Importance of biotic interactions for the fitness and activity of rhizosphere biocontrol pseudomonads

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„All novelty is but oblivion“

Francis Bacon

Contents

Summary

This work investigates the ecology of biocontrol bacteria in the rhizosphere of crop plants. It focuses on biotic interactions influencing the fitness and the activity of these bacteria, and on defence mechanisms increasing their competitiveness against other bacteria.

A number of soil bacterial present antagonistic activity against soil borne plant pathogens by producing antibiotics and enzymes. Thereby they bear potential for developing environmentally friendly management of crop diseases, as an alternative to conventional fungicides or fumigants. The application of such biocontrol bacteria, however, is still limited by the lack of consistency in their survival and antagonistic activity. Introduced bacteria often fail to establish in soil or remain in an inactive state. Biotic interactions are central for the fitness of introduced strains. Bacteria in soil compete with indigenous microorganisms present in high density and diversity. Further, they are exposed to a complex community of predators, in particular protozoa and nematodes. In order to successfully use bacterial inoculants under field conditions there is a need to better understand which interactions are the most relevant for the survival of introduced strains, and which defence mechanisms help bacteria to establish stable and persisting populations. Especially toxins play an important role. Antibiotics responsible for phytopathogen inhibition are often inhibitors of bacterial growth, and are highly toxic against protozoan predators.

We used as model organism the biocontrol bacterium *Pseudomonas fluorescens* CHA0, an efficient coloniser of crop plants with a strong antagonistic activity against fungal pathogens and root knot nematodes. We tested if bacterial toxicity enhances competitiveness against other rhizosphere bacteria and improve resistance against predation pressure, and if bacteria alter the production of toxins in response to predator chemical cues or to signal molecules involved in plant - bacteria communication

The first two experiments investigated the impact of bacterial toxins and microfaunal predation on intra- and interspecific competition among bacteria in the rhizosphere. We used gnotobiotic or semi-natural simplified microcosms with and without predators. Predation favoured toxic phenotypes and increased their competitiveness against other rhizobacteria such as non-toxic spontaneous mutants. This suggests that toxins of biocontrol bacteria primarily function as antipredator defence, and that microfaunal predators promote toxic bacteria thereby enhancing soil suppressiveness.

The third and fourth experiments investigated the chemical ecology of biocontrol bacteria. By using green fluorescent protein (gfp) reporter fusions reflecting the expression of the main biocontrol genes, we followed changes in toxin production in response to chemical cues from predators and the host plant. The results demonstrated that bacteria sense chemical cues from free living amoebae, and respond by increased toxin production. Bacterial toxicity was also influenced by the host plant, which modulated the expression of antifungal genes upon infection with a root pathogen. The results suggest

that bacteria adjust the production of toxins in response to a wide range of environmental parameters in order to optimise the costs and benefits of defence mechanisms.

The fifth experiment explored the integration of introduced biocontrol bacteria in soil food webs by RNA Stable Isotope Probing (SIP). In this experiment wildtype and gacS$^-$ strains of *P. fluorescens* CHA0 were labelled with ^{13}C and introduced in an agricultural soil. Microfaunal predators consuming both strains were resolved by T-RFLP and RT-qPCR of the 18S rRNA. The results indicate that carbon is transferred rapidly to higher trophic levels, and that toxic bacteria were consumed by a distinct and more restricted eukaryote community than bacteria without defence mechanisms.

In conclusion, the production of extracellular toxins by biocontrol bacteria appear thus to be crucial for their competitiveness in the soil. This overlapping of antipredator and crop protecting traits opens promising possibilities of improvement of the efficiency of microbial biocontrol agents by manipulating the predation regime.

Zusammenfassung

Diese Arbeit untersucht die Ökologie von Biokontroll-Bakterien in der Rhizosphäre von Nutzpflanzen. Der Schwerpunkt liegt dabei auf biotischen Interaktionen, die die Fitness und Aktivität dieser Bakterien beeinflussen, und auf Verteidigungsmechanismen, die ihre Wettbewerbsfähigkeit gegenüber anderen Bakterien steigern.

Viele Bodenbakterien zeigen antagonistische Aktivität gegen Pflanzenpathogene im Boden, indem sie Antibiotika und Enzyme produzieren. Dadurch enthalten sie Potenzial für die Entwicklung von umweltfreundlicher Behandlung von Nutzpflanzen-Krankheiten, als eine Alternative zu konventionellen Fungiziden. Die Anwendung solcher Biokontroll-Bakterien ist jedoch immer noch begrenzt durch die mangelnde Beständigkeit ihres Überlebens und ihrer antagonistischen Aktivität. Inokulierte Bakterien können sich oft nicht im Boden etablieren oder verbleiben in einem inaktiven Status. Biotische Interaktionen sind zentral für die Fitness inokulierter Stämme. Bakterien im Boden konkurrieren mit in grosser Dichte und Diversität präsenten einheimischen Mikroorganismen. Weiter sind sie einer komplexen Prädatorengemeinschaft ausgesetzt, insbesondere Protozoen und Nematoden. Um erfolgreich Bakterien-Impfungen unter Feldbedingungen anwenden zu können, muss besser verstanden werden, welche Interaktionen die relevantesten für das Überleben der inokulierten Stämme sind, und welche Verteidigungsmechanismen den Bakterien helfen, stabile und beständige Populationen zu bilden. Besonders Giftstoffe spielen eine wichtige Rolle. Phytopathogenhemmende Antibiotika wirken oft hemmend auf das Bakterienwachstum und sind hoch giftig gegen prädatorische Protozoen.

Wir verwendeten als Modellorganismus das Biokontroll-Bakterium *Pseudomonas fluorescens* CHA0, ein effizienter Besiedler von Nutzpflanzen mit einer starken antagonistischen Aktivität gegen Pilzbefall und parasitische Nematoden. Wir testeten, ob die Giftigkeit der Bakterien die Wettbewerbsfähigkeit gegenüber anderen Rhizosphären-Bakterien erhöht und ob sie die Resistenz gegen Prädationsdruck verbessert, und ob Bakterien die Produktion von Giftstoffen in Reaktion auf chemische Signale der Prädatoren oder auf Moleküle, die in die Kommunikation zwischen Pflanze und Bakterien involviert sind, verändern.

Die ersten beiden Experimente untersuchen den Einfluss von Bakterien-Giftstoffen und mikrofaunaler Prädation auf die intra- und interspezifische Konkurrenz zwischen Bakterien in der Rhizosphäre. Wir verwendeten gnotobiotische oder halb-natürliche vereinfachte Mikrokosmen mit und ohne Prädatoren. Prädation begünstigte toxische Phänotypen und erhöhte deren Wettbewerbsfähigkeit gegenüber anderen Rhizobakterien wie ungiftigen spontanten Mutanten. Dies legt nahe, dass Giftstoffe von Biokontroll-Bakterien primär als Verteidigung gegen Prädatoren wirken, und dass mikrofaunale Prädatoren giftige Bakterien begünstigen, wodurch die natürliche Eigenschaft des Bodens, die Entwicklung von Pflanzenpathogenen zu hemmen, gefördert wird.

Das dritte und das vierte Experiment untersuchen die chemische Ökologie von Biokontroll-Bakterien. Durch die Verwendung von auf grün fluoreszierenden Proteinen (gfp) basierenden Reporterfusionen, die die Expression der Hauptbiokontrollgene spiegeln, verfolgten wir Veränderungen der Giftproduktion in Reaktion auf chemische Signale von Prädatoren und der Wirtplanze. Die Ergebnisse zeigten, dass Bakterien chemische Signale von freilebenden Amöben erkennen und darauf mit erhöhter Giftproduktion reagieren. Die Bakteriengiftigkeit wurde ebenfalls beeinflusst durch die Wirtpflanze, die die Expression antifungaler Gene auf die Infektion mit Wurzelpathogenen abstimmte. Die Ergebnisse zeigen, dass Bakterien die Giftproduktion in Reaktion auf ein breites Spektrum an Umweltfaktoren anpassen, um Kosten und Nutzen der Verteidigungsmechanismen zu optimieren.

Das fünfte Experiment untersucht die Integration inokulierter Bakterien in Bodennahrungsketten durch RNA Stable Isotope Probing (SIP). In diesem Versuch wurden wildtype und gacS-Stämme von *P. fluorescens* CHA0 mit ^{13}C markiert und in einen Ackerboden inokuliert. Mikrofaunale Prädatoren wurden durch T-RFLP und RT-qPCR der 18S rRNA nachgewiesen. Die Ergebnisse zeigen, dass Kohlenstoff schnell auf höhere trophische Ebenen transferiert wird, und dass die Produktion von Giftstoffen die Anzahl Bakterienkonsumenten reduziert.

Zusammenfassend ist festzuhalten, dass die Produktion extrazellulärer Gifte durch Biokontroll-Bakterien als entscheidend für deren Wettbewerbsfähigkeit im Boden erscheint. Diese Überlappung von antiprädatorischen und pflanzenschützenden Eigenschaften eröffnet vielversprechende Möglichkeiten zur Verbesserung der Effizienz von mikrobiellen Biokontroll-Agenten durch die Manipulation des Prädationsdrucks.

List of Publications

Publications presented in this work

- **Jousset A**, Scheu S, Bonkowski M (2008). Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. *Functional Ecology* **22:** 714-719.
- **Jousset A,** Rochat L, Keel C, Pechy-Tarr M, Scheu S, Bonkowski M (2009). Predators promote toxicity of rhizosphere bacterial communities by selective feeding on non-toxic cheaters. *ISME Journal* **in press**.
- **Jousset A**, Rochat L, Keel C, Scheu S, and Bonkowski M *(in prep)* Predator-prey chemical warfare determines the antifungal activity of rhizosphere pseudomonads.
- **Jousset A**, Rochat L, Keel C, Lanoue A, Scheu S, and Bonkowski M *(in prep)* Plants manipulate antifungal compound production by rhizobacteria upon pathogen infection.
- **Jousset A**, Euringer K, Bonkowski M, Scheu S, Lueders T (*in prep*). Extracellular toxin production changes the integration of biocontrol bacteria in soil foodwebs.

Other publications

- Schebb NH, Vielhaber T, **Jousset A**, Karst U (2009). Development of a new comprehensive screening methodology for proteases. *Journal of Chromatography A* **in press**.
- Henkes G, **Jousset A**, Bonkowski M, Thorpe M, Scheu S, Lanoue A, Schurr U, Rose U. (*submitted*) Systemic response of barley to the biocontrol bacterium *Pseudomonas fluorescens* and the root pathogen *Fusarium graminearum* induces rapid changes in root carbon allocation.
- Neidig N, **Jousset A**, Paul R, Scheu S (*in prep*). Nematostatic and repellant effect of bacterial toxins from the biocontrol bacterium *Pseudomonas fluorescens* CHA0 against *Caenorhabditis elegans*.
- Neidig N, **Jousset A**, Paul R, Scheu S (in prep): Mutual toxicity determines the fitness of the nematode *Caenorhabditis elegans* and the amoeba *Acanthamoeba castellanii*.

Chapter 1

Introduction

1. Introduction

Current intensive agricultural practices rely on a high input of fertilizers and agrochemicals to maintain high yields and prevent crop diseases. Crop plants can be infected by a range of soil borne microbial pathogens (Schroeder and Paulitz, 2006). Infections such as the head blight fusarose (caused by *Fusarium* spp.), the take all disease (caused by *Gaeumannomyces sp.*), or damping off (caused by the oomycete *Pythium* spp. or *Rhizoctonia solanii*) cause considerable yield losses in the agriculture, and new diseases constantly appear or re-emerge (De Cock *et al.*, 2008; McMullen *et al.*, 1997). The breed of resistant plant varieties only brought limited results, partly due to the lack of information available on the traits involved in pathogen resistance (Bai and Shaner, 2004). Current management strategies, based on fungicide application or fumigation can control disease development (McMullen *et al.*, 2008). However, the re-emergence of resistant pathogens (Goswami and Kistler, 2004) the high costs of the current disease control practice and the concerns about their environmental toxicity call for cheap and environmentally sound alternative to control phytopathogens.

1.1. Biocontrol of plant diseases

Biological control of soil borne pathogens using microorganisms received increasing attention during the last decades as an alternative to conventional fungicide treatments (Weller, 2007). Various microorganisms with antagonistic activity against phytopathogens have been isolated from suppressive soils. In these soils pathogens are either unable to persist or cause low damage to plants, and antagonistic microorganisms account for a large part of the natural attenuation or elimination of plant diseases (Mazzola, 2002; Postma *et al.*, 2008). Biocontrol organisms inhibit plant pathogens by producing inhibitory toxins (Haas and Defago, 2005), but also lytic enzymes like proteases, chitinases or lipases (van den Broek *et al.*, 2003). Most characterised bacterial biocontrol strains belong to actinomycetes (El-Tarabily and Sivasithamparam, 2006), the genera *Bacillus* (Ongena and Jacques, 2008), *Burkholderia* (Mahenthiralingam *et al.*, 2005) and *Pseudomonas* (Weller and Raaijmakers, 2002). The genus *Pseudomonas* has been intensively investigated, and is one of the best characterised group of biocontrol microorganisms (Weller, 2007).

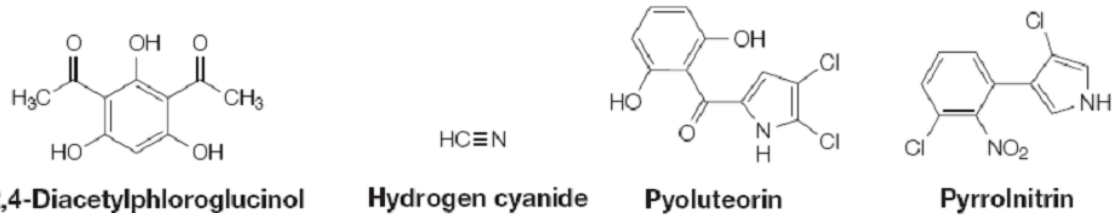

Figure 1 Some secondary metabolites produced by fluorescent pseudomonads with antifungal activity (Adapted from Haas and Defago, 2005).

Autecology (Lugtenberg *et al.*, 2001) and genetic regulation mechanisms involved in toxin production are well known for many strains (Haas and Keel, 2003; Raaijmakers *et al.*, 2002), and the genome of the reference strain *P. fluorescens* pf-5 now is completely sequenced and annotated (Loper and Gross, 2007), making pseudomonads a useful model to study biocontrol interactions.

1.1.1. Mode of action of biocontrol bacteria

Biocontrol bacteria colonise the root system of plants and in some cases penetrate into plant tissue (Coombs *et al.*, 2004; Troxler *et al.*, 1997), and protect the host plant in different ways (Compant *et al.*, 2005).

Direct pathogen inhibition

Biocontrol bacteria produce extracellular toxins, such as 2,4, diacetylphloroglucinol (DAPG), pyrrolnitrin, phenazine or hydrogen cyanide (Haas and Defago, 2005) or lytic enzymes (Sacherer *et al.*, 1994). These exoproducts act synergistically (Dunne *et al.*, 1998) to inhibit or kill pathogens. For example, hydrogen cyanide is a potent inhibitor of the respiratory cascade with a broad range of action (Blumer and Haas, 2000), and DAPG profoundly damages the hyphae and propagules of the oomycete *Pythium ultimum* (de Souza *et al.*, 2003). Many rhizosphere bacteria share the ability to produce antagonistic compounds (Adesina *et al.*, 2007; Berg *et al.*, 2006), and probably present an important hurdle for pathogens.

Elicitation of plant defences

Beneficial bacteria can improve plant resistance to infection by triggering plant immune response (van Wees *et al.*, 2008). This priming of plant defence confers a partial or total immunity against a broad range of pathogens, lessening the severity of infection (Conrath *et al.*, 2006), and is elicited by microbe-associated-molecular-patterns (MAMPs), such as bacterial lipopolysaccharides (LPS), toxins and bacterial produced plant hormones, *e.g.* salicylic acid (Van Loon *et al.*, 1998). This Induced Systemic Resistance (ISR) is mediated by jasmonic acid and ethylene pathways (Pieterse *et al.*, 2003) and results in similar plant responses to pathogen induced salicylic acid mediated Systemic Acquired Resistance (SAR). As a response to defence priming, plants accumulate pathogen related proteins(PRs), including chitinases and peroxidases, which suppress potential pathogens (Duijff *et al.*, 1998). The different ways of ISR elicitation can act synergistically, and induction of plant defences may be at least as important as direct toxicity against the pathogen for disease suppression by beneficial bacteria (Henkes *et al.*, submitted).

1.1.2. Restrictions to the use of biocontrol bacteria

Despite of the range of economical and ecological advantages of microbial biological control of crop diseases, applications of biocontrol bacteria are still limited (Fravel, 2005; Walsh *et al.*, 2001), and introduced population often decline under field conditions (Bennett and Whipps, 2008). In order to promote dense and active populations of biocontrol bacteria, factors determining their fitness in the field require better understanding. The perception and response to environmental stress need to be better characterised (Walsh *et al.*, 2001). Moreover, the biotic interactions with indigenous microorganisms must be better understood, since they probably are most important in determining the realised niche of bacteria (Begon *et al.*, 1996).

1.2. Ecology of rhizosphere bacteria

1.2.1. The rhizosphere

The rhizosphere is the zone of soil surrounding plant roots, and a hot spot of microbial activity. Plants allocate a substantial part of the fixed carbon to the root system, and release about 20% of the net fixed carbon as root exudates (Nguyen, 2003). Exudates primarily are composed of amino-acids, organic acids, sugars and phenolic compounds, and form the basis of intensive interaction between plants and soil bacteria (Bais *et al.*, 2006). Plant exudates add a considerable amount of easily available resources to the rhizosphere where they may exert a "priming effect": soil bacteria are usually carbon limited while rhizosphere soil supports a high carbon turnover and a bacterial community up to 30 times more abundant than in bulk soil (Kuzyakov, 2002).

This host specific (Costa *et al.*, 2006; Kowalchuk *et al.*, 2002; Wieland *et al.*, 2001) and highly active commensal microbial community provides various services to the plant. Some rhizobacteria function as biofertilisers increasing plant growth by improving nutrient acquisition (Vessey, 2003a). Symbiotic *Rhizobia* and actinomycetes (Van Rhijn and Vanderleyden, 1995; Wall, 2000), but also free living diazotrophs fix N_2 thereby providing bioavailable ammonium (Dobbelaere *et al.*, 2003). Nitrogen uptake is facilitated by root associated ammonium oxidisers. These bacteria convert ammonium into nitrate, which can readily be taken up by the plant (Briones *et al.*, 2003). Microorganisms also increase plant phosphorus uptake. Phosphate solubilisers degrade insoluble mineral and organic phosphorus sources like phosphate rock or phytate into forms which can be assimilated by plants (Rodriguez *et al.*, 2002), and mycorrhizal fungi improve nutrient acquisition by extending the soil regions from which nutrients can be taken up (Artursson *et al.*, 2006). Another growth promoting effect is associated with phytohormone producing bacteria which modify root structure and branching (Costacurta and Vanderleyden, 1995). These bacteria produce hormones like auxins (Barazani and Friedman, 1999), or downregulate the stress related ethylene pathway (Saleem *et al.*, 2007). Many crop plants also select

antifungal producing bacteria that protect against soil borne pathogens (Bergsma-Vlami *et al.*, 2005; Mazzola *et al.*, 2004) and monocultures surprisingly tend to be free of soil borne pathogens, as in the case of take-all decline (TAD) in wheat fields (Weller, 2007).

Interestingly, plants regulate the amount of nutrients leached (Phillips *et al.*, 2003) in response to nutrient availability (Paterson *et al.*, 2006) or the presence of beneficial bacterial metabolites such as the antifungal compounds phenazines or 2,4-DAPG (Phillips *et al.*, 2004). This suggests that bacteria providing a service to the plant may be rewarded by a local increase of exudation thereby improving the competitiveness and cooperation of bacteria which otherwise may turn inefficient or parasitic (Denison *et al.*, 2003; Kiers and Denison, 2008). Presumably, the ability of plants to communicate and control root associated microbial populations is essential for their fitness.

1.2.2. Fundamental and realised niche: Rhizosphere competence of biocontrol bacteria

The rhizosphere as fundamental niche

The prerequisite for rhizobacteria is the potential to colonize the rhizosphere niche. This rhizosphere competence involves first the ability to successfully establish a population on plant roots or in their vicinity. The rhizosphere therefore can be considered as part of the fundamental niche of soil bacteria, *i.e.* the combination of conditions and resources that allow the species to maintain a viable population (Hutchinson, 1957). A number of bacterial traits are required for root colonization. First, bacteria need to detect the presence of roots and migrate toward them, which requires a chemotactic (Kumar *et al.*, 2007) or electrotactic (Van West *et al.*, 2002) behaviour. Active motility enhances the root colonisation although it is not indispensable for it (Czaban *et al.*, 2007). Further, bacteria must attach to the roots. Exopolysaccharides (EPS; Bianciotto *et al.*, 2001) or pili (Lugtenberg *et al.*, 2001) facilitate the adhesion to the roots, and specific lipopolysaccharide (LPS) structures are required for plant lectins mediating recognition and adhesion (Burdman *et al.*, 2000). The establishment of rhizosphere populations further is improved by biofilm formation (Koutsoudis *et al.*, 2006), a multicellular structure that offers optimal condition for cell-to-cell communication (Gantner *et al.*, 2006). To be competitive on the roots, rhizobacteria must adapt their metabolism to the composition of exudates (Mark *et al.*, 2005), and efficiently use exudates as an energy source (de la Fuente *et al.*, 2007). The rhizosphere environment is spatially and temporally highly variable (Marschner *et al.*, 2002), as reflected by the heterogeneous colonisation of its different parts (Watt *et al.*, 2006). Rapid adaptation therefore is necessary for survival in this changing niche; indeed many bacteria use recombination and phase variation to colonise microenvironments (van den Broek *et al.*, 2005a; Martinez-Granero *et al.*, 2005; Sanchez-Contreras *et al.*, 2002) like root tips (Achouak *et al.*, 2004).

However, the potential of bacteria to colonize roots not necessarily relates to their competitiveness in a complex environment. The realised niche, *i.e.* the range of environmental conditions where a given species is indeed competitive, only corresponds to a fraction of the fundamental niche. In the field factors such as soil properties (Ownley *et al.*, 2003; Verma *et al.*, 2007) or host plant genotype (Mazzola *et al.*, 2004) affect bacterial colonisation, suggesting fine tuned adaptation of bacteria to environmental cues. Moreover, introduced bacteria have to compete successfully with indigenous microorganisms. The local microbial consortium presumably represents the most crucial factor for the dimensions of the realised niche. In order to successfully apply biocontrol bacteria these interactions therefore need to be understood.

1.2.3. Biotic interactions in the rhizosphere

The rhizosphere is the fundamental niche of a highly diverse and specialized microbial community (Wieland *et al.*, 2001) and the base of a complex food web (Pollierer *et al.*, 2007). Introduced biocontrol strains exert a transient effect on soil protozoan, fungal and bacterial communities (Andersen and Winding, 2004; Girlanda *et al.*, 2001; Johansen *et al.*, 2005; Johansen *et al.*, 2002), but often decline in the long term (Bennett and Whipps, 2008), suggesting intense forces restricting the rhizosphere niche.

Rhizobacteria face two main types of antagonists. First, they must compete with the other species for nutrients (bottom-up regulation), principally root exudates but also mineral nutrients, and second they are attacked by predators, in particular protists and nematodes (top-down regulation).

Bottom-up control

Compared to the bulk soil, the rhizosphere is a hot spot of microbial activity, where a commensal community feeds on plant exudates (Haichar *et al.*, 2008). Competition for plant derived resources nonetheless is intense, and ribosomal RNA content analysis revealed that most rhizosphere bacteria are starving (Ramos et al., 2000a), suggesting that bacterial communities are controlled by bottom-up forces. Rhizosphere bacteria also compete with other heterotrophic microbes for plant resources, e.g. mycorrhizal fungi (de Boer *et al.*, 2005). The energy invested in mycorrhizal fungi diminishes the resources available to rhizosphere bacteria, and symbiosis with *Glomus* spp. reduces the colonisation of roots by bacteria (Marschner *et al.*, 1997). To improve their competitiveness, rhizosphere bacteria developed strategies such as the synthesis of broad spectrum antimicrobial compounds (Compant *et al.*, 2005; Dubuis *et al.*, 2007), or growth in tight biofilm structures (Rudrappa *et al.*, 2008). On the contrary, other bacteria specialised in motility (Czaban *et al.*, 2007) allowing to rapidly colonize new root regions. Expression of most antibiotic traits is regulated by cell-signalling, mostly under the control of the gac/rsm cascade or N-acyl homoserine lactone (AHLs) derivatives (Lapouge *et al.*, 2008;

Somers *et al.*, 2004). Interestingly, some strains evolved the potential to eavesdrop communication of competitors (Dubuis and Haas, 2007), or even to interfere with this communication by degrading the signal molecules (Dong and Zhang, 2005). Depending on the root region and the composition of the leached compounds, certain growth and nutrient acquisition strategies are adopted (Folman *et al.*, 2001). For example, rapid growth may allow to conquer uncolonised root tips, while biofilm formation and antibiosis is more advantageous to colonise older roots where competition among bacteria is more severe (Achouak *et al.*, 2004).

