# Genomics and Transcriptomics of Plant Beneficial *Serratia* spp.

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Cover: Scanning electron micrographs of *S. plymuthica* AS9, AS12, AS13 and *S. proteamaculans* S4
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## Abstract

Plant growth stimulation and antagonism against phytopathogens by rhizobacteria are widely recognised phenomena, but the variation in the underlying causal mechanisms between and within different bacterial taxa is still poorly understood. Some bacteria of the genus *Serratia* are known to be associated with plant roots, and have potential as possible biocontrol agents in agriculture. This thesis provides the detailed genomic structure of three *Serratia plymuthica* and one *S. proteamaculans* isolates with their ability to promote oilseed rape plant growth and to inhibit its fungal pathogen, *Rhizoctonia solani* AG 2-1. Differences in the composition of these genomes were reflected in different phenotypes associated with antagonism and plant growth stimulation observed in different experiments. Their genomes contain the basic genetic traits required for survival in the rhizosphere, root colonisation, antibiosis, induced systemic resistance and production of phytohormones and other factors involved in plant growth promotion. Genetically *S. proteamaculans* is highly diverged from *S. plymuthica* isolates and was a better plant growth promoter. The three *S. plymuthica* genomes are almost identical, however significant variation was observed in plant growth promotion and antagonism by these three isolates. A possible explanation could be due to mutation in one or more putative genes.

Genome-wide expression profiling of *S. plymuthica* and *S. proteamaculans* revealed that they responded to *R. solani* by activating genes associated with antibiosis, competition and defence mechanisms. Antibiosis seems to be a major mode of action in *S. plymuthica* AS13 since genes for pyrrolnitrin biosynthesis and transporters were highly up-regulated while in *S. proteamaculans* S4, combination of competition, defense mechanism and antibiosis play a role in antagonism. The results suggest that there is much variation in bacterial antagonism at the species level.



*Keywords: Serratia*, genome, transcriptome, plant growth promotion, biocontrol,


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*To my Grandmother*

# Contents

# List of Publications

This thesis is based on the work described in the following papers, referred to by Roman numerals in the text:

I Neupane, S., Högberg, N., Alström, S., Lucas, S., Han, J., Lapidus, A., Cheng, J.F., Bruce, D., Goodwin, L., Pitluck, S., *et al.,* (2012a). Complete genome sequence of the rapeseed plant-growth promoting *Serratia plymuthica* strain AS9. *Stand Genomic Sci* 6: 54-62.

II Neupane, S., Finlay, R.D., Alström, S., Goodwin, L., Kyrpides, N.C., Lucas, S., Lapidus, A., Bruce, D., Pitluck, S., Peters, L., *et al.,* (2012b). Complete genome sequence of *Serratia plymuthica* strain AS12. *Stand Genomic Sci* 6: 165-173.

III Neupane, S., Finlay, R.D., Kyrpides, N.C., Goodwin, L., Alström, S., *et al.*, (2012c). Complete genome sequence of the plant-associated *Serratia plymuthica* strain AS13. *Stand Genomic Sci* 7: 22-30.

IV Neupane, S., Finlay, R.D., Kyrpides, N.C., Goodwin, L., Alström, S., *et al.*, (2013). Non-contiguous complete genome sequence of the plant-associated *Serratia proteamaculans* S4. (submitted)

V Neupane, S., Guy, L., Högberg, N., Alström, S., Andersson, S. and Finlay, R.D. (2013). Comparative genomics of plant associated *Serratia* species. (manuscript).

VI Neupane, S., Finlay, R.D., Alström, S., Elfstrand, M. and Högberg, N., (2013). Genome-wide transcriptome profiling of *Serratia* spp. reveals species-specific response to *Rhizoctonia solani*. (manuscript)

**Additional Publication**

Neupane, S., Andersson, B., Högberg, N., Ihrmark, K., and Alström, S., (2013). Fungal communities associated with field grown oilseed rape (*Brassica napus* L.) – their possible role in early crop establishment. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science* 63: 241-252.

The contribution of Saraswoti Neupane (SN) to the papers included in this thesis was as follows:

I **Papers I – IV** SN extracted and prepared the DNA, conducted the phenotypic characterization and planned and wrote the articles, with suggestions and comments from the supervisors and sequencing and annotation carried out by co-authors from the Joint Genome Institute (JGI).

II **Paper V -** SN analysed the genome data and planned and wrote the paper, with comments and suggestions from the co-authors.

III **Paper VI** - SN planned and conducted the interaction experiments, extracted the RNA, analysed the transcriptome data and wrote the paper, with comments and suggestions from the co-authors.

# 1 Background

Modern agriculture faces major challenges with respect to food security. The excessive use of chemical fertilisers and pesticides in intensive agriculture reduces sustainability. These practices also adversely affect the environment and human health. Soil microorganisms are key determinants of soil fertility and plant health, but the natural roles of these organisms have been marginalized in intensive agriculture. The use of plant beneficial microorganisms to achieve sustainable increases in crop yields is one possible alternative to chemical fertilisers and pesticides.

Many scientific challenges exist in the successful use and application of these microorganisms. Recent development of genomic and high-throughout sequencing approaches allows the exploration of the genomic signatures of organisms of interest, the evolution of genetic traits and their effect in various areas such as physiology, pathogenesis and adaptation. In addition to these, their mode of action in key functional mechanisms in plant growth promotion and antagonism against pathogens is of importance. Increasing numbers of genome sequences of different microorganisms (both plant pathogens and their antagonists) and economically important plants are now available, and provide the foundation with which to exploit newly discovered genetic traits underlying complex interactions between plants and microbes.

Rhizobacteria-mediated control of plant pathogens and plant growth promotion are widely accepted approaches, but the variation in the underlying mechanisms, between and within the different bacterial species, is still poorly understood. This is mainly due to high genetic diversity within and between the bacterial species, which has been shown in various *Pseudomonas* spp. (Loper *et al.*, 2012). This thesis provides an overview of four *Serratia* isolates, their direct and indirect beneficial effects on oilseed rape growth, their genomes and the genetic traits underlying plant growth promotion and antagonism against the oilseed rape pathogen *Rhizoctonia solani*.

# 2 Introduction

## 2.1 Plant growth and health

Plants are the primary producers of most terrestrial ecosystems and fix atmospheric carbon for structural materials and metabolic products that are used by other organisms including humans. Plant roots release up to 30 % of their photosynthates into the soil (Morgan *et al.*, 2005; Lynch & Whipps, 1990), resulting in elevated numbers of microorganisms, the phenomenon first described as the "rhizosphere" by Hiltner (1904).

Plant roots acquire essential nutrients and water, required for growth. Bioavailability of various macro- and micro- nutrients is limited in soil and plants face various environmental stresses. To cope with these problems, plants have evolved different strategies such as mutualistic association with microorganisms, to which the plant provides carbon, in return for nutrients and protection from stresses such as drought, salinity, temperature shock, oxygen deficiency and pathogens. The most ancient of these symbioses is arbuscular mycorrhizal symbiosis (Parniske, 2008). Bacterial symbiosis is common in legume plants in the form of nitrogen-fixing rhizobia (Kiers *et al.*, 2003). Other associations between bacteria and plants may be ancient as well, but there is a lack of fossil evidence.

Under both natural and agricultural conditions, plants are exposed to a wide array of potential pathogens and pests. These include fungi, oomycetes, bacteria, viruses, nematodes, and insects that cause disease or physical damage to the plant. They affect plant health by consuming, degrading, or killing tissues and suppressing the host defense system. Chemical pesticides to control diseases are commonly used in agriculture but integrated pest management necessitates use of other non-chemical alternatives such as crop rotation, resistant cultivars or other cultural practices, as well as biological control.

