Nora Spanberger, BSc

Biocontrol potential of *Serratia plymuthica* 3Rp8 towards human pathogens on lettuce

MASTER THESIS

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Univ.-Prof. Dipl.-Biol. Dr. rer. nat. Gabriele Berg

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Abstract

Leafy green vegetables, in particular lettuce species such as *Lactuca sativa* L. and its varieties are an important part of a healthy diet, by providing the consumer with vitamins, minerals and dietary fibers. However, they have also been reoccurringly reported as sources of severe pathogenic outbreaks. Foodborne outbreaks due to the consumption of raw or minimal processed vegetables contaminated with bacterial human pathogens (HPs) are a food safety concern worldwide. Many outbreaks occur each year and especially enteric pathogens, *e.g*. *Salmonella enterica* or *Escherichia coli* pathovars O157:H7 / O104:H4 cause severe epidemics and can result in death of the patients. Currently no biological control method is available to address HPs. Therefore, the objective of this master thesis was to evaluate *Serratia plymuthica*, which was identified as promising biocontrol agent against plant pathogens, as biocontrol agent against HPs. *Serratia plymuthica* 3Rp8 was studied for its i) *in vitro* activity against model pathogens including *Enterobacteriaceae* ii) *in vivo* activity related to lettuce colonisation, germination and growth in pot experiments and iii) *ad planta* effect on the indigenous lettuce microbiome under greenhouse conditions. Additional genome analyses and 16SrRNA amplicon sequencing of the lettuce microbiome facilitate the holistic understanding of the biocontrol effect of *S. plymuthica*. It was demonstrated that 3Rp8 is a highly effective antagonist towards bacterial HPs and lettuce foliage-associated pathogens. 3Rp8 showed significant plant growth promoting activities when applied in combination with *Pseudomonas fluorescens* WCS 365. Furthermore, significant negative spearman co-occurrence correlations ($p<0.01$) reinforced that it is a strong effector in the lettuce-associated microbial community. The application of *Serratia plymuthica* 3Rp8 to seeds and harvested plants for the purpose of inhibiting the growth and survival of bacterial HPs may be a useful food safety intervention for both conventional and organic vegetable growers.

Kurzfassung

Blattgemüse und insbesondere Salat (*Lactuca sativa* L.) sind durch den hohen Gehalt an Vitaminen, Mineralien und Ballaststoffen, ein wichtiger Bestandteil einer ausgewogenen und bewussten Ernährung. Da diese pflanzlichen Nahrungsmittel meist roh verzehrt werden, ist der Konsument lebensmittelassoziierten Pathogenen ausgesetzt, welche schon in geringer Menge einen großen Einfluss auf die Gesundheit haben können. Von humanpathogenen Ausbrüchen bei roh verzehrtem und minimal verarbeitetem Blattgemüse wird weltweit jährlich berichtet. Speziell enterische Pathogene wie *Salmonella enterica* und *Escherichia coli* Pathovare O157:H7 / O104:H4 verursachen Epidemien und können zum Tod der Betroffenen führen. Derzeit gibt es keine biologische Kontrollmethode für humanpathogene Keime. Daher wurde in dieser Masterarbeit das vielversprechende Biokontrollagenz (Biocontrol Agents = BCAs) *Serratia plymuthica*, welches in früheren Studien bereits hohes antagonistische Potential gegenüber Phytopathogenen zeigte, als BCA gegen Humanpathogene evaluiert. *Serratia plymuthica* 3Rp8 wurde im Bezug auf i) seine *in vitro* Aktivität gegen Modelpathogene, inklusive Pflanzenpathogenen und natürlich vorkommende Enterobakterien getestet, ii) seine *in vivo* Kolonisierungseigenschaften, Keimunterstützung und pflanzenwachstumsfördernde Wirkung auf *Lactuca sativa* L. var *capitata* „Gelber Winter“ in Gewächstöpfen überprüft und iii) seine Auswirkungen auf das pflanzenassoziierte Mikrobiom von *L. sativa* im Gewächshaus evaluiert. Anhand der erhaltenen Daten von Genomsequenzierung und 16SrRNA Amplikonsequenzierung des Mikrobioms von *L. sativa* konnten zusätzliche Einblicke in den Wirkmechanismus von *S. plymuthica* gewonnen werden. Wir konnten zeigen, dass 3Rp8 über sehr hohes antagonistische Potential gegen Humanpathogene und Pathogene welche die Phyllosphere von Salat besiedeln verfügt. Des Weiteren konnte die pflanzenwachstumsfördernde Wirkung von 3Rp8 in Kombination mit *Pseudomonas fluorescens* WCS365 auf *L. sativa* bestätigt werden und Netzwerkanalysen bestätigten 3Rp8 als starken Effektor der Salat-assoziierten bakteriellen Gemeinschaft. Zusammenfassend bietet der starke Einfluss von 3Rp8 auf potenzielle Salat-assoziierte humanpathogene Bakterien eine neue Möglichkeit im Einsatz gegen Lebensmittelinfektonen. Das

Aufbringen von 3Rp8 auf Samen oder Pflanzen nach der Ernte, könnte die Lebensmittelsicherheit erhöhen und eine sinnvolle Strategie für konventionelle und biologische Gemüsebauern darstellen.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AHL	N-acyl-homoserin-lactone
Aqua dest.	distilled water
BCA	biological control agent
BLAST	basic local alignment search tool
BNF	biological nitrogen fixation
bp	base pair
CDC	United States Centers for Disease Control and Prevention
CFU	colony forming units
CLSM	confocal laser scanning microscopy
CPAs	confrontation plate assay
CS	cropping system
DMSO	dimethylsulfoxid
et al.	lat: et alteri
EDTA	ethylendinitrilotetraaceticacid
EHEC	enterohaemorrhagic *E. coli*
FAO	Food and Agriculture Organization of the United Nations
FISH	fluorescence *in situ* hybridisation
Gb	giga base pairs
HACCP	Hazard analyses and critical control point
HPs	human pathogens
HUS	haemolytic uraemic syndrome
IAA	indol-3-acetic acid
INT	iodonidtetrazoliumchloride
IPM	integrated pest management
ISR	induced systemic resistance
LB	Luria-Bertani-Medium
MC	MacConkey agar
NA	Nutrient agar
NaOCl	sodium hypochloride
NaOH	sodium hydroxide
NCBI	national center for biotechnology information

NRPs	non ribosomal peptides
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDA	Potato dextrose agar
PFA	paraformaldehyde
PGPB	plant growth promoting bacteria
PGPR	plant growth promoting rhizobacteria
PKs	polyketides
PNA	peptide nucleic acid
PRR	pattern recognition receptors
PTI	PAMP-triggered immunity
QS	quorum sensing
rpm	rounds per minute
STEC	Shiga toxin-producing *Escherichia coli*
TAE	tris-acetat-EDTA
TBE	tris-borat-EDTA
TCVAs	two clamp volatile organic compound assays
Tris	2-amino-2-(hydrosymethyl)-propan-1,3-diol
PFA	paraformaldehyd
PBS	phosphate buffered saline
VOC´s	volatile organic compounds

1. Introduction

1.1. Global production of leafy greens

1.1.1 Importance and production

Leafy green vegetables, especially lettuce species such as *Lactuca sativa* L. and its varieties, are an important part of a healthy diet by providing the consumer with vitamins, minerals and dietary fibers. Daily consumption of such leafy greens can reduce the risk for certain cancers and cardiovascular diseases and therefore production of these superfoods is expected to continue growing (reviewed in Taban et al., 2011). Lettuce is the most important leafy vegetable nowadays, it is produced in open field and greenhouse farms and its global yield increments every year (Katz, 2003; Holvoet et al., 2014). In 2014 the world production of lettuce and chicory was 24.9 million tonnes. China produced with 13.6 Mio t the vast bulk of it, followed by the United States of America with 3.8 Mio t and India with 1.1 Mio t (Table 1) (FAO, http://faostat3.fao.org/browse/Q/QC/E).

Table 1 Ranking of the top lettuce-producing countries in 2014 (FAO, http://faostat3.fao.org/browse/Q/QC/E)

Country	Millions of tons produced in 2014
China	13.6
Unites States of America	3.8
India	1.1
Spain	0.9
Italy	0.7

1.1.2 Pathogenic outbreaks attributed to leafy greens

Despite its benefits for human health, leafy greens have been involved in several pathogenic outbreaks of human gastroenteritis. Between 2010 and 2014, 120 multistate foodborne outbreaks have been reported to the United

States Centers for Disease Control and Prevention (CDC). The main species involved in multistate foodborne outbreaks were *Salmonella* (63 outbreaks), Shiga toxin-producing *Escherichia coli* (STEC) (34), and *Listeria monocytogenes* (12). Among the 34 STEC outbreaks, 41% were linked to leafy greens and 59% were caused by the serogroup O157 (Crowe et al., 2014). In May 2011 the latest widespread outbreak of enterohaemorrhagic *E. coli* (EHEC) was reported in Germany (Frank et al., 2011). Here, the serotype 104:H4 caused a large outbreak of bloody diarrhea and haemolytic uraemic syndrome (HUS) via contaminated sprouts. This outbreak gave a vivid insight, how an infectious agent can harm and raise fears in a whole country. More than 3000 EHEC cases were observed between May and September 2011 and 18 deaths were observed among the EHEC patients (Frank et al., 2011). Fenugreek seeds (*Trigonella foenum-graecum*) used for growing sprouts were finally identified to be responsible for the outbreak (Appel et al., 2011). Table 2 provides a list of noteworthy foodborne outbreaks of infections associated with leafy greens within the last three decades.

Table 2 List of outbreaks of infections associated with lettuce and vegetables

Microorganism	Year	Location	Type of product	Reference
Clostridium botulinum	1987	USA	Cabbage	Solomon et al., 1990
Hepatitis A	1988	USA	Lettuce	Rosenblum et al., 1990
Salmonella Javiana	1990	USA	Tomatoes	Beuchat et al., 2002
E. coli	1993	USA	Carrots	Beuchat et al., 2002
Shigella sonnei	1994	Norway	Lettuce	Kapperud et al., 1995
E. coli O157:H7	1995	USA	Lettuce	Beuchat et al., 2002
Salmonella Thompson	2004	Norway	Lettuce	Nygard et al., 2008
E. coli O157	2005	Sweden	Lettuce	Söderström et al., 2008
E. coli O104:H4	2011	France	Fenugreek seeds	King et al., 2012
E. coli O104:H4	2011	Germany	Fenugreek seeds	Buchholz et al., 2011
Salmonella enterica	2015	USA	Cucumber	Kozyreva et al., 2016
E. coli O157	2016	USA	Alfalfa sprouts	CDC, 2016

Contamination of leafy greens can occur through direct or indirect interactions with animals, insects, water, soil, dirty equipment and human handling, but also the application of manure or compost as fertilizers to open field or greenhouse production systems, are a major sources of contamination (Suslow et al., 2003; Johannessen et al., 2004; Islam et al., 2005; Erickson et al., 2010). Lettuce production in greenhouses is assumed to be more easily controllable than in open fields, as these offer multiple contamination sources (Bracket et al., 1999, Holvoet et al., 2015). Furthermore climatic conditions, such as rainfall and temperature can have an impact on growth and survival of pathogenic microorganisms within production fields (Holvoet et al., 2014). Detailed transmission routes that allow widespread pathogen colonization of plants still remain to be identified.

1.1.3 Pathogen suppression strategies on leafy greens

Pathogenic outbreaks associated with leafy greens have forced producers and processors to establish certain guidelines to eliminate pathogens at all stages of the food supply chain. Most guidelines are focusing on the source of contamination, such as irrigation, processing water or worker hygiene (Hald et al., 2012). Hazard analyses and critical control point (HACCP) principles have been implemented, but are not applicable to production in an open field (Gil et al., 2015). Currently there are several pre- and post-harvest decontamination strategies, such as chemical and physical sanitizers. These can reduce the risk of contamination with pathogenic bacteria, but safety concerns, effects on quality and cost intensive processes limit their use (Allende et al., 2008; Gil et al., 2009). Furthermore decontamination strategies to avoid pathogens on leafy greens can only minimise the microbial risk but not eliminate it (reviewed in Allende et al., 2015). Currently, there is no strategy to counteract such contamination with beneficial microorgansims. Biological control targeting human pathogens could offer hereby an attractive and environmentally friendly amendment for implemented pest management systems (IPM).

1.1.1. Biocontrol agents (BCAs) and plant growth promoting bacteria (PGPB)

1.2.1 Application of BCAs and PGPB

Damaging and unfavourable growth conditions as well as pathogenic bacteria, fungi and pest organisms can be a menace for plant growth. Approximately 25% of the worlds crop yield is lost every year, mainly due to diseases caused by fungi, other pathogens and by pests. To fight such diseases many products are available on the market; mainly chemical ones. The use of such chemicals can pollute the environment and can harm the health of consumers. An attractive alternative is the use of beneficial microorganisms, which allows sustainable crop production (Lugtenberg et al., 2015a). Such microorganisms are known as plant growth promoting bacteria (PGPB) and biological control agents (BCA). PGPB are naturally occurring soil bacteria, which colonise plant roots and provide growth promotion by diverse mechanisms. Furthermore, particular plant associated microorganisms are able to enhance stress tolerance, provide disease resistance, aid nutrient availability and uptake, and promote biodiversity (Lugtenberg et al., 2009, Morrissey et al., 2004). PGP can occur by direct interaction with the plant by providing nutrients or phytohormones to the host. Currently there are several plant growth promoting rhizobacteria (PGPR) inoculants commercialised, which promote plant growth by improved nutrient uptake (biofertilizers), suppression of plant disease (bioprotectants) or phytohormone production (biostimulans). Such microbial inoculants are safer, show reduced environmental damage compared to pesticides and therefore they show a potential smaller risk to human health. They decompose more quickly than conventional chemical pesticides, resistance development is reduced due to several mechanisms and they can be also used in conventional or integrated pest management systems (Berg, 2009). On the other hand, many bacterial strains that promote plant growth and antagonise plant pathogens can colonise human tissues and therefore can potentially cause diseases in humans (Berg et al., 2005b). In contrast to PGPB, BCAs provide benefits by indirect interactions that target plant pathogens. They can be mediated through direct antagonism against pathogens, the competition

for special niches or by triggering the plant hosts defence system (Mercado-Blanco, 2015). Until now research focused on the development of bioinoculants, which are able to suppress phytopathogens, but there is no biological method available to protect plants against bacterial HPs. Lately, there is accumulating evidence that BCAs could combine more advantageous properties. They could be used to enhance the plants fitness, promote plant growth and to lower the risk of foodborne pathogenic outbreaks, by producing compounds or metabolites, which suppress potential human pathogenic bacteria. For instance Schikora et al., (2016) showed in their study how N-acyl-homoserine lactone (AHL) producing bacteria (*Sinorhizobium meliloti*) enhanced the host plants immune system and additionally, that AHLs are necessary to avoid the risk of plant-originated pathogenic infections of salmonellosis. AHLs are part of the quorum sensing (QS) signalling within foodborne pathogens. QS is known to play an important role in biofilm formation, which is necessary for the manifestation of many foodborne pathogens. Diverse bacteria depend on QS to colonise natural habitats and to interact with eukaryotic hosts (Koutsoudis et al., 2006; Masák et al., 2014). Therefore blocking the quorum sensing (QS) signals through a compound, metabolite or bacterium, could be an attractive alternative to conventional procedures and to regulate the virulence of foodborne pathogens (Bjarnsholt et al., 2007). Several studies have shown, that metabolites (urolithins), phytochemicals and bacteria (*Bacillus thurienginsis*) can inhibit QS in potential HPs (Zhang et al., 2007; Truchado et al., 2012; Giménez-Bastida et al., 2012). BCAs that directly antagonize human-pathogenic bacteria could be a further improvement of integrated farming practices.

1.2.2 Specific mechanisms of plant growth promotion

Plant growth promotion can occur by direct interaction of beneficial microbes with their host plant, but also indirectly by antagonistic activity against plant pathogens. Several mechanisms are known to promote plant growth and biocontrol, but the most widely recognised mechanism is the competition for an ecological niche or substrate by which PGPB protect the plant from

phytopathogens (Bloemberg et al., 2001). The induction of systemic resistance (ISR) in the host plant to a wide spectrum of pathogens is another phenomenon caused after applying certain PGPB (reviewed in Compant et al., 2005). Also an important scheme is the biological nitrogen fixation (BNF). BNF is the enzyme-catalysed fixation of atmospheric dinitrogen into ammonia by nitrogenase, an enzyme complex known as *nif* genes. Fixed nitrogen is a limiting resource for plant growth, which can be overcome by nitrogen fixation of many free living microorganisms. It is an environmentally and economically friendly alternative to synthetic nitrogen fertilizers, which are very cost intensive (de Bruijn, 2015). Furthermore, the mobilisation of phosphate, the production of phytohormones and the signal mediated stress control by beneficial microorganisms result in plant growth promoting activity. The bacterial genera *Bacillus, Pseudomonas, Serratia, Stenotrophomonas, Streptomyces* and the fungal genera *Ampelomyces, Coniothyrium* and *Trichoderma* are examples for such promotion of plant health (Berg, 2009).

1.2.3 The potential of *Serratia* species as BCAs

Serratia are Gram-negative bacteria belonging to the family of *Enterobacteriaceae*. *Serratia* spp. are ubiquitously distributed and their natural habitats range from water, soil and plants to insects, humans and vertebrates. Some *Serratia* spp. are involved in infections caused in immunocompromised humans (Gavini et al., 1979, Grimont, 2006). However, they are also associated with plants and have been isolated amongst others from the rhizosphere of wheat, oat, cucumber, maize, oilseed rape and potato (Berg, 2000). *Serratia* spp. are well known for their biocontrol activity with broad-spectrum antagonism against phytopathogens and their plant growth promoting abilities. Representatives of this species show high antagonistic activity against *Rhizoctonia solani* Kühn [teleomorphe: *Thanatephorus cucumeris* (A.B. Frank) Donk; *Basidiomycetes*] (Scherwinski et al., 2008), against *Verticillium* wilt, *Fusarium* wilt and *Phytophtora* root rot and also show plant growth promoting activity for strawberries, hops and oilseed rape (Kurze et al., 2001, Berg et al., 2005a; Müller et al., 2009; Rossmann et al., 2012; Maurer et al., 2013;

Rybakova et al., 2016). Mechanisms for their antagonistic and plant growth promoting activity include antibiosis, competition for niches, production of phytohormones, rhizosphere competence and induction of systemic resistance within the host plant (Whipps, 2001; Lugtenberg et al., 2002). Furthermore functional genes involved in antagonism and plant growth promotion were identified within *Serratia* spp. (Adam et al., 2016).

1.2. Plant-microbe interactions

1.3.1 The implications of plant-associated microbial communities

The technological advances and intensive research over the last half century - especially in terms of developing and improving sequencing technologies - changed the field of plant-microbe research and made it possible to analyse holistically plant-associated microbial community structures and functions (Schlaeppi and Bulgarelli 2015; Heather and Chain 2016). Approaches to analyse complex DNA mixtures (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics) and metabolites (meta-metabolomics) from a given habitat, led to novel insights regarding the functioning of specific community members (Riesenfeld et al., 2004). The plant microbiome is one of the key determinants for plant health, fitness and productivity (Berg et al., 2015). It is undergoing a tight symbiotic relationship with its host, the composition is breed and plant organ specific and it fulfils multiple functions, which are important for plant and human health (Berg, 2009; Schlaeppi et al., 2014; Hirsch et al., 2012; Lundberg et al., 2012). Plant microbiota promote germination, support plant growth, are involved in pathogen suppression and the stimulation of the plants immune system, protect plants against biotic and abiotic stress, they are influencing the composition of secondary metabolites and they are involved in many more essential functions (reviewed in Berg et al., 2015). In the lettuce microbiome, *Alpahproteobacteria*, *Betaproteobacteria*, as well as *Gammaproteobacteria* make up a considerable portion of the lettuce-associated microbiome with a high proportion of *Enterobacteriaceae* (Rastogi et al., 2012, Erlacher et al., 2014). Potentially human-pathogenic bacteria found on lettuce

belong to the genera of *Flavobacterium*, *Burkholderia*, *Escherichia*, *Pantoea, Salmonella*, *Shigella*, *Staphylococcus*, *Stenotrophomonas*, *Cladosporium* and *Aspergillus* (Bert et al., 2014; Kapperud et al., 1995; Nygard et al., 2008; Scherwinski et al., 2008; Söderström et al., 2008). The class of *Gammaproteobacteria* harbours many members that are pathogenic to humans, animals and plants. Interestingly, they have been shown to be a solid part of the plant microbiome in general, and therefor it is important to investigate and assess the pathogenic potential of plant-associated bacteria and their interaction with PGPB and BCAs (Brandl et al., 2006; Dworkin et al., 2006; Katz, 2003; Erlacher et al., 2014). On the other hand, there´s evidence, that potential human pathogenic bacteria and in particular *Enterobacteriaceae* function as an immune stimulating system for humans, for example by enhancing the expression of anti-inflammatory cytokines and by raising the microbial diversity in the gut microbiome in humans (reviewed in Berg et al., 2014).

1.3.2 Microbial life on plants

Plants in their natural environment are encompassed by a large number of microorganisms. Some microbes directly interact with the plant, whereas others only colonise the plant for their own benefit. Plant-microbe interactions can be beneficial, neutral or pathogenic. The soil contains many beneficial microorganisms, which are enriched in the rhizosphere and provide mineral nutrients and fixed nitrogen for the plants in exchange for carbon (Lugtenberg et al., 2009). The rhizosphere was defined more then 100 years ago by Lorenz Hiltner as “the soil compartment influenced by the roots of growing plants”. It is 10- to 100-fold richer in microbes than the surrounding soil due to the ‘rhizosphere effect’. To allow this enrichment 6-21% of the carbon that is fixed by the plant is secreted by its roots (Lugtenberg et al., 2015b). Interactions in the rhizosphere occur in various combinations; between fungus-plant, bacterium-plant, bacterium-bacterium, bacterium-fungus and within many other organisms. Some of such interactions have been already successfully visualised by Confocal Laser scanning microscopy (CLSM) and scanning

electron microscopy (reviewed in Lugtenberg et al., 2015b). Communication between organisms occurs by chemical signalling (chemotaxis) and many compounds secreted by plant roots and fungal hyphae, such as organic acids, sugars and amino acids, can function as signals for chemoattraction, which guide bacteria that are starving for nutrients in the rhizosphere, towards these compounds (de Weert et al., 2002). Colonisation finally occurs on the target organism and more specialised communication between different organisms such as nodulation, gene transfer, pathogenesis and the production of antibiotics can start (Lugtenberg et al., 2015b).

In contrast, microbial life on leaves is characterised by less diversity and abundance, because the phyllosphere is known to be an extreme habitat for microbes. Microorganisms have to be well adapted to such harsh conditions, for example to protect themselves against ultraviolet radiation, to be able to repair DNA damages caused by such ultraviolet radiation, to withstand drought and to form biofilms in order to overcome leaf stresses (Leveau, 2015). The occurrence of specific microorganisms in the phyllosphere of leafy greens is of great interest and scientific research offered new insights into the sources of disease outbreaks in humans by raw consumed leafy greens such as spinach and lettuce (Leveau, 2015). It was shown that foodborne microbes survive the stomach passage and are metabolically active in the gut (David et al., 2014). Yet little is known about the interactions of host plants and HPs with other microbes on the leafy surface and how such interactions affect the abundance and activity of pathogens. However, correlations between pathogen presence and microbial community structure have been reported (Rastogi et al., 2012, Erlacher et al., 2014).