Top down control

Rhizosphere bacteria face an intense predation pressure from bacteriophages (Keel *et al.*, 2002; Stephens *et al.*, 1987), *Bdellovibrio* bacterial predators (Lambert *et al.*, 2006; Lueders *et al.*, 2006) and microfaunal predators (protists and nematodes). The latter include nematodes and protists and constitute the main predator group of soil bacteria, consuming up to 50% of the bacterial productivity (Foissner, 1999). Predation is a major cause of bacterial mortality (Pernthaler, 2005). It exerts a considerable selective pressure on rhizosphere microbial communities (Blanc *et al.*, 2006; Rønn *et al.*, 2001; Rosenberg *et al.*, 2009), and predators massively consume introduced bacteria (Danso and Alexander, 1975; Sorensen *et al.*, 1999) The ability to cope with top-down forces is thus a determining factor of bacterial fitness, and grazing resistant phenotypes are favoured by high predation regimes (Corno and Jurgens, 2006; Queck *et al.*, 2006). Most microfaunal predators feed selectively (Jezbera *et al.*, 2005) according to surface properties (Wildschutte *et al.*, 2004; Wootton *et al.*, 2007), prey size (Posch *et al.*, 2001) or metabolic state (Gonzalez *et al.*, 1993). Further, in soil the effect of predators depends on habitat structure (Postma *et al.*, 1990). While flagellates and ciliates are restricted to the water pellicle, amoebae are able to access prey deep in soil microaggregates (Coûteaux and Darbyshire, 1998), and nematodes can occupy larger pores by carrying their water film with them (Bamforth, 1988).

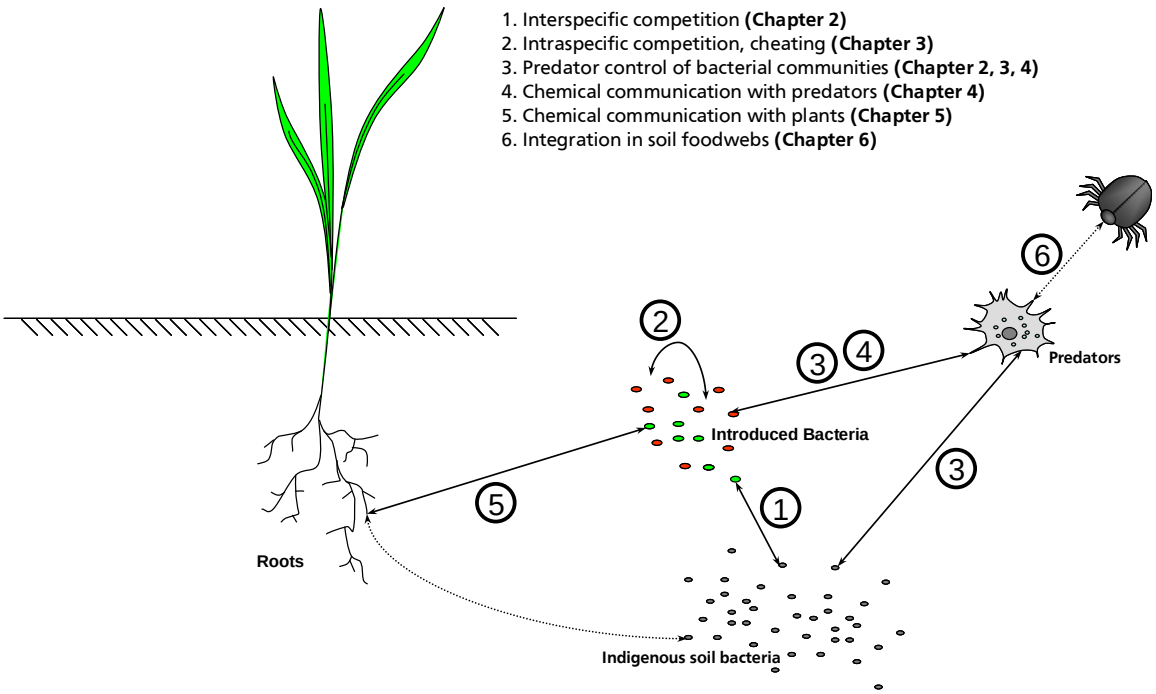

Figure 2 Schematic representation of biotic interactions affecting the fitness of introduced bacteria into the rhizosphere; chapter numbers in bold refer to the chapters of this work.

By releasing the nutrients locked in bacterial biomass, microfaunal predators play a central role for nutrient cycling (Kuikman *et al.*, 1991), and contribute to 14-66% and 20-40% of C and N mineralisation, respectively (Ekelund and Ronn, 1994; Griffiths, 1994). Nematodes and protozoa also increase nutrient cycling in the rhizosphere and keep rhizosphere bacteria in an active state (Bonkowski et al., 2000; Ferris et al., 2004). The increased turnover of microbial populations is referred to as microbial loop and improves plant growth (Bonkowski, 2004) and even influences the aboveground food web (Moore *et al.*, 2003). Moreover, some studies suggest that bacterivores may affect plant growth by favouring hormone producing bacteria (Bonkowski and Brandt, 2002; Mao *et al.*, 2007).

1.2.4. Bacterial defence strategies: importance for biocontrol

Bacteria developed sophisticated defence strategies to avoid predation (Matz and Kjelleberg, 2005). Many species present morphologic adaptations, form inedible filaments (Hahn *et al.*, 1999), microcolonies or biofilms (Matz *et al.*, 2004a), or secrete an extracellular matrix for protection (Hahn *et al.*, 2004). However, the efficiency of these morphological adaptations depend on the type of predator (Weitere *et al.*, 2005). Another potent defence strategy is the production of toxins. Defensive toxins can be intra- (Matz *et al.*, 2004b) or extracellular (Jousset *et al.*, 2006), or be directly injected into the predator cell using a type three secretion system (TTSS; Matz *et al.*, 2008a). Intracellular

toxins can be very effective, and the ingestion of a few violacein producing cells is sufficient to kill the predator (Matz *et al.*, 2004b). Moreover, extracellular toxins may act as deterrent for predators thereby redirecting them to alternative prey (Neidig *et al.*, in prep). However, repelling substances are only useful if the predator is able to select its prey (Pedersen *et al.*, 2009). TTSS injected toxins may also be useful in more complex interactions such as the intracellular growth inside of the predator (Declerck *et al.*, 2007). These defence traits are often regulated by cell signalling (Queck *et al.*, 2006), which may ensure that the investment in defence compounds preferentially benefits genetically related neighbours (Keller and Surette, 2006), but may be vulnerable to cheating (Denison *et al.*, 2003). These different antipredator strategies are not mutually exclusive, but, depending on the predation pressure and the bacterial community structure, occur simultaneously or follow one after the other (Salcher *et al.*, 2005). Interestingly, antifungal toxins from biocontrol bacteria are also very potent against protozoa (Jousset *et al.*, 2006), and predation increases the suppressiveness of the soil (Pussard *et al.*, 1994), suggesting that selective grazing may indirectly increase crop protection by fostering an antifungal bacterial community.

1.2.5. Objectives of this thesis

This work aims at understanding the biotic interactions involving biocontrol bacteria, and the impact of these interactions on the fitness and biocontrol efficiency of the introduced strains. In particular, it focuses on the role of secondary metabolites in interactions between biocontrol strains and indigenous microorganisms, especially microfaunal predators.

1.2.6. Organisms

Bacteria

In this work we used the model biocontrol strain *Pseudomonas fluorescens* CHA0 (Voisard *et al.*, 1994). This strain was isolated from the rhizosphere of tobacco of a Swiss suppressive soil. It produces an array of secondary metabolites (Figure 1), including 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolonitrin (PRN), and hydrogen cyanide (HCN). It is a potent biocontrol agent against various fungal phytopathogens (Haas and Defago, 2005) and possibly insect pests (Pechy-Tarr *et al.*, 2008). Moreover, this strain efficiently colonizes the rhizosphere and is highly resistant to predation (Jousset *et al.*, 2006). It is physiologically well characterized (Haas *et al.*, 2002) and genetically easy to investigate thanks to its similarity to the sequenced strain *P. fluorescens* pf-5 (de la Fuente *et al.*, 2006). Its secondary metabolism is finely regulated at the transcriptional and posttranscriptional level (Haas and Keel, 2003). Secondary metabolism is controlled at the transcriptional level by gene specific transcription factors (Baehler *et al.*, 2005; Schnider-Keel *et al.*, 2000) and by global regulators such as sigma factors (Pechy-Tarr *et al.*, 2005; Schnider *et al.*, 1995). Moreover, secondary metabolism is

regulated at the posttranscriptional level through the Gac/rsm regulatory cascade (Figure 3), which is involved in cell- to-cell signalling (Lapouge et al., 2008).

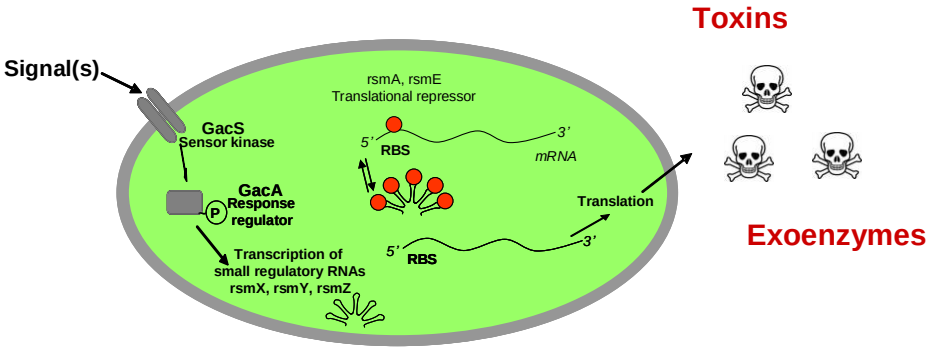

Figure 3: Simplified model of the post transcriptional regulation pathways of secondary metabolism by *Pseudomonas fluorescens* CHA0. Activation of the GacS sensor kinase by unknown signals triggers the phosphorylation and activation of the GacA response regulator. Phosphorylated GacS positively controls the expression of the small regulatory RNAs rsmX, rsmY and rsmZ. These RNAs bind to the translational inhibitors rsmA and rsmE that block the ribosomal binding site of the mRNAs, activating their translation.(Adapted fromHaas and Keel, 2003)

Recently, different *gfp* based reporter fusions have been developed for this strain, allowing easy screening of factors affecting secondary metabolism at the transcriptional (Baehler *et al.*, 2005; Rochat *et al.*, in prep; de Werra *et al.*, 2008) and post-transcriptional level (Dubuis *et al.*, 2006).

Amoebae

We used the naked amoeba *Acanthamoeba castellanii* (Figure 4) as a model predator in the different experiments. Naked amoebae constitute an important part of soil protozoa communities and play a major role in soil ecology by regulating bacterial density (Clarholm, 1981) and community structure (Kreuzer *et al.*, 2006), thereby linking primary consumers with higher trophic levels (Rodriguez-Zaragoza, 1994).

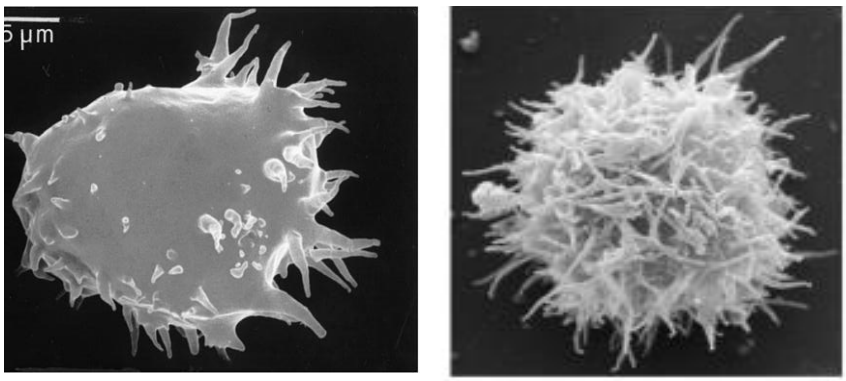

Figure 4: Scanning electron microscope pictures from trophozoites of the free living amoeba *Acanthamoeba castellanii* (Foissner, 1999; Khan, 2006).

A. castellanii is a generalist predator feeding on a wide range of bacteria (Pickup *et al.*, 2007) and fungi (Levrat *et al.*, 1991), and even on biofilm matrices (Parry, 2004). It produces extracellular

bacteriolytic enzymes such as glucanases, serine- and metalloproteases (Alfieri *et al.*, 2000; Serrano-Luna *et al.*, 2006; Weekers *et al.*, 1995). These enzymes help in digesting prey and detritus outside of the cell thereby widening the food spectrum. It harbours a diverse intracellular bacterial community which is in part presumably mutualistic (Horn and Wagner, 2004), although many pathogenic bacteria evade predation by growing inside of amoebae (Hilbi *et al.*, 2007). The genome of this species is widely sequenced, which allows getting important informations on its physiology and the receptors involved in processes like phagocytosis (Anderson *et al.*, 2005). The used *A. castellanii* strain was isolated from a woodland soil (Bonkowski and Brandt, 2002) and cultivated axenically (Rosenberg *et al.*, 2009). The use of axenic cultures allows cultivating amoebae at high density and avoiding artefacts from co-cultivated bacteria.

Plant

For investigating interactions of bacteria with plants barley (*Hordeum vulgare* L. cv. Barcke) was used as model system. Barley germinates and grows rapidly forming a large root system which allows setting up split root microcosms easily. The exudates of the barley variety used have been characterised recently (Lanoué et al, *submitted*), allowing to analyse potential molecules involved in the communication between plant and bacteria.

1.2.7. Experiments

The first experiment (**Chapter 2**) investigated the role of secondary metabolites in the interaction of the introduced bacteria with the indigenous microbial community. We compared the survival of the strain CHA0 with the one of its isogenic *gacS*$^-$ mutant CHA19. The latter strain is deficient in cell signalling and lacks production of all major exoproducts. This allows evaluating the importance of toxicity for bacterial competitiveness. The bacteria were inoculated in microcosms containing a natural bacterial community and the model bacterivorous amoeba *A. castellanii*. This design permits to determine the relative advantage of cell signalling and toxicity against competitors and predators.

The second experiment (**Chapter 3**) addressed the question of intraspecific competition and cheating by rhizosphere bacteria. pseudomonads coordinate the production of toxins by cell-signalling. However, signal-blind mutants spontaneously appear in pseudomonad populations, and profit from extracellular metabolites of the wild type population, potentially reducing biocontrol efficiency. We set up populations of *P. fluorescens* CHA0 containing increasing proportions of *gacS*$^-$ mutants. Populations were consumed by the amoeba *A. castellanii* and the nematode *Caenorhabditis elegans*. Experiments were carried out under batch conditions and in the rhizosphere.

- The third experiment (**Chapter 4**) investigated the adaptation of toxin production by *P. fluorescens* in response to predation risk. Toxicity efficiently protects the bacteria from predation but has a high metabolic cost. Consequently, bacteria may optimise the investment in defence compounds according to the predation risk. We used reporter transcriptional fusions in which the *gfp* gene was fused to the promoter of the first genes of the operons responsible for the biosynthesis of DAPG, PRN, PLT and HCN. The reaction of bacteria to *A. castellanii* or its supernatant was assayed in vitro and on the roots of barley. Fluorescence was determined either in a plate reader or by flow cytometry, and serve to infer the expression of the investigated toxic genes.

In the fourth experiment (**Chapter 5**) we set up a split root system (Figure 5) to determine if plants can manipulate the metabolism of rhizosphere bacteria to improve their needs. The established split root system allowed separating pathogens from biocontrol bacteria. Consequently, the only communication between the two compartments is plant mediated. On one side of the roots barley plants were challenged with the phytopathogen oomycete *Pythium ultimum*, while the other root part was inoculated with a mix of two reporter fusions of *P. fluorescens* CHA0 reporting the expression of bacterial growth as indicated by regulation of the ribosomal RNA operon and the antifungal gene *phlA*.

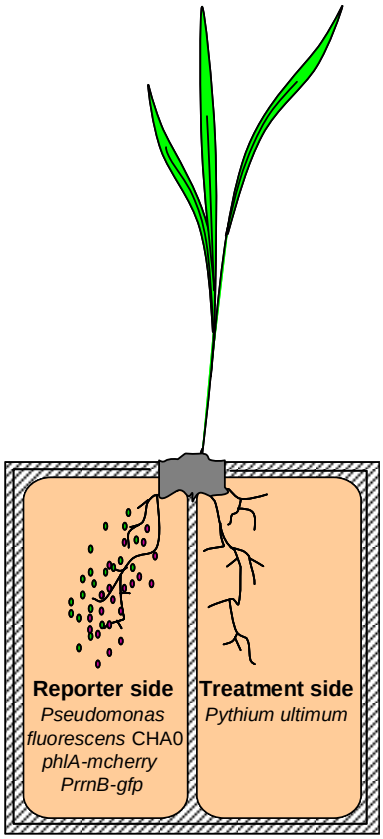

Figure 5: Schematic representation of the split root system used to investigate modulation of the metabolism of Plant Growth Promoting Rhizobacteria by plants

The expression of these two genes was measured simultaneously by flow cytometry. The use of the two genes permits to calibrate changes in bacterial metabolism upon plant infection, and to exclude purely nutritional effects (due for example to modifications of the plant carbon flow upon infection). In order to better understand the chemical signalling between plant and bacteria, a second split root experiment was carried out to investigate changes in root exudation upon infection with *P. ultimum*, and the exudates from infected and healthy plants were analysed by HPLC.

In the fifth experiment (**Chapter 6**) we followed the role of cell signalling for the integration of *P. fluorescens* into the soil food web by Stable Isotope Probing (SIP). Bacteria were labelled with ^{13}C and inoculated into non-sterile soil. After incubation, microbial ribosomal RNA was isolated and fractionated by ultracentrifugation. The eukaryotic community was determined by T-RFLP fingerprinting and the heavy rRNA fractions, including the organisms which incorporated C from the ^{13}C labelled bacteria, were subsequently cloned.

Chapter 2

Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria

2. Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria

2.1. Abstract

Rhizosphere bacteria live in close contact to plant roots feeding on root exudates and rhizodeposits. By producing toxic exoproducts rhizobacteria may inhibit plant pathogens thereby functioning as biocontrol agents and increasing plant fitness. However, the evolutionary basis why rhizobacteria protect plants is little understood. To persist toxin production needs to improve the competitiveness of the bacteria themselves.

We investigated the importance of secondary metabolite production for the establishment of the model soil biocontrol bacterium *Pseudomonas fluorescens* CHA0 in the rhizosphere of rice. We compared the performance of this toxin producing strain and its isogenic *gacS* deficient mutant defective in secondary metabolite production. The bacteria were added to the rhizosphere of rice where they had to compete with the indigenous flora for resources and to resist predation by the protist *Acanthamoeba castellanii*.

Secondary metabolite production strongly enhanced the establishment of the inoculated bacteria by improving competitive strength and predator resistance. The fitness gain due to attenuation of predation exceeded that due to competition by a factor of 2-3, confirming the importance of grazing resistance for rhizosphere bacteria

Biocontrol properties of PGPR such as *P. fluorescens* therefore gain a new dimension. Toxicity primary plays a role in the interaction with competitors and especially predators, and not in the protection of the host plant. Thus, establishment and efficiency of biocontrol bacteria may be improved by fostering predator defence via toxin production.

Keywords

Pseudomonas fluorescens / biocontrol bacteria / interspecific competition / protozoa / top-down

2.2. Introduction

Plants roots are colonized by specific bacterial communities living on root exudates and rhizodeposits. Some rhizobacteria produce exometabolites, which are toxic to many organisms, including plant soil borne pathogens. Toxin production makes them interesting as biocontrol organisms, and they are increasingly seen as a green alternative to agrochemicals (Weller, 2007). However, the evolutionary basis of the plant protective activity is still puzzling, since the effect appears to lack reciprocity. Toxin production is costly for the bacteria and a reward in form of more root exudates would be too unspecific to select for this trait (Denison *et al.*, 2003). Consequently, we hypothesize that there exist direct feedbacks of toxins produced by bacteria improving bacterial fitness. In particular, toxin-producing bacteria may benefit from reduced competition for resources by indigenous microflora and from increased resistance against predation. Indeed, bacteria lacking toxin production are impaired in their ability to colonize the rhizosphere of plants (Natsch *et al.*, 1994), suggesting that there is an intense selective pressure favouring toxic bacteria. Further, there is evidence that bacterial toxins indeed impair predator pressure (Jousset *et al.*, 2006). However, both reduced competition by competitors and exposure to predators lacks experimental proof from rhizosphere systems resembling those in the field.

For testing these hypotheses under natural conditions we established a model rhizosphere system with plant seedlings in soil containing a natural bacterial community with and without predators. The role of toxins for increasing competitiveness and reducing predation was investigated using the model biocontrol organism *Pseudomonas fluorescens* CHA0 which is widespread in the rhizosphere of mono- and dicotyledonous plants. It owes its biocontrol ability to diverse secondary metabolites, including cyanhidric acid, DAPG and exoproteases (Haas and Keel, 2003) which inhibit fungal pathogens, such as *Pythium ultimum* (Keel et al., 1992b) and *Fusarium oxysporum* (Zuber *et al.*, 2003). As in many pseudomonads, the production of secondary metabolites in *P. fluorescens* is controlled by a two-component gacS/gacA receptor system (Heeb and Haas, 2001) which is involved in the response to density-sensing signals. Activation of the gac cascade up-regulates the production of secondary metabolites through the production of three small RNAs, rsmX, rsmY and rsmZ. These molecules bind to the posttranscriptional inhibitor rsmA(Valverde *et al.*, 2003), thereby inducing translation of the corresponding mRNAs. Interestingly, spontaneous gacS/gacA deficient mutants occur at high frequency (Martinez-Granero *et al.*, 2005). These mutants are defective in secondary metabolite production and are less competitive in non-sterile environments (Chancey *et al.*, 2002; Natsch *et al.*, 1994) but not when bacterial diversity is low (Schmidt-Eisenlohr *et al.*, 2003), suggesting that toxins increase bacterial fitness by modulating biotic interactions.

For testing if secondary metabolites indeed increase the competitive strength of *P. fluorescens* CHA0 we compared the performance of the wild-type *P. fluorescens* CHA0 producing an arsenal of secondary metabolites with its isogenic *gacS* deficient mutant defective in secondary metabolite production. To determine the role of toxins for improving the competitiveness against other rhizobacteria, we followed the differential ability of these two strains to establish populations in the rhizosphere of plants in absence of predators. For investigating the role of toxins in attenuating predator pressure we compared the establishment of wild type bacteria in presence and absence of protozoan predators (*Acanthamoeba castellanii*).

2.3. Materials and methods

2.3.1. Experimental system

Natural rhizosphere systems were established in soil microcosms planted with rice. The microcosms were inoculated with a predator-free soil bacterial assemblage. *Acanthamoeba castellanii*, a ubiquitous soil bacterivorous amoeba, was added as model predator in a factorial design. Soil from a pasture near Heteren, the Netherlands (84.6% sand, silt 8.2%, clay 6.2%, carbon content 2.1%, C/N ratio 16.7)(van der Putten *et al.*, 2000) was sieved (2 mm), autoclaved and washed twice with a threefold volume of tap water on a 100 μm mesh to remove nutrients and toxins released by autoclaving. The washed soil was dried for 72 h at 70°C and rewetted to water holding capacity with distilled water (200 ml kg^{-1}). In order to keep moisture conditions constant during the experiment, 50 mg kg^{-1} soil of a water retaining polymer were added (Grain d'Eau, La Celle St-Cloud, France). A total of 50 g wet weight soil was filled in 3 x 20 cm glass tubes (Schott, Mainz, Germany) and autoclaved (121°C, 30 min).

Each tube was inoculated with a protozoa free soil bacteria assemblage at a concentration of 10^8 bacteria g^{-1} soil (see below), and incubated in the dark at room temperature in order to allow the bacterial population to grow and equilibrate. After 5 days 10^6 amoeba g^{-1} soil (total volume 1 ml) were added to the amoeba treatment. The control samples received 1 ml sterile Neff's modified amoeba saline (AS; Page, 1988). Five days later one rice seedling was transferred aseptically into each tube, and 12 h later the plants were inoculated with 10^6 *P. fluorescens* g^{-1} soil. Plants were grown at a constant temperature of 22°C and 16 h of light (500 μmol s^{-1} m^{-2}). The tubes were randomized daily.

2.3.2. Organisms and culture conditions

Bacteria for inoculation of the sterilized soil were isolated from the experimental soil as described previously (Kreuzer *et al.*, 2006) with few modifications. Briefly, 10 g of soil were suspended in 100 ml of NMAS and filtered through a paper filter. The filtrate was successively filtered through 5 μm and 1.2 μm membranes (Millipore, Schwalbach, Germany) to remove protists. The resulting filtrate was mixed in a 1:1 ratio with a diluted nutrient solution (0.8 g l^{-1} nutrient broth in NMAS) and incubated in 10 ml

tissue culture flasks. Cultures were checked after 4 and 6 days with an inverted microscope at 100x magnification for contamination by flagellates. Bacterial cultures were harvested by centrifugation (13,000 RPM for 5 min) and washed in NMAS prior to inoculation.