## 2.2 Plant growth-promoting bacteria

Plant growth-promoting bacteria represent a wide variety of bacteria, which can establish a mutualistic relationship with host plants and exert various beneficial effects on the plant. Rhizosphere inhabiting, plant growth-promoting bacteria utilise diverse mechanisms during plant-microbe interaction (Berg, 2009; Compant *et al.*, 2005; Whipps, 2001). These bacteria may facilitate plant growth directly by providing plant growth hormones and facilitating nutrient acquisition (Richardson *et al.*, 2009; Spaepen *et al.*, 2007; Salamone *et al.*, 2001; Glick, 1995) or indirectly, either by inhibiting the growth of well known disease causing plant pathogens, or by reducing deleterious effects of minor pathogens (Whipps, 2001).

The common feature of all plant growth-promoting rhizobacteria is root colonisation (Lugtenberg *et al.*, 2002). Many bacteria can exhibit a non-specific adhesion to the root by adhering with adhesive factors such as pili or fimbriae and the others exhibit specific adhesion and establish themselves endophytically. However, specific adhesion is not an absolute requirement for colonisation. Once they colonise the host root, the different interactions result in plant growth stimulation, either directly or indirectly.

### 2.2.1 Direct plant growth promotion

Plant-associated bacteria utilise many mechanisms that directly influence plant growth. Different bacteria enhance plant growth directly by supplying nutrients through fixing nitrogen from the atmosphere or solubilising and mineralising phosphorus from inorganically and organically bound phosphates (Berg, 2009; Richardson *et al.*, 2009; Kiers *et al.*, 2003). In addition to nitrogen and phosphorus, many soil bacteria are able to provide iron and vitamins to plants (Richardson *et al.*, 2009). Several plant growth-enhancing bacteria are able to synthesize and transport different plant growth hormones such as auxins, gibberellins, cytokinins and ethylene, and directly regulate physiological processes of the plant (Berg, 2009; Loper & Schroth, 1986). The positive effect of indole-3-acetic acid (IAA), an auxin, produced by bacteria such as *Azospirillum* and *Pseudomonas*, is directly involved in enhancing plant growth (Spaepen *et al.*, 2007; Loper & Schroth, 1986). Besides production of plant growth hormones, these bacteria also influence the hormonal balance within the plant. Some bacteria produce 1-amincyclopropane-1-carboxlate deaminase that degrades ACC, a precursor of ethylene, reducing the negative effect of ethylene in response to stresses such as drought and salinity (Glick *et al.*, 2007). In addition to plant hormones, certain rhizobacteria such as *Bacillus subtilis*, *B. amyloliquefaciens* and *Pseudomonas fluorescens* are able to produce volatiles such as acetoin and 2,3-butanediol, that are important in

modulating the endogenous signals to trigger the plant growth (Ryu *et al.*, 2004; Ryu *et al.*, 2003).

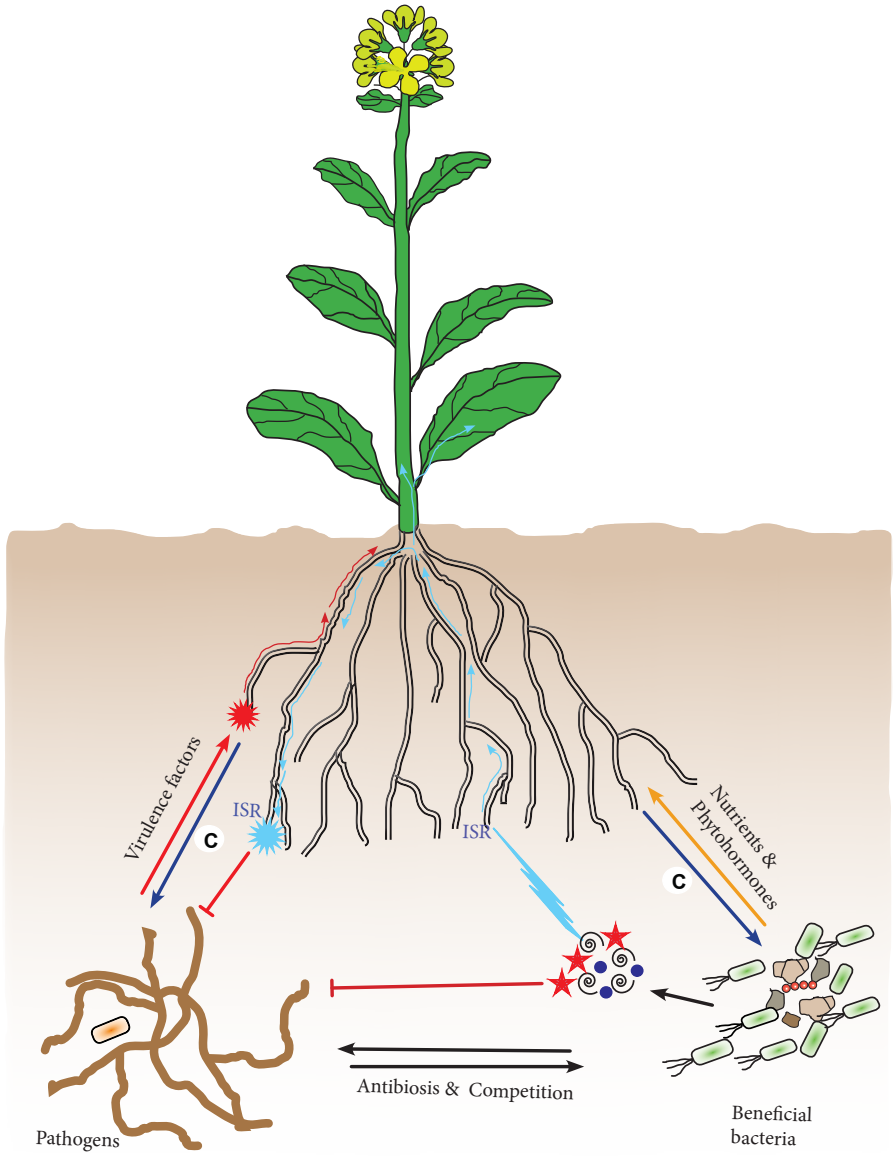


*Figure 1.* Schematic representation of the interactions between plants, plant beneficial bacteria and plant pathogens.

### 2.2.2 Indirect plant growth promotion

Indirect plant growth promotion can occur through antagonism against plant pathogens, competition and induction of systemic resistance, and is termed biocontrol-mediated plant growth. Antagonism may involve suppression of plant pathogens through parasitism mostly used by fungi such as *Trichoderma* species (Harman *et al.*, 2004), and antibiosis. Several plant growth-promoting bacteria including *S. plymuthica, S. marcescens* and *Aeromonas* sp., release exoenzymes such as chitinases and glucanases, that may play a role in degrading the cell wall of the fungal pathogens (Inbar & Chet, 1991; Ordentlich *et al.*, 1988; Tanaka & Phaff, 1965). Besides exo-enzymes, many bacteria produce siderophores that can bind with ferric ($Fe^{+3}$) ions in the soil and subsequently deplete this micronutrient from the environment, thus suppressing pathogen growth. Antagonistic ability of bacteria may rely on production of antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, phenazine, hydrogen cyanide, and proteases, that also play a determining role for competition for space and nutrients (Loper *et al.*, 2012; Raaijmakers *et al.*, 2002; Whipps, 2001).

#### *2.2.2.1 Antagonism*

Diverse groups of bacterial species have the potential to control pathogens either directly or indirectly. The bacterial control of pathogen growth occurs directly by production of antimicrobial compounds against other microorganisms competing for nutrients and space. The processes are known as antibiosis and competition.

Bacterial antibiotics play an important role in suppression of plant pathogens and may indirectly promote plant growth. A wide range of antibiotics produced by different rhizospheric, plant growth-enhancing bacteria has been identified and characterized. Members of the genera *Pseudomonas* and *Serratia* are known for their ability to produce antibiotics - phenazine, pyrrolnitrin and 2,4-diacetylphloroglucinol are important examples (Müller *et al.*, 2009; Raaijmakers *et al.*, 2002; Défago, 1993) and utilise these to inhibit different plant pathogens.