The interior part of the plant provides another habitat for microbes. Distinct bacteria and fungi that are able to live inside the plant are called endophytes (Stone et al., 2000). Endophytes live in healthy plants and do not cause diseases to their host. Moreover, they can play an important role in the plant's fitness and development. The inner plant parts are not as stressful as the phyllosphere and they provide a constant source of nutrients for microbes. The majority of endophytes belongs to soil-inhabitants and their population density

is usually higher in the roots than in other plant parts. Endophytic colonisation occurs usually by invading the roots at cracks or wounds, but the mechanisms used to enter the plant are largely unknown (Rosenblueth et al., 2006). Many endophytes are of great interest because of their biotechnological potential for plant growth promotion (PGP) and disease suppression within the plant.

1.4 Aims and rationale of this study

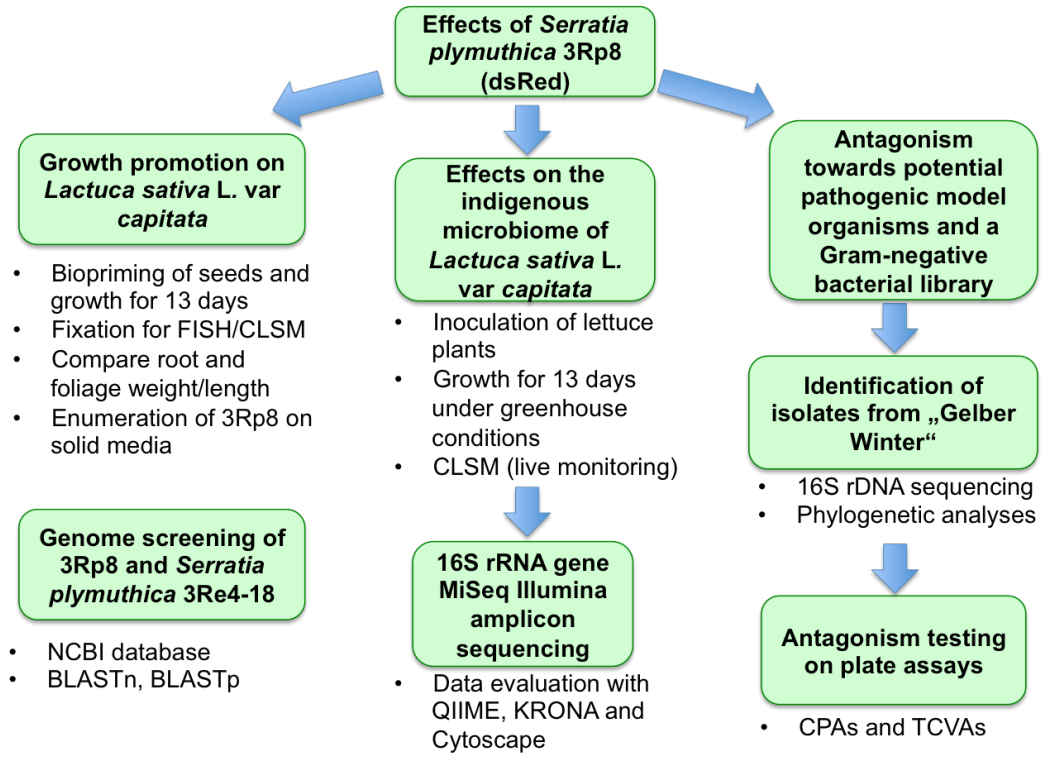
Leafy green vegetables, in particular lettuce species such as *Lactuca sativa* L. and it´s varieties are an important part of a healthy diet, by providing the consumer with vitamins, minerals and dietary fibers. However, they have also been reoccurringly reported to be the resource of severe foodborne pathogenic outbreaks worldwide. Especially enteric pathogens, e.g. *Salmonella enterica* and *Escherichia coli* pathovars O157:H7 / O104:H4 are involved in many outbreaks worldwide and can cause epidemics and death. Currently there is no biological control method available to control these pathogens. In order to develop alternative methods that could reduce outbreaks in the future, it is necessary to investigate how biological control agents (BCAs) interact with bacterial HPs and how they affect the plant and its natural microbiome. The objective of this master thesis was to evaluate *Serratia plymuthica*, which was identified as promising biocontrol agent against plant pathogens, as biocontrol agent against HPs. Therefore we studied *Serratia plymuthica* 3Rp8 for its i) *in vitro* activity against model pathogens and lettuce-associated *Enterobacteriacea* in dual-culture assays, ii) *in vivo* activity on lettuce colonisation, germination and growth in pot experiments and iii) *ad planta* effect on the indigenous lettuce microbiome under greenhouse conditions. By exploiting results obtained by 16SrRNA amplicon sequencing of the lettuce microbiome, we developed a model to understand the biocontrol effect of *S. plymuthica* 3Rp8. Therefore screening for antagonism of three promising bacterial strains on pathogen inoculated solid media - via direct interaction and via indirect interaction by the production of bioactive volatiles - was performed. Rhizosphere competence and plant growth promotion of 3Rp8 was evaluated using sterilised seeds treated with the bioinoculant individually and in combination with the plant growth

promoting strain and the competitor *Pseudomonas fluorescens* WCS 365 in steady state pots under gnotobiotic conditions. Confocal laser scanning microscopy combined with fluorescence *in situ* hybridization was applied to investigate the colonisation success and response of the DsRed fluorescence protein tagged strain 3Rp8 along whole lettuce plantlets. The effects on the community structure of *Lactuca sativa* L. var *capitata* mediated through the application of the biocontrol agent 3Rp8 was revealed by 16S rRNA gene MiSeq Illumina amplicon sequencing. Received data were analysed with QIIME and visualised with Cytoscape and KRONA.

2. Materials and Methods

2.1. Flow sheet

The illustrated flow sheet provides an overview about the performed work in this thesis.



2.2 Sources of supply

Austrosaat (Vienna, Austria), Biorad (Vienna, Austria), Biometra (Göttingen, Germany), Bitplane (Zürich, Swizerland), Fermentas (St. Leon-Rot, Germany), genXpress (Wiener Neudorf, Austria), Gramoflor (Vechta, Germany), Greiner Bio-One (Frickenhausen, Germany), Invitrogen (Lofer, Austria), Lactan (Graz, Austria), Leica Microsystems (Mannheim, Germany), Life Technologies (Carlsbsd, California USA), MP Biomedicals (Illkirch, France), New England Biolabs (Frankfurt, Germany), Promega (Madison, WI, USA), Roth (Karlsruhe,

Germany), Sifin (Berlin, Germany), Sigma-Aldrich (St. Louis, USA), Thermo Scientific (Wilmington, DE, USA), Thermo Fischer Scientific (Vienna ,Austria) Qiagen (Vienna, Austria)

2.3 Growth Media

All used media and solutions were autoclaved at 121°C for 15 minutes to assure sterility.

Nutrient bouillon (NB; Sifin, Germany):

Peptone from casein	3.5 g
Peptone from meat	2.5 g
Peptone from gelatine	2.5 g
Yeast extract	1.5 g
Sodium chloride	5.0 g
Aqua dest.	ad 1000 ml
pH 7.0 ± 0.2	

Potato dextrose agar (PDA; Sifin, Germany):

Potato-Infus	6.5 g
Glucose*1H_2O	20.0 g
Agar	15.0 g
Aqua dest.	ad 1000 ml
pH 5.6 ± 0.2	

MacConkey Agar (Roth, Karlsruhe, Germany):

Pancreatic digest of gelatine	7.0 g
Poly peptone	3.0 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Aqua dest.	ad 1000 ml
pH 7.1 ± 0.2	

Nutrient Agar II (NA; Sifin, Germany):

Peptone from casein	3.5 g
Peptone from meat	2.5 g
Peptone from gelatine	2.5 g
Yeast extract	1.5 g
Sodium chloride	5.0 g
Agar	15.0 g
Aqua dest.	ad 1000 ml
pH 7.0 ± 0.2	

LB-Medium (Roth, Karlsruhe, Germany):

Tryptone	10.0 g
Yeast extract	5.0g
Sodium chloride	10.0g
Agar	15.0 g
Aqua dest.	ad 1000 ml
pH 7.0 ± 0.2	

For selective media, the medium was autoclaved for 15 minutes and after cooling-down, the antibiotic Trimethoprim (Sigma Aldrich) was added to a final concentration of 50mg/ml. Trimethoprim was dissolved in dimethylsulfoxid (DMSO).

2.4 Buffers, solutions and chemicals

TAE [50x]:

Tris 99.9 %	242.0 g
Acetic acid 100%	57.0 ml
EDTA [0.5 M] pH 8	100.0 ml
Aqua dest.	ad 1000 ml

TBE-Puffer [5x]:

Tris [99.9 %]	54.0 g
Boric acid [99.8 %]	27.5 g
EDTA [0.5 M] pH 8	20.0 ml

0.85 % Sodium chloride peptone:

Peptone from meat	1.0 g
Sodium chloride	8.5 g
Aqua dest.	ad 1000 ml

10 % Glycerine solution:

Glycerine	10.0 ml
Aqua dest.	ad 1000 ml

Phosphate buffer:

NaH_2PO_4 [200mM]	20 % (v/v)
Na_2HPO_4 [200mM]	80 % (v/v)
Aqua dest.	ad 1000 ml

pH 7.2 – 7.4

Phosphate buffered saline (PBS) [1x]:

Phosphate buffer	5 % (v/v)
NaCl	8.0 g
Aqua dest.	ad 800 ml

pH 7.2 – 7.4

PBS [3x]:

Phosphate buffer	15 % (v/v)
NaCl	24.0 g
Aqua dest.	ad 800 ml

pH 7.2 – 7.4

Tris-HCl [1M]:

Tris hydroxymethyl-	
aminomethan [99.9 %]	24.2 g
concentrated HCl to adjust pH 8.0	
Aqua dest.	ad 1000 ml

6x Loading Dye:

Bromphenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)
Glycerol	30.0% (v/v)
0.5 M EDTA-NA_2*$2H_2O$	30.0 mM

1% Sodium hypochloride solution (NaOCl):

12% NaOCl-stock	
solution	25 ml
Aqua dest.	275 ml

4 % Paraformaldehyde (PFA) solution:

Aqua dest.	33.0 ml
PFA	2.0 g
Sodium hydroxide (NaOH) until PFA is dissolved	
PBS [3x]	16.6 ml
pH 7.2 – 7.4	

For 50 ml PFA solution, 33 ml distilled water was heated to 65°C. 2.0 g PFA was added while stirring and NaOH was added until the PFA was dissolved.

16.6 ml PBS [3x] was added and the solution was cooled to room temperature. The pH was adjusted between 7.2 – 7.4.

EDTA [0.5M]:

Ethylendiaminetetraaceticacid 9.306 g/50.0 ml

Addition of NaOH pellets to a final pH of 8.0

Electron acceptor Iodonidtetrazoliumchloride (INT) [95 %] (Fig. 1)

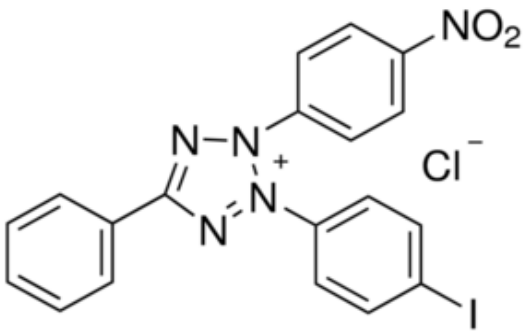


Figure 1 Molecular structure of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride. Common synonyms for this structure are *p*-Iodonitrotetrazolium violet and INT. source: www.sigmaaldrich.com.

INT was dissolved in ethanol [99.9 %] and added to the cooled down NA II agar at a final concentration of 0.2 mg/ml.

2.5 DNA Standards

For all gel electrophoreses, Generuler DNA ladder mix 1kb or 100 bp (Life Technologies, Carlsbad, California USA) at a concentration of 0.01 g/l was used. Between three to five microliter, depending on the thickness of the gel, were used for each preparation (Fig. 2).

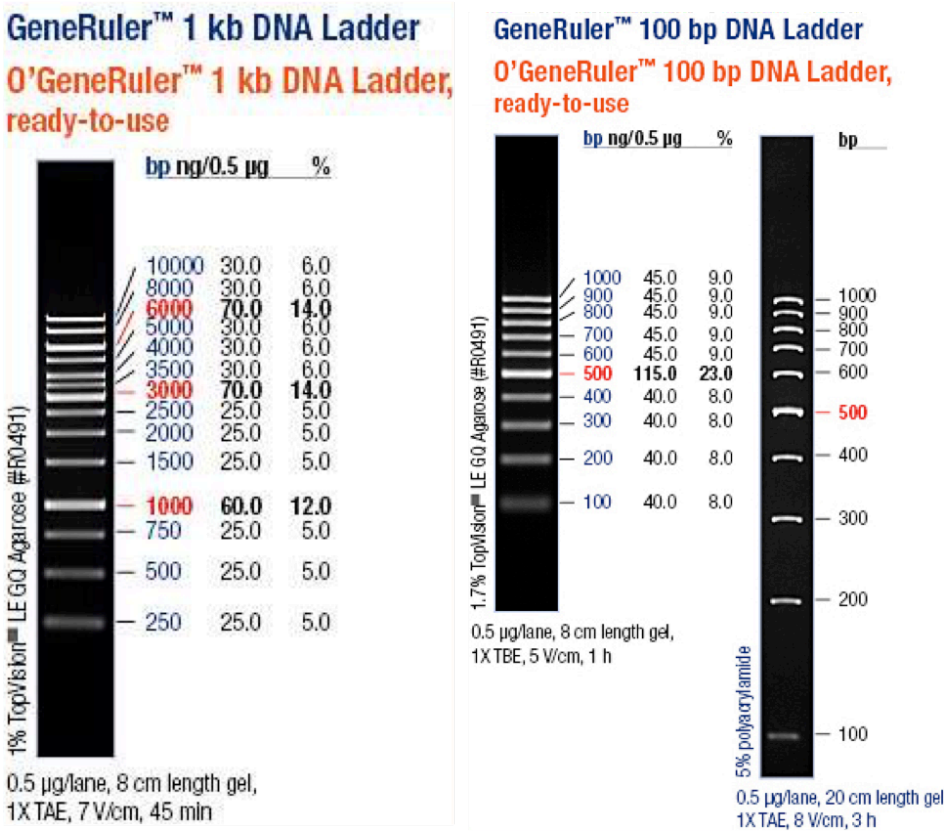


Figure 2 Generuler 1kb and 100 bp DNA Ladder (Life Technologies). Between three and five microliter were used for each gel electrophoresis.

2.6 Bacterial strains used in this thesis

Various pathogenic model organisms and biological control agents (BCAs) were used in this study and are listed in Table 3.

Table 3 Strains utilized in this study as model pathogens and biological control agents

Strain	Description	Reference
Serratia plymuthica 3Re4-18	BCA[1]	Berg et al., 2005a
Serratia plymuthica 3Rp8(dsRed)	BCA DsRed[2]	Berg et al., 2002 Vergunst et al., 2010

E. coli K12	MP[3]	Strain collection
Serratia marcescens	MP[3]	Strain collection
Pseudomonas fluorescens WCS 365	MP/RC[4]	Bollwerk et al., 2003
Enterobacter agglomerans 4Rx13	MP[3]	Berg et al., 2002
Salmonella typhimurium LT2	MP[3]	Strain collection
Klebsiella pneumoniae	MP[3]	Strain collection
Bacillus subtilis Co1-6	MP[3]	Köberl et al., 2011

[1] BCA – biological control agent; [2] BCA DsRed – biological control agent tagged with DsRed fluorescence protein (plasmid pIN69); [3] MP – model pathogen from the strain collection of the Institute of Environmental Biotechnology, of the Technical University of Graz; [4] MP/RC – model pathogen used as rhizosphere competitor from the strain collection of the Institute of Environmental Biotechnology, of the Technical University of Graz.

Strains of the established Gram-negative bacteria library isolated from *Lactuca sativa* L. var *capitata* from three different locations, which were used as potential pathogens for antagonistic screening are listed in Table 4.

Table 4 Strains of the Gram-negative bacteria library used for antagonistic screening. Strains listed below were isolated from different *Lactuca sativa* L. var *capitata* cultivars from three different locations and were used as model pathogens for antagonistic screening with *Serratia plymuthica* 3Re4-18, *Serratia plymuthica* 3Rp8 and *Serratia plymuthica* 3Rp8 tagged with DsRed fluorescent protein.

Strain	**Cultivar isolated from**
Achromobacter spanius	Maikönig[1]
Pseudomonas putida	Iceberg[2]
Moellerella wisconsensis	Iceberg[3]
Serratia liquefaciens	Iceberg[3]
Pantoea wallisii	Iceberg[2]
Pantoea rodasii	Iceberg[2]
Enterobacter aerogenes	Iceberg[2]
Enterobacter ludwigii	Iceberg[2]
Pantoea eucalypti	Iceberg[2]
Pantoea ananatis	Iceberg[2]

Pantoea agglomerans	Iceberg[3]
Curtobacterium plantarum	Iceberg[3]

[1] Maikönig - *L. sativa* L. var *capitata* from a private garden; [2] Iceberg - *L. sativa* L. var *capitata* from a local market; [3] Iceberg - *L. sativa* L. var *capitata* from a commercial shop.

2.7 Screening for bioactive volatiles of 3Rp8(dsRed), 3Re4-18 and Co1-6 by two clamp volatile organic compound assays (TCVAs)

The TCVAs were performed as described in Cernava et al., 2015. 6-well plates (Greiner Bio-One, Frickenhausen, Germany) containing Nutrient Agar (NA, Sifin, Germany) were used to streak out the antagonists. The plates were pre-incubated for 24 h at 30 °C. The model strains for pathogens (Table 3, Table 4) were cultured in fluid Nutrient Broth (NB, Sifin, Berlin, Germany) to an OD_{600} of 0.5. 8 ml aliquots were transferred to 80 ml NA, supplemented with 0.2 mg/ml INT (Fig. 1) and 5 ml of this suspension were immediately transferred into each well of the 6-well plates. After solidification of the pathogen containing plates, silicone foils were placed between the two plates, of which one contained the model pathogen and one contained the antagonists. As a positive control, a disinfectant was used in the 6-well plates instead of antagonist. For the negative control 6-well plates containing only NA were used. The plates were fixed with clamps and incubated with the possible antagonist on top at 21 °C for 48 hours (Fig. 3). After 24 hours of incubation the plates were checked for indicator change and were compared to the positive and negative controls.

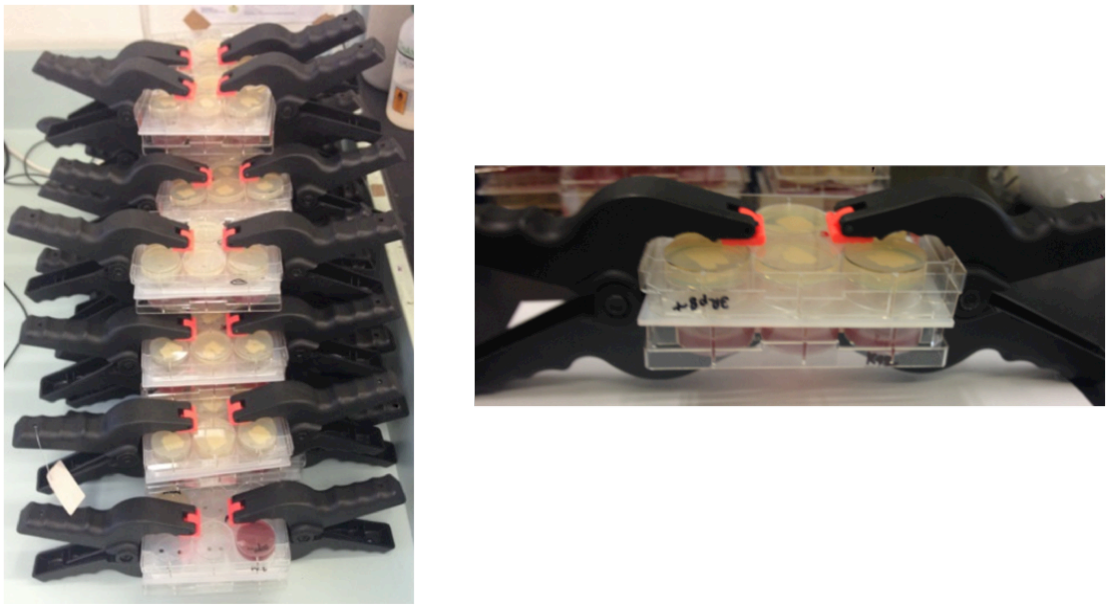


Figure 3 Two clamp volatile organic compound assays (TCVAs) with 3Rp8(dsRed) and 3Re4-18. The plates were fixed with clamps and incubated with the antagonists on top at 21°C for 48 hours. After 24 hours of incubation the plates were checked for indicator change and were compared to positive and negative controls.

2.8 Confrontation plate assays (CPAs) with 3Rp8(dsRed), 3Re4-18 and Co1-6 against model pathogens

The potential antagonists were screened for antagonism *in vitro* against model pathogens from the strain collection from the Institute of Environmental Biotechnology, of Graz University of Technology (Table 3) and against a Gram-negative bacteria library (Table 4) isolated from two different *Lactuca sativa* L. var. capitata cultivars (Iceberg lettuce and “Maikönig”), which originated from three different locations (from a local market, grown in a home garden and from a commercial shop). It was also tested if there is a difference between the tagged and the not tagged strain 3Rp8 in antagonistic activity. The Gram-negative bacteria library, was created by Olivia Laggner during her Bachelor´s thesis and the isolates were identified by 16S rDNA sequencing in this work (section 2.12). The potential antagonistic strains were streaked out on NA and incubated for 12 h at 30 °C. The model pathogens and the model pathogens of the Gram-negative bacteria library, were cultured in fluid Nutrient Broth (NB, Sifin, Berlin, Germany) to an OD_{600} of 0.5. 8 ml aliquots were transferred to 80

ml NA and 5 ml of this suspension were immediately transferred into each well of 6-well plates (Greiner Bio-One, Frickenhausen, Germany). After solidification of the model pathogen-containing plates, each well was inoculated with one of the potential antagonists by three single streaks of a single colony in the centre of the well, using a tooth pick. The plates were incubated for 48 hours at 30 °C and at 21 °C. The edge of the zone of inhibition was measured with a ruler after 12 and 24 hours.

2.9 Genome screening of 3Rp8(dsRed) and 3Re4-18 for plant growth promoting and antagonistic mechanisms

The genomes of both *Serratia* strains were kindly provided by Dr. Henry Müller (Institute of Environmental Biotechnology; Graz University of Technology). Both genomes were screened for potential antagonistic and plant growth promoting genes and proteins against bacteria and fungi. Sequences of pre-selected genes were aligned and compared with sequences from the NCBI database with the BLASTn and BLASTp algorithms (http://www.ncbi.nlm.nig.gov).

The abovementioned strains were tested against the fungal model pathogens *Rhizoctonia solani* and *Botrytis cinerea,* provided by the in-house strain collection (Institute of Environmental Biotechnology, Graz University of Technology), to compare genome analyses with *in vivo* activity. It was also tested if there is a difference in the antagonistic potential between the tagged and non-tagged strain 3Rp8. The pathogenic fungi were first cut with a cork borer, equally sized plugs, and transferred with a sterile tooth pick onto PDA plates. The possible antagonists were streaked out between the fungal samples as shown in Fig. 4. Plates were incubated at room temperature for 48 hours and evaluated according to the method described by Berg and Bahl (1996).

