Endophytic bacteria with plant growth promoting and biocontrol abilities

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Proefschrift

ter verkrijgen van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. C.J.J.M. Stolker
volgens besluit van het College voor Promoties
te verdedigen op woensdag 10 April 2013
klokke 15:00 uur

door

Natalia V. Malfanova

geboren te Staraya Russa, Rusland
in 1984

Promotie commissie

Promotor:	Prof. Dr. E.J.J. Lugtenberg (Universiteit Leiden)
Overige leden:	Prof. Dr. H.P. Spaink (Universiteit Leiden)
	Prof. Dr. C.A.M.J.J. van den Hondel (Universiteit Leiden)
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Cover: Designed by Natalia Malfanova and Gerben Voshol
Printed by: Ridderprint BV, Ridderkerk
ISBN number: 978-90-9027408-9
Funding organization: The research described in this thesis took place as part of a Dutch - Russian Research Cooperation in the Centre of Excellence “Interaction of Plants and Beneficial Bacteria” and was subsidized by NWO/RFBR (NWO project number 047.018.001).

To *Gerben* and *Sabina*

Contents

List of abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AFM	antifungal metabolite
AHL	*N*-acyl homoserine lactone
AMF	arbuscular mycorrhizal fungi
BCA	biological control agent
BNF	biological nitrogen fixation
c-LP	cyclic lipopeptide
CLSM	confocal laser scanning microscopy
CNN	competition for nutrients and niches
DMDS	dimethyl disulfide
EPS	exopolysaccharide
ET	ethylene
FA	fusaric acid
FAO	food and agriculture organization
FISH	fluorescent *in situ* hybridization
Forl	*Fusarium oxysporum* f.sp. *radicis-lycopersici*
GB	gibberellin
GFP	green fluorescent protein
IAA	indole-3-acetic acid
ISR	induced systemic resistance
LC-MS	liquid chromatography mass spectrometry
LCO	lipochitooligosaccharide
LPS	lipopolysaccharide
MHB	mycorrhiza helper bacteria
P&P	predation and parasitism
PCN	phenazine-1-carboxamide
PGP	plant growth promotion
Phl	2,4-diacetyl phloroglucinol
QS	quorum sensing
ROS	reactive oxygen species
SL	strigolactone
TFRR	tomato foot and root rot

TLC	thin layer chromatography
TTSS	type three secretion system
VOC	volatile organic compound

General introduction

It is estimated that the world's population will increase by 2.3 billion (or 34%) by 2050. Moreover, the average food consumption is also expected to rise from 2000 to 3070 kcal per person per day. To meet these demands, agricultural production has to be increased by 60% over the next 40 years (FAO, 2012). This can be achieved by the expansion of the amount of farmland and/or by increasing agricultural productivity. Expanding agricultural land is difficult because this possibility is limited by a number of important constraints such as competing with urban growth and scarcity of fresh water. Therefore, improvement of agricultural productivity will be the key approach for reducing the global food insecurity over the coming decades.

It is possible to increase agricultural productivity by stimulating plant yield and by protecting crops from phytopathogens. Commercial fertilizers and pesticides, which are commonly used for these practices, are dominated by synthetic products. However, because of growing concern about the negative impact of chemical fertilizers and pesticides on human and environmental health, farmers are encouraged to use more environmentally friendly alternatives [Directive 2009/128/EC, Regulation (EC) 1107/2009]. Biofertilizers and biopesticides may become the preferred substitutions for some conventional synthetic products. Since such biopreparations are based on non-pathogenic life microorganisms, they can substantially contribute to the sustainable production of environmentally friendly and low chemical residue products.

At present, the majority of the registered bacterial products in Europe is based on species of *Bacillus* and *Pseudomonas* (EU Pesticides Database, 2012). Members of both genera are predominant in soil and plant microenvironments, presumably due to their high growth rate and simple nutritional requirements. These species are widely known for their versatile metabolic activity and diverse beneficial effects on plant vigor and health. Moreover, their beneficial action can be expressed on a large range of plants which places these bacteria among the best candidates for the development of biopreparations. However, despite these positive characteristics, bacterial products can show some inconsistency between trials (Montesinos, 2003). This is assumed to be due to the short persistence of bacterial cells in the rhizosphere/soil environment and their susceptibility to unfavorable environmental conditions.

One possible way to overcome these drawbacks is to develop biopreparations based on beneficial *endophytic* bacteria. Since bacterial endophytes colonize the plant

interior, which is a stable and protected environment, their interaction with a plant can grow into a longer relationship. In addition to housing endophytic bacteria, plants provide them with nutrients and, in turn, some endophytes recompense their hosts by stimulating plant growth and suppressing phytopathogens. If, after production, such beneficial strains can be re-introduced into an endophytic stage, a sustainable and effective crop production system can be achieved.

Aims of the thesis

This Ph.D. thesis focuses on the isolation and characterization of novel beneficial endophytic bacteria with plant growth promotion and biocontrol abilities. Aims were as follows:

1. To isolate endophytic bacteria from different plants of agricultural and horticultural importance
2. To characterize potential plant-beneficial traits of the isolated endophytes
3. To test the most promising isolates for their ability to promote plant growth and to control plant disease, and
4. To characterize the endophytic lifestyle of selected strains.

Outline of the thesis

Chapter 1 contains a brief introduction to the main aims of the thesis. **Chapters 2**, **3** and **4** give a detailed overview of the three most relevant topics treated in this thesis. **Chapter 2** provides an introduction to endophytic bacteria with specific emphasis on how they enter a plant, live inside and contribute to plant health. Mechanisms of plant growth promotion and biocontrol which were found for endophytes in *in planta* studies are discussed in detail. This chapter ends with the evaluation of available genomic, metagenomic and postgenomic tools to get a deeper insight into plant-endophyte beneficial interactions. **Chapters 3** and **4** describe our knowledge of known mechanisms of plant growth promotion and biocontrol, respectively. In **Chapter 3**, examples are given of microbes which provide a plant with essential nutrients, secrete phytohormones and other plant growth promoting substances and increase plant resistance to abiotic stresses. **Chapter 4** describes biocontrol bacteria and their secondary metabolites involved in various biocontrol mechanisms.

In **Chapter 5**, the isolation of endophytic bacteria from different plants of agricultural and horticultural importance is described. The isolated endophytes were subsequently characterized with regard to their plant-beneficial traits and ability to

promote plant growth and control plant diseases. This resulted in the selection of a novel beneficial strain, namely *Bacillus subtilis* HC8 from giant hogweed. This strain is able to produce a wide range of bioactive compounds, a trait which probably contributes to the beneficial effect mediated by HC8. The secondary metabolites produced by *B. subtilis* HC8 include cyclic lipopeptides (c-LPs) which were further characterized in **Chapter 6** using liquid chromatography mass spectrometry (LC-MS) followed by *in vitro* bioactivity tests. Endophytic bacteria with biocontrol properties were also isolated and characterized as described in **Chapter 7**. Those isolated strains, which were identified as members of the *Pseudomonas* genus, were compared with rhizospheric pseudomonads with respect to their abilities to utilize various carbon sources. This resulted in identifying the carbon source L-arabinose as a nutrient which might be important for the endophytic lifestyle of *Pseudomonas* species.

Chapter 8 is a general discussion on the results obtained in this thesis in comparison with the literature. Moreover, additional information is provided on plant growth promotion, biocontrol and the endophytic lifestyle of some strains. Concluding remarks and future prospects complete this chapter. **In Chapter 9**, a summary is given of the major findings of the thesis, in both English and Dutch.

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Bacterial endophytes: who and where, and what are they doing there?[1]

Natalia Malfanova, Ben Lugtenberg, and Gabriele Berg

Abstract

Bacterial endophytes are ubiquitous colonizers of the inner plant tissues where they do not normally cause any substantial morphological changes and disease symptoms. In this chapter we will give an overview of which bacterial species can live as endophytes, and how they enter a plant and live inside. We will also describe various bacterial traits which are required for a successful colonization of the plant's interior by endophytes. Some endophytes can promote plant growth and/or protect their host against phytopathogens. Many mechanisms of their beneficial action are predicted, but we will focus on those for which experimental support *in planta* was reported. Genomic analysis can give a deeper insight into the capabilities of endophytes and their possible role in plant growth and health. We will end our chapter with a brief discussion of available postgenomic tools and their utility in understanding the functionality of endophytic bacteria in plants.

[1] To be published as a chapter in the book "Molecular Microbial Ecology of the Rhizosphere" (2013), Frans J. de Bruijn (ed), Wiley-Blackwell.

Introduction

Virtually all plants are inhabited by diverse bacteria known as endophytes. Endophytic bacteria are referred to as those which can be detected at a particular moment within the tissues of apparently healthy plant hosts (Hallmann et al. 1997; Schulz and Boyle, 2006). Most of the endophytes colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls and xylem vessels. Some of them are able to colonize reproductive organs of plants, e.g. flowers, fruits and seeds. Inside a plant these bacteria do not normally cause any substantial morphological changes like root-nodule symbionts do. They also do not cause any disease symptoms, in contrast to phytopathogens. Many endophytic bacteria possess a number of plant-beneficial traits *in vitro*; few of those exhibit them *in planta* and only a small number of endophytes proved to be very effective plant-growth promoting and/or biocontrol agents under agricultural conditions (Scherwinski et al., 2008; Berg, 2009).

In the following paragraphs we will discuss a number of important issues about endophytes. We will begin with a description of which bacteria were found as endophytes. Subsequently, colonization strategies used by endophytes will be described. How do they get inside plants? Which molecular traits are important for endophytic colonization? How do they escape the plant's immune response? Once they have established themselves in a plant, some endophytes can have a number of beneficial effects on their hosts. What are the mechanisms of their beneficial influence on plants? Here we will focus on those mechanisms which have been verified *in planta*, e.g. by a mutational study. Finally, we will try to get a deeper insight into the capabilities of endophytic bacteria and their possible role in plant health and development by evaluating a genomic approach. The utility of metagenomic and postgenomic approaches to study the structure and function of the endophytic community will complete the discussion of this chapter

Which bacteria can be found as endophytes?

Since the first reliable reports about the isolation of endophytic bacteria from surface-sterilized plants (Samish et al., 1960; Mundt and Hinkle, 1976) more than 200 bacterial genera from 16 phyla have been reported as endophytes. These include both culturable and unculturable bacteria belonging to Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Cholorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Spirochaetes and Verrucomicrobiae (Sun et al., 2006; Berg and

Hallmann, 2006; Mengoni et al., 2009; Manter et al., 2010; Sessitsch et al., 2012). However, the most predominant and studied endophytes belong to three major phyla (Actinobacteria, Proteobacteria and Firmicutes) and include members of *Azoarcus* (Krause et al., 2006), *Acetobacter* (renamed as *Gluconobacter*) (Bertalan et al., 2009), *Bacillus* (Deng et al., 2011), *Enterobacter* (Taghavi et al., 2010), *Burkholderia* (Weilharter et al., 2011), *Herbaspirillum* (Pedrosa et al. 2011), *Pseudomonas* (Taghavi et al., 2009), *Serratia* (Taghavi et al., 2009), *Stenotrophomonas* (Ryan et al., 2009) and *Streptomyces* (Suzuki et al., 2005). Species of these genera are ubiquitous in the soil/rhizosphere which represents the main source of endophytic colonizers (Hallmann and Berg, 2006). Other possible sources of endophytes include the phyllosphere, the anthosphere and seeds (Compant et al., 2010). Naturally occurring endophytes can be visualized by FISH (fluorescence *in situ* hybridization) combined with confocal laser scanning microscopy using specific probes (Amann et al. 1990; Loy et al. 2007). In **Fig. 1** examples are shown for the phyllosphere and rhizosphere of plants (Bragina et al., 2011 a, b).

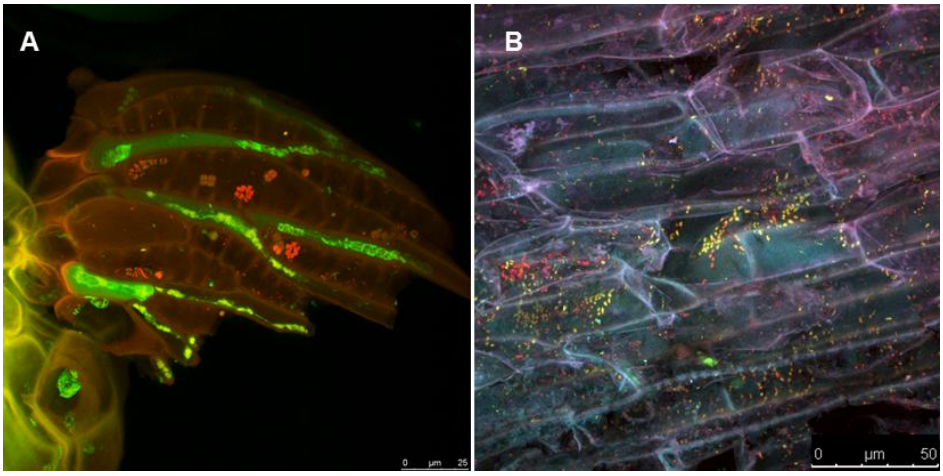

Fig. 1. Localization of endophytic bacteria by fluorescence *in situ* hybridization combined with confocal laser scanning microscopy in the phyllosphere of a moss gametophytes of *Sphagnum fallax* (A) and in the rhizosphere of *Lolium perenne* (B). Images show colonization of hyaline leave cells of *S. fallax* by Bacteria (red) and Alphaproteobacteria (yellow) (A) and of root cells of *L. perenne* by Bacteria (red), Alphaproteobacteria (pinkish), and Gammaproteobacteria (yellow) (B).

1. Colonization of plants by endophytic bacteria

There is a number of ways by which endophytic bacteria can get access to a plant's interior. In this section we will follow their main colonization route from the rhizosphere and give a brief description of alternative ways of plant colonization by endophytes.

1.1. Rhizoplane colonization

Colonization of the plant's interior by bacteria generally starts with their establishment in the rhizosphere. The early events of this process such as recognition and chemotaxis have been extensively reviewed by Lugtenberg et al. (2001) and Lugtenberg and Kamilova (2009). They will not be covered here. Following rhizosphere colonization, bacteria attach to the rhizoplane, i. e. the root surface. A number of mutational studies showed that attachment of bacterial cells to the root is a crucial step for subsequent endophytic establishment. Several bacterial surface components can be involved in this process. For *Azoarcus* sp. BH72, an endophytic diazotroph of rice, type IV pili encoded by *pil*AB are required for attachment to the root surfaces (Dörr et al., 1998). A mutant impaired in the expression of *pil*AB fails to successfully colonize roots and shoots of rice plants (Reinhold-Hurek et al., 2006). The attachment of another diazotrophic endophyte, *Herbaspirillum seropedicae*, to root surfaces of maize depends on LPS (liposaccharide) (Balsanelli et al., 2010). A mutant strain with changed monosaccharide composition in the core domain of LPS showed a hundred-fold lower root adhesion and endophytic spreading compared to the wild type. A similar study showed that EPS (exopolysaccharide) is necessary for rhizoplane and endosphere colonization of rice plants by *Gluconacetobacter diazotrophicus* (Meneses et al., 2011). Since none of these mutant strains completely lost their ability for adhesion, it can be expected that other bacterial surface components are also involved in this process.

1.2. Bacterial entry

The preferable sites of bacterial attachment and subsequent entry are the apical root zone with the thin-walled surface root layer such as the cell elongation and the root hair zone (zone of active penetration), and the basal root zone with small cracks caused by the emergence of lateral roots (zone of passive penetration) (**Fig. 2**). At these sites bacteria are often arranged in microcolonies comprising several hundreds of cells (Zachow et al., 2010). For active penetration, endophytic bacteria have to be well-equipped with cellulolytic enzymes which hydrolyze the plant's exodermal cell

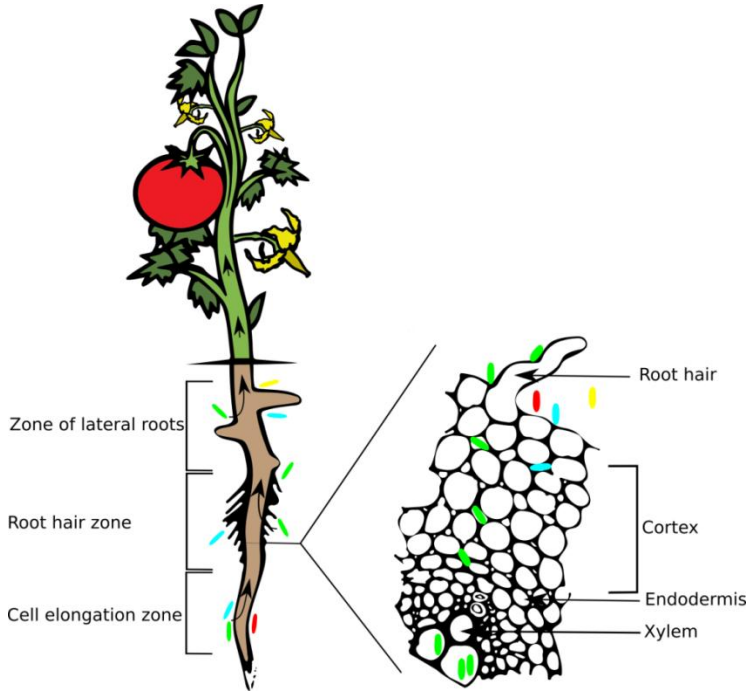

Fig. 2. The main plant colonization routes by endophytic bacteria. Bacteria can enter a plant at several root zones as indicated above. Endophytes can either remain at the site of entry (indicated in blue) or move deeper inside and occupy the intercellular space of the cortex and xylem vessels (indicated in green). Red and yellow represent rhizospheric bacteria which are unable to colonize inner plant tissues.

walls. *In vitro* production of these enzymes has been reported for many endophytes (Compant et al., 2005; Reinhold-Hurek et al., 2006). The expression of endoglucanase, the main cellulase responsible for hydrolysis of β(1→4) linkage in cellulose, was detected *ad planta* at the primary sites of entry of *Azoarcus* sp. BH72 (Reinhold-Hurek et al., 2006). Moreover, the role of endoglucanase in its endophytic colonization has been confirmed by mutational analysis. An *egl*A mutant failed to efficiently invade plant cells and to systemically colonize the plant, in contrast to the wild type strain and the mutant complemented with *egl*A.

Bacterial cell-wall degrading enzymes are also known to be involved in the elicitation of defense pathways in plants as many proteins which are involved in defense and repair are associated with plant cell walls (Norman-Setterblad et al., 2000). Induction of such a response usually results in decreasing the spread of pathogens inside a plant (Iniguez et al., 2005). Since this is not the case for endophytes, endophytic bacteria must be able to escape the plant immune response or even reduce it to some extent. Genomic analysis of sequenced endophytes

confirmed this notion (see section Genomic and postgenomic view of plant-endophyte interactions). The exact mechanism of this process remains to be elucidated.

By entering a plant through natural cracks at the region where the lateral roots appear, bacteria remain "invisible" for the plant's immune system. This mode of entry (often combined with active penetration) has been suggested for *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek, 1998) and *Burkholderia vietnamiensis* (Govindarajan et al., 2007) in rice, *B. phytofirmans* PsJN in grape (Compant et al., 2005), *B. subtilis* Lu144 (Ji et al., 2008) and *B. cepacia* Lu10-1 (Ji et al., 2010) in mulberry, *Gluconacetobacter diazotrophicus* Pal5 in sugar cane (James et al., 1994) and *Herbaspirillum seropedicae* Z67 in rice (James et al., 2002).

1.3. Colonization of the plant cortex

Once bacterial cells have crossed the exodermal barrier, they can remain at the site of entry as it has been shown for *Paenibacillus polymyxa* in *Arabidopsis* (Timmusk et al., 2005) or move deeper inside and occupy the intercellular space of the cortex (James et al., 1994; Roncato-Maccari et al., 2003; Compant et al., 2005; Gasser et al., 2011) (**Fig. 2**). It is uncommon for endophytic bacteria to penetrate plant cells and cause formation of specific morphological structures like root-nodule bacteria do. However, recently Huang et al. (2011) showed that *Bacillus subtilis* GXJM08 colonizes the root of the leguminous plant *Robinia pseudoacacia* L. in a mode similar to that used by rhizobia. The most dramatic changes include (i) deformation of the root hair (swelling, dichotomous branching), (ii) development of infection threads with bacteria between the cell walls of root cortical cells, and (iii) formation of bacteroids inside plant cortical cells. It is unknown whether this strain could fix N like the root-nodule bacteria do. It would also be of interest to determine whether other non-symbiotic bacteria can induce similar morphological changes in this plant.

1.4. Colonization of the xylem

Only a few bacteria can penetrate the endodermal barrier and invade the xylem vessels (James et al., 2002; Roncato-Maccari et al., 2003; Compant et al., 2005; Gasser et al., 2011) **(Fig. 2).** This usually happens through unsuberized endodermal cells in the apical root zone and/or in the basal root zone, where the emerging lateral roots interrupt the continuity of the Casparian band in the wall of endodermal cells. The long-distance transport of water, ions and low-molecular weight organic compounds, such as sugars, organic and amino acids, takes place in the xylem (Sattelmacher, 2001).

Though the concentration of available nutrients is relatively low and represents 0.006 - 0.034 µmol/g of fresh weight for some sugars (Madore and Webb, 1981), it has been calculated that they are sufficient to support the growth of endophytic bacteria (Sattelmacher, 2001; Bacon and Hinton, 2006). Direct evidence that bacterial endophytes feed on plant nutrients came from several radioactive labeling experiments. For example, after incubation of potato plants with $^{13}CO_2$, Rasche et al. (2009) detected the isotope label first in the plant's photosynthetic metabolites and subsequently in diverse bacterial endophytes.

Several attempts were made to find carbon sources which might be important or crucial for the endophytic lifestyle (Shishido et al., 1999; Krause et al., 2011; Malfanova et al., 2013). Shishido et al. (1999) compared carbon oxidation profiles of the endophytic *Paenibacillus polymyxa* strain Pw-2R and *Pseudomonas fluorescens* Sm3-RN with those of rhizospheric strains, which were unable to colonize spruce endophytically. Strains Pw-2R and Sm3-RN were able to metabolize D-sorbitol and D-galacturonic acid while their rhizospheric colleagues could not. In our recent study (Malfanova et al., 2013) we found that, in contrast to most rhizospheric *Pseudomonas* spp., endophytic pseudomonads isolated from cucumber plants were able to utilize L-arabinose, one of the most abundant available sugars in the xylem fluid of various plants (Iwai et al., 2003). In another study Krause et al. (2011) detected induced expression of several bacterial alcohol dehydrogenases inside rice roots during their colonization by *Azoarcus* sp. BH72. Mutant strains with disrupted genes coding for alcohol dehydrogenases colonized the root interior less efficiently than the wild type. Since ethanol is abundant in waterlogged rice, these data suggest that it might be one of the major carbon sources for strain BH72 cells inside the plant. Taking together, these studies show that the ability of bacteria to utilize certain plant metabolites might be a prerequisite for their successful endophytic establishment.

1.5. Colonization of the reproductive organs

It is likely that the concentration of available nutrients in xylem is decreasing along the plant axis. This can explain the facts that the diversity and population density of endophytic bacteria decreases with the distance from the root and that only a small number of bacteria reaches the upper parts of shoots, the leaf apoplast and reproductive organs, such as flowers, fruits and seeds (Compant et al., 2010; Fürnkranz et al. 2011). The presence of endophytic bacteria in reproductive organs of plants was confirmed by cultivation (Samish and Etinger-Tulczynska, 1963; Mundt and Hinkle,

1976; Graner et al., 2003; Okunishi et al., 2005; Fürnkranz et al., 2011) and by microscopic visualization (Coombs and Franco, 2003; Compant et al., 2011). Most likely, bacterial cells enter the reproductive organs through the plant's vascular tissues. For example, many bacterial and fungal phytopathogens infect the developing seeds via vascular tissues of the funiculus and chalaze region as well as via the stigma and micropyle (Agarwal and Sinclair, 1996). It is also possible that if one of the reproductive cells (egg cell or male gametes) carries a microbe, the resulting embryo and endosperm may be colonized. This could explain the transfer of endophytes from plants to seeds. However, so far the invasion of reproductive tissues (ovule, megaspore mother cell, stamens, and pollen mother cells) has been shown only for viruses (Agarwal and Sinclair, 1996). The exact mechanism of transmission of endophytic bacteria from the vascular tissues to the reproductive organs and subsequently to the new plant generation still remains to be established.

1.6. Other ways of plant colonization

Although the rhizosphere is assumed to be the main source of endophytic colonizers, other sites of entry cannot be ignored. Some bacteria are able to enter a plant through stomata as has been shown for *Gluconobacter diazotrophicus* on sugarcane (James et al., 2001) and for *Streptomyces galbus* on rhododendron (Suzuki et al., 2005). In the latter case, production of non-specific wax-degrading enzymes might have facilitated the leaf surface colonization and the subsequent endophytic establishment of this microbe (Suzuki et al., 2005). Bacteria can also enter a plant through flowers, fruits and seeds. However this is mostly known for specialized phytopathogens and was not shown for (non-pathogenic) bacterial endophytes.

2. Beneficial endophytic bacteria and their effects on a plant

After establishing in a plant, endophytes can positively influence plant growth and its resistance to different stresses. For detailed overviews of their beneficial actions the reader is referred to Ryan et al. (2008), Hardoim et al. (2008) and Berg (2009). In the following section we will restrict ourselves to the plant growth-promoting effects mediated by endophytic bacteria. These can be grouped as direct PGP (plant growth promotion) and biocontrol of phytopathogens. A variety of PGP and biocontrol mechanisms can be expected for endophytic bacteria based on those described for rhizobacteria (see Chapters 3 and 4, respectively). However, only a few mechanisms have been proven to occur *in planta* (**Fig. 3**).

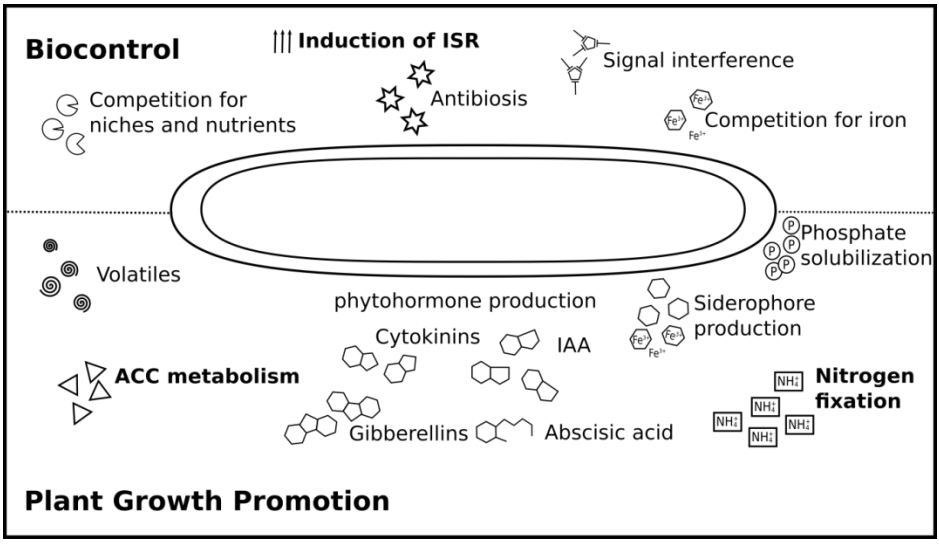

Fig. 3. Illustration of the main mechanisms of PGP and BC mediated by endophytic plant-beneficial bacteria. Indicated in bold are the mechanisms used by endophytes as shown by experimental studies. Other mechanisms are putatively involved based on genomic data.

2.1. Plant growth promotion by endophytic bacteria

PGP has been shown for many endophytic bacteria (Zachow et al., 2010; Gasser et al., 2011; Malfanova et al., 2011). Direct PGP mediated by endophytes is mostly based on providing essential nutrients to plants and production and/or regulation of phytohormones.

After water, nitrogen is the major limiting compound for crop production. Many plants can obtain nitrogen through a process known as BNF (biological nitrogen fixation). For details see Chapter 3. BNF by legumes is based on a symbiosis with root-nodule nitrogen-fixing bacteria while other agriculturally important plants such as maize, rice, sugar cane and wheat can benefit from the association with diverse endophytic diazotrophs. The best studied endophytic diazotrophs include members of *Azoarcus*, *Burkholderia*, *Gluconobacter, Herbaspirillum* and *Klebsiella* (James, 2000).

The ability of endophytic diazotrophs to fix N_2 *in planta* was demonstrated in several studies. This was done by monitoring the expression of nitrogenase genes in nitrogen-fixing cells at the endophytic stage (Egener et al., 1999; Roncato-Maccari et al., 2003; You et al., 2005) and by isotope analysis (Sevilla et al., 2001; Elbeltagy et al., 2001). $^{15}N_2$ incorporation experiments showed that sugar cane plants inoculated with *G. diazotrophicus* Pal5 obtained up to 0.6% of total N from BNF over a 24-h period (Sevilla et al., 2001); for rice plants harboring *Herbaspirillum* sp. B501 this value was

0.14% (Elbeltagy et al., 2001), indicating that diazotrophic endophytes can contribute a significant amount of N to a plant. Other studies suggested that plants can get up to 70% of the required nitrogen through BNF mediated by endophytic diazotrophs (James, 2000).

Nitrogen fixation is regulated by the concentration of oxygen and the availability of nitrogen. In *Herbaspirillum* sp. B501 the expression of nitrogenase was repressed in free air (21% O_2) and induced under microoxic conditions (2% O_2) (You et al., 2005) suggesting that the plant's interior is a suitable environment for BNF. Sevilla et al. (2001) have demonstrated that under N-deficient conditions sugarcane plants inoculated with wild type *G. diazotrophicus* Pal5 have significantly greater shoot mass and N content than plants inoculated with a mutant unable to fix N_2, suggesting that BNF is the likely cause of PGP. It is interesting to note that N starvation can also derepress the biosynthesis of the plant hormone IAA (indole-3-acetic acid). For example, Brandi et al. (1996) demonstrated that IAA synthesis in the culture supernatant of *Erwinia herbicola* 299R was over 10-fold higher under nitrogen-limiting conditions. IAA was detected in the culture supernatant of *G. diazotrophicus* (Fuentes-Ramirez et al., 1993; Bastian et al., 1998). Therefore, it is likely that some diazotrophic bacteria stimulate plant growth both by supplying N and by production of phytohormones, in particular IAA. This possibility is further supported by the observation that when N was not limiting, both wild type *G. diazotrophicus* Pal5 and its fix^- mutant strains were able to increase the biomass of sugar cane (Sevilla et al., 2001). The *in vitro* production of IAA and its possible involvement in PGP has been reported for many other endophytic bacteria (Govindarajan et al., 2008; Rothballer et al., 2008; Jha and Kumar, 2009; Malfanova et al., 2011). However, the principal role of IAA in PGP was confirmed only for rhizobacteria, using mutational studies (Patten and Glick, 2002; Spaepen et al., 2008). For a more detailed overview of the microbial production of auxins and its role in the interaction with plants the reader is referred to Spaepen and Vanderleyden (2011).