Antibiotic production is influenced by the overall metabolic status of cells that in turn is influenced by nutrient availability and other environmental stimuli. The production of different antibiotics by rhizospheric bacteria is regulated by one or two-component system involving membrane proteins and a cytoplasmic factor (Haas & Keel, 2003). Genes for regulatory cascades such as GacS/GacA, GrrA/GrrS, PhzI/PhzR, RopD, RopS and auto-regulators in different bacterial strains such as *Pseudomonas* and *Serratia*, are involved in biosynthesis and transportation of diverse antibiotics (Selin *et al.*, 2012;

Fineran *et al.*, 2005; Ovadis *et al.*, 2004; Haas & Keel, 2003). In addition to the regulatory system for antibiotic production, several genes and gene clusters responsible for antibiotic biosynthesis and secretion have recently been described (Fineran *et al.*, 2005; Thomson *et al.*, 2000; Kraus & Loper, 1995).

*2.2.2.2 Competition*

Bacterial survival and proliferation in the rhizosphere are the primary traits, required to exert various functions. The rhizosphere is a reservoir of energy-rich carbon sources excreted by the plant, and microorganisms, including pathogens, residing in the soil are attracted towards this environment. Rhizosphere competence therefore implies that plant growth-promoting bacteria are well adapted to utilise these carbon resources (Lugtenberg & Kamilova, 2009).

Iron is one of the essential elements required by living organisms, but in nature its bioavailability is often low. To circumvent this problem most microorganisms have diverse mechanisms for iron acquisition. They produce siderophores, low molecular weight secondary metabolites, which form a soluble complex with insoluble iron (Loper & Henkels, 1999) in soil and subsequently take up the iron complex using efficient transport systems. Besides their own siderophores, several plant growth-enhancing bacteria have the ability to utilise siderophores produced by other species of bacteria and fungi (Loper & Henkels, 1999; Dean *et al.*, 1996) placing their competitors at a competitive disadvantage and depriving them of iron.

*2.2.2.3 Induced systemic resistance*

All plants have evolved constitutive and inducible defense mechanism to protect themselves from pathogen infection. Inducible defense mechanism is activated by various biotic and antibiotic agents (Kloepper *et al.*, 1992) and root-colonising non-pathogenic rhizobacteria are known for their ability to produce several different signalling molecules such as jasmonic acid, ethylene, sideorphores, cyclic lipoproteins, acetoin and 2,3-butanediol, that trigger induced systemic resistance (ISR) of the host plant (Ongena *et al.*, 2007; Pieterse *et al.*, 1998; van Loon *et al.*, 1998). Once induced the ISR results in altered physiology and metabolism, strengthening the cell wall and plant defence systems (Figure 1). An example of this is the plant growth promoting *Bacillus* species producing 2,3-butanediol that triggers ISR in *Arabidopsis* and eventually leads to a significantly lower level of disease incidence by pathogenic *Erwinia carotova* in *Arabidopsis* (Ryu *et al.*, 2004).

## 2.3 Genomes

The recent progress in next generation sequencing technologies such as Illumina (Bennett, 2004), 454 pyrosequencing (Margulies *et al.*, 2005), Ion Torrent (Rusk, 2011) and PacBio (Eid *et al.*, 2009), provide us with increasing opportunities to explore genomes of various organisms. Numerous whole genome sequences of all three domains of life are already available and more and more are becoming available. This information has provided significant advances in understanding the physiology, evolution and ecology those organisms. Genome sequences of many plant growth promoting and biocontrol bacteria, including many bacterial isolates from the genera *Pseudomonas*, *Enterobacter*, and *Bacillus* are available (Loper *et al.*, 2012; Taghavi *et al.*, 2010; Silby *et al.*, 2009; Taghavi *et al.*, 2009; Chen *et al.*, 2007; Loper & Gross, 2007). These data can contribute to advancing the knowledge on plant growth promotion and the effects of bacteria on plant health and productivity.

## 2.4 Oilseed crop

Oilseed rape (*Brassica napus L.*) is one of a main oilseed crops and accounts for approximately 12% of global oilseed production (FAO, 2010). Due to its use in biofuel production and human consumption, its global demand is increasing rapidly. Intensification of its cropping is associated with is increased loss due to pests and diseases. Some pathogens that have been reported to cause yield losses in oilseed rape are fungi such as, *Rhizoctonia solani*, *Verticillium dahliae*, *Leptosphaeria maculans, Sclerotinia sclerotiorum* and *Alternaria brassicae*. Poor emergence and early seedling establishment have sometimes been observed in oilseed rape fields and have often been attributed to abiotic factors (Blake *et al.*, 2004). However, the fungal pathogen *R. solani* that causes damping off, has been suggested to be a possible cause of poor emergence and early establishment of oilseed crops (Neupane *et al.*, 2013).

## 2.5 *Rhizoctonia solani* – the pathogen

*Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) represents a large species complex of saprophytic, parasitic (symbiotic) and pathogenic strains of soil-borne basidiomycetes (Ogoshi, 1996; Sneh *et al.*, 1996; Ogoshi, 1987). Currently, this species complex includes 14 anastomosis groups (AG, AG1 – AG13 and AGBI), which are categorized on the basis of hyphal anastomosis reactions on culture media (Guillemaut *et al.*, 2003; Gonzalez *et al.*, 2001; Ogoshi, 1996). However, some groups have been further divided into subgroups based on their pathogenicity, host range, biochemical

characteristics and molecular characteristics (Cubeta & Vilgalys, 1997; Ogoshi, 1987). The saprophytic and parasitic nature of *R. solani* allows it to survive as mycelium by colonising plants, as well as soil organic matter (Agrios, 2005). Moreover, under harsh conditions, the fungus can survive for several years as dormant vegetative structures called, sclerotia (Sneh *et al.*, 1996).

*Rhizoctonia solani* is a soil-borne fungal pathogen of a wide range of economically important plants that causes economic losses worldwide (Sneh *et al.*, 1996). Some anastomosis groups of *R. solani* can attack a wide range of plant species while others show host specificity (Ogoshi, 1987). AG 4 can cause root rot disease in crucifers including oilseed rape and in soybean this group cause both damping off and root rot. Another group, AG 2-1 (Figure 2) is responsible for damping off disease in oilseed rape. Isolates from this group cause both pre- and post-emergence damping off independent of cultivar type and can possibly involved in patchiness in the field (Neupane *et al.*, 2013).

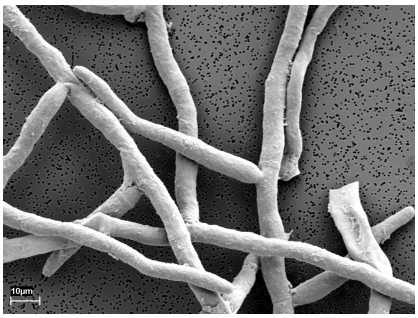


*Figure 2.* Scanning electron micrograph of *R.solani*.

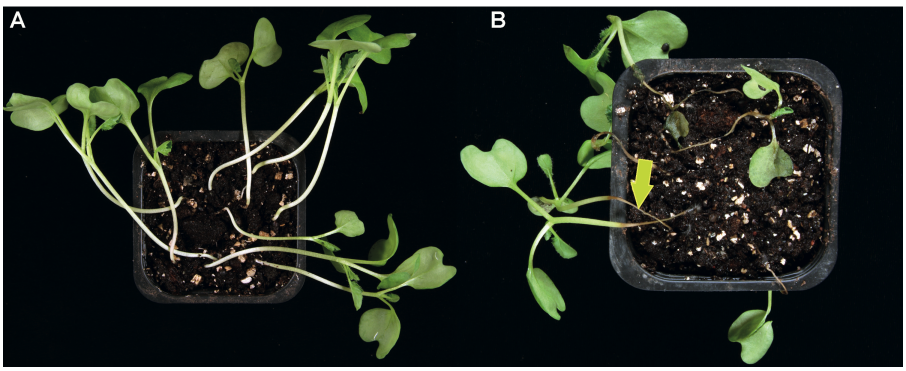


*Figure 3.* Damping off symptoms (yellow arrow) in oilseed rape seedlings caused by *R. solani*.