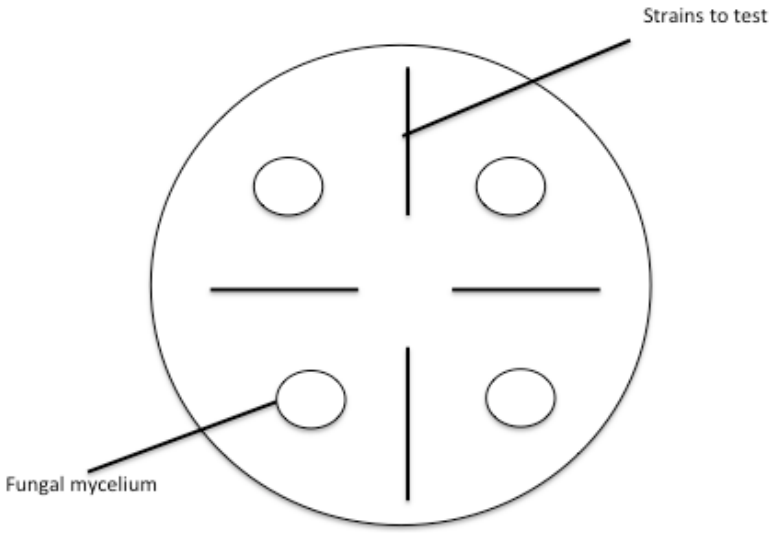


Figure 4 Dual culture scheme with potential antagonists and fungal pathogens. Fungal test strains and potential antagonists (3Rp8 and 3Re4-18) were transferred with a sterile tooth pick. Subsequently, the PDA plates were incubated for 48 hours.

Additionally, a test for quorum sensing detection was applied to compare genome analyses with *in vivo* activity. The production of the quorum sensing molecule AHL (N-acyl-homoserin-lactone) was assessed with the sensor strain *Chromobacterium violaceum* CV026 and the reference strain *Serratia plymuthica* 3Re4-18. The test is based on the production of short chain C4 to C6 quorum sensing molecules. If these substances are produced, the sensor strains change to a purple colour at the contact point. Fig. 5 illustrates the experiment setup.

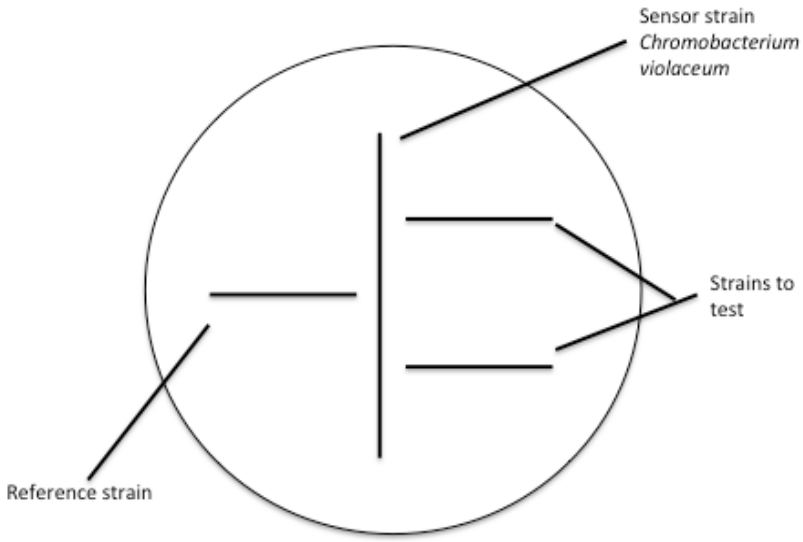


Figure 5 Scheme of the quorum sensing test. The sensor strain *Chromobacterium violaceum* was applied with the reference strain *Serratia plymuthica* 3Re4-18 and *Serratia plymuthica* 3Rp8 and 3Rp8(dsRed).

2.10 Identification of isolates obtained from *Lactuca sativa* L. var. capitata by 16S rDNA sequencing

DNA from pure cultures was amplified with primer pair 27F/1492r according to Weisburg et al., 1991. The PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) followed by Sanger sequencing (LGC Genomics, Berlin, Germany). The sequences were aligned with BLASTn (http: //www.blastn.ncbi.nlm.nih.gov/). Identification of the closest match (query cover between 98-100%) was applied for the retrieved results. For the preparation of a phylogenetic tree, representative sequences for each isolate were picked from the NCBI database (http://www.ncbi.nlm.nih.gov/) and as outgroups two Gram-positive strains (*Bacillus subtilis* and *Paenibacillus pabuli*) were selected. The multiple sequence alignment was created with Clustal W version 2.0 (Larkin et al., 2007). The alignment was visualised with UGENE for manual adjustment (Okonechnikov et al., 2012). Phylogenetic trees were built using the software PYHLIP (Felsenstein et al., 1989). A distance matrix was created by DNADist and processed by the neighbor-joining method. For visualisation and editing of the phylogenetic tree, the software FigTree was used (Schneider et al., 2000).

2.11 PGP effects of 3Rp8(dsRed) on *Lactuca sativa* L. var *capitata* "Gelber Winter"

2.11.1 Tests with 3Rp8 tagged with DsRed2 fluorescent protein

The strain 3Rp8(dsRed) was kindly provided by Eveline Adam from the Institute of Environmental Biotechnology of Graz University of Technology. The strain carries the plasmid pIN69, which is a non-mobilisable pBBR-derived plasmid with a broad host range. It contains Trimethoprim as the selectable marker gene, an origin of transfer (OriT), an origin of replication (rep) and expresses DsRed2 from the *tac* promotor sequence (Fig. 6; Vergunst et al., 2010).

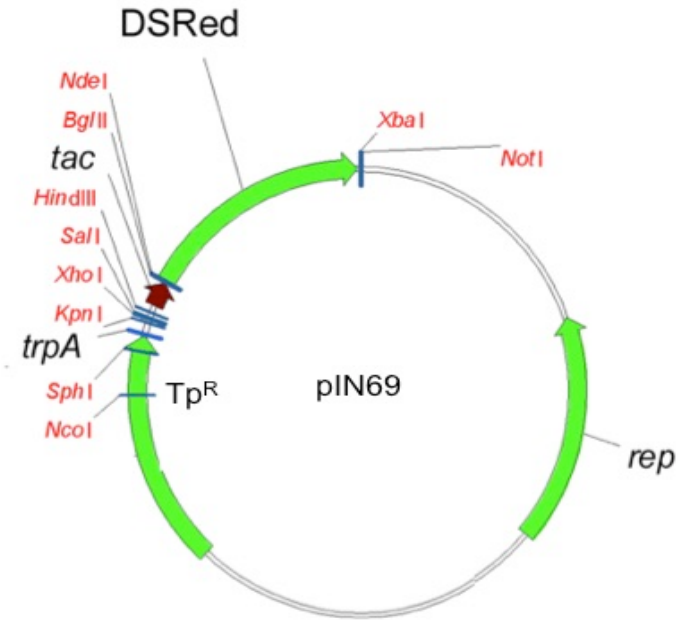


Figure 6 DsRed2 pIN69 expression plasmid transformed into *Serratia plymuthica* 3Rp8 (layout). The vector construct pIN69 is a non-mobilisable version and derived from a pBBR-broad host range plasmid. Abbreviations: Tp^R, trimethoprim selectable marker; OriT, origin of transfer; rep, required for replication; DsRed, gene encoding red fluorescent protein, DsRed2; *tac, tac* promotor sequence, *trpA*, termination sequence of *trpA* gene.

The expression of DsRed2 fluorescent protein in 3Rp8 allows fast monitoring of inoculated lettuce plants with a confocal laser scanning microscope (CLSM) at an excitation wavelength of 532 nm.

2.11.2 Inoculation of lettuce seeds and growth experiment setup

Soil composed of one part quartz sand and three parts peat soil (Gramoflor, moderate to strong decomposition) was utilised. The trays were filled with 162 g soil and irrigated with 50 ml tap water. After autoclaving the moisture content was measured with a gravimetric moisture content analyser. Lettuce seeds were sterilised in 50 ml tubes with 15 ml of a 1% NaOCl-solution for five minutes on a rotary shaker at 120 rpm. After sterilising, the seeds were washed four times with autoclaved water and dried overnight in a laminar flow cabinet. For the preparations of starter cultures, single colonies of each bacterial strain were inoculated in 6 ml nutrient broth II medium and grown at 30 °C and 120 rpm overnight. To ensure that the bacterial strains are in an optimum growth phase (exponential phase), the main cultures were inoculated with an optical density of 0.05. All main cultures were incubated at 30 °C and 120 rpm for two hours. Cell counts were determined with a haemocytometer. The sterilised lettuce seeds were inoculated in 15 ml nutrient broth with a cell count of 10^7 cells per millilitre on a rotary shaker for 20 minutes. Following inoculations were prepared: seeds with 3Rp8(dsRed); seeds with WCS365; seeds with WCS365+3Rp8(dsRed) and seeds without any inoculated bacteria. For each treatment 48 seeds were inoculated and after incubation washed three times with sterilised H_2O, dried for half an hour and planted half a centimetre deep in the autoclaved soils. The planting pattern of the bioprimed seeds is shown in Fig. 7. Six inoculated lettuce seeds were planted in each sterilised seed tray.

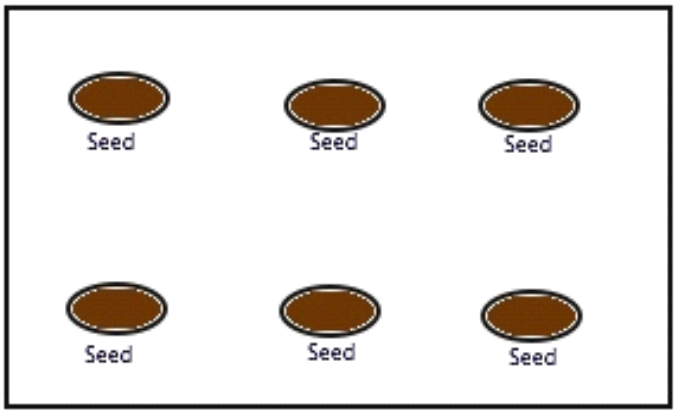


Figure 7 Planting pattern of inoculated lettuce seeds. The seeds were inoculated with a cell count of 10^7 in 15 ml nutrient broth II. After incubation for 20 minutes on a rotary shaker they were washed three times with sterile water, dried under a laminar flow hood and planted with a sterile tweezer.

The trays were arranged in a randomised design in a green house at 28 °C during the day and 18 °C at night, with a 14 h light period followed by a 10 h dark period.

2.11.3 Fixation of lettuce plants for FISH/CLSM, determination of colony forming units and measurement of the lettuce plants

Seed germination was recorded on the fourth day. After 13 days of growth, six trays of each treatment were collected for measurements of the plants. Two trays of each treatment were used for the determination of colony forming units per ml and one plant of each treatment was fixed for FISH. Plants were carefully extracted from the trays with a sterile tweezer and roots and phyllosphere were separated with a sterile scalpel. Roots were additionally freed from the adhering soil and samples were collected in sterile 50 ml tubes for fixation. 2 ml of 1xPBS were added to all samples. 1xPBS was immediately removed and 4 ml of 4% PFA and 1x PBS at a ratio of three to one were added. The samples were incubated overnight at 4 °C. After incubation the solution was removed and all samples were washed four times with 2 ml of 1xPBS. At the first washing step PBS was immediately removed again. For the second step samples were incubated for 10 minutes and for the third and fourth washing step samples

were incubated for 5 minutes. The samples were overlaid with 2 ml 96% ethanol and 2 ml 1xPBS and stored at -20 °C until further use. For determination of the CFU/ml, phyllosphere and rhizosphere samples were separated in sterile plastic bags and weighed on an analytical balance. The plant parts were disrupted with a sterile pestle and mortar and dissolved in 3 ml of a sterile 0.85% NaCl-Peptone solution. Serial dilutions between 10^0 and 10^{-5} were prepared and 10 µl of the dilutions were transferred on nutrient agar (Sifin, Berlin, Germany). The plates were incubated at 30 °C for 12 hours. Colonies were counted on a plate counter and CFU/ml were determined with following equation:

$$\frac{CFU}{mL} = \frac{counted\ colonies}{mL\ solved * dilution\ factor}$$

The length of roots, leaves and stems of the plants, which were inoculated as previously described, were measured with a ruler. Roots and foliage were analysed together and fresh and dry weight of each treatment was determined on an analytical balance.

2.11.4 Statistical analysis

PASW Statistics 18 software (SPSS Inc. 2009) was used to calculate significant correlations between plant growth after different treatments. Data were checked for homogeneity of variance and for normal distribution. With data that followed a normal distribution, a one-way ANOVA analysis was performed. Furthermore a post hoc test was applied. Non-homogenous data were analysed with a Games-Howell analysis and homogeneous data were analysed with Tukey and Sheffé tests. Figures containing the analysed data were labelled with letters to match the corresponding groups.

2.12 High-throughput sequencing of total community DNA

2.12.1 Growth of lettuce plants under advanced greenhouse conditions

A total of 28 lettuce seedlings were grown until they reached a four to eleven leave stadium under greenhouse conditions in pots (Fig. 8). Soil was composed of one part quartz sand and three parts peat soil (Gramoflor, moderately to strongly decomposed) was utilised. Seeds ("Neusiedler Gelber Winter", Austrosaat, Vienna, Austria) were grown under greenhouse conditions. For irrigations before the inoculation, tap water was used as required. The pots were placed in the atrium of the greenhouse for 47 days. Seven days before inoculation the pots were placed in a greenhouse at 28 °C during the day and 18 °C at night, with a 14 h light period followed by a 10 h dark period.

Figure *8 Lactuca sativa* L. var capitata grown to a four to eleven leave stadium under greenhouse conditions. 28 lettuce seedlings "Neusiedler Gelber Winter" were grown till a four to eleven leave stadium under controlled greenhouse conditions in pots. Therefore soil composed of one part quartz sand and three parts peat soil was used (Gramoflor, moderately to strongly decomposed) was utilised. Seeds commercially available from "Austrosaat" were grown under greenhouse conditions.

2.12.2 Inoculation of lettuce plants with *Serratia plymuthica* 3Rp8(dsRed)

After 54 days of growth the lettuce plants were bedded-out and inoculated with 10^6 cells of 3Rp8(dsRed). Six replicates with the following treatments were prepared: application of *Serratia plymuthica* to the rhizosphere (Dip+), application of *Serratia plymuthica* to the phyllosphere (Spray+) and application of sterile inoculum to phyllosphere (Spray-) and rhizosphere (Dip-). For monitoring the accumulation of 3Rp8(dsRed) on rhizosphere and phyllosphere, four additional plants were inoculated either on phyllosphere or rhizosphere with bacteria and a control inoculum. For preparing the bacterial inocula for the plant treatments, 3Rp8(dsRed) was grown in nutrient broth (Sifin, Germany) for 12 h at 30 °C. To ensure that the cells are in an optimal growth phase, the main culture was started with an optical density of 0.05 and was incubated at 30 °C and 120 rpm for two hours. Cell counts were determined with a haemocytometer. The inocula were prepared with a 1:100 dilution of the main culture with autoclaved water. For the controls, the main cultures were centrifuged at 5000 rpm for 15 minutes, the resulting supernatant was sterile filtered with a 0.2 µm syringe filter and diluted in the same factor as the positive controls before application.

The phyllosphere was inoculated by spraying 25 ml of the inoculum on each lettuce head with a sprayer (100 ml sprayer with Erlenmeyer flask) as shown in Fig. 9 A. The rhizosphere was inoculated by dipping. Therefore the roots, which were previously freed from adhering soil by shaking virgously, were dipped in the inoculum for 30 minutes (Fig. 9 B). The CFU/ml of the utilized inocula were determined on nutrient agar (Siffin, Germany). The inoculated plants were kept in the greenhouse at 28 °C during the day and 18 °C at night, with a 14 h light period followed by a 10 h dark period. Irrigation was done with 250 ml of autoclaved tap water per plant immediately one day after inoculation and further watering was done on every fifth day.

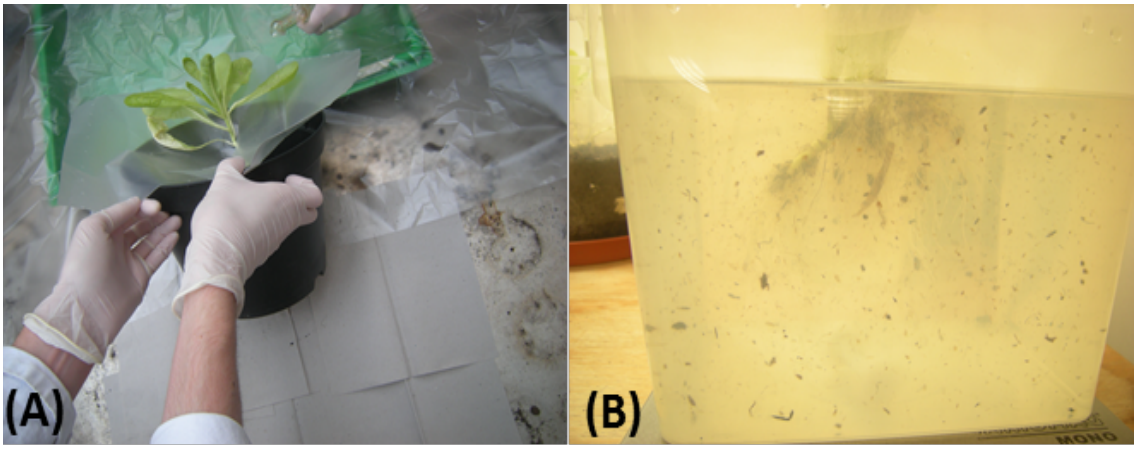


Figure 9 Inoculation of the lettuce phyllosphere (A) and rhizosphere (B) with 10^6 cells of *Serratia plymuthica* 3Rp8(dsRed). (A) shows the inoculation of the phyllosphere by spraying, where 25 ml of the inoculum were applied with a sprayer (100 ml sprayer with Erlenmeyer flask) onto each lettuce plant; (B) shows the inoculation of the rhizosphere, where the roots which were previously freed from adhering soil by shaking virgously, were dipped in the well mixed inoculum for 30 minutes

2.12.3 Monitoring of inoculated lettuce plants

The inoculated control plants of each treatment were grown for two days, bedded out and phyllosphere and rhizosphere were separated in sterile plastic bags. The plant parts were weighed, disrupted with a pestle and mortar and dissolved in 3 ml of a 0.85% NaCl-Peptone solution. Serial dilutions between 10^{-1} and 10^{-4} were prepared and plated out on LB-Trimethoprim plates and incubated at 30 °C for 14 hours. CFU/ml and gram (CFU/ml*g) were determined according to the equation in 2.11.3. To check for fluorescence of the colonies, the plates were analysed with a CCD-camera at 532 nm with the Gel Doc^{TM} XR^{+} System (Biorad, Austria) and the corresponding software Image Lab^{TM}.

2.12.4 Sampling of inoculated lettuce plantlets

After 13 days of growth of the inoculated lettuce plants, the plants were bedded out and phyllosphere and rhizosphere were separated in sterile plastic bags and petri dishes. From each treatment rhizosphere and phyllosphere samples from three plants were fixed for FISH as described in 3.7.2. The CFU/ml*g of phyllosphere and rhizosphere from three plants of each treatment were

determined on LB-Trimethoprim, two plants of each treatment were prepared for confocal laser scanning microscopy and DNA was extracted from six plants of each treatment for total metagenomic community analyses.

2.12.5 Total community DNA extraction

Rhizosphere

Between 0.36 g and 6.70 g of roots with surrounding soil were weighed in plastic bags. The material was disrupted with liquid nitrogen and ground with a pestle. Samples were homogenized with 5 - 25 ml of a sodium chloride peptone solution (0.85%). The supernatant was transferred into 2.0 ml tubes in duplicates and centrifuged for 25 minutes with 13500 rpm at 4 °C. The supernatant was discarded and the resulting pellet was stored until further analyses at -70 °C.

Phyllosphere

Between 0.87 g and 5.36 g of leaves were weighed in plastic bags. The material was disrupted with liquid nitrogen and ground with a pestle. Samples were homogenized with 5 - 25 ml of a sodium chloride peptone solution (0.85%). The supernatant was transferred into 2.0 ml tubes in duplicates and centrifuged for 25 minutes with 13500 rpm at 4°C. The supernatant was discarded and the resulting pellet was stored until further analyses at -70 °C.

All six replicate samples of each treatment were picked and used for DNA extraction. DNA from rhizosphere and phyllosphere samples was extracted from between 180 mg and 660 mg plant tissue using a Fast DNA SPIN Kit for soil (MP Biomedicals, Eschwege, Germany), according to the manufacturers protocol. The extracted DNA was dissolved in 100 µl nuclease free water, quantified with a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA) at 260 nm and stored at -70 °C until further use.

2.12.6 Quantification of liveable bacteria from inoculated plants

From each treatment (Spray+, Spray-, Dip+, Dip-) three plants were used for the determination of colony forming units per ml and gram. Therefore, the plant samples (rhizosphere, phyllosphere) were separated with a sterile scalpel and weighed in sterile plastic bags on an analytical balance. Each plant part was dissolved in 15 ml of a 0.85% NaCl-Peptone solution and homogenised in a bag mixer for 30 seconds. Serial dilutions between 10^{-1} to 10^{-4} CFU/ml for the phyllosphere and between 10^{-1} to 10^{-6} CFU/ml for the rhizosphere were prepared with a 0.85% NaCl-Peptone solution. 10 µl of each dilution were transferred to LB-Trimethoprim plates and incubated for 14 hours at 30 °C. CFU/ml and gram were determined according to the equation in section 2.11.3. To check for fluorescence of the colonies, the plates were analysed with a CCD-camera at 532 nm with the Gel Doc^{TM} XR^{+} System (Biorad, Austria) and the corresponding software Image Lab^{TM}.

2.12.7 Barcoded MiSeq Illumina sequencing of 16S rRNA gene amplicons

MiSeq Illumina sequencing is a high-throughput method that generates 1.5 Gb per day from 5 million 150-base paired-end reads (Caporaso et al., 2012). Illumina sequencing is a next-generation sequencing (NGS) technology, where flow cell adaptors and sequencing primers are added to the target sample. Sequencing occurs first by bridge amplification of the target DNA followed by sequencing by synthesis, where each incorporated base emits a unique fluorescent signal as it is added to the growing strand, which is used to determine the order of the DNA sequence (www.illumina.com/technology/next-generation-sequencing.html). Using this method the hypervariable V4 region of the 16S rRNA gene was amplified with the primer pair 515f and 806r that include the flow cell adaptors for Illumina and the specific barcodes for sample identification. Sequences were analysed with the QIIME software version 1.9.1 (Caporaso et al., 2010). To minimise primer bias, a PCR was performed using the molecular tagging with peptide nucleic acid (PNA) PCR clamps protocol described in Lundberg et al., 2013.

2.12.8 Bacterial 16S rRNA gene amplification for MiSeq sequencing

The amplification was performed using molecular tagging with peptide nucleic acid (PNA) PCR clamps as described in Lundberg et al., 2013. The hypervariable V4 region was amplified using the region-specific bacterial/archaeal primer 515f/806r that include the Illumina flow cell adaptors and are listed in Table 4 (Caporaso et al., 2012). Barcodes (BC1-BC48) were located on the reverse primer 806r. All PCR reactions were prepared on ice, and amplified in triplicates with a PCR cycler (Biometra, Germany). Illumina PCR reactions including a mixture with anti-mitochondria PNA (mPNA) and anti-plastid (pPNA) for each sample was composed of 6.0 µl 5x Taq & Go (MP Biomedicals, Illkirch, France), 1.2 µl of the forward Primer 515f_BC (5.0 µM), 1.2 µl of the reverse Primer 806r_BC (5.0 µM), 0.45 µl mixed PNAs (1:1 mix of 100 µM pPNA and 100 µM mPNA stocks), 19.15 µl PCR water and 2.0 µl DNA (~1.5 ng in 30 µl). Following temperature program for amplification was carried out:

1. Initial Denaturation……. 96°C, 05 min
2. Denaturation……..... 96°C, 01 min
3. PNA annealing…… 78°C, 05 s
4. Primer annealing…….. 54°C, 01 min
5. Elongation…… 74°C, 01 min
6. Repeat steps 2-5 30 times
7. Final Elongation……... 74°C, 10 min
8. Hold…… 4°C

Table 5 Primers for MiSeq Illumina sample amplification and barcoding. 515f (forward primer) PCR primer sequence: blue - 5´Illumina adapter; black - Forward primer pad; dark purple - Forward primer linker; green - Forward primer. 806r (reverse primer) PCR primer sequence (each sequence contains different barcode): blue - Reverse complement of 3' Illumina adapter; red - Golay barcode; black - Reverse primer pad; dark purple - Reverse primer linker; green - Reverse primer.

