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Stenotrophomonas: genomic and transcriptomic studies to understand the mechanisms behind plant growth promotion and the biocontrol effect and the role of quorum sensing therein

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Abstract

The genus *Stenotrophomonas* includes species with entirely different habitats and economic importance. While clinical strains of *S. maltophilia* are opportunistic human pathogens, *S. rhizophila* species includes significant biocontrol and plant growth promoting agents. Thus far, several *S. maltophilia* genomes have been sequenced and a few also characterized, but there is nothing known regarding the genetic information, genome characterization or gene expression in *S. rhizophila*. Moreover, many questions also remain unanswered regarding the regulation of positive plant-bacteria interactions including the potential impact of the community-driven population-regulatory mechanism through quorum sensing. In this work, the role of the *rpf*/DSF quorum sensing system in the beneficial plant-associated *S. maltophilia* R551-3 model strain was studied, which revealed that positive interactions with the plant host were significantly controlled by the *rpf*/DSF system. The DSF-controlled effects on the host plant relied upon the regulation of a great number of physiologically crucial genes and were evident in seed germination, plant growth promotion, and plant tissue colonization. In another part of this work, the genome of the plant growth promoting and biocontrol strain *S. rhizophila* DSM14405^T was characterized regarding both general and specific characteristics and biological functions. Moreover, whole-genome comparisons with the plant-associated beneficial *S. maltophilia* R551-3 model strain and the clinical *S. maltophilia* K279a were performed which revealed a great degree of similarity among all three strains. The great genome-wide similarity found between *S. rhizophila* and the clinical *S. maltophilia* K279a is very intriguing, as these species have entirely different habitats, lifestyles and economic significance. Nevertheless, a great number of physiologically crucial genes were revealed to be specific to *S. rhizophila* DSM14405^T. These genes play a crucial role in enabling *S. rhizophila* to function as a distinguished plant growth promoting and biocontrol agent, as transcriptomic studies revealed. Furthermore, the impact of salt stress on *S. rhizophila*, which is known to be highly resistant against salinity, was studied using transcriptomics which confirmed the production and excretion of glucosylglycerol (GG) as the main salient substance responsible for salt stress protection. All in all, this work significantly contributed to achieving a better understanding of the mechanisms behind biological control and the plant growth promoting effect by *Stenotrophomonas* strains and the regulatory role the *rpf*/DSf quorum sensing system plays therein.

Zusammenfassung

Die Gattung *Stenotrophomonas* beherbergt verschiedene Arten, welche sich in Bezug auf das Habitat und die wirtschaftliche Bedeutung stark unterscheiden. Beispielsweise während zahlreiche *S. maltophilia*-Stämme klinischer Herkunft und somit opportunistisch humanpathogen sind, gehören die isolierten Stämme der Spezies *S. rhizophila* hingegen zu den ‚Plant Growth Promoting' (PGP)- Bakterien und sind ‚biocontrol agents' ohne klinisches Potential. Während in den letzten Jahren das Genom einiger klinisch relevanten *S. maltophilia*-Spezies sequenziert und in manchen Fällen charakterisiert wurde, liegen hingegen keine Studien über die Genom-Information, Charakterisierung bzw. Genexpression von *S. rhizophila* vor. Außerdem ist es weiterhin unklar ob und welche Rolle einem selbstregulatorischen Mechanismus mittels Quorum-Sensing in Hinblick auf die positive Pflanzen-Bakterien-Interaktionen zuzuschreiben ist. Ein Teil dieser Arbeit untersuchte die potentielle Rolle des *rpf*/DSF Quorum-Sensing hinsichtlich dieser positiven Interaktionen im Beispiel des Modellstammes *S. maltophilia* R551-3, welcher pflanzenassoziiert vorkommt und PGP-Wirkung aufweist. Zum einen konnte es bewiesen werden, dass die positiven Interaktionen mit der Wirtspflanze stark vom DSF-System reguliert werden. Zum anderen zeigte sich, dass das System im Speziellen über die Regulation einer Reihe von physiologisch bedeutenden Genen funktioniert, was letztendlich zur starken Aufkeimung der Samen, Besiedlung der Pflanzenteile sowie PGP-Effekt führt. Ein weiterer Teil dieser Arbeit beschäftigte sich mit der funktionellen Charakterisierung des Genoms von *S. rhizophila* DSM14405^T, welcher einen ausgeprägten PGP-Effekt aufweist und eine strake Biokontroll-Wirkung besitzt. Des Weiteren wurde das Genom von *S. rhizophila* mit dem von *S. maltophilia* R551-3 sowie den klinischen *S. maltophilia* K279a verglichen, was eine starke Ähnlichkeit und genetische Gemeinsamkeit aller drei Genome feststellen ließ. Insbesondere, ist die hohe genetische Gemeinsamkeit mit *S. maltophilia* K279a interessant, denn diese stellt die Frage, welche genetischen und regulatorischen Faktoren für die gänzlich kontroverse Entwicklung der beiden Bakterien in Bezug auf den Lebensraum und die wirtschaftliche Bedeutung zuständig sind. Weiters ließen die Genomvergleiche eine Reihe *S. rhizophila* spezifischer Gene feststellen, welche für die Rolle von *S. rhizophila* als ein PGP-Stamm und ‚biocontrol agent' entscheidend sind. In der weiteren Folge, wurden Transkriptom-Untersuchungen durchgeführt, um den Effekt von Salzstress zu untersuchen. Hier ließ sich unter anderem feststellen, dass die Gene für die Synthese und Ausscheidung des Osmolyts glucosylglycerol (GG) eine wichtige Rolle bei der hohen Salzresistenz von *S. rhizophila* DSM14405^T spielen. Zusammengefasst trug diese Arbeit

zu einem besseren Verständnis in Bezug auf die für die Biokontrolle und PGP-Effekt zugrundeliegenden Mechanismen in der Gattung *Stenotrophomonas* sowie die entsprechende regulatorische Rolle von *rpf*/DSF-Quorum-Sensing-System bei.

Introduction

Growing agricultural challenges and the role of biocontrol and osmoprotection

Today's world agriculture faces an increasing threat by phytopathogens which can hardly be overcome by conventional methods of pest management. In addition to the limited efficiency of chemical-based pesticides, consumers are increasingly concerned about the environment, food safety and food quality. On the other hand, insufficient food and deficiencies of vitamins and micronutrients are widely spread in many developing countries and compensation requires extensive and expensive agricultural efforts. In many of these areas, soil salinization – originally caused by humidification due to the clearing of trees in order to expand agricultural land and intensified by salt brought in by ground water and strong irrigation – is an immense additional problem. In 1999, 42% of the arable land in Asia and 31% in the Near East and North Africa was irrigated while the area under irrigation in developing countries is estimated to increase by 27% between 1996 and 2030. Irrigation results in soil salinization which is estimated to reduce the world's irrigated area by 1-2% each year, hitting hardest in the arid and semi-arid regions (FAO 2005). Moreover, soil salinization causes saline and water unbalance stress to plants which consequently become more vulnerable to diseases caused by pathogens such as fungi. In addition to the issue of soil salinization, fumigation with methyl bromide and related compounds was the standard method for disease control in soils for many decades. However, the undifferentiated destruction of microbial communities leads to a vacuum effect in the soil allowing uncontrolled spread of pathogens unaffected by methyl bromide treatment or brought on the fields via plant seeds or seedlings (Ibekwe et al., 2001). Furthermore, methyl bromide is a greenhouse gas and the bromine released from methyl bromide depletes ozone in the stratosphere 60 times more severely than chlorine does (WMO, 1998). Considering the fast-growing challenges noted above, it has become increasingly compelling to apply environmentally and consumer-friendly biologicals as a sustainable solution in combination with the reduction of chemicals.

Biologicals based on naturally occurring antagonists are an environmentally friendly alternative to control soil-borne pathogens in the rhizosphere (Lugtenberg and Kamilova, 2009; Berg, 2009). In biotechnology, these root-associated beneficial microorganisms are applied as biological control agents (BCAs) and plant growth promoting rhizobacteria (PGPR) for the

biological control of plant pathogens, and growth promotion, respectively, with many of these being active in salinated soils too, due to their role as osmoprotectants (Egamberdieva et al., 2011). Others enhance stress tolerance and are harnessed as biofertilizers and phytostimulators or as rhizoremediators (Berg 2009). While the exploration has just started to detect bacterial inocula that provide resistance against salt stress and have a plant growth promotion effect on plants in saline soils, first reports have already proven promising (Mo et al., 2006; Egamberdieva et al., 2008; Nadeem et al., 2010).

Screening strategies for biocontrol and plant growth promoting bacteria

One important strategy to screen for bacteria capable of biological control and plant growth promoting activity includes salt tolerance tests and was suggested by Egamberdieva et al. (2011) who found that the majority of rhizobacterial strains were highly tolerant to salt. This finding can be explained by the permanently changing osmotic conditions due to exudation in the rhizosphere. In another study, fifty-two beneficial and salt-tolerant bacteria from all over the world were screened for their ability to promote growth and/or to control diseases caused by the soil-borne fungus *Fusarium solani* on cucumber and tomato plants under saline conditions (Berg, G., pers. commun.). The five best strains were used in large-scale greenhouse trials. Four of the five strains significantly controlled cucumber foot and root rot, reducing the percentage of diseased plants from 54% in the negative control to between 10 and 29% in the plants inoculated with bacteria. Furthermore, all five strains increased the dry weight by between 29 and 62%. In two consecutive years, all five strains significantly increased the plant height (by 4 to 15%) and the fruit yield (by 12 to 32%). The results with tomato were similar. The conclusion was that many beneficial bacteria isolated from plants grown in non-salinated soil are perfectly able to promote plant growth and control plant diseases in salinated soil. Moreover, *Stenotrophomonas rhizophila* strain DSM14405^{T}, which was originally isolated from the rhizosphere of oilseed rape, was one of the most effective strains in this study.

Another strategy to screen for bacteria promoting plant growth includes the synthesis of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase. ACC deaminase degrades the well-known plant stress hormone ethylene which results in plant growth enhancement. An

assay based on the ACC deaminase activity was used by Nadeem et al. (2010) to evaluate the plant growth promoting effect of rhizobacterial strains under saline conditions by conducting a jar experiment at 1 (original), 5, 10, and 15 dS m^{-1}. The four most effective strains were further evaluated in a pot trial at salinity levels of 1.46 (original), 5, 10, and 15 dS m^{-1}. In general, salinity depressed the growth of wheat, but inoculation improved the growth and yield of wheat compared with the non-inoculated controls. At the high salinity level (15 dS m^{-1}), plant height, root length, plant biomass, and grain yield increased up to 37, 70, 116, and 111%, respectively, compared with the control. Moreover, this study showed *Pseudomonas putida* W2 and *P. fluorescens* W17 to be the most effective strains for alleviating salt stress even at higher salinity levels.

Major mechanisms of plant growth promotion and biocontrol

Rhizosphere-associated bacteria with antagonistic activity against detrimental bacteria and fungi interact using various mechanisms including the inhibition of pathogens by producing antibiotics, toxins and bio-surfactants (antibiosis), competition over colonization sites and nutrients, minerals, e.g. iron through production of siderophores or efficient siderophore-uptake systems, degradation of pathogenicity factors of the pathogen such as toxins, the production of extracellular, cell wall-degrading extracellular enzymes such as chitinases and β-1,3 glucanases (Raaijmakers et al. 2009; Lugtenberg and Kamilova 2009), the synthesis and excretion of antifungal metabolites such as antibiotics, toxins and bio-surfactants (Jacobi et al., 1996, Berg and Ballin, 1994, Kobayashi et al., 1995, Dunne et al., 2000), and the synthesis of volatile organic compounds (VOCs) (Alström 2001, Wheatley 2002). Interestingly, in addition to direct antagonism, plant-associated bacteria can induce a systemic response in the plant that results in the activation of plant defence mechanisms (Pieterse et al. 2003). This response includes the innate immune system which harnesses general microbe-associated molecular patterns (MAMPs) of plants for the recognition of microbes, and the recently identified bacterial quorum sensing signalling molecules that induce systemic resistance against biotrophic plant pathogens (Schuhegger et al. 2006; Schikora et al. 2011; Schenk et al. 2012). The positive effect on plant growth is caused both indirectly through the mechanisms described above and directly through

the synthesis of auxins such as phytohormone indole-3-acetic acid (IAA) and other metabolites and enzymes such as ACC deaminase.

Stenotrophomonas rhizophila: a model bacterium for plant growth promotion and biological control under saline conditions

Stenotrophomonas isolates have a great potential for applications in biotechnology and biological control due to the high capacity to promote plant growth and their antagonism against various phytopathogenic fungi (Ryan et al., 2009). While the species *S. maltophilia* has become important as a multidrug-resistant nosocomial human pathogen which is associated with significant case/fatality ratios, particularly in patients who are severely debilitated or immunosuppressed, no pathogenic potential has ever been observed for the closely related species *S. rhizophila* (Wolf et al., 2002). Both species can be easily distinguished due to the production of osmoprotective substances and the occurrence of multidrug-efflux pumps (Ribbeck-Busch et al., 2005).

Several studies have reported the significant positive impact of *S. rhizophila* on plant growth which however was shown to be dose-dependent (Wolf et al. 2002, Suckstorff and Berg 2003). Furthermore, the treatment of plant seeds including wheat, tomato, lettuce, sweet pepper, melon, celery and carrot grown in the highly salinated soils of Uzbekistan with *S. rhizophila* DSM 14405^T resulted in significant plant growth promotion which was evident in both higher germination rates and longer shoots and roots. For example in tomato, the germination rate, the growth of shoot and root was at 180%, 120%, and 142%, respectively. Although plant species-specific effects were observed with increasing salinity under greenhouse conditions, the plant growth promotion effect was, in general, more pronounced in non-sterile soil, and decreased with the degree of salinity. In non-sterile soil, a positive effect of *S. rhizophila* DSM14405^T was consistent across all salinities, and plant growth was in fact described in a linear regression model with soil salinity (Egamberdieva et al., 2011).

The mechanisms underlying the strong biocontrol activity of *S. rhizophila* against soil-borne fungi such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium* sp. were studied. In

addition to the synthesis of antifungal metabolites, chitinases and proteases, it was shown that it also produces VOCs that proved to inhibit the mycelial growth of the soil-borne pathogen *R. solani* to more than 90% in dual culture tests. From a vast diversity of VOCs produced by *S. rhizophila*, two, namely dodecanal and α-phenylethanol were identified by GC-MS (Kai, 2007).

S. rhizophila DSM14405^T also produces the osmolytes trehalose and glucosylglycerol (GG) as response to salt stress (Roder et al., 2005). Osmolytes are highly soluble compounds with no net charge at physiological pH which are compatible with cellular functions e.g. DNA replication, DNA-protein interactions and cellular metabolism, but their major role in providing resistance against salinity stress is suggested to lie in their role as effective stabilizers of enzymes in maintaining the osmotic balance under saline conditions (Lippert and Galinski, 1992; Welsh, 2000).

Pathogenicity and biocontrol: the same potential exploited for different purposes (?)

There are a great number of mechanisms and strategies that are harnessed by both BCAs and pathogenic bacteria to successfully inhabit their niche in various stages of the life cycle such as invasion, colonization and growth (Rahme et al., 1995; Cao et al., 2001). Moreover, several human-pathogenic bacteria have been revealed to be able to colonize plants. For instance, extensive invasion and endophytic colonization of plants were demonstrated for food-borne pathogens such as *Salmonella enterica* pv. *Typhimurium* in barley (Kutter et al., 2006) and the aggressive human pathogen *Burkholderia pseudomallei* in diverse plants in Northern Australia (Kaestli et al., 2011). Various plants were demonstrated to be colonized endophytically by clinical isolates of the food borne pathogen *Cronobacter* (former *Enterobacter*) *sakazakii*, which is associated with cases of meningitis, necrotizing enterocolitis and sepsis in neonates and immune-compromised infants (Schmid et al. 2009). Strains of *C. sakazakii* were isolated from the rhizosphere of Salicornia and other plants (Jha et al 2012; Schmid et al. 2009). Tan et al. (1999) reported of the utilization of siderophore uptake systems and extracellular enzymes by both BCAs and human-pathogenic bacteria, and Dörr et al. (1998) found that the type IV pili of the plant-associated *Azoarcus* sp. BH72, which are responsible for the adhesion on both plant and fungal cells, showed a high amino acid sequence similarity to those of the human-pathogenic *P.*

aeruginosa and *Neisseria gonorrhoeae* strains. Another example includes the components of the well-known type III secretion system (T3SS) that have been discovered in both pathogenic bacteria and plant-associated bacteria with biocontrol activity (Preston et al. 2001). In a study published by Alonso et al. (1999) it was shown that environmental and clinical isolates of *P. aeruginosa*, the latter a major causal agent for morbidity and mortality of patients with cystic fibrosis, share several phenotypic traits with respect to both virulence and environmental properties.

One important mechanism by which harmless bacteria can behave as pathogens is change of host or host niche, upon which their virulence potential is frequently released to its full extent. This mechanism is clearly relevant for opportunistic pathogens from plant-associated habitats. In addition, other mechanisms such as structural changes of the bacterial chromosome due to gene acquisition and loss, recombination and mutations can lead to bacterial pathogenicity (Hacker et al. 2003). Genes responsible for pathogenicity or fitness of bacteria often occur as genomic islands, which are blocks of DNA with signatures of mobile genetic elements (Hacker and Carniel 2001). These are called "fitness islands" or "pathogenicity islands" according to their function.

Furthermore, several studies support the view that the environmental strains are indistinguishable from those arising from clinical sources in terms of genotypic, taxonomic or metabolic properties (Kiewitz and Tümmler 2000; Finnan et al. 2004; Morales et al. 2004). In addition, differences between environmental strains and those that cause infections are suggested to be rather due to the regulation of genes, than their mere presence or absence (Parke and Gurian-Sherman 2001). In this regard, similar studies with focus on *P. aeruginosa*, *S. maltophilia* (reviewed in Ryan et al. 2009) and *Burkholderia cepacia* (Parke and Gurian-Sherman 2001) have been published. Nevertheless, antagonism studies and biocontrol effects were reported for all mentioned species, and one product derived from *B.* (*ceno*)*cepacia* was successfully launched onto the market (Hebbar et al. 1998; Nakayama et al. 1999; Dunne et al. 2000, Govan et al. 2000). Although all so far known species are common inhabitants of the rhizosphere, they are ordered to be excluded from direct biotechnological applications due to their medical relevance, and grouped into risk group 2 by the public databases, e.g. that run by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de). Rhizosphere-associated bacteria with a high capacity for biocontrol can be potentially dangerous for human health. Therefore, it is important to understand the mode of action and specific properties of the

BCA. It is well known that antagonistic properties and underlying mechanisms are highly strain-specific (Berg et al. 2002), but the currently performed identification of bacteria is mainly based on 16S-rDNA sequencing. However, based on the sequence information of the ribosomal RNA, which is a central and well-conserved housekeeping gene, it is impossible to draw conclusions about the potential pathogenicity: neutral bacterial strains can be dangerous due to pathogenicity islands or pathogenic bacteria can be harmless because of the absence of any pathogenicity factor.

In conclusion, findings gained through genome analysis, physiological and molecular studies reveal that there are numerous mechanisms shared by both plant-associated bacteria and those reported as human pathogens. Genes encoding molecular systems and proteins with a potential ambivalent role are very often highly homologous, sometimes even nearly identical among these two groups of bacteria. Nevertheless in some cases, specific critical physiological features from plant-associated bacteria give a hint whether these could potentially pose a threat with regard to causing diseases in humans. *S. rhizophila*, for instance, shares several virulence-associated genes and gene clusters with human/animal pathogenic bacteria (Alavi et al.:Manuscript), but it is incapable of growth at 37 ºC, which is a crucial prerequisite for successful survival and virulence in the human body. The beneficial plant-associated *S. maltophilia* R551-3 strain, on the contrary, can grow at 37 ºC but it lacks genetic potentials similar to those possessed by *S. rhizophila*. In addition, several studies have shown that plant and human-associated bacteria may harbour similar "interaction factors", but their mere occurrence in the genome is not necessarily an evidence for pathogenicity. Furthermore, proteomics and interaction studies seem to be more appropriate to assess the potential risk of bacterial strains than genomics, as there is a better correlation at protein level as shown for 14 epidemic bacterial killers ("badbugs") (Georgiades and Raoult 2011). Nevertheless, it should also be noted that naturally occurring gene communication mechanisms among bacteria such as horizontal gene transfer and recombination events could potentially equip the bacterial genome arsenal with new genetic information that could lead to the development of novel physiological traits.

rpf*/DSF system: The quorum sensing mechanism in *Stenotrophomonas

In 1997, Barber and colleagues reported of a novel regulatory system showing density-dependent regulation similar to that of N-acyl derivatives of homoserine lactones (N-AHLs) which are responsible for quorum sensing in Gram-negative bacteria. The regulatory system detected proved to require the diffusible signal factor (DSF), was shown to be essential for the pathogenicity of the plant pathogenic *Xanthomonas campestris* pv. *campestris* strain 8004 and suggested to be dependent for its synthesis on the *rpfF* of a seven-gene locus in the chromosome of *X. campestris* pv. *campestris*. Wang et al. (2004) provided experimental evidence for DSF in *X. campestris* pv. *campestris* be of fatty acid nature, a cis-11-methyl-2-dodecenoic acid, a novel α, β unsaturated fatty acid in fact, (Fig. 1). The detection of the *rpf*/DSF system presents a new family of bacterial quorum sensing.

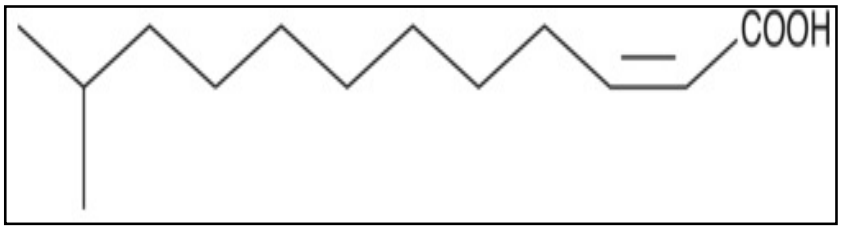

Fig. 1: The structure of the diffusible signal factor (DSF) in *X. campestris* pv. *campestris* (Wang et al., 2004)

In addition to *rpfF*, other genes present in the *rpf* (regulation of pathogenicity factors) gene locus include *rpfB*, *rpfC* and *rpfG*, each having a specific function regarding synthesis and perception of DSF (Dow and Daniels, 1994). RpfB, a putative long-chain fatty acyl CoA ligase, is believed to be involved in the biosynthesis of DSF through providing substrates for interaction with DSF-synthase, RpfF, an enzyme similar to enoyl CoA hydratase. RpfC is a sensor kinase while *rpfG* codes for a protein with a receiver domain. RpfC/RpfG form together a two-component system responsible for DSF perception and signal transduction (Tang et al., 1991; Slater et al., 2000). *rpfH* is present in the *rpf* locus of *X. campestris* pv. *campestris*, but it is absent from other xanthomonads, and it is unclear whether it fulfills a specific function in the synthesis or perception of DSF (Barber et al., 1997). In the plant-associated beneficial strain *S.*

maltophilia R551-3, the *rpf* gene locus consists of four genes: *rpfB*, *rpfF*, *rpfC* and *rpfG* which is similar to that of the clinical *S. maltophilia* K279a strain, while the plant growth promoting *S. rhizophila* DSM14405^T possesses one additional gene between *rpfF* and *rpfB*.

Thus far the *rpf*/DSF system has been detected in numerous xanthomonads including plant-pathogenic *Xanthomonas* species and the clinical human-pathogenic *S. maltophilia* K279a. In contrast to originally assumed, the DSF-driven quorum sensing is not confined to xanthomonads as similar molecules with the same role were detected in other bacteria such as *B. cenocepacia* (Boon et al., 2008). Furthermore, *rpf*/DSF-driven quorum sensing proved to transgress the species-boundaries. For instance, interspecies signalling through the *rpf*/DSF system was reported by Ryan et al. (2008) who found that, in mixed biofilms consisting of the two nosocomial pathogens, DSF produced and released by the clinical *S. maltophilia* plays a crucial role in the development of extended filaments of *P. aeruginosa*, the pathogen causing cystic fibrosis in human lungs. Cross-kingdom signalling of DSF was reported by Wang et al. (2004) who found that bacterial DSF could mimic the fungal key signal molecule, farnesoic acid in preventing the formation of *Candida albicans* mycelia.

Deletion of *rpfF* results in the total loss of the DSF synthesis which leads to the collapse of the *rpf*/DSF quorum sensing system. Numerous studies generating *rpfF* mutants found that a high number of cellular mechanisms are under control of DSF in pathogenic bacteria. These studies showed that in almost all cases, the break-down of the *rpf*/DSF system due to *rpfF* deletion resulted in significant loss of virulence and pathogenicity through affecting virulence mechanisms. For instance, the deletion of *rpfF* resulted in reduced activity of extracellular enzymes such as proteases, endoglucanase and extracellular polysaccharide (EPS) in *X. campestris* (Barber et al., 1997). In the human-pathogenic *S. maltophilia* K279a, the loss of DSF signal due to the deletion of *rpfF* led to debilitated swimming motility, altered LPS structure, the loss of dispersed lifestyle and microcolony formation, and an –in some cases- significant increase in sensitivity against numerous antibiotics and heavy metals including ampicillin, nalidixic acid, rifampin, Zinc, Copper, and Nickel in addition to the reduction in the synthesis of extracellular proteases and endoglucanase (Fouhy et al., 2007). *Xylella fastidiosa,* which causes Pierce's disease in grapevine, utilizes an insect vector to infect its plant host. Deletion of *rpfF* in *X. fastidiosa* reduced both its capability to colonize and form biofilm in the insect vector, but the mutant strain caused the Pierce's disease symptoms more severely than the wild-type when it was

mechanically inoculated into grapevine-plants (Newman et al., 2004). The impact of DSF on biofilms was shown to be both species-specific and dependent on the culture medium used, as there have been reports of both positive and negative regulation of biofilm formation. For instance, Dow et al. (2003) showed biofilm formation in *X. campestris* to be positively regulated in rich medium by the *rpf*/DSF system while the system showed a negative impact on it when the culture was grown in the minimal Y medium Torres et al. (2007). The *rpf*/DSF mechanism regulates a vast number of virulence-associated characteristics in plant and human-pathogenic bacteria, as described above. While thoroughly studied in pathogenic species, especially in pathogenic xanthomonads, the *rpf*/DSF system has however so far remained unknown in beneficial plant-associated bacteria.

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The goals of the Ph.D. thesis

Study the role of *rpf*/DSF quorum sensing system in the beneficial plant-associated *S. maltophilia* R551-3 model strain (Publication 1).

Overall, effects regarding positive interactions with the plant host were detected to be significantly controlled by the *rpf*/DSF quorum sensing system in *S. maltophilia* R551-3 .Oilseed rape seeds treated with the *S. maltophilia* R551-3 wild-type strain showed a statistically significant increase in germination rate compared with those treated with the *rpfF* mutant. Similarly, the wild-type strain exhibited better plant growth promotion, a greater efficiency in colonizing oilseed rape and the ability to form biofilm compared to the mutant strain. Furthermore, gene transcription analyses showed that numerous genes known to play a role in plant colonization (e.g. chemotaxis, cell motility, biofilm formation, multidrug efflux pumps) are controlled by the *rpf*/DSF system in *S. maltophilia*. In addition, new potential functions for spermidine were detected, primarily regarding both growth promotion and stress protection.

Investigate the response of the endophytic bacterium *S. rhizophila* DSM14405^T to changing environmental conditions associated with osmotic stress including salt stress and root exudates to understand the stress protection effect for plant roots delivered by *S. rhizophila* against changing osmotic conditions (Publication 2, Bulletin).

The transcriptome of *S. rhizophila* DSM14405^T changed drastically in response to both salt shock and root extracts. A notable similarity regarding the response towards both stress factors was detected including general stress protection, energy production, and cell motility. Nevertheless, unique changes were also evident. Production and excretion of glucosylglycerol (GG) was found as salient substance responsible for the stress protection. The treatment of *S. rhizophila* with root exudates resulted in a shift from the planktonic lifestyle to a sessile one, as expressed in the down-regulation of the flagellar-driven motility. Moreover, export genes for the plant growth regulator spermidine, which is known to strongly promote plant growth, were up-regulated.

S. rhizophila DSM14405^T genome announcement, characterization and comparison with clinical and environmental *Stenotrophomonas* model strains (Manuscript)

The genome of the plant growth promoting and biocontrol agent *S. rhizophila* DSM14405^T was characterized regarding both general and specific characteristics as well as functional annotation. Furthermore, it was also compared with the genome of the plant-associated beneficial *S. maltophilia* R551-3 and the clinical *S. maltophilia* K279a. Many genes were detected to be shared among all three strains. The striking genomic similarity between *S. rhizophila* and *S. maltophilia* K279a is highly interesting as to which genes and gene regulation mechanisms

account for the entirely different niches and lifestyles of the two *Stenotrophomonas* species. Nevertheless, a great number of physiologically crucial genes were revealed to be specific to *S. rhizophila* DSM14405^{T} which are responsible for its distinguished capabilities regarding the interactions with the host plant.

Pseudomonas poae RE*-1-1-14 genome announcement, characterization

The genome of the biocontrol agent *Pseudomonas poae* RE*-1-1-14 was sequenced and characterized (Publication 3).

Publication 1 (in Press)

The DSF quorum sensing system controls the positive influence of *Stenotrophomonas maltophilia* on plants

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ABSTRACT

The interaction of the Gram-negative bacterium *Stenotrophomonas maltophilia* with eukaryotes can improve overall plant growth and health, but can also cause opportunistic infections in humans. While the quorum sensing molecule DSF (diffusible signal factor) is responsible for the regulation of phenotypes in pathogenic *Stenotrophomonas*, up until now, no beneficial effects were reported to be controlled by it. Our objective was to study the function of DSF in the plant growth promoting model strain *S. maltophilia* R551-3 using functional and transcriptomic analyses. For this purpose, we compared the wild-type strain with a mutant deficient in the *rpfF* (regulation of pathogenicity factors) gene that is essential for the synthesis of DSF. Oilseed rape seeds treated with the wild-type strain showed a statistically significant increase in germination rate compared with those treated with the *rpfF* mutant. Similarly, the wild-type strain exhibited better plant growth promotion and a greater efficiency in colonizing oilseed rape compared to the mutant strain. Moreover, only the wild-type was capable of forming structured cell aggregates both *in vitro* and in the rhizosphere, a characteristic mediated by DSF. Gene transcription analyses showed that numerous genes known to play a role in plant colonization (e.g. chemotaxis, cell motility, biofilm formation, multidrug efflux pumps) are controlled by the *rpf*/DSF system in *S. maltophilia*. In addition, we detected new potential functions of spermidine, primarily for both growth promotion and stress protection. Overall, our results showed a correspondence between the regulation of DSF and the positive interaction effect with the plant host.

Introduction

Stenotrophomonas maltophilia (syn. *Pseudomonas* and *Xanthomonas maltophilia*) is a type species within Gammaproteobacteria [1]. Although the species was isolated from diverse environments, plants are one of its main reservoirs [2]. In Brassicaceae oilseed rape, for example, these bacteria dominate the plant microbiome [2, 3]. *S. maltophilia* can be both seed-borne and occur with an endophytic lifestyle [2, 4]. The species is characterized by an extremely high intra-

species diversity on the physiological and molecular level, especially within the environmental populations [1, 5]. Nevertheless, *S. maltophilia* strains are also nosocomial opportunistic pathogens. These clinical strains can cause disease with significant case/fatality ratios, especially in immunocompromised patients [6, 7]. Despite different approaches it was not possible to differentiate between environmental and clinical strains [8, 9, 10]. Interestingly, the mutation rate of *S. maltophilia* strains was the key to divide both groups. Clinical strains have a higher mutation rate than those from the environment and also contain hypermutators to help them quickly adapt once inside the fluctuating human body [11]. Sequence analysis of the first two known genomes of *S. maltophilia* is in favor of this assumption: the gene homology between *S. maltophilia* R551-3 and its clinical counterpart *S. maltophilia* K279a is approximately 85% [6]. While *S. maltophilia* belongs to the group of growth promoting rhizobacteria with biocontrol activity [12, 13], little is known about the mode of beneficial plant-microbe interactions [14]. Many strains are distinguished by unique mode of actions. Several strains are able to produce the phytohormone indole-3-acetic acid [15], while other *S. maltophilia* strains are free-living nitrogen-fixing bacteria [16]. Others can produce antifungal antibiotics [17, 18] or bioactive volatiles [19]. However, there is no past or current research concerning the regulation of these metabolites.

Quorum sensing systems based on N-acyl derivatives of homoserine lactones (N-AHLs) are often responsible for the regulation of various phenotypic characteristics in numerous plant-associated bacteria [20, 21]. AHL-based quorum sensing was not detected in *Stenotrophomonas*, but a diffusible signal factor (DSF)-based system, a novel quorum sensing system used by numerous xanthomonads [22], has been identified in clinical *S. maltophilia* K279a. In addition, structurally related systems have been detected in *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* as well [23, 24]. DSF is a quorum sensing molecule of fatty acid nature that was first detected in *Xanthomonas* [25]. The *rpf* (regulation of pathogenicity factors) gene cluster is responsible for the synthesis and perception of DSF [25], and the *rpfF* gene product, known as DSF synthase, is essential for the synthesis of DSF in all known bacteria with this *rpf*/DSF system [23]. Each of the other members of the *rpf* gene locus (*rpfC*, *rpfG* and *rpfB*) fulfill a particular function with the RpfC/RpfG two-component system responsible for DSF perception and signal transduction [25, 26]. The *rpf*/DSF mechanism regulates a number of virulence-associated characteristics such as synthesis of extracellular enzymes, extracellular

polysaccharides, and biofilm formation in various pathogenic strains [25, 26, 27]. While thoroughly studied in pathogenic species, especially in pathogenic xanthomonads, the *rpf*/DSF system is still unexamined in beneficial plant-associated bacteria.

The objective of this study was to investigate the role of DSF in the beneficial plant-associated *S. maltophilia* R551-3 model strain, originally isolated from the endosphere of poplar [14]. To address this question, we generated a DSF signal deficient mutant strain and investigated the role of the *rpf*/DSF signaling system with respect to bacteria-plant interactions by comparing the *S. maltophilia* R551-3 wild-type to the *rpfF* mutant strain.

