Phenotypic variation and molecular signaling in the interaction of the rhizosphere bacteria *Acidovorax* sp. N35 and *Rhizobium radiobacter* F4 with roots

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To my family and to my Xu

Contents

Abbreviations

ACC	1-aminocyclopropane-1-carboylate
Amp^R	ampicilin resistance
BP	band pass (filter type for fluorescence microscope)
Cand.	candidatus
Cm^R	chloramphenicol resistance
Cy3	cyanine dye3
Cy5	cyanine dye5
dem.	deionized
DMSO	dimethylsulfoxid
DNA	dioxyribonucleic acid
DSMZ	Deustsche Sammlung von Mikroorganismen und Zellkuluren GmbH
EDTA	ethylendiamintetraacetat
Em^R	erythromycin resistance
et al.	*et alteri,* and others
$EtOH_{abs}$	absolute ethanol
Fig.	figure
Fluos	5(6)-carboxyfluorescein-N-hydroxysuccinimidester
Gm^R	gentamycin resistance
i.e.	that is
kb	kilo base pairs
Km^R	kanamycine resistance
Lm^R	lincomycin resistance
LP	long pass (filter type for fluorescence microscope)
MCS	multi cloning site
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
Rp^R	rifamycin resistance
Sm^R	Streptomycin resistance
$Tell^R$	tellurite resistance
Tm^R	trimethophrim resistance
Tc^R	tetracycline resistance
Tris	tris(hydroxymethyl)-aminomethan
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 Introduction

1.1 Plant-microorganisms interactions in the rhizosphere

1.1.1 The rhizosphere

The rhizosphere theory was proposed by Prof. Lorenz Hiltner in 1904, and he also emphasized the important role of microorganisms in the rhizosphere. According to the theory, the rhizosphere has been defined as the zone of soil which is influenced by plant roots and their releasing compounds (Hartmann *et al.*, 2008). Roots are able to secrete exudates, including carbohydrates, carboxylic acids, phenolics, amino acid as well as inorganic ions, which are an important source of substrates available to rhizosphere microorganisms (Hartmann *et al.*, 2009). Root exudates bind to water and form a hydrated environment for roots and rhizosphere microorganisms. But root exudates are not the only substance to stimulate the growth of microorganisms in the rhizosphere. The rhizodermis cells lyse after living for about 3 weeks, their lysates also offer nutrition for the rhizosphere microbial community (Sørensen and Sessitch, 2007). During the growth of roots, sloughed-off cortex cells (particularly at the root tip), root death (root hair cells and epidermis cells in older root parts) and the root exudates are the outstanding substrates for microorganisms (Hartmann *et al.*, 2009). Emerging lateral roots provide more substrates for microorganisms via cell lysis at the point of root emergence. Mature roots produce less mucilage and fewer lysates, which support slow-growing microorganisms like fungi and actinomycetous bacteria. In contrast, the developing young roots generally support fast-growing microorganisms like r-type bacteria and fungi (Beattie, 2007; Sørensen and Sessitch, 2007). Through the quality of exudates, the plant selects a more or less specific rhizosphere microflora. Mycorrhizal symbioses are common traits in almost land plants and the mycorrhizae are divided into two main groups: endomycorrhizae (AM) and ectomycorrhiza (EM) (Hartmann *et al.*, 2009). Thus, the rhizosphere concept has been extended to mycorrhizosphere, which this term includes the physical zone influenced not only by the root, but also by the mycorrhizal fungus mycelium or hyphosphere (Hartmann *et al.*, 2009; Rambelli, 1973). Furthermore, the growth of roots changes the soil parameters, including pH, soil texture, water potential and salinity.

1.1.2 Effects of microorganisms on plants

On the one hand, the rhizosphere represents a nutrient rich habitat for microorganisms, on the other hand, the microbial colonization of the rhizosphere also affects the whole plant (Hartmann *et al.*, 2009). Kloepper and Schroth (1978) suggest the term "plant growth promoting rhizobacteria (PGPR)" for an important group of rhizosphere bacteria that have beneficial effects on plant growth when colonizing roots. Such effects are earlier seedling emergence, and increased vigor, biomass, yield, as well as proliferation of the root system in various plants. *Bacillus* and *Pseudomonas* sp. are identified as dominant PGPR and a few of them are commercially proposed, e.g. *B. amyloliquefaciens* FZB42 (Abitep, Berlin, Germany). PGPR enhance plant growth due to various factors, among which the release of phytohormones, nitrogen fixation, regulation of ethylene production in roots, solubilizing nutrients such as phosphate, siderophore production, promoting mycorrhizal function and decreasing heavy metal toxicity (Whipps, 2001) are the most important. Some fungi have also plant growth promoting effect, e. g. *Trichoderma virens* can enhance the biomass production and promote the lateral root growth in *Arabidopsis* (Contreras-Cornejo *et al.*, 2009). *T. harzianum* T-22 has been wildly developed for plant growth promoting products (Bioworks, NY, USA).

Rhizosphere microorganisms, which are able to eliminate or reduce other pathogenic microorganisms, have been defined as biocontrol agents. Important mechanisms of microbial antagonism to plant pathogens are antibiosis, parasitism and competition for nutrients and/or induced host defense responses (Podile and Kishore, 2007). Furthermore, complexation of ferric iron by siderophores may play a role (Sørensen and Sessitch, 2007).

Successful root colonization is an essential criterion for PGPR to display beneficial effects on plant growth. Bacterial traits, such as pili, outer membrane proteins and flagella, are involved in the PGPR adherence to plant root surfaces. Not only the surface of roots is colonized but also inner tissues of the plant. This endophytic colonization by non-pathogentic bacteria causes no substantial damage to the host. These endophytes have been isolated from a variety of plants, including trees and agricultural crops such as potato or gramineous plants (Garbeva *et al.*, 2001; Rothballer *et al.*, 2008). The following genera of endophytes isolated from agricultural crops harbor PGPR-active strains: *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* (Hallmann *et al.*, 1997). Some endophytes such as *B. pumilus* SE34 and *P. fluorescens* 63-28 grow both on the root surface and intercellularly in pea roots (Benhamou *et al.*, 1996; M'piga *et al.*, 1997). The ability to

enter the root interior might help these microorganisms to evade the highly competitive rhizosphere habitat (Whipps, 2001).

1.1.3 Molecular microbial techniques to study microbe-plant interactions

In the last twenty years, ribosomal RNA gene (16S rRNA or 23S rRNA) of bacteria has been applied as a universal molecular marker for phylogenetic studies (Olsen *et al.*, 1986). The fluorescence *in situ* hybridization (FISH) method uses fluorescent oligonucleotide probes, which is able to bind to the conserved or more or less variable sequence of 16S and 23S rRNA gene, to identify and characterize the prokaryotic cells (Amann *et al.*, 1995; Schmid *et al.*, 2007). This method combination with confocal laser scanning microscope (CLSM) is an ideal approach for *in situ* identification of microorganisms in the rhizosphere (Schmid *et al.*, 2004) and characterization the spatial arrangement of the target microorganisms in their habitat (Wagner *et al.*, 2003).

Biosensors based on the whole bacterial cells, which produce a measurable response in the presence or absence of a compound or condition, have been applied for *in situ* investigation of microbe-plant interactions. The advantage of the biosensor is to report immediately the real conditions in the rhizosphere (Poulsen *et al.*, 2007). Steidle *et al.* (2001) have been described using green fluorescence protein (GFP) based biosensors for *in situ* visualization of AHL-mediated communication between individual cells in the rhizosphere of tomato.

CLSM is a powerful technique for qualitative analyses of cell distributions in the rhizosphere. In CLSM, the imaging thin optical sections in living and fixed specimens can range in thickness up to 100 μm, which have a crucial meaning for the root samples. In combination with fluorescence staining or tagging methods, CLSM has been wildly exploited in the analysis of microbe-plant interactions (Lawrence *et al.*, 2001; Schmid *et al.*, 2004).

1.2 Cell-cell communication in bacteria

1.2.1 Quorum sensing in Gram-negative bacteria

Bacteria are able to communicate with each other via a cell density dependent regulation of gene expression, which has been defined as quorum sensing (Fuqua *et al.*, 2001; Fuqua *et*

al., 1994; Whitehead *et al.*, 2001). In Gram-negative bacteria, frequently N-acyl-homoserine lactones (AHLs) are used as signal molecules (Fuqua *et al.*, 1994). *V. harveyi* uses not only AHL (called AI-1) but also a second autoinducer, named AI-2 as a signal molecule to regulate the quorum sensing (Bassler *et al.*, 1993; Bassler *et al.*, 1994). Pesci *et al.* (1999) have reported that *P. aeruginosa* produces 2-heptyl-3-hydroxy-4-quinolone and this *Pseudomonas* quinolone signal can function as an intracellular signal. A diffusible signal factor (DSF) produced by a pathogenic bacterial *Xanthomonas campestris* regulates virulence factor through quorum sensing (Barber *et al.*, 1997). Schaefer *et al.* (2009) described the use of *p*-coumaroyl-HSL for quorum sensing signals synthesized from environmental *p*-coumaric acid in *Rhodopseudomonas palustris*, *Bradyrhizobium* sp. and *Silicibacter pomeroyi*. The best investigated example is *Vibrio fisheri* (formerly *Photobacterium fisheri*) LuxI/LuxR quorum sensing system (Nealson, 1977; Fuqua *et al.*, 1994). In this system the *luxI* gene encodes AHL synthase and *luxR* encodes the regulatory protein. When AHL accumulation reaches a threshold level in the environment surrounding of the bacteria, the AHL/LuxR receptor complex activates or represses one or several target genes (Fig. 1.1). Homologs of LuxI/LuxR quorum sensing system have been identified in a large number of Gram-negative bacteria.

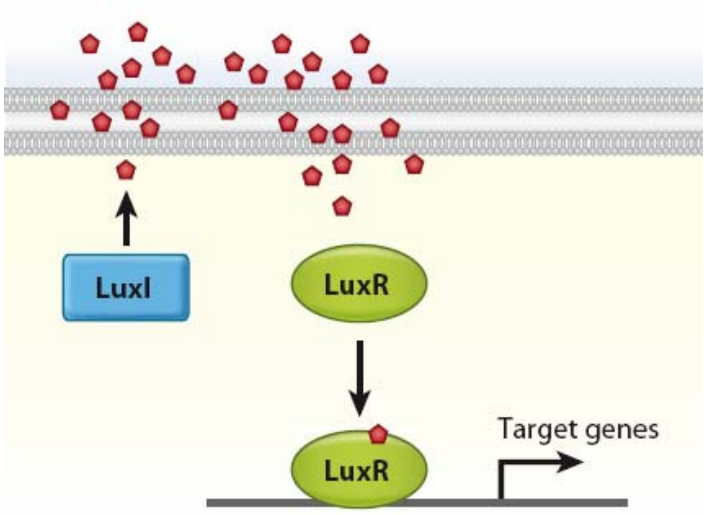


Fig. 1.1. LuxI/LuxR-type quorum sensing system. Red pentagons denote AHL quorum sensing signals (Ng and Bassler, 2009).

1.2.2 Quorum sensing in plant-associated bacteria

Many plant-associated bacteria utilize quorum sensing systems to regulate the expression of a diversity of genes, which are involved in interactions between microbes and plants (Loh *et al.*, 2002; Pierson III *et al.*, 1998). The best known system is TraI/TraR of *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*), which regulates transfer of oncogenic genes from its Ti plasmid to the plant host (Fuqua and Winans, 1994; Hwang *et al.*, 1995; Hwang *et al.*, 1994). Quorum sensing is also involved in special phenotype expression of PGPR, such as biofilm formation, siderophore production or antibiotic production (Fuqua and Greenberg, 2002; Somers *et al.*, 2004), and thus influences rhizosphere competence of PGPR. For example, the quorum sensing system in *P. putida* IsoF isolated from tomato rhizosphere influences biofilm structural development (Steidle *et al.*, 2002). In biocontrol agent *Pseudomonas aureofaciens* strain 30-84, two separate quorum sensing systems of PhzI/PhzR and CsaI/CsaR regulate the production of antibiotics, secondary metabolites, exoprotease activity and cell surface features (Wood and Pierson III, 1996; Zhang and Pierson III, 2001).

1.2.3 Cross-kingdom signaling

Quorum sensing signal molecules serve not only as an intraspecies bacterial signal but also as an inter-kingdom signal to their hosts. Plants possess the ability to detect and response to the bacterial AHL signals. *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF colonizing tomato roots, produce AHLs in the rhizosphere and thereby increase systemic resistance of tomato plants against a fungal leaf pathogen (Schuhegger *et al.*, 2006). *Arabidopsis thaliana* roots can take up bacterial C6-HSL which allows systemic distribution throughout the plant (von Rad *et al.*, 2008). Quorum sensing signal 3-oxo-C12-HSL of *P. aeruginosa* suppresses the fungus filamentation in human pathogen fungus *Candida albicans*. On the other hand, the *C. albicans* quorum sensing molecule farnesol regulate the quorum sensing signal quinolone of *P. aeruginosa* and thus, pyocyanin production (Cugini *et al.*, 2007). In *P. aeruginosa*, an opportunistic human pathogen, quorum sensing signal molecule 3-oxo-C12, has immunomodulatory activity in human (Telford *et al.*, 1998).

1.3 Phase variation in bacteria

1.3.1 Phase variation in bacteria mediated by genome modification

Phase variation or phenotypic variation is an adaptive process used by several bacterial species to generate population diversity, which increases bacterial fitness during fluctuating environmental conditions (van den Broek *et al.*, 2005; Wisniewski-Dyé and Vial, 2008). In contrast to the spontaneous mutations, phase variation occurs at a high frequency of more than 10^{-5} switches per cell per generation (Henderson *et al.*, 1999). In phase variation, the expression of a given gene is either ON or OFF. These events are usually reversible (ON↔OFF), but in some cases it is irreversible (ON→OFF or OFF→ON). Various DNA regulatory mechanisms are involved in phase variation: site specific inversion, recombinational deletion, transpositions, spontaneous duplication and mutations, slipped-strand mispairing, genomic rearrangements and differential methylation. These DNA changes lead to phenotypically heterogeneous populations (Henderson *et al.*, 1999; van den Broek *et al.*, 2005). In addition, according to Van der Wound and Baumler (2004) changes in the expression of global regulatory proteins lead to an alteration in the expression of several operons, thus resulting in phase variation.

1.3.2 Phase variation in human pathogens

Phase variation events are important e. g. in *Neisseriae*, and other mucosal human pathogens for adaptation to immune response and the unpredictable environment in the host. Over thirty surface-exposed molecules such as lipopolysaccharide, reporters for iron acquisition, capsule, pili and adhesions were expressed in phase variations of *Neisseria meningitides* (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). The repeats near or within the coding region are involved in shifting of reading frames or altering the strength of promoters, thus affecting gene expression (Maxon *et al.*, 1994). In *Haemophilus influenzae*, to give another example, mutations of tetranucleotide repeat tracts also invoke phase variation (Bayliss *et al.*, 2001; Hood *et al.*, 1996).

1.3.3 Phase variation in rhizosphere bacteria

Phase variation is not only a phenomenon associated with host-pathogen interaction, but also appears in rhizobacteria. In rhizosphere bacteria, different forms of phase variation

generate a certain amount of diversity in bacterial colony morphology and physiology, forming specific subpopulations. This phase variation is a broadly active phenomenon affecting root colonization, biocontrol activity, immune invasion, the expression of exoenzymes and secondary metabolites. In *Pseudomonas* spp., two mechanisms have been described, one of them a so far unknown mechanism affecting specific characters such as epitopes, expression of exoenzymes or flagella; the other mechanism is based on mutation of *gacA/S* (van den Broek *et al.*, 2005). In *Azospirillum brasilense*, a plant growth promoting rhizobacterium, various polysaccharides have been identified to be different in wild type and phenotype variant. The variant also shows a different colonization behavior as compared to the wild type (Katupitiya *et al.*, 1995). In *A. lipoferum*, the loss of a 750 kb plasmid is associated with forming phase variants (Vial *et al.*, 2006).

The role of host signals involved in phase variation has also been investigated. The expression of phase variation associated genes *sss* and *xerD* in *P. fluorescens* is induced by the plant. The expression of these two genes is enhanced by bacteria colonizing the roots. The chemical nature of this plant signal is unknown (Martinez-Granero *et al.*, 2005).

1.4 The Genus *Acidovorax*, a phenotypically diverse Gram-negative bacterium

On the basis of 16S rRNA gene sequence analysis, the genus *Acidovorax* belongs to the class of β-proteobacteria, family *Comamonadaceae* (Willems and Gillis, 2005). The genus *Acidovorax* now contains 13 recognized species, which were isolated from soil and water habitats: *Acidovorax facilis*, *Acidovorax delafieldii*, *Acidovorax temperans* (Willems *et al.*, 1990), *Acidovorax defluvii* (Schulze *et al.*, 1999), *Acidovorax caeni* (Heylen *et al.*, 2008), *Acidovorax soli* (Choi *et al.,* 2010). It includes also some opportunistic pathogens from clinical origin, as well as the phytopathogenic species *Acidovorax conjaci* (Willems *et al.*, 1992), *Acidovorax avenae*, *Acidovorax cattleyae*, *Acidovorax citrulli*, *Acidovorax oryzae* (Schaad *et al.*, 2008), *Acidovorax anthurii* (Gardan *et al.*, 2000) and *Acidovorax valerianellae* (Gardan *et al.*, 2003).

These Gram-negative bacteria are straight or slightly curved rods, aerobic and motile due to one or rarely three polar flagella, with a cell size of 0.8-5.0 μm in length and 0.2-1.2 μm in diameter. They are oxidase positive, chemoorganotrophic and grow well on a variety of

carbohydrates, organic acids, amino acids, and peptone. G+C content of the DNA is 62-70 mol%. Major fatty acids are $C_{16:0}$, $C_{16:1}$, $C_{18:1\omega7cis}$ (Willems and Gillis, 2005).
Owen and Zdor (2001) described that inoculation with a liquid suspension of *A. delafieldii* ATH2-2RS/1, a hydrogen cyanide (HCN) producing strain, on velvetleaf and corn plants reduce growth of velvetleaf, but not corn plants. Soil and water inhabitants of *Acidovorax* strains are able to biodegrade some commercial polyesters, e. g. *A. facilis* and *A. delafieldii* are able to degrade poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) in *vivo* (Mergaert and Swings, 1996). Some phytopathogenic *Acidovorax* species have antibacterial and antifungal activities, like for example *A. avenae* and *A. cattleyae*, that show biocontrol activity against *Rhodotorula mucilaginosa* (Hu and Young, 1998). This gives pathogenic bacteria an advantage, as they are more competitive in colonization and survival on plants. Until now, no particular root associated PGPR within *Acidovorax* has been described.

1.5 *Rhizobium radiobacter*, associated closely with the plant growth promoting fungus *Piriformospora indica*

1.5.1 Fungus associated bacteria

In some recent studies it has been described that mycorrhizal fungi are colonized by bacteria. These bacteria, such as some *Rhizobium* and *Pseudomonas* species, are able to attach to geminated fungal spores and hyphae (Bianciotto *et al.*, 1996). Bacterial cells are also present inside the fungal cells as endobacteria. Endocellular bacteria are reported in *Glyomeromycota* species and in several members of the *Gigasporaceae* family, where they show a certain type of specificity (Artursson *et al.*, 2006). Garbaye (1994) suggest the term "mycorrhization helper bacteria" for mycorrhizal associated rhizobacteria, which promote mycorrhizal development. These bacteria have a beneficial effect on the associated mycorrhizal fungi; they can directly promote the germination and growth rate as well as hyphal branching (Aspray *et al.*, 2006; Carpenter-Boggs *et al.*, 1995; Frey-Klett and Garbaye, 2005). Bacteria have been also shown to support the mycorrhizal symbiosis, including enhancement of colonization levels in roots or enhancement of the recognition process between root and fungus. Bacterial strains from diverse Gram-negative and Gram-positive bacterial genera, such as *Rhizobium, Azospirillum, Pseudomonas, Bacillus and Paenibacillus* form beneficial mycorrhizal associations (Artursson *et al.*, 2006; Bertaux *et*

al., 2005; Bertaux *et al.*, 2003; Garbaye, 1994). However the mechanisms of the association between mycorrhizal fungi and bacteria are not very well understood.

1.5.2 *Piriformospora indica* - a plant growth promoting fungus

Fungi of the order Sebacinales in *Basidiomycota* are involved in a broad range of symbioses with a variety of plants. *Piriformospora indica*, an axenically cultivable root endophyte isolated in the Indian Thar dessert, is a model organism of this order (Varma *et al.*, 1999). In many studies the plant growth promoting effects of *P. indica* for a variety of crop plants have been described. This fungus is able to increase biomass and grain yield of crop plants. In barley (*Hordeum vulgare* L.), *P. indica* induces root resistance against *Fusarium culmorum* and systemic resistance to barley powdery mildew. Furthermore, *P. indica* is able to protect barley from salt stress (Waller *et al.*, 2005).

1.5.3 *Rhizobium radiobacter* - an endofungal bacterium

The α-proteobacterium *Rhizobium radiobacter* (form. *Agrobacterium tumefaciens*) is a well known soil-borne bacterium. Strains of *Agrobacterium tumefaciens* can be significant plant pathogens infecting a wide range of plants and causing crown gall disease. Infected plants develop tumors which are induced by the integration of a bacterial virulence plasmid (Ti) into the plant genome (Escobar and Dandekar, 2003; Gelvin, 2003; Tomlinson and Fuqua, 2009). The completion of the genome sequencing and annotation of *A. tumefaciens* C58 affirm the presence of four replicons: a circular chromosome, a linear chromosome and plasmids pAtC58 and pTiC58 (Allardet-Servent *et al.*, 1993; Goodner *et al.*, 2001; Piper *et al.*, 1999).

Sharma et al. (2008) described an endofungal bacterium isolated from plant growth promoting fungus *Piriformospora indica*. This bacterium was identified as α-proteobacterium *Rhizobium radiobacter*. The biological activity of isolated *R. radiobacter* has been tested with barley as model plant. Most interestingly, *R. radiobacter* has similar effect on plants as the fungus *P. indica*, which has been found to promote plant growth and to increase pathogen resistance against powdery mildew. In addition, this isolated *R. radiobacter* is devoid of *isopentenyltransferase* (*ipt*) gene, which is involved in cytokinin biosynthesis. Thus, although the Ti plasmid is present in *R. radiobacter*, this bacterium has no harmful effect to the plant (Sharma *et al.*, 2008).

1.6 Aim of the work

In this study, the rhizobacterial strain *Acidovorax* sp. N35 isolated from surface sterilized wheat roots (*Triticum aestivum*) and the fungus-associated rhizobacteria *Rhizobium radiobacter* F4 and F7 were investigated to identify their interactive mechanisms with plants. Barley (*Hordeum vulgare*) was used as the model plant to investigate the rhizobacteria-plant interactions, since barley is a world wide important crop plant and has the simplest genome in *Triticeae*.

As strain N35 undergoes phenotypic variation, a characterization of the two phenotypic forms should be achieved, elucidating their possible role in the association with plants. Therefore, the plant growth stimulating effect of the two phenotypes in barley should be investigated. Furthermore, the two forms should be labeled with fluorescent genetic markers. Their colonization behavior should be investigated in monoxenic and soil systems. To investigate the mechanisms and genetic basis behind this phenotypic variation, the possible differences of both types at genetic level should be clarified using whole genome sequence data obtained from the latest Titanium version of 454 pyrosequencer (Roche). In addition, a phylogenetic and taxonomic characterization of isolate N35 should be performed.

The α-proteobacteria *R. radiobacter* F4 and F7, isolated from *P. indica* DSM 11827 and showing plant growth promoting effect on barley, were obtained from a cooperating working group in Giessen. This fungus associated *R. radiobacter* has similar effect on plants as the fungus *P. indica*, which was found to promote plant growth and to increase pathogen resistance against powdery mildew (Sharma *et al.*, 2008). In this study, the localization of endofungal bacterium *R. radiobacter* on or in barley roots should be examined with fluorescence in *situ* hybridization (FISH) using specific oligonucleotide probes and confocal laser scanning microscope (CLSM). The interaction of the plant growth promoting fungus *P. indica* with other rhizosphere bacteria, e.g. *Pseudomonas* and *Bacillus* strains should be performed using plate confrontation assays.

The other aim of this study was to identify the compounds and signals involved in rhizobacteria-plant interactions. First of all, the quorum sensing signals in two phenotypes of isolate N35 and fungus associated *R. radiobacter* should be identified using AHL-biosensors and Fourier transform ion cyclotron resonance - mass spectrometry (FT-ICR-MS) as well as ultra performance liquid chromatography (UPLC). In order to investigate the possible role of quorum sensing signals involved in microbe-plant interactions, *R.*

radiobacter and N35 will be genetically modified to abolish their AHL production. In the case of N35 with the help of the genome data obtained by 454 sequencing the genes responsible for AHL production can be identified, an AHL negative mutant of isolate N35 shall be constructed and characterized in comparison to the wild type. In case of *R. radiobacter* the introduction of a lactonase expressing plasmid should lead to an AHL negative phenotype, which can then be characterized as well.

2 Material and methods

For preparing the buffers and solutions in this study ultra pure water (Milli-Q Plus, Millipore, Schwabalbach, Germany) was used, media to cultivate microorganisms and plants were prepared with deionized water ($H_2O_{dem.}$).

All chemicals used in this study were obtained from Sigma-Aldrich (Steinheim, Germany) if not indicated otherwise. Heat stable solutions, buffers and media were heated for 20 min at 121 ºC and $1.013x10^5$ Pa gage pressure in an autoclave (Systec D65/V65/50, Systec GmbH, Wettenberg, Germany). Heat unstable substances were sterile filtered (sterile filter, pore size 0.22 µm, Millipore, Eschborn, Germany). pH was adjusted with NaOH or HCl if not indicated otherwise.

2.1 Cultivation of microorganisms

2.1.1 Bacterial strains and plasmids

Table 2.1. Strains specifications

Organisms	Relevant Characters	Reference and/or source
Escherichia coli TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1*nup*G	Invitrogen, Carlsbad, USA
Escherichia coli HB101	*recA thi pro leu hsdR$^-$M$^+$*Cm^R	Kessler *et al.*, 1992
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F$^-$ *proAB lac1^q ZΔM15* Tn10 (Tc^R)]	Stratagene, La Jolla, USA
"*Cand.* Acidovorax radicis" N35	wild type isolated from surface sterilized wheat roots, rough colony surface, flocculation in liquid medium	Klein, 2003

"*Cand.* Acidovorax radicis" N35v	Phenotype variant of N35, smooth colony surface, no flocculation in liquid medium	this study
"*Cand.* Acidovorax radicis" N35 GFP & N35v GFP	Km^R, chromosomally labeled with GFP (green fluorescent protein)	this study
"*Cand.* Acidovorax radicis" N35 YFP & N35v YFP	Km^R, labeled with YFP (yellow fluorescent protein)	this study
Acidovorax facilis DSM 649	type strain	Willems *et al.*, 1990; DSMZ, Braunschweig
Acidovorax defluvii DSM 12644	type strain	Schulze *et al.*, 1999; DSMZ, Braunschweig
Acidovorax delafieldii DSM 64	type strain	Willems *et al.*, 1990; DSMZ, Braunschweig
Acidovorax defluvii DSM 12578	wild type	Schulze *et al.*, 1999; DSMZ, Braunschweig
Rhizobium radiobacter F4 & F7	isolated from *Piriformospora indica* DSM 11827	Sharma *et al.*, 2008; Prof. Kogel, Uni. Giessen
Piriformospora indica	wild type	Prof. Kogel; Uni. Giessen
Pseudomonas putida IsoF	wild type, isolated from tomato roots	Steidle *et al.*, 2001; Prof. Eberl, Uni. Zürich
Serratia liquefaciens MG1	wild type; Amp^R & Tc^R	Givskov *et al.*, 1988 Prof. Eberl, Uni. Zürich
Azospirillum brasilense Sp245	wild type	Baldani *et al.*, 1987
Pseudomonas fluorescens SS101	wild type, Rp^R	Raaijmakers *et al.*, 2006; Prof. Raaijmakers, Uni. Wageningen

Pseudomonas fluorescens SS101 10.24	biosurfactant massetolideA deficient mutant, Rp^R & Km^R	Mazzola *et al.*, 2007; Prof. Raaijmakers, Uni. Wageningen
Bacillus amyloliquefaciens FZB42	wild type	Prof. Borriss, HU Berlin; Rhizo PlusR, Abitep, Berlin
Bacillus amyloliquefaciens FZB42 CH3	lipopeptides and polyketides double deficient mutant, Em^R & Lm^R	Chen *et al.*, 2006; Prof. Borriss, HU Berlin
Bacillus amyloliquefaciens FZB42 AK1	bacillomycin D deficient mutant, Em^R & Lm^R	Koumoutsi *et al.*, 2004; Prof. Borriss, HU Berlin
Bacillus amyloliquefaciens FZB42 AK2	fengyin deficient mutant, Cm^R	Koumoutsi *et al.*, 2004; Prof. Borriss, HU Berlin
Bacillus amyloliquefaciens FZB42 AK3	bacillomycine D & fengyine double deficient mutant, Em^R, Lm^R & Cm^R	Koumoutsi *et al.*, 2004; Prof. Borriss, HU Berlin
Serratia liquefaciens MG44	AHL negative mutant, host for AHL biosensor pBAH9, Amp^R, Tc^R & Sm^R	Eberl *et al.*, 1996 Prof. Eberl, Uni. Zürich
"*Cand.* Acidovorax radicis" N35 ΔaraI	AHL negative mutant, Tc^R	this study
Rhizobium radiobacter F4 NM13	AHL negative transformant, $Tell^R$	this study
Rhizobium radiobacter F7 NM13	AHL negative transformant, $Tell^R$	this study

Table 2.2. Plasmids

Plasmids	Relevant Characters	Reference
pCR2.1-TOPO	Amp^R, Km^R; *lacZ*α, cloning vector	Invitrogen, Carlsbad, USA
pEYFP	Amp^R, *lacZ, eYFP* carrier vector	Clontech, CA, USA
pJBA28	Amp^R, Km^R; carrier plasmid for mini-Tn5-Km-$P_{A1/04/03}$-RBSII-*gfp*mut3*-T_0-T_1	Andersen *et al.*, 1998
pBBR1MCS-2	Km^R; *lacZ*α, cloning vector	Kovach *et al.*, 1995
pBAH9	Km^R, green fluorescent AHL sensor plasmid for C4-C14-HSL	Huber, unpublished
pEX18Gm	Gm^R, $oriT^+$, $sacB^+$, gene replacement vector with MCS from pUC18	Hoang *et al.*, 1998
pEX18Tc	Tc^R, $oriT^+$, $sacB^+$, gene replacement vector with MCS from pUC18	Hoang *et al.*, 1998
pRK600	Cm^R, ColE1 *oriV* RP4 tra^+ RP4 *oriT*, helper strain for conjugation mating	Figurski and Helinski, 1979
pMLBAD-aiiA	$Tell^R$, *aiiA* lactonase gene in expression vector, lactonase activity induced by 0.2% arabinose	Wopperer *et al.*, 2006

2.1.2 Media and buffers

Unless otherwise noted all solid media were prepared with 15 g agar pro liter and for semisolid agar with 7.5 g agar pro liter. pH was adjusted with 0.1 M NaOH or 0.1 M HCl.

NB (Nutrient Broth) medium:	meat peptone	5 g
(Nr. 4, Fluka, Steinheim, Germany)	meat extract	3 g
	ad $H_2O_{dem.}$	1000 ml
	pH 7.0	

LB (Luria-Bertani) medium:	peptone from casein	10 g	
(Bertani, 1951, modified)	yeast extract	5 g	
	NaCl	5 g	
	ad $H_2O_{dem.}$	1000 ml	
	adjust pH to 7.0		
SOC medium:	yeast extract	0.5 g	
(Sambrook *et al.*, 1989)	casein hydrolysat	2 g	
	$MgCl_2x6H_2O$	0.2 g	
	$MgSO_4x7H_2O$	0.25 g	
	Glucose	0.36 g	
	ad $H_2O_{dem.}$	100 ml	
M9 mineral medium:	solution A:	Na_2HPO_4	4.8 g
(Sambrook *et al.,* 1989, modified)		KH_2PO_4	5 g
		NH_4Cl	1 g
		ad $H_2O_{dem.}$	900 ml
	solution B:	$CaCl_2$	0.011 g
		ad $H_2O_{dem.}$	40 ml
	solution C:	$MgSO_4$	0.24 g
		ad $H_2O_{dem.}$	50 ml
	solution D:	20% Glucose	9 ml
	pH 6.8		

Solutions A, B and C were autoclaved separately and solution D was sterile filtered. Finally solutions B, C and D were added to solution A.

M461 mineral medium:	solution A:	Na_2HPO_4	1.45 g
(Nagel and Andreesen, 1989,		KH_2PO_4	0.8 g
modified)		ad $H_2O_{dem.}$	800 ml
	pH 6.9		
	solution B:	$CaCl_2$	0.01 g
		$MgSO_4x7H_2O$	0.50 g
		$MnSO_4$	0.01 g
		NH_4Cl	0.30 g
		NaCl	0.05 g
		ad $H_2O_{dem.}$	100 ml
	pH 6.9		
	solution C:	Vitamine B12	50 mg
	(10x)	Pantothenic acid	50 mg
		Riboflavin	50 mg
		Pyridoxal-HCl	10 mg
		Biotin	20 mg
		Folic acid	20 mg
		Nicotinic acid	25 mg
		Nicotine amide	25 mg
		Tiamine-HCl	50 mg
		ad $H_2O_{dem.}$	100 ml
	solution D:	Glucose	20 g
		ad $H_2O_{dem.}$	100 ml

Solutions A and B were autoclaved separately. Solutions C and D were sterile filtered. Finally, 100 ml solution B, 500µl solution C and 100 ml solution D were added to 800 ml solution A.

BUG agar:	BUG agar	57 g
(Biolog, CA, USA)	ad $H_2O_{dem.}$	1000 ml

BUG agar was dissolved in microwave, pH was adjusted to 7.3.

CAS medium:	solution A:	KH_2PO_4	0.3 g
(Schwyn and Neilands, 1987)		K_2HPO_4	0.2 g
		fructose	2 g
		glucose	2 g
		NH_4Cl	0.5 g
		NaCl	15 g
		ad ultra pure water	800 ml
		adjust pH to 6.0	
	solution B:	$MgSO_4x7H_2O$	0.2 g
		ad ultra pure water	2 ml
	solution C:	$CaCl_2$	0.02 g
		ad ultra pure water	2ml
	solution D:	Mops	10.46 g
		ad ultra pure water	100 ml
		adjust pH to 6.8	
	solution E:	a: CAS	0.061g
		ad ultra pure water	50 ml
		b: HDTMA	0.073 g
		ad ultra pure water	40 ml
		c: $FeCl_3x6H_2O$	0.135 g
		ad 1N HCl	5 ml
		ad ultra pure water	495 ml
	mix 50 ml solution A, 40 ml solution B and 10 ml solution C		

Solutions A and E were autoclaved separately. Solutions B, C and D were sterile filtered. Finally solution B, C, D and solution E were added in solution A.

Skim milk plate:	solution A:	casein hydrolysate	10 g
(Frank and Yousef, 2004,		yeast extract	5 g
modified)		NaCl	4 g
		agar	15 g
		ad $H_2O_{dem.}$	500 ml
	solution B:	skim milk puder	20 g
		ad $H_2O_{dem.}$	500 ml

Solution A was autoclaved normally, solution B was autoclaved 10 min at 121 ºC and $1.013x10^5$ Pa gage pressure. Finally solution B was added to solution A.