Strains of *P. fluorescens* CHA0 and its isogenic *gacS* deficient mutant CHA19 tagged with *gfp* were used (Jousset *et al.*, 2006). The strains were routinely kept on nutrient agar (blood agar base 40 $g \cdot l^{-1}$, yeast extract 5 $g \cdot l^{-1}$). Prior to inoculation a single colony was picked and incubated overnight in NYB medium (nutrient broth 25 $g \cdot l^{-1}$, yeast extract 5 $g \cdot l^{-1}$) at 28°C and agitation of 300 rpm. Cultures were washed twice in phosphate buffer saline (PBS) and resuspended in NMAS. Concentration of bacteria was determined on the base of the OD_{600} and checked under an epifluorescence microscope as described below.

Acanthamoeba castellanii was isolated from a woodland soil (Bonkowski, 2002) and kept axenically on PYG medium (peptone 20 $g \cdot l^{-1}$, yeast extract 5 $g \cdot l^{-1}$, glucose 10 $g \cdot l^{-1}$). Prior to inoculation 10 ml of a stationary phase culture were collected and washed twice by centrifugation (100 g, 10 min). The pellet was resuspended in NMAS, and the cell concentration was determined with a Neubauer counting chamber.

Rice seeds (*Oryza sativa* cv. Zhonghua11) were dehusked by grinding lightly with a pestle in a mortar, and surface sterilized by soaking in 96% ethanol for 1 min, and for 30 min in a solution containing 30 $g \cdot l^{-1}$ NaCl, 13 $g \cdot l^{-1}$ NaClO, 1 $g \cdot l^{-1}$ Na_2CO_3 and 1.5 $g \cdot l^{-1}$ NaOH (Hurek *et al.*, 1994). Seeds were separately pre-germinated in the dark at 28°C, in a 96-well microtiter plate containing 100 µl NMAS $well^{-1}$. After six days germinated seeds were checked for sterility with an inverted microscope (Nikon Diaphot, 100x magnification). Only sterile seeds were used for the experiment.

2.3.3. Analyses

On day 4, 8 and 16 after the set up the microcosms were destructively sampled. At harvest, shoots were cut at the base, dried (70°C, 72 h) and milled. Shoot carbon and nitrogen concentrations were analyzed from 3-5 mg of shoot material with an elemental analyser (EA 1108, Carlo Erba, Milan, Italy).

The roots were gently removed by shaking off the soil and rhizosphere bacteria were subsequently extracted (Normander *et al.*, 1999). Briefly, the roots were gently rinsed with sterile PBS to remove soil aggregates and placed in 8 ml PBS buffer. Samples were vortexed for 1 min, bath sonicated on ice for 2 x 30 s and again vortexed for 1 min. Samples (1 ml) were then fixed in 3% formaldehyde, filtered on a 0.22 µm TGTT membrane (Millipore, Schwalbach, Germany), and stained with 2.5 μg ml^{-1} DAPI; gfp tagged cells and total bacteria were enumerated with a Zeiss Axioscop 2 epifluorescence microscope at 400x magnification. The filter settings for DAPI and GFP counts were as follows:

excitation filter 365 and 470 nm, dichroic filter 395 and 493 nm and emission filter 397 and 505-530 nm, respectively. Images were taken in triplicate using a mounted Canon Powershot G5 digital camera. Bacteria were automatically counted using the ImageJ software (http://rsb.info.nih.gov/ij/). In order to check the accuracy of the automated counts some samples were counted by eye.

Soil microbial respiration, microbial biomass and the respiratory quotient (qO_2, i.e. the ratio of respiration to biomass) were determined using a substrate induced respiration method (SIR; (Anderson and Domsch, 1978; Beck *et al.*, 1997) with an automated respirometer (Scheu, 1992).

Amoebae were enumerated using a modified most probable number method (Darbyshire *et al.*, 1974). Five g of soil were gently shaken for 15 min in 20 ml NMAS, and 100 µl aliquots (4 replicates per sample) were successively diluted threefold in a suspension of 10^7 *E. coli* ml^{-1} in NMAS in 96-well microtiter plates and incubated in the dark at 15°C. Wells were checked for presence of amoebae after 3, 7 and 14 d under an inverted microscope (Nikon Diaphot, 100x magnification).

2.3.4. Statistical analyses

The experiment followed a 2×2 factorial setup investigating the effects of the inoculated bacterial strains (Wild type and *gacS* mutant) and predators (presence and absence of *A. castellanii*), with seven replicates per treatment. The results were analyzed by analysis of variance using the general linear model (GLM) procedure and type III sum of squares. The factors investigated the effect of the inoculated strain ("Strain"), and of the presence and absence of predators ("Amoeba"). Two contrasts were set up to further analyse the role of secondary metabolite production for the establishment of *P. fluorescence* strains: (1) The importance of toxicity in the interspecific competition with other rhizobacteria was evaluated by comparing the performance of the Wt and *gacS* strain in absence of predators ("Competition"); (2) the importance of toxicity against predators was inspected by comparing the performance of the Wt strain in treatments with and without predators ("Predation"). The relative importance of secondary metabolite production in these two interactions was estimated by comparing the proportion of the total variance explained by these two contrasts. The expected negative effect of the toxic Wt strain on the density of amoebae was evaluated by a one tailed Student's t-test comparing the density of amoebae in the microcosms inoculated with the two *P. fluorescens* strains. Prior to analyses the variables were inspected for homogeneity of variance, total bacterial densities were log-transformed and percent values were arcsin square root transformed. Statistical analyses were carried out using SAS 9.1 (SAS Institute, Cary, USA).

2.4. Results

Amoebae

At the end of the experiment, the abundance of amoebae (active and encysted) was two times higher in treatments containing the *gacS* strain, reaching 4.2 10^6 ind. g^{-1} soil, compared to 2.1 10^6 ind. g^{-1} soil in treatments with the Wt strain (t-test, p=0.006), suggesting that Wt bacteria negatively affected predator growth.

Total bacteria

Total bacterial density (DAPI counts g^{-1} root) increased from 5.0 10^8 ind. g^{-1} roots on day 4 to 6.9 10^8 ind. g^{-1} roots on day 8, and then decreased to 4.3 10^8 ind. g^{-1} roots on day 16, suggesting an active growth phase until day 8. Amoebae significantly reduced bacterial density by 37% on day 16 (
Table 1, Figure 6).

Table 1: ANOVA Table of F- and p-values on the effect of Strain (Wt or gacS) and Amoebae (with and without) on the density of total rhizobacteria and on the relative density of Pseudomonas fluorescens (as percentages of total bacteria) 4, 8 and 16 days after inoculation. In addition to effects of main factors and their interaction, contrasts have been calculated to evaluate the importance of competition and predation on the relative density of Wt or gacS strains of P. fluorescens. Significant effects (p<0.05) are highlighted in bold.

		Day 4		Day 8		Day 16	
Total bacterial density	d.f.	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Factors							
Strain	1	0·9	0·362	10·1	**0·004**	6·9	**0·014**
Amoebae	1	1·5	0·239	0·5	0·488	16·1	**< 0·001**
Strain × Amoebae	1	1·3	0·269	2·3	0·144	5·2	**0·031**
Relative density of *P. fluorescens*							
Factors							
Strain	1	2·1	0·166	38·2	**< 0·001**	43·9	**< 0·001**
Amoebae	1	0·3	0·602	14·2	**< 0·001**	13·0	**0·001**
Strain × Amoebae	1	0·6	0·433	6·21	**0·019**	9·8	**< 0·001**
Contrasts							
Competition	1	0·2	0·658	11·5	**0·002**	6·6	**0·016**
Predation	1	0·8	0·381	17·4	**< 0·001**	22·6	**< 0·001**

Interestingly, this effect was less pronounced in presence of the Wt strain (significant Amoeba x Strain interaction; Table 1) suggesting that the presence of toxic bacteria protected to some extent the whole bacterial community from grazing (Figure 6). Similar to total microbial numbers, amoebae reduced soil microbial respiration from 1.003 to 0.802 μl O_2 h^{-1} g^{-1} soil (ANOVA, $F_{1,28}$=5.7, p= 0.02) with the reduction tending to be more pronounced in the *gacS* treatment (-28%) than in the Wt treatment (-10%; ANOVA, $F_{1,28}$=3.6, p= 0.07). Microbial biomass (260.6 ± 55.0 μg C g^{-1} soil) did not significantly differ between treatments.

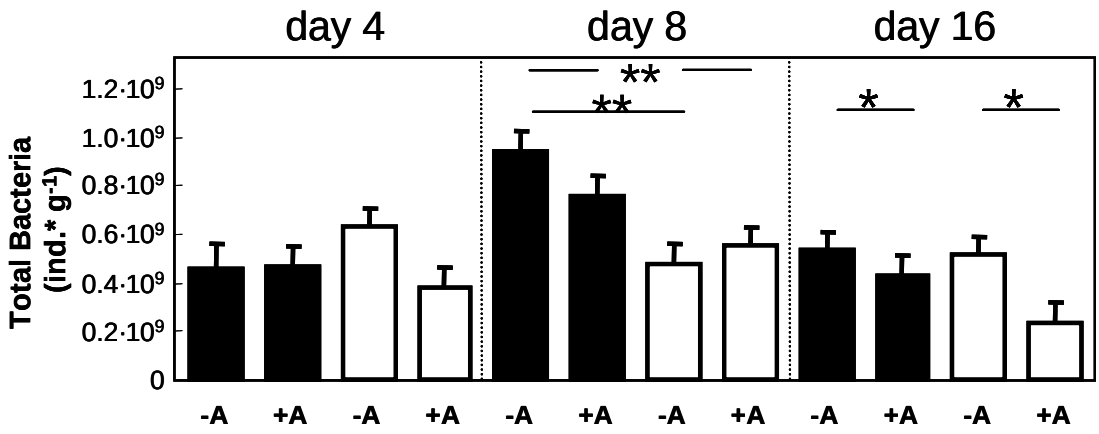

Figure 6 : Effect of inoculation with *Pseudomonas fluorescens* Wt and *gacS* strains and the presence of predators (*Acanthamoeba castellanii*; -A, without; +A, with) on total density of bacteria (DAPI counts; ind. g^{-1} fresh weight of roots) in the rhizosphere of rice. Black bars: Wild type (Wt) strain, white bars: *gacS* strain. Error bars represent ± SE. Horizontal bars show significant effect between treatments (* p<0.05, ** p<0.01, *** p< 0.001) as described in Table 1.

Pseudomonas fluorescens

Root colonization by *P. fluorescens* (GFP counts g^{-1} root) rapidly increased during the experiment, with the differences between treatments being most pronounced at the end of the experiment. Numbers of *gacS* mutant bacteria remained low during the whole experiment. Their relative densities decreased slowly from 3.4% of the total rhizobacteria on day 4 to 1.7 and 2.0 % on day 8 and 16 (Figure 7).

The Wt strain more successfully colonized the roots. In absence of predators its density significantly exceeded that of the *gacS* strain by factors of 2.9 and 1.7 at day 8 and 16, respectively (effect of "Competition"; Table 1, Figure 7), suggesting that the toxin-mediated increase in competitive strength against other rhizobacteria was most pronounced on day 8 when the number of total bacteria was at a maximum.

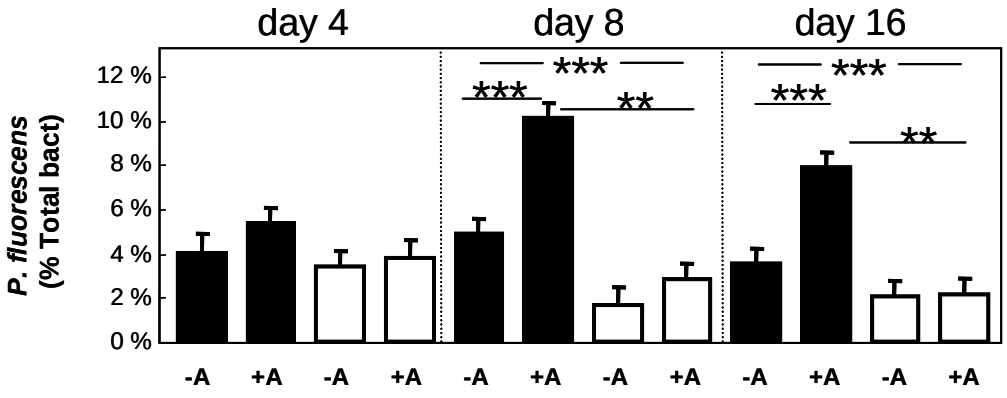

Figure 7: Relative colonization of the rhizosphere of rice by *Pseudomonas fluorescens* Wt and *gacS* strains in absence (-A) or presence (+A) of predators (*Acanthamoeba castellanii*); data are expressed as percentages of the total number of rhizosphere

bacteria in respective treatments (see Fig. 1). Black bars: Wild type (Wt) strain, white bars: *gacS* strain. Error bars represent ± SE. Horizontal bars show significant effect between treatments (* p<0.05, ** p<0.01, *** p< 0.001) as described in Table 1.

The increase in density of the Wt strain was even more pronounced in presence of amoebae. Compared to the *gacS* strain it was significantly increased by factors of 3.7 and 3.7 at day 8 and 16, respectively (significant effect of "Predation"; Table 1, Fig. 2), suggesting that compared to increasing competitiveness secondary metabolites confer an even stronger advantage by attenuating predator pressure. Indeed, the effect of "Predation" were not significant at day 4 (Table 1), but explained 19 and 24% of the total variance in density of *P. fluorescens* at day 8 and 16, respectively, whereas the effect of "Competition" only explained 11 and 5% (Table 1)

2.4.1. Discussion

Using a semi-natural rhizosphere system, we investigated for the first time the role of bacterial secondary metabolites in modifying the two major structuring forces of food webs, i.e. bottom-up (competition for resources) and top-down (predation) control. Conform to our hypothesis and in agreement with past observations in non-sterile soil (Chancey *et al.*, 2002), bacteria lacking secondary metabolite production were less competitive than toxin producing wild type bacteria. Other traits associated with gac deactivation, such as increased siderophore production (Heeb and Haas, 2001) unlikely contributed to reduced competitiveness. This suggests that secondary metabolites of biocontrol bacteria indeed primarily function in improving bacterial fitness via targeting other rhizosphere organisms rather than improving plant growth and pathogen resistance. In addition to affecting competitors, bacterial toxins from *P. fluorescens* also reduced predator pressure by protists. Remarkably, the gain in fitness (measured as increase in relative density) of toxic bacteria by avoiding predation on themselves and increasing predation on competing bacteria exceeded that caused by improved competitive strength. Further, the advantage due to attenuated predator pressure increased faster than that due to increased competitive strength. Top-down control is an important factor for the establishment of bacterial populations in the rhizosphere (Christensen *et al.*, 2007). Our results suggest that toxin production is an efficient strategy to alleviate losses from predation and by increasing predation on competitors gaining in competitiveness. In fact, for improving competitiveness this strategy may be more efficient than inhibiting competing bacteria via e.g. investing in bacteriotoxic substances.

This functioning of metabolites in attenuating predator pressure sheds new light on the classical theory of antibiosis, according to which the primary function of bacterial toxins is to damage competing microorganisms (Clardy *et al.*, 2006). The limited advantage of wild type strain bacteria in absence of predators in the present and previous experiments (Johansen *et al.*, 2002) and the reduction in predator pressure suggest that in fact secondary metabolites primarily target against predators rather

than against competing bacteria and fungi. Rhizobacteria in fact generally appear to suffer heavily from predation, especially by protists and nematodes (Bonkowski, 2004), supporting our conclusion that attenuating the impact of predators is vitally important.

Protozoan predation is known to structure bacterial communities in aquatic and terrestrial habitats including the rhizosphere of plants (Bonkowski, 2004; Matz and Kjelleberg, 2005; Pernthaler, 2005). Consequently, adaptations for reducing predation by microfaunal predators, such as biofilm formation and toxin production, are widespread among bacteria (Matz *et al.*, 2004a; Queck *et al.*, 2006), including *P. fluorescens*, which have been shown previously to harm eukaryotic predators (Jousset *et al.*, 2006).

Results of the present study indicate that repelling predators not only reduces losses from predation, but even allows toxic bacteria to increase in numbers. This suggests that toxic bacteria take benefit of predators, presumably by redirecting them towards neighbouring bacteria. The potential enemy is thus transformed into an ally and toxic strains profit three times. They avoid losses due to predation, they reduce the density of competitors and thereby, through excretion, release nutrients locked up in the cells of competitors, making them available for growth of toxin producing bacteria.

Predators are driving agents of the community composition of rhizosphere bacteria (Rønn *et al.*, 2002). High density of toxic bacteria therefore likely alters the impact of predators on the whole bacterial community. Supporting this assumption, total bacterial densities were less affected by predators in presence of the *P. fluorescens* Wt strain, and in turn, the density of amoebae was lower. This supports the above suggestion that via changing predator pressure toxin producing bacteria indirectly affect the structure of the whole bacterial community. As indicated by reduced microbial respiration in presence of the *gacS* mutant, toxin-mediated attenuation of predator pressure not only affects microbial community structure and increases total bacterial density but also increases microbial community functioning.

The colonization of the *P. fluorescens* Wt and mutant strain increasingly diverged during the experiment, suggesting that the advantage of producing secondary metabolites increased with time. This indicates that biotic interactions are of minor importance during early phases of rhizosphere colonization, but that in mature communities of older roots biotic interactions predominate, thereby favouring toxin-producing strains. This is in agreement with the preferential colonization of root tips by toxin-deficient mutants of *Pseudomonas* (Achouak *et al.*, 2004) a niche with reduced competition for resources and exposure to predators (Folman *et al.*, 2001).

2.5. Conclusions

The results of the present study challenge previous views on the role of secondary metabolite production by plant growth promoting rhizobacteria. Conform to our hypotheses, secondary

metabolites of biocontrol rhizobacteria primarily improved bacterial fitness by manipulating biotic interactions with other rhizosphere organisms. Secondary metabolites improved the competitive strength against other rhizobacteria but in particular they attenuated losses due to protozoan predators. Thus, bacterial fitness and biocontrol properties are linked in a causal way providing an evolutionary explanation for the biocontrol activity of rhizosphere bacteria such as *P. fluorescens*. Since predators may promote the establishment of *P. fluorescens* strains, knowledge on soil protozoan predators and their interactions with bacterial prey may allow improving management strategies employing plant growth promoting rhizobacteria in arable systems. Biotic interactions between biocontrol bacteria and their predators have been traditionally neglected, the effects on soil protozoa being seen as mere side effects of inoculation (Winding *et al.*, 2004). In contrast to this view, results of the present study suggest that the functioning of biocontrol bacteria can only be understood considering biotic interactions in particular top-down forces. Potentially, the biocontrol activity of bacterial secondary metabolites exerted on plants itself results from side effects of the toxins due to the fact that they evolved for manipulating eukaryotic organisms (predators).

2.6. Acknowledgements

We thank Dr. Claudio Valverde (Universidad Nacional de Quilmes, Argentina) for his helpful advices, Dr. Xin Ke (Institute of Crop Breeding and Planting, Chinese Academy of Agricultural Sciences, China) for providing the rice seeds, and Katja Rosenberg (TU Darmstadt, Germany) for providing axenic *Acanthamoeba* cultures. This work was partially funded by the fellowship program of the German Federal Foundation for the Environment (DBU).

Chapter 3

Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters

3. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters

3.1. Abstract

Soil pseudomonads increase their competitiveness by producing toxic secondary metabolites which inhibit competitors and repel predators. Toxin production is regulated by cell-cell signalling and efficiently protects the bacterial population. However, cell communication is unstable, and natural populations often contain signal blind mutants displaying an altered phenotype defective in exoproduct synthesis. Such mutants are weak competitors, and we hypothesized that their fitness depends in natural communities on the exoproducts of wild-type bacteria, especially defence toxins. We established mixed populations of wild-type and signal blind, non-toxic *gacS* deficient mutants of *Pseudomonas fluorescens* CHA0 in batch and rhizosphere systems. Bacteria were grazed by representatives of the most important bacterial predators in soil, nematodes (*Caenorhabditis elegans*) and protozoa (*Acanthamoeba castellanii*). The *gacS* mutants showed a negative frequency dependent fitness and could reach up to one third of the population, suggesting that they rely on the exoproducts of the wild-type bacteria. Both predators preferentially consumed the mutant strain, but populations with a low mutant load were resistant to predation, allowing the mutant to remain competitive at low relative density. The results suggest that signal blind *Pseudomonas* increase their fitness by exploiting the toxins produced by wild-type bacteria, and that predation promotes the production of bacterial defence compounds by selectively eliminating non-toxic mutants. Therefore, predators not only regulate population dynamics of soil bacteria but also structure the genetic and phenotypic constitution of bacterial communities.

Keywords
Amoebae / cheating / nematodes / *Pseudomonas fluorescens* / protozoa / soil

3.2. Introduction

Fluorescent pseudomonads are ubiquitous rhizosphere bacteria, and many species produce toxic exoproducts that increase their resistance to microfaunal predators and improve their competitiveness against other bacteria (Jousset *et al.*, 2008). These toxins often inhibit plant pathogens, making pseudomonads potent biological control agents in agricultural systems (Haas and Defago, 2005). Toxin production in *Pseudomonas* is controlled at the post-transcriptional level by the gac/rsm cascade (Lapouge *et al.*, 2008). Activation of the GacS/GacA two component system by unknown signals leads to the production of small RNAs which remove the translational inhibition of secondary metabolites. This cell-to-cell signalling allows the bacteria to coordinate their behaviour, activating the secondary metabolism at high bacterial density (Dubuis *et al.*, 2007; Haas and Keel, 2003).

Pseudomonas fluorescens CHA0 is an effective colonizer of the rhizosphere of mono- and dicotyledonous plants possessing remarkable biocontrol efficiency (Voisard *et al.*, 1994) and high resistance to predation (Jousset *et al.*, 2006) due to the production of an array of secondary metabolites including toxins such as hydrogen cyanide (HCN), 2, 4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) (Haas *et al.*, 2002).

Initially rhizosphere bacteria originating from a single cell contribute equally to toxin production (de Werra *et al.*, 2008), but cell signalling is genetically unstable, with *gacS/gacA* genes being frequently mutated and inactivated (van den Broek *et al.*, 2005b; Martinez-Granero *et al.*, 2005). The resulting mutants are impaired in cooperative group traits, such as extracellular enzymes and toxin production (Lapouge *et al.*, 2008), but under laboratory conditions show an improved growth rate compared to their wild-type ancestors due to the cessation of secondary metabolism (Bull *et al.*, 2001). Despite being weak competitors when inoculated alone (Natsch *et al.*, 1994), *gacS/gacA* mutants multiply rapidly within soil pseudomonad populations (Chancey *et al.*, 2002; Martinez-Granero *et al.*, 2005; Sanchez-Contreras *et al.*, 2002). Consequently, we hypothesised that these mutants gain advantage by exploiting the exoproducts of the wild-type population, with their competitiveness being highest at low frequency in a dense wild-type population (Velicer *et al.*, 2000). In particular mutants may benefit from the production of defence toxins that protect them from predators.

Predation significantly affects bacterial communities (Blanc *et al.*, 2006; Rønn *et al.*, 2002), and many bacteria evolved defence mechanisms such as the production of toxins. Toxic exoproducts protect bacteria by repelling predators, resulting in prey-switching towards more palatable prey (Jezbera *et al.*, 2006; Liu *et al.*, 2006), and at high toxin concentrations inhibit or even kill the predators (Matz *et al.*, 2004b). Predators therefore affect the fitness of bacteria at the individual level by selective feeding on non toxic cells, and at the population level by preferentially consuming bacteria from populations that are little protected by toxins, i.e. those containing few toxin producing bacteria. Therefore, signal blind, non toxic *gacS/gacA* mutants should be preferentially consumed by predators, resulting in a

reduced fitness. Mutants in mixed populations neither contribute to autoinducer signals nor to toxin production (Dubuis and Haas, 2007) and high mutant load has been shown to reduce toxin-mediated biocontrol activity of *P. fluorescens* (Duffy and Defago, 2000).Consequently, increasing frequency of *gacS/gacA* mutants may result in a lower predation resistance of the whole population.

We established populations of *P. fluorescens* CHA0 containing an increasing proportion of signal blind non-toxic *gacS* mutants in batch and rhizosphere systems. We exposed these populations to representatives of the two major groups of bacterial predators in soil, naked amoebae and nematodes, to investigate if toxins differentially affect bacterial predators (Weitere *et al.*, 2005). If *gacS* mutants exploit the exoproducts of the wild type they should perform better at low density, and we tested if this strain is subject to negative frequency dependent selection. The lack of defence compounds should however make bacteria vulnerable to predation, and we tested if predators of different functional groups (amoebae and nematodes) preferentially consume non-toxic mutants. Since *gacS* mutants do not contribute to the defence of the population, and we tested if populations with higher loads of *gacS* mutants suffer from increased predation pressure by the different predators.