Materials and Methods

Bacterial isolates and growth conditions

Stenotrophomonas maltophilia R551-3 was originally isolated from the endosphere of poplar [14]. Unless otherwise stated, the *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains were cultivated at 37 ºC in Luria Bertani broth (Carl Roth, Germany). Overnight cultures were obtained by incubating the bacterial strains in LB medium at 37 ºC and 120 rpm for 18-20 h.

Generation of the *S. maltophilia* R551-3 *rpfF* mutant strain

Generation of the *S. maltophilia* R551-3 *rpfF* mutant strain was performed according to Hoang et al. [28]. The *S. maltophilia* R551-3 *rpfF* gene sequence and its flanking regions were obtained from the genome database and specific primers were designed, as described below, to allow and confirm the generation of the *rpfF* knock-out strain. A DNA construct of 861 nucleotides consisting of the upstream and downstream flanking regions with respect to the *rpfF* gene in *S. maltophilia* R551-3 genome (NCBI Accession Nr.:CP001111) was obtained over a two-step PCR. In the first PCR, two amplicons were generated separately using primers R551-Up-F (cggaattcCAACCCGATTGCTGGAAGTAT) and R551-Up-R (cccctcactcctctccgtGACCAGTGCATTCCTGCC) which delivered the upstream flanking region of approximately 400 bp. Also, primers R551-Down-F

(ggcaggaatgcactggtcACGGAGAGGAGTGAGGGG) and R551-Down-R (cccaagcttGCTTCAACGTGTACCCGAAC) were used to deliver the downstream flanking region of approximately 450 bp. The second PCR step was performed using primers R551-Up-F and R551-Down-R, delivering the desired gene construct, referred to as fragment C to maintain lucidity, which was subsequently cut with restriction endonucleases *Eco*RI and *Hin*dIII and ligated into the suicide vector pEX18Tc [28].

The suicide vector pEX18Tc possesses the tetracycline selectable (*tet*) marker and *sacB*, the sucrose counter-selectable marker. The vector was transferred into *S. maltophilia* R551-3 cells using tripartite mating [29]. The first and second crossover events were selected using tetracycline [20 µg ml^{-1}] and 10% [w/ v] sucrose, respectively. Generation of the *rpfF* mutant strain was confirmed, as described below, by performing separate PCRs, each with particular primers designed to confirm the recombination event and the generation of the *S. maltophilia* R551-3 knock-out *rpfF* mutant strain.

The primer pairs used for this purpose include R551-Up-F/ R551-Down-R that would deliver a fragment consisting of the upstream and downstream regions and the *rpfF* gene in the *S. maltophilia* R551-3 wild-type strain while the mutant strain would solely deliver fragment C. Similarly, primers Deletion Check –F (CCAGGTTGCTGCCTCCAGCG) and Deletion Check –R (GTGATACGCCCGCCCCGTAAG) would only deliver a ca. 300 nucleotide-long inner part of the desired fragment C in the mutant strain while the wild-type would yield a fragment consisting of the *rpfF* gene and shorter-than-original flanking upstream and downstream regions attached to it. Primers R551-RpfF-F (GGTCGAACAGCACCTCCGGC) and R551-RpfF-R (ATCATCACCCGCCCCTCGCT) were specific for the *rpfF* gene and would deliver the product only in the wild-type. Moreover, primers Tet1 (AGCTGTCCCTGATGGTCGTC)/ Tet2 (GAGCCTTCAACCCAGTCAGC) were used to confirm the elimination of the pEX18Tc suicide vector after the recombination event. All PCR results were sequenced in addition to confirmation on electrophoresis gel. Moreover to study the possible impact of the deletion of *rpfF* on the general growth of *S. maltophilia* R551-3, cultures of the wild-type and *rpfF* mutant strain were grown in liquid LB under 120 rpm at 37 ºC, and growth was assessed up to the mid-stationary phase. The deletion of the *rpfF* gene showed no impact on the growth rate of *S. maltophilia* R551-3.

Confirmation of the incapability of the *S. maltophilia* R551-3 *rpfF* strain to produce DSF using glucanase plate assay

The incapability of the *S. maltophilia* R551-3 *rpfF*-deficient strain to produce DSF was confirmed using an assay based on the finding by Barber et al. [25] that showed the glucanase synthesis by *X. campestris* pv. *campestris* 8004 is DSF-dependent. The assay was performed on Petri dishes containing tryptone soy agar (TSA) (Carl Roth, Germany) supplemented with 1 g L^{-1} AZCL-Barley Beta-Glucan (Megazyme, Ireland). 1.5-cm-long streaks of the *Xanthomonas campestris* pv. *campestris rpfF* mutant strain were put on the plates, and supplemented with either sterile water (control), 100 µM cis-Δ2-11-methyl-Dodecenoic acid (Cayman Chemical Company, MI, USA) as synthetic DSF, supernatant extracts of a *S. maltophilia* R551-3 *rpfF*-deficient culture or supernatant extracts of a *S. maltophilia* R551-3 wild-type culture. The plates were incubated at 30 ºC for approximately 22 h and observed for glucanase production through formation of blue zones around the streaks.

To prepare supernatant extracts used in the glucanase assay from *S. maltophilia* R551-3 wild-type and *rpfF* mutant, 50 ml of 24-hour-incubation cultures were centrifuged at 10,000 x *g* for 10 min. The supernatant was extracted twice with 1/5 vol. ethyl acetate (Carl Roth, Germany). For efficient phase separation, the samples were centrifuged at 7,500 x *g* for 15 min. The ethyl acetate extract was completely evaporated and dissolved in 200 µl of sterile deionized water.

RNA extraction and transcriptomic analyses

Quantitative sequencing of mRNA was used to assess gene expression. This characterization of bacterial transcriptomes based on ssRNA-seq is a novel and effective approach described by Perkins et al. [30]. To extract RNA, cultures of *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains were grown in LB at 37 ºC and 120 rpm until the early stationary phase was reached, as the DSF concentration has been revealed to be highest at this point [25]. Cells were harvested using centrifugation at 5000 x *g* for 3 min. RNA was extracted with the RNAprotect® Bacteria Reagent (Qiagen, Hilden, Germany), and rRNA was removed with MICROBExpress Kit (Invitrogen, Carlsbad, USA). The enriched mRNA was sequenced by LGC Genomics (Berlin, Germany) and data collections were performed by MicroDiscovery (Berlin, Germany).

Plant experiments

Oilseed rape (cv. Californium, Kwizda, Austria) was used for plant assays. Prior to bacterial inoculation, seeds were surface sterilized using the seed infiltration approach described by Müller and Berg [31]. Seeds (0.7 g) were treated with 3% sodium hypochlorite (NaOCl) solution for 5 min and subsequently washed three times with sterile water. For inoculation, surface sterilized seeds were placed in a Petri dish and incubated in 10 ml of 0.85 % NaCl solution containing 10^6 CFU ml^{-1} of the bacterial culture on an orbital shaker at 85 rpm at room temperature for 3.5 hours. Colony forming units (CFU) ml^{-1} had been determined previously by diluting and plating 100 µl aliquots of 18-hour-old overnight cultures of the bacterial strains on LB plates. Colonies were counted after incubating the plates at 37 ºC for 48 h.

The impact of *S. maltophilia* R551-3`s on seed germination, plant growth, and the colonization of rhizosphere (root system) and aboveground plant parts was studied. To this end, two gnotobiotic systems were applied: seed germination pouches (mega international, MN, USA) and gnotobiotic soil. For microscopy, plant experiments were performed in seed germination pouches. In the approach using pouches, sterile pouches were loaded with inoculated seeds (5 each pouch) and moistened with 20 ml of sterile deionized water. The control group consisted of seeds incubated with 0.85% NaCl without bacteria. To avoid dehydration, the pouches were placed in sterile, covered polypropylene containers and incubated in a greenhouse at 23 ± 2°C under artificial lighting (16 h light period) for 11 days. In the approach using the gnotobiotic soil system, polypropylene containers (4 L) were filled with 1.5 L of standard propagation soil (Empfinger Rindenmulch, Austria) and autoclaved to reduce the amount of soil-borne microorganisms and make possible the subsequent re-isolation of the strains. To test sterility, around 1 g of soil was suspended in 5 ml of sterile 0.85% NaCl solution and aliquots thereof were plated on LB Petri dishes which revealed that although significantly reduced in both the diversity and number of soil-borne microorganisms compared to non-autoclaved soil, the autoclaved soil was not absolutely sterile. Inoculated seeds were planted in the autoclaved soil (11 seeds pot^{-1}) and watered with sterile water. The control group consisted of seeds incubated with 0.85% NaCl without bacteria. The plastic beakers were covered with sterile transparent lids and incubated in a greenhouse at 23 ± 2°C under artificial lighting (16 h light period).

Bacterial colonization was assessed after 20 days. *S. maltophilia* wild-type and *rpfF* mutant strains were re-isolated from the 20-day-old oilseed rape plants using plant sections (roots with adhering soil, stem or leaves) sampled in sterile Whirlpack® bags (Carl Roth, Germany), supplemented with 5 ml 0.85% NaCl solution, and rigorously disintegrated using pestle and mortar. Subsequently, serial dilutions of the extract were plated onto LB plates. After incubation for 48 h at 37 °C, the number of colonies was counted and the CFU g^{-1} plant material was assessed. The impact of the *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains on seed germination was checked for as of the next day after sowing. The plant growth promoting effect was studied by measuring the fresh weight [g] of oilseed rape plants twice, after 5 and 20 days, respectively.

GFP labeling of *S. maltophilia* R551-3 wild-type and *rpfF* strains

To study the capability of *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains to form biofilm, the GFP-expressing plasmid pSM1890 [32] was introduced into the strains using tripartite mating as described by De Lorenzo and Timmis [29]. *gfp*-labeled cultures were grown in LB at 37 ºC and 120 rpm to OD_{600} of about 1, and then transferred into chambers purchased from Lab-Tek® II CC2™ Chamber Slide™ System (Thermo Fisher Scientific, NY, USA). These static cultures were then incubated at 37 ºC for three days. To remove the unbound bacteria, the medium was discarded and the glass slides were washed three times. This procedure was repeated four times. To complement the *rpfF* mutant strain, the culture medium was supplemented with 100 µM of synthetic DSF (cis-Δ2-11-methyl-Dodecenoic Acid, Cayman Chemical Company, MI, USA). Microscopic images were captured using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with the Leica ACS APO 63X OIL CS objective (NA: 1.30). A z-step of 0.4–0.9 µm was applied to acquire confocal stacks. Photomultiplier gain and offset were individually optimized to improve the signal/ noise ratio, and the 3D analysis of the stacks generated by confocal laser scanning microscopy (CLSM) was performed using Imaris 7.0 software (Bitplane, Zurich, Switzerland).

Fluorescent *in situ* hybridization (FISH)

To study the ability of *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains to colonize oilseed rape plants, FISH in combination with CLSM was used. To this end, oilseed rape roots which were colonized with the bacterial strains and grown in seed germination pouches were fixed with 4% paraformaldehyde/ phosphate buffered saline (PBS) (3:1 vol/vol). The control group contained roots with no bacterial treatment grown in the seed germination pouches. The fixed samples were then stored in PBS/ 96% ethanol (1:1) at -20 ºC. The FISH probes were purchased from genXpress® (Wiener Neudorf, Austria), and the in-tube FISH was performed as described by Cardinale et al. [33]. The FISH probes used for the hybridization step were labeled with the fluorescent dye Cy3 and included EUB338 [34], EUB338 II, and EUB338 III [35], all directing eubacteria. An equimolar ratio of the FISH probes was used for the hybridization step to detect *S. maltophilia* wild-type and *rpfF* mutant strains. In this step, 15% formamide was added to the samples which were then subsequently incubated in a water bath (46 ºC) for 90 min. After hybridization, the samples were washed at 42 ºC for 15 min. Microscopy and image capturing were performed using a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) with the Leica ACS APO 63X OIL CS objective (NA: 1.30). A z-step of 0.2–0.9 µm was applied to acquire confocal stacks.

Results

Characterization of the *S. maltophilia* R551-3 *rpfF* mutant using glucanase plate assay and transcriptomic studies

In a first step, the *S. maltophilia* R551-3 *rpfF* mutant was characterized using the glucanase assay which proved that the *S. maltophilia* R551-3 *rpfF* mutant strain is incapable of producing DSF. The *X. campestris rpfF* mutant strain, when supplemented with either supernatant extracts from the *S. maltophilia* R551-3 wild-type culture or 100 µM synthetic DSF (cis-Δ2-11-methyl-dodecenoic acid), formed a blue zone due to the restored glucanase activity that leads to the degradation of the AZCL-Barley beta-glucan (Supplementary Fig. S1). Conversely, no glucanase activity was observed after supplementing the *Xanthomonas rpfF* mutant with supernatant

extracts from the *S. maltophilia* R551-3 *rpfF* mutant strain. Furthermore, transcriptomic analyses proved that the *rpfF* gene was transcribed only in the *S. maltophilia* R551-3 wild-type strain, as no *rpfF* mRNA could be detected for the *S. maltophilia* R551-3 *rpfF* mutant strain (locus tag: Smal_1830, Supplementary Table 1).

The effect of the *rpf*/DSF system on seed germination and plant growth promotion in *S. maltophilia* R551-3

Oilseed rape seeds were inoculated with *Stenotrophomonas* (wild-type and *rpfF* mutant), planted into soil, and grown under greenhouse conditions. The number of germinated seeds was then compared one day after sowing (Fig. 1). The germination rate of the oilseed rape seeds treated with *S. maltophilia* R551-3 wild-type was twice that of seeds incubated with the *rpfF* mutant strain. Seeds which had not been inoculated with either of the strains (control seeds) showed no germination after 24 hours of incubation (Fig. 1).

However, the effect of the *rpf*/DSF system was confined to seed germination and, as described below, the early phase of plant growth. After five days of incubation in a greenhouse, the young oilseed plants were cut from the point of contact with the soil and weighed. The mean weight of 5-day-old plants inoculated with the wild-type strain was 0.154 (± 0.012) g, which is notably higher than those treated with the *rpfF* mutant (0.100 [± 0.014] g) and the non-inoculated control group (0.08 [± 0.01] g). At the end of the 20-day period, the plant growth promotion effect was less pronounced with no statistically significant difference (wild-type, 3.85 [± 0.67] g, the *rpfF* mutant strain, 3.49 [± 0.35] g and control, 3.06 [± 0.35] g). Moreover, the impact of *S. maltophilia* R551-3 on seed germination and plant growth was confined to the gnotobiotic soil system, as no effect was observed in the approach using sterile germination pouches.

The effect of the *rpf*/DSF system on the plant colonization ability of *S. maltophilia* R551-3

Oilseed rape plants were treated with *Stenotrophomonas* cells using seed-priming and cultivated in the gnotobiotic soil systems for 20 days to investigate the plant colonization efficiency of *S. maltophilia* R551-3 and to discern the possible role of the *rpf*/DSF system in plant colonization. Prior to sowing, the cell density attached to and within the seeds was evaluated. The seed

inoculation was similar for both strains and resulted in 1.44 x 10^6 (± 1.55 x 10^5) and 1.48 x 10^6 (±7.8 x 10^4) CFU seed $^{-1}$ for the wild-type and *rpfF* mutant strain, respectively. At the end of the incubation period, the plant sections (rhizosphere, stems, leaves) were dissected and *Stenotrophomonas* cells were subsequently re-isolated. In general, both strains were capable of colonizing the rhizosphere and phyllosphere (stem and leaves), however the *S. maltophilia* R551-3 wild-type strain showed a significantly higher colonization of all parts of the oilseed rape plant in comparison with the *rpfF* mutant (Fig. 2). In addition to the soil system, plants were grown in sterile seed germination pouches. The cell count obtained from the stem of plants inoculated with wild-type was higher (7.8 x 10^7 [± 2.7 x 10^7] CFU g^{-1}) than that of the *rpfF* mutant strain (3.4 x 10^7 [± 1.3 x 10^7] CFU g^{-1}), as well as from the leaves of the oilseed rape plants, respectively, (9.8 x 10^7 [± 2.6 x 10^7] CFU g^{-1} and 4.8 x 10^7 [± 2.0 x 10^7] CFU g^{-1}).

In addition, the colonization of the oilseed rape rhizosphere by *S. maltophilia* R551-3 wild-type and the *rpfF* mutant strain was also investigated using FISH combined with CLSM (Fig. 3). We found similar results to those achieved in re-isolation assays in which the wild-type strain colonized oilseed rape more intensely than the *rpfF* mutant strain (Fig. 3). In addition, we observed a compact organization of the wild-type cells in the oilseed rape rhizosphere. The *rpfF* mutant strain, however, sparsely colonized the rhizosphere with bacterial cells scattered throughout the oilseed rape root (Fig. 3).

The impact of DSF on the formation of biofilm-like structures

In order to investigate the possible role of the *rpf*/DSF system in *S. maltophilia* R551-3, *gfp*-labeled wild-type and *rpfF* mutant strains were generated and cultivated in glass chambers. The CLSM and 3D analysis of the glass slides showed that the *S. maltophilia* R551-3 wild-type strain formed structured, surface-covering cell architecture with a particular texture consisting of several cell layers. Conversely, the *rpfF* mutant strain solely constructed an unstructured and unconnected monolayer film of cells. The *rpfF* mutant strain supplemented with 100 µM DSF (cis-Δ2-11-methyl-Dodecenoic Acid), however, constructed the same structure observed for the wild-type. Fig. 4 represents the 3D CLSM images captured from *gfp*-labeled wild-type, *rpfF* mutant, and the *rpfF* mutant strain supplemented with DSF.

The impact of the *rpf*/DSF system on the genome expression of *S. maltophilia* R551-3

Comparing the transcriptomic data of the *S. maltophilia* R551-3 wild-type to the *rpfF* mutant strain revealed that the *rpf*/DSF system regulates the expression of numerous key genes that both directly and indirectly are involved in plant growth promotion and biocontrol including those coding for cell motility, chemotaxis, LPS structure, biofilm formation, iron transport, antibiotic resistance, and also stress resistance through the synthesis of chaperone proteins (Table 1). Of the total genes either up or down-regulated, only those showing fold changes greater than or equal to 1.5 and less than or equal to 0.5 were considered significantly impacted. The complete list of genes with a significant transcription fold change is provided in Supplementary Table 1.

Regarding cell motility, genes coding for the flagellar machinery as well as chemotaxis in *S. maltophilia* R551-3 are strongly controlled by the *rpf*/DSF system, as they are positively regulated by DSF as shown in Table 1. Some of these genes, Smal_1868 (coding for flagellar biosynthetic protein; expression fold change of 17.9) and Smal_1869 (coding for flagellar export protein; expression fold change of 35.1) for instance, are significantly up-regulated by DSF. Regarding plant growth promotion, the *S. maltophilia* R551-3 *rpfF* mutant strain showed a slight decrease in the expression of the spermidine synthase gene, coding for a well-known growth regulator. In addition, the expression of two adjacent genes, Smal_2304 and Smal_2305, that code for spermidine export proteins is highly DSF-dependent, as the corresponding expression fold changes of 43.8 and 5.4, respectively suggest. As for surface adherence, genes that play a role in LPS biosynthesis and biofilm formation are positively regulated by the *rpf*/DSF system with the exception of Smal_2717, a polysaccharide deacetylase gene that affects biofilm formation, which shows a wild-type/*rpfF* mutant expression fold change of -5.5. Furthermore, genes involved in iron transport, antibiotic resistance, and those responsible for stress resistance through biosynthesis of chaperones are also positively regulated by DSF in *S. maltophilia* R551-3, as presented in Table 1.

Discussion

Stenotrophomonas maltophilia is known for its ambiguous interaction with eukaryotic hosts. We found that the quorum sensing system DSF is involved in many beneficial interactions such as plant growth promotion as well as plant colonization in *S. maltophilia* R551-3. The plant growth promoting effect, however, is not a result of the regulation of indole-3-acetic acid (IAA) synthesis, extracellular proteases, or volatile organic compounds as the respective physiological tests showed no significant difference between the wild-type and *rpfF* mutant strains (data not shown). However, the transcriptomic analysis indicated that numerous genes crucial for bacteria-plant interactions are regulated by the *rpf*/DSF quorum sensing in *S. maltophilia* R551-3. With this new information, we can analyze the function of these DSF-dependent cellular mechanisms that underlie both plant colonization and growth promotion in *S. maltophilia*. However, it should be noted that although the transcriptomic analyses were very helpful in understanding the role of the *rpf*/DSF system in *S. maltophilia* R551-3, more investigations also under different conditions are needed to understand the whole functioning.

Flagella-dependent motility and chemotaxis are important factors in biofilm formation [36] and plant colonization [37]. As represented in Fig. 4, only *S. maltophilia* R551-3 wild-type formed the structured surface-covering and multi-layer biofilm-like cell architecture on glass slides. According to numerous studies, the *rpf*/DSF system has been shown to play an important role in forming cell aggregates, surface attachment, and surface adherence in various pathogenic xanthomonads [22, 35]. Furthermore, biofilm formation was found both negatively [38] and positively [39] regulated by the *rpf*/DSF system. Our findings on quorum sensing-dependent biofilm formation in the plant-associated *S. maltophilia* R551-3 are similar to those by Torres et al. [39]. Moreover, the transcriptomic analyses indicated that the expression of flagellar apparatuses as well as biofilm formation are controlled by the *rpf*/DSF system. Given these results from physiological and transcriptomic approaches the *rpf*/DSF quorum sensing system controls the genes responsible for biofilm formation and plant colonization, which in turn play an important role in the interaction between *S. maltophilia* R551-3 and plants.

S. maltophilia R551-3 promotes seed germination and plant growth, however it is controversial which mechanism(s) causes this positive interaction. Only low levels of the plant growth hormone indole-3-acetic acid (IAA) are produced by the bacterium, [14] and no

difference was observed for IAA synthesis between the wild-type and the *rpfF* mutant strain. In addition, the *S. maltophilia* R551-3 genome does not contain typical plant growth promoting genes that are found in other plant-beneficial bacteria, such as genes for the metabolism of plant signal molecules (e.g.: γ-amino butyric acid and phenyl acetic acid) or those for the synthesis of acetoin [6]. Nevertheless, our transcriptomic analyses revealed that a gene with significant homology to spermidine synthase was down-regulated in the *S. maltophilia* R551-3 *rpfF* mutant strain (wild-type/mutant fold change of 1.5; Table 1). Spermidine is a well-known plant growth regulator and has been recently shown to strongly promote the growth of arugula plants [40]. Furthermore, spermidine affects biofilm formation in various bacterial species via multiple pathways that involve both transport and signaling networks [41]. In addition to spermidine synthase, two adjacent genes (Smal_2304 and Smal_2305) in the chromosome of *S. maltophilia* R551-3 are also strongly regulated by the *rpf*/DSF system, as they were down-regulated by 43.8 and 5.4 folds (Table 1) respectively in the *rpfF* mutant strain. Although originally annotated as multidrug-coding genes with unknown functions, the amino acid sequences of Smal_2304 and Smal_2305 showed significant similarity to the spermidine export protein of the plant growth promoting and biocontrol agent *S. rhizophila* DSM14405^{T}. In this way, a higher level of spermidine synthase combined with highly active spermidine export proteins could result in a notably higher spermidine concentration in oilseed rape seeds, thus leading to enhanced germination and growth promotion.

Biological control of pathogens can indirectly result in plant growth promotion [42]. According to the transcriptomic data and the finding that the *rpf*/DSF-dependent seed germination and plant growth promoting effects are present only in the gnotobiotic soil system, but not the sterile germination pouches, biocontrol can indirectly be involved in causing seed germination and plant growth promotion in oilseed rape by *S. maltophilia* R551-3. Numerous studies have focused on mechanisms underlying biocontrol [43, 44, 45], and according to the transcriptomic analyses, a number of these mechanisms that are indicated to be DSF-dependent in *S. maltophilia* R551-3, are briefly discussed here. For instance, competing over iron through its efficient transport is a biocontrol mechanism [45] that is positively regulated by the *rpf*/DSF system in *S. maltophilia* R551-3. Another important biocontrol mechanism is the ability to compete over nutrients and niches through plant colonization [43, 44]. In this regard, the *S. maltophilia* R551-3 *rpfF* mutant strain is impaired in root/plant colonization that could be driven

by the down-regulation of the flagellar machinery. Other biocontrol mechanisms that are indicated to be DSF-dependent in *S. maltophilia* R551-3 include the biosynthesis of antibiotic (beta-lactamase) and multidrug efflux pumps.

In conclusion, this study demonstrated that the significance of the *rpf*/DSF quorum sensing system is not confined to virulence caused by pathogenic bacteria [23, 46], but also used by the plant-associated biocontrol agent *S. maltophilia* R551-3 that underlies its role in positive plant-microbe interactions. Furthermore, the dual role of quorum sensing systems as beneficial and harmful is of relevance for understanding the interactions of both opportunistic and beneficial bacteria with their hosts in hospitals and in the field.

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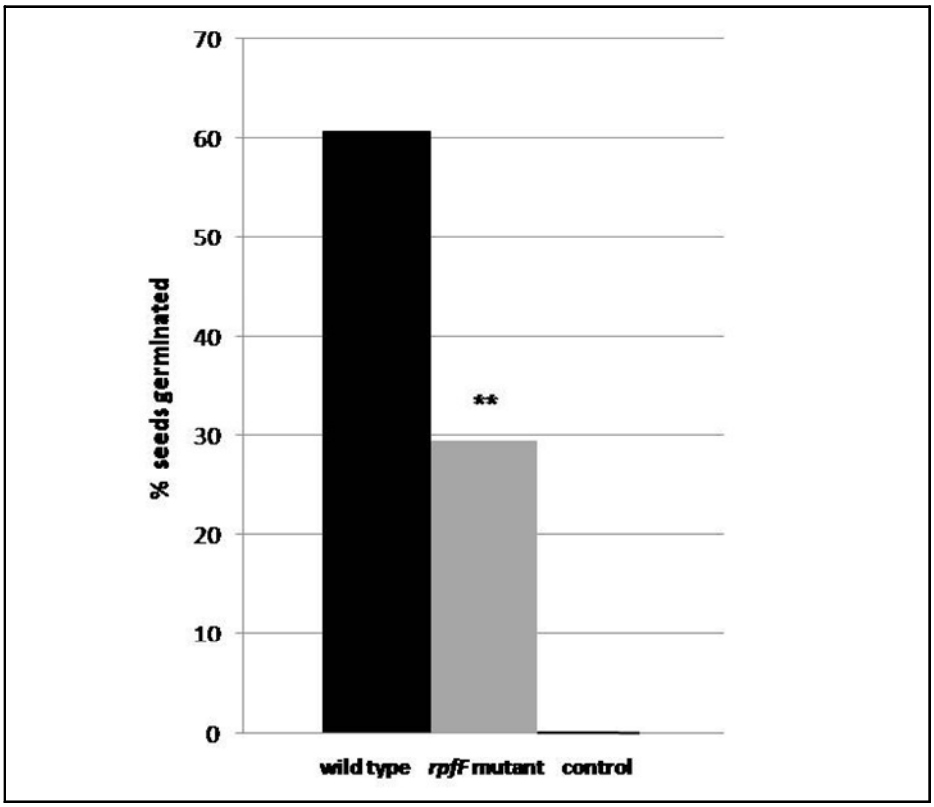

Fig. 1: The role of the *S. maltophilia* R551-3 *rpf*/DSF system in seed germination. Bio-primed oilseed rape seeds were treated with 10^6 CFU ml^{-1} *S. maltophilia* R551-3 wild-type or the *rpfF* mutant strain. The control plants were not treated with either of the bacterial strains, and showed no germination at all after 24 h of incubation. Oil seed rape seeds were planted into autoclaved soil and incubated under greenhouse conditions. The seed germination data represented here was obtained after 24 h of incubation. Data are presented as the mean values of germinated seeds of eight independent replicates. Each replicate consists of eleven surface sterilized, bio-primed oilseed rape seeds planted into soil. There was no seed germination for the control group after 24 h of incubation. ** : $p < 0.05$.

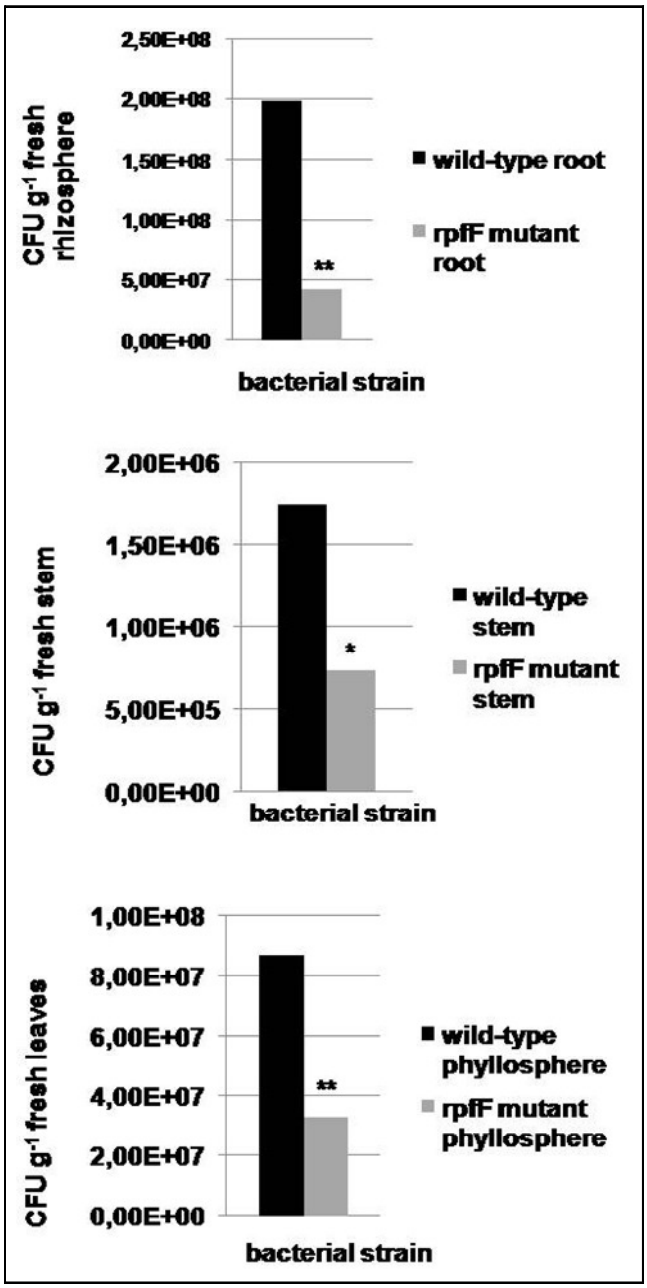

Fig. 2: Colonization of oilseed rape plants by *S. maltophilia* R551-3 wild-type and *rpfF* mutant strains. Bacteria were re-isolated from 20-day-old oilseed rape plants grown in gnotobiotic soil systems under greenhouse conditions. For re-isolation, plant sections (roots with adhering soil, stem or leaves) were supplemented with 0.85% NaCl solution and rigorously disintegrated with pestle and mortar. Serial dilutions of the extract were then plated onto LB plates. After incubation at 37 °C for 48 h, cell counts were determined and CFU g^{-1} plant material was calculated. Data are presented as the mean values of at least four replicates. * : $p < 0.1$; ** : $p < 0.05$.

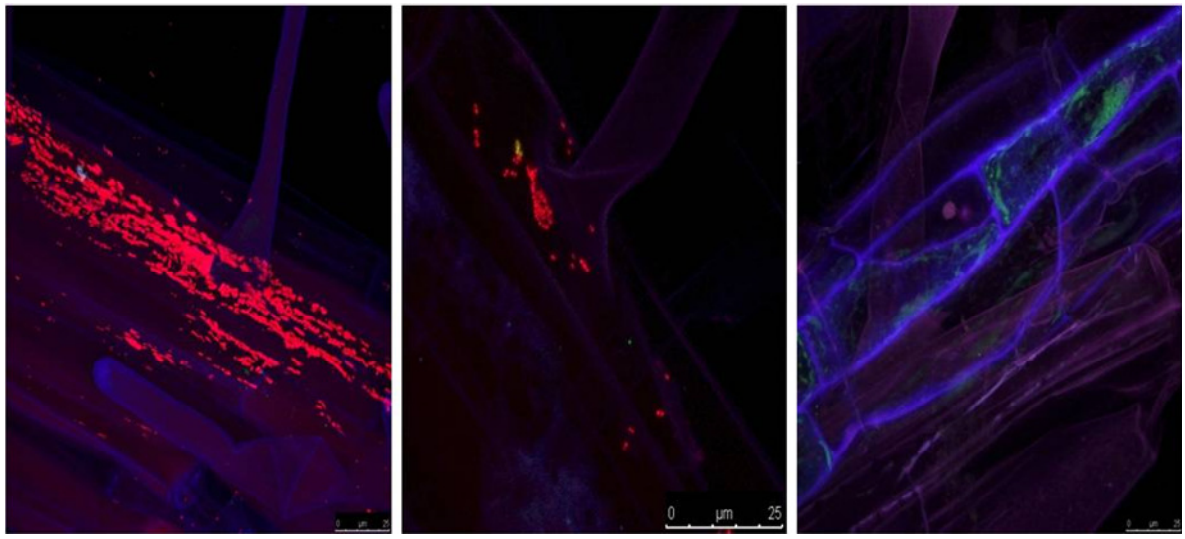

Fig. 3: Colonization of the 11-day-old oilseed rape rhizosphere by the wild-type (left) and the *rpfF* mutant strain (middle) visualized by fluorescent *in situ* hybridization (FISH). The image on the right-hand side corresponds to the seeds without bacterial inoculation (control). An equimolar ratio of the FISH probes EUB338, EUB338 II and EUB338 III labeled with the fluorescent dye Cy3 was used in the hybridization step for the detection of *S. maltophilia* wild-type and *rpfF* mutant strains. Microscopic images were captured using a Leica TCS SPE confocal microscope. The Leica ACS APO 63X OIL CS objective (NA: 1.30) was used to acquire confocal stacks by applying a z-step of 0.2–0.9 µm. Same colonization pattern was obtained for at least four samples from separate replicates.