Glyceryl tributyrate plate:	tributyrin agar	23 g
(Lawrence *et al.*, 1967)	glyceryl tributyrate	10 ml
	ad $H_2O_{dem.}$	1000 ml

Before autoclaving, tributyrin agar and glyceryl tributyrate were dissolved by heating to 100 ºC and mixed together.

10x PBS:	Na_2HPO_4	11.5 g
(Fluka)	KH_2PO_4	2 g
	NaCl	80 g
	KCl	2 g
	ad ultra pure water	1000 ml
	adjust pH to 7.2	
10x TE:	Tris/HCl	12.1 g
	EDTA	3.72 g
	ad ultra pure water	1000 ml
	adjust pH to 7.5	

2.1.3 Selective agents

For the preparation of selective media antibiotics and other supplements were sterile filtered and added after the medium cooled to 50 ºC.

Table 2.3. Antibiotics and medium supplements

Medium supplements	Abbre-viation	Activity mechanisms	Solvent	Concen-tration
Ampicillin	Amp	β-lactam antibiotic, inhibition of the synthesis of peptidoglycan	50% ethanol	100 mg/l
Kanamycin	Km	aminoglycoside, inhibition of protein synthesis by binding to 30S ribosomal subunit	ultra pure water	50 mg/l
Tetracycline	Tc	inhibition of protein synthesis by binding to 30S ribosomal subunit	methanol	20 mg/l
Rifampicin	Rp	inhibition of RNA synthesis by binding to the RNA polymerase	DMSO	100 mg/l
Chloramphenicol	Cm	inhibition of the formation of peptide bonds by binding to 50S ribosomal subunit	50% ethanol	10 mg/l
Thrimethophrim	Tm	interference with the production of tetrahydrofolic acid	DMSO	100 mg/l
Gentamycin	Gm	inhibition of protein synthesis by binding to the 50S ribosomal subunit	ultra pure water	20 mg/l
Lincomycin	Lm	inhibition of 30S ribosomal subunit, stimulation of nonenzymatic formation of 70S ribosomal subunit	ultra pure water	25 mg/l
Erythromycin	Em	inhibition of protein synthesis in proliferating organism	ultra pure water	1 mg/ml
Streptomycin	Sm	inhibition of protein synthesis by binding to 30S ribosomal subunit	ultra pure water	50 mg/l, 750mg/l*
Tellurite	Tell	oxidizing agent	ultra pure water	100 mg/l
5-bromo-4-chloro-3-indolyl-beta-D-Galactopyranosid	X-gal	substrate for ß-galactosidase	N,n-dimethyl-formamide	40 mg/l
p-coumaric acid	CA	natural component of plant	$EtOH_{abs.}$	320 mg/l (1 mM)

***** 50 mg/l streptomycin was added to medium and 750 mg/ml was used in seeds sterilization protocol.

2.1.4 Cultivation of microorganisms

Sterile wire loops and tooth picks were used for inoculation of bacterial strains in liquid and solid media. A 1 ml pipette tip was used for inoculation of *P. indica* on solid medium. All microorganisms were re-cultivated every 3-4 weeks and stored at 4 ºC. Glycerol stocks were used for long time preservation. For preparation of glycerol stocks the microorganisms were grown over night or 5 days for *P. indica* in liquid medium and were then transferred to 20% glycerol. The strains were stored at -80 ºC.

2.2 Cultivation of barley

Barley (*Hordeum vulgare* L.) variety "Barke" used in this study was purchased from the company "National Breed Josef Breun GdbR" (Herzogenaurach, Germany).

2.2.1 Barley in monoxenic system

2.2.1.1 Monoxenic system

The monoxenic system is suitable for the inoculation of defined bacterial strains on plant roots. Here the barley seedlings were grown in glass tubes (Ø 30 mm, Schott glass, Mainz, Germany) filled 5-6 cm in height with sterilized quartz sand (Ø 1.0-2.5 mm, Sakret, Ottobrunn). 10 ml MS medium was supplied for plant nutrition.

MS medium	Murashige and Skoog Basal Salt Mixture	4.3 g
	add $H_2O_{dem.}$	1000 ml

Plants were grown at 16 ºC/12 ºC day/night cycle, 50% relative humidity and a photo period of 12 h. Barley plants could be cultivated for a maximum of 3-4 weeks in this system.

2.2.1.2 Seed sterilization

Seeds used in the monoxenic system were surface sterilized to avoid contamination with fungi and bacteria. Barley seeds were shaken in 70% ethanol for 2 min and incubated in 2% NaOCl for 15 min. Then the seeds were washed with $H_2O_{dem.}$ 5 times and incubated in 600 mg/l penicillin and 250 mg/l streptomycin solution for 30 min. At last the seeds were incubated on NB plates at 30 ºC, letting them germinate for 3 days. After this period they were inspected for contaminations and only uncontaminated seedlings were selected for inoculation.

2.2.2 Barley in soil system

The sandy and acidic soil (pH 5) from Neumarkt in Oberpfalz, Germany, was used for cultivation of barley seedlings in soil system. The pots were filled with 2 l soil and 50ml deionized water was added. The barley seeds were incubated on moist filter paper and germinated for 3 days at room temperature (non sterile conditions). Then the seedlings were inoculated with GFP-labeled "*Cand.* A. radicis" N35 or N35v (2.3.1). Per pot 3 plants were cultivated for 3-4 months. Plants were grown as in the monoxenic system. The plants were watered 2 times with 50 ml deionized water per week in the first month and 3 times per week in the second and third month. Throughout the experiment the plants were fertilized two times with 150 ml of 0.7% Wuxal Top N (Aglukon Spezialdünger GmbH, Düsseldorf, Germany) and 150 ml of 0.02 M $MgSO_4$ per pot.

2.3 Localization of bacteria on barley roots

2.3.1 Inoculation of barley roots with bacteria

The bacterial strains "*cand.* A. radicis" N35 and N35v labeled with GFP or YFP, and "*cand.* A. radicis" N35 ΔaraI as well as *R. radiobacter* F4 were used for the inoculation of barley root. An overnight culture of different bacterial strains was harvested at 5000x g (Eppendorf 5417R, Eppendorf, Hamburg, Germany) for 5 min at RT and the supernatant was discarded. The cells were washed 2 times with 10ml of 1x PBS and thereafter resuspended in 10 ml 1x PBS. The optical density (OD) of cells was measured at a wave length of 435 nm using a spectral photometer (CE3021, Cecil, Cambridge, England). The

cell density was adjusted to an OD_{435nm} of 0.7-0.8 for "*cand.* A. radicis" N35v and *R. radiobacter* or 1.5 for "*cand.* A. radicis" N35 and "*cand.* A. radicis" N35 ΔaraI, respectively. These culture densities corresponded to about 10^8 cells/ml using the spread plate method on NB medium. For the inoculation of single bacterial strains, the seedlings were incubated in the bacterial suspension for 1 h at room temperature. At last, the seedlings were transferred to a monoxenic or soil system.

2.3.2 Root harvesting

The roots of barley were harvested after 3 weeks growth in monoxenic system and 16 weeks in soil system. Quartz sand or soil were carefully removed from the plant roots. The roots were separated from the shoots with sterilized scissors and washed several times with 1x PBS in order to remove only loosely attached bacteria or particles. Then the roots were stored for later applications in 1x PBS, or dried at 70 ºC for 2 days for the determination of dry weight. Dry weight of shoots was determined using the same method.

2.3.3 Preparation of roots for microscopic detection of bacteria

For fluorescence in *situ* hybridization (FISH, 2.7.1), the roots were washed as described in 2.3.2. Then, the roots were incubated in 4% PFA as described in 2.7.1.2 at 4 °C overnight. After hybridization the roots were observed with a CLSM (2.4.2).

For the localization of GFP or/and YFP-labeled bacterial cells, the roots were harvested and washed as described in 2.3.2. The intact pieces of the roots were put on the slide and embedded with citifluor-AF1 (Citifluor Ltd., London, England) in order to prevent photo bleaching. At last the cover slip was placed carefully on the slide and fixed with tape.

After SYTO orange 81 staining, intact root pieces (2.7.13) were also prepared as described above with citifluor-AF1 and observed with CLSM.

2.4 Microscopy

2.4.1 Epifluorescence microscopy

The epifluorescence microscope Axionplan 2 (Zeiss, Oberkochen, Germany) was used for detection of bacteria in pure culture. Here an oil immersion objective (C-Apochromat, 100x1.2oil Korr) with 100 times magnification was used. The light source was a mercury short arc reflector lamp. The data for the used filters are shown in the following table 2.4.

Table 2.4. Data of filter systems by epifluorescence microscopy

Fluorophores	Excitation filter	Beam splitter	Emission filter
Green fluorescens; fluorescein and GFP	BP 470/40	FT 495	BP 525/50
Orange red fluorescens; Cy3	BP 545/25	FT 570	BP 605/70
Cy5	BP 640/30	FT 660	BP 690/50

2.4.2 Confocal laser scanning microscopy (CLSM)

In this study the confocal laser scanning microscope LSM 510 META (Carl Zeiss, Oberkochen, Germany) was used for detection of fluorescent labeled bacteria on roots. Here a water immersion objective C-Apochromat 63x1.2W Korr (aperture number 1.2) with 63 times enlargement was used. In λ-mode the use of a wavelength specific detector (META module) allows the identification and separation of even rather similar emission spectra of fluorophores, such as GFP with a maximum emission wavelength of 510 nm versus YFP with 530 nm.

The image analysis was done using Zeiss software LSM Image Browser Version 3.5. Other data of the used CLSM are shown in the following table 2.5.

Table 2.5. Characteristics of fluorophores, as well as filter and laser system

Fluorophores	Excitation maximum (nm)	Emission maximum (nm)	Laser Type (nm)	Beam splitter	Filter
GFP	498	509	Argon 488	NFT490 HFT488/543	BP 500-550
YFP	514	527	Argon 488	NFT490 HFT488/543	BP 500-550
Fluos	490, 494	520, 525	Argon 488	NFT490 HFT488/543	BP 500-550
Cy3	514, 552, 554	566, 570	Helium-Neon 543	HFT 488/543	LP 560
Cy5	649	666, 670	Helium-Neon 633	HFT UV/488/543/633	LP 650

2.4.3 Scanning electron microscopy

For scanning electron microscopy, 1 ml cells grown overnight in NB medium were harvested and washed twice with 1x PBS. Then, the cells were fixed with 2 ml 1% glutaraldehyde in 1x PBS (pH7.4) overnight at 4 ºC. The cells were dehydrated through a series of ethanol solutions with increasing concentrations (50, 70, 80, 95 and 100% ethanol). Liquid CO_2 was used to replace the ethanol, and the cells were dried in a critical-point drier. The cells were sputter-coated with platinum and observed in a scanning electron microscope (JSM 6300F; JEOL).

2.5 Rhizobacteria-fungi interactions

Interactions between different rhizobacteria and *P. indica* were determined using confrontation assays. Here the rhizobacteria and defined mutants were used to test the effect on *P. indica*. A 5 mm mycelial plug of *P. indica* was incubated on NB agar at 30°C for three days. Bacterial strains were streaked around the mycelium in 1cm distance from the mycelium in order to avoid the overgrowth of both cultures (Fig. 2.1 a). Cultures were incubated at 30 °C for 2 days and then the interactions were examined.

The effect of isolated cyclic lipopeptide masstolide A was also tested. The substance was dissolved in ultra pure water at a concentration of 10 mg/ml, 1 mg/ml and 100 µg/mg, respectively. *P. indica* grew on agar plate as described above. 10 µl of the dissolved substance was dripped around the mycelium in 1 cm distance (Fig. 2.1 b). *P. indica* was incubated at 30 °C for 2 days and then the effects were examined.

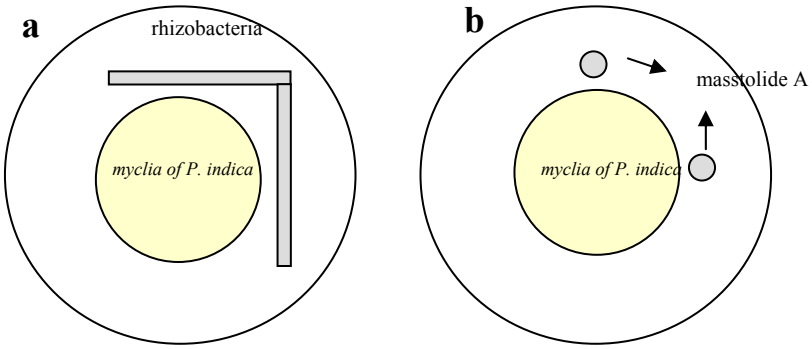


Fig. 2.1. Confrontation assays performed with different rhizobacteria (a) and masstolide A (b)

2.6 Metabolic analyses of bacteria

2.6.1 Detection and characterization of N-acyl-homoserine lactones (AHLs)

2.6.1.1 Detection of AHLs via biosensor

Green fluorescent AHL sensor plasmid pBAH9 harbored by *S. liquefaciens* MG44 had been constructed to detect broad range C4-C14 homoserine lactones (HSLs, Huber, unpublished). In this study, AHL production of "*cand.* A. radicis" and *R. radiobacter*, as well as the AHL negative mutants of "*cand.* A. radicis" and lactonase transconjugates of *R. radiobacter* were examined using sensor plasmid pBAH9. The sensor strain was streaked onto the center of an LB agar plate, and the test bacterial strains were cross-streaked near the biosensor. The cultures were incubated at 30 °C for 24-48 h. The detection of AHL production was observed by binocular Lumar V12 (Zeiss, Oberkochen, Germany).

2.6.1.2 Detection and characterization of AHLs via Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and ultra performance liquid chromatography (UPLC)

For FT-ICR-MS and UPLC a careful sample pretreatment was required to remove the matrix components of the biological samples and to optimize the concentrations of AHLs for analysis. For preparing the supernatant, "*cand.* A. radicis" N35 and N35v were incubated in M9 medium at 30 °C for 24 h and *R. radiobacter* F4 and F7 were incubated in M461 medium with or without *p*-coumaric acid at 30 °C for 48 h. All of the media were buffered to a pH of 6.8 to avoid abiotic degradation of AHLs (Englmann *et al.*, 2007). After OD_{435} reached 0.6-0.8, the bacterial suspension (50 ml) was centrifuged by 5000x g for 15 min at RT. Briefly, 5 ml of 10% acetonitrile/water (v/v) was added to 45 ml of sample and then, 50 ml supernatant was induced into a solid phase extraction catridge (C18 Bond Elut Varian, Darmstadt, Germany). The cartridge was washed with 15% methanol/water (v/v), dried under vacuum and the solutes were eluted with 25% hexan/propanol (v/v). After drying with nitrogen, the eluate was dissolved again in 10% acetonitrile/water (v/v). The samples were filtered with a polytetrafluoroethylene filter (pore size 0.2 µm, VWR, Darmstadt, Germany) and stored at -20 °C.

To prepare the supernatant for quantitative determination of AHL production, strains N35 and N35v grew in M9 medium and samples were taken after 18 h incubation as well as then every two hours. The medium were filtered with a cellulose filter (Millipore) and dry weight of the cells was recorded. The solid phase extraction was carried out as described above.

Mass spectrometry (MS) is a technique generally used for structure recognition. In MS the mass:charge ratio (m/z) is determined utilizing the differences of the "movement" ability of the gaseous ion in the electric and/or magnetic field under vacuum (Fekete *et al.*, 2010). In this study, the high mass accuracy instrument FT-ICR-MS (Bruker Daltonics, Bremen, Germany) was used to identify the AHL production by different bacterial strains. UPLC (Waters, Darmstadt, Germany) was used to quantify the AHL signaling substances and the homoserines (Li *et al.*, 2006; Englmann *et al.*, 2007) produced by bacteria. The measurement was carried out by Dr. Fekete, Agnes and Fonseca, Juliano R. in the institute of ecological chemistry in Helmholtz Zentrum München according to the published methods (Li *et al.,* 2006; Fekete *et al.*, 2007).

2.6.2 Phenotypic characterization of bacterial strains

Swarming tests for both types of "*cand.* A. radicis" N35 were performed on NB semisolid agar plates at 30 °C for 48 h (Daniels *et al.*, 2006). Protease activity was examined on skim milk agar plates resulting in the hydrolysis of 1% (w/v) casein hydrolysate and 2% (w/v) skim milk (Frank and Yousef, 2004). Lipase activity was indicated by the formation of a halo on 1% (v/v) glyceryl tributyrate agar plates (Lawrence *et al.*, 1967). Siderophore production was revealed by the formation of orange halos on CAS agar plate (Schwyn and Neilands, 1987). Here 5 µl of bacterial cells in exponential phase (OD_{435}=0.6) were dripped on an agar plate and the cultures were incubated at 30 °C for 24-48 h.

Switch frequency:

For calculation the switch frequency of wild type N35 to phenotype variant N35v, the wild type cells were grown in NB liquid medium and the total colony numbers as well as the numbers of colonies with switched phenotype were counted on NB solid agar plate at 0 h and 16 h.

The switch frequency was calculated using a minimising algorithm provided by Prof. Dr. Kuttler, Christina (IBB, Helmholtz Zentrum München):

$$P_0 = a + (1-a) \cdot p$$

$$P_{16} = 1 - (1-a)(1-p)^{x_{16}} + (1-a)(1-p)^{x_{16}} \cdot p$$

("p" denotes the switch probability per cell per generation; "a" denotes the proporation of switched cells in the total population; "P_0" is the proportion of switched colony numbers in the total population at 0 h; "P_{16}" is the proportion of switched colony numbers in the total population at 16 h; "x_{16}" denotes the bacterial generations took place in the 16 h interval.)

Metabolism of substrates:

Metabolism of a variety of substrates was tested using the substrate utilization test with GN2 microplates (Biolog, CA, USA; Tang *et al.*, 1998). The GN2 plate is designed for identification and characterization of the substrate utilization of a wide range of aerobic Gram-negative bacteria. Briefly, bacterial cells were grown on BUG agar plates at 30 °C for 48 h. The bacteria were swabbed from the surface of the agar plate and suspended to OD_{460}=0.354 in GN/GP inoculating fluid (Biolog, CA, USA). 150 µl of bacterial suspension were pipetted to each well of a microplate and the plate was incubated at 30°C

for 48h. The color reaction was recorded for positive utilization of the substrate by the bacteria. All the bacterial strains were tested in at least three replications.

Fatty acid analysis:

For cellular fatty acid analysis, 250 ml cells grown in NB medium were harvested and about 1.5 g wet weight cell pellets were washed twice with 1x PBS. Lipid extraction was performed as described by Zelles (1997). Saturated and polyunsaturated fatty acid methyl esters (FAMEs) were measured in 100 µl internal standard (nonadecanoic acid methyl ester in isooctane). Monounsaturated and hydroxysubstituted FAMEs were measured in 100 µl of internal standard after derivatization with dimethyl disulfide (DMDS) and trimethyl chlorsilane (TMSI). All fractions were analysed on a GC/MS (Trace MS, Thermo Finnigan, United States) according to Zelles (1999). Fatty acids were identified using FAME and BAME standards (Supelco^{TM}, United States) as well as established fatty acid libraries (CH 6500 Solvit, Switzerland). In this study the measurements and analysis were implemented by Dr. Esperschütz, Jürgen (IBOE, Helmholtz Zentrum München).

2.7 Molecular genetic methods

2.7.1 Fluorescence *in situ* hybridization (FISH)

FISH is a phylogenetic technique used to detect, localize and identify the presence of single microbial cells in a microhabitat. This method uses fluorescent oligonucleotide probes that bind to the conserved or more or less variable sequence of ribosomal RNA of microorganisms. According to the specificity of probes this technique can differentiate bacteria at phylum group, genus and species level (Amann *et al.*, 1995).

Material

All the buffers applied in the FISH experiments were obtained from AppliChem (Darmstadt, Germany).

4% paraformaldehyde (PFA) solution:

First, 45 ml $H_2O_{dem.}$ was warmed to 60-65 °C, and 2 g PFA was added in this 45 ml $H_2O_{dem.}$ 10N NaOH was added to the mixture till the solution is clear. At last, 5 ml 10x PBS was added to the solution. The 4% PFA solution was sterile filtered with 0.45 µm filter (Millipore) and stored at 4 °C for maximum one week.

2.7.1.1 Oligonucleotide probes

Table 2.6 shows the oligonucleotide probes used in this study. Most of the probes can be labeled with different fluorophores. The excitation and emission wavelength of fluorophores are showed in table 2.5. All of the probes labeled with fluorescein (Fluos), Cy3 and Cy5 in this study were obtained from Thermo Electron (Ulm, Germany). The concentration of the stock solution was determined with Nanodrop ND-1000 (Nanodrop, Wilmington, USA; 2.7.4). The working solution was diluted with nuclease free water to the following concentrations:

Cy3, Cy5 → 30 µg/ml

Fluos → 50 µg/ml

The stock and working solutions were stored at -20 °C.

Table 2.6. Oligonucleotide probes

Probe	Specificity	Binding position[1]	Probe sequence 5'-3'	FA[2] %	Reference
EUB338I[3]	Bacteria without *Planctomycetales, Verrucomicrobiales*	16S, 338-355	GCTGCCTC CCGTAGGA GT	var.	Amann *et al.*, 1990
EUB338II[3]	*Planctomycetales*	16S, 338-355	GCAGCCAC CCGTAGGT GT	var.	Daims *et al.*, 1999
EUB338III[3]	*Verrucomicrobiales*	16S, 338-355	GCTGCCAC CCGTAGGT GT	var.	Manz *et al.*, 1992
Rhi1247	*Rhizobium* sp., *Agrobacterium* sp., *Ochrobacterium* sp., some *Azospirillum* sp., few *Sphingomonas* sp.	16S, 1247-1252	TCGCTGCC CACTGTG	35	Sharma *et al.*, 2008
ACISP145	*Acidovorax radicis* N35 and N35v, *Acidovorax defluvii*, *Acidovorax facilis*	16S, 145-162	TTTCGCTC CGTTATCC CC	35	Schmid and Rothballer, unpulished

[1] Position in ribosomal nucleotides of *E.coli* (Brosius *et al.*, 1981)

[2] % Formamide in hybridization buffer

[3] For using, EUB338I, II, III were mixed in equal molars

2.7.1.2 Cell fixation with paraformaldehyde

Paraformaldehyde (PFA) induced cross linking of cell wall components, which causes the cells to stay in stable form during storage. Here 2 ml overnight bacterial culture was centrifuged at 5000x g for 3 min at RT. The cells were resuspended in 800 µl PBS/PFA solution (1x PBS:4% PFA= 1:3) and fixed at 4 °C for at least 1 h or overnight. At last, the cells were washed twice with 1x PBS and stored in 50% PBS/$EtOH_{abs}$ (v/v) at -20 °C.

The roots prepared in 2.3.2 were fixed in 4% PFA at 4 °C overnight. Then, the roots were washed with ultra pure water. At last the roots were stored in 50% PBS/$EtOH_{abs}$ (v/v) at -20 °C.

2.7.1.3 Hybridization with oligonucleotide probes

For hybridization of pure cultures, 1-3 µl fixed bacterial suspension was pipetted on epoxy coated slides (Roth, Karlsruhe, Germany). The roots were cut into 2 cm pieces and all the other steps were performed in 2 ml Eppendorf tubes.

Bacterial cells and roots were dehydrated through a series of ethanol solutions with increasing concentrations (50, 80 and 100% ethanol) for 5 min, respectively. The stringency of the hybridization is determined by the formamide concentration in the hybridization buffer; this means, the more formamide is included in the buffer, the more stringent are the hybridization conditions. The formamide concentration was empirically determined depending on the oligonucleotide probe (Tab. 2.6). The composition of the hybridization buffer was as follows:

Hybridization buffer:	NaCl (5M)	360 µl
	Tris/HCl (1M, pH 8)	40 µl
	Formamide	table 2.7
	Ultra pure H_2O	table 2.7
	SDS (10%w/v)	2 µl

Table 2.7. Formamide concentration in hybridization buffer by hybridization temperature of 46 °C

Formamide (%)	Formamide (µl)	Ultra pure water (µl)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800

45	900	700
50	1000	600
55	1100	500
60	1200	400
65	1300	300
70	1400	200
75	1500	100
80	1600	0

Pure cultures were hybridized on a slide with a mixture of 1 µl probe solution and 8 µl hybridization buffer. Roots were hybridized in an Eppendorf tube with a mixture of 15 µl probe solution and 120 µl hybridization buffer. For the hybridization of two probes with the same formamide concentration, the probes were added in equal amounts to the same reaction. The slides with hybridization buffer were then transferred into a 50 ml tube. In order to avoid the evaporation, the tissue with the rest of hybridization buffer was also transferred together into the tube. The samples were incubated at 46 °C for at least 1 h 30 min.

After hybridization a washing step under stringent conditions was performed to remove unspecifically bound probe molecules. The composition of the washing buffer was as follows:

Washing buffer:	Tris/HCl (1M, pH8.0)	1 ml
	Na-EDTA (0.5M, pH 8.0)*	500 µl
	NaCl (5M)	table 2.8
	ad ultra pure water	50 ml
	SDS (10% w/v)	50 µl

Table 2.8. NaCl concentration in wash buffer at 48°C

Formamide in hybridization buffer (%)	NaCl (mol)	5M NaCl (µl)
0	0.900	9000
5	0.636	6300
10	0.450	4500
15	0.318	3180

20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	0.010	-
70	0.007	-
75	0.005	-
80	0.0035	-

* For 20-65% formamide, 500 µl Na-EDTA solution should be added to the wash buffer; for 70%, 75% and 80% formamide, 350 µl, 250µl and 175 µl Na-EDTA solution were added to the wash buffer, respectively.

The slides were incubated in pre-warmed wash buffer at 48 °C for 15 min, and then carefully washed with ultra pure water and air dried. The roots were treated with the same procedure in an Eppendorf tube. The samples were embedded with citifluor-AF1 in order to prevent photo bleaching. At last, the cover slip was carefully placed on the slide and fixed with tape, and the samples were analyzed using epifluorescence microscopy (2.4.1) or CLSM (2.4.2)

2.7.2 DNA isolation

2.7.2.1 Plasmid DNA isolation

High quality plasmid DNA for applications, such as sequencing, PCR or enzymatic manipulation was isolated with NucleoSpin Plasmid Kit (Machery-Nagel, Düren, Germany). All of the required buffer and solutions were supplied by the manufacturer. The principle of this isolation method is DNA liberation via alkaline lysis and purification of DNA using column chromatography. The procedure of isolation was carried out according to the manufacturer's manual. At last, plasmid DNA binding to the silica membrane was

eluted with elution buffer or nuclease free water (Promega, Madison, USA). The isolated plasmid DNA was stored at -20 °C for at least one year.

2.7.2.2 Chromosomal DNA isolation

The isolation of high molecular DNA from a pure culture of bacteria was carried out with FastDNA SPIN Kit for soil (MP biomedicals, IIIkirch, France). The principle of this isolation method is DNA liberation via mechanical shearing by lysing matrix E containing a mixture of ceramic and silica particles, homogenization and protein solubilization, and at last DNA purification using a silica matrix. DNA was eluted with DNA elution solution provided by the manufacturer and stored at -20 °C for at least one year.

2.7.3 DNA purification

Purification and rebuffering of DNA after enzymatic manipulation, direct purification of PCR products, as well as purification of DNA from TAE agarose gels were carried out with NucleoSpin Extract II kit (Machery-Nagel). In this method DNA binds in the presence of chaotropic salt to a silica membrane. All of the required buffers and protocols were supplied by the manufacturer.

2.7.4 Quantification of DNA concentration

DNA concentrations were determined with NanoDrop ND-1000 (NanoDrop, Wilmington, USA). This spectrophotometer (UV and visible spectrum, 220-750 nm) is used for measuring absorbance of DNA, RNA, proteins and dyes. The absorbance of 2 µl DNA samples absorbance was measured at 230 nm, 260 nm and 280 nm; the absorbance values, concentrations and the ratios at 230/260 and 260/280 for evaluating purity of DNA were calculated by the software ND-1000 V3.1.

For measurement of oligonucleotide probes, the stock solutions were diluted 1:200 and 2 µl of the probes was measured at 495 nm for Fluos, 550 nm for Cy3 and 650 nm for Cy5, respectively. The probes were measured in three replications and the average concentration was used to dilute the stock solution.

2.7.5 Enzymatic DNA modifications

2.7.5.1 Digestion of DNA with restriction enzymes

The restriction enzymes used in this work were obtained from Fermentas (St. Leon-Rot, Germany) and New England BioLabs (Frankfurt am Main, Germany). The recognition sequence, reaction buffer and incubation conditions are supplied by the manufacturer.

For analysis, 200 ng DNA in a total volume of 10 µl with 2 to 5 U restriction endonuclease and the buffer were incubated at an enzyme specific temperature (mostly 37 °C) for 4-16 h. After digestion, the different sizes of DNA fragments were separated by gel electrophoresis (2.7.6).

For preparation of the restriction fragments for cloning, 1-5 µg DNA in a total volume of 20-50 µl with 5-10 U restriction endonuclease were incubated for 4-16 h. After digestion, the enzyme was either inactivated through heat or separated by gel electrophoresis (2.7.6).

2.7.5.2 Dephosphorylation of linear DNA

Phosphatase catalyzes the release of 5'- and 3'- phosphate groups from DNA and RNA to inhibit the re-ligation of linear DNA to vector DNA. The dephosphorylation reaction can be performed directly after the restriction digestion. For this, a total of 50 µl reaction mixture contained digestion samples, 5 µl 10x buffer and 1 U alkaline phosphatase (Fermentas). The reaction was performed at 37 °C for 30 min and finally the enzyme was deactivated at 85 °C for 15 min.

2.7.5.3 Ligation of DNA

T4 DNA ligase (Fermentas) catalyzes the formation of a phosphodiester bond between juxtaposed 5'- phosphate and 3'- hydroxyl termini. Therefore, 50-400 ng vector DNA and three times the molar amount of insert DNA were mixed. To this mixture, ligation buffer and 1-5 U ligase was added. The reaction was incubated at 22 °C overnight. The ligase was inactivated at 65 °C for 10 min.

2.7.6 Gel electrophoresis

Gel electrophoresis is a technique for separation of DNA molecules using an electric field. The migration speed of DNA from the negative to the positive pole in an agarose gel is related to the molecular weight of linear DNA. After electrophoresis, DNA can be made visible via ethidium bromide staining. The size of unknown fragments is determined by comparison to standard DNA (λ-standard) containing a mixture of fragments of known size. In this work, a 1 kb DNA ladder and a 100 bp DNA ladder from Fermentas were used as standard gene ruler.

Here the horizontal electrophoresis system of Peqlab (Erlangen, Germany) was used. 1x TAE buffer (made from 50x TAE buffer, AppliChem) was applied for the preparation of 1-2% gels. 0.5 µg/ml ethidium bromide (Roth, Karlsruhe, Germany) was directly added to the agarose gel. The DNA solution was mixed with 6x loading dye solution (Fermentas) and separated by electrophoresis at 80-120 mA. DNA bands were detected by UV light (λ=312 nm) in a trans-illuminator (Biostep, Johnsdorf, Germany) and documented with the Argus X1 documentation system (Biostep, Johnsdorf, Germany).

The DNA fragments could be excised from the agarose gel and purified for later cloning or ligation. The agarose pieces containing the desired DNA fragment were cut with an x-tracta (Biozym, Oldendorf, Germany) and eluted according to the description of 2.7.3.

2.7.7 Amplification of specific DNA fragments via PCR

2.7.7.1 PCR primer

The primers used in this work were synthesized by Sigma Genosys (Steinheim, Germany). The annealing temperature depends on the length and nucleotide composition of the primer. The annealing temperature was determined by the "2+4 rule" (Suggs *et al.*, 1981):

$$\mathbf{T_M\ (°C) = 2(A+T) + 4(G+C)}$$

Some of the primers were designed to have a recognition site of a restriction enzyme at the 5' end. In order to ensure a reliable recognition of the binding site by the restriction enzyme, three additional bases were added at 5' end. The sequences of the applied primers and the appropriate annealing temperatures are provided in Table 2.9.

The lyophilized primers were dissolved in nuclease free water (NFW), so that the concentration of the stock solution was 100 pmol/µl. The stock solution and the working solution of 50 pmol/µl were stored at -20 °C.

Table 2.9. PCR and sequencing primers

Name	Sequence (5'-3')	Fragments	T_M (ºC)
M13F	GTAAAACGACGGCCAG	insertion fragment in pCR2.1-TOPO vector	50
M13R	CAGGAAACAGCTATGAC		50
TcR-s	AAAGTCTACTCAGGTCGAGG	TcR (*tet* gene)	55
TcR-as3	AAAGTAGACGACGAAAGGC		55
MutL-s	AACTGTTCTTCTCCACCC	MutL (*mutL* gene of "*cand.* A. radicis" N35)	55
MutL-as	TCACGAAGGGTGCGGC		55
AHLsyn-s2	GCCAGCTTGTCATAGGACTC	AHL synthase (*araI* gene of "*cand.* A. radicis" N35)	55
AHLsyn-as2	ATGCACCTCCAGAAAACG		55
eYFP-for	CGCCCAATACGCAAACC	eYFP	50
eYFP-rev	GTTGGAATTCTAGAGTCG		50

2.7.7.2 Standard PCR

For amplification of DNA fragments the thermocycler Primus 96 (Ebersberg, Germany) was used. The standard PCR reaction volume was 50 µl. Reaction mixture using *Taq* DNA polymerase contained 1x Taq buffer (Fermentas), 200 µM of each dNTP (Fermentas), 0.3 mM $MgCl_2$ (Fermentas), 10-100 ng of template DNA and 2.5 U *Taq* DNA polymerase. Reaction mixture using top *taq* DNA polymerase contained 0.5 µM primer of each, 1x PCR buffer (Qiagen), 200 µM of each dNTP (Fermentas), 1x coralload (Qiagen), 100ng of template DNA and 1.0 U top *Taq* DNA polymerase.

Colony PCR was used for direct amplification from the sample without previous DNA extraction. For this, the tip of a toothpick was dipped in a bacterial plate culture and the adhering bacteria were dissolved in 100 µl PBS. 1µl cell suspension was used for PCR amplification and the reaction mixture was applied as described above.

The standard PCR cycle program consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles of 94 °C for 30 sec, annealing at 55 °C (or the optimal temperature according to the primer) for 30 sec and elongation at 72 °C for 45 sec. A final elongation at 72 °C for 10 min concluded the program. The elongation time depended on the length of the amplificate and ranged from 45 sec for fragments smaller than 1 kb to 2 min 30 sec for fragments larger than 3 kb.

2.7.7.3 Cloning of PCR amplicons

For the cloning of PCR amplicons, the TOPO TA Cloning kit (Invitrogen, Carlsbad, USA) with chemically competent cells was applied. The supplied linear cloning vector pCR2.1-TOPO carries a single 3'-T overhang and a covalently bound topoisomerase I. *Taq* polymerase has a non-template dependent terminal transferase activity that adds a single A to the 3' end of PCR products. This allows PCR inserts to ligate efficiently with linear vectors with 3'-T overhangs. The cloning procedure was carried out according to the manufacturer's hand book.

50-100 µl transformation suspension was spread on LB agar plate plus 50 mg/l kanamycin and 40 mg/l X-gal and incubated overnight at 37 °C. Blue/white screening allowed to selectively pick clones containing a vector with insert (white colonies). 10 white colonies were picked for preparation of plasmid DNA and restriction enzyme analysis.

2.7.8 DNA sequence analysis

2.7.8.1 DNA sequencing with ABI 3730 sequencer

Sequencing of purified PCR products or plasmid DNA was carried out using the BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, USA) with different sequencing primers and annealing temperatures (Table 2.9). Sequencing reactions for the ABI 3730 sequencer (Applied Biosystems) were prepared and purified by ethanol precipitation according to the manufacturer's instructions.

