23:72-85.

Grube, M., Cardinale, M., Castro, Jr. J.V., Müller, H. & Berg, G. 2009: Species-specific structural and functional diversity of bacterial communities in lichen symbiosis *The ISME J* 3: 1005-1015.

Liba, C.M., Ferrara, F.I.S., Mangio, G.P., Fantinatti-Garboggini, F., Albuquerque, R.C., Pavan, C., Ramos, P.L., Moreira-Filho, C.A. & Barbosa, C.R. 2006: Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J Appl Microb*.101: 1076–1086.

Nash, T. 2008: Lichen biology. Cambridge Univ. Press.

Sheu, D.S., Wang, Y.T. & Lee, C.Y. 2000: Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. *Microbiology* 146: 2019-2025.

Manuscript I

Analysis of ecosystem function and endophytic life style of the polyhydroxybutyrate-producing Burkholderia terricola *ZR2-12*

Ilona Gasser[1], Massimiliano Cardinale[1], Henry Müller[1], Stefanie Heller[2], Leo Eberl[2], Nicole Lindenkamp[3], Chlud Kaddor[3], Alexander Steinbüchel[3] and Gabriele Berg[1]*

[1]Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

[2]Institute of Plant Biology, University of Zurich, Zurich, Switzerland

[3]Institute of Molecular Microbiology and Biotechnology, Westfälische Wilhelms-Universität Münster, Münster, Germany

*Corresponding author

E-mail: gabriele.berg@tugraz.at

Short title: Life style of *Burkholderia terricola* ZR2-12

Keywords: Polyhydroxyalkanoates, *Burkholderia terricola,* CLSM, plant growth promotion,

Submitted to FEMS Microbiology Ecology

Abstract

Members of the genus *Burkholderia* are versatile bacteria with ambivalent interactions with eukaryotes and a high biotechnological potential. To study ecosystem function and life style of *B. terricola*, a so far less-studied species, we analysed the interaction with plants and in soil as well as the mechanisms behind. *B. terricola* ZR2-12 treatment resulted in enhanced growth of sugar beet, biomass was more than doubled in comparison to the non-treated control. The strain is an extraordinary good root coloniser, which can be found in rhizosphere as well as endorhiza of sugar beet plants up to 10 $\log_{10}$ CFU g^{-1} root fresh weight (fw). Using DsRed labelled cells in confocal laser scanning microscopy (CLSM) to study colonisation pattern, we found *Burkholderia* cells in 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. Colonisation patterns strongly differed between older and younger parts of the roots. ZR2-12 was not able to fix nitrogen, produced no indole acetic acid or antagonistic activity but showed 1-aminocyclopropane-1-carboxylate deaminase activity and stored polyhydroxybutyrate (PHB) up to 70% w/w of cell dry mass. The role of PHB was analysed using a PHB-negative mutant in comparison to the wild type. The ability to produce PHB enhanced the strength of competition for space, which was demonstrated for root colonization. According to our results, *B. terricola* ZR2-12 is a promising plant growth promoting agent.

Introduction

The genus *Burkholderia* described by Yabuuchi *et al.* (1992) combines a diverse group of Betaproteobacteria with currently 64 species, and includes bacteria that undergo bivalent interactions with animals, plants and human hosts (Vandamme *et al.,* 1997). Members of the genus *Burkholderia* are able to colonise a wide range of niches including bulk soil, rhizosphere, water and also urban environments. *Burkholderia* is well known for its pathogenic representatives like *B. mallei*, *B. pseudomallei* or *B. cepacia* (Godoy *et al.,* 2003; Mahenthiralingam *et al.,* 2005). On the other side, *Burkholderia* species are known for important ecosystem functions in soil like biodegradation of complex (xenobiotic) compounds (Chang and Zylstra 1998; Diaz 2008), and for plants: plant growth promotion (Sessitsch *et al.,* 2005) as well as pathogen defence against plant pathogenic fungi (McLoughlin *et al.,* 1992; Hebbar *et al.,* 1998; Tran Van *et al.,* 2000). Especially during the recent years, a growing number of *Burkholderia* species with beneficial interactions with their host plants have been reported, e.g. nitrogen-fixing *Burkholderia* strains like *B. vietnamensis* (Gillis *et al.,* 1995) and *B. silvatlantica* (Perin *et al.,* 2006), nodulating strains from different plants (Vandamme *et al.,* 2002; Chen *et al.,* 2006), endosymbionts of leaf galls of *Psychotria* plants (van Oevelen *et al.,* 2002), species with an intimate interaction with bryophytes (Vandamme *et al.,* 2007), and endophytes with a high activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase able to influence the ethylene level in plants (Sessitsch *et al.,* 2005). Many *Burkholderia* species exhibit antifungal activity, which was identified as quorum sensing-regulated function (rev. in Eberl 2006; Sokol *et al.,* 2007, Schmidt *et al.,* 2008). The life style as well as the ecosystem function of *Burkholderia terricola,* a species described by Goris *et al.* (2002), are largely unknown (Coenye & Vandamme 2003). While the type

strain was isolated from agricultural soil degrading a wide range of carbon sources, we isolated a strain from a plant-associated habitat: the rhizosphere of sugar beet (Gasser *et al.,* 2009). The strain *Burkholderia terricola* ZR2-12 was characterised by its polyhydroxybutyrate (PHB) production.

In comparison to bulk soil, in the rhizosphere the abundance and activity of microorganisms is enhanced as result of rich root exudation (Sørensen 1997; Bais *et al.,* 2006). Especially bacteria with antagonistic activity against plant pathogens are enriched in the rhizosphere of different plants (Berg *et al.,* 2002, rev. in Berg & Smalla 2009) but also PHB producing bacteria (Gasser *et al.,* 2009). Inclusion of storage substances such as PHBs is a bacterial strategy to increase survival in changing surroundings such as in the rhizosphere (Müller *et al.,* 1999) but also to store energy (Steinbüchel *et al.,* 2002¸ Steinbüchel & Valentin 2006). Little is known about the relevance of PHBs for the life style of *Burkholderia*. Interestingly, for free-living as well as symbiotic nitrogen-fixing bacteria of the genera *Azospirillum, Azotobacter, Herbaspirillum* and *Rhizobium,* a relationship between PHB-accumulation and rhizosphere colonisation was described (rev. in Castro-Sowinski *et al.,* 2010). Understanding the role of PHB as storage material is important for agricultural applications like bacterial inoculants for soils, seeds and plants (Kadouri *et al.,* 2005).

To investigate in a first step ecosystem function and the life style of *B. terricola* in terrestrial ecosystem, the interaction of *Burkholderia terricola* ZR2-12 (Gasser *et al.,* 2009) with sugar beet plants and in soil was analysed. The ability to colonise the root was compared with *Pseudomonas fluorescens* WCS365, which is known to be an extraordinary root coloniser (Dekkers *et al.,* 1998). Furthermore, using DsRed labelled *Burkholderia* cells in confocal laser scanning microscopy (CLSM), colonisation pattern were studied in detail. Due to the plant growth promotion and

antagonistic activity, the role of PHB in root colonisation was analysed by using a PHB-negative mutant in a polyphasic approach.

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. No differences in growth rate were found between mutant and wild type. For competition assays, *Pseudomonas fluorescens* WCS365 was used as model strain for an excellent root coloniser (Dekkers *et al.,* 1998).

Seed priming, plant growth conditions and root colonization experiments

Seeds of sugar beet (cv. Calida, KWS SAAT AG, Einbeck, Germany) were surface sterilised by washing with water for 5 min and incubated with 70% ethanol for another 5 minutes followed by an additional washing step in sterile water. For seed priming, 1 g of seeds was incubated for 6 h in liquid culture of the respective strains grown in mineral salt medium (MM) (Schlegel *et al.,* 1961) for 24 h. For control treatment, 1 g of seeds was incubated with sterile MM medium. After priming, inoculated seeds were dried and sown in 12 cm pots or in culture tubes type ‘De Wit’ (Duchefa, Haarlem, The Netherlands) containing sterile soil (Gramoflor, Vechta, Germany) or in seed germination pouches (Mega International, Minneapolis, MN, USA) moistened with sterile water. Plants were grown under greenhouse conditions with 14 h of light

and 20°C. For root colonisation competition assay sugar beet seeds were primed with *B. terricola* ZR2-12 and *P. fluorescens* WCS365 (Dekkers *et al.,* 1998) at the same time and germinated in sterile soil in 'De Wit' culture tubes. A spontaneous rifampicin resistant mutant of ZR2-12 was used for this experiment. After 10 days of growth, cell numbers per g root (fresh weight) of both strains were determined. For root colonisation competence assays sugar beet seeds were primed with spontaneous rifampicin resistant mutants of strains ZR2-12 or ZR2-12_PHB$^-$ and germinated in autoclaved soil in 12 cm pots. After three weeks bacterial cell numbers present on roots were determined. For determination of colonisation velocity non primed sugar beet seeds were set into soil inoculated with either ZR2-12 or ZR2-12_PHB$^-$. Inoculated soil was prepared as described under drought stress experiment. Suspensions were filtered, bacterial cell number of soil was determined, and soil was placed into culture tubes. In each culture tube two non primed sugar beet seeds were set. After 10 days bacterial cell number of roots was determined. Experiment was repeated three times.

Confocal Laser Scanning Microscopic (CLSM) analyses

For microscopic analyses of ZR2-12 and its PHB negative Mutant (ZR2-12_PHB$^-$) on sugar beet roots (cv. Calida), transconjugants of both strains expressing the fluorescent protein DsRed were used. Conjugative plasmid transfer by triparental mating was done as described by de Lorenzo und Timmis (1994). For both strains, the pIN69 Vector (Vergunst 2010) with a Trimethoprim resistance (100 µg ml^{-1}) was used. One gram of surface sterilised seeds was incubated for 6 hours in liquid culture of ZR2-12 [pIN69] and ZR2-12_PHB$^-$ [pIN69] grown in MM to an OD of 1.5. After priming, the seeds were dried and applied to seed germination pouches with 10 ml sterile water. This was done in 3 replicates (6 seeds per pouch). The experiments

were conducted under greenhouse conditions (14 h of light, 20 °C). After one, two and three 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).

Plant growth promotion experiment

Seeds were inoculated with a spontaneous rifampicin resistant mutant of *B. terricola* ZR2-12 and applied to autoclaved soil in 12 cm pots. After one, two, and three weeks, plantlets were harvested and fresh weight of all plant parts was determined gravimetrically. Experiment was done in three replicates each with six seeds per pot and repeated three times.

Determination of bacterial cell numbers

About 0.4 g (week 1), 3.5 g (week 2) and 13 g (week 3) of root material was sampled into sterile plastic bags and homogenised with mortar after adding 3 ml of NaCl solution (0.9%). Suspensions were serially diluted and plated on nutrient agar II (Sifin, Berlin, Germany). After three days of incubation at 30°C colony forming units (CFU) were determined and calculated to CFU per gram root fresh weight. In experiments using the spontaneous rifampicin resistant mutant of *B. terricola* ZR2-12, medium was supplemented with 80 µg ml^{-1} rifampicin (Fluka, St. Louis, MO, USA).