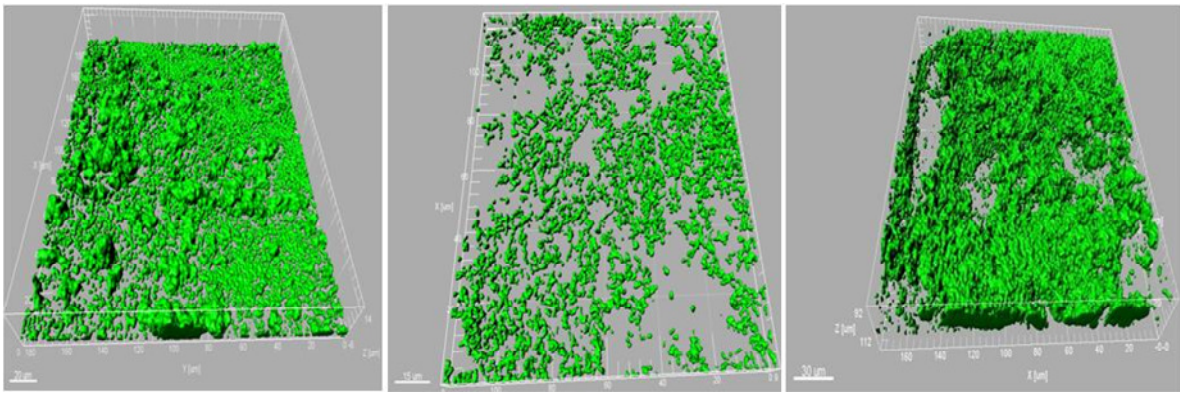

Fig.4: 3D CLSM images were captured from the *gfp*-labeled *S. maltophilia* wild-type (left), *rpfF* mutant strain (middle) and the *rpfF* mutant strain supplemented with 100 µM DSF (right). While the wild-type strain formed structured, surface-covering cell architecture with a particular texture consisting of several cell layers, the *rpfF* mutant strain constructed an unstructured and unconnected monolayer film of cells. The *rpfF* mutant strain supplemented with 100 µM DSF (cis-Δ2-11-methyl-Dodecenoic Acid), however, formed the same structure observed for the wild-type. *gfp*-labeled wild-type and *rpfF* mutant strain cultures as well as the *rpfF* mutant strain culture supplemented with 100 µM synthetic DSF molecule were grown in LB medium up to OD_{600} of 1. The cultures were then placed into the chambers of the Lab-Tek® II CC2™ Chamber Slide™ System and incubated at 37 ºC for three days. To capture the microscopic images a Leica TCS SPE confocal laser scanning microscope was used. The confocal stacks were acquired with the Leica ACS APO 63X OIL CS objective (NA: 1.30) by applying a z-step of 0.4–0.9 µm. The 3D analysis of the CLSM stacks was performed using the software Imaris 7.0. The assay was performed at least four times.

Publication 2

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Root-microbe systems: the effect and mode of interaction of Stress Protecting Agent (SPA) *Stenotrophomonas rhizophila* DSM14405^T

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Stenotrophomonas rhizophila has great potential for applications in biotechnology and biological control due to its ability to both promote plant growth and protect roots against biotic and a-biotic stresses, yet little is known about the mode of interactions in the root-environment system. We studied mechanisms associated with osmotic stress using transcriptomic and microscopic approaches. In response to salt or root extracts, the transcriptome of *S. rhizophila* DSM14405^T changed drastically. We found a notably similar response for several functional gene groups responsible for general stress protection, energy production, and cell motility. However, unique changes in the transcriptome were also observed: the negative regulation of flagella-coding genes together with the up-regulation of the genes responsible for biofilm formation and alginate biosynthesis were identified as a single mechanism of *S. rhizophila* DSM14405^T against salt shock. However, production and excretion of glucosylglycerol (GG) were found as a remarkable mechanism for the stress protection of this *Stenotrophomonas* strain. For *S. rhizophila* treated with root exudates, the shift from the planktonic lifestyle to a sessile one was measured as expressed in the down-regulation of flagellar-driven motility. These findings fit well with the observed positive regulation of host colonization genes and microscopic images that show different colonization patterns of oilseed rape roots. Spermidine, described as a plant growth regulator, was also newly identified as a protector against stress. Overall, we identified mechanisms of *Stenotrophomonas* to protect roots against osmotic stress in the environment. In addition to both the changes in life style and energy metabolism, phytohormons, and osmoprotectants were also found to play a key role in stress protection.

Keywords: plant-microbe interaction, oilseed rape, PGPR, SPA, transcriptomics, root exudates, FISH–CLSM

INTRODUCTION

Crop cultivation in salinated soils is one of the major challenges facing agriculture today. Salinated areas are increasing world-wide and plants growing under saline or water-imbalance stress are more vulnerable to diseases caused by soil-borne pathogens (FAO, 2005). Biocontrol using salt-tolerant, plant growth-promoting rhizobacteria (PGPR) to protect plant roots against high salinity and pathogens offers sustainable solutions for plant protection, and *Stenotrophomonas rhizophila* is a model bacterium for a rhizosphere- and phylloplane-competent, salt-tolerant PGPR (Ryan et al., 2009; Berg et al., 2010, 2013). While the species *S. maltophilia* has become important as a nosocomial human pathogen, no pathogenic potential for humans has ever been observed in the related species *S. rhizophila* (Wolf et al., 2002). Moreover, both species can be easily distinguished by the production of the osmoprotective substance glucosylglycerol (GG) (only present in *S. rhizophila*) and the occurrence of specific multidrug-efflux pumps (only present in *S. maltophilia*) (Ribbeck-Busch et al., 2005).

Plant growth promotion by *S. rhizophila* strain DSM14405^T (syn. strain e-p10) was observed under greenhouse conditions (Schmidt et al., 2012) and in the highly salinated soils of Uzbekistan at levels up to 180% (Egamberdieva et al., 2011). Use of classical physiological and biochemical methods unveiled the mechanisms of plant growth promotion and biocontrol against soil-borne pathogens (Berg and Ballin, 1994; Kobayashi et al., 1995; Jacobi et al., 1996; Dunne et al., 2000; Suckstorff and Berg, 2003) as well as the production of high amounts of osmolytes trehalose and GG in response to salt stress (Roder et al., 2005). Next generation sequencing techniques have allowed for new possibilities to study plant-microbe interaction. For example, genome sequencing has given new insight into the genetic sources that provide beneficial plant-associated bacteria with traits such as plant growth promotion, protection against phytopathogens, and osmoprotection. In general, the described mode of action could be confirmed due to the presence of genes possibly responsible in the genome of *S. rhizophila* DSM14405^T (Berg et al., 2013). For example, *S. rhizophila* possesses genes

responsible for the synthesis and transport of osmoprotective molecules out of the cell. In addition, it contains a number of genes involved in the biocontrol of soil-borne pathogens and important genes that aid in the competition for nutrients and niches as well. Additionally, *S. rhizophila* is equipped with several genes which may play a role in root colonization, such as those that encode the O-antigen, capsule polysaccharide biosynthesis pathways, hemagglutinin, and outer membrane adhesion proteins. However, despite this knowledge, there is still no evidence that these genes are involved in successful root-microbe interactions under salinated conditions. In addition to the high salinity, the role of root exudates for this interaction was pointed out in other studies (González-Pasayo and Martínez-Romero, 2000; rev. in Bais et al., 2006). The ability of cells to respond appropriately to changing environmental conditions can be investigated using a transcriptomic approach. This technique offers a new and powerful tool to evaluate these hypothetical mechanisms *in situ*, as shown already by van de Mortel et al. (2012) for the *Pseudomonas-Arabidopsis* and by López-Guerrero et al. (2012) for the *Rhizobium-Phaseolus* interaction.

The objective of our study was to investigate the response to changing environmental conditions associated with osmotic stress (1) salt stress and (2) root exudates to understand stress protection against changing osmotic conditions of roots by the endophytic bacterium *S. rhizophila* DSM14405^T in more detail. We hypothesized that there is a general response to changing osmolarities, but also a specific answer to each other of the two parameters which are important for colonizing the root system of plants.

MATERIALS AND METHODS

TREATMENT WITH OILSEED RAPE EXUDATES

Root exudates were collected from oilseed rape cultivar Californium (Kwizda, Austria) and grown for 14 days in gnotobiotic systems of 50 ml of sterilized vermiculite packaged in pots and covered with lids (Metro, Austria). Prior to sowing the seeds, about 50 ml of tap water was amended with 1/10 [v/v] of minimal medium (Gamborgs B5 basal salt mixture; Duchefa), and the seeds were surface-sterilized in sodium hypochlorite (10% wt/wt) for 10 min and washed successively with sterile water under sterile conditions. No seeds were sown in the control system. Plant and control systems were arranged in a replicate randomized block design and maintained at 20°C under 16-h light and 8-h dark conditions. After 14 days, plants were removed and the root exudates and liquid from the control system were collected in sterile bags and squeezed. To corroborate sterility, both root material and exudates were plated on nutrient agar. Root exudates were centrifuged (10 min, 5000 × *g*), and the supernatant was collected, filter-sterilized (first 0.45 µm, second 0.22 µm filter, Millipore), and stored at −20°C in the dark until use. *S. rhizophila* DSM14405^T was cultivated under agitation in 40 ml CAA (per liter: 5.0 g casamino acids, 1.54 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$) and supplemented with 10 ml of the root exudates and the control liquid, respectively, at 30°C for 48 h. Cells were harvested using centrifugation at 2500 × *g* for 1 min for RNA extraction.

SALT SHOCK

S. rhizophila DSM14405^T was cultivated in 50 ml CAA under agitation at 30°C for 13 h (per liter: 5.0 g casamino acids, 1.54 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$) until an optical density of OD_{600} 0.8 was reached. A final salt (NaCl) concentration of 3% in the medium was reached by using a sterile concentrated sodium chloride stock solution (0.3 g l^{-1}). After 2.7 h cultivation in the medium containing 3% salt, the *S. rhizophila* DSM14405^T culture ($OD_{600} = 0.9$) was used for RNA extraction. Two independent replicates were performed as described above.

RNA EXTRACTION AND TRANSCRIPTOMIC ANALYSES

RNA was extracted using the RNAprotect® Bacteria Reagent (Qiagen, Hilden, Germany). Total rRNA was removed and mRNA was enriched using the MICROBExpress kit, according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). The mRNA was sequenced using LGC Genomics (Berlin, Germany), and data collection was performed using MicroDiscovery (Berlin, Germany). The data used for assessing the changes in gene transcription correspond to normalized values for the number of reads that uniquely mapped to each CDS. Transcription fold change for each CDS was assessed by dividing the corresponding value from the cells that were either treated with root exudates or exposed to salt shock by those from the control group. Of the total genes either up or down-regulated, only those showing fold changes greater than or equal to 1.5 and less than or equal to 0.6 were considered significantly impacted.

GERMINATION POUCH COLONIZATION ASSAY

A batch of 200 oilseed rape seeds were surface-sterilized with 40 ml of 3% NaOCl for 1 min and subsequently washed twice with 40 ml of water for 1 min each time. Surface-sterilized seeds were inoculated with *S. rhizophila* DSM14405^T by incubating in a 2 ml cell suspension containing 10^7 CFU ml^{-1}. The control included seeds treated with 0.85% NaCl. Twelve seeds per treatment were placed into 2 (6 seeds per pouch) sterile Cyg™ germination pouches (Mega International, West St. Paul, MN, USA) wetted with 10 ml of sterilized deionized water or 1.25% NaCl solution. Germination pouches were then placed in sterile, aseptically sealed containers and placed in a growing chamber for 9 days with controlled day and night settings (12 h of light at 25°C and 12 h of dark at 20°C). After 9 days of growth, roots of 3 seedlings were combined for determination of cell counts resulting in 4 replicates per individual treatment. All root material was cut and transferred to Whirlpak® bags (Carl Roth, Karlsruhe, Germany) containing 2 ml of 0.85% NaCl solution. The roots in the bags were then crushed using a pestle to form a homogenous suspension, which was subsequently serially diluted and drop-streaked onto LB Petri dishes. The plates were then incubated at 30°C for 24 h.

FLUORESCENT *in situ* HYBRIDIZATION (FISH)

To study the oilseed rape colonization ability of *S. rhizophila* DSM14405^T using confocal microscopy, the oilseed rape roots grown in seed germination pouches were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) (3:1 vol/vol). The control group contained roots without bacterial treatment.

The fixed samples were then stored in PBS/ 96% ethanol (1:1) at −20°C. The FISH probes were purchased from genXpress® (Wiener Neudorf, Austria), and the in-tube FISH was performed as described by Cardinale et al. (2008). The FISH probes used for the hybridization step were labeled with the fluorescent dye Cy3 and included EUB338 (Amman et al., 1990), EUB338 II, and EUB338 III (Daims et al., 1999), all directing eubacteria. An equimolar ratio of the FISH probes was used for the hybridization step to detect *S. rhizophila* DSM14405^T. In this step, 30% formamide was added to the samples which were then subsequently incubated in a water bath (43°C) for 90 min. After hybridization, the samples were washed at 44°C for 15 min. Microscopy and image capturing were performed using a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) with the Leica ACS APO 63X OIL CS objective (NA: 1.30). A z-step of 0.4–0.8 μm was applied to acquire confocal stacks.

RESULTS

TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO SALT STRESS

Under salt stress of 3% NaCl, a total number of 912 and 1521 genes of *S. rhizophila* DSM14405^T were significantly up and down-regulated, respectively. The impact of salt shock on the transcription of *S. rhizophila* DSM14405^T with respect to various functional gene groups is shown in **Figure 1**. The majority of functional groups were strongly affected, such as up-regulated genes involved in translation, synthesis of the cell wall, outer or cytoplasm membrane, nucleotide and amino acid transport and metabolism, and the production and conversion of energy. In contrast, genes involved in cell motility, secretion, intracellular trafficking, defense mechanisms, and the transport and metabolism of carbohydrates and inorganic ions are down-regulated. Moreover, genes responsible for lipid metabolism and hypothetical genes are somewhat ambiguously affected by salt stress as some are up while others down-regulated.

Of the genes that are significantly impacted by salt stress in *S. rhizophila* DSM14405^T (**Table 1**, **Tables S1A, S1B**), a number of those responsible for general and specific stress responses are up-regulated. While *surA* and *dnaJ* code for general stress chaperones, *ggpS* and *ycaD* build a well-known salt stress response mechanism in *S. rhizophila* DSM14405^T through the synthesis of the osmolyte GG and show a fold change of 8.3, and 7.7, respectively (Hagemann et al., 2008). Moreover, two genes responsible for cold shock, *deaD* and *cspA*, are also strongly up-regulated under salt stress. In addition, the transcription of *ousA* that codes for an osmotic stress protein is positively affected as well. Cellular ion exchange mechanisms and some iron uptake genes are also up-regulated in *S. rhizophila* DSM14405^T as the result of salt stress.

Although unable to synthesize xanthan, *S. rhizophila* DSM14405^T possesses some of the up-regulated xanthan-coding genes including *xanA*, *xanB*, and *rmlAC*. These genes are involved in biofilm formation in addition to their role in xanthan biosynthesis (Huang et al., 2006). Likewise, the alginate coding gene *algJ* shows a fold change of 3.2 as a result of salt shock. Alginate is an exopolysaccharide involved in the development and architecture of biofilms that protect bacteria from antibiotics and other harmful environmental factors (Monday and Schiller, 1996; Stapper et al., 2004). Furthermore, specific secretion and transport systems such as those that code for the type VI secretion system (TVISS) are strongly up-regulated in *S. rhizophila* DSM14405^T under salt shock, however, it should be noted that closely related plant-associated *Stenotrophomonas* strains such as *S. maltophilia* R551-3 lack the TVISS. Moreover, genes involved in the conversion and transport of substances through the cell wall and those responsible for cell division are also up-regulated.

Genes responsible for flagellar apparatus and fimbriae-biosynthesis genes are comparatively down-regulated in *S. rhizophila* DSM14405^T under salt shock. Similarly, salt shock also negatively impacted the predicted capsule biosynthesis genes.

TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO ROOT EXUDATES

A total of 763 and 246 genes were significantly up and down-regulated, respectively, as a result of the addition of oilseed-rape root exudates (**Figure 2**). In general, the effect of root exudates on the functional groups is indeterminate, as some genes of a particular group are up-regulated while others are transcribed in lesser numbers. However, some functional groups are equally affected by plant root exudates, as the transcription of almost all corresponding gene sequences is either up or down-regulated. For instance, as shown in **Figure 2**, root exudates have only a positive effect on the transcription of genes responsible for amino acid, nucleotide, and carbohydrate transport and metabolism, as well as biogenesis of cell membranes, transport of substances through the cell, and the genes responsible for the transport of secondary metabolites and coenzymes. Conversely, genes involved in the secretion, transport, and metabolism of inorganic ions as well as in cell motility are mainly down regulated in response to root exudate stress.

Of those genes with a significant transcription fold change discussed above, some code for products with a known physiological function and are presented in **Table 2**. The complete list of *S. rhizophila* DSM14405^T genes with a significant transcription fold change is presented in **Tables S2A, S2B**. Cell wall breakdown and cell adherence are early and crucial steps in host-plant colonization. As presented in **Table 2**, the treatment of *S. rhizophila* DSM14405^T cells with oilseed rape seedling exudates resulted in enhanced expression of *cbg-1* and *xynB* that code for beta-glucosidase and xylanase B, respectively, and are involved in cell wall breakdown. Furthermore, both these genes are conserved among plant-associated *Stenotrophomonas* strains as they are present in both *S. rhizophila* DSM14405^T and the plant-benefiting *S. maltophilia* R551-3, but absent from the human-pathogenic *S. maltophilia* K279a. In addition, Sr14405 2818, which is also up-regulated by 2.4 folds, codes for an adhesin protein and is homologous to the haemagglutinin-like protein coding gene from the human-pathogenic *S. maltophilia* K279a (**Table 2**). Other up-regulated genes include two adjacent genes, *mdtI* and *mdtJ* that both code for spermidine export proteins. Spermidine is a plant growth regulator and has been recently shown to strongly promote the growth of arugula plants (Al-Whaibi et al., 2012). Moreover, several genes that code for multidrug resistance pumps, efflux transporters, heavy metal transport systems, and

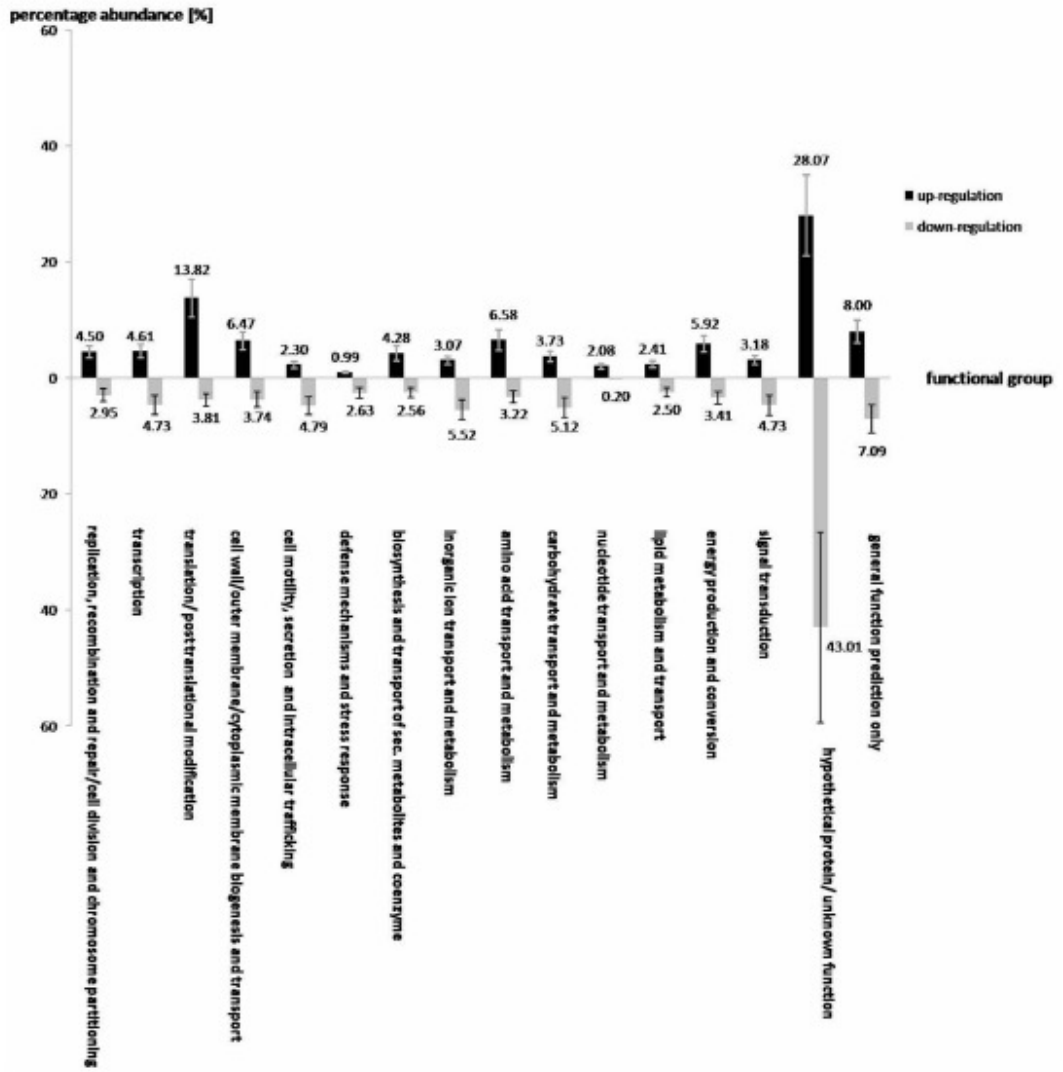

FIGURE 1 | The effect of salt shock on the gene expression of various functional gene groups in *S. rhizophila* DSM14405^T. A total of 912 and 1521 genes were significantly up and down-regulated, respectively. The impact of salt stress on most functional gene groups is clearly pronounced, as a given functional group shows either an increase or decrease in the transcription of genes belonging to that group. Genes involved in translation, synthesis of the cell wall, outer or cytoplasm membrane, nucleotide and amino acid transport and metabolism, energy production and conversion are up-regulated. In contrast, genes involved in cell motility, secretion, and intracellular trafficking, defense mechanisms, and transport and metabolism of carbohydrates and inorganic ions are down-regulated. Genes involved in lipid metabolism, and the hypothetical genes are rather ambiguously affected by salt stress, as some of these are up and others down-regulated. The values above each column correspond to the percentage abundance of the corresponding functional group relative to the total count of the up and down-regulated genes. The transcription fold change for each CDS corresponds to the ratio calculated for *S. rhizophila* under salt shock compared with the control. Data are presented as the mean value of two independent replicates. The error bar shown on each functional group corresponds to the mean value of errors for all genes belonging to that functional group.

resistance against antibiotics are positively affected by seedling exudates.

S. rhizophila DSM14405^T contains two flagella-encoding gene blocks that are almost entirely negatively affected by the addition of oilseed rape seedling exudates. The complete list of the flagellar apparatus-coding genes that are down-regulated is not confined to those noted in **Table 2**, and is presented in **Tables S2A, S2B**. Likewise, the

Table 1 | Selected *S. rhizophila* DSM14405^T genes with known biological roles impacted by salt shock.

Gene	(Putative) Product	Transcription fold change	Biological function
ggpS	Glucosylglycerol-phosphate synthase	8.3	Salt shock response protein
ycaD	MFS-type transporter	7.7	Salt shock response protein transporter
xanA	Hosphohexane mutases	3.0	Xanthan biosynthesis; biofilm formation
xanB	Xanthan biosynthesis protein xanB	2.9	Xanthan biosynthesis; biofilm formation
rmlC	dTDP-4-dehydrorhamnose 3,5-epimerase	2.3	Xanthan biosynthesis; biofilm formation
algJ	Alginate biosynthesis protein	3.2	Alginate biosynthesis
Sr14405 2749	TVISS effector, Hcp1 family protein	2.6	Type VI secretion system
Sr14405 2755	Rhs element Vgr protein	4.2	Type VI secretion system
Sr14405 2761	Rhs element Vgr protein	3.4	Type VI secretion system
icmF	TVISS protein	5.0	Type VI secretion system
Sr14405 2781	TVISS-associated protein, ImpA family	2.4	Type VI secretion system
Sr14405 2791	Rhs element Vgr protein	6.8	Type VI secretion system
deaD	Cold-shock DEAD box protein A homolog	8.4	Cell shock response
cspA	Major cold shock protein	4.3	Cell shock response
Sr14405 1916	Beta-lactamase L2 protein	6.3	Antibiotic resistance
tetA	Tetracycline resistance protein	3.8	Antibiotic resistance
Sr14405 1293	Bacterioferritin-associated ferredoxin	2.1	Iron uptake and transport
bfr	Bacterioferritin	5.9	Iron uptake and transport
hisI	Histidine biosynthesis bifunctional protein	4.6	Histidine biosynthesis
ousA	Osmoprotectant uptake system protein	4.2	Osmotic stress response
surA	Chaperone protein	3.8	Cellular stress response
dnaJ	Chaperone	3.1	Stress response
ompW	Outer membrane protein	3.6	Transport
oprF	Outer membrane protein	2.6	Transport
ftsQ	Cell division protein	3.4	Cell division
ftsA	Cell division protein	2.3	Cell division
ftsY	Cell division protein	2.6	Cell division
ftsZ	Cell division protein	2.0	Cell division
lptF	Lipopolysaccharide export system permease protein	4.5	Cell wall transport
lptG	Lipopolysaccharide export system permease protein	3.3	Cell wall transport
Sr14405 2454	Peptidoglycan-associated outer membrane lipoprotein	2.5	Cell wall protein
mltD	Muramidase	3.2	Bacterial cell wall biodegradation
Sr14405 1936	Peptidoglycan-associated lipoprotein	2.8	Cell wall structure protein
Sr14405 4324	Cell morphology protein	2.7	Unknown
clcA	H(+)/Cl(−) exchange transporter	2.7	Ion regulation
kefA	Potassium efflux system	2.3	Ion regulation
flgA	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
flgC	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
flgG	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
flgF	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
flIF	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
flhA	Flagellar biosynthesis	0.3	Flagellar-driven motility
flhB	Flagellar biosynthesis	0.3	Flagellar-driven motility
cfaB	CFA/I fimbrial subunit B	0.3	Fimbriae synthesis
cfaC	CFA/I fimbrial subunit C	0.4	Fimbriae synthesis
csoB	Fimbrial subunit B	0.2	Fimbriae synthesis
Sr14405 3215	Capsule polysaccharide biosynthesis protein	0.1	Capsule biosynthesis
Sr14405 3217	Putative UDP-glucose 4-epimerase	0.2	Capsule biosynthesis
wzc	Tyrosine-protein kinase	0.2	Capsule biosynthesis

The values for fold changes correspond to the S. rhizophila DSM14405^T subjected to the 3% NaCl shock compared to the control.

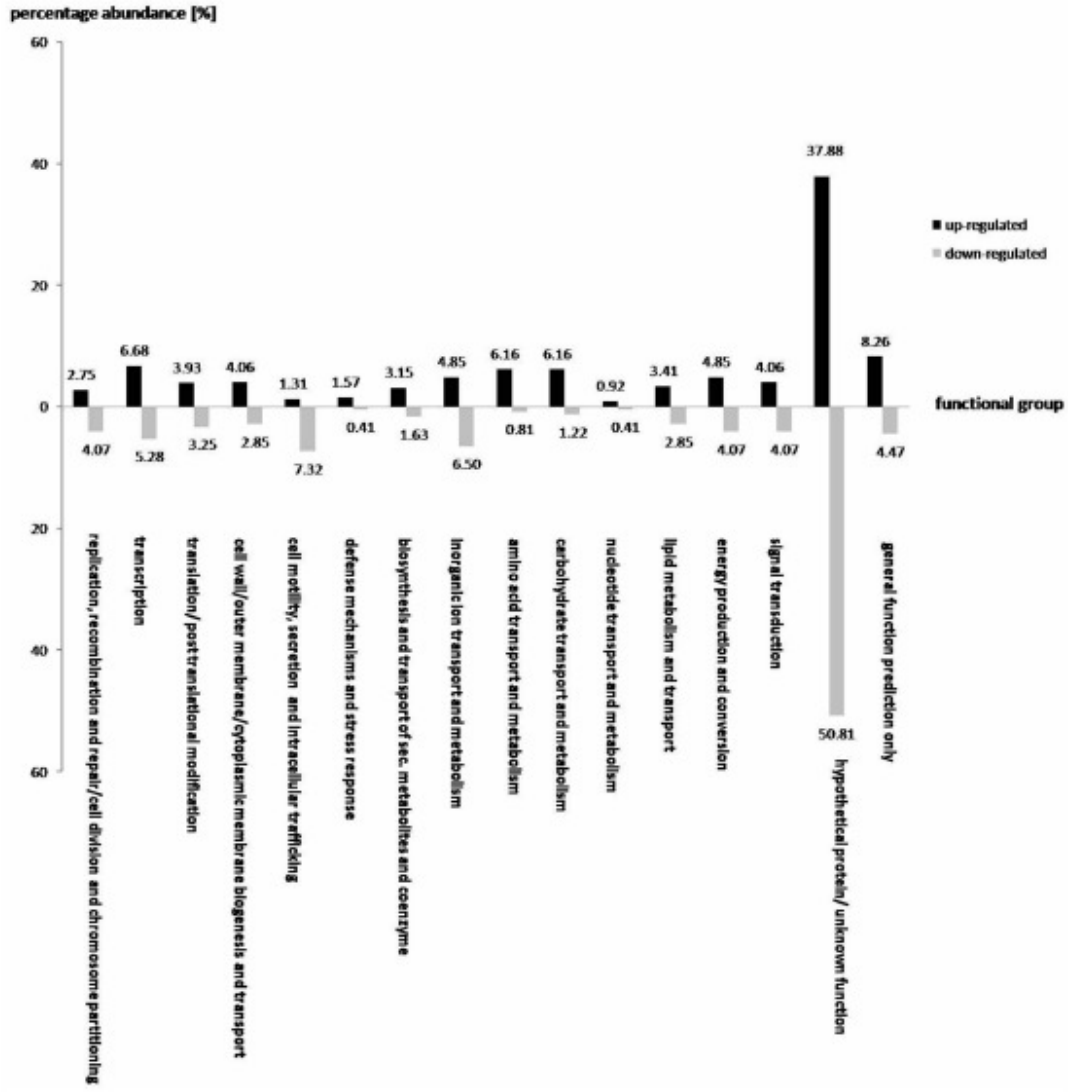

FIGURE 2 | The effect of oilseed rape seedling exudates on gene expression of various functional gene groups in *S. rhizophila* DSM14405^T. A total of 763 and 246 genes were significantly up and down-regulated, respectively. While some functional groups are both positively and negatively regulated by root exudates, others show a clear and pronounced alteration, as the majority of the corresponding genes are either up or down-regulated. For example, genes responsible for amino acid, nucleotide, and carbohydrate transport and metabolism, and those involved in cell wall, outer-membrane or cytoplasmic membrane biogenesis and transport as well as genes responsible for the transport of secondary metabolites and coenzymes are mainly up-regulated. In contrast, genes involved in cell motility and secretion, and those responsible for the transport and metabolism of inorganic ions are mainly down-regulated. The value above each column corresponds to percentage abundance of the corresponding functional group in the total count of the up or down-regulated genes.

expression of the genes responsible for fimbriae-driven cell motility, such as *cfaB* and *csoB* is negatively impacted by seedling exudates. Moreover, genes involved in the uptake, transport, and bioavailability of iron are also down-regulated.

SIMILARITIES IN THE TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO SALT AND ROOT EXUDATES

In response to both oilseed rape root exudates and salt shock, *S. rhizophila* DSM14405^T copes with osmotic stress in a surprisingly similar way through the alteration of gene

Table 2 | Selected *S. rhizophila* DSM14405^T genes with known biological roles impacted by plant root exudates.

Gene	(Putative) Product	Transcription fold change	Biological function
mdtI	Spermidine export protein	6.3	Export of the plant growth regulator spermidine
mdtJ	Spermidine export proteins	7.6	Export of the plant growth regulator spermidine
Sr14405 2818	Adhesin	2.4	Host cell surface attachment/colonization
cbg-1	Beta-glucosidase	1.7	Plant cell wall biodegradation/colonization
xynB	Xylanase B	1.6	Plant cell wall biodegradation/colonization
Sr14405 4324	Cell morphology protein	2.2	Unknown
Sr14405 1672	Generally characterized MFS-type transporter	3.0	Antibiotic resistance
Sr14405 1673	Multidrug synthesis protein	8.8	Antibiotic resistance
Sr14405 2718	Multidrug synthesis protein	3.5	Antibiotic resistance
tetX	Tetracycline resistance protein	3.5	Antibiotic resistance
Sr14405 4658	Acriflavin resistance protein	1.6	Antibiotic resistance
Sr14405 2827	Heavy metal transport and detoxcification protein	2.0	Heavy metal efflux system
Sr14405 1538	Efflux transporter	1.5	Efflux of unknown target
figA	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
figC	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
figG	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
figF	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
fliF	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
flhA	Flagellar biosynthesis	0.6	Flagellar-driven motility
flhB	Flagellar biosynthesis	0.5	Flagellar-driven motility
cfaB	CFA/I fimbrial subunit B	0.5	Fimbriae synthesis
csoB	Fimbrial subunit B	0.4	Fimbriae synthesis
Sr14405 1293	Bacterioferritin-associated ferredoxin	0.6	Iron uptake and transport
Sr14405 1746	Heme oxygenase	0.4	Iron bioavailability
fpvA	Ferripyoverdine	0.5	Iron uptake and transport
Sr14405 4245	Outer-membrane hemin receptor	0.4	Iron uptake and transport

The values for fold changes correspond to the S. rhizophila DSM14405^T treated with plant root exudates compared to the control.

expression. Numerous functional gene groups are up-regulated in response to osmotic stress factors and include those involved in energy production, as well as those involved in the synthesis and transport of cell wall, outer membrane, and cytoplasmic membrane, and those responsible for the metabolism and transport of amino acids, nucelotides, and secondary metabolites (**Figure 3**). Conversely, genes responsible for cell motility, secretion, intracellular trafficking, and the transport and metabolism of inorganic ions are down-regulated under both salt stress and treatment with root exudates.