In the soil, *R. solani* can survive as mycelium or sclerotia and is able to infect new seeds and seedlings by colonising them. However, *R. solani*

infection by basidiospores is rare. The fungus grows very rapidly so the infection and symptoms appear within a few days e.g. pre-emergence damping off in oilseed rape (Figure 3). Once the fungus kills the plant, it can survive in the dead plant debris and soil for several months to years.

## 2.6 The genus *Serratia*

The history of existence of *Serratia* can be traced back to 332 B.C. (Quintus Curtius Rufus) and 60 B.C. (Diodorus of Sicily), who reported that during the siege of Tyre, Macedonian soldiers noticed drops of blood dropping out of broken pieces of bread (Gaughran, 1969). The ability of this group of bacteria to grow in bread and to produce a red pigment has been evoked by a medieval Eucharist to institute the Feast of Corpus Christi. However, the bacterium attracted scientific attention in 1819, when an Italian pharmacist Bartolomeo Bizio found drops of red coloured substance oozing out from damp statues and communion wafers. He found that a bacterium was responsible for it and named it *Serratia* (Bizio, 1823) in honour of the physicist Serafino Serrati.

The genus *Serratia* belongs to the bacterial family Entererobacteriaceae, and the class Gammaproteobacteria. The members of the genus are widely distributed in nature and commonly found in soil, water and in plants, insects and humans (Alström & Gerhardson, 1987; Grimont & Grimont, 1978) and are efficient colonisers of various biotic and abiotic surfaces (Grimont & Grimont, 2006). The genus includes several closely related species that have similar phenotypic and genotypic characteristics. Currently, 16 validly named *Serratia* species have been described such as *S. entomophila, S. ficaria, S. fonticola, S. grimesii, S. liquefaciens, S. marcescens, S. nematodiphila*, *S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea*, *S. symbiotica* and *S. ureilytica* (Garrity *et al.*, 2005).

Some species of the genus *Serratia* produce a red-pink coloured non-diffusible pigment, prodigiosin, (Grimont & Grimont, 2006). The colour of the pigment depends on the composition of the growth substrate, temperature and pH of the culture medium (Alström & Gerhardson, 1987). Several species use flagella for their movement and can live as facultative anaerobes (Grimont & Grimont, 2006).

The genus also includes biologically and ecologically diverse species – from those that are beneficial to economically important plants, to pathogenic species that are harmful to humans. The plant-associated species comprise both endophytes and free-living species such as *S. proteamaculans* and *S. plymuthica*. Strains of these species include antagonists to soil borne pathogens of different plant species (Alström, 2001; Berg, 2000; Kalbe *et al.*, 1996), plant

growth enhancers (Taghavi *et al.*, 2009) and insect pathogens. However, some other species such as *S. marcescens* have been implicated in nosocomial varieties of infection in humans (Grimont & Grimont, 2006). The genus includes the insect endosymbiont, *S. symbiotica* colonising the aphid gut, the biology of which is important for understanding of how bacteria evolved from a facultative to an obligate life style (Burke & Moran, 2011; Lamelas *et al.*, 2011).

Currently, several fully sequenced genomes are available for the genus *Serratia* from different environments such as free-living, facultative endosymbionts and endophytic member of the genus. Examples of such *Serratia* isolates include *S. proteamaculans*, *S. marcescens* and *S. symbiotica* isolated from poplar trees, humans and aphids respectively (Wang *et al.*, 2012; Burke & Moran, 2011; Lamelas *et al.*, 2011; Taghavi *et al.*, 2009). However, so far, there have been few detailed analyses of plant root-associated *Serratia* speices in relation to genetic traits that may be beneficial for plant growth. This thesis presents the genomes of plant growth promoting, and rhizospheric *Serratia* isolates and their genetic traits underlying biocontrol and plant growth promoting activity.

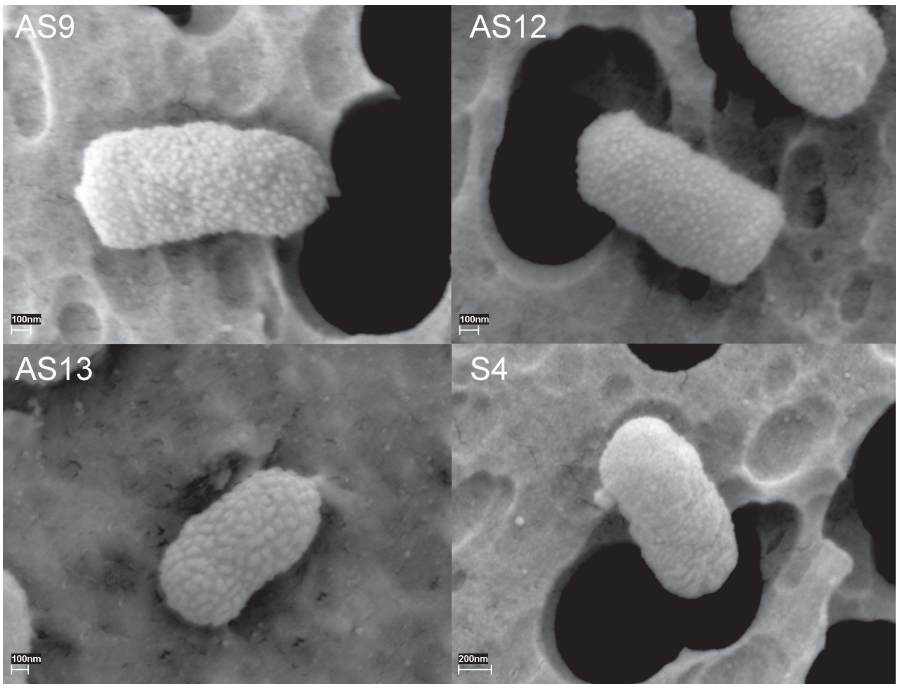


*Figure 4.* Scanning electron micrographs of *S. plymuthica* AS9, AS12, AS13 and *S. proteamaculans* S4

# 3 Objectives

Plant growth promotion by rhizobacteria is a widely accepted phenomenon but the variation in underlying causal mechanisms between and within different bacterial taxa is still poorly understood. This is due to the genetic diversity, within and between the species, horizontal gene transfer among taxa and variation in the traits that are involved in different interactions. The aim of the studies described in this thesis was to elucidate the genetic traits of four *Serratia* isolates and their involvement in biocontrol and plant growth promotion. The detailed objectives were:

- To characterise the genomes of four plant-beneficial *Serratia* isolates (Paper I – IV).
- To investigate the importance of genetic variability of the four *Serratia* isolates in relation to plant growth promotion (Paper V).
- To elucidate the intra-specific variation underlying the plant growth-promoting effects of *Serratia* spp. (Paper V).
- To explore the differentially expressed genes of two *Serratia* isolates during interaction with *R. solani* and to elucidate the genetic traits underlying the antagonism (Paper VI).

# 4 Materials and Methods

## 4.1 Bacterial isolates and growth conditions

Three of the isolates, *Serratia* spp. AS9, AS12 and AS13, were selected on the basis of their ability to inhibit fungal pathogens of oilseed rape, in particular *Verticillium longisporum* (earlier *V. dahliae*) (Alström, 2001). The fourth isolate, *Serratia* sp. S4 showed a similar inhibition of fungal growth (Alström and Andersson, unpublished). The bacterial isolates and their origin are summarised in Table 1.