Many IAA-producing endophytes possess ACC (1-aminocyclopropane-1-carboxylate)-deaminase activity which is involved in lowering the level of plant ethylene (Long et al., 2008). Elevated levels of ethylene caused by some stresses (see Chapter 3) are known to inhibit root elongation and lateral root emergence (Ivanchenko et al., 2008). According to the model proposed by Glick (2005) bacterial IAA activates ACC-synthase of plants resulting in the production of ACC, the ethylene precursor. Some bacteria can use ACC as a nutrient source and thereby decrease the

synthesis of ethylene in plants. ACC-deaminase activity was described for plant growth-promoting endophytic strains of *Burkholderia* (Sun et al., 2009; Gasser et al., 2011), *Herbaspirillum* (Rothballer et al., 2008) and *Pseudomonas* (Long et al., 2008). The role of ACC-deaminase in plant growth promotion has been further confirmed in a mutational study by Sun et al. (2009). Deletion of the *acd*S gene, coding for ACC-deaminase, in *B. phytofirmans* PsJN resulted in a decrease of the root length of canola seedlings by 32%.

Other phytohormones produced by endophytic bacteria include ABA (abscisic acid) (Cohen et al., 2008), cytokinins (Sgroy et al., 2009) and GBs (gibberellins) (Lucangeli and Bottini, 1997; Malfanova et al., 2011). Inoculation of maize with a GB-producing endophytic *Azospirillum* spp. increased the level of GA3 in plant roots and resulted in promotion of plant growth (Lucangeli and Bottini, 1997). An enhanced ABA content in plants has been detected after inoculation of *A. thaliana* with an ABA-producing strain of *Azospirillum* (Cohen et al., 2008). However, whether endophytic bacteria directly contribute to the increase of the plant phytohormone pool remains to be elucidated.

2.2. Biocontrol of phytopathogens by endophytic bacteria

While the biocontrol effect of endophytic bacteria is well known (Berg and Hallmann, 2006; Scherwinski et al., 2008; Malfanova et al., 2011), the mechanisms of biocontrol mediated by endophytes are less well elucidated. Biocontrol of phytopathogens can be based on several mechanisms which include antibiosis, CNN (competition for nutrients and niches) and ISR (induced systemic resistance) (**Fig. 3**). For more mechanisms, see Chapter 4. So far, only the role of ISR in biocontrol mediated by endophytes has been confirmed *in planta*. This was done by microscopic observations of endophytic bacteria inside the plant, where they induce morphological changes associated with ISR and reduce disease symptoms at locations where the endophyte itself is absent. For example, Melnick et al. (2008) evaluated the ability of several Bacilli to colonize cacao plants and reduce the symptoms of black pod rot caused by *Phytophthora capsici*. Inoculation of leaves with a suspension of vegetative cells resulted in a local colonization of plants. A small subpopulation (5-15%) of bacteria was recovered from the inner leaf tissues and no bacteria were detected in vascular tissues or in newly-developed leaves, indicating that bacteria were unable to systemically colonize the plant. Significant biocontrol was observed 26 days after inoculation on newly developed, non-colonized leaves, suggesting the induction of systemic resistance of cacao plants by bacilli.

Colonization of plants by biocontrol endophytes induces several cell-wall modifications, such as deposition of callose, pectin, cellulose and phenolic compounds leading to the formation of a structural barrier at the site of potential attack by phytopathogens (Benhamou et al., 1998; Benhamou et al., 2000). Another common response of bacterized plants challenged with a pathogen is an induction of defense-related proteins such as peroxidases, chitinases and β-1,3-glucanases (Fishal et al., 2010). These reactions result in a substantial reduction of pathogen spreading in a plant. For example, in *Pythium*-infected cucumber plants the hyphal growth was mainly restricted to the outer root tissue five days after oomycete inoculation (Benhamou et al., 2000). Moreover, 80% of the oomycete hyphae which penetrated the epidermis barrier were distorted. Significant disease suppression was also reported for wheat plants endophytically colonized with *B. subtilis* (Liu et al., 2009) and for banana plants pre-inoculated with endophytic *Pseudomonas* and *Burkholderia* (72 days before pathogen challenge) (Fishal et al., 2010).

Most likely, a combination of several mechanisms is exhibited by many biocontrol endophytic bacteria. This notion is supported by the fact that some antimicrobial compounds are involved in both antibiosis and triggering ISR (Ongena et al., 2007). The presence of other mechanisms such as competition for iron and for colonization sites is proposed for some endophytes based on the analysis of their genomes (see below). However this has not yet been confirmed *in planta.*

3.Genomic and postgenomic view of plant-endophyte interactions

In recent years a number of genomes of endophytic bacteria has been sequenced (**Table 1**). All beneficial traits which are discussed above (N fixation, IAA, ACC deaminase, etc.) are reflected in their genomes. Moreover, analysis of their genomes also revealed the existence of a high number of genes involved in iron uptake and metabolism. For example, the genome of *Enterobacter* sp. 638 has nine ABC transporters for siderophore complexes in contrast to four in *E. coli* K12 (Taghavi et al., 2010). *Azoarcus* sp. BH72 has 22 iron TonB receptor genes, which is twice as much as its free-living soil colleague EbN1 (Krause et al., 2006). These data suggest that endophytic bacteria are well-equipped to survive in a low-iron environment and can efficiently compete for this element with other microorganisms, including phytopathogens.

In addition to the above-mentioned plant beneficial traits, a number of genes involved in QS (quorum sensing) have been identified in the endophytic genomes. For

Table 1. Sequenced bacterial endophytes and mechanisms of their beneficial action

Endophyte	Phylum	Plant of origin	Mechanisms of beneficial action[a]	References
Azoarcus sp. BH72	ß-Proteobacteria	Kallar grass	**BNF** and competition for iron	Krause et al., 2006
Azospirillum sp. B510	α-Proteobacteria	Rice	ACC metabolism, BNF, ISR, production of IAA and siderophores	Kaneko et al., 2010
Bacillus subtilis BSn5	Firmicuta	Konjac	Antibiosis *in vitro*, production of lipopeptides and polyketides	Deng et al., 2011
Burkholderia phytofirmans PsJN	ß-Proteobacteria	Onion	**ACC metabolism**, production of IAA and siderophores	Weilharter et al., 2011
Enterobacter sp. 638	Y-Proteobacteria	Poplar	Competition for iron, production of antimicrobials, IAA, siderophores and volatiles	Taghavi et al., 2010
Gluconacetobacter diazotrophicus Pal5	α-Proteobacteria	Sugarcane	Antibiosis, **BNF**, phosphate and zinc solubilization, production of GB, IAA and volatiles	Bertalan et al., 2009
Herbaspirillum seropedicae SmR1	ß-Proteobacteria	Sorghum	ACC metabolism, BNF, competition for iron, production of IAA	Pedrosa et al., 2011
Klebsiella pneumoniae 342	Y-Proteobacteria	Maize	BNF	Fouts et al. 2008
Methylobacterium populi BJ001	α-Proteobacteria	Poplar	Unknown	Copeland et al., *unpublished*
Pseudomonas putida W619	Y-Proteobacteria	Poplar	Production of IAA	Taghavi et al., 2009
Pseudomonas stutzeri A1501	Y-Proteobacteria	Rice	BNF	Yan et al., 2008
Serratia proteamaculans 568	Y-Proteobacteria	Poplar	Production of volatiles	Taghavi et al., 2009
Stenotrophomonas maltophilia R551-3	Y-Proteobacteria	Poplar	Antibiotic production	Taghavi et al., 2009
Variovorax paradoxus S110	ß-Proteobacteria	Potato	ACC metabolism, competition for iron, signal interference	Han et al., 2011

[a] Both confirmed (in bold) and suspected from genomic analysis and experimental studies

example, 24 *lux*R QS genes are present in the genome of *Serratia proteamaculans* 568. In the related endophytic strain *S. plymuthica* G3 QS controls important colonization-related traits such as swimming motility and biofilm formation (Liu et al., 2011). Interestingly, in some free-living *Serratia* spp. these traits are QS-independent, suggesting that the precise role of QS depends on the bacterium's lifestyle.

Further genome analysis revealed genes which might be important for the endophytic lifestyle. For example, the genome of diazotrophic *K. pneumoniae* (Kp) 342 contains genes for superoxide dismutases, putative catalases, peroxidases and reductases which are involved in the protection of bacterial cells against plant ROS (reactive oxygen species) (Fouts et al., 2008). Additionally, genome analysis revealed the ability of Kp342 to metabolize a wide range of plant sugars, carbohydrates and hemicellulosic substrates. Furthermore, a comparison of the genome of Kp342 with that of the clinical isolate MGH78578 revealed a major difference in their metabolism, surface attachment and secretion. These data suggest that Kp342 is well adapted to escape plant defense reactions and successfully establish itself inside a plant.

Metagenomic analysis of the most abundant endophytic bacteria of rice verified traits which are shared among endophytes and are therefore potentially important for their interactions with plants (Sessitsch et al., 2012). These include (i) a whole set of specialized secretion systems, except the type III secretion system which was not highly conserved among rice endophytes, (ii) cellulolytic and pectinolytic enzymes, (iii) flagellins, (iv) enzymes involved in ROS degradation, (v) receptors and transporters for iron uptake, (vi) QS systems, (vii) metabolic pathways for degradation of plant compounds, and (viii) numerous plant-growth promoting and biocontrol traits (ACC-deaminase activity, BNF, production of antimicrobial compounds, phytohormones and volatiles).

Applying postgenomic approaches, such as metaproteomics, metaproteogenomics and metatranscriptomics, can link the genomic potential with function and therefore give a deeper insight into plant-endophyte interactions. These tools deal with global expression of proteins (metaproteomics) or mRNA (metatranscriptomics) from microbial communities. Metaproteogenomics links the proteome and the genome of the environmental sample. This allows identification of more proteins (functions) than proteomics alone. Recently, a metaproteogenomic approach was used to study microbial communities in the phyllosphere and rhizosphere of rice (Knief et al., 2011). The results showed that despite the presence of *nifH* genes in both microenvironments, dinitrogenase reductase was exclusively identified in the

rhizosphere. If such an approach could be applied to study the endosphere, more significant data regarding the endophyte functionality can be collected.

Acknowledgements

We would like to thank Anastasia Bragina, Bernhard Hofbauer and Massimiliano Cardinale (Graz) for providing images for Fig. 1. We would also like to thank Gerben Voshol (Leiden) for his help with Figs. 2 and 3.

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Plant growth promotion by microbes[1]

Ben Lugtenberg, Natalia Malfanova, Faina Kamilova, and Gabriele Berg

Abstract

Since the world's population is still growing, food production should be increased. This should be done without damaging the environment further and with a decreased input of chemical hormones and fertilizers. This realization has resulted in an increasing interest in the use of microbes as sustainable and inexpensive alternatives for agrochemicals. In this chapter, we will describe three classes of microbial alternatives for agrochemicals, namely a) general microbial plant growth promoters, b) microbial fertilizers for specific nutrients, and c) microbial plant growth regulators which act through a hormonal mechanism. The latter class includes microbial stress controllers.

[1] To be published as a chapter in the book "Molecular Microbial Ecology of the Rhizosphere" (2013), Frans J. de Bruijn (ed), Wiley-Blackwell.

Introduction

The world's population is assumed to increase from 7 billion now to 8.3 billion in 2025. The world will need 70 to 100 percent more food by 2050 (Godfray et al., 2010). Therefore, the production of cereals, especially wheat, rice and maize, which accounts for half of the human's calorie intake, has to be increased. Currently, plant growth is enhanced by the input of chemicals which act as plant growth regulators (using a hormonal mechanism) and as nutrients. Of the nutrients added to the soil, nitrogen and phosphorous are the major ones. They are, together with potassium, applied as chemical fertilizers to improve grain yield. According to Roberts (2009) the present global annual use of chemical nitrogen, phosphorous, and potash fertilizer is 130, 40, and 35 million tonnes, respectively.

The high input of chemicals raises a number of concerns such as water contamination leading to euthrophication and health risks for humans. Moreover, it results in soil degradation and loss of biodiversity. In this chapter we will describe beneficial microbes which can act as environmentally friendly alternatives for agrochemicals. Their application will increase the sustainability of agriculture.

We will sub-divide these beneficial microbes in the following groups. A. General plant growth promoters. These microbes stimulate plant growth through a variety of known mechanisms or by one or more unknown mechanisms. B. Microbial fertilizers for specific nutrients, the most important ones being N, P and Fe^{3+}. C. Microbial plant growth regulators. These secrete hormones or hormone-like substances which stimulate plant growth in extremely low concentrations. This sub-division is not perfect since one microbe can combine several mechanisms.

The major global nutrition processes will be illustrated in Figures, whereas the PGP traits of some species will be listed in Table 1.

A. General microbial plant growth-promoters

Some microbes and molecules have a general plant growth promoting effect. They can stimulate for example plant establishment and enhance plant vigor. They will be treated in this section. Other microbes have a more specific effect for a certain nutrient. They will be discussed in section B**.**

A.1. Arbuscular Mycorrhizal Fungi

Approximately 90% of the land plants live in symbiosis with AMF (**Fig. 1** and **Table 1A**). AMF are not host-specific. Combinations of AMF and plant roots can form enormous

underground networks. Since exudates from fungal hyphae solubilise more P than root exudates alone, it was suggested that mycorrhizae contribute to the increase of P-uptake through P-solubilisation. AMF can enhance plant establishment and increase water and nutrient uptake, especially of P, Zn and Cu (Clark and Zeto, 2000; see **Fig. 1; Table 1A**). AMF also protect plants against biotic and abiotic stresses and can improve soil structure (Smith and Read, 2008). Since AMF perform similar functions as roots, they functionally extend the root system. Therefore, the area around roots with attached AMF is called mycorrhizosphere. Because of their smaller diameter, the fungal hyphae are able to reach places where roots cannot penetrate. AMFs are also beneficial for soil structure, because they cause aggregate formation.

SLs (strigolactones), the recently discovered class of shoot branching inhibiting hormones, are involved in early stages of the plant-AMF interaction (see **Fig. 1**). They

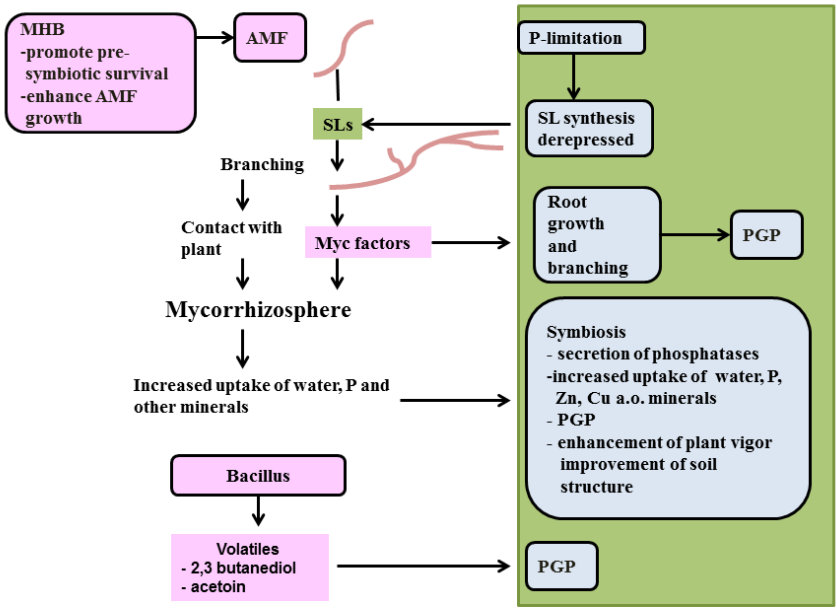

Fig. 1. Role of Arbuscular Mycorrhizal Fungi (AMF) in PGP. For explanation, see text and Table 1A. Colours: green, plant; pink, microbes; blue: processes.

Table 1. Selection of PGP microbes and their relevant PGP

A. Arbuscular Mycorrhizal Fungi	
Trait	**Reference**
Functions as extension of the root system	Parniske, 2013
AMF branching and contact formation with roots stimulated by SLs	Lopez-Raez, 2013
AMF secrete Myc-factors which stimulate root growth and branching	Maillet et al., 2011
Uptake of water, P, Zn, Cu and other nutrients	Clark and Zeto, 2000
Improve of soil structure	Smith and Read, 2008
Protection against (a)biotic stresses	Smith and Read, 2008
Mycorrhiza helper bacteria promote pre-symbiotic survival and fungal growth	Frey-Klett et al., 2007
B. TRICHODERMA	
Trait	**Reference**
Can act as endophyte; increases uptake of water and nutrients; increases solubilization of soil nutrients; increase of nitrogen use efficiency; enhancement of plant vigor; enhanced growth and development of roots and above-ground plant parts; increases root hair formation; causes deeper rooting; improved photosynthetic efficiency	Harman, 2006 ; Lorito et al., 2010; Shoresh et al., 2010; Hermosa et al., 2012
Degrades phenolic compounds secreted by plants.	Ruocco et al., 2009
Produces auxin	Contreras-Cornejo et al., 2009
Accelerates seed germination	Mastouri et al., 2010
Increase of plant resistance, especially under sub-optimal growth conditions	Lorito et al., 2010
Amelioration of abiotic stress; alleviation of physiological stresses, e.g. seed aging	Mastouri et al., 2010 ; Shoresh et al., 2010
The secondary metabolite harzianic acid promotes plant growth	Vinale et al., 2009
C. BACILLUS	
Trait	**Reference**
N_2-fixer	Borriss, 2011
Phosphate solubilizer	Rodríguez et al., 2006; Borriss, 2011
Release of Pi from phytate	Idriss et al., 2002
Potassium solubilizer	Wu et al., 2005
D. PSEUDOMONAS	
Associative N-fixer	Dobbelaere et al., 2003
Phosphate solubilizer	Rodríguez et al., 2006
Siderophore producer	Lemanceau et al., 2009
Auxin producer	Kamilova et al., 2006
Cytokine producer	García de Salmone et al., 2001
ACC deaminase producer	Glick et al., 2007a

[a]Note that not all strains of the mentioned species have the listed traits and that all listed traits are not present in a single strain.

are present in the root exudates of both mono- and dicotyledonous plants. Their synthesis is upregulated by phosphate limitation. SLs from root exudate cause branching of neighbouring AMF spores, thereby increasing their chances to encounter a plant root. SLs also influence auxin transport. In principle, SLs or some of their analogues have the potential to be used for weed control: they are able to induce germination of spores of the weed Striga, which causes massive crop losses of cereals in developing countries. If this induction takes place in the absence of crop plants, the Striga will die (see Schachtschabel and Boland, 2009).

AMF produce diffusible symbiotic signals, recently identified as lipochitooligosaccharides and designated as Myc factors (see **Fig 1**; **Table 1A**). They stimulate root growth and branching. It is expected that (derivatives of) these compounds will be used in future agriculture (Maillet et al., 2011).

Some bacteria help AMF (MHB's; Frey-Klett et al., 2007; Frey-Klett et al., 2011; see also **Fig. 1**). In the case of the *Pseudomonas fluorescens* helper bacterium strain BBc6R8 it was shown that this bacterium promotes the pre-symbiotic survival and growth of the fungus (Deveau et al., 2007).

A.2. *Trichoderma*

Although the soil fungus *Trichoderma* is mainly known as a biocontrol agent (Harman et al., 2004; Lorito, 2010), it has also a large set of direct plant growth-promoting properties (see **Table 1B**). *Trichoderma* is claimed to increase plant resistance under sub-optimal growth conditions, to increase nutrient uptake, to increase nitrogen use efficiency, to enhance solubilization of soil nutrients, to enhance growth, vigor, photosynthetic efficiency, and development of roots and above-ground plant parts, to increase root hair formation and to enhance deeper rooting (Harman, 2006; Shoresh et al., 2010; Lorito et al., 2010; see **Table 1B**). Moreover, it can reduce abiotic and physiological stresses. The latter may be due to ACC deaminase (Viterbo et al., 2010). The secondary metabolite harzianic acid has been identified as a plant growth promoter (Vinale et al., 2009; see **Table 1B**). We conclude that *Trichoderma* has properties similar to those of AMF. However, *Trichoderma* has the advantage that it can be grown in pure culture. Products with *Trichoderma* as the active ingredient have been commercialized.

B. Biofertilisers for specific nutrients.

Plant growth-promoting microbes which fix N_2, solubilise phosphate, and/or produce siderophores are classified as biofertilisers, since they increase the availability of these nutrients to plants (Fuentes-Ramirez and Caballero-Mellado, 2006).

B.1. Nitrogen fixation.

N_2 is abundant in the atmosphere, but is unavailable to plants. Plants receive their nitrogen in the form of ammonium (NH_4^+) and nitrate (NO_3^-). Uptake of NO_3^- occurs together with influx of protons whereas uptake of NH_4^+ occurs together with release of protons. These processes therefore cause alcalinization and acidification of the rhizosphere, respectively, and substantially influence rhizosphere processes.

Conversion of atmospheric N_2 to ammonium is known as the process of biological nitrogen fixation or diazotrophy. The ability to fix nitrogen is widespread among prokaryotes with representatives in both bacteria and archaea (Dekas et al., 2009). This reaction is catalyzed by the nitrogenase enzyme complex which in most bacteria contains molybdenum-iron (Mo-Fe) as the cofactor. Some bacteria have an additional

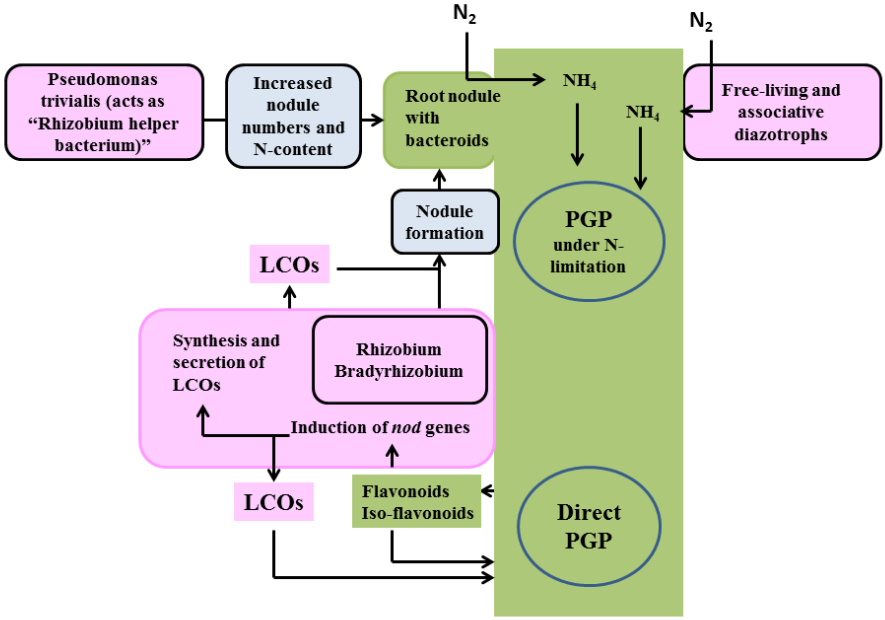

Fig. 2. Microbial contribution to plant N-nutrition. For explanation, see text. Colours: green, plant; pink, microbes; blue: processes.

nitrogenase containing vanadium (Robson et al., 1986) or only iron (Chisnell et al., 1988). However, the alternative nitrogenases have a lower efficiency of nitrogen fixation compared with the conventional ones (Joerger and Bishop, 1988).

Many diazotrophic bacteria are able to establish a symbiotic relationship with plants. The best studied symbiotic diazotrophs belong to the gram-negative rhizobia which induce nodules on leguminous plants (Fabales; see **Fig. 2**). The only exception is the genus *Parasponia* which belongs to Rosales but is nodulated by rhizobia (Markmann and Parniske, 2009).

The rhizobium-legume symbiosis is considered to be the major source of fixed nitrogen. It has been estimated that this symbiosis contributes more than 45 million metric tons of N per year to the terrestrial ecosystems (Vance, 2001). The current taxonomy of rhizobia includes 12 genera with more than 90 species (Weir, 2011) and it is still expanding. The best known rhizobia are those of the α–subclass of Proteobacteria (*Allorhizobium*, *Azorhizobium*, *Rhizobium*, *Mesorhizobium*, *Ensifer* (former *Sinorhizobium*) and *Bradyrhizobium*). In addition, several beta-proteobacteria belonging to *Burkholderia* and *Cupriavidus* have been shown to nodulate plants (Moulin et al., 2001). Rhizobia and other N-fixing bacteria share essential *nod* and *nif* genes encoding nodulation and nitrogen fixation functions, respectively (Zehr and Turnet, 2001). These genes are often carried on symbiotic plasmids which are highly transferable (Brom et al., 2004). Moreover, recipient bacteria are able to obtain a symbiotic function after being transformed with these plasmids (Rogel et al., 2001). Since this can happen under both laboratory and field conditions, it might partly explain the diversity of root-nodulating bacteria.

The symbiosis is initiated by root exudate components, flavonoids or isoflavonoids, which, upon uptake by the bacterium, activate *nod* genes in the bacterium (see **Fig. 2**). The bacterial answer in this molecular dialogue is secretion of products encoded by the *nod*-genes, the NOD factors. NOD factors are lipo-chitin oligosaccharides differing from each other in the length of the chitin fragment, in the unsaturation of their fatty acyl chain and in the presence of several molecular decorations. This makes NOD factors major determinants of the host-specificity of the symbiosis (Spaink et al., 1998; see also **Fig. 2**). Specific perception of NOD factors by plants results in activation of a set of plant genes leading to the formation of root nodules and entry of bacteria (Geurts and Bisseling, 2002). However, certain photosynthetic bradyrhizobia lacking *nod* genes rely on a different, yet to be characterized, strategy for plant signaling (Giraud et al., 2007). *nod* genes have also not been detected in the genome of *Frankia*, Gram-positive

bacteria from the family of Actinobacteria which nodulate non-leguminous plants belonging to the Rosales, Fagales and Cucurbitales. These interesting findings represent a promising source for developing nitrogen-fixing cereals.

Rhizobia can interact with other plant-associated bacteria in the rhizosphere. Such a cooperation can have a beneficial effect on plant growth. For example, Egamberdieva et al. (2010) recently showed that co-inoculation of fodder galega with *Rhizobium* and biocontrol pseudomonads improves shoot and root dry matter of the plant. One of these strains, the cellulase-producing *Pseudomonas trivialis* 3Re27 (Scherwinski et al., 2008), significantly increased nodule numbers and nitrogen content of the co-inoculated plant. The authors coined the term "*Rhizobium* helper bacteria" for this biocontrol strain (see **Fig. 2**).

In addition to symbionts, there are also free-living and associative diazotrophs; these include bacteria from a number of genera: *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobact*er, *Bacillus* (**Table 1C**), *Beijerincka*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, *Pseudomonas* (**Table 1D**), and *Stenotrophomonas* (Dobbelaere et al*.,* 2003; see **Fig. 2**). Using mutants unable to fix nitrogen, Hurek et al. (2002) showed that the beneficial effects of the endophytic diazotrophic bacteria *Azoarcus* sp. on Kaller grass are directly associated with their nitrogen-fixing ability and this is also true for *Acetobacter diazotrophicus* on sugarcane (Sevilla et al., 2001).

Klebsiella pneumoniae and *Azospirillum* are free-living nitrogen-fixing rhizosphere bacteria. In the past, the plant growth-promoting properties of *Azospirillum* were thought to be due to its N_2-fixing property but recent developments show that this property is mainly due to its ability to produce the root architecture influencing hormone auxin. See section C.1 in this Chapter.

B.2. Phosphate solubilization.

After water and nitrogen, phosphorus is the third plant growth-limiting compound. Phosphorus plays a role in numerous plant processes including energy generation, nucleic acid synthesis, photosynthesis, respiration and cellular signaling (Vance et al., 2003).

Plants can absorb phosphorus only as $H_2PO_4^-$ and HPO_4^{2-} ions. Most soils contain amounts of phosphate which are in principle sufficient to support plant growth. However, many of these organic and inorganic forms are not accessible for the plant. Also phosphorus added to the soil as a soluble chemical fertiliser can be rapidly fixed

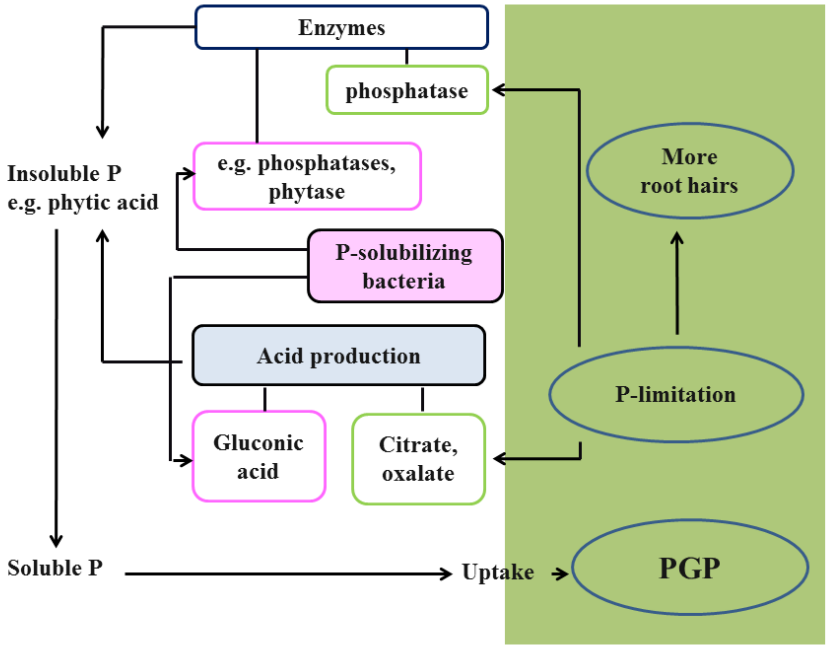

Fig. 3. Microbial contribution to plant P-nutrition. For explanation, see text. Colours: green, plant; pink, microbes; blue: processes.

into insoluble forms and thus made unavailable to plants (Rodriguez and Fraga, 1999; Igual et al., 2001; Smyth, 2011).

Plants react to P-limitation by acidification of the rhizosphere, by increased growth of roots towards unexploited soil zones, by increasing the number of root hairs, and by secreting phosphatases. Acidification is the result of secretion of organic anions together with protons. Organic anions, with citrate and oxalate being more effective than others, can directly facilitate the mobilisation of phosphate (Richardson, 2009; see **Fig. 3**).

Phosphorus is widely applied as a chemical fertilizer, and the excessive and unmanaged application of phosphorus can have negative impacts on the environment, including the eutrophication and hypoxia of lakes and marine estuaries (Smyth, 2011).

Some bacteria, referred to as phosphate-solubilising bacteria (Igual et al., 2001; Kim et al., 1998) are able to solubilise bound phosphorous from organic or inorganic molecules, thereby making it available for the plant (Lipton et al., 1987; see **Fig. 3**). Phosphate-solubilizing bacteria are ubiquitous and *Bacillus* **(Table 1C**), *Enterobacter*, *Erwinia* and *Pseudomonas* spp. **(Table 1D)** are among the most potent species.