COLONIZATION PATTERNS OF *S. rhizophila* DSM14405^T ON ROOTS UNDER STRESS

S. rhizophila DSM14405^T intensely colonizes oilseed rape plants, as revealed by the cell count of $\log_{10}$ 9.47 CFU g^{-1} root fresh weight ($\pm$0.08) for seeds treated with deionized water. The treatment of seeds with 1.25% NaCl, however, decreased the colonization ability by nearly half resulting in a cell count of $\log_{10}$ 9.09 CFU g^{-1} root fresh weight ($\pm$0.18). Furthermore, microscopic images captured using FISH combined with confocal laser scanning microscopy (CLSM) also revealed a significant decrease in the colonization of oilseed rape roots treated with 1.25% NaCl (**Figure 4**).

DISCUSSION

We studied the response of *S. rhizophila* DSM14405^T to osmotic changes in the form of plant root exudates and salt shock at both the physiological and molecular level. Even though we found a notable similarity in how the cell copes with these stressors, the individual responses included a great deal of specificity at the gene level thus. The response of *S. rhizophila* DSM14405^T to both oilseed rape root exudates and salt corresponds with several functional gene groups including those responsible for the synthesis and transport of cell wall, outer membrane, and cytoplasmic membrane, the metabolism and transport of amino acids, nucleotide, and secondary metabolites, energy production, cell motility, secretion and intracellular trafficking, and the transport and metabolism of inorganic ions. For *S. rhizophila* treated with root exudates, however, the shift from the planktonic lifestyle to a sessile one as expressed in the down-regulation of flagellar-driven motility is targeted to colonize the plant host, and is well in accordance with the observed positive regulation of host colonization genes. In addition to the changes in behavior and lifestyle of the bacterium, several bioactive substances were identified as key factors in stress protection. The first among them is the plant growth regulator, spermidine. Although this substance is known to strongly promote growth, this is the first evidence to show its involvement in stress protection of roots. The second group includes osmoprotective substances which were both produced

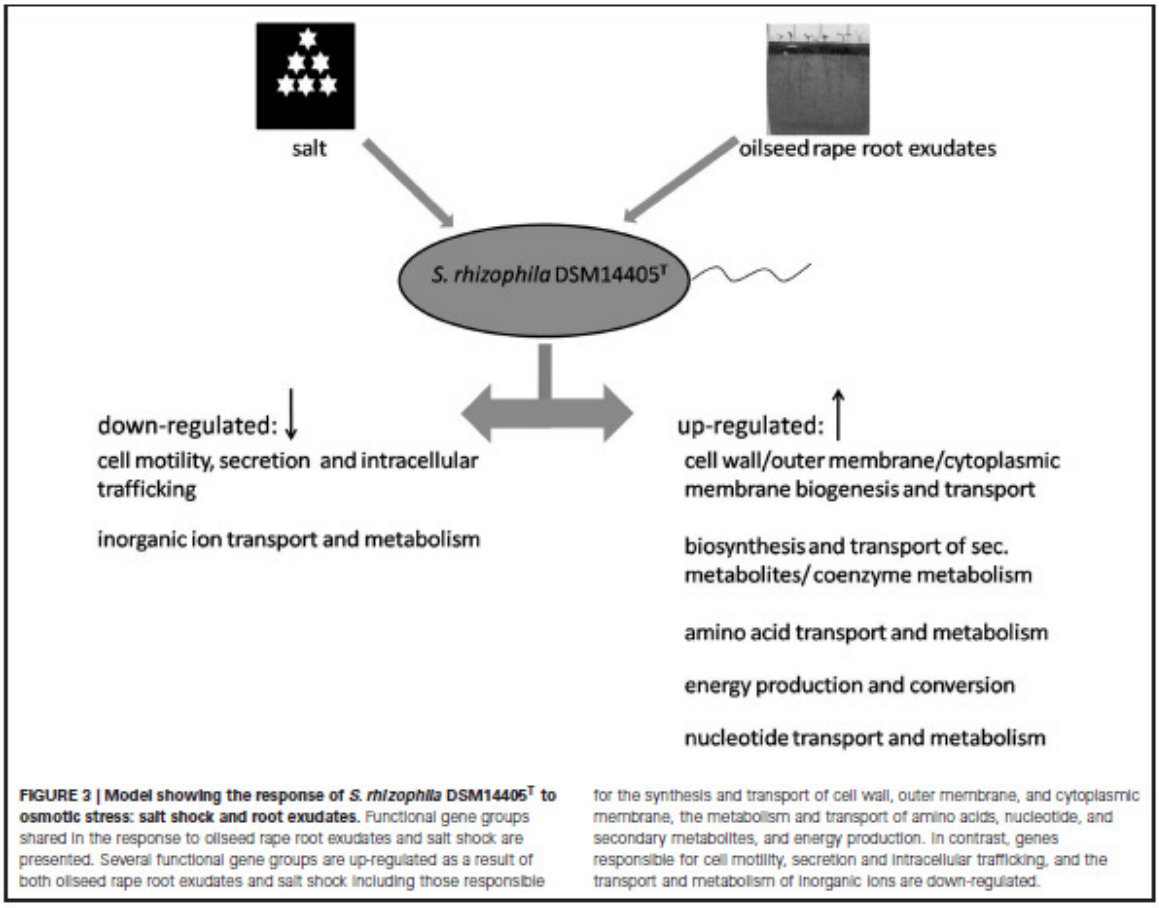

FIGURE 3 | Model showing the response of *S. rhizophila* DSM14405^T to osmotic stress: salt shock and root exudates. Functional gene groups shared in the response to oilseed rape root exudates and salt shock are presented. Several functional gene groups are up-regulated as a result of both oilseed rape root exudates and salt shock including those responsible for the synthesis and transport of cell wall, outer membrane, and cytoplasmic membrane, the metabolism and transport of amino acids, nucleotide, and secondary metabolites, and energy production. In contrast, genes responsible for cell motility, secretion and intracellular trafficking, and the transport and metabolism of inorganic ions are down-regulated.

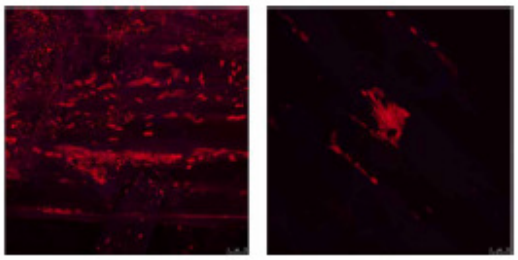

FIGURE 4 | The impact of salt stress on the capability of *S. rhizophila* DSM14405^T to colonize the oilseed rape rhizosphere visualized using FISH-CLSM. *S. rhizophila* DSM14405^T intensely colonizes the oilseed rape rhizosphere (left) while the treatment of seeds with 1.25% NaCl (right) severely decreases the colonization capability. An equimolar ratio of the FISH probes EUB338, EUB338 II, and EUB338 III labeled with the fluorescent dye Cy3 was used in the hybridization step. Microscopic images were captured using a Leica TCS SPE confocal microscope. The Leica ACS APO 63X OIL CS objective (NA: 1.30) was used to acquire confocal stacks by applying a z-step of 0.4–0.8 µm.

and excreted in high volumes as described earlier (Roder et al., 2005).

Spermidine is a well-known plant growth regulator and has been revealed to play a critical role in plant embryo development (Imai et al., 2004). Moreover, it has been recently shown to strongly promote the growth of arugula plants (Al-Whaibi et al., 2012). In addition, spermidine affects biofilm formation in various bacterial species via multiple pathways that involve both transport and signaling networks (McGinnis et al., 2009). As a result, enhanced biofilm formation or possible plant growth regulation resulting from the up-regulation of *S. rhizophila* spermidine export genes would well-serve the lifestyle shift that ultimately leads to efficient colonization of the plant host in the presence of oilseed rape exudates. Spermidine was found to prolong the life span of several eukaryotic model organisms including yeasts, nematodes, flies, and plants as well as significantly reduce age-related oxidative protein damage in mice which could indicate a potential universal anti-aging drug for eukaryotes (Imai et al., 2004; Eisenberg et al., 2009).

GG and trehalose are well-studied general osmoprotective substances that protect cells from high salt

concentrations (Ferjani et al., 2003; Hincha and Hagemann, 2004). While both species produce trehalose, GG is synthesized exclusively in *S. rhizophila* thus distinguishing itself from the pathogenic *S. maltophilia* (Ribbeck-Busch et al., 2005; Roder et al., 2005). In *S. rhizophila* DSM14405^T, *ggpS* and *ycaD* are both strongly up-regulated under 3% salt and are essential for the synthesis and transport of GG. This finding corresponds completely with both the general role of GG as a cell protector and previous findings that the amount of GG excreted into the medium increases substantially in comparison with intracellular GG content resulting from a shift of lower (less than 2%) to higher salt concentrations (Roder et al., 2005). Thus, GG production is the specific mechanism of *S. rhizophila* DSM14405^T to cope with salt stress.

TVISS genes represent a novel key virulence system used by many important pathogenic bacteria in eukaryotic host infection (Bingle et al., 2008; Pieper et al., 2009) and are intensely up-regulated under salt shock. In addition, plant growth promotion increased up to 180% in the highly salinated soils of Uzbekistan in the presence of *S. rhizophila* DSM14405^T (Egamberdieva et al., 2011). Similarly, Schmidt et al. (2012) reported that this plant growth promotion effect was more pronounced in soil than under gnotobiotic conditions suggesting it is due to the control of diseases and deleterious microorganisms. Together with the absence of TVISS genes from the other known plant-beneficial *Stenotrophomonas* strains with no plant growth promoting effect under saline conditions, these findings imply that the salt-stimulated *S. rhizophila* DSM14405^T TVISS is indirectly harnessed to promote plant growth by eliminating harmful and deleterious microorganisms in soil.

The treatment of *S. rhizophila* DSM14405^T with oilseed rape exudates resulted in the down-regulation of iron uptake and transport genes. This change is possibly due to the fact that once treated with oilseed rape exudates, *S. rhizophila* is provided with biologically available iron bound with plant siderophores resulting in less demand for the synthesis of bacterial siderophores to bind and uptake biologically unavailable iron ions present in the medium. Moreover, treatment with root exudates resulted in the up-regulation of several multidrug resistance pumps thus demonstrating that the role of the multidrug pumps is not confined to the export of antibiotics out of the cell, but also includes a more general function with the transport of other substances once in a hyperosmotic environment.

Several questions still remain, such as the reason for positive regulation of several genes responsible for iron uptake and transport, as well as the reason for cell division under salt shock or the role of other remaining genes that are significantly up or down-regulated by osmotic stress factors. However, this work has shed light on the so far unknown mode of action of *S. rhizophila* DSM14405^T to a-biotic changes by unveiling the mechanisms that are harnessed to establish in highly salinated plant root ecosystems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Functional_Plant_Ecology/10.3389/fpls.2013.00141/abstract

Table S1A | Significantly up-regulated genes in *S. rhizophila* DSM14405^T under salt shock.

Table S1B | Significantly down-regulated genes in *S. rhizophila* DSM14405^T under salt shock.

Table S2A | Significantly up-regulated genes in *S. rhizophila* DSM14405^T under treatment with root exudates.

Table S2B | Significantly down-regulated genes in *S. rhizophila* DSM14405^T under treatment with root exudates.

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ORIGINAL PAPER

Stenotrophomonas rhizophila DSM14405^T promotes plant growth probably by altering fungal communities in the rhizosphere

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Abstract *Stenotrophomonas rhizophila* DSM14405^T is of high biotechnological interest as plant growth stimulator, especially for salinated conditions. The objective of this study was to determine the effect of plant species (cotton, tomato, and sweet pepper) on colonisation and plant growth promotion of this beneficial bacterium in gnotobiotic systems and in non-sterile soil. All plant structures (leaves, stems, and roots) were densely colonised by DSM14405^T reaching up to 10^9 cells g^{-1} fresh weight; under gnotobiotic conditions the abundances were 4–5 orders of magnitude higher than in non-sterile soil. Under non-sterile conditions and ambient humidity, tomato shoots were more densely colonised than shoots of sweet pepper and cotton. *S. rhizophila* DSM14405^T was shown to grow endophytically and colonise the vicinity of root hairs of tomato. Plant growth promotion was particularly apparent in tomato. In general, the impact of plant species on colonisation and plant growth promotion was more pronounced in soil than under gnotobiotic conditions and likely due to the control of diseases and deleterious microorganisms. *S. rhizophila* DSM14405^T was shown to control diseases in sweet pepper and in cotton. Molecular profiling via single strand conformation polymorphism of internal transcribed spacers and 16S rRNA genes (PCR-single strand conformational polymorphism (SSCP)) revealed that *S. rhizophila* DSM14405^T strongly affected fungal, but not bacterial communities in the rhizosphere of tomato and sweet pepper. Major SSCP bands related to uncultured fungi and *Candida subhashii*, disappeared in tomato rhizosphere after *Stenotrophomonas* treatment. This suggests an indirect, species-specific plant growth promotion effect of *S. rhizophila* via the elimination of deleterious rhizosphere organisms.

Keywords Plant growth promoting bacteria · *Stenotrophomonas* · Rhizosphere communities · Fungal communities · Sweet pepper · Tomato · Cotton

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Introduction

With increasing efforts to develop sustainable agriculture, the use of microbial inocula for promotion of crop growth and for the biological control of plant diseases becomes ever more attractive to reduce the current dependency of agriculture on fossil fuels and environmentally harmful synthetic pesticides. A particular challenge limiting plant production in arid areas is soil salinity, increased by inappropriate irrigation techniques (Tanji and Kielen 2002; FAO 2005). Soil salinisation does not only damage crops directly, but also renders the plants more susceptible to soil-borne pathogens adapted to these conditions (Howell et al. 1994). The exploration of bacterial inocula for relief of salt stress and plant growth promotion in saline soils has just started, but first reports are promising (Mo et al. 2006; Egamberdiyeva et al. 2008; Nadeem et al. 2010).

Bacteria of the genus *Stenotrophomonas* are of increasing biotechnological interest due to their ubiquitous occurrence and versatility (Ryan et al. 2009). Their plant growth-promoting properties and their antagonistic behaviour

against soil-borne plant pathogens are well-documented (Berg et al. 1994; Dunne et al. 1998; Ryan et al. 2009), but development for commercial application of *Stenotrophomonas maltophilia*, the most intensively studied species, has been hampered by its potential as opportunistic human pathogen in immune-suppressed patients (Berg et al. 2005a; Hagemann et al. 2006). Within a broad range of isolates of environmental and clinical origin, classified at the time as *S. maltophilia*, a distinguished genomovar consisting only of environmental isolates could be separated (Minkwitz and Berg 2001), and further characterisation led to their description as a separate species, *Stenotrophomonas rhizophila* (Wolf et al. 2002). Unlike *S maltophilia*, *S. rhizophila* does not have human pathogenic traits (Ribbeck-Busch et al. 2005; Hagemann et al. 2006). The species has a lower temperature optimum than *S. maltophilia* (Wolf et al. 2002), and is therefore safe to use. The synthesis of an additional osmolyte, glucosyl glycerol, confers a greater degree of salt resistance in vitro (Hagemann et al. 2008) and makes it an ideal candidate for application in saline soil conditions. In vitro, isolates of this species produce fungal cell wall degrading enzymes, siderophores (Minkwitz and Berg 2001) and volatile antifungal compounds (Kai et al. 2007); antifungal activity against soil-borne plant pathogens has been demonstrated (Minkwitz and Berg 2001). Also, production of the plant growth hormone indol-acetic acid (IAA), and direct growth promotion of strawberry plants in vitro has been shown (Suckstorff and Berg 2003). These studies indicate the potential of *S. rhizophila* to directly promote plant growth as well as to inhibit plant pathogens. Growth of a wide variety of crops was strongly promoted in the saline soil of Uzbekistan by *S. rhizophila* strain DSM14405^T (=e-p10^T and =P69^T) (Egamberdieva et al. 2011); these promising results raise the question whether the observed effects were due to direct plant growth promotion by the strain or to the suppression of deleterious flora and soil-borne pathogens in the rhizosphere. No studies on the survival and population distribution on different plant species have been done.

The aim of this study was to assess the influence of the plant species on root colonisation and plant growth promotion of *S. rhizophila* DSM14405^T. Comparison of plant growth promotion under sterile (gnotobiotic) and non-sterile conditions should shed light on the question whether direct or indirect effects (elimination of deleterious rhizosphere flora) contributed to the plant growth promoting effect of this beneficial bacterium *in planta*. To assess the influence of the strain on native rhizosphere microbial communities, single strand conformational polymorphism (SSCP) profiles of amplified 16S rRNA genes, and internal transcribed spacers (ITS) from the rhizosphere with and without presence of *S. rhizophila* DSM14405^T were compared.

Materials and methods

Plant seeds and bacterial strains

Tomato seeds (*Solanum lycopersicum* cv. 'Avicenne', cv. 'Petto 86'), sweet pepper (*Capsicum annuum* cv. 'Zdorové'), and cotton seeds (*Gossypium* sp., non-specified cultivar) were obtained from Dilfuza Egamberdiyeva (Centre of Agroecology, Tashkent State University of Agriculture, Uzbekistan). Two sweet pepper varieties ('Californian Wonder', 'Chinese Giant') were purchased from www.bobby-seeds.com (Floveg GmbH; Rheinbach-Wormersdorf, Germany). Unless stated otherwise, the sweet pepper cv. 'Zdorovje' and the tomato cv. 'Avicenne' were used in all experiments. Seeds of cotton and sweet pepper cv. 'Californian Wonder' were partially infected with endophytic seed-borne pathogens which could not be eliminated by the seed surface sterilisation procedure described below; in sweet pepper, the pathogens could be identified as *Alternaria* sp.

The bacterial isolate *S. rhizophila* DSM14405^T was isolated from oilseed rape (Minkwitz and Berg 2001; Wolf et al. 2002) and originated from the stock culture collections of the Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria.

Surface sterilisation and pregermination of seeds

In experiments in gnotobiotic systems, tomato and sweet pepper seeds where surface sterilised according the protocol of Götz et al. (2006). For sweet pepper, the concentration of NaOCl was lowered from 12 to 2.5 %. Cotton seeds were delinted in concentrated H_2SO_4 according to Da Silva et al. (2006). After neutralising in 10 % Na_2CO_3, they were washed in water for 30 min and surface sterilised following the protocol of Gould and Magallenes-Cedeno (1998). Surface-sterilised seeds were imprinted on Luria Bertani agar (Roth GmbH+Co KG; Karlruhe, Germany) to check for sterility and then placed in Petri dishes between two sterile, moistened filter papers; the Petri dishes were sealed with Parafilm. Seeds were pre-germinated for 2–3 days at a temperature of 30°C (cotton and tomato) or 2–7 days at a temperature ~20°C (sweet pepper). The surface sterilisation procedure did not kill pathogens present inside the seeds.

Treatment of seeds and plants with *S. rhizophila* DSM14405^T

S. rhizophila DSM14405^T was grown over night in Luria Bertani broth (Roth GmbH+Co KG) or Nutrient Broth II (SIFIN; Berlin, Germany) for 22 h. Of the rifampicin, 100 μg ml^{-1} (50×stock solution in dimethyl sulfoxide) was added to the medium for the rifampicin-resistant mutants. Bacterial cultures were centrifuged (5,000×*g*), the

cells washed once in 0.85 % NaCl and finally resuspended in an equivalent volume of 0.85 % NaCl (OD_{600nm}=1.2, 200 µl suspension in 96-well microplates). In gnotobiotic test systems (plant agar), 10 µl suspension was dripped onto both the root and the hypocotyl of freshly transplanted plantlets; the suspension was applied undiluted (10^8 cells $plant^{-1}$) or diluted (10^4 cells $plant^{-1}$). Ten thousand cells per plant had been shown to be the optimal dose for plant growth promotion of strawberry in such systems (Suckstorff and Berg 2003).

When seeds were sown into soil directly after soaking, the bacterial suspension was diluted 100-fold (10^5 cells $seed^{-1}$ in tomato and 10^7 cells $seed^{-1}$ in cotton). For bio-priming, the seeds were soaked in the undiluted bacterial suspension for 5 h, according to Müller and Berg (2008), in order to compensate for losses occurring during seed drying. Primed seeds where then dried in the laminar flow cabinet for additional 9–10 h, and stored at room temperature. Seeds for the control treatments were soaked or primed with sterile 0.85 % NaCl in exactly the same way. Populations in seeds were determined by grinding 10 seeds (or five in cotton) in 0.85 % NaCl in Whirlpack® bags (Carl Roth GmBH) and plating of serial dilutions.

Plant experiments under gnotobiotic conditions

Seedlings were grown under gnotobiotic conditions as described by Suckstorff and Berg (2003), with following modifications. Medium consisted of 6 % plant agar (Duchefa Biochemie BV, Haarlem, Netherlands) with 1/10×Gamborg salts B5 (0.31 gL^{-1}; Duchefa) in demineralised water. In tentative small-scale experiments, tap water was used instead of 1/10×Gamborg medium and in order to test salt resistance, NaCl was added at 0.8 and 1.5 %. Plants were either grown in culture tubes 'de Vit' (Duchefa) in 15 ml medium (6 cm medium height) or in 50 ml plastic centrifuge tubes in 40 ml medium (cotton). Aseptically pre-germinated seedlings were planted into the medium. Lids were removed from the 50-ml centrifuge tubes to allow light access and plant growth and they were placed in autoclaved plastic beakers (19.2 mm diameter, 21 cm height; FUERST GmbH; Hallerndorf, Germany) which were closed with a lid. Incubation was at 20−25°C at a 16/8 h day/night cycle. After 5 weeks growth time, the number of side roots (assessed as number of root tips), the root length, and number of leaves were determined in gnotobiotic systems (plant agar), as described for strawberry in Suckstorff and Berg (2003). In addition, shoot length was measured to detect a possible effect of IAA production by *S. rhizophila* DSM14405^T. If seed-borne diseases were apparent, they incidence was assessed according to following scale: 0=plant healthy, 1=plant diseased, 2=plant killed. One plant constituted one replicate in the assessments of plant growth promotion; 45–52 replicate plants per treatment were assessed. They were then grouped and pooled into three replicates consisting of ~15 plants each which were dissected into roots, stems, and leaves to determine population sizes of *S. rhizophila* DSM14405^T on plants.

Plant experiments in soil

Peat-based potting compost was used as substrate in all experiments under non-sterile conditions (Profi-Substrat, Grammoflor GmbH+Co KG 'Topf Pikier M+Ton+FE', Vechta, Germany; 250 g moist mass; gravimetric water content (GWC)=1.3). This substrate is raised bog peat, amended with clay (90 gL^{-1}) and iron (50 mg FE L^{-1}), nitrogen content is 50–300 mg N L^{-1}, phosphorus 35–130 mg P L^{-1}, potassium 60–330 mg K L^{-1}; pH ($CaCl_2$)=5.8, and salt content <1.5 g/L (manufacturers specifications). This compost was filled into transparent plastic beakers (195 mm diameter, 210 mm height; FUERST). Added to the soil were 200 ml demineralised H_2O to adjust the soil to a GWC (dry weight based) of 3.0−3.5, i.e. 75 % of the resulting substrate wet weight was soil water. Seeds were soaked in bacterial suspension (see above) or 0.85 % NaCl and sown into the compost (10 seeds per pot). Alternatively, bio-primed seeds were used. The rifampicin-resistant mutant RifR5 was employed in experiments on phytosphere colonisation. Lids of the microcosms were removed as soon as the cotton plants touched them (~2 weeks); ambient humidity was 25 %. The plants were incubated at 20−25°C at a 16 h/8 h day/night cycle for 4 weeks. Three replicates of two plants were harvested per treatment; two experiments, one with bio-primed seeds, and another one with seeds soaked in bacterial suspension, were performed. In the experiment on plant growth promotion, seeds primed with *S. rhizophila* DSM14405^T wild type were used. In order to observe more pronounced effects of treatment with *S. rhizophila* DSM14405^T, plants were grown until they had developed inflorescences. After 35 days growth, each was planted into a separate pot and grown for further 53 days, resulting in a total growth time of 87 days. Twenty replicate single pots (7 cm diameter) with one plant were set up per treatment; each plant constituted one replicate in the assessments of plant growth promotion. Plants were fertilised with liquid fertiliser (SUBSTRAL Pflanzennahrung; N/P/K 6:3:6, with trace elements Cu, Fe, Mn, Mo, Zn; Scotts Celarflor Handelsgesellschaft, Salzburg, Austria). Shoot height and number of secondary leaves, and number of shoots and number of both inflorescences and single flower buds were recorded.

Recovery of *S. rhizophila* DSM14405^T RifR5 from plants

Plants were grouped into three replicate samples. Each replicate consisted of ~15 plants in gnotobiotic experiments,

three plants (first experiment done with bioprimed seeds in soil) or two plants (second experiment done with soaked seeds in soil). Plants were dissected into fractions of roots, stems, and leaves. From root samples of plants grown in non-sterile soil, a small proportion of each replicate was set aside for rhizosphere-DNA isolation in the first experiment done with bioprimed seeds (see above). For recovery of the epiphytic plant fraction, 20 ml (or, if necessary, up to 45 ml) of 0.85 % NaCl were added to the plant sections, and samples were vortexed and then sonicated for 3 min at a strength of 100 (Elma Transsonic Digital, Schalltec GmbH, Mörfelden-Walldorf, Germany). Serial dilutions were plated on LB Agar+ 100 ppm rifampicin. The plant parts were then surface sterilised by immersion into 2.5 % NaOCl for 5 min, rinsed three times with sterile water and imprinted on Luria Bertani agar to check sterility. They were subsequently placed in Whirlpack® bags (Karlsruhe, Germany) and crushed with pestle and mortar in 5 ml 0.85 % NaCl. Serial dilutions of the extract where plated to quantify the endophytic population. Non-treated control plants were also prepared in the same way to confirm the identity of the recovered bacteria as *S. rhizophila* DSM14405^T. Although some rifampcin-resistant soil bacteria could be observed in the controls, they differed in morphology from the typical yellow colonies of *S. rhizophila* DSM14405^T which were only detectable in preparations from treated plants. The detection limit of this method was 10^3 cells per gram freshweight.

Fluorescence in situ hybridisation and confocal laser scanning microscopy

Sample preparation from gnotobiotic plants for fluorescent in situ hybridisation (FISH) was carried out as described in Daims et al. (2005). Samples were fixed in 4 % paraformaldehyde and stored in a 1:1 mixture of phosphate buffered saline and 96 % ethanol. FISH was performed according to the protocol of Cardinale et al. (2008). Samples were hybridised first with the probe Gam42a in hybridisation buffer containing 40 % formamide and an unlabelled Bet42a probe was applied as competitor to ensure specificity (Manz et al. 1992). Both probes hybridise at the positions 1027–1043 of the 23S-rRNA gene of beta-proteobacteria or gamma-proteobacteria, respectively. Samples were then hybridised with probes EUB338, EUB338II, and EUB338III, which bind to the nucleotides 338–355 of the eubacterial 16S rRNA gene (Amann et al. 1990; Daims et al. 1999); for this, hybridisation buffer containing 10 % formamide was used. Hybridisations were performed at 41°C for 120 min and followed by a washing step with appropriate washing buffer at 42°C for 15 min. Samples were visualised with a Leica TCS solid-phase extraction confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany). An appropriate number of 0.8–1.0 μm depth optical slices were applied to visualise the sections of roots, shoots, and leaves (confocal stacks). Up to five scans per optical slice were averaged to improve the image resolution and to reduce noise. Three-dimensional models consisting of isosurfaces were obtained from the confocal stacks with the software Imaris 7.0 (Bitplane, Zürich, Switzerland).

Microbial fingerprints performed by single-strand conformation polymorphism of 16S rRNA and ITS genes

DNA from rhizosphere samples of tomato and sweet pepper was isolated using the BIO 101 FastDNA® spin kit according to the manufacturer's instructions (Qbiogene, Carlsbad, CA, USA). Samples were ribolysed 2×20 s at speed 6.5 and cooled on ice for at least 5 min between these three steps (Ribolyser Fast Prep™, Qbiogene). The DNA was finally eluted in 100 μl H_2O. The ITS region of total fungi was amplified in a nested PCR with the primers ITS1f (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4r (5′ TCC TCC GCT TAT TGA TAT GC 3′) in the first round, ITS1f and ITS2rP (5′ GCT GCG TTC TTC ATC GAT GC 3) in the second round (White et al. 1990). Ascomycete DNA was amplified using the primers ITS1F (=ITS1A, 5′ CTT GGT CAT TTA GAG GAA GTA A 3′) and ITSA4 (5′ CGC CGT TAC TGG GGC AAT CCC TG 3′) in the first round, ITS1F and ITS2rP (5′ GCT GCG TTC TTC ATC GAT GC 3′) in the second round (Larena et al. 1999). The 16S rRNA genes were amplified from the total eubacterial DNA with primers Unibac-II-515f (5′ GTG CCA GCA GCC GC 3′) and Unibac-II-927rP (5′ CCC GTC AAT TYM TTT GAG TT′ 3; Berg et al. 2005b). Gammaproteobacterial 16S rRNA genes was amplifed using the primers γ-prot 595f and γ-prot 871rP (Mühling et al. 2008). For amplification with ITS primers in the first round, Phusion TAQ and buffer (Finnzymes Oy, Espoo, Finland) were used. For all other PCRs, the TAQ & Go system (Qbiogene) was employed. PCR preparations and PCR conditions were as described in Fürnkranz et al. (2009), except that 10 μg bovine serum albumin was added to the PCR mixture for bacterial DNA to protect the TAQ polymerase against inhibitors present in DNA isolated from soil; for amplification of eubacterial DNA with TAQ & Go, additional $MgCl_2$ (up to 3 mM) was added. PCR products were cleaned using the WIZARD® SV Gel and PCR clean-up system (Promega GmbH, Mannheim, Germany), made single stranded by digestions with λ-exonuclease and then snapped on ice. SSCP was performed in a TGGE apparatus (Biometra, Göttingen, Germany) according to Schwieger and Tebbe (1998). Fungal PCR products and bacterial PCR products were loaded in 9 and 10 % acrylamide gels, respectively. Gels were run at 400 V and 26°C for 17 h (fungal DNA) or 26 h (bacterial DNA). The gels were silver stained as described by Bassam et al. (1991), and scanned at 600 dpi resolution. Scanned images were

converted to 8-bit monochrome .tif files for analysis. Selected bands were excised, crushed, and eluted in 40 ml of sterile water overnight. One millilitre of supernatant was then used for re-amplification with respective primers. PCR products were purified with the GENECLEAN Turbo kit (BIO101 Systems; Qbiogene) and sequenced using the BigDye Terminator Ready Reaction kit (Applied Biosystems, Norwalk, CT, USA) and an ABI 310 automated sequencer (Applied Biosystems). The GenBank database (NCBI–BLASTN; Altschul et al. 1997) was used to retrieve closely related sequences and to calculate percentage similarity. GenBank accession numbers of submitted sequences are JQ260863–JQ260868.

For statistical analysis of data (analysis of variance (ANOVA) and Tukey's test) the statistical package SPSS (SPSS Inc., Chicago, IL, USA) was used. This package also allowed the analysis of root colonisation data with a mixed ANOVA model with plant section as within-subject factor and all other factors as independent factors.

Results

Survival of *S. rhizophila* DSM14405^T on seeds

Soaking of seeds in bacterial suspensions and direct sowing yielded start populations of ~10^5 CFU seed^{-1} (10^7 cells seed^{-1} in the larger cotton seeds). Biopriming of tomato, sweet pepper, and cotton seeds resulted in populations of 10^3–10^6 CFU seed^{-1}, irrespective whether the wild type or the rifampicin-resistant mutant RifR5 was used. Populations remained stable for 30–45 days. However, after 4 months storage at room temperature, no *S. rhizophila* DSM14405^T could be detected in bio-primed seeds with dilution-plating methods except for one batch of cottonseeds; there, low numbers of the rifampicin-resistant mutant DSM14405^T RifR5 (10^2 CFU seed^{-1}) could be re-isolated. After 4 months, the cells had either died or had entered the viable-but-non-culturable state.

Colonisation of different plant species by *S. rhizophila* DSM14405^T

In gnotobiotic systems, there was a significant effect of plant section and an interaction between plant species and plant section (roots, stems, and leaves), but no significant effect of plant species itself, except for leaves, where cell numbers on sweet pepper were significantly lower than on cotton (Fig. 1, left side). *S. rhizophila* DSM14405^T established high populations of 10^8–10^9 cells g^{-1} fresh weight on the roots and stems of all host plants from diverse families (Fig. 1). Populations on the leaves were lower (10^7–10^8 cells g^{-1} fresh weight). Population distribution on cotton was different; here the stems bore the lowest proportion of

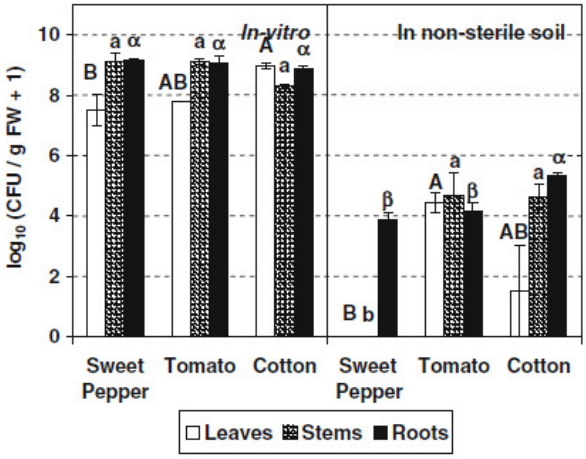

Fig. 1 Colonisation of different plant species (tomato, sweet pepper, and cotton) in vitro and in non-sterile soil by *S. rhizophila* DSM14405^T RifR5. Plants were either grown in Gamborg agar medium (in vitro) or in commercial potting compost (Grammoflor 'Profi Substrat') and harvested after 4 weeks growth. Tomato variety 'Avicenne' and sweet pepper varieties 'Californian Wonder' (in vitro) and 'Zdorové' in non-sterile soil were used. Soil moisture was kept at field capacity. Plantlets in vitro were treated with 10^4 cells after planting; for the experiment in soil, seeds were soaked in suspension of *S. rhizophila* DSM14405^T RifR5 (see 'Materials and methods' for details), resulting in ~10^5 cells seed^{-1} (10^7 cells seed^{-1} in cotton), respectively. Different letters indicate significantly different means at $p<0.05$ (Tukey's test); note that comparison were made only within the categories 'in vitro' and 'non-sterile soil, and within the plant region (root, shoot, leaves), respectively. There was a significant effect of plant species ($p<0.001$ in soil, not significant in vitro), plant section ($p=0.01$ in soil, $p<0.001$ in vitro) and a significant interaction between plant species and plant section ($p<0.03$)

DSM14405^T population (Fig. 1, left side). Application of a higher initial dose (10^8 cells plant^{-1}) did not result in higher cell densities on the plants. This indicates that the carrying capacity of the host rather than the application dose determines the final population density.