2.7.8.2 Genomic DNA sequencing with a 454 pyrosequencer

The whole genomes of "*cand.* A. radicis" N35 and N35v were sequenced using a 454 GS FLX pyrosequencer (Roche Diagnostics-Applied Science, Mannheim, Germany) with a GS FLX Titanium kit (Roche). The sequencing included three steps: DNA library preparation for generation of single stranded fragments with ligated adaptors, emulsion PCR (emPCR) to amplify single DNA fragments bound to beads, and the sequencing run using a special CCD camera to record the chemoluminescent signals which marks the incorporation of one or more nucleotides into the DNA.

At first, genomic DNA was isolated as described in 2.7.2.2 and the concentration was quantified (2.7.4). A special quality of DNA was needed for optimal results: A260/A280

should be ≥ 1.8, the concentration of DNA should be ≥ 300 ng/µl and the total volume of DNA should be ≥ 20 µl.

The procedure for DNA library preparation was carried out with the standard protocol provided by the manufacturer using a GS FLX Titanium library kit (Roche). The DNA library quality assessment and quantitation was determined by a RNA 6000 Pico Chip (Agilent Technologies, Böblingen, Germany). For a nebulized DNA library, the average fragment length of DNA should be between 500-800 bp, < 10% of DNA fragments were supposed to be below 350 bp or above 1000 bp and the total DNA yield should be ≥ 3 ng. 2 µl of the library was diluted to $1x10^8$ molecules/µl in TE buffer and the library was stored at -20 °C.

Next, a small volume emPCR was implemented for library titration. This step was carried out to determine the amount of library to be used in a large volume emPCR amplification. This small volume emPCR using a GS FLX Titanium emPCR kit consisted of 6 steps: DNA library capture, emulsification, amplification, bead recovery, bead enrichment and sequencing primer annealing. At last, the enrichment values of the library were determined with a Coulter Counter (Coulter Electronics, Luton, England). Enrichment values of 5-10% were indicative of libraries yielding good sequencing results.

Large volume emPCR was carried out to amplify single DNA fragments bound to a bead. The correct amount of library used here was determined by the small volume emPCR. The procedure consisted of the same steps as the small volume emPCR but a large volume GS FLX Titanium emPCR kit was used following the manufacturer's protocol. Also here it was important that the enrichment value of enriched beads was between 5-10% after sequencing primer annealing. These enriched beads with immobilized, clonally amplified DNA fragments from the used library could be stored at 4 °C and remained sequenceable for at least 1 month.

The DNA carrying beads were loaded into the wells of a PicoTiterPlate device (PTP device) with one single DNA bead per well. After inserting the loaded PTP device into the Genome Sequencer FLX Instrument, the sequencing run was started and sequencing reagents were sequentially flowed over the plate. The sequencing reactions were automatically performed and monitored in all the wells of the PTP device simultaneously.

2.7.8.3 Sequencing data analysis

The sequencing data obtained by ABI 3730 sequencer were analyzed with the Lasergene software version 7.1.0 (DNAstar, Madison, USA). The data resulting from the 454

pyrosequencer were assembled with the software GS FLX 2.0.01 (Roche). The 16S rRNA gene sequences were analyzed using the ARB software package (TU München, Germany). Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Olsen *et al.*, 1994) and maximum-parsimony (Felsenstein, 1981) algorithms.

"BLAST" (http://blast.ncbi.nlm.nih.gov/) was used to search for sequence homologies to known sequences (Altschul *et al.*, 1997). The program ORF Finder (open reading frame finder, http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify all open reading frames. The Swiss Institute of Bioinformatics (ExPASy, http://www.expasy.ch/tools/dna.html) provided the tool for the translation of DNA to a protein sequence.

2.7.9 DNA-DNA hybridization

Bacterial strains were incubated in pre-culture overnight and were microscopically checked for contaminations using FISH. When no contamination could be detected 250 ml NB medium was inoculated and after overnight cultivation at 30 °C about 3 g cell materials was harvested. The cells were washed twice with 1x PBS and suspended in 10 ml 50% 2-propanol. All other steps were accomplished by the DSMZ as described in protocols of Cashion *et al.* (1977), DeLey *et al.* (1970) and the modified protocol of Huss *et al.* (1983).

2.7.10 DNA transfer into Gram-negative bacteria

2.7.10.1 Electrocompetent cells after Hanahan (1983, modified)

1 l culture of the receptor strain (e.g. isolate N35) was incubated overnight till OD_{600} reached 0.5-0.7. All following steps were carried out on ice. The culture was cooled on ice for 15 min and centrifuged at 5000x g and 4 °C for 15 min. The cells were washed twice with cold ultra pure water and one time with 10% cold glycerol. At last, the cells were resuspended in 2 ml 10% cold glycerol and cells were aliquoted in small volumes e.g. 60 µl. The electrocompetent cells were stored at -80 °C.

2.7.10.2 Electroporation after Dower *et al.* (1988)

Plasmid DNA can be transferred into bacterial cells through a local perforation of the cell wall caused by an externally applied electrical field. High salt contents in DNA samples

(e.g. ligation products) were reduced by drop dialysis against ultra pure water (nitrocellulose membrane, pore size 0.025 µm, Millipore) for 30-60 min.
The electrocompetent cells were chilled on ice. 300-500 ng DNA solution was added to the cells and the mixture was incubated on ice for 30 min. The mixture was pipetted into a cold electroporation cuvette (electrode distance 2 mm, Peqlab, Erlangen, Germany). The electroporation was accomplished using a Gene Pulser (Bio-Rad, Munich, Germany) with a voltage of 2.5 kV for 4.5-5.5 msec. Immediately after electroporation, 1 ml SOC or NB medium was added to the bacterial suspension. The bacteria were incubated at optimal growth temperature for 1 h or more to allow the recovery of the cells and expression of antibiotic resistance markers. At last, the cells were spread on selective agar plates.

2.7.10.3 DNA transfer into E. coli via heat shock

Chemically competent *E. coli* Top 10 cells (Invitrogen, Carlsbad, USA) were used for heat shock transfer of DNA. The procedure was performed in principle using the same protocol as for electroporation. However, the perforation of the cell wall was carried out at 42 °C for 30 sec. Next, 250 µl of the SOC medium was added to the cells and the following steps were done as described above.

2.7.10.4 DNA transfer via conjugation after Kristensen *et al.* (1995, modified)

Plasmid DNA can also be transferred into cells via conjugation, in which plasmid types, characteristics of the donor as well as the recipient cells and reaction conditions affect the rate of conjugation (Stotzky *et al.*, 1990). *E. coli* HB101 pRK600 (Figurski and Helinski, 1979) was used as helper strain for transferring plasmid DNA from the donor strain into the recipient strain. Figure 2.2 schematically presents the conjugation procedure of bacteria.

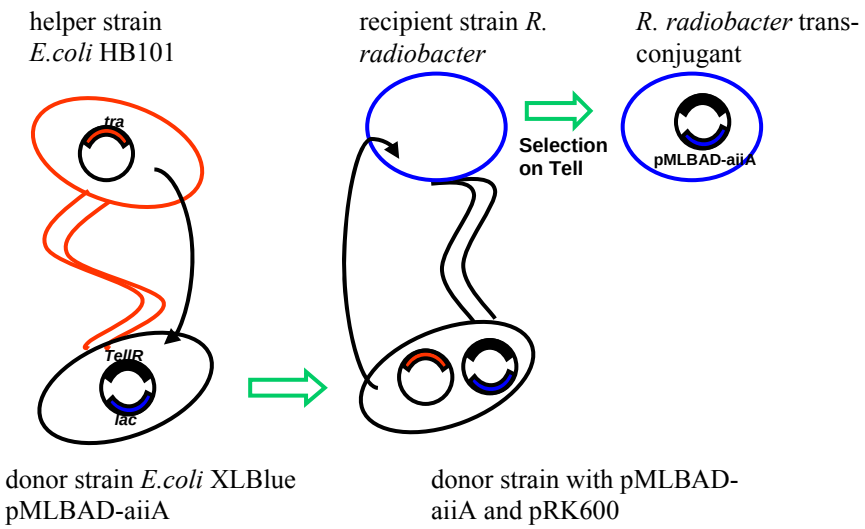


Fig. 2.2. Conjugational transfer via triparental mating. As example the transfer of plasmid pMLBAD-aiiA into *R. radiobacter* was selected. *tra*: transfer gene; *Tell*^{R}: tellurite resistance gene; *lac*: lactonase gene

In this work the conjugational transfer was used for transferring plasmid DNA into *R. radiobacter*. The conjugational transfer was carried out on agar plates. Donor, recipient and helper strains were incubated in appropriate medium overnight. 1 ml culture of donor and helper cells were centrifuged at 5000x g for 3 min at RT. The cells were resuspended in NB medium and washed twice with 1 ml NB medium. Next, the cells were resuspended in 250 µl NB medium. Donor cells were pipetted to helper cells and the mixture was incubated for 30 min at RT. 5 ml recipient cells were centrifuged at 5000x g for 3 min at RT and washed twice with 1 ml NB, if incubated in selective medium. Then the cells were resuspended in 250 µl NB medium. The recipient cells (250 µl) were added to the mixture of donor/helper cells (500 µl). From this mixture, 15 drops (50 µl each) were dripped on a NB agar plate and incubated at 30 °C for 4 h. The cells were then resuspended with 2 ml 1x PBS. 100 µl of this suspension were spread on a selective agar plate and incubated for 48 h at 30 °C.

2.7.11 Plasmid constructions

First of all, plasmid construction was carefully planned with the help of the program Clone Manager Version 5.02 (Scientific & Education software, Durham, USA). With this software it was possible to perform restriction and ligation analyses with known sequences,

as well as to display all possible recognition sites of various restriction enzymes. According to 2.7.2.1 plasmid DNA was isolated and digested with restriction enzymes (2.7.5.1). The desired fragment was obtained through separation, excision and purification of the DNA fragment in agarose gel (2.7.6). The obtained vector and insert DNA were ligated according to the description in 2.7.5.3 and the ligated product was transferred into competent cells via electroporation (2.7.10.2) or heat shock (2.7.10.3). After plating on selective agar plates, resulting clone colonies were picked, grown in selective liquid medium and subjected to a plasmid isolation (2.7.2.1). This insert DNA was controlled by restriction analysis.

2.7.12 GFP- and YFP-labeling of isolate N35

2.7.12.1 GFP labeling of N35 and N35v by transposon tagging

The mini-Tn5 derivative located on a pUT vector (pJBA28) was used for chromosomal GFP-labeling of "*cand.* A. radicis" N35 and N35v cells. This transposon cassette includes a selective marker for kanamycin and a constitutively expressed GFP reporter gene. The pUT vector bases on the plasmid R6K and exhibits a *pir* dependent replication origin, therefore the plasmid could not replicate after transfer into strain N35.

The plasmid pJBA28 was isolated and transferred into electrocompetent cells of N35 via electroporation (2.7.10.2). After transposition of the mini-Tn5 transposon chromosomally GFP labeled bacteria were selected on kanamycin selective medium and their GFP fluorescence was verified with a binocular providing wavelength specific excitation at 488 nm (Zeiss, Lumar V12).

2.7.12.2 YFP labeling of N35 and N35v by stable plasmid transformation

The broad host range vector pBBR1MCS-2 was used for this purpose, as it is known to be stable in a large number of hosts even without antibiotic pressure. PCR amplified eYFP was cloned into this vector and the resulting construct pBBR1MCS-2-eYFP was transferred into electrocompetent cells of "*cand.* A. radicis" N35 and N35v via electroporation. The YFP-labeled bacteria were selected on kanamycin selective medium and cells were inspected for YFP specific fluorescence via the λ- mode of the CLSM (2.4.2).

2.7.13 SYTO staining of bacterial cells

SYTO orange 81 (Molecular Probes, Leiden, Netherlands) which has a maximum emission wavelength of 544 nm, is a cell-permeant nucleic acid stain and can stain most live and permeabilized bacteria. The SYTO orange 81 staining bacterial cells could be differentiated from the GFP-labeled bacterial cells by different emission wavelength using λ-mode of CLSM (2.4.2). Hereby, the plant roots co-inoculated with GFP-labeled bacterial cells and non-labeled bacterial cells (2.3.1) and grown in the monoxenic system were harvested and washed twice in 1x TE buffer. The roots were incubated in SYTO orange 81 (1 µM solution) for 5 min and subsequently washed twice in 1x TE buffer. The samples were prepared as described in 2.3.3 and observed with the CLSM

2.7.14 Knock-out mutagenesis via a gene replacement vector

For knock-out mutagenesis in "*cand.* A. radicis" N35, the *sacB* based gene replacement vector pEX18Gm described by Hoang *et al.* 1998 was employed.
At first, a constructed DNA cassette, which carried an antibiotic marker, was cloned into the gene replacement vector pEX18Gm (2.7.11). Subsequently, this constructed gene replacement plasmid was transferred into electrocompetent "*cand.* A. radicis" N35 cells via electroporation. In the target cell a homologous recombination event occurred after pairing of the constructed DNA cassette with the homologous region in the genome of "*cand.* A. radicis" N35, which led to an insertion of the whole constructed pEX18 plasmid into the genome of N35. The bacteria with integrated plasmid were selected on NB medium containing antibiotics. Merodiploids were resolved by plating on NB medium containing 5% sucrose, which led to cell death when the *sacB* gene was expressed. Only cells where the *sacB* gene together with the gentamycin selective marker was removed from the genome of N35 by a second homologous recombination could survive on sucrose containing medium. The resulting mutants carried the mutant allele instead of the original target gene.

2.8 Statistics

The harvested root and shoot samples from the green house were at first analyzed with the table calculation program Microsoft Office Excel 2003 (Microsoft, Remond, WA, USA).

Statistic analysis for determination of significance and assessment of difference was accomplished with sigma plot 10.0 (Systat, Erkrath, Germany). A standard t test was implemented. This t-test is used to assess whether the means of two groups are statistically different from each other.

3 Results

3.1 Characterization of *Acidovorax* sp. N35

3.1.1 Growth conditions and phenotypic characterization of isolate N35 and its phenotype variant N35v

Isolate N35 grew at 10-35ºC, 0-2% NaCl and pH 5.0-9.0. The optimal growth condition for strain N35 was at 30ºC, pH 7.0 without NaCl.

Surprisingly, phenotypic variation was observed in strain N35 when grown on NB plates; two different colony types could be observed. One type showed characteristic rough colonies on agar plates and formed flocks in liquid medium, while the other type grew in smooth colonies and without flocculation in liquid medium (Fig. 3.1 a and b). The rough colony type could switch to the smooth type at a rate of $3.2 \cdot 10^{-3}$ per cell per generation on NB solid medium. This was calculated from $2 \cdot 10^{6}$ switched colonies in $3.8 \cdot 10^{7}$ total colonies at 0 h and $7.2 \cdot 10^{7}$ switched colonies in $1.1 \cdot 10^{9}$ total colonies at 16 h using the formulas in 2.6.2. There was no reversion detectable back from the smooth type to the rough type. Because of these observations, strain N35 was considered to perform phenotypic variation, with the rough colony type being the wild type and the smooth colony type being a phenotypic variation thereof, named N35v. In addition, strain N35 showed swarming on soft agar plate; however strain N35v could not swarm (Fig. 3.1 c).

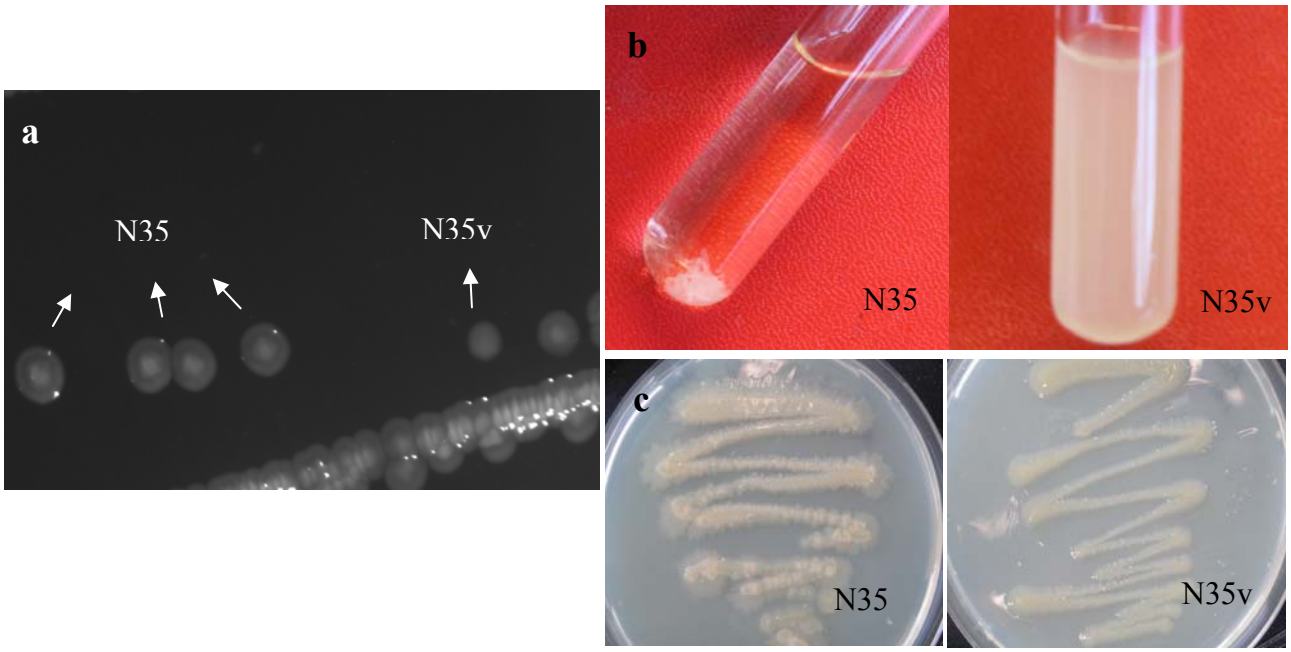


Fig. 3.1. Phenotypic comparison between wild type N35 and variant N35v. (a) Wild type N35 (rough colonies) could switch to phenotype variant N35v (smooth colony) on NB agar plate; small white spots: light reflections. (b) Growth distinction between wild type N35 and variant N35v in NB liquid medium. (c) Swarming motility of both types of N35 and N35v on NB semisolid agar plate.

The cells of both types were observed by light microscopy (2.4.1) and scanning electron microscopy (SEM, 2.4.3). Cells of strain N35 are motile, straight rods with the length of 1.5-2 µm and a diameter of 0.3-0.5 µm. SEM images revealed that wild type cells were often arranged in clusters with filaments (extracellular microfibrils, Fig. 3.2 c), whereas phenotype variant cells were found to be separate from each other. In contrast to the cells of the wild type N35 where one polar flagella and rapid movement through 1x PBS could be observed, variant type N35v cells apparently lost their flagella (Fig. 3.2 a and b) and were not able to swim.

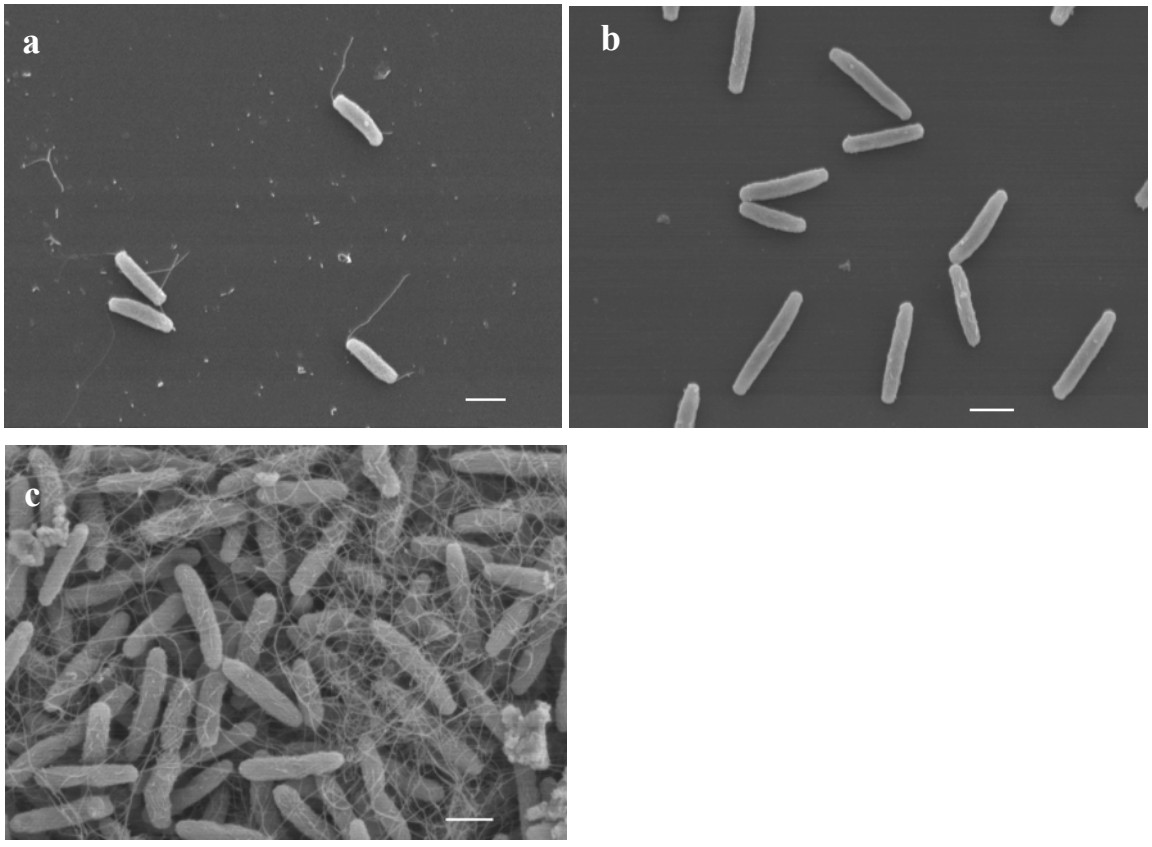


Fig. 3.2. Scanning electron micrographs of strain N35 and phenotype variant N35v. In a, one polar flagella could be seen for strain N35; while in b, the phenotype variant N35v had no flagella. In c, N35 cells occurred in cluster. Bars, 1 µm.

3.1.2 Metabolism of various substrates

A halo surrounding the colony of both phenotypes could be observed on glyceryl tributyrate agar plates, indicating that both bacterial strains possess lipase activity. An orange halo was observed surrounding colonies of both types on CAS agar indicating the release of siderophores and ability for iron scavenging. There was no protease activity observed by both phenotypes. The following sugars, alcohols as well as fatty acids and amino acids could be metabolized by strain N35: Tween 40, Tween 80, L-arabinose, L-arabitol, D-frucose, L-fucose, D-galactose, α-D-glucose, D-mannitol, D-mannose, D-psicose, D-sorbitol, methyl pyruvate, mono-methyl-succinate, acetic acid, formic acid, D-gluconic acid, α- hydroxy butyric acid, β-hydroxy butyric acid, p-hydroxy phenylacetic acid, α-keto butyric acid, D,L-lactic acid, propionic acid, quinic acid, sebacic acid, succinic acid, bromo succinic acid, succinamic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxyl-L-proline, L-leucine, L-phenylalanine, L-proline, L-threonine, γ-amino butyric acid, urocanic acid and glycerol. The only difference between the wild type and variant type was that N35 could readily metabolize L-fucose and formic acid, whereas N35v lost the ability to utilize these two substrates. However, both types could clearly be distinguished from the other phylogenetically closely related type strains of *Acidovorax* species by the utilization of various substrates; comparison of N35/N35v to the other three species *A. defluvii*, *A facilis* and *A. delafieldii* with 35, 23 and 7 differences, respectively (Table 3.1).

Table 3.1. Utilization of different carbon sources in a Biolog GN2 microplate by strain N35 as well as phenotype variant N35v and pylogenetically closely related species.
Strains: 1, N35 (wild type); 2, N35v (phenotype variant); 3, *Acidovorax defluvii* DSM 12578; 4, *Acidovorax facilis* DSM 649^T; 5, *Acidovorax delafieldii* DSM 64^T. +, positive; –, negative; w, weakly positive. All strains were positive for utilization of Tween 40, Tween 80, L-arabinose, D-galactose, D-mannitol, D-mannose, methyl-pyruvate, mono-methyl-succinate, β-hydroxy butyric acid, α-keto butyric acid, D,L-lactic acid, sebacic acid, succinamic acid, L-alanine, L-proline, glycerol. All strains were negative for water (negative control), α-cyclodextrin, glycogen, N-acetyl-D-galactosamine, adonitol, D-cellobiose, i-erythritol, gentiobiose, m-inositol, α-D-lactose, lactulose, maltose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, D-trehalose, xylithol, citric acid, D-galactonic acid lactone, D-glucosaminic acid, D-glucoronic acid, itaconic

acid, D-saccharic acid, glucuronamide, D-serine, inosine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, glucose-1-phosphate.

Carbon source	1	2	3	4	5
Dextrin	–	–	+	–	–
N-Acetyl-D-glucosamine	–	–	–	+	–
D-Arabitol	+	+	–	+	+
D-Fructose	+	+	–	+	+
L-Fucose	+	–	–	–	–
α-D-Glucose	+	+	–	+	+
D-Psicose	+	+	–	+	+
D-Sorbitol	+	+	–	+	+
Sucrose	–	–	+	–	–
Turanose	–	–	+	–	–
Acetic acid	+	+	–	–	+
Cis-aconitic acid	–	–	–	–	+
Formic acid	+	–	–	–	+
D-Galacturonic acid	–	–	+	–	–
D-Gluconic acid	+	+	–	–	+
α-Hydroxy butyric acid	+	+	+	–	+
γ-Hydroxy butyric acid	–	–	+	+	–
p-Hydroxy phenylacetic acid	+	+	–	–	+
α-Keto glutaric acid	–	–	+	w	w
α-Keto valeric acid	–	–	+	+	–
Malonic acid	–	–	–	w	–
Propionic acid	+	+	–	–	+
Quinic acid	+	+	–	–	+
Succinic acid	+	+	–	w	+
Bromo succinic acid	+	+	-	+	+
L-Alaninamide	–	–	+	–	w
D-Alanine	+	+	–	w	w
L-Alanyl-glycine	w	w	–	+	+
L-Asparagine	+	+	w	–	+

L-Aspartic acid	+	+	w	–	+
L-Glutamic acid	+	+	+	–	+
Glycyl-L-aspartic acid	–	–	–	+	–
Glycyl-L-glutamic acid	+	+	–	–	+
L-Histidine	+	+	–	–	+
Hydroxy-L-proline	+	+	–	–	+
L-Leucine	+	+	–	w	+
L-Ornithine	–	–	+	–	–
L-Phenylalanine	+	+	–	+	+
L-Pyroglutamic acid	–	–	–	+	+
L-Serine	–	–	+	+	+
L-Threonine	+	+	–	–	+
D,L-Carnitine	–	–	–	+	–
γ-Amino butyric acid	+	+	–	+	+
Urocanic acid	+	+	–	–	+
2,3-Butanediol	–	–	+	–	–
D,L-α-Glycerol phosphate	–	–	w	–	w
Glucose-6-phosphate	–	–	–	–	+

3.1.3 Phylogenetic characterization of isolate N35

3.1.3.1 Genome sequencing

The whole genomes of strains N35 and N35v were sequenced with a 454 pyrosequencer (Roche) using the GS FLX Titanium chemistry and the software package GS FLX 2.0.01 (Roche) for the assembly (2.7.8.2). After sequencing, 31 contigs for N35 and 41 contigs for N35v were obtained from the assembly and the average reading coverage for both types are 40 times. The genome size of both types predicted by the software was the same, nearly 5.5 Mb.

3.1.3.2 16S rRNA gene sequence

For determination of G+C ratio, a DNA statistics calculation function of the software Lasergene 7.1.0 (DNAstar) was carried out using the whole genome sequence data. The

G+C ratio of genomic DNA of strain N35 was 64.9 mol%. 16S rRNA gene sequences were also taken from this genomic data and analyzed using the ARB software package version 5.1 (Ludwig *et al.*, 2004; Strunk and Ludwig, 1997). The phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Olsen *et al.*, 1994) and maximum-parsimony (Felsenstein, 1981) algorithms.

The nucleotide sequence of the 16S rRNA gene of N35 and N35v were 100% identical and showed 97.2-99.0% similarities to the other environmental *Acidovorax* species (Fig. 3.3). The closest phylogenetic neighbor of strain N35 was the type strain *A. defluvii* DSM 12644^T with a sequence similarity of 99.0%; other close relatives are *A. facilis* DSM 649^T (98.5%), *A. delafieldii* DSM 64^T (98.2%), *A. temperans* DSM 7270^T (98.1%), *A. soli* BL21^T (97.6%) and *A. caeni* R-24608^T (97.2%). Ludwig *et al.* (1998) considered that a value of 16S rRNA gene sequence similarity of 95% or more indicated the same genus, these results provided clear evidence that the isolate N35 belongs to the genus *Acidovorax*.

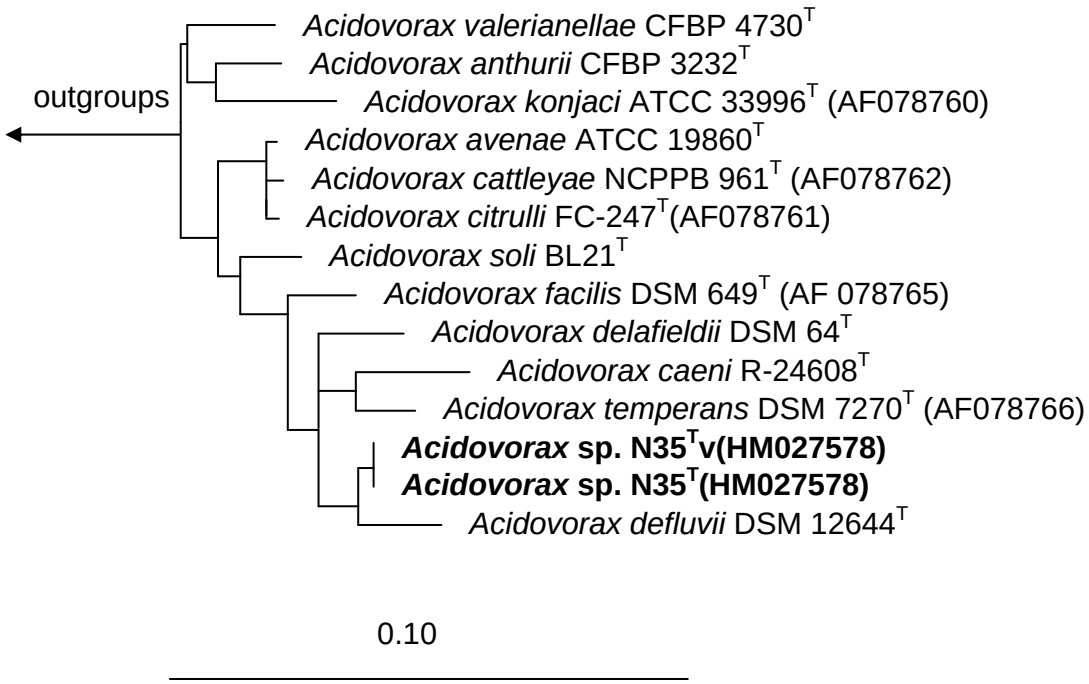


Fig. 3.3. Phylogenetic tree calculated using the neighbor-joining, maximum-likelihood and maximum-parsimony method based on 16S rRNA gene sequence data. NCBI accession numbers for the sequence data were given in parentheses. The tree included all validly described members of the genus *Acidovorax*. Bar, 10% sequence divergence.

3.1.3.3 DNA-DNA hybridization

DNA-DNA hybridizations were performed to analyze DNA-DNA relatedness between strain N35 and the three phylogenetically most closely related type strains *A. defluvii*, *A. facilis* and *A. delafieldii* (2.7.9).

DNA-DNA hybridization value between N35 and N35v was 100% similarity. According to the 16S rRNA gene sequence data and DNA-DNA hybridization, both types were genetically very closely related. These results confirmed that N35v was not an independent different strain (contaminant), but instead a phenotypic variation of the wild type N35.

The values for DNA-DNA hybridization of strain N35 were found to be 25.3% similarity with *A. defluvii* DSM 12644^T, 40.3% similarity with *A. delafieldii* DSM 64^T and 55.7 % similarity with *A. facilis* DSM 649^T. According to the accepted criterion for the species definition by Wayne *et al*. (1987), similarity values of over 70% in DNA-DNA hybridizations are characteristic for members of the same species. Therefore, the isolate N35 belongs to none of the compared three species.

3.1.4 Fatty acids analysis

The fatty acids of wild type N35 and phenotype variant N35v as well as the phylogenetically closely related strains were determined (2.6.2).

The dominant fatty acids detected in strain N35 were $C_{16:0}$ (51 mol%), $C_{16:1\omega7cis/trans}$ (37 mol%) and $C_{18:1\omega7cis/trans}$ (9.5 mol%) (Table 2); N35v has identical fatty acids profiles as wild type N35. All of the other fatty acids detected were present at <2 mol%, which is summarized in table 3.2. We could not resolve the $C_{16:1\omega7cis}$ and $C_{16:1\omega7trans}$ as well as $C_{18:1\omega7cis}$ and $C_{18:1\omega7trans}$. However, comparison of the fatty acid data of genus *Acidovorax* by Willems and Gillis (2005) and our data suggested $C_{16:1\omega7cis}$ and $C_{18:1\omega7cis}$ as the dominant fatty acids. Strain N35 was found to have different fatty acid profiles from the three phylogenetically most closely related species. Cyclopropane-substituted methylene-hexadecanoic acid ($C_{17:0cyclo}$) was not detected in strain N35 and other differences were found in $C_{16:0}$, $C_{16:1\omega7cis/trans}$, and $C_{18:0cyclo}$ and $C_{19:0cyclo}$.

Table 3.2. Fatty acid composition of strain N35 and phylogenetically closely related species. Strains: 1, wild type N35; 2, phenotype variant N35v; 3, *Acidovorax defluvii* DSM 12644^T; 4, *Acidovorax facilis* DSM 649^T; 5, *Acidovorax delafieldii* DSM 64^T. All data were obtained in this

study. Mean values of three measurements, ± range of values are given in mol%. ND, not detected; Tr, trace component (less than 0.1 %).

Fatty acid	1	2	3	4	5
14:0	0.8±0.1	1.2±0.4	0.4±0.0	1.0±0.1	0.5±0.1
14:1ω5*cis*	ND	ND	ND	Tr	0.6±0.0
14:0 3OH	ND	0.6±0.1	ND	Tr	ND
15:0	ND	1.7±0.1	7.9±0.1	2.1±0.1	ND
15:0 α	0.6±0.2	0.1±0.0	0.4±0.0	ND	ND
15:0iso	0.5±0.0	ND	0.1±0.0	ND	ND
15:1ω5*cis*	ND	0.3±0.0	ND	0.5±0.0	ND
15:1ω6*cis*	ND	ND	1.9±0.1	ND	0.2±0.0
16:0	50.7±2.8	49.0±2.9	36.2±0.6	34.5±1.5	24.0±1.4
16:1ω7*cis/trans*[1]	36.8±1.3	35.9±1.7	38.7±0.5	5.7±0.4	26.0±0.6
17:0	0.4±0.0	0.5±0.0	3.9±0.1	1.2±0.1	0.4±0.0
17:0iso	ND	0.1±0.0	0.2±0.0	Tr	ND
17:0bra	ND	ND	ND	Tr	ND
17:0cyclo	ND	ND	0.6±0.1	37.9±0.4	0.5±0.0
17:1ω6*cis*	ND	ND	0.3±0.1	Tr	ND
17:1ω7*cis*	0.2±0.1	0.7±0.0	0.4±0.1	ND	ND
17:1ω8*cis*	ND	ND	0.1±0.0	ND	ND
18:0	0.4±0.0	1.2±0.1	0.3±0.0	2.5±0.2	0.2±0.0
18:0cyclo	ND	ND	ND	ND	34.7±0.5
18:1ω7*cis/trans*[2]	9.5±1.5	8.9±1.0	8.6±0.3	7.7±0.5	12.8±0.2
19:0cyclo	ND	ND	ND	5.9±0.9	ND

1 16:1ω7*cis/trans* contains 16:1ω7*cis* and 16:1ω7*trans*.