Primer	Sequence 5' - 3'	Target Organism	Reference
515f	AATGATACGGCGACCACCG AGATCTACAC TATGGTAATT GT GTGCCAGCMGCCGCGGTAA	*Eubacteria*/*Archaea*	Caporaso et al., 2012
806r	CAAGCAGAAGACGGCATAC GAGAT XXXXXXXXXXXX AGTCAGTCAG CC GGACTACHVGGGTWTCTAA T	*Eubacteria*/*Archaea*	Caporaso et al., 2012

PCR amplification was checked with agarose gel electrophoresis. Thereafter, triplicate products from each sample were pooled during PCR purification according to the manufacturer´s protocol (Wizard® SV Gel and PCR Clean-Up System; Promega, Madison, WI, USA). DNA concentrations were measured with a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA) at 260 nm and samples were combined equimolar for MiSeq Illumina sequencing (LGC, Berlin, Germany). Grouping of the samples from the four different treatments with corresponding barcodes:

BC_01-BC_06: Dip^- Rhizosphere

BC_07-BC_12: Dip^- Phyllosphere

BC_13-BC_18: Dip^+ Rhizosphere

BC_19-BC_24: Dip^+ Phyllosphere

BC_25-BC_30: $Spray^-$ Rhizosphere

BC_31-BC_36: $Spray^-$ Phyllosphere

BC_37-BC_42: $Spray^+$ Rhizosphere

BC_43-BC_48: $Spray^+$ Phyllosphere

Amplicon sequencing was performed on the Illumina MiSeq platform at LGC Genomics (Berlin, Germany). Sequencing reads were assigned to each sample according to the unique barcode of each sample.

2.12.9 Bioinformatic analyses and taxonomic assignments

Sequences were analysed with the QIIME software version 1.9.1 (Caporaso et al., 2010). Sample replicates of each treatment and habitat were bioinformatically pooled during QIIME analyses. Barcodes were removed, minimum sequence length was adjusted to ≥200 nucleotides, the phred quality score was set to 20, chloroplasts and mitochondria were filtered and singletons were dislodged. Chimera checking was done with usearch within QIIME. Reads were assigned to operational taxonomic units (OTUs) by using the open reference OTU picking protocol of QIIME, where uclust was applied to search sequences against a subset of the greengenes database filtered at 97% identity (Caporaso et al., 2012). For core diversity analyses the data of rhizosphere samples were normalised to 3970 reads per sample and for the phyllosphere it was normalised to 4453 reads per sample. Ring charts were created using the Krona software package version 2.2 (Ondov et al., 2011).

Co-occurrence patterns were determined at 97% similarity. Spearman's correlation and corresponding significance was calculated in R version 3.2.0 using the Hmisc package (http://www.r-project.org/; R core team, 2013). Spearman's correlations ($\rho \geq 0.6$ and $\rho \leq -0.6$), which were statistically significant (p-value < 0.001), were considered as positive or negative correlations. The correlation- and statistic matrices were reassembled using pivot tables. Co-occurrence patterns were visualized as networks using Cytoscape version 3.2.1 (Shannon et al., 2003).

2.13 Confocal Laser Scanning Microscopy (CLSM) analysis of BCA-inoculated lettuce

2.13.1 CLSM imaging of phyllosphere and rhizosphere samples of inoculated lettuce plants

Two plants of each treatment were prepared for the microscopy. Phyllosphere and rhizosphere samples were separated with a scalpel and cut into small pieces. Leave and root parts were placed on a microscopy slide and immediately mounted with sterile PCR water. CLSM imaging was performed with a Leica TCS SPE confocal microscope (Leica Microsystems, Germany), followed by picture reconstructions with the software Imaris 7.3 (Bitplane, Swizerland). Fluorescent protein DsRed, which is expressed by the transformed plasmid pIN69 (Vergunst et al., 2010) in 3Rp8, was sequentially excited with 532 nm laser beams. The emitted light was detected in a emission window between 556 and 607 nm and an additional channel at 405 nm was applied for the auto fluorescence of the phototrophic plant cells. Photomultiplier gain and offset were individually optimised for every channel to improve the signal/noise ratio. Confocal stacks were acquired with a Leica ACS APO 40 OIL CS objective (NA: 1.15) by applying a Z-step size of 0.8 mm (Bragina et al., 2012).

2.13.2 Fixation of plant samples

From each treatment three plants were fixed with 4% Paraformaldehyd (PFA). Therefore the plants were carefully extracted from the pots with a tweezer and roots and phyllosphere were separated with a scalpel. Roots were freed from the adhering soil and samples were collected in sterile 50 ml tubes. Samples were washed with 2 ml of 1xPBS, which was immediately discarded and 4 ml of 4% PFA and 1x PBS at a ratio of three to one were added. The samples were incubated overnight (12 h) at 4 °C. After incubation the solution was removed and samples were washed four times with 2 ml of 1xPBS. At the first washing step PBS was immediately removed again. For the second step samples were incubated for 10 minutes and for the third and fourth washing step samples

were incubated for 5 minutes. Then samples were overlaid with 2 ml 96% ethanol and 2 ml 1xPBS and stored at -20 °C.

2.13.3 Fluorescence *in situ* hybridisation (FISH) of fixed phyllosphere and rhizosphere samples

Fixed phyllosphere and rhizosphere samples of the lettuce plants inoculated with 3Rp8(dsRed) and plant parts, which were fixed after growth promotion testing, were stained with the in-tube FISH method (Cardinale et al., 2008). The samples were hybridised with four different rRNA-targeting probes (genXpress, Wiener Neudorf, Austria) specific for *Enterobacteria*, *Gammaproteobacteria* and with universal bacterial probes, which are listed in Table 6. Selected pyllosphere and rhizosphere samples were additionally stained with calcofluor white (Sigma-Aldrich, St. Louis, USA) after hybridisation to increase the contrast of plant structures. The hybridization was carried out at 41 °C. Further information about stringency conditions, specificity and the fluorescent dyes is listed in Table 6. The negative control was labelled with the same fluorescent dyes as the corresponding positive FISH probes (Table 6).

Table 6 Nucleotide probes utilized for the FISH experiments

FISH Probe	Sequence 5' - 3'	Specificity	References	Formamide concentration (%)[a]	Dye
EUB338*	GCTGCCTCCCG TAGGAGT	Most bacteria	Amann et al., 1990	25	Cy3
Gam42a*****	GCCTTCCCACA TCGTTT	*Gamma-Proteobacteria*	Manz et al., 1992	45	Cy5
Gam42a-competitor	GCCTTCCCACT TCGTTT	*Beta-Proteobacteria*	Manz et al., 1992	45	/
Enterobac_D	TGCTCTCGCGA GGTCGC TTCTCTT	*Enterobacteriaceae*	Ootsubo et al., 2002	40	ATT O488
NONEUB	ACTCCTACGGG AGGCAGC	/	Wallner et al., 1993	-[b]	-[c]

[a] Stringency condition for hybridisation at 41 °C, [b] Probe used as a negative control at the same stringency conditions applied for the positive FISH probe, [c] Probe used for negative control was labeled with the same fluorophore as the corresponding FISH probe.

2.13.4 CLSM imaging of FISH-labeled phyllosphere and rhizosphere samples

Fixed FISH samples were placed on a microscopy slide and immediately dried with soft compressed air and mounted with ProLong® Gold antifadent (Thermo Fischer Scientific, Vienna, Austria). CLSM was performed with a Leica TCS SPE confocal microscope (Leica Microsystems, Germany), followed by picture reconstructions with the software Imaris 7.3 (Bitplane, Swizerland). Fluorescent dyes were sequentially excited with 532 nm, 488 nm and 635 nm laser beams. The emitted light was detected between 508-556 nm, 556-607 nm and 657-709 nm and an additional channel at 405 nm was applied for the auto fluorescence of the lettuce cells and the calcofluor white stained tissues. Photomultiplier gain and offset were individually optimised for every channel to improve the signal/noise ratio. Confocal stacks were acquired with a Leica ACS APO 40 OIL CS objective (NA: 1.15) by applying a Z-step size of 0.8 mm (Bragina et al., 2012).

3. Results

3.1 Screening for bioactive volatiles emitted by 3Rp8(dsRed), 3Re4-18 and Co1-6

The two clamp VOCs assay (TCVA) was performed to detect bioactive volatiles, produced by *Serratia plymuthica* 3Rp8(dsRed), *Serratia plymuthica* 3Re4-18 and *Bacillus subtilis* Co1-6, which can inhibit the growth of potential HPs. A colour change of the indicator INT, which was utilised to detect dehydrogenase activity of viable cells, was visually inspected after 24 hours of incubation. This was done for the combinations *E. coli* K12 with 3Rp8(dsRed), *E. coli* Op50 with 3Rp8, for *Serratia marcescens* with 3Rp8, and for *Salmonella typhimurium* with 3Rp8 (Fig. 10). After 48 hours of incubation a difference between the colour change of the negative control compared to the incubated samples could not be observed any more. Compared to the positive (disinfectant) and the negative control (NA), there was no increase of dead cells after 48 hours caused by bioactive volatiles produced by 3Rp8(dsRed), 3Re4-18 and Co1-6. The results for the 24 h incubation are shown in Figure 10.

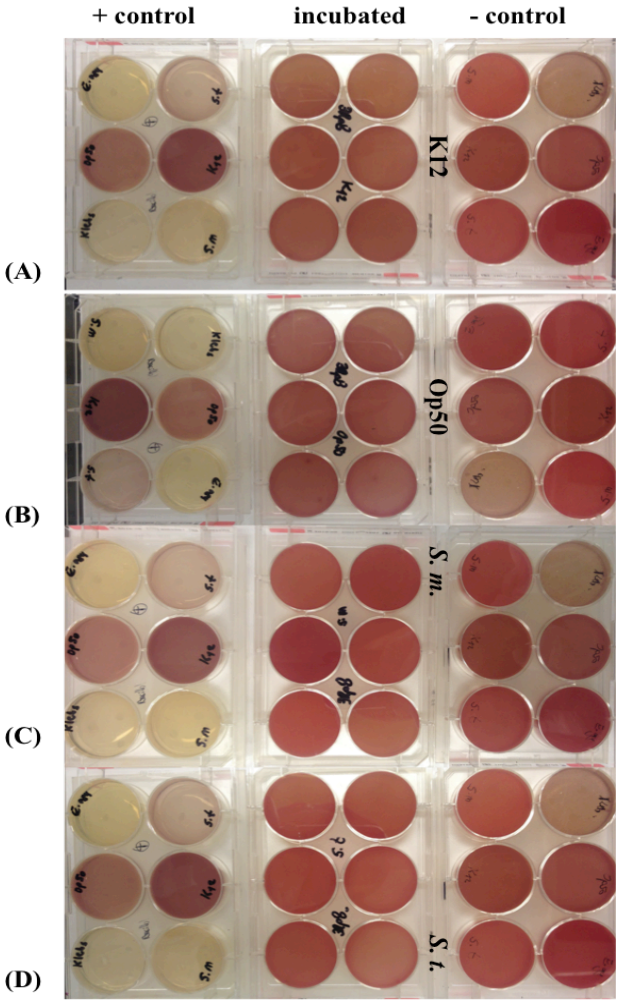


Figure 10 VOCs assay with 3Rp8(dsRed), 3Re4-18 and Co1-6. Inhibition of growth is shown by indicator colour change from deep red to brighter red incubated with 3Rp8(dsRed) for 12 hours. Negative control - samples incubated with sterile medium; Positive control - samples incubated with disinfectant; (A) *E. coli* K12 inhibited by 3Rp8(dsRed); (B) *E. coli* Op50 inhibited by 3Rp8(dsRed); (C) *S. m.* = *Serratia marcescens* inhibited by 3Rp8(dsRed); (D) *S.t.* = *Salmonella typhimurium* inhibited by 3Rp8(dsRed); A-D – growth inhibition after 24 h of incubation with 3Rp8; After incubation for 48 h no growth inhibition could be observed for any approach.

3.2 Screening for direct antagonistic activity of 3Rp8(dsRed), 3Re4-18 and Co1-6 against model pathogens

To assess the antagonistic potential against certain potential human pathogenic bacteria, a confrontation plate assay was performed. The potential antagonists were screened for antagonism *in vitro* against model pathogens of the strain collection from the Institute of Environmental Biotechnology (Graz University of Technology; Table 2) and against the established Gram-negative bacteria

library (Table 3), including isolates from two different *Lactuca sativa* L. var *capitata* types (iceberg lettuce and “Maikönig”), which originated from three different locations (local market, grown in a home garden, and commercial shop). The pathogen containing plates were inoculated with one of the potential antagonists by three single streaks of a single colony placed in the center of the well, using a toothpick. The plates were incubated for 48 hours at 30 °C and at 21 °C. The edge of the zone of inhibition was measured with a ruler after 12 and 24 hours. Figure 11 shows one of the confrontation plate assays with *E.coli* K12 incubated at room temperature for 24 h with the potential antagonists *Serratia plymuthica* 3Rp8 (dsRed), *Serratia plymuthica* 3Re4-18 and *Bacillus subtilis* Co1-6. The edge of the zone of inhibition is visible in A (*Serratia plymuthica* 3Rp8), B (*Serratia plymuthica* 3Re4-18) and C (*Serratia plymuthica* 3Rp8(dsRed). *Bacillus subtilis* Co1-6 showed no inhibition on *E. coli* K12 and on all other tested model pathogens.

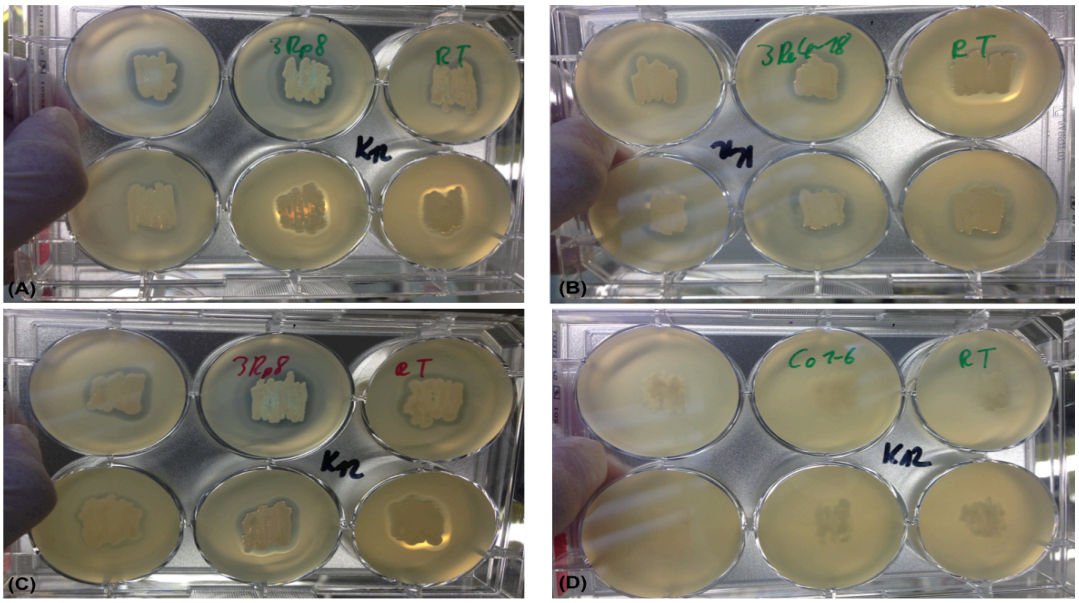


Figure 11 Confrontation plate assay (CFP) with *E. coli* K12 as model pathogen and *Serratia plymuthica* 3Rp8(dsRed), *Serratia plymuthica* 3Re4-18 and *Bacillus subtilis* Co1-6 as potential antagonists. (A) *Serratia plymuthica* 3Rp8; (B) *Serratia plymuthica* 3Re4-18, (C) *Serratia plymuthica* 3Rp8(dsRed) and (D) *Bacillus subtilis* Co1-6; in A, B and C the edge of the zone of inhibition is clearly visible; D shows no inhibition zone.

In Figure 12 the antagonism of 3Rp8(dsRed) and 3Re4-18 on model pathogens from the strain collection from the Institute of Environmental Biotechnology (Table 2) in regard to growth inhibition is shown. Co1-6 was not included in this figure, because it showed no antagonism towards the tested pathogens. The other tested antagonists showed higher inhibition at room temperature. The strongest inhibition was observed with 3Rp8 and *E. coli* Op50 at room temperature. 3Rp8(dsRed) showed no antagonism on *Serratia marcescens* at room temperature, but showed antagonism at 30 °C. The antagonists inhibited the growth of all tested strains at room temperature and at 30 °C, except the *Serratia marcescens.* This isolate was not inhibited at room temperature.

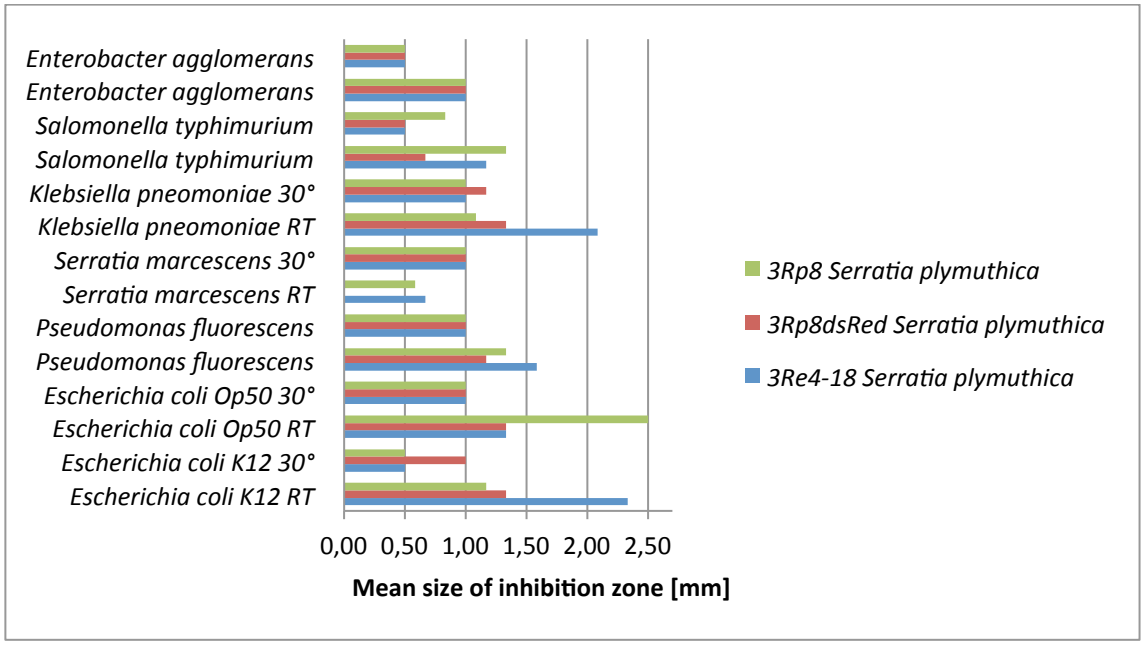


Figure 12 Growth inhibition of 3Rp8 (dsRed), 3Re4-18 on the model pathogens. The mean sizes in mm of the zones of inhibition are compared. A higher antagonism is shown at room temperature for all tested antagonists. The highest antagonism was observed with *Serratia plymuthica* 3Rp8 and *E. coli* Op50 at room temperature. *Serratia plymuthica* 3Rp8(dsRed) showed no antagonism on *Serratia marcescens* at room temperature but showed antagonism at 30 °C. The growth of all tested strains was inhibited by the potential antagonists at room temperature and at 30°C, except for the growth of *Serratia marcescens* at room temperature.

Figure 13 shows the antagonistic activity of 3Rp8 (dsRed) and 3Re4-18 on the Gram-negative bacteria collection, which was isolated from two different *Lactuca sativa* L. var *capitata* types (iceberg lettuce and “Maikönig”) and

originated from three different locations (from a local market, grown in a home garden and from a commercial shop). None of the antagonists showed any inhibition of growth on *Achromobacter spanius* after incubation at 30 °C. *Serratia plymuthica* 3Re4-18 showed no inhibition of *Achromobacter spanius* at room temperature (RT) and at 30 °C, no inhibition of *Pseudomonas putida* at RT and 30 °C, no inhibition of *Pantoea rodasii* at RT, no inhibition of *Enterobacter aerogenes* and *Enterobacter ludwigii* at 30 °C and no inhibition of *Pantoea agglomerans* at RT. The growth of *Pseudomonas putida* was only inhibited by *Serratia plymuthica* 3Rp8 at RT. *Serratia plymuthica* 3Rp8 exclusively inhibited the growth of *Enterobacter aerogenes* at 30 °C. The growth of *Curtobacterium plantarum* was inhibited by *Serratia plymuthica* 3Rp8(dsRed) and *Serratia plymuthica* 3Re4-18 but not by *Serratia plymuthica* 3Rp8 not tagged with the plasmid pIN69. In ten combinations (*Moellerella wisconsensis* RT, *Pantoea wallisii* RT+30 °C, *Pantoea rodasii* RT, *Enterobacter aerogenes* RT, *Enterobacter ludwigii* RT, *Pantoea eucalypti* RT+30 °C, *Pantoea ananatis* RT and *Pantoea agglomerans* 30 °C) *Serratia plymuthica* 3Rp8(dsRed) inhibited the growth more strongly than the untagged strain. The untagged strain inhibited the growth more strongly than the tagged strain in three combinations (*Pseudomonas putida* RT, *Enterobacter aerogenes* 30 °C, *Pantoea agglomerans* 30 °C).