Colonisation of the phytosphere in non-sterile soil was examined in two experiments, the first one with bio-primed seeds (initial population 10^3–10^6 CFU seed^{-1}, presented in Fig. 1) and the second one with seeds soaked in suspension before sowing (initial population ~10^5 CFU seed^{-1}), respectively. Both experiments yielded similar results. Plant species and plant section affected population density significantly ($p<0.001$ and $p=0.01$, respectively) and also affected the shape and distribution of *S. rhizophila* populations, which was apparent in a significant interaction between plant species and plant section ($p<0.03$; Fig. 1, right side). Population density of *S. rhizophila* DSM14405^T RifR5 in the rhizosphere was uniformly high in all three plant species (~10^4–10^5 CFU g^{-1} root fresh weight) albeit 4–5 orders of magnitude lower than in gnotobiotic in vitro systems (Fig. 1). The differences between the plant species

became apparent in the above-ground sections. In tomato (cv. 'Avicenne'), populations tended to increase from bottom to top with the highest population density on the leaves (Fig. 1, right side). Cotton and sweet pepper (cv. 'Zdorové') showed the opposite trend with highest population density in the rhizosphere. This was particularly pronounced in sweet pepper, where population density declined sharply down to the to the detection limit of 10^2–10^3 cells per gram plant fresh weight in all aboveground plant parts (Fig. 1). Colonisation of two other sweet pepper varieties ('Californian Wonder', 'Chinese Giant') was not significantly different from that on the variety shown here ('Zdorové'). On cotton, stems were colonised but a significant decline occurred on the leaves (Fig. 1).

No endophytic *S. rhizophila* DSM14405^T could be isolated from surface-sterilised plant parts, neither in vitro plants nor in plants grown in non-sterile soil, although the exposure time and concentration of the surface sterilant NaOCl had already been reduced to the minimum necessary for successful surface sterilisation.

However, light micrographs of *S. rhizophila* on gnotobiotic tomato roots indicated a colonisation around and possibly also inside the root hairs (Fig. 2a) and FISH–confocal laser scanning microscopy of root-associated bacteria (stained with the bacterial EUB338MIX FISH probe) revealed that *S. rhizophila* DSM14405^T colonised the inside of tomato roots (Fig. 2b and c).

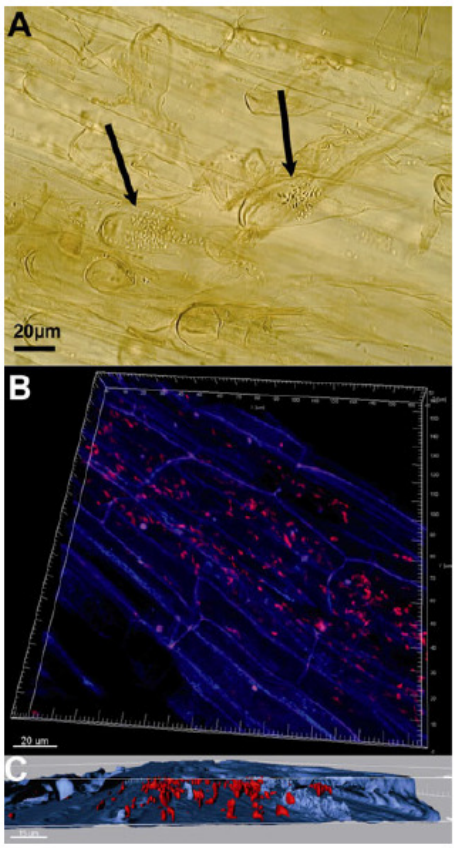

Fig. 2 Cells of *S. rhizophila* DSM14405^T in tomato roots (gnotobiotic in vitro plants, cv. 'Avicenne'). **a** Light micrograph viewing on top of the root surface with root hairs growing towards the viewer; the bacterial cells form colonies between and possibly within the root hairs (*arrows*). **b** Volume rendering of a confocal laser scanning microscopy Z-stack showing FISH stained bacterial cells (red signal, probe EUB338MIX) within the roots of tomato (*blue/pink* signal). **c** Cut plane of the isosurface reconstruction of panel B (*red* bacteria, *blue* root tissue) showing endophytic colonies of *S. rhizophila* DSM14405^T

Plant growth promotion of cotton, sweet pepper, and tomato in gnotobiotic systems

In cotton, treatment with low doses of *S. rhizophila* DSM14405^T (10^4 cells plant^{-1}) increased the length of the main root; no other significant direct effects on plant growth could be detected (Table 1). Treatment with *S. rhizophila* DSM14405^T significantly reduced incidence and severity of seed-borne diseases (most likely *Fusarium* spp., Table 1). Application of high doses of *S. rhizophila* DSM14405^T (10^8 cells plant^{-1}) reduced the number and length of side roots in cotton and in tomato (Table 1).

In vitro plants of Tomato (cv. 'Avicenne') were negatively affected by *S. rhizophila* DSM14405^T. A significant reduction in the number of side roots was already observed at the lower initial dose (10^4 cells plant^{-1}); the effect increased with dose (Table 1). The effect on the number of secondary leaves was strongly dose dependent. Low initial doses of *S. rhizophila* DSM14405^T had a positive influence; high doses had a negative effect (Table 1).

S. rhizophila DSM14405^T had no direct plant growth promoting effect on sweet pepper (cv. 'Californian Wonder'). However, a biocontrol effect was observable similar to cotton. Incidence of seed-borne fungal diseases (identified as *Alternaria* spp.) was significantly reduced by the treatment with *S. rhizophila* DSM14405^T (Table 1).

Tentative experiments in tap water instead of 1/10×Gamborg salts and at different salinitites (0.5 % and 1.0 % NaCl) indicated that the plant growth promoting effect of *S. rhizophila* DSM14405^T on tomato and sweet pepper was not increased under nutrient limiting conditions and that the strain did not alleviate salt stress of in vitro plants (data not shown).

Plant growth promotion of cotton, sweet pepper, and tomato in non-sterile soil

In order to allow for more subtle effects of *S. rhizophila* DSM14405^T to become apparent, single plants were grown in 10 cm pots (20 replicate pots per treatment) for a longer

Table 1 Effect of *S. rhizophila* DSM14405^T on sweet pepper (cv. 'Californian wonder'), tomato (cv. 'Avicenne') and cotton (*Gossypium* sp.) in vitro

Plant species	Parameter monitored	Experiment 1 (low dose)[a]			Experiment 2 (high dose)[b]		
		Control	*S. rhizophila* DSM14405^T 10^4 cells plant^{-1}	Significance (direction of the effect)[c]	Control	*S. rhizophila* DSM14405^T 10^8 cells plant^{-1}	Significance (direction of the effect)[c]
Cotton[d]	No. of root tips	50±3.8	48±2.3	ns	14±1.9	8.0±1.6	0.019 (−)
	Length of main root (cm)	nd	nd	nd	1.5±0.18	1.7±0.17	ns
	Length of side roots (cm)	nd	nd	nd	38±5.3	21±6.5	0.056 (−)
	Shoot length (cm)	10.4±0.4	10.8±0.2	ns	5.7±0.61	4.3±0.63	ns
	No. of leaves (healthy)	2.4±0.3	2.8±0.2	ns	nd	nd	nd
	Disease severity[e]	0.7±0.12	0.19±0.07	<0.001 (−)	nd	nd	nd
Tomato (cv. Avicenne)	No root tips	17±1.2	13±0.9	<0.001 (−)	16±2.7	8.0±1.7	0.015 (−)
	Root length (cm)	66±4.2	57±4.0	0.078 (−)	51±7.9	25±6.2	0.017 (−)
	Shoot length (cm)	2.5±0.15	2.5±0.16	ns	3.1±0.26	5.0±1.7	ns
	No. of secondary leaves	3.0±0.1	3.4±0.1	0.029 (+)	3.0±0.31	2.1±0.36	0.056 (−)
Sweet pepper (cv. Californian Wonder)	No root tips	19.9±0.82	19±0.9	ns	nd	nd	
	Length of main root (cm)	3.0±0.13	3.4±0.14	0.058(+)	nd	nd	
	No of secondary leaves	3.8±0.11	3.7±0.11	ns	nd	nd	
	Disease severity[e]	0.21±0.07	0.04±0.04	0.041 (−)	nd	nd	

Plants were grown in plant agar+1/10×Gamborg salts B5 for 5 weeks. Upon transfer to plant agar, sterile seedlings were inoculated with *S. rhizophila* DSM14405^T cells suspended in 0.85 % NaCl. Control plants were inoculated with 0.85 % NaCl only; 16 h/8 h day night cycle; growth temperature was 18–25°C

ns non significant, *nd* not determined

[a] Experiment 1 (low dose) : 35 replicates per treatment

[b] Experiment 2 (high dose): 15 replicates per treatment

[c] Significance assessed by *t* test

[d] Cotton in experiment 2 (higher dose) was assessed at 3 weeks growth time (before development of secondary leaves); all other plants and cotton in experiment 1 were assessed after 5 weeks, when first secondary leaves were developed symptoms, two plants killed by the fungus

[e] Incidence and severity of diseases (re-isolated fungus identified as *Alternaria* sp. in sweet pepper; not identified in cotton); 0=healthy, 1= diseased, 2=killed

period of 51 days. By that time, the sweet pepper plants were already fully developed and in bloom, and the tomatoes had developed their first flower buds. No significant effect of *S. rhizophila* DSM14405^T could be observed in sweet pepper (cv. 'Zdorové'), although the average shoot height, number of leaves and flower buds tended to be higher (Table 2). However, *S. rhizophila* DSM14405^T significantly increased shoot height and shoot weight of tomato (cv. 'Avicenne'). Flower development of tomato was not increased; however, it has to be noted that the harvest was done just after appearance of the first flower buds. In cotton, no plant growth promoting effect was visible and *S. rhizophila* DSM14405^T reduced the number of side buds significantly (Table 2).

Effect of *S. rhizophila* DSM14405^T on the native rhizosphere flora of tomato and sweet pepper

Plant growth promotion of tomato by *S. rhizophila* DSM14405^T was more apparent in non-sterile soil and a suppressive effect on seed-borne pathogens was observed in vitro; this indicates a more indirect effect via the suppression of pathogens and deleterious microbes rather than a direct plant growth promotion effect. Therefore, the influence of *S. rhizophila* DSM 14405^T on the community profiles of selected microbial taxa of the rhizosphere in the rhizosphere of tomato and sweet pepper was investigated. Total fungi and *Ascomycetes* were chosen because these groups contain many plant pathogens; as *S. rhizophila* DSM14405^T

Table 2 Effect of *S. rhizophila* DSM14405^T on sweet pepper (cv. 'Zdorovjé'), tomato (cv. 'Avicenne') and cotton (*Gossypium* sp.) in non-sterile soil

Plant species	Parameter monitored	Treatment[a]		
		Control	*S. rhizophila* DSM14405^T Bioprimed (□10^4 CFU seed^{-1})	Significance (direction of the effect)
Tomato (cv. Avicenne)	Shoot length (cm)	30.5±1.5	35±1.4	0.028 (+)[b]
	No. of secondary leaves	9±0.6	11±0.6	0.07 (+)
	No. of inflorescences	1.2±0.16	1.8±0.5	ns
	No. of single flowers	3±0.6	3.7±0.7	ns
Sweet pepper (cv. Zdorové)	Shoot length (cm)	19±1.0	20.5±0.7	ns
	No. of shoots	3.1±0.34	2.9±0.26	ns
	No. of secondary leaves	22±1.4	24±1.1	ns
	No. of flowers	6±0.8	8±1.0	ns
Cotton	Shoot length (cm)	28±0.9	28±1.0	ns
	No. of secondary leaves	7.6±0.42	7.3±0.34	ns
	No. of side buds	2.9±0.48	1.25±0.33	0.009 (−)

Plants were grown in potting compost (Grammoflor 'Profi Substrat' Grammoflor Co & KG Vechta, Germany) for 51 days. Seeds were bioprimed with *S. rhizophila* DSM14405^T cells suspended in 0.85 % NaCl (see text for details). Controls were primed with 0.85 % NaCl only; 16/8 h day/night cycle, growth temperature was 18–25°C

ns non significant

[a] Twenty replicate plants per treatment × plant species

[b] Significance assessed by *t* test

belongs to the *Gammaproteobacteria*, within the bacteria, it appears likely that these groups are also affected by competition effects; therefore profiles these groups were also compared.

Profiles of *Ascomycetes* appeared largely similar irrespective of plant species and treatment (Fig. 3, right gel). A few minor exclusive bands appeared in the rhizosphere of tomato and sweet pepper plants treated with *S. rhizophila* DSM14405^T (Fig. 3, bands B_{asco} 1 and B_{asco} 2). Sequencing of those bands showed a close relationship to fungi of the genus *Cladosporium* (Table 3). Cluster analysis did not reveal a significant influence of *S. rhizophila* DSM14405^T on the total *Ascomycetes* profile (data not shown).

Striking differences were visible in the total fungal community of tomato (Fig. 3 left gel). Major bands almost disappeared in the treatment with *S. rhizophila* DSM14405^T (Fig. 3, bands B1 B3, B4 and B5); some weaker bands also appeared less pronounced or less frequent in treatments with *S. rhizophila* (B2, B7, B8). With around 80 % similarity, the sequences represented by the major bands were distantly related to those of *Mortierella* or other yet uncultured fungi, from soil or forest habitats (B2 and B3, respectively; Table 3), or even to ITS sequences of the green algae *Scenedesmus* (Johnson et al. 2007) or soil ciliates (B4 and B1, respectively; Table 3). Most remarkably, the sequence obtained from band B5 showed a very close relationship (96 %) to the human pathogen *Candida subhashii* (Adam et al. 2009; Table 3). Another dominant band, B6, appeared to increase due to the treatment with *S. rhizophila* DSM14405^T; unexpectedly, it proved to be closely related to *Dictyochloris* sequences (*Chlorophyceae*; Shoup and Lewis 2003) and only more distantly related to other yet uncultured soil fungi (82 % similarity; Table 3). Sequences related to *Ascomycetes* isolated from mosses and liverworts (Table 3; band B8) could be recovered more frequently in the control samples than in samples treated with *S. rhizophila* DSM14405^T; however, their presence was generally on the border of the detection limit (Fig 3). The influence of *S. rhizophila* DSM14405^T on total fungal communities of sweet pepper was less apparent than in tomato; thus we did not excise and sequence single bands here.

The community profiles of *Gammaproteobacteria* and all bacteria were largely similar, irrespective of plant species and treatment (Fig. 4). Interestingly, a band corresponding to *S. rhizophila* DSM14405^T was only present in total eubacterial profiles, but not in gamma-proteobacterial profiles (Fig. 4).

Discussion

Plant-associated microorganisms fulfil important functions for plant growth and health. Direct plant growth promotion by microbes is based on improved nutrient acquisition and

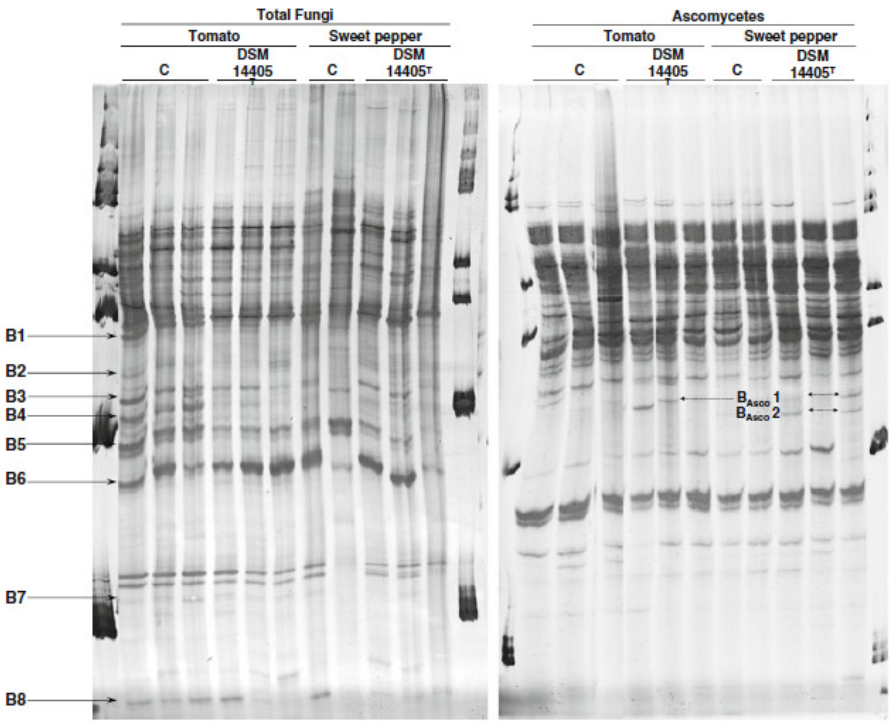

Fig. 3 Impact of *S. rhizophila* DSM14405^T on fungal community profiles in the rhizosphere of sweet pepper and tomato detected by PCR-SSCP. Fungal DNA was detected with ITS1 and ITS2 primers specific for the respective groups. *Arrows* indicate an influence of *S. rhizophila* DSM14405^T on the banding pattern; dotted arrows indicate bands present only in one treatment but not in all replicates; *solid arrows* indicate bands consistently present or significantly stronger only on one treatment

hormonal stimulation (Berg 2009). Diverse mechanisms are involved in suppression of plant pathogens (Mazzola 2002; Weller 2007; Lugtenberg and Kamilova 2009; Barret et al. 2011), which is often indirectly connected with plant growth. Isolates of *S. rhizophila* directly inhibited plant pathogenic fungi in vitro (Wolf et al. 2002); and produced antifungal volatiles (Kai et al. 2007). In this study, plant growth-promoting effects of *S. rhizophila* DSM14405^T were most pronounced in non-sterile soil and only subtle at best in gnotobiotic systems, despite populations being 4–5 orders of magnitude lower in non-sterile soil. Also, plant growth promotion in artificially salinated soil was only observable in native, not in sterilised soils (Berg et al. 2012). Positive effects on in vitro plantlets were largely due to the biocontrol of seed-borne diseases, which were systemically present within the seeds and could not be eliminated via surface sterilisation. This is the first demonstration of the biocontrol efficacy of this strain in cotton and in solanaceous crops. In vitro antagonistic activity of *S. rhizophila* DSM14405^T against the important soil-borne plant pathogens *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* has been demonstrated earlier (Minkwitz and Berg 2001). These observations, together with the proven capability of the strain to produce the antifungal volates β-phyenylethanol, dodecanal, siderophores, and fungal cell wall-degrading enzymes (Kai et al. 2007; Minkwitz and Berg 2001) point towards biocontrol of deleterious microorganisms and pathogens present in the soil substrate being responsible for plant growth promotion by *S. rhizophila* DSM14405^T. This is also likely to be the case in the Uzbek soils as there was a strong presence of soil-borne pathogens (*Fusarium solani* and *V. dahliae*; Egamberdiyeva, personal communication). Consequently, the plant growth promotion by *S. rhizophila* DSM14405^T in these soils was much stronger than observed in our study (Egamberdieva et al. 2011). Based on those results and our findings, we suggest that *S. rhizophila* DSM14405^T promotes plant growth indirectly through shaping the fungal rhizosphere community.

Unexpectedly, none of the fungal sequences in our SSCP profiles visibly affected by *S. rhizophila* DSM14405^T was related to known plant pathogens; many of the closest related fungal sequences were indeed from yet uncharacterised fungi inhabiting pristine environments. They may belong to the so-called deleterious microorganisms or deleterious rhizobacteria, which affect plant growth negatively but do not necessarily parasitize the plant tissue. Their deleterious activities include alterations of the supply of water, ions, and plant growth substances by changing root functions and/or by limiting root growth (Schippers et al. 1987; Kremer and Kennedy 1996) and their effects can be even plant species-specific (Mejri et al. 2010). Furthermore, the activity of *S. rhizophila* against human pathogenic genera like *Candida* (Adam et al. 2009) may have positive implications for food safety. Unexpectedly, some sequences whose relative abundance in the rhizosphere was affected by *S. rhizophila* bore similarity to those of green algae

Table 3 Identification and characteristics of the closest BLAST matches of fungal DNA sequences obtained by SSCP from tomato rhizosphere

Band no.	Similarity (%)	Accession no.	Description	Habitat
B_{Asco} 1	97	EU570258	*Cladosporium sphaerospermum* CPC 14016, *Capnodiales Ascomycota*	Wheat (*Triticum aestivum*)
B_{Asco} 2	97	AM159631	*Cladosporium sphaerospermum* clone K7	Paintings in the castle of Schönbrunn, Vienna
	98	GU721592	Uncultured fungus clone f3Fc57	HVAC filter dust
B5	**100**	**AB663086**	***Candida sp.* NY7122, *Saccharomycetales*, *Ascomycota***	**Soil, Ibaraki, Tsukuba, Japan**
	97	**EU836707**	***Candida subhashii* UAMH 10744**	**Human host, peritoneum**
B2	71	GQ517296 FJ761879.1	Uncultured fungus clones	*Quercus macrocarpa* phyllosphere
		AY354234.1	*Davidiella tassiana* isolate	Betula pendula xylem (live stem), Lithuania
B8	93	AM397675	Uncultured ascomycete clone BuxP4-1	Bryophyte *Buxbaumia aphylla*
	93	AM397689 AM397688.1	Uncultured ascomycete clones	*Bazzania trilobata* (riverbanks) Rhineland-Palatinate, Germany
B7	96	FJ873574	*Cryptococcus sp.* EN14M04 *Filobasidiales, Basidiomycota*	Mushrooms of Thailand
	96	AF042417	*Tremella foliacea* CCJ1396, *Tremellales, Basidiomycota*	–
B3	**81**	**GU997894 GU997756**	**Uncultured *Mortierella* clones, *Mortierellales*, *Zygomycota***	Ectomycorrhiza root tip, *Betula nana*, Toolik Lake, AK, USA
	78	**HQ630296.1, HQ630295.1**	***Mortierella parvispora* strains**	
B4	**80**	**DQ417571**	***Scenedesmus* sp. Pic 6/16 T–1 W (*Chlorophyceae*) *Chlorococcales*Chorophyta**	Picnic Pond, Itasca State Park, MN, USA
	78	**JN660672.1 JN660634**	**Uncultured organism clones ciidir**	Tomato rhizosphere cv. 'Gabriella' Valle de Guasave, Sinaloa, Mexico
B6	**96**	**AF367862, AF367860**	***Dictyochloris* (*Chlorophyceae*), *Chlorococcales, Chorophyta***	–
B1	**89**	**FJ554355.1**	**Uncultured *stichotrichid* clone**	–
	87	AF508762	*Oxytricha granulifera* (soil ciliate)	–

Bold major bands. B1–8 originate from tomato rhizosphere, rthe ascomycete bands B_{asco} 1 and 2 originate from the rhizosphere of sweet pepper. For position of bands, see Fig. 3

(*Chlorophyceae*: *Dictyochloris*, *Scenedesmus*), and the question arises how these phototrophic organisms receive sufficient light for growth in the rhizosphere. Fujita and Nakahara (2006) detected large numbers of viable microalgae, among them *Chlorella* and *Scenedesmus*, in the darkness of subsurface soil and facultative heterotrophy could be demonstrated in *Dictyochloris* when grown in darkness (Parker et al. 1961).

Plant species as a factor shaping rhizosphere communities is well documented in earlier studies (Berg and Smalla 2009; Hartmann et al. 2009) and differences may exist even between members of the same plant family; three different gramineous hosts shared only about one tenth of all computed protein sequences in their rhizosphere microbiomes (Barret et al. 2011). Here, we could demonstrate that two closely related plant species (both within the family of *Solanaceae*) displayed significant differences in their fungal, but not bacterial rhizosphere communities. Contrarily, an effect of biocontrol agents on microbial and fungal communities that becomes visible in banding patterns generated by SSCP or DGGE analysis is rarely reported; a majority of studies showed no or only subtle short-term changes (Götz et al. 2006; Scherwinski et al. 2008; Berg and Zachow 2011). This profound effect of *S. rhizophila* DSM14405^T on the fungal communities observed in our study is even more noteworthy as the inoculant contributes only a minor proportion to the total rhizosphere flora. *S. rhizophila* DSM14405^T was not detectable in profiles of gamma-proteobacteria. Its population density of 10^4–10^5 cells g^{-1} root fresh weight in non-sterile soil is only minute compared to a total of 10^8–10^{11} CFU g^{-1} root fresh weight, which was observed even in saline, mineral soils (Egamberdiyeva et al. 2008). Total populations per plant declined in non-sterile soil with 10^5 cells applied per seed and ~10^4 cells recovered per plant (10^7 applied/10^5 recovered in cotton). This is in remarkable contrast to the strong plant competence in gnotobiotic systems where the population rose by several orders of magnitude in all plant parts. We observed a similar increase in our soil substrate when it was sterilised (unpublished data). This indicates that the strain is probably

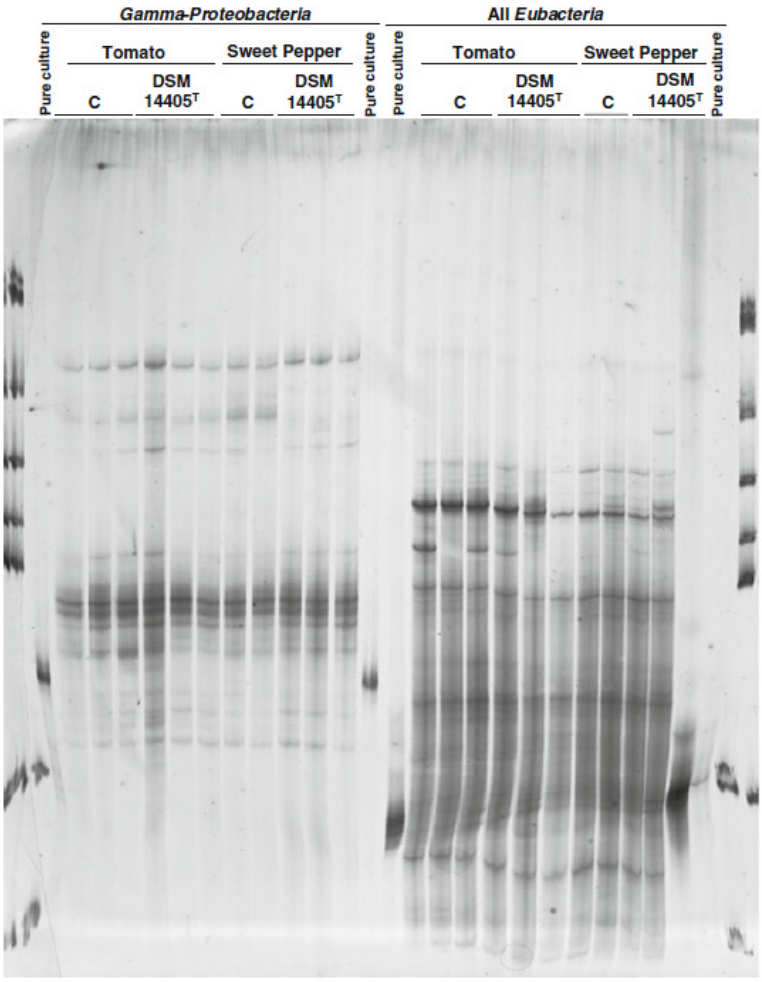

Fig. 4 Impact of *S. rhizophila* DSM14405^T on bacterial community profiles in the rhizosphere of sweet pepper and tomato detected by PCR-SSCP. Gammaproteobacterial communities were detected with primers γ-prot 595f and γ-prot 871rP, eubacterial communities were detected with primers Unibac-II-515f and Unibac-II-927rP. Pure culture= DNA from pure culture of *S. rhizophila* DSM14405^T amplified with respective primers co-separated for control of its presence in environmental profiles

outcompeted by indigenous soil microbes. *S. rhizophila* DSM14405^T may face even stiffer competition from indigenous rhizobacteria in the organic nutrient-rich substrate used here than in the nutrient-poor salinated mineral soils of Usbekistan (calcisols), where it displayed stronger plant growth promoting capability (Egamberdieva et al. 2011). There, populations of indigenous microbes are 1,000-fold lower than in a non-salinated agricultural soil (Ji and Wilson 2002; Egamberdiyeva et al. 2008). The question remains on how *S. rhizophila* DSM14405^T could exert such a strong effect on the fungal communities while representing only such a minute compound in the rhizosphere. Nutrient release from dying surplus inoculum could have favoured indigenous antagonists as nutrients, notably nitrogen, can be a limiting factor in the rhizosphere (Jensen and Nybroe 1999). However, this effect should have been confined to the seed were the inoculum was directly applied, whereas the change in fungal communities occurred in colonised roots; also, we did not apply any additional nutrients or culture medium. It should be borne in mind that population size of applied inocula and their antagonistic activity need not to be correlated. Formulation additives deleterious to the growth of applied inocula (trace elements and carob, respective) increased antagonistic activity of *Pseudomonas fluorescens* and *Pantoea agglomerans*, respectively, whereas additives supportive of their growth (*N* compounds) decreased efficacy (Wiyono et al. 2008; Schmidt et al. 2001). This gives rise to the assumption that adverse conditions, such as increased competition in this case, might induce an increase in antibiotic production of microbial inocula. Furthermore, the gaseous nature of the antifungal substances produced by *S. rhizophila* DSM14405^T (Kai et al. 2007) may ensure a very fast, effective distribution in the soil pores, even at low population densities. On the other hand, synthesis of antifungal compounds in rhizosphere bacteria is largely regulated by quorum sensing and therefore requires a certain population threshold (Müller et al. 2009; Williams 2007). The possibility exists, however, that signal molecules provided by resident bacteria in the rhizosphere may help to reach the threshold necessary for quorum sensing. Interspecies

signalling is recorded in biocontrol pseudomonads (Dubuis and Haas 2007; Dubuis et al. 2007) as well as for the diffusible signal factor system found in *Stenotrophomonas* and *Xanthomonas* (Ryan et al. 2008). Furthermore, signalling distances between in the rhizosphere exceeded the size of the individual cells up to 30-fold suggesting that induction via quorum sensing requires much lower population thresholds than previously thought (Gantner et al. 2006).

In vitro systems, Suckstorff and Berg (2003) had shown a strong dose-dependency of plant growth promotion by *S. rhizophila* in strawberries, and doses above 10^4 CFU plant^{-1} had deleterious effects; these results could be confirmed in tomato and cotton. This dose dependency may also explain the negative effect of the strain on tomato in vitro, while its effect in non-sterile soil was beneficial. Even with low initial doses of 10^4 CFU plant^{-1}, populations in vitro rose to high densities of 10^8 CFU g^{-1} root fresh weight in gnotobiotic systems, which were four- to fivefold higher than in soil. However, these final population sizes were not deleterious in other crops. The time–course of population build-up appears to be the crucial element here; the non-germinated or germinating seed may represent a particular sensitive stage, which is particularly susceptible to phytotoxic effects of high initial populations. In this context, it is noteworthy that oilseed rape (*Brassica napus*), was particularly resistant against the phytotoxic effects of high initial doses of *S. rhizophila* DSM14405^T but was also not positively affected by the strain (data not shown). This may be the result of long-term adaptation to *S. rhizophila*, as oilseed rape was the source of the strain (Minkwitz and Berg 2001; Wolf et al. 2002)

Our work demonstrated that *S. rhizophila* DSM14405^T, when applied to the seed, does also colonise the above-ground parts of the plants. However, it does appear to need high humidity, as the shoots of sweet pepper plants were densely colonised only in the water-saturated atmosphere of closed microcosms (unpublished results), but not in open top microcosms with ambient humidity. Populations on tomato shoots were significantly higher than on all other plant species at ambient humidity. This may be attributed to their dense coverage with trichomes which may provide for a moister more sheltered micro-environment close to the plant surface, ensuring a better survival of *S. rhizophila*. *Stenotrophomonas* isolates have also been shown to colonise plants endophytically (Berg et al. 2005b; Ryan et al. 2009); therefore, the isolate may settle into the even more sheltered environment in the inside of the trichomes. In this study, we could demonstrate that *S rhizophila* DSM14405^T microcolonies are located in the vicinity of and possibly also inside root hairs. However, our results obtained by serial dilution plating appear to be contrary to that, as almost all of the population of *S. rhizophila* DSM14405^T was recovered in the ectophytic fractions (surface washings) and no cells could be recovered from the inside of plant parts after surface sterilisation. Possibly, the root hairs (and trichomes) break during vortexing and sonication, releasing their inhabitants into the surrounding solution or exposing them to the hypochloride treatment during the later operations for surface sterilisation. Cells of *S. rhizophila* growing endophytically in plant hairs may therefore end up in the surface washing or may be killed during surface sterilisation. Therefore, they may be falsely attributed to the epiphytic population in plating experiments.

Colonisation of above-ground plant parts raises the question whether the strain poses a health risk if it colonises harvestable products, especially as the species is closely related to *S. maltophilia*, an opportunistic human pathogen. However, *S rhizophila* forms a clearly distinguished clade consisting only of non-clinical isolates (Minkwitz and Berg 2001; Ribbeck-Busch et al. 2005) and lacks important traits making the latter a harmful pathogen such as the multidrug efflux pump SmeDEF and the gene for a zonula occludens-like toxin (Ribbeck-Busch et al. 2005; Hagemann et al. 2006). Colonisation of the smooth and waxy surfaces of sweet pepper and tomato fruit (Suslow 2004) may be absent or reduced at lower humidity, as was observed on sweet pepper leaves. However, colonisation of aboveground plant parts by *S. rhizophila* deserves further attention, especially as it also may also have positive implications and highlights a possible potential of the strain for the control of aerial pathogens such as tomato blight (*Phytophthora infestans*).

In conclusion, the plant growth promoting effect of *S. rhizophila* DSM14405^T appears to be largely based on antagonism towards deleterious and pathogenic rhizosphere microorganisms. This is suggested by (1) a greater effect in non-sterile soil than in sterile systems and (2) the profound effect of the isolate on the rhizosphere community composition. This effect should be further investigated in different soil types, and under conditions of increasing salinity, as the *S. rhizophila* DSM14405^T is envisaged to be used under saline conditions (Egamberdieva et al. 2011).