2 18:1ω7*cis/trans* contains 18:1ω7*cis* and 18:1ω7*trans*.

Thus, according to the 16S rRNA gene sequence data and DNA-DNA relatedness analysis as well as substrate use profiles and cell wall fatty acid profiles, the strain N35 is classified as representative of a new species within the genus *Acidovorax,* and the name *Acidovorax radicis* sp. nov. is suggested (manuscript in version).

3.2 Plant growth promoting effect of "*cand.* A. radicis" N35

To assess the effect of isolated bacteria "*cand.* A. radicis" N35 and N35v on barley, roots of 3-day-old, no-sterile barley seedlings were inoculated with a suspension of N35 or N35v (10^8 cells/ml) for 1 h (2.2.2). The plants were harvested after 16 weeks growth in agricultural soil in a green house and subsequently, the dry weight of roots and shoots was determined (2.3.2). Hereby, barley plants inoculated with N35 or N35v or non-inoculated controls from eight different pots with three barley plants per pot, respectively, were harvested. 24 plants of each treatment were analyzing the plant stimulating effect of "*cand.* A. radicis" N35 and N35v on barley.

After 16 weeks, root dry weight of wild type N35 inoculated plants was significantly ($p < 0.05$) increased relative to control plants. Similar comparison for root dry weight of phenotype variant N35v inoculated plants showed also some increase, but the difference was not statistically significant. Plants treated with N35 showed an increase in root dry weight of 40% over non-inoculated control plants, demonstrating the significant growth promoting effect of this bacterium. Increases of N35v inoculated root dry weight was over 13% in comparison with non-inoculated control (Fig. 3.4).

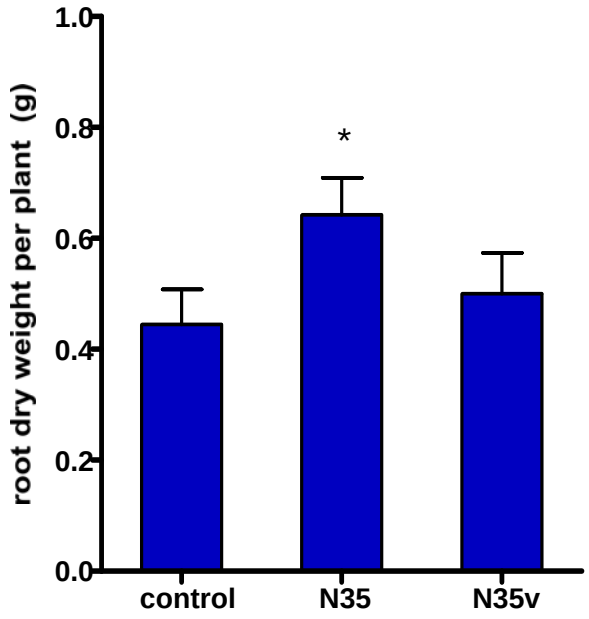


Fig. 3.4. Root dry weight per plant of barley after inoculation with "*cand.* A. radicis" N35 and N35v as well as non-inoculated control. Values were means of 24 plants from eight different pots. Error bars, standard error. * indicated statistically significant differences between roots of inoculated and non-inoculated plants (t-test, $p < 0.05$).

The shoot dry weight also increased significantly ($p < 0.05$) when barley was inoculated with wild type N35, showing 20% more biomass in comparison with non-inoculated control. The shoot dry weight showed a slight increase by inoculation of phenotype variant N35v in comparison with non-inoculated plants (Fig. 3.5).

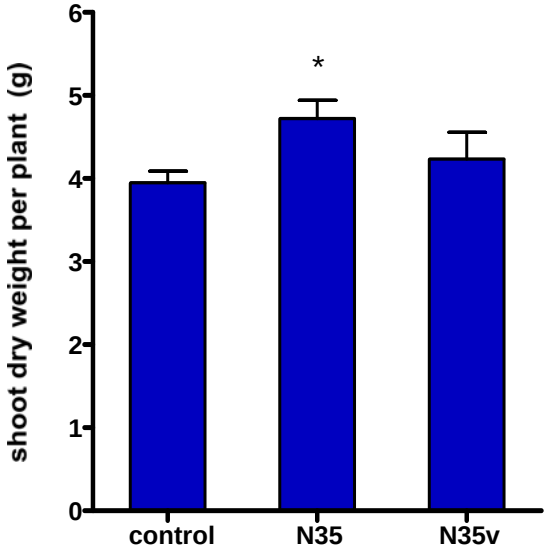


Fig. 3.5. Shoot dry weight per plant of barley after inoculation with "*cand.* A. radicis" N35 and N35v as well as non-inoculated control. Values were means of 24 plants from eight different pots. Error bars, standard error. * indicated statistically significant differences between shoots of inoculated and non-inoculated plants (t-test, $p < 0.05$).

Thus, the significant plant growth promoting effect on barley was observed by wild type strain N35 both in roots as well as in shoots. In comparison to the wild type N35, the phenotype variant N35v had no significant plant growth promoting effect on barley.

3.3 Colonization behavior of "*cand.* A. radicis" N35 and N35v on barley roots

The localization of "*cand.* A. radicis" N35 and N35v on barley roots was examined both in monoxenic system and soil system. Next, to determine whether the colonization behavior of "*cand.* A. radicis" N35 was associated to its ability of phenotypic variation, co-colonization of both phenotype cells on barley roots was investigated in a monoxenic

system. To this aim, cells of wild type N35 and phenotype variant N35v were labeled with GFP and YFP and their colonization behavior was examined on barley roots using CLSM.

3.3.1 Labeling of "*cand.* A. radicis" N35 and N35v cells with GFP

pJBA28, a carrier plasmid for mini-Tn5 transponson carrying a stable mut3 variant of the *gfp* gene and a selective marker for kanamycin, was used for chromosomal GFP labeling of "*cand.* A. radicis" N35 and N35v cells. The resulting chromosomally GFP-labeled N35 and N35v transformants were selected on kanamycin containing medium and by binocular Lumar V12 (2.7.12.1).

Kanamycin resistant colonies of both types showed green fluorescence when exposed to light with the excitation wavelength of 488 nm. The single cells of both types were also observed using the λ-mode of the CLSM (2.4.2), GFP-labeled cells showed a maximum emission wavelength of 510 nm (Fig. 3.7 a and b).

3.3.2 "*Cand.* A. radicis" N35 and N35v cells labeled with YFP

As for YFP no satisfactory fluorescence could be obtained with the chromosomal labeling, a plasmid based labeling method had to be applied. For this, the broad host range vector pBBR1MCS-2 was used to construct an YFP expressing vector, as it is known to be stable in a large number of hosts even without antibiotic pressure.

First, the expression vector of pEYFP with enhanced yellow fluorescent protein (Clontech, CA, USA) was isolated. The eYFP fragment was amplified with primer pair eYFP-for and eYFP-rev and cloned into a pCR2.1-TOPO vector. The resulting plasmid pTOPO-eYFP was digested with *EcoR*I and eYFP fragment was gel purified. To obtain pBBR1MCS-2-eYFP, pBBR1MCS-2 was digested with *EcoR*I and dephosphorylated. Subsequently, the eYFP fragment was ligated to the *EcoR*I fragment of pBBR1MCS-2 vector (Fig. 3.6).

Next, YFP expression vector pBBR1MCS-2-eYFP was transferred into both phenotypes of N35 via electroporation (2.7.12.2). Colonies with Km^R marker were selected. The expression of YFP and also the intensity of its fluorescence were analyzed using one-week-old pure cultures. YFP-labeled single cells of both types showed a maximum emission wavelength of 530 nm by CLSM. (Fig. 3.7 a and c). Therefore, the YFP- and/or

GFP-labeled "*cand.* A. radicis" N35 and N35v cells were detectable and differentiable using the λ-mode of the CLSM.

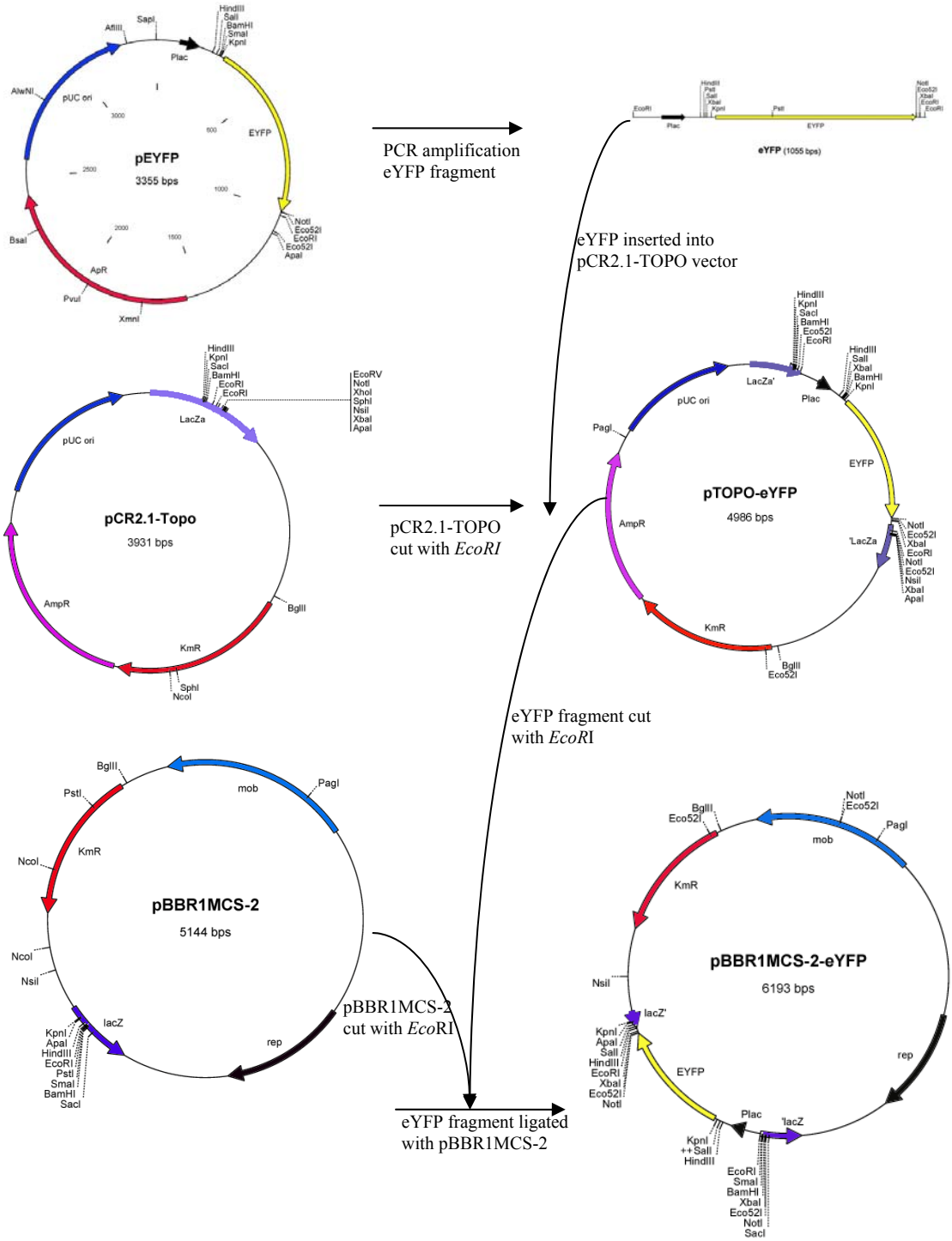


Fig. 3.6. Construction of pBBR1MCS-2-eYFP vector. The location of genes, their transcriptional orientations and only selected restriction enzyme cleavage sites are shown. *KmR*, kanamycin resistance gene; *AmpR*, ampicillin resistance gene; pUC ori, pUC plasmid replication origin; P*lac*, *lac* promoter; *EYFP*, enhanced yellow fluorescent protein gene; *lacZα*, β-galactosidase gene; *lacZ`*

and `*lacZ*, part of inactive *lacZ* gene; *mob*, gene for plasmid mobilization; *rep*, gene for plasmid replication.

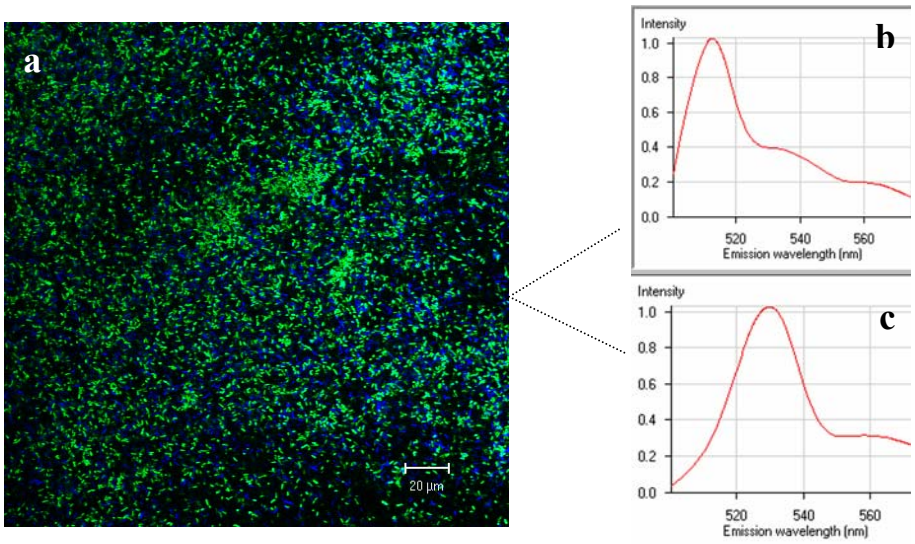


Fig. 3.7. (a) CLSM image (λ-mode) of a mixture of YFP-labeled N35 cells (blue) and GFP-labeled N35v cells (green) from one-week-old agar plate. (b) GFP-labeled bacterial cells showed a maxmium emission wavelength of 510 nm and (c) YFP-labeled bacterial cells showed a maximum emission wavelength of 530 nm.

3.3.3 Colonization of GFP-labeled "*cand.* A. radicis" N35 and N35v on barley roots

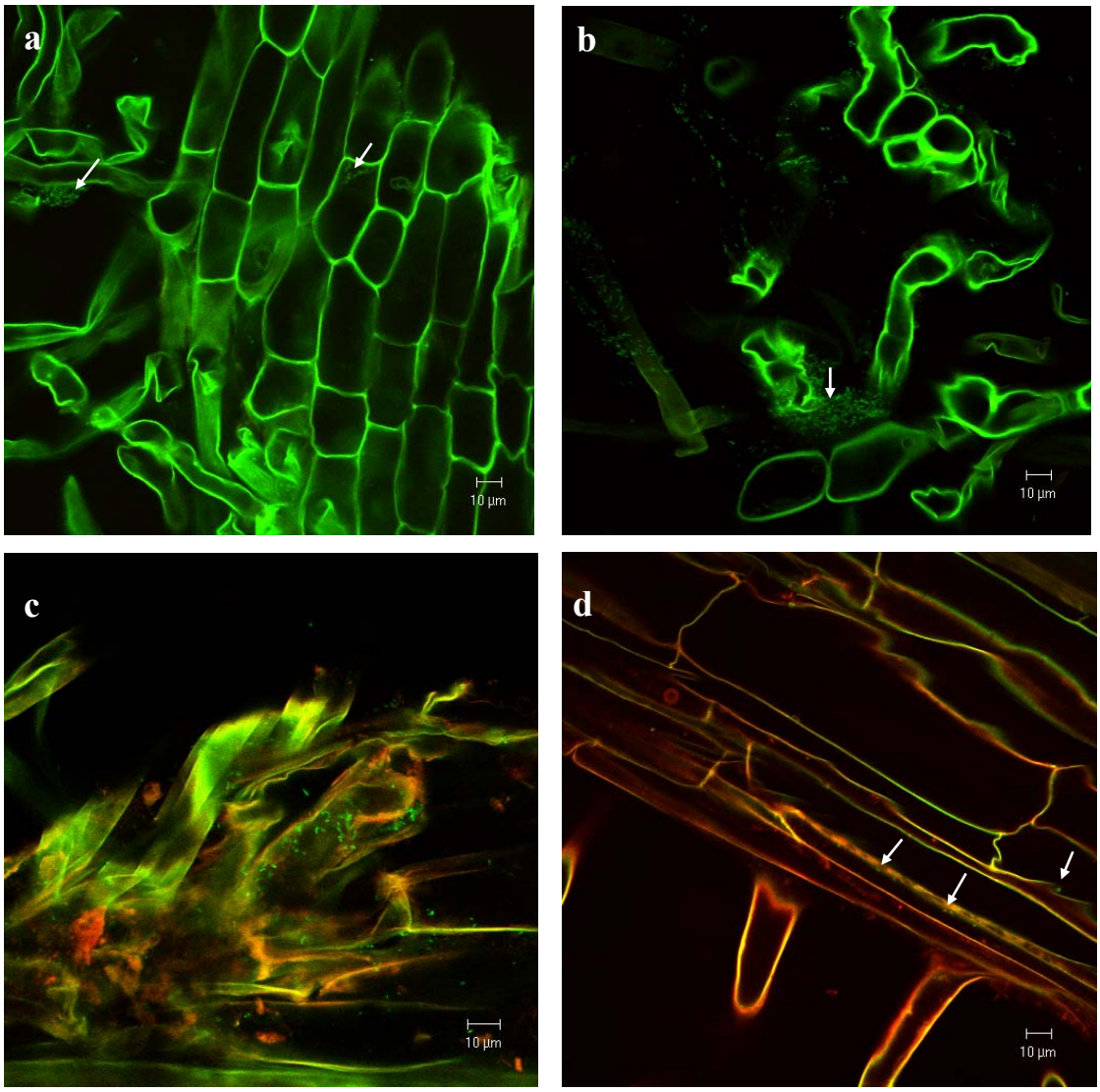
For the colonization assay, barley roots were inoculated with GFP-labeled N35 and N35v cells. For observation, root samples were taken after 3 weeks in monoxenic system, or 2 weeks and 12 weeks in soil system, respectively.

In the monoxenic system, the basal part of barley roots was colonized by both types 3 weeks after inoculation. Both types could colonize the surface of main roots and root hairs. N35 and N35v cells colonized the surface of root hairs in large numbers, forming biofilm-like aggregates (Fig. 3.8 a and b). No differences in colonization behavior were found between wild type N35 and phenotype variant N35v on barley roots in the monoxenic system.

In the soil system, both types were also observed primarily in the basal part of the roots 2 weeks after inoculation. The bacterial cells could colonize the surface of main roots and root hairs of barley (Fig. 3.8 c and d). After 12 weeks grown in agricultural soil, both types were observed mostly in the middle part of barley roots. A different colonization pattern

was observed after 12 weeks inoculation in the soil system, where both types proliferated inside barley cells and occurred in aggregates (Fig. 3.8 e and f).

Thus, both types of N35 could endophytically colonize barley roots 12 weeks after inoculation in the soil system, and no apparent differences in their colonization behavior were found between wild type N35 and the phenotype variant N35v on barley roots both in monoxenic and soil system.



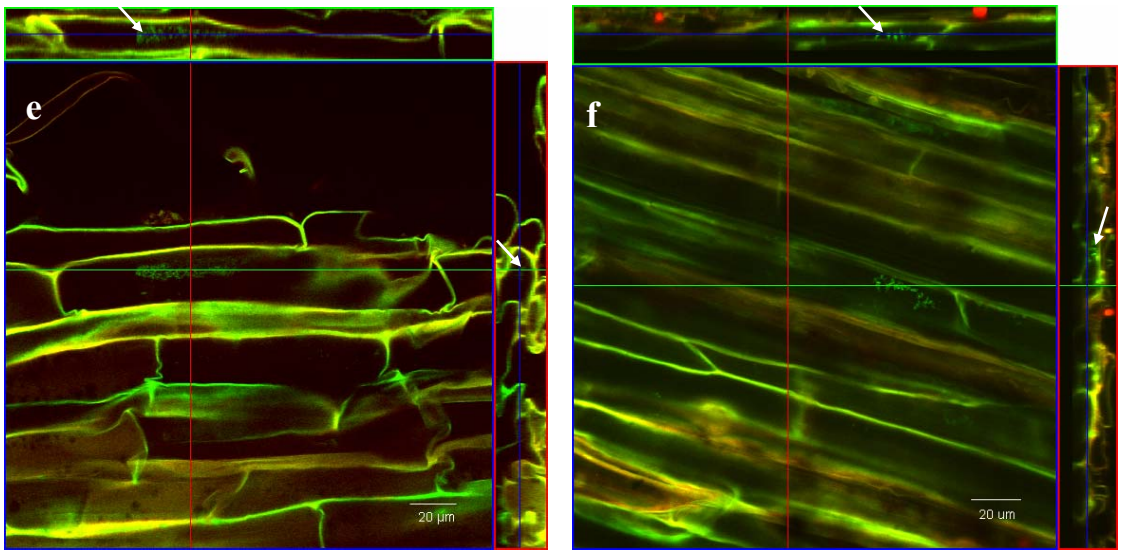


Fig. 3.8. CLSM image showing the localization of GFP-labeled "*cand.* A. radicis" N35 and N35v on barley roots in monoxenic and soil system. GFP-labeled bacteria are shown in green.
3-week-old basal root part after inoculation of GFP-labeled "*cand.* A. radicis" N35 (a) and GFP-labeled "*cand.* A. radicis" N35v (b) in the monoxenic system. Both GFP-labeled N35 and N35v cells colonize barley roots forming biofilm-like aggregates (white arrows).
(c) Basal root part after inoculation of GFP-labeled "*cand.* A. radicis" N35 after 2 weeks in soil system. Rod-shaped, GFP-labeled N35 cells colonize root hairs. (d) Basal part of roots after inoculation with GFP-labeled "*cand.* A. radicis" N35v after 2 weeks in soil system. Several bacterial cells are observed on the surface of roots (white arrows).
Middle part of roots after inoculation of GFP-labeled "*cand.* A. radicis" N35 (e) and GFP-labeled "*cand.* A. radicis" N35v (f) after 12 weeks in soil system. Orthogonal view of a 3D confocal image created from a z-stack of xy-scans. The top view, framed in blue, shows one picture from the middle of this z-stack. The red and green lines represent vertical optical cuts through the stack, which results in the side view images framed in red and green, respectively. In these side views the blue line marks the vertical position, where the top view image is located within the z-stack. For both types, the GFP-labeled bacteria are observed in aggregates in the top view, the white arrows in the side view show the bacteria colonizing the inside of the root cells.

3.3.4 Co-colonization of "*cand.* A. radicis" N35 and N35v on barley roots

For co-colonization investigation, barley seedlings were inoculated with a 1:1 mixture of GFP-labeled N35 and YFP-labeled N35v cells or YFP-labeled N35 and GFP-labeled N35v cells and grown in the monoxenic system (2.3.1). Co-colonization of both phenotypes on barley roots were investigated 3 weeks after inoculation using the CLSM.

At all growth stages of the roots, bacterial cells colonized mostly the basal part of the roots 3 weeks after inoculation, but the phenotype variant colonized barley roots differently. GFP-labeled wild type N35 colonized root surfaces in high numbers forming aggregates, whereas only a few YFP-labeled N35v cells could be observed (Fig. 3.9 a). These results proved to be independent of the fluorescent protein used, as when the bacteria were labeled with the respective other fluorescent protein the dominant colonization pattern of N35 wild type could still be demonstrated (Fig. 3.9 b). In fig. 3.9 c and d, the N35 cells are shown colonizing the roots endophytically in much higher numbers than N35v.

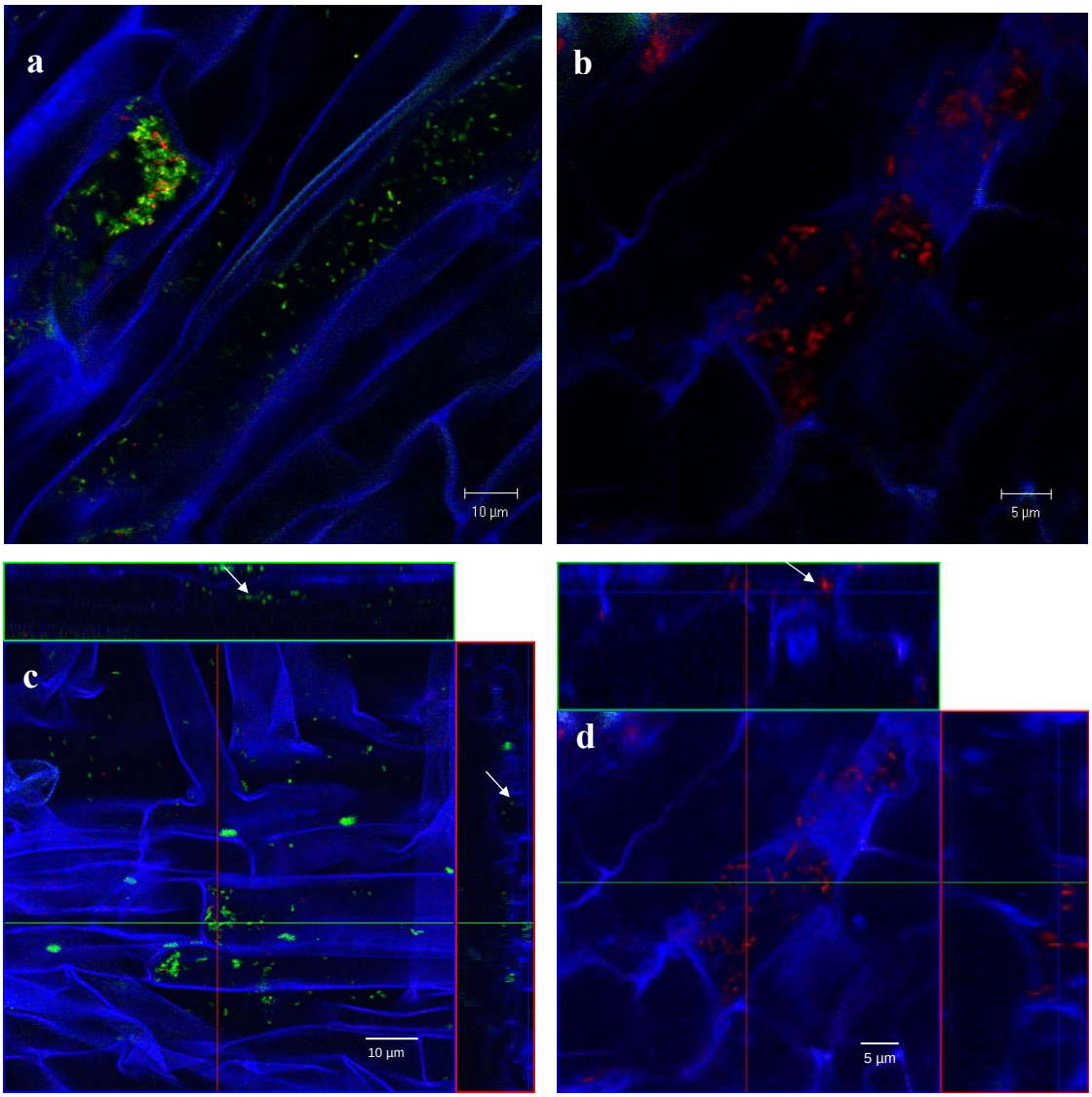


Fig. 3.9. CLSM images of co-colonization of both types of "*cand.* A. radicis" N35 on barley roots in monoxenic system. GFP-labeled bacterial cells are shown in green, YFP-labeled bacterial cells in red and the roots in blue. The samples were taken from 3-week-old barley roots. (a) Co-inoculation of GFP-labeled N35 and YFP-labeled N35v on barley roots. GFP-labeled N35 cells colonize the roots in a large number forming aggregates, where YFP-labeled N35v are observed

only in a few cells. (b) Co-inoculation of YFP-labeled N35 and GFP-labeled N35v on barley roots. YFP-labeled N35 cells constitute the vast majority, while only one GFP-labeled N35v cell can be observed. (c) Orthogonal view of co-colonization of GFP-label N35 and YFP-labeled N35v on barley roots. GFP-labeled N35 colonize inside the roots (white arrows). (d) Orthogonal view of co-colonization of YFP-labeled N35 and GFP-labeled N35v on barley roots. YFP-labeled N35 cells proliferate inside the root cells (white arrows).

3.4 Identification of *mutL* gene influencing phenotypic variation in "*cand.* A. radicis" N35

In order to find possible differences on a genetic level between the both types, the genomes of N35 and N35v were compared.

A sixteen nucleotides deletion region was identified in the genome sequence of phenotype variant N35v as compared to wild type N35. This region is predicated to encode the mismatch repair protein MutL. This protein is truncated in N35v, because the frame shift caused by the 16 nucleotides deletion leads to the insertion of a genetic stop codon in the coding gene. In contrast, this mismatch repair protein MutL is active and responsible for specific gene expression in N35. Subsequently, six colonies of wild type N35 and ten colonies of the phenotype variant N35v were independently picked on NB agar plates and the *mutL* gene region was amplified using primer pair MutL-s and MutL-as. This sixteen nucleotides deletion event was observed by all of the colonies of N35v in *mutL* gene region, while the *mutL* gene of the wild type N35 harbored the entire *mutL* gene.

The PFAM active site method (Mistry *et al.*, 2007) was applied on the two proteins of N35 and N35v, which was able to identify three highly significant matches for N35 and two highly significant matches for N35v. One of the domains is identical to HATase_c, which is a histidine kinase-, DNA gyrase B-, and HSP90-like ATPase; the other one is DNA_mis_repair, which is a short form for DNA mismatch repair protein. These two domains complement the third domain, MutL_C domain, a MutL C-terminal dimerisation domain, which is only detected in N35 (Fig. 3.10).

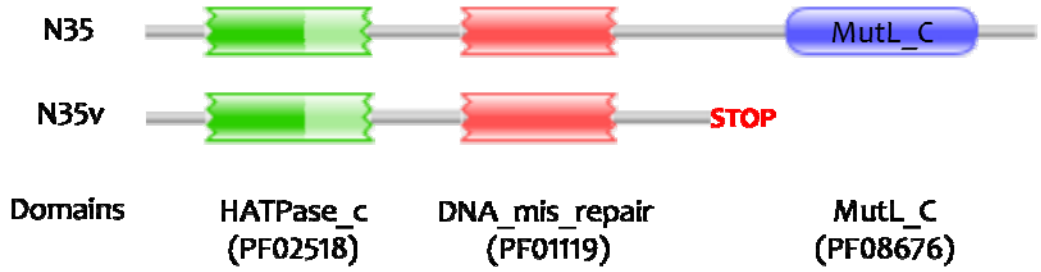


Fig. 3.10. The domains of "*cand.* A. radicis" N35 and N35v *mutL* gene: HATPase and DNA_mis_repair domains are found in both types, while the MutL_C dimerisation domain is deletion in N35v because of an insertion of a stop codon.

3.5 Detection and characterization of N-acyl-homoserine lactone (AHL) synthesis in "*cand.* A. radicis" N35 and N35v

3.5.1 Detection of AHLs via biosensor

GFP sensor construct pBAH9 located in *S. liquefaciens* MG44, responsive to a broad range of HSLs (C4-C14), was used to detect the AHL production in "*cand.* A. radicis" N35 and N35v (2.6.1.1).

The sensor pBAH9 was similarily activated by the AHL production in N35 and N35v, which resulted in a green fluorescence when excited at 488nm (Fig. 3.11 a and b).

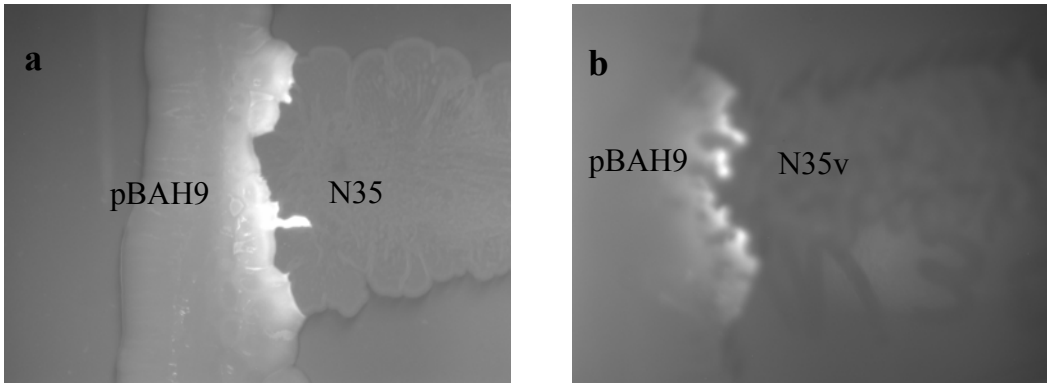


Fig. 3.11. Detection of AHLs using sensor plasmid pBAH9 in *S. liquefaciens* MG44. "*cand.* A. radicis" N35 (a) and N35v (b) were streaked perpendicularly near the sensor plasmid pBAH9. The sensor activated by AHLs showed green fluorescent when excited at 488nm.

3.5.2 Characterization of AHL production in "*cand.* A. radicis" N35 and N35v using FT-ICR-MS and UPLC

To characterize the AHL signals produced by N35 and N35v, concentrated extracts of culture supernatants were analyzed by FT-ICRMS and UPLC (2.6.1.2). By using FT-ICR-MS a mass of 294.1676 was calculated for the compound in the active fraction for both types, which was in agreement with a molecular formula of $C_{14}H_{25}O_4Na$ (Fig. 3.12). This formula is consistent with 3-hydroxy-decanoyl homoserine lactone, 3-OH-C10-HSL. To confirm the identification, the same sample was separated by UPLC. The UPLC measurements supported the result of FT-ICR-MS that both types of "*cand.* A. radicis" N35 produced 3-OH-C10-HSL as major AHL compound.

However, no significant quantitative differences were observed for 3-OH-C10-HSL production between the two phenotypes during the exponential phase, concentrations of 3-OH-C10-HSL per gram cells were 116.61 µg/ml for N35 versus 113.47 µg/ml for N35v.