3.3. Methods

3.3.1. Organisms

The wild-type strains *P. fluorescens* CHA0 (Voisard *et al.*, 1994) and its isogenic *gacS* deficient mutant CHA19 (Zuber *et al.*, 2003) were tagged with two different fluorescent proteins allowing easy detection. A DsRed-tagged derivative CHA0-*r* was constructed as follows: A 0.7-kb *Sma*I-*Hin*dIII fragment from pDsRed.T3_S4T, containing the *dsred.T4_S4T* gene which encodes a rapidly maturing red fluorescent protein variant with strongly enhanced brightness (Sorensen *et al.*, 2003), was cloned under the control of the constitutive P_{tac} promoter in pME6552. A 1.5-kb *Mlu*I-*Hin*dIII fragment from the resulting plasmid carrying the P_{tac}-*dsred.T3_S4T* fusion was then cloned into the mini-Tn*7*-Gm carrier plasmid pME3280b (Zuber *et al.*, 2003). The construct obtained, pME7160, and the Tn*7* transposition helper plasmid pUX-BF13 (Bao *et al.*, 1991) were co-electroporated (Baehler *et al.*, 2006) into the recipient strain CHA0. The *gacS* mutant CHA19 (Zuber *et al.*, 2003) was tagged with a green fluorescent protein (GFP) as follows: a mini-Tn*7*-*gfp*2 cassette carrying the *gfp*mut3 gene controlled by a modified P_{lac} promoter (Koch *et al.*, 2001) was introduced into the chromosome of strain CHA19 using the transposition helper pUX-BF13, giving CHA19-*g*. The strains were kept routinely on nutrient agar plates (blood agar base 40 g L^{-1}, yeast extract 5 g L^{-1}) containing 8 μg mL^{-1} gentamycine sulfate (Sigma-Aldrich, Munich, Germany). Preliminary experiments under batch experiment conditions showed that labelling did not affect the growth characteristics of the two strains, and that no spontaneous *gac* mutants of the DsRed-tagged wild-type strain could be detected in mixed communities in the tested time frame (data not shown).

Prior to inoculation bacteria were grown in NYB medium (nutrient broth 25 g L^{-1}, yeast extract 5 g L^{-1}) at 30°C with agitation. Late exponential phase bacteria were harvested by centrifugation (13,000 rpm for 2 min) and washed three times in Neff's modified amoeba saline (AS; Page, 1988). The concentration of bacteria was determined by measuring optical density at 600 nm (OD_{600}) and checked under a Zeiss Axioscope 2 epifluorescence microscope at 400x magnification.

The naked amoebae (*Acanthamoeba castellanii*) isolated from a woodland soil (Bonkowski and Brandt, 2002) were grown axenically on PYG medium (peptone 20 g L^{-1}, yeast extract 5 g L^{-1}, glucose 10 g L^{-1}). Cells were harvested by centrifugation (100 g, 10 min) and washed twice in AS prior to inoculation.

The bacterivorous nematode *Caenorhabditis elegans* was grown axenically in liquid medium (peptone 30 g L^{-1}, yeast extract 30 g L^{-1}, haemoglobin 500 mg L^{-1}, cholesterol 1 mg L^{-1}). Prior to inoculation juveniles and adults were collected on a 5 μm mesh, washed with sterile PBS and resuspended in AS.

3.3.2. Experimental systems and setup

Two experimental systems of different complexity were established. In a simple batch system the relative fitness of non-toxin producing *P. fluorescens* mutant bacteria was investigated at six levels of mutant frequency. In complex rhizosphere systems the fitness of wild-type and non-toxin producing bacteria affected by both protozoan and nematode predators was investigated at three levels of mutant frequency.

Batch experiment: The experiment was set up in 96-well microtiter plates with mixed populations of *P. fluorescens* containing an initial frequency of 1, 10, 33, 66, 90 and 99% *gacS* mutants. Bacteria were inoculated at an initial concentration of 5 x 10^7 mL^{-1} in AS containing 300 mg L^{-1} PYG medium, in presence or absence of *A. castellanii* (5000 ind. mL^{-1}). Plates were grown at 22°C under agitation (150 rpm). Preliminary experiments indicated no biofilm formation on the walls of the plate under the tested conditions (data not shown).

Rhizosphere experiment: The experiments were set up in microcosms filled with quarz sand and planted with rice. Quartz sand (grain size 100 μm - 1.4 mm) was dried (72 h, 60°C) and rewetted with 10% (w/w) of a modified Yoshida hydroponic nutrient solution (pH 6.0) containing 1.43 mM NH_4NO_3, 0.37 mM NaH_2PO_4 x 2 H_2O, 0.5 mM K_2SO_4, 1 mM $CaCl_2$ and 1.6 mM $MgSO_4$ x 7 H_2O (Walia *et al.*, 2005). The microcosms consisted of 30 x 200 mm glass tubes (Schott, Mainz, Germany) filled with 50 g of the wet autoclaved sand (30 min, 121°C).

Rice seeds (*Oryza sativa* cv. Zhonghua11) were dehusked by careful grinding with a pestle in a mortar, and surface sterilized by soaking in 96% ethanol for 1 min and for 30 min in a solution containing 30 g L^{-1} NaCl, 13 g L^{-1} NaClO, 1 g L^{-1} Na_2CO_3 and 1.5 g L^{-1} NaOH (Hurek *et al.*, 1994). Seeds were separately germinated in 96-wells microtiter plates containing 100 µl AS. After 6 days sterility was

checked under a Nikon Diaphot inverted microscope at 100x magnification. Sterile seedlings were aseptically transferred to the microcosms, and the plants were grown at a constant temperature of 22°C and a 16 h photoperiod (500 μmol s^{-1} m^{-2}).

Three different mixtures of the bacterial strains *P. fluorescens* CHA0-*r* (wild-type) and CHA19-*g* (*gacS* mutant) were set up, with an initial frequency of 10% 50% and 90% *gacS* mutants, and inoculated at a total concentration of 10^6 bacteria g^{-1} sand (dry weight). Three predator treatments were established: a control without predators (Ctrl), addition of the bacterivorous amoeba *A. castellanii* (Amo), and addition of the bacterivorous nematode *C. elegans* (Nem). Amoebae were inoculated at a density of 10^4 g^{-1} sand and nematodes at a density of 50 g^{-1} sand which correspond to their natural densities in the soil. Seven replicates were set up per treatment. Negative controls consisted of non-inoculated sterile rice seedlings.

3.3.3. Measurements

In the batch experiment 100 μl aliquots were collected after 48 h and fixed in 3% formaldehyde. The rhizosphere experiment was terminated after 12 days. Roots were removed from the sand, fixed in 8 ml of ice-cold PBS buffer containing 3% formaldehyde. Rhizosphere bacteria were detached by vortexing the roots for 2 min, sonicating two times for 1 min and vortexing again for 2 min.

To enumerate bacteria 100 µl aliquots of bacterial suspensions were filtered on a 0.2 µm polycarbonate membrane and counted under a Zeiss Axioscop 2 plus epifluorescence microscope at 400x magnification. The filter settings for GFP and DsRed counts were, respectively, an excitation filter of 470 and 546 nm, a dichroic filter of 493 and 580 nm, and an emission filter of 505-530 and 590 nm. Images were taken in triplicate using a Canon Powershot G5 digital camera and bacteria were automatically counted using the ImageJ software (http://rsb.info.nih.gov/ij/). Red and green bacteria were counted separately. Unspecific excitation of the fluorescent proteins was assayed first with pure cultures of the two strains and did not interfere with the counts (data not shown).

Active nematodes were extracted in Baermann funnels, fixed in a 6% formaldehyde solution and enumerated at 25x magnification. Amoebae were enumerated at the end of the experiment using a modified most probable number method (Darbyshire *et al.*, 1974). Briefly, 5 g of sand were gently shaken for 15 min in 20 ml AS, and 100 µl aliquots (4 replicates per sample) were successively threefold diluted in a suspension of 10^7 *E. coli* mL^{-1} in AS in 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany) and incubated in the dark at 15°C. Wells were checked for presence of active and encysted amoebae after 3, 7 and 14 days using an inverted microscope at 100x magnification.

3.3.4. Statistical analyses and modelling of populations dynamics

The relative fitness ν of the *gacS* mutant was calculated by comparing its frequency at the beginning and end of the experiment as described by Ross-Gillespie *et al.* (2007):

$\nu = X_2*(1-X_1)/X_1*(1-X_2)$

with X_1 the initial and X_2 the final frequency of the strain. The stable frequency of *gacS* mutants in the population was defined according to the regression slope as $\nu=1$.

The predation pressure *p* on the roots was estimated as

$p = (Y_c-Y_p)/Y_c$

where Y_c is the bacterial density in the control treatment, and Y_p the bacterial density in the tested sample. The food selectivity index *s* of predators was calculated by dividing the percentages of *gacS* mutants in the diet of the predators and in the population. These two proportions were defined as the contribution of the *gacS* mutants to the total predation pressure ponderated by the percentage of mutants in non grazed populations, respectively

$s = (Z_c-Z_p)/(Y_c-Y_p)*X_c$

where X_c is the proportion of *gacS* mutants and Z_p and Z_c the total numbers of *gacS* mutants in the predator and control treatment, respectively. An index of 1 indicates absence of preference for one of the two strains, an index > 1 preference for the *gacS* mutant.

The relative fitness of the *gacS* mutant, the predation pressure and the predator selectivity were analysed with a general linar model (GLM; type III sum of squares) investigating the effect of predator treatment (factor) and the original mutant frequency (linear descriptor) in a full factorial design. Data were analyzed using Statistica 7.0 (StatSoft Inc, Tulsa, USA). Prior to statistical analyses data were checked for homogeneity of variances and log-transformed if necessary; percentage values were arcsin square root transformed prior to analysis to meet assumptions of homoscedasticity.

3.4. Results

Fitness of the gacS mutant

The *gacS* deficient mutant was subject to negative frequency dependent selection both in batch and rhizosphere systems (Figure 8 & 2). Its relative fitness (ν) decreased in parallel with its frequency in the population, suggesting that the mutant was not able to displace wild-type populations, but instead required an elevated wild-type density for maximum fitness. In the batch system pure cultures of *gacS* mutant grew better than the wild type in the exponential phase, but reached lower densities (see Fig. S1 in supplementary information).

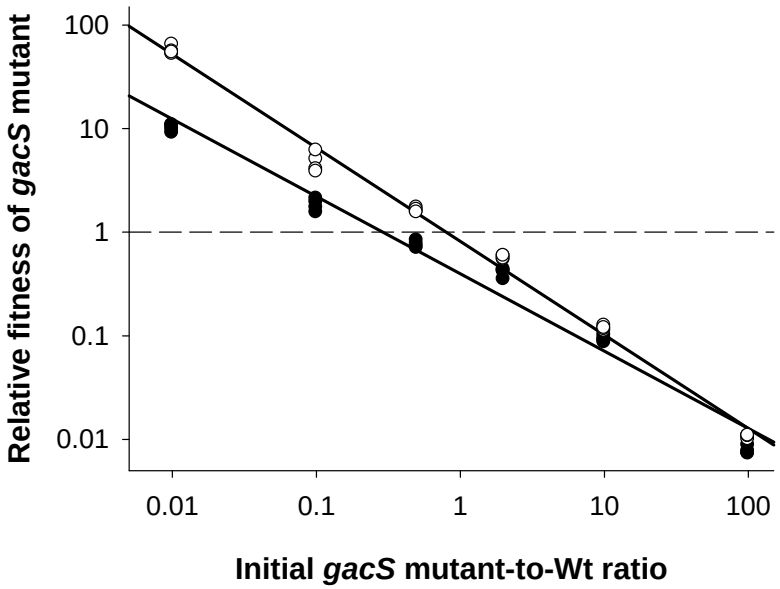

Figure 8 Relative fitness of the *gacS* mutant of *Pseudomonas fluorescens* CHA0 in the batch experiment after 48 h as a function of its initial frequency (initial *gacS* mutant-to-wild-type ratio) in presence (black circles) or absence (open circles) of the predator *Acanthamoeba castellanii.* The relative fitness of the *gacS* mutant is expressed as ratio between its final and initial frequency; the two strains are at equilibrium at a relative fitness of 1.

In mixed cultures the relative fitness of the mutant was negatively correlated with its initial frequency, showing a strong decline at high initial densities (r^2=0.96, p<0.001; Fig. 1). In absence of amoebae the *gacS* mutant was fitter than the wild-type at frequencies below 35%, where both strains were at equilibrium (ν=1). Amoebae rapidly reduced the fitness of the mutant strain (see Fig. S2), especially at low mutant frequency ($F_{1,44}$=34.7, p<0.001 for the interaction between predator and mutant frequency; Figure 8), suggesting food preference for the non-toxic strain. Predators thus shifted the relationship between frequency and relative fitness of the *gacS* mutant, reducing its equilibrium frequency to only 20% ($F_{1,44}$=6.37, p=0.015). Similar shifts occurred in the rhizosphere (Figure 9); likewise the fitness of the *gacS* mutant was negatively correlated with its initial frequency ($F_{2,49}$= 295.4, p<0.001).

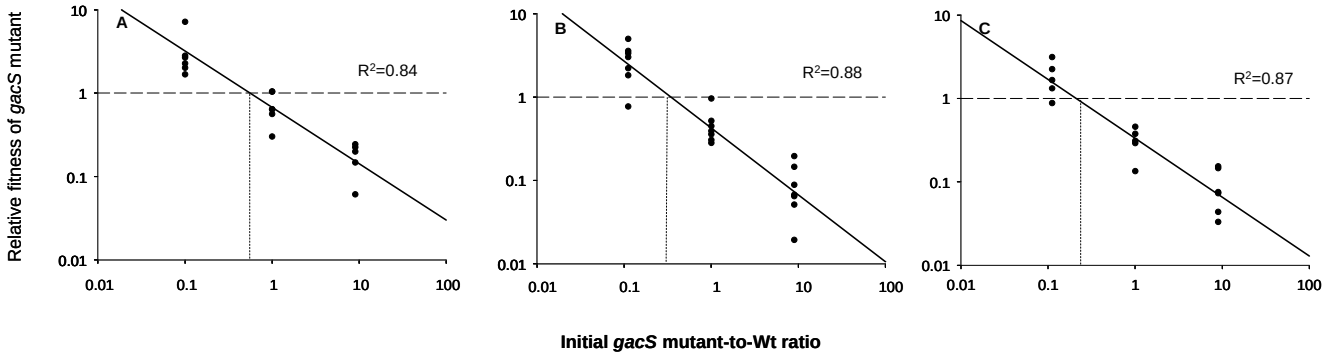

Figure 9: Relative fitness of the *gacS* mutant of *Pseudomonas fluorescens* CHA0 in the rhizosphere of rice in relation to the initial *gacS* mutant-to-wild-type ratio without predators (A), with *Caenorhabditis elegans* (B) or *Acanthamoeba castellanii* (C). The vertical dashed lines indicate the frequency at which both strains are at equilibrium.

Both predators reduced the fitness of the *gacS* mutant strain. They significantly shifted the intercept of the regression lines between *gacS* mutant frequency and relative fitness lowering the equilibrium mutant frequency from 37% in the control treatment to 26% and 18% in the nematode and amoebae treatments, respectively ($F_{2,49}=7.2$, $p=0.0015$, Figure 10). Linear regressions explained well the observed changes in mutant frequency ($r^2=0.84$, 0.88 and 0.87, $p<0.001$ for control, nematode and amoeba treatments, respectively).

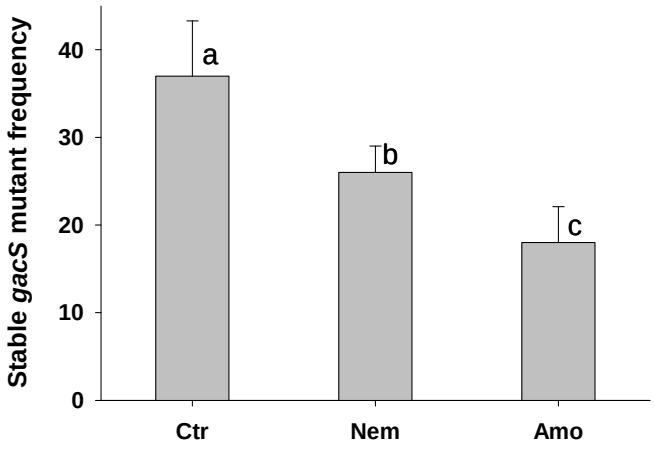

Figure 10: Predicted frequency at which the wild-type and *GacS* mutants of *Pseudomonas fluorescens* CHA0 are at equilibrium in bacterial populations without predator (Ctr), with *Caenorhabditis elegans* (Nem) or *Acanthamoeba castellanii* (Amo). Error bars indicate ± SE (N=6) for the intercept of the regression slopes (see Figure 9); different letters indicate significant differences between the intercept of the regression slopes (Tukey's HSD test, $\alpha=0.05$).

Prey selection

Both predators preferentially consumed *gacS* mutants in the rhizosphere, with preferences being more pronounced at low mutant frequency ($F_{2,32}=9.5$, $p=0.001$), suggesting active food selection. However, food selectivity strongly differed between predators ($F_{1,32}=4.2$, $p=0.025$). The nematodes were less

selective (Figure 11), and their diet composition was only marginally influenced by the frequency of the two bacterial strains. In contrast, prey selection of amoebae decreased parallel to the increase in mutant frequency. The preference index indicates that amoebae preferentially consumed the mutant strain when present at low frequency (s=2.4), but discriminated little between bacterial strains at the initial mutant frequency of 90% (s=1.16, Figure 11).

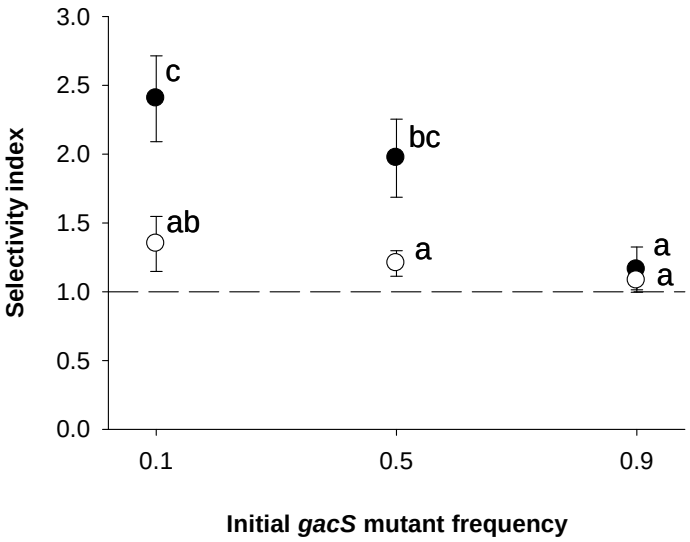

Figure 11: Food selectivity of *Acanthamoeba castellanii* (black circles) and *Caenorhabditis elegans* (white circles) at different frequencies of *gacS* mutants of *Pseudomonas fluorescens* CHA0. Values above 1 indicate preference for *gacS* mutants. Error bars indicate ± SE; different letters indicate significant differences between means at p<0.05 (Tukey's HSD test).

Predation intensity

In absence of predators bacteria reached in the rhizosphere an average total density of 5.5 x 10^6 cells (root system)$^{-1}$ irrespective of the initial mutant frequency ($F_{2,48}$=1.14, p=0.328), suggesting that the carrying capacity of the system was independent of the initial mutant frequency. Nematodes and amoebae strongly reduced total bacterial numbers at the end of the experiment ($F_{2,48}$=24.4, p<0.001), with the effect of nematodes exceeding that of amoebae. Increasing *gacS* mutant frequency differently affected the predation pressure of the two predators ($F_{2,32}$=3.7, p=0.035 for the interaction between predator type and initial mutant frequency). Nematodes grazed on bacterial populations at all densities, thereby reducing bacterial numbers by 56, 58 and 69% in 10, 50 and 90% mutant treatments. In contrast, predation by amoebae increased with the frequency of *gacS* mutants, reducing bacterial numbers by 11, 36 and 56% in 10, 50 and 90% mutant treatments (Figure 12). This suggests that bacterial toxins efficiently protected the population against amoebae but less against nematodes. Increasing mutant frequency resulted in a loss of this protection, confirming that the *gacS* mutant did not contribute to defence at the population level.

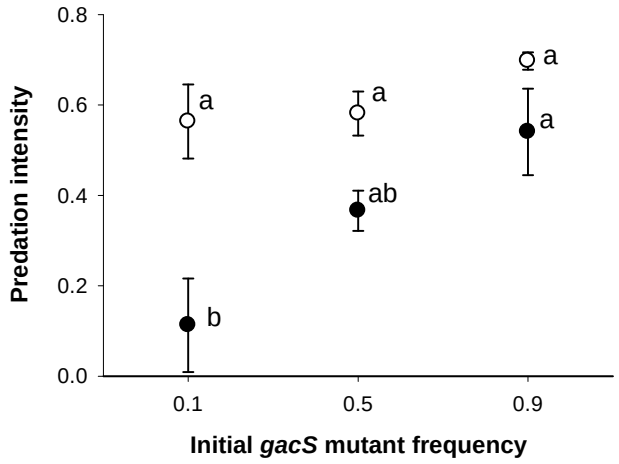

Figure 12: Predation intensity on the total bacterial population by *Acanthamoeba castellanii* (black circles) and *Caenorhabditis elegans* (white circles) at different initial frequencies of *gacS* mutants of *Pseudomonas fluorescens* CHA0. Predation intensity is expressed as percentage reduction in bacterial numbers compared to the respective treatment without predators at the end of the experiment. Error bars indicate ± SE; different letters indicate significant differences between means at $p<0.05$ (Tukey's HSD test).

Predator response

The density of amoebae at the end of the experiment was independent of the initial frequency of *gacS* mutants and reached on average 1.34 ± 0.89 10^4 ind. g^{-1} sand ($F_{2,16}=0.40$, $p=0.700$), suggesting low mortality even at high frequency of toxic bacteria, although more amoebae may have been encysted due to increased toxin concentrations. In contrast, nematode density tended to increase with initial mutant frequency ($F_{2,16}=2.90$, $p=0.082$), with actively swimming nematode numbers of 2.9, 4.5 and 6.5 ind. g^{-1} sand in 10, 50 and 90% mutant treatments, respectively, suggesting an inhibitory effect of the toxic wild-type bacteria on nematodes.

3.5. Discussion

Frequency dependent fitness of gacS mutant bacteria

Our results demonstrate that spontaneous *gacS* mutation in rhizosphere bacteria is an effective strategy to increase fitness by exploiting secondary metabolism of wild-type neighbours. Both in batch and rhizosphere systems the fitness of *gacS* deficient mutants proved to be negatively frequency dependent. This suggests that mutant bacteria are favoured within wild-type populations, where they may contribute up to 40% of the population.

In microorganisms the coordinated production of common goods, such as siderophores (Ross-Gillespie *et al.*, 2007), extracellular enzymes (Greig and Travisano, 2004) or multicellular structures (Velicer *et al.*, 2000), may strongly improve bacterial fitness but is vulnerable to cheating. Typically, signal blind

mutants rapidly evolve inside cooperating populations (Sandoz *et al.*, 2007), reaping the benefits of the shared trait while sparing the associated costs. Cell signalling in *P. fluorescens* coordinates the production of exoproducts such as enzymes and toxins (Haas and Keel, 2003). Extracellular proteases, chitinases and lipases (van den Broek *et al.*, 2003) contribute to the breakdown and assimilation of organic material. Moreover, our results show that secondary metabolites protect bacteria from predation (*A. castellanii*) or significantly reduce the fitness of the predators (*C. elegans*). Secondary metabolites, including enzymes and toxins, therefore can be considered as a common good beneficial to the whole population.

The negative frequency dependent fitness of the *gacS* mutant suggests that this strain exploits the exoproducts of the wild-type strain. Cell signalling deficient mutants indeed may have higher maximum growth rates, but in pure culture reach lower density than the wild-type strain, lack extracellular enzymes like phospholipases or proteases (Sacherer *et al.*, 1994) and are more vulnerable to predation. However, the latter is not true in dense wild-type populations, indicating that the benefits of exploiting the exoproducts of neighbouring cells may outweigh the disadvantage of the altered phenotype.

Interestingly, a range of random and spontaneous mutations in the *gacS/gacA* genes, such as deletions, insertions and inversions, frequently switch off the response to population density signals (van den Broek *et al.*, 2005a; Martinez-Granero *et al.*, 2005). We propose that these high mutation rates result from the advantage of individual bacteria switching of cell-signalling first. Since the fitness of signal blind mutants depends on the frequency of wild-type bacteria, mutants evolving inside of an otherwise wild-type population receive greater benefit than bacteria mutating later when the relative frequency of wild-type bacteria has decreased. Remarkably, most mutations are reversible and cell signalling can be restored later (van den Broek *et al.*, 2005b), suggesting increased fitness when reverting to the original phenotype in populations loaded with mutants.

Selective feeding on gacS mutant bacteria

Predation exerts a major selective pressure on free living bacteria (Pernthaler, 2005) and plays a key role in driving bacterial microevolution (Meyer and Kassen, 2007; Wildschutte *et al.*, 2004). Consequently, bacteria evolved sophisticated defence strategies, including the production of toxins that repel or inhibit their predators (Andersen and Winding, 2004; Jousset *et al.*, 2006; Matz *et al.*, 2004b). The production of these toxins often is density dependent resulting in increased protection of related neighbouring cells (Matz and Kjelleberg, 2005).