Production of organic acids such as gluconic acid is a major factor in the release of phosphorous from mineral phosphate (Rodríguez et al., 2006). Also the release of a range of enzymes results in the generation of phosphate forms which can be taken up by the plant (see **Fig. 3**). These include non-specific phosphatases that dephosphorylate phosphor-ester and/or phosphoanhydride bonds in organic matter, phytases that release phosphorus from phytic acid (Idriss et al., 2002), and phosphonatases and C-P lyases that dissociate C-P bonds in organophophonates (Rodriguez et al., 2006). Vyas and Gulatti (2009) showed that phosphate-solubilising *Pseudomonas* spp. are able to increase both the growth and phosphorus content of maize. Sundara et al. (2002) showed that a phosphate-solubilising *Bacillus megaterium* increases both the amount of plant-available phosphorus as well as the yield of sugarcane. De Freitas et al. (1997) showed that phosphate-solubilising *Bacillus* spp. increase the yield of canola. Using molecular techniques, it was possible to identify a possible new mechanism involved on P solubilization: assessing a genomic library of *Pseudomonas fluorescens* B16, pyrroloquinoline quinone (PQQ) biosynthetic genes were identified responsible for plant growth promotion in this strain (Choi et al., 2008).

AMF were initially thought to provide the plant with phosphorous only. Since it is now known that AFM has a more general function, AFM has been described under section A.1.

B.3. Fe and siderophores.

Iron is an essential element for all organisms. Iron is an abundant element on the earth crust but it is hardly soluble and therefore not suitable for uptake by living organisms. The concentration of Fe^{3+}, the form of iron ions available for living organisms, is only 10^{-18} M.

Plants produce and excrete chelators and/or phytosiderophores which bind Fe^{3+} and transport it to the root surface where it is either reduced to Fe^{2+}, that is subsequently taken up by the plant, or it is absorbed as a Fe^{3+}-phytosiderophore complex by the plant (Lemanceau et al., 2009; see **Fig. 4**).

Bacteria growing under low Fe^{3+} concentrations also produce a variety of siderophores which bind this ion with high affinity (see **Fig. 4**). A number of plant species can absorb bacterial Fe^{3+}-siderophores complexes, but it is unclear whether the uptake of these complexes has any significance to plant iron nutrition and/or direct plant growth promotion (Zhang et al., 2008).

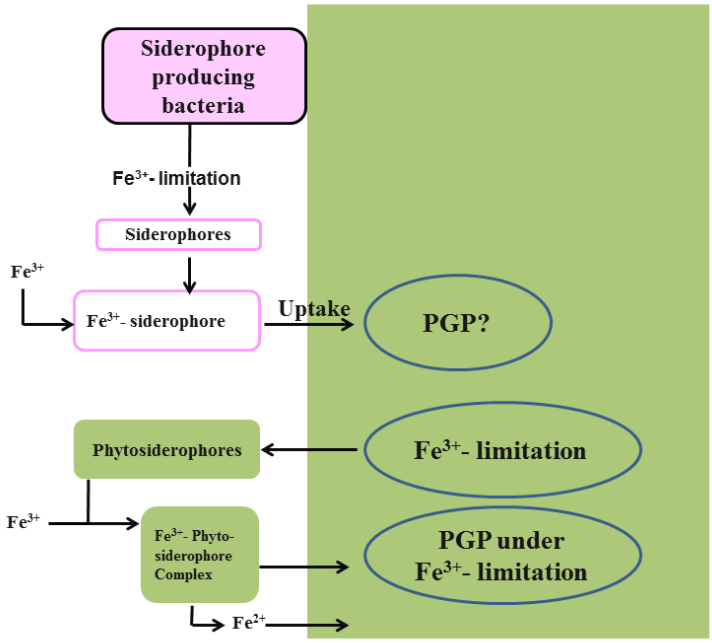

Fig. 4. Possible microbial contributions to plant Fe-nutrition. For explanation see text. Colours: green, plant; pink, microbes; blue: processes.

B.4. Mixures of biofertilizers

Wu et al. (2005) performed a thorough greenhouse study to evaluate the effect of a mixture of four biofertilizers, namely an AMF (*Glomus mossae* or *Glomus intraradices*), an N-fixer (*Azobacter chroococcum*), a P-solubiliser (*Bacillus megaterium*) and a K-solubilizer (*Bacillus mucilaginous*) on growth of *Zea* mays and soil properties. Controls were no fertilizer, chemical fertilizer, organic fertilizer, and two types of biofertilizers. The mixture of the four microbes significantly increased the growth of Z*. mays* and resulted in the highest biomass and seedling height. It also increased assimilation of N, P and K. Moreover, soil properties such as organic matter and total N in soil were improved. The presence of the bacteria in the inoculum resulted in an at least 5-fold higher root infection rate by AMF.

C. Microbial plant growth regulators

Plants produce phytohormones or plant growth regulators, i.e. compounds which at concentrations lower than 1 µM can regulate plant growth and development. There are six classes of plant hormones, namely auxins, brassinosteroids, cytokinins,

gibberellins, abscisic acid, ethylene, and the recently discovered strigolactones. Phytohormones regulate processes such as cell division, cell expansion, differentiation, shoot branching and cell death. Phytohormone pathways and cross-talk between them plays a key role in process coordination and cellular responses (Moller and Chua, 1999; Santner et al., 2009).

Many rhizosphere bacteria can produce plant growth regulators *in vitro*, such as auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Zahir et al., 2003). Bacteria which produce abscisic acid, and ethylene are known as stress controllers. As far as presently known, brassinosteroids and strigolactones are not produced by bacteria or fungi.

Phytohormone production by microbes can modulate the endogenous plant hormone levels and consequently can have an enormous influence on plant growth and development (Gray, 2004; van Loon, 2007). For details on hormones produced by plants and rhizosphere bacteria, the reader is referred to excellent reviews by García de Salome et al. (2006), and Spaepen et al. (2009).

C.1. Auxins.

Nonconjugated indole-3-acetic acid (IAA) is the most abundant member of the auxin family. The concentration of auxin and the ratio of auxin to other hormones are critical for the physiological response of the plant (Lambrecht et al., 2000).

It has been estimated that up to 80% of the rhizosphere bacteria can synthesize IAA (Khalid et al., 2004; Patten and Glick, 1996). Bacteria which produce IAA can add to, or influence, the levels of endogenous plant auxin (Patten and Glick, 1996). It is assumed that plant growth promotion by exogenously added auxin acts by increasing root growth, length and surface area, thereby allowing the plant to access more nutrients and water from the soil (Vessey, 2003; see **Fig. 5**).

Rhizosphere bacteria can use several different pathways for IAA biosynthesis. Most of them use tryptophan, secreted by the plant as a component of root exudate, as a precursor (Costacurta and Vanderleyden, 1995; Spaepen et al., 2007; Spaepen et al., 2009; see **Fig. 5**). Indeed, Kamilova et al. (2006) observed that *P. fluorescens* biocontrol strain WCS365, which produces IAA in the presence of tryptophan, is able to stimulate root growth of radish, a plant which secretes high amounts of tryptophane in its exudate, but not of tomato, sweet pepper or cucumber plants which secrete at least 10-fold less tryptophan.

Azospirillum brasilense is an N_2-fixer which promotes plant growth by increasing its

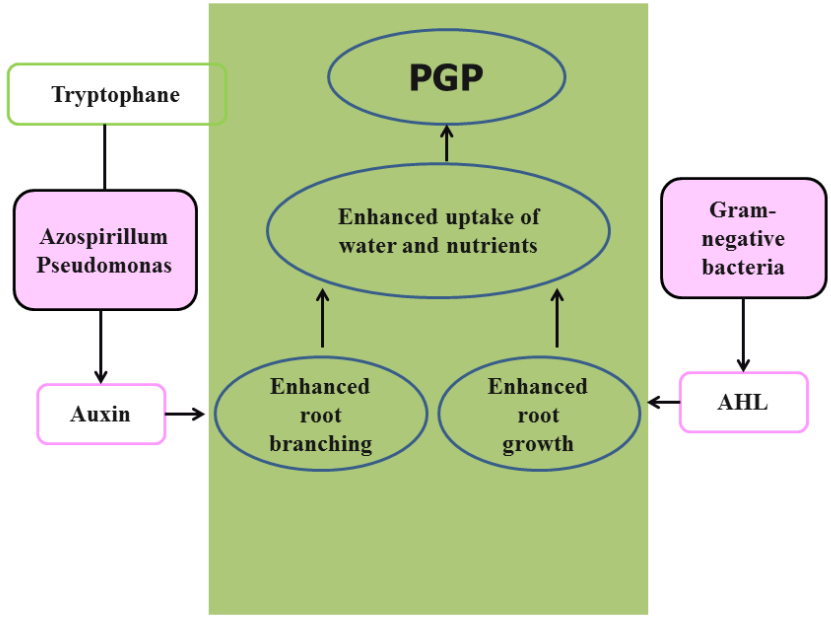

Fig. 5. Stimulation of root branching and growth by auxin. For explanation, see text. Colours: green, plant; pink, microbes; blue: processes.

root surface through shortening the root length and enhancing root hair formation. It has been thought for a long time that its plant growth-promoting ability was based on N_2 fixation. However, the present notion is that auxin production is the major factor responsible for its root changes and therefore for its plant growth-promoting properties (Pliego et al., 2011; see **Fig. 5**). This notion is based on the following observations. (i) Dobbelaere et al. (1999) showed that the effect of the wild type strain on the root can be mimicked by the addition of pure auxin. (ii) A mutant strain strongly reduced in IAA production did not induce the root changes and, (iii), a strain constitutive for IAA production showed the same effect on the root changes as the wild type strain but already at lower bacterial cell concentrations (Spaepen et al., 2008). Interestingly, when the amount of root exudate becomes limiting for bacterial growth, *Azospirillum brasilense* increases its IAA production, thereby triggering lateral root and root hair formation which results in more exudation and, therefore in further bacterial growth. In this way, a regulatory loop is created which connects plant root proliferation with bacterial growth stimulation (Spaepen et al., 2009).

C.2. Cytokinins

Zeatin is the major representative of a group of molecules called cytokinins. Cytokinins have the capacity to induce division of plant cells in the presence of auxin. Starting from callus tissue, the ratio between the amounts of auxin and cytokinin determines whether callus differentiates in root or shoot: high auxin promotes root differentiation whereas high cytokinin promotes shoot morphogenesis. Equimolar concentrations induce cell proliferation.

Cytokinin production is linked to callus growth of tobacco. A test based on this principle can be used as a screening method for cytokinin-producing bacteria. Many rhizosphere bacteria can produce cytokinins in pure culture, e.g. *Agrobacterium, Arthrobacter, Bacillus, Burkholderia, Erwinia, Pantoea agglomerans*, *Pseudomonas, Rhodospirillum rubrum, Serratia* and *Xanthomonas* (reviewed in García de Salome et al., 2001). The spectrum of cytokinins produced by rhizobacteria is similar to that produced by the plant (Barea et al., 1976; García de Salome et al., 2001; Frankenberger and Arshad, 1995) of which isopentenyladenine, trans-zeatin, cis-zeatin and their ribosides as the most commonly found.

García de Salome et al. (2001) provided evidence for a role of cytokinin of rhizosphere bacteria in plant growth promotion. They used mutants of *P. fluorescens* strain G20-18 which produce reduced amounts of cytokinin and normal amounts of auxin. In contrast to the wild type strain, the mutants appeared to be unable to promote growth of wheat and radish plants (García de Salome et al., 2006).

Concerning the mechanism of action of cytokinins, one speculates that cytokinin produced by rhizosphere bacteria becomes part of the plant cytokinin pool, and thus influences plant growth and development.

The ability to produce auxins and cytokinins is a virulence factor for the pathogen *Agrobacterium tumefaciens* which produces crown galls. This bacterium can transfer the genes for production of auxins and cytokinins to the plant and incorporate these genes in the plant's DNA (see Spaink et al., 1998). Another bacterium from this genus, *A. rhizogenes*, modifies cytokinin metabolism, resulting in the appearance of masses of roots - instead of callus- from the infection site (Hamill, 1993).

C.3. Gibberellins (GAs)

These hormones consist of a group of terpenoids with 20 carbon atoms, but active GAs only have 19 carbon atoms. This group of compounds consists of over 130 different molecules (Dodd et al., 2010). GAs are mainly involved in cell division and cell

elongation within the subapical meristem, thereby playing a key role in internode elongation. Other processes affected by these hormones are seed germination, pollen tube growth and flowering in rosette plants. Like auxins and cytokinins, GAs mainly act in combination with other hormones.

Bacteria which produce gibberellins, such as *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azospirillum brasilense*, *A. lipoferum, Azotobacter*, *Bacillus*, *Bradyrhizobium japonicum, Clostridium*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Rhizobium* and *Xanthomonas*, secrete it in the rhizosphere (Frankenberger and Arshad, 1995; Gutiérrez Manero et al., 2001; Rademacher, 1994; Tsavkelova et al., 2006). Hardly anything is known about gibberellin synthesis in rhizosphere bacteria.

Kang et al. (2009) showed that culture suspensions of GA-producing *Acinetobacter calcoaceticus* were able to increase the growth of cucumber, Chinese cabbage and crown daisy. The mechanism of plant growth stimulation by gibberellins is still rather obscure. It is thought that bacteria may increase GA levels *in planta* by either producing GAs, deconjugating GAs from root exudates or hydroxylating inactive GA to active forms (Bottini et al., 2004). Fulchieri et al. (1993) speculate that gibberellins increase root hair density in root zones involved in nutrient and water uptake.

C.4. Abcisic Acid (ABA)

ABA is a 15-carbon compound which, like ethylene, is involved in plant responses to biotic and abiotic stresses. It inhibits seed germination and flowering. It is involved in protection against drought, salt stress and toxic metals. It also induces stomatal closure (Smyth, 2011).

ABA can be produced in culture media by several bacteria such as *Azospirillum brasilense* (Cohen et al., 2008; Perrig et al., 2007) and *Bradyrhizobium japonicum* (Boiero et al., 2007). ABA levels *in planta* have been increased in *Arabidopsis thaliana* by *Azospirillum brasilense* Sp25 (Cohen et al., 2008).

The effect of inoculation with ABA-producing bacteria on plant growth is experimentally poorly underpinned. Since ABA inhibits the synthesis of cytokinins (Miernyk, 1979) it was speculated that ABA increases plant growth by interfering with the cytokinin pool (Spaepen et al., 2009). It could also alleviate plant stress by increasing the root/shoot ratio (Boiero et al., 2007).

C.5. Ethylene (ET) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

Ethylene is a gaseous hormone best known for its ability to induce fruit ripening and flower senescence. ET affects numerous plant developmental processes including root growth, root hair formation, flowering, fruit ripening and abscission, and leaf and petal senescence and abscission (Dugardeyn et al., 2008). ET usually inhibits both primary root elongation and lateral root formation but it can promote root hair formation (Dodd et al., 2010). It generally inhibits stem elongation in most dicots favouring lateral cell expansion and leading to swelling of hypocotyls. ET also breaks seed and bud dormancy. ET production is typically up-regulated in plants in response to pathogen attack, heat and cold stress, waterlogging, drought, excess heavy metals, high soil salinity and soil compaction (Dodd et al., 2010; Glick, 2005).

ET is synthesised under biotic stress conditions following infection by pathogens, as well as by abiotic stress conditions such as drought. It is therefore also known as the stress hormone. In the plant, ethylene is produced from S-adenosylmethionine (SAM) which is enzymatically converted to ACC and 5'-deoxy-5'methylthioadenosine (MTA) by ACC synthase (Giovanelli et al., 1980; see **Fig. 6**).

The enzyme ACC deaminase is present in many rhizosphere bacteria, such as *Achromobacter*, *Pseudomonas*, and *Variovorax* and in the fungus *Trichoderma*. Such microbes can take up ACC secreted by the plant root and convert it into α-ketobutyrate and ammonia (Glick et al., 2007a) **(Fig. 6)**. This results in the decrease of ACC levels, and therefore also of ethylene levels, in the plant and in decreased plant stress. Inoculation of plants with ACC deaminase producing bacteria can protect plants against stress caused by flooding, salination, drought, waterlogging, heavy metals, toxic organic compounds and pathogens (Berg, 2009; Glick, 2005; Glick et al., 2007a; Glick et al., 2007b; Belimov et al., 2005). ACC deaminase activity has been found in fungi such as *Trichoderma* (Viterbo et al., 2010) and in free-living soil bacteria, endophytes, and rhizobia from a wide range of genera, and there have been many correlations between ACC deaminase activity in a range of bacteria and their ability to promote plant growth under various conditions, for example in wheat (Zahir et al., 2009), maize (Shaharoona et al., 2006), and tomato (Grichko and Glick, 2001; Mayak et al., 2004a; Mayak et al., 2004b).

In addition to a direct role of ethylene on plant growth, this hormone can also act as a virulence factor and a signalling molecule in plant protection against pathogen attack. Ethylene production was reported to act as a virulence factor for bacterial

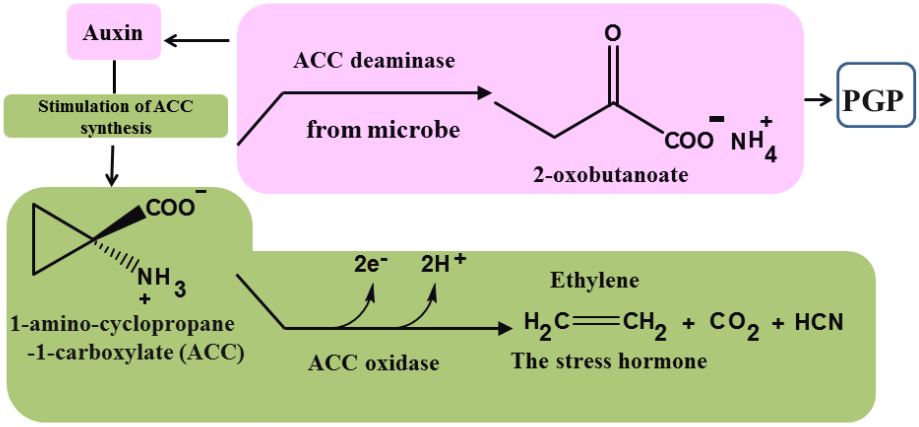

Fig. 6. Role for microbial ACC deaminase in plant stress control. For explanation, see text. The figure is mainly based on papers by the group of B. Glick. According to his hypothesis (Glick et al., 1998), bacterial auxin activates plant ACC synthase. The produced ACC can be used by some microbes as an N-source, thereby decreasing ethylene levels. In order to explain how the ACC produced by the plant is converted by ACC deaminase from the bacterial cytoplasm, Glick et al. (1998) assumed that a significant portion of ACC is exuded from plant roots and seeds and then taken up by the microbe. We would like to suggest the following alternative explanation, namely that the microbe uses the TTSS (type three secretion system) for this purpose since it has been proposed earlier that a beneficial bacterium uses the needle of the TTSS to suck nutrients from the plant root (De Weert et al., 2007). Another possibility is that the bacterium uses its TTSS to deliver the enzyme into the plant. In the case of *Trichoderma*, one can imagine that its endophytic localization facilitates contact between enzyme and substrate.
Colours: green, plant; pink, microbes; blue: processes.

pathogens e.g. *P. syringae* (Weingart and Völksch, 1997; Weingart et al., 2001). Furthermore, ethylene acts as a signalling compound in induced systemic resistance caused by some rhizobacteria (Van Loon et al., 2007).

C.6. Volatiles

Bacteria can produce a wide range of volatiles. While the biological function of most of these volatiles is not fully understood, it is assumed they are involved in a number of processes including cell-cell signaling, inter-species signaling, a possible carbon release valve and that these compounds can promote plant growth and act as microbial inhibiting agents (Wheatley, 2002; Vesperman et al., 2007; Kai et al., 2009).

Bacterial volatiles produced by *Bacillus* spp. have been shown to promote plant growth in *A. thaliana*. The highest level of growth promotion was observed with 2, 3 butanediol and its precursor acetoin (Ryu et al., 2003).

Farag et al. (2006) identified 38 volatile compounds from rhizobacteria. Blom et al. (2011) screened 42 strains grown in four different growth media on the growth response of *A. thaliana*. Under at least one of these conditions each strain showed significant volatile-mediated plant growth modulation. Only one strain, a *Burkholderia pyrrocinia,* showed significant plant growth-promotion on all four media. The volatiles indole, 1-hexanol and pentadecane showed plant growth promotion but the results suggested that this occurred only under stress conditions.

C.7. A-HSLs

N-acyl homoserine lactones (A-HSLs) are signal molecules secreted by many bacteria. When their extracellular concentration reaches a certain value, the quorum, they play a role in many processes such as secretion of antibiotics and exo-enzymes (Vivanco, 2013). In terms of growth promotion, it was shown recently that 10µm C6-AHL and C8-AHL increase root growth in *A. thaliana* (see **Fig. 5**). This is accompanied by an increase in the auxin/cytokinin ratio and in increased expression of over 700 genes in the roots and of a lower number in the stem (von Rad et al., 2008).

C.8. *nod* gene inducers and LCO's (Nod-factors)

The *nod* genes of (*Brady)Rhizobium* are induced by flavonoids or isoflavonoids. LCO's signal molecules are the products of *nod* genes. They initiate root hair curling and subsequent steps in the nodulation of leguminous plants by (*Brady)rhizobium* bacteria (see section B.1).

Interestingly, both the inducers as well as the products of the *nod* genes promote plant growth and this effect is not restricted to leguminous plants (see **Fig. 2**). See http://www.bioag.novozymes.com. For example, one product is based on isoflavonoids and is claimed to activate mycorrhizae before the plant does so, resulting in enhancing nutrient uptake, which in turn leads to lateral root development, and stress tolerance. Formulations for soybean, peanut, alfalfa and pea/lentil, combining the respective LCO and rhizobia, have also been commercialized. When LCOs were applied on seeds of the non-legumes corn, cotton, and wheat, increased plant growth as well as yield increase in the field was observed. In furrow application as well as foliar sprays have similar effects. Possible explanations given are enhanced germination, early seedling growth, increased photosynthesis, enhanced nutrient uptake and enhanced LCO-stimulated mycorrhizal root colonization (Smith et al., 2011).

Conclusions

Nitrogen and phosphorous are the major chemical fertilizers applied to enhance crop yield. This raises a number of concerns such as water contamination leading to euthrophication and health risks for humans. Moreover it results in soil degradation and loss of biodiversity. Presently, the cost for nitrogen fertilizer is steeply increasing as a consequence of the increasing energy prices. The amount of available phosphorous is limited. For these reasons, the interest in sustainable fertilization, using microbes, is strongly increasing. In this chapter we have discussed many microbes which can be applied for a more environmentally friendly agriculture.

Acknowledgement

The authors thank Dr. Doreen Schachtschabel for discussions about strigolactones and her help with **Fig. 6.**

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Microbial control of plant diseases[1]

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Abstract

Most plant diseases are caused by fungi and oomycetes. Presently, the major method for controlling plant diseases is the use of agrochemicals. However, this practice raises health and environmental concerns among consumers and politicians. An alternative for chemicals is the application of products based on natural enemies of the pathogen. Several of such BCAs (Biological Control Agents) with bacteria or fungi as the active ingredient are already on the market. In this review we describe the discovery of such microbes as well as methods for their isolation. Using microscopy, we visualized biocontrol at the cellular level. Furthermore, we describe the role of root colonization by the BCA in biocontrol. Finally, mechanisms of biocontrol at the molecular level are described and the risk of resistance towards BCAs is discussed.

[1] To be published as a chapter in the book "Molecular Microbial Ecology of the Rhizosphere" (2013), Frans J. de Bruijn (ed), Wiley-Blackwell.

Introduction

Some practical aspects of biocontrol

For recent reviews on microbial control of plant root diseases the reader is referred to Berg (2009), Compant et al. (2005), Haas and Défago (2005), Lorito et al. (2010); Lugtenberg and Kamilova (2009), Pliego et al. (2011), and Raaijmakers et al. (2009). The concept of microbial control of plant root diseases originates from the discovery of disease-suppressive soils (Schroth and Hancock, 1982) and is explained in detail in excellent reviews by Weller et al. (2002) and Haas and Défago (2005). Briefly, disease-conducive soils contain pathogens and therefore cause plant disease. In contrast, there are soils which also contain pathogens but hardly cause disease. These so-called disease-suppressive soils contain microbes which suppress the action or growth of the pathogen or even kill the pathogen (Mendes et al., 2011). The disease-suppressive trait can be transferred to conducive soils by mixing the latter with a small amount of disease-suppressive soil. Details on factors influencing transfer of disease-suppressiveness can be found in Haas and Défago (2005).

Plant diseases are responsible for annual crop losses at a total value of more than 200 billion Euro (Agrios, 2005). Major root diseases are caused by fungi, oomycetes and nematodes. Also some bacteria are responsible for root diseases. The fungi include *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, and *Thielaviopsis*. The major oomycetes are *Phytophthora* spp. and *Pythium* spp. The pathogenic bacteria include *Erwinia amylovora, Ralstonia solanacearum* and *Streptomyces scabies*, whereas *Meloidogyne incognita* is an example of a root-pathogenic nematode.

The major form of crop protection is the use of chemicals. However, this practice raises health and environmental concerns among public and politicians. As a result, many chemicals have been banned and more will follow. Also, some supermarket chains put pressure on fruit and vegetable producers by requiring zero tolerance.

An attractive alternative to chemical crop protection products, or more realistically, for the reduction of chemical input, is the use of disease-suppressing microbes. These are found among natural enemies of the pathogens. In principle, the use of these microbes is an environmentally friendly and safe way to replace or reduce chemicals. In case these microbes produce antibiotics, these molecules are produced in only minute amounts and only at the site where they are needed, i.e. on the plant surface. In contrast to this form of precision agriculture, most chemicals are applied in much higher amounts and a significant fraction of the applied molecules does not even reach

the plant surface. A disadvantage of biologicals is that they are often less efficient than chemicals and their action is less consistent than that of chemicals. Therefore a major challenge for biocontrol scientists and producers of microbial products is to create more efficient products. In order to sell a product, the producer should make clear that the product is safe and effective. Despite the strong public and political demand for biological alternatives for chemicals, there are no specific registration procedures for biologicals but they are regulated as general plant protection products which are designed for chemicals.

Although most crops are grown in soil, many greenhouse vegetables are nowadays grown on other substrates such as stonewool. New stonewool is practically sterile. This means that pathogens which invade the young plants can have a devastating effect on the whole plant population because the buffering capacity of indigenous microbes, which is strong in healthy soil, is absent in new stonewool. However, biocontrol microbes such as *Pseudomonas putida* strain PCL1760, added to new stonewool before planting, can protect the plantlets very efficiently against pathogens (Validov et al., 2009). Cells of this strain appeared to stick tightly to stonewool and remain the dominant microbe on the root for at least 3 weeks (Validov et al., 2007). This suggests that addition of such microbes to stonewool can replace indigenous microbes with respect to buffering capacity against pathogens.

Life style of microbes in the rhizosphere

The rhizosphere is defined as the soil area around the root which is influenced by the root (Hiltner, 1904). It is 10 to 1,000 times richer in microbes than bulk soil. This so-called rhizosphere effect is assumed to be caused by nutrients for microbes secreted by the root and by residues of dead roots or root cells. It has been estimated that 5 to 21 percent of the carbon fixed by the plant is secreted, mainly as exudate (Marschner, 1995).

The simplest nutrients in root exudate, which are the most attractive food sources for rhizosphere microbes, are organic acids, sugars, and amino acids. In addition, a large variety of compounds such as enzymes, fatty acids, nucleotides, osmoprotectants, putrescine, sterols and vitamins have been detected in root exudate, as well as signal molecules playing a major role in communication between different microbes and also between microbes and other organisms (see later on in this chapter). The exudate composition is the net result of secretion, conversion by soil

enzymes, and uptake by microbes and plant. For reviews on exudates, the reader is referred to Lugtenberg and Bloemberg (2004) and Uren (2007).

BCAs which are added to the soil have to compete for nutrients and niches on the plant root with indigenous microbes, such as bacteria and fungi, and with predators such as nematodes and protozoa. Microbes living in the rhizosphere usually live under nutrient-starvation conditions since the nutrient concentration is much lower than that in laboratory media (Lugtenberg and Kamilova, 2009). The doubling time of pseudomonads in the rhizosphere is 3 to 6 hours, i.e. ten times slower than in rich laboratory media (Haas and Défago, 2005). Also osmotic stress may play a role in the life of a rhizosphere microbe since the osmotic conditions may vary due to drought and rainfall. This is probably the reason why many rhizosphere microbes produce osmoprotectants (Berg et al., 2013).

BCAs may communicate with other organisms through a variety of signal molecules. We will restrict ourselves here to AHLs because they are relevant for biocontrol. AHLs are molecules secreted by many Gram-negative bacteria. They can sense the level of other bacteria of the same kind. When the concentration of these bacteria reaches a certain level (the quorum), as sensed by the extracellular concentration of AHLs, they start to produce many secondary metabolites and exo-enzymes (Uroz et al., 2009).

Visualisation of biocontrol

GFP can be visualized using CLSM. Since *gfp* mutants with different colors exist, several microbes labeled with GFP and derivatives can be visualized simultaneously in the same preparation against the autofluorescent plant root (Bloemberg et al., 2000). Using the combination of *gfp*-labeled microbes and CLSM, the process of biocontrol of TFRR was visualized, first by following the behavior of BCA and fungus on the root separately, later with all players present. After application on the seed and subsequent germination, the microbe starts to colonize the root collar, followed by colonization of the root, first as single cells and later as micro colonies or biofilms **(Fig. 1**) (Chin-A-Woeng et al., 1997; Bloemberg and Lugtenberg, 2004). The first step carried out by the causal agent of TFRR, *Forl,* is attachment of hyphae to root hairs (**Fig. 1a**). Subsequently the hyphae colonize preferentially the grooves along the junctions of the epidermal cells (**Fig 1b**), penetrate the epidermal cells (**Fig. 1c**) and overgrow the root completely (**Fig. 1d**) (Lagopodi et al., 2002). *Pseudomonas* BCAs, applied on the seed,

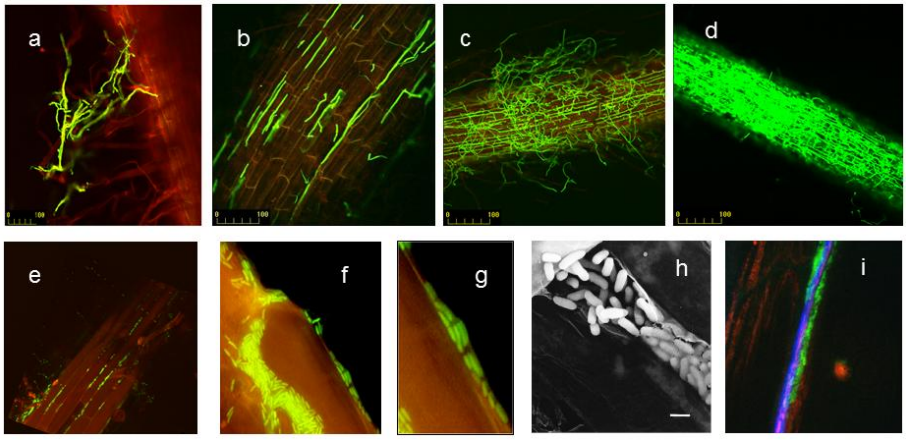

Fig. 1. Visualisation of biocontrol. CLSM (confocal laser scanning microscopy) (a-g and i) and scanning electron microscopy (h) were used to visualize control of TFRR caused by *Forl* (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) by *Pseudomonas* biocontrol bacteria. For CLCM, bacteria and fungi were labeled using mutants of the *gfp* (green fluorescent protein) gene. The tomato root is autofluorescent. The infection process by the pathogen starts with attachment of hyphae to the root hairs (a) followed by colonization of the grooves between the junctions of the epidermal cells (b), penetration of the root cells (c) and overgrowth of the internal root (d). Upon seed germination, bacteria coated on the seed multiply, colonize the grooves between plant cells (e) and form biofilms on part of the root (f). Note that the bacteria in biofilms are covered by a mucoid layer (see g, which is a detail of f, and 1h in which the mucoid layer is broken open) which creates an ideal condition for quorum sensing and processes dependant on QS, such as F-mediated DNA transfer, and the syntheses of antibiotics and exo-enzymes. The bacteria also colonize the hyphae extensively (i).