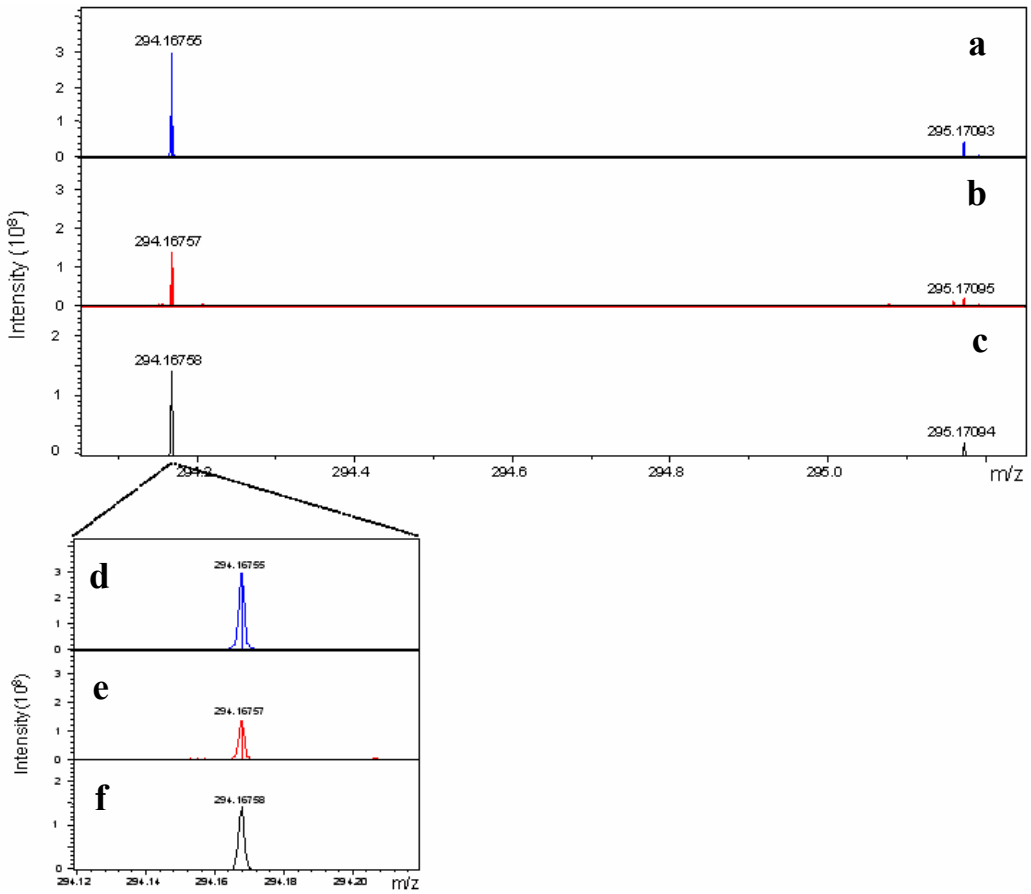


Fig. 3.12. FT-ICR-MS spectra of AHL signals produced by N35 and N35v. m/z range was between 294.2 and 295.2 (a, b and c), 294.12 and 294.20 (d, e and f). (c) and (f) Simulated 3-OH-C10-HSL showed the profiles at the m/z of 294.1676. (a) and (d) The peak at the m/z of 294.1676 was dominant for N35, same as simulated 3-OH-C10-HSL. (b) and (e) The same peak at the m/z of 294.1676 was found for N35v .

3.5.3 Identification of *araI-araR* locus in the genome of "*cand.* A. radicis" N35 and N35v

In order to determine the gene(s) directing synthesis of the 3-OH-C10-HSL signal molecules, the obtained whole genome sequence data was compared with the sequence data of autoinducer synthase gene in the databank (2.7.8.3). A 555 nucleotides sequence containing a significant open reading frame (ORF) in N35/N35v revealed homology to an autoinducer synthases gene of the *luxI* family. This 555 bp fragment, designated *araI*, is predicted to encode a protein that is homologous to the members of the LuxI family of AHL synthases. Using the BLAST analysis program, the AraI protein exhibits 54%

identity (81% similarity) to *Acidithiobacillus ferrooxidans* ATCC 23270 AfeI (Farah *et al.*, 2005; Barreto *et al.*, 2003), 53% identity (81% similarity) to autoinducder synthase of *Ralstonia solanacearum* GMI1000 (Salanoubat *et al.*, 2002) and 53% identity (81% similarity) to *Acinetobacter baumannii* AB0057 AbaI (Adams *et al.*, 2008). A 779 nucleotides sequence containing the other ORF in N35/N35v, designated *araR*, is located upstream of *araI* and is transcribed in the contrary direction. The araR encodes a protein which has 55% identity (82% similarity) to transcriprional activator of quorum sensing of *Ralstonia solanacearum* GMI1000 (Salanoubat *et al.*, 2002), 54% identity (79% similarity) to LuxR type transcriptional regulator of *Burkholderia pseudomallei* (Holden *et al.*, 2004) and 51% identities (81% similarity) to *Acidithiobacillus ferrooxidans* AfeR (Farah *et al.*, 2005; Barreto *et al.*, 2003). Both types of "*cand.* A. radicis" N35 possess the same AraI/AraR quorum sensing system.

3.5.4 Knock-out mutagenesis of AHL synthase

3.5.4.1 Construction of gene replacement vector

The goal of this experiment was to construct a gene replacement vector for knock-out mutagenesis of the AHL synthase in "*cand.* A. radicis" N35.

First, the AHL synthase gene (*araI* gene, about 1.2 kb) of N35 was PCR amplified using the primer pair AHLsyn-s2 and AHLsyn-as2 and cloned into a pCR2.1-TOPO vector. The resulting plasmid pTOPO-AHL was digested with restriction enzyme *Acc*I. Tetracyclin resistance gene (*tet* gene, about 1.5kb) was also amplified from pEX18Tc with primer pair TcR-s and TcR-as3 by PCR and cloned into a pCR2.1-TOPO vector, the resulting plasmid pTOPO-TcR was digested with restriction enzyme *Acc*I. The *AccI* fragment of the *tet* gene was gel purified and ligated to the *Acc*I fragment of pTOPO-AHL. The resulting plasmid was named pTOPO-N35AHL-TcR.

To obtain the gene replacement vector pEX18Gm-N35AHL-TcR, an *EcoR*I fragment of N35AHL-TcR digested from pTOPO-N35AHL-TcR was gel purified and ligated to the large *EcoR*I fragment of pEX18Gm (Fig. 3.13). Cells harboring the recombinant plasmid were selected on LB medium containing Tc and Gm. The resulting plasmid pEX18Gm-N35AHL-TcR contained the selectable Tc^R marker and a *sacB* based gene replacement system as well as an AHL synthase mutant (Δ*araI*) allele, which was obtained by the insertion of a tetracycline resistance cassette flanked by the homologous regions of AHL synthase gene.

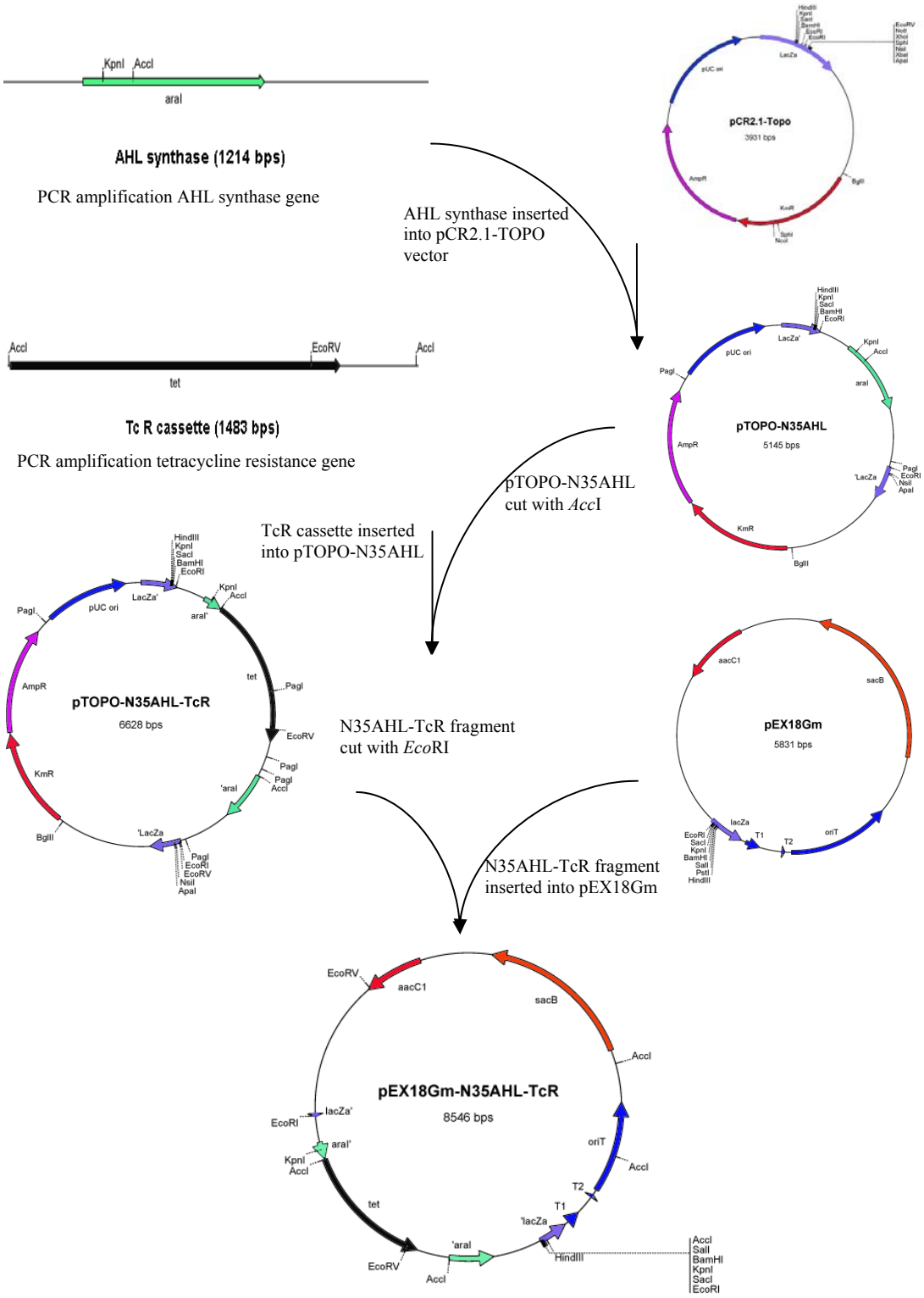


Fig. 3.13. Construction of gene replacement vector pEX18Gm-N35AHL-TcR. The location of genes, their transcriptional orientations and selected restriction enzyme cleavage sites are shown. *KmR*, kanamycin resistance gene; *AmpR*, ampicillin resistance gene; *tet*, tetracycline resistance gene; *aacC1*, gentamicin acetyl transferase gene; *araI*, AHL synthase gene; *araI`* and *`araI*, part of inactive *araI* gene; *lacZα*, β-galactosidase gene; *lacZα`* and *`lacZα*, part of inactive *lacZα* gene; pUC ori, pUC plasmid replication origin; *sacB*, levansucrase precursor gene; *oriT*, origin of transfer; T1 and T2, terminator of transcription.

3.5.4.2 Isolation of "*cand.* A. radicis" ΔaraI mutants

The recombinant plasmid pEX18Gm-N35AHL-TcR was transferred into "*cand.* A. radicis" N35 via electroporation. Homologous recombination occurred in pairing of the Δ*araI* mutant allele with the homologous regions in the genome of "*cand.* A. radicis" N35, which led to an insertion of the whole constructed pEX18 plasmid into the genome of N35. The plasmid integrants were selected on NB medium containing Tc. Merodiploids were resolved by plating on NB medium containing 5% sucrose. Only cells where the *sacB* gene together with the gentamycin selective marker was removed from the genome of N35 by a second homologous recombination could survive on sucrose containing medium. The AHL synthase mutant, named "*cand.* A. radicis" N35 ΔaraI, should be resistant to sucrose and Tc (Fig. 3.14).

The success of gene replacement in strain N35 ΔaraI was verified by colony PCR analysis (2.7.7.2) using primer pair AHLsyn-s2 and AHLsyn-as2. The presence of a 2.7 kb fragment (*araI* gene fragment 1.2 kb plus *tet* gene fragment 1.5 kb) revealed the putative Δ*araI* mutant allele in "*cand.* A. radicis" N35 (Fig. 3.15 a, line 2). In the positive control in lane 3 with wild type N35 as template a fragment at about 1.2 kb was observed, which revealed the AHL synthase gene.

Finally, the putative AHL synthase mutant of "*cand.* A. radicis" N35 was tested with AHL biosensor pBAH9. AHL sensor plasmid pBAH9 was not activated when screened with "*cand.* A. radicis" N35 ΔaraI (Fig. 3.15 b).

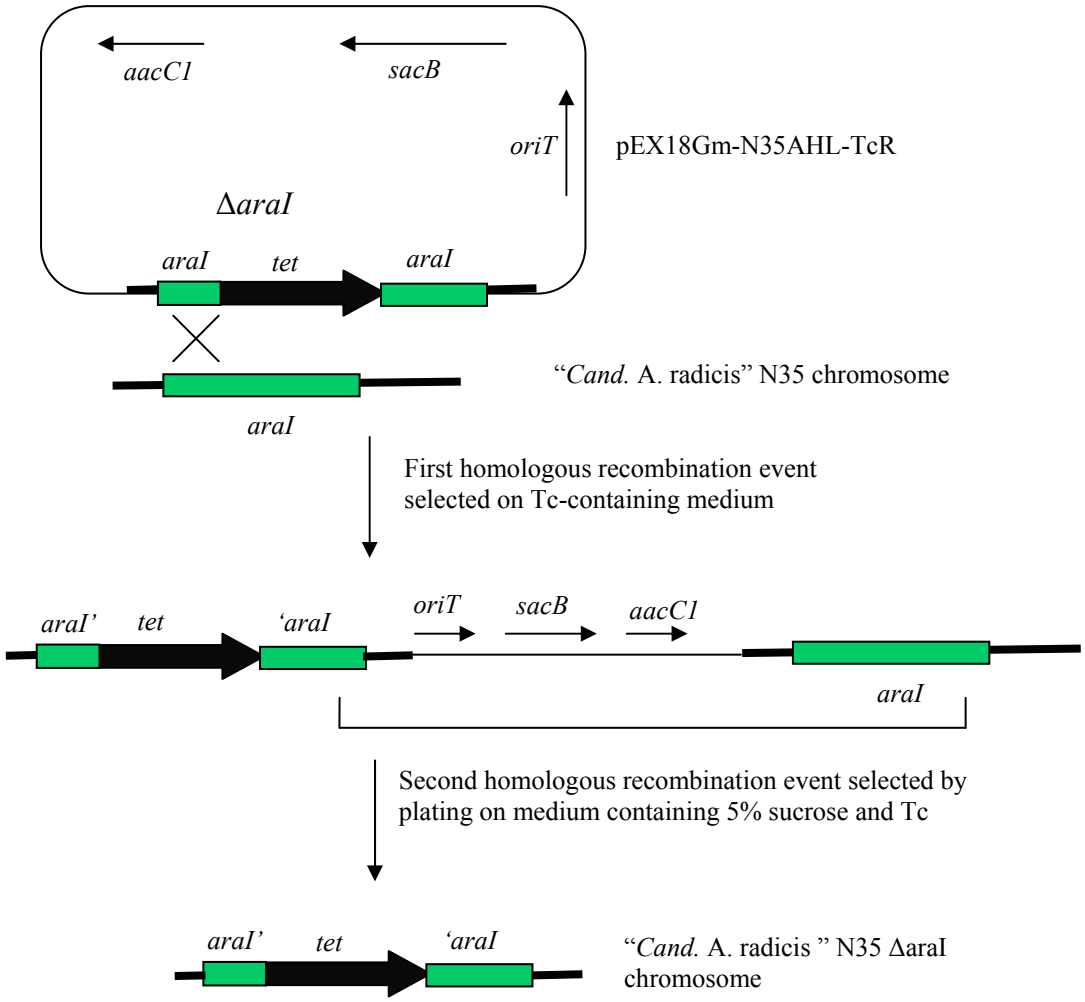


Fig. 3.14. Strategy for isolation of *ΔaraI* mutant of "*cand.* A. radicis" N35. *araI*, AHL synthase gene; *araI`* and *`araI*, part of inactive *araI* gene; *oriT*, origin of transfer; *sacB*, levansucrase precursor gene; *aacC1*, gentamicin acetyl transferase gene.

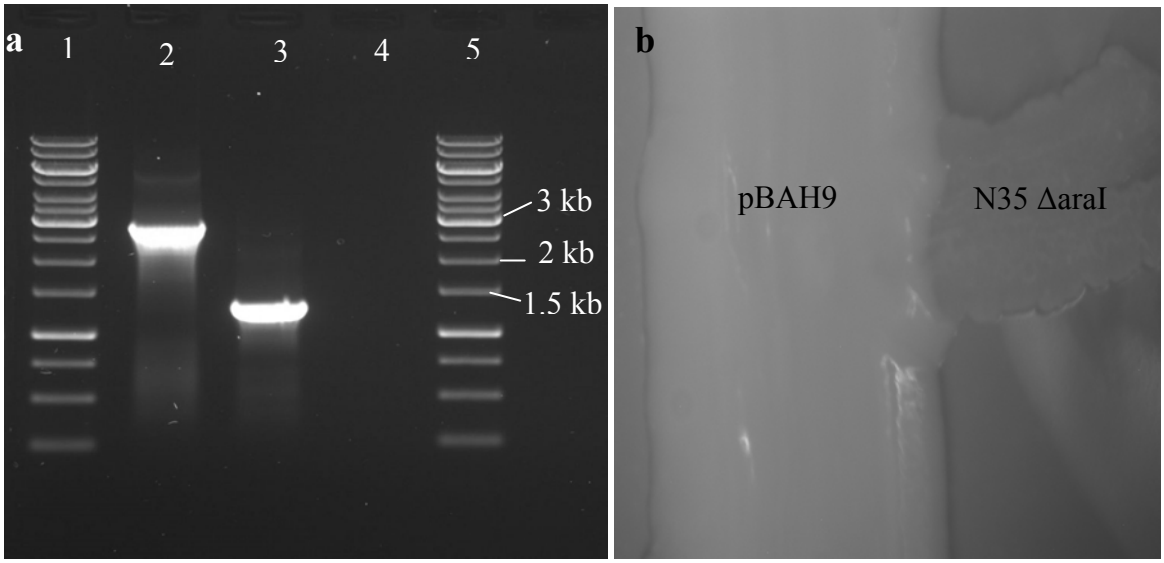


Fig. 3.15. (a) Colony PCR to reveal AHL synthase mutant. Line 1 and 5, 1 kb DNA ladder; line 2, "*cand.* A. radicis" N35 ΔaraI; lane 3, "*cand.* A. radicis" N35; line 4, negative control. (b) "*cand.* A. radicis" N35 ΔaraI screened with AHLs sensor plasmid pBAH9 in *S. liquefaciens* MG44, which sensor plasmid showed no active.

3.5.4.3 Influence of quorum sensing on root colonization

Many rhizobacteria utilize quorum sensing systems to regulate the expression of a diversity of genes, which are involved in microbe-plant interactions (Loh *et al.*, 2002). In order to find the role of quorum sensing signals in root colonization, the wild type N35 and AHL negative mutant N35 ΔaraI were co-inoculated on barley roots in a monoxenic system. DNA binding dye SYTO orange 81 was used as counter stain, so that GFP-labeled N35 cells also labeled with SYTO orange showed a double peak with a maximum emission wavelength of 510 nm and 550 nm and non-GFP labeled N35 ΔaraI cells showed only one peak at 550 nm.

GFP-labeled wild type N35 colonized the roots in high numbers, whereas only a few N35 ΔaraI cells could be observed (Fig. 3.16). This experiment in a monoxenic system showed a competitive root colonization behavior by co-inoculation with wild type N35 and AHL negative mutant N35 ΔaraI, with wild type N35 showing dominant colonization on barley roots as compared to AHL negative mutant N35 ΔaraI.

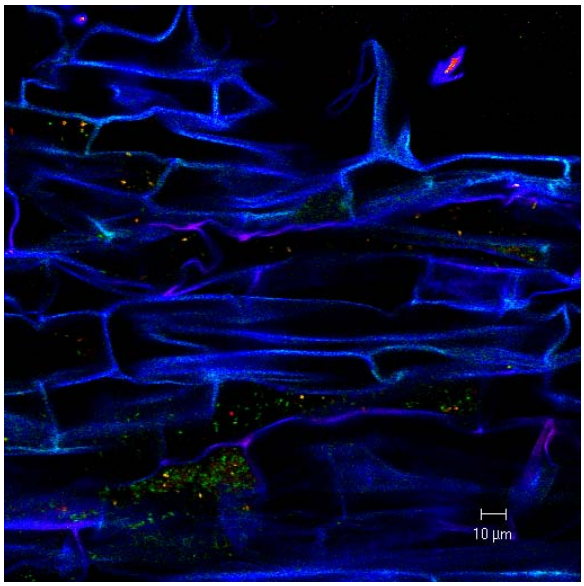


Fig. 3.16. CLSM image of GFP-labeled wild type N35 and non-labeled AHL negative mutant N35 ΔaraI stained with SYTO orange 81. GFP-labeled N35 cells are shown in green, non-labeled N35 ΔaraI cells in red and root cells in blue, signals in orange are ambigous. GFP-labeled N35 cells colonize the roots at high numbers where only a few non-labeled N35 ΔaraI cells can be observed.

3.5.4.4 Influence of quorum sensing signal on the other phenotypic properties of N35

The phenotypic properties including lipase activity, siderophore production and swarming ability was also performed in AHL negative mutant "*cand.* A. radicis" N35 ΔaraI.

The AHL negative mutant N35 ΔaraI had a similar swarming ability as the wild type N35 on soft agar plate. The siderophore production and lipase activity were also not affected by quorum sensing signal in N35, where a round halo surrounding the colony of N35 ΔaraI on glyceryl tributyrate agar plates and an orange halo on CAS agar plates could be observed, indicating the lipase activity and the release of siderophore in AHL negative mutant N35 ΔaraI.

3. 6 Rhizobacteria-fungal interactions

In the rhizosphere, bacteria and fungi are present in close vicinity. Commercial inoculants may influence each other in a positive or negative way. In some cases, bacteria and fungi are living in close association.

Stimulatory and inhibitory influences as a result of co-cultivation of the plant growth promoting fungus (PGPF) *P. indica* with diverse rhizobacteria were studied in confrontation assays on NB agar plates using a collection of strains and mutants (2.5). The commercial plant growth promoting and biocontrol strain *B. amyloliquefaciens* FZB42 and its various lipopeptide and antibiotic mutants as well as biocontrol strain *P. fluorescens* SS101 and its lipopeptide biosurfactant massetolide A deficient mutant 10.24 were used for the investigation of rhizobacteria-PGPF interactions. PGPR strains *Azospirillum brasilense* SP245 and *Serratia liquefaciens* MG1 as well as fungus growth promoting strain *P. putida* IsoF were also tested for their association with *P. indica*. Eventually, "*cand.* A. radicis" N35 and N35v as well as *R. radiobacter* F4 and F7 isolated from *P. indica* were also tested in confrontation assays.

The wild type strain FZB42 severely inhibited the growth of *P. indica*, the mutants CH3, AK1 and AK3, lacking lipopeptide and polyketide synthesis or bacillomycin D production respectively, had no inhibitory effect any more. In contrast, the mutant AK2, deficient only in fengyin production, was still inhibitory. This pointed to the fact, that bacillomycin is the most effective inhibitory metabolite in the interaction of FZB42 with *P. indica*. The biocontrol strain *P. fluorescens* SS101 inhibited growth of *P. indica*, while its lipopeptide biosurfactant massetolide A deficient mutant 10.24 had clearly no inhibitory effect on hyphal growth of *P. indica.* Accordingly, the isolated cyclic lipopeptide massetolide A inhibited the growth of *P. indica* down to a concentration of 100 µg/ml in the confrontation assay. *R. radiobacter* F4 and F7 isolated from *P. indica* had no effect on the host in the confrontation assay (Table 3.3, Fig. 3.17).

Table 3.3. Influence of rhizobacteria on growth of *P. indica*

Bacterial strains	Characters	Impact
B. amyloliquefaciens FZB42	wild type; commercial inoculant "Rhizo-plus" (Abitep)	inhibition
B. amyloliquefaciens FZB42 CH3	lipopeptide and polypeptide deficient mutant	no inhibition
B. amyloliquefaciens FZB42 AK1	bacillomycin deficient mutant	no inhibition
B. amyloliquefaciens FZB42 AK2	fengycin deficient mutant	slight inhibition
B. amyloliquefaciens FZB42 AK3	fengycin and bacillomycin deficient mutant	no inhibition
P. fluorescens SS101	wild type; biocontrol strain	inhibition
P. fluorescens SS101 10.24	biosurfactant massetolideA deficient mutant	no inhibition
S. liquefaciens MG1	PGPR	inhibition
P. putida IsoF	fungus growth promoting bacteria	stimulation
Azospirillum brasilense Sp245	PGPR	no effect
R. radiobacter F4 and F7	wild type; isolated from *P. indica*	no effect
"*Cand*. A. radicis" N35 and N35v	wild type	no effect

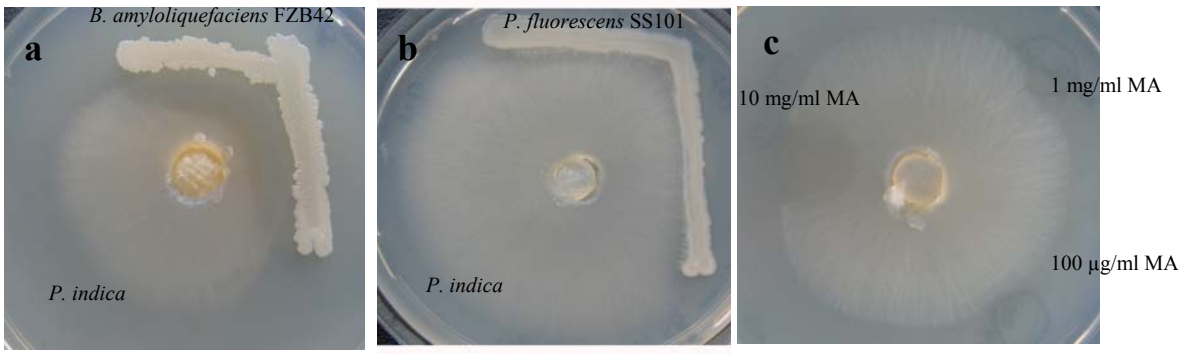


Fig. 3.17. Confrontation assays of *P. indica* with rhizobacteria. (a) Interaction of *P. indica* with *B. amyloliquefaciens* FZB42. Growing stage of mycelia of *P. indica* was inhibited by *B. amyloliquefaciens* FZB42. (b) Interaction of *P. indica* with *P. fluorescens* SS101. A slight inhibition effect on *P. indica* could be observed, which at the contact zone of both cultures formed an indentation of the mycelium of *P. indica.* (c) Effect of masstolide A (MA) on *P.*

indica. Cyclic lipopeptide masstolide A had an inhibitory effect on the mycelia of *P. indica* even at a concentration of 100 µg/ml.

3.7 Endofungal rhizobacteria *Rhizobium radiobacter* F4 and F7

R. radiobacter F4 and F7, isolated in parallel from *P. indica* DSM 11827, were obtained from a cooperative working group in Giessen (Sharma *et al.*, 2008). In this study, the localization of endofungal *R. radiobacter* on the plant roots, the AHL production as well as the role of the AHL in *R. radiobacter* were investigated.

3.7.1 Localization of *R. radiobacter* on barley roots

FISH (2.7.1) was used for the localization of *R. radiobacter* on barley roots. Surface sterilized barley seedlings were inoculated with *R. radiobacter* F4 and grown in a monoxenic system for 3 weeks (2.3.1). The roots were harvested, fixed, fluorescently labeled with EUB338mix and Rhi1247 and colonization of *R. radiobacter* was detected with the CLSM (2.4.2).

The cells of *R. radiobacter* F4 could be observed on the surface of the root hair zone of barley roots, where they colonized in high numbers and formed aggregates (Fig.3.18).

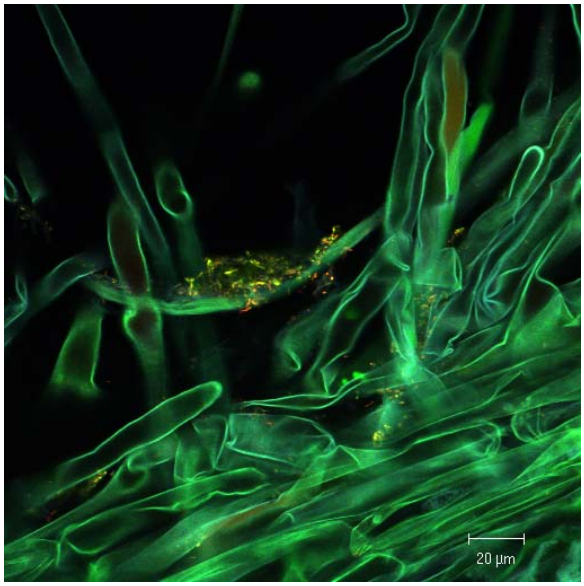


Fig.3.18. CLSM image of *R. radiobacter* F4 3 weeks after inoculation on barley root hairs in monoxenic system. Roots were hybridized with EUB338mix (green) and Rhi1247 (red) according to the FISH protocol. The binding of both probes resulted in an orange color of target cells.

3.7.2 Detection and characterization of AHL production by *R. radiobacter*

3.7.2.1 Detection of AHLs via biosensor

Green fluorescent AHL sensor pBAH9 was used to detect the AHL production in *R. radiobacter* F4 and F7. The presence of AHL production was observed by binocular Lumar V12.

The sensor pBAH9 was activated by the AHL production in *R. radiobacter* F4 and F7 and therefore showed a bright-green fluorescence when excited at 488nm (Fig.3.19 a and b).

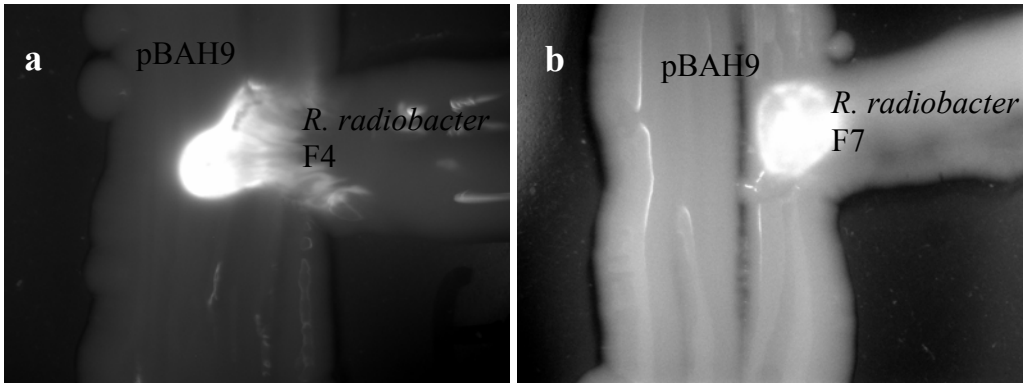


Fig.3.19. Detection of AHLs using sensor plasmid pBAH9 in *S. liquefaciens* MG44.

3.7.2.2 Characterization of AHL production in *R. radiobacter* F4 and F7

For further characterization of AHL production in strains F4 and F7, concentrated extracts of culture supernatants were measured by FT-ICR-MS (2.6.1.2).

Endofungal bacterium *R. radiobacter* was able to synthesize a variety of oxo- and hydroxyl-C8- to C12-HSL compounds. Table 3.4 summarizes the detected putative AHLs in strains F4 and F7. Differences were found in 3-oxo-C12-HSL and 3-OH-C12-HSL, which were only detected in *R. radiobacter* F4.

Most interestingly, *R. radiobacter* also produced *p*-coumaroyl-HSL type quorum sensing signaling molecules, when *p*-coumaric acid (CA, 1mM) was supplied in the medium (Fig. 3.21). Three chemical structures were found in strains F4 and F7 (Fig. 3.20): *p*-coumaroyl HSL, methylated *p*-coumaroyl-HSL and ethylated *p*-coumaroyl-HSL, according to the extract mass peaks in FT-ICR-MS.

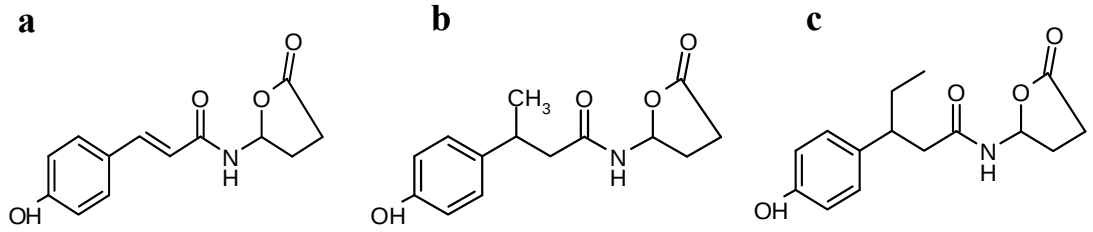


Fig. 3.20. Chemical structure of detected coumaroyl-HSL type quorum sensing signals produced by *R. radiobacter*. (a) *p*-coumaroyl-HSL (m/s: 270.0737); (b) metylated *p*-coumaroyl-HSL (286.1050); (c) ethylated *p*-coumaroyl-HSL (m/s: 300.1206)

3.7.2.3 Lactonase degradation of AHLs in *R. radiobacter*

To express the *aiiA* lactonase gene, the broad-host-range expression vector pMLBAD-aiiA carrying a tellurite selective marker was conjugated into *R. radiobacter* F4 and F7 (2.7.10.4). In pMLBAD-aiiA, the *aiiA* gene was transcribed from the P_{BAD} promoter of *E. coli* and thus expression was induced with 0.2% arabinose (Wopperer *et al.*, 2006).

The expression of the *aiiA* lactonase gene in the transformants, designated *R. radiobacter* F4 NM13 and F7 NM13, was examined both with sensor plasmid and FT-ICR-MS.

The sensor plasmid pBAH9 was not active and showed no signal at an excitation of 488nm when the transformants F4 NM13 and F7 NM13 were cross-streaked on agar plates. In contrast, sensor plasmid pBAH9 was activated by strains F4 and F7. This result demonstrated, that the expression of *aiiA* lactonase gene induced the degradation of AHLs as well as *p*-coumaroyl-HSLs in the transformants.

Furthermore the activity of *aiiA* in transformants F4 NM13 and F7 NM13 was examined by FT-ICR-MS. Transfer of plasmid pMBALD-aiiA into the *R. radiobacter* strains caused a dramatic reduction of AHL accumulation. The AHLs detected in wild type strains F4 and F7 were not found in transformants F4 NM13 and F7 NM13 (Tab. 3.4). The presence of pMLBAD-aiiA also abolished *p*-coumaroyl-HSL production in transformants F4 NM13 and F7 NM13 even though *p*-coumaric acid was added to the medium (Fig. 3.21).

According to these results, the *aiiA* gene located on plasmid pMBALD-aiiA was successfully transferred and expressed in *R. radiobacter* strains. By the expression of the *aiiA* gene, the AHLs as well as the *p*-coumaroyl-HSL were totally degraded in *R. radiobacter* strains.