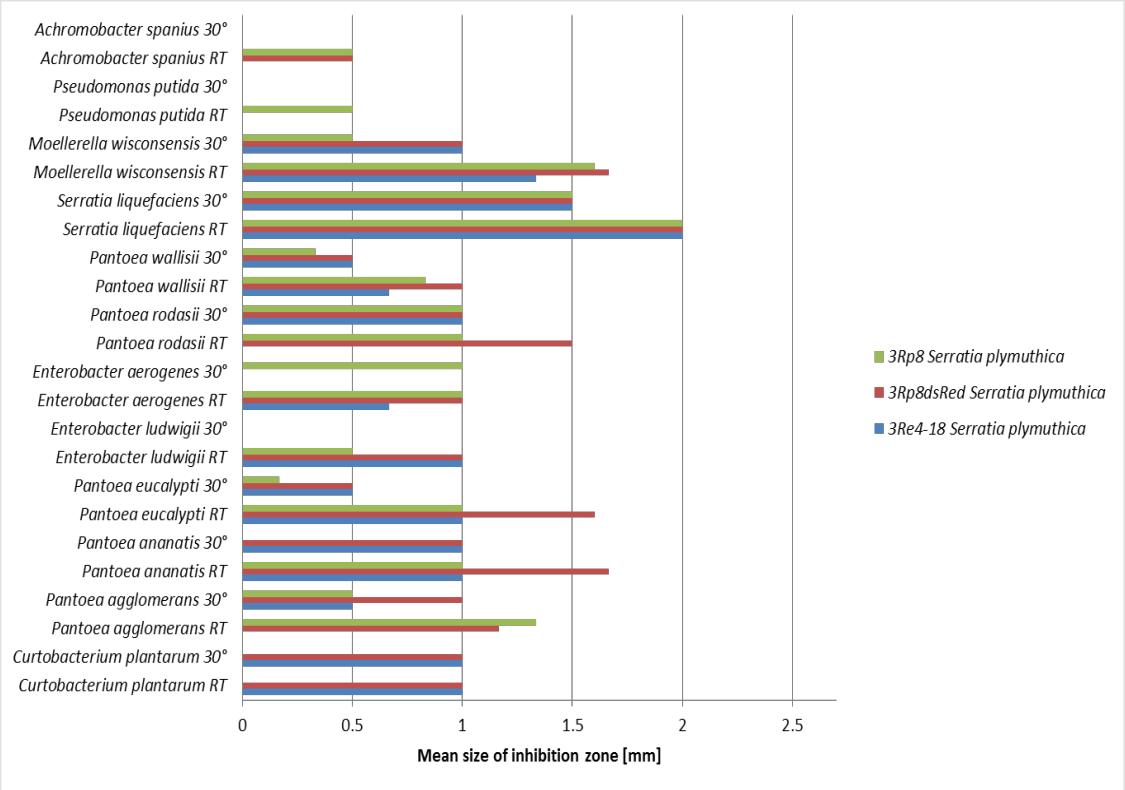


Figure 13 Growth inhibition of *Serratia plymuthica* 3Rp8(dsRed), *Serratia plymuthica* 3Re4-18 on possible pathogens of the Gram-negative bacteria collection. Mean size in mm of the zones of inhibition are compared. RT= room temperature for 24 hours; 30 °C = 30°C for 24 hours; *Enterobacter ludwigii* (30 °C), *Pseudomonas putida* (30 °C) and *Achromobacter spanius (*30 °C) showed no growth inhibition when incubated with *Serratia plymuthica* 3Rp8 (dsRed), *Serratia plymuthica* 3Re4-18. In ten cases (*Moellerella wisconsensis* RT, *Pantoea wallisii* RT+30 °C, *Pantoea rodasii* RT, *Enterobacter aerogenes* RT, *Enterobacter ludwigii* RT, *Pantoea eucalypti* RT+30 °C, *Pantoea ananatis* RT and *Pantoea agglomerans* 30 °C) Serratia plymuthica 3Rp8(dsRed) inhibited the growth more strongly than the untagged strain. The untagged strain inhibited the growth more strongly than the tagged strain in three cases (*Pseudomonas putida* RT, *Enterobacter aerogenes* 30 °C, *Pantoea agglomerans* 30 °C).

In Figure 14, the distribution of the antagonism against the tested model pathogens is shown. *Serratia plymuthica* 3Rp8 without tag showed a higher antagonism against 16% of the tested isolates. The tagged *Serratia plymuthica* 3Rp8(dsRed) showed a higher antagonism against 21% and *Serratia plymuthica* 3Re4-18 against 10% of the tested isolates. Interestingly, 53% of the tested pathogens were inhibited by more than one antagonist.

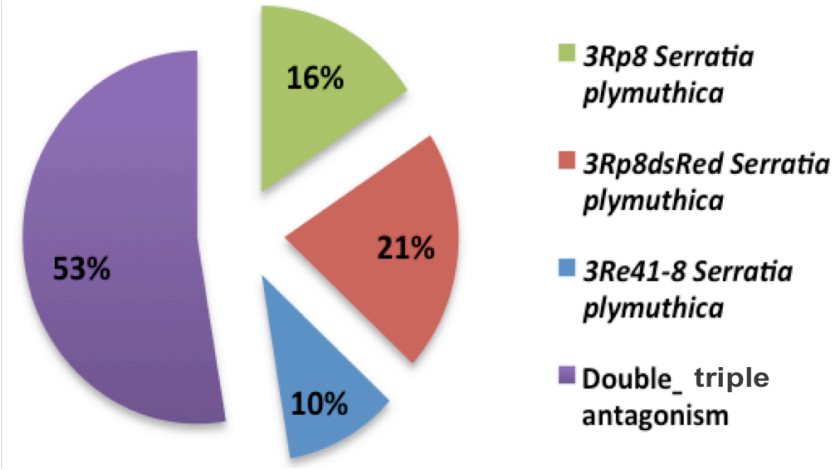


Figure 14 Summary of growth inhibition against model pathogens. Percentage of isolates, which showed a higher inhibition by one specific antagonist; double_triple antagonism = the growth inhibition, was the same for at least two antagonists. 16% (6 isolates out of 38 isolates) showed strongest inhibition with the untagged *Serratia plymuthica* 3Rp8; 21% (8 isolates out of 38 isolates) showed strongest inhibition with the tagged *Serratia plymuthica* 3Rp8(dsRed); *Serratia plymuthica* 3Re4-18 inhibited 16% of the tested isolates the strongest.

3.3 Genome screening of 3Rp8(dsRed) and 3Re4-18 on genome/protein level compared to *in vivo* mode of action

The genomes of the isolates were sequenced in order to identify genes that are responsible for its beneficial effects. In Table 7 potential antagonistic proteins and plant growth promoting proteins of 3Rp8 and 3Re4-18 are listed.

Table 7 Genes coding for specific proteins in 3Rp8 and 3Re4-18 that are involved in PGP and antagonistic activity. PGP= plant growth promotion; AA= antagonistic activity.

Protein/gene	PGP/AA activity	Identified in strain
pksE	Encodes for polyketide synthase operon (AA)	3Rp8, 3Re4-18
Spermidine synthase	PGP activity	3Rp8, 3Re4-18
Indole-3-pyruvate-decarboxylase	PGP activity	3Rp8, 3Re4-18
Acyl-homoserine-lactone synthase	PGP activity	3Rp8, 3Re4-18

Chitinase	AA activity	3Rp8, 3Re4-18
Protease	AA activity	3Rp8, 3Re4-18
Endoglucanase	AA activity	3Rp8, 3Re4-18
Nonribosomal peptide synthase	AA and PGP activity	3Rp8, 3Re4-18
Phosphatase	PGP activity	3Rp8, 3Re4-18

Dr. Henry Müller of the Institute of Environmental Biotechnology kindly provided the assembled genomes of both strains. As published in Adam et al. (2016) the whole genome of 3Rp8 consists of a main chromosome with 5,046,042 bp. The main chromosome contains 5,823 gene coding sequences (CDS) and has a G+C content of 56.1%. The whole genome of 3Re4-18 consists of a main chromosome of 5,439,575 bp. The main chromosome contains 5,622 CDS and has a G+C content of 56.2 % (UniproUGENE). In both strains genes, which are coding for enzymes such as chitinases, proteases and glucanases were found. These proteins are putatively involved in fungal cell wall degradation. The *in vivo* dual culture plate assay with *Rhizoctonia solani* and *Botrytis cinerea* confirmed the antifungal activity of both strains. This was also shown for the tagged and non-tagged 3Rp8 strain (Fig. 15).

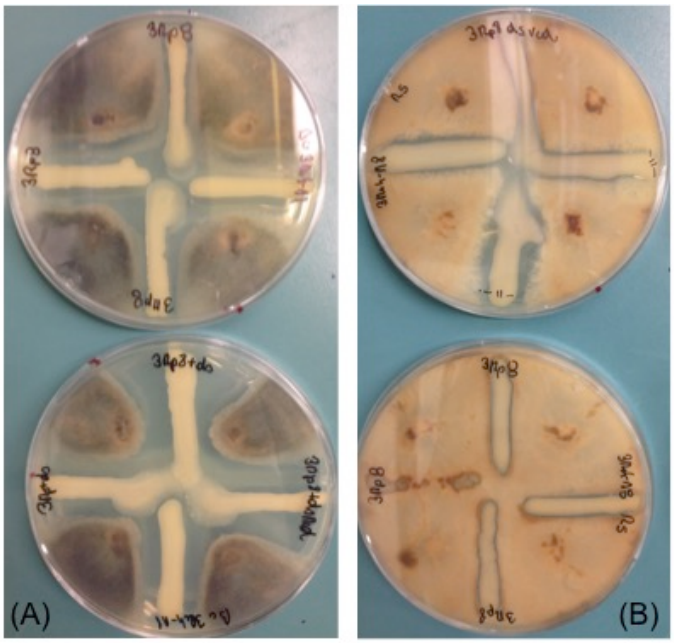


Figure 15 Dual culture plate assay against *Rhizoctonia solani* and *Botrytis cinerea.* (A) upper side shows *Rhizoctonia solani* against 3Rp8 and the reference strain 3Re4-18; lower side shows *Rhizoctonia solani* against 3Rp8(dsRed) and the reference strains 3Re4-18; (B) upper side shows *Botrytis cinerea* against 3Rp8(dsRed) and the reference strain 3Re4-18; lower side shows *Botrytis cynerea* against 3Rp8 and the reference strain 3Re4-18. Both strains are inhibiting the growth of the tested fungi. *Rhizoctonia solani* incubated with the tagged 3Rp8 shows lower growth inhibition compared to the untagged strain.

Three non-ribosomal peptide synthase operons and two polyketide synthase operons were identified in 3Rp8 and 3Re4-18. Two operons showed 100% identity and 100% query cover with the zeamine operon (NCBI Acc. HE995400.1) of *Serratia plymuthica* with BLASTn on http://www.ncbi.nlm.nig.gov/ (Masschelein et al., 2013). In addition, genes coding for enzymes, which are involved in quorum sensing were identified in both strains, such as Acyl-homoserine-lactone synthase as well as genes, coding for enzymes, which are involved directly in plant growth promotion (PGP), such as spermidine synthase and the corresponding export protein MdtJ, indole-3-pyruvate-decarboxylase responsible for the production of indole-3-acetic acid (IAA) and enzymes involved in the acetoin production. The production of AHL was also tested *in vivo* and confirmed the production of such molecules (Fig. 16). In addition, genes, which are coding for enzymes, which are involved in phosphate mobilisation, such as phosphatases and inorganic phosphate transporters were found in both strains.

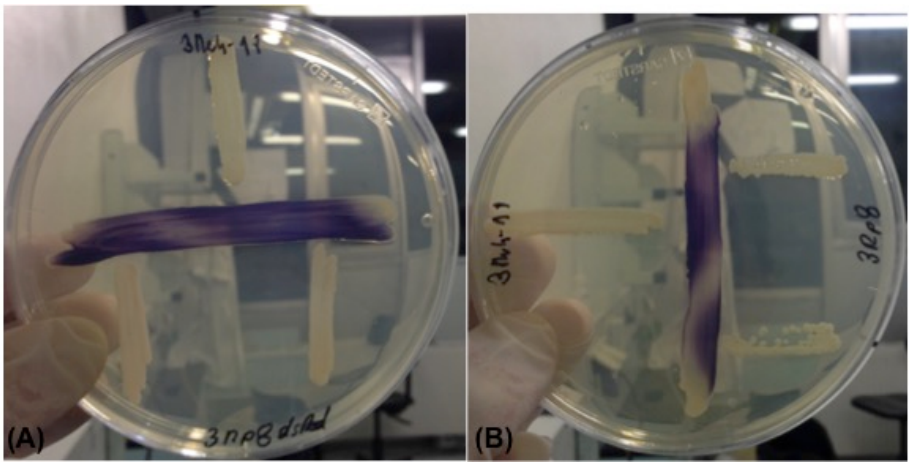


Figure 16 AHL production test. The production of AHL of 3Rp8 tagged with DsRed protein (A) and 3Rp8 (B), was tested with the reference strain 3Re4-18 and the sensor strain *Chromobacterium violaceum* CV026. The sensor strains turn into a purple colour at the contact point if production occurs in the tested strains. Both strains produce AHL. The genomic analyses also support this result.

3.4 Identification of isolates originated from *Lactuca sativa* L. var *capitata* by 16S rRNA gene sequencing

To identify the isolates of *Lactuca sativa* L. var *capitata*, 16S rDNA sequencing analyses were performed. The sequences were subjected to phylogenetic analyses as described in section 2.12. The phylogenetic tree constructed with PHYLIP was visualised with the software FigTree (Schneider et al., 2000, Fig. 17). Five families were identified - *Pseudomonadaceae*, *Moraxellaceae*, *Alcaligenaceae*, *Microbacteriaceae* and *Enterobacteriaceae*. Most isolates belong to the family of *Enterobacteriaceae*, followed by *Pseudomonadaceae* and *Microbateriaceae*. The highest diversity on the family level was observed with the isolates of the home garden grown lettuce (A). *Moraxellaceae* were only isolated from the lettuce of a commercial supermarket (S) and *Pseudomonadaceae* were prevalently isolated from the lettuce of a commercial market (M).

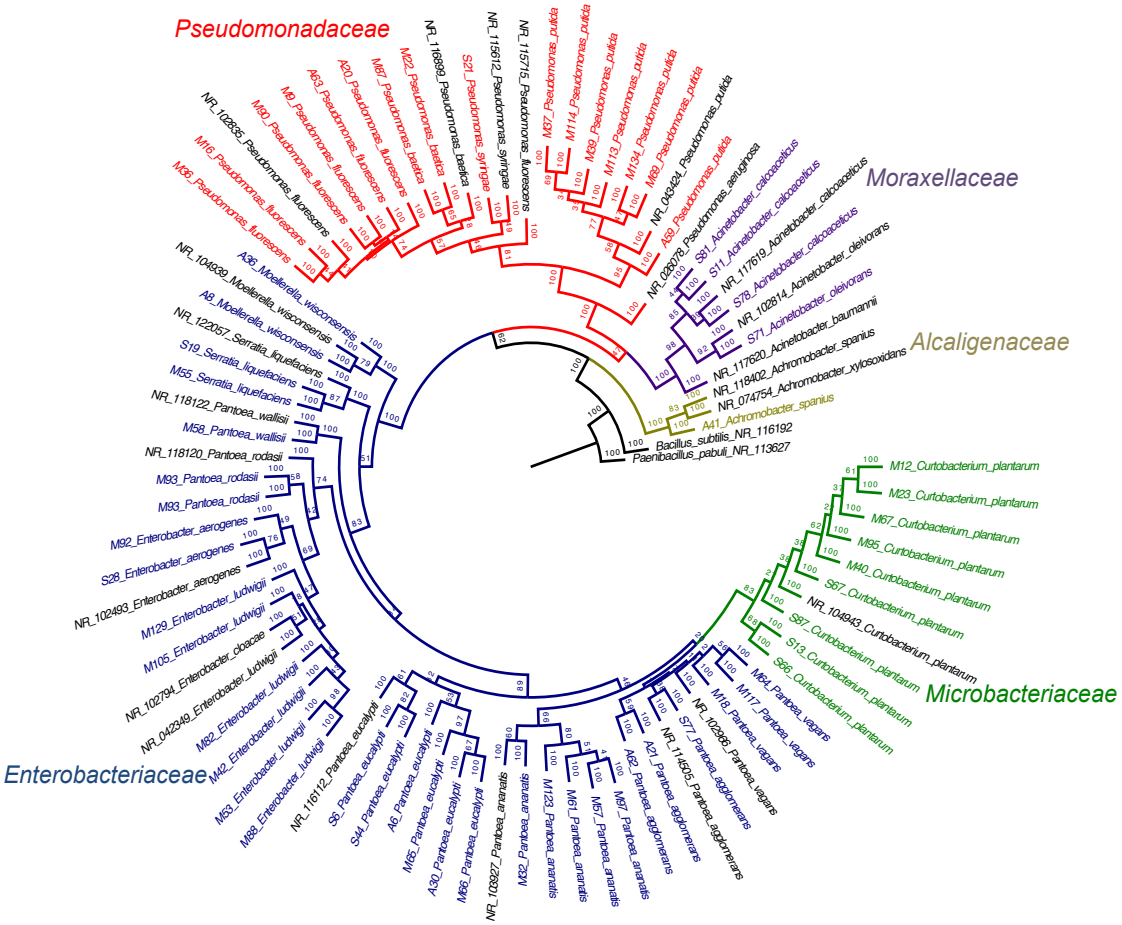


Figure 17 Phylogenetic tree of isolated microorganisms of *Lactuca sativa* L. var *capitata* of three different locations. The tree was created using the software PYHLIP (Felsenstein et al., 1989). A distance matrix was created by DNADIST and processed by the neighbor-joining method. For visualisation and editing of the phylogenetic tree the software FigTree was used (Schneider et al., 2000). Members of five bacterial families were identified: *Pseudomonadaceae* (red), *Moraxellaceae* (purple), *Alcaligenaceae* (brown), *Microbacteriaceae* (green) and *Enterobacteriaceae* (blue). Outgroups (*Bacillus subtilis* and *Paenibacillus pabuli)* and reference sequences are shown in black.

3.5 PGP ability of 3Rp8(dsRed) on *Lactuca sativa* L. var *capitata* "Gelber Winter"

3.5.1 Rhizosphere competence by bacterial abundance

In-pot experiments under gnotobiotic conditions were performed to analyse the growth promotion effects of *Serratia plymuthica* 3Rp8 tagged with the fluorescent protein DsRed against the strong rhizosphere competitor *Pseudomonas fluorescens* WCS365. Therefore, sterilised seeds with NaOCl

were primed with 10^7 cells of *Serratia plymuthica* 3Rp8(dsRed) individually and in combination with *Pseudomonas fluorescens* WCS365 and planted into sterile soil. The competence of *Serratia plymuthica* 3Rp8(dsRed) to colonise roots, was tested by re-isolation on NA plates. The rhizosphere was colonised with $\log_{10}$ 8.2 CFU g^{-1} root fresh weight (RFW, Fig. 18). The phyllosphere showed an abundance of *Serratia plymuthica* 3Rp8 with $\log_{10}$ 6.4 CFU g^{-1} foliage fresh weight (FFW).

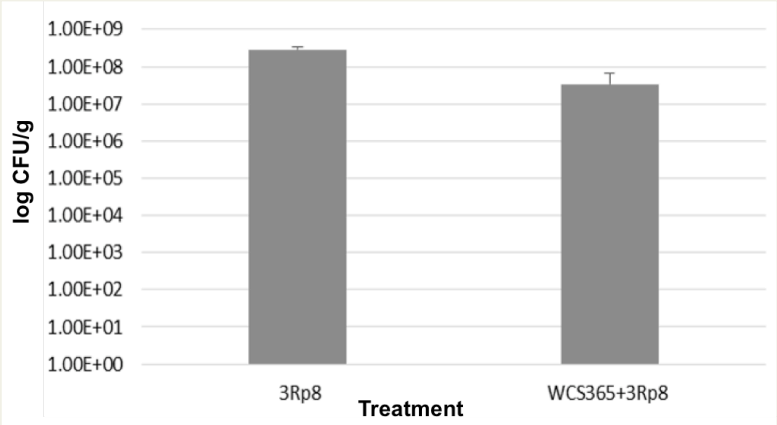


Figure 18 CFU/g root fresh weight. The competence of *Serratia plymuthica* 3Rp8(dsRed) to colonise roots was tested by re-isolation on NA plates. The rhizosphere was colonised with $\log_{10}$ 8.2 CFU g^{-1} root fresh weight. Phyllosphere was colonised with 6.4 CFU g^{-1} root fresh weight.

3.5.2 Germination support

The germination rates were recorded on the fourth day and are shown in Fig. 19. The control (unprimed seeds) showed a germination rate of 54%; seeds primed with *Pseudomonas fluorescens* WCS365 in combination with *Serratia plymuthica* 3Rp8(dsRed) germinated at 89%; seeds only primed with WCS365 showed a rate of 59% and seeds primed only with 3Rp8(dsRed) showed a rate of 64% (Fig. 19).

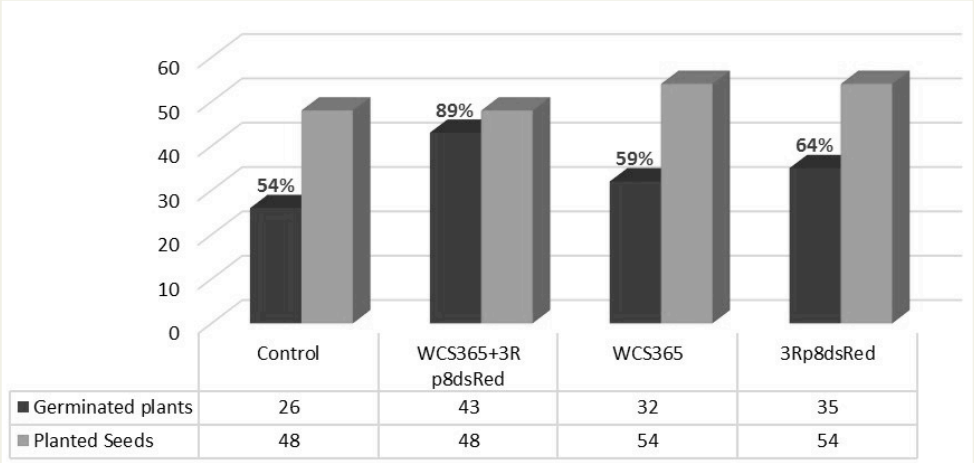


Figure 19 Germination rate of *Lactuca sativa* L. var *capitata* plants. Germination rates were evaluated at the fourth day after planting. The control shows the germination rate of unprimed seeds with 54%; Seeds primed with *Pseudomonas fluorescens* WCS365 in combination with *Serratia plymuthica* 3Rp8(dsRed) germinated with 89%; Seeds only primed with WCS365 showed a rate of 59% and seeds primed only with 3Rp8(dsRed) showed a rate of 64%.

3.5.3 Effects of bacterial treatment on plant growth

After 13 days of growth, six trays of each treatment were collected for length evaluations of the plants (Fig. 20). The length of roots, leaves and stems, which were inoculated as previously described, were measured with a ruler. Plantlets were carefully extracted from the trays with a sterile tweezer and roots and foliage were separated to determine fresh and dry weight of each approach.

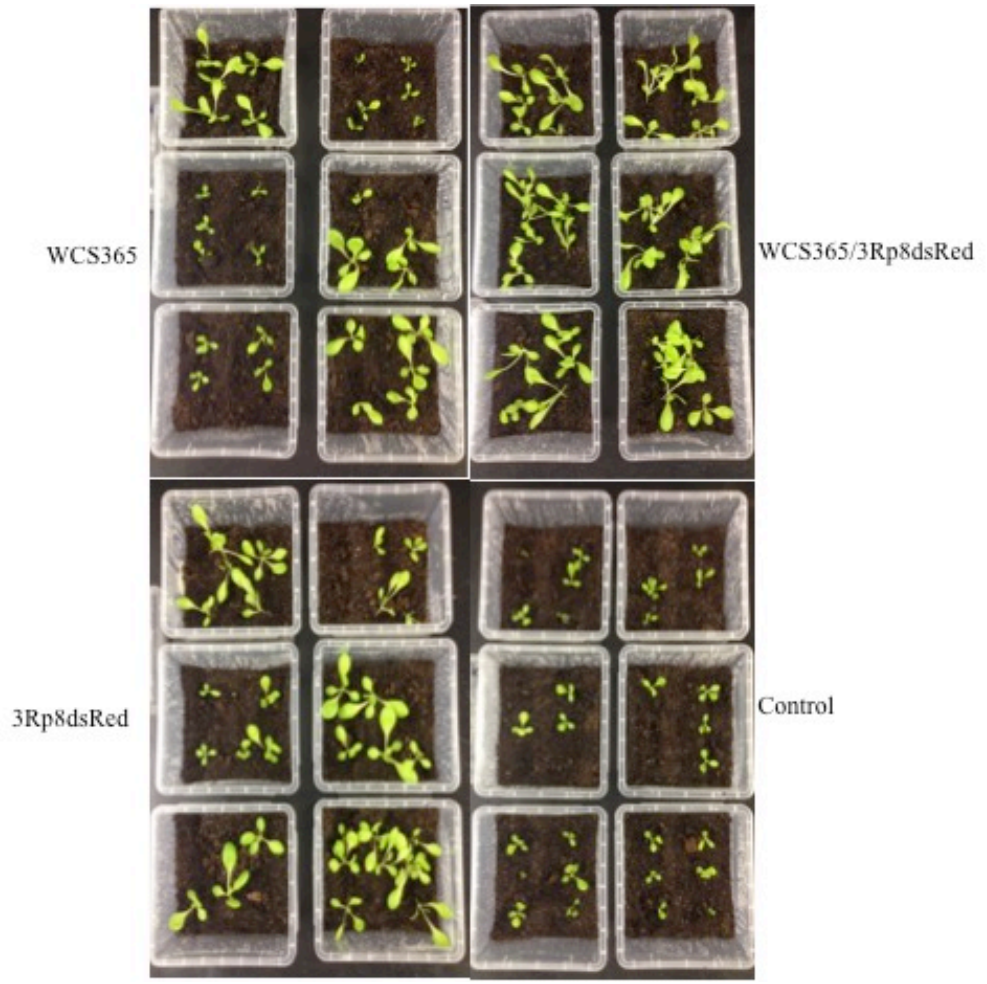


Figure 20 Growth promotion effects of *Serratia plymuthica* 3Rp8(dsRed) on *Lactuca sativa* L. var *capitata* "Gelber Winter". After 13 days of growth, the bioprimed plantlets and the control plantlets were harvested as described in the text. The primed plantlets inoculated with WCS365, 3Rp8(dsRed) and combined inoculated with both strains, show growth promotion effects compared to the control.