Panels a, c, and d were reproduced from Lagopodi et al. (2002), panel b from Bolwerk et al. (2003), and panel h from Chin-A-Woeng et al. (1997). Panel e is from Bolwerk, Lagopodi and Bloemberg, *unpublished*. Panels f and g are from Bloemberg et al., (1997); Copyright © American Society for Microbiology.

multiply extensively upon germination, start to colonize the root surface, first the grooves along the junctions between root cells (**Fig. 1e**), form biofilms **(Fig. 1f)** which are covered by a mucoid layer **(Fig. 1g, h)** and eventually reach the root tip. The bacteria also colonize the fungal hyphae **(Fig. 1i)** (Lagopodi et al., 2002; Bolwerk et al., 2003). The observation that the two microbes colonize the same niche on the root, initially suggested to us that they therefore have a fair chance to interact, which would be beneficial for biocontrol. However, it could be that even smaller micro-niches are required for BCA and pathogen to meet (see section f. Competition for nutrients and niches).

Under the tested biocontrol circumstances, i. e. BCA applied on the root and pathogen mixed through the sand, the BCA reaches the root first. In addition, it colonizes the hyphae extensively (Lagopodi et al., 2002; Bolwerk et al., 2003). The

metabolic basis of the initiation of the interactions during the colonization processes was unraveled: *Pseudomonas* is chemotactically attracted to the root by root exudate components, in particular malic acid and citric acid (De Weert et al., 2003), and chemotactically attracted to the fungus by fusaric acid (De Weert et al., 2002).

Detailed colonization studies suggest that each BCA is characterized by a more or less specific colonization pattern and mode of interaction with pathogens and plant hosts (Zachow et al., 2010; Compant et al., 2011).

Competitive root colonization by biocontrol microbes

Since root colonization is the delivery system of beneficial microbes and their products, effective biocontrol microbes should be rhizosphere competent. This has been proven for the mechanisms antibiosis (Chin-A-Woeng et al., 2000) and competition for nutrients and niches (Kamilova et al., 2005; Validov et al., 2007). For the mechanism ISR it seems to be sufficient that the microbe is present on part of the root although full root colonization provides better protection (Dekkers et al., 2000).

In order to identify traits involved in root colonization, a gnotobiotic competitive tomato root colonization system was developed in which bacteria from two different strains are applied on the seed and, upon germination, compete for nutrients by moving chemotactically towards the root tip. The ratio in which the microbes were found on the root tip was used as the criterion for effective competitive root colonization (Simons et al., 1996). This system was not only used for comparison of the competitive root colonization abilities of wild type strains, but also for the screening for competitive colonization mutants. After complementation analysis and after confirmation of the colonization defect of the putative mutants in a soil system, traits playing a role in competitive root colonization were identified. These traits include phase variation, motility, adhesion to the root, utilization of organic acids from exudate, the syntheses of amino acids, nucleotides, uracil, vitamin B1, and the LPS O-antigenic side chain, and the TTSS (Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001; Lugtenberg and Bloemberg, 2004; Lugtenberg and Kamilova, 2009). In the following we will discuss some important traits in detail, namely chemotaxis of the BCA towards the root, utilization of root exudate nutrients, and the role of TTSS in competitive colonization.

Not surprisingly, it turned out that not motility in general but chemotaxis towards specific root exudate components, especially malic acid and citric acid, is crucial for

effective competitive tomato root colonization by *Pseudomonas* (De Weert et al., 2003).

In an early stage of the colonization research fast growth on root exudate components was shown to be important. Consistent with this notion was the observation that mutants impaired in the utilization of the major group of exudate nutrients, organic acids, were impaired in competitive root colonization whereas mutants impaired in the utilization of sugars, which are present is lower amounts in tomato root exudate, showed practically normal behavior (Simons et al., 1997; Lugtenberg et al., 1999).

Since mutants impaired in their TTSS are poor in competition with the parental strain for root colonization, it was concluded that type three secretion plays a role in competitive root colonization. Since the presence of the parental cells did not compensate the colonization defect of the mutants, it was suggested that the needle of the TTSS in wild type cells was not used to release nutrients from the plant cells into the environment because that would have resulted in phenotypic complementation. Rather, the presence of the needle gives the wild type a competitive growth advantage. Apparently, the needle was used to tap nutrients from the plant cell directly into the bacterium. Based on this result it was hypothesized that early in evolution the TTSS needle was developed to give the bacterial cell access to nutrients present in the plant cell and that the system later evolved to inject bacterial molecules into the plant cell (De Weert et al., 2007).

Antibiotics and biocontrol

Up to one third of rhizosphere bacteria produce AFMs and therefore may play a role in the control of diseases caused by fungi (Opelt et al., 2007). This has to be confirmed by mutational analysis followed by complementation studies. The best known antibiotics involved in biocontrol by Gram-negative bacteria are Phl, phenazines, pyoluteorin, pyrrolnitrin and the volatile HCN. The possible modes of action of several of these antibiotics are discussed by Haas and Défago (2005). Some bacilli can produce zwittermycin A (Emmert et al., 2004) and kanosamine (Milner et al., 1996). More recently, BCAs were discovered which produce the antibiotics D-gluconic acid (Kaur et al., 2006), 2-hexyl-5-propyl resorcinol (Cazorla et al., 2006) and the volatiles 2,3-butanediol (Ryu et al., 2003), 6-pentyl-α-pyrone (Lorito et al., 2010) and DMDS (Dandurishvili et al., 2011). The role of the volatile HCN in biocontrol has been known

for a long time (Haas and Défago, 2005) and it was recently discovered that also other volatiles can play a role in biocontrol (Ryu et al., 2003; Dandurishvili et al., 2011).

A class of antibiotics which was studied in great detail during the last decade is that of the c-LPs. These compounds are produced by several bacterial species, including *Bacillus* (Borriss, 2011; Chen et al., 2009; Ongena et al., 2007; Romero et al., 2007) and *Pseudomonas* (Raaijmakers et al., 2006; Raaijmakers et al., 2010). *Bacillus* c-LPs belong to three major families, the iturins (bacillomycins, iturins and mycosubtilins), the fengycins (plipastatins) and the surfactins (bamylocin A, esperins, lichenysins, pumilacidins and surfactins). These c-LPs are composed of seven (iturins and surfactins) or ten (fengycins) amino acids of both D- and L-configuration which form a ring linked to either a β-hydroxy (fengycins and surfactins) or a β-amino (iturins) fatty acid. Both the peptide moiety and the fatty acyl chain are essential for the biological functions of c-LPs (Jacques, 2011). All three major families of cLPs are key effector molecules of biological control. The mechanism of their beneficial action is based on direct antibiosis of phytopathogens and/or triggering ISR (Borriss 2011; Raaijmakers et al., 2010; Pérez-García et al., 2011). Iturins and fengycins are originally known for their strong antifungal activity against a wide range of phytopathogens while surfactins are mostly antibacterial (Ongena and Jacques, 2008). Recently Zeriouh et al. (2011) provided strong evidence for a major role of iturins in inhibition of the Gram-negative bacterial phytopathogens *Xanthomonas campestris* and *Pectobacterium carotovorum*. This is an interesting finding since the antibacterial activity of iturins was initially thought to be restricted to only a few Gram-positive species (Besson et al., 1978). Several mutational analysis studies have shown a role of iturins in biocontrol of both fungal and bacterial phytopathogens (Leclère et al., 2005; Arrebola et al., 2010; Zeriouh et al., 2011). Touré et al (2004) presented strong evidence for the involvement of fengycins in biocontrol of *Botrycis cinerea* on apple. They detected fengycins in infected tissues in inhibitory concentrations. Using mutational analysis, Yánez-Mendizábal et al (2011) showed a major role for fengycins in suppression of peach brown rot. Surfactins are very effective against *Pseudomonas syringae* on *Arabidopsis* plants (Bais et al. 2004). Fengycins and surfactins trigger defense pathways in bean and tomato plants (Ongena et al., 2007; Henry et al., 2011). Furthermore, when different families of c-LPs are co-produced they can interact in a synergistic manner resulting in more effective plant protection (Ongena et al., 2007; Romero et al., 2007). c-LPs and particularly surfactins are not only directly responsible for biocontrol, they are also

involved in motility and biofilm formation (Bais et al., 2004) and in cell differentiation and cannibalism (López et al., 2009).

Mechanisms of biocontrol

For major reviews about biocontrol and its mechanisms the reader is referred to **Table 1**. An overview of the microbes most used for biocontrol of root diseases, their traits and mechanisms of action is presented in **Table 2**.

1. Antibiosis

Since antibiotic-producing bacteria occur frequently, are easy to isolate, and are interesting for molecular studies on biosynthesis and regulation, they are the best known class of BCAs. The production of antibiotics is very dependent on environmental conditions such as temperature, pH and the levels of various metal ions, particularly of Zn^{2+} (Duffy and Défago, 1999; van Rij et al., 2004). Tripartite interactions and signaling among plants, pathogens, and bacteria is involved in the regulation of antifungal traits of *Pseudomonas* (Jousset et al., 2011) Moreover, the effect of environmental conditions is strain-dependent (van Rij et al., 2004). Therefore, and because efficient colonization is required for antibiosis (Chin-A-Woeng et al., 2000; Dekkers et al., 2000), it is not surprising that some strains which show anti-fungal activity on plates, do not act as biocontrol agents *in vivo*. The identification and quantification of the antibiotics which are produced during biocontrol *in situ* is a challenge and has been shown only for a few cases (Tomashow and Weller, 1996).

The slow growth rate of bacteria in the rhizosphere favors the production of secondary metabolites (Haas and Défago, 2005). It is also very likely that the presence of bacterial biofilms under a mucoid layer (**Fig. 1 g,h**) is favorable for quorum sensing (Chin-A-Woeng et al., 1997), a prerequisite for the production of many antibiotics.

A risk of using an antibiotic-producing BCA in practice is that cross-resistance can occur with antibiotics used in human or animal practice. Another risk is that genes encoding the antibiotic production ability can be transferred to related strains (Zhang et al., 1993). This is a realistic possibility since some forms of conjugative transfer require quorum sensing which requires a high density of microbes. This is the case on the root where pseudomonads form micro colonies under a mucoid layer **(Fig 1g,h)** (Chin-A-Woeng et al., 1997). Indeed, it has been shown by van Elsas et al. (1988) that genetic material is exchanged at a high frequency in the rhizosphere. These facts form

Table 1. Major reviews about biocontrol and its mechanisms

Topic	References
Biocontrol general	Schroth and Hancock, 1982; Compant et al., 2005; Haas and Défago, 2005; Berg, 2009; Lugtenberg and Kamilova, 2009; Mendes et al., 2011
Biocontrol by Bacillus	Raaijmakers et al., 2006; Borriss, 2011; Pérez-Garcia et al., 2011
Biocontrol by Pseudomonas	Haas and Défago, 2005; Raaijmakers et al., 2006; Validov, 2007; Pliego et al., 2011
Biocontrol by Trichoderma	Harman et al., 2004 ; Lorito et al., 2010
Antibiosis	Thomashow and Weller, 1996; Opelt et al., 2007
CNN	Lugtenberg and Kamilova, 2009; Pliego et al., 2011
Ferric iron ion acquisition	Leong, 1986
Induced systemic resistance	Van Loon, 2007; Van Wees et al., 2008
Predation and parasitism	Harman et al., 2004; Lorito et al., 2010
Root colonization	Chin-A-Woeng et al., 1997; Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001; Bolwerk et al., 2003; De Weert et al., 2007

Table 2. Major microbes used for biocontrol, their traits and mechanisms of action

A. Bacillus	
Traits / mechanisms of action	**References**
Root colonization	Fan et al., 2011
Antibiosis	Romero et al., 2007 ; Ongena and Jacques, 2008 ; Chen et al., 2009 ; Raaijmakers et al., 2010; Borriss, 2011
Induced systemic resistance	Kloepper et al., 2004; Ongena et al., 2007
Signal interference	Dong et al., 2004
B. Trichoderma	
Root colonization	Harman et al., 2004; Harman, 2006
Antibiosis	Lorito et al., 2010
CNN	Lorito et al., 2010
Induced systemic resistance	Lorito et al., 2010
Predation and parasitism	Lorito et al., 2010
C. Pseudomonas and some other Gram-negatives	
Traits / mechanisms of action	**References**
Root colonization	Simons et al., 1996; Lugtenberg et al., 2001; Lagopodi et al., 2002; Berg, 2009; Lugtenberg and Kamilova, 2009
Antibiosis	Thomashow and Weller, 1996; Chin-A-Woeng et al., 1998; Haas and Défago, 2005; Compant et al., 2005; Cazorla et al., 2006; Raaijmakers et al., 2010; Egamberdieva et al., 2011
Predation and parasitism	Ordentlich et al., 1998
Induced systemic resistance	Audenaert et al., 2002; Iavicoli et al., 2003; Shuhegger et al., 2006; Van Wees et al., 2008
Competition for Nutrients and Niches	Kamilova et al., 2005; Pliego et al., 2007; Validov, 2007
Colonization of hyphae	Bolwerk et al., 2003; De Weert et al., 2003
Ferric iron ion acquisition	Kloepper et al., 1980; Leong, 1986

risks for human health and represent reasons why registration of products based on antibiotic-producing microbes is difficult.

2. Signal interference

Several pathogens perform their action by hydrolyzing the cell walls of cells of their target plant. The production of many exo-enzymes is regulated by quorum sensing. One way to control exo-enzymes of pathogens is to inactivate the AHL molecule required for exo-enzyme production. This mechanism has been designated as signal interference (Dong et al., 2004). Two classes of AHL-inactivating enzymes have been identified, namely AHL-lactonases which hydrolyse the lactone ring, and AHL-acylases which break the amide linkage. For a review on these two enzymes, on AHL modifying enzymes, and on abiotic factors influencing the stability of AHLs, the reader is referred to Uroz et al. (2009).

In the pathosystem *Verticillium dahliae*-oilseed rape, the essential role of AHL-mediated signaling for disease suppression, including production of AFMs and VOCs, in *Serratia plymuthica* HRO C48 was demonstrated (Müller et al., 2009). Dandurishvili et al. (2011) reported that VOCs produced by rhizospheric strains *P.fluorescens* B-4117 and *S. plymuthica* IC1270 might be involved in the suppression of crown gall disease in tomato plants caused by *Agrobacterium*. Recently, Chernin et al. (2011) showed that VOCs emitted by cells of these strains, as well as the pure volatile DMDS, can cause significant suppression of transcription of AHL synthase genes *phzI* and *csaI*. Since AHLs play a role in conjugational transfer of *A. tumefaciens* Ti plasmids to the plant (Zhang et al., 1994), which is an essential step in crown gall formation, the volatile DMDS may control crown gall disease through signal interference.

3. Predation and parasitism

Since the cell walls of many fungi contain chitin, ß-1,3 glucan and protein, BCAs which produce exo-enzymes which degrade these compounds, alone or in combination, are often successful in killing the pathogen. This biocontrol mechanism is called P&P. It is used by some strains of *Trichoderma* (Harman et al., 2004) and *Serratia marscescens* (Ordentlich et al., 1998).

4. Induced systemic resistance

ISR is a broad spectrum plant immune response that is activated by some plant-beneficial bacteria that live on plant roots (Kloepper et al., 2004; van Wees et al., 2008;

Pieterse et al., 2009), such as *P. fluorescens* strains WCS417R (van Loon and Bakker, 2003; van Wees et al., 1997) and WCS365 (Kamilova et al., 2005). Immunized plants become potentiated to mobilize infection-induced defense responses faster and stronger after pathogen or insect attack, resulting in an enhanced level of protection. ISR microbes induce resistance systemically, i.e. also in distant plant parts such as leaves (Van Peer et al., 1991; Wei et al., 1991). The outcome of ISR can be a broad range of protection but it is also somewhat unpredictable. ISR can protect the plant against several pathogenic bacteria, fungi and viruses (van Loon et al., 1998; van Loon, 2007). The success of ISR-inducing strains depends on the plant species and cultivar (van Loon and Bakker, 2003; van Wees et al., 1997). The hormones jasmonic acid and ethylene are key regulators of ISR (van Wees et al., 2000). It was suggested that ISR resembles innate immunity and uses Toll like receptors (de Weert et al., 2007).

ISR does not require complete root colonization persé as was shown using competitive colonization mutants (Dekkers et al., 2000). In addition to live microbes, such as *Bacillus*, *Pseudomonas* and *Trichoderma*, ISR can be triggered by dead microbes and even by bacterial molecules and organelles such as siderophores, lipopolysaccharides, flagella, salicylic acid, the combination of pyocyanin and pyochelin (Audenaert et al., 2002), the volatile 2,3-butanediol (Ryu et al., 2003), the signal molecule AHL (Schuhegger et al., 2006), the antibiotic phloroglucinol (Iavicoli et al., 2003) and some c-LPs (Ongena et al., 2007; Pérez-García et al., 2011)

5. Competition for ferric iron ions

All organisms need Fe^{3+} for growth. Under conditions of Fe^{3+}-limitation, many bacteria secrete Fe^{3+}-chelating compounds, called siderophores. The siderophore-Fe^{3+} complex is subsequently bound to Fe^{3+}-limitation-inducible outer membrane protein receptors and the Fe^{3+} ion is transported into the bacterial cell, in which it becomes biologically active as Fe^{2+}. An example of a siderophore is pyoverdin or pseudobactin, the pigment responsible for the fluorescence of fluorescent pseudomonads. Fe^{3+} is poorly soluble under aerobic conditions at neutral and alkaline pH. Some bacteria produce siderophores which are sufficiently strong to bind Fe^{3+} to the extent that fungi in their neighbourhood cannot grow anymore under iron limitation and siderophore-producing bacteria can then act as biocontrol agents (Leong, 1986), as examplified by the control of *Erwinia carotovora* by *P. fluorescens* strains (Kloepper et al., 1980).

6. Competition for nutrients and niches

Kamilova et al. (2005) showed that CNN is a mechanism for biocontrol. They selected enhanced root tip colonizers from a crude mixture of rhizosphere bacteria. Approximately half of the selected enhanced colonizers appeared to be able to control TFRR caused by *Forl*. They showed that such strains out compete other microbes in competition for exudate nutrients and in competition for niches on the root (Kamilova et al., 2005; Validov et al., 2007). The observation that not all enhanced colonizers are BCAs can be explained by a discovery of Pliego et al. (2008) who found that two very similar *Pseudomonas* strains, selected for their efficient colonizing abilities, colonized different micro-niches on the root. This difference was used as an explanation why one strain is able to control the disease avocado white root rot whereas the other strain could not.

Bacteria controlling disease using CNN as a mechanism have several advantages. (i). CNN is the only mechanism for which strains can be selected. So, such strains can be isolated from a soil, a plant and a climate of preference. (ii). Most CNN strains do not produce antibiotics which is an advantage for registration since regulatory authorities do not like the introduction of antibiotic-producing strains in the environment. (iii). In case antibiotic production is considered to be an advantage, strains can be selected which use a combination of CNN and antibiosis as mechanisms (Pliego et al., 2007). (iv). Resistance against BCAs using CNN as their biocontrol mechanism is hard to imagine. The same applies for biocontrol strains which use both CNN and antibiosis as mechanisms since pathogens resistant to one mechanism can be controlled by the other mechanism.

7. Interference with activity, survival, multiplication, germination, sporulation and spreading of the pathogen

Studies with biocontrol strain *P. fluorescens* WCS365 have shown that this strain shows a series of activities which contribute to control of TFRR. (i). Cells of the strain are attracted to FA secreted by the hyphae. Subsequently they colonize the hyphal surface of the pathogen extensively, resulting in the formation of micro colonies or biofilms **(Fig. 1i)** (de Weert et al. 2003). This is probably the first step in an attempt to use the fungus as a food source. It is likely that colonization of hyphae makes the fungus less virulent, inhibits its activity and is detrimental for its survival and multiplication**.** (ii). Microconidia of *Forl* germinate in tomato root exudate. Germination is inhibited by biocontrol strain *P. fluorescens* WCS365, presumably because of nutrient deprivation.

(iii). When hyphae are grown in tomato root exudate, microconidia are formed. These are spores that can spread the pathogen through the environment. The presence of WCS365 reduces spore formation and therefore reduces pathogen spread (Kamilova et al., 2008). In conclusion, *P. fluorescens* WCS365 bacteria inhibit activity, survival, multiplication, germination, sporulation and spreading of the pathogen. We have not studied other bacteria or BCAs on these traits, which therefore may not be unique for *P. fluorescens* WCS365.

Resistance towards biocontrol microbes

Several mechanisms of resistance towards BCAs have been discovered in fungi (Duffy et al., 2003) which resemble resistance mechanisms used by bacteria against antibiotics. (i). Inhibition of antibiotic production. The secondary metabolite FA, secreted by many Fusarium strains (Notz et al., 2002), previously shown to be a chemoattractant for biocontrol strain *P. fluorescens* WCS365 (De Weert et al., 2003), inhibits the synthesis of Phl in the biocontrol bacterium *P. fluorescens* CHA0 by repression of the *phlA* promoter (Duffy and Défago, 1997). FA also inhibits the synthesis of another antibiotic, PCN, in another biocontrol bacterium, namely *P. chlororaphis* PCL1391. In this case a different inhibition mechanism is used, namely at or before the level of AHL production (van Rij et al., 2005). Note that AHL is required for the synthesis of PCN but not for that of Phl. (ii). Detoxification of the antibiotic. Between 18 and 25 percent of the isolated *Fusarium* strains were tolerant to Phl. Deacetylation of the antibiotic to the mono-acetyl form is the major mechanism of action (Schouten et al., 2004). Another form of detoxification is acetylation, which is used by biocontrol strain *Bacillus subtilis* strain UW85, the producer of the antibiotic zwittermycin A (Milner et al., 1996). (iii). The presence of phenazine induces an efflux pump for this compound in *Botrytis cinerea.* Mutants lacking the pump are more sensitive to the antibiotic (Schoonbeek et al., 2002).

In order to avoid resistance in biocontrol, it is preferable to use a BCA which uses more than one mechanism. Alternatively, a combination of BCAs with different mechanisms of action can be used. If a pathogen is resistant to one mechanism it can still be inactivated by a second one. Suitable microbes would for example be *Trichoderma* spp. (Lorito et al., 2010), which use at least mechanisms a, c and d (see section Mechanisms of biocontrol), and *P. fluorescens* WCS365, which uses at least mechanisms d, e, f and g, and some bacilli, which use mechanisms a and d. A summary of processes in which signal molecules and nutrients play a role in the rhizosphere

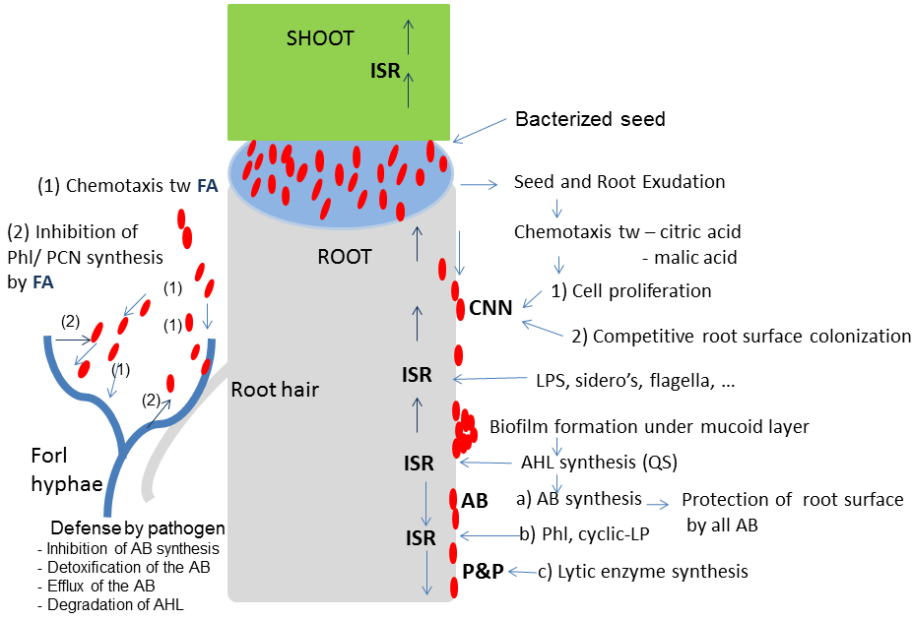

Fig. 2. Nutrients and molecules involved in biocontrol of TFRR. Cells of a hypothetical biocontrol bacterium applied on the seed proliferate on nutrients from seed exudate. Subsequently they are attracted to the root by citric acid and malic acid from root exudate and successfully compete for root exudate nutrients and niches in case the mechanism is CNN. Specific cell surface components and secondary metabolites of the BCA can induce the mechanism ISR. Upon formation of biofilms, the resulting quorum results in AHL synthesis. AHL in turn leads to synthesis of antibiotics, some of which also cause ISR, and of exo-enzymes which are required for the mechanism P&P. When the pathogen *Forl* arrives close to the root, cells of a BCA can be chemo-attracted to FA secreted by the hyphae, and subsequently colonize the hyphae. Whether syntheses of the AFFs phenazine and Phl are inhibited by FA or whether the cells of the BCA damage or kill the hyphae will depend on timing and concentrations of the metabolites and organisms and on whether the fungus is resistant and, if so, by which mechanism. Additional abbreviations: AB, antibiotic; LP, lipopeptide.

during biocontrol is shown in **Fig. 2**.

Conclusions

Phytopathogenic fungi and oomycetes cause enormous crop losses. Presently, chemical agents are the major way of disease control but they have the disadvantages that i) many of them are detrimental for health and environment, and ii) that resistance occurs rather fast. In this review, we discuss that products which contain natural microbial enemies of these pathogens are a realistic alternative and addition to chemical pesticides. The quality of these BCAs can be further increased by using fundamental knowledge to improve methods for their production and to increase their shelf life. In addition, the fast development of very advanced techniques in microbial

ecology and a focus on mechanisms of actions make improvement of strain selection feasible.

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Microb Biotechnol 2011 (4): 523-532

Characterization of *Bacillus subtilis* HC8, a novel plant-beneficial endophytic strain from giant hogweed

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Abstract

Thirty endophytic bacteria were isolated from various plant species growing near Saint-Petersburg, Russia. Based on a screening for various traits, including plant-beneficial properties and DNA fragment patterns, potential siblings were removed. The remaining isolates were taxonomically identified using 16S rDNA sequences and potential human and plant pathogens were removed. The remaining strains were tested for their ability to promote radish root growth and to protect tomato plants against tomato foot and root rot (TFRR). One strain, *Bacillus subtilis* HC8, isolated from the giant hogweed *Heracleum sosnowskyi* Manden, significantly promoted plant growth and protected tomato against TFRR. Metabolites possibly responsible for these plant-beneficial properties were identified as the hormone gibberellin and (lipo)peptide antibiotics, respectively. The antibiotic properties of strain HC8 are similar to those of the commercially available plant-beneficial strain *B. amyloliquefaciens* FZB42. However, thin layer chromatography profiles of the two strains differ. It is speculated that endophytes such as *B. subtilis* HC8 contribute to the fast growth of giant hogweed.

Introduction

Bacteria which associate with plants include rhizobacteria, epiphytic bacteria and endophytic bacteria. Endophytic bacteria are defined as bacteria that can be detected within the tissues of apparently healthy plants (Schulz and Boyle, 2006). Although the majority of research on plant-associated bacteria has been focused on rhizobacteria, interest in the diversity and role of endophytic bacteria is increasing. The main reason for the interest in endophytes is the realization that, if these bacteria can be re-introduced in the endophytic stage, a more stable relationship can be established between plant-beneficial endophytic bacteria and plants than for rhizospheric or epiphytic bacteria and plants. Therefore, endophytes with the plant-beneficial traits are potentially excellent plant growth promoters and/or biological control agents for sustainable crop production (Di Fiore and Del Gallo, 1995; Strobel, 2006).

The best studied host plants of bacterial endophytes are species of agricultural importance, such as rice (Baldani et al., 2000; Okunishi et al., 2005), maize (McInroy and Kloepper, 1995; Rijavec et al., 2007), cotton (Misaghi and Donndelinger 1990; McInroy and Kloepper, 1995), potato (Sturz et al., 1998; Krechel et al., 2002), and sugar cane (Rennie et al., 1982; James and Olivares, 1997). The most common taxa of isolated heterotrophic endophytes include *Bacillus* (Bai et al., 2003), *Enterobacter* (Torres et al., 2008), *Pseudomonas* (Reiter et al., 2003; Rai et al., 2007), *Serratia* (Gyaneshwar et al., 2001; Berg et al., 2005), and *Streptomyces* (Sessitsch et al., 2002; Coombs and Franco, 2003).

It is assumed that bacterial endophytes use the same mechanisms of biological control and plant growth promotion as their rhizospheric counterparts (Berg and Hallmann, 2006). Widely recognized mechanisms of biocontrol mediated by plant growth-promoting microbes are antibiosis (Thomashow and Weller, 1995; Chin-A-Woeng et al., 1998; Haas and Défago, 2005; Lugtenberg and Kamilova, 2009), induced systemic resistance (Van Peer et al., 1991; Kloepper et al., 2004; Van Loon, 2007), competition for niches and nutrients (Kamilova et al., 2005; Validov, 2007) and predation and parasitism (Ordentlich et al., 1998; Harman et al., 2004).

Beneficial bacterial endophytes which use the above-mentioned mechanisms of biocontrol include (i) *Bacillus* sp. CY22 which produces the antibiotic iturin A and suppresses root rot of balloon flower caused by *Rhizoctonia solani* (Cho et al., 2003), (ii) *B. pumilus* SE 34 which induces systemic resistance against *Fusarium* wilt of tomato (Benhamou et al., 1998), and (iii) *P. fluorescens,* carrying the chitinase-encoding gene *chi*A, which is able to control the phytopathogenic fungus *Rhizoctonia solani* on bean

seedlings (Downing and Thomson, 2000). In addition to protecting against pathogens, a number of endophytic bacteria is supposed to promote plant growth directly by the production and/or modulation of plant hormones (Bastian et al., 1998; Spaepen et al., 2009), by fixing atmospheric nitrogen (Baldani et al., 2000; Oliveira et al., 2002) and by solubilization of bound phosphates (Verma et al., 2001; Kuklinsky-Sobral et al. 2004). Using these mechanisms, some endophytic bacteria can significantly contribute to the growth of plants on low-fertility soils (Sevilla et al., 2001).

The main aims of this study are: (i) to collect different endophytic bacteria from different plants, (ii) to screen these bacteria for a number of plant-beneficial traits, such as secretion of the exo-enzymes chitinase, cellulase, β-glucanase and protease, and production of hormones and antifungal metabolites, (iii) to test the selected potentially beneficial strains for their abilities to promote growth of radish and to control tomato foot and root rot (TFRR) caused by the fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*), and (iv) to evaluate the putative compounds responsible for the plant growth promotion and antifungal activities of (a) selected endophytic strain(s). The results are reported in this paper.