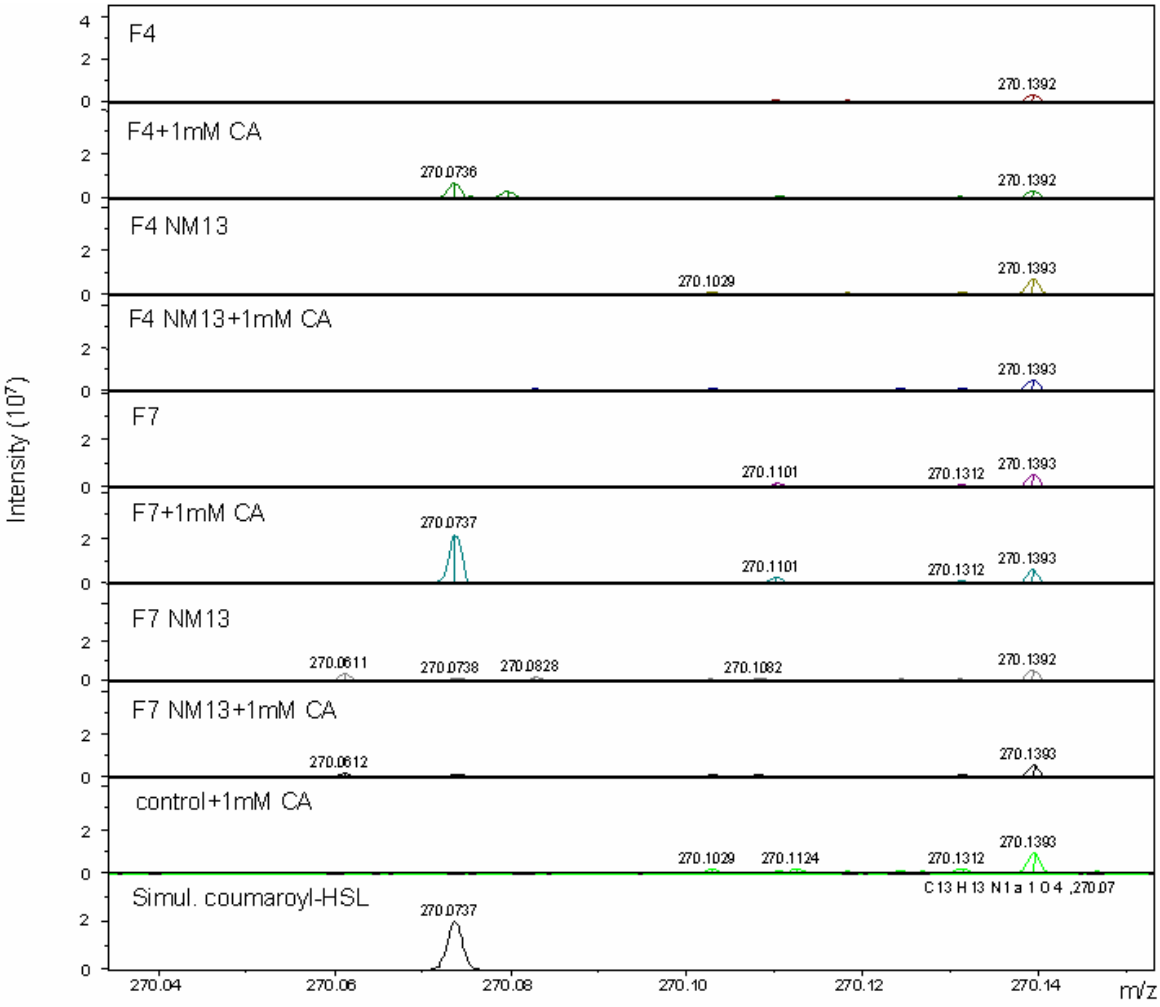


Fig. 3.21. FT-ICR-MS spectra of *p*-coumaroyl-HSL signal produced by *R. radiobacter* F4 and F7 when 1mM *p*-coumaric acid was added, and missing in the lactonase expressing transformants F4 NM13 and F7 NM13. m/z range was between 270.04 and 270.14. The same peak as detected for F4 and F7 at the m/z of 270.0737 was simulated for *p*-coumaroyl-HSL.

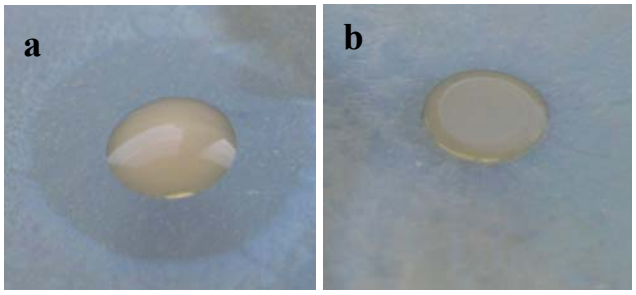
Table 3.4. Putative AHLs detected in *R. radiobacter* F4 and F7 and its lactonase expressing transformants F4 NM13 and F7 NM13. "+" AHL production detected in the tested strain, "ND" AHLs was not detected in the tested strain.

Measured mass	Putative AHLs	F4	F4NM13	F7	F7NM13
264.1206	3-oxo-C8-HSL	+	ND	+	ND
266.1363	3-OH-C8-HSL	+	ND	+	ND
292.1519	3-oxo-C10-HSL	+	ND	+	ND
294.1676	3-OH-C10-HSL	+	ND	+	ND
306.2042	3-C12-HSL	+	ND	+	ND
320.1832	3-oxo-C12-HSL	+	ND	ND	ND
322.1991	3-OH-C12-HSL	+	ND	ND	ND

3.7.2.4 Role of quorum sensing signals in *R. radiobacter* strains

To test whether the lipase activity was generally AHL-regulated in *R. radiobacter* strains, wild type strains and transformants grown in liquid medium were point inoculated onto glyceryl tributyrate agar plates (2.6.2). Clearing halos, which were indicative for lipase activity, were observed in strains F4 and F7 after 48 h incubation. However, transformants F4 NM13 and F7 NM13 did not exhibit lipase activity (Fig. 3.22).

The siderophore production of wild type strains of *R. radiobacter* and transformants were examined using CAS agar plates (2.6.2). The orange halos surrounding the colonies, which indicated the release of siderophores, were observed for strains F4 and F7. On the other hand, the production of siderophore in the lactonase transformants F4 NM13 and F7 NM13 were greatly reduced (Fig. 3.23).



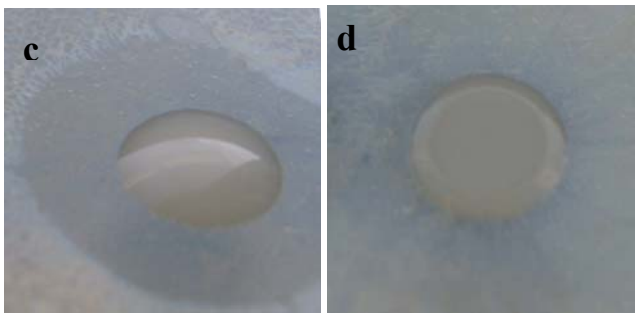


Fig. 3.22. AHLs degradation in *R. radiobacter* strains reduced lipase activity. Clear halos indicated the lipase activity. (a) *R. radiobacter* F4 (b) *R. radiobacter* F4 NM13 (c) *R. radiobacter* F7 (d) *R. radiobacter* F7 NM13

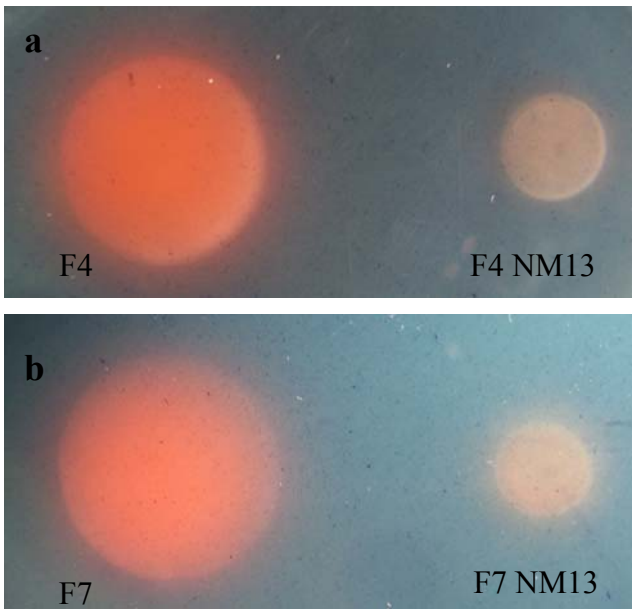


Fig. 3.23. AHLs degradation in *R. radiobacter* strains reduced siderophore production. Orange halos surrounding the colonies indicated the release of siderophores. (a) *R. radiobacter* F4 and lactonase transformant F4 NM13 (b) *R. radiobacter* F7 and lactonase transformant F7 NM13

4 Discussion

4.1 Phylogenetic characterization of isolate N35

For the characterization and classification of newly isolated bacteria, a polyphasic approach including phylogenetic analysis based on 16S rRNA gene sequences, determination of DNA-DNA similarity and G+C mol% as well as the description of various phenotypic characteristics is essential (Wayne *et al.*, 1987). According to the 16S rRNA gene data, the isolate N35 showed closest sequence similarities (97.2-99.0%) to the environmental species of the genus *Acidovorax*. Ludwig *et al.* (1998) considered that a value of 16S rRNA gene sequence similarity of 95% or more indicates the same genus; these results provided evidence that the isolate N35 belongs to the genus *Acidovorax*. However, due to the highly conserved nature of the 16S rRNA gene, analysis of 16S rRNA gene sequences alone could not provide sufficient evidence for resolving at species level (Rossello-Mora and Amann, 2001). Thus, the DNA-DNA relatedness between strain N35 and the three phylogenetically most closely related type strains *A. defluvii*, *A. facilis* and *A. delafieldii* had to be compared. DNA-DNA hybridization is a standard technique for bacterial species delineation (Rossello-Mora and Amann, 2001). DNA-DNA hybridization results showed that the strain N35 had 25.3, 40.3 and 55.7 % similarities with *A. defluvii, A. delafieldii* and *A. facilis* type strains, respectively. According to the accepted criterion for species definition (Wayne *et al.*, 1987), similarity values of over 70% in DNA-DNA hybridizations are characteristic for members of the same species. Therefore, the strain N35 belongs to none of the compared three species.

The phenotypic methods, such as composition and relative ratio of fatty acids as well as metabolization of different substrates are also important for classification of bacterial strains (Wayne *et al.*, 1987), because the observed differences of phenotypic characters are the results of different genetic potentials and independent from cultivation conditions, as long as comparable growth conditions are used for the tested strains (Rossello-Mora and Amann, 2001). In strain N35, phenotypic properties, such as substrate metabolization profiles (Table 3.1) and cell wall fatty acid profiles concerning the fatty acids $C_{16:0}$, $C_{16:1\omega7cis/trans}$, $C_{17:0cyclo}$ and $C_{18:0cyclo}$ and $C_{19:0cyclo}$ (Table 3.2), facilitated also the differentiation from its closest relatives.

Summarizing, the comparison of the phylogenetic and phenotypic analysis of strain N35 with the three phylogenetically most closely related type strains *A. defluvii*, *A. facilis* and *A. delafieldii*, provides sufficient evidence to conclude, that isolate N35 represents a new species within the genus *Acidovorax*, and the name *Acidovorax radicis* sp. nov. is suggested. This is the first description of a root endophytic bacterial species within the genus *Acidovorax*, which interestingly also harbors opportunistic human pathogens, e.g. some isolates of *A. delafieldii* and *A. temperans*.

4.2 Phenotypic variation in "*cand.* A. radicis" N35

According to the definition of phase or phenotypic variation in literature, it is considered as an event that arises at higher frequency than spontaneous mutation, i. e. higher than 10^{-5} switches per cell per generation. It is involved in various DNA changes or expression patterns, which lead to a phenotypically heterogeneous population (Henderson *et al.*, 1999; Saunders *et al.*, 2003;Wisniewski-Dyé and Vial, 2008). In case of "*cand.* A. radicis" N35, the rough colony type switched to the smooth colony type at a frequency of about $3.2 \cdot 10^{-3}$ per cell per generation on nutrient medium, according to the calculation in 2.6.2. The 16S rRNA gene sequence data and DNA-DNA hybridization values of both phenotypes were found to be 100% identical, indicating that both phenotypes were genetically almost identical. These results confirmed that N35v is not a co-cultivated or contaminating microorganism in nutrient medium, but is instead a very closely related to the wild type N35. Therefore, "*cand.* A. radicis" N35 can be considered to perform phase or phenotypic variation.

In rhizosphere bacteria, phase variation generates intra-population diversity in bacterial colony morphology and physiology, forming specific subpopulations, which increase bacterial fitness and niche adaptation. This phase variation affects root colonization, biocontrol activity, immune invasion, the expression of exoenzymes and production of secondary metabolites (van den Broek *et al.*, 2005, Wisniewski-Dyé and Vial, 2008). In plant growth promoting rhizobacteria *Azospirillum brasilense* Sp7, the phenotype variant shows a modified colonization behavior and superior nitrogen fixation on wheat compared to the wild type (Katupitiya *et al.*, 1995). A phenotype variant in *P. fluorescens* WCS365 is impaired in competitive root tip colonization of tomato, wheat, radish and potato in comparison with the wild type (Dekkers *et al.*, 1998). In chemolithoautotrophic bacteria *Acidithiobacillus ferrooxidans* ATCC 19859 (formerly *Thiobacillus ferrooxidans*), even

the major metabolism is regulated by phase variation, by which the phenotype variant lacks the ability of iron oxidation (Cabrejos *et al.*, 1999; Schrader and Holmes, 1988). In "*cand.* A. radicis" N35, as discussed in detail below, the phenotypic variation influenced some cell morphologies, utilization of carbon sources, root colonization behavior and also plant growth promoting effect. The wild type N35 was more competitive compared to the phenotype variant N35v in root colonization. The phenotype variant N35v could be considered as a planctonic culture type, as some important surface colonization abilities are missing, including mobility by flagella and swarming, aggregation ability as well as the ability to utilize formic acid and fucose present as exudates in the rhizosphere. Colony heterogeneity within the bacterial population may provide flexibility in response to environmental changes, i. e. planctonic versus attached or nutrient rich versus nutrient poor life style. It was reported in some studies that an increasing proportion of phenotype variants was generated during colonization of roots. For example in *P. fluorescens* F113, phenotypic variation is taking place during alfalfa rhizosphere colonization (Sánchez-Contreras *et al.*, 2002) and also Achouak *et al.* (2004) describe that phase variation in *P. brassicacearum* NFM42 occurs during *A. thaliana* and *Brassica napus* root colonization both *in vitro* and in soil. However, in this study no rhizosphere stimulated increase of phase variation in "*cand.* A. radicis" N35 on barley was observed.

Phase variation mechanisms are described to be involved in gene modification, such as site specific inversion, recombinational deletion, transpositions, spontaneous duplication and mutations, slipped-strand mispairing, genomic rearrangements or epigenetic mechanisms such as differential methylation (Henderson *et al.*, 1999; van den Broek *et al.*, 2005). Usually in phase variation, the variants are unstable and able to reverse to the original phenotype, but in some cases the switch may be irreversible (Wisniewski-Dyé and Vial, 2008). The plant phytopathogen *Ralstonia solanacearum* GMI1000 undergoes a phenotypic conversion from a pathogenic wild type to a non-pathogenic variant *in vitro*, however the reversion to the pathogenic form was only observed in planta or in the presence of tomato root exudates (Poussier *et al.*, 2003). In the case of "*cand.* A. radicis", according to preliminary results, no reversion of the phenotype variant N35v to the wild type N35 was observed by multiple subculturing in laboratory culture conditions as well as in the rhizosphere of barley. Vial *et al.* (2009) described the generation of irreversible phenotype variant cells by phase variation of *B. ambifaria* HSJ1, where the variants proved to be stable even after re-isolation from the eukaryotic hosts. In "*cand.* A. radicis" N35, the phenotypic variation was shown to be based on at least one mutation event (3.1.1), i. e. a

16 nucleotides deletion in the *mutL* gene in N35v (3.4). However, deletions in a definite gene are not necessarily irreversible, as e.g. for human pathogen *Campylobacter coli* UA585 reversible phenotypic variation is described. Reversible switching between the single polar flagellum, motile phenotype and the non-flagellum, non-motile phenotype of *C. coli* UA585 is correlated with high frequency, reversible insertion and deletion in a short homopolymetric tract of thymine residues located in *flhA* gene. The mechanism(s) involved in this reversible insertion and deletion of T residues of *flhA* gene is unknown (Park *et al.*, 2000). Therefore, the question whether phase variation of N35 is reversible or not, cannot be finally answered, as a switch back to the wild type form might depend on very specific niche conditions. But at least in all conditions tested throughout this work N35v proved to be stable.

4.3 Plant growth promoting effect of "*cand.* A. radicis" N35 and its phenotype variant on barley

This study provides clear evidences for plant growth stimulating effects of the two phenotypes of "*cand.* A. radicis" N35 in soil as demonstrated in pot experiments in the green house (3.2). A significant plant growth promoting effect of wild type N35 on barley could be observed after 16 weeks growth in a soil system. The phenotype variant N35v also showed a plant growth promoting effect, but compared to the wild type N35, phenotype N35v had no significant growth stimulating effect on barley (Fig. 3.4 and 3.5). Phenotypic variation has been described in many PGPR such as *Azosprillum lipoferum* 4B (Vial *et al.*, 2004) and *Burkholderia ambifaria* HSJ1 (Vial *et al.*, 2009), however, until now, no particular difference in plant growth promoting effect has been described in phenotype variants of PGPR.

As one possible factor in plant growth promotion, the siderophore production in N35 and N35v cells, was tested using CAS agar plates. The production of siderophores in "*cand.* A. radicis" N35 and N35v could be detected under laboratory conditions (3.1.2). Iron is an essential micronutrient of plants used as a cofactor of many enzymes, however in soil most of the iron exists in insoluble form as ferric hydroxide (Podile and Kishore, 2007). Several strains of PGPR have the ability to produce siderophores, Fe^{3+}-chelating compounds, which have high affinity and are also taken up by plants, and thereby enhance plant iron nutrition directly (Kloepper *et al.*, 1991; Sharma and Johri, 2003). Siderophore production

by PGPR has been broadly studied in *Pseudomonas* sp., e.g. a cold resistant strain of *P. fluorescens* CRPF9 with 17-fold siderophore production shows an increased rhizosphere colonization and growth promoting effect on mungbean (Katiyar and Goel, 2004). Therefore, the production of siderophores in "*cand.* A. radicis" N35 and N35v could be involved in the mechanism of plant growth promotion on barley by enhancing Fe^{3+} nutrition of the plant.

Furthermore, PGPR produced siderophores, which are able to scavenge Fe^{3+} from soil, also potentially inhibit pathogenic microorganisms by iron starvation in the rhizosphere (Podile and Kishore, 2007). Vandenburgh and Gonzalez (1984) have described a siderophore over-producing mutant of *P. putida* NRRL-B-12537 which is more effective in the suppression of pathogenic fungus *Fusarium* in tomato compared to the wild type. De Weger *et al.* (1986) have investigated more than 30 siderophore producing *Pseudomonas* strains, all of them exhibiting antagonistic activity towards the tested organisms *in vivo*. In addition, purified siderophores have a similar inhibitory effect on pathogens as the producing strains (Kloepper *et al.*, 1980; Neilands and Leong, 1986). Accordingly, "*cand.* A. radicis" N35 and N35v could compete for iron with deleterious microorganisms and pathogens in the rhizosphere. The root pathogens might be inhibited by iron limitation, which provides an indirect plant growth promoting effect on barley by "*cand.* A. radicis" N35 and N35v. The siderophore production of microorganisms in the rhizosphere is influenced by the nutrition of plants, and also the pH and redox conditions of the soil (Crowley and Kraemer, 2007). Thus, to understand the environmental factors regulating growth promoting mechanisms by PGPR is an essential step to realize their promoting activity (Podile and Kishore, 2007).

In general, rhizobacteria usually do not use a single mechanism to promote plant growth, but two or more mechanisms are involved (Glick *et al.*, 1999). Other mechanisms such as production of phytohormones like auxin, cytokinins, the reduction of exogenous plant ethylene by ACC-deamination, the capacity of solubilization of phosphate and nitrogen fixation as well as decreasing heavy metal toxicity could be involved in the plant growth promoting effect of rhizobacteria (Whipps, 2001). For further studies, research on these possible mechanisms involved in this plant growth promoting effect of rhizobacterium "*cand.* A. radicis" N35 and N35v should contribute to the better understanding of the beneficial association between bacteria and plants.

In comparison to the wild type N35, the phenotype variant N35v had no significant plant growth promoting effect on barley. The possible mechanisms involved in this observed

difference in the plant growth promoting effect of the two phenotypes will be discussed in the following chapters.

4.4 Colonization behavior of "*cand.* A. radicis" N35 and N35v

One of the aims of this study was to investigate the colonization as well as co-colonization behavior of both types of "*cand.* A. radicis" N35 on barley roots both in the monoxenic system and the soil system. Therefore, the cells of both types were first labeled with GFP. GFP, obtained from the jellyfish *Aequorea victoria*, is stable in the tagged cells and its fluorescence does not depend on cellular energy or additional substrates. Thus GFP is sufficient for the detection of single cells (Nybroe *et al.*, 2007). The *gfp* marker gene technology has been widely used in microbe-plant interactions, making GFP-labeled bacterial cells detectable in the rhizosphere by CLSM. GFP mutant protein, GFPmut3, is characterized by a 20 times higher fluorescence intensity than wild type GFP when excited at 488nm, and shows weak fluorescence when excited by UV light (Cormack *et al.*, 1996). Andersen *et al.* (1998) have described a stable variant of *gfp*mut3 gene expressed in *P. putida* KT2442, the half-life of matured GFPmut3 protein being more than 1 day in vivo. In this work, the *gfp*mut3 gene was introduced into the chromosomal DNA of "*cand.* A. radicis" N35 and N35v cells, resulting in a stable GFP label without antibiotic pressure. Thus both chromosomally GFP labeled phenotypes of N35 were suitable for the long-term investigation of colonization behavior in soil. In addition, the GFP labeled cells were easily separated from auto-fluorescent signals of roots and soil particles (Fig. 3.8 c).

A successful colonization of plant roots by introduced bacterial cells is a critical step for an effective bacteria-plant interaction. Plant growth promoting rhizobacteria are known for their high colonization ability (Chebotar *et al.*, 2001). High microbial abundance and activity in the rhizosphere are always found in positions where high concentrations of root exudates are present, e. g. lateral roots and root hairs (Rovira and Davey, 1974) as well as directly on the root surface (rhizoplane) (Hartmann *et al.*, 2009). Accordingly, GFP labeled N35 and N35v cells could colonize barley roots both in monoxenic and soil system (Fig. 3.8), and colonized the surface of root hairs equally well and in large numbers (Fig. 3.8 a and b); they were unevenly distributed and formed biofilm-like aggregates, probably reflecting root exudation profiles.

Most interestingly, an endophytic colonization in barley roots could be observed 12 weeks after inoculation with GFP-labeled N35 and N35v cells in the soil system (Fig. 3.8 e and f) without any obervious detectable damage to root cells. Endophytic bacteria are capable of

developing within the roots, where the bacteria have a more intimate contact to the host giving them a better chance of efficient signaling and nutrient exchange than on the surface and are also protected from biotic and abiotic stresses in the rhizosphere (Rosenblueth and Martinez-Romero, 2006; Whipps, 2001). Endophytic colonization has been recorded in many strains of PGPR. Endophyte *Azoarcus* sp. BH72 isolated from Kallar grass in Pakistan can fix N_2 in N-limited rice (Reinhold-Hurek *et al.*, 1993a; Reinhold-Hurek *et al.*, 1993b). *Azospirillum brasilense* Sp245 colonizes wheat roots endophytically and has a plant growth promoting effect on different wheat varieties (Rothballer *et al.*, 2003). Plant endophyte *Herbaspirillum frisingense* GSF30(T) produces indole-3-acetic acid and has ACC-deaminase activity (Rothballer *et al.*, 2008). However, it is still not clear if endophytic bacteria are better plant growth promoters than the rhizoplane bacteria (Rosenblueth and Martinez-Romero, 2006), but in any case endophytic colonization causes no substantial damage to plants (Hallmann *et al.*, 1997). Endophytes have more intimate contact to the host giving better chance of efficient signaling and nutrient exchange.

For the examination of competitive colonization behavior between the N35 and N35v, in addition to GFP, eYFP was applied for discrimination of the two types in co-inoculation experiments using CLSM. eYFP is an enhanced yellow-green fluorescence protein variant of GFP from *Aequorea victoria*. The fluorescence emission maximum of eYFP is 527 nm, which is clearly different from GFP with a maximum at 510 nm. The fluorescence intensity observed from eYFP is usually roughly equivalent to GFP (Clontech). However, because the fluorescence intensity of eYFP in chromosomal DNA of "*cand.* A. radicis" N35 and N35v was too low, the eYFP was cloned into a broad host range vector pBBR1MCS-2 (Fig. 3.6). This vector is known to be stable in a large number of hosts without antibiotic pressure (Kovach et al., 1995), so the constructed plasmid pBBR1MCS-2-eYFP was suitable for labeling N35 for the co-colonization investigation in a monoxenic system. The characteristic emission spectra of GFP and YFP labeled N35 and N35v could be differentiated using the λ-mode of the CLSM Meta (Fig. 3.7).

A competitive colonization behavior could be observed after co-inoculation of GFP and/or YFP-labeled wild type N35 and phenotype variant N35v, where wild type N35 cells were dominant on barley roots after 3 weeks growth in a monoxenic system (Fig. 3.9). This can be explained e. g. by the loss of motility of N35v due to the absence of flagella demonstrated by SEM analysis (Fig. 3.2 b) and its incapability of swarming (Fig. 3.1 c). The presence of flagella is required in rhizobacteria for the effective colonization of roots, as e. g. for *P. fluorescens* WCS374 colonization of potato roots (de Weger *et al.*, 1987).

Previous studies have shown that flagella mediated chemotaxis towards plant root exudates plays a major role in competitive root colonization of rhizobacteria. This chemotaxis driven colonization could be considered as a most important initial stage of colonization (de Weert *et al.*, 2002; Zhulin and Armitage, 1992).

Some studies have also shown that swarming is important for pathogenic bacteria in tissue colonization, e. g. *Proteus mirabilis* during urinary tissue colonization (Mobley and Belas, 1995). Swarming is bacterial group motility across a surface, which is driven by lateral bacterial flagella (Butler *et al.*, 2009; Harshey, 2003). For swarming on a surface more flagella are needed than for swimming in liquid medium (Harshey, 2003). If a bacterial colony grows on swarming medium, the cells differentiate into specialized swarmer cells, which are characterized by an increased number of elongated flagella (Alison and Hughes, 1991). Alexandera *et al.* (1999) considered that swarming across root surfaces may be responsible for long-term colonization of *A. lipoferum* 4B. According to this, swarming motility of cells in wild type N35 can be presumed as an additional mechanism of bacterial movement, which plays a role during colonization of barley roots. The variant phenotype N35v cells, which lack the flagella and swarming ability, are devoid of the ability to move on root surfaces and are therefore less competitive in barley root colonization.

Finally, wild type N35 was nutritionally also more versatile than the phenotype variant. Using a Biolog GN2 assay, the wild type N35 was found to readily metabolize fucose and formic acid as carbon source, whereas the phenotype variant N35v lost the ability to utilize these two substrates. Fucose is a monosaccharide in plant root secreted mucilage, present in varying ratios in different plants (Basic *et al.*, 1986). Formic acid is also one of the monocarboxylic acids which are possible constituents of plant root exudates, e. g. in spruce and birch root mucilage (Sandnes *et al.*, 2005). Fucose and formic acid provided by plant root exudates could be utilized as carbon sources by N35 and consequently wild type N35 is probably better adapted to the rhizosphere in comparison to phenotype variant N35v, which is another competitive advantage for root colonization.

4.5 Genetic mechanisms behind phenotypic variation of "*cand.* A. radicis" N35

To investigate the mechanisms and genetic basis behind this phenotypic variation of "*cand.* A. radicis" N35, the possible differences of both types at genetic level were investigated

using whole genome sequence data. Analyzing 454-sequence data of wild type and variant strain, the only difference was found in the C-terminal part of the *mutL* gene, which encodes the mismatch repair protein MutL. This protein is truncated in N35v, because of the frame shift caused by a 16 nucleotides deletion leading to the formation of a stop codon in the coding gene, while in N35 MutL protein is intact. Mismatch repair proteins MutS and MutL are evolutionarily conserved and are involved in DNA mismatch repair by correcting replication errors (Modrich and Lahue, 1996). In *E. coli*, MutS recognizes mispaired or mis-inserted bases and binds to MutL in the presence of ATP forming a MutS-MutL-DNA mismatch repair complex (Grilley *et al.*, 1989; Habraken *et al.*, 1998; Junop *et al.*, 2003; Schofield *et al.*, 2001).

MutL contains a highly conserved N-terminal ATPase domain and a less conserved C-teminal domain (Guarné *et al.*, 2004). The MutL C-terminal domain plays a key role for the homodimerization of MutL in prokaryotes, while the activity of MutL protein is regulated by the ATP dependant dimerization of MutL C-terminal domain (Kosinski *et al.*, 2005; Guarné *et al.*, 2004). In the "*cand.* A. radicis" *mutL* gene product, two domains were identified in both wild type N35 and phenotype variant N35v, namely HATase_c and DNA_mis_repair, while the third domain MutL_C (MutL C-terminal domain) was only detected in N35 not in N35v (Fig. 3.10). Because MutL C-terminal domain mediates the dimerization of MutL protein, the absence of MutL C-terminal domain abolishes the activity of MutL protein, which points towards a loss of DNA repair function in "*cand.* A. radicis" N35v. To verify that the impaired MutL protein was not an individual event randomly occurring in one studied colony, ten colonies of N35v were independently selected and the *mutL* gene region was sequenced. The results showed that all of the colonies of N35v had the same mutation in their *mutL* gene.

The mismatch repair system has been reported to influence the phase variation frequency in human pathogenic bacteria *Neisseria meningitidis*, where an inactivation of the *mutL* and *mutS* gene increases switching frequency (Richardson and Stojiljkovic, 2001). In another human pathogen, *Haemophilus influenzae*, mismatch repair genes influence the dinucleotide repeat-mediated phase variation rate (Bayliss *et al.*, 2004). In this study, the impaired mismatch repair protein MutL in N35v might be directly or indirectly responsible for the phenotypic variation in "*cand.* A. radicis" N35. The impaired MutL protein in N35v abolishes the mismatch repair function during DNA replication, which results in a high frequency of mutations in a given cell at one or more sites. If the mutation is advantageous for the bacteria, a new mutant colony (variant) can arise and the phenotypic traits of this

mutant are inherited to the next generation. Consequently, the impaired MutL protein could induce the other mutations, which allow the observed adaptation to the changed nutrient and colonization conditions. However, stable point mutations could not be found in N35v.
Changes in the expression of global regulator proteins leading to phase variation have also been reported in *Streptococcus pyogenes* (Bormann and Cleary, 1997) and *E. coli* (Blyn *et al.*, 1990). The changes of a single regulatory protein can lead to phase-variable expression of multiple cellular proteins through regulating initiation of transcription at the main promoter of the operon (van der Woude and Baumler, 2004). MutL also possesses a non-specific DNA-binding activity (Guarné *et al.*, 2004). This DNA-binding activity of MutL might lead to down-regulation of expression of multiple specific proteins resulting in phenotypic variation in "*cand.* A. radicis" N35. In this study, the regulation or repair mechanism of MutL might be responsible for the phenotypic variation of N35. However, the impaired repair mechanism of MutL usually leads to unspecific and undirected mutations, which did not match with the observed specific phenotype of N35v. Therefore, the phenotypic variation in "*cand.* A. radicis" N35 might be mediated by the MutL down-regulating of specific protein expression.
Further work is needed to investigate the possible mechanisms behind this *mutL* gene deletion. Two major questions arise: Is there any genetic factor(s) modulating this mutation of *mutL* gene region in wild type N35? Why does this mutation of *mutL* gene region always occur in the same position in N35?

4.6 Quorum sensing signals mediating rhizobacteria-plant interactions

Another aim of this study was to identify the compounds involved in rhizobacteria-plant interactions, and therefore the role of quorum sensing signals in "*cand.* A. radicis" N35 and *R. radiobacter* F4 and F7 was investigated in the association with their hosts. A LuxI/LuxR type quorum sensing system could be identified and characterized in "*cand.* A. radicis" N35 and N35v. The autoinducer synthase protein AraI and putative signal receptor AraR of N35 and N35v have a high similarity to the quorum sensing system of *Acidithiobacillus ferroxidans* ATCC 23270 with protein pair AfeI/AfeR (Barreto *et al.*, 2003; Farah *et al.*, 2005) and quorum sensing system of *R. solanacearum* GMI1000 (Salanoubat *et al.*, 2002). AfeI/AfeR is a LuxI/LuxR type quorum sensing system in

chemolithoautotrophic, gama-proteobacterium *Acidithiobacillus ferroxidans*. This microorganism produces various AHLs including also 3-OH-C10-HSL (Farah *et al.*, 2005), which is also the major AHL signal in "*cand.* A. radicis" N35 and N35v.

Some studies have shown that phase variation is directly or indirectly regulated by quorum sensing. LuxS in *Salmonella enterica* serovar Typhimurium, which catalyzes the synthesis of quorum sensing molecule AI-2, also regulates the flagellar phase variation, which is not influenced by the quorum sensing signals themselves (Karavolos *et al.*, 2008). The quorum sensing regulator ValR is involved in colony phase variation in *Vibrio alginolyticus* ZJ51 (Chang *et al.*, 2010). However, quorum sensing was not found to mediate the phenotypic variation in "*cand.* A. radicis" N35, as the two phenotypes produced nearly the same concentrations of 3-OH-C10-HSL during the exponential growth phase.

In recent years, many evidences have accumulated that a large number of plant-associated bacteria synthesize AHL signal molecules. Cha *et al.* (1998) have reported that the majority of plant-associated Gram-negative bacteria produces AHLs as signal molecules. Most strains of *Agrobacterium*, *Rhizobium*, *Pantoea* and half of the strains belonging to the *Erwinia* and *Pseudomonas* genera can synthesize detectable amounts of acyl-HSLs. Steidle *et al.* (2001) have screened over 300 strains isolated from the rhizosphere of tomato and the results showed that approximately 12% of the strains were able to activate one or more AHL biosensors. These studies point to the fact that quorum sensing signals play an important role in microbe-plant interactions. AHL mediated quorum sensing by rhizobacteria regulates the expression of a diversity of genes, which are involved in interactions between microbial populations and their plant hosts (Loh *et al.*, 2002; Pierson III *et al.*, 1998). Quorum sensing is involved in the biocontrol and plant growth promoting activity of bacteria towards their host plants. The PcoI/PcoR quorum sensing system of *P. fluorescens* 2P24 influences the rhizosphere competence, where the *pcoI*-minus mutant has a significant defect in its colonization ability on wheat roots (Wei and Zhang, 2006). LasI/R and PhlI/R quorum sensing systems in *P. aeruginosa* PUPa3 are involved in regulation of plant growth promotion and important for bacterial colonization of the rice rhizosphere (Steindler *et al.*, 2009). The result of co-inoculation of "*cand.* A. radicis" wild type N35 and its AHL negative mutant N35 ΔaraI showed that N35 wild type had a dominant colonization behavior on barley roots compared to N35 ΔaraI (Fig. 3.16). Altered cell flagellation and swarming ability, two traits determining rhizosphere competence and influenced by quorum sensing in other bacteria (Wei and Zhang 2006), could not provide an explanation for the observed AHL-negative mutant phenotype, as this was not found to

be regulated by quorum sensing in "*cand.* A. radicis" N35 (3.5.4.4). Instead, N35 might use quorum sensing signaling molecules not only as an intraspecies bacterial signal but also for an inter-kingdom communication to host plants. In *Serratia liquefaciens* MG1, the AHLs molecules act as mediators of communication between prokaryotes and eukaryotes (Schuhegger *et al.*, 2006). In roots of *Medicago truncatula*, more than 150 proteins of plants had responses to C12- to C16-HSLs (Mathesius *et al.*, 2003). The AraI/AraR quorum sensing system of "*cand.* A. radicis" N35 might play an important role in colonization of plant roots, where plants give a positive feedback to the quorum sensing signal produced by wild type N35 cells during the bacterial colonization of barley rhizosphere. The AHL negative mutant N35 ΔaraI would then suffer a severe disadvantage during root colonization. However, it remains a further challenge beyond the scope of this work to determine the exact mechanisms of quorum sensing regulated root colonization in "*cand.* A. radicis" N35 and the possible role of AHLs in the interaction with the plant.