Table 1. General information of bacterial isolates

| **Bacterial Isolate** | **Origin** | | **Source** | **Paper** |
|---|---|---|---|---|
| | **Year** | **Location** | | |
| *Serratia* sp. AS9 | 1998 | Uppsala, Sweden | Oilseed rape rhizosphere | I and V |
| *Serratia* sp. AS12 | 1998 | Uppsala, Sweden | Oilseed rape rhizosphere | II and V |
| *Serratia* sp. AS13 | 1998 | Uppsala, Sweden | Oilseed rape rhizosphere | III, V and VI |
| *Serratia* sp. S4 | 1980 | Uppsala, Sweden | *Equisetum* rhizosphere | IV, V and VI |

The frozen bacterial cells were routinely cultured, either on diluted tryptic soy agar (TSA, 20 g/l TSA supplemented with 7.5 g/l agar, Oxoid, UK), Luria-Bertani broth (LB), or in Kings B broth (KB), depending on the experiment. The optimum growth temperature for all four *Serratia* isolates was assayed and found to be 28 °C. However, the bacterial culture conditions varied.

### 4.1.1 Phenotypic characterization

Basic phenotypic characteristics such as Gram staining, pH tolerance, effect of temperature, osmotic pressure and nutrient utilisation abilities have been described. Cell morphology and motility were observed using an Epifluorescence microscope (Leica, Germany) at 1000x and the scanning

electron micrographs were taken at The Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

In addition to the above-mentioned phenotypic characters of these bacterial isolates, their role in plant growth promotion and antagonism against plant pathogens (paper V and VI) has been studied *in vitro* and in greenhouse experiments, as described in papers V and VI. In addition to these general characters, the phenotypic traits that may be directly and indirectly involved in plant growth promotion, such as inhibition of pathogen growth by the production of antifungal compounds such as exoenzymes, plant hormones and siderophores, were evaluated using *in vitro* assays. These assays include chitinolytic activity, proteolytic activity, haemolytic activity, cellulolytic activity, indole-3-acetic acid (IAA) production and siderophore production.

## 4.2 Fungal isolate, growth conditions and identification

The fungus was isolated from diseased oilseed rape seedlings and routinely grown in diluted potato dextrose agar (PDA, 19.5 g/l PDA supplemented with 7.5 g/l agar, Oxoid, UK) at 20 °C. Its identification was carried out by sequencing the intergenic region of ribosomal DNA using ITS1F and ITS4 primers (White *et al.*, 1990) followed by comparing the sequence with the most recent database from GenBank using NCBI-BLAST (Altschul *et al.*, 1997) under default settings.

The fungal pathogen, *R. solani*, was selected because of its effects on pre- and post- emergence of oilseed rape under greenhouse conditions (Neupane *et al.*, 2013).

## 4.3 Plant material

Poor and failed establishment frequently observed in oilseed rape (*Brassica napus*) in the field was the motive for the selection of this plant. In addition to oilseed rape, a model plant, belonging to the same family (Brassicaceae), *Arabidopsis thaliana* ecotype *Col-0* was also used for certain experiments.

The effects of these bacteria on plant growth promotion and pathogen inhibition were evaluated in the greenhouse experiments (paper V).

## 4.4 Genome sequencing

Genome sequences provide a broad base for understanding species, their genetic constitution and ability to adapt in different environments. Recent progress in next generation sequence technologies such as Illumina (Bennett,

2004), 454 pyrosequencing (Margulies *et al.*, 2005), Ion Torrent (Rusk, 2011) and PacBio (Eid *et al.*, 2009), provides us with new opportunities to explore the genetic constituents of organisms and to understand the genetic traits that are involved in various physiological and metabolic functions.

The genomes of four *Serratia* isolates were sequenced in collaboration with the Department of Energy - Joint Genome Institute (DOE-JGI), USA (papers I – IV). The protocol used to extract DNA from bacteria growing at optimal temperature and early stationary phase is available at DOE-JGI (http://www.jgi.doe.gov/). Sequencing of the genomes was carried out by using a combination of Illumina GAii and 454 pyrosequencing techniques and the detailed methods for genome sequencing, assembly and annotation are described in papers I – IV and available at http://www.jgi.doe.gov/.

### 4.4.1 Bacterial identification

In papers I – III, *Serratia* sp. AS9, AS12 and AS13 have been described as *S. plymuthica* isolates AS9, AS12 and AS13 and in paper IV *Serratia* sp. S4 has been identified as *S. proteamaculans* S4. The identification was carried out by comparing 16S rRNA gene sequences with the most recent GenBank databases using the NCBI-BLAST (Altschul *et al.*, 1997) tool and the comparison of whole genome sequences by using digital DNA-DNA hybridization (Auch *et al.*, 2010b) with the reference genome sequences using the Genome-to-Genome Distance Calculator (GGDC) web server (Auch *et al.*, 2010a).

## 4.5 Genome comparison

In the natural environment, bacterial genomes undergo various evolutionary processes that result in a broad genetic diversity within similar groups. Genome rearrangement due to recombination and introduction of alien genes due to horizontal gene transfers are important factors contributing to bacterial evolution (Ochman *et al.*, 2000). Comparative genomics is a fascinating approach with which to investigate the variability in genetic traits underlying particular phenotypes.

In paper V, the genomes of three *S. plymuthica* isolates AS9, AS12 & AS13 and *S. proteamaculans* S4 were chosen on the basis of the variation in their efficacy to enhance oilseed rape growth and inhibit a fungal pathogen of oilseed rape, *R. solani*. To understand the key genetic traits involved in plant growth promotion, their genomes, genes and gene clusters were analysed using various software suites such as MAUVE (Darling *et al.*, 2004), MUMmer (Kurtz *et al.*, 2004), Artemis and Artemis Comparison Tool (ACT) (Carver *et al.*, 2005). Various functions were inferred using the Integrated Microbial

Genomes (IMG) System (Markowitz *et al.*, 2006), MicrobeOnLine web server (Alm *et al.*, 2005), KEGG databases (Kanehisa *et al.*, 2004) and antiSMASH program (Medema *et al.*, 2011).

## 4.6 Gene expression profile

Strong phenotypic variability related to antagonism was prevalent in the studied *Serratia* isolates. The genomes of these bacteria contain several putative genetic traits that are involved in biocontrol mechanisms (paper V). However, functional studies are required to understand their mode of action during antagonism. Recent development of RNA sequencing techniques provides an opportunity to explore and examine many simultaneously expressed genes (Wang *et al.*, 2009). In paper VI, RNA sequencing technique was used to study the bacterial transcriptional response to the fungal pathogen *R. solani*. *Serratia plymuthica* AS13 and *S. proteamaculans* S4 were selected due to their strong inhibitory effect on *R. solani* growth. The detailed methods are described in paper VI.

### 4.6.1 Bacterial RNA extraction and cDNA synthesis

Two biological replicates of bacterial samples from microcosms containing the bacteria alone (control) and microcosms containing bacteria confronted with *R. solani* (treatment) growing for 48 hours, were subjected to RNA extraction. Removal of rRNA from the total RNA was carried out using a capture hybridization method. This method removes a large portion of the 16S and 23S rRNA and the remaining mRNA-enriched samples were amplified with T7 based linear amplification of RNA (van Gelder *et al.*, 1990). The amplified RNA was subjected to cDNA preparation.

### 4.6.2 cDNA sequencing and data analysis

Four µg/sample cDNA was submitted to the Uppsala University Sequencing Centre http://molmed.medsci.uu.se/SNP+SEQ+Technology+Platform. Two cDNA libraries (650 bp and 350 bp) for each biological replicate were prepared and cDNA was sequenced using Illumina GAii (Bennett, 2004). The details of library preparation and sequencing are available at the sequencing centre.

The paired-end sequence reads were mapped on to the respective reference genomes by using the EDGEpro software (Magoc *et al.*, 2013). The resulting count datasets for each expressed gene of individual samples were exported to the DESeq package in the program R (Anders & Huber, 2010), where the count datasets were normalized and pair-wise differential expression of

transcript levels were determined. The details of sequence analysis and statistics for the expression profile are described in paper VI.