Both predators preferentially consumed *gacS* mutant bacteria, documenting that amoebae and nematodes can select their prey and that extracellular toxins act as repellent. Prey selection is crucial for bacterivores, such as protozoa and nematodes, since the quality of ingested bacteria directly affects

their fitness (Blanc *et al.*, 2006; Jousset *et al.*, 2006; Zubkov and Sleigh, 2000). In particular amoebae were highly selective and preferentially consumed the non-toxic mutant in both the batch and rhizosphere experiment. Despite being less selective, nematodes also negatively affected the *gacS* deficient mutant. Grazing by nematodes reduced total bacterial density thereby decreasing the size of the wild-type population. As the production of secondary metabolites is density dependent this likely impairs protection of the mutant strain. Generally, the results confirm the vulnerability of *gacS* mutant bacteria to predation and support our hypotheses that they gain protection by toxins from wild-type bacteria.

Predation and population level defence

Predation was markedly reduced in populations with low *gacS* mutant frequency, suggesting that in addition to the individual level bacteria benefit from the dominance of wild-type bacteria at the population level. Vulnerability to predation at high mutant frequency is in agreement with past observations that the biocontrol function of *P. fluorescens* collapses with increasing frequencies of *gacS* mutants (Duffy and Defago, 2000). The *gacS* mutant bacteria neither produce toxins nor auto-inducers (Lapouge *et al.*, 2008), thus interfering with toxin production of wild-type bacteria. Consequently, proliferation of the mutant compromises the population level traits from which this strain benefits. If bacterial populations become overloaded with mutants, as is the case in presence of hypermutable strains (Harrison and Buckling, 2005), the whole population may be eliminated by predators, suggesting that in spatially structured habitats predation may enforce cooperation, i.e. toxin production, at the group level by counter selecting defenceless populations. Overall, toxin-mediated attenuation of predation by wild-type bacteria may help explain why keeping the ability to produce secondary metabolites in *P. fluorescens* is evolutionary stable despite the associated costs and the frequent loss of this function.

Conclusions

Results of the present study suggest that by selectively feeding on non-toxic bacteria and reducing predation on bacterial populations loaded with mutants, predators reinforce toxin production of wild-type bacteria at both the individual and population level. The study therefore provides an ecological and evolutionary explanation for the spontaneous deactivation of cell signalling by pseudomonads. At low frequency signal blind toxin deficient mutants profit from the exoproducts of coexisting wild-type bacteria, suggesting that they improve their fitness by cheating. Predators, especially amoebae, reduced the frequency of non toxic phenotypes than nematodes, suggesting that protozoa efficiently reinforce toxicity of bacterial communities. Intriguing bacterial traits, such as the spontaneous deactivation of the GacS/GacA-mediated toxin production in pseudomonads, likely evolved in the

context of predation pressure. Predator–prey interactions therefore not only allow a mechanistic understanding of the factors driving population dynamics and evolution of cooperating and cheating in *Pseudomonas* strains but also may allow developing strategies for the successful establishment of biocontrol bacteria in the rhizosphere of plants.

3.6. Acknowledgements

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Chapter 4

Chemical warfare between predator and prey determines the toxicity of rhizosphere pseudomonads

4. Chemical warfare between predator and prey determines the toxicity of rhizosphere pseudomonads

4.1. Abstract

Soil pseudomonads reduce the impact of predation by producing toxic exometabolites. These toxins repel and kill microfaunal grazers, and significantly enhance bacterial survival in soil. Toxicity has however an important metabolic cost, and should therefore be regulated to the predation risk in order to optimize the costs to benefits balance of this trait. We investigated the response of the biocontrol strain *Pseudomonas fluorescens* CHA0 to a common predator, the free living amoeba *Acanthamoeba castellanii*. We followed the expression of the biosynthetic genes *phlA*, *prnA*, *pltA*, and *hcnA*. These genes are involved in the production of the major toxins DAPG, pyrrolnitrin, pyoluteorin and hydrogen cyanide, respectively. Bacteria reacted rapidly to predator chemical cues by upregulating all tested genes but *pltA*. This reaction was triggered essentially by high Mr molecules (>3kDa) secreted by the predator. Amoebae could however counteract bacterial defences by specifically repressing toxin genes. The results suggest a permanent eavesdropping process between rhizosphere organisms, and that bacteria finely tune their toxicity in response to biotic stresses.

Keywords
chemical communication / toxicity / biocontrol / predation / rhizosphere / DAPG

4.2. Introduction

Predation is a major shaping force for the structure of microbial communities (Pernthaler, 2005). In soil, predation by protists is a main cause of mortality for soil bacteria (Clarholm, 1981), and the competitiveness of bacteria strongly depends on their ability to avoid predation (Jousset *et al.*, 2008; Pedersen *et al.*, 2009). Consequently, bacteria developed defence mechanisms such as morphological plasticity, microcolony formation and toxicity (Matz *et al.*, 2004a). Toxicity is a potent defence strategy for reducing predator impact by killing or directing them to alternative prey (Jousset *et al.*, 2006; Matz *et al.*, 2008b).

Defence mechanisms, however, are costly and defence theory predicts that prey species should optimise the investment in defence according to the resources available and the predation risk (Steiner, 2007). Typically, prey species activate defence strategies in presence of predators or predator-associated chemical cues (Bergkvist *et al.*, 2008; Fyda *et al.*, 2005). At the microbial level, bacteria respond to flagellate grazing by the formation of inedible morphotypes or microcolonies (Salcher *et al.*, 2005; Weitere *et al.*, 2005; Yang *et al.*, 2006). Surprisingly, there is no information on antipredator toxin production by bacteria, one of most powerful defence strategies.

We investigated the mutual perception between the bacterium *Pseudomonas fluorescens* CHA0 and the bacterivorous amoeba *Acanthamoeba castellanii*. *Pseudomonas fluorescens* CHA0 is a soil bacterium that efficiently colonises roots of crop plants (Voisard *et al.*, 1994) and protect them against soil-borne phytopathogens. It produces a variety of extracellular toxins including pyrrolnitrin (PRN), 2,4-diacetyl-phloroglucinol (DAPG), hydrogen cyanide (HCN) and pyoluteorin (PLT) (Haas and Keel, 2003). In addition to their biocontrol properties, these toxins increase the competitiveness of the bacteria in the rhizosphere and are especially efficient in antipredator defence (Jousset *et al.*, 2008).

The production of secondary metabolites is a dynamic process depending on environmental factors such as nutrient availability (Duffy and Defago, 1999) and cell density (Haas *et al.*, 2002). Moreover, *P. fluorescens* can adapt its toxin production in response to signals from plants (de Werra *et al.*, 2008), phytopathogens (Notz *et al.*, 2001) or other bacteria (Dubuis and Haas, 2007). We hypothesised that *P. fluorescens* is able to sense predators and responds by increasing its toxin production. As model predator we used the bacterivorous amoeba *Acanthamoeba castellanii* (Bonkowski and Brandt, 2002), an ubiquitous protist (Rodriguez-Zaragoza, 1994) able to grow on a wide range of bacterial species (Pickup *et al.*, 2007). *Acanthamoeba castellanii* may strongly alter the composition of soil bacterial communities via top-down forces (Kreuzer *et al.*, 2006; Rosenberg *et al.*, 2009) resulting in increased frequency of toxic bacterial strains (Jousset *et al.*, 2008). Organisms targeted by toxins of antagonists often adopt counterstrategies to repress their production. For example, eukaryotes can interfere with bacterial quorum sensing (Gonzalez and Keshavan, 2006), which regulates toxin production in many

bacteria (Matz *et al.*, 2004a; Matz *et al.*, 2004b), and in soil the fungal pathogen *Fusarium* can inhibit the production of DAPG by pseudomonads (Notz *et al.*, 2002b). Consequently, we hypothesised that *A. castellanii* counteracts the defence strategy of its prey by inhibiting bacterial toxin production.

We investigated the effects of predator - prey interactions on the regulation of the production of extracellular toxins (DAPG, PLT, PRN and HCN) of *P. fluorescens* CHA0 with a previously developed set of autofluorescent GFP and mCherry-based reporter fusions (Baehler *et al.*, 2005; Rochat *et al.*, in prep). Autofluorescent reporter fusions allow the non-destructive measurement of gene expression and have been applied to monitor the regulation of antifungal genes in the rhizosphere (de Werra *et al.*, 2008). We also monitored the metabolism of the bacteria with a GFP reporter fusion for the rRNA operon (Lambertsen *et al.*, 2004). The regulation of this housekeeping gene is growth rate-dependant and reflects the metabolic state of the bacteria (Boldt *et al.*, 2004), thus, permitting to distinguish between bacterial inhibition by predators and interference with secondary metabolism. We analysed the response of the bacteria to predator chemical cues in batch experiments and under natural settings on the roots of barley.

4.3. Material and Methods

Organisms and plasmids used

All organisms and plasmids are listed in Table 2. Bacterial strains.were kept routinely on nutrient agar (NA) plates amended with 125 μg mL^{-1} tetracycline, 50 μg mL^{-1} kanamycine, or 8 μg mL^{-1} gentamycine, if required (all chemicals from Sigma-Aldrich, St-Louis, USA).

Table 2: Plasmids used in this study

Plasmids	Properties	Reference
pME7100	*phlA-gfp* transcriptional fusion, Tc^r	(Baehler *et al.*, 2005)
pME7116	*pltA-gfp* transcriptional fusion, Tc^r	(Baehler *et al.*, 2005)
pME7144	*prnA-gfp* transcriptional fusion, Km^r	(Baehler *et al.*, 2005)
pME7155	*hcnA-gfp* transcriptional fusion, Tc^r	(Rochat *et al.*, in prep)

pME9012	*phlA-mcherry* transcriptional fusion, Km^r	(Rochat *et al.*, in prep)
psm1973	miniTn7 $P_{rrnB\ P1}$ *gfp*-a	(Lambertsen *et al.*, 2004)
pUX-BF13	Helper plasmid for Tn7-based transposon mutagenesis containing the transposition functions; R6K replicon; Ap^r	(Bao *et al.*, 1991)

Acanthamoeba castellanii, isolated from a woodland soil (Bonkowski and Brandt, 2002) was cultivated axenically in PGY medium (peptone 20 g L^{-1}, glucose 10 g L^{-1}, yeast extract 5 gL^{-1}) as described previously (Rosenberg *et al.*, 2009). Prior to the experiment, cells were harvested by gentle centrifugation (100 g, 2 min) and washed three times in Page's Amoeba Saline (AS, Page, 1988). Cell density was measured in a Neubauer counting chamber and adjusted to a 10^6 cells mL^{-1} in AS, and incubated at 17°C in the obscurity for three days. Supernatant of the amoebae culture medium was obtained by separating the cells by gentle centrifugation. The supernatant was sterile filtered (0.22 μm) and fractionated with a vivaspin 20 column (MWCO 3000; Sartorius, Aubagne, France) according to the manufacturer's instructions. The concentrated larger fraction (Mr >3 kDa) was re-diluted to the original volume in AS, the eluted fraction (Mr <3 kDa) was used directly. Raw and fractionated supernatant were snap frozen in liquid nitrogen and stored at -80°C until use.

Tagging of bacteria with transcriptional reporter fusions

Midi-prep extractions of plasmids from *E. coli* were carried out with a JetStar 2.0 Plasmid Midiprep kit (Genomed, Löhne, Germany). Plasmids containing the transcriptional fusions *phlA-gfp, pltA-gfp, prnA-gfp* and *hcnA-gfp* were electroporated into *P. fluorescens* CHA0 as described in (Baehler *et al.*, 2005; Schnider-Keel *et al.*, 2000). The mini Tn7 shuttle vector containing the *rrnB* P1-*gfp* fusion (Lambertsen *et al.*, 2004) was inserted at the Tn7 chromosomal neutral site by co-electroporation with the PUX BF13 helper vector (Bao *et al.*, 1991). Recombinants were selected on NA plates with the appropriate antibiotics (Table 1).

In vitro assays of gene expression

Assays to monitor expression of the GFP-based reporter fusions were performed in 96 well microtiter plates (Nunc, Langenselbold, Germany) using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland). Bacteria from a single colony were grown for 12 h at 26°C in 5 mL Luria broth on a rotary shaker (250 rpm). These pre-cultures were centrifuged (8 krpm, 1 min), washed twice in 0.9%

NaCl and adjusted to an OD_{600} of 0.1 in minimal medium (OSG, Schnider-Keel *et al.*, 2000) containing 0.5% (w/v) glycerol as sole carbon source. Ninety μL of bacterial suspensions were mixed with 10 μL of washed amoebae or fractionated amoebae supernatant (see above); the control treatment received 10 μL AS. Eight replicates were set up per treatment. Plates were incubated with orbital agitation (∅ 1.5 mm) in the plate reader at 26°C for 10 h. At 10 min intervals the OD_{600} and the green fluorescence (excitation 485 nm, emission 518 nm, gain 90) were recorded. Green fluorescence was expressed as relative fluorescence units (RFU) by dividing the total fluorescence signal by the OD_{600} (Baehler *et al.*, 2005). The fluorescence signal of non-tagged *P. fluorescens* CHA0 was used for background correction.

Expression patterns of bacteria on roots

We assessed the effect of *A. castellanii* on the extracellular toxin production of *P. fluorescens* CHA0 on the roots of barley (*Hordeum vulgare* cv. Barke). In order to distinguish between modulations of the secondary metabolism and variation of the global activity state of the bacteria, we used a mix of two reporter strains covering the expression of ribosomal RNA (PrrnB-*gfp*) and the DAPG biosynthetic gene *phlA* (*phlA-mcherry*), the most strongly expressed toxin gene in the rhizosphere (de Werra *et al.*, 2008). The diverging excitation and emission spectra of GFP and mCherry (Shaner *et al.*, 2004) avoids cross excitation of the fluorophores, allowing the simultaneous detection of both signals by flow cytometry (see below).

Barley seeds were surface-sterilised by scarification in 50% H_2SO_4, immersion in 2% $AgNO_3$ solution for 20 min and five washing cycles in 1% NaCl and H_2O (Henkes *et al.*, 2008). Seeds were germinated in the dark for 48 h on 1.5% water agar and transferred individually into autoclaved growth pouches (Mega International, St. Paul, USA) containing 15 mL of a 1/5 Hoagland hydroponic solution. Then, the pouches were inoculated with 500 μL of a 1:1 mixture of the two reporter strains (total 10^8 bacteria) suspended in AS and 10^6 amoebae, if required. Ten replicates were set up for each treatment and time point. The pouches were covered with aluminium foil and plants were grown at 16 h light (22°C, 500 μmol s^{-1} m^{-2}), 8 h darkness (18°C) and 75% relative humidity for six days.

FACS analysis of gene expression in the rhizosphere

After 4, 8 and 16 days the plants were harvested. Bacteria adhering to roots were collected and fixed by vigorously shaking the roots in 6 mL phosphate buffer saline (PBS) containing 0.8% formaldehyde. This concentration was found to be optimal to fix the cells without affecting the fluorescence signal (data not shown). *In situ* expression of the reporter fusions for *phlA* and *PrrnBP1* was analysed simultaneously with a FACScalibur flow cytometer (Becton and Dickinson, San Jose, USA) equipped with a 15 mW, air-cooled argon ion laser excitation light source (488 nm). GFP and mCherry fluorescence emissions were measured with FL1-H and FL3-H channels, respectively. Forward scatter

(FSC) signals were collected using a photodiode with an amplification factor of ten, a threshold of 253 and a log gain. Side scatter (SSC) signals were measured using a photomultiplier tube set at 350 V, a threshold of 72 and a log gain. Green fluorescence was detected at 515-530 nm by the FL1-H detector set at 505 V and a log gain. Cherry fluorescence was detected at 670 nm by the FL3-H detector set at 690 V and a log gain.

Data were collected using CellQuest™ software (Becton and Dickinson, San Jose, USA) and analysed with WinMDI 2.8 (http://facs.scripps.edu/software.html). Selection of the counted events was done on the base of the FCS and SSC signals. Fluorescence signals for each fluorophore were gated on the FL1-H and FL3-H histograms using root washes of plants inoculated with the complementary reporter strains as blank in order to avoid measuring potential unspecific signals from root particles or bacteria expressing the other fluorophore. FL1-H signals between 100 and 1023 and FL3-H signals between 50 and 1023 were taken into account.

Bacterial colonisation was determined by epifluorescence counting to recheck FACS counts (Jousset *et al.*, 2008).

Statistical analyses

Geometric means from green and cherry fluorescence from the FACS measurement and the bacterial density were log- transformed prior to analyses. Data were analysed with a general linear model function and type III sum of squares investigating the effect of density of amoebae and the supernatant fraction on the relative fluorescence (RFU) in the batch experiment, and the presence of amoebae and the time post inoculation on the FACS data and the bacterial colonization in the root experiment. Analyses were performed with Statistica 7.1 (Statsoft, Tulsa, USA).

4.4. Results

Bacterial response in vitro

Incubation of *P. fluorescens* CHA0 with *A. castellanii* supernatant or its different fractions elicited a strong response in the expression of all tested extracellular toxin genes compared to the control treatment mixed with fresh AS ($F_{3,101}$ =32.0, P< 0.001). The four tested genes reacted differently ($F_{3,101}$=202.2, p<0.001), and differed in their sensitivity to the distinct fractions ($F_{9,101}$=28.0, p< 0.001 for Gene x Treatment interaction). The DAPG and pyrrolnitrin biosynthetic genes *phlA* and *prnA*, respectively, were most upregulated in response to amoeba supernatant (Figure 13).

The dialysed fraction larger than 3 kDa exhibited a similar effect on the expression of *phlA* as did the crude supernatant, and its effect on *prnA* even exceeded the effect of the crude supernatant. The

fraction smaller than 3 kDa still activated these two genes, albeit to a lower extent. The expression of

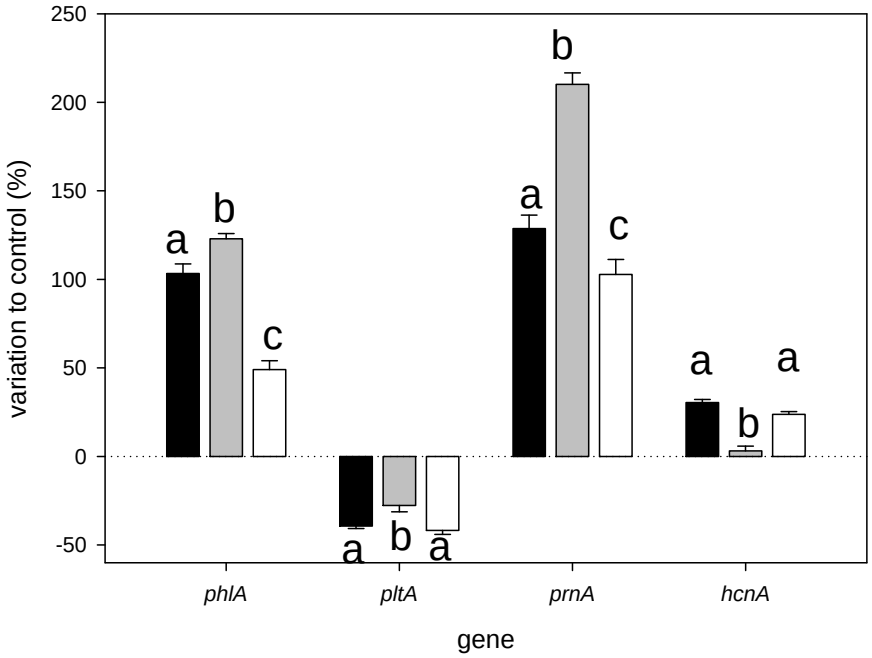

Figure 13: Effect of co-incubation with supernatant of *Acanthamoeba castellanii* culture (black bars), and the fraction larger than 3 KDa (grey bars) and smaller than 3 KDa of the supernatant (white bars) on the expression of extracellular toxin genes of *Pseudomonas fluorescens* CHA0. Bacteria were incubated for 5 h in OSG-glycerol medium (see Methods). Effects are expressed as percentages of the control treatment (culture medium without *A. castellanii*), errors bars show ± SE. Different letters indicate significant differences between the treatments (Tukey's HSD, α =0.05; separate analysis for each gene).

Supernatant of *A. castellanii* also induced a moderate increase in the expression of the *hcnA* gene. Interestingly, the fraction larger than 3 kDa had no effect on the expression of this gene, while the smaller than 3 kDa fraction induced a similar increase in the gene expression as the supernatant (Figure 13). *pltA* was the only gene that was downregulated in presence of the supernatant or its fractions. The fraction larger than 3 kDa had a lower inhibitory effect than the raw supernatant and the smaller fraction. Co-cultivation of *P. fluorescens* CHA0 with *A. castellanii* resulted in contrasting gene expression patterns as compared to the supernatant. Increased density of amoebae reduced the expression of *phlA*, *hcnA*, *prnA* and *pltA* genes after 5 h ($F_{6,177}$=25.1, p<0.001; Figure 14). The different genes were distinctly inhibited ($F_{3,177}$=3.3, p=0.02): The expression of *phlA* amd *pltA* was affected most; amoebae at a concentration of 10^6 mL^{-1} reduced the specific fluorescence of the *phlA-gfp* and *pltA-gfp* reporter fusions by up to 66% and 55%, respectively (Figure 14).

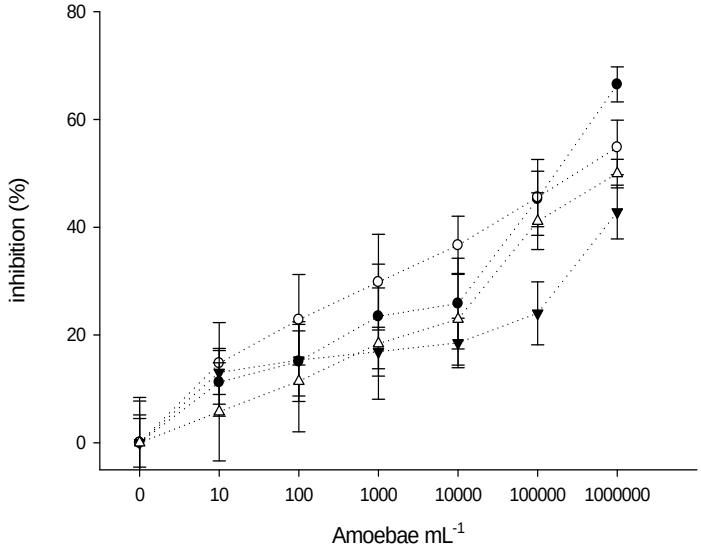

Figure 14: Effect of co-cultivation of *Pseudomonas fluorescens* CHA0 with *Acanthamoeba castellanii* on the expression of *phlA* (●), *hcnA* (▼), *prnA* (△) and *pltA* (○) genes of *P. fluorescens*. Bacteria were grown during 5 h in OSG-glycerol medium; Inhibition is expressed as the relative reduction of the specific fluorescence (RFU) compared to control treatment. Error bars show ± SE.

Neither amoebae nor the supernatant of amoebae culture medium affected bacterial growth throughout the experiment (data not shown), suggesting specific inhibition of secondary metabolism regulatory pathways rather than a global inhibition of bacterial growth.

Gene expression in the rhizosphere

The density of *P. fluorescens* CHA0 increased during the experiment ($F_{2,54}$= 4.51, p=0.015) from $2.99*10^7$ bacteria per root system on day 4 to 3.58 and $3.51*10^7$ bacteria per root system on day 8 and 16, respectively. The density was not significantly affected by amoebae ($F_{1,54}$= 2.28, p=0.16), indicating low consumption of bacteria by the amoebae. The green fluorescence signal, coupled with the expression of the growth dependent promoter PrrnB1, decreased by 16% during the experiment ($F_{2,54}$= 29.66, p<0.001), but was not affected by the presence of amoebae ($F_{1,54}$= 1.20, p=0.277,Figure 15), suggesting that amoebae did not affect the basal metabolism of the bacteria. During the experiment, however, amoebae increasingly affected the mCherry signal reporting *phlA* gene expression ($F_{2,54}$= 7.43, p=0.0015 for the Amoeba x Time interaction) until reducing it by 25 % after 16 days (Figure 15).

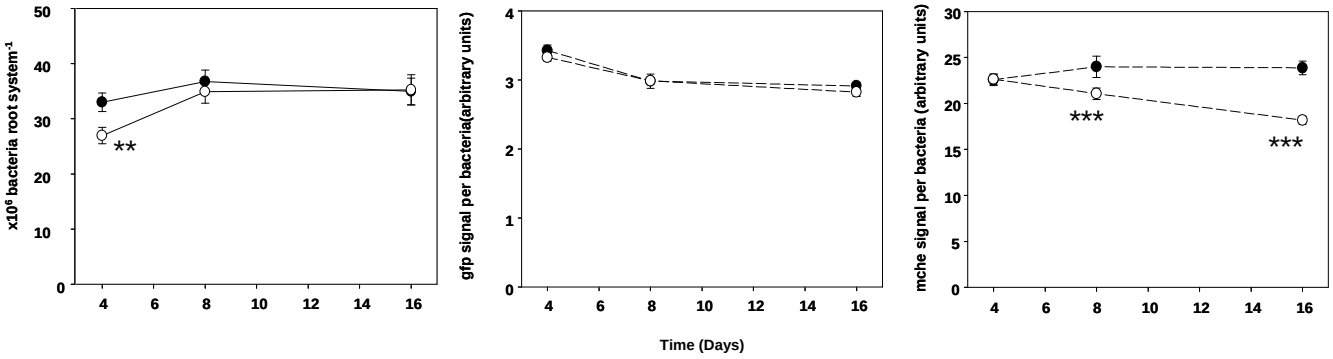

Figure 15: Barley root colonisation (A) and expression of the reporter fusions *PrrnB1-gfp* (A) and *phlA-gfp* (B) by *Pseudomonas fluorescens* CHA0 in absence (●) and presence of *Acanthamoeba castellanii* (○). Activity was measured by flow cytometry 4, 8 and 16 days after inoculation and expressed as geometric mean of the fluorescence signal per bacterial cell. Error bars show ± SE, asterisks indicate significant differences to the control treatment (Tukey's HSD, ** P<0.01, *** P<0.001).