Phenotypical and biochemical characterization of *Burkholderia terricola* ZR2-12

ACC (1-aminocyclopropane 1-carboxylate) deaminase activity was tested on Brown & Dilworth (BD) minimal medium (Brown and Dilworth, 1975) containing 0.7 g ACC L-1 as a sole nitrogen source. BD plates containing 0.7 g NH4Cl L-1 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 (Vandamme *et al.,* 2007). Auxin production was tested by colorimetric estimation of indole-3-acetic acid in liquid culture according to Sawer and Kremer (1995). Formation of pink colour was measured photometrically at 530 nm and quantified with a dilution row of indole-3-acetic acid (Sigma-Aldrich, St. Louis, USA) ranging from 5 µg ml-1 to 60 µg ml-1. Production of C4-C6 AHLs was checked by plate assays according to McClean *et al*. (1997). Test strains were cross-streaked against *Chromobacter violaceum* CV026. Development of purple colour due to violacein production of *C. violaceum* evaluated. For assessment of production of extracellular proteolytic enzymes, ZR2-12 was screened by plating on nutrient agar II containing 10% skim milk. Clearance halos indicating protease activity were measured after an incubation time of 3 days at 30°C. Antifungal activity was determined by a dual-culture *in vitro* assay on Waksman agar (WA). Zone of inhibition was measured after 5 days of incubation at 20°C by the method of Berg (1996). Fungi used in this bioassay included Rhizoctonia solani DSMZ 63010 and Verticillium dahliae V25 (strain collection Graz University of Technology). Additionally Rhizoctonia solani was used for an *in vitro* biocontrol experiment with three days old seedling of sugar beet `Calida` was done according to Keijer *et al*. (1997). Siderophore production was assayed by the method of Schwyn & Neilands (1987). Clearance halos indicating siderophore production were measured after 3 days of incubation at 30 °C. Nitrogen

fixation was tested with nitrogen free medium according to Brown and Dilworth (1975). Ability to grow on nitrogen free medium was assessed after one week. Thin layer chromatography was performed to analyse triglyceride (4 mg ml-1 glyceryl trioleate, fatty acids (2 mg ml-1 oleic acid) und wax ester (1 mg ml-1 oleyl oleate). As eluent hexane, diethyl ether and acetic acid in a ratio 90:7.5:1 were used (Wältermann *et al.,* 2000; Uthoff *et al.,* 2005).

Construction and characterization of a PHB-negative mutant

A PHB negative mutant of *Burkholderia terricola* ZR2-12 was produced by chemical mutagenesis with Ethylmethansulfonat (EMS, Merck, Darmstadt, Germany) according to Schlegel *et al.* (1970). Cell material of 1 ml liquid culture (grown for 24 hours) was pelleted by centrifugation (2 min, 8000 rpm) and washed with phosphate buffered saline (PBS, pH 7.2). Pellet was suspended with 1 ml PBS containing 30 µg of EMS and incubated for 30 min. Afterwards pellet was washed two times with PBS again and resuspended in MM medium and plated onto MM Agar. After a 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). Lower fraction was taken for further processing (Steinbüchel *et al.*, 2003). After a phenotypic screening, the PHB negative mutant (ZR2-12_PHB$^-$) was confirmed with nile red staining, microscopic analysis and GC analysis (Spiekermann *et al.,* 1999; Timm and Steinbüchel 1990). No differences in growth rate in nutrient broth media and MM (24 h, 30 °C) were found between mutant and wild type.

Starvation and drought stress experiment

Autoclaved soil (15 g) was placed in a Petri dish and inoculated with 420 µl of a 24 h culture grown in MM medium of ZR2-12 and ZR2-12_PHB$^-$. After incubation for 48 h

at 30°C, soil was suspended to a final volume of 100 ml with physiological NaCl solution and stirred for 30 min. Suspensions were filtered and soil with adhering bacterial cells was air dried for 24 h. 0.5 g of dried soil were resuspended in three ml of physiological NaCl solution, plated on nutrient agar II and cell number was determined.

Quantitative and qualitative analyses of Polyhydroxybutyrate Acid

Cells of ZR2-12 and ZR2-12_PHB$^-$ grown on MM with different NH4Cl concentrations (for inclusions 0.1% (w/v); for growth conditions 1% (w/v) were harvested by centrifugation (15 min, 6000 *g*, 4°C) and washed in 0.9% (w/v) sodium chloride, then lyophilized for 24 h. The PHB contents of the cells were determined upon methanolysis of 5 to 10 mg lyophilized cells in presence of 85% (v/v) methanol and 15% (v/v) sulfuric acid. 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; Brandl *et al.,* 1988). Lyophilized cells were subjected to methanolysis. For molecular analysis of the 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 and analysis of PHB for characterization was done by extracting the lyophilised PHB with chloroform (50 ml) by shaking for 24 h and precipitating with 10 x ice cold Ethanol.

Statistics

Plant experiments and colonisation experiments were done in three replicates and repeated three times. Results shown represent mean values of all replicates.

Statistical spread was calculated with confidence interval at $P \leq 0.05$ or with standard deviation. Significances of differences were calculated using Student's t-test.

Results

Effect of *B. terricola* treatment on plant growth

The effect of bacterial treatment on growth of sugar beet plants was monitored in autoclaved soil. 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 Figure 1., *B. terricola* ZR2-12 inoculants induced a positive reaction on growth of sugar beet seedlings. Three weeks post-inoculation, *Burkholderia*-primed seeds resulted in statistically significant higher weight of seedlings ($P = 0.0019$), which was more than double than the non-treated control (1.26 g and 0.53 g on average per plant, respectively). Plant weight was in correlation with the leaf length: in *B. terricola* treated plants, leaves were on average 1.4 cm longer than in non-treated control plants. In addition, experiments were conducted under non-sterile conditions in a climate chamber, in which the same plant growth promoting effect of *B. terricola* ZR2-12 but to a negligible lesser extend was observed (data not shown).

Comparative analysis of root competence

Root colonisation competence, which expresses the ability to establish in the root system of a plant (Sørensen 1997), was assessed by monitoring abundances of *B. terricola* ZR2-12 in the sugar beet root system. This experiment was carried out in comparison to *Pseudomonas fluorescens* WCS365, which is known to be an

extraordinary root colonising strain (Dekkers *et al.,* 1998). Seeds were primed with an overnight culture of bacteria, which yielded in a cell number of 3.87 ± 0.27 log_{10} CFU $seed^{-1}$. After 10 days, bacterial treatment resulted in comparable numbers for both strains (Fig. 2.). Sugar beet roots were colonised with 9.86 ± 0.97 log_{10} CFU g^{-1} 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 in the root competence of both strains (P = 0.30) was calculated.

Microscopic analyses of root colonisation pattern

To analyse the colonisation pattern of *B. terricola* ZR2-12 in detail, CLSM (Confocal Laser Scanning Microscopy) was used. For this approach, seeds primed with DsRed tagged strains of ZR2-12 (ZR2-12 [pIN69]) were applied. The roots were analysed after three weeks grown under gnotobiotic conditions. Surprisingly, cells of ZR2-12 [pIN69] were not only found in the root, also in the endorhiza. Indeed, analysis with Imaris software revealed an extremely dense colonisation of the endorhiza as well as rhizosphere by *B. terricola* (Fig. 3.). 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 root tips mainly single cells were present.

Phenotypic characterization of *Burkholderia terricola*

Knowledge about the mode of action is important for understanding the ecological role of plant associated microorganisms. Therefore several *in vitro* assays were performed to analyse the physiological and biochemical properties of *B. terricola* ZR2-12 (Tab.1). Strains ZR2-12 and ZR2-12_PHB^- were able to grow on medium containing ACC as sole nitrogen source suggesting ability of ACC deaminase activity

for both strains. We found neither auxin production nor nitrogen fixation. No antifungal as well as antagonistic activity against the soil-borne plant pathogenic fungi *Verticillium dahliae* and *Rhizoctonia solani* were shown. *B. terricola* ZR2-12 was not able to control *R. solani* in a plant protection assay according to Kejger *et al.* (2003). Furthermore, no evidence for production of extracellular proteases, and resistance to high salinity was provided. After three days of incubation on siderophore detection medium, clearance halos of 1 mm diameter were formed, suggesting production of siderophores.

Under accumulation conditions, *B. terricola* is capable of producing PHB up to 70% w/w of cell dry mass by feeding with 1% (w/v) glucose and 0.05% (w/v) NH_4Cl. Also under growth conditions in MM with 0.1% (w/v) NH_4Cl and 1 (w/v) Glucose PHB level of up to 32% PHB w/w of cell dry weight was measured. Using TLC common fatty acids could be detected, but no wax ester and triglyceride (data not shown).

Construction of PHB negative mutant *Burkholderia terricola* ZR2-12_PHB$^-$

In order to investigate the role of PHB synthesis/accumulation, which was an outstanding ability of the strain, in root colonisation ability of *B. terricola*, we constructed a PHB-negative mutant of *B. terricola* ZR2-12. After chemical mutagenesis, a phenotypic screening of above 3000 strains resulted in six strains, which were subjected to GC analysis, nile red staining and microscopic analysis. Five of these strains produced an amount of in average 10.3% PHB. One strain (ZR2-12_PHB$^-$) was verified by GC to produce no PHB. This was verified after few months with same results. Microscopic and nile red staining affirmation was done continuously. No difference in growth rate was found between PHB$^-$ mutant and wild

type. Furthermore, the mutant ZR2-12_PHB$^-$ showed no differences to the wild type in all other mechanisms investigated (Table 1).

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

To assess whether the ability of PHB-production influences the colonisation competence of *B. terricola,* different experiments were conducted to analyse differences between wild type strain ZR2-12 and its PHB-negative derivate ZR2-12_PHB$^-$. Sugar beet roots grown from primed seeds were colonised in similar abundances by wild type and mutant: 6.05 ± 0.59 and 6.03 ± 0.57 $\log_{10}$ CFU g^{-1} fw root (Table 2, experiment A). As production 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. Densely colonised soil (Table 2, experiment B) was filtered and air dried for 24 h. After drought stress, survival was determined by plating resulting in a cell number reduction 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). To assess whether PHB-production ability may facilitate faster colonisation of roots, sterile seeds of sugar beet were set into *B. terricola* colonised soil (Table 2, experiment D). After 10 days, cell numbers of roots of germinated seedlings were determined resulting in a strong colonisation of 9.45 and 9.69 $\log_{10}$ CFU g^{-1} fw root for wild type and PHB$^-$-mutant respectively. These data suggest no advantage to colonise plant habitats of wild type producing PHB strain towards its PHB$^-$ derivative in non-competitive systems.

Therefore, a competitive assay was used to determine the effect 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 for up to three weeks. When the PHB-negative mutant was DsRed tagged, no signal could be found, revealing ZR2-12_PHB$^-$ [pIN69] is suppressed by the not tagged PHB producing wild type ZR2-12. When ZR2-12 is DsRed tagged a dense colonisation could be observed. Colonisation patterns differed between older and younger parts of the root (Fig. 4.). Whereas dense colonies were present in the apoplastic spaces of the older root parts (Fig. 4., A-C), small colonies and single cells were found in the middle part of the roots (Fig. 4., D-F). In the vicinity of the root tip, only single cells were present (Fig. 4., G-I). Colonisation density increased with plant age (Fig. 4.). Older, middle aged and younger parts of roots from three weeks old plants were stronger colonised than those from two weeks or one week old plants, indicating a succession of colonisation in dependence of plant age and plant part. Analysis with Imaris software displays a dense colonisation of root surface and root endosphere by *B. terricola.* By serially diluting of crushed roots and plating on nutrient agar an average cell number of 9.86 $\log_{10}$ CFU g^{-1} fw root was determined.