### 4.6.3 Quantitative real time PCR (qRT-PCR)

A set of differentially expressed genes was selected based on their putative function involved in bacterial antagonism against *R. solani* for further verification. cDNA prepared from the total bacterial RNA was used as a template for the qRT-PCR reactions. The relative expression of differentially expressed genes was analysed using REST (Pfaffl, 2001).

# 5 Results and Discussion

## 5.1 General characteristics of *Serratia spp.* (Paper I - IV)

The identity of three isolates AS9, AS12 & AS13, was confirmed as *S. plymuthica* while S4 was identified as *S. proteamaculans*. The phylogenetic tree (Figure 5) shows their relationship with the other members of the family Enterobacteriaceae, where two sister groups *S. plymuthica* and *S. proteamaculans* clustered separately. These Gram-negative, rod shaped (Figure 4), motile bacteria have an optimal growth temperature of 28 °C and can tolerate a wide range of pH and osmotic pressure. These characteristics are probably required to survive in different environments. All three *S. plymuthica* isolates formed red to pink colonies due to production of the red pigment, prodigiosin (Grimont & Grimont, 1978) while *S. proteamaculans* S4 formed pale yellowish colonies. However, the intensity of pigment formation depends on composition of the medium, pH and growth temperature (Alström & Gerhardson, 1987). In common with other rhizospheric and plant beneficial bacteria such as *Pseudomonas* (Loper et al., 2012), all four *Serratia* isolates were able to utilise a wide range of carbon and nitrogen sources, and were also able to resist several antibiotics (Neupane *et al.,* unpublished).

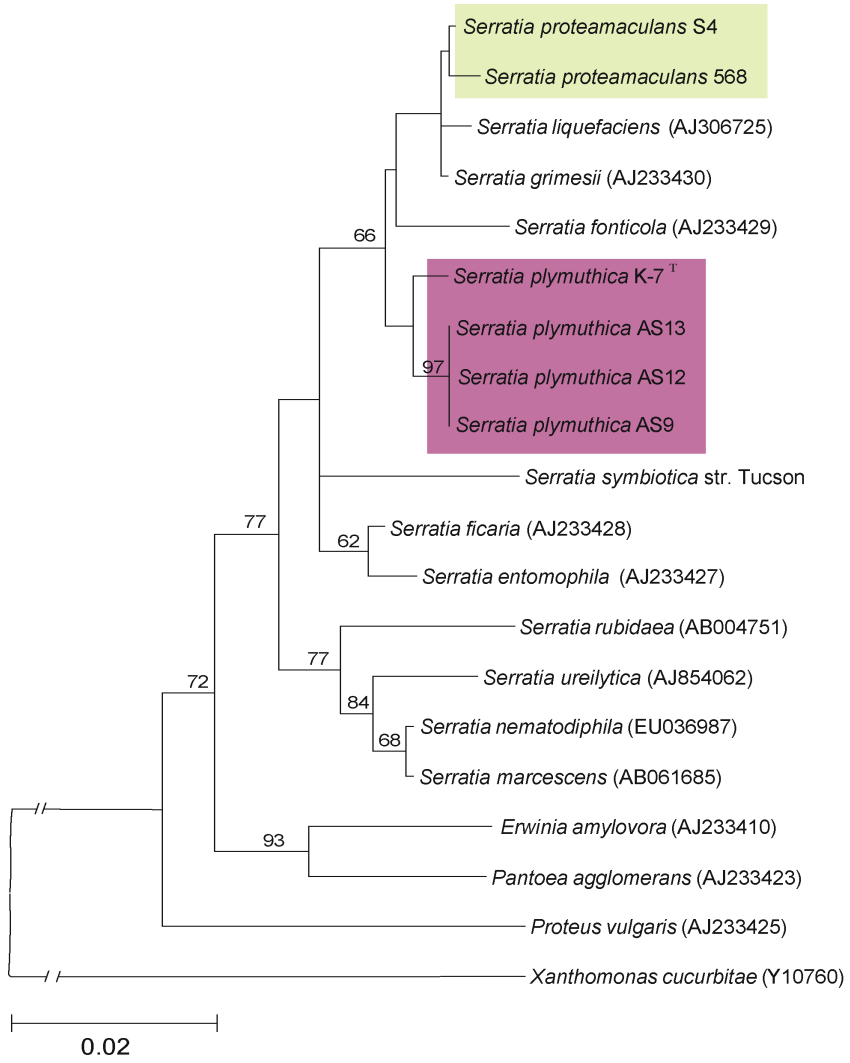


*Figure 5.* Phylogenetic tree highlighting the position of *S. plymuthica* isolates AS9, AS12 & AS13 and *S. proteamaculans* S4 in the family *Enterobacteriaceae*. The tree was based on 16S rRNA gene sequences and was constructed under the maximum likelihood criterion. The branches are scaled based on the expected number of substitutions per site. The numbers above branches are support values from 1000 bootstrap replicates if larger than 60%. The branches to *Xanthomonas* and the *Enterobacteriaceae* have been shorted for readability.

### 5.1.1 Antagonism (paper VI)

The reduced radial growth of *R. solani* mycelium during the interaction with *Serratia* isolates was used as a parameter to estimate the antagonistic activity of these bacterial isolates. This method was based on the hypothesis that either diffusible or volatile compounds, produced by the bacterium in response to the

fungus, were responsible for inhibition of fungal growth. The results revealed that *S. proteamaculans* S4 showed a strong antagonistic activity against *R. solani* compared to the *S. plymuthica* isolates. Variability in the antagonistic activity was observed between the three *S. plymuthica* isolates where the inhibitory effect of *S. plymuthica* AS13 was higher in comparison with the other two *S. plymuthica* isolates (Figure 6). There is evidence that volatile compounds produced by bacteria such as *Serratia* spp. *Pseuodomonas* spp. and *Bacillus* spp. are involved in the suppression of *R. solani* growth in *in vitro* (Kai *et al.*, 2007). In this study, diffusible compounds produced by *Serratia* isolates seem to play a major role in the inhibition of *R. solani* growth, as there was no evidence of involvement of volatile metabolites. *In vitro* functional variability is common in rhizobacteria with biocontrol ability, which may be explained by horizontal gene transfer (HGT) due to mobile genetic elements (MGE) in functional behaviour (van Elsas *et al.*, 2003; Ochman *et al.*, 2000).

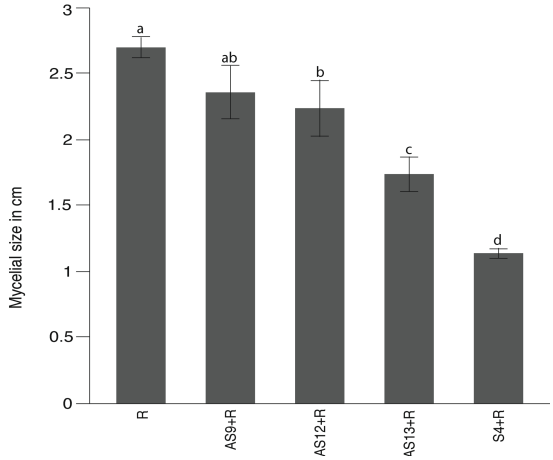


*Figure 6.* Effect of *Serratia* isolates on *R. solani* mycelial growth. R = *R. solani* only, and AS9+R = *S. plymuthica* AS9 + *R. solani*, AS12+R = *S. plymuthica* AS12 + *R. solani*, AS13+R = *S. plymuthica* AS13 + *R. solani*, S4+R = *S. proteamaculans* S4 + *R. solani*. Bars represent standard errors (n = 5).