4.5. Discussion

The results of this study suggest that toxin production by P. fluorescens is modulated by chemical perception of predators. Bacteria were able to sense chemical cues and react by boosting the expression of toxicity related genes, suggesting a permanent eavesdropping on other rhizosphere organisms. Amoebae could however counteract this defence strategy, pointing to a reciprocal perception of predator and prey.

Perception of amoebae by bacteria

Pseudomonas fluorescens sensed and rapidly responded to the presence of amoebae. Addition of amoebae culture supernatant led to a rapid increase in the expression of each of the studied genes regulating the biosynthesis of extracellular toxins. The response in absence of amoebae suggests that predator perception by bacteria is mediated by water soluble chemical compounds as is the case in aquatic systems (Pohnert *et al.*, 2007).

Since bacteria are consumed as whole cells, early perception of predators and fast upregulation of secondary metabolite production is crucial for effective chemical defence. Expression of anti-predator traits negatively impacts the fitness of the producer and is only advantageous at certain conditions, i.e. at high predator density and high nutrient availability (Friman *et al.*, 2008). The advantage of defence mechanisms depends on the feeding behaviour of predators and prey population density (van Donk, 2007; Jeschke, 2006). Continuous production of secondary metabolites by bacteria likely impedes their competitiveness against fast-growing bacterial neighbours, especially at low predator density (Jousset *et al.*, 2009), and variation in the production of toxins in presence of predator clues suggests that bacteria optimise investment in defence compounds in response to predation risk. Modulating defence traits according to their costs and benefits is advantageous for prey species in general (Agrawal, 2007;

Zangerl, 2003) and this also applies to bacteria (Pernthaler, 2005). Bacteria have evolved strategies to confer grazing resistance which can be grouped in structural and chemical defences (Matz and Kjelleberg, 2005). Responses to predators are widespread (Bergkvist *et al.*, 2008; Fyda *et al.*, 2005) and can be predator-specific (Bergkvist *et al.*, 2008). In the rhizosphere, where microcolonies of different bacterial species are closely associated (Normander *et al.*, 1999), the production of repelling toxins may be especially efficient.

To our knowledge this is the first study reporting that bacteria are able to respond to predator signals and adapt the production of toxins for improving predator defence. Interestingly, the studied bacterial toxin genes responded strongly and differentially to chemical cues of the predator, and the response was related to the efficiency of the toxins against predators. The expression of *phlA* responsible for the production of DAPG, the most potent anti-protozoa toxin of *P. fluorescens* CHA0 (Jousset *et al.*, 2006), showed the strongest response. In contrast, the expression of *pltA*, coding for the less toxic PLT, was reduced. Since the production of DAPG and PLT is negatively co-regulated (Baehler *et al.*, 2005; Schnider-Keel *et al.*, 2000), the reduced expression of *pltA* may be considered a trade-off in favour of the production of the most effective toxin. HCN is a broad-spectrum toxin inhibiting cytochrome *c* oxidase activity which for example results in paralytic death of nematodes (Gallagher and Manoil, 2001). Interestingly, *Acanthamoeba* species posses cyanide-resistant terminal oxidases (Edwards and Lloyd, 1978). The low response of *hcnA* to predator cues may thus be linked to its low efficiency against *A. castellanii*. Similar to *phlA*. *prnA* was strongly upregulated in presence of chemical clues of *A. castellanii.* Similar to HCN, pyrrolnitrin is a potent inhibitor of the respiratory pathway (Tripathi and Gottlieb, 1969) affecting a wide spectrum of fungi and protists (Carlone and Scannerini, 1974; Chernin *et al.*, 1996), although few data are available on the role of this toxin in antagonistic interactions with other soil organisms. The prn operon is widespread in bacterial phyla, suggesting that it frequently was transferred horizontally providing ecological benefit to the host (Costa *et al.*, 2009). The response of the supernatant fraction larger than 3 kDa elicited a similar (DAPG, HCN) or stronger (PRN) response than the crude supernatant, although the smaller fraction still presented some activity. This implies that the perception of amoebae by bacteria relies on larger molecules such as peptides, in agreement with previous experiments that demonstrated that predatory amoebae induce defence reaction by the ciliate *Euplotes* sp. through high mocular weight proteins (Kusch, 1999). A detailed fractionation and analysis of the exoproducts of *A. castellanii* may help elucidating the structure of the kairomones involved in this interaction.

Counteraction of prey defence by amoebae

Compared to the supernatant, presence of amoebae contrastingly affected gene expression of bacteria; it strongly reduced the expression of all the investigated exoproducts inducing genes of *P. fluorescens*

both in vitro and in the rhizosphere. *Acanthamoeba castellanii* produces extracellular enzymes which may disturb bacterial metabolism (Serrano-Luna *et al.*, 2006; Weekers *et al.*, 1995). Surprisingly, however, reduced expression of defence genes in *P. fluorescens* by amoebae did not affect bacterial growth in both the batch and rhizosphere experiment. Moreover, basal bacterial metabolism, as reflected by the expression of rRNA genes, was not influenced by the predators in the rhizosphere. This suggests that amoebae specifically inhibited the production of secondary metabolites of their prey. Inhibition of DAPG, as most potent anti-predator toxin of *P. fluorescens* CHA0 (Jousset *et al.*, 2006), is likely to strongly benefit the amoebae. Similar responses have been reported for soil fungi that hydrolyse DAPG (Bottiglieri and Keel, 2006; Schouten *et al.*, 2004), or inhibit DAPG production (Duffy and Defago, 1999; Notz *et al.*, 2002b; Schnider-Keel *et al.*, 2000). Since the production of toxins by pseudomonads is regulated by cell signalling (Dubuis *et al.*, 2007; Heeb and Haas, 2001), amoebae may interfere with bacterial communication. In fact, both prokaryotes and eukaryotes can interfere with bacterial signalling (Gonzalez and Keshavan, 2006; Hentzer *et al.*, 2002; Mathesius *et al.*, 2003; Teasdale *et al.*, 2009). Potentially, the effect of amoebae on gene expression of bacteria occurs upstream rather than directly at the level of the studied promoters.

Since supernatant of the culture medium of amoebae resulted in increased bacterial secondary metabolism, the inhibitory effect of amoebae co-cultured with bacteria on bacterial gene expression probably is induced by prey signals. Eukaryotes use conserved receptors, such as Toll like receptors or mannose-binding receptors, to sense bacteria with the detection being based on general molecular patterns, such as flagellin or lipopolysaccharides (Wildschutte *et al.*, 2004). Moreover, microfauna predators secrete opsonins (Sakaguchi *et al.*, 2001) to detect and localize bacterial prey based on diffusible chemical cues (Willard and Devreotes, 2006). Detection of bacterial cues appeared early in the evolution of eukaryotes and permitted the evolution of selective phagocytosis (Cavalier-Smith, 2009). Our results show that recognition mechanisms not only control phagocytosis, but also the general adaptation to toxic prokaryotic prey.

4.6. Conclusions

Toxin production is a key feature for the fitness of rhizosphere pseudomonads and their biocontrol ability. We demonstrated that the production of extracellular toxins by pseudomonads is a dynamic process driven by biotic stressors and optimises investment in defence traits. Moreover, we showed that microfauna predators are able to disarm their prey or at least counteract their defence by inhibiting the production of toxins. This mutual perception and response resembles chemical warfare between bacterivorous protozoa and bacterial prey species. Since the same toxins are known to function as biocontrol agents, bacteria - protozoa interactions are likely to also affect fungal pathogens by modifying the abundance of toxic bacterial strains and by affecting the exoproduct production of

bacteria. Understanding and manipulating predator – prey interactions in the rhizosphere therefore may allow to improve biocontrol of soil born diseases in agricultural systems.

4.7. Aknowledgements

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Chapter 5

Plants manipulate antifungal compound production by rhizobacteria upon pathogen infection

5. Plants manipulate antifungal compound production by rhizobacteria upon pathogen infection

5.1. Abstract

The rhizosphere is a place of intense interaction between plant roots and a diverse microbial community of symbiotic, opportunistic and pathogenic root colonizers. Pseudomonads are important bacterial colonizers of plant roots known to produce antifungal compounds that can protect the roots from infection by soil borne pathogens, but their toxins may inhibit plant growth at high concentrations. By controlling bacterial toxin production, plants therefore may optimize the benefit they gain from associated rhizobacteria. In this study we show that plants actively communicate with beneficial bacteria and adjust their metabolism according to their needs. We set up a split-root system where on the one side the roots of barley were challenged with the phytopathogen *Pythium ultimum*, while the other side of the root system was colonized by reporter strains of the biocontrol bacterium *Pseudomonas fluorescens* CHA0. We followed the expression of the housekeeping gene *PrrnB* coding for rRNA and the antifungal gene *phlA* involved in the production DAPG. Infection of one root side resulted in a distortion of bacterial gene expression patterns on the other, separated root side. Bacteria allocated more resources to antifungal toxin production at the expense of their basal metabolism. The observed effects correlated with modifications of the root exudation pattern of phenolic compounds, suggesting that the production of antifungal compounds in the rhizosphere is regulated by a complex communication between plants and beneficial rhizobacteria.

Keywords

tri-partite interaction / biocontrol / exudates / chemical communication / DAPG

5.2. Introduction

Plant roots continuously interact with a diverse community of soil microorganisms. Plants allocate considerable resources to the root system, and exude large amounts of mono- and polysaccharides, organic acids, phenolic compounds, amino acids and proteins (Bais *et al.*, 2006). These exudates function as resources feeding and selecting a specific bacterial community (Kowalchuk *et al.*, 2002; Mazzola, 2002). In return, this commensal flora provides a number of services to the plant, such as mobilising soil nutrients (Vessey, 2003b), protecting the plant against pathogens by producing antifungal toxins (Raaijmakers *et al.*, 2002) or eliciting plant defence (Compant *et al.*, 2005; Iavicoli *et al.*, 2003b).

In order to fully profit from these beneficial properties, plants need to modify the interaction with their rhizosphere associated microflora to suit their own needs. Plant regulate the quantity and composition of root exudation according to environmental factors (Phillips *et al.*, 2003), such as the presence of root pathogens and symbionts, bacterial products and nutrient availability (Paterson *et al.*, 2006; Phillips *et al.*, 2004). In particular phenolic compounds have been found to play important roles as plant signal molecules in communication between plants and rhizosphere microorganisms (Hirsch *et al.*, 2003), e.g. between plants and rhizobia (Mishra *et al.*, 2006; Stacey, 1995) and mycorrhiza (Steinkellner *et al.*, 2007). Plant phenolics therefore probably structure the rhizosphere bacterial community (Shaw *et al.*, 2006), and host dependent exudate composition has been found to change the gene expression pattern of root associated bacteria (Mark *et al.*, 2005).

In this study we investigate a tripartite interaction between host plant, fungal pathogen and biocontrol bacteria. We set up a split root system with barley, where one side of the root system was infected with the oomycete *Pythium ultiumum*, a fungal pathogen causing damping-off disease (Martin and Loper, 1999). The other side was inoculated with the biocontrol bacterium *Pseudomonas fluorescens* CHA0. This strain is an efficient rhizosphere coloniser and protects crop plants against pathogenic fungi by producing antifungal compounds (Voisard *et al.*, 1994). In particular, 2,4-diacetylphloroglucinol (DAPG) plays a key role in the biocontrol properties of this strain due to its high antifungal activity and its potential to elicit induced systemic resistance (ISR) in the host plant (Iavicoli *et al.*, 2003a). The biosynthetic locus *phlA* of *P. fluorescens* CHA0 is the most expressed antifungal gene on plant roots (de Werra *et al.*, 2008), and DAPG is a potent inhibitor of *Pythium* (de Souza *et al.*, 2003). In this study we tested if the host plant can manipulate root associated biocontrol bacteria and increase their antifungal activity upon infection. The used split root design allowed monitoring systemic effects of the infection on the activity of rhizobacteria. We used two reporter fusions in order to follow the expression patterns of the house keeping gene *PrrnaB* and the DAPG biosynthetic gene *phlA*. In addition, we analysed the exudates produced by healthy and infected barley plants to screen for putative signal molecules.

5.3. Methods

Bacterial strains, plasmids and culture conditions

The strains were kept on nutrient agar plates (NA) with the corresponding antibiotics (Table 3). Prior to inoculation, bacteria from a single colony were grown in liquid LB (Sambrook and Russell, 2001) for 12 h at 25°C with agitation (200 RPM). Early exponential phase bacteria (OD_{600} nm= 0.3) were harvested by centrifugation (4'600 RPM, 4°C, 10 min), washed in 0.9 % NaCl and resuspended in Neff's modified amoeba saline (Page, 1988).

Table 3: Strains and plasmids used in this study

Strain or plasmid	*Genotype and phenotype*	*Reference*
P. fluorescens		
CHA0	Wild type	(Voisard *et al.*, 1994)
Plasmids		
pME9408	0.7 kb *Sal*I-*Hind*III fragment of pMQ64-mChe cloned into pME7100 for substitution of *gfp* sequence	This study
Psm1973	MiniTn7(Kmr) PrrnBP1 gfp-a	(Lambertsen *et al.*, 2004)
pMQ64-mChe	7.4 kb vector carrying the sequence of mCherry	(Shanks *et al.*, 2006)
pME 7100	pPROBE-TT with a 1.1 kb *Hind*III-*Xba*I fragment of pME6257, containing a *phlA-gfp* transcriptional fusion	(Baehler *et al.*, 2005)
Primers		
Mche1 (5'-3')	ACGCGTCGACGTCTAACTAACTAAAGATTAACTTTAATAA GGAGGAAAAACATATGGTTTCCCAAGGGCGAGGAG	This study
Mche2(5'-3')	CCCAAGCTTGTTATTTGTACAGCTCATCCATGCCACC'	This study

DNA manipulation

Chromosomal DNA extraction was prepared as described elsewhere (Sambrook and Russell, 2001). Small-scale plasmid preparations were performed with the CTAB method, large-scale plasmid preparations were carried out with the Jetstar 2.0 kit (Genomed, Basel, Switzerland). Standard techniques were used for restriction, agarose gel electrophoresis and ligation. Polymerase chain reaction (PCR) product and restriction fragments were purified from agarose gel using MinElute Gel Extraction kit or QIAquick Gel Extraction kit (Qiagen, Basel, Switzerland). Electroporations of competent cell with plasmid DNA were performed as described elsewhere (Farinha and Kropinski,

1990). PCR were carried out with thermostable GoTaq DNA polymerase (promega, Switzerland), according to manufacturer's recommendations. Nucleotides sequencing were determined on both strands by Microsynth (Balgach, Switzerland). Nucleotide and deduced amino acid were analysed with program Chromas Lite version 2.01 (freely available under http://www.technelysium.com.au/chromas_lite.html).

Construction of mCherry based phlA reporter fusion

The mCherry sequence was amplified from pMQ64-mche (table 1) with the PCR primers Mche1 and Mche 2, reaching a 0.7 kb *Sal*I-*Hind*III fragment. The PCR fragment was cloned into the pME7100 vector by substitution of the *gfp* [mut3] sequence yielding to pME9408. The plasmid pME7100 (Baehler et al, 2005), contain the CHA0 *phlA* promoter region, this sequence contain the entire region between *phlF* and *phlA*, cloned in the pPROBE-TT vector (Miller et al., 2000) The final construction pM9408 contain the *mCherry* sequence under the control of the *phlA* promoter. Activity of the bacteria was assessed by using a reporter mini Tn7 *gfp* insert under the control of the growth rate dependant promoter rPrrnB P1 (Lambertsen et al, 2004).

Plants

Barley seeds (*Hordeum vulgare* cv. Barke) were dehusked by immersion for 90 min in 50% H_2SO_4 with agitation, and washed twice in a solution of $NaHCO_3$ 0.5 M to neutralize the acid. Seeds were sterilized by soaking in 70% ethanol for 2 minutes and 2% $AgNO_3$ for 30 minutes under agitation. Silver residues were removed by 5 successive washing cycles in NaCl 0.9 % and distilled water of 10 min each. The seeds were germinated individually on diluted nutrient agar (TSB 0.3 g l^{-1}, Agar 8 g l^{-1}). After 6 days, the plantlets were checked for contamination, transferred to the split root microcosm (see below) and inoculated according to treatment.

Pythium ultimum was kept routinely on 20% V8 Agar (V8 200 ml l^{-1} CaCO3 10 gl^{-1}, Agar 20 g l^{-1}). An actively growing inoculum of mycelium was prepared by cultivating the fungus on sterile millet seeds as described by Maurhofer et al. (2002). Plates were incubated at room temperature in the dark for 7 days and regularly mixed to ensure a homogenous distribution.

Microcosms construction and inoculation

Barley plants were grown as follows: The split-root microcosms used were constructed from 250 x 160 x 15 mm polycarbonate plates (Fig. S1). One plant seedling was placed in each microcosm and the roots were equally distributed in the two split-root chambers. Prior to establishment of the seedlings the microcosms were autoclaved and each chamber (100 x 150 x 5 mm) was filled with 15 ml of an

agarised (2% agar) and sterile 1/5 Long Ashton nutrient solution (Hewitt, 1966). Seedlings were added after cooling of the microcosm,.

The treatment side of the split-root system was infected with 500 mg of the *Pythium* inoculum; the control plants received an equal quantity of sterile millet seeds. The reporter side was inoculated with one ml of a 1:1 mixture of both reporter strains of *P. fluorescens* CHA0, adjusted to an OD600 of 0.1. The microcosms were covered with a 6 mm thick polycarbonate lid and held tight with paper clamps. The lid was closed and the opening of the microcosm sealed with sterile Terostat VII sealing mass (Henkel, Düsseldorf, Germany). Microcosms for HPLC analysis of root exudates were built of two 12 mL glass bottles with PE lid (Neolab, Heidelberg, Germany). Bottles were washed twice in ethyl acetate, autoclaved, glued together and filled with 10 mL HPLC H_20 (Roth, Karlsruhe, Germany). Seedlings were introduced in the split root as described above. One side of the root system was infected with chopped *Pythium* mycelium, and the opening was sealed with autoclaved silicon grease. Plants were grown with a 16 h light phase (22°C, 500 μmol s^{-1} m^{-2}) and 8 h darkness (18°C) at 60 % relative humidity for 6 d. Eight replicates were set up per treatment, and the experiment was repeated twice.

Measurement of bacterial activity

Six days after inoculation, the microcosms were destructively sampled. Roots inoculated with *P. fluorescens* were placed in 10 ml of cold PBS buffer containing 0.8% formaldehyde, and root associated bacteria were extracted by vigorously shaking the roots for 20 minutes. Samples were kept at 4°C until analysis. *In situ* expression of the reporter fusions for the *phlA* and *PrrnBP1* was analysed with a FACScalibur flow cytometer (Becton and Dickinson, San Jose, USA) equipped with a 15mW, air-cooled argon ion laser excitation light source (488nm). Gfp and mCherry emission were measured at 530 and 661 nm, respectively, and amplified with a logarithmic gain. FSC signals were collected using a photodiode with an amplification factor of 10, a threshold of 253 and a log gain. SSC signals were measured using a photomultiplier tube set at 350 V, a threshold of 72 and a log gain.

Data were collected using CellQuest™ software (Becton and Dickinson) and analysed with WinMDI 2.8 (http://facs.scripps.edu/software.html). Gating was done on the base of the FSC signal. Fluorescence signals were gated using the complementary single fluorescent strains as blank in order to eliminate the background fluorescence.

Extraction of root exudates

To extract root exudates, samples of the media (5 mL) from each of the treatments were filtered through a 0.22 μm filter (Millipore, Bedford, MA), acidified to pH 2 with 1 N HCl and extracted in 5 mL ethyl acetate on an orbital shaker at 290 rpm (Bühler, Hechingen, Germany) at room temperature

for 1 h. The organic phase was evaporated under nitrogen and the residue taken up in 70 µL of methanol for HPLC analyses.

HPLC-DAD analyses

The HPLC apparatus used consisted of a Waters system equipped with a gradient pump (Waters 600 controller, Milford, MA, USA), a cooled autosampler (Waters 717 plus) and a UV–visible photodiode-array detector (Waters 996) set to acquire data from 200 to 400 nm. Empower 2 software from Waters was used for instrument control, data acquisition and data processing. Analyses were performed on 3 µm column (250 × 4 mm, Multospher 120 RP18HP; CS-Service, Langerwehe, Germany) at room temperature (21°C). The mobile phase consisted of aqueous phosphoric acid (0.1 % w/v; eluent A) and acetonitrile (eluent B) pumped at 0.5 mL min^{-1} into the HPLC system (Lanoué *et al.*, submitted). The gradient started at 5 % B and increased linearly to 72.5 % in 60 min, followed by washing and reconditioning the column. Wavelength detection was set up at 219 nm for vanillic acid, 310 nm for *p*-coumaric acid and 210 nm for fumaric acid. Compounds in extracts were identified according to their UV spectra and retention time by comparison with authentic standards.

Chemicals

Standard compounds vanillic acid, *p*-coumaric acid and fumaric acid were purchased from Sigma-Aldrich (St Louis, MI, USA). Acetonitrile (LiChrosolv), methanol (LiChrosolv), ethyle acetate (LiChrosolv) were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore Milli-Q water purification system.

Statistical analyses

Geometrical means for FL1 and FL3 signals were normalized prior to analysis. Gene expression of the bacteria was analysed using a General Linear Model (GLM) with a full factorial design. The model investigated the effect of the infection by *P. ultimum* and of the root size on the normalised *phlA* value and on the relationship between the expression level of both genes. The relationship between primary and secondary metabolism was estimated by the same method, predicting the expression of *phlA* as a function of the expression of *rRNA* and of the treatment. Vanillic acid, *p*-coumaric acid and fumaric acid concentrations were tested by a factorial ANOVA investigating the effect of infection and root side on the exudation level. All statistical analyses were performed in STATISTICA 7.1 (Statsoft inc., Tulsa, USA).

5.4. Results

Plant growth

Plant growth

The plant fresh weight was 112.9 ± 6.2 (SE) mg at harvest. Infection by *P. ultimum* did not significantly affect plant biomass of *P. fluorescens* inoculated plants ($F_{(1;14)}$ = 0.1; p = 0.748), but reduced plant biomass by 30% in non-inoculated plants ($F_{(1,14)=}$26,6; p=0.001). Also, neither the shoot-to-root ratio ($F_{(1;14)}$ = 1.26; p = 0.280), nor the ratio between the two root halves ($F_{(1;14)}$ = 0.30; p = 0.590) were affected by the infection, suggesting that the plant-pathogen interaction did not result in directed resource allocation toward the uninfected side in plants inoculated with *P. fluorescens*.

Changes in exudation

HPLC analyses of methanolic extracts of root exudates of roots attacked by *P. ultimum* were compared to root exudates from plants grown in uninfected split root systems. Only vanillic, fumaric and *p*-coumaric acids were detected. The infection modified the concentrations of these molecules ($F_{(3, 18)}$=3.7822, p=0.02893 for Infection x Root side); concentrations in the infected side were not affected by *P. utlimum* (Table 4), whereas concentrations of vanillic, coumaric and fumaric acid increased by a factor 2.7 in the uninfected side.

Table 4: Concentrations (± SE) of the exudates vanillic acid, p-coumaric acid and fumaric acid in uninfected barley plants and plants infected with *Pythium* in gnotobiotic split-root systems. Local, *Pythium* infected side; distal, sterile side. Different letters indicate significantly different concentrations (Tukey´s HSD test, α=0.05)

Root side	Treatment	Vanillic acid (μM)	Coumaric acid (μM)	Fumaric acid (nM)
distal	infected	60.94 ± 9.12 a	23.11 ± 8.59 a	16.79 ± 5.1 b
distal	control	21.74 ± 8.23 b	9.69 ± 4.43 ab	10.29 ± 2.48 ab
local	infected	7.19 ± 2.65 b	3.07 ± 3.07 b	4.6 ± 0.98 a
local	control	18.98 ± 8.42 b	10.79 ± 7.27 ab	13.58 ± 4.76 ab

Plant impact on bacterial metabolism:

Roots were colonized by on average 5.28 ± 1.1 (SE) 10^7 bacteria g^{-1} root with the colonization being not influenced by *P. ultimum* infection ($F_{(1, 25)}$<0.01, p=0.98), suggesting that plant infection did not result in enhanced root exudation. However, gene expression in root colonizing bacteria was drastically modified upon plant infection. In control plants bacterial expression of *phlA* was negatively correlated with the expression of rRNA (R= -0.75, p=0.001), suggesting a trade-off in resource allocation between bacterial investment in growth vs. secondary metabolite production. In contrast, gene expression in bacteria colonising infected plants was reversed ($F_{(1,25)}$=20.1, p<0.001, treatment × rRNA interaction; Figure 16). In infected plants the expression of the two bacterial genes was

strongly positive correlated (R=0.62, p= 0.016), suggesting drastic changes in the resource allocation of the bacteria following pathogen infection of a distant root compartment.