Discussion

Beside plant and human pathogens, the genus *Burkholderia* harbours diverse species, which are able to enhance plant growth and health. This study showed that the so far less-studied *B. terricola* belongs to the group of plant beneficial bacteria. *B. terricola* ZR2-12, which was originally isolated from the rhizosphere of sugar beet, was able to promote plant growth and showed an endophytic life style with extremely high colonisation abilities. During the last decade, endophytes defined as microorganisms living inside of plant organs and tissues without causing disease symptoms (Hallmann *et al.,* 1997) have become interesting models to study plant-

microbe interaction (Mei & Flinn 2010). Besides *B. phytofirmans* (Compant *et al.,* 2008), *B. terricola* can be suggested as model for investigating polyhydroxybutyrate-producing endophytes.

Plant-associated *Burkholderia* strains with beneficial plant-microbe interaction are shown to be free-living or endophytic nitrogen fixers or they showed high activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase able to influence the ethylene level in plants (Sessitsch *et al.,* 2005). Activity of ACC deaminase found in *B. terricola* ZR2-12 could contribute at least in part to the observed plant growth promoting effect. No antifungal or other antagonistic activity, which was also described for *Burkholderia* species with a positive plant interaction, was found (Schmidt *et al.,* 2008). Many Gram-negative bacteria have been shown to produce *N*-acyl-homoserine lactone (AHL) signal molecules, which are utilized by the bacteria to monitor their own population densities in a process known as 'quorum sensing' (QS; Fuqua *et al.,* 1996). Most *Burkholderia* species investigated so far produce *N*-octanoyl-homoserine lactones (C8-HSL), which is synthesized by the AHL synthase CepI. Previous works has identified several QS-regulated functions in strains of the genus *Burkholderia*, including the production of extracellular proteases, chitinases, a polygalacturonase and siderophores, swarming motility and biofilm formation as well as antibiotic production (for reviews see Eberl 2006; Sokol *et al.,* 2007, Schmidt *et al.,* 2008). For *B. terricola* ZR2-12 we could not find production of C4-C6 AHLs. The outstanding ability of ZR2-12 was the production of PHBs up to 70% w/w of cell dry mass. Therefore we decided to study this mechanisms and the involvement in root competence more in detail using a PHB-negative mutant.

When tested in separate systems, no statistically significant differences between wild type strain and PHB-negative mutant were observed in root competence, drought stress tolerance, survival ability and colonisation velocity.

However, when both strains were tested in a competitive assay, *B. terricola* ZR2-12 was able to occupy root and endosphere, and completely outcompete the PHB-negative mutant. These findings are in accordance with results obtained for nitrogen-fixing bacteria of the genera *Azospirillum, Azotobacter, Herbaspirillum* and *Rhizobium,* which showed a positive relationship between PHB accumulation and root colonisation (rev. in Castro-Sowinski *et al.,* 2010). It has to be discussed that this mutant was constructed chemically. Although we have shown that the phenotype does not produce PHBs anymore, we were not able to fully characterise the genotype (data not shown). Reasons for this are the missing genetic information and the low level of phylogenetic similarity to other PHB producing bacteria. Using Tn5 mutagenesis, we were not able to produce a defined mutant without any PHB production. Here, reasons can be the higher copy number of genes. However, molecular knowledge of PHB synthesising and degrading genes in *Burkholderia* is necessary to fully understand their ecosystem function.

Microbial inoculants on the basis of plant growth promoting or biocontrol agents have a big potential for sustainable and environmentally friendly agriculture (rev. in Berg 2009). In addition, microorganisms can act as stress-protecting agents, which shows that plant associated bacteria are an important factor influencing 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) showed endophytic life style, iii) can well survive in plant-associated microhabitats with changing conditions, iv) possesses the ability of stress-protection, and v) its PHB production may be of advantage for formulation. The interaction between plant-associated microorganisms among themselves as well as with their host plants is highly complex (Sørensen 1997). Here we showed a first step

to identify this complex network for *B. terricola,* which is important to understand the ecological function 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 PHBs.

References

Ajub N, Pettinari M and Ruiz J (2004) A polyhydroxyalkanoate-producing *Pseudomonas sp.* Isolated from Antarctic environments with high stress resistance. *Curr Microbiol* 49: 170-174.

Bais HP, Weir TL, Perry LG, Gilroy S and 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 (1996) Rhizobacteria of oilseed rape antagonistic to *Verticillium dahliae*. *J Plant Dis Protect* 103: 20-30.

Berg G and 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, Krechel A, Ditz M, Faupel A, Ulrich A and Hallmann J (2005) Comparison of endophytic and ectophytic potato-associated bacterial communities and their antagonistic activity against plant pathogenic fungi. *FEMS Microb Ecol* 51: 215-229.

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

Brandl H, Gross RA, Lenz RW and Fuller RC (1988) Pseudomonas oleovorans as a source of poly(ß-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* 66: 2117-2124.

Brown CM and Dilworth MJ (1975) Ammonia Assimilation by *Rhizobium* Cultures and Bacteroids. *J Gen Microbiol* 86: 39-48.

Castro-Sowinski S, Burdman S, Matan O and Okon Y (2010) Natural Functions of Bacterial Polyhydroxyalkanoates. Microbiology Monographs Verlag, Springer Berlin / Heidelberg 14: 39-61.

Chang HK and Zylstra GJ (1998) Novel Organization of the Genes for Phthalate Degradation from *Burkholderia cepacia* DBO1. *J Bacteriol* 180: 6529-6537.

Chen WM, James E K, Coenye T, Chou JH, Barrios E, de Faria SM, Elliott GN, Sheu SY, Sprent JI and Vandamme P (2006) *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. *Int J Syst Evol Microbiol* 56: 1847–1851.

Coenye T and Vandamme P (2003) Diversity and significance of Burkholderia species occupying diverse ecological niches. *Environ Microbiol* 5: 719-29.

Compant S, Kaplan H, Sessitsch A, Nowak J, Ait Barka E and Clément C (2008) Endophytic colonization of Vitis vinifera L. by Burkholderia phytofirmans strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiol Ecol* 63: 84-93.

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

Cook RJ (1990) Twenty-five years of progress towards biological control. In Biological Control of Soil-Borne Pathogens. Hornby, D. Walingford, UK: CAB International, 1–14.

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

Dekkers LC, Bloemendaal CJ, De Weger LA, Wijffelman CA, Spaink HP and Lugtenberg BJ (1998) A two-component system plays an important role in the

root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. *Mol Plant Microbe Interact* 11: 45-56.

Diaz E (2008) Microbial Biodegradation: Genomics and Molecular Biology (1st ed. ed.). Caister Academic Press.

Eberl L (2006) Quorum sensing in the genus Burkholderia. *Int J Med Microbiol* 296: 103-110.

Fuqua C, Winans SC and Greenberg EP (1996) Census and Consensus in Bacterial Ecosystems: The LuxR-LuxI Family of Quorum-Sensing Transcriptional Regulators. *Annu Rev Microbiol* 50: 727-751.

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

Gillis M, Van Van T, Bardin R, Goor M, Hebbar P and Willems A (1995) Polyphasic taxonomy in the genus Burkholderia leading to an emended description of the genus and proposition of Burkholderia vietnamiensis sp. nov. for N_2-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* 45: 274-289.

Godoy D, Randle G and Simpson AJ (2003) Multilocus Sequence Typing and Evolutionary Relationships among the Causative Agents of Melioidosis and Glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *J Clin Microbiol* 41: 2068–2079.

Goris J, Dejonghe W, Falsen E, De Clerck E, Geeraerts B and 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.

Hallmann J, Quadt-Hallmann A, Mahaffee WF and Kloepper JW (1997) Bacterial endophytes in agricultural crops. *Can. J. Microbiol* 43: 895-914.

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

Kadouri D, Jurkevitch E, Okon Y and Castro-Sowinski S (2005) Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates. *Crit Rev Microbiol* 31: 55-67.

Kang Y, Carlson R, Tharpe W and Schell MA (1998) Characterization of Genes Involved in Biosynthesis of a Novel Antibiotic from *Burkholderia cepacia* BC11

and Their Role in Biological Control of *Rhizoctonia solani*. *Appl Environ Microb* 64: 3939-3947.

Keijer J, Korsman MG, Dullemans AM, Houterman De Bree J and Van Silfhout CH (1997) *In vitro* analysis of host plant specificity in *Rhizoctonia solani*. *Plant Pathol* 46: 659–669.

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M and van der Lelie D (2002) Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 21: 583–606.

Mahenthiralingam E, Urban T and Goldberg J (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* 3: 144–56.

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GSAB and 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 and Bookland R (1992) *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. *Appl Env Microbiol* 58: 1760–1763.

Mei C and Flinn BS (2010) The Use of Beneficial Microbial Endophytes for Plant Biomass and Stress Tolerance Improvement. *Recent Patents on Biotechnology* 4: 81-95.

Müller S, Bley T and Babel W (1999) Adaptative responses of *Ralstonia eutropha* to feast and famine conditions analysed by flow cytometry. *J Biotechnol* 75: 81-97.

Okon Y and Itzigsohn R (1992) Poly-ß-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. *FEMS Microbiol Rev* 103: 131-140.

Pavez P, Castillo JL, Gonzáles C and Martínez M (2009) Poly-ß-Hydroxyalkanoate Exert a Protective Effect Against Carbon Starvation and Frozen Conditions in *Sphingopyxis chilensis. Curr Microbiol* 59: 636-640.

Perin L, Martínez-Aguilar L, Paredes-Valdez G, Baldani JI, Estrada-de los Santos P, Reis VM and 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.

Poonguzhali S, Madhaiyan M and Sa T (2007) Quorum-sensing signals produced by plant-growth promoting *Burkholderia* strains under *in vitro* and *in planta* conditions. *Res Microbiol* 158: 287-294.

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

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

Schlegel HG, Lafferty R and Krauss I (1970) Isolation of mutants not accumulating poly-beta.-hydroxybutyric acid. *Arch.Mikrobiol* 71: 283-94.

Schmidt S, Blom JF, Pernthaler J, Berg G, Baldwin A, Mahenthiralingam E and Leo Eberl (2008) Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex. *Environmental Microbiology Reports* 11: 1422-1437.

Schwyn B and 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 and 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 and Eberl L (2007) Communication systems in the genus Burkholderia: global regulators and targets for novel antipathogenic drugs. *Future Microbiology* 2: 555-563.

Sørensen J (1997) The rhizosphere as a habitat for soil microorganisms. In J. D. Van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc, New York, N.Y. 21-45.

Spiekermann P, Rehm BHA, Kalscheuer R, Baumeister D and 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.

Stein S, Levitsky A, Fateev O and Mallard G (1998) The NIST Mass Spectral Search Program. Windows-Software Version 1.6d.

Steinbüchel A and Oppermann-Sanio FB (2003) Mikrobiologisches Praktikum. Springer-Verlag Berlin Heidelberg New York.

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

Steinbüchel A, DeBaets S and Vandamma EJ (2002) Biopolymers. Wiley-VCH, Weinheim.

Tabacchioni S, Bevivino A, Chiarini L, Visca P and Del Gallo M (1993) Characteristics of two rhizosphere isolates of *Pseudomonas cepacia* and their potential plant-growth-promoting activity. *Microb Rel* 2: 161-168.

Timm A and A Steinbüchel (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.

Tran Van V, Berge O, Ngo Ke S, Balandreau J and Heulin T (2000) Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. *Plant Soil* 218: 273–284.