Significant differences between treatments and their growth effects were analysed with PASW Statistics 18 software (SPSS Inc.). In order to compare the growth effects with other BCAs, previous achieved data from in-pot experiments with *Serratia plymuthica* 3Re4-18 and *Bacillus subtilis* Co1-6 were compared in our statistical analyses. Fig. 21 shows the growth promoting effects on the average length of leaves from *L. sativa* inoculated with the previously described strains. The analysed data was labelled with letters to match the comparable groups, which are significantly different according to Games-Howell test ($P \geq 0.05$). It is clearly visible that all tested BCAs have growth promoting effects on "Gelber Winter" compared to the control, which was

not primed. The highest statistically significant growth promoting effect on the average leave length is shown in the boxplots labelled with “c”, which represents the treatments with *Serratia plymuthica* 3Re4-18, *Bacillus subtilis* Co1-6, *Bacillus subtilis* Co1-6 combined with *Pseudomonas fluorescens* WCS365 and *Serratia plymuthica* 3Rp8(dsRed) combined with WCS365. *Serratia plymuthica* 3Rp8(dsRed) primed individually showed significantly lower PGP effects compared to the other applications but in combination with WCS365 there was no significant difference (Fig. 21).

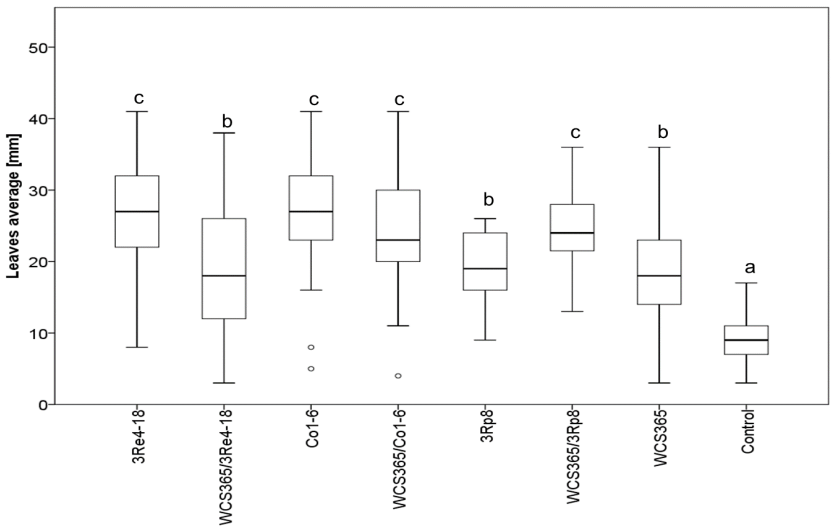


Figure 21 Growth promotion effects of *Serratia plymuthica* 3Re4-18, *Bacillus subtilis* Co1-6, *Pseudomonas fluorescens* WCS365 and *Serratia plymuthica* 3Rp8(dsRed) on *Lactuca sativa* L. All tested BCAs showed growth promoting effects on “Gelber Winter” compared to the control. The highest growth promoting effects are depicted in the boxplots marked with “c”, which represent the treatments with *Serratia plymuthica* 3Re4-18, *Bacillus subtilis* Co1-6, *Bacillus subtilis* Co1-6 combined with *Pseudomonas fluorescens* WCS365 and *Serratia plymuthica* 3Rp8(dsRed) combined with WCS365. The analysed data are labelled with letters a, b and c to match the comparable groups which are significantly different according to Games-Howell test (P ≥ 0.05).

Figure 22 shows the plant growth promoting effects of the previously described BCAs on the rhizosphere and stem length. The data were analysed in the same way as described before. The letters in the box plot mark the comparable groups with significant differences according to Games-Howell test (P ≥ 0.05). The tested strain 3Rp8 shows no significant difference compared to the other

BCAs, which were individually primed. A significant difference can only be seen on the stem length compared to the combined primed plantlets with Co1-6 and WCS365 (Fig. 22).

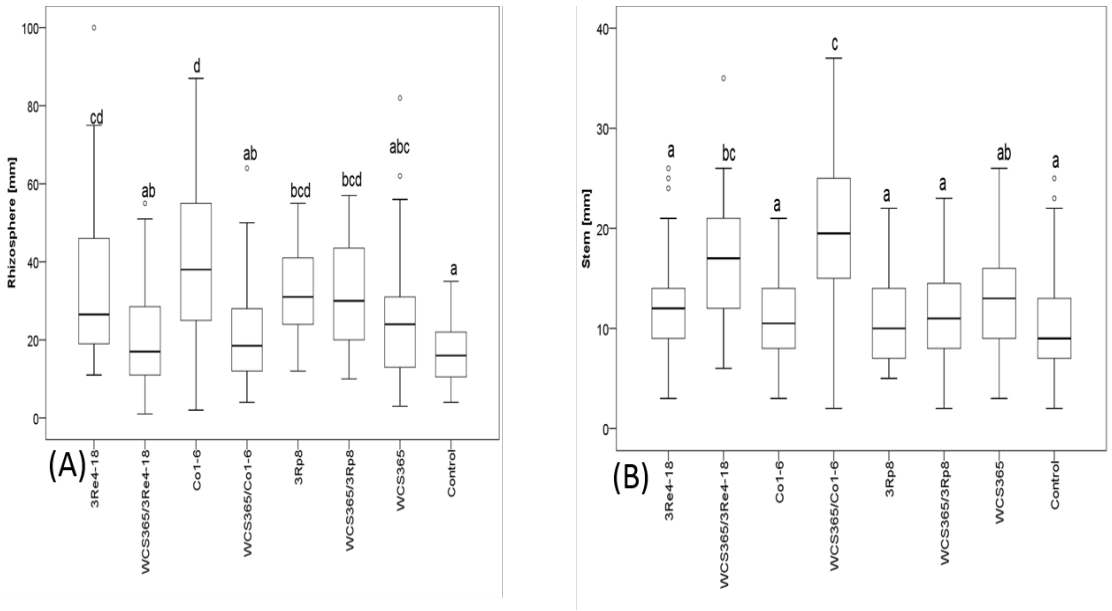


Figure 22 Growth promotion effects of *Serratia plymuthica* 3Re4-18, *Bacillus subtilis* Co1-6, *Pseudomonas fluorescens* WCS365 and *Serratia plymuthica* 3Rp8(dsRed) on *Lactuca sativa* L. Resulting data were analysed with PASW Statistic 18 software and compared with previous achieved data from in-pot experiments. (A) No significant difference can be observed in the tested strain 3Rp8 compared to other plant growth promoting bacteria. (B) Significant differences of PGP effects of 3Rp8 were observed compared to plantlets primed with WCS365 combined with Co1-6.

3.5.4 Colonisation patterns observed by FISH/CLSM

To visualise the colonisation patterns in the rhizosphere, the combination of FISH with CLSM imaging was used. The colonisation patterns were analysed after 13 days of growth under gnotobiotic conditions. One plant of each treatment was chemically fixed for FISH/CLSM. Fixed phyllosphere and rhizosphere samples of the primed lettuce plants with *Serratia plymuthica* 3Rp8(dsRed) - individually and in combination with *Pseudomonas fluorescens* WCS365 - were stained following the in-tube FISH protocol (Cardinale et al., 2008). The samples were hybridised with four different rRNA-targeting probes (genXpress,Wiener Neudorf, Austria) specific for *Enterobacteriaceae*,

Gammaproteobacteria and with universal bacterial probes. A high density of WCS365 cells (yellow) was found in the rhizosphere near damaged cell parts (Fig. 23,A, B). *Serratia plymuthica* 3Rp8 (white) formed small colonies on the rhizoplane (Fig. 23, A, B). Small pink (*Serratia plymuthica* 3Rp8) colonies distributed all over the phyllosphere were observed (Fig. 23, C,D).

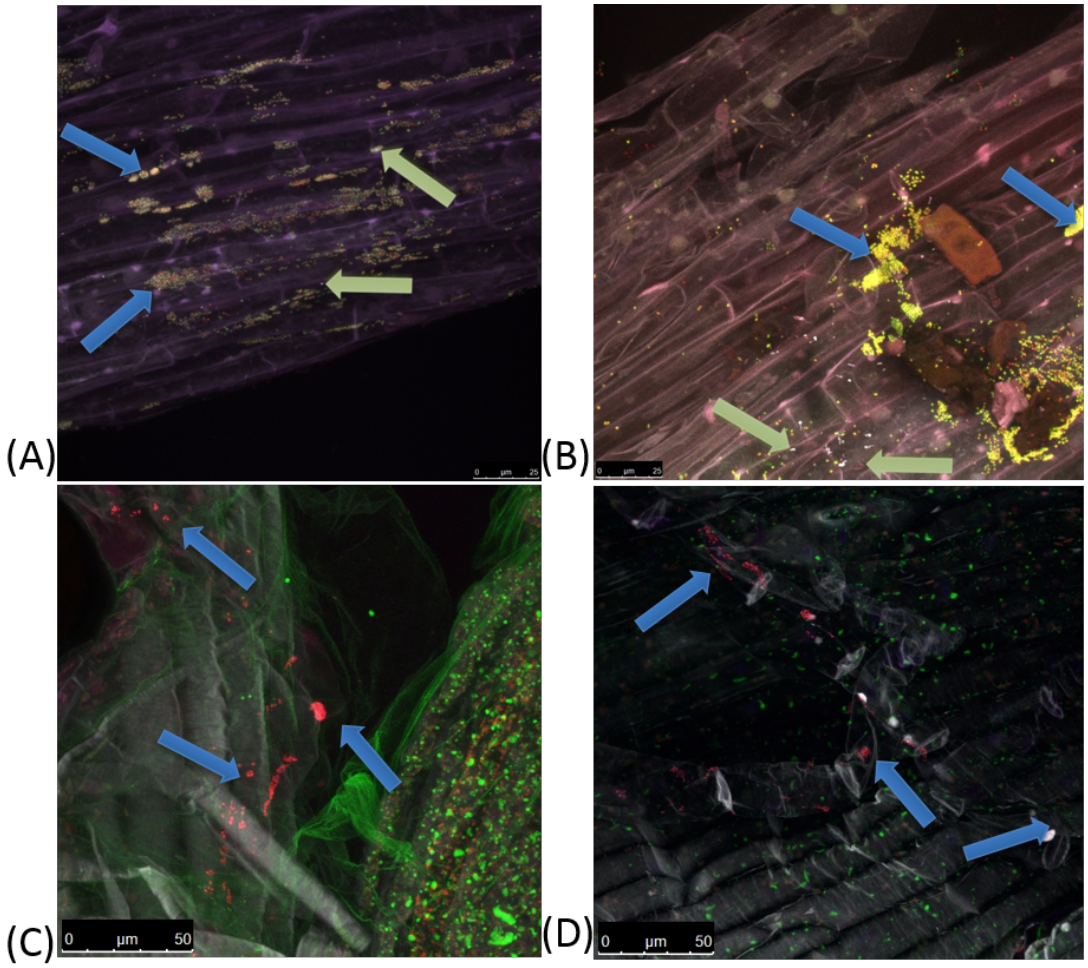


Figure 23 Colonisation pattern of FISH-labelled bacteria in the root system and on the phyllosphere of "Gelber Winter" primed with *Serratia plymuthica* 3Rp8 and *Pseudomonas fluorescens* WCS365. To visualise the colonisation patterns in the rhizosphere and phyllosphere, FISH coupled with CLSM was performed. To avoid autofluorescense of the phyllosphere plant parts, the plant material was additionally stained with Calcoflour white. (A, B) a high density on the root surface of WCS365 cells (yellow, blue arrow); single microcolonies formed by 3Rp8 (white, green arrow) distributed all over the rhizosphere. (C, D) shows small colonies of *Serratia plymuthica* 3Rp8 (pink, blue arrow) on the phyllosphere.

3.6 Visualisation and reisolation of *Serratia plymuthica* 3Rp8(dsRed) from inoculated phyllosphere and rhizosphere

In order to visualise the colonisation patterns of the inoculated strain *Serratia plymuthica* 3Rp8(dsRed), live CLSM imaging was performed. The fluorescent protein DsRed, which is expressed by the plasmid pIN69 in *Serratia plymuthica* 3Rp8(dsRed), was detected at 532 nm with a Leica TCS SPE confocal microscope (Leica Microsystems, Germany). Figure 24 shows the inoculated strain 3Rp8 expressing the fluorescent protein DsRed on lateral roots of lettuce (var. *capitata*).

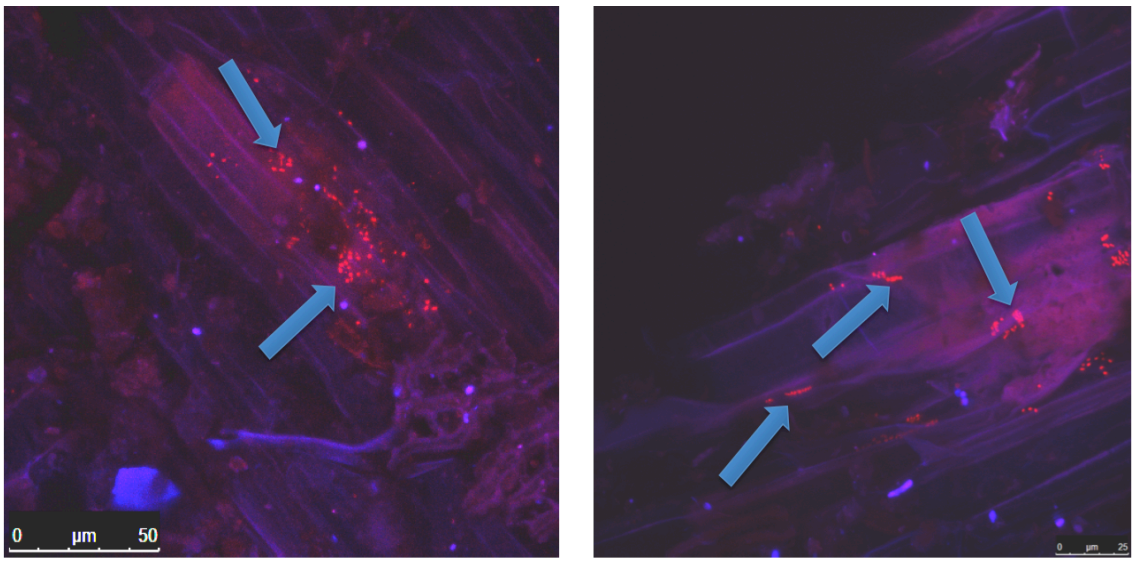


Figure 24 Colonisation patterns of *Serratia plymuthica* 3Rp8 tagged with pIN69DsRed on lateral roots of lettuce (var. *capitata*). CLSM was performed with a Leica TCS SPE confocal microscope. The expressed fluorescent protein DsRed was sequentially excited with 532nm laser beams. Confocal stacks were acquired by applying a Z-step size of 0.8 µm. Cells of 3Rp8 could be detected distributed all over the rhizosphere arranged in small colonies.

To validate population densities of 3Rp8(dsRed), the strain was reisolated on LB-Trimethoprim plates. Because non-sterilised soil was used for the experimental setup, other than just 3Rp8 cells were isolated. Figure 25 shows the population densities in CFU/g fresh weight of phyllosphere and rhizosphere. Between $9.6x10^5$ CFU/g fresh weight of rhizosphere and $1.97x10^7$ CFU/g fresh weight of rhizosphere were reisolated (Fig. 25).

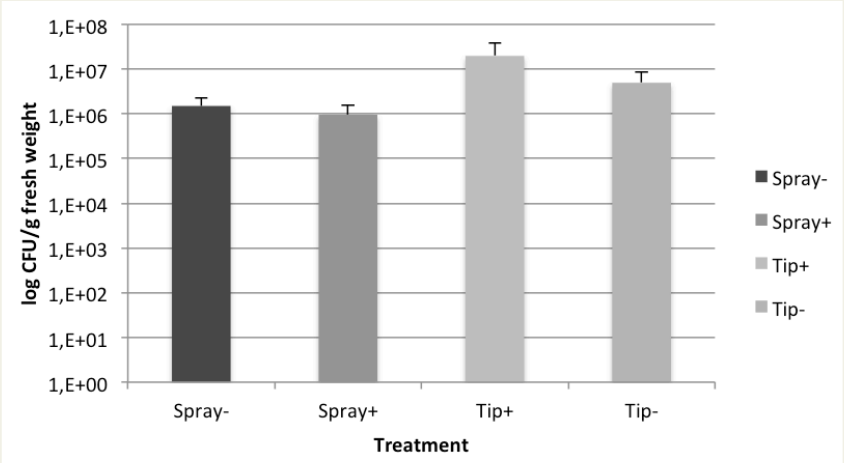


Figure 25 Population densities of isolated bacteria on LB-Trimethoprim plates. (Spray-)- shows CFU/g fresh weight of rhizosphere of plantlets, which were inoculated by control spraying on the phyllosphere; (Spray+)- shows CFU/g fresh weight of rhizosphere of plantlets, which were inoculated by spraying with 3Rp8(dsRed) cells on the phyllosphere; (Tip+)- shows CFU/g fresh weight of rhizosphere inoculated by dipping with 3Rp8(dsRed) cells; (Tip-)- shows CFU/g fresh weight of rhizosphere inoculated by dipping with the control medium; A difference between dipped and sprayed plantlets can be observed.

Additionally, the plates were screened for fluorescence at 532nm with a CCD-camera at 532 nm with the Gel Doc^{TM} XR^{+} System (Biorad, Austria) and the corresponding software Image Lab^{TM} Plates with cells of the not inoculated rhizosphere showed no fluorescence at 532 nm. The plates of the inoculated rhizosphere with 3Rp8(dsRed) showed fluorescence at 532 nm (Fig. 26).

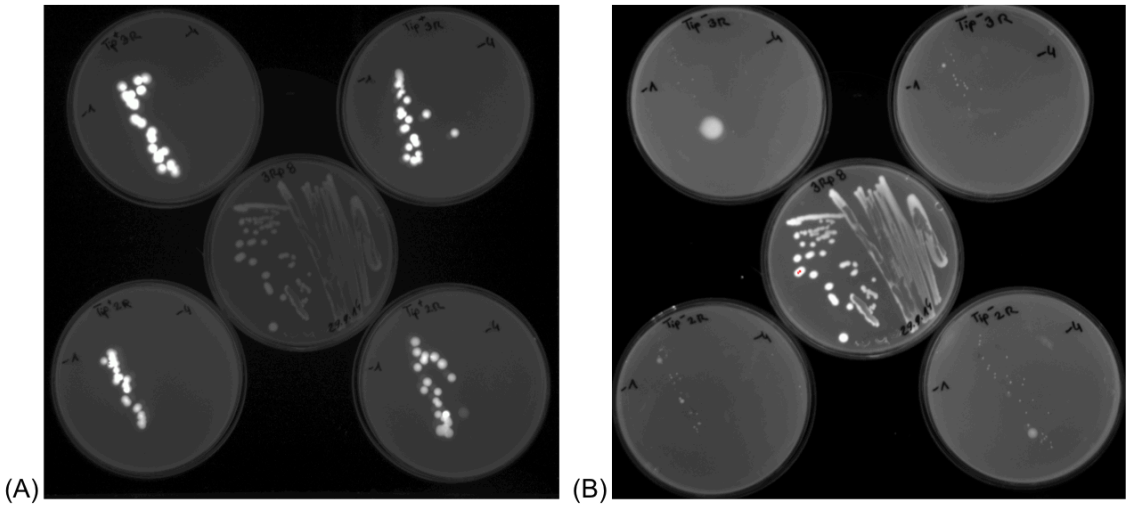


Figure 26 Fluorescence of reisolated bacteria. Fluorescence was checked at 532 nm with a CCD-camera with the Gel Doc™ XR+ System (Biorad, Austria) and the corresponding software Image Lab™. (A) Reisolated cells of the inoculated rhizosphere with 3Rp8(dsRed); (B) Plates of the reisolated cells of the control inoculated rhizosphere with no cells. The plate in the middle is the negative control, which shows no fluorescence at 532 nm.

3.7 Impact of *Serratia plymuthica* 3Rp8(dsRed) on the bacterial community of lettuce revealed by barcoded MiSeq Illumina sequencing of 16S rRNA gene amplicons

To analyse the impact of *Serratia plymuthica* 3Rp8(dsRed) on the bacterial community of lettuce, an amplicon sequencing approach using Illuminas MiSeq platform at LGC Genomics was performed. Molecular tagging in combination with peptide nucleic acid (PNA) PCR clamps (Lundberg et al., 2013) of the treated and non-treated lettuce plants was used. The number of reads obtained by amplicon sequencing ranged from 3,974 to 60,589 for the rhizosphere samples and from 4,453 to 17,061 for the phyllosphere samples (Supplementary Table 1). Based on the taxonomic classification of representative sequences from all OTUs, the composition of bacterial communities was analysed at phylum, class, family and genus level. The bacterial phyla in rhizosphere and phyllosphere samples were predominated by *Proteobacteria* (32.50%-49.11%), *Bacteriodetes* (9.39%-13.76%), *Actinobacteria* (8.25%-9.08%) and by *Acidobacteria* (5.08%-6.00%) (Fig. 27). Less abundant phyla, which were detected in rhizosphere and phyllosphere,

were *Verrucomicrobia* with 4.48% in the rhizosphere and 5.53% in the phyllosphere and *Planctomycetes* with 5.05% in the rhizosphere and 3.38% in the phyllosphere. *Firmicutes* were present at with only 0.45% in the rhizosphere and 0.07% in the phyllosphere. A small portion of *Crenarchaeota* (*Archaea*) with 0.05% in the rhizosphere and 0.04% in the phyllosphere samples could be also detected.