In this context a second rhizobacterium, *R. radiobacter*, was investigated. The two tested strains *R. radiobacter* F4 and F7 have been isolated from plant growth promoting fungus *P. indica*. This endofungal *R. radiobacter* has a similar biocontrol activity as its host, which promotes plant growth and decreases powdery mildew pustules in barley (Sharma *et al.*, 2008). *R. radiobacter* F4 and F7 are able to produce a variety of C8- to C12- oxo- and hydroxyl-HSLs as quorum sensing signals (Table 3.4). AHL molecules produced by *Serratia liquefaciens* MG1 and *P. fluorescens* IsoF induced the systemic resistance of tomato plants against the fungal pathogen *Alternaria alternata* (Schuhegger *et al.*, 2006). AHL molecules can also modify the hormonal balance in *Arabidopsis* (von Rad *et al.*, 2008). Preliminary results of the corporation group of Prof. K.-H. Kogel at Uni. of Giessen showed that 3-OH-C14-HSL induced the systemic resistance of *Arabidopsis* against the shoot pathogen *P. syringae* DC3000. It is not yet known, if the quorum sensing signal molecules in *R. radiobacter* F4 and F7 could induce the systemic resistance against these pathogens; the analysis of induced systemic resistance using AHL-negative phenotypes, *R. radiobacter* F4 NM13 and F7 NM13, are required for future studies.

R. radiobacter F4 and F7 could produce *p*-coumaroyl-HSL, when *p*-coumaric acid was supplied in the medium (Fig. 3.20 and 3.21). *p*-coumaric acid (synonym *p*-coumarate) is a aromatic acid produced by plants as a major precursor compound of plant polymer lignin (Whetten and Sederoff, 1995). Plants produce also *p*-coumaric acid in stress conditions such as tissue damage (Dixon and Palva, 1995). Interestingly, it has been demonstrated, that plant derived *p*-coumaric acid can repress the expression of the type III secretion

system, which is a major virulence factor in many Gram-negative plant pathogens (Li *et al.*, 2009). As the production of *p*-coumaroyl-HSL in *R. radiobacter* F4 and F7 requires plant secreted *p*-coumaric acid as a source, a tight relationship might be present between *R. radiobacter* F4 and F7 and their host plants, and *p*-coumaroyl-HSL signaling might be involved in this relationship. It could also be possible, that the formation of coumaroyl-HSL from *p*-coumaric acid by *R. radiobacter* F4 and F7 may reduce inhibitory influences of *p*-coumaric acid in the rhizosphere towards *R. radiobacter* and its host *P. indica*. The production of *p*-coumaroyl-HSL using *p*-coumaric acid in *Rhodopseudomonas palustris*, *Bradyrhizobium* sp. BTAi1 and *Silicibacter pomeroyi* DSS3 has been first described by Schaefer *et al.* (2009). According to this study, *Bradyrhizobium* sp. BTAi1 is a photosynthetic, nitrogen fixing bacterium and can form root and stem nodules on *Aeschynomene* plant. As mentioned in 1.2.3, that quorum sensing signals can serve as a cross-kingdom communication signal to their hosts (Cugini *et al.*, 2007; Schuhegger *et al.*, 2006; von Rad *et al.*, 2008), the *p*-coumaroyl-HSL could possibly act as an inter-kingdom signal to plants and/or its original host *P. indica*. The role of the bacteria produced *p*-coumaroyl-HSL in plants is still unknown, however since coumaric acid is a natural compound of plant origin in the rhizosphere, this may represent a new group of signaling compounds with yet unknown function in plant-bacteria-fungus interactions. Indeed, it has been shown already that AHL compounds could interact with fungi in a specific manner. For example, quorum sensing molecule *cis*-2-dodecenoic acid (BDSF) produced by *Burkholderia cenocepacia* strongly inhibited germ tube formation in human pathogen *Candida albicans* (Boon *et al.*, 2007).

The lactonase expressing transformants F4 NM13 and F7 NM13, which are phenotypically AHL negative, were found to have abolished lipase and siderophore activity (Fig. 3.22 and 3.23). The production of extracelluar lipase in rice pathogen *B. glumae* AU6208 (Devescovi *et al.*, 2007) as well as siderophores in *B. cepacia* K56-2 (Lewenza *et al.*, 1999) and *B. ambifaria* BC-F (Zhou *et al.*, 2003) have been described to be also under the control of quorum sensing. As mentioned in 4.2, siderophores produced by PGPR are an important plant growth promoting effector (Kloepper *et al.*, 1991; Sharma and Johri, 2003). Extracellurlar lipase activity is a property related to the plant growth promoting effect of *P. fluorescens* RAF15 (Park *et al.*, 2009) and promoted the spore germination of biocontrol fungus *Nomuraea rileyi* MJ (Supakdamrongkul *et al.*, in press). According to these studies, quorum sensing signaling molecules regulating the production of siderophore and lipase

might contribute to the plant growth promoting effect of *R. radiobacter* F4 and F7 on barley.

A tight association of *R. radiobacter* F4 and F7 with *P. indica* has been found, as the *R. radiobacter* strains could not be cured from the host (Sharma *et al*., 2008). Thus, the endofungal bacterium could have an important function in the interaction with the fungus and the plant. The possibility is that the bacterium assists the fungus in its interaction with the plant by producing signaling molecules including a variety of AHLs and coumaroyl-HSLs which modify the plant response. The possible involvement of quorum sensing signals in the tripartite symbiosis of *P. indica*, *R. radiobacter* and barley derserves further studies.

4.7 Interactions of rhizobacteria and *Piriformospora indica*

Several rhizobacteria produce siderophores, antifungal metabolites, hydrogen cyanide (HCN), ammonia and lytic enzymes like chitinases and β-1,3-glucanases and have been implicated in suppression of fungal growth (Raaijmakers *et al.*, 2009). In this study, the intense interaction between plant growth promoting fungus *P. indica* and economically important rhizobacteria, namely plant growth enhancing rhizobacterium *B. amyloliquefaciens* FZB42 and biocontrol rhizobacterium *P. fluorescens* SS10 were investigated. While some rhizobacteria could promote growth and root colonization of the fungus or behave neutral in the interaction with *P. indica*, others sincerely inhibit its development.

In this study, *B. amyloliquefaciens* FZB42 and *P. fluorescens* SS10 were shown to inhibit the growth of *P. indica* (Fig. 3.17 a and b). Antibiotic bacillomycine and lipopeptides produced by *B. amyloliquefaciens* FZB42 and masstolide A excreted by *P. fluorescens* SS101 were shown to be responsible for the observed inhibition of *P. indica* (Table 3.3 and Fig. 3. 17).

Piriformospora indica has a plant growth promoting effect, which may be at least in part related to *R. radiobacter*, the constitutively associated endofungal bacterium (Sharma *et al.*, 2008). They benefit the plant in nutrient uptake, surviving under water, temperature and salt stress, inducing system resistance to pathogenic microorganisms (e.g. *Blumeria*) and stimulating seed production (Oelmueller *et al.*, 2009). Plant growth enhancing rhizobacteria *B. amyloliquefaciens* FZB42 is a commercially proposed PGPR (Abitep) and *P. fluorescens* SS101 has been also investigated for its biocontrol capability (Raaijmakers

et al., 2006). However, these biocontrol agents produce the antifungal metabolites which can also affect plant growth promoting fungi (Raaijmakers *et al.*, 2009). Plant growth promoting fungus *P. indica* is being exploited as biocontrol agent in the area of agriculture, forestry and field. Thus, when combined application of *P. indica* with other plant growth promoting rhizobacteria is desired, it should be tested beforehand, that an inhibitory effect of these rhizobacteria on *P. indica* does not occur.

5 Summary

The aim of this doctoral thesis was to investigate the factors relevant in plant interaction of two plant growth promoting rhizobacteria (PGPR). For this, the strain *Acidovorax* sp. N35 isolated from surface sterilized wheat roots and the two strains F4 and F7 of *Rhizobium radiobacter*, a bacterium associated with the plant growth promoting fungus *Piriformospora indica*, were chosen. First of all, the isolate N35 was characterized using phylogenetic and taxonomic methods. The 16S rRNA gene sequence analysis showed that strain N35 has the closest sequence similarities (98.2, 98.5 and 99.0 %) to the environmental *Acidovorax* species *A. delafieldii, A. facilis* and *A. defluvii*. The DNA-DNA hybridization values clearly separated the isolate from these three species. Additionally, phenotypic properties, such as substrate metabolization profiles as determined by a Biolog GN2 assay and cell wall fatty acid profiles concerning the fatty acids $C_{16:0}$, $C_{16:1\omega7cis/trans}$, $C_{17:0cyclo}$ and $C_{18:0cyclo}$ and $C_{19:0cyclo}$, facilitated the differentiation of the newly isolated strain N35 from its closest relatives. Thus, the strain N35 was classified as representative of a new species within the genus *Acidovorax,* and the name *Acidovorax radicis* sp. nov. is suggested.

"*Cand.* A. radicis" N35 undergoes an irreversible phenotypic variation, resulting in different colony shapes on agar plate. In soil system, both phenotypes showed a plant growth promoting effect both on barley roots and shoots. The wild type N35 (rough colony type) had a better plant growth promoting effect on barley in comparison with phenotype variant N35v (smooth colony type). Wild type and phenotype variant cells of "*cand.* A. radicis" N35 were labeled with GFP and/or YFP and their separate and co-colonization behavior was investigated in a monoxenic system and a soil system using a CLSM for detection. Both types of N35 could endophytically colonize barley roots after 12 weeks inoculation in the soil system. Competitive root colonization behavior was observed after co-inoculation with differentially labeled wild type N35 and phenotype variant N35v bacteria, where the wild type showed dominant colonization of barley roots compared to the phenotype variant. Moreover, the variant N35v lost its motility due to missing flagella and swarming ability. The differences of both types at genetic level were investigated using whole genome sequence data obtained from 454 pyrosequencing (Roche) using the GS FLX Titanium chemistry. As only difference in the genome sequence, a 16 nucleotides deletion was identified in the *mutL* gene, which encodes for the mismatch repair protein

MutL. In phenotype N35v, the frame shift caused by this deletion leads to the formation of a stop codon in the coding gene, resulting in a truncated MutL protein with a missing functional MutL C-terminal domain. This mutation occurred in exactly the same way in all investigated phenotype variants. These results suggest that MutL might be directly or indirectly responsible for the phenotypic variation in "*cand.* A. radicis" N35.

Quorum sensing signaling molecules produced by "*cand.* A. radicis" N35 were identified using biosensors as well as Fourier transform ion cyclotron resonance - mass spectrometry (FT-ICR-MS) and ultra performance liquid chromatography (UPLC). Both types of "*cand.* A. radicis" N35 possess the same AraI/AraR quorum sensing system, which belongs to the LuxI/LuxR type. The two N35 phenotypes produced nearly the same amount of 3-OH-C10-HSL in the exponential growth phase. A co-inoculation experiment of AHL producing wild type N35 and a constructed AHL negative mutant N35 ΔaraI showed that wild type N35 had a dominant colonization behavior compared to the AHL negative mutant on barley roots in a monoxenic system. These data indicate that quorum sensing is involved in regulation of root colonization by "*cand.* A. radicis" N35.

The second examined PGPR, *R. radiobacter*, which occurs naturally as endofungal bacterium in the plant growth promoting fungus *P. indica*, was demonstrated to colonize the surface of barley roots with fluorescence in *situ* hybridization (FISH) in a monoxenic system. The interaction of *P. indica* harboring *R. radiobacter* with other rhizobacteria was investigated using plate confrontation assays. Antibiotics and lipopeptides produced and excreted by the plant growth enhancing rhizobacterium *Bacillus amyloliquefaciens* FZB42 and the biocontrol rhizobacterium *Pseudomonas fluorescens* SS101 were shown to be responsible for the observed inhibition of *P. indica* by these bacteria. *R. radiobacter* F4 and F7 were able to synthesize a variety of oxo- and hydroxyl-C8- to C12-HSL compounds. In addition, both strains also produced coumaroyl-HSL when coumaric acid was supplied in the medium. The lactonase expressing transformants F4 NM13 and F7 NM13, which are the AHL negative phenotypes, abolished the lipase and siderophore activity. Considering this, quorum sensing influences the production of metabolites including lipase and siderophores in *R. radiobacte*r F4 and F7. Further work should be directed to the question whether quorum sensing also plays a role in the interaction of the bacterium with fungus and/or plant.

Zusammenfassung

Ziel der vorliegenden Arbeit war es die einflussnehmenden Faktoren bei der Pflanzeninteraktion zweier "plant growth promoting rhizobacteria" (PGPR) zu untersuchen. Dabei handelte es sich um den von oberflächensterilisierten Weizenwurzeln isolierten Stamm *Acidovorax* sp. N35, sowie um den mit dem Pilz *Piriformospora indica* assoziierten Stämme *Rhizobium radiobacter* F4 und F7. Zunächst wurde das Isolat N35 mittels phylogenetischer und taxonomischer Methoden charakterisiert. Die Sequenzanalyse des 16S rRNA Gens zeigte, dass Stamm N35 die höchsten Ähnlichkeiten (98.2, 98.5 und 99.0 %) mit den Sequenzen der Umweltisolate *A. delafieldii, A. facilis* and *A. defluvii* aufweist. Die Werte der DNA-DNA-Hybridisierung erlaubten eine klare Differenzierung des Isolats N35 von den drei Spezies. Des Weiteren konnte mittels phänotypischer Eigenschaften, wie der Metabolisierung unterschiedlicher Substrate in einer Biolog GN2 Analyse und Fettsäure-Profilen der Zellwände für die Fettsäuren $C_{16:0}$, $C_{16:1\omega7cis/trans}$, $C_{17:0cyclo}$, $C_{18:0cyclo}$ und $C_{19:0cyclo}$, eine Unterscheidung des neu isolierten Stamms N35 von seinen nächsten Verwandten erfolgen. Der Stamm N35 wurde daher als eine neue Spezies innerhalb der Gattung *Acidovorax* eingeordnet und der Name *Acidovorax radicis* sp. nov. vorgeschlagen.

Bei "*Cand.* A. radicis" N35 trat eine irreversible Phänotypenvariation auf, die zu unterschiedlichen Kolonieformen auf Agarplatten führte. Im Bodensystem zeigten beiden Typen eine wachstumsstimulierende Wirkung sowohl auf Gerstenwurzeln als auch auf den Spross. Der N35 Wildtyp (rauer Kolonie-Typ) hatte eine bessere wachstumsfördernde Wirkung auf Gerste als die Variante N35v (glatter Kolonie-Typ). Die Zellen von "*Cand.* A. radicis" N35 Wildtyp und der Phänotypenvariante wurden mit GFP und/oder YFP markiert und ihr Kolonisierungsverhalten separat wie auch zusammen im monoxenischen System und Bodensystem mittels konfokaler Laser-Scanning-Mikroskopie untersucht. Beide Typen von N35 konnten nach 12 Wochen im Bodensystem endophytisch in Gerstenwurzeln nachgewiesen werden. Eine kompetitive Wurzelbesiedlung konnte nach Co-Inokulierung mit den unterschiedlich markierten N35 Typen beobachtet werden, bei der der Wildtyp N35 eine dominante Besiedlung auf den Gerstenwurzeln zeigte. Außerdem verlor die Variante N35v ihre Motilität, da die Geißel und Fähigkeit zum Schwärmen fehlten. Die Unterschiede der beiden Typen auf genetischer Ebene wurden mit Hilfe von Sequenzdaten der gesamten Genome aufgeklärt, die mittels 454 Pyrosequenzierung (Roche) unter Anwendung der FLX Titanium Chemie erhalten wurden. Als einziger Unterschied in

der genomischen Sequenz wurde eine 16 Nukleotide lange Deletion im *mutL* Gen festgestellt, welches für das Mismatch-Reparatur-Protein MutL codiert. Die durch diese Deletion hervorgerufene Verschiebung des Leserahmens erzeugt ein Stopcodon im codierenden Gen, wodurch das MutL-Protein verkürzt wird, so dass die funktionelle MutL C-terminale Domäne fehlt. Die Mutation trat an gleicher Position in allen untersuchten Phänotypvarianten auf. Diese Ergebnisse deuten darauf hin, dass das MutL-Protein direkt oder indirekt verantwortlich für die phänotypische Variation bei "*cand.* A. radicis" N35 sein könnte.

Von "*cand.* A. radicis" N35 produzierte Quorum-Sensing Signalmoleküle wurden mittels Biosensoren sowie "Fourier Transform Ion Cyclotron Resonance - Mass Spectrometry" (FT-ICR-MS) and "Ultra Performance Liquid Chromatography" (UPLC) identifiziert. Beide Typen von "*cand.* A. radicis" N35 besitzen das gleichen AraI/AraR Quorum-Sensing System, welches zum Typ LuxI/LuxR gehört. Die beiden Typen von N35 produzierten fast die gleiche Menge 3-OH-C10-HSL in der exponentiellen Wachstumsphase. Ein Co-Inokulationsexperiment mit dem AHL-produzierenden Wildtyp N35 und der konstruierten AHL-negativen Mutante N35 ΔaraI zeigte, dass der Wildtyp N35 eine dominante Besiedlung im Vergleich mit der AHL-negativen Mutante N35 ΔaraI auf Gerstenwurzeln im monoxenischen System hatte. Diese Daten legen nahe, dass Quorum-Sensing an der Regulation der Wurzelbesiedlung durch "*cand.* A. radicis" N35 beteiligt ist.

Bei dem zweiten untersuchten PGPR, *R. radiobacter,* der als endofungisches Bakterium in der Natur mit dem Pflanzenwachstum stimulierenden Pilz *P. indica* vergesellschaftet ist, konnte mittels Fluoreszenz *in situ* Hybridisierung (FISH) die Fähigkeit zu Besiedlung von Wurzeloberflächen im monoxenischen System gezeigt werden. Die Interaktion von *R. radiobacter* besiedeltem *P. indica* mit anderen Rhizobakterien wurde mittels Platten-Konfrontations-Assay untersucht. Antibiotika und Lipopeptide, die von dem Pflanzenwachstum stimulierenden Rhizobakterium *Bacillus amyloliquefaciens* FZB42 und dem Biokontroll-Rhizobakterium *Pseudomonas fluorescens* SS101 produziert und ausgeschieden werden, erwiesen sich als ursächlich für die beobachtete Hemmung von *P. indica*. *R. radiobacter* F4 and F7 konnten eine Vielfalt von oxo- und hydroxyl-C8- bis C12-HSL Komponenten synthetisieren. Außerdem konnten die beiden Stämme Coumaroyl-HSL produzieren, wenn im Medium Coumarsäure vorhanden war. Die Lactonase-Transformanden F4 NM13 und F7 NM13, bei denen es sich um AHL negative Phänotypen handelt, zeigten keinerlei Lipase- und Siderophore-Aktivität mehr. Demnach

beeinflusst Quorum-Sensing die Produktion von Metaboliten und von Enzymen wie Lipase und Siderophore in *R. radiobacter* F4 und F7. Weitere Arbeiten sollten sich mit der Frage beschäftigen, ob Quorum-Sensing auch bei der Interaktion dieses Bakteriums mit Pilz und/oder Pflanze eine Rolle spielt.

6 References

Achouak, W., Conrod, S., Cohen, V. and Heulin, T. (2004). Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Molecular Plant-Microbe Interactions* **17**, 872-879.

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., MacDonald, I. J., Martin, K. M., Russo, T., Campagnari, A. A., Hujer, A. M., Bonomo, R. A. and Gill, S. R. (2008). Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *Journal of Bacteriology* **190**, 8053-8064.

Alexandre, G. and Bally, R. (1999). Emergence of a laccase-positive variant of *Azospirillum lipoferum* occurs via a two-step phenotypic switching process. *FEMS Microbiology Letters* **174**, 371-378.

Alison, C. and Hughes, C. (1991). Bacterial swarming: an example of prokaryotic differentiation and multicellular behaviour. *Science Progress* **75**, 403-422.

Allardet-Servent, A., Michaux-Charachon, S., Jumas-Bilak, E., Karayan, L. and Ramuz, M. (1993). Presence of one linear and one circular chromosome in the *Agrobacterium tumefaciens* C58 genome. *Journal of Bacteriology* **175**, 7869-7874.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Amann, R. I., Krumholz, L. and Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative and environmental studies in microbiology. *Journal of Bacteriology* **172**, 762-770.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbilogical Reviews* **59**, 143-169.

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. and Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and Environmental Microbiology* **64**, 2240-2246.

Artursson, V., Finlay, R. D. and Jansson, J. K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth *Environmental Microbiology* **8**, 1-10.

Aspray, T. J., Frey-Klett, P., Jones, J. E., Whipps, J. M., Garbaye, J. and Bending, G. D. (2006). Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. *Mycorrhiza* **16**, 533-541.

Baldani, V. L. D., Baldani, J. I. and Döbereiner, J. (1987). Inoculation of field grown wheat with *Azospirillum brasilense* spp. *Biology and Fertility of soils* **4**, 37-40.

Barber, C. E., Tang, J. L., Feng, J. X., Pan, M. Q., Wilson, T. J. G., Slater, H., Dow, J. M., Williams, P. and Daniels, M. J. (1997). A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Molecular Microbiology* **24**, 555-566.

Barreto, M., Quatrini, R., Bueno, S., Arriagada, C., Valdes, J., Silver, S. J., Jedlicki, E. and Holmes, D. S. (2003). Aspects of the predicted physiology of *Acidithiobacillus ferrooxidans* deduced from an analysis of its partial genome sequence. *Hydrometallurgy* **71**, 97-105.

Basic, A., Moody, S. F. and Clarke, A. E. (1986). Structure analysis of secreted root slime from maize (*Zea mays* L.). *Plant Physiology* **80**, 771-777.

Bassler, B. L., Wright, M., Showalter, R. E. and Silverman, M. R. (1993). Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Molecular Microbiology* **9**, 773-786.

Bassler, B. L., Wright, M. and Silverman, M. R. (1994). Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Molecular Microbiology* **13**, 273-286.

Bayliss, C. D., Field, D. and Moxon, E. R. (2001). The simple sequence contingency loci of *Haemophilus influenzae* and *Neisseria meningitidis*. *The Journal of Clinical Investigation* **107**, 657-666.

Bayliss, C. D., Sweetman, W. A. and Moxon, E. R. (2004). Mutations in *Haemophilus influenzae* mismatch repair genes increase mutation rates of dinucleotide repeat tracts but not dinucleotide repeat-driven pilin phase variation rates. *Journal of Bacteriology* **186**, 2928-2935.

Beattie, G. A. (2007). Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: *Plant-associated bacteria*. Edited by S. S. Gnanamanickam, eds. Springer, Dordrecht.

Benhamou, N., Kloepper, J. W., Quadt-Hallman, A. and Tuzun, S. (1996). Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. *Plant Physiology* **112**, 919-929.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**, 293-300.

Bertaux, J., Schmid, M., Hutzler, P., Hartmann, A., Garbaye, J. and Frey-Klett, P. (2005). Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Environmental Microbiology* **7**, 1786-1795.

Bertaux, J., Schmid, M., Prevost-Boure, N. C., Churin, J. L., Hartmann, A., Garbaye, J. and Frey-Klett, P. (2003). In situ identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Applied and Environmental Microbiology* **69**, 4243-4248.

Bianciotto, V., Minerdi, D., Perotto, S. and Bonfante, P. (1996). Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria *Protoplasma* **193**, 123-131.

Blyn, L. B., Braaten, B. A. and Low, D. A. (1990). Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. *the EMBO Journal* **9**, 4045-4054.

Boon, C., Deng, Y., Wang, L.-H., He, Y., Xu, J.-L., Fan, Y., Pan, S. Q. and Zhang, L.-H. (2007). A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *ISME Journal* **2**, 27-36.

Bormann, N. E. and Cleary, P. P. (1997). Transcriptional analysis of *mga*, a regulatory gene in *Streptococcus pyogenes*: identification of monocistronic and bicistronic transcripts that phase vary. *Gene* **200**, 125-134.

Brosius, J., Dull, T. L., Sleeter, D. D. and Noller, H. F. (1981). Gene organization of primary structure of a ribosomal operon from *Escherichia coli*. *Journal of Molecular Biology* **148**, 107-127.

Butler, M. T., Wang, Q. and Harshey, R. M. (2009). Cell density and mobility protect swarming bacteria against antibiotics. *Proceedings of the National Academy of Sciences USA* **107**, 3776-3781.

Cabrejos, M.-E., Zhao, H.-L., Guacucano, M., Bueno, S., Levican, G., Garcia, E., Jedlicki, E. and Holmes, D. S. (1999). IST1 insertional inactivation of the *resB* gene: implications for phenotypic switching in *Thiobacillus ferrooxidans*. *FEMS Microbiology Letters* **175**, 223-229.

Carpenter-Boggs, L., Loynachan, T. E. and Stahl, P. D. (1995). Spore germination of *Gigaspora margarita* stimulated by volatiles of soil-isolated actinomycetes. *Soil Biology and Biochemistry* **27**, 1445-1451.

Cashion, P., Hodler-Franklin, M. A., McCully, J. and Franklin, M. (1977). A rapid method for base ratio determination of bacterial DNA. *Analytical Biochemistry* **81**, 461-466.

Cha, C., Gao, P., Chen, Y.-C., Shaw, P. D. and Farrand, S. K. (1998). Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Molecular Plant-Microbe Interactions* **11**, 1119-1129.

Chang, C., Jing-Jing, Z., Chun-Hua, R. and Chao-Qun, H. (2010). Deletion of *valR*, a homolog of *Vibrio harveyi's luxR* generates an intermediate colony phenotype between opaque/rugose and translucent/smooth in *Vibrio alginolyticus*. *Biofouling: The Journal of Bioadhesion and Biofilm Research* **26**, 595 - 601.

Chebotar, V. K., Asis Jr, C. A. and Akao, S. (2001). Production of growth-promoting substances and high colonization ability of rhizobacteria enhance the nitrogen fixation of soybean when coinoculated with *Bradyrhizobium japonicum Biology and Fertility of soils* **34**, 427-432.

Chen, X.-H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., Koumoutsi, A., Hitzeroth, G., Grammel, N., Strittmatter, A. W., Gottschalk, G., Sussmuth, R. D. and Borriss, R. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *Journal of Bacteriology* **188**, 4024-4036.

Contreras-Cornejo, H. A., Macias-Rodriguez, L., Cortes-Penagos, C. and Lopez-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiology* **149**, 1579-1592.

Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33-38.

Crowley, D. E. and Kraemer, S. M. (2007). Function of siderophores in the plant rhizosphere. In: *The Rhizosphere: Biochemistry and organic substances at the soil plant interface*, pp. 173-200. Edited by P. Pinto, Z. Varanini and P. Nannipieri, eds. CRC Press, Boca Raton.

Cugini, C., Calfee, M. W., Farrow III, J. M., Morales, D. K., Pesci, E. C. and Hogan, D. A. (2007). Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Molecular Microbiology* **65**, 896-906.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology* **22**, 434-444.

Daniels, R., Reynaert, S., Hoekstra, H., Verreth, C., Janssens, J. K., Braeken, K., Fauvart, M., Beullens, S., Heusdens, C., Lambrichts, I., De Vos, D. E., Vanderleyden, J., Vermant, J. and Michiels, J. (2006). Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *Proceedings of the National Academy of Sciences USA* **103**, 14965-14970.

De Ley, J., Cattoir, H. and Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *European Journal of Biochemistry* **12**, 133-142.

de Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., De Mot, R. and Lugtenberg, B. J. J. (2002). Flagella-Driven Chemotaxis Towards Exudate Components Is an Important Trait for Tomato Root Colonization by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions* **15**, 1173-1180.

de Weger, L. A., van Boxtel, R., van der Burg, B., Gruters, R. A., Geels, F. P., Schippers, B. and Lugtenberg, B. (1986). Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing *Pseudomonas* spp. *Journal of Bacteriology* **165**, 585-594.

de Weger, L. A., van der Vlugt, C. I., Wijfjes, A. H., Bakker, P. A., Schippers, B. and Lugtenberg, B. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *Journal of Bacteriology* **169**, 2769-2773.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. and Lugtenberg, B. J. J. (1998). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proceedings of the National Academy of Sciences USA* **95**, 7051-7056.

Devescovi, G., Bigirimana, J., Degrassi, G., Cabrio, L., LiPuma, J. J., Kim, J., Hwang, I. and Venturi, V. (2007). Involvement of a quorum-sensing-regulated lipase secreted by a clinical Isolate of *Burkholderia glumae* in severe disease symptoms in rice. *Applied and Environental Microbiology* **73**, 4950-4958.

Dixon, R. A. and Palva, N. L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell* **7**, 1085-1097.

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988). High efficiency transfermation of *Escherichia coli* by high voltage electroporation. *Nucleic Acids Research* **16**, 6127-6145.

Eberl, L., Winson, M. K., Sternberg, C., Stewart, G. S. A. B., Christiansen, G., Chhabra, S. R., Bycroft, B., Williams, P., Molin, S. and Givskov, M. (1996). Involvement of *N*-acyl-l-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Molecular Microbiology* **20**, 127-136.

Englmann, M., Fekete, A., Kuttler, C., Frommberger, M., Li, X., Gebefügi, I., Fekete, J. and Schmitt-Kopplin, P. (2007). The hydrolysis of unsubstituted N-acylhomoserine lactones to their homoserine metabolites. Analytical approaches using ultra performance liquid chromatography. *Journal of Chromatography A* **1160**, 184-193.

Escobar, M. A. and Dandekar, A. M. (2003). *Agrobacterium tumefaciens* as an agent of disease. *Trends in Plant Science* **8**, 380-386.

Farah, C., Vera, M., Morin, D., Haras, D., Jerez, C. A. and Guiliani, N. (2005). Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. *Applied and Environmental Microbiology* **71**, 7033-7040.

Fekete, A., Frommberger, M., Rothballer, M., Li, X., Englmann, M., Fekete, J., Hartmann, A., Eberl, L. and Schmitt-Kopplin, P. (2007). Identification of bacterial N-acylhomoserine lactones (AHLs) with a combination of ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors. *Analytical and Bioanalytical Chemistry* **387**, 455-467.

Fekete, A., Rothballer, M., Hartmann, A. and Schmitt-Kopplin, P. (2010). Identification of Bacterial Autoinducers - *Methods Chapter*. In: *Bacterial Signaling*, pp. 95-111. Edited by R. Krämer and K. Jung, eds. Wiley-VCH Verlag, Weinheim.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368-376.

Figurski, D. H. and Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences USA* **76**, 1648-1652.

Frank, J. F. and Yousef, A. E. (2004). Tests for groups of microorganisms. In: *Standard methods for the examination of dairy products*, pp. 227-226. Edited by J. F. Frank and H. M. Wehr, eds. American Public Health Association, Washington DC.

Frey-Klett, P. and Garbaye, J. (2005). Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal-bacterial interactions. *New Phytologist* **168**, 4-8.

Fuqua, C. and Greenberg, E. P. (2002). Listening in on bacteria: acyl-homoserine lactone signaling. *Nature Reviews: Molecular Cell Biology* **3**, 685-695.
Fuqua, C., Parsek, M. R. and Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annual Review of Genetics* **35**, 439-468.
Fuqua, W. C. and Winans, S. C. (1994). A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *Journal of Bacteriology* **176**, 2796-2806.
Fuqua, W. C., Winans, S. C. and Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* **176**, 269-275.
Garbaye, J. (1994). Helper bacteria: a new dimension to the mycorrhizal symbiosis (Tansley Review, 76). *New Phytologist* **128**, 197-210.
Garbeva, P., van Overbreek, L. S., van Vuurde, J. W. L. and van Elsas, J. D. (2001). Analysis of endophytic bacterial communities of potato by plating and denaturating gradient gel electrophoresis (DGGE) of potato of 16S rDNA based PCR fragments. *Microbial Ecology* **41**, 369-383.
Gardan, L., Dauga, C., Prior, P., Gillis, M. and Saddler, G. (2000). *Acidovorax anthurii* sp. nov., a new phytopathogenic bacterium which causes bacterial leaf-spot of anthurium. *International Journal of Systematic and Evolutionary Microbiology* **50**, 235-246.
Gardan, L., Stead, D. E., Dauga, C. and Gillis, M. (2003). *Acidovorax valerianellae* sp. nov., a novel pathogen of lamb's lettuce [*Valerianella locusta* (L.) Laterr.]. *International Journal of Systematic and Evolutionary Microbiology* **53**, 795-800.
Gelvin, S. B. (2003). *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiololgy and Molecular Biology Reviews* **67**, 16-37.
Givskov, M., Olsen, L. and Molin, S. (1988). Cloning and expression in *Escherichia coli* of the gene for extracellular phospholipase A1 from *Serratia liquefaciens*. *Journal of Bacteriology* **170**, 5855-5862.
Glick, B. R., Patten, C. L., Holguin, G. and Pebrise, D. M. (1999). *Biochemical and genetic mechanisms used by plant growth-promoting bacteria*. London: Imperial College Press.
Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B. S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, C. and Slater, S. (2001). Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**, 2323-2328.
Grilley, M., Welsh, K. M., Su, S. S. and Modrich, P. (1989). Isolation and characterization of the *Escherichia coli mutL* gene product. *Journal of Biological Chemistry* **264**, 1000-1004.
Guarné, A., Ramon-Maiques, S., Wolff, E. M., Ghirlando, R., Hu, X., Miller, J. H. and Yang, W. (2004). Structure of the MutL C-terminal domain: a model of intact MutL and its roles in mismatch repair. *the EMBO Journal* **23**, 4134-4145.
Habraken, Y., Sung, P., Prakash, L. and Prakash, S. (1998). ATP-dependent assembly of a ternary complex consisting of a DNA mismatch and the yeast MSH2-MSH6 and MLH1-PMS1 protein complexes. *Journal of Biological Chemistry* **273**, 9837-9841.
Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F. and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* **43**, 895-914.
Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Molecular Microbiology* **166**, 557-580.

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annual review of microbiology* **57**, 249-273.
Hartmann, A., Rothballer, M. and Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil* **312**, 7-14.
Hartmann, A., Schmid, M., van Tuinen, D. and Berg, G. (2009). Plant-driven selection of microbes. *Plant and Soil* **321**, 235-257.
Henderson, I. R., Owen, P. and Nataro, J. P. (1999). Molecular switches - the ON and OFF of bacterial phase variation. *Molecular Microbiology* **33**, 919-932.
Heylen, K., Lebbe, L. and De Vos, P. (2008). *Acidovorax caeni* sp. nov., a denitrifying species with genetically diverse isolates from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* **58**, 73-77.
Hiltner, L. (1904). Über neue Erfahrung und Problems auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. *Ara Dtsch Landwirt Gellschaft* **98**, 59-78.
Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. and Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77-86.
Holden, M. T. G., Titball, R. W., Peacock, S. J. and Parkhill, J. (2004). Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences USA* **101**, 14240-14245.
Hood, D. W., Deadman, M. E., Jennings, M. P., Bisercic, M., Fleischmann, R. D., Venter, J. C. and Moxon, E. R. (1996). DNA repeats identify novel virulence genes in *Haemophilus influenzae*. *Proceedings of the National Academy of Sciences USA* **93**, 11121-11125.
Hu, F.-P. and Young, J. M. (1998). Biocidal activity in plant pathogenic *Acidovorax*, *Burkholderia*, *Herbaspirillum*, *Ralstonia* and *Xanthomonas* spp. *Journal of Applied Microbiology* **84**, 263-271.
Huss, V. A. R., Festl, H. and Schleifer, K.-H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Systematic and Applied Microbiology* **4**, 184-192.
Hwang, I., Cook, D. M. and Farrand, S. K. (1995). A new regulatory element modulates homoserine lactones-mediated autoinducer of Ti plasmid conjugal transfer. *Journal of Bacteriology* **177**, 449-458.
Hwang, I., Li, P. L., Zhang, L., Piper, K. P., Cook, D. M., Tate, M. E. and Farrand, S. K. (1994). TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer *Proceedings of the National Academy of Sciences USA* **91**, 4639-4643.
Junop, M. S., Yang, W., Funchain, P., Clendenin, W. and Miller, J. H. (2003). *In vitro* and *in vivo* studies of MutS, MutL and MutH mutants: correlation of mismatch repair and DNA recombination. *DNA Repair* **2**, 387-405.
Karavolos, M. H., Bulmer, D. M., Winzer, K., Wilson, M., Mastroeni, P., Williams, P. and Khan, C. M. A. (2008). LuxS affects flagellar phase variation independently of quorum sensing in *Salmonella enterica* serovar Typhimurium. *Journal of Bacteriology* **190**, 769-771.
Katiyar, V. and Goel, R. (2004). Siderophore mediated plant growth promotion at low temperature by mutant of fluorescent pseudomonad. *Plant Growth Regulation* **42**, 239-244.
Katupitiya, S., Millet, J., Vesk, M., Viccars, L., Zeman, A., Lidong, Z., Elmerich, C. and Kennedy, I. R. (1995). A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. *Applied and Environental Microbiology* **61**, 1987-1995.