Materials and Methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from several plant species. These include four vegetable plants [beet (*Beta vulgaris* L.), carrot (*Daucus carota* L.), potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* L.)], two grasses [maize (*Zea mays* L.) and millet (*Panicum miliaceum*)], and the weed plant *Heracleum sosnowskyi* Manden. Plants were collected from experimental fields of the All-Russia Research Institute for Agricultural Microbiology (ARRIAM) which is located near Pushkin, Saint-Petersburg, Russia.

To isolate endophytes, different surface sterilization procedures were developed (see Results) which are modifications of previously published ones (Misaghi and Donndelinger, 1990). Briefly, plant samples were disinfected and subsequently crushed with a pestle in a mortar under sterile conditions. Aliquots of 100 µl of the resulting plant juices were plated on 1/20 tryptic soy agar (TSA, Difco Laboratories, MI, USA) plates. The sterility check consisted of aliquots of water from the last rinsing which were plated on 1/20 TSA. Plates were incubated at 28°C for 3 days. Colonies derived from plant juice were further analyzed.

Microbial strains and growth conditions

All isolated bacterial strains were grown in, and maintained on, full strength TSA. Strain *Bacillus amyloliquefaciens* FZB42 (Idriss et al., 2002), and its mutants AK1 and AK2 (Koumoutsi et al., 2004) were purchased from the *Bacillus* Genetic Stock Center (BGSC, http://www.bgsc.org/). Strain FZB42 was used for comparison studies as a known antibiotic-producing *Bacillus* strain. This strain is also commercialized as a biofertilizer, biocontrol and plant-growth promoting agent (RhizoVital®, ABiTEP, Berlin, Germany). Its two mutants, AK1 (Δ*bmy*A, defective in the production of bacillomycin D) and AK2 (Δ*fen*A, defective in the production of fengycin), were used to attempt to localize these antibiotics on TLC plates.

The fungi *Aspergillus niger, Forl*, *F. solani* and the oomycete *Pythium ultimum* were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories). To obtain spores of *Forl* to be used in biocontrol experiments, the fungus was grown in Czapek-Dox liquid medium (Difco Laboratories) for 4 days at 28°C at 150 rpm.

For the extraction of antibiotics and gibberellins, strains were grown in Brain Heart Infusion broth (BHI, Difco Laboratories, MI, USA). To extract cytokinins and to check the ability of strains to sulubilize phosphates, bacteria were grown in minimal medium (MM) containing per liter of distilled water: NH_4Cl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; glucose, 10 g; yeast extract, 50 mg, and agar, 18 g. For the evaluation of ACC (1-aminocyclopropane-1-carboxylate) utilization, bacteria were grown in sucrose-malt extract-yeast extract medium (SMY) which has the following composition (weight/L): glucose, 1.2 g; KH_2PO_4, 0.4 g; K_2HPO_4, 2.0 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; $FeSO_4$, 5.0 mg; H_3BO_3, 2 mg; $ZnSO_4$, 5.0 mg; Na_2MoO_4, 1.0 mg; $MnSO_4$, 3.0 mg; $CoSO_4$, 1.0 mg; $CuSO_4$, 1.0 mg; $NiSO_4$, 1.0 mg; yeast extract, 50 mg; pH 6.4.

Characterization of exo-enzymes produced by endophytic bacteria

Production of the exo-enzymes cellulase, chitinase, β-glucanase, and protease was judged as the appearance of clear zones around the growth of a bacterium on the following solid media. Cellulase activity was tested on 1/20 TSA agar plates supplemented with 1% carboxymethylcellulose (Hankin and Anagnostakis, 1977). Production of chitinase were evaluated on 1/20 TSA agar plates supplemented with 1% colloidal chitin (Wirth and Wolf, 1990). ß-glucanase activity was detected on 1/20 TSA agar plates supplemented with 0.1% lichenan (Walsh et al., 1995). Protease secretion was evaluated after growing the strains for 48 hours on 1/20 TSA supplemented with 5% skimmed milk according to Brown and Foster (1970).

Characterization of antifungal metabolites produced by bacteria

To test production of antifungal metabolites *in vitro,* a plug of mycelium, 5-mm in diameter, was taken from an actively growing culture on solid medium and stabbed in the center of a PDA agar plate which was subsequently inoculated with up to 6 individual bacterial strains at a distance of 3 cm from the fungus. All plates were incubated at 28°C for one week and subsequently scored for inhibition of fungal growth.

The method of Chittara et al. (2002) was used with some modifications to extract antibiotics produced by *Bacillus subtilis* HC8*.* Briefly, the strain was grown in BHI medium for 60 h. Subsequently cells were removed by centrifugation at 13 000 rpm for 10 min. The supernatant fluid was divided into two equal parts, one part (100 ml) was freeze-dried and the other was acidified to pH 2.0 with concentrated HCl. The resulting dry biomass and precipitate, respectively, were extracted twice with methanol. The methanolic extract was concentrated by vacuum evaporation, dissolved in methanol and stored at -20°C.

The methanolic extracts were analyzed by thin layer chromatography (TLC) on silica gel 60 F254 plates with a 20 x 2,5 cm concentrating zone (Merck, Darmstadt, Germany). Plates were developed in chloroform/methanol/water 65:25:4 (v/v/v) for 1.5 h at room temperature. After drying, the pattern of compounds on the developed plate was visualized using UV_{254} and stained in an iodine chamber for 5 min at room temperature followed by dipping in 1% aqueous starch. The putative antifungals were preliminarily characterized by their Retention factor (R_f) values. Pure iturin A from *B. subtilis* (Sigma-Aldrich, Steinheim, Germany) was used as a reference.

To analyze the antifungal activity of the different spots, TLC plates were run in duplicate, one was used for staining and the other one to recover the fractions by extraction. To extract the spots, silica regions were scratched off the plate and were extracted with methanol. The activity of the individual extracts was tested against *Forl* in *in vitro* assay as follows. A plug of mycelium was placed in the center of a PDA plate and pre-grown for 2 days. Subsequently six wells, 8 mm in diameter, were made in the agar plate at a distance of 1.0 cm from the growing fungus. The bottom of the wells was sealed using melted agar, and each of the wells was filled with 80µl of an individual extract. Methanol was used as a control. The plates were incubated for 2 days at 28°C and the inhibition of the *Forl* growth was judged. All experiments were carried out at least three times. To compare the activity and R_f values of the HC8 extract with those of a known antibiotic-producing strain, methanolic extracts of *B.*

amyloliquefaciens FZB42 were prepared and profiled on TLC plates as described for strain HC8.

Characterization of bacterial phytohormone production

The production of auxin (IAA, indole-3-acetic acid) was determined as described by Kamilova et al. (2005) using Salkowski reagent (Gordon and Weber, 1951). A modified method of Gutierrez-Manero et al. (2001) was used for the extraction of gibberellins from the supernatant fluid of *Bacillus subtilis* HC8. Bacteria were grown in 100 ml BHI medium for 60 h at 28°C at 150 rpm. Bacterial cells were removed by centrifugation for 15 min at 5 000 rpm and the supernatant fluid was subsequently filtered through a 0.22-µm Millipore filter. Bacteria-free supernatant was then acidified to pH 2.5 with concentrated HCl and partitioned four times with water-saturated ethyl acetate (v/v). The organic phase, containing the gibberellins, was dried by vacuum evaporation and subsequently dissolved in water-saturated ethyl acetate and stored at -20°C. A modified method of Jones and Varner (1967) was used for the evaluation of the biological activity of the crude extract. Briefly, seeds of barley cv. Triumph, 1989 harvest, were transversely cut in half and the embryo part was removed. The embryo-free halves were then surface-sterilized with 70% ethanol for 2 min followed by 4% sodium hypochlorite for 2 min and several rinses with sterile water. The disinfected half seeds were stored in sterile water at +4°C for 2 days. For gibberellin assays, 10 half seeds were transferred to a 100 ml Erlenmeyer flask with 6 ml of test solution containing: (i) 20 mM sodium succinate buffer, pH 5.3, (ii) 20 mM $CaCl_2$, and (iii) the sample to be assayed. Chloramphenicol at a final concentration 10µg/ml was added to each flask to prevent bacterial growth. After incubation for 27 h at 25°C in the dark, 1.0 ml of the solution was added to a tube containing 1.0 ml of starch reagent (Jones and Varner, 1967) and incubated for 10 min at room temperature. The reaction was stopped by adding 1.0 ml of iodine reagent (Jones and Varner, 1967). A volume of 2.0 ml of distilled water were added and, after mixing, the intensity of blue colour was measured at 620 nm. The gibberellin concentration in the crude extract was determined by using a calibration curve with pure gibberellic acid (GA_3) as a standard. The experiment was performed three times.

A modified method of Vereecke (*personal communication*) was used for the extraction of cytokinins secreted by *B. subtilis* HC8. Briefly, bacteria were grown in 50 ml MM for 96 h at 28°C and 150 rpm. Subsequently the bacterial cells were removed by centrifugation for 20 min at 10 000 rpm and subsequently filtering the supernatant

through a 0.22-µm Millipore filter. The cell free supernatant fluid was transferred to a Sep-Pak®Plus C18 column (Waters, USA), which had previously been activated with 5 ml 100% methanol and equilibrated with 0.1% acetic acid. Subsequently the cytokinins were eluted with 3 ml of 80% methanol-2% acetic acid, concentrated *in vacuo* and re-suspended in water before further use. The method of Biddington and Thomas (1973) was used for the evaluation of the biological activity of the eluate. Briefly, seeds of *Amaranthus caudatus* L. (purchased from Sluis Garden http://www.gardenseeds.nl/) were allowed to germinate on wet filter paper at 25°C in the dark for 72 h. The seed coats and the roots were subsequently removed and ten explants consisting of cotyledons and the upper part of the hypocotyl were placed on filter paper which had been moistened with 2 ml 0.2 M phosphate buffer (pH 6.3) containing 1 mg/ml tyrosine and the sample to be tested. After an incubation period of 18 h at 25°C in the dark the seedlings were placed in 1.0 ml distilled water. Betacyanin was extracted from the samples by 3 cycles of freezing and thawing and the optical density of the supernatant fluids was measured at 542 nm. The amount of cytokinins was determined by using a calibration curve with pure trans-zeatin as a standard. The experiment was performed three times.

Utilization of ACC and solubilization of bound phosphates by endophytic bacteria

The ability of bacteria to utilize ACC as the sole nitrogen source was monitored by screening for growth on plates according to Belimov et al. (2005).

The ability of bacteria to solubilize phosphates was evaluated on hydroxyapatite medium as described by Kim et al. (1997) with some modifications. Briefly, endophytic strains were grown in MM in which the phosphorus was present in the form of hydroxyapatite ($Ca_5HO_{13}P_3$, Sigma-Aldrich, Steinheim, Germany) at 12 g/L and the pH was adjusted to 7.0. Plates were incubated at 28°C for one week. Phosphorus-solubilizing activity was judged as the appearance of clear zones around the growth area of a bacterial sample spotted on the plate.

Molecular characterization of endophytic strains

Amplified ribosomal DNA restriction analysis (ARDRA) in combination with phenotypic characterization was applied to eliminate putative siblings as described by Validov et al. (2007). Briefly, portions of the 16S rRNA genes were obtained via PCR amplification with primers 27 fm (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') (Weisburg et al., 1991). The amplified DNA fragments were

subsequently digested with the four nucleases TaqI, BsuRI, HinfI and Hin6I. The resulting fragments were subsequently separated on a 2% agarose gel and the profiles of the endophytic strains were compared.

For nucleotide sequence determination, PCR products were separated on a 1% agarose gel, recovered and purified from agarose using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed by ServiceXS (Leiden, The Netherlands). Similarity searches in GenBank were performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/; Altschul et al., 1990).

Plant growth promotion

Endophytic bacteria were tested for their ability to promote the growth of radish plants. To do this, seeds of radish cv. Duro (Russkiy Ogorod – NC, Moscow, Russia) were allowed to germinate for 24 hours on moist filter paper at room temperature. The germinated seeds were then soaked in a suspension of bacterial cells in 0.85% NaCl adjusted to 10^6 cfu/ml for 15 minutes. As a negative control, seedlings were treated with 0.85% NaCl without added bacteria. The treated seedlings were subsequently planted in non-sterile potting soil (Terravita, Russia) mixed with field podsol soil in the ratio 4:1 and grown under agroindustrial conditions in the summer greenhouse of ARRIAM. Each variant consisted of four replicates with five seedlings each. After 31 days of growth, the fresh weight of the roots was determined.

Biocontrol of tomato foot and root rot

Biocontrol of TFRR was carried out in stonewool substrate as described by Validov et al. (2007). Briefly, 120 stonewool plugs were soaked in 1.0 L of commercial Plant Nutrient Solution (PNS, Wageningen UR Greenhouse Horticulture, Bleiswijk, the Netherlands) supplemented with *Forl* spores (10^7 spores/L) and bacterial cells (10^6 cfu/ml). In the negative control PNS was supplemented with spores only. Seeds of tomato cv. Carmello (Syngenta, B.V., Enkhuizen, the Netherlands) were placed in the stonewool plugs (one seed per plug) and grown for 14 days under greenhouse conditions at 80% humidity and 16 h of daylight. The plants were then removed from the stonewool and examined for symptoms of foot and root rot. Only roots without any brown spots or lesions were referred to as healthy. Dead plants, wilting plants or plants with symptoms of foot and root rot were considered as diseased. All experiments were performed twice.

Statistics

Homogeneity of variance and analysis of variance (ANOVA) at P = 0.05 were conducted with the program DIANA (Saint-Petersburg, Russia) and SPSS software (Chicago, IL, USA) for the plant-growth promotion and biocontrol assays, respectively.

Results and Discussion

Isolation and preliminary characterization of endophytic bacteria

Procedures of chemical sterilization of plant parts from different plants were developed (Table 1) to kill non-endophytic microorganisms. Validation of the surface sterilization procedure was done by culturing aliquots of water from the last rinsing onto nutrient media. Bacterial growth was never detected on such control plates, indicating the efficiency of the developed sterilization protocols.

A total of 30 morphologically different strains was chosen from a larger collection of isolates obtained after plating plant juices on 1/20 TSA. The strategy described by Validov et al. (2007) was used for the elimination of siblings and potential pathogens.

Table 1. Origin of endophytes and protocols for surface sterilization of plant samples[a]

Host plant	Part of isolation[b]	Sterilization procedure[c]
Beta vulgaris L. (beet) *Daucus carota* L. (carrot) *Lycopersicon esculentum* L. (tomato) *Solanum tuberosum* L. (potato)	Beetroot Taproot Fruit Tuber	A: 1. tap water for 30 sec 2. 70% ethanol for 5 min 3. 15% H_2O_2 for 10 min 4. sterile water 2 min ×5
Heracleum sosnowsky Manden[d] (hogweed)	Stem	B: 1. 70% ethanol for 10 min 2. 15% H_2O_2 for 15 min 3. sterile water 2 min ×5
Panicum miliaceum (millet)[d] *Zea mays* L. (maize)[d]	Stem Stem	C: 1. tap water for 30 sec 2. 70% ethanol for 7 min 3. 15% H_2O_2 for 10 min 4. sterile water 2 min ×5

[a] Plant samples were collected from experimental fields of St-Petersburg suburbs.

[b] The disinfected plant samples were crushed with a pestle in a mortar under sterile conditions.

[c] Validation of the surface sterilization procedure was done by culturing aliquots of water from the last rinsing onto nutrient medium. Bacterial growth was never detected on control plates.

[d] Plants were analyzed at the stage of flowering.

To eliminate siblings, the 30 strains were compared for their motility, their ARDRA patterns and production of the exo-enzymes chitinase, cellulase, β-glucanase and protease. Strains originating from the same sample which were indistinguishable with respect to these mentioned traits were considered as likely siblings. Eighteen isolates were removed from the collection as possible siblings. This left us with 12 strains for further analysis.

Characterization of potential plant- beneficial traits

The 12 remaining strains were screened for their antagonistic activity towards four phytopathogens, their ability to produce auxin, their growth on ACC as the sole N-source and their ability to solubilize bound phosphates (Table 2).

Three strains, namely BT18, HC8 and MZ3 show strong antifungal activity against all four tested pathogens. These strains also have cellulase, glucanase and protease activity. Strain ML15 is antagonistic only towards *P. ultimum* and does not secrete cellulases and glucanases. None of the strains showed chitinase activity.

Table 2. Overview of potential plant-beneficial traits of the selected endophytic strains[a]

Strain	Host plant	Antifungal activity[b]	Exo-enzymes[c]	Auxin[d/e]	ACC[f]/PO_4[g]
BT18	*Beta vulgaris* L. (beet)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
CAR2	*Daucus carota* L.(carrot)	-	C,βG	+++/-	-/-
HC2	*Heracleum* sp. (hogweed)	-	-	+/+	-/+
HC8	*Heracleum* sp. (hogweed)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
ML15	*Panicum miliaceum* (millet)	Pu	P	+/-	-/-
ML16	*Panicum miliaceum* (millet)	-	C,βG	++/-	-/-
TM1	*L. esculentum* L. (tomato)	-	-	+++/+	-/-
TM2	*L. esculentum* L. (tomato)	-	-	-/-	-/-
PT19	*Solanum tuberosum* L. (potato)	-	P	-/-	-/-
PT20	*Solanum tuberosum* L. (potato)	-	-	-/-	-/-
MZ3	*Zea mays* L.(maize)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
MZ4	*Zea mays* L.(maize)	-	-	-/-	-/+

[a] After elimination of siblings.

[b] A, *Aspergillus niger*; *Forl*, *Fusarium oxysporum f.sp. radicis-lycopersici*; Fs, *Fusarium solani*; Pu, *Pythium ultimum.*

[c] C, cellulase; βG, β-glucanase; P, protease.

[d] Auxin level after growth in medium supplemented with tryptophan: +++ >60 µg/ml, ++ >30 µg/ml, + >10 µg/ml, - < 10 µg/ml.

[e] Auxin level after growth in medium without tryptophan: + >10 µg/ml, - < 10 µg/ml.

[f] ACC, 1-aminocyclopropane-1-carboxylate.

[g] Solubilization of bound phosphates.

Two strains, HC2 and TM1, produce detectable amounts of auxins in the presence and absence of tryptophan in the medium. The level of auxin secreted by strain HC2 is less than 30 µg/ml. In the case of TM1, the auxin level in the media without and with tryptophan is less than 30 µg/ml and higher than 60 µg/ml, respectively. Three strains, namely CAR2, ML15, and ML16, produce different auxin levels and only in the medium supplemented with tryptophan.

None of the twelve strains was able to utilize ACC as the sole nitrogen source. However, all of them, except ML15 and TM2, showed a poor to good growth on N-free medium. Two strains, namely HC2 and MZ4, were able to solubilize hydroxyapatite in an *in vitro* plate assay.

Molecular identification of endophytic strains

BLAST searches in the GenBank database using 16S rDNA sequences revealed that the strains belong to different bacterial species (Table 3). To see whether these strains are safe to be applied in the field as bioocontrol and/or plant-growth promoting strains, we evaluated to which risk group (Anonymous, 1998) they belong. Of the twelve

Table 3. Molecular identification of endophytic strains and risk group classification[a]

Strain	Bacterial species and accession number[b]	Phylum	Risk group[c]
BT18	*Bacillus subtilis* HQ667318	Firmicutes	1
CAR2	*Enterobacter agglomerans* HQ667319	γ-Proteobacteria	2
HC2	*Rahnella aquatilis* HQ667320	γ-Proteobacteria	1
HC8	*Bacillus subtilis* HM441224	Firmicutes	1
ML15	*Bacillus cereus* HQ667321	Firmicutes	2
ML16	*Enterobacter agglomerans* HQ667322	γ-Proteobacteria	2
MZ3	*Bacillus subtilis* HQ667323	Firmicutes	1
MZ4	*Acinetobacter baumannii* HQ667324	γ-Proteobacteria	2
PT19	*Serratia sp.* HQ667325	γ-Proteobacteria	1
PT20	*Enterobacter amnigenus* HQ667326	γ-Proteobacteria	2
TM1	*Enterobacter agglomerans* HQ667327	γ-Proteobacteria	2
TM2	*Kocuria* sp. HQ667328	Actinobacteria	2

[a] Based on comparison of their 16S rDNA sequences with those in the GenBank database sharing at least 99% homology.

[b] All sequences have been submitted to GenBank. Sequences were obtained by sequencing the 5'end using primer 27fm for HC8 and the 3'end using primer R1522 for all other strains. Sequences are between 600 and 800 bp long.

[c] Risk group 1 includes bacteria which are safe to be applied in the field; risk group 2 includes potential human and plant pathogens.

remaining strains as many as seven strains belong to risk group 2 (Table 3), indicating a high percentage of potential human and/or plant pathogens among these endophytes. Therefore, they were excluded from further experiments. High levels of potential pathogens have been found earlier for rhizosphere bacteria (Berg et al., 2005; Egamberdiyeva et al., 2008).

The remaining five endophytic strains were BT18, HC8 and MZ3, identified as *Bacillus subtilis*, HC2 (*Rahnella aquatilis*), and PT19 (*Serratia* sp.). All of them have been found earlier as endophytes (Bai et al., 2003; Berg et al., 2005; Torres et al., 2008). Of these, strains BT18, HC8 and MZ3, which possess strong antifungal activity *in vitro* against *A. niger*, *Forl*, *F. solani* and *P. ultimum* as well as strain HC2, which produces auxin, can be considered as potential beneficial strains.

Plant growth promotion by B. subtilis HC8 and possible mechanism of action

Four endophytic strains, namely BT18, HC2, HC8 and MZ3 were tested for their ability to promote the growth of radish plants in non-sterile potting soil (Fig. 1). Radish was chosen as the model plant because its roots secrete a high level of tryptophan (Kamilova et al., 2006) which can be used by many beneficial bacteria as the precursor of auxin. The only tested strain which was able to increase the root weight of radish plants was *Bacillus subtilis* HC8 (Fig. 1). The root weight was chosen since this is the commercially interesting plant part. Strain HC8 significantly enhanced fresh root biomass, with as much as 46% compared with uninoculated control plants. Inoculation

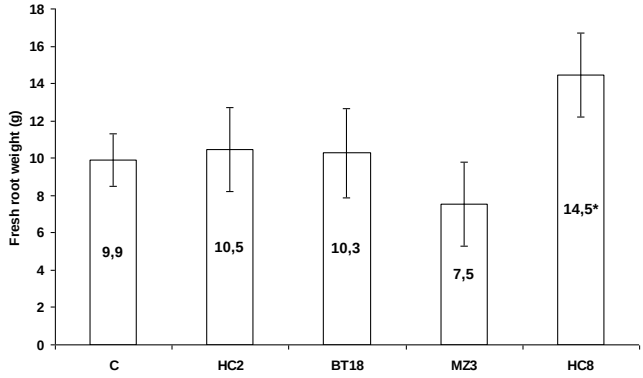

Fig. 1. Plant growth promotion mediated by endophytic bacteria. Seedlings of radish were inoculated with a suspension of bacterial cells except for the control (C) and planted in soil. Each variant consisted of four replicates with five seedlings each. Numbers inside the columns represent the mean fresh weight of the root system scored 31 days after inoculation. Bars indicate confidence interval (p = 0.05). The asterisk indicates a significantly different value.

with the auxin-producing strain *Rahnella aquatilis* HC2 and with *B. subtilis* BT18 did not show a significant increase of root growth. *B. subtilis* strain MZ3 decreased the root biomass, but not significantly.

One of the mechanisms of stimulation of plant growth by bacteria involves the production of phytohormones, such as auxins, gibberellins and cytokinins. Auxins are known to be essential for plant physiology directly affecting the root and shoot architecture (Spaepen et al., 2009). Since HC8 did not produce auxin in the tested laboratory media (Table 2) its ability to produce the plant hormones cytokinin and gibberellin was tested. Indeed, gibberellin but not cytokinin was found to be produced by HC8 (150 ng per 10^9 cells). Previously, microbial production of similar amounts of gibberellins (appr. 200 ng per 10^9 cells) has been reported for *B. lichenoformis* and *B. pumilus* (Gutierrez-Manero et al., 2001). Gibberellin is not known to enhance root growth directly (Spaepen et al., 2009). A possible explanation of the results is that gibberellin acts synergistically with another, unknown compound.

Biocontrol of TFRR by B. subtilis HC8 and possible mechanism of action

The three *B. subtilis* strains, BT18, HC8 and MZ3, were selected as the best antagonists (Table 2). Therefore, their ability to control TFRR was evaluated. Seed bacterization with only HC8 significantly decreased disease symptoms, from 91 to 42% (Fig. 2a). Significant biocontrol activity of HC8 was also found in a second experiment (Fig 2b).

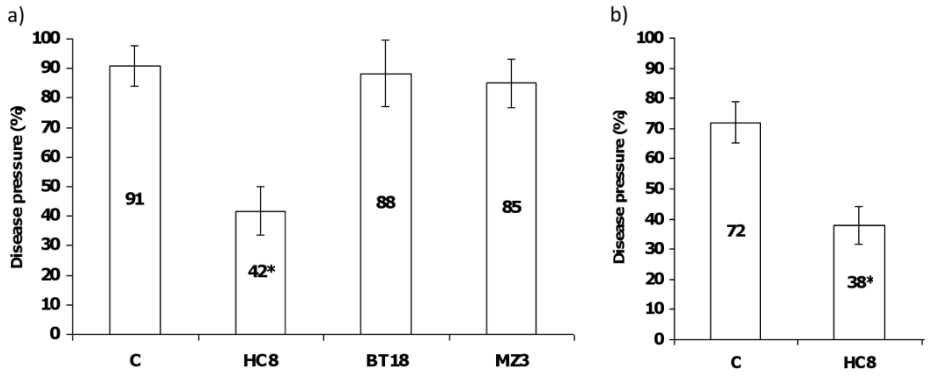

Fig. 2. Biocontrol of TFRR in stonewool substrate by endophytic bacteria. Tomato seeds were inoculated with a suspension of bacterial cells except for the control (C) and grown in stonewool plugs with added spores. Each variant consisted of 4 replicas with 30 plants each. Numbers inside the columns present the percentage of sick plants scored 2 weeks after inoculation. Bars indicate confidence interval ($p < 0.05$). Statistically different values are indicated with asterisks. (a) and (b) represent different experiments.

Although the two other antagonistic strains, MZ3 and BT18, did not show significant biocontrol of TFRR (Fig. 2a) plants bacterized with these strains did show reduced disease severity (results not shown).

For the detection of one or more compounds responsible for the antifungal activity, and therefore probably for biocontrol, the crude methanolic extracts from the dried and acid precipitated supernatant fluid of *B. subtilis* HC8 were profiled on thin layer chromatography (TLC) plates, using iturin A as a reference antibiotic (Fig. 3a). We have also profiled *B. amyloliquefaciens* FZB42 to evaluate the similarity/difference between two beneficial strains.

The iodine-starch pattern of supernatant fluids of FZB42 and HC8 are very similar. Dried supernatant fluids and acid precipitated supernatant fluids had indistinguishable patterns (results not shown). We found for both HC8 and FZB42 major spots in positions ***t***, ***u***, ***w***, ***x*** and ***y***. The R_f values of these spots shown in Fig. 3a are ***t***, 0.10; ***u***, 0.16; ***w***, 0.21; ***x***, 0.23; and ***y***, 0.26. Although the two strains produce very similar antibiotic patterns, there are also clear differences, not only in taxonomy. Spot ***v*** (R_f = 0.18) is present in HC8 but always missing in FZB42. In addition, FZB42 lacks spot ***z*** with the R_f value similar to that of iturin A (Rf_z=0.47). Spot ***s*** (Rf=0.31) of strain FZB42 is not visible in HC8 material. To test which spots are active against *Forl*, we extracted the whole HC8 and FZB42 strips (major and minor spots as well as the regions without visible spots) and checked their antibiotic activity against *Forl in vitro* (Fig. 3b). Also for biological activity dried supernatant fluids and acid precipitated supernatant fluids have indistinguishable patterns. We found for both HC8 and FZB42 that four spots, namely ***t***, ***u*** (in case of HC8 it was ***u***/***v*** since spot ***v*** sometimes migrates very close to spot ***u***, which makes it difficult to analyze them separately), ***w*** and ***x***, clearly inhibit the growth of *Forl*. Spot ***y*** does not show any antibiotic activity. Interestingly, both spot ***z*** of HC8 and spot ***s*** of FZB42 had very low antibiotic activities when re-extracted from TLC plate.

Based on R_f values and activity against *Forl*, spot ***z*** could be iturin. We used mutant strains of FZB42 which do not produce bacillomycin D and fengycin, to see which FZB42 spots, and possibly HC8 spots, correspond with these antibiotics. The results (Fig 3a) showed that the fengycin-deficient mutant lacks two spots, ***t*** and ***w***, both of which are present in both HC8 and FZB42. These spots are also still present in the fengycin producing mutant strain Δ*bmy*A indicating that compounds in positions ***t*** and ***w*** represent (derivatives of) fengycin. The bacillomycin D lacking mutant Δ*bmy*A does

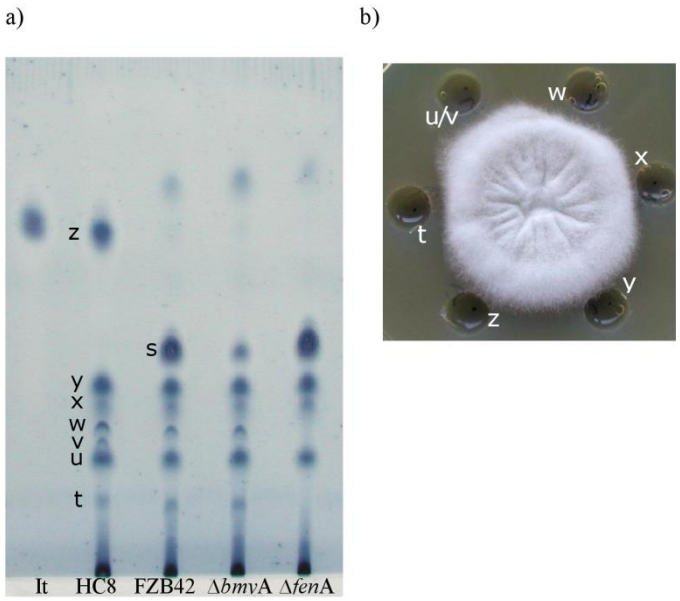

Fig. 3. Evaluation of antifungal metabolites produced by *B. subtilis* HC8 and B. *amyloliquefaciens* FZB42.
a) TLC analysis of methanol extract of the supernatant fluids of *Bacillus* strains. The plate was developed in chloroform/methanol/water 65:25:4 (v/v/v) for 2,5 hours. For visualization, the developed plate was stained in iodine followed by dipping in 1% aqueous starch. Pure iturin A (It) was used as a reference. HC8, endophytic strain *B. subtilis* HC8; FZB42, *Bacillus amyloliquefaciens* FZB42; Δ*bmy*A, mutant of FZB42 unable to produce bacillomycin D; Δ*fen*A, mutant of FZB42 unable to produce fengycin; ***t*-*z***, major spots of the HC8 crude extract; ***z***, likely correspond to iturin; ***s***, fraction likely to contain bacillomycin D; ***w*** and ***t*** likely to contain fengycin.
b) Antifungal activity of individual fractions of crude extract from *B. subtilis* HC8 towards *Forl in vitro.* ***t*-*z***, major fractions corresponding to spots in a).

produce spot ***s*** but in lower amounts than its wild type strain FZB42, therefore this spot probably contains bacillomycin D. No information on the identity of spots ***u***, ***x***, and ***y*** from HC8 was generated.