Uthoff S, Stöveken T, Weber N, Vosmann K, Klein E, Kalscheuer R and 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 and 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 and 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, Holmes B, Vancanneyt M, Coenye T, Hoste B, Coopman R, Revets H, Lauwers S, Gillis M, Kersters K and Govan JRW (1997) Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* 47: 1188-1200.

Vandamme P, Opelt K, Knochel N, Berg C, Schonmann S, DeBrandt E, Eberl L, Falsen E and 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 Micr* 57: 2228–2235.

Vergunst AC (2010) *Burkholderia cenocepacia* creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. *IAI* 19 January.

Wältermann N, Luftmann H, Baumeiser D, Kalscheuer R and Steinbüchel A (2000) *Rhodococcus opacus* strain PD 630 as a new source of high-value single cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiology* 146: 1143-1149.

Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T and 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.

Tab. 1. Phenotypic and biochemical characterization of *B. terricola* ZR2-12 and its PHB$^-$ mutant. Different *in vitro* and *ad planta* tests were conducted in order to investigate properties related to plant-microbe interaction and ecological function. Positive or negative results are pictured with + or -. When experiment was not determined it is abbreviated with n.d.

	Antifungal activity and Biocontrol						Plant-microbe-interaction			Storage compounds			PHB production in ZR2-12[7]	
	Antifungal activity[1]	Plant protection[2]	Growth on 3 % NaCl[3]	Siderophore production[3]	AHL[3]	Proteolytic activity[3]	Plant growth promotion[4]	Nitrogen fixation[3]	Auxin[5]	Wax ester[6]	Triglyceride[6]	PHB production[7]	under growing conditions	under accumulation condititons
ZR2-12	-	-	-	+	-	-	+	-	-	-	-	+	32 % PHB w/w	70 % PHB w/w
ZR2-12_PHB$^-$	-	-	-	+	-	-	+	-	-	n.d.	n.d.	-	-	-

[1] Dual culture plate assay against *Rhizoctonia solani* and *Verticillium dahliae*

[2] Plant protection assay against *Rhizoctonia solani* according to Keijer *et al.* (1997).

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

[4] Elevated increase in plant biomass compared to non primed seeds

[5] Colorimetric assay of liquid culture supernatants according to Gordon *et al.* (1951)

[6] Determined by thin layer chromatography

[7] Determined by gas chromatography

Tab. 2. Comparison of colonisation competence between wild type strain *B. terricola* ZR2-12 and its PHB negative mutant ZR2-12_PHB$^-$. Experiments A and E show abundance of *B. terricola* on roots (log cfu/g root ± SD), experiments B-D show abundance in soil (log_{10} CFU g^{-1} soil ± SD) also for both, wild type and mutant.

	Experimental design	**ZR2-12**	**ZR212_PHB$^-$**
A	Colonisation competence[1)]	6.05 ± 5.98	6.03 ± 5.78
B	Soil before drought stress2)	9.64 ± 9.06	9.56 ± 8.77
C	Soil after drought stress2)	5.49 ± 5.07	5.40 ± 5.10
D	Soil without seedlings[3)]	8.53 ± 7.31	8.50 ± 7.33
E	Roots after 10 days[3)]	9.45 ± 9.27	9.69 ± 9.67

[1)] Sugar beet seeds were primed with the respective strain and germinated in sterile soil. Bacterial cell numbers per g root freshweight were determined after three weeks.

[2)] Sterile soil was inoculated with the respective strain, incubated in physiological NaCl solution, filtered and air dried. Cell number was determined before and after air drying (cfu per g soil)

[3)] Non primed sugar beet seeds were set into soil inoculated with the respective strain. Cell numbers of soil before setting of seeds (CFU per g soil) and cell numbers per g root fresh weight after 10 days were determined.

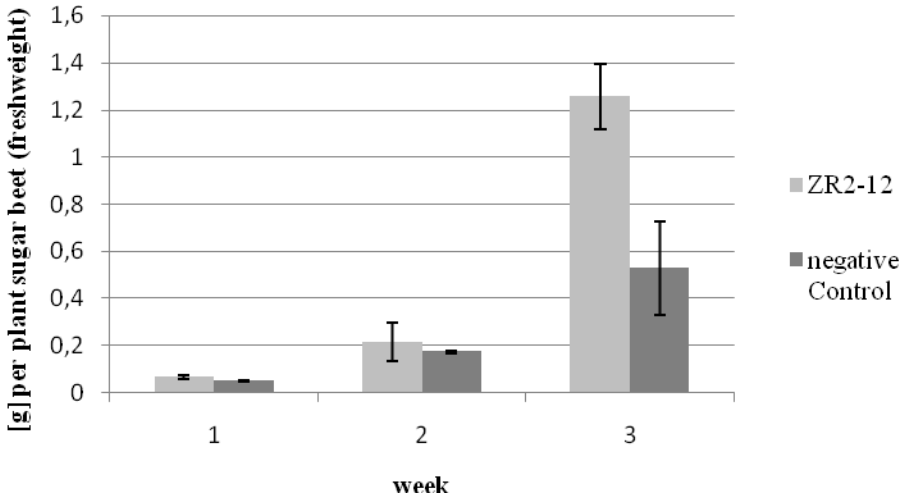

Fig. 1. Plant growth promoting effect of *B. terricola* ZR2-12 . Seeds of sugar beet were primed with strain ZR2-12 before setting and fresh weight of seedling shoots was examined after one, two and three weeks. Control seeds (negative control) were not primed. Y-axis shows fresh weight per plant in g. Error bars represent confidence intervals at $P \leq 0.05$. Significance of difference between ZR2-12 and negative control in week 3 was 0.0019.

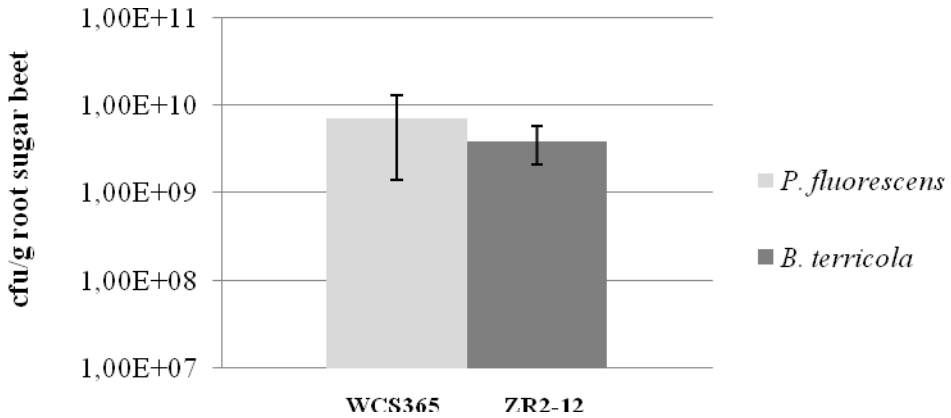

Fig. 2. Root colonisation competence of *Burkholderia terricola* ZR2-12 compared to *Pseudomonas fluorescens* WCS365 in a competitive assay. Seeds of sugar beet cv. 'Calida' were primed with both strains at the same time and cell numbers of roots were determined after 10 days of growth. Y-axis shows CFU g^{-1} root (fresh weight). Error bars represent confidence intervals at $P \leq 0.05$.

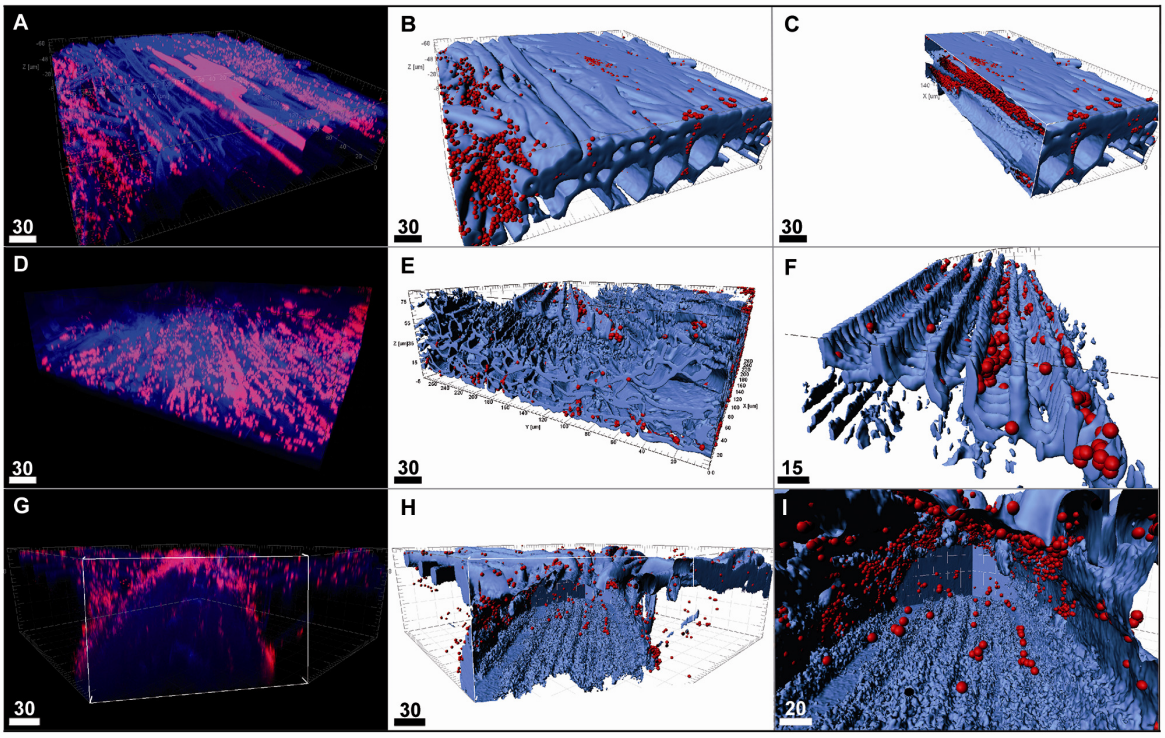

Fig. 3. Endophytic colonisation of sugar beet roots (*Beta vulgaris*) by *Burkholderia terricola* ZR2-12 [pIN69], observed by confocal laser scanning microscopy. A-C: dense colonies of *B. terricola* ZR2-12 following the direction of root apoplastic spaces in the older part of the root, three weeks after plant germination; D-F: small colonies and single cells of *B. terricola* ZR2-12 colonizing the inside of the xylematic vessel in the middle part of the root, two weeks after plant germination; G-I: endophytic cells of *B. terricola* ZR2-12 in the vicinity of the root tip, three weeks after plant germination. (A, D and G: volume rendering; B, C, E, F, H and I: 3D-reconstructions).