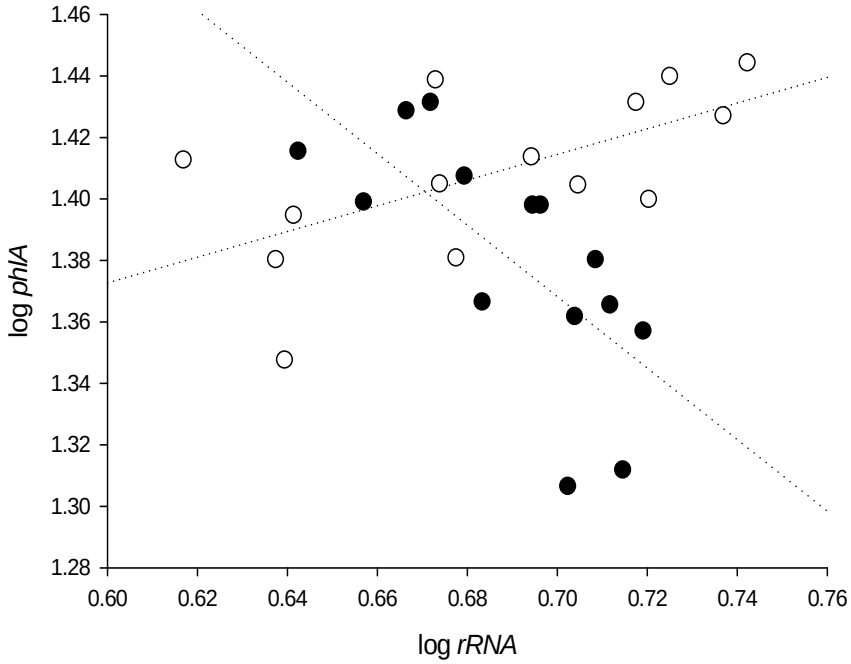

Figure 16: Relationship between the expression of the rRNA *rrnB P1* and *phlA* genes of *Pseudomonas fluorescens* CHA0 on the roots uninfected (closed symbols) and infected with *Pythium* (open symbol). Dashed lines show the respective regression lines for the two treatments. For statistical analysis see text.

The relative expression of *phlA* increased in parallel with root size in non-infected plants ($F_{(1,25)}$=12.27, p=0.004, Figure 17). However, the expression of *phlA* on average was 18% higher in the infected plants ($F_{(1,\ 25)}$=9.35, p=0.004), and the ratio between relative *phlA* expression and root weight of infected plants increased tenfold compared to control plants ($F_{(1,12)}$=8.39, p=0.013, root × treatment interaction, Figure 17).

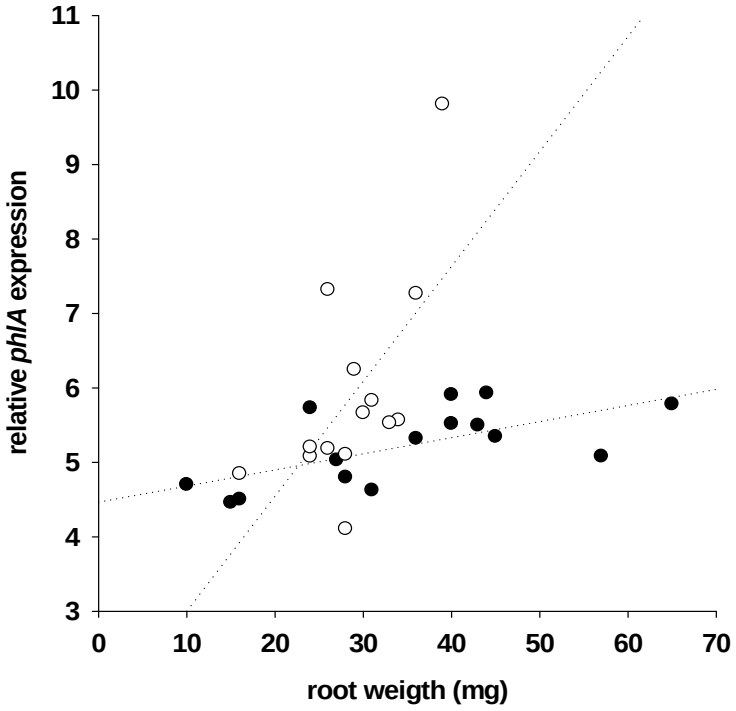

Figure 17: Relative expression of the *phlA* gene varying with the size of the reporter root system uninfected (closed symbols) and infected with *Pythium* (open symbols). Dashed lines show linear regressions for each treatment, for statistical results see text.

5.5. Discussion

In the split-root system used biocontrol bacteria were physically separated from the phytopathogen allowing to study plant-mediated alterations of the activity and antifungal compound production of biocontrol bacteria. The two reporters fusions used permitted to follow the expression of the antifungal *phlA* gene (Baehler *et al.*, 2005) and the housekeeping rRNA *rrnB P1* gene (Lambertsen *et al.*, 2004) and investiate their interrelationship. The rRNA *rrnB P1* reporter fusion allows to monitor the nutritional and growth status of *Pseudomonas* species *in vitro* (Ramos et al., 2000b) and in the rhizosphere (Boldt *et al.*, 2004), thus providing information on the general activity of the bacteria. Expression of the *phlA* gene is directly linked to the production of DAPG, the most potent antifungal toxin produced by *P. fluorescens* (Fenton *et al.*, 1992; Keel *et al.*, 1992a; Vincent *et al.*, 1991; Weller *et al.*, 2007).

Rhizosphere bacteria have limited resources at their disposal (Normander *et al.*, 1999), and the production of DAPG by pseudomonads on uninfected plants was characterized by a trade-off between bacterial growth and secondary metabolism, as reflected by the negative correlation between rRNA *rrnB P1* and *phlA* expression. This relationship was reversed by fungal infection of plant roots, suggesting that plant exudates were used for DAPG production. The contrasting investment in DAPG

production upon fungal infection of distant roots is remarkable as it is unlikely to increase the fitness of the bacteria but rather that of the plants.

Communication between plants and bacteria has been intensively investigated in context of interactions between legumes and their root-infecting nitrogen-fixing symbionts (Gray and Smith, 2005). Although it has been shown that plants also respond to metabolites of non-infecting rhizosphere bacteria (Lugtenberg *et al.*, 2002) the tight tri-partite interaction between pseudomonads and their host plant upon infection with a fungal pathogen discovered in our study is novel. The tenfold increase in the relative expresion of *phlA* at equivalent root size in infected plants demonstrates that plants can alter the expression patterns of pseudomonads even in roots not in contact with the pathogen, resulting in increased investment in the production of the anti-fungal toxin DAPG. *In situ* experiments recently showed an increase of antifungal genes expression in *Pythium* infected roots (Notz *et al.*, 2002a). However as the three organisms were not spatially separated, the role of the plant in these interactions could not be discriminated from pathogen effects such as nutrients leaking from wounded roots. Interestingly, leaf-attack by *Botrytis cinerea* resulted in downregulation of antifungal gene expression in rhizosphere *P. fluorescens* (de Werra *et al.*, 2008). Our results give strong evidence that plants actively communicate with beneficial rhizosphere bacteria and modulate their behaviour in order to produce antifungal toxins upon encounter with pathogenic microorganisms. This suggests that interactions between plants and free-living rhizosphere bacteria are much more specific and more important than previously assumed.

Since infected roots and roots inoculated with pseudomonads were physically separated, the results demonstrate that the switch in bacterial behaviour must have been activated by a systemic plant signal. Potentially, bacteria and pathogens can also indirectly influence each other via changes in plant carbon flow (Duffy *et al.*, 2004; Henkes *et al.*, submitted). The expression of *PrrnB* and the colonisation of the roots by bacteria were not influenced by the infection status of the plant, suggesting that no major quantitative shifts in carbon exudation occurred. Therefore, the observed changes in bacterial gene expression likely were caused by qualitative rather than quantitative changes in plant exudation.

Root exudates affect the structure and activity of the rhizosphere bacterial community (Somers *et al.*, 2004), and signal molecules like flavonoids are perceived and metabolised by a large number of rhizosphere bacteria (Shaw *et al.*, 2006). Some plants induce AHL mediated quorum sensing by soil bacteria (Degrassi *et al.*, 2007), and the composition of carbohydrates in exudates affects the production of DAPG (Standing *et al.*, 2008). As shown by the expression of *phlA* in this study, bigger plants support similar bacterial density on the roots, but increase the production of antibiotics as compared to smaller roots. Notably, in infected plants the expression of secondary metabolites increased more rapidly in bigger plants, suggesting that if needed larger plants more efficiently foster

the production of antibiotics by rhizosphere bacteria than smaller plants; presumably, the latter lack resources which can be allocated for improving plant defence. Exudation is costly for the plant, and is strictly regulated according to the needs of the plant as triggered by environmental conditions such as nutrient availability (Paterson *et al.*, 2006). The low bacterial toxin production on small plants, especially the infected ones, supports this theory and points to a depletion of plant resources upon infection.

Exudation

Infection by *Pythium* only marginally influenced the exudation of the tested compounds at the local scale, but fostered the exudation in the distal root part. In an earlier study investigating barley root exudates after attack of *Fusarium graminearum* we also found increased exudation of *t*-cinnamic, ferulic, syringic, indoleacetic, 4-hydroxyphenylacetic and benzoic acids on the non-infected side (Lanoue et al., unpublished), but these compounds were not detected upon *P. ultimum* infection in the present study. Root infection with *P. ultimum* specifically increased the amounts of vanillic, coumaric and fumaric acid almost threefold in non-infected roots, suggesting that these phenolic compounds are involved in root-bacteria communication.

5.6. Conclusion

Results of this study suggest that plants modulate the metabolism of rhizosphere bacteria and manipulate them to produce chemical compounds which improve plant fitness. Increased production of the fungal toxin DAPG by the bacteria reduced bacterial investment in growth suggesting that at least in short term the service of the bacteria to the plants impeded bacterial fitness. Since DAPG is also phytotoxic and inhibits root growth (Brazelton *et al.*, 2008), plants may gain advantage by turning down DAPG production if no longer required. A more detailed understanding of the communication between plants and rhizosphere bacteria is needed for improving crop protection by biocontrol rhizobacteria.

5.7. Acknowledgments

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Chapter 6

Toxicity change the integration of biocontrol bacteria in soil foodwebs

6. Toxicity change the integration of biocontrol bacteria in soil foodwebs

6.1. Abstract

The fitness of soil bacteria is mainly determined with their ability to compete for nutrients and avoid predation. Introduced bacterial populations are often massively consumed by microfaunal predators, and rapidly decline in non sterile soil. In this work, we follow the fate of the biocontrol bacterium *Pseudomonas fluorescens* CHA0 in non sterile soil, and the role of bacterial toxins in avoiding predation. We compared the fate of the wildtype, toxic strain CHA0 and an isogenic gacS deficient mutant lacking secondary metabolites. We labelled the bacteria with ^{13}C glucose, and revealed the eukaryotic consumers of the introduced bacteria by RNA-Stable Isotope Probing. Results show a rapid consumption of non-toxic bacteria by a diverse eukaryotic community dominated by amoebae, cercomonads and fungi, suggesting that predation is a major factor affecting bacterial survival. Toxin production conferred an efficient protection to the bacteria, and the wild type strain was consumed at a lower rate and by a less diverse community. This indicates that only few organisms adapted to toxic preys, and that toxicity improves bacterial fitness by limiting the numbers of potential predators. Resistance to predation appears thus as a key feature that allows introduced bacteria maintaining stable populations in soils.

Keywords
Biocontrol bacteria / predation / stable isotope probing / protozoa

6.2. Introduction

Predation is one of the major forces structuring soil bacterial communities. Microfaunal predators, in particular protozoa and nematodes, consume a major part of bacterial production (Griffiths, 1994), and shape the structure of bacterial communities (Blanc *et al.*, 2006; Rønn *et al.*, 2002; Rosenberg *et al.*, 2009). Bacteria developed various strategies to cope with predation pressure. Morphological adaptations, such as formation of microcolonies and filaments, prevent consumption by flagellates (Hahn *et al.*, 2004; Hahn *et al.*, 1999), and toxin production reduces prey mortality by repelling or killing predators (Jousset *et al.*, 2006; Matz *et al.*, 2004b).

Bacterial defences are often controlled by cell signalling (Jousset *et al.*, 2006; Matz *et al.*, 2008a; Queck *et al.*, 2006). Bacteria produce autoinducer molecules, which concentration depends on population density and environmental factors (Muller et al., 2006). At a certain threshold of autoinducer concentration defensive traits are activated (Williams, 2007). This coordinated cell signalling allows bacteria to optimise their investment in secondary metabolism, and to activate antipredator strategies at high population density where toxicity is more advantageous (Jeschke, 2006). In pseudomonads, secondary metabolism is controlled at the posttranscriptional level by the gac/rsm cascade: Activation of the two component system GacS/GacA by unknown signals results in the production of the small RNAs rsmX, rsmY and rsmZ (Kay et al., 2005), which relieve post-transcriptional inhibition of mRNAs by removing proteins blocking the ribosome binding site (Lapouge et al., 2007).

Resistance to protozoan grazing strongly increases the fitness of soil bacteria (Jousset et al., 2008). However, defence strategies do not work equally for all predators (Pedersen *et al.*, 2009; Weitere *et al.*, 2005). Predator species differ in their sensitivity to bacterial toxins (Jousset et al., 2006), and some predators such as the naked amoeba *Acanthamoeba castellanii* developed detoxification mechanisms helping to overcome prey toxicity (Edwards and Lloyd, 1978). This suggests that some species may even specialise on unpalatable or highly toxic prey species. Consequently, toxicity may strongly affect the integration of bacteria into soil foodwebs by modifying the intensity of predation pressure as well as the identity of the main predators.

We investigated the impact of antipredator traits on the fate of the biocontrol bacteria *Pseudomonas fluorescens* CHA0 in natural soil. This bacterial strain produces various toxic exometabolites including 2,4,-Diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and cyanhidric acid (Haas and Keel, 2003). These toxins inhibit a wide range of plant pathogens and present high potential for the biological control of crop diseases (Haas and Defago, 2005). The toxins also inhibit protozoa (Jousset et al., 2006) and nematodes (Cronin et al., 1997; Gallagher and Manoil, 2001), and confer resistance to protozoan grazing (Jousset et al., 2006). We investigated the role of toxin-production for the integration of *P.*

fluorescens into the soil foodwebs by comparing the fate of the toxin-producing wild type strain CHA0 with its isogenic *gacS*$^{-}$ mutant CHA19 defective in exoproduct formation (Zuber et al., 2003).

Incorporation of bacterial carbon into the soil foodwebs was followed by RNA Stable Isotope Probing (RNA SIP; Whiteley et al., 2006). Bacteria were labelled with ^{13}C, and the predators actively feeding on the two strains were identified by T-RFLP fingerprints and by cloning of the ^{13}C labelled RNA fractions. RNA SIP has been successfully applied to investigate predators consuming methylotroph bacteria (Murase and Frenzel, 2007) and marine cyanobacteria (Frias-Lopez et al., 2009). In this study we used RNA SIP to analyse the influence of bacterial toxins on the fate of introduced bacteria into agricultural systems. We hypothesized that bacterial exoproducts will reduce predation and thus limit the transfer of carbon to higher trophic levels, and that toxic bacteria will be consumed by a different predator community.

6.3. Material and Methods

Bacterial strains and ^{13}C labelling

The biocontrol bacterium *Pseudomonas fluorescens* CHA0 and its isogenic *gacS*$^{-}$ mutant CHA19 were chromosomally tagged with gfp at the tn7 neutral site (Jousset et al., 2006). Bacteria were stored at -80°C. Prior to the experiment, bacteria were grown (24 h, 28°C) on NA plates supplemented with 50 μg mL^{-1} kanamycine sulphate (Sigma-Aldrich, St- Louis, USA).

Bacteria were fully labelled with ^{13}C as follows: One colony was picked and grown (12 h, 20°C, 400 RPM) in 10 mL OSG minimal medium (Schnider-Keel et al., 2000) supplemented with 100 mM $^{13}C_6$ glucose as sole carbon source. The culture was pelleted and resuspended in Phosphate Buffer Saline (PBS) at a density of 10^8 bacteria mL^{-1}. 100 μL of this suspension served as starter for a new 10 mL culture in OSG-Glucose. Late exponential phase cultures were pelleted by centrifugation (8000 g, 5 min), washed twice in Amoeba Saline (AS; Page, 1988). Resuspended bacteria were left starving for 6 h at 17 °C in order to avoid oversecretion of labelled extracellular products in the soil, pelleted and resuspended in AS at a density of $5x10^9$ bacteria mL^{-1}. Non-labelled control cultures for each strain were grown under the same conditions but with ^{12}C glucose as sole carbon source.

Isotopic signatures of labelled bacteria (atom-%) were analysed with an EA/IRMS elemental analyzer (Euro EA, Eurovector, Milan, Italy) coupled with a MAT 253 isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany).

Microcosms

Alluvial soil from an experimental field in Jena, Thüringen (Baade, 2001; Roscher et al., 2004) was sieved (1 mm mesh) and homogeneously inoculated with washed *P. fluorescens* CHA0 or CHA19 cells at a density of 6.5 * 10^7 CFU g^{-1} soil. Sterile 30 x 200 mm glass tubes (Schott, Mainz, Germany) were

filled with 50 g of the inoculated soil, taped with sterile cotton wool and incubated at 17°C in the obscurity (80% relative humidity). Three replicates were set up for each of the four treatments (CHA0 ^{12}C, CHA0 ^{13}C, CHA19 ^{12}C, CHA19 ^{13}C) and time point.

Sampling

After 3 and 6 days microcosms were destructively sampled. For bacteria counts, 1 g soil was shaken vigorously for 10 min in 10 mL AS, and bacteria were enumerated by the drop plate method (Chen et al., 2003) on NA plates supplemented with 25 μg mL^{-1} kanamycine. For nucleic extraction, 3 x 0.5 gram soil portions were taken with a clean metal spatula and total nucleic acids were extracted according to a previously described protocol (Lueders et al., 2006). Briefly, soil was mixed with a detergent suspension amended with β-mercaptoethanol, cells were lysed by bead beating and RNA extracted with subsequent Phenol–Chloroform (pH 5.0) method, and purified in PEG 6000. DNA was digested with DNAse I (Promega, Madison, USA), and RNA samples were stored at -20 °C prior to further processing.

RNA fractionation and analysis

Five hundred ng of Ribogreen (Invitrogen, Carlsbad, CA, USA) quantified RNA were fractionated on a self generated Cesium Trifluoroacetate (CsTFA) density gradient by ultracentrifugation (45'000 g, 72h) on a vertical rotor according to an established protocol (Whiteley et al., 2007). For each gradient twelve fractions were collected by displacement with water (Manefield et al., 2002) using a Perfusor V syringe pump (B. Braun, Melsungen, Germany) at a flow rate of 1 ml min^{-1}. The density of each fraction was determined by refractometry (Lueders et al., 2004a); RNA was purified by precipitation in 1 vol. isopropanol and resuspended in TE Buffer (pH 8.0).

Bacterial and eukaryal rRNA copies in the different fractions were quantified by reverse transcriptase quantitative PCR (RT-qPCR) as follows: 16S rRNA was amplified with the general eubacterial primer set Ba519F/ba907r (Lueders et al., 2004a). 18S rRNA was amplified with the primer set Euk20F/ Euk516R as described elsewhere (Euringer and Lueders, 2008). Bacterial template was quantified using dilution series (10^8-10^1 copies μL^{-1}) of the 16S rRNA gene from *E. coli* (Lueders et al., 2006). Eukaryal template was quantified using a dilution series (10^7-10^0 copies μL^{-1}) of almost full-length amplicons of the *Bodo designis* 18S rRNA gene amplified with the Euk20F/Euk1179R primer set (Euringer and Lueders, 2008).

Terminal restriction fragment length polymorphism (T-RFLP) patterns were obtained as published elsewhere (Euringer and Lueders, 2008). Briefly, rRNA from fractions covering the four gradients was amplified by a during 25 cycles were amplified with a FAM labelled forward primer and digested with the Bsh1236 enzyme. Electrophoresis of terminal restriction fragments was carried out with POP-7 polymer in a 50-cm capillary array with 10 s injection time, 2 kV injection voltage, 7 kV run voltage,

66°C run temperature and 63-min analysis time. Electropherograms were analyzed using the GeneMapper 3.5 software package (Applied Biosystems, Carlsbad, USA).

18S rRNA from the heavy fraction of the CHA19 13C gradient was amplified with the Euk20F/Euk516R primer set (Euringer and Lueders, 2008), cloned into competent Escherichia coli with the PCR cloning kit (Qiagen, Germantown, USA) according to manufacturer's instructions. Hundred clones were randomly picked and partially sequenced with the M13F universal primer (Macrogen, Seoul, Korea).

Statistical analyses

T-RFLP patterns were binned with the T-REX software (Culman et al., 2008). Peaks appearing in less than two samples were discarded. Three fractions from the "heavy" and "light" regions were chosen according to the peaks in the qPCR analyses and used for statistical analysis. Diversity in the different fractions was estimated with the Shannon index. Normalised T-RFLP patterns were ordinated by detrended correspondence analysis (DCA). Statistical analyses were carried out with R 2.8.0 (www.r-stats.org).

6.4. Results

Bacterial survival

Both introduced bacterial strains differed in their competitiveness in the soil ($F_{1,30}$=55.0, p<0.001) and their respective populations developed differently during the experiment ($F_{1,30}$=4.4, p=0.02 for Strain x Time interaction). The population of the wildtype strain *P. fluorescens* CHA0 remained stable at around 6 * 10^7 CFU g^{-1} soil, whereas the population of the CHA19 strain decreased by up to 66% after 9 days (Figure 18).

Isotope-analysis of the labelled bacteria before inoculation confirmed full labelling of the two strains *P. fluorescens* CHA0 and CHA19 grown on $^{13}C_6$ glucose (≥95% ^{13}C). RT-qPCR analysis of eubacterial 16S rRNA and eukaryotic 18S rRNA revealed a sharp peak in the heavy region (1.8245 g mL^{-1}) of the gradients in the ^{13}C treatments (Figure 19), demonstrating successful labelling and integration of labelled bacterial carbon into consumers.

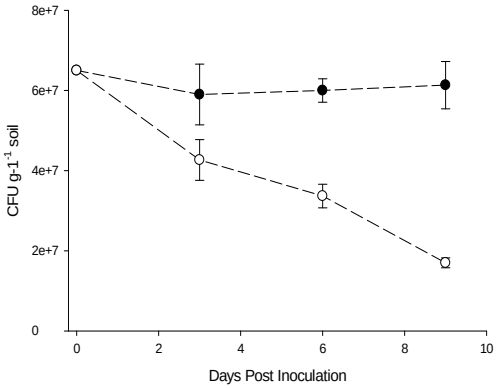

Figure 18: Survival of introduced Pseudomonas fluorescens CHA0 (closed symbols) or its isogenic gacS- mutant CHA19 (open symbols) in non- sterile agricultural soil. Error bars show ± SE.

Inoculation with the non toxic strain *P. fluorescens* CHA19 resulted in a sharp ^{13}C peak, suggesting selective feeding of consumers on this strain (Figure 19). Inoculation with the toxic wild-type strain CHA0 resulted in a smaller ^{13}C peak, and in a ^{12}C peak which was shifted to the heavy fractions (Figure 19), indicating that only few eukaryote species specifically consumed this strain, whereas these bacteria constituted only a minor part in the diet of the bulk of the remaining consumers.

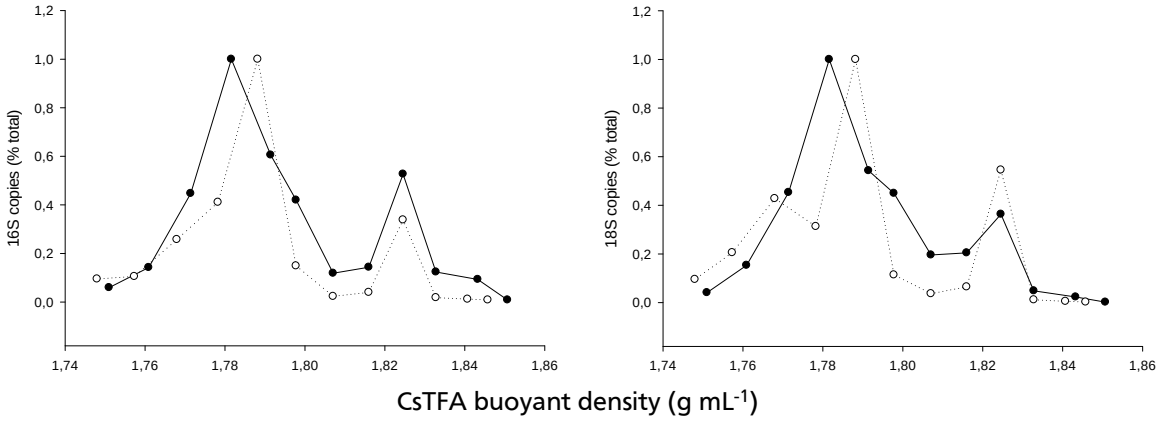

Figure 19: Quantitative profile of bacterial 16S and eukaryotic 18S rRNA along CsTFA density gradients from soil microcosms inoculated with 13C labelled bacteria. Microcosms were inoculated with 108 CFU g-1 Pseudomonas fluorescens CHA0 (solid lines) or its isogenic gacS- mutant CHA19 (dashed lines) and incubated for three days at 17°C.