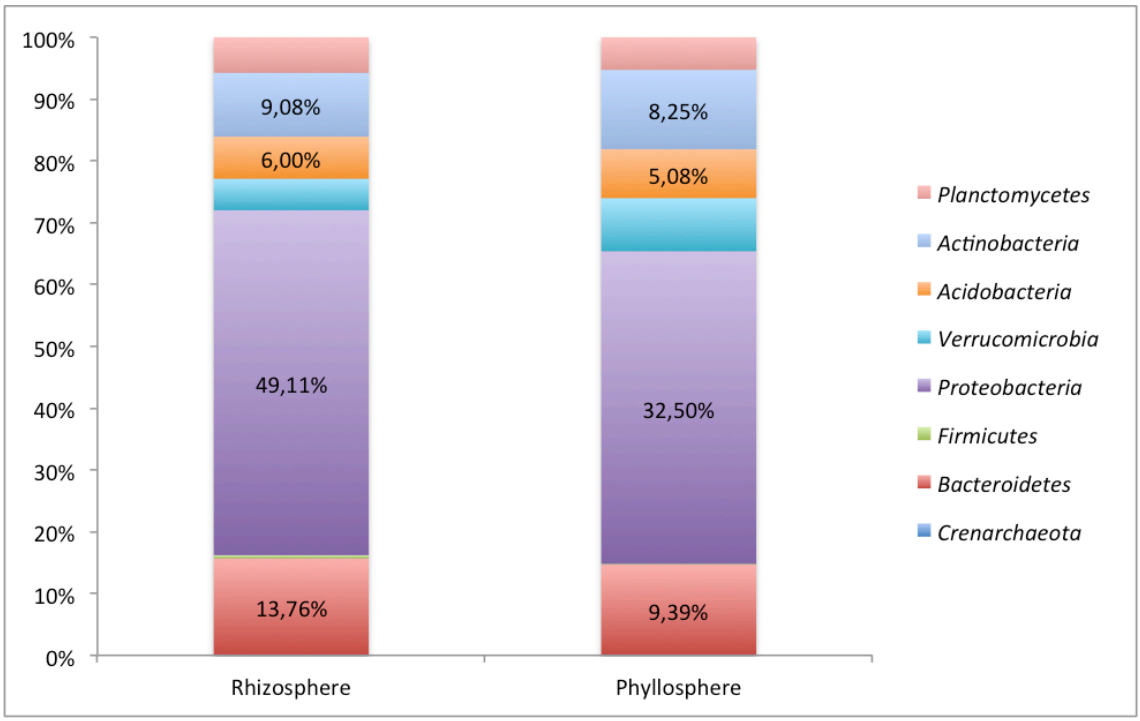


Figure 27 Structure of the microbial communities in the rhizosphere and phyllosphere of *Lactuca sativa* L. var *capitata* revealed by Illumina MiSeq 16S rRNA gene amplicon sequencing at phylum level. The rhizosphere was dominated by *Proteobacteria* (49.11%), *Bacteriodetes* (13.76%) and *Actinobacteria* (9.08%). The phyllosphere was dominated by *Proteobacteria* (32.50%), *Bacteriodetes* (9.38%) and *Actinobacteria* (8.25%).

Structure of the microbial community within the different inoculation types of phyllosphere and rhizosphere are shown in Fig. 28. Rhizosphere and phyllosphere, were predominately colonized by *Proteobacteria* (39.69%-48.71%) followed by *Bacteriodetes* (9.39%-14.67%) and *Actinobacteria* (6.81%-7.53%).

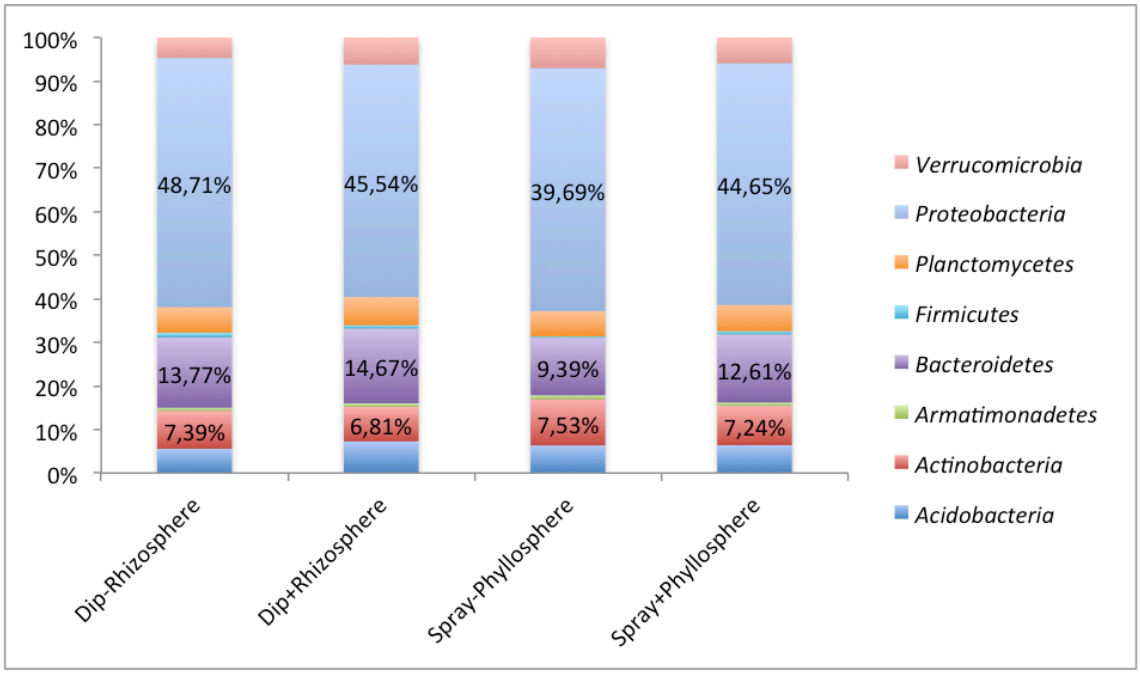


Figure 28 Structure of the microbial communities in the rhizosphere (Dip- and Dip+) and phyllosphere (Spray- and Spray+) of *Lactuca sativa* L. var capitata revealed by Illumina MiSeq 16S rRNA gene amplicon sequencing at phylum level. Dip- (not inoculated rhizosphere); Dip+ (inoculated rhizosphere); Spray- (not inoculated phyllosphere); Spray+ (inoculated phyllosphere).

Ring charts based on the Krona software package version 2.2 (Ondov et al., 2011) were used for a deeper insight into changes in the bacterial structure within the inoculated and non-inoculated phyllosphere and rhizosphere samples. In particular, the gammaproteobacterial diversity was analysed more deeply. Its abundance on the phyllosphere shifted from 15% within all bacteria of the non-inoculated samples to 23% at the inoculated samples. Also within the gammaproteobacterial diversity, a shift from 20% *Enterobacteriaceae* at the non-inoculated samples to 38% to the inoculated samples was observed. On family level, the inoculated phyllosphere was dominated by *Enterobacteriaceae* (38%) and *Sphingomonadaceae* (8%). The non-inoculated phyllosphere samples were dominated by *Enterobacteriaceae* (20%) and *Sphingomonadaceae* (11%) (Fig. 29).

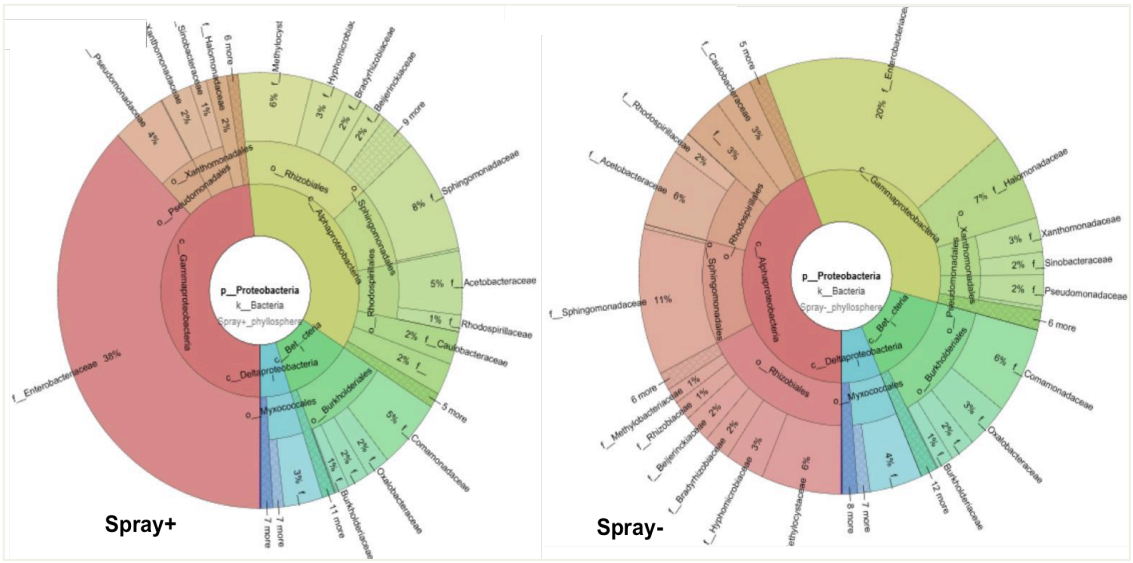


Figure 29 Ringcharts of proteobacterial diversity of inoculated and non-inoculated phyllosphere samples. RDP classifier analysis shows: Spray+:six phyllosphere samples inoculated by spraying with 3Rp8 with a cell count of 10^6 (i.); Spray-:six phyllosphere samples inoculated with sterile filtered medium (n.i). Taxonomic ranks are shown by the ring and columns represent distinct taxa (g-genus; f-family; c-class; p-phylum). Other-not taxonomically assigned.

On rhizosphere level, a shift within the gammaproteobacterial abundance was observed from 5% in non-inoculated (n.i) to 21% in inoculated (i.) samples for *Enterobacteriaceae* and a shift from 28% (n.i) to 16% in inoculated (i.) samples for *Legionellales*. Also, a shift from 11% (n.i) to 7% (i.) for *Pseudomonadales* was detected. The dominating genus on the inoculated rhizosphere within the *Gammaproteobacteria* was not taxonomically assigned at genus level (19%) and belongs to the family of *Xanthomonadaceae*, followed by 17% of an unassigned genus belonging to the family of *Enterobacteriaceae*. The non-inoculated rhizosphere samples were also dominated by an unassigned genus (22%) of the family *Xanthomonadaceae* followed by 10% of an unassigned genus of the family *Legionellaceae* (Fig. 30).

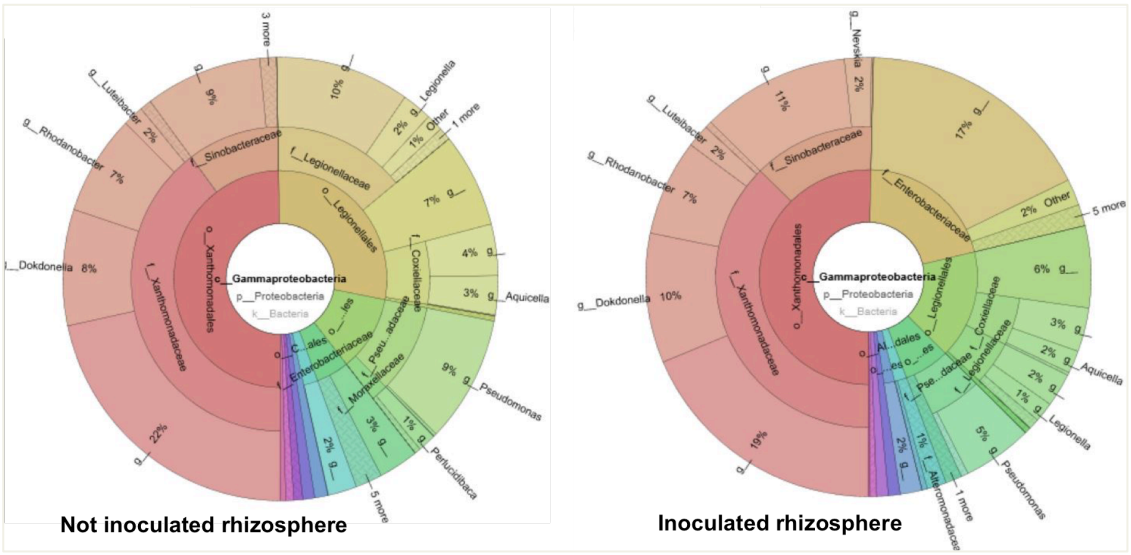


Figure 30 Ringcharts of gammaproteobacterial diversity of inoculated and non-inoculated rhizosphere samples. The RDP classifier analysis is shown from six inoculated rhizosphere samples and from six non-inoculated rhizosphere samples. Taxonomic ranks are shown by the ring and columns represent distinct taxa (g-genus; f-family; c-class; p-phylum). Other-not taxonomically assigned.

Fig. 31 shows the RDP classifier analyses on gammaproteobacterial community structure of the phyllosphere samples (n.i). A shift from 3% (i.) to 19% (n.i) was detected on genus level for *Candidatus Portiera. Buchnera* increased from 19% to 28% in the inoculated samples. On family level, a shift of *Enterobacteriaceae* was detected from 57% (n.i) to 78% (i.).

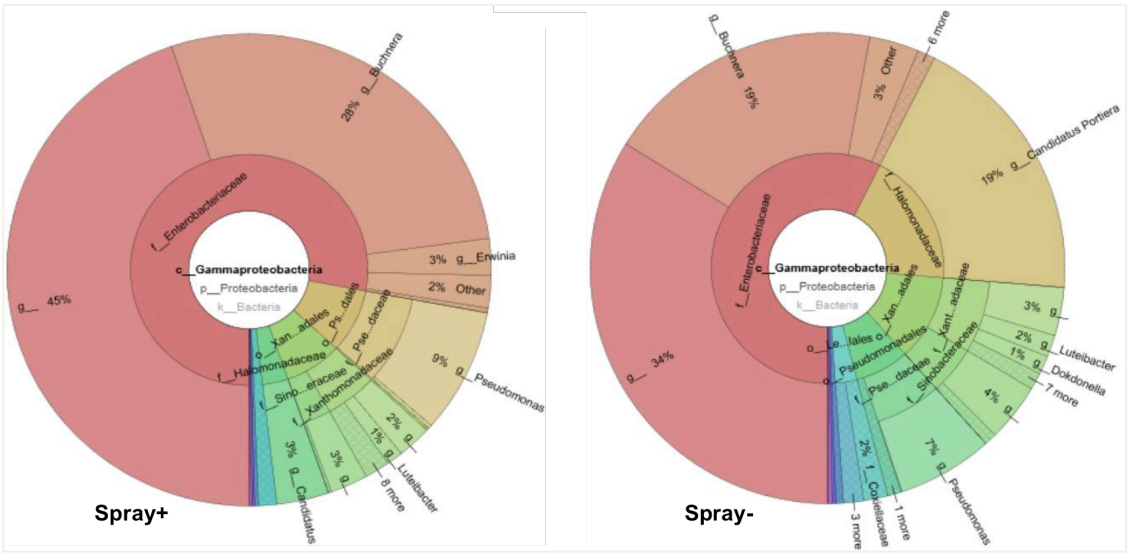


Figure 31 Ringcharts of gammaproteobacterial diversity on the inoculated and non-inoculated phyllosphere. The RDP classifier analysis for six inoculated phyllosphere samples (Spray+) and six non-inoculated phyllosphere samples (Spray-) is shown. Taxonomic ranks are shown by the ring and columns represent distinct taxa (g-genus; f-family; c-class; p-phylum). Other-not taxonomically assigned.

Also in the phyllosphere samples, which were inoculated by dipping either with 3Rp8 or sterile medium, a shift for *Gammaproteobacteria* from 16% (n.i) to 22% (i.) was observed. The dominating genera within the dipped phyllosphere samples were *Candidatus Portiera* (16%), *Pseudomonas* (16%) and a taxonomically unassigned genus of the family of *Enterobacteriaceae* (15%). The phyllosphere samples, which were rhizosphere dipped with sterile medium, were dominated by 18% of *Buchnera* and 15% of an unassigned genus of *Gammaproteobacteria*. On the inoculated samples *Candidatus Blochmannia* is less abundant with 0.8% compared to 16%. The genus *Erwinia* was present on all samples except for the phyllosphere of the rhizosphere medium dipped samples, on which no representative of *Erwinia* were detected. *Klebsiella* on the phyllosphere samples was negatively affected, either by dipping with 3Rp8 and spraying with 3Rp8. *Klebsiella* disappeared from the 3Rp8 dipped phyllosphere samples completely.

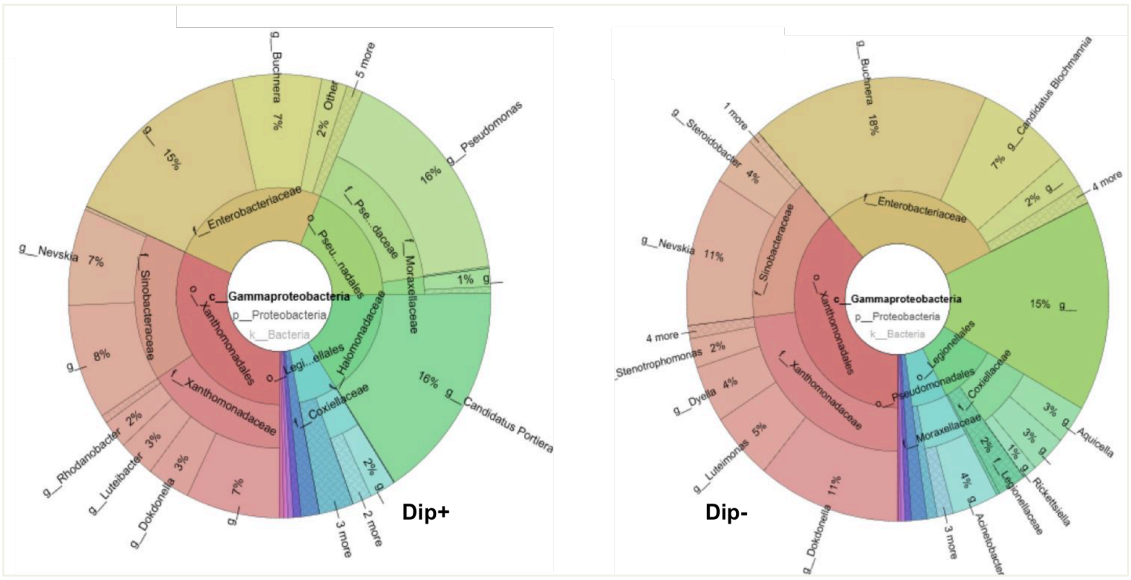


Figure 32 Ringcharts of phyllosphere samples inoculated by dipping with 3Rp8 or sterile medium. The RDP classifier analysis is shown from six inoculated phyllosphere samples by rhizosphere dipping with 3Rp8 (Dip+) and six non-inoculated phyllosphere samples where rhizosphere was dipped with sterile medium (Dip-). Taxonomic ranks are shown by the ring and columns represent distinct taxa (g-genus; f-family; c-class; p-phylum). Other-not taxonomically assigned.

To analyse how *Enterobacteriaceae* and *Serratia plymuthica* 3Rp8 interact and how they affect the bacterial community in lettuce, Spearman co-occurrence networks have been created with the r package and Cytoscape version 3.2.1 (Shannon et al., 2003). In Fig. 33 the positive (green edges) and negative (red edges) effects are shown. A negative effect is shown with *Legionellales*, *Xanthomonadaceae* and *Chromatiales*. Positive effects were shown with *Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* and *Halomonadaceae*.

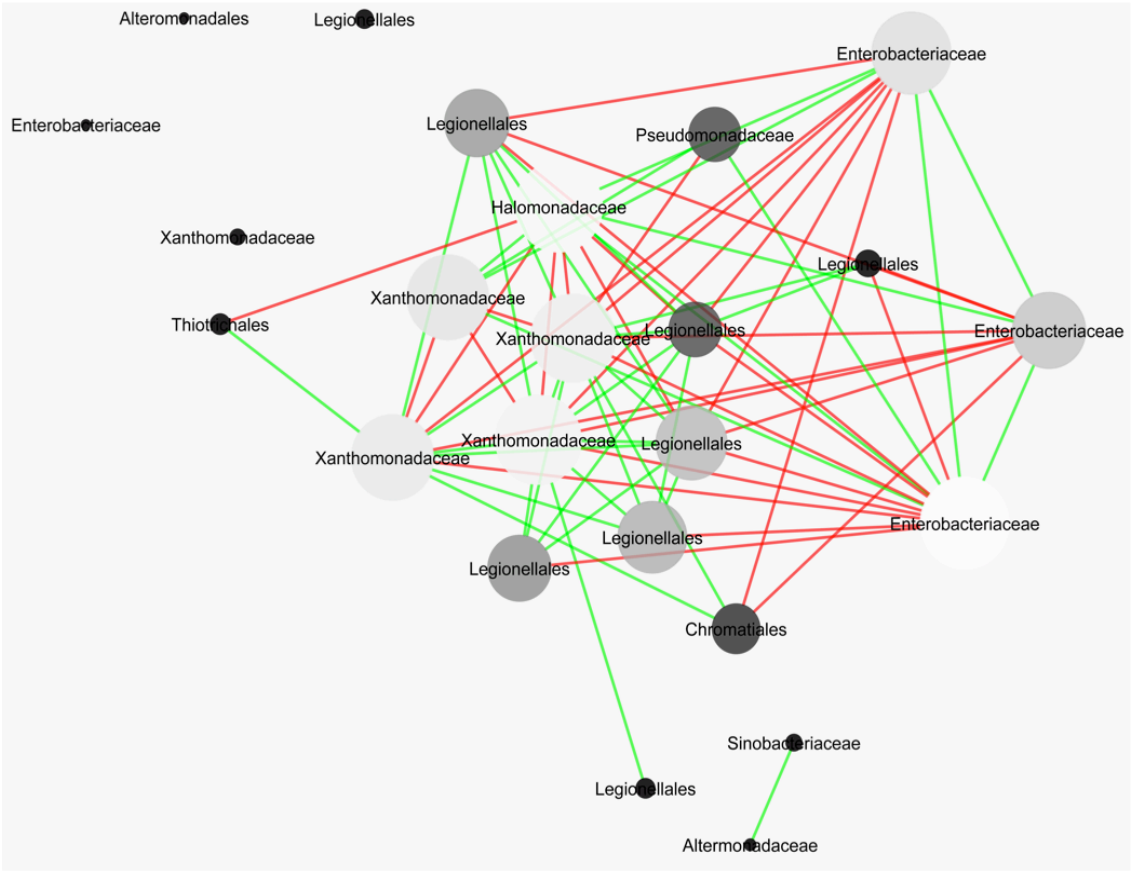


Figure 33 Network analyses of effects of *Enterobacteriaceae* on the community. Network analyses were created with Cytoscape version 3.2.1 (Shanon et al., 2003). Red edges - negative effect; green edges - positive effect. Size of the circles represents the proportion of affect. Circles without lines -no effects.

The next neighbour effects of 3Rp8 on the bacterial community are shown in Fig. 34. Positive effects are represented by green edges and negative effects are represented by red edges. The size of the circle shows how strong 3Rp8 affects the community. A very high portion of the microbiome is negatively affected and only a few colonizers are positively influenced.

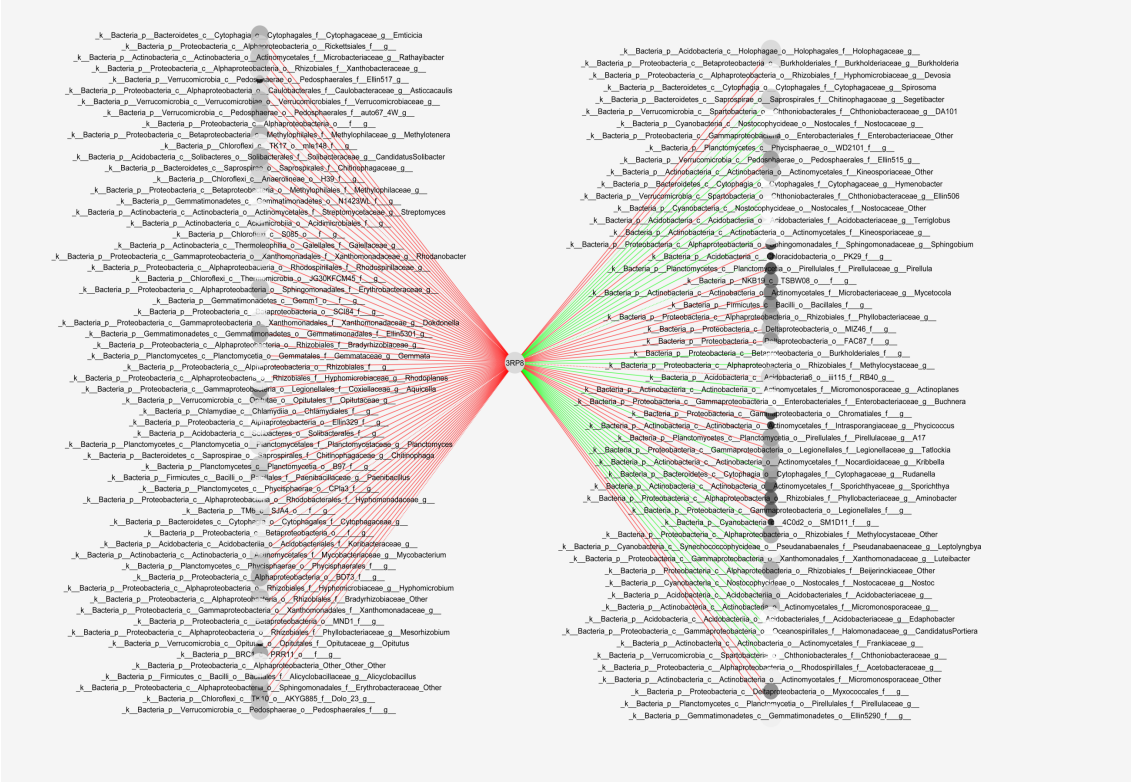


Figure 34 Network analyses of effects of 3Rp8 on the bacterial community on lettuce. Red edges represent negative effects; green edges represent positive effects.