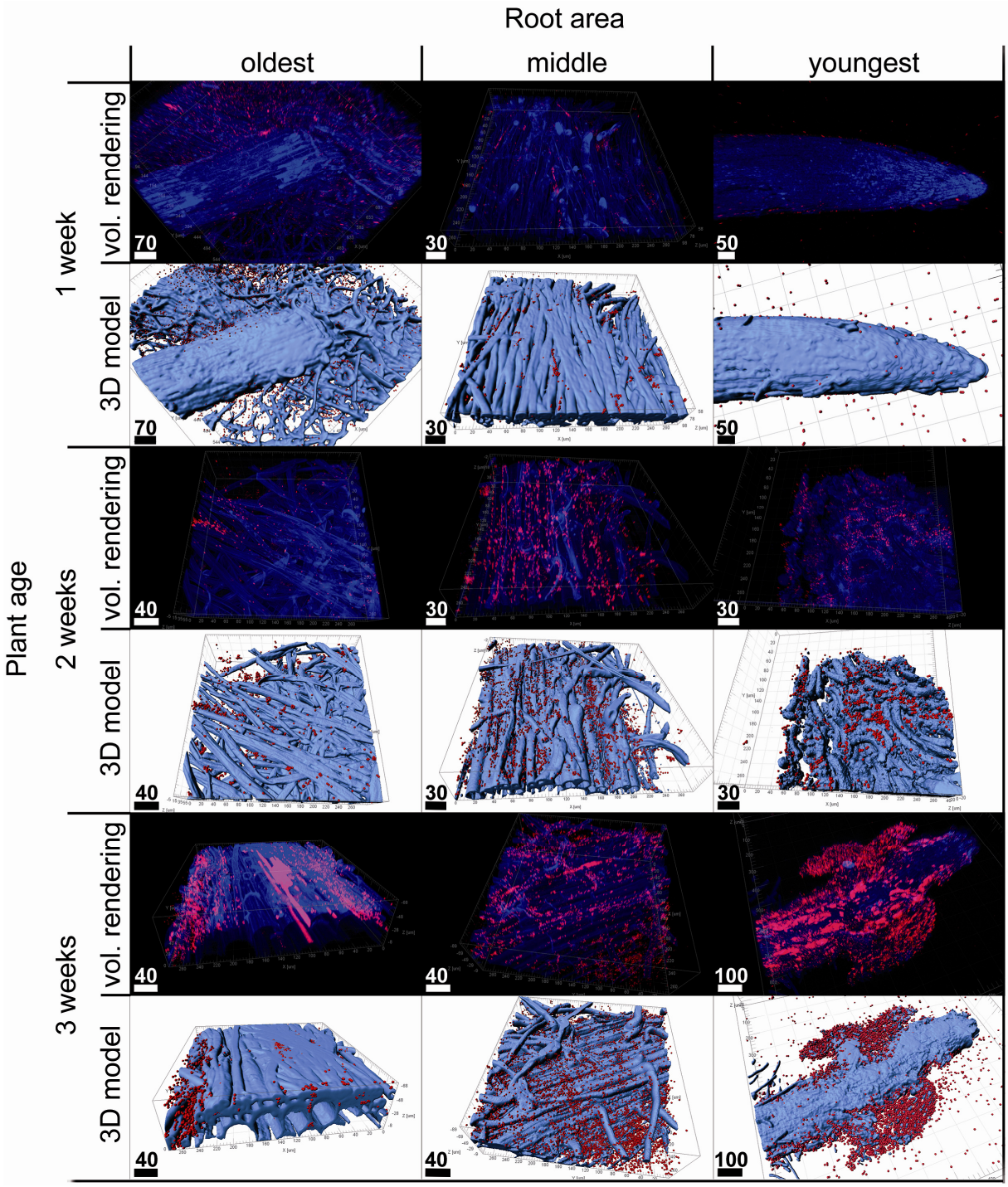

Fig. 4. Endophytic colonisation in different parts of sugar beet roots (oldest, middle, and youngest) by *Burkholderia terricola* ZR2-12 [pIN69] investigated by Confocal Laser Scanning Microscopy over a time period of one to three weeks. Volume rendering and 3-D reconstructions with Imaris software display a dense colonization of root surface and endorhiza.

Manuscript II

Minireview Part I (in progress)

Environmental habitats and ecology of PHA producing bacteria

Ilona Gasser, Florian Schmid, Henry Müller, Martin Koller und Gabriele Berg

Introduction

Production of poly-β-hydroxyalkanoates (PHA) by bacteria was first reported by Lemoigne in 1923 (Lemoigne 1923). It is well established that PHA are synthesized by bacteria as intracellular storage compounds to serve as carbon and energy source under unfavourable conditions (Macrae and Wilkinson 1958). PHA producing microorganisms can take advantage of their ability to accumulate storage compounds in environments of fluctuating availability and limitation of nutrients. Fluctuating availability of carbon sources is challenged by the production of PHA in times of carbon excess and its degradation and utilization in times of carbon limitation. PHA-producers also can overcome limitation of other essential nutrients such as nitrogen, phosphate, oxygen etc. and concomitant sufficient availability of carbon. The storage compounds produced under these conditions can be utilized as carbon source as soon as the limiting nutrient is available again. Ecosystems, where PHA producers are to be expected, exhibit the mentioned properties – fluctuation of carbon availability or transient limitation of essential nutrients. PHA producing bacteria have been found in both aquatic and terrestrial environments.

Occurrence and importance of PHA-producers in aquatic ecosystems

As fluctuations of available nutrients are strongly pronounced in marine environments, a lot of studies have investigated the PHA producing microorganisms, the amount of PHA present and the composition of different PHA producing species. For example the nature of PHAs extracted from estuarine sediments is diverse. At least 11 different short chain PHAs have been detected in sediments sampled in Florida. Microcosm studies using these sediments show that mechanic perturbations and treatment with run-off water coming from adjacent pine woods leads to an increased production of PHA (Findlay and White 1983). In the latter case, run-off water acts as a chelating compound leading to a decrease in essential nutrients and by this way producing a relative excess of carbon. This triggers PHA synthesis of bacteria capable of PHA production present in the sediment. Mechanic perturbations lead to a disruption of the redox potential of the sediment releasing available carbon sources. In anaerobic layers of marine sediments PHA is detected only after aeration (Herron *et al*. 1978). As it seems to be a common phenomenon that PHA amounts are increased by perturbation of environments, it has been established that occurrence of PHA is an indication for an unbalanced growth of microorganisms in disturbed environments (Nickels *et al*. 1979).

Another interesting habitat characterised by fluctuations of carbon availability are estuarine microbial mats. A high proportion of carbon is synthesized by anoxic photosynthesis (Martinez-Alonso *et al*. 2004), which requires special adaptations for microorganisms colonising this habitat, of which one is the production of storage compounds. In microbial mats of the Ebro delta, PHA amounts fluctuate in a diel manner, reaching its maximum at 18:00 o'clock and being degraded to a minimum level at 9:00 o'clock (Navarrete *et al*. 2000). Carbonic compounds produced during

the day in excess are intracellularly stored by PHA producing bacteria and utilized during the night as carbon and energy source suggesting a syntrophic interaction between PHA producing and photosynthetic microorganisms. From these mats PHA producing strains of *Halomonas* and *Labrenzia* have been isolated (Villanueva *et al*. 2010). Combination of PHA-production and photosynthesis is not only limited to interspecies syntrophic effects, as shown by the example of *Rhodospirillum rubrum*, a photosynthetic bacterium producing PHA during light phase and utilizing it as energy and carbon source in the dark (Stanier *et al.* 1959). Also some cyanobacteria are known to produce PHA (Capon *et al*. 1983). Recently the ability of PHA production also has been shown for the marine cyanobacterium *Spirulina subsalsa* (Shrivastav *et al*. 2010).

In aquatic ecosystems, occurrence of PHA producing bacteria is not limited to marine environments. Also freshwater sludges exhibit conditions favourable for PHA producing bacteria. The well-investigated model strain *Ralstonia eutropha* H16 was isolated from freshwater sludge of the Weende Quelle in Göttingen, Germany (Schlegel *et al*. 1961). It is used for industrial production of a copolymer consisting of poly-β-hydroxybutyric acid and poly-β-hydroxyvaleric acid (Holmes 1985; Steinbüchel 1989; Holmes, European patent application 0 052 459, Oct. 1981). Abundance of PHA synthase genes also has been detected in river water of the Rio de la Plata, Buenos Aires in Argentina (Lopez *et al*. 1997). Another important habitat for PHA producing bacteria is groundwater sediment, where availability of phosphate and other nutrients is fluctuating with the quality of percolating water. PHB accumulation is shown for an isolate from these sediments as a response to fluctuation from starving conditions to nutrient rich conditions (Bengtsson 1991). PHA accumulating bacteria present in groundwater sediment may also be interesting for bioremediation of pollutants (Nichols and White, 1989).

Several man made aquatic systems offer appropriate living conditions for the accumulation of PHA producing bacteria. From activated sludge a mixture of different PHA species, ranging from C4 to C7 monomer chain lengths, have been isolated using chloroform extraction (Lowell *et al*. 1974). Oshiki *et al.* (2008) have found PHA producing representatives of the genera *Dechloromonas, Accumulibacter, Thauera, Zoogloea, Comamonas, Competibacter* and a novel cluster of Betaproteobacteria in activated sludge. PHA producing strains of *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Aeromonas* and *Chromobacterium* have been found in sewage treatment plants (Vishnuvardhan *et al.* 2008). Two novel PHA producing gram positive species, belonging to the high G+C group have been isolated from an alternated anaerobic/aerobic (A/O) activated sludge system (Liu *et al.* 2000). Especially in A/O activated sludge systems PHA producing bacteria play an important role in nutrient and particularly phosphorous elimination from wastewaters. In the first, anaerobic, stage phosphate is released from particles of activated sludge. In this stage PHA producing bacteria accumulate PHA as a consequence of oxygen limitation but presence of sufficient organic carbon. The second stage is characterised by aeration, where these bacteria can utilize the stored carbon source and generate polyphosphate from the soluble phosphate in the wastewater. In this process called "*enhanced biological phosphorous elimination"'* it is important to enrich bacteria, which are able to undergo both processes, PHA-accumulation and polyphosphate synthesis (Serafim, 2002).

Whereas the mentioned habitats require the action of PHA producing bacteria for remediation of unwanted substances from different wastewaters, the following habitats should give examples for cheap substrates, which can be used as raw material for industrial production of PHA. The finding of cheap raw materials for PHA production is essential in order to keep production costs in a competitive range. For

example palm oil mill effluents and waste water from date syrup production display both carbon rich and cheap raw materials, where PHA producing bacteria have been found (Redzwan and Tan 1997; Ataei *et al*. 2008). Also nitrogen poor pulp and paper wastewaters can be sources of interesting PHA producing strains. In New Zealand the strain *Novosphingobium nitrogenifigens* has been isolated from such effluents (Addison *et al*. 2007).

Occurrence and importance of PHA-producers in terrestrial ecosystems

Being a well known source of biotechnologically interesting microorganisms (Handelsman *et al*. 1998; Daniel 2004), soil is also a promising source for PHA producing bacteria. For marine sediments it is shown that presence of PHA is an indicator for unbalanced growth (Nickels *et al*. 1979). Evidences, which support this finding to be true also for terrestrial ecosystems, come from reports of presence of PHA producing bacteria in somehow disturbed soils. PHA producing *Bacillus* strains have been isolated from gas field soil (Tajima *et al*. 2003). Kung *et al*. (2007) have found PHA producing bacteria in oil contaminated soils, industrial waste drainage sites and agricultural soils. In soil of an intensively cultivated hop field a higher PHA : phospholipid fatty acid ratio than in grassland and a crop rotation field has been detected. This can be traced back to the fact that higher amounts of fungicides and more rigorous farming practices in the hop field lead to an unbalanced microbial growth (Zelles *et al*. 1994).