### 5.1.2 Plant growth promotion (paper V)

The plant growth-promoting efficacy of the four *Serratia* isolates was investigated in the presence and absence of *R. solani* under greenhouse conditions. The results from different experiments revealed that all four bacterial isolates had a significant effect on seed germination and seedling establishment of both oilseed rape and *Arabidopsis* (Figure 7 & 8). These results highlight the importance of pathogen suppression ability during bacterium-fungus-plant interactions in the soil environment and are consistent

with previous observations from a field study by Alström and Andersson (unpublished data). The variability in plant growth promotion and pathogen inhibition was observed both *in vitro* and in greenhouse experiments, and *S. proteamaculans* S4 proved to enhance plant growth to a greater extent than the *S. plymuthica* isolates. Interestingly, *S. plymuthica* isolates being less effective antagonists *in vitro*, they efficiently promoted oilseed rape growth, in both the presence and absence of *R. solani* in the greenhouse experiments. However, the mechanisms and genetic regulation underlying antagonism against plant pathogens and plant growth promotion by these bacteria are not as extensively studied as in other plant growth promoting bacteria such as *Pseudomonas* spp. (Loper *et al.*, 2012; de Bruijn *et al.*, 2007; Haas & Defago, 2005; Haas & Keel, 2003; Keel *et al.*, 1992) and *Bacillus* (Ongena *et al.*, 2007; Kloepper *et al.*, 2004).

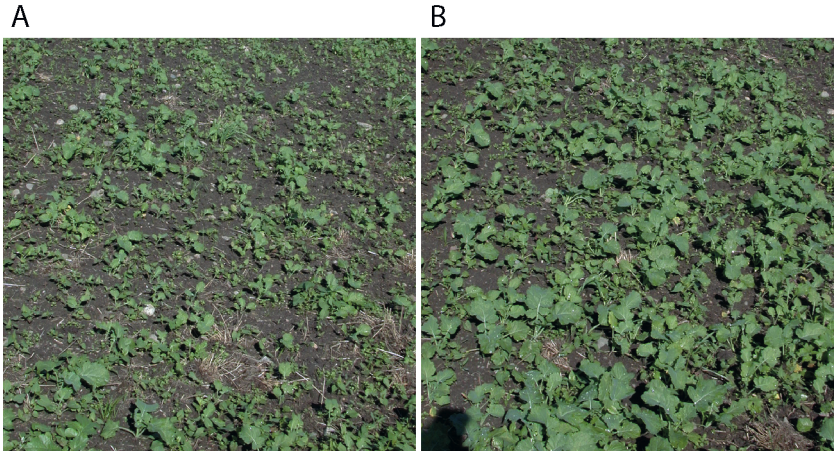


*Figure 7.* Improved emergence and growth of field grown winter oilseed rape when seeds were bacterized with *S. proteamaculans* S4 (B). (Photo by Alström S.)

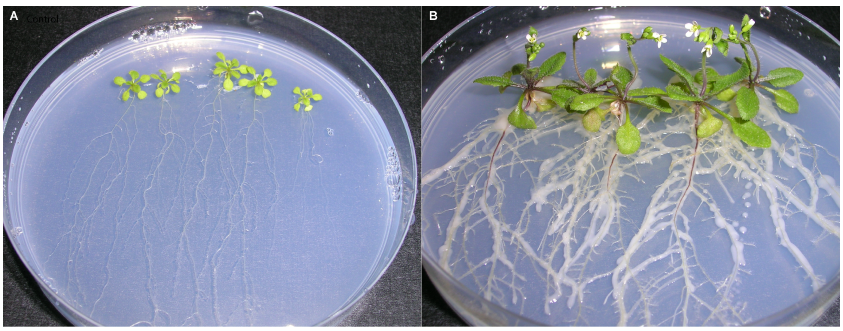


*Figure 8. In vitro* enhancement of plant growth by *S. proteamaculans* S4 in *Arabidopsis thaliana* (ecotype – Col)

### 5.1.3 Genomes of plant-associated *Serratia* spp. (Paper I - IV)

The finished genome sequences of three *S. plymuthica* isolates AS9, AS12 and AS13 were obtained and the genome sequence of *S. proteamaculans* was non-contiguously finished. The genome sizes of these bacteria are about 5.4 Mb, within the range of average genome size of most free-living rhizosphere bacteria. The numbers of protein coding genes in the three *S. plymuthica* isolates are almost identical but the total number of such genes is higher in *S. proteamaculans* S4. Protein-coding genes were assigned to functional categories based on clusters of orthologous group (COG). The numbers of genes in COG categories for carbon, energy, amino acid and nucleic acid metabolism were found to be high and similar in all four *Serratia* isolates, revealing their efficient carbon, amino acid and nucleotide metabolism. This is an indication of their efficiency to utilise a wide range of nutrients that enables them to survive in different natural environments.

## 5.2 Genetic traits involved in plant growth promotion (Paper V)

Genetic traits required for bacterial survival in the natural environment, their ability to colonise the rhizosphere, establish themselves in this particular niche, production of compounds that stimulate plant growth, induce systemic resistance, inhibit host pathogens and enable the bacteria to promote plant growth were compared in the studied genomes.

### 5.2.1 Survival in the rhizosphere

Because of their rhizospheric origin, *S. plymuthica* isolates AS9, AS12 & AS13 and *S. proteamaculans* S4 are expected to survive in the rhizosphere. Their genomes contain many genes and pathways for carbohydrate metabolism, amino acid metabolism, energy metabolism, iron uptake and several transporters, which are crucial for survival (Table 1). Their genomes contain central pathways for carbohydrate metabolism such as the tricarboxylic acid cycle (TCA) and pentose phosphate pathway. In addition to major metabolic pathways, they are able to utilise a wide range of carbon sources that include several plant-derived compounds such as arbutin, salicin, sucrose, cellobiose, maltose and glucose. The genomes of all four *Serratia* spp. contain many ABC transporters that may, not only help them to acquire nutrients efficiently, but also help them to compete with other microbes in their environment.

The genomes of all four bacteria contain complete gene clusters for chemotaxis, flagellar biosynthesis and assembly, which are identical in all four *Serratia* isolates. This suggests that these bacteria efficiently and rapidly

respond to changes in the chemical composition of their environment, in order to move towards favourable environments. The presence of genetic traits for movement and different types of primary metabolism supports the conclusion that they are efficient competitors in the rhizosphere.

### 5.2.2 Adhesion, root colonization and establishment

Adhesion to plant roots is an important prerequisite for colonisation and establishment of plant-associated bacteria in the rhizosphere. Bacterial adhesion in the root occurs through adhesion factors. Both pathogenic and non-pathogenic bacteria utilise different adhesion factors such as flagellin, pilin and haemagglutinin for their specific and non-specific adhesion. The genomes of all four *Serratia* isolates contain genes putatively encoding flagellin, pilin and haemagglutinin. The genomes of *Serratia* isolates contain gene clusters for type I and type IV, flagella and haemagglutinin, however there is a lack of homology and variability in numbers of genes between *S. plymuthica* isolates and *S. proteamaculans* S4. As an adhesive structure, the pili play a crucial role during host-bacteria interactions, colonisation, biofilm formation, motility and signalling procedures (Kline *et al.*, 2009; Proft & Baker, 2009). The better performance of *S. proteamaculans* S4 as a plant growth promoter in the field and *in vitro* experiments may partly be explained by its effective genetic traits that support adhesion and colonization in the rhizosphere.

Once they are adhering to biotic or abiotic surfaces in the rhizosphere, only certain bacteria are able to colonise and establish themselves in this niche. Biofilm formation is essential for successful establishment in the nutrient rich rhizosphere. It is a highly regulated process involving a number of molecular mechanisms including chemotaxis, motility and adhesion, involving regulation by quorum sensing (Labbate *et al.*, 2007). Cellulose and various polysaccharides are essential components of the biofilms and the genomes of *S. plymuthica* isolates and *S. proteamaculans* S4 contain the gene cluster for cellulose biosynthesis. However, the *in vitro* biofilm formation was more pronounced in *S. plymuthica* AS13 and *S. proteamaculans* S4 than the other isolates. Biofilms have permeable water channels that allow exchange of nutrients and toxins (Costerton *et al.*, 1999) and provide protection from different environmental stresses or toxins and facilitate nutrient acquisition and metabolic activity in a cooperative manner (Davey & O'toole, 2000). It is possible that AS13 and S4 produce biofilms in the natural environment and protect themselves, as well as the host plant, from its pathogens.