Beta diversity (pairwise sample dissimilarity) based on weighted UniFrac distances revealed clear differences between habitats (phyllosphere, rhizosphere), but also between samples with different inoculation methods. Higher variation was observed among the samples from the rhizosphere. Alpha diversity indices based on the observed OTUs showed a higher diversity in the rhizosphere than in the phyllosphere. Based on the inoculation method, the highest diversity could be observed within the rhizosphere samples, which were inoculated by dipping (Fig. 35). In Fig. 35 (A, B) no curve reaches a plateau, which means that with further sequencing more species would have been detected. Analysed sequences are plotted on the x-axis against the observed operational taxonomic units (OTUs) on the y-axis.

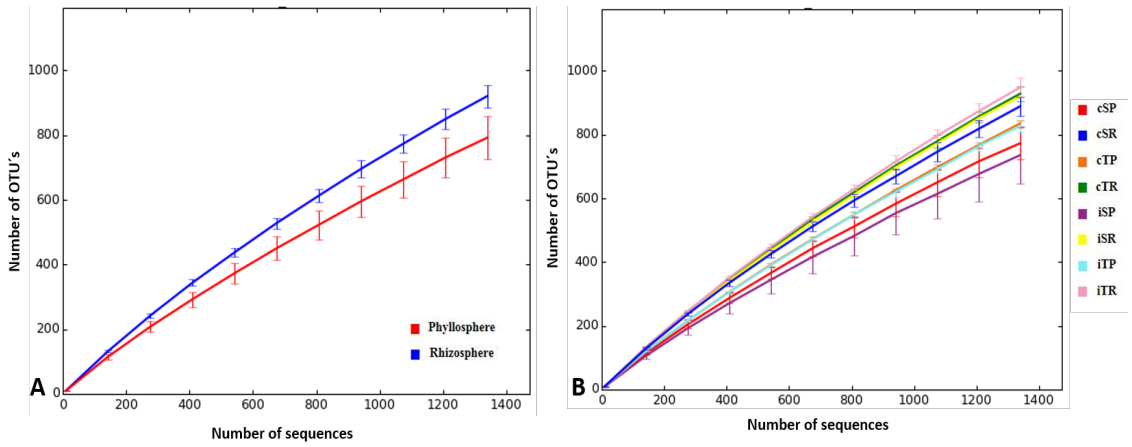


Figure 35 Rarefraction analyses comparing overall diversity of the investigated lettuce samples (var. *capitata*). Rarefaction curves show saturation of the combined datasets that were clustered at 97% sequence similarity. The curves are supported by 95% confidence intervals. The overall diversity was more highly affected in the rhizosphere. Abbreviations: c-inoculated with sterile medium; S-inoculated by spraying; P-phyllosphere; R-rhizosphere; i-inoculated with 3Rp8; T-inoculated by dipping.

The alpha rarefraction plots were generated with QIIME and the output files were visualised based on a html protocol for interactive alpha rarefraction plots (Caporaso et al., 2010).

4. Discussion

Novel biological control strategies to minimise food safety concerns and to protect human health are becoming more important for agriculture. Nowadays, studies are focusing on beneficial bacterial inoculants for plants. For BCAs, the biocontrol activity against phytopathogens is accessed *in vitro* and *in vivo*. The aim of this thesis was to evaluate the promising BCA *S. plymuthica* 3Rp8 as a control mechanism against potential HPs on lettuce. It was shown that *S. plymuthica* 3Rp8 is a highly effective antagonist of potential human pathogenic bacteria and lettuce foliage-associated pathogenic bacteria when compared to two well-known BCAs (*Serratia plymuthica* 3Re4-18 and *Bacillus subtilis* Co1-6). Furthermore, its ability for plant growth promotion could be reinforced when applied with *Pseudomonas fluorescens* WCS365. The effects of 3Rp8 on the indigenous microbiome of lettuce revealed it as a strong keyplayer within the lettuce associated community.

4.1 3Rp8(dsRed) and 3Re4-18 confirmed as highly effective antagonists

Two of the three tested bacterial antagonists showed promising antagonistic activity against potential human pathogenic bacteria. In contrast, *Bacillus subtilis* Co1-6 showed no antagonistic activity against the tested bacteria (mainly *Enterobacteria*) in this study, although it had shown promising antagonistic activity against various soilborne pathogens in previous studies (Köberl et al., 2013). A slight decrease in the antagonistic activity from the tagged to the untagged 3Rp8 was observed. The tagged strain inhibited the growth more strongly than the untagged strain in ten cases (Fig. 12, 13), which was expected conversely. Usually the expression of foreign DNA in a host organism changes the metabolism of an organism, which may impair normal cellular functions by generating a metabolic load. A consequence of the metabolic load can be the loss of a portion of plasmid DNA or sometimes a decrease in cell growth. While cells are growing during high metabolic load, they usually have a decreased level of energy sources for cellular functions and

therefore energy-intensive metabolic processes, such as protein synthesis or nitrogen fixation are reduced (Glick et al., 2010). 3Rp8(dsRed) did not show the negative effects of high metabolic load in this study. The cells still carried the plasmid after reisolation and expressed the protein DsRed after 13 days. For robust validation of the results, a tripartite approach including live monitoring with CLSM at 532 nm, a CCD-camera at 532 nm with the Gel Doc^{TM} XR^{+} System (Biorad, Austria) and reisolation on LB-Trimethoprim plates was carried out. Overall, 3Rp8(dsRed) showed promising antagonistic activity against the tested potential HPs via direct interactions on plate assays and via the production of bioactive volatiles within the VOCs assay. The antibacterial activity of both strains could be attributed to the production of polyketides (PKs) and nonribosomal peptides (NRPs), which are part of a large family of complex metabolites that include many antimicrobial substances. Both are synthesised by large multifunctional enzymes known as polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs), which link amino acids and acyl-CoAs by a cascade of condensation reactions. Three NRPSs and two PKSs operons were identified in both strains. These operons showed a high query cover to the zeamine operon, which was identified by Masschelein et al., in 2013. They found that this PKS/NRPS/fatty acid synthase (FAS) hybrid gene cluster from *Serratia plymuthica* RVH1 encodes for the biosynthesis of three zeamine-related antibiotics. Zeamine is a broad spectrum-antibiotic and was recently shown to also kill the nematode *Caenorhabditis elegans* (Helleberg et al., 2015). It exhibits potent bactericidal activity against a broad spectrum of Gram-positive and Gram-negative bacterial strains, including multidrug resistant pathogens and appears to be quorum-sensing regulated in *S. plymuthica* RVH1, thereby providing the strain with a selective advantage in mixed species biofilms (reviewed in Masschelein et al., 2015). Both tested strains (3Rp8 and 3Re4-18) showed antagonistic activity against plant pathogenic fungi. Analyses of the genomes revealed the production of chitinases, which are a group of inducible enzymes that degrade chitin and the have also been assumed to be required for egg infection of nematodes (Huang et al., 2015). Chitin is a basic component of fungal cell walls and provides a pathogen-associated molecular pattern (PAMP) for plants. Plants can recognise PAMPs by pattern recognition

receptors (PRRs) and thereby mediate a PAMP-triggered immunity (PTI), which protects plants against potential microbial pathogens (reviewed in de Wit et al., 2015). Proteases, Chitinases and Endoglucanases are known as cell-wall hydrolases and play an important role in bacterial control agent-pathogen interactions, either by inhibiting spore germination, mycelial growth and tube elongation (reviewed in Kamensky et al., 2003, Marco et al., 2003). The *in vivo* dual culture plate assay with *Rhizoctonia solani* and *Botrytis cinerea* confirmed this antifungal activity of both strains.

4.2 Potential pathogens within the lettuce microbiome

Within this thesis, several HPs including *Shigella* spp., *Listeria monocytogenes* and *Salmonella* spp. were found on the phyllosphere of fresh and partial processed lettuce. These bacteria are connected with serious disease outbreaks (Davis et al., 1988; Loncarevic et al., 2005). Within the family of *Enterobacteriaceae* some potential pathogenic bacteria, which can cause infections in immunocompromised individuals, were detected. The highest diversity within the family of *Enterobacteriaceae* was observed with the isolates that originated from the iceberg lettuce of a local market. Among these were *Enterobacter aerogenes* and *Enterobacter cloacae,* which are potential HPs*,* which rarely cause diseases in healthy individuals but can do so in immunocompromised individuals (Sanders et al., 1997). Interestingly *Moellerella wisconsensis* was only isolated from the home garden grown "Maikönig". *Moellerella wisconsensis* is a member of the *Enterobacteriaceae*, and while its native habitat is not well known and the pathogenic role remains unclear, they have been associated with diarrhoea, acute cholecystitis, and peritonitis. In 2009 *Moellerella wisconsensis* was involved in a case of a 46-year-old Belgian cirrhotic patient with acute cholecystitis who was found to have bacteraemia caused by *M. wisconsensis* (Cardentey-Reyes et al., 2009). Among the family of *Pseudomonadaceae* the dominating genus was *P. fluorescens* followed by *P. putida*, which was also mainly isolated from the lettuce obtained from the local market. *P. fluorescens* is rarely pathogenic and it is not growing well at 37 °C, but was shown to induce systemic resistance in

Arabidopsis thaliana (Madigan et al., 2009, Iavicoli et al., 2003). *P. putida* can cause nosocomial infections and infections in animals, but is also a PGPB, which enhances seedling growth on canola, tomato and various agriculturally important plants (Hall et al., 1996; Yang et al., 1996; Glick et al., 1997; Altinock et al., 2006). *Curtobacterium plantarum* was isolated from the lettuce obtained from the local market and from the commercial market. *C. plantarum* belongs to the family of *Microbacteriaceae* and is ubiquitous in plant leaves (Dunleavy et al., 1989). The highest diversity at family level was shown for the home garden grown “Maikönig”. Overall, it is of great importance to understand the ecology of potential HPs in the soil and plant environment and to figure out, how bacteria change their physiology and motility when they are in contact with plants (Fornefeld et al., 2017). Future studies should target on transcriptional and metabolic changes of HPs, in order to understand and prevent foodborne outbreaks and diseases, especially on fresh produce.

4.3 3Rp8(dsRed) in combination with WCS365 showed promising growth promoting effects on *Lactuca sativa* L. var *capitata*

The highest germination support was shown by the plantlets inoculated with 3Rp8(dsRed) in combination with WCS 365 in this study (89%). FISH coupled with CLSM revealed that both strains are present on the rhizoplane and that *Serratia plymuthica* 3Rp8/(dsRed) also colonised areas of the phyllosphere. Statistical analyses showed that the combination of WCS365 and 3Rp8 produced the highest growth effects on *Lactuca sativa* L. var *capitata* with regard to average length of leaves. Compared to previous studies, the combination of WCS365 and 3Rp8(dsRed) showed no significant difference in growth effects in regard to average length of leaves, but showed significant differences in growth effects on the average length of rhizosphere and average length of stems. Compared to previous experiments, the rhizosphere growth was influenced most by *Bacillus subtilis* Co1-6 and the stem growth by WCS365 in combination with Co1-6. 3Rp8 shows growth promoting effects on *Lactuca*

sativa L. var *capitata* compared to the control plants. In combination with WCS365 and compared to 3Re4-18 and Co1-6, 3Rp8 showed a high potential of plant growth promoting effects.

On genome and protein level, several genes and proteins were identified in 3Re4-18 and 3Rp8, which are known for their plant growth promoting activity. Several phosphatases were identified in both strains, which are known for their phosphate solubilising activity. Phosphorus (P) is one of the major macronutrients for biological growth and development. Bacteria, which solubilise and mineralise inorganic phosphate, can have plant growth promoting activity by making P available to the plant (reviewed in Rodriguez et al., 1999). The mineral phosphate solubilisation occurs in microorganisms by production of organic acids, which results in acidification of the surrounding of the microbial cell. For this process, P must be released from a mineral phosphate by proton substitution for Ca^{2+} (Goldstein et al., 1994). Furthermore, a spermidine synthase and a spermidine transporter are present in both of the analysed strains. Spermidine is a well-known growth regulator and showed promising growth promoting effects on *Eruca Sativa* Mill (Abd et al., 2004, Al Whaibi et al., 2012). Additionally, the expression of Indole-3-pyruvate-decarboxylase was detected in both strains. Indole-3-pyruvate-decarboxylase is a key enzyme in the production of indole-3-acetic acid (IAA), which is by far the most common and studied auxin. IAA influences plant cell division, extension and differentiation; stimulates seed and tuber germination; affects photosynthesis, pigment formation, biosynthesis of various metabolites and resistance to stressful conditions. IAA produced by bacteria may be involved in plant-bacterial interactions, possibly by plant growth promotion and root nodulation. Furthermore there are several reports, which indicate that some PGPB, which do not synthesise the enzyme 1-aminocyclopropane-1-carboxylate (ACC), which prevents plant ethylene levels from becoming inhibitory to growth, are also able to protect the plant against destructive effects by abiotic stress (rev. Glick et al., 2012).

4.4 3Rp8 was revealed as a strong keyplayer within the lettuce microbiome

Live monitoring with CLSM showed, that *Serratia plymuthica* 3Rp8(dsRed) colonised the plant in spatially segregated colonies distributed all over the rhizosphere. *Proteobacteria*, *Bacteriodetes*, *Acidobacteria* and *Actinobacteria* were identified as the most abundant bacterial phyla on phyllosphere and rhizosphere of lettuce. The gammaproteobacterial diversity, which is a major part of the plant microbiome and especially associated with the lettuce microbiome, was considered in particular. Many *Gammaproteobacteria* can cause infections in immunocompromised individuals and are also involved in severe foodborne outbreaks (reviewed in Erlacher et al., 2014). It was expected, that the gammaproteobacterial diversity as well as the diversity within the *Enterobacteriaceae*, will show a shift in the BCA-inoculated samples. The inoculated rhizosphere was dominated by an unassigned genus of *Xhantomonadaceae* and by an unassigned genus of *Enterobacteriaceae*, of which is assumed to represent the applied strain 3Rp8. This is confirmed by the result that the non-inoculated samples. They were dominated by an unassigned genus of *Xanthomonadaceae* followed by an unassigned genus of *Legionellaceae*. A shift from 3% to 19% in the non-inoculated phyllosphere samples of *Candidatus Portiera* and a balance of 19% of *Candidatus Portiera* and *Buchnera* were not observed on the non-inoculated phyllosphere although a missidentification of *Buchnera* has to be considered. Bacterial taxa with the designation '*Candidatus'* are bacteria, which are yet poorly characterised and are not cultivable under laboratory conditions at this point. *Candidatus Portiera* is taxonomically assigned to the *Gammaproteobacteria* and contains a single species *Candidatus Portiera aleyrodidarum*, which is known to be an obligate endosymbiotic bacterium of whiteflies (Murray et al., 1994; Thao et al., 2004). *Buchnera* are the primary endosymbionts (P-endosymbionts) of aphids and they are almost unknown apart from them. Beside that, they are uncultivable (Douglas et al., 1998). Inside aphids they are known to bind viral coat proteins and thereby help them with survival, which enables the virion to further infect other plants by feeding aphids (Banerjee et al., 2004). However, it must be considered that the bioinformatic evaluation of high-throughput sequencing data

can be biased due to wrong assignments (Edgar, 2017). Compared to the non-treated phyllosphere samples, *Enterobacteriaceae* increased from 25% and 29% for the non-treated phyllosphere to 78% for the BCA-inoculated samples and to 57% for the medium-inoculated ones. Not only when the strain 3Rp8 was applied, but also when the phyllosphere was treated with sterile medium, *Enterobacteriaceae* increased. This indicates the lettuce associated microbiome is not only affected by applying the strain, but also by the inoculation method. *Xanthomonadales* decreased in the treated phyllosphere samples from 32% and 39% to 8% and 12%, respectively. These negative effects of 3Rp8 on *Xanthomonadales* could be also shown in the network analyses. *Xanthomonadaceae* are known to cause bacterial leaf spot on lettuce (reviewed in Rastogi et al., 2012). Furthermore, negative correlations of 3Rp8 were shown with *Legionellales*, *Xanthomonadaceae* and *Chromatiales*. Positive correlations were shown with *Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* and *Halomonadaceae*. Positive and negative effects can occur when the applied microorganism antagonises certain microbes and thereby provides new ecological niches for other microbes.

4.5 *S. plymuthica* 3Rp8 as a promising tool for biological control

To fight chemical usage, growers are interested in environmental friendly biological control strategies and these could play an important role in integrated pest management systems (IPM). Many models for IPM are implemented for various crops every year including good cultural practice, cropping systems (CS) and the assessment of agricultural performance on CS level (Wolf et al., 2002; Liu et al., 2005; Ahuja et al., 2016; Lechenet et al., 2017). 3Rp8 showed in this thesis promising antagonistic activity against possible human and plant bacterial pathogens and pathogenic fungi. The application of a BCA, which minimises the risk of human and plant pathogenic outbreaks on plants and simultaneously enhances the plants fitness by plant growth promotion and microbial shifts, could be an attractive amendment and alternative to

conventional pathogen suppression strategies on leafy greens. 3Rp8 could combine even more beneficial effects by protecting plants and human health, promoting plant growth, enhancing the plants fitness and thereby offering an environmental friendly tool for biological control in IPM. Figure 36 shows the beneficial and promising effects of 3Rp8 that were confirmed in this thesis.

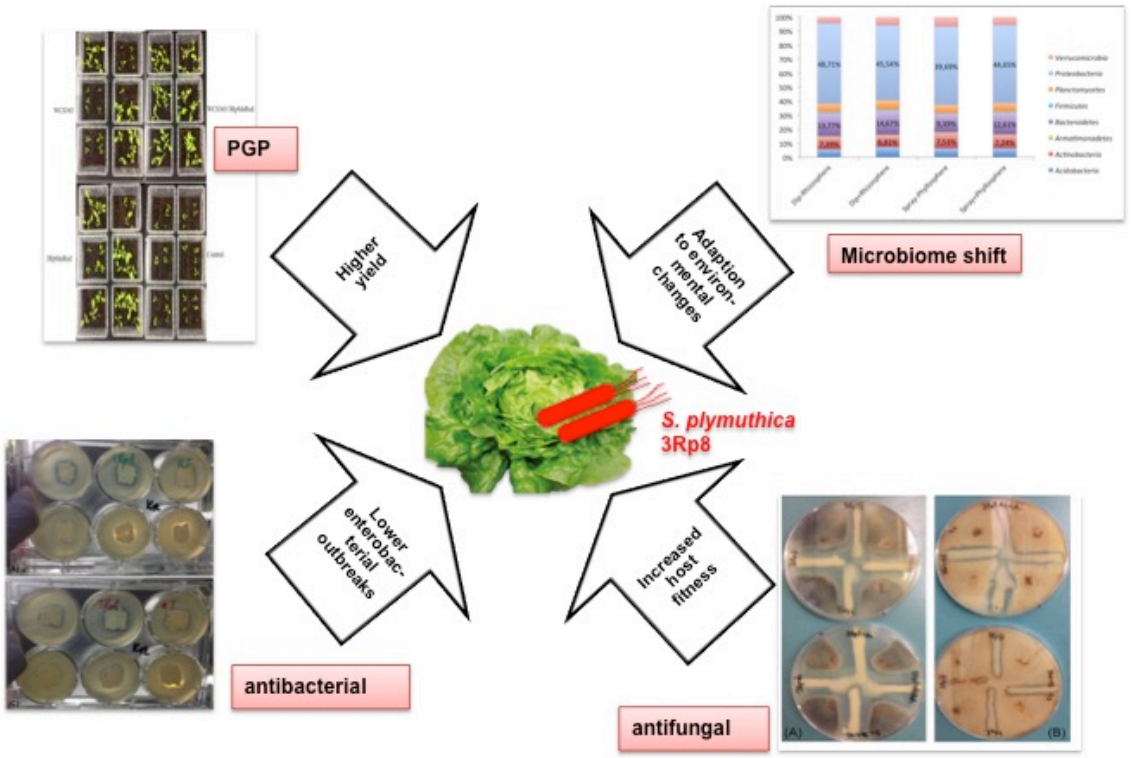


Figure 36 The potential of 3Rp8 for application as a BCA in agriculture. In this thesis 3Rp8 showed antibacterial and antifungal activity against tested model pathogens, which can harm plant and human health. It also showed promising plant growth promoting activities on *Lactuca sativa* L. var *capitata.* In addition, it was revealed as a strong keyplayer in the lettuce microbiome.

5. Conclusions

This study revealed that *Serratia plymuthica* 3Rp8 and 3Re4-18 are promising BCAs with a strong ability to antagonise potential plant and human pathogenic bacteria, via direct or indirect interactions. Their plant growth promoting activity and the genes present in their genomes, which are known to produce substances for plant growth promotion and antagonistic activity, facilitate their use as BCAs. MiSeq Illumina sequencing revealed that 3Rp8 is a dominant key player and coloniser within the native microbiome of lettuce and also has an impact on non-target bacterial populations. 3Rp8 is a promising alternative for chemical pesticides for minimising the risk of pathogenic outbreaks within lettuce. Further experiments need to be performed to optimize the application technique, concentrations and growth stage. Furthermore the ability of 3Rp8 to colonise the host plant and its role as a strong key player within the microbiome of “Gelber Winter” was demonstrated. The removal of distinct native colonizers creates new niches that can be colonised by other taxonomic groups. This makes it an interesting candidate for future targeted studies on pathogen suppression and the biocontrol of lettuce.

6. Supplementary material

Supplementary Table 1 Read counts for phyllosphere and rhizosphere sample obtained by amplicon sequencing; Counts per phyllosphere sample (left column) ranged from 4453 to 17061 reads with a mean value of 8559.348 and a median of 8175.000. Counts per rhizosphere sample (right column) ranged from 3974 to 60589 reads with a mean value of 18720.174 and a median of 16039.000.

BC11:	4453	BC17:	3974
BC44:	4651	BC14:	4720
BC19:	5205	BC18:	6086
BC43:	5277	BC06:	6264
BC09:	5519	BC39:	8686
BC08:	5971	BC28:	11085
BC12:	6553	BC02:	12086
BC46:	6974	BC01:	12918
BC48:	7218	BC26:	13005
BC21:	7268	BC27:	13761
BC33:	7925	BC15:	14158
BC20:	8175	BC30:	16039
BC24:	8332	BC04:	16161
BC10:	8382	BC25:	16627
BC31:	8698	BC05:	16815
BC23:	9245	BC03:	17839
BC45:	10826	BC41:	22081
BC22:	10968	BC13:	22975
BC47:	10996	BC42:	30084
BC35:	11010	BC29:	32383
BC36:	12352	BC38:	34908
BC32:	13806	BC40:	37320
BC34:	17061	BC37:	60589

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