The antibiotics iturin and bacillomycin D belong to the same family of cyclic lipopeptides which comprises iturins A, C, D and E, bacillomycin D, F and L, bacillopeptin and mycosubtilin (Moyne et al., 2004). Iturins interact with the cytoplasmic membrane of the target cells forming ion-conducting pores (Magnet-Dana and Peypoux, 1994). These antifungals appeared to work synergistically with other lipopeptides, such as surfactins and fengycins. For example, Chen et al. (2009) report that the fungicidal activity of FZB42 is due to synergistic action of bacillomycin D and fengycin since without fengycin the antifungal effect of this strain is less profound. This

may explain why the iturin and bacillomycin D fractions almost lack biological activity in our experiments.

Taking together all these data suggest that *B. subtilis* HC8 produces several (lipo)peptide antibiotics, some of them are different from FZB42 and may be important for antifungal and biocontrol activity of HC8.

Do endophytes play a role in the growth of the giant hogweed?

In this study we have isolated the novel biocontrol and plant growth promoting strain *B. subtilis* HC8 from the giant hogweed *H. sosnowskyi.* This plant can grow in low nutritional environments while reaching a high biomass. This observation has led us to speculate that microbes colonizing the inner plant tissues of *Heracleum* have beneficial traits which may contribute to its enormous growth. Strain HC8 appears to have the ability to produce a large variety of bioactive compounds that might play a role in biocontrol and plant growth promotion mediated by this strain. Although it was isolated from *H. sosnowskyi*, it is able to promote the growth of radish and reduce TFRR in tomato plants. This will facilitate its application as a bioinoculant. Endophytes from the plant *Heracleum* have never been isolated previously. It may be interesting to evaluate the entire endophytic microbial content of *Heracleum* in more detail.

Acknowledgements

The research described here was supported by the Netherlands Organization for Scientific research (NWO, project number 047.018.001), by Russian Foundation for Fundamental Investigations (RFFI, project 06.04.89000 NVOC_a “Plants interaction with beneficial bacteria”) and by Goskontract Minobrnauki (project number P760 from 20.05.2010 "Development of microbial preparations on the basis of endophytes and rhizobacteria for cultivation of agricultural crops in conditions of technogenic disasters) and represents part of the Russian-Dutch collaboration project “Center of Excellence”. We thank Dr. Bernadette Kroon (Syngenta B.V., Enkhuizen, the Netherlands) for providing us with tomato seeds. We thank Prof. Dr. Bert van Duijn (Fytagoras B.V., Leiden, the Netherlands) for providing us with aged barley seeds. We thank Dr. Danny Vereecke for giving us a protocol for cytokinin determination. We thank Gerben Voshol for his help in some experiments and for critically reading the manuscript.

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Arch Microbiol 2012 (194): 893-899

Cyclic lipopeptide profile of the plant-beneficial endophytic bacterium *Bacillus subtilis* HC8

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Abstract

In a previous study (Malfanova et al. 2011) we described the isolation and partial characterization of the biocontrol endophytic bacterium *B. subtilis* HC8. Using thin-layer chromatography we have detected several bioactive antifungal compounds in the methanolic extract from the acid-precipitated supernatant of HC8. In the present study we have further analyzed this methanolic extract using liquid chromatography-mass spectrometry (LC-MS). Based on the comparison of retention times and molecular masses with those of known antifungal compounds we identified three families of lipopeptide antibiotics. These include four iturins A having fatty acyl chain lengths of C14 to C17, eight fengycins A (from C14 to C18 and from C15 to C17 containing a double bond in the acyl chain), four fengycins B (C15 to C18) and five surfactins (C12 to C16). Evaluation of the antifungal activity of the isolated lipopeptides showed that fengycins are the most active ones. To our knowledge this is the first report of an endophytic *Bacillus subtilis* producing all three major families of lipopeptide antibiotics containing a very heterogeneous mixture of homologues. The questions remain open which of these lipopeptides (i) are being produced during interaction with the plant and (ii) are contributing to the biocontrol activity of HC8.

Introduction

Endophytes are plant-associated microbes which are able to colonize plants internally. Due to the nature of their endophytic lifestyle they establish a long-lasting stable relationship with a plant. In this symbiotic association the plant provides nutrients and shelter for the microbes and, in turn, the endophyte can help the plant by protecting it against phytopathogens or by promoting its growth. One of the mechanisms of such a protection includes production of bioactive secondary metabolites which either can be directly involved in antibiosis (Thomashow and Weller 1995; Haas and Défago 2005; Lugtenberg and Kamilova 2009) and/or in triggering induced systemic resistance (ISR) (Tran et al. 2007; Ongena et al. 2007). *Bacillus* spp. are known to produce a wide range of secondary metabolites including cyclic lipopeptides(c-LPs), some of the most powerful ones with regard to their antifungal and biosurfactant activity (Ongena and Jacques 2008; Jacques 2011).

Secondary metabolites produced by *Bacillus* spp. consist mainly of three families of non-ribosomally synthesized c-LPs. These are the iturins, the fengycins and the surfactins. These c-LPs contain a peptide ring with seven (iturins and surfactins) or 10 (fengycins) amino acids linked to a β-hydroxy (fengycins and surfactins) or β-amino (iturins) fatty acid. Each lipopeptide family is further sub-divided into groups based on its amino acid composition. For example, the fengycin family comprises fengycin A and fengycin B, which differ in a single amino acid in the sixth position (D-alanine and D-valine, respectively). Within each group there are homologues differing in the length, branching and saturation of their acyl chain (Ongena and Jacques 2008). Members of the iturin family range from C14 to C17, fengycins from C14 to C19 and surfactins from C12 to C17. Both iturins and fengycins are mainly known for their anti-fungal properties, while surfactins are mostly anti-viral and anti-bacterial. When different families are co-produced, their interaction can become synergistic and enhances each of their respective activities (Maget-Dana et al. 1992; Ongena et al. 2007; Romero et al. 2007).

In our previous work (Malfanova et al. 2011) we have described the isolation and partial characterization of the plant-beneficial endophytic bacterium *B. subtilis* HC8. This strain shows strong *in vitro* antifungal activity against various fungal phytopathogens. When applied to seeds, *B. subtilis* HC8 is able to significantly decrease symptoms of tomato foot and root rot which is caused by the phytopathogen *Forl*. The crude methanolic extract from the acid-precipitated supernatant fluid of this strain contains several bioactive compounds which behave similar to some known

lipopeptide antibiotics on a TLC plate. Taking together, all these data suggested that *B. subtilis* HC8 produces several lipopeptide antibiotics which might be important for its antifungal and biocontrol activities. Therefore, the aims of this study were (i) to identify the putative lipopeptides produced by the beneficial endophytic strain *Bacillus subtilis* HC8, (ii) to characterize the antifungal activity of the isolated c-LPs families against *Forl* in an *in vitro* bioassay, (iii) to test whether there is synergistic activity between the families of c-LPs towards *Forl in vitro*, and (iv) whether active c-LPs affect hyphal morphology.

Materials and Methods

Extraction of antifungal compounds

The extraction of antifungal compounds was performed as described in our previous study (Malfanova et al. 2011). Briefly, *B. subtilis* HC8 was grown in Brain Heart Infusion broth (BHI, Difco Laboratories, MI, USA) for 60h at 28°C. Subsequently, cells were removed by centrifugation at 13 000 r.p.m. for 10 min. The supernatant fluid was acidified to pH 2.0 with concentrated HCl. The resulting precipitate was extracted twice with methanol, the combined extracts were concentrated by vacuum evaporation and the resulting material was subsequently dissolved in $1/50^{th}$ of the initial culture volume of methanol.

Identification of c-LPs using LC-MS analysis

Putative c-LPs were identified as described by Arguelles-Arias and colleagues (2009) using LC-MS analysis. Briefly, the crude methanolic extract was analyzed by reverse-phase high pressure liquid chromatography (Waters Alliance 2695/diode array detector) coupled to a quadrupole mass analyzer on an X-Terra MS 150*2.1 mm, 3.5 µm C8 column (Waters, Milford, MA, USA). Lipopeptides were eluted using a two component solvent system of which solvent A is water and solvent B is acetonitrile, both acidified with 0.1% formic acid. We used four different elution programs including one general program to elute all lipopeptides and three family-specific programs to get a better separation and quantification of the different lipopeptides within each family (Table 1). All elution programs used a flow rate of 0.5 ml/min and detection occurred using the positive ion mode.

Identification of lipopeptides was based on the comparison of retention times and molecular masses with those of known cyclic lipopeptides (Ongena et al. 2005; Ongena and Jacques 2008). As a control, the 95% pure authentic standards for each family

were used. The fengycin A and B lipopeptides with identical molecular mass and retention time were distinguished as described by Sun et al. (2006) based on the formation of specific product ions upon mild conditions of fragmentation of molecular ions. Product ions with mass-to-charge value (m/z) 966 and 1080 correspond to fengycin A while those at m/z 994 and 1108 correspond to fengycin B. Amount of each lipopeptide family present in the sample was calculated based on calibration curves of purified iturins, fengycins and surfactins available in the laboratory.

Evaluation of antifungal activity of the isolated c-LPs

Antifungal activity of the isolated c-LPs was evaluated in the 96-well microtiter plate assay against *Forl*. To do this, the suspension of fungal spores, adjusted to a density of $5x10^5$ spores/ml was combined either with single compounds dissolved in methanol ranging from 3 to 100 µg/ml or with their combination according to the co-production profile (iturins 47%, fengycins 36% and surfactins 17%) in a final volume of 150 µl of half strength Potato Dextrose Broth (PDB, Difco Laboratories, MI, USA). In the positive control, c-LPs were replaced with the corresponding volume of methanol. In the negative control, no spores and no c-LPs were added. Inoculated plates were incubated for 25 h at 30°C and subsequently the fungal growth was determined by measuring the optical density (OD) at 620 nm with a microplate reader. To see the impact of c-LPs on fungal morphology, fungal hyphae treated with 100 µg/ml of c-LPs were observed with an Axioskop2-type microscope using a 40x objective (Carl Zeiss Jena GmbH, Germany). All experiments were performed at least twice.

Results and Discussion

LC-MS was performed on a crude methanolic extract of the acid precipitated supernatant fluid of *B. subtilis* HC8. To elute all putative c-LPs we used a general elution program (Table 1a). This program uses a gradient of increasing amounts of acetonitrile (a polar solvent) and thus the first eluents include the less polar iturins followed by the increasingly polar fengycins and surfactins (Fig 1). Iturins and fengycins are less separated compared to surfactins due to the similar polarity of the biggest iturins and the smallest fengycins. Based on calibration curves for standard c-LPs, iturins represent the most abundant family of the lipopeptides (65 µg/ml culture supernatant, followed by fengycins (50 µg/ml) and surfactins (23 µg/ml).

Before the elution of surfactins (20-22 min) several peaks appeared which correspond to unknown compounds that could be related to surfactin lipopeptides

Table 1. Elution programs used in HPLC[a]

a General Elution Program				**b Iturin Specific Elution Program**			
Time	**A%**	**B%**	**Curve**	**Time**	**A%**	**B%**	**Curve**
0	57	43	1	0	62	38	1
1,5	57	43	1	20	55	45	6
17	37	63	6	25	50	50	6
17,5	20	80	6	27	0	100	6
26	0	100	6	32	0	100	6
27	57	43	6	33	62	38	6
35	57	43	6	40	62	38	6
c Fengicyn Specific Elution Program				**d Surfactin Specific Elution Program**			
Time	**A%**	**B%**	**Curve**	**Time**	**A%**	**B%**	**Curve**
0	60	40	1	0	22	78	1
20	35	65	6	20	22	78	1
21	0	100	6				
26	0	100	6				
27	60	40	6				
35	60	40	6				

[a] Solvent A is water, acidified with 0.1% formic acid and solvent B is acetonitrile, acidified with 0.1% formic acid. The curve indicates the rate at which the solvent is changed to the new compositions, curve 1 is exponential and curve 6 is linear.

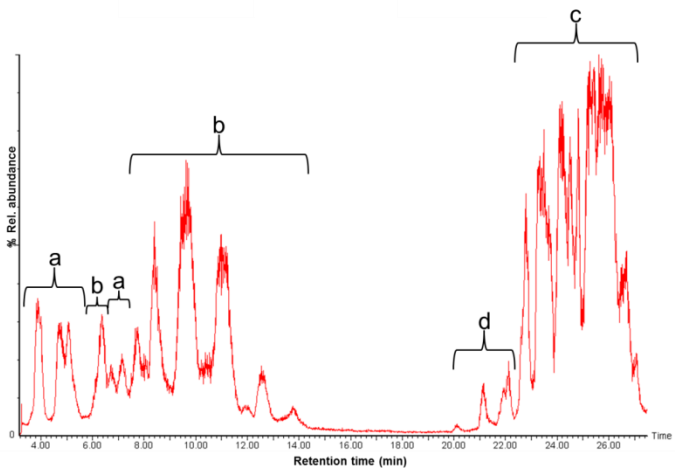

Fig. 1. LC-MS analysis of the crude methanolic extract from the acid-precipitated supernatant fluid of *B. subtilis* HC8. Analysis was performed as described by Arguelles-Arias et al. (2009). a, iturins A; b, fengycins; c, surfactins; d, unknown compounds.

based on their chromatographic behavior and fragmentation pattern. Preliminary analysis of their product ions (results not shown) indicates that these compounds contain unusual amino acid(s) in their peptide moiety. The presence of unknown surfactin-like compounds can be either specific for *B. subtilis* HC8 or due to relative abundance of certain amino acids in the medium. A possible influence of the composition of the medium was shown in several studies (Peypoux et al. 1994; Grangemard et al. 1997). For example, addition of L-alanine to the growth medium resulted in incorporation of this amino acid in the fourth position of the peptide ring of surfactins instead of the usual amino acid L-valine (Peypoux et al. 1994). This can be explained by the non-specificity of the adenylation domain of some non-ribosomal peptide synthetases involved in surfactin biosynthesis (Jacques 2011). Additional culturing in various growth media, purification and analysis would be required to elucidate the exact composition and structure of these minor unknown surfactin-like compounds detected in the present study.

To obtain a better separation and quantification of the different lipopeptides of the same family, we ran three family-specific programs (Tables 1b, c and d). The iturin-specific program revealed the presence of all four members of iturin A, having fatty acyl chain lengths from C14 to C17 (Table 2; Fig 2a). The most abundant homologue is C15, followed by C14, C16 and C17. The fatty acid chain length of iturins is known to be important for their antifungal activity which increases with increasing number of carbon atoms (Bonmatin et al. 2003; Shai et al. 2006; Tabbene et al. 2011). For example, it has been shown that the C16 homologue of bacillomycin D-like compound, which is a member of the iturin family, displayed the strongest fungicidal activity *in vitro* against *Candida albicans* whereas C14 and C15 homologues showed weak and moderate activity, respectively (Tabbene et al. 2011). This is supposed to be due to the fact that long chain iturins are more hydrophobic and therefore may interact more effectively with ergosterol-containing membranes of fungi and yeasts. Moreover, Malina and Shai (2005) suggested that the length of the acyl chain can also affect the specificity of lipopeptide-cell membrane interactions. They synthesized several lipopeptides with increasing acyl chain lengths of 10, 12, 14 and 16 carbons to the peptides. Lipopeptides with short fatty acid chains (C10 and C12) displayed both antibacterial and antifungal activity, whereas those with long chains (C14 and C16) were active only against fungi. A possible explanation of this result is that long chain c-LPs more readily form oligomers and thus interact easier with the fungal membrane than with the bacterial one (Malina and Shai 2005). This might partly explain the strong

Table 2. c-LPs production by *B. subtilis* HC8 as detected by LC-MS[a]

Cyclic lipopeptide family	Molecular mass $(M-H)^+$	Homologue
Iturin A	1043,7	C-14
	1057,74	C-15
	1071,75	C-16
	1085,71	C-17
Fengycin A[a]	1436,18	C-14
	1450,16	C-15
	1464,14	C-16
	1478,12	C-17
	1492,16	C-18
	1448,15	C=15[c]
	1462,19	C=16[c]
	1476,1	C=17[c]
Fengycin B[b]	1478,05	C-15
	1492,16	C-16
	1506,2	C-17
	1521,22	C-18
Surfactin	994,21	C-12
	1008,75	C-13
	1022,33	C-14
	1036,87	C-15
	1050,92	C-16

[a] c-LPs were identified by comparing both their molecular masses and their retention times with those from the literature (Ongena et al., 2005; Ongena and Jacques, 2008).

antifungal and the limited antibacterial properties of iturins.

Using the fengycin-specific program, we found eight fengycins A and four fengycins B (Table 2, Fig 2b). Fengycins A consist of the saturated C14 to C18 homologues and C15 to C17 containing a single double bond in the fatty acyl chain. Fengycins B comprise C15 to C18 homologues with a saturated acyl chain. Fengycins A are present in our sample in a larger quantity compared to fengycins B. The C17 fengycin A is the most abundant homologue while the C15-C17 homologues with unsaturated acyl chain appear to be the least abundant ones. Among fengycins B, C17 is the most abundant homologue and C18 is the least. Although fengycin homologues have long fatty acyl chains, they are less hemolytic than iturins and more active towards filamentous fungi

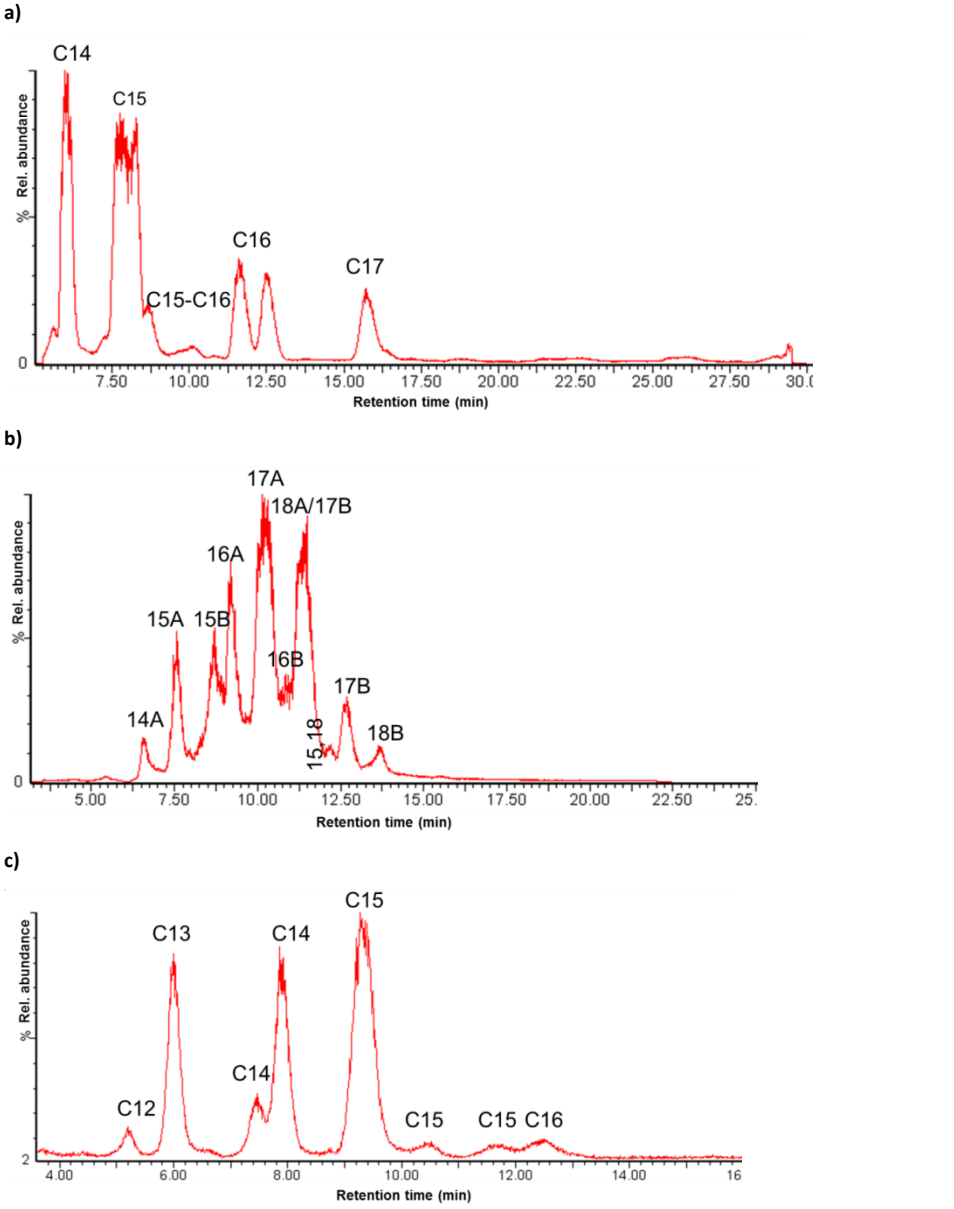

Fig. 2. Family-specific LC-MS analysis of the methanolic extract of *B.subtilis* HC8. Each lipopeptide family was characterized using specific elution gradients as described elsewhere (Toure et al., 2004).

a) Iturin-specific analysis. The homologues of iturin A contain C14-C17 acyl chains.

b) Fengycin-specific analysis. A, fengycin A; B, fengycin B; 15,18 include C15 fengycin A homologue with an unsaturated fatty acyl chain and the saturated C18 homologue. Fengycins A C16 and C17 homologues with a double bond elute together with 18B.

c) Surfactin-specific analysis. The homologues of surfactin contain C12-C16 acyl chains.chains (up to C19).

(Jacques 2011). Indeed, in our study we found that fengycins are bioactive at all tested concentrations while iturins display an inhibitory effect only at high concentrations (30 and 100 µg/ml) (Fig 3). Moreover, fengycins alone are significantly more active than the mix of the three c-LP's suggesting that fengycins are the major antifungal compound against *Forl*. This notion is supported by the microscopic observation of more severe growth restriction of fungal hyphae incubated with fengycins than with any of the other compounds (Fig 4). The result with the mixture also shows that there is no (strong) synergy in the action of the various c-LP's (Fig 4).

The surfactin-specific program revealed the presence of six out of seven known surfactins with an acyl chain from C12 to C16 and the amino acid leucine at the seventh position of the peptide ring (Table 2; Fig 2c). The most abundant homologue is C15 followed by C14, C13, C12 and C16. Numerous studies showed that C14 and C15 surfactin homologues are the most bioactive ones with respect to their antiviral activity (Kracht et al. 1999), insecticidal activity (Assié et al. 2002), triggering several plant defense mechanisms (Jourdan et al. 2009) and foaming properties (Razafindralambo et al. 1998). Although surfactins do not show significant antifungal

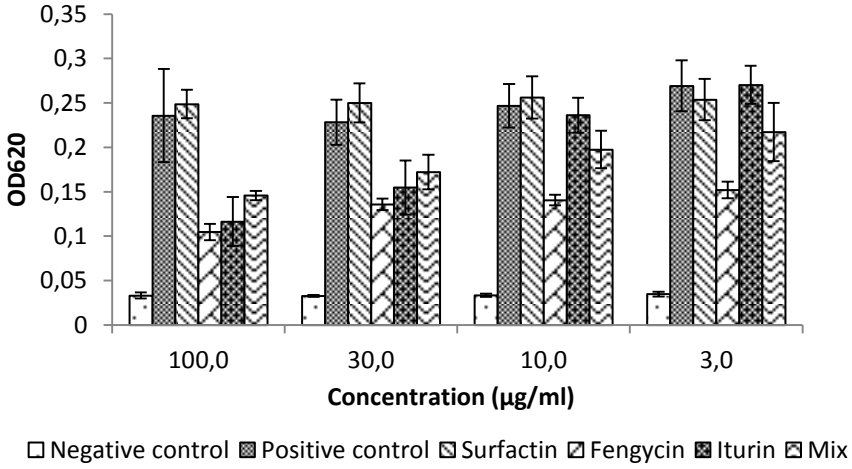

Fig. 3. Evaluation of antifungal activity of c-LPs against *Forl*. The fungal spores were incubated with c-LPs at four different concentrations and with a combination of iturins, fengycins and surfactins. The inhibition of the fungal growth was judged as the decrease in OD620 compared to the control. Bars indicate confidence intervals.

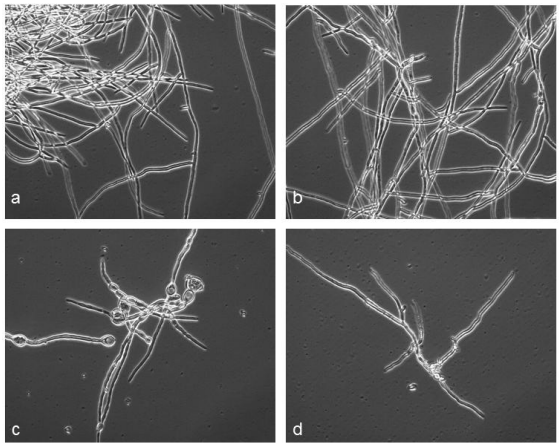

Fig. 4. Visualization of the inhibitory effect of c-LPs. a. methanol; b. surfactins; c. fengycins; d, iturins. Fungal spores were treated with a 100 µg/ml of each c-LP and incubated at 30°C for 25 hours.

activity at the concentrations tested (see Fig 3 and 4), they can favor establishment and spreading of biocontrol bacteria in internal host tissues. LP has been shown to be implicated in a flagella-independent surface motility (Kinsinger et al. 2003; Leclère et al. 2006) and in the formation of biofilms (Hofemeister et al. 2004) thereby globally contributing to the ability of some bacilli to efficiently colonize surfaces of plant roots (Bais et al. 2004).

In this study we show for the first time that an endophytic *B. subtilis* strain is able to produce all three families of c-LPs of which the fengycins displayed the strongest antifungal activity against *Forl*. Production of fengycins A and B was also reported for the endophytic bacteria *B. amyloliquefaciens* ES-2 (Sun et al. 2006) and *B. subtilis* B-FS01 (Hu et al. 2007). However, in contrast to HC8, neither of these strains co-produces significant amounts of surfactin, fengycin and iturin. Moreover, a very heterogeneous mixture of homologues was detected in the methanolic extract of our strain. Whether the same c-LPs and homologues are being produced during interaction of *B. subtilis* HC8 with plants and which of them are involved in its biocontrol activity remains to be established.

Acknowledgements

The research described here was supported by the Netherlands Organization for Scientific research (NWO, project number 047.018.001) and by Goskontract Minobrnauki (project number P760 from 20.05.2010) and represents part of the Russian-Dutch collaboration project “Center of Excellence”. We thank Gerben Voshol

for his help in some experiments and for critically reading the manuscript. This work also received financial support from the Program Crédit aux chercheurs n° 1.5.192.08F (National Funds for Scientific Research, F.R.S.-FNRS, Belgium). M. Ongena is Research Associate at the F.R.S.-FNRS.

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Arch Microbiol 2013 (195): 9-17

Is L-arabinose important for the endophytic lifestyle of *Pseudomonas* spp.?

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Abstract

Twenty endophytic bacteria were isolated from surface-sterilized stems and roots of cucumber plants. After removal of potential siblings and human pathogens, the remaining seven strains were identified based on their 16S rDNA as *Pseudomonas fluorescens* (2 strains) and *P. putida* (5 strains). Three strains, namely *P. fluorescens* CS1, *P. fluorescens* CR2 and *P. putida* CR3, were able to suppress tomato foot and root rot (TFRR). Special attention was paid to the characterization of the BIOLOG carbon oxidation profiles of the isolated pseudomonads in order to identify nutrients which might be important for their endophytic lifestyle. Comparative analysis of the profiles of these seven strains with those of seven rhizospheric *Pseudomonas* spp. revealed that endophytes were able to oxidize L-arabinose and 2,3-butanediol significantly more often than the rhizospheric group. An independent growth experiment performed in tubes using L-arabinose and 2,3-butanediol as sole carbon sources showed the same results as seen using BIOLOG for L-arabinose, but not for 2,3-butanediol. Since L-arabinose is one of the most abundant sugars in xylem of cucumber plants and was not detected in their rhizosphere, our data suggest that utilization of L-arabinose might be a trait contributing to the endophytic lifestyle of the isolated *Pseudomonas* endophytes.

Introduction

Plants live in association with many bacteria which can be classified as rhizobacteria, epiphytic bacteria and endophytic bacteria. Endophytic bacteria are referred to as those which are able to colonize plants internally without causing any apparent harm. Due to their endophytic lifestyle, bacterial endophytes establish a more stable and long-lasting relationship with a plant than other plant-associated bacteria do (Hardoim et al. 2008). In addition, some endophytic bacteria have beneficial effects on plants. Therefore, bacterial endophytes with plant-beneficial traits are considered to be promising bio-inoculants for agricultural application (Strobel 2006).

Once endophytes establish themselves inside a plant, some of them can stimulate plant growth and/or protect plants against phytopathogens. Endophytic bacteria are able to promote plant growth directly by the secretion of phytohormones (Spaepen et al. 2008; Sgroy et al. 2009), by nitrogen fixation (You et al. 2005; Pedraza 2008) and by phosphate solubilization (Taurian et al. 2009; Lopez et al. 2011). In addition, several beneficial bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the precursor of the plant hormone ethylene, to NH_3 and α-ketobutyrate (Glick 2005). The bacteria utilize NH_3 as a source of N and thereby decrease the ACC and ethylene levels within the plant and, as a result, can stimulate plant growth (Sun et al. 2009). The ACC-deaminase activity of endophytic bacteria and its responsibility for growth promotion of *Solanum nigrum* was reported for various strains of *Pseudomonas* (Long et al. 2008).

Endophytic bacteria can also promote plant growth indirectly via biocontrol of phytopathogens. Known mechanisms of biocontrol mediated by endophytic bacteria include (i) antibiosis through the production of antibiotics (Cho et al. 2003) or exo-enzymes (Downing and Thomson 2000) and (ii) induction of systemic resistance (Van Wees et al. 2008; Yasuda et al. 2009).

Endophytic bacteria are able to colonize intercellular spaces of the cell walls and xylem vessels which together form the plant apoplast (Compant et al. 2010). Biochemical studies of nutrients which are present in the apoplast indicate that this niche contains sugars, alcohols, amino acids, organic acids, growth factors, and mineral elements (Bacon and Hinton 2006). The question remains open of which carbon sources are utilized by endophytic bacteria in the plant apoplast.

The main aims of the present study were: (i) to isolate and identify the major culturable heterotrophic bacterial endophytes from stems and roots of cucumber plants grown in a greenhouse, (ii) to select novel beneficial strains based on their

abilities a) to promote growth of radish and/or b) to control TFRR caused by the fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*), and (iii) to characterize and compare oxidation profiles of carbon sources of endophytic and rhizospheric *Pseudomonas* spp. in an attempt to identify compounds which might be involved in the endophytic lifestyle.

Materials and Methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from stems and roots of cucumber (*Cucumis sativus* L.) plants collected from greenhouses in the Tashkent area, Uzbekistan. Plant samples were treated with 70% ethanol for 3 min, followed by 4% sodium hypochlorite for 5 min, and several rinses with sterile water. To verify adequate surface sterilization, aliquots of water from the last rinsing were plated on 1/20 strength TSA control plates. Subsequently, the surface-sterilized plant samples were crushed under sterile conditions and the resulting juices were plated on 1/20 strength tryptic soy agar (TSA, Difco Laboratories, MI, USA) plates. After incubation at 28°C for 3 days colonies originating from plant juice with empty control plates were used for further analysis.