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Publication 4

Genomic/transcriptomic studies to optimize the biocontrol effect of *Stenotrophomonas rhizophila*

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Abstract The genus *Stenotrophomonas* is of high medical, ecological and biotechnological interest due to the versatility of the different species. For example, *Stenotrophomonas rhizophila* is a model for a rhizosphere- and phylloplane- competent, salt-tolerant biocontrol agent. One of the most effective strains *S. rhizophila* DSM 14405^T showed biocontrol activity on various crops (e.g. pepper, oilseed rape, cucumber) under salinated conditions in greenhouse and field trials. Strain DSM 14405^T does not only show rhizosphere competence and antagonistic activity; it also produces high amounts of osmoprotective substances allowing it to survive under saline conditions. New insights into its mode of action are presented from transcriptomic studies based on the genome. Furthermore, this information will be used to optimise the fermentation, formulation and efficiency of the biocontrol agent.

Keywords: biocontrol, genomics, mode of action

Introduction

Bacteria of the genus *Stenotrophomonas* are of increasing biotechnological interest due to their ubiquitous occurrence and versatility (Ryan et al., 2009). Their plant growth promoting

properties and their antagonistic activity against soil-borne plant pathogens are well-documented (Berg et al.,1994; rev. in Ryan et al.,2009), but development for commercial application of *S. maltophilia*, the most intensively studied species, has been hampered by its potential as opportunistic human pathogen in immune-suppressed patients (Hagemann et al., 2006). Within a broad range of isolates of environmental and clinical origin, classified at the time as *S. maltophilia*, a distinguished genomovar consisting only of environmental isolates could be separated (Minkwitz & Berg, 2001), and further characterisation led to their description as a separate species, *Stenotrophomonas rhizophila* (Wolf et al., 2002). Contrary to *S maltophilia, S. rhizophila* does not have human pathogenic traits (Ribbeck-Busch et al., 2005; Hagemann et al., 2006), has a lower temperature optimum than *S. maltophilia* (Wolf et al., 2002), and is therefore safe to use. The synthesis of an additional osmolyte, glucosyl glycerol, confers a greater degree of salt resistance *in vitro* (Hagemann et al., 2008) and makes it an ideal candidate for application in saline soil conditions. These studies indicate the potential *of S. rhizophila* to directly promote plant growth as well as to inhibit plant pathogens. Growth of a wide variety of crops was strongly promoted in the saline soil of Uzbekistan by *Stenotrophomonas rhizophila* strain DSM14405^T (=e-p10T, =P69T) (Egamberdiyeva et al., 2011). Plant species-specific effects were also observed for *S. rhizophila* DSM 14405^T at increasing salinities under greenhouse conditions (Schmidt et al., 2012). Plant growth promotion was particularly apparent in solanaceous crops (tomato and sweet pepper) in contrast to cotton. Under greenhouse conditions, a positive effect of *S. rhizophila* DSM14405T on the growth of sweet pepper was consistent across all tested salinity values. In general, plant growth promotion by *S. rhizophila* DSM14405T was more pronounced in non-sterile (greenhouse and field conditions) than in sterile soil, which can be explained by the interaction with indigenous plant-associated microorganisms (Schmidt et al., 2012).

Genomic/transcriptomic studies can help to understand the mode of action of the biocontrol agent but also to optimise its production process. Our aim was to sequence the genome of *Stenotrophomonas rhizophila* strain DSM14405^T and to exploit this information for a biocontrol and plant growth promotion strategy. Here, we present new insights on the mechanisms responsible for antifungal activity and plant growth promotion based on the genome information.

Material and methods

The genome of *S. rhizophila* DSM 14405^T was sequenced with a combination of next generation sequencing methods (Roche 454 GS, Sanger sequencing, Illumina paired-end sequencing). The Illumina reads were aligned to the draft genome with CLC Genomics Workbench 4.7.2 (CLC, Aarhus, Denmark). Genes were identified with the Prodigal gene finder (Hyatt et al., 2010) ARAGORN (Laslett & Canback., 2004) and RNAmmer 1.2. Homology analyses were performed using the Blastp tool provided by NCBI (National Center for Biotechnology Information).

Results and discussion

Insights into the mode of action of *Stenotrophomonas rhizophila*

Use of classical physiological and biochemical methods unveiled the mechanisms of plant growth promotion and biocontrol by *Stenotrophomonas* against soil-borne fungi like *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Verticillium dahliae*. Biocontrol involves the excretion of antifungal metabolites such as antibiotics, toxins and bio-surfactants, and the production of a wide range of extracellular enzymes (Jacobi et al., 1996, Berg & Ballin, 1994, Kobayashi et al., 1995, Dunne et al., 2000). The excretion of soluble antibiotics and enzymes as well as the production of volatile organic compounds (VOCs) by soil bacteria like *Stenotrophomonas* can negatively influence the growth of fungi (Alström 2001, Wheatley 2002). Recently, it has been shown that the VOCs of *S. maltophilia* and *S. rhizophila* inhibit mycelial growth of the soil-borne pathogen *R. solani* by more than 90% in dual culture tests. Out of a vast diversity of VOCs produced by *S. rhizophila*, two, namely dodecanal and □-phenylethanol could be identified by GC-MS (Kai et al., 2007). It has been shown that *S. rhizophila* has a great potential as a plant growth promoting bacterium although positive effects are dose-dependent (Wolf et al., 2002, Suckstorff & Berg, 2003). *S. rhizophila* is able to grow at salt concentrations up to 4% and produces the osmolytes trehalose and glucosylglycerol (GG) in response to salt stress (Roder et al., 2005). Osmolytes are compounds compatible with cellular functions, e.g. DNA replication, DNA-protein interactions

and cellular metabolism. Osmolytes are highly soluble; they have no net charge at physiological pH and do not interact with proteins. Furthermore, they function on osmotic balance and are effective stabilizers of enzymes (Lippert & Galinski, 1992; Welsh, 2000).

Plant growth promotion

Genome sequencing resulted in new insights into the genetic sources providing beneficial plant-associated bacteria with traits such as plant growth promotion, antagonisms towards phytopathogens as well as osmoprotection. Here, we discuss some of these genetic sources for *S. rhizophila* DSM14405^T. *S. rhizophila* produces high levels of the auxin phytohormone indole-3-acetic acid (IAA) (Suckstorff & Berg, 2003). Bacteria synthesize IAA through various pathways (Spaepen et al., 2007). Our BLASTn investigations revealed that the genes encoding the regular pathways of IAA synthesis such as tryptophan-2-monooxygenase and tryptophan decarboxylase are not present in the genome of *S. rhizophila*. However, the presence of the nitrilase gene suggests that the biosynthesis of IAA in *S. rhizophila* could be through the indole-3-acetonitrile pathway. Moreover, *S. rhizophila* possesses genes responsible for the synthesis and transport of osmoprotective molecules (osmolytes) out of the cell. Glucosylglycerol-phosphate synthase (*ggpS*) gene has been shown to be essential for the synthesis of the osmolyte glucosylglycerol (GG), which has been suggested to be transported into the environment by a transporter encoded by the *ycaD* gene located upstream of *ggpS* (Hagemann et al., 2008).

Antagonistic activity towards fungi

In regard to the importance of *S. rhizophila* DSM14405^T as a biocontrol agent, numerous genes have been detected, which code for products with biocontrol relevance. For instance, two putative chitinase genes have been detected, as both are located on the leading DNA strand at positions 1502459…1503634 and 3857478…3859580, respectively. In respect of the protease activity observed for *S. rhizophila* (Minkwitz & Berg, 2001), four genes with locus tag annotations 1680, 2119, 4184 and 4348 were detected to code for extracellular proteases. In addition, the coding DNA sequence (CDS) 2947 was predicted to code for a putative exo-1,3/1,4-beta-glucanase, due to its significant amino acid sequence similarity. Yet another CDS in the genome of *S. rhizophila* DSM14405^T, 1262, reveals homology to a *Xanthomonas*-origin putative exported glucanase.

Furthermore, in addition to the possession of ordinary antibiotic-encoding genes as a trait shared among many *Stenotrophomonas*, *S. rhizophila* possesses a novel gene homologous to the bacterial lanthionine synthetase C-like gene (LanC). The bacterial *lanC* is responsible for the synthesis of peptide antibiotics (lantibiotics). Subtilin from *Bacillus subtilis* ATCC 6633 and epidermin from *Staphylococcus epidermidis* are two other well-studied examples for bacterial metabolites with antibiotic activity against other bacteria (Chung et al., 1992, Schnell et al., 1992).

Life style and fitness

Aside from well-known products such as IAA and VOCs there exist also further, rather backbone mechanisms leading to plant growth promotion and biocontrol (Egamberdieva et al., 2011). The bacterial competitiveness for colonizing niches and utilizing nutrients are important examples of these mechanisms (Kamilova et al., 2005). *S. rhizophila* possesses several genes of great importance with regard to competition for nutrients and niches, which are not present in other plant-associated *Stenotrophomonas* spp. such as *S. maltophilia* R551-3. Examples are genes responsible for pectin degradation, xylan degradation and the pectate lyase gene. Adhesion to the host-plant cell surface and the ability to form biofilms are also important features for out-competing other microorganisms during the competition for niches. To this end, *S. rhizophila* is equipped with several genes which may play a role in root colonization, such as those encoding the O-antigen and capsule polysaccharide biosynthesis pathways, genes encoding hemagglutinin, outer membrane adhesion protein, etc. Furthermore, there are also amino acid biosynthesis genes present in the genome of *S. rhizophila* such as one encoding chorismate mutase, which plays a crucial role in the biosynthesis pathway of tyrosine and phenylalanine (Guo et al., 2001). In addition, our BLASTp analyses have revealed that *S. rhizophila* possesses a gene homologous to the bacterial cardiolipin synthetase (*cls*), which plays an important role in the adaptation to elevated osmotic stress due to environmental changes (Romantsov et al., 2008).

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Manuscript (in preparation)

Running title: Genome announcement and characterization of *Stenotrophomonas rhizophila* DSM14405^T

Bacterial strain

S. rhizophila DSM14405^T was isolated from the rhizosphere of oilseed rape in Rostock, Germany (**Minkwitz and Berg, 2000**). The strain is also referred to as e-p10 and p69.

Genome Sequencing, assembly, annotation of *S. rhizophila* DSM14405^T

The genome of *S. rhizophila* DSM14405^T was sequenced using a combination of next generation sequencing platforms. A first draft assembly based on 905,689 reads of an 8kbp paired-end library (Roche 454 GS, FLX Titanium, Helmholtz Center Munich, Germany) with a total of 167.1 Mbps (36-fold coverage) was generated with Newbler 2.6 (Roche Diagnostics, Penzberg, Germany). This assembly consisted of 175 contigs, 122 of which could be joined into a single circular scaffold. Gaps resulting from repetitive sequences were resolved by *in silico* gap filling, remaining gaps were closed by PCR followed by Sanger sequencing or by long reads from a Pacific BioSciences sequencing run (PacBio RS, 150,305 reads, 174.8 Mbps, 38-fold coverage, GATC, Konstanz, Germany), yielding a draft genome of 4,648,936 bps. To improve the quality of the sequence by eliminating 454 sequencing artefacts in homopolymer stretches, the genome was subsequently sequenced using the Illumina paired-end method (Illumina HiSeq 2000, 15,086,654 reads, 1508 Mbp; 324-fold coverage, Ambry Genetics, Aliso Viejo, CA, USA). The Illumina reads were aligned to the draft genome with CLC Genomics Workbench 4.7.2 (CLC bio, Aarhus, Denmark). The final consensus sequence was derived by counting instances of each nucleotide at a position and then letting the majority decide the nucleotide in the consensus sequence. Genes were identified with the Prodigal gene finder **(4)**, ARAGORN **(6)**, and RNAmmer 1.2 (**5**). Functional annotation of the predicted genes was performed using BASys (**8)**, which provides annotations with respect to Clusters of Orthologous Groups (COG, **7**), Pfam (**2)** and Gene Ontology (GO, **3**). The final genome includes 4,648,976 bases with a GC content of 67.26%.

Comparative genomics and bioinformatic analyses

Whole genome comparisons between S. rhizophila DSM14405T, S. maltophilia R551-3 and K279a were performed using Mauve 2.3 (**Darling et al., 2010**) and Artemis Comparison Tool (ACT) (**Carver et al., 2005**). In the approach using Mauve, the Progressive Mauve algorithm was used to score the genome alignment.

DNAPlotter (**Carver et al., 2009**) was used for circular genome visualization.

Orthologous coding DNA sequences (CDS) shared between *S. rhizophila* DSM14405^T and the other two *Stenotrophomonas* were assessed by performing reciprocal BLASTp best hits with an identity and evalue threshold of 30% and 10^{-6}, respectively.

Results

Table 1 General genomic characteristics of *S. rhizophila* DSM14405^T, *S. maltophilia* R551-3 and *S. maltophilia* K279a.

	S. rhizophila DSM14405^T *	*S. maltophilia* R551-3 **	*S. maltophilia* K279a**
Number of bases	4,648,976 bp	4,573,969 bp	4,851,126 bp
G+C content (%)	67.26	66.3	66.3
Number of CDSs	4,033	4,039	4,386
Coding percentage	88.5	89.4	88.8
Average ORF length	1,020 bp	1,013 bp	983 bp
rRNA	12 (genes)	13 (genes)	12 (genes)
tRNA	72	73	74

* Information presented here corresponds to the original annotation. Alterations could occur due to possible updates.
** According to the genome information provided in the corresponding NCBI gbk-data.

General genomic features of *S. rhizophila* DSM14405^T were compared to the plant-associated *S. maltophilia* R551-3 and the human pathogenic *S. maltophilia* K279a, and are presented in table 1. The genome of *S. rhizophila* DSM14405^T consists of 4,648,976 nucleotides with a GC content of 67.26%, and has been predicted to code for 4,033 CDSs. Compared with *S. maltophilia* R551-3 and *S. maltophilia* K279a with each 4,573,969 n (4,039 CDSs) and 4,851,126 n (4,386 CDSs),

respectively, the size of the genome and the predicted number of CDSs are slightly smaller. There is no plasmid present in *S. rhizophila* DSM14405^T.

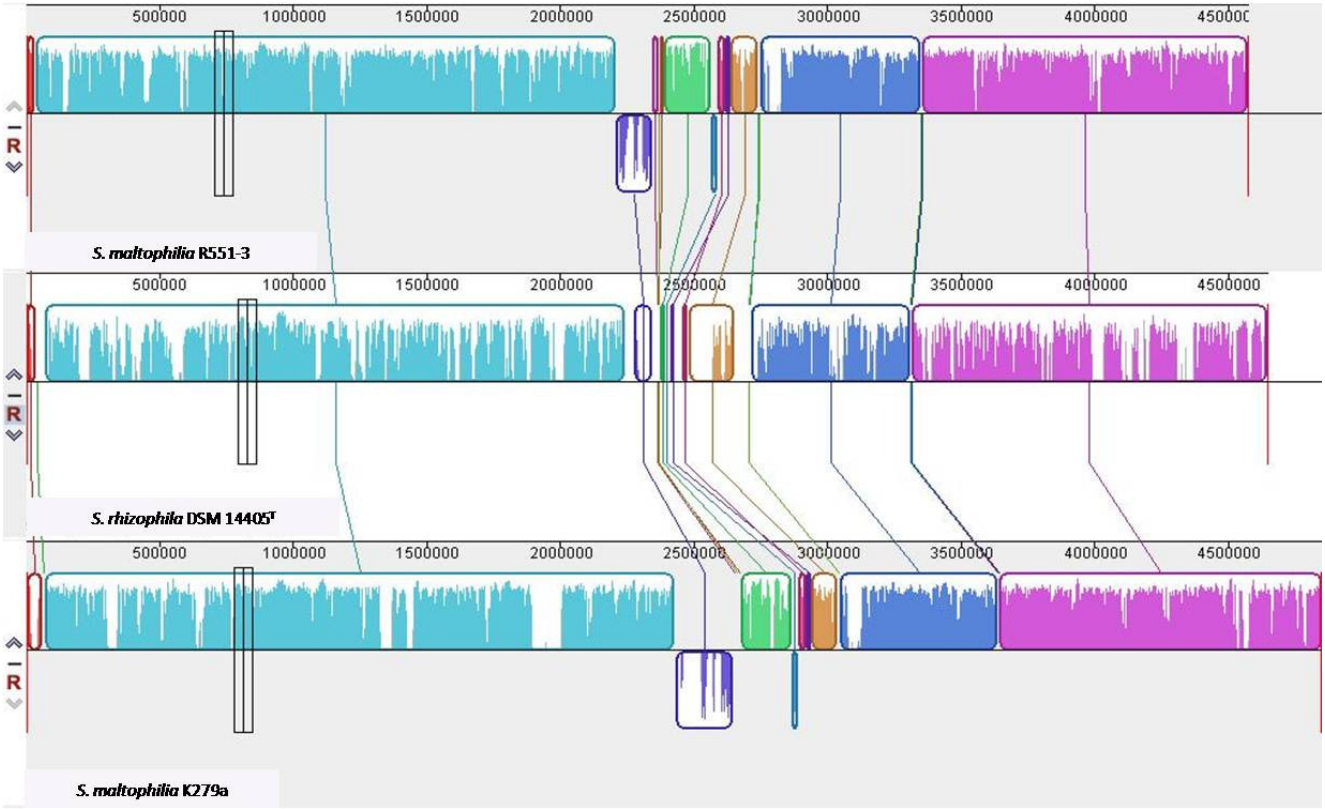

Fig. 1: Genome-scale comparison for the plant-beneficial strains *S. maltophilia* R551-3 (top), *S. rhizophila* DSM14405^T (middle), and the human pathogenic *S. maltophilia* K279a (bottom). The original genomic sequence of *S. rhizophila* DSM14405^T was converted into its reverse complement counterpart to achieve the same direction for all three genomes. Homologous DNA segments among the strains are marked by boxes with the same color, while gaps correspond to non-homologous regions. There are vast regions of homology between the genome of *S. rhizophila* DSM14405^T and both *S. maltophilia* R551-3 and K279a. The figure was generated using nucleotide sequences of the genomes compared by Mauve 2.3.

Fig. 1 shows genomic comparisons between the genome of *S. rhizophila* DSM14405^T, *S. maltophilia* R551-3, and *S. maltophilia* K279a. Overall, there is a very high degree of sequence similarity between the genome of the plant growth-promoting *S. rhizophila* and both *S.*

maltophilia R551-3 and the human-pathogenic K279a. The homology boxes are however separated by non-homologous regions. The whole genome sequence comparison between the three genomes was also performed using ACT which revealed the same result as in Fig. 1 and is presented in the supplementary Fig. 1

Fig. 2 represents circular genomic map of *S. rhizophila* DSM14405^T, wherein genes are colored according to the product's function in the cell. In addition, the figure depicts the position of tRNA and rRNA genes, and other characteristics of the *S. rhizophila* DSM14405^T genome such as GC content as well as the excess of C over G throughout the whole genome (GC skew). Furthermore gene orthology analyses were performed, where *S. rhizophila* genes were compared with those of *S. maltophilia* R551-3 and K279a to assess orthologous genes among genomes (Fig. 3). In addition to orthologous genes, Fig. 3 depicts the position and color-coded function of the *S. rhizophila* DSM14405^T-specific genes. The numbers of orthologous and strain-specific unique genes are shown in the Venn diagrams (Fig. 4a). The number of CDSs shared between *S. rhizophila* and *S. maltophilia* R551-3 or *S. maltophilia* K279a is 3171 and 3149, respectively (Fig. 4a). There are 862 and 884 *S. rhizophila*-specific CDSs, as orthology analyses against *S. maltophilia* R551-3 and *S. maltophilia* K279a, respectively, reveal. 762 CDSs are unique to *S. rhizophila* DSM14405^T (Fig. 4a), as these CDSs are absent from both *S. maltophilia* R551-3 and *S. maltophilia* K279a. The percentage distribution of *S. rhizophila*-unique CDSs with regard to their predicted cellular functions has been presented in Fig. 4b. Of 762 *S. rhizophila*-unique CDSs, those encoding proteins of unknown function or hypothetical proteins have the greatest abundance (71%). The CDSs whose functions could only be generally predicted (4.46%), those that are involved in carbohydrate transport and metabolism (4.07%), and those playing a part in cell wall/ outer membrane/ cytoplasmic membrane biogenesis (3.02%) are also relatively abundant among the uniques CDSs to *S. rhizophila* DSM14405^T.

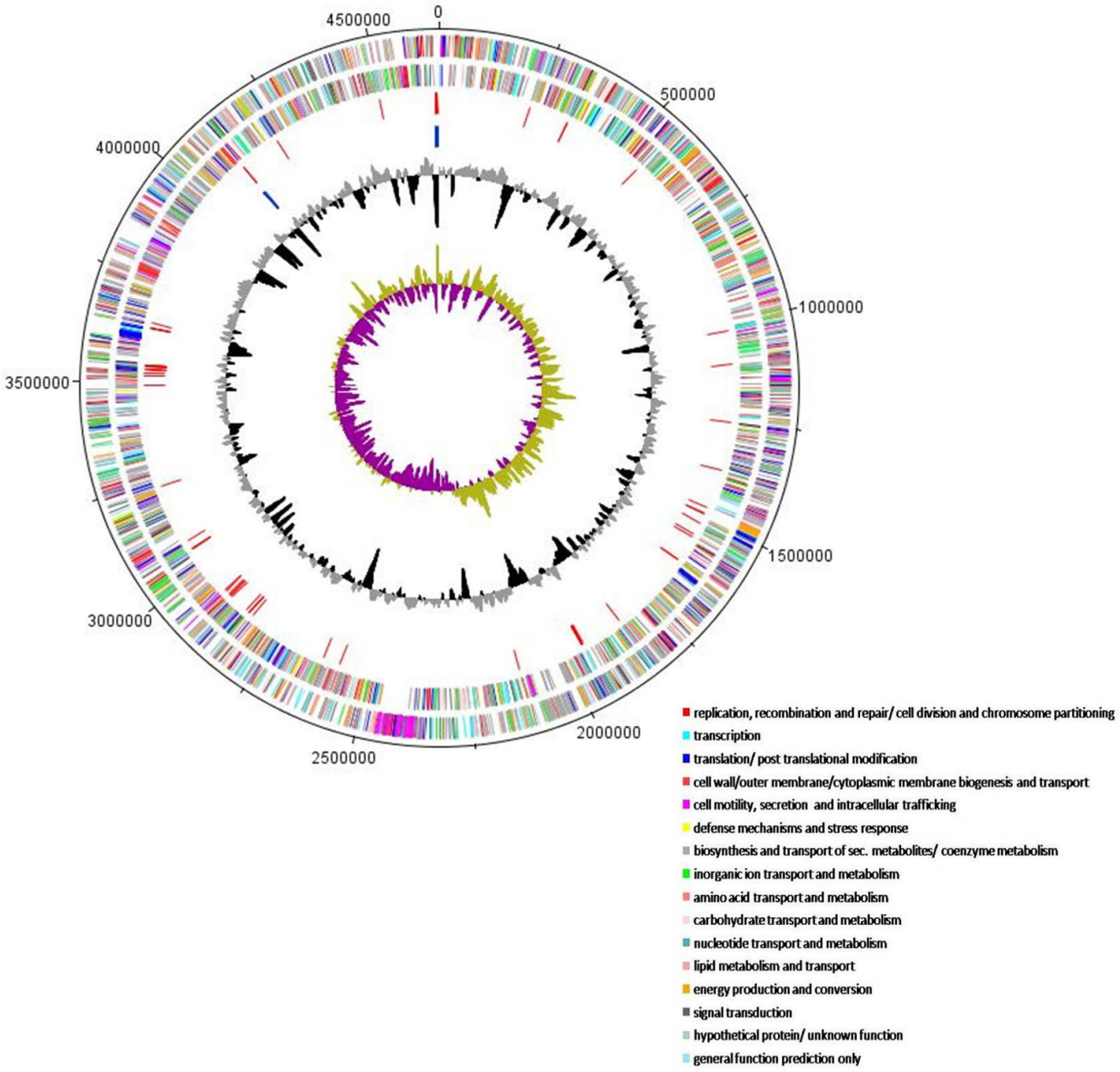

Fig. 2: Circular genome map of *S. rhizophila* DSM14405^T; Predicted coding sequences (CDSs) are assigned various colors with respect to cellular functions. The circles show from the outermost to the innermost: 1. DNA coordinates; 2, 3. Function-based color coded mapping of the CDSs predicted on the forward and reverse strands. Various functions are assigned different colors. tRNA genes; 5. rRNA genes; 6. GC plot with regions above and below average in gray and black, respectively; 7. GC skew showing regions above and below average in dark yellow and magenta, respectively (window size: 10,000 bp). The circular genome map was constructed using DNAPlotter [4].

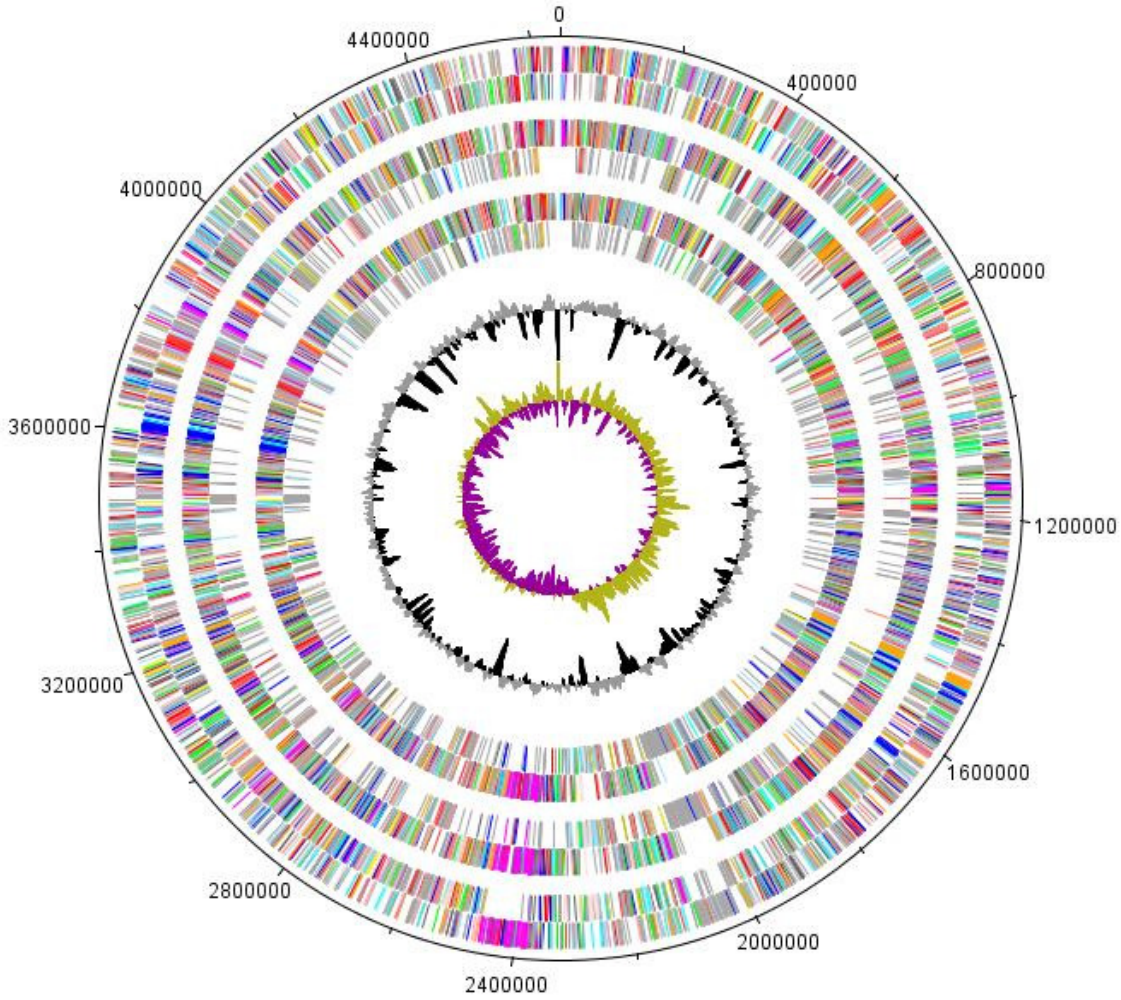

Fig. 3: Gene orthology analyses between *S. rhizophila* DSM14405^T, *S. maltophilia* R551-3 and the clinical *S. maltophilia* K279a; Circles show from the outermost to the innermost: 1. DNA coordinates; 2, 3. Function-based color-coded mapping of the CDSs predicted on the forward and reverse strands of the *S. rhizophila* DSM14405^T genome, respectively. 4. Orthologous CDSs shared between *S. rhizophila* DSM14405^T and *S. maltophilia* R551-3. 5. *S. rhizophila* specific CDSs, compared with *S. maltophilia* R551-3. 6. Orthologous CDSs shared between *S. rhizophila* and *S. maltophilia* K279a. 7. *S. rhizophila* specific CDSs, compared with *S. maltophilia* K279a. 8. GC plot depicting regions above and below average in gray and black, respectively; 9. GC skew showing regions above and below average in yellow green and magenta, respectively (window size: 10,000 bp). The assessment of orthologous CDSs was carried out using the reciprocal best BLASTp hit approach with an identity threshold of 30% and evalue of 10^{-6}.

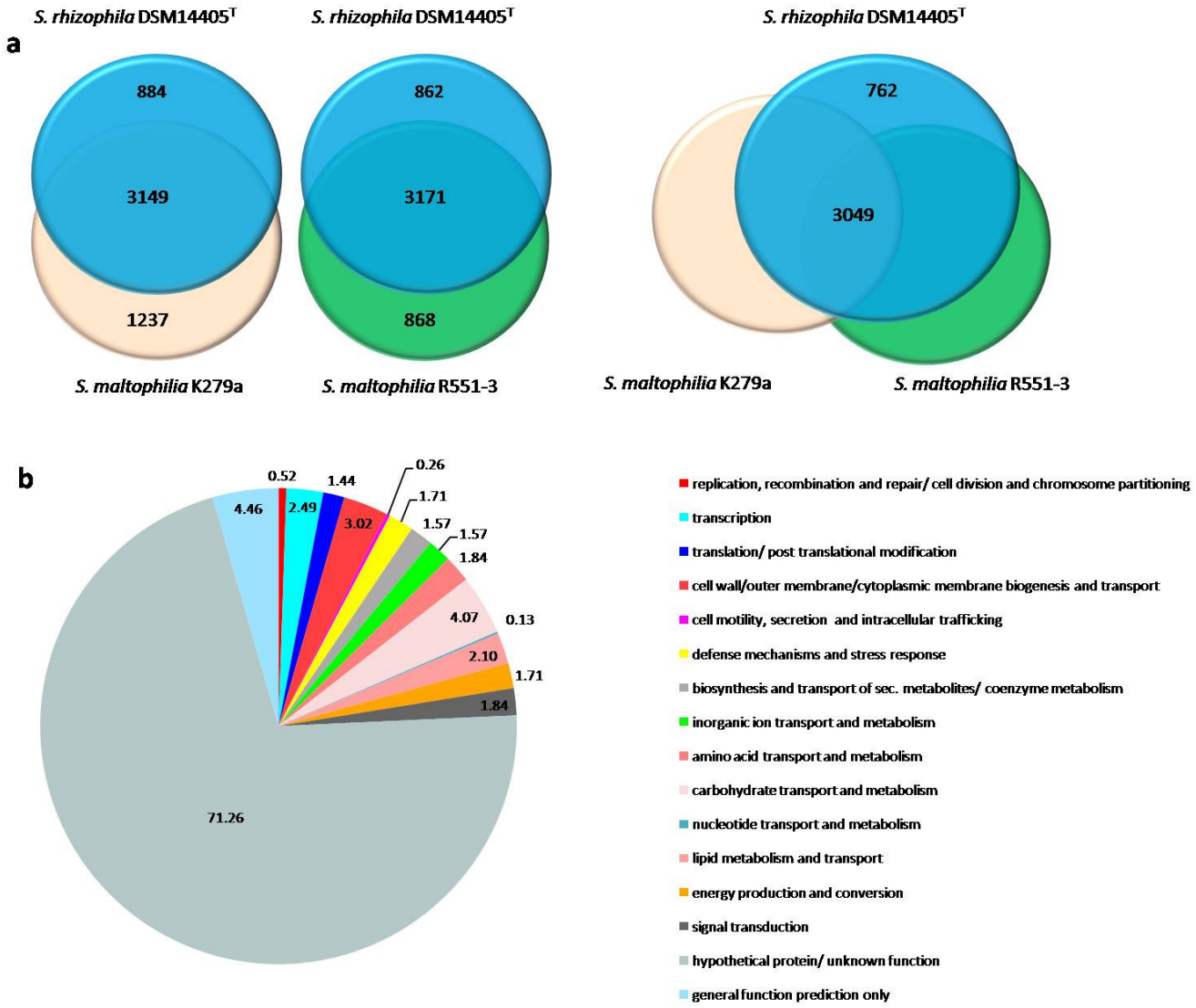

Fig 4a: Venn diagram showing the number of CDSs shared between *S. rhizophila* DSM14405^T (reference genome), the plant-associated *S. maltophilia* R551-3 and the opportunistic human pathogenic *S. maltophilia* K279a. 3171 and 3149 CDSs are shared between *S. rhizophila* and *S. maltophilia* R551-3 and K279a, respectively. 3049 CDSs are shared between all three strains, as a trio-analysis of the three genomes revealed. 762 CDSs are absent in both *S. maltophilia* R551-3 and *S. maltophilia* K279a, and hence unique to *S. rhizophila*. 4b: Diagram showing the percentage distribution of the 762 *S. rhizophila*-specific CDSs with regard to the predicted cellular functions. Most of these are hypothetical genes (71.26%) or CDSs of general function (4.46%). Other *S. rhizophila* unique genes showing a relative abundance are involved in carbohydrate transport and metabolism (4.07%), and cell wall, outer membrane, and cytoplasmic membrane biogenesis (3.02%).