Contrary reports about PHA producing bacteria and PHA amounts associated with the rhizosphere are found in literature. The rhizosphere is defined as the volume of soil adjacent to and influenced by plant root and as a habitat of great microbial diversity (Sørensen 1997, Berg and Smalla 2009). On the one hand, several studies show a high abundance of PHA producing bacteria or high amounts of PHA in the

rhizosphere, sometimes in comparison to bulk soil. In the rhizosphere of rice plants a higher amount of PHA has been found in comparison to bulk soil (Reichardt *et al*. 1997). Sugarcane fields are a rich source for PHA producing microorganisms (de Lima *et al*. 1999; Romo *et al*. 2007) and rhizosphere of sugar beet, oilseed rape and wheat harbour high amounts of PHA producing microorganisms as determined by screening of isolates, community DNA fingerprints and qPCR (Gasser *et al*. 2009). On the other hand, a study with seven rhizospheric gram-negative strains on roots of oilseed rapes have shown a lower PHA content at the rhizosphere in comparison to sand alone (Tunlid *et al*. 1985). From a clay-loam soil, where, besides of oligothrophic growth, accumulation of storage material is thought to be one survival strategy of microorganisms, a high percentage of PHA accumulating isolates has been retrieved (Wang and Bakken 1996). Reasons for these different findings could be the fact, that different parameters were tested in some studies (PHA amount, abundances of PHA producing isolates or PHA synthase genes) and different experimental setups were used (*in vitro* plantlets, field trial, field sampling). However presence of PHA producing bacteria in rhizosphere soil can be explained by the fact that roots on the one hand limit nutrient access for rhizospheric bacteria by consumption of essential inorganic nutrients, and on the other hand enrich rhizospheric soil with fluctuating root exudates mainly consisting of organic compounds. Root exudates mainly consist of organic secondary metabolites and their composition and amount is specific for plant species and fluctuates with plant's physiological state (reviewed in Flores *et al*. 1999).

By this way growing conditions suitable for PHA producing bacteria (limitation of essential nutrients and concomitant supply of carbon) are generated. It is remarkable that especially carbohydrate and oil producing crop plants display frequent and intense association with PHA producing bacteria (de Lima *et al*. 1999; Romo *et al*.

2007; Gasser *et al*. 2009). Since decades it is known that PHA is produced by bacteroids of root nodules of legumes and also of free living nitrogen fixing bacteria (Wong and Evans 1971; Stam *et al*. 1986), as though the role of PHA in the process of nitrogen fixation is not completely understood. Data suggest that accumulated PHA may prolong the period of nitrogen fixation in the dark (Bergersen *et al*. 1991) or may help the bacterial cell in maintaining its redox potential by energy production and NADH consumption (Dunn 1998; Encarnación *et al*. 2002). Recently PHA producing microorganisms have been detected in the microbiota of lichens (Gasser *et al*. 2010).

With the aim of keeping production costs for industrial PHA low and by this way develop an attractive alternative to petroleum based products the search for new PHA producing bacterial strains still remains of interest. Important features of such strains could be also the tolerance towards extreme conditions. A PHA producing isolate of *Bacillus* isolated from gas field soil can accumulate PHA at temperatures of up to 45°C (Tajima *et al*. 2003). From hypersaline marine mats, PHA producing strains of the genera *Sphingomonas* and *Bacillus* have been isolated (Villanueva *et al.* 2010). Photoautotrophic and diazotrophic PHA producers may be helpful in utilization of certain nutrient poor substrates for the industrial production of PHA.

Methods for examination of PHA and PHA producing organisms

First analytic methods to determine PHB content in bacterial cells are based on gravimetric methods. PHB is extracted with chloroform from lyophilized bacterial cells and precipitated with acetone or diethylether (Lemoigne 1926). Data about PHA amounts also can be generated by turbidity measurement following lysis of cell material in alkaline sodiumhypochlorite (Williamson and Wilkinson 1958). For analysis of the polymer it is necessary to produce derivates of the polymer, which can be detected with subsequent methods. Hydrolysis with sulfuric acid (Law and

Slepecky 1961), methanolysis (Braunegg *et al*. 1978) and propanolysis (Riis and Mai 1988) are common methods for generation of derivates of the extracted polymer. These derivates can be analysed using IR spectrometry (Jüttner *et al*. 1975), GC (Braunegg *et al.* 1978), GC/MS (Wallen and Rohwedder 1974), HPLC (Karr *et al*. 1983) and fast atom bombardmend mass spectrometry (Ballistreri *et al.* 1989). Also a test kit for enzymatic PHA measurement in waste water treatments is available (Hesselmann *et al*. 1999). In signature lipid biomarker analysis amounts of different lipid molecules is measured using GC/MS. A high ration of PHA : phospholipid fatty acids is suggested as a marker for an unbalanced environment (White and Ringelberg 1997). These methods require break down of cell material, but are commonly used for quantitative and qualitative analyses of PHA in environmental samples. Methods which can measure PHA in intact cells following specific staining are flow cytometry and spectrofluorometry (Degelau *et al*. 1995; Vidal-Mas *et al.* 2001), two-dimensional fluorescence spectroscopy and flow cytometry (Gorenflo *et al*. 1999) and fourier-transform infrared spectroscopy (Hong *et al*. 1999).

For intracellular visualization of PHA granules staining methods such as sudan black B, nile blue A or nile red followed by light or fluorescent microscopy are used (Schlegel *et al*. 1970; Ostle and Holt 1982; Spiekermann *et al.* 1999). These dyes are more soluble in PHB and other lipophilic storage materials than in stain solution (Bartholomew 1981; Murray *et al.* 1994). Further information about PHA producing cells can be provided by combining staining methods with the usage of fluorescence labelled oligonucleotide probes and subsequent fluorescence microscopy. By this way new PHA producing microorganisms have been found in activated sludge (Liu *et al.* 2001). PHA staining can also be combined with staining of other inclusion bodies, such as poly phosphate (Rees *et al*. 1992; Kämpfer *et al*. 1996). For a rapid direct screening of environmental samples it is possible to include nile red into solid media

at a concentration of 0.5 µg/ml and detect presence of PHA positive strains in viable colonies. Nile red does not district growth of cells, but it is not applicable for gram positive bacteria. Intensity of fluorescence can also be used to differentiate different amounts of PHB inside the cells (Spiekerman *et al*. 1999). For tracking carbon fluxes within a community *in vivo* NMR is useful. Pereira *et al.* (1996) fed activated sludge reactors with ^{13}C and investigated carbon fluxes with the involvement of PHA.

Investigating PHA qualitatively or quantitatively in environmental samples with chemical extraction or staining approaches requires conditions which favour production of PHA at the actual sampling time point. Also, when isolates are screened for PHA production it is necessary to find cultivation conditions favourable for PHA accumulation, when using traditional approaches (Madison and Huisman 1999). This can be avoided using a genotypic approach by confirming presence of PHA synthase genes (Kung *et al.* 2007). Colony PCR represents a rapid and reliable method to detect PHA producing strains from environmental habitats.

A broad range of short and medium chain length polyhydroxyalkanoate-producing microorganisms can be screened by PCR. PHA synthases (PhaC) are key enzymes for producing PHAs (Rehm and Steinbüchel 1999). López *et al* (1997) first published the possibility of PCR to detect PHA producing bacteria in river water. There are primers available for all four classes of PHA synthase genes and methods of detection reviewed by Solaiman and Ashby (2005). PHA synthase gene amplifying primers are also useful to differentiate the different classes of PHA synthase genes. Solaiman *et al.* (2000) designed specific primers by multiple sequence alignment on basis of highly conserved sequences in regions of class II PHA synthase genes to detect class II PHA genes. Sheu *et al.* (2000) developed primers for colony PCR detection of class I and II PHA synthase genes of bacteria isolated from environment

by aligning highly conserved sequences of class I and II genes of 13 gram negative bacteria. For detection of class IV synthase genes Shamala *et al.* (2003) published primers basing on class IV phaC genes of *Bacillus megaterium*. Hai *et al.* (2004) developed a method to detect class III PHA genes of sulfate reducing- and cyanobacteria. These primers also can be used successfully in combination with community DNA fingerprint techniques such as single strand conformation polymorphism (SSCP). By this way specific patterns showing different *phaC* species for rhizosphere of sugar beet, oilseed rape, and wheat have been revealed (Gasser *et al*. 2009). Ciesielski *et al.* (2008) used some of these primers for construction of a *phaC* clone library from activated sludge samples and monitored *phaC* expression levels in a laboratory scale plant using Reverse-Transcription qPCR.

There is a broad range of currently used cultivation independent methods for investigating PHA and PHA producing microorganisms in environmental samples as shown in Fig. 1. These methods provided a detailed insight into single processes correlated in PHA accumulation. To improve further understanding and to give deep and encompassing insights into the complex role of PHA producing microorganisms in different habitats and processes, it is necessary to apply combined approaches, covering measurement of PHA amounts, investigation of microbial diversity and assessment of enzymatic and microbial activity.

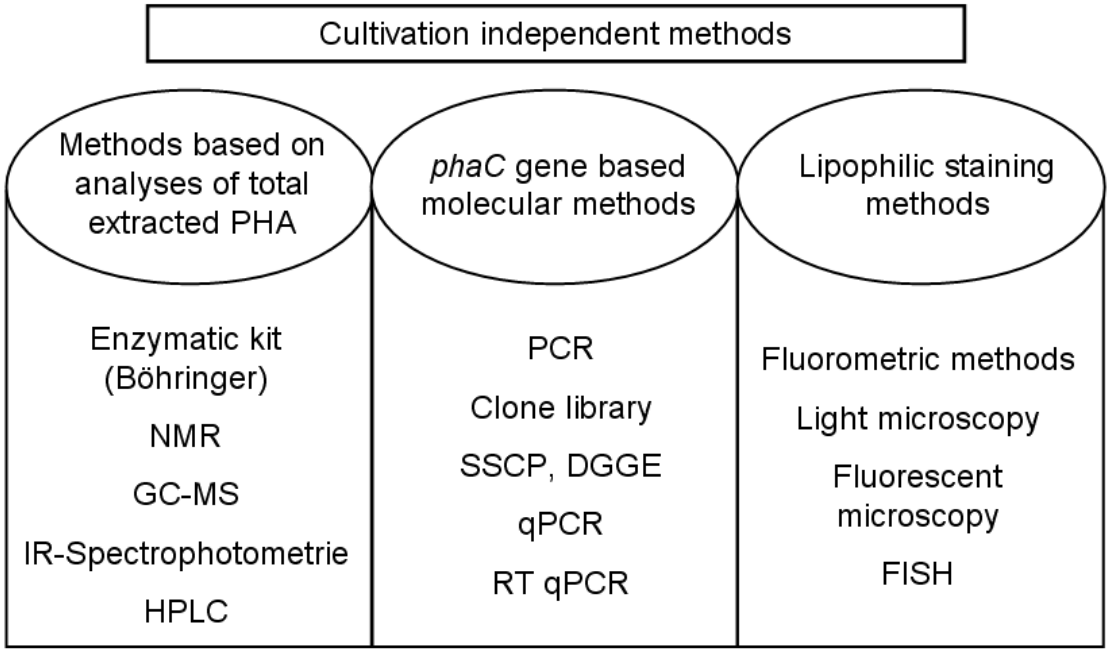

Fig 1. Summary of currently used methods for detection of PHA and PHA producing bacteria in environmental samples. NMR: nuclear magnetic resonance, GC/MS: gas chromatography-mass spectrometry, IR: infrared, HPLC: High-performance liquid chromatography, SSCP: single strand conformation polymorphism, DGGE: denaturing gradient gel electrophoresis, qPCR: quantitative PCR, RT-qPCR: reverse transcriptase quantitative PCR, FISH: fluorescence in situ hybridisation.