Microbial strains and growth conditions

All isolated bacterial strains and eight *Pseudomonas* rhizospheric strains (*P. fluorescens* WCS365, PCL1444 and PCL1751 and *P. putida* PCL1760, PCL1759, PCL1758, PCL1603 and PCL1445), which belong to the collection of Institute of Biology Leiden, were grown and maintained on full strength TSA. The rhizospheric strains used for a comparative analysis originate from the following plants: avocado (*P. putida* 1603 and 1760), Barmultra grass (*P. fluorescens* 1444 and P. putida 1445), tomato (*P. putida* 1758 and 1759) and potato (*P. fluorescens* WCS365). *P. fluorescens* PCL1751, which is an excellent root colonizer (Kamilova et al. 2005) and is naturally resistant to kanamycin, was used as a reference strain for competitive cucumber root tip colonization experiments. Kanamycin was used at the final concentration of 50 µg ml^{-1}.

The fungi *Aspergillus niger*, *Forl, F. solani* and the oomycete *Pythium ultimum* were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories). To obtain spores for biocontrol experiments, *Forl* was routinely grown on Czapek-Dox liquid medium (Difco Laboratories) and incubated on a rotary shaker at 150 rpm at 28 °C.

Molecular characterization of endophytic strains

Amplified ribosomal restriction analysis (ARDRA) and identification of endophytic strains was performed as described previously (Malfanova et al. 2011). Briefly, 16S rRNA gene was amplified, cut with four different restriction enzymes and the resulting fragments were separated using a 2% agarose gel. Those strains which gave a unique restriction pattern and appeared morphologically distinct were sent for sequencing to Service XS, Leiden, the Netherlands. Species they belonged to were identified as sharing at least 99% homology with those of known species.

Characterization of potential plant-beneficial traits

Characterization of potential plant-beneficial traits such as the production of exo-enzymes (β-glucanase, cellulase, chitinase, lipase and protease), antifungal metabolites (AFM) and auxins was performed as described previously (Malfanova et al. 2011). The presence of ACC deaminase was judged by growth on 1-aminocyclopropane-1-carboxylate (ACC) as the sole N-source according to Belimov et al. (2005).

Plant growth promotion

Endophytic bacteria were tested for their ability to promote the growth of radish plants. This was done by soaking seeds of radish in a bacterial cell suspension (adjusted to 10^8 cfu/ml) in phosphate buffered saline solution (PBS) for 15 minutes. As a negative control, seeds were treated with sterile PBS. The treated seeds were subsequently planted in non-sterile potting soil and grown under greenhouse conditions at 80% humidity and 16 h of daylight. Each variant consisted of four replicates with five seeds each. After two weeks of growth, the fresh weight of the roots was determined. All experiments were performed at least twice.

Biocontrol of tomato foot and root rot

Biocontrol of TFRR in soil was performed according to Kamilova et al. (2005) with small modifications. Briefly, tomato seeds of cultivar Carmello (Syngenta, Enkhuizen, the Netherlands) were coated with bacteria by dipping the seeds in a suspension of 1% (w/v) methylcellulose (Sigma, St Louis, MO, USA) in PBS containing 10^8 cfu/ml. The treated seeds were then planted in non-sterile potting soil supplemented with 10^7 *Forl* spores per kg. For each treatment, 96 plants were tested in eight trays of 12 plants each. Plants were grown in a greenhouse at 21–24°C, 70% relative humidity and 16 h light. After 3 weeks of growth, plants were removed from soil and examined for

symptoms of foot and root rot, such as brown spots, lesions, wilting or even death. Only roots without any of these symptoms were referred to as healthy and all others were scored as diseased. All experiments were performed at least twice.

Biocontrol of TFRR in stonewool substrate was conducted as described by Validov et al. (2007). Briefly, tomato seeds were placed in stonewool plugs which had been soaked in advance in Plant Nutrient Solution (PNS) (Wageningen UR Greenhouse Horticulture, Bleiswijk, the Netherlands) supplemented with *Forl* spores (10^7 spores/L) and bacterial cells (10^6 cfu/ml). In the negative control PNS was supplemented with spores only. Plants were grown for 14 days under greenhouse conditions at 80% humidity and 16 h of daylight. The plants were then removed from the stonewool and examined for symptoms of foot and root rot. All experiments were performed twice. Homogeneity of variance and analysis of variance (ANOVA) at p = 0.05 were conducted with SPSS software (Chicago, IL, USA).

Carbon oxidation/utilization assay

Seven *Pseudomonas* strains isolated from roots and stems of cucumber plants and seven rhizospheric strains of *Pseudomonas* spp. of different plant origin were tested for their ability to oxidize various carbon sources using BIOLOG GN2 Microplates (Biolog Inc., Hayward, CA, USA). Bacteria were grown overnight at 28 °C under aeration (150 rpm), harvested by centrifugation and subsequently resuspended in 0.85% (w/v) NaCl solution to a final OD_{590} of 0.15. Aliquots of 150 µl were inoculated in each well of a 96-wells microplate using a multichannel pipette. Plates were covered with a lid and incubated statically at 28˚C for 48 hours. The appearance and intensity of a purple color, caused by the reduction of the tetrazolium salt, was read using a microplate reader at OD_{590} and by direct observation. Only results with values of $OD_{590} > 0.3$ and the visible presence of the purple color were scored as positive. In the case of border values between 0.29-0.3 and/or absence of purple color in the test wells, the results were scored as "+/-". To check whether there is a significant difference in carbon oxidation abilities between the endophytic and rhizospheric group, the Chi-square test (p=0.05) was performed using SPSS software.

To verify results obtained using the Biolog assay, we performed independent growth experiments. Endophytic and rhizospheric bacteria were grown overnight in LB broth and washed twice with 0.85% NaCl to remove traces of extracellular carbon. Subsequently bacteria were inoculated in M9 minimal medium containing (i) 0.2% (w/v) L-arabinose or 2,3-butanediol (experiment), (ii) 0.2% (w/v) glucose (positive

control), and (iii) no added carbon source (negative control) to a final OD_{590} of 0.15. Falcon tubes containing 5 ml of each suspension were incubated for 24 h at 28˚C and 150 rpm. Subsequently the bacterial growth was scored spectrophotometrically at OD_{590}. All experiments were performed at least twice.

Competitive cucumber root tip colonization

In order to verify the ability of rhizospheric pseudomonads to efficiently colonize the rhizosphere of cucumber plants, competitive root tip colonization experiments were performed as described by Kamilova et al. (2005). Briefly, surface-sterilized cucumber seeds were inoculated with a 1:1 mixture of two bacterial strains (experiment vs. reference strain). The treated seeds were then planted in a gnotobiotic sand system (Simons et al. 1996) and grown for 7 days under greenhouse conditions. Subsequently, one cm of the root tip was cut off and vigorously shaken for 15 min to remove the adhered bacteria. Suspensions with bacterial cells were then diluted and plated on TSB with and without Km. The number of Km-sensitive (experiment) and Km-resistant (reference) colonies was determined and the average of each group was calculated. All colonization experiments were performed in five replicates.

Results

Isolation and characterization of endophytic bacteria

Endophytic bacteria were isolated from stems and roots of greenhouse-grown cucumber plants. The controls showed that all living microorganisms on the plant surface were killed or became nonculturable.

A total of 20 strains were randomly chosen from the colonies obtained after plating plant juices on 1/20 strength TSA. To eliminate potential siblings, these strains were compared for their colony morphology, motility, their ARDRA patterns and production of the exo-enzymes β-glucanase, cellulase, chitinase, lipase and protease. Eleven strains which were indistinguishable with respect to the mentioned traits were considered as likely siblings and not further studied.

The nine remaining strains were screened for their antagonistic activity towards four pathogens, their ability to produce auxin, and their growth on ACC as the sole N-source (Table 1). Strain CR3 is antagonistic towards *P. ultimum*, but not *A. niger*, *F. solanum* or *Forl* and does not secrete any exo-enzymes. None of the other strains showed any antagonism against these phytopathogens or production of exo-enzymes

Table 1. Overview of plant-beneficial traits of selected endophytic bacteria

Strain	Antifungal activity[a]	Exo-enzymes[b]	Auxin[c]	ACC[d]
CS1	-	P	-	+
CS4	-	-	+	-
CS5	-	-	++	-
CS6	-	-	-	-
CS8	-	-	+	-
CR2	-	-	-	-
CR3	Pu	-	+	-
CR6	-	-	+	-
CR9	-	-	-	-

[a] Pu, *Pythium ultimum.*

[b] P, protease.

[c] Auxin level after growth in medium supplemented with tryptophan: ++ >80 µg/ml, + >10-20 µg/ml, - < 10 µg/ml. Auxin level after growth in medium without tryptophan was zero for all strains.

[d] ACC **(**1-aminocyclopropane-1-carboxylate), growth on ACC as the sole N-source.

except for CS1 which showed protease activity.

Five strains, namely CR3, CR6, CS4, CS5 and CS8 produce detectable amounts of auxins, but only in the presence of tryptophan in the growth medium. The level of auxin secreted by strain CS5 is more than 80 µg/ml. In the case of CS4 and CR3, the auxin level in the media with tryptophan is more than 15 µg/ml. Two other strains, namely CR6 and CS8 produce appr. 10 µg/ml of auxins.

The only strain able to utilize ACC as the sole nitrogen source is CS1.

Molecular identification of endophytic strains

The strains were identified based on comparison of their rRNA sequences with database sequences from correctly identified strains (Table 2). The following species were isolated from stems and roots of cucumber plants: one *Bacillus cereus* strain, two *P. fluorescens* strains, five *P. putida* strains and one *Stenotrophomonas maltophilia*. To see whether these strains are safe to be applied in the field as biocontrol and/or plant-growth promoting strains, we evaluated to which risk group (Anonymous 1998) they belong. Two strains, namely *B. cereus* and *S. maltophilia* belong to risk group 2, representing potential human pathogens. Therefore, they were excluded from further experiments. This left us with seven pseudomonads.

Table 2. Molecular identification of endophytic strains[a] and risk group classification[b]

Strain	Bacterial species[c]	Identity (%)	Phylum	Risk group
CS1	*Pseudomonas fluorescens*	99	β-Proteobacteria	1
CS4	*Pseudomonas putida*	99	β-Proteobacteria	1
CS5	*Pseudomonas putida*	99	β-Proteobacteria	1
CS6	*Stenotrophomonas maltophilia*	99	β-Proteobacteria	2
CS8	*Bacillus cereus*	99	Firmicutes	2
CR2	*Pseudomonas fluorescens*	100	β-Proteobacteria	1
CR3	*Pseudomonas putida*	99	β-Proteobacteria	1
CR6	*Pseudomonas putida*	99	β-Proteobacteria	1
CR9	*Pseudomonas putida*	99	β-Proteobacteria	1

[a] After elimination of siblings.

[b] Risk group 1 includes bacteria which are safe to be applied in the field; risk group 2 includes potential human and/or plant pathogens (Anonymous, 1998).

[c] Sequences have been submitted to GenBank under accession numbers JX010776-JX010784.

Plant growth promotion

Four auxin-producing strains, namely CR3, CR6, CS4 and CS5, and one ACC-utilizing strain, CS1, were tested for their ability to promote radish growth under greenhouse conditions. Radish was chosen as the model plant because its roots secrete a high level of tryptophan on filter paper (Kamilova et al. 2006) which can be used by many beneficial bacteria as the precursor of auxin. However none of the tested strains showed plant growth promotion (results not shown).

Biocontrol of tomato foot and root rot

All seven *Pseudomonas* spp. were tested for their ability to suppress TFRR in soil and stonewool. Two strains, namely *P. fluorescens* CR2 and *P. putida* CR3, significantly decreased disease symptoms of tomato plants in soil from 38% in the non-inoculated control to 22% and 24%, respectively (Fig 1a). Significant biocontrol activity of these strains was also found in the second soil experiment. In the stonewool biocontrol experiments strains CR2 and CR3 were not significantly active but another strain, *P. fluorescens* CS1, was able to suppress TFRR symptoms from 71% to 41% in the first and from 58% to 36% in the second experiment (Fig 1b).

Carbon oxidation assay

Seven endophytic and rhizospheric *Pseudomonas* spp. were characterized and compared in respect to their carbon oxidation profiles in order to identify carbon sources which might be important for their endophytic lifestyle (Table 3). Out of 95

Table 3. Carbon sources oxidized by endophytic and rhizospheric pseudomonads[a]

Carbon source (GN2)	R[b]	E[b]	Carbon source (GN2)	R[b]	E[b]
Dextrin	2	2	D-glucuronic acid	6	7
Glycogen	2	2	Alpha-hydroxybutyric acid	6	6
NAC-D-glucosamine	3	1	Gamma-hydroxibutyric acid	1	1
L-arabinose*	**2**	**7**	**P-hydroxiphenylacetic acid***	**5**	**1**
D-arbitol	3	2	Itaconic acid	0	1
D-fructose	7	6	Alpha-keto- butyric acid	4	3
D-galactose	2	2	Malonic acid	4	5
M-inositol	1	2	Sebacic acid	1	0
D-mannitol	3	2	Succinamic acid	5	3
D-mannose	7	6	Glucuronamide	5	5
D-psicose	2	2	L-alanil-glycine	7	6
D-sorbitol	1	2	Glyciyl-L-Glutamic acid	1	0
Sucrose	1	3	D-serine	5	5
D-trehalose	1	2	L-threonine	6	7
Succinic acid mono-methyl ester	5	3	Urocanic acid	3	5
Acetic acid	6	7	Inosine	6	6
Formic acid	6	7	Uridine	2	2
D-galactonic acid lactone	2	2	Phenylethylamine	6	5
D-galacturonic acid	6	6	**2,3-butanediol***	**1**	**6**
D-glucosaminic acid	2	2	D,L-alpha-glycerol phosphate	1	2

[a] Does not include carbon sources which oxidized by all or none of the tested strains (see Results).

[b] Number of strains out of the seven tested pseudomonads which oxidized the carbon source.

[*] Carbon sources which were differently oxidized between the rhizospheric (R) and the endophytic (E) group based on Chi-square analysis ($p < 0.05$).

different carbon sources, as many as 34 were oxidized by all tested strains. These include one sugar (alpha-D-glucose), 12 organic acids (cis-acetonic, citric, D-gluconic, beta-hydroxybutyric, alpha-ketoglutaric, alpha-ketovaleric, D,L-lactic, propionic, quinic, D-saccharic, succinic and bromosuccinic acid), 13 amino acids (D-alanine, L-alanine, L-asparagine, L-aspartic, L-glutamic, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, L-serine and gamma-amino butyric acid) and eight other compounds. A total number of 21 carbon sources were not oxidized by any of the strains. These comprise 11 sugars (D-cellobiose, L-fucose, gentiobiose, alpha-D-lactose, lactulose, maltose, D-melibiose, beta-methyl-D-glucoside, D-raffinose, L-rhamnose and turanose), three sugar alcohols (adonitol, i-erythritol and xylitol), one amino acid (L-phenylalanine), one amino acid derivative (glycil-L-aspartic acid) and five other compounds.

Three out of the 40 remaining carbon sources gave significantly different oxidation

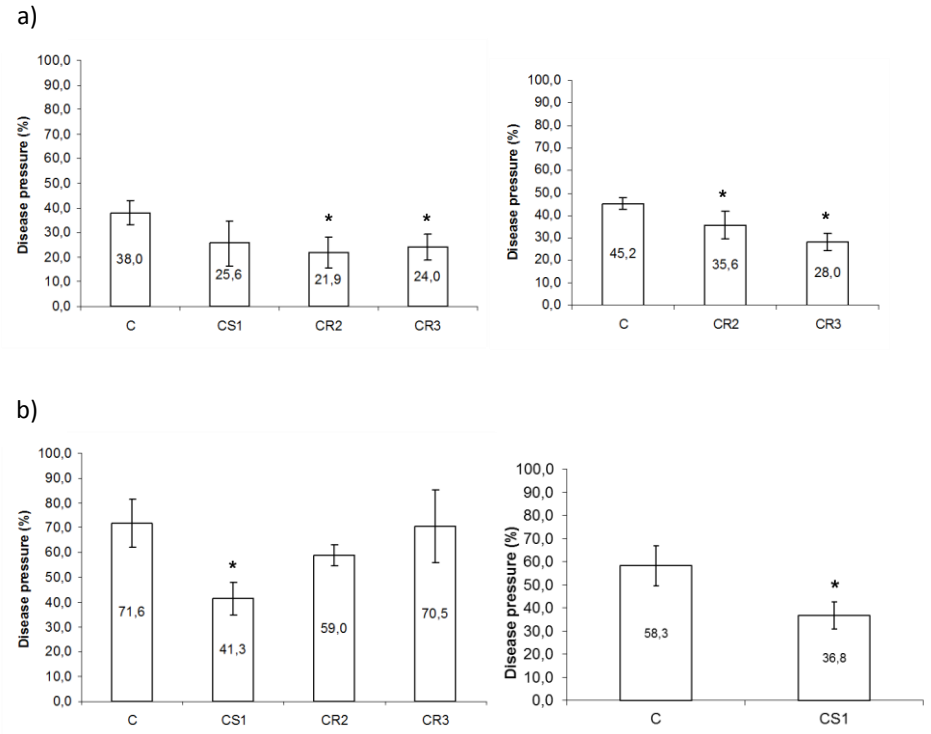

Fig. 1. Biocontrol of TFRR by endophytic *Pseudomonas* spp. a) in soil; b) in stonewool substrate. Numbers inside the columns present the percentage of sick plants. Bars indicate confidence interval ($p < 0.05$). Statistically different values are indicated with asterisks. C, uninoculated control.

profiles between the endophytic and rhizospheric group, namely L-arabinose, 2,3-butanediol and p-hydroxyphenylacetic acid (Table 3). L-arabinose was oxidized by all seven endophytes and only by two rhizospheric strains, namely *P. fluorescens* strains WCS365 and PCL1444 ($p < 0.05$). Six out of seven endophytic pseudomonads (*P. fluorescens* CS1 and CR2 and *P. putida* CS5,CR3, CR6 and CR9) and one out of seven rhizospheric ones (*P. fluorescens* WCS365) were able to oxidize 2,3-butanediol ($p < 0.05$). Para-hydroxyphenylacetic acid was oxidized by five rhizospheric and one endophytic strain ($p < 0.05$).

The results of an independent growth experiment, as judged by an increase in the optical density (590 nm) compared to M9 medium without added carbon source, using L-arabinose as the sole carbon source showed the same results as the BIOLOG experiments obtained for L-arabinose. However for 2, 3-butanediol as the sole carbon source, growth was measured for all endophytic strains and for six out of the seven rhizosperic strains.

Competitive cucumber root tip colonization experiment

To check whether the tested rhizospheric strains which originate from different plant hosts are cucumber rhizosphere competent and therefore can serve as suitable controls for the cucumber endophytes, we evaluated these strains in a competitive root tip colonization experiment against *P. fluorescens* PCL1751 (Kamilova et al. 2005), an excellent root colonizer. It appeared that all strains colonized the cucumber root tip in competition with *P. fluorescens* PCL1751 and therefore are cucumber rhizosphere competent (Table 4).

Discussion

General remarks about the isolated endophytes

After developing the protocol for the isolation of endophytic bacteria from cucumber plants, we used a similar strategy as described by Validov et al. (2007) for the elimination of siblings and potential pathogens. The fact that 11 out of the 20 strains are siblings indicates that the diversity among the isolated endophytes is low. Out of the nine remaining strains, two strains belong to risk group 2 (Table 2), indicating the presence of potential human pathogens among these endophytes. This phenomenon has been reported previously for both rhizospheric (Berg et al. 2005; Egamberdiyeva et al. 2008) and endophytic bacteria (Malfanova et al. 2011).

Table 4. Competitive cucumber root tip colonization experiment.

Competing strains[a]	cfu/cm of root tip[b]	
	Test strain	**Reference strain**
365 vs 1751	$(3{,}52\pm0{,}43)*10^4$	$(4{,}27\pm0{,}39)*10^4$
1444 vs 1751	$(2{,}60\pm1{,}41)*10^5$	$(4{,}03\pm0{,}48)*10^5$
1445 vs 1751	$(9{,}81\pm4{,}1)*10^3$	$(1{,}35\pm1{,}64)*10^5$
1603 vs 1751	$(5{,}43\pm4{,}2)*10^3$	$(2{,}21\pm0{,}37)*10^5$
1758 vs 1751	$(2{,}00\pm0{,}35)*10^4$	$(7{,}65\pm2{,}78)*10^4$
1759 vs 1751	$(2{,}40\pm0{,}54)*10^4$	$(4{,}90\pm0{,}72)*10^4$
1760 vs 1751	$(5{,}14\pm0{,}66)*10^4$	$(3{,}38\pm0{,}39)*10^5$

[a] The tested strains were inoculated on cucumber seeds in a 1:1 ratio with the reference strain *P. fluorescens* PCL1751.

[b] The average number of Km-sensitive (test) and Km-resistant (reference) colonies after plating the suspension with bacterial cells washed from the cucumber root tip.

Seven remaining bacteria were identified as *Pseudomonas* spp. of which members have been found as endophytes of different plants (Mercado-Blanco and Bakker 2007; Ramesh et al. 2008). Several representatives of this group, namely *P. fluorescens* and *P. putida*, are widely known for their various plant-beneficial traits which include production of antifungal metabolites and exo-enzymes, ACC-deaminase activity and secretion of phytohormones (Mercado-Blanco and Bakker 2007). In our study only a few identified pseudomonads displayed these characteristics (Table 1). The most common beneficial trait was the production of auxin in the presence of its precursor L-tryptophan.

Plant growth promotion

Auxin-producing strains were further tested under greenhouse conditions for their ability to promote the growth of radish roots. Auxins are known to be essential for plant physiology because they affect the root and shoot architecture (Spaepen et al. 2009). To our surprise, none of the tested pseudomonads had a significant effect on the root biomass of radish plants. The same results were obtained on tomato and cucumber plants (data not shown). The amount of L-tryptophan secreted by cucumber and tomato plants is low (1.8 and 7.4 ng per seedling, respectively), while the amount secreted by radish plants exceeds 0.29 µg per seedling (Kamilova et al. 2006) and is sufficient to stimulated microbial IAA production in nutrient rich medium (Kravchenko et al. 2004). However in nutrient poor soil, addition of high amounts of either L-tryptophan or IAA (up to 3 mg per kg soil) does not lead to a significant increase in radish root weight (Frankenberger et al. 1990). This may explain the absence of plant growth promotion in our experiments and highlights the importance of the substrate used during such investigations.

Biocontrol of TFRR

Biocontrol results (Fig 1) indicated that *P. fluorescens* strains CS1 and CR2 and *P. putida* strain CR3 have a strong ability to control TFRR. However, the biocontrol effect of these strains was substrate-dependent. Apparently, the biocontrol mechanisms used by the different strains do not necessarily function well in both substrates. A similar effect was reported previously by Validov et al. (2009) who found that flagellar motility, which is a key trait for the biocontrol ability of PCL1751 in potting soil, is not important during colonization of stonewool by *P. putida* PCL1760.

Despite the fact that the selected biocontrol strains do not produce exo-enzymes and antifungal metabolites against *Forl in vitro*, they were able to suppress the disease caused by the fungus *in vivo*. This observation is in agreement with other reports (Berg and Hallmann 2006; Malfanova et al. 2011), indicating that *in vitro* and *in vivo* beneficial activity of some bacteria is not necessarily correlated. Possible mechanisms of biocontrol include induction of systemic resistance (ISR) and competition for niches and nutrient (CNN) (Lugtenberg and Kamilova 2009). It is tempting to speculate that CNN is likely to be involved in biocontrol mediated by the selected endophytic pseudomonads. This mechanism has been proven for *P. fluorescens* PCL1751 and *P. putida* PCL1760 which also do not inhibit *Forl* in the plate assay but show significant biocontrol of TFRR in stonewool (Kamilova et al. 2005; Validov et al. 2007; Validov et al. 2009). Since the three strains which showed biocontrol did only so on one of the two substrates, we did not study the mechanism(s) of action.

Utilization of carbon sources by endophytic and rhizospheric pseudomonads

Out of the 95 different carbon sources tested, three were significantly differentially oxidized between the rhizospheric and the endophytic group ($p<0.05$). An independent growth experiment using M9 medium confirmed the BIOLOG result in the sense that L-arabinose is utilized by all endophytic pseudomonads while only two out of the seven tested rhizospheric strains could use it as their sole carbon source. Interestingly, L-arabinose is one of the major sugars present in the apoplast of different plants, including cucumbers (Iwai et al. 2003). Therefore our results suggest that utilization of L-arabinose might be a trait contributing to the endophytic lifestyle of the *Pseudomonas* endophytes isolated from cucumber plants.

Our suggestion about the role of L-arabinose may be extended to other endophytes and plants because Prakamhang et al. (2009) found that all 51different endophytes isolated from rice were able to use L-arabinose as well as glucose as their sole carbon sources. L-arabinose was not detected in the root exudate of cucumber plants (Kamilova et al. 2006). This fact together with the results from the competitive cucumber colonization experiment, which showed that all tested rhizospheric strains are able to reach the tip of the root at high density (see Table 4), suggests that the root tip colonization ability of the rhizospheric strains is not dependent on utilization of L-arabinose. The results also suggest that those rhizosperic strains which are able to utilize L-arabinose have an enhanced possibility of becoming endophytes.

Further analysis of the BIOLOG results showed that 2, 3-butanediol was differentially oxidized. However this could not be fully verified in an independent growth experiment although the endophytic strains were able to reach a higher cell density level than the rhizospheric strains (data not shown). It is interesting to note that 2, 3-butanediol is a volatile signaling molecule involved in plant growth regulation and triggering of ISR (Ryu et al. 2003). As can be expected from signal molecules, they can be degraded and several pseudomonads apparently are able do so.

In conclusion, the results of the present study show that among the seven isolated *Pseudomonas* cucumber endophytes three strains have biocontrol activity. Moreover, we also found that, in contrast to most rhizospheric *Pseudomonas* spp., endophytic pseudomonads were able to utilize L-arabinose, one of the most abundant sugars in the xylem fluid of various plants.

Acknowledgements

The research described here was supported by the Netherlands Organization for Scientific research (NWO, project number 047.018.001) and represents part of the Russian-Dutch collaboration project “Center of Excellence”. We thank Dr. Bernadette Kroon (Syngenta B.V., Enkhuizen, the Netherlands) for providing us with tomato and cucumber seeds. We thank Gerben Voshol for his help in some experiments and for critically reading the manuscript.

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General discussion

Limitations for the isolation of endophytic bacteria

Endophytic bacteria can be found in virtually all parts of a plant, including roots and stems (Chapters 5 and 7) as well as reproductive and storage organs (Chapter 5). Since various plant tissues react differently to chemicals which are used for surface sterilization, each plant and plant organ require adjusted sterilization procedures. A harsh treatment of delicate plant samples can reduce quantitative and qualitative evaluation of bacterial endophytes, particularly of vegetative cells. On the other hand, a too mild surface sterilization may result in the isolation of epiphytic microorganisms which would be incorrectly identified as endophytes and this is why a proper control on sterility is required. Therefore, selecting an adequate sterilization protocol is a crucial step in endophytic research. We demonstrated that certain variations of established disinfection protocols (concentration of chemicals and incubation time) result in efficient sterilization of plant surfaces and allow the isolation of various phylogenetic groups of endophytic bacteria (Chapter 5 and 7).

From a technological point of view, cultivability and fast multiplication of microorganisms are important prerequisites for the production of effective bioproducts. In this context, use of the appropriate nutrient media and growth conditions can favor growth of some species over others and, as a result, increase the possibility of obtaining useful strains. For this reason, bacterial endophytes were isolated on general nutrient medium which supports the growth of fast-growing heterotrophic bacteria, such as those belonging to the phyla Firmicutes and Proteobacteria which harbor the majority of the species of agricultural importance (Chapters 5 and 7). To recover bacteria from a low-nutrient environment such as plant tissues, diluted synthetic media have been successfully used (Chapters 5 and 7; Adams and Kloepper, 2002). However, even after simulating the natural habitat, up to 99% of the bacteria cannot be isolated due to their (yet-) unknown growth requirements (Donachie et al., 2007). Recent progress in applying metagenomic tools and analysis of sequence information from the entire population might facilitate in finding an optimal cultivation strategy for (yet-) uncultured microorganisms and therefore result in increasing the number of biotechnologically promising strains (Handelsman, 2004).

Siblings, plant-beneficial strains and human pathogens

Isolation of bacteria in pure culture was followed by several initial screenings in order to discard siblings (Chapters 5 and 7). This procedure considerably decreased the number of strains for subsequent analysis and therefore made the remaining part of the screening process less laborious and time-consuming. Identification of the remaining strains showed the numerical prevalence of gram-negative Gammaproteobacteria (71%) over gram-positive Firmicutes (24%) and Actinobacteria (5%) (Table 3 in Chapter 5; Table 2 in Chapter 7). This finding confirms some of the earlier reports that members of the Gammaproteobacteria head the list of the most abundant culturable endophytes of many different plants (Kuklinsky-Sobral et al., 2004; Khan and Doty, 2009; Taghavi et al., 2009). As many as seven different genera were identified within these phyla, with *Bacillus* and *Pseudomonas* being the most frequently isolated ones (Chapters 5 and 7).

With regard to the plant-beneficial traits, 76% of the isolated strains had at least one of the tested beneficial properties indicating the occurrence of a large proportion of possible plant-beneficial strains among the isolated endophytic bacteria (Table 2 in Chapter 5; Table 1 in Chapter 7). The most common endophytic traits were (i) secretion of auxin (48%) and (ii) production of fungal cell-wall degrading enzymes (38%). The proportion of endophytic bacteria with antagonistic properties towards one or more fungal pathogens was 24% which is comparable with values reported by Berg et al. (2005) for endophytic bacteria of potato roots. Interestingly, the percentage of bacterial antagonists in the endosphere (21%) was always higher than found in the rhizosphere (14%). This fact, together with the notion that the root endosphere is the primary site attacked by most soilborne pathogens, allows the suggestion that plants can harbour specific endophytic bacterial groups in response to environmental stress. This suggestion is further supported by work of Siciliano et al (2001) who found that a number of bacterial genotypes containing catabolic genes for the degradation of petroleum hydrocarbons and nitrotoluenes increased in the interior of plant roots in response to soil pollution and that this response was contaminant-dependent. Understanding to which extent plants can regulate their bacterial inhabitants, remains an interesting research direction.

Along with agriculturally important strains, the identified phyla are known to comprise a number of well-known human and plant pathogens. For this reason it is important to evaluate the biosafety risk of the isolated bacteria and to do this at an early stage of the screening for bioinoculant agents. Indeed, we found that all analyzed

plants, except *Heracleum sosnowskyi*, harbor close relatives of species associated with human and plant diseases (Chapters 5 and 7). Moreover, these species account for 43% of the diversity of the entire bacterial collection indicating a remarkably high incidence of potential pathogens among endophytes. This finding supports previous reports that pathogenic bacteria are widespread in many natural environments and that they use plants as alternative hosts and an important source of transmission (Berg et al., 2005; Tyler and Triplett, 2008). Although some of these potential pathogens have interesting plant-beneficial traits (Table 2 in Chapter 5 and Table 1 in Chapter 7) and might miss some of the virulence factors present in clinical isolates (Dong et al., 2003), they are prohibited for agricultural application due to their possible threat to human and environmental health. Therefore, they were excluded from further studies.