### 5.2.3 Plant growth promotion

Plant beneficial rhizobacteria utilise various mechanisms to survive and proliferate in the rhizosphere. They influence plant growth directly by producing phytohormones, and their regulators, that modulate metabolism and plant physiology. There has been some evidence for the involvement of auxin and cytokinin produced by rhizobacteria in the enhancement of plant growth and health (Spaepen *et al.*, 2007; Salamone *et al.*, 2001). The results presented in paper V revealed that all four *Serratia* genomes contain genes encoding for the biosynthesis of phytohormones. Besides phytohormones, these bacterial genomes contain putative genes encoding for different volatiles such as acetoin and 2,3-butanediol that directly influence plant growth by signalling (Ryu *et al.*, 2003). Indirect influence on plant growth through pathogen suppression, mediated either through direct antagonism or through ISR, has been shown for many rhizobacteria (Kloepper *et al.*, 2004; Whipps, 2001; Weller, 1988). Bacteria produce diverse compounds such as antibiotics, exo-enzymes, siderophores and lipopolysaccharides that either directly or indirectly suppress the growth of phytopathogens and their secretion. The genomes of *Serratia* isolates contain the genetic traits required for biosynthesis of these compounds. However, the genes required for biosynthesis of prodigiosin are conserved only in *S. plymuthica* strains. The variability in genetic traits for type II and VI secretion systems was observed between *S. plymuthica* isolates and *S. proteamaculans*. Presence of more than one type of secretion system (I, II and VI) in the genome of *S. proteamaculans* S4 is evidence that S4 possesses more efficient translocation systems for secreted compounds across the inner and outer membrane of the cells.

## 5.3 Response of *Serratia* spp. to *R. solani* (Paper VI)

Genome sequencing and comparison provide a platform to unravel the mechanisms and genetic traits that are involved either directly or indirectly in plant growth promotion. Variability in different genetic traits was found in the genomes of *S. proteamaculans* and *S. plymuthica* isolates. Paper VI explores the genetic traits underlying antagonistic activity of two *Serratia* species and their mode of action by means of genome-wide transcriptome profiling of *S. proteamaculans* S4 and *S. plymuthica* AS13 during interaction with *R. solani*.

The results revealed that antibiosis is apparent in *S. plymuthica* AS13, where the genes, *prnABCD,* for an antibiotic, pyrrolnitrin biosynthesis (Hammer *et al.*, 1997), non-ribosomal protein synthase and several genes for ABC transporters and type secretion systems were up-regulated during interaction with *R. solani*. This result is consistent with the involvement of

pyrrolnitrin produced by *Serratia* spp. and *Pseudomonas* spp. to inhibit various fungal plant pathogens (Müller *et al.*, 2009; Raaijmakers *et al.*, 2002; Hammer *et al.*, 1997). On the other hand, *S. proteamaculans* S4 responded to *R. solani* through a combination of competition, defence mechanisms and antibiosis. The gene expression profile showed that genes for general metabolism, including carbon, energy, amino acids and nucleic acids, and their transporters, including siderophore transporters, were highly up-regulated. In addition genes for ascorbate utilization, a compound possibly derived from *R. solani*, were up-regulated. These results are consistent with the finding by Mela *et al.,* (2011) that *Collimonas* bacteria are able to utilise oxalate produced by *Aspergillus* as a defence mechanism during interaction. The overall results revealed that the mode of action underlying bacterial antagonistic activity is isolate-specific.

# 6 Conclusions

The genomes of all four *Serratia* isolates are similar in size and contain genes for all essential functions that are required for a free-living life style. Larger inter-specific genetic variation existed between the *S. plymuthica* and *S. proteamaculans* isolates, and intra-specific variation within the *S. plymuthica* isolates was remarkably small.

The comparison of *Serratia* genomes reveals that the general processes involved in plant growth promotion are similar across widely related phyla, but that the genes and pathways underlying these processes are variable not only among widely separated groups such as *Pseudomonas* and *Serratia* but also within the genus *Serratia*.

The detailed mode of action underlying plant growth promoting ability depends on variation in genetic traits, and the regulatory apparatus in different bacterial isolates. Better performance of *S. proteamaculans* S4 as an antagonist and plant growth promoter may be due to the presence of unique genes for colonisation and different types of secretion system. Moreover, intra-species variability in the phenotypic characteristics of *S. plymuthica* isolates may be due to mutations in at least two putative genes.

Genome-wide transcriptional profiling revealed that the two *Serratia* species utilise different modes of action during the process of antagonism. Transcriptional responses of two species differed irrespective of their genetic constitution. *Serratia plymuthica* AS13 utilises antibiosis against *R. solani* while *S. proteamaculans* S4 utilises competition for nutrients as well as antibiosis.

# 7 Future perspectives and implications

## 7.1 Future perspectives

The results presented in this thesis provide a genomic picture of plant growth promoting *Serratia* isolates. The genetic traits underlying plant growth promotion and biocontrol mechanisms described in this thesis can be used as milestones for further development of more effective biocontrol agents and also provide a platform within which to investigate the evolution of plant growth promotion and biocontrol mechanisms in these different bacterial isolates.

The successful use of microorganisms in sustainable agriculture requires improved understanding of the processes and modes of action underlying their ability to directly and indirectly stimulate plant growth. Better understanding of the possible impact of biocontrol organisms on indigenous microbial communities is also required. The use of metatranscriptomics and metagenomic approaches will be valuable for assigning the functional traits involving in different cellular and extracellular processes in different taxa and their mode of action under natural conditions.

The results presented in this thesis provide us with information about the variation in genes involved in different putative physiological and metabolic processes. Several hypothetical genes were differentially expressed but their function is still unknown and multiple simultaneous processes seem to be involved in antagonism. This suggests the involvement of novel compounds and hitherto unidentified mechanisms in antagonism, providing a basis for future innovations in developing efficient biocontrol strategies. To verify the functional traits, a combination of mutagenesis, proteomics and localization studies will be crucial. These methods will allow us to assign the function of putative genes and pathways for new proteins and subsequently to understand their functional roles.

The importance of the self-defence ability of different pathogens cannot be underestimated, since they use many strategies to defend themselves against competitors and plant defence systems (Duffy *et al.*, 2003). It is therefore important to understand how pathogens interact with bacteria and plants. The recently available genome of the pathogenic *R. solani* AG 3 will provide a platform with which to explore the different genes for different defence and pathogenicity mechanisms of other *R. solani* strains. The use of different "-omics" tools in addition to other molecular tools including gene mutagenesis and protein localization will help to explore the full picture of interactions between microbes, pathogens and plants.

## 7.2 Implications

### 7.2.1 Bioenergy and environmental issues

One of the consequences of industrialisation and rapidly increasing global populations is increased demand for energy. Public concern about environmental pollution and negative impacts on natural ecosystems due to fossil fuels and safety concerns related to nuclear energy have increased the demand for agricultural products that can be used for sustainable production of bioenergy. Oilseed rape is an example of such bioenergy crop. As demand is increasing, cultivation is also increasing. However, increased cultivation may lead to a build up of pathogen pressure on this crop. Climate change may, in the future, cause abiotic stresses in the natural environment resulting in reduced crop yield. *Serratia* isolates may then be used to retain soil fertility and increase the crop yield, in an environmentally friendly manner.

### 7.2.2 Legislation

The EU directive on sustainable use of pesticides is to be implemented in all member countries by 2014. According to this directive, IPM (Integrated Pest Management) is mandatory for all agricultural production and natural pest control mechanisms should be preferred before chemical pesticides. The role of natural (non genetically-modified) microorganisms in biological control and IPM is therefore of increasing importance.

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