References

Addison SL, Foote SM, Reid NM and Lloyd-Jones G (2007) *Novosphingobium nitrogenifigens* sp. nov., a polyhydroxyalkanoate-accumulating diazotroph isolated from a New Zealand pulp and paper wastewater. *Int J Syst Evol Micr* 57: 2467-2471

Ataei SA, Vasheghani-Farahani E, Shojaosadati SA and Tehrani HA (2008) Isolation of PHA Producing Bacteria from Date Syrup Waste. *Macromolecular Symposia* 269: 11-16

Ballistreri A, Garozzo D, Giuffrida M, Impallomeni G and Montaudo G (1989) Sequencing bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate) by partial methanolysis, HPLC fractionation, and fast-atom-bombardment mass spectrometry analysis. *Macromolecules* 22: 2107-2111

Bartholomew JW (1981) Stains for Microorganisms in Smears. In: G. Clark, Editor, Staining Procedures (4th edn. ed.), Williams and Wilkins, Baltimore 375-440

Bengtsson G. (1991) Bacterial exopolymer and PHB production in fluctuating ground-water habitats. *FEMS Microbiol Ecol* 86:15-24

Berg G and 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

Bergersen FJ, Peoples MB and Turner GL (1991) A role for poly-β-hydroxybutyrate in bacteroids of soybean root nodules. *Proc. R. Soc. London B* 245: 59-64

Braunegg G, Sonnleitner B and Lafferty RM (1978) A rapid gas chromatographic method for the determination of poly-*β*-hydroxybutyric acid in microbial biomass. *Appl Microbiol Biot* 6: 29-37

Capon RJ, Dunlop RW, Ghisalberti EL and Jefferies PJ (1983) Poly-3-hydroxyalkanoates from marine and freshwater cyanobacteria. *Phytochemistry* 22: 1181-1184

Ciesielski S, Pokoj T and Klimiuk E (2008) Molecular insight into activated sludge producing polyhydroxyalkanoates under aerobic–anaerobic conditions. *J Ind Microbiol Biotechnol* 35: 805-814

Daniel R (2004) The soil metagenome – a rich resource for the discovery of novel natural products. *Curr Opin Biotech* 15: 199-204

De Lima TCS, Grisi BM and Bonato MCM (1999) Bacteria isolated from a sugarcane agroecosystem: their potential production of polyhydroxyalcanoates and resistance to antibiotics. *Rev Microbiol* 30: 214-224

Degelau A, Scheper T, Bailey JE and Guske C (1995) Fluorometric measurement of poly-β hydroxybutyrate in Alcaligenes eutrophus by flow cytometry and spectrofluorometry. *Appl Microbiol Biot* 42 (5)

Dunn MF (1998) Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS Microbiol Rev* 22:105-123

Encarnación S, Vargas MC, Dunn MF, Dávalos A, Mendoza G, Mora Y and Mora J (2002) AniA Regulates Reserve Polymer Accumulation and Global Protein Expression in *Rhizobium etli*. *J Bacteriol* 184: 2287-2295

Findlay RH and White DC (1983) Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Appl Environ Microbiol* 45: 71-78

Flores HE, Vivanco JM and Loyola-Vargas VM (1999) 'Radicle' biochemistry: the biology of root-specific metabolism. *Trends Plant Sci* 4: 220-226

Gasser I, de Castro Junior JV, Müller H and Berg G (2010) Lichen-associated bacteria antagonistic to phytopathogens and their potential to accumulate polyhydroxyalkanoates. *IOBC/wprs bulletin, Biological Control of Fungal and Bacterial Plant Pathogens* „Climate change: Challenge or Threat to Biocontrol?" (in press)

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

Gorenflo V, Steinbüchel A, Morose S, Rieseberg M, Scheper T (1999) Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining. *Appl Microbiol Biotechnol* 51: 765-772

Hai T, Lange D, Rabus R and Steinbüchel A (2004) Polyhydroxyalkanoate (PHA) Accumulation in Sulfate-Reducing Bacteria and Identification of a Class III PHA Synthase (PhaEC) in *Desulfococcus multivorans*. *Appl Environ Microb* 70: 4440-4448

Handelsman J, Rondon MR, Brady SF, Clardy J and Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* 5: 245-249

Herron JS, King JD and White DC (1978) Recovery of poly-p-hydroxybutyrate from estuarine microflora. *Appl Environ Microbiol* 35: 251-257

Hesselmann RPX, Fleischmann T, Hany R, Zehnder AJB (1999) Determination of polyhydroxyalkanoates in activated sludge by ion chromatographic and enzymatic methods. *J Microbiol Meth* 35: 111-119

Holmes PA (1985) Applications of PHB - a microbially produced biodegradable thermoplastic. *Phys Technol* 16: 32-36

Hong K, Sun S, Tian W, Chen GQ and Huang W (1999) A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Appl Microbiol Biot* 51 (4)

Hong K, Sun S, Tian W, Chen GQ and Huang W (1999) A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Appl Microbiol Biot* 51: 523-526

Jüttner RR, Lafferty RM and Knackmuss HJ (1975) A simple method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *Eur J Appl Microbiol* 1: 233–237

Kämpfer P, Erhart R, Beimfohr C, Böhringer J, Wagner M and Amann R (1996) Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecol* 32: 101-121

Karr DB, Waters JK, Emerich DW (1983) Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Appl Environ Microbiol* 46: 1339– 1344

Kung SS, Chuang YC, Chen CH and Chien CC (2007) Isolation of polyhydroxyalkanoates-producing bacteria using a combination of phenotypic and genotypic approach. *Lett Appl Microbiol* 44: 364–371

Law JH, Slepecky RA (1961) Assay of poly-β-hydroxybutyric acid. *J Bacteriol* 82: 33-36

Lemoigne M (1923) Production d'acide β-oxybutyrique par certaines bacteries du groupe du *B. subtilis*. *CR Acad Sci*, 176: 1761

Lemoigne M (1926) Produits de deshydration et de polymerisation de lacide β-oxybutyrique. *Bull Soc Chim Biol* 8: 770-782

Liu WT, Mino T, Matsuo T and Nakamura K (2000) Isolation, characterization and identification of polyhydroxyalkanoate-accumulating bacteria from activated sludge. *J Biosci Bioeng* 90: 494-500

Liu WT, Nielsen AT, Wu JH, Tsai CS, Matsuo Y and Molin S (2001) *In situ* identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. *Environ Microbiol* 3: 110-122

López NI, Pettinari MJ and Méndez BS (1997) Detection of reserve polymer synthesis genes in natural bacterial populations. *FEMS Microbiol Ecol* 22: 129-136

Macrae RM and Wilkinson JF (1958) Poly-β-hyroxybutyrate Metabolism in Washed Suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J Gen Microbiol* 19: 210-222

Madison LL and Huisman GW (1999) Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiol Mol Biol R* 63: 21-53

Martínez-Alonso M, Mir J, Caumette P, Gaju N, Guerrero R and Esteve I (2004) Distribution of phototrophic populations and primary production in a microbial mat from the Ebro Delta, Spain. *Int Microbiol* 7:19-25

Murray RGE., Doetsch RN and Robinow CF (1994) Determinative and cytological light microscopy. *Methods for General and Molecular Bacteriology*, (Gerhardt P., Murray R. G. E., Wood W. A., and Krieg N. R., eds) 21–41. American Society for Microbiology, Washington DC

Navarrete A, Peacock A, Macnaughton SJ, Urmeneta J, Mas-Castellà J, White DC and Guerrero R (2000) Physiological Status and Community Composition of Microbial Mats of the Ebro Delta, Spain, by Signature Lipid Biomarkers. *Microbial Ecol* 39: 92-99

Nichols PD and White DC (1989) Accumulation of poly-β-hydroxybutyrate in a methane-enriched, halogenated hydrocarbon-degrading soil column: implications for microbial community structure and nutritional status. *Hydrobiologia* 176-177: 369-377

Nickels JS, King JD and White DC (1979) Poly-3-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. *Appl Environ Microbiol* 37: 459-465

Oshiki M, Onuki M, Satoh H and Mino T (2008) PHA-accumulating microorganisms in full-scale wastewater treatment plants. *Water Sci Technol* 58:13-20

Ostle AG and Holt JG (1982) Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Appl Environ Microbiol* 44: 238-241

Pereira H, Lemos PC, Carrondo MJT, Crespo JPSG, Reis MAM and Santos H (1996) Model for carbon metabolism in biological phosphorus removal processes based on *in vivo* 13C-NMR labelling experiments. *Water Res* 30: 2128–2138

Redzwan G, Gan SN and Tan IKP (1997) Isolation of polyhydroxyalkanoate-producing bacteria from an integrated-farming pond and palm-oil mill effluent ponds. *World Journal of Microbiology and Biotechnology* 13: 707-709

Rees GN, Vasiliadis G, May JW, Bayly RC (1992) Differentiation of polyphosphate and poly-β-hydroxybutyrate granules in *Acinetobacter sp*. isolated from activated sludge. *FEMS Microbiol Lett* 94: 171– 174

Rehm BHA and Steinbüchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int J Biol Macromol* 25: 3-19

Reichardt W, Mascarin G, Padre B and Doll J (1997) Microbial Communities of Continuously Cropped, Irrigated Rice Fields. *Appl Environ Microb* 63: 233–238

Riis V and W Mai (1988) Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. *J Chromatogr* 445: 285–289

Romo DMR, Grosso MV, Solano NCM and Castaño DM (2007) A most effective method for selecting a broad range of short and mediumchain-length polyhidroxyalcanoate producing microorganisms. *Electronic Journal of Biotechnology* 10 (3)

Schlegel HG, Gottschalk G and Von Bartha R (1961) Formation and Utilization of Poly-β-Hydroxybutyric Acid by Knallgas Bacteria (*Hydrogenomonas*). *Nature* 191: 463-465

Schlegel HG, Lafferty R and Kraus I (1970) The isolation of mutants not accumulating poly-ß-hydroxybutyric acid. *Arch Mikrobiol* 71: 283-294

Serafim L, Lemos PC, Levantesi C, Tandoi V, Santos H and Reis MAM (2002) Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J Microbiol Meth* 51: 1-18

Shamala TR, Chandrashekar A, Vijayendra SVN and Kshama L (2003) Identification of polyhydroxyalkanoate (PHA) producing *Bacillus* spp. using the polymerase chain reaction (PCR). *J Appl Microbiol* 94: 369-374

Sheu DS, Wang YT and Lee CY (2000) Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. *Environ Microbiol* 146: 2019-2025

Shrivastav A, Mishra SK and Mishra S (2010) Polyhydroxyalkanoate (PHA) synthesis by *Spirulina subsalsa* from Gujarat coast of India*. Int J Biol Macromol Mar* 1; 46:255-60

Solaiman DKY and Ashby RD (2005) Rapid Genetic Characterization of Poly(hydroxyalkanoate) Synthase and Its Applications. *Biomacromolecules* 6: 532–537

Solaiman DKY, Ashby RD and Foglia TA (2000) Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Appl Microbiol Biot* 53: 690-694

Sørensen J (1997) The rhizosphere as a habitat for soil microorganisms. In JD Van Elsas, JT Trevors, and EMH Wellington (ed.), *Modern soil microbiology*. Marcel Dekker, Inc, New York, N.Y. 21-45