Gene characteristics of *S. rhizophila* DSM14405^T

Quorum sensing

Similar to various xanthomonads, *S. rhizophila* DSM14405^T does not possess a homoserine lactone-based quorum sensing system, but instead uses the *rpf*/DSF system for quorum sensing and cell-cell communication. The *rpf* (regulation of pathogenicity factors) gene cluster is responsible for the synthesis and perception of the DSF molecule which is a quorum sensing regulatory molecule of fatty acid nature with similarity to enoyl-CoA hydratase, and was first detected in *Xanthomonas* (**Barber et al., 1997; Wang et al., 2004**). The *rpfF* gene product, known as DSF synthase, is essential for the synthesis of DSF (**Deng et al., 2011; Ryan and Dow, 2011**). Other members of the *rpf* gene locus (*rpfC*, *rpfG* and *rpfB*) have been revealed to each fulfill a particular function, with the RpfC/RpfG two-component system consisting of a sensory (RpfC) and regulatory (RpfG) component that are responsible for DSF perception and signal transduction, respectively (Barber et al., 1997; **Slater et al., 2000**).

Similar to *Stenotrophomonas* strains K279a and R551-3, the core of the *rpf* gene locus of *S. rhizophila* DSM14405^T consists of four genes: *rpfB*, *rpfF*, *rpfC* and *rpfG*. Fouhy et al. (1997) described the positions of these in the human-pathogenic *S. maltophilia* K279a, which we found to be similar to those in the plant-associated *S. maltophilia* R551-3. Moreover, in both K279a and R551-3, *rpfB* and *rpfF* are located on the lagging strand while *rpfC* and *rpfG* are located on the leading strand. In *S. rhizophila* DSM14405^T, however, the *rpfB* and *rpfF* genes are located on the leading strand while *rpfC* and *rpfG* are on the lagging strand. In addition, in the genome of *S. rhizophila*, there is a 228 nucleotide gene of unknown function on the lagging strand between *rpfF* and *rpfB* , which extends from 2469447 to 2469674 and was annotated as Sr14405 3111. In addition, Sr14405 3111 is transcribed in the cell, as we detected the corresponding mRNA in a whole genome expression analysis approach (data not shown). There is no homologue to Sr14405 3111 in either *S. maltophilia* R551-3 or K279a, and its gene function in *S. rhizophila* DSM14405^T remains to be elucidated.

Flagella, fimbriae

Flagella and fimbriae-driven motility are crucial for biofilm formation and host-plant colonization by bacteria (**Pratt & Kolter, 1998; Van de Broek et al., 1998; De Weert et al., 2002**). *S. rhizophila* DSM14405^T possesses several genes coding for motility. A gene block encoding a flagellar apparatus includes 22 genes, and extends over the genome from Sr14405 3034 to 3055 with most genes located on the leading strand. Another flagella-encoding gene block includes 26 genes and is located between Sr14405 3063 and 3086. Furthermore, two gene clusters, Sr14405 1802-1805 and Sr14405 1812-1816, code for fimbriae; other fimbriae-coding genes are scattered throughout the genome.

Chitinase, extracellular proteases, antibiotic and salinity resistance

S. rhizophila DSM14405^T is a biocontrol agent capable of synthesizing extracellular enzymes with antipathogenic activity such as chitinase and extracellular proteases, and is antagonistic against important fungal pathogens such as *Verticillium dahlia* and *Rhizoctonia solani* (**Minkwitz and Berg, 2001**; **Wolf et al., 2002**). In addition to its direct effect, *S. rhizophila* DSM14405^T is thought to also indirectly promote plant growth through biological control (**Schmidt et al,, 2012**). In the genome, Sr14405 4351 codes for an extracellular chitinase gene, and Sr14405s 1680, 2219, 4184, 4348 are predicted to code for extracellular proteases.

In general, *Stenotrophomona*s species are known to show resistance against a broad range of antibiotics (**Minkwitz and Berg, 2001; Ryan et al., 2009**). There are numerous resistance genes against various antibiotics in the genome of *S. rhizophila* DSM14405^T, some code for general resistance, while others provide resistance against particular classes of antibiotics. The gene cluster extending from Sr14405 1308 to 1310 was predicted to code for a multidrug export system. Another two multidrug resistance gene clusters were detected from Sr14405 1414 to 1416 and from 3642 to 3644. A number of single multidrug resistance genes, such as *mdtN*, *mdtA* and Sr14405 1673 are scattered throughout the genome as well. Moreover, *macA* and *macB* code for the macrolide-specific efflux protein and a macrolide export ATP-binding/permease, respectively. Other identified genes include: Sr14405 2268 and *ampH* that code for β-lactamases, Sr14405 2600 that codes for an aminoglycoside efflux pump, and a transposon tetracycline resistance gene (*tetX*).

S. rhizophila DSM14405^T possesses both *ggp*S and *ycaD*, which are essential for the synthesis and transport of the important osmolyte glucosylglycerol, which provides tolerance against salinity and salt stress (**Roder et al., 2005**). Both *ggpS* and *ycaD* are absent in *S. maltophilia* R551-3 and K279a.

Surface polysaccharides

Homologs to *xan*A, *xanB,* and *rmlA*C were detected in *S. rhizophila* DSM14405^T. These genes are not only involved in the biosynthesis of the *Xanthomonas* well-known surface polysaccharide xanthan, but they are also important in biofilm formation (**Hunag et al., 2006**).

The bacterial capsule is an extracellular structure usually composed of polysaccharides which is considered an important virulence factor in surface adherence, antibiotic resistance, and preventing phagocytosis (**Smith et al., 1999; Boyce and Adler, 2000**). **Reckseidler et al.** (2001) demonstrated that the ability to synthesize capsule is crucial for virulence in the human pathogenic *Burkholderia*. In *S. rhizophila* DSM14405^T, a gene block from Sr14405 3205 to 3220 is homologous to a capsule biosynthesis gene cluster of *Pseudomonas pseudomallei*, described by **Cuccui et al., 2012**. This gene block includes genes that code for proteins of various functions such as signal transduction, transport, and biosynthesis of capsule polysaccharide components. None of the genes present in the *S. rhizophila* capsule gene block were detected in *S. maltophilia* R551-3 and K279a.

Alginate, an exopolysaccharide, is involved in the development and architecture of biofilms and protects bacteria from antibiotics and other antibacterial mechanisms (**Monday and Schiller, 1996; Lyczak et al., 2002; Stapper et al., 2004**). Alginate biosynthesis genes *algI* and *algJ* code for the poly (beta-D-mannuronate) O-acetylase and thealginate biosynthesis protein, respectively. While both were detected in *S. rhizophila* DSM14405^T, neither the plant-associated strain *S. maltophilia* R551-3 nor the human pathogenic *S. maltophilia* K279a contained either of these genes. *AlgI* is preceded by four genes which are also absent from both *S. maltophilia* R551-3 and K279a, with one of these being homologous to a gene coding for a cell morphology protein from the biocontrol agent *P. fluorescens* SBW25.

Secretion systems

While type II and V secretion system genes were identified in *S. rhizophila* DSM14405^T, there is no type III secretion system present, as this is typical of *Stenotrophomonas*. Although there are several genes belonging to the type IV secretion system, a complete gene set was not detected in *S. rhizophila* DSM14405^T. Furthermore, a gene block extending from Sr14405 2737 to 2791 was identified in *S. rhizophila*, which includes numerous genes of the type VI secretion system (T6SS) such as *icmF*, *impA*, genes belonging to the Hcp1 family, and genes coding for proteins with a T6SS Rhs element. With the exception of Sr14405 2775, 2786, and 2790, there were no homologs in *S. maltophilia* K279a and R551-3 to any of the genes of the *S. rhizophila* type VI secretion system block.

One genus, two entirely different habitats and life-styles: Genome comparison between *S. rhizophila* DSM14405^T and *S. maltophilia* K279a

All genes of the plant growth-promoting environmental *S. rhizophila* DSM14405^T and the human pathogenic clinical *S. maltophilia* K279a were compared. While absent from *S. maltophilia* K279a, numerous *S. rhizophila*-specific genes play a role in host-plant colonization. Some of these genes, as described earlier, are crucial for surface attachment, biofilm formation, secretion systems-driven molecular mechanisms, and tolerance of environmental stress such as high soil salinity. In addition, another *S. rhizophila*-specific gene codes for spermidine synthase (*speE*). Spermidine is a plant growth regulator and has been recently shown to strongly promote the growth of arugula plants (**Al-Whaibi et al., 2012**). There are also *S. rhizophila*-specific genes that are involved in the biodegradation of bacterial and plant cell wall. m*ltD*, located closely to the predicted *S. rhizophila* type VI secretion system gene block, codes for muramidase that plays an important role in the bacterial cell wall breakdown. Furthermore, a gene block located from Sr14405 2941 to 2946 codes for several genes involved in the breakdown of plant cell walls.

As a next step, the *S. rhizophila* DSM14405^T having a counterpart in the plant-associated *S. maltophilia* R551-3, but with no homologous genes in the clinical *S. maltophilia* K279a were studied. Of these 88 genes, several help with the need to adapt to plant and rhizosphere as the natural habitat. For instance, the endo-1,4-beta-xylanase B gene (*xynB*) is involved in plant cell wall biodegradation. Other genes conserved in *S. rhizophila* DSM14405^T and *S. maltophilia*

R551-3 are the ferrichrome receptor genes, *fcuA* and *fhuA* which code for siderophore receptors, and the outer membrane adhesin-like gene (Sr14405 3894).

Table 2 presents a list of selected *S. rhizophila* DSM14405^T–specific genes with no homologs in the human pathogenic *S. maltophilia* K279a, together with their biological role. The complete list of the 884 *S. rhizophila* DSM14405^T-specific genes which are not present in *S. maltophilia* K279a is provided in the supplementary Table 1.

Table 2: selected *S. rhizophila* DSM14405^T–specific genes revealing no homologs in the human pathogenic *S. maltophilia* K279a with their role in coping with the environment and bacteria-plant interactions

Biological role	Gene/ Locus tag	(putative) Product
Plant growth promotion		
	speE	spermidine synthase
Secretion system		
	2746	lipoprotein
	2749	type VI secretion system effector
	2755	Rhs element Vgr protein with a type VI secretion system protein domain
	icmF	type VI secretion system protein
	2781	type VI secretion system system-associated protein, ImpA family
	2785	Rhs element Vgr protein with a type VI secretion system protein domain
Bacterial and plant cell wall breakdown		
	mltD	muramidase
	xsa	xylosidase/arabinosidase
	cbg1	beta-glucosidase
	2944	sialate O-Acetylesterase
	aguA	alpha-glucuronidase
	xynB	endo-1,4-beta-xylanase B

Resistance toward antibiotics and salinity		
	mdtABC	three multidrug resistance proteins; form together a multidrug resistance protein channel
	2268	beta-lactamase
	tetX	transposon tetracycline resistance protein
	ampH	beta-lactamase
	2600	aminoglycoside efflux protein
	4792, *mdtA*, *mdtN*	multidrug resistance proteins
	4793	transposon tetracycline repressor protein
	ggpS	glucosylglycerol-phosphate synthase; essential for the synthesis of the osmolyte glucosylglycerol
	ycaD	glucosylglycerol transporter
Surface attachment and biofilm formation		
	yccZ	capsule polysaccharide export protein
	ymcC	lipoprotein
	3215	capsule polysaccharide biosynthesis protein
	wcaJ	colanic biosynthesis UDP-glucose lipid carrier transferase
	3894	adhesin-like protein
	algI	poly(beta-D-mannuronate) O-acetylase
	algJ	alginate biosynthesis protein AlgJ
Iron uptake		
	fcuA, *fhuA*	ferrichrome receptor proteins

Next, the genomes of *S. rhizophila* DSM14405^T and *S. maltophilia* K279a were compared using the latter as the reference, which revealed that 1230 genes are specific to the human pathogenic K279a with no homologs in the plant-growth promoting *S. rhizophila*. While many of these genes are hypothetical or have unknown protein function, others play a specific role. Of the genes with a known or predicted function, many are involved in pathogenicity and virulence. For example,

the gene block extending from Smlt 2997 to 3005 codes for proteins of the type IV secretion system, which is known to have a dual role of both horizontal gene transfer and pathogenicity. Other *S. maltophilia* K279a-specific virulence genes are Smlt 3048, 3683, and 4452, which code for an outer membrane-located adhesin, hemolysin, and hemagluttinin, respectively. Furthermore, Smlt 4391, and *afaD* code for an exopolysaccharide, and adhesin, respectively. In addition, a K279a-specific fimbriae-coding gene block (Smlt 706-709) codes for fimbrial adhesin proteins and their chaperones.

Moreover, there are also several *S. maltophilia* K279a heat shock and chaperone-encoding genes that have no homologs in *S. rhizophila* DSM14405^T, such as Smlt 1818 and Smlt 4629-4631, as both code for heat shock chaperone proteins. Synthesis of chaperones to cope with temperature-caused stress is fundamental for natural habitat conditions of *S. maltophilia* K279a as a human pathogenic strain.

Various antibiotic resistance genes specific to *S. maltophilia* K279a were identified, such as β-lactamase genes, Smlt 4159 and 4211. Other antibiotic resistance genes are Smlt 1071 and Smlt 4474-4476, which code for a fluoroquinolone resistance protein and a multidrug efflux pump, respectively. In addition, there are ABC-type transporters such as Smlt 2642, which codes for a macrolide-specific ABC-type efflux transporter.

Other *S. maltophilia* K279a-specific genes include those that code for TonB-dependent receptors, which are involved in siderophore transport, and phage-coding genes, which are present as both in gene blocks and scattered throughout the genome. Several heavy metal resistance genes for the transport of arsenate, mercuric, and copper are other genomic characteristic of the human pathogenic *S. maltophilia* K279a. Table 3 shows various *S. maltophilia* K279a genes with no homologs in the plant growth-promoting *S. rhizophila* DSM14405^T together with their products and biological roles. The complete list of the 1230 *S. maltophilia* K279a-specific genes is presented in supplementary Table 2.

Table 3: Selected human pathogenic *S. maltophilia* K279a–specific genes, which are involved in virulence and pathogenicity, and reveal no homologs in *S. rhizophila* DSM14405^T

Biological role	Gene/ Locus tag	(putative) Product
Virulence		
	smf-1, 0709	fimbrial adhesin proteins
	0707	pili chaperone protein
	mrkC	outer membrane usher protein
	3048	Hep Hag family adhesin
	wbpV (3683)	hemolysin protein
	4391	exopolysaccharide synthesis protein
	afaD	non-fimbrial adhesin
	4452	cell surface haemagluttinin protein
Secretion system-mediated pathogenicity, horizontal gene transfer		
	2997, 3000	type IV secretion system transmembrane proteins
	2999, 3002, 3003	type IV secretion conjugal transfer proteins
	3005	VirB9 protein
Heat shock resistance, chaperones		
	1818	heat shock chaperone protein
	hscC	chaperone heat shock Hsp70 protein
	4630, 4631	heat shock chaperone proteins

Antibiotic resistance	*qnrB*	fluoroquinolone resistance protein
	2642	macrolide-specific ABC-type efflux carrier
	4159, 4211	beta-lactamase
	smeC (*oprM*)	multidrug efflux system outer membrane protein
	smeB	multidrug efflux protein
	smeA (*acrA*)	drug resistance efflux protein

Fig. 5 represents a model that summarizes crucial specific mechanisms harnessed by *S. rhizophila* DSM14405^{T} and *S. maltophilia* K279a to best adapt to their particular habitats together with the corresponding genes, and those important mechanisms that are used by both species to ensure survival by outcompeting other microorganisms present in the environment.

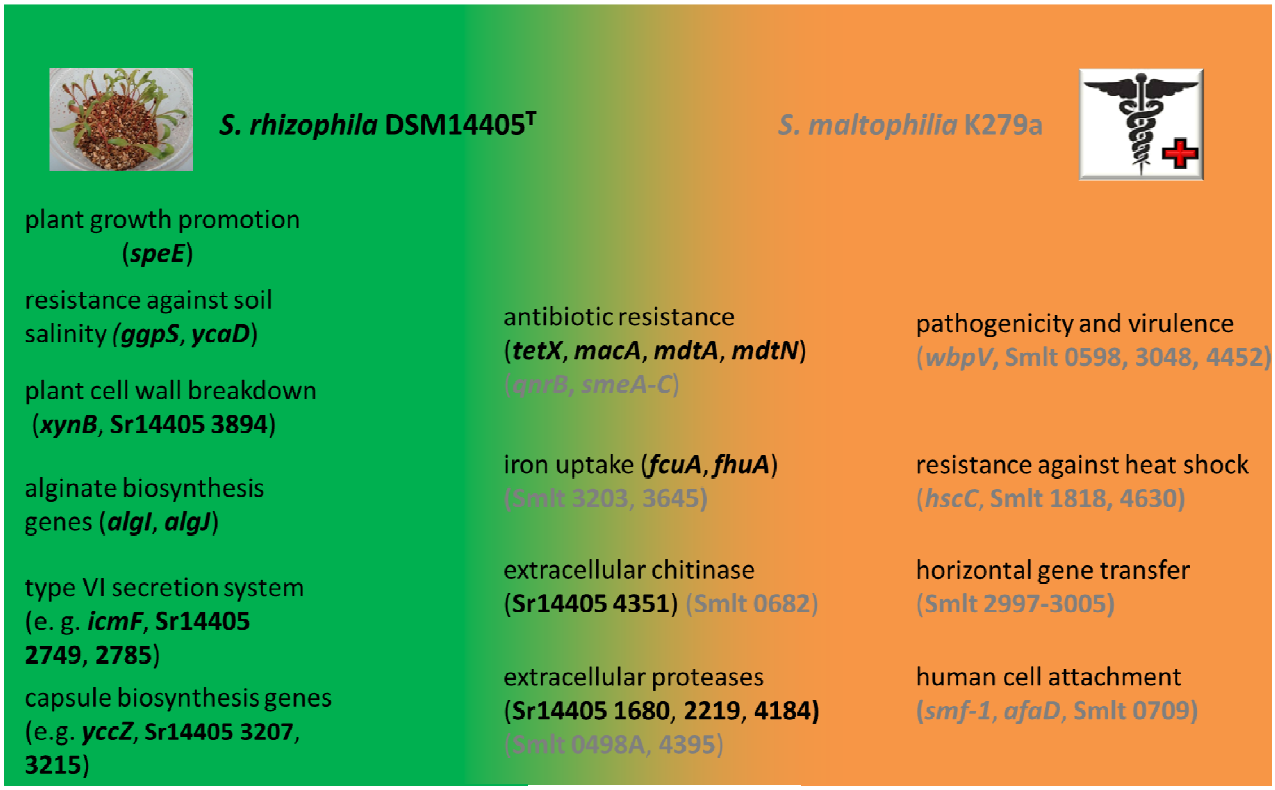

Fig. 5: The plant growth promoting and biocontrol agent *S. rhizophila* DSM14405^T and the human-pathogenic clinical *S. maltophilia* K279a each harness particular mechanisms relying on species-specific genes to best adapt and perform in their habitats. The *S. rhizophila*-specific genes are shown in black while those genes in gray are specific to *S. maltophilia* K279a. Nevertheless, other crucial mechanisms such as ensuring access to biologically available iron and resistance against antibiotics are shared between both species (middle).

Book Chapter 1

Biocontrol and Osmoprotection for Plants under Saline Conditions

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Abstract

Crop cultivation in salinated soils is one of the major agricultural challenges: plants under saline or water unbalance stress become more vulnerable to diseases caused by soil-borne pathogens. Biocontrol using salt-tolerant, plant growth promoting rhizobacteria (PGPR) to protect plant roots against high salinity and pathogens offers sustainable solutions for plant protection. Screening strategies for specific PGPRs were presented and evaluated. *Stenotrophomonas rhizophila* is a model for a plant competent, salt-tolerant PGPR. Besides rhizosphere competence and antagonistic activity, the strain *S. rhizophila* DSM 14405^T is characterized by the production of high amounts of osmoprotective substances. New insights into the mode of action are presented from genomic information.

Introduction

Today's world agriculture faces an increasing threat by phytopathogens which hardly can overcome by conventional methods of pest management. In addition to the limited efficiency of chemical-based pesticides consumers concern more and more about the environment, food safety and food quality. On the other hand insufficient food and deficiencies of vitamins and micronutrients are widely spread in many developing countries and compensation requires extensive and expensive agricultural efforts. In many of these areas soil salinization – originally caused by humidification due to clearing of trees for agriculture and amplified by salt brought in by ground water and strong irrigation – is an immense additional problem. In 1999, 42% of arable land in Asia was irrigated, 31% in the Near East and North Africa and irrigated land in developing countries is estimated to increase by 27% between 1996 and 2030. But soil salinization is reducing the world's irrigated area by 1-2% every year, hitting hardest in the arid and semi-arid regions [FAO 2005]. As a result of soil salinization, plants are under saline or water unbalance stress and become more vulnerable to diseases caused by pathogens such as fungi.

For many decades, fumigation with methyl bromide and related compounds was the standard method for disease control in soils. However, the undifferentiated destruction of microbial communities leads to a vacuum effect in the soil allowing uncontrolled spread of pathogens unaffected by methyl bromide treatment or brought on the fields via plant seeds or seedlings [Ibekwe et al., 2001]. Furthermore, methyl bromide is a greenhouse gas and the bromine released from methyl bromide depletes ozone in the stratosphere 60 times more efficient than chlorine [WMO, 1998]. It is obvious that the problems mentioned above can only be solved by reduction of chemicals in combination with the application of environmentally and consumer friendly biologicals.

Biologicals based on naturally occurring antagonists are an environmentally friendly alternative to control soil-borne pathogens in the rhizosphere [Lugtenberg and Kamilova, 2009; Berg, 2009]. Under salinated conditions, root-associated beneficial microorganisms can help improve plant growth and nutrition. The exploration of bacterial inocula for relief of salt stress and plant growth promotion in saline soils has just started, but first reports are promising [Mo et al., 2006; Egamberdiyeva et al., 2008; Nadeem et al., 2010]. While the mode of action for biocontrol agents (BCAs) is well-investigated [Compant et al., 2005; Lugtenberg and Kamilova,

2009], less is known about the osmoprotective function of rhizosphere microorganisms. In this chapter we describe i) screening strategies for salt-tolerant BCAs, ii) examples for their biocontrol and osmoprotective effects, and iii) the effect and mode of action of *Stenotrophomonas rhizophila*, a model BCA for salinated conditions in more detail.

Screening strategies, biocontrol and osmoprotective effects of salt-competent BCAs

Inconsistent effects under field conditions are one of the main problems in translation of biocontrol studies into practical approaches. To overcome this hurdle, ecological knowledge about BCAs with a-biotic and biotic factors is necessary [Köberl et al., 2011]. This knowledge should be included already screening strategies.

There are several examples published, in which different screening strategies were reported and assessed. Egamberdieva et al., (2008) isolated salt-tolerant bacteria from the rhizosphere of Uzbek wheat with potentially beneficial traits. Eight strains which positively affect the growth of wheat plants *in vitro* were salt tolerant and had plant growth-beneficial properties. Surprisingly, after identification by partial sequencing of the 16S rDNA, the eight new isolates were identified as potential human pathogens, e.g. *Pseudomonas aeruginosa, Staphylococcus saprophyticus, Bacillus cereus, Enterobacter hormaechei, Pantoae agglomerans* and *Alcaligenes faecalis*. The occurrence of potential human pathogens under extreme conditions is known from other studies too.

Another strategy was suggested in Egamberdieva et al. (2011) including salt tolerance tests in the first step. Interestingly, the majority of rhizobacterial strains were highly tolerant to salt. This can be explained by the permanently changing osmotic conditions due to exudation in the rhizosphere. Fifty two beneficial and salt-tolerant bacteria from collections of our institutes from all over the world were screened for their ability to promote growth and/or to control diseases caused by the soil-borne fungus *Fusarium solani* on cucumber and tomato plants. The five best strains were used in large scale greenhouse trials. Four out of five strains significantly controlled cucumber foot and root rot., reducing the percentage of diseased plants from 54% in the negative control to between 10 and 29% in bacterized plants. All five strains increased the dry weight, by between 29 and 62%. In two consecutive years, all five strains significantly increased the plant height (by 4 to 15%) as well as the fruit yield (by 12 to 32%). Tests of plant-beneficial traits suggest that auxin production, antibiosis and competition for nutrients and niches are

mechanisms involved in the observed plant growth stimulation and biocontrol. The results with tomato were similar. The conclusion was that many beneficial bacteria isolated from plants grown on non-salinated soil are perfectly able to promote plant growth and control plant diseases in salinated soil. *Stenotrophomonas rhizophila* strain DSM14405T, which was originally isolated from the rhizosphere of oilseed rape, was one of the most effective strains in this study.

ACC (1-aminocyclopropane-1-carboxylic acid)-deaminase activity is another interesting target for screening strategies of BCAs active under salt stress. This was used by Nadeem et al. (2009) to evaluate rhizobacterial strains by conducting a jar experiment under axenic conditions at 1 (original), 5,10, and 15 dS m^{-1}. The four most effective strains were further evaluated in a pot trial at salinity levels of 1.46 (original), 5, 10, and 15 dS m^{-1}. In general, salinity depressed the growth of wheat, but inoculation improved the growth and yield of wheat compared with the non-inoculated controls. At the high salinity level (15 dS m-1$^{)}$, plant height, root length, plant biomass, and grain yield increased up to 37, 70,116, and 111%, respectively, compared with the control. Results indicated that inoculated plants had higher K^+/Na^+ ratios, relative water contents and chlorophyll content; however, relatively low proline contents compared with controls. The results also showed that intensity of the classical triple response decreased due to inoculation with these strains. *Pseudomonas putida* W2 and *P. fluorescens* W17 were the most effective strains for alleviating salt stress even at higher salinity levels. The results suggest that the assay for ACC-deaminase activity could be an efficient approach to screen effective PGPR for increasing the growth and yield of wheat under salt-stressed conditions.

For biocontrol approaches in salinated soils, specific BCAs are required. Salt tolerance is an important requirement for these BCAs [Príncipe et al., 2007; Egamberdieva et al., 2011]. Beside antagonistic traits, production of osmoprotective substances or ACC desaminase is essential for successful introduction into salinated soils. However, the study of Egamberdieva et al. (2011) showed that salinization does not seem to be a threat for the application of presently used plant-beneficial bacteria because many BCAs belonging to the generalists are salt-tolerant and can be applied under salinated conditions. This was also shown for the two main BCAs *Pseudomonas* [Rangarajan et al., 2003] and *Bacillus* [Bochow et al., 2001]. Due to the fact that there are hints that especially salinated soils contain potential human pathogenic pathogens, biosafety aspects should be integrated at an early stage of product development. Here, biological assays, which indicate human pathogenicity like the *Caenorhabditis elegans* assay, can be used for risk assessment [Zachow et al., 2008].

Stenotrophomonas rhizophila a model bacterium for salt-tolerant BCA

Stenotrophomonas isolates have a great potential for applications in biotechnology and biological control due to the high capacity to promote plant growth and their antagonism against different phytopathogenic fungi [Ryan et al., 2009]. While the species *S. maltophilia* has become important as a nosocomial multidrug-resistant human pathogen associated with significant case/fatality ratios particularly in patients who are severely debilitated or immunosuppressed, the closely related species *S. rhizophila* [Wolf et al., 2002] no pathogenic potential for humans has ever been observed. Both species can be easily distinguished by production of osmoprotective substances and the occurrence of multidrug-efflux pumps [Ribbeck-Busch et al., 2005].

Plant growth promotion was also observed in the highly salinated soils of Uzbekistan; statistically significant effects of *Stenotrophomonas rhizophila* strain DSM14405T treatment were observed for wheat, tomato, lettuce, sweet pepper, melon, celery and carrot: the treatment resulted in higher germination rates as well as in longer shoots and roots. For example, in tomato the germination rate was 180%, the growth of the shoot 120% and root 142% enhanced in comparison to the untreated control [Egamberdiyeva et al., 2011]. Plant species-specific effects were also observed for *S. rhizophila* DSM 14405^T at increasing salinities under greenhouse conditions. The plant growth promoting effect produced an increase in shoot length as well as in the number of secondary leaves (Fig. 1). In general, plant growth promotion by *S. rhizophila* DSM14405^T was more pronounced in non-sterile soil, and decreased with salinity. Significant increase in shoot length as well as the number of secondary leaves was observable in non-sterile soil at 0% salinity. With increasing salinity, this effect became less pronounced and was not always detectable using the Mann-Whitney test. However, in non-sterile soil, a positive effect of *S. rhizophila* DSM14405^T was consistent across all salinities, and therefore both plant growth parameters could be described in a linear regression model with soil salinity and *S. rhizophila* DSM14405^T (0=absent, 1=present) as significant factors: Shoot length [cm]= - 2.4 × salinity [% NaCl] + 0.7 × *S. rhizophila* DSM14405^T + 4.2 (R^2 = 0.393, p<0.001); No. of secondary leaves = - 2.1 × salinity [% NaCl] + 0.7 × *S. rhizophila* DSM14405^T + 2.4 (R^2 = 0.334, p<0.001 (R^2 = 0.393, p<0.001 for shoot length, R^2 = 0.334, p<0.001 for number of secondary leaves). The p-value of the factor "*S. rhizophila* DSM14405^T" was 0.016 for shoot length and 0.018 for number of secondary leaves. Contrary to non-sterile soil, no plant growth promotion by *S. rhizophila* 14405^T was observable in sterile soil under saline conditions. Shoot length was slightly increased

(p<0.1) only in non-saline soil. The number of leaves was slightly increased at zero and medium salinity, but the effect was not significant using Mann-Whitney test. Since there was no consistent effect across all treatments, no linear regression model could be fitted in sterile soil.

The mechanisms behind plant growth promotion and biocontrol by *Stenotrophomonas* against soil-borne fungi like *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Verticillium* are well-investigated by physiological methods. The latter include the excretion of antifungal metabolites (AFM; antibiotics, toxins and bio-surfactants) and the production of a wide range of extracellular enzymes [Jacobi et al., 1996, Berg and Ballin, 1994, Kobayashi et al., 1995, Dunne et al., 2000]. Besides the excretion of soluble AFMs and enzymes also volatile organic compounds (VOCs) produced by soil bacteria like *Stenotrophomonas* can negatively influence growth of fungi [Alström 2001, Wheatley 2002]. Recently, it has been shown that the VOCs of *S. maltophilia* and *S. rhizophila* inhibit mycelial growth of the soil-borne pathogen R. solani to more than 90% in dual culture tests. Out of a vast diversity of VOCs produced by *S. rhizophila*, two, namely dodecanal and □-phenylethanol could be identified by GC-MS [Kai, 2007]. It has been shown that *S. rhizophila* has a high-potential for plant growth promotion although positive effects are dose-dependent [Wolf et al. 2002, Suckstorff and Berg 2003]. *S. rhizophila* is able to grow at salt concentrations up to 4% and produces the osmolytes trehalose and glucosylglycerol (GG) in response to salt stress [Roder et al., 2005]. Osmolytes are compounds compatible with cellular functions e.g. DNA replication, DNA-protein interactions and cellular metabolism. Osmolytes are highly soluble; they have no net charge at physiological pH and do not interact with proteins. Furthermore, they function on osmotic balance and are effective stabilizers of enzymes [Lippert and Galinski, 1992; Welsh, 2000].

Genome sequencing resulted in new insights into genetic sources, which provide beneficial plant-associated bacteria with traits such as plant growth promotion, antagonisms towards phytopathogens as well as osmoprotection.

Here we discuss some of these genetic sources for *S. rhizophila* DSM14405T.

S. rhizophila produces high levels of the auxin phytohormone indole-3-acetic acid (IAA) (Suckstorff and Berg 2003). Bacteria synthesize IAA through several various pathways (Spaepen et al., 2007). Based on our BLASTn investigations the genes encoding the regular pathways of IAA synthesis such as tryptophan-2-monooxygenase and tryptophan decarboxylase are not

present in the genome of *S. rhizophila*. However, the presence of the nitrilase gene suggests that the biosynthesis of IAA in *S. rhizophila* could be through the indole-3-acetonitrile pathway.

Moreover, *S. rhizophila* possesses genes responsible for the synthesis and transport of osmoprotective molecules (osmolytes) out of the cell. Glucosylglycerol-phosphate synthase (*ggpS*) gene has been shown to be essential for the synthesis of the osmolyte molecule glucosylglycerol (GG), which has been suggested to be transported into the environment by a transporter encoded by *ycaD* gene located upstream to *ggpS* (Hagemann et al. 2008).
S. rhizophila also possesses a number of genes known to be involved in the biocontrol of soil-borne pathogens such as metalloprotease and antibiotic synthesis genes. Furthermore, our genome sequence investigations have revealed that *S. rhizophila* possesses a novel gene homologous to the one coding for the bacterial lanthionine synthetase C-like protein (LanC). The bacterial *lanC* is responsible for the synthesis of peptide antibiotics (lantibiotics). Subtilin from *Bacillus subtilis* ATCC 6633 and epidermin from *Staphylococcus epidermidis* are two well-studied examples for bacterial lantibiotics with antibiotic activity against other bacteria (Chung et al. 1992, Schnell et al. 1992).

Aside from notorious products such as IAA and VOCs there exist also further, rather backbone mechanisms leading to plant growth promotion and biocontrol (Egamberdieva et al. 2011). The bacterial competitiveness in regard to colonizing niches and utilizing nutrients are important examples of these mechanisms (Kamilova et al. 2005). *S. rhizophila* possesses several genes of great importance in regard to competition for nutrients and niches, which are not present in other plant-associated *Stenotrophomonas* spp. such as *S. maltophilia* R551-3. Examples for this are genes responsible for pectin degradation, xylan degradation and the pectate lyase gene. Adhesion to the host-plant cell surface and the ability to form biofilms are also important features for out-competing other microorganisms during the competition process over niches. Also in these terms *S. rhizophila* is equipped with several genes such as those encoding the O-antigen and capsule polysaccharide biosynthesis pathways, genes encoding hemagglutinin, outer-membrane adhesion protein, etc. Furthermore, there are also amino acid biosynthesis genes present in the genome of *S. rhizophila* such as chorismate mutase, which plays a crucial role in the biosynthesis pathway of tyrosine and phenylalanine (Guo et al. 2001). In addition, our BLASTp analyses have revealed that *S. rhizophila* possesses a gene homologous to the bacterial cardiolipin synthetase (*cls*), which plays an important role in the adaptation to elevated osmotic stress due to environmental changes

(Romantsov et al. 2008). Figure 2 demonstrates an overview over the features possessed by *S. rhizophila* DSM14405T, which play a role in the plant growth promotion, biocontrol and plant osmoprotection.