Endophytic bacteria promoting plant growth

Auxin production is the best documented mechanism of plant growth promotion used by various plant-associated bacteria, including endophytes (Chapters 2 and 3; Spaepen and Vanderleyden, 2011). Inoculation of plants with auxin-producing strains can result in increasing the total root absorption surface and subsequent nutrient uptake leading to enhanced plant growth and biomass production. However, we did not observe the expected phytostimulating effect of the auxin-producing endophytic bacteria after their application on radish, tomato or cucumber plants (Chapters 5 and 7). Moreover, the IAA-producing strain *P. fluorescens* WCS365 which shows plant growth promotion of these plants also failed to stimulate the plant biomass in our experiments (Malfanova et al., *unpublished*). Taking this fact into consideration, our results can possibly be explained by assuming increased plant sensitivity to exogenous auxins under certain growth conditions, particularly when P is limited. For example, it has been shown that when P is not limiting, even low concentrations of exogenous IAA (up to 100 nM) increase nodule numbers as well as dry shoot and root weight of common bean (Remans et al., 2007). However, when P is low, the same concentrations of IAA can have a negative effect on nodule formation. In agreement with this, the increase in nodulation induced by the well-known IAA-producing strain *Azospirillum brasilense* Sp245 detected under high P was not detected under low P (Remans et al., 2007).

Overall, these results indicate that soil fertility and plant nutrition status can determine the outcome of plant-microbial interactions. This suggestion is further supported by the fact that when NPK fertilizer was added to soil, two IAA-producing endophytic strains, namely *Pseudomonas putida* CR3 and *Rahnella aquatilis* HC2 were

able to stimulate growth of radish and some cereal plants (Zaplatkin et al., *unpublished*). Moreover, the phytostimulating effect of these strains was also recorded in salinated and heavy metal-contaminated soils (Fig. 1).

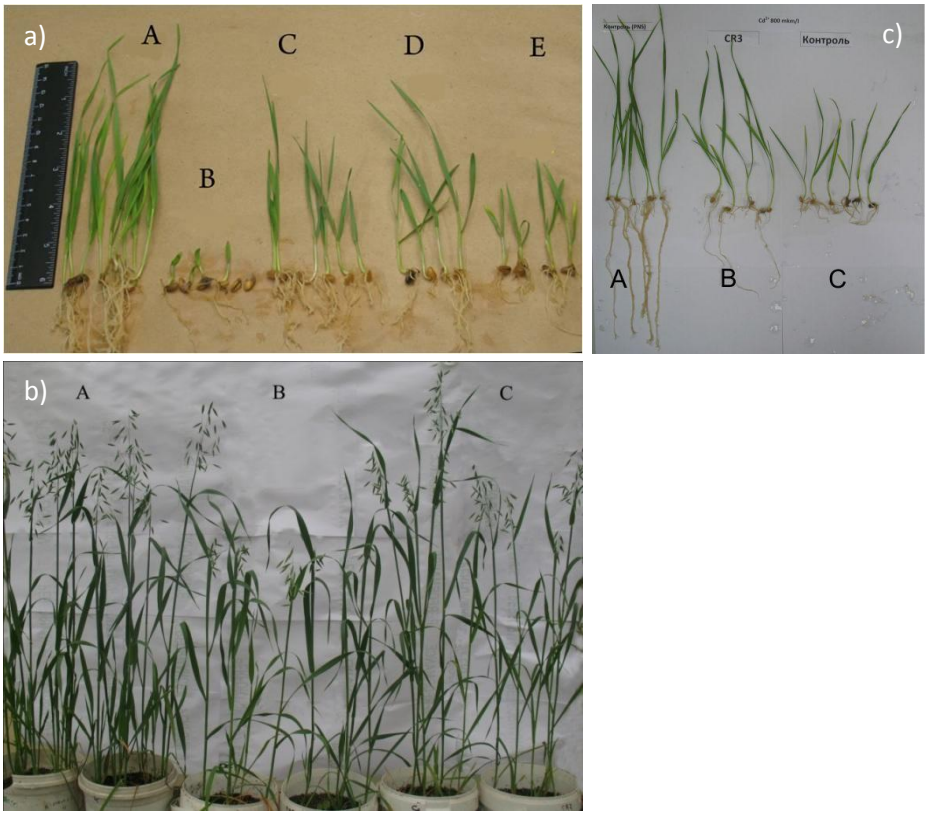

Fig. 1. The phytostimulating effect of endophytic bacteria on growth of cereal plants under high salt and heavy metal conditions.

a) Plant growth promotion of wheat by *Pseudomonas fluorescens* CR2 in the presence of 1% of salt. A – control without bacteria or salt added, B – control with 1% NaCl, C,D,E – three replicates of the experiment in which the bacterium and salt were present. A positive effect was also observed for the bacteria *Bacillus subtilis* HC8, *P. putida* CR3 and *Rahnella aquatilis* HC2.

b) Effect of *R. aquatilis* HC2 on growth of oat plants in the presence of 0.25% of salt.

A – control without salt or bacteria added; B – control with 0.25% NaCl added; C – plants inoculated with bacteria and salt.

c) Plant growth promotion of wheat by *P. putida* CR3 in the presence of 800 µM Cd^{2+}.

A – control without Cd^{2+}; B – plants inoculated with bacteria and Cd^{2+}; C – control with Cd^{2+}. A positive effect was also observed for CR2, HC2 and HC8.

All experiments and photos are from A. Zaplatkin, ARRIAM, Saint-Petersburg, Russia.

Along with auxins, other phytohormones are known to have a stimulatory effect on plant growth (Chapters 2 and 3). Since *Bacillus subtilis* HC8 is capable of promoting plant growth of both radish (Chapter 5) and wheat plants (Zaplatkin et al., *unpublished*) but does not produce detectable amounts of auxin, we tested this strain for its ability to produce cytokinins and gibberellins. While cytokinin production was not observed, a high amount of bioactive gibberellin was found to be secreted by *B. subtilis* HC8. Generally, GB-producing bacteria increase the endogenous content of these phytohormones and promote shoot and root growth of plants (Joo et al., 2005). However, since we did not detect GB in the tested plants, we cannot exclude an effect of other phytostimulatory compounds on growth promotion. Evidence for this notion was obtained by finding that *B. subtilis* HC8 was able to influence the growth of Arabidopsis seedlings in a split-plate assay when bacteria and plants were physically separated from each other (Malfanova et al., *unpublished*). This is likely due to the production of volatile compounds which have been shown to stimulate growth of various plants (Ryu et al., 2003).

Endophytic *Bacillus* and *Pseudomonas* with biocontrol properties

Species of *Bacillus* have attracted considerable attention because they produce many potent antibiotics. Among them c-LPs present the structurally most diverse group due to the variation in the amino acid composition of the peptide ring as well as in the length, branching and saturation of the acyl chain (Chapter 4). We found that *B. subtilis* HC8 is able to produce all three major families of c-LPs, namely iturin A, fengycin A and B and surfactin (Table 2 in Chapter 6). Within each family, we detected a remarkable high number of different homologues – more than has been reported for other beneficial bacilli, including for the commercial strain *B. amyloliquefaciens* FZB42 (Koumautsi et al., 2004). Moreover, these homologues comprise variants with long fatty acyl chains which are assumed to be more bioactive (Ongena and Jacques, 2008; Raaijmakers et al., 2010). In case of fengycins, long C18 homologues of fengycin A and B secreted by HC8 have been described only for a limited number of strains (Ongena et al., 2005; Nihorimbere et al., 2012).

These results suggest that HC8 is a powerful producer of c-LPs, a trait which can favor broad biotechnological applications of this strain, for example in the control of phytopathogens. Indeed, HC8 shows biocontrol in both stonewool substrate (Chapter 5) as well as in soil (Malfanova et al., *unpublished*) and is able to reduce TFRR of tomato plants by almost 50 and 25%, respectively. It is interesting to note that the two

other c-LP-producing endophytic bacilli which lack some of the c-LP fractions present in HC8 (*unpublished*), failed to show significant biocontrol in any of the tested substrates (Chapter 5).

There are three main modes of action by which endophytic bacteria can exert biocontrol of phytopathogens. These are antibiosis, CNN and ISR (Chapter 4). We found that fengycin and iturin LPs are responsible for most, if not all, antifungal activity of HC8 in *in vitro* assays against *Forl* (Chapter 6). Whereas these metabolites can suppress growth of *Forl* in soil, we expect that antibiosis is not powerful in stonewool because of diffusion of c-LPs in PNS. In addition to antagonistic activity, c-LPs are known to trigger ISR. Particularly, fengycins and surfactins induced significant protection in bean and tomato leaves against *Botrytis cinerea* following root treatment (Ongena et al., 2007). c-LPs, and more specifically surfactins, can also stimulate motility (Bais et al., 2004) and solubilization of plant nutrients (Lindow and Brandl, 2003), the two processes important for CNN (Lugtenberg and Kamilova, 2009). These data suggest that production of c-LPs can be the molecular basis of at least three different disease control mechanisms.

Other examples of shared determinants of different biocontrol mechanisms include volatiles (Ryu et al., 2003) and hydrolytic enzymes (Connelly et al., 2004). These compounds are also produced by HC8 (Chapters 5 and 8) and could also be involved in the biocontrol of TFRR by this strain. Moreover, since c-LPs, hydrolytic enzymes and volatiles are involved in different modes of action, we can speculate that HC8 can use a combination of different disease control mechanisms. Therefore, it is not very likely for a pathogen to acquire resistance to HC8, which makes this strain a strong candidate for the development of a biofungicide. Also, its ability to interact with multiple plants (see the previous section) and to express a beneficial effect under different growth conditions can facilitate its use in multifaceted plant protection.

Three other promising biocontrol strains characterized in our study are *P. fluorescens* CS1 and CR2 and *P. putida* CR3 (Chapter 7). Unlike *B. subtilis* HC8, these strains do not produce any exo-enzymes or antifungal metabolites, except for CR3 which secretes protease and inhibits the oomycete *P. ultimum in vitro* (Table 1 in Chapter 7). Nevertheless, strains CR2 and CR3 appeared to be able to significantly reduce TFRR symptoms in soil and had a biocontrol effect comparable to HC8 (Fig. 1b in Chapter 7). Whereas CS1 failed to show biocontrol in soil, it was effective against TFRR in stonewool substrate where the other two pseudomonads were not active.

Endophytic lifestyle

With regard to plant growth promotion and biocontrol, endophytism is in principle advantageous since endophytic biological agents can form a long-lasting association with plants in a relatively safe environment. Therefore, it is important to demonstrate the endophytic lifestyle of promising strains after production outside the plant and subsequent application. This can be done by visualization of labeled bacteria in inner plant tissues using confocal laser scanning microscopy (CLSM). Despite numerous attempts, we could not label *B. subtilis* HC8, neither by transforming it with reporter plasmids, nor by hybridizing it with fluorescent probes *in situ*. However we were able to show an endophytic lifestyle for *P. fluorescens* CS1 in gnotobiotic tomato plants using FISH followed by CLSM (Fig. 2). Microcolonies of CS1 have been detected in cortex tissues of seven days-old tomato roots as can be clearly observed in 3D projections (Fig. 2d-h).

Microscopic results of endophytic colonization of tomato plants by CS1 were further supported by the introduction of a rifampicin-resistant derivative of this strain inside tomato seeds followed by its re-isolation on antibiotic-containing medium (Malfanova et al., *unpublished*). Introduction of a biological agent inside a seed is generally referred to as seed biopriming and its aim is physiological enhancement of seed germination and plant vigor (Müller, 2006). Colonization of inner seed structures by bacterial cells is likely to be initiated by the process of water uptake by dormant seeds (imbibition) and release of growth substances which are readily used by bacteria (Bewley and Black, 1993). After four hours of incubation (imbibition time of tomato seeds) and two hours of air-drying, the population density of bacteria on the seed surface varies between 10^2-10^3 CFU per seed. Similar values were obtained for endophytic populations of rifampicin-resistant CS1^r following 36 h germination of seeds under gnotobiotic conditions. This value increases to 10^5 cfu for 7-days old tomato seedlings. These results indicate that CS1 is not only able to maintain itself, but can also thrive within a growing plant.

For successful endophytic colonization, bacteria should be capable of efficient utilization of carbon sources available inside a plant. In Chapter 7 we suggested that utilization of L-arabinose by endophytic pseudomonads might be important for their endophytic lifestyle in cucumber plants. It is unlikely that L-arabinose utilization is the only trait relevant for this complex life style. For example, other major xylem nutrients could also play a role, certainly in crops with a different xylem composition as well as for other endophytic bacterial species.

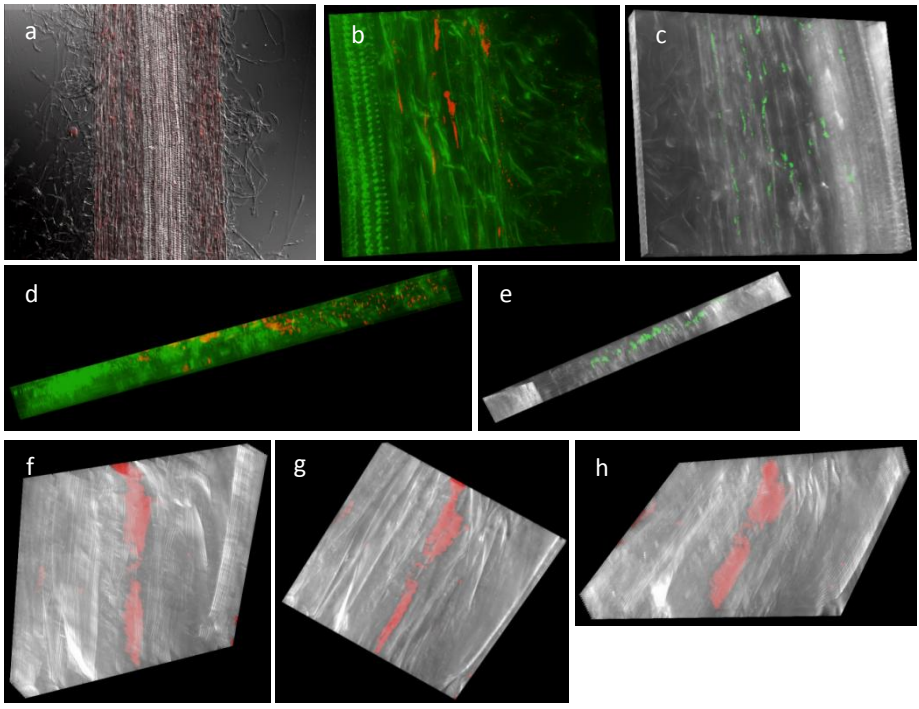

Fig. 2 Endophytic colonization of tomato roots by *Pseudomonas fluorescens* CS1 revealed by FISH followed by CLSM.

Tomato seeds were surface-sterilized using twice a 4% sodium hypochlorite treatment for 10 min followed by 10 washes with sterile water. Sterile seeds were soaked for 30 min in a bacterial suspension of CS1 adjusted to 10^8 cfu/ml. The treated seeds were then placed in a quartz sand gnotobiotic system and allowed to germinate for seven days. Subsequently, plant roots were aseptically removed and treated according to the FISH protocol described by Cardinale et al., 2008.

a – 10× magnified (objective) section of a tomato root heavily colonized with CS1 (bacterial signal shown in red);

b,c – 20× magnified (objective) sections of a tomato root with fluorescent signal in the cortex (bacterial signal is in red (b) and green (c).

d,e – z-stack of sections b and c, respectively, viewed from the top

f,g,h – z-stack of 200× magnified (objective) section of tomato root with macrocolonies of CS1.

Experiment and photos are from A. Shcherbakov, ARRIAM, Saint-Petersburg, Russia.

Concluding remarks and future prospects

In this research project we succeeded in the isolation of many endophytic strains with plant-growth promoting and biocontrol abilities. Among them, *B. subtilis* HC8 isolated from giant hogweed proved to be the most versatile promising bioinoculant since it expressed its beneficial effect on diverse plants under different growth conditions. It was also shown that this strain, so far, is the only endophytic *Bacillus* – and one out of a few *Bacillus* strains – which is capable of producing excessively high levels of a large variety of c-LPs. The high and varied production of c-LPs and other metabolites by HC8 might partly explain its excellent plant growth-promoting and biocontrol properties and favor its broad biotechnological application. This and other beneficial endophytic bacteria are currently being tested in pilot trials under various environmental conditions to select the most effective strains with a wide spectrum of action. For final bioproduct development, a number of important aspects still need to be further investigated, including evaluation of toxicity and environmental impact, fermentation, preservation, storage and formulation.

Our results as well as literature data suggest that the plant response to endophytic bacteria is a very complex process which involves interplay of many known and (yet-) undefined biotic and abiotic factors. Therefore, understanding mechanisms of beneficial action of selected endophytes as well as of plant growth parameters, such as soil type and nutritional status, can help to develop a better formulation and application strategy. With regard to plant growth conditions, additional greenhouse and field experiments are required to provide more conclusive information about the potential of using auxin-producing strains for plant inoculation in soil with limited nutrient resources, for example in combination with other beneficial bacteria.

Attempts were made to shed light on the endophytic lifestyle of several isolated strains. For *P. fluorescens* CS1, we confirmed its endophytic nature by a combination of microscopy studies and re-isolation of the introduced bacterium. These results are promising for further studies, e.g. to use multiple time points to examine entry sites for bacterial cells and to follow bacterial spreading inside the plant. Whereas such colonization studies are normally conducted under gnotobiotic conditions, it would be more relevant to investigate whether the beneficial strains also have the endophytic lifestyle of under practical conditions.

We found that, in contrast to most rhizospheric *Pseudomonas* spp., endophytic pseudomonads isolated from cucumber plants were able to utilize L-arabinose, one of the most abundant available sugars in the xylem fluid of various plants. This and other

(yet-) unidentified traits could contribute to the complex interaction of endophytic bacteria and plants. To further test the role suggested for L-arabinose, it would be interesting to use an L-arabinose utilization-negative mutant of one of the isolated pseudomonads and compare the abilities of wild type and mutant to live endophytically.

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Summary

In **Chapter 1** I give a brief introduction to the topic of my Ph.D. thesis and its main aims. Since global food insecurity is one of the major problems faced by humanity, there is a necessity to increase plant productivity. For this, biofungicides and biofertilizers present an ecologically friendly alternative to their chemical counterparts. Among these bioinoculants, endophytic bacteria with plant growth promotion and biocontrol abilities are the most promising candidates due to their ability to colonize the plant's interior and establish a stable, long-lasting relationship with a plant.

In **Chapter 2** I give a detailed overview of endophytic bacteria and their interactions with plants. According to the current knowledge, more than 200 different bacterial genera from 16 phyla can be found as endophytes, with Actinobacteria, Firmicutes and Proteobacteria being the best studied phylogenetic groups. They colonize virtually all plants and plant organs and can enter a plant through the root zone, phylosphere and antosphere, or can be vertically transmitted from one generation to another. For successful rhizosphere colonization and subsequent endophytic establishment, bacteria have to possess a number of "competence" traits, such as expression of surface components (e.g. pili, LPS, EPS) as well as production of cell-wall degrading enzymes and the ability to utilize certain plant compounds. Inside a plant, endophytic bacteria can positively influence plant growth through providing plants with N and/or modulating the phytohormone level. Some endophytes protect plants from pathogens via ISR or, most likely, by using a combination of different biocontrol mechanisms. Analysis of the 13 sequenced endophytic genomes revealed the existence of additional traits which are possibly involved in endophytic colonization and in beneficial interactions with a plant.

In **Chapter 3** we describe different plant growth promoting microbes and their modes of beneficial action. For example, Arbuscular Mycorrhizal Fungi and *Trichoderma* have a general growth promoting effect on plants through a variety of mechanisms. Other microbes, e.g. *Bacillus*, *Pseudomonas*, *Serratia* etc. have a more specific effect by providing a plant with certain nutrients (e.g. N, P and Fe^{3+}). The well-recognized mechanisms mediated by these bacteria include biological nitrogen fixation, phosphate solubilization and siderophore production. The third class of beneficial

microbes includes microbial plant growth regulators which secrete hormones (e.g. auxins, cytokinins, gibberellins, etc.) or hormone-like substances (e.g. volatiles, *N*-Acyl Homoserine Lactones) which stimulate plant growth in extremely low concentrations.

In **Chapter 4** we describe different mechanisms of disease control by microbes. The majority of plant-associated bacteria produce various antifungal metabolites (e.g. c-LPs of *Bacillus* spp.) which can play a role in antibiosis and ISR. In addition to antifungal metabolites, ISR can also be triggered by bacterial molecules and organelles such as flagella, LPS, siderophores etc. Some beneficial bacteria are capable of signal interference with a pathogen by production of AHL-lactonases and AHL-acylases, which destroy AHLs which are required for the expression of pathogenicity factors. Moreover, biocontrol bacteria can interfere with activity, survival, multiplication, germination, sporulation and spreading of a pathogen. Other biocontrol mechanisms include CNN, predation and parasitisms and competition for Fe^{3+}.

In **Chapter 5** I describe the isolation and partial characterization of the novel biocontrol and plant-growth promoting endophytic bacterium *Bacillus subtilis* HC8 from giant hogweed. This strain exhibits a high number of plant-beneficial traits including *in vitro* production of the phytohormone gibberellin, of cyclic lipopeptide antibiotics and fungal cell-wall degrading enzymes. When applied to seeds, *B. subtilis* HC8 is able to stimulate plant growth and suppress TFRR. Moreover, this strain has the ability to express its beneficial effect on different plant hosts which is an important prerequisite of an efficient bio-inoculant agent.

In **Chapter 6** I describe the identification of the lipopeptide antibiotics produced by *B. subtilis* HC8 and evaluation of their antifungal effect against *Forl in vitro*. We showed for the first time that an endophytic *B. subtilis* is able to produce all three major families of lipopeptides with a remarkably wide range of different homologues. Among them, fengycins are the most potent lipopeptides with regard to their fungicidal activity. We expect that the wide range of different homologues strongly contributes to the excellent disease control properties of the strain. Surprisingly, we did not find any synergism between different groups of lipopeptide antibiotics in contrast to some claims in the literature.

In **Chapter 7** I describe novel biocontrol endophytic *Pseudomonas* spp. isolated from cucumber plants. The selected biocontrol strains do not exhibit any of the potential plant-beneficial traits *in vitro*. Nevertheless, when applied to seeds, they were able to significantly suppress TFRR caused by *Forl* in a growth substrate-dependent way. Perhaps most interesting is the observation that utilization of L-arabinose by endophytic pseudomonads might be important for their endophytic lifestyle in the cucumber plants.

In **Chapter 8** I discuss the major findings of this thesis and included some unpublished results. For example, I showed that *B. subtilis* HC8 and *Rahnella aquatilis* HC2 from giant hogweed as well as *P. fluorescens* CR2 and *P. putida* CR3 from cucumber not only reduce stress of cereal plants caused by salinity and heavy metals, but have a stimulatory effect on plant growth as well. I also presented strong evidence of endophytic colonization of tomato plants when the biocontrol strain *P. fluorescens* CS1 was applied.

Samenvatting

In **Hoofdstuk 1** presenteer ik een korte inleiding over het onderwerp van mijn proefschrift evenals de belangrijkste doelstellingen. Omdat de behoefte aan voedsel in de wereld sterk zal toenemen is het nodig de productiviteit van gewassen te vergroten. Daarvoor zijn biologische antischimmel middelen en biologische meststoffen ecologisch vriendelijke alternatieven voor chemische producten. Onder deze biologische middelen zijn endofytische bacteriën met ziekteonderdrukkende en/of groeibevorderende eigenschappen de meest belovende kandidaten omdat ze zich binnenin de plant vestigen om daar een stabiele, langdurende relatie met de plant op te bouwen.

In **Hoofdstuk 2** presenteer ik (met enkele co-auteurs) een gedetailleerd overzicht over endofytische bacteriën en hun interacties met planten. Volgens onze huidige kennis worden meer dan 200 verschillende bacteriële genera van 16 phyla gevonden als endofyten met Actinobacteria, Firmicutes en Proteobacteria als de best bestudeerde fylogenetische groepen. Ze koloniseren bijna alle planten en plantenorganen en zijn in

staat de plant binnen te gaan via de wortelzone, de fylosfeer en de antosfeer, of ze kunnen verticaal worden doorgegeven van de ene generatie naar de andere. Voor succesvolle kolonisatie van de rhizosfeer en de daaropvolgende vestiging als endofyt, dienen bacteriën over een aantal competenties te beschikken, zoals expressie van oppervlaktecomponenten (bv. pili, lipopolysaccharide, exopolysaccharide etc.) alsmede de productie van schimmelcelwand-afbrekende enzymen en het vermogen bepaalde planten bestanddelen te gebruiken als voedsel. Binnenin de plant kunnen endofytische bacteriën een positieve invloed hebben op de groei van de plant door deze te voorzien van stikstof of door het nivo van hun hormonen te veranderen. Sommige endofyten beschermen de plant tegen ziekteverwekkers via ISR (induced systemic resistance) of (vaker) door een combinatie van verschillende mechanismen. Analyse van dertien genomen van endofyten liet het bestaan zien van nóg meer eigenschappen die mogelijk betrokken zijn bij endofytische kolonisatie en van hun gunstige interacties met de plant.

In **Hoofdstuk 3** beschrijven mijn co-auteurs en ik verschillende plantengroeibevorderende bacteriën alsmede de manier waarop ze hun gunstige werking uitvoeren. AMF (Arbuscular Mycorrhizal Fungi) en *Trichoderma* vertonen bijvoorbeeld een algemeen groeibevorderend effect op planten via een aantal verschillende mechanismen. Andere micro-organismen zoals bv. *Bacillus*, *Pseudomonas* en *Serratia* hebben een meer specifiek effect door de plant van bepaalde voedingsstoffen te voorzien (bv. N, P en Fe^{3+}). De bekende mechanismen die door deze bacterien worden gebruikt zijn o.a. biologische stikstoffixatie, fosfaat oplossen en productie van sideroforen. De derde groep van gunstige micro-organismen zijn de microbiële plantengroeibevorderaars die hormonen (bv. auxines, cytokinines, gibberellines, etc) of hormoonachtige stoffen (bv. vluchtige organische stoffen en AHLs (*N*-acyl homoserine lactones) uitscheiden die de plantengroei in extreem lage concentraties stimuleren.

In **Hoofdstuk 4** beschrijven mijn co-auteurs en ik verschillende mechanismen van ziekte beheersing door micro-organismen. De meerderheid van de plant-geassocieerde bacteriën produceert verschillende anti-schimmel metabolieten (bv. c-LPn (cyclische lipopeptiden) van *Bacillus* spp.) die een rol kunnen spelen bij antibiose en/of ISR. ISR kan worden veroorzaakt door anti-schimmel metabolieten en door bacteriele organellen en moleculen zoals flagellen, LPS en sideroforen. Sommige gunstige

bacteriën veroorzaken “signal interference” door productie van AHL-lactonases en/of AHL-acylases die AHLs inactiveren. AHLs zijn noodzakelijk voor de productie van vele pathogeniteitsfactoren. Ziekteonderdrukkende bacteriën kunnen ook interfereren met activiteit, overleving, vermeerdering, kieming, sporulatie en verspreiding van een pathogeen. Andere biocontrole mechanismen zijn CNN (competition for nutrients and niches), predatie en parasitisme, en competitie voor Fe^{3+}.

In **Hoofdstuk 5** beschrijf ik de isolatie en gedeeltelijke karakterisatie van de unieke biocontrole en plantengroeistimulerende bacterie *Bacillus subtilis* HC8, geïsoleerd uit de plant *Heracleum sosnowskyi* Manden. Deze stam heeft een groot aantal voor de plant gunstige eigenschappen zoals de productie van het hormoon gibberelline, van c-LP antibiotica en van schimmelcelwand afbrekende enzymen. Na op zaad te zijn aangebracht is *B. subtilis* HC8 in staat plantengroei te bevorderen en de ziekte TFRR (tomato foot and root rot) te reduceren. Bovendien kan deze stam haar gunstige effect tot expressie brengen op diverse waardplanten, hetgeen een belangrijke eigenschap is voor een commercieel efficiënt product.

In **Hoofdstuk 6** beschrijf ik de identificatie van de c-LP antibiotica van *B. subtilis* HC8 en de evaluatie van hun antischimmel werking tegen *Forl in vitro*. We toonden aan dat *B. subtilis* HC8 het eerste voorbeeld is van een endofytische *Bacillus* die alle drie belangrijke families van c-LPs produceert en dat tevens doet met een opvallend breed scala aan verschillende analogen van elk van deze drie families. Onder deze c-LPn zijn de fengicines de meest potente antischimmel agentia. We verwachten dat het brede scala aan verschillende analogen sterk bijdraagt aan de voortreffelijke ziekteonderdrukkende eigenschappen van deze stam. Tot onze verbazing vonden we geen synergie tussen de verschillende groepen c-LP antibiotica, in tegenstelling tot sommige claims daarover in de literatuur .

In **Hoofdstuk 7** beschrijf ik nieuwe endofytische *Pseudomonas* soorten geïsoleerd uit komkommerplanten. *In vitro* experimenten met de geselecteerde *Pseudomonas* biocontrolestammen leverden geen aanwijzingen voor potentiële voor de plant gunstige eigenschappen. Desondanks zijn ze in staat om, na coaten op zaad, de door *Forl* veroorzaakte ziekte TFRR significant te onderdrukken, op een substraatafhankelijke wijze. De mogelijk meest interessante waarneming is dat het

vermogen van endofytische pseudomonaden om L-arabinose als koolstofbron te gebruiken belangrijk zou kunnen zijn voor de endofytische levenswijze in komkommer.

In **Hoofdstuk 8** bediscussieer ik de belangrijkste resultaten uit dit proefschrift en beschrijf ik sommige recentere resultaten. Ik laat bv. zien dat zowel *B. subtilis* HC8 en *Rahnella aquatilis* HC2 van *Heracleum sosnowskyi* Manden als *P. fluorescens* CR2 en *P. putida* CR3 van komkommerplanten niet alleen de door zout en zware metalen veroorzaakte stress bij granen verminderen, maar de groei van de plant ook nog stimuleren. Tenslotte beschrijf ik sterke aanwijzingen voor endofytische kolonisatie van tomaat door de biocontrolestam *P. fluorescens* CS1.

Curriculum vitae

Natalia Vitalyevna Malfanova was born on the 16th of November 1984 in Staraya Russa, Russian Federation. In 2002 she finished high school and moved to Saint-Petersburg where she continued her study as a biology student at the Saint-Petersburg State University. In 2005 she became a visiting researcher of the All-Russia Research Institute for Agricultural Microbiology (ARRIAM) where she conducted her Bachelor and Master projects under supervision of Dr. V. Chebotar. In 2007, within the Russian-Dutch "Centre of Excellence" cooperation project she visited the Department of Molecular Microbiology at the Institute of Biology of Leiden University where she worked with plant beneficial bacteria under the supervision of Prof. Dr. E. (Ben) J.J. Lugtenberg and Dr. Faina Kamilova. In 2008 she graduated from the Saint-Petersburg State University with a Master degree in Microbiology and started working on her PhD thesis "Endophytic bacteria with plant growth promoting and biocontrol abilities" under supervision of Prof. Dr.E.J.J. Lugtenberg.

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