Spiekermann P, Rehm BHA, Kalscheuer R, Baumeister D and 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

Stam H, Devries W, Stouthamer AH and Vanverseveld HW (1986) Utilization of poly-b-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571. *FEMS Microbiol Lett* 35: 215–220

Stanier RY, Doudoroff M, Kunisawa R and Contopoulou R (1959) The role of organic substrates in bacterial photosynthesis. *Proc Natl Acad Sci USA* 45: 1246-1260

Steinbüchel A. (1989) Poly(hydroxyalkanoates)-storage materials of bacteria: biosynthesis and genetics. *Forum Mikrobiol* 12:190-198

Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y and Munekata M (2003) Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil. *J Biosci Bioeng* 95: 77-81

Tunlid A, Baird BH, Olson S, Findlay RH, Odham G and White DC (1985) Determination of phospholipid ester-linked fatty acids for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* L. *Can J Microbiol* 31: 1113-1119

Vidal-Mas J, Resina-Pelfort O, Haba E, Comas J, Manresa A and Vives-Rego J (2001) Rapid flow cytometry–Nile red assessment of PHA cellular content and heterogeneity in cultures of *Pseudomonas aeruginosa* 47T2 (NCIB 40044) grown in waste frying oil. *Ant Leeuw* 80: 57-63

Villanueva L, Del Campo J and Guerrero R (2010) Diversity and physiology of polyhydroxyalkanoate-producing and -degrading strains in microbial mats. *FEMS Microbiol Ecol* Epub ahead of print

Vishnuvardhan Reddy S, Thirumala M, Kishore Reddy TV and Mahmood SK (2008) Isolation of bacteria producing polyhydroxyalkanoates (PHA) from municipal sewage sludge. *World Journal of Microbiology and Biotechnology* 24: 2949-2955

Wallen LL and Rohwedder WK (1974) Poly-beta-hydroxyalkanoate from activated sludge. *Environ Sci Technol* 8- 576-579

Wang JG and Bakken LR (1996) Screening of Soil Bacteria for Poly-β-Hydroxybutyric Acid Production and Its Role in the Survival of Starvation**.** *Microbial Ecol* 35: 94-101

White DC and Ringelberg DB (1997) Utility of the Signal Lipid Biomarker Analysis in Determining the in situ Viable Biomass, Community Structure, and Nutritional/Physiologic Status of Deep Subsurface Microbiota. *The Microbiology of the Terrestrial Deep Subsurface* (Amy P.S. and Haldeman D.L., eds) 119-133. CRC Press LLC, Corp., Boca Raton, Florida

Williamson DH and Wilkinson JF (1958) The isolation and estimation of poly-β-hydroxybutyrate inclusions of *Bacillus* species. *J Gen Microbiol* 19: 198-209

Wong PP and Evans HJ (1971) Poly-3-hydroxybutyrate Utilization by Soybean (*Glycine max* Merr.) Nodules and Assessment of Its Role in Maintenance of Nitrogenase Activity. *Plant Physiol* 47: 750-755

Zelles L, Bai QY, Ma RX, Rackwitz R, Winter K and Beese F (1994) Microbial biomass, metabolic activity and nutritional status determined from fatty acid patterns and poly-hydroxybutyrate in agriculturally-managed soils. *Soil Biology and Biochemistry* 26: 439-446

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …… ……..

(Unterschrift)

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… ……..

date (signature)

Acknowledgements

Curriculum vitae

Persönliche Daten

Name:	Ilona Bernadette Gasser
Geburtsdatum und- ort	29. Oktober 1983 in Innsbruck
Wohnadresse:	Raiffeisenstr. 124/8 8041 Graz – Liebenau
Staatsbürgerschaft:	Österreich

Berufserfahrung

2006-2007:	Laborassistentin Fa.Eccocell, Graz: verantwortlich für den Bereich ID-Check (Vaterschaftstests), Erfahrung in HPV- und Laktoseintoleranztests, Genetik
2007-2010:	Wissenschaftliche Assistentin mit Lehrverpflichtung, TU Graz, Institut für Umweltbiotechnologie

Schule und Ausbildung

1990-1994:	Volksschule Angergasse Innsbruck
1994-2002:	Neusprachliches Gymnasium Meinhardinum Stams
2002-2005:	Bakkelaureat Biologie, Universität Innsbruck
2005-2007:	Magisterstudium Molekulare Mikrobiologie, Universität Graz, Erhalt des Leistungsstipendium 2006/2007
2007-2010:	Doktoratsstudium der Naturwissenschaften; Technische Chemie, TU Graz;
2009:	Auslandssemester am *Institut für Molekulare Mikrobiologie und Biotechnologie* an der Westfälischen Wilhems-Universität Münster, Deutschland in der Arbeitsgruppe von Prof. Dr. Alexander Steinbüchel

Abschlussarbeiten

Diplomarbeit: Etablierung einer Drosophila „Knock-out" Linie für das Autismus Kandidatengen *IMMP2L* (am Institut für Medizinische Biologie und Humangenetik, Prof. Dr. Michael Speicher, Medizinische Universität Graz)

Dissertation: Biopolymer producing bacteria and analysis of ecosystem function (am Institut für Umweltbiotechnologie, Prof. Dr. Gabriele Berg, TU Graz)

Jobs zur Finanzierung des Studiums

- Ferialjob auf Alpenvereinshütte am Großvenediger (Sommer 2002)
- Animateurin bei Hannes-Kids-and-Family-Events für Kindergeburtstage (2002-2003)
- Bürogehilfe Fa. Metalwork, Innsbruck (2003-2005)
- Vigilantin Kunsthaus Graz (2005-2006)
- Ehrenamtlich: Kursleiter Ausdauertraining ASV Inzing (Winter 2004, 2005)
- Volunteer Mediateam WinterUniversiade Innsbruck/Seefeld 2005

Weitere Kenntnisse und Fähigkeiten

Fortbildung

- Kompetenztraining für StudienassistentInnen: Gruppenführung, Kommunikation, Teamarbeit und Motivation
- Unterrichtspraxis
- Selbst- und Zeitmanagement
- Ersthelfer
- Kommunikation, Moderation
- Durchsetzungs- und Verhandlungsstrategien. Konfliktmanagement
- Leiten von Gruppen und Teams. Motivation. Einflussnehmen – Mitgestalten
- Projektmanagement auf der methodischen Grundlage des Systems Engineering-Konzepts

Praxis

- Im Organisationsteam *Lange Nacht der Forschung 2009 („Was haben FreChe-Materialien mit Frauen zu tun?“, „Was haben Bakterien mit dem Erdbeeraroma zu tun?“*
- Im Organisationsteam *IOBC Congress (Climate change: challenge or threat to biocontrol?)*
- Betreuung T³UG Ferialpraktikantinnen (Sommer 2008, 2009) FiT Mentoring TU Graz

Sportliche Erfolge:

- Rennrad: Nationalteammitglied 2000-2005, Österreichische Meisterin Straße, Teilnahme B-WM, Weltcuprennen …
- Wintertriathlon: Österreichische Meisterin, 4. Rang bei Europameisterschaften

Sprachkenntnisse:

- Englisch fließend in Wort und Schrift
- Italienisch (4 J.)
- Latein (8 J.)

Publication list

Publications and Manuscripts

Ilona Gasser, Henry Müller and Gabriele Berg (2009) Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants. *FEMS Microbiol Ecol 70: 142–150*

Ilona Gasser, João Vieira de Castro Junior, Henry Müller H. & Gabriele Berg (2010) Lichen-associated bacteria antagonistic to phytopathogens and their potential to accumulate polyhydroxyalkanoates. *Bulletin IOBC 2010*

Ilona Gasser, Massimiliano Cardinale, Henry Müller, Stefanie Heller, Leo Eberl, Nicole Lindenkamp, Chlud Kaddor, Alexander Steinbüchel and Gabriele Berg (2010) Analysis of ecosystem function and endophytic life style of the poly-ß-hydroxybutyrate-producing *Burkholderia terricola* ZR2-12. *(in progress)*

Ilona Gasser, Florian Schmid, Henry Müller, Martin Koller und Gabriele Berg (2010) Review: Environmental habitats and ecology of PHA producing bacteria. *(in progress)*

Talks

Ilona Gasser, Henry Müller and Gabriele Berg Wie Bakterien unser Müllproblem lösen könnten... - Die Wunderbare Welt der Bakterien. *Lange Nacht der Forschung, Graz am 07.11.2009*

Ilona Gasser Umweltbiotechnologie: TU was für deine Zukunft. *Schülerinfo TU Graz am 13.04. 2010*

Ilona Gasser, Henry Müller and Gabriele Berg Die wunderbare Welt der Bakterien; *Schülerinfo TU Graz am 27.01.2010*

Ilona Gasser, Henry Müller and Gabriele Berg Pflanzen als Bioresource für Biopolymer-produzierende Bakterien. *„Projekt fFORTE Wissenschafterinnekolleg " Leibnitz/Kaindorf am: 13.11.2009*

Ilona Gasser, Henry Müller and Gabriele Berg Pflanzen-assoziierte Bakterien als Biopolymer-Produzenten. *„Projekt fFORTE Wissenschafterinnekolleg " Leibnitz/Kaindorf am: 22.11.2008*

Abstracts and poster

Massimiliano Cardinale, Christin Zachow, Ilona Gasser, Jamshid Fatehi, Leo Eberl, Gabriele Berg (2010) Confocal Laser Scanning Microscopy to Study the Rhizosphere Competence of Beneficial Microorganisms. *Confocal and multifoton microscopy, 17th International Microscopy Congress.* Rio, Brazil

Ilona Gasser, Henry Müller und Gabriele Berg (2009) Diversity of PHA producing bacteria associated with crop plants. *Doc Day*. Graz (21 – 21)

Ilona Gasser, Henry Müller und Gabriele Berg (2009) Diversity of PHB producing bacteria associated with crop plants. *Vereinigung für Allgemeine und Angewandte Mikrobiologie,* Bochum (142 – 142)

Ilona Gasser, Henry Müller und Gabriele Berg (2008) Biopolymers: Plant-associated bacteria as PHB producer. *1st Joint Austrian & Slovenian Polymer Meeting ASPM 2008 and 9th AustrianPolymerMeeting,* Graz

Ilona Gasser, Henry Müller und Gabriele Berg (2008) Biopolymers: Plant-associated bacteria as PHB-producer. *12. ISME International Society for Microbial Ecology Symposia, Microbial Diversity - Sustaining the Blue Planet,* Cairns, Australia

Ilona Gasser, Henry Müller und Gabriele Berg (2008) Biopolymers: Plant-associated bacteria as PHB-producers. *Workshop "Functional Genomics and Industrial Biotechnology",* Seggau (14 – 14)

Joao Castro Jr, Ilona Gasser, Henry Müller , Massimiliano Cardinale, Martin Grube, Gabriele Berg (2008) Biotechnological Potential of Bacteria Associated to Lichens. *Life Sciences, the joint meeting LIFE SCIENCES of the ÖGBM, ÖGGGT, ÖGBT and ANGT.* Graz (72 – 72)

Ilona Gasser, Henry Müller und Gabriele Berg (2008) Plants as bio-resource for PHB producing bacteria. *Life Sciences, the joint meeting LIFE SCIENCES of the ÖGBM, ÖGGGT, ÖGBT and ANGT.* Graz

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.