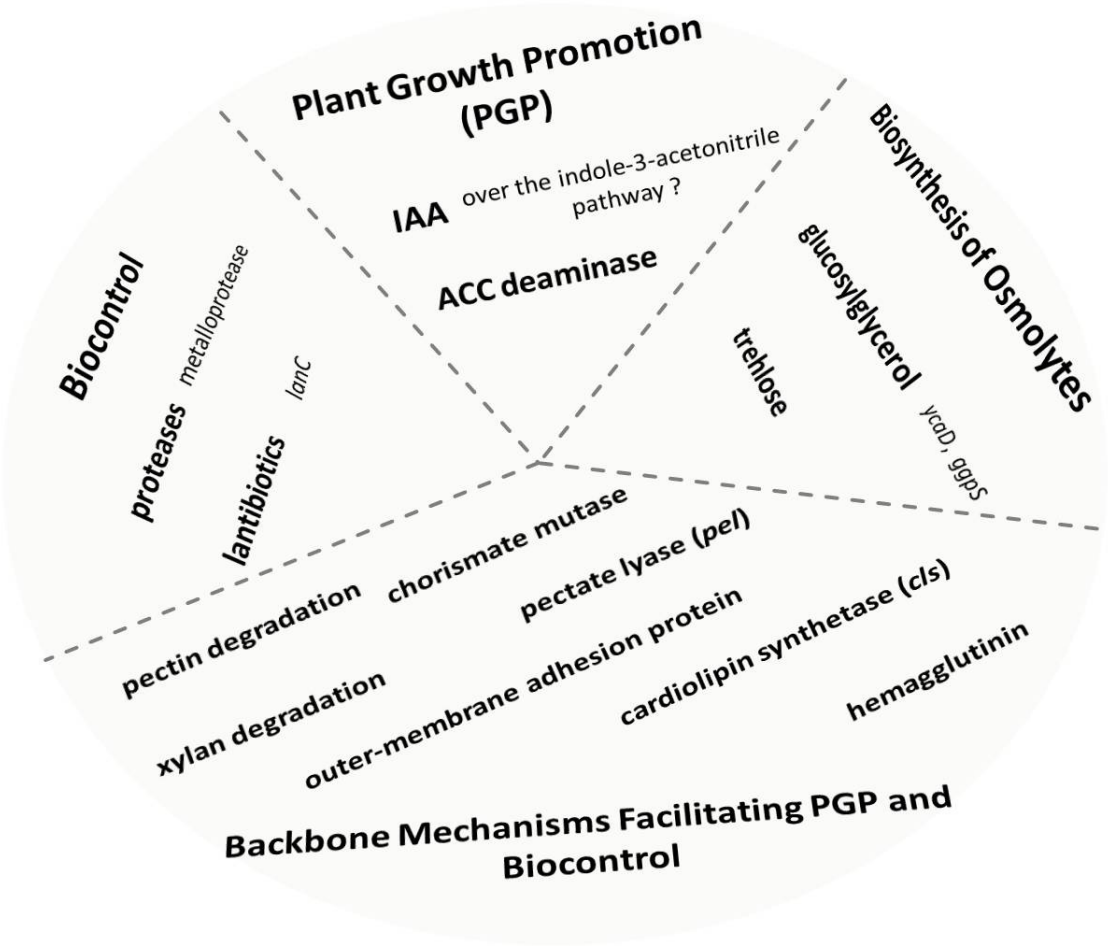

Fig. 2: Overview of features, which play a role in the plant growth promotion, biocontrol and plant osmoprotection delivered by S. rhizophila DSM14405T. Features and the corresponding genes were detected through genome sequence investigations, BLASTp analysis as well as using references cited in this work.

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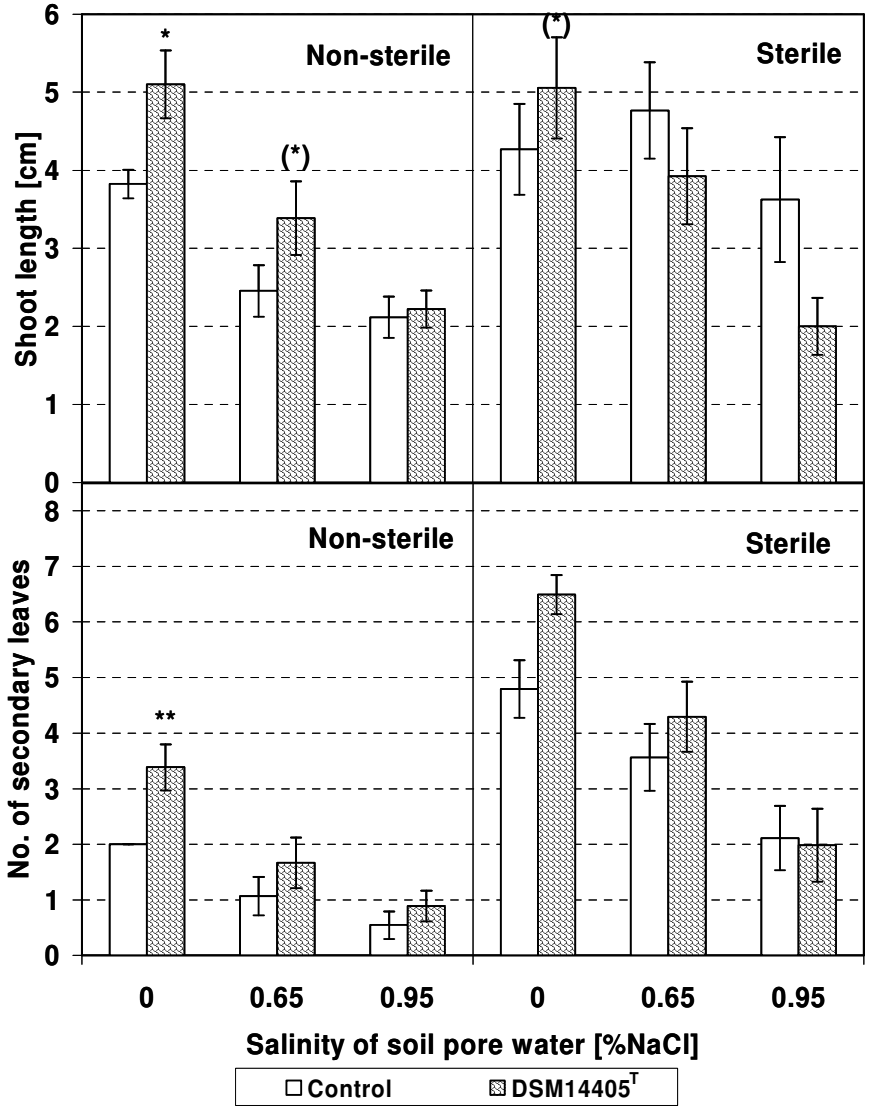

Fig. 1: Influence of soil salinity on plant growth promotion of sweet pepper by *S. rhizophila* DSM14405^T in non-sterile soil (top) and sterile (autoclaved) soil (bottom). Plants were grown in "Profi Substrat", GWC set to 3.5. Seeds were soaked with suspension of *S. rhizophila* DSM14405^T RifR5 in 0.85% NaCl before sowing, resulting in ~10^5 CFU seed^{-1}. Data of 3 experiments (non-sterile soils) or 2 experiments (sterile soils) were pooled. Asterisks indicate a significant effect of *S. rhizophila* DSM14405^T at $p<0.01$ = ** $p<0.05$ = *, $p<0.1$=(*) (Mann-Whitney-Test).

Book Chapter 2

The Rhizosphere as a Reservoir for Opportunistic Human Pathogenic Bacteria

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Abstract

The rhizosphere is, due to root exudates and the resulting high nutrient content, a unique microenvironment in terrestrial ecosystems characterized by high microbial density and activity. Rhizosphere-associated bacteria have a great potential in diverse areas of biotechnology, e.g. as biological control agents (BCAs) in plant protection. Although many of them have a positive interaction with their host plants, they can interact with other eukaryotic hosts like humans in a pathogenic way. This review presents an overview about these bacteria that have bivalent interactions with plant and human hosts. We discuss mechanisms of the interactions and their behavior and ecology. Furthermore, new insights from genome research of such ambivalent bacterial species are discussed in detail for *Stenotrophomonas* and diazotrophic plant growth promoting bacteria.

Introduction

The interface between soil and plant roots – the rhizosphere – is, due to root exudates and the resulting high nutrient content, a unique microenvironment in terrestrial ecosystems (Sørensen 1997; Raaijmakers *et al.* 2009). Cultivation-independent methods on the basis of DNA/RNA, such as microbial fingerprinting techniques, fluorescence-in-situ-hybridization (FISH) and pyrosequencing gave interesting insights into the structure of rhizosphere-associated bacterial communities (reviewed in Smalla 2004; Hartmann *et al.* 2009; Mendes *et al.* 2011). But what do we know about the functions of plant-associated bacteria? Firstly, bacteria play a role in plant growth. They can support nutrient uptake, enhance the availability of phosphorous and produce a broad range of phytohormones (Costacurta and Vanderleyden 1995). An interesting phenomenon is the enhancement of stress tolerance by lowering the ethylene level (Glick 1998). Another important function is the involvement of plant-associated bacteria in pathogen defense. Many pathogens attack plants, especially fungi, oomycetes and nematodes, and cause yield losses of more than 30% worldwide. Whereas resistance against leaf pathogens is often encoded in the plant genome, it is difficult to find resistance genes against soil-borne pathogens. Cook *et al.* (1995) suggest that antagonistic rhizobacteria fulfill this function. Interestingly, besides direct antagonism, plant-associated bacteria can induce a systemic response in the plant, resulting in the activation of plant defense mechanisms (Pieterse *et al.* 2003). Besides the general microbe-associated molecular patterns (MAMPs) for recognition of microbes by the plants, innate immune system, more recently bacterial quorum sensing signaling molecules were identified to induce systemic resistance against biotrophic plant pathogens (Schuhegger *et al.* 2006; Schikora *et al.* 2011; Schenk *et al.* 2012).

To study plant-associated bacteria and their structure and functions is important not only for understanding their ecological role and the interaction with plants and plant pathogens, but also for any biotechnological application. In biotechnology, plant-associated bacteria can be applied directly for biological control of plant pathogens as biological control agents (BCAs), for growth promotion as Plant growth promoting rhizobacteria (PGPR) and enhancement of stress tolerance as biofertilizers and phytostimulators or as rhizoremediators (Whipps 2001; Berg 2009). To avoid any risk of the application of these microbial inoculants for human health, it is important to understand the mode of interaction with eukaryotic hosts (Berg *et al.* 2010). On the

other hand, the knowledge over the pathogenic potential of rhizobacteria could help understand and discover newly emerging pathogens.

The rhizosphere as reservoir for opportunistic human pathogenic bacteria

During the last few years, it has been shown that plants, especially in the rhizosphere, can harbor not only beneficial bacteria, but also those that can potentially cause diseases in humans (rev. in Berg *et al.* 2005). Opportunistic pathogens can only cause diseases in patients with a strong predisposition to illness (Parke and Gurian-Sherman 2001; Steinkamp *et al.* 2005). This group of bacteria cause the majority of bacterial infections associated with significant case/fatality ratios in susceptible patients in Europe and Northern America (Vincent *et al.* 1995) and their importance is still increasing world-wide.

Many plant-associated genera, including *Burkholderia, Enterobacter, Cronobacter, Herbaspirillum, Azospirillum, Ochrobactrum, Pantoaea, Pseudomonas, Ralstonia, Serratia, Staphylococcus* and *Stenotrophomonas* contain root-associated and endophytic bacteria that engage in bivalent interactions with plant and human hosts. Several strains of these genera show plant growth promoting as well as excellent antagonistic properties against plant pathogens and were therefore applied as BCAs or PGPRs (Whipps 2001). However, many strains also successfully colonize human organs and tissues and thus cause diseases as shown for members of the *Burkholderia cepacia* complex (Govan *et al.* 2000; Parke and Gurian-Sherman 2001). This underlines the importance of thorough risk assessment studies prior to registration of microbial inoculants.

Factors involved in eukaryote-microbe interaction

Rhizosphere-associated bacteria with antagonistic activity against eukaryotes are able to interact with their hosts using various mechanisms. These mechanisms include i) recognition and adherence, i) inhibition of pathogens by antibiotics, toxins and bio-surfactants [antibiosis], ii) competition for colonization sites and nutrients, iii) competition for minerals, e.g. for iron through production of siderophores or efficient siderophore-uptake systems, iv) degradation of

pathogenicity factors of the pathogen such as toxins, and v) parasitism that may involve production of extra-cellular, cell wall-degrading enzymes such as chitinases and □-1,3 glucanases (Raaijmakers *et al.* 2008; Lugtenberg and Kamilova 2009). Other factors that contribute to rhizosphere fitness include the ability to use seed and root exudates as carbon sources or, more generally, ecological and nutritional versatility. Steps of pathogenesis are similar and include invasion, colonization and growth, and several strategies to establish virulence (the relative ability of a pathogen to cause disease in the host). Each of the involved factors was referred to as "virulence factor" or "pathogenicity factor" (Dobrindt *et al.* 2004). Many mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for pathogenicity of bacteria (Rahme *et al.* 1995; Cao *et al.* 2001). Extensive invasion and endophytic colonization of plants have been demonstrated for food-borne pathogens like *Salmonella enterica* pv. Typhimurium in barley (Kutter *et al.*, 2006) and for the severe human pathogen *Burkholderia pseudomallei* in diverse plants in Northern Australia (Kaestli *et al.,* 2011). Also for the food borne pathogen *Cronobacter* [former *Enterobacter] sakazakii*, which is associated with cases of meningitis, necrotizing enterocolitis and sepsis in neonates and immunocompromised infants, diverse plants were demonstrated to be colonized endophytically by clinical isolates (Schmid *et al.* 2010). Strains of *C. sakazakii* were isolated from the rhizosphere of *Salicornia* and other plants (Jha *et al* 2012; Schmid *et al.* 2010).

Several studies provided evidence that similar or even identical functions are responsible for beneficial interactions with plants and virulence in humans. For example, the involvement of siderophore-uptake systems or extra-cellular enzymes is common to both beneficial bacteria and human pathogens (Tan *et al.* 1999). Dörr *et al.* (1998) reported that type IV pili of the plant-associated *Azoarcus* sp. BH72 are responsible for the adhesion on plant and fungal cells. Furthermore, the amino acid sequence of the pilus showed a high similarity to pili of the human-associated strains of *P. aeruginosa* and *Neisseria gonorrhoeae*. Type III secretion systems are responsible for the introduction of effectors into eukaryotic host cells; they have been discovered for pathogenic bacteria as well as plant-associated bacteria with beneficial effects on host plants (Preston *et al.* 2001).

In a study published by Alonso *et al.* (1999) it was shown that clinical and environmental isolates of *P. aeruginosa*, which is the major causal agent for morbidity and mortality of patients with cystic fibrosis, share several phenotypic traits with respect to both virulence and

environmental properties. Several studies support the view that the environmental strains are indistinguishable from those arising from clinical sources in terms of genotypic, taxonomic or metabolic properties (Kiewitz and Tümmler 2000; Finnan *et al.* 2004; Morales *et al.* 2004). In addition, differences between environmental strains and those that cause infections might rather be due to the regulation of genes, than their mere presence or absence (Parke and Gurian-Sherman 2001). In this regard, similar studies with focus on *P. aeruginosa, Stenotrophomonas maltophilia* (reviewed in Ryan *et al.* 2009) and *Burkholderia cepacia* (Parke and Gurian-Sherman 2001) have been published. Nevertheless, antagonism studies and biocontrol effects were reported for all mentioned species, and one product derived from *B. (ceno)cepacia* was on the market (Hebbar *et al.* 1998; Nakayama *et al.* 1999; Dunne *et al.* 2000, Govan *et al.* 2000). All species are common inhabitants of the rhizosphere; yet due to their medical relevance, they are grouped into risk group 2 in the public databases, e.g. those by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de), and should be excluded from direct biotechnological applications.

An important mechanism by which harmless bacteria can behave as pathogens is change of host or host niche, upon which their virulence potential is frequently released to its full extent. This mechanism is clearly relevant for opportunistic pathogens from plant-associated habitats. In addition, other mechanisms such as structural changes of the bacterial chromosome due to gene acquisition and loss, recombination and mutations can lead to bacterial pathogenicity (for a review see Hacker *et al.* 2003). Genes responsible for pathogenicity or fitness of bacteria often occur as genomic islands, which are blocks of DNA with signatures of mobile genetic elements (Hacker and Carniel 2001). They are called “fitness islands” or “pathogenicity islands” according to their function.

Rhizosphere-associated bacteria with a high capacity for biocontrol can be potentially dangerous for human health. Therefore, it is important to understand the mode of action and specific properties of the BCA. It is well known that antagonistic properties and underlying mechanisms are highly strain-specific (Berg *et al.* 2002) but identification of bacteria is based mainly on 16S-rDNA sequencing. However, based on the sequence information of the ribosomal RNA, which is a central and well conserved housekeeping gene, it is impossible to draw conclusions about potential pathogenicity: neutral bacterial strains can be dangerous due to

pathogenicity islands or pathogenic bacteria can be harmless because of the absence of any pathogenicity factor.

Stenotrophomonas: an ambivalent global player from the rhizosphere

Bacteria of the genus *Stenotrophomonas* are of increasing biotechnological interest due to their ubiquitous occurrence and versatility (Ryan *et al.* 2009). Their plant growth promoting properties and their antagonistic behavior against soil-borne plant pathogens are well-documented (rev. in Ryan *et al.* 2009), but development for commercial application of *S. maltophilia*, the most intensively studied species, has been hampered by its potential as opportunistic human pathogen in immune-suppressed patients (Hagemann *et al.* 2006). Within a broad range of isolates of environmental and clinical origin, classified at the time as *S. maltophilia*, a distinguished genomovar consisting only of environmental isolates could be separated (Minkwitz and Berg 2001), and further characterization led to their description as a separate species, *Stenotrophomonas rhizophila* (Wolf *et al.* 2002). Contrary to *S. maltophilia*, *S. rhizophila* does not have human pathogenic traits (Ribbeck-Busch *et al.* 2005; Hagemann *et al.* 2006), has a lower temperature optimum than *S. maltophilia* (Wolf *et al.* 2002), and is therefore safe to apply in biotechnological approaches. In addition, the synthesis of an additional osmolyte, glucosyl glycerol, confers a greater degree of salt resistance *in vitro* (Hagemann *et al.* 2008) and makes it an ideal candidate for applications under saline soil conditions. *In vitro*, isolates of this species produce fungal cell wall degrading enzymes, siderophores (Minkwitz and Berg 2001) and volatile antifungal compounds (Kai *et al.* 2007); antifungal activity against the soil-borne plant pathogens has been demonstrated (Minkwitz and Berg 2001). Also, production of the plant growth hormone indolacetic acid (IAA) and direct growth promotion of strawberry plants *in vitro* have been shown (Suckstorff and Berg 2003). Furthermore, growth of a wide variety of crops was strongly promoted in the saline soil of Uzbekistan by *S. rhizophila* strain DSM14405^T (=e-p10^T, =P69^T) (Egamberdiyeva *et al.* 2011). These studies indicate the potential of *S. rhizophila* to directly promote plant growth as well as to inhibit plant pathogens.

All these information based on physiological and targeted molecular investigations – but how does this information correlate with results obtained by genome sequencing? Although *S. rhizophila* is grouped in risk group 1 and no human infection has ever been reported till now,

genome sequencing of DSM14405T revealed numerous potential virulence factors. For example, type VI secretion system (T6SS), a bacterial transport system recently discovered in a number of important human, animal and plant pathogenic Gram-negative bacteria, has dragged attention of many scientists in the past years due to its role in causing virulence and pathogenesis in the host organisms. Some clinically important pathogenic bacteria, which use T6SS for secretion of virulence factors into the cytosol of human cells are: *Burkholderia mallei*, *Yersinia pestis*, *Salmonella typhimurium* and *Legionella pneumophila* (Purcell and Shuman 1998; Parsons *et al.* 2005; Schell *et al.* 2007). Also *Pseudomonas aeruginosa*, an opportunistic but also plant-associated bacterium possesses T6SS (Mougous *et al.* 2006). Surprisingly, our genome analysis investigations have revealed that DSM14405^T also possesses a gene cluster with at least 19 genes being directly related to the structure and function of T6SS. Genes known to make the backbone of T6SS are conserved among bacteria with a functioning type VI secretion system. The most important members are *icmF*, *clpV* encoding the corresponding ATPase, *hcp* encoding the hemolysin coregulated protein and the *Vgr*-related genes (Bingle *et al.* 2008). The *S. rhizophila* DSM14405^T T6SS gene cluster possesses homologs to all these genes. Also other genes known to belong to T6SS in various other bacteria are present in the *S. rhizophila* T6SS gene cluster such as an *impA*-related N-terminal family gene which finds a homolog in *Burkholderia pseudomallei* 7894, the pathogenic agent for Melioidosis in humans. Figure 1 shows the T6SS gene cluster in *S. rhizophila* DSM14405^T with the most important components noted above being highlighted. The physiological role of T6SS in *S. rhizophila* as a BCA and plant growth promoting agent is still unknown but there is a great chance for its involvement in the secretion of a broad range of effectors and other molecules with importance for bacteria-host plant and bacteria-plant pathogen interactions. Aside from type VI secretion system, the genome analysis investigations have revealed that the genome of *S. rhizophila* DSM14405^T harbors other genes encoding proteins/systems of ambivalent significance with occurrence in both plant-associated and human/animal pathogenic bacteria. For instance, there are pilin synthesis and assembly genes present in the genome of *S. rhizophila*. An example therefor, is a gene which is homologous to *pilE* (pilin protein) from *Legionella longbeachae*, a soil bacterium known to be capable of causing legionellosis in humans (Fields *et al.* 2002). Moreover, *S. rhizophila* possesses a non-hemolytic phospholipase C gene (*plC*), which finds homologs in the clinical *Stenotrophomonas maltophilia* K279a as well as other pathogenic bacteria such as *P. aeruginosa* and *B. pseudomallei*. PLCs are virulence factors, which degrade cell membrane phospholipids. In *P.*

aeruginosa they play a role in bacterial survival in the human endothelial cells (Plotkowski & Meirelles 1997). Another group of virulence factors important for invasion of host cells are bacterial metalloproteases (Justice *et al.* 2008). The *S. rhizophila* genome harbors a gene encoding a metalloprotease, which finds homologs in *S. maltophilia* K279a (Smlt1595), the beneficial plant-associated *Stenotrophomonas maltophilia* R551-3 and *P. aeruginosa*. Another important virulence feature of many human pathogenic bacteria is their resistance towards a broad range of antibiotics (Walsh 2003). The genome of *S. rhizophila* DSM14405^T is shielded with some antibiotic resistance genes. For instance, it harbors a gene coding for an aminoglycoside phosphotransferase, which is homologous to Smlt0191 from the clinical strain *S. maltophilia* K279a and Stemr_0151 from the beneficial endophytic strain *S. maltophilia* R551-3. *Stenotrophomonas maltophilia*, however, happens in general to more strongly share the feature of possessing antibiotic resistance genes with human pathogenic bacteria, as our genome comparison analyses revealed. There are a number of antibiotic resistance genes and gene clusters present in both *S. maltophilia* R551-3 and K279a strains, which don't exist in the genome of *S. rhizophila*. The gene clusters Smlt4474-76 and Smlt2796-8 from *S. maltophilia* K279a (Stemr_3899-3901 and Stemr_2294, Stemr_2297 from *S. maltophilia* R551-3) encoding RND-type tripartite efflux system and multidrug/fusaric acid resistance channel, respectively (Ryan *et al.* 2009) are examples therefor.

Diazotrophic plant growth promoting rhizobacteria (PGPR)

Diazotrophic bacteria with plant growth promotion and biocontrol activity related to the *Burkholderia cepacia* complex are regularly isolated from diverse rhizospheres, especially from the rhizosphere of rice (Jha *et al.* 2009). *Burkholderia vietnamensis* has been known since a long time as diazotrophic PGPR, but being a member of the *Burkholderia cepacia* complex and thus suspected for harboring pathogenicity features has resulted in the banning of *Burkholderia vietnamensis* from application. However, many plant associated strains were isolated, which belong to a separate branch of the genus *Burkholderi.*

Within the genus *Herbaspirillum,* which includes *H. seropedicae* as a typical endophytic plant growth promoting rhizobacterium, Baldani *et al.* (1996) described *H. rubrisubalbicans* as a mild phytopathogen and *Herbaspirillum* species 3 which harbored mostly isolates from clinical

specimen. In the meantime, clinical isolates characterized as *Herbaspirillum seropedicae* (e.g. strain 14010) are known (Helisson Faoro and Fabio Pedrosa, personal communication) for having opportunistic clinical characteristics. Recent preliminary DNA-sequencing data revealed that this strain lacks the *nif*- genes and type III secretion system but harbors type 3 fimbriae similar to pathogenic *Klebsiella pneumoniae*. Certainly, further studies on the proteomic level are needed to clarify this comparison to *H. seropedicae*.

Within the genus *Azospirillum,* no phylogenetically related clinical isolates were known until recently. For several decades various strains of *Azospirillum* (mostly *A. brasilense* and *A. lipoferum* strains) have been successfully used as biofertilizers in millions of ha worldwide to enhance growth of wheat, maize and other crops (Hartmann and Bashan, 2010) without any reported adverse impact on human health. In 2004, Cohen *et al.* published evidence on the isolation and characterization of *Azospirillum* spp. from wheat rhizosphere, *Rhizoctonia solani* mycelia, and human skin wounds and reported a very close relationship of these isolates to *Roseomonas*, a genus described in 1993 as being associated with bacteremia and other human infections (Rihs *et al.* 1993). In particular, a close resemblance to *Roseomonas fauriae* and *Roseomonas* genomospecies 6 was found. This even led Helsel *et al.* (2006) to the suggestion, that *Roseomona fauriae* Rihs *et al.* 1998 had to be reclassified as *Azospirillum brasilense* Tarrand *et al.* 1979. The evidence provided were a series of biochemical tests and molecular phylogenetic data, which apparently could not differentiate between the two taxa. A thorough reexamination of this comparison by Hartmann and coworkers (unpublished results) revealed, that indeed the whole sequences of 16S-rRNA- and 23S-rRNA-genes, the ITS1-region of the rRNA-operon, as well as the *nifH*- and the *rpoB*-genes of *Azospirillum brasilense, Roseomonas fauriae* and *Roseomonas* genomospecies 6 are indeed highly similar. Their sequence similarity ranged from 95% (ITS1 region) to 98-99% for the 16S and 23S rDNA. However, when the % DNA-DNA relatedness was examined, a value of 12% was found between *Roseomonas fauriae* KACC11694^T and *Azospirillum brasilense* Sp7^T and 25% between *Roseomonas* genomospeciea 6 CCUG 22010 and A. brasilense Sp7^T. This clearly demonstrates, that these bacteria are obviously separate possibly even on the genus level, although the ribosomal and other housekeeping genes are very similar. The examination of the fatty acid content and the physiological markers according to BIOLOG Microplate Systems^R revealed also clear differences (Kinzel *et al.*, unpublished); 9-12 (*R. fauriae*) and 5-6 (R. genomospecies 6) differences were found in the

biochemical utilization patterns of carbon substances in these strains as compared to different *Azospirillum brasilense* strains. Therefore, *Roseomonas fauriae* and *Azospirillum brasilense* have some relatedness, but this is much more distant as could be aligned from phylogenetically used molecular markers. Whole genome sequencing is in progress to reveal more details – especially concerning traits present in the opportunistic pathogens as compared to the plant-associated strains.

Conclusions

In conclusion, results obtained by genome analysis investigations support findings gained through physiological and molecular studies discussed in the beginning of this chapter in terms of mechanisms shared by both plant-associated bacteria and those known as human pathogens. Genes encoding molecular systems and proteins with a potential ambivalent role are very often highly homologous, sometimes even nearly identical among these two groups of bacteria. Nevertheless, in some cases specific physiological features from plant-associated bacteria give a hint over a possible threat these bacteria could pose in terms of causing diseases in humans. *Stenotrophomonas rhizophila*, for instance, does certainly share several virulence-associated genes and gene clusters with human/animal pathogenic bacteria as described above but it is incapable of growth at 37 ºC, which is a crucial prerequisite for successful survival and virulence in the human body. The beneficial plant-associated *S. maltophilia* R551-3 strain, on the contrary, can grow at 37 ºC but it lacks genetic potentials similar to those discussed from *S. rhizophila*. However, naturally occurring gene communication mechanisms among bacteria such as horizontal gene transfer and recombination events could always equip the bacterial genome arsenal with new genetic information leading to development of novel physiological traits. On the other hand, several studies have shown that plant and human-associated bacteria harbor similar "interaction factors". Their mere occurrence in the genome is not necessarily an evidence for pathogenicity; In addition, there is a better correlation at protein level as shown for 14 epidemic bacterial killers ("badbugs") (Georgiades and Raoult 2011). Therefore, proteomics and interaction studies seem to be more appropriate to assess the potential risk of bacterial strains than genomics.

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Figure 1

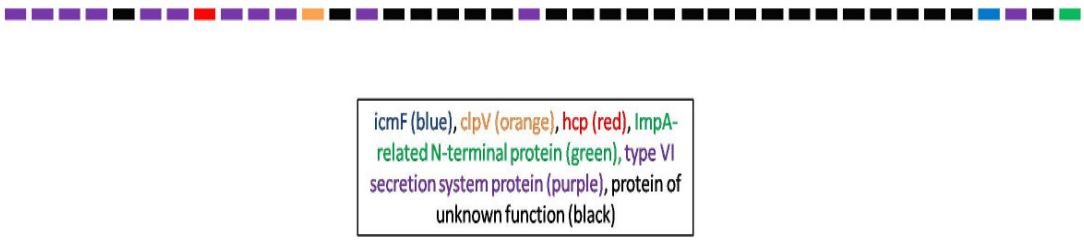

Fig. 1: Gene organization in the type VI secretion system cluster of *S. rhizophila* DSM14405T yielded through protein BLAST analysis investigations, the most crucial genes have been highlighted. Some genes with unknown functions in other bacteria could play as strain specific effector molecules or belong to the structural component set of type VI secretion system in *S. rhizophila* DSM14405T.

Associated Publication

genomeA
Genome Announcements

Complete Genome Sequence of the Sugar Beet Endophyte *Pseudomonas poae* RE*1-1-14, a Disease-Suppressive Bacterium

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The endophytic bacterium *Pseudomonas poae* RE*1-1-14 shows broad antagonistic activity and is applied to seeds as a biocontrol agent to suppress late root rot in the sugar beet. The completely sequenced 5.5-Mb genome reveals genes that putatively contribute to this antagonistic activity and the intimate plant-microbe interaction.

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Pseudomonas poae RE*1-1-14 is a member of the group of pseudomonads that interact beneficially with plants. Based on the 16S rRNA gene sequence, *P. poae* RE*1-1-14 is closely related to strains *Pseudomonas trivialis* DSM 14937^T and *P. poae* DSM 14936^T, distinguished by their ability to utilize sucrose (1). RE*1-1-14 was isolated from the endorhiza of the sugar beet and exhibits antagonistic activity against the phytopathogens *Phoma betae*, *Rhizoctonia solani* AG2-2IIIB, *R. solani* AG4, and *Sclerotium rolfsii* (2). Reintroduced to sugar beet seeds, *P. poae* RE*1-1-14 was demonstrated to densely colonize emerging roots, a primary requirement for the effective suppression of root pathogens (2). In field trials performed over six consecutive years, this isolate was proven to control late root rot caused by *R. solani* (unpublished data).

The genome of *P. poae* RE*1-1-14 was sequenced using a combination of next-generation sequencing platforms. A first-draft assembly based on 882,576 reads of an 8-kbp paired-end library (Roche 454 GS FLX Titanium) (Center for Medical Research [ZMF], Medical University of Graz, Austria) with a total of 171.1 Mb (31-fold coverage) was generated with Newbler 2.6 (Roche Diagnostics, Penzberg, Germany). This assembly consisted of 144 contigs, 66 of which could be joined into a single circular scaffold. Gaps resulting from repetitive sequences were resolved by *in silico* gap filling, and the remaining gaps were closed by PCR followed by Sanger sequencing, yielding a draft genome of 5,512,225 bp. To improve the quality of the sequence by eliminating 454 sequencing artifacts in homopolymer stretches, the genome was subsequently sequenced using the Illumina paired-end method (Illumina HiSeq 2000; Ambry Genetics, Aliso Viejo, CA) (6,973,734 reads, 697 Mb; 128-fold coverage). The Illumina reads were aligned to the draft genome with CLC Genomics workbench 4.7.2 (CLC bio, Aarhus, Denmark). The final consensus sequence was derived by counting the instances of each nucleotide at a particular position and then letting the majority decide the nucleotide for the consensus sequence.

Genes were identified with the Prodigal gene finder (3), ARAGORN (4), and RNAmmer 1.2 (5). Functional annotation of the predicted genes was performed using BASys (6), which provides annotations with respect to the Clusters of Orthologous Groups (COG) (7), Pfam (8), and Gene Ontology (GO) (9) databases. The final genome includes 5,512,241 bases, with a G+C content of 60.85%. The number of putative genes totals 4,854, of which 4,768 are protein coding. There are five instances of the ribosomal 5S-23S-16S cluster, an additional 5S rRNA gene, and 70 tRNAs. *P. poae* RE*1-1-14 additionally harbors a plasmid consisting of 6,375 bp and carrying 15 putative genes.

A cursory search of the genome sequence revealed the presence of gene clusters putatively involved in antagonistic activity, including genes for the synthesis and exudation of hydrolytic exoenzymes and cyclic lipopeptides. Various types of secretion systems and genes encoding the 1-aminocyclopropane-1-carboxylate deaminase indicate an ability to interact closely with plants. Despite its somewhat high similarities to the genomes of other sequenced plant-associated *Pseudomonas* strains, *P. poae* RE*1-1-14 possesses a genome with a unique assemblage of accessory genes.

Nucleotide sequence accession number. The genome sequence for *P. poae* RE*1-1-14 has been deposited at EMBL/GenBank under the accession no. CP004045.

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Statutory Declaration

I hereby declare that I wrote this thesis on my own with no help other than the literature and auxiliary means that have been explicitly mentioned.

Signature .....

27.05.2013