Kessler, B., de Lorenzo, V. and Timmis, K. N. (1992). A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Molecular Genetics and Genomics* **233**, 293-301.

Klein, I. (2003). Besiedlung von Weizen- und Gerstenwurzeln durch endophytisch Rhizophärenbakterien. In Mikrobiology, pp. 100. Munich: Ludwig-Maximilians-Universität München.

Kloepper, J. W., Leong, J., Teintze, M. and Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**, 885-886.

Kloepper, J. W. and Schroth, M. N. (1978). Plant growth promoting rhizobacteria on radishes. In: *Proceedings of the fourth International Conference on Plant Pathogenic Bacteria*, pp. 879-892, Argers, France: Station de Pathologie Vegetale et Phytobacteriologyie, INRA.

Kloepper, J. W., Zablokovicz, R. M., Tipping, E. M. and Lifshitz, R. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizer. In: *The rhizosphere and plant growth* pp. 315-326. Edited by D. L. Keister and P. B. Cregan, eds. Kluwer Academic Publishers, The Netherlands.

Kosinski, J., Steindorf, I., Bujnicki, J. M., Giron-Monzon, L. and Friedhoff, P. (2005). Analysis of the quaternary structure of the MutL C-terminal Domain. *Journal of Molecular Biology* **351**, 895-909.

Koumoutsi, A., Chen, X.-H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *Journal of Bacteriology* **186**, 1084-1096.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II. and Peterson, K. M. (1995). Four new derivatives of the broad-host-range vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-176.

Kristensen, C. S., Eberl, L., Sanches-Romero, J. M., Givskov, M., Molin, S. and de Lorenzo, V. (1995). Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. *Journal of Bacteriology* **177**, 52-58.

Lawrence, J. R., Korber, D. R., Wolfaardt, G. M., Caldwell, D. E. and Neu, T. R. (2001). Analytical imaging and microscopy techniques. In: *Manual of environmental microbiology*, pp. 39-61. Edited by C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney and L. D. Stetzenbach, eds. American Society for Microbiology, Washington, DC.

Lawrence, R. C., Fryer, T. F. and Reiter, B. (1967). Rapid method for the quantitative estimation of microbial iipases. *Nature* **213**, 1264-1265.

Lewenza, S., Conway, B., Greenberg, E. P. and Sokol, P. A. (1999). Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI Homologs CepRI. *Journal of Bacteriology* **181**, 748-756.

Li, X., Fekete, A., Englmann, M., Gotz, C., Rothballer, M., Frommberger, M., Buddrus, K., Fekete, J., Cai, C., Schroder, P., Hartmann, A., Chen, G. and Schmitt-Kopplin, P. (2006). Development and application of a method for the analysis of *N*-acylhomoserine lactones by solid-phase extraction and ultra high pressure liquid chromatography. *Journal of chromatography* **1134**, 186-193.

Li, Y., Peng, Q., Selimi, D., Wang, Q., Charkowski, A. O., Chen, X. and Yang, C.-H. (2009). The plant phenolic compound *p*-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. *Applied and Environmental Microbiology* **75**, 1223-1228.

Loh, J., Pierson, E. A., Pierson III, L. S., Stacey, G. and Chatterjee, A. (2002). Quorum sensing in plant-associated bacteria. *Current Opinion in Plant Biology* **5**, 285-290.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. and Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554-568.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K. H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Research* **32**, 1363-1371.

M'piga, P., Belanger, R. R., Paulitz, T. C. and Benhamou, N. (1997). Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. *Physiological and Molecular Plant Pathology* **50**, 301-320.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclass of *Proteobacteria*: problems and solutions. *Systematic and Applied Microbiology* **15**, 593-600.

Martinez-Granero, F., Capdevila, S., Sanchez-Contreras, M., Martin, M. and Rivilla, R. (2005). Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*. *Microbiology* **151**, 975-983.

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anollés, G., Rolfe, B. G. and Bauer, W. D. (2003). Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proceedings of the National Academy of Sciences USA* **100**, 1444-1449.

Maxon, E. R., Rainey, P. B., Nowak, M. A. and Lenski, R. E. (1994). Adaptive evolution of highly mutatble loci in pathogenic bacteria. *Current Biology* **4**, 24-33.

Mazzola, M., Zhao, X., Cohen, M. F. and Raaijmakers, J. M. (2007). Cyclic lipopeptide surfactant production by *Pseudomonas fluorescens* SS101 is not required for suppression of complex *Pythium* spp. populations. *Phytopathology* **97**, 1348-1355.

Mergaert, J. and Swings, J. (1996). Biodiversity of microorganisms that degrade bacterial and synthetic polyesters *Journal of Industrial Microbipology and Biotechnology* **17**, 463-469.

Mistry, J., Bateman, A. and Finn, R. (2007). Predicting active site residue annotations in the Pfam database. *BMC Bioinformatics* **8**, 298.

Mobley, H. L. and Belas, R. (1995). Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends in Microbiology* **3**, 280-284.

Modrich, P. and Lahue, R. (1996). Mismatch Repair in Replication Fidelity, Genetic Recombination, and Cancer Biology. *Annual Review of Biochemistry* **65**, 101-133.

Nagel, M. and Andreesen, J. R. (1989). Molybdenum-dependent degradation of nicotinic acid by Bacillus sp. DSM 2923. *FEMS Microbiolology Letters* **59**, 147-152.

Nealson, K. H. (1977). Autoinduction of bacterial luciferase. *Archives of Microbiology* **112**, 73-79.

Neilands, J. B. and Leong, S. A. (1986). Siderophores in relation to plant growth and disease. *Annual Review of Plant Physiology* **37**, 187-208.

Ng, W.-L. and Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annual Review of Genetics* **43**, 197-222.

Nybroe, O., Brandt, K. K., Nicolaisen, M. H. and Sørinson, J. (2007). Methods to detect and quantify bacteria in soil. In: *Modern soil microbilogy*, pp. 283-316. Edited by J. Dirk van Elsas, J. K. Janssens and J. T. Trevors, eds. CRC Press, Boca Raton.

Oelmueller, R., Sherameti, I., Tripathi, S. and Varma, A. (2009). *Piriformospora indica*: a novel multifunctional symbiotic fungus. *Symbiosis* **49**, 1-18.
Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R. and Stahl, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annual review of microbiology* **40**, 337-365.
Olsen, G. J., Matsuda, H., Hagstrom, R. and Overbeek, R. (1994). fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applications in the Biosciences* **10**, 41-48.
Owen, A. and Zdor, R. (2001). Effect of cyanogenic rhizobacteria on the growth of velvetleaf (*Abutilon theophrasti*) and corn (*Zea mays*) in autoclaved soil and the influence of supplemental glycine. *Soil Biology and Biochemistry* **33**, 801-809.
Park, K. H., Lee, C. Y. and Son, H. J. (2009). Mechanism of insoluble phosphate solubilization by *Pseudomonas fluorescens* RAF15 isolated from ginseng rhizosphere and its plant growth-promoting activities. *Letters in Applied Microbiology* **49**, 222-228.
Park, S. F., Purdy, D. and Leach, S. (2000). Localized reversible frameshift mutation in the *flhA* gene confers phase variability to flagellin gene expression in *Campylobacter coli*. *Journal of Bacteriology* **182**, 207-210.
Parkhill, J., Achtman, M., James, K. D., Bentley, S. D., Churcher, C., Klee, S. R., Morelli, G., Basham, D., Brown, D., Chillingworth, T., Davies, R. M., Davis, P., Devlin, K., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Leather, S., Moule, S., Mungall, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Simmonds, M., Skelton, J., Whitehead, S., Spratt, B. G. and Barrell, B. G. (2000). Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* **404**, 502-506.
Pesci, E. C., Milbank, J. B. J., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. and Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonasaeruginosa*. *Proceedings of the National Academy of Sciences USA* **96**, 11229-11234.
Pierson III, L. S., Wood, D. W. and Pierson, E. A. (1998). Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annual Review of Phytopathology* **36**, 207-225.
Piper, K. R., Beck von Bodmann, S., Hwang, I. and Farrand, S. K. (1999). Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. *Molecular Microbiology* **32**, 1077-1089.
Podile, A. R. and Kishore, G. K. (2007). Plant growth-promoting rhizobacteria. In: *Plant-associated bacteria*, pp. 195-230. Edited by S. S. Gnanamanickam, eds. Springer, Dordrecht.
Poulsen, P. H. B., Hansen, L. H. and Sørensen, S. J. (2007). Biosensors to monitor soil health or toxicity. In: *Modern soil microbilogy*, pp. 435-454. Edited by J. Dirk van Elsas, J. K. Jansson and J. T. Trevors, eds. CRC Press, Boca Raton.
Poussier, S., Thoquet, P., Trigalet-Demery, D., Barthet, S., Meyer, D., Arlat, M. and Trigalet, A. (2003). Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from non-pathogenic to pathogenic forms via alterations in the *phcA* gene. *Molecular Microbiology* **49**, 991-1003.
Raaijmakers, J. M., de Bruijn, I. and de Kock, M. J. D. (2006). Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Molecular Plant-Microbe Interactions* **19**, 699-710.
Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., C., A. and Moenne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms *Plant and Soil* **321**, 341-361.
Rambelli, A. (1973). The rhizosphere of mycorrhizae. In: *Ectomycorrhizae*, pp. 299-343. Edited by L. Mg and T. T. Koslowski, eds. Academic Press, New York.

Reinhold-Hurek, B., Hurek, T., Claeyssens, M. and van Montagu, M. (1993a). Cloning, expression in *Escherichia coli*, and characterization of cellulolytic enzymes of *Azoarcus* sp., a root-invading diazotroph. *Journal of Bacteriology* **175**, 7056-7065.

Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K. and DeLey, J. (1993b). *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *International Journal of Systematic Bacteriology* **43**, 574-584.

Richardson, A. R. and Stojiljkovic, I. (2001). Mismatch repair and the regulation of phase variation in *Neisseria meningitidis*. *Molecular Microbiology* **40**, 645-655.

Rosenblueth, M. and Martinez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions* **19**, 827-837.

Rossello-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *FEMS microbiology reviews* **25**, 39-67.

Rothballer, M., Eckert, B., Schmid, M., Fekete, A., Schloter, M., Lehner, A., Pollmann, S. and Hartmann, A. (2008). Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*. *FEMS Microbiology Ecology* **66**, 85-95.

Rothballer, M., Schmid, M. and Hartmann, A. (2003). *In situ* localization and PGPR-effect of *Azospirillum brasilense* strains colonizing roots of different wheat varieties. *Symbiosis* **34**, 261-279.

Rovira, A. D. and Davey, C. B. (1974). Biology of the rhizosphere. In: *The plant root and its environment*, pp. 153-204. Edited by E. W. Carson, eds. University of Virginia Press, Charlottesville.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. and Boucher, C. A. (2002). Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* **415**, 497-502.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, New York, USA Cold Spring Harbor Loboratory Press.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. and Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfafa root colonization by *Pseuodmonas fluorescens*. *Journal of Bacteriology* **184**.

Sandnes, A., Eldhuset, T. D. and Wollebæk, G. (2005). Organic acids in root exudates and soil solution of Norway spruce and silver birch. *Soil Biology and Biochemistry* **37**, 259-269.

Saunders, N. J., Moxon, E. R. and Gravenor, M. B. (2003). Mutation rates: estimating phase variation rates when fitness differences are present and their impact on population structure. *Microbiology* **149**, 485-495.

Schaad, N. W., Postnikova, E., Sechler, A., Claflin, L. E., Vidaver, A. K., Jones, J. B., Agarkova, I., Ignatov, A., Dickstein, E. and Ramundo, B. A. (2008). Reclassification of subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Systematic and Applied Microbiology* **31**, 434-446.

Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Juhaszova, K., Sufrin, J. R. and Harwood, C. S. (2008). A new class of homoserine lactone quorum-sensing signals. *Nature* **454**, 595-599.

Schmid, M., Rothballer, M., Aßmus, B., Hutzler, P., Schloter, M. and Hartmann, A. (2004). Detection of microbes by confocal laser scanning microscopy. In: *Molecular Microbial Ecology Manual II*, pp. 875-910. Edited by G. A. Kowalchuk, F. H. de Bruijn, I. M. Head, A. D. L. Akkermans and J. D. van Elsas, eds. Kluwer Academic Publishers, Dordrecht.

Schmid, M., Rothballer, M. and Hartmann, A. (2007). Analysis of microbial communities in soil microhabitats using fluorescence *in situ* hybridization. In: *Modern soil microbilogy*, pp. 317-335. Edited by J. Dirk van Elsas, J. K. Jansson and J. T. Trevors, eds. CRC Press, Boca Raton.

Schofield, M. J., Nayak, S., Scott, T. H., Du, C. and Hsieh, P. (2001). Interaction of *Escherichia coli* MutS and MutL at a DNA mismatch. *Journal of Biological Chemistry* **276**, 28291-28299.

Schrader, J. A. and Holmes, D. S. (1988). Phenotypic switching of *Thiobacillus ferrooxidans*. *J Bacteriol* **170**, 3915-3923.

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A. and Langebartels, C. (2006). Induction of systemic resistance in tomato by *N*-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant, Cell and Environment* **29**, 909-918.

Schulze, R., Spring, S., Amann, R., Huber, I., Ludwig, W., Schleifer, K. H. and Kampfer, P. (1999). Genotypic diversity of *Acidovorax* strains isolated from activated sludge and description of *Acidovorax defluvii* sp. nov. *Systematic and Applied Microbiology* **22**, 205-214.

Schwyn, B. and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**, 47-56.

Sharma, A. and Johri, B. N. (2003). Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. *Microbiological Research* **158**, 243-248.

Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., Kampfer, P., Domann, E., Schafer, P., Hartmann, A. and Kogel, K. H. (2008). Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. *Cellular Microbiology* **10**, 2235-2246.

Somers, E., Vanderleyden, J. and Srinivasan, M. (2004). Rhizosphere bacterial signaling: a love parade beneath our feet. *Critical Review in Microbiology* **30**, 205-240.

Sørensen, J. and Sessitch, A. (2007). Plant-associated bacteria-lifestyle and molecular interactions. In: *Modern soil microbilogy*, pp. 211-236. Edited by J. Dirk van Elsas, J. K. Jansson and J. T. Trevors, eds. CRC Press, Boca Raton.

Steidle, A., Allesen-Holm, M., Riedel, K., Berg, G., Givskov, M., Molin, S. and Eberl, L. (2002). Identification and characterization of an N-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Applied and Environmental Microbiology* **68**, 6371-6382.

Steidle, A., Sigl, K., Schuhegger, R., Ihring, A., Schmid, M., Gantner, S., Stoffels, M., Riedel, K., Givskov, M., Hartmann, A., Langebartels, C. and Eberl, L. (2001). Visualization of N-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. *Applied and Environmental Microbiology* **67**, 5761-5770.

Steindler, L., Bertani, I., De Sordi, L., Schwager, S., Eberl, L. and Venturi, V. (2009). LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants. *Applied and Environmental Microbiology* **75**, 5131-5140.

Stotzky, G., Devanas, M. A. and Zeph, L. R. (1990). Methods for studying bacterial gene transfer in soil by conjugation and transduction In: *Advances in Applied Microbioliogy*, pp. 57-169. Edited by S. L. Neidleman and A. I. Laskin, eds. Academic Press, San Diego.

Strunk, O. and Ludwig, W. (1997). ARB: A software environment for sequence data [www.document]. *URL http://wwwmikrobiologietu-muenchende*.

Suggs, S. V., Hirose, T., Miyake, T., Kawashima, E. T., Johnson, M. J., Itakura, K. and Wallace, R. B. (1981). Use of synthetic oligodeoxyribonucleotides for the isolation of cloned DNA sequences. In: *Developmental Biology Using Purified Genes*, pp. 683-693. Edited by D. D. Brown and C. F. Fox, eds. Akademic Press, New York.

Supakdamrongkul, P., Bhumiratana, A. and Wiwat, C. Characterization of an extracellular lipase from the biocontrol fungus, *Nomuraea rileyi* MJ, and its toxicity toward *Spodoptera litura*. *Journal of Invertebrate Pathology* **In Press**.

Tang, Y. W., Ellis, N. M., Hopkins, M. K., Dodge, D. E. and Persing, D. H. (1998). Comparison of phenotypic and genotypic techniques of identification of unusual aerobic pathogenic Gram negative Bacilli. *Journal of Clinical Microbiology* **36**, 3674-3679.

Telford, G., Wheeler, D., Williams, P., Tomkins, P. T., Appleby, P., Sewell, H., Stewart, G. S. A. B., Bycroft, B. W. and Pritchard, D. I. (1998). The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infection and Immunity* **66**, 36-42.

Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J., Nelson, W. C., Gwinn, M. L., DeBoy, R., Peterson, J. D., Hickey, E. K., Haft, D. H., Salzberg, S. L., White, O., Fleischmann, R. D., Dougherty, B. A., Mason, T., Ciecko, A., Parksey, D. S., Blair, E., Cittone, H., Clark, E. B., Cotton, M. D., Utterback, T. R., Khouri, H., Qin, H., Vamathevan, J., Gill, J., Scarlato, V., Masignani, V., Pizza, M., Grandi, G., Sun, L., Smith, H. O., Fraser, C. M., Moxon, E. R., Rappuoli, R. and Venter, J. C. (2000). Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* **287**, 1809-1815.

Tomlinson, A. D. and Fuqua, C. (2009). Mechanisms and regulation of polar surface attachment in *Agrobacterium tumefaciens*. *Current Opinion in Microbiology* **12**, 708-714.

van den Broek, D., Bloemberg, G. V. and Lugtenberg, B. (2005). The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. *Environmental Microbiology* **7**, 1686-1697.

van der Woude, M. W. and Baumler, A. J. (2004). Phase and antigenic variation in bacteria. *Clinical Microbiolology Review* **17**, 581-611.

Vandenbergh, P. A. and Gonzalez, C. F. (1984). Method for protecting the growth of plants by employing mutant siderophore producing strains of *Pseudomonas putida*. *U S Patent* **No. 4479**, 936.

Varma, A., Verma, S., Sudha, S., Sahay, N., Bütehorn, B. and Franken, P. (1999). *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Applied and Environental Microbiology* **65**, 2741-2744.

Vial, L., Cuny, C., Gluchoff-Fiasson, K., Comte, G., Oger, P. M., Faure, D., Dessaux, Y., Bally, R. and Wisniewski-Dye, F. (2006). *N*-acyl-homoserine lactone-mediated quorum-sensing in *Azospirillum*: an exception rather than a rule. *FEMS Microbiology Ecology* **58**, 155-168.

Vial, L., Groleau, M.-C., Lamarche, M. G., Filion, G., Castonguay-Vanier, J., Dekimpe, V., Daigle, F., Charette, S. J. and Deziel, E. (2009). Phase variation has a role in *Burkholderia ambifaria* niche adaptation. *The ISME Journal* **4**, 49-60.

Vial, L., Pothier, J. F., Normand, P., Moënne-Loccoz, Y., Bally, R. and Wisniewski-Dyé, F. (2004). Construction of a recA mutant of *Azospirillum lipoferum* and involvement of recA in phase variation. *FEMS Microbiology Letters* **236**, 291-299.

von Rad, U., Klein, I., Dobrev, P., Kottova, J., Zazimalova, E., Fekete, A., Hartmann, A., Schmitt-Kopplin, P. and Durner, J. (2008). Response of *Arabidopsis thaliana* to *N* -

hexanoyl-dl -homoserine-lactone, a bacterial quorum sensing molecule produced in the rhizosphere. *Planta* **229**, 73-85.

Wagner, M., Horn, M. and Daims, H. (2003). Fluorescence *in situ* hybridisation for the identification and characterisation of prokaryotes. *Current Opinion in Microbiology* **6**, 302-309.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P. and Kogel, K.-H. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences USA* **102**, 13386-13391.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. and Trüper, H. G. (1987). Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**, 463-464.

Wei, H.-L. and Zhang, L.-Q. (2006). Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24. *Antonie van Leeuwenhoek* **89**, 267-280.

Whetten, R. and Sederoff, R. (1995). Lignin Biosynthesis. *Plant Cell* **7**, 1001-1013.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52**, 487-511.

Whitehead, N. A., Barnard, A. W., Slater, H. and Silmond, G. P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS microbiology reviews* **25**, 365-404.

Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., Vandamme, P., Gillis, M., Kersters, K. and De Ley, J. (1990). *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **40**, 384-398.

Willems, A. and Gillis, M. (2005). Genus II. *Acidovorax* Willems, Falsen, Pot, Jantzen, Hoste, Vandamme, Gillis, Kersters and De ley 1990, 394^{vp}. In: *Bergey's Manual of Systematic Bacteriology*, pp. 696-703. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley and G. M. Garrity, eds. Springer, New York.

Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K. and De Ley, J. (1992). Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *International Journal of Systematic and Evolutionary Microbiology* **42**, 107-119.

Wisniewski-Dyé, F. and Vial, L. (2008). Phase and antigenic varition mediated by genome modifications. *Antonie van Leeuwenhoek* **94**, 493-515.

Wood, D. W. and Pierson III, L. S. (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49-53.

Wopperer, J., Cardona, S. T., Huber, B., Jacobi, C. A., Valvano, M. A. and Eberl, L. (2006). A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex. *Applied Environmental Microbiology* **72**, 1579-1587.

Zelles, L. (1997). Phospholipid fatty acid profiles in selected members of soil microbial communities. *Chemosphere* **35**, 275-294.

Zelles, L. (1999). Identification of single cultured micro-organisms based on their whole-community fatty acid profiles, using an extended extraction procedure. *Chemosphere* **39**, 665-682.

Zhang, Z. and Pierson III, L. S. (2001). A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Applied and Environmental Microbiology* **67**, 4305-4315.

Zhou, H., Yao, F., Roberts, D. and Lessie, T. (2003). AHL-deficient mutants of *Burkholderia ambifaria* BC-F have decreased antifungal activity *Current Microbiology* **47**, 174-179.

Zhulin, I. B. and Armitage, J. P. (1992). The role of taxis in the ecology of *Azospirilum*. *Symbiosis* **13**, 199-206.

7 Appendix

7.1 16S rRNA gene sequence of "*cand.* A. radicis" N35 and N35v

(The Genbank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N35 is HM027578)

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACG
GTAACAGGTCTTCGGATGCTGACGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCC
GATCGTGGGGGATAACGGAGCGAAAGCTTTGCTAATACCGCATACGATCTACGGATGAAAG
CAGGGGACCGCAAGGCCTTGCGCGGACGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGG
ATAAAAGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGA
AAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTtGT
ACGGAACGAAAAGACTCTGGTTAATACCTGGGGTCCATGACGGTACCGTAAGAATAAGCAC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGTGCGCAGGCGGTTATATAAGACAGATGTGAAATCCCCGGGCTCAACCT
GGGAACTGCATTTGTGACTGTATAGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGT
AGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTG
TACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCCTAAACGATGTCAACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGT
GAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACC
CGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTT
GACATGTACGGAATCCTTTAGAGATAGAGGAGTGCTCGAAAGAGAACCGTAACACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTCATTAGTTGCTACATTTAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATA
CAATGGATGGTACAAAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCATAAAGCCATTCG
TAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGAT
CAGAATGTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAG
CGGGTTCTGCCAGAAGTAGTTAGCCTAACCGCAAGGAGGGCGATTACCACGGCAGGGTTCG
TGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT
TCTG

7.2 Sequence of *araI-araR* gene in "*cand.* A. radicis" N35 and N35v

7.2.1 Sequence of *araI* gene in "*cand.* A. radicis" N35 and N35v

ATGCGCATCACCTCCGGCTGCTCTGCTGAACTGCATCCACCACTGATGACCCGCATGGCCC
GGTACCGGCACCGTGTTTTTGTGGAAAAACTGGGCTGGCAACTTCATTGCCGCGATGCCCT
GGAGCTGGACCAGTTTGACCGGGACGACACCGTCTACGTCATCGCACAAAACGAAGACGGC
GAGGTCATCGGCACCGCCCGGCTGCTGCCCACCACCCGCCCCTACCTGCTGGCCGAGGTGT
TCCCGCAACTGCTCAACGGCGCCCCGGCACCGAACGCACCCGAGGTGTGGGAACTGTCCCG
CTTTGCCTCGATGGACCTTTCGGGGCCTACGGGCTCCGCGCTTGACCAGTTCTCCTCGCCC

CTCACCACCGGCCTGCTGCAGGCGGCCAGCCGCTGCGCCATGGCGCGCGGTGCCCAGCGCA
TGGTCACCGTCTCACCGCTGGGGGTGGAACGCCTGCTGCGCCGCACGGGTTTTGAATCCTA
CCGCCTGGGGCCACCGACCGTGGTCCAGGGCCATCCGCTTTTTGCGTGCGCCATCCGGTGC
CAGTGA

7.2.2 Sequence of *araR* gene in "*cand.* A. radicis" N35 and N35v

CATCAGCCCAGCAACCCCAGCATGGCGGCACGGACGGTGGCGGCCGTCTTGTTGGTGGTTT
GCAGCTTGGTCACGGCATTCTTGACATGGAAGTTCACGGTGTTTTCCGACACATCCAGCAG
CGAGGAGATGTCGGCAGAGGTCTTGCCGTCGGCGGTCCATTTGAGCACCTCGATTTCTCTG
GCCGTCAGATGCGGCTGCATCAGTTCGGCCTGGCGGGTCGTAAAGATGCGCGACAGGATCA
CGTGCGCAATGTGCGCCAGCCAGCGCATCTTCATCTCACTGTGGGCAATCTCTTCGGGTGT
CAGGGCCTCGGACGGACGCGCCAGCGTCAGCATGCCACGCACCCCGCCGCCGTCAATGCAC
GATTGCGCCCAACCAAAACGCAATCCGAACGATCGTGCGTCTTCCCAGAAATTCTGGGCCG
ATGCAAACACATCGTCCGACCAGACCAGCGGGGTCTGCTTGCGGCATCCGTGGAGCACCGT
CGGATCGATCTGCACATAGCCCTGTTGCGCATACCGTTCCTGCAGGCCAAGGGGTAGTTGT
TTAACAAAATGGTCTTTGGGTTGGACAAGGGCATGGGTACCCGCAGGCCATAGGCGCAGTA
CTCAAACCCCAGAGCGTGCGCAGCGAGCTCGATTTTGCAAAAAACCTCTTGCTCTGACTGC
GCGCGGTCCATCACCCCCAAAAGATCTTCTTGCCAGCTTGTCATAGGACTCCAACACCGAG
CACCACCCCACAGAGACTGACCATCCGCACGCGAATTACGCGCCAT

7.3 *mutL* gene sequence of "*cand.* A. radicis" N35 and N35v

7.3.1 *mutL* gene sequence of "*cand.* A. radicis" N35

ATGCCCCTGCGCAGCCCTCGCCCTAAAATCTGCCGGGTGAACGACACCATCCCCACCCTCG
CTGCCGCACAGCCTGCCCCCCCCcGCCACCCGCCGCCCCATCCGCGACCTGCCCGACGAGCT
GATCAGCCAGATTGCTGCGGGCGAAGTGGTGGAACGCCCCGCCTCGGTGGTGCGCGAGCTG
GTGGACAACGCGCTCGATGCGGGCGCCACGCAGATCACCGTGCGCCTGTCGGCCGGCGGCG
TGCGGCTGATCACCGTGGAAGACGATGGCGGCGGCATTCCACACGAAGAGCTGCCCGTGGC
CCTGCGCCGCCATGCCACCAGCAAGATCACCAACCTCAACGACCTGGAAACCGTGGCCACC
ATGGGCTTTCGGGGCGAGGCGCTGGCGGCGATTGCCTCGGTGTCGGAGATGGCCCTGCTCT
CACGCCCGGCCGCACAGGCCAGCGCGTTCCTGCTGGACGCCCGCAGCGGCGAACTGCGCCC
CGCCGCACGCAGCACCGGCACGACGGTGGAGGTGAAGGAACTGTTCTTCTCCACCCCGGCG
CGCCGCAAGTTCTTGAAGACCGACGCCACCGAATTGGCGCACTGCATTGAATCGGTGCGCC
GCCATGCCCTGGCGCGGCCCGATGTGGGCTTTGCCATCTGGCACGAAGGCAAGCTGGTGGA
GCAATGGCGCGCGACTTTCATTCCTGGCCAAGGCACCACCGACGCCGTGCAGGATGCGCTG
GCCCGCCGCCTCTCGGACGTGCTGGGCGAAGACTTCGTCACGCAATCGGTCGCCGTACAGC
ACCGCGTGGGCCCTGTCATCGTGACCGGCCGTGCAGGCCTGCCCGATGCCGCCCGCTCGCG
CCCCGACCACCAGTATTGCTACGTCAACGGCCGCTTCGTGCGCGACAAGGTGCTCACCCAC
GCCGCCCGCAGCGCGTATGAAGACGTGCTGCACGGCCACAAGCAGCCCATTTATGCGCTGT
ATGTCGAGATCGACCCGGCCCGCGTGGACGTGAACGTGCACCCCACCAAGATCGAAGTGCG
CTTTCGGGACAGCCGCGAGGTGCACCAGGCCGTGCGCCACGCCGTGGAAAACGCGCTGGCC
GCGCCGCGCGCCGCTGCGCTGGCGGCAGCAGGGGCTGGCGCATCAGCAGACACAGGCTCCT
CGGCACAGGGCAGCATCGAAAATTTCAAGCCAAATCAGCCCCTGGCGCAAAACTGGCAAGC
GCAAGCAGCTATCAAATTTGAAGAACGTGGCCACCATGTGGCCGACCTGCAGGCCCTGTGG
GGgCCCCGGAAAGACAACGCAGCCACCGTGCCTTCTGGCCCCGCCGCACCCTTCGTGAACT

GGGGCACTGCGGCCAACACCGCTGAGGCAGACGCCATGCCGCAGCCCTTTGCGCCCGCCTC
GTCTATCCCCCCTGCCGCAGCAGGCGCTGTGGCCTGGCCCAGCAACAATACCGGACATGAA
GCCGCCTGGCCCCAGGGCCAGGGCGGCAACGACATCGCCTGGCCGCTGGGCAAGGCCGTGG
CCCAGTTGCACGGTGTGTACATCCTGGCTGAAAACGCCCAAGGCATGGTCATCGTGGACAT
GCACGCCGCGCACGAACGCATCGTCTACGAGCGCCTGAAATCCCAGGTGGACAGCGGCGCC
CGCATTGCGAGCCAGCCCCTGCTGATCCCCGCCACCTTTGCCGCCACCCCGCAGGAAGTGG
CTACCGCCGAAGAGTCGGCCGAGGTGCTGGCCACGCTGGGCCTGGAGGTCGTGCCTTTCTC
CCCCAAGACCCTGGCCGTGCGCGCCGTGCCCACCACGCTGGCCCAGGGCGACCCCGTGGAA
CTGGCCCGCAGCGTGCTGGCCGAACTGGCCGCACACGACGCCACCACCGTGGTGCAACGCG
CCCGCAATGAAATCCTGGGCACCATGGCTTGCCACGGCGCCGTGCGCGCCAACCGCAAGCT
CACCATCGACGAGATGAACGCCCTTCTGCGGCAAATGGAAACCACGGACCGGTCGGACCAG
TGCAACCACGGAAGGCCAACGTGGCGGCAGCTGTCGATGAAAGAGCTGGACGCGTTGTTTT
TGCGCGGCCGTTAA

7.3.2 *mutL* gene sequence of "*cand.* A. radicis" N35v

ATGCCCCTGCGCAGCCCTCGCCCTAAAATCTGCCGGGTGAACGACACCATCCCCACCCTCG
CTGCCGCACAGCCTGCCCCCCCCGCCACCCGCCGCCCCATCCGCGACCTGCCCGACGAGCT
GATCAGCCAGATTGCTGCGGGCGAAGTGGTGGAACGCCCCGCCTCGGTGGTGCGCGAGCTG
GTGGACAACGCGCTCGATGCGGGCGCCACGCAGATCACCGTGCGCCTGTCGGCCGGCGGCG
TGCGGCTGATCACCGTGGAAGACGATGGCGGCGGCATTCCACACGAAGAGCTGCCCGTGGC
CCTGCGCCGCCATGCCACCAGCAAGATCACCAACCTCAACGACCTGGAAACCGTGGCCACC
ATGGGCTTTCGGGGCGAGGCGCTGGCGGCGATTGCCTCGGTGTCGGAGATGGCCCTGCTCT
CACGCCCGGCCGCACAGGCCAGCGCGTTCCTGCTGGACGCCCGCAGCGGCGAACTGCGCCC
CGCCGCACGCAGCACCGGCACGACGGTGGAGGTGAAGGAACTGTTCTTCTCCACCCCGGCG
CGCCGCAAGTTCTTGAAGACCGACGCCACCGAATTGGCGCACTGCATTGAATCGGTGCGCC
GCCATGCCCTGGCGCGGCCCGATGTGGGCTTTGCCATCTGGCACGAAGGCAAGCTGGTGGA
GCAATGGCGCGCGACTTTCATTCCTGGCCAAGGCACCACCGACGCCGTGCAGGATGCGCTG
GCCCGCCGCCTCTCGGACGTGCTGGGCGAAGACTTCGTCACGCAATCGGTCGCCGTACAGC
ACCGCGTGGGCCCTGTCATCGTGACCGGCCGTGCAGGCCTGCCCGATGCCGCCCGCTCGCG
CCCCGACCACCAGTATTGCTACGTCAACGGCCGCTTCGTGCGCGACAAGGTGCTCACCCAC
GCCGCCCGCAGCGCGTATGAAGACGTGCTGCACGGCCACAAGCAGCCCATTTATGCGCTGT
ATGTCGAGATCGACCCGGCCCGCGTGGACGTGAACGTGCACCCCACCAAGATCGAAGTGCG
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GCGCCGCGCGCCGCTGCGCTGGCGCATCAGCAGACACAGGCTCCTCGGCACAGGGCAGCAT
CGAAAATTTCAAGCCAAATCAGCCCCTGGCGCAAAACTGGCAAGCGCAAGCAGCTATCAAA
TTTGAAGAACGTGGCCACCATGTGGCCGACCTGCAGGCCCTGTGGGGGCCCCGGAAAGACA
ACGCAGCCACCGTGCCTTCTGGCCCCGCCGCACCCTTCGTGA

List of pulications

Li, D., Rothballer, M., Schmid, M., Esperschütz, J. and Hartmann, A. *Acidovorax radicis* sp. nov., a rhizosphere bacterium isolated from wheat roots. *International Journal of Systematic and Evolutionary Microbiology*, (in version)

Li, D., Rothballer, M., Schmid, M., Engel, M., Hoser, J., Schmidt, T. and Hartmann, A. Phenotypic variation influences the colonization efficiency in plant growth promoting *Acidovorax radicis* N35. *Environmental Microbiology*, (in preparation)

Varma, A., Fekete, A., Srivastava, A., Saxena, A. K., Frommberger, M., Li, D., Gschwendtner, S., Raha, M., Sherameti, I., Oelmueller, R., Schmitt-Kopplin, P. and Hartmann, A. Rhizobacteria interact with cultivable symbiotic fungus *Piriformospora indica*. *Environmental Microbiolology,* (in preparation)

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