Consumers communities

The diversity of eukaryotic assemblages (H'=2.49 ± 0.15) in the light fractions of the gradients were identical for both bacterial strains, suggesting that inoculation affected the structure of the bulk community only little. The diversity in the heavy fractions was lower than in the light fractions ($F_{1,7}$=25.9, p=0.001), showing that only part of the soil eukaryotic community was involved in the consumption of the inoculated bacteria. Eukaryotic diversity in the heavy fractions was 25% lower in

the CHA0 ^{13}C treatment (H'=1.61 ± 0.09) compared to the CHA19 ^{13}C treatment ($F_{1,7}$=6.7, p=0.03 for Strain x Fraction interaction), suggesting that carbon from the wild type bacteria was available only to a restricted consumer community.

T-RFLP fingerprints revealed different peaks specific to the heavy fraction (Figure 20). The wild type strain CHA0 supported a less diverse community in the heavy fraction than the gacS$^-$ mutant CHA19, suggesting that fewer species were able to consume this strain.

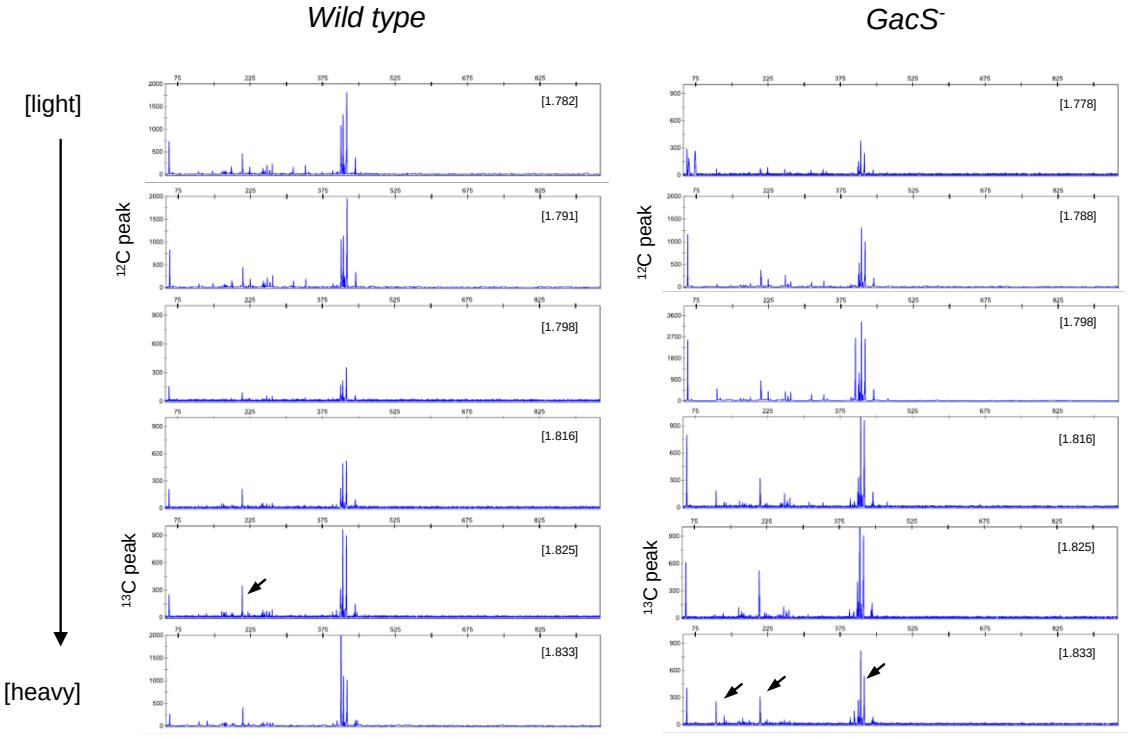

Figure 20: T-RFLP patterns of eukaryotic communities along CsTFA gradients from soil inoculated with 13C labelled Pseudomonas fluorescens CHA0 (left) or CHA19 (right). CsTFA density (g mL-1) of the fractions is given in brackets. Arrows show peaks characteristic for the heavy fractions.

The heavy fraction from the CHA19 ^{13}C treatment showed an increased abundance of the 118, 209 and 210 bp T-RFs (Figure 20). In contrast, the heavy fractions from the CHA0 ^{13}C treatments showed an increased abundance of the 209 bp T-RF, suggesting that the corresponding species consumed both strains. Moreover, an increase in the 418 and 425 bp fragments in the CHA0 ^{13}C treatment but not in the CHA19 treatment, suggests the existence of a specialist consumer adapted to consume the toxic bacteria. Eukaryotic communities from the light fractions were dominated by T-RFs associated with fungi, suggesting that protozoa constituted only a minor part of the total eukaryotic biomass in the tested soil. In contrast, clone libraries of the heavy fractions from the CHA19 ^{13}C treatment revealed high abundance of clones related to bodonids, cercozoa and heterolobosea (Table 5). Almost no ciliate sequences were retrieved, but surprisingly about 5% of all sequences corresponded to the soil alga *Heterothrix debilis*, from which no bacterivorous activity is known.

Table 5: Eukaryotic clones amplified from the heavy gradient fractions of the microcosm inoculated with ^{13}C labelled *Pseudomonas fluorescens* CHA19.

Affiliation /predicted T-RF (bp)	Closest NCBI match	Accession number	% homology
Cercomonads			
124	*Cercomonas longicauda*	AF411270.1	98%
181	Uncultured cercozoan clone 13-2.7	AY620300.1	92%
425	Uncultured cercozoan clone 10-3.2	AY620291.1	87%
419	*Arachnula impatiens* strain BaikalA155	EU567294.1	98%
Bodonids			
193	*Neobodo designis* strain HFCC330	DQ207586.1	100%
222	*Neobodo designis* isolate Panama	AY753617.1	95%
Amoebozoa			
177	*Lobosea* sp.	AB425950.1	91%
178	*Acanthamoeba* sp. EIDS3	AM408802.1	95%
426	*Lobosea* sp.	AB425950.1	94%
429	Uncultured Euamoebida clone D0570_60_S	EU646945.1	94%
Heterolobosea			
195	*Hartmannella* sp. 2 4/3Da/10	AY680841.1	96%
213	*Heterolobosea* sp. OSA	DQ388520.1	95%
257	*Heterolobosea* sp. OSA	DQ388520.1	95%
261	*Heterolobosea* sp. OSA	DQ388520.1	95%
353	Hartmannellidae environmental	EF023499.1	99%
429	Hartmannellidae environmental	EF023499.1	94%
Xanthophyceae			
373	*Heterothrix debilis*	U43277.1	99%
424	*Heterothrix debilis*	U43277.1	99%
497	*Heterothrix debilis*	U43277.1	99%
Ciliates			
259	*Colpoda magna*	EU039896.1	99%
420	Oxytrichidae environmental sample	EF024827.1	97%

Fungi				
	67	*Basipetospora chlamydospora*	AB024045.1	100%
	67	*Fusarium oxysporum*	AB110910.1	100%
	182	*Syncephalis depressa*	AB016011.1	97%
	228	*Scotiellopsis terrestris*	AB012847.1	99%
	406	Uncultured Pucciniomycotina	EU647044.1	99%
	407	Uncultured fungus	AM114819.1	99%
	416	*Lecythophora hoffmannii*	AJ496245.1	98%
	416	*Glyphium elatum*	AF346419.1	97%
	422	Uncultured fungus clone	EU733583.1	94%
	428	*Diversispora* sp.	AM713421.1	96%
	432	*Magnaporthe grisea*	AB026819.1	97%

6.4.1. Discussion

Results of this study suggest that cell-signalling controlled toxicity plays a key role in the interaction of rhizosphere bacteria with eukaryotic bacterivores, causing significant changes in the integration of introduced bacteria in agricultural soil foodwebs.

Predation intensity

The toxic wild type strain *P. fluorescens* CHA0 maintained high population density in the inoculated soils while populations of the gacS$^-$ strain CHA19 rapidly declined. This is in agreement with past studies showing that cell signalling is an important factor for the competitiveness of pseudomonads in soil (Chancey *et al.*, 2002; Natsch *et al.*, 1994), and by reducing predation pressure in particular (Jousset et al., 2008). Our results show that bacterial toxicity profoundly modifies the integration of bacterial strains into soil foodwebs. Most soil bacteria are nutrient- and energy-limited and exist in a starving state (Ramos et al., 2000a). Therefore, the introduced bacteria presumably were much more attractive to bacterivores which are known to preferentially feed on actively growing bacteria (Gonzalez et al., 1993). In agreement with this assumption, carbon of the non-toxic strain was rapidly transferred to higher trophic levels, suggesting fast response of soil protozoa. Recent studies showed that protozoan populations rapidly increase upon soil amendment with palatable bacteria (Rønn et al., 2001), and isotope gradients of 18S rRNA after three days of incubation could be clearly separated into ^{12}C and ^{13}C labelled populations, showing that the signal was neither blurred by assimilation of unlabelled soil resources by the introduced bacteria nor by transfer of labelled carbon to other bacteria. The distinct peak of eukaryotic rRNA in the heavy fractions of the ^{13}C CHA19 treatment

suggests that this palatable strain supported the growth of an abundant and diverse eukaryotic community. This demonstrates that pseudomonads lacking toxin-production are highly palatable prey, massively consumed when introduced in soil, and that toxicity help them to reduce overall predation pressure. Recent investigations in model systems demonstrated that toxin production is an efficient strategy for bacteria to escape predation (Matz and Kjelleberg, 2005). In particular, *P. fluorescens* has been shown to increase its fitness by inhibiting a wide range of microfaunal predators (Jousset et al., 2006; Jousset et al., 2008). Our results demonstrate that these findings also apply to natural soils. Toxin production by bacteria is a highly efficient strategy to resist predation pressure, since protozoan predators will preferentially consume the less toxic bacterial competitors (Jousset et al. 2008). Already after three days, inoculation of microcosms with the toxic strain *P. fluorescens* CHA0 resulted in a different repartition of eukaryotic rRNA across isotope gradients. A distinct, albeit smaller than for *P. fluorescens* CHA19, peak of fully labelled eukaryotic rRNA could be observed for *P. fluorescens* CHA0, suggesting that a limited number of organisms was able to obtain energy from this strain. In addition, the peak in the ^{12}C region of the gradient was shifted towards fractions of intermediate buoyant density, suggesting that this strain contributed only little to the diet of other eukaryotes.

Diversity of predator communities

The T-RFLP patterns of labelled fractions indicate that the wild-type strain *P. fluorescens* CHA0 was consumed by a less diverse community of predators than the non-toxic strain CHA19. The efficiency of bacterial defence mechanisms varies between predators (Weitere et al., 2005); some predators developed resistance to bacterial toxins such as hydrogen cyanide (Edwards and Lloyd, 1978). The lower number of predators feeding on the wild-type strain *P. fluorescens* CHA0 than on the mutant strain CHA19 suggests, however, that few soil protozoa species that evolved resistance mechanisms against bacterial toxins were present in the soil used.

Eukaryotic communities from the light fractions were dominated by T-RFs associated with fungi, suggesting that protozoa constituted only a minor part of the total eukaryotic biomass in the tested soil. In contrast, clone libraries of the labelled fractions indicated high abundance of clones related to bodonids, cercozoa and heterolobosea (Table 1). These taxa comprise many *r* selected species (Bamforth, 1988) which likely are able to grow rapidly on introduced bacteria. Only few sequences from ciliated protozoa were found. Ciliates are common in soil and potentially important bacterial consumers in wetlands (Murase and Frenzel, 2007). However, they may contribute little to bacteria – protozoa interactions in agricultural soils (Ekelund et al., 2002). Surprisingly, 5% of all clones from the heavy fractions were related to the soil algal protist *Heterothrix debilis*, suggesting that this species may be mixotrophic, a strategy used by various unicellular algae to improve their nutrient status (Kamjunke et al., 2007).

Fungal related T-RFs were found in all fractions of the gradients. Previous experiments have shown that fungi can absorb labelled bacterial carbon (Lueders et al., 2004b). Presumably, fungi took up carbon from dead bacteria. The presence of fungal T-RFs in the heavy fractions of the ^{12}C treatments suggests, however, that fungal abundance has been overestimated, potentially due to different buoyant density of fungal rRNA fragments, related to their G+C content (De Ley, 1970). Nonetheless, the potential transfer of bacterial carbon to fungi indicates that the complexity of microbial foodwebs may be higher than indicated by current models.

Conclusions

This work revealed for the first time how cell-signalling regulated toxin production improves bacterial fitness by reducing the global impact of predation and by reducing the number of consumers. The non-toxic strain was very vulnerable to predation and consumed by a diverse community. However, production of toxins by the wild-type strain resulted in better survival. Toxicity strongly reduced the diversity of consumers feeding on bacteria and subsequently reduced their overall consumption.

Production of toxic secondary metabolites by soil pseudomonads has been primarily characterised as a feature inhibiting plant pathogens (Raaijmakers et al., 2002). Our results show that the same exoproducts involved in the biocontrol of plant pathogens profoundly affect the interaction of the bacteria with the soil microfaunal consumer/predator community. The less pronounced integration of toxic bacteria into the first predator trophic level is likely to result in a divergence in the flow of bacterial carbon to higher trophic levels, and therefore in the long-term affect the fitness of introduced biocontrol bacteria in complex soil systems. Long-term isotope analysis of higher level predators, such as predatory nematodes or mites (Griffiths et al., 2006), is necessary for evaluating the role of bacteria as basal resource of soil foodwebs, and open new possibilities to manage soil conditions fostering antagonistic strains and therefore the susceptibility of soils in harbouring pathogens of plants.

6.5. Acknowledgements

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Chapter 7

General discussion

7. General discussion

This study aimed at understanding better the fitness of rhizosphere bacteria, with a special focus on the fate of inoculated biocontrol *Pseudomonas*. Antibiotics involved in antagonist activity of these bacteria against plant pathogens are well characterised (Raaijmakers *et al.*, 2002), and the impact of introduced biocontrol bacteria on indigenous microbial communities has been extensively investigated (Girlanda *et al.*, 2001; Johansen *et al.*, 2005; Johansen *et al.*, 2002). However, surprisingly few data on the ecology of pseudomonads are available, and most describe the influence of abiotic factors (Duffy and Defago, 2000; Ownley *et al.*, 2003) on bacterial fitness. Studies on the impact of biotic interactions on bacterial fitness and toxin production are still limited (Notz *et al.*, 2001; Pedersen *et al.*, 2009), but the importance of these interactions is now being increasingly recognised (Dubuis *et al.*, 2007).

This work investigated the effect of antibiotics production by *Pseudomonas fluorescens* CHA0 on bacterial fitness, and the regulation of toxicity in response to biotic constraints. Toxic exoproducts play a major role in the antagonism against plant pathogens (Haas and Defago, 2005) and reduce predation pressure in gnotobiotic systems (Jousset *et al.*, 2006), suggesting an overlapping function of these toxins. However, the importance of toxicity for bacterial fitness in natural conditions is poorly understood. To efficiently protect the plants, biocontrol organisms have to persist in the rhizosphere and reach a threshold concentration on the roots (Weller and Raaijmakers, 2002), and understanding the features improving competitiveness in field conditions are primordial for a reliable use of microbial biological control agents (Fravel, 2005).

7.1. Predator-prey interaction and bacterial fitness

Our results demonstrate that predation is a major determinant of the fitness of rhizosphere bacteria. Microfaunal predators (nematodes and protozoa) structure soil bacterial communities (Blanc *et al.*, 2006; Rønn *et al.*, 2002), and can negatively affect populations of susceptible bacteria (Ramirez and Alexander, 1980). On the other side, they promote resistant phenotypes, which are often toxic (Matz and Kjelleberg, 2005).

In absence of predators, toxin production only marginally improves the competitiveness of rhizosphere pseudomonads. Investment in toxic exometabolites can even turn out to be a burden for the producer, and bacterial populations rapidly become overloaded with non-toxic mutants. This suggests that production of antibiotics by rhizosphere bacteria is not a stable trait, and that without selective pressure rhizosphere populations would tend to become less toxic. This is well illustrated by the genetic instability of rhizosphere Pseudomonads. These bacteria spontaneously mutate and deactivate the GacS/GacA regulatory pathway (van den Broek *et al.*, 2005b), resulting in signal blind mutants. Since these mutants do not produce toxins, they may reduce the biocontrol ability of the population,

and their high frequency in laboratory and soil culture may be a threat to the reliable use of bacterial inoculants in crop protection (Chancey *et al.*, 2002; Duffy and Defago, 2000).

Predation however dramatically increased the fitness of the biocontrol bacteria. By preferentially consuming non-toxic phenotypes, predators increase the fitness of toxic bacteria by improving their competitive advantage at the intra- and interspecific level. According to our results, root colonisation of *P. fluorescens* CHA0 was more than tripled in presence of bacterivorous amoebae, and predators reduced by 50% the numbers of non-toxic mutants. This suggests that bacterial secondary metabolites responsible for crop protection are a key feature of bacterial competitiveness in the soil. These toxins function by repelling predators rather than inhibiting competitors, and there is thus an overlap between biocontrol traits and antipredator defence. This is in agreement with the broad spectrum toxicity of exoproducts produced by fluorescent pseudomonads. For example, 2,4, DAPG, a toxin produced by a wide range of biocontrol pseudomonads (de la Fuente *et al.*, 2006) can inhibit organisms from nearly all eukaryotic groups including fungi (Girlanda *et al.*, 2001), heterokonts (de Souza *et al.*, 2003), heterolobosea, alveolata and euglenozoa (Jousset *et al.*, 2006), as well as chloroplastida (Brazelton *et al.*, 2008). Stable isotope probing analysis revealed that cell-signalling regulated traits such as toxicity change the integration of introduced bacteria in the existing soil food webs. The non-toxic strain was rapidly consumed by a diverse protozoan community, and declined rapidly. This is in agreement with past observations that inoculated bacteria lacking defence mechanisms can not maintain stable populations in the soil (Postma et al., 1990; Sorensen et al., 1999), and that toxin deficient fluorescent pseudomonads are a good food source for protozoa (Jousset et al., 2006). Interestingly, toxicity not only reduced the total predation pressure, but also reduced the diversity of organisms able to grow on this strain, suggesting that only few organisms evolved resistance mechanisms against bacterial toxins allowing them to feed on unpalatable preys. Since different predators may have distinct effects on the competitiveness of toxic bacteria, manipulating soil conditions to favour the species that actively consume soil bacteria but avoid the toxic inoculated biocontrol strains may help improve the establishment of bacterial populations in soil.

7.2. Chemical communication and the regulation of toxicity

The rhizosphere is a place of intense chemical communication which modulates bacterial toxin production (Dubuis *et al.*, 2007). By producing autoinducers bacteria communicates with neighbouring cells of the same strain (Gantner *et al.*, 2006) or of different species (Dubuis and Haas, 2007). Bacterial communication can be eavesdropped by the plant (Schuhegger *et al.*, 2006), which can in turn emit or degrade bacterial signals (Degrassi *et al.*, 2007; Dong and Zhang, 2005). Bacteria can moreover increase their toxin production in response to plant pathogens (Notz *et al.*, 2001), and the pathogens can in turn inhibit this reaction (Notz *et al.*, 2002b; Notz *et al.*, 2001).

By using a split root system we could demonstrate that plants, in presence of root pathogens, can stimulate the production of antifungal compounds by pseudomonads in distal parts of the root system, indicating a plant-bacteria crosstalk as part of the defence reaction. Bacterial primary metabolism was unaffected, suggesting a specific elicitation of antifungal activity. In healthy plants a trade-off could be observed between expression of the *phlA* gene involved in 2,4-DAPG production, and the housekeeping rRNA operon, but this relation was inverted in presence of pathogens. We conclude that upon infection plant lead bacteria to invest more resources in antifungal compounds than in their own growth. Plant can select a cultivar-specific antibiotics producing bacterial community (Mazzola *et al.*, 2004), and our results show that they can moreover manipulate the activity of the root associated bacteria. Phenolic composition of root exudates also changed upon plant infection, and these compounds are likely involved in the communication with associated rhizobacteria. Since antifungal toxins also negatively affect plant growth (Brazelton *et al.*, 2008), the optimal strategy for the plant may be to keep bacterial secondary metabolism at a low activity level in normal times, and activate it upon infection. Consequently, selection of crop varieties able to better communicate with root- associated pseudomonads and to stimulate their antifungal activity may be a promising way to improve plant resistance against soil borne pathogens in a low input agricultural system.

We showed moreover that soil bacteria can react to biotic stress like the presence of predators by adapting their toxin production. *Pseudomonas fluorescens* CHA0 responds to chemical cues from bacterivorous amoebae by boosting the expression of defence genes, suggesting that bacteria eavesdrop other rhizosphere organisms and secrete a cocktail of antipredator compounds that may be predator specific.

Toxin production is a key feature for the fitness of rhizosphere pseudomonads and their biocontrol ability. We demonstrated that pseudomonads optimise their investment in defence mechanisms according to the predation risk. Moreover, we showed that microfauna predators are able to counteract prey defence by inhibiting the production of toxins. The production of extracellular toxins by pseudomonads is thus a dynamic process driven by chemical communication with other soil organisms. Since the same toxins are known to function as well as defensive compounds and as well as inhibitors of plant pathogens, bacteria - protozoa interactions likely impact on the antagonistic activity of rhizosphere pseudomonads by by modifying synthesis of antifungal compounds by the bacteria. The potential of soil pseudomonads communities to inhibit plant pathogens may thus depend on the perception and response to microfaunal predators.

7.3. Consequences for crop protection

In this study we showed that biotic interactions are a key determinant for the survival and biocontrol activity of inoculated bacteria. Predator prey interaction appears to be particulary important. In

contrast to the negative effects of predation on rhizobial populations (Ramirez and Alexander, 1980), we demonstrated that microfaunal predators improve the competitiveness of toxic bacterial strains by eliminating competing bacteria, and may even be required for the conservation of biocontrol traits by rhizosphere bacteria. Models of PGPR survival (Strigul and Kravchenko, 2006) need thus to be adjusted according to the defence mechanisms of the strain of interest. Not all toxins are equally efficient against predators, and biocontrol strains that produce toxins efficient against pathogens and predators will probably be more competitive in non-sterile soil. Moreover, since toxic bacteria are consumed by a different predator community compared to strains lacking defence mechanisms, the structure of the soil protozoa community may also profoundly influence the survival of biocontrol bacteria. Bacteria reacted to the presence of predators by increasing the expression of antifungal genes, which implies that optimisation of bacterial defence under high predation risk may be beneficial for the plants by creating hostile conditions against soil born pathogens. Predation pressure on soil bacteria thus likely promotes soil suppressiveness against plant pathogens by fostering a toxic bacterial community, and manipulating predation regime on soil microbial communities may help improve the future applications of biocontrol bacteria in the agriculture.

7.4. Perspectives

This worked showed that biotic interactions are a major determinant of the success of bacterial inoculants in soil, and opens new perspectives for the improvement of biological control of crop diseases in the agriculture. In order to achieve this goal, different aspects of microbial ecology deserve further investigation.

The experiments of this work used a model organism, *Pseudomonas fluorescens* CHA0, to answer the importance of secondary metabolites for bacterial fitness, and to explore the chemical communication of biocontrol bacteria with other rhizosphere organisms. This approached allowed using functional mutants and reporter fusions to fit mechanistic models. The next step toward applications of the findings for biological control of plant disease is a generalisation of the results to the entire bacterial community. Different bacterial strains may vary in their response to predators, and the functional overlap between biocontrol activity and predator defence may vary according to the produced toxins and the present predators. Investigation of toxin production by the whole bacterial community under different predation regimes may provide insights in the role of top-down pressure in shaping the functional structure of soil bacterial communities and fostering antagonistic phenotypes. In this purpose, we are currently developing a real-time PCR protocol to quantify the expression of the different biocontrol genes in the soil.

Understanding how chemical cues from diverse soil organisms influence the regulation of bacterial toxicity can also help manipulate the expression of biocontrol traits. In particular, bacteria may

eavesdrop other prokaryotes. First results indicate that *P. fluorescens* CHA0 can respond to AHLs signals from other species, and interaction between different bacterial strains may be thus also a determinant of toxin production *in situ* and deserve further investigation.

In order to better understand the population dynamics and the modulation of genes expression, we need to include a spatial dimension in the experiments. In a structured environment like the rhizosphere, spatial organisation of the bacterial populations has important consequences on resource acquisition, as well as on bacterial communication and defence. Because of the complex structure of root systems, sampling is however tedious and it is extremely complicated to retrieve quantitative results on the spatial population structure. The use of simplified models like biofilm systems simulating root carbon flow, may allow following the effect of predators on the structure of bacterial communities, and to monitor the spatial distribution of gene expression at the microcolony scale.

We showed that bacteria are integrated in complex food webs, and establishing response curves of the principal predator species will allow modelling the bacterial interactions with the predator community and the effect of predation on the functional structure of bacterial populations. Further, understanding the links between the first predators and the higher trophic levels will certainly help managing soil condition to favour “inoculant friendly” predators.

Finally, since resistance to predation increases bacterial fitness in soil, introduction of genes coding for defence toxins may improve the competitiveness of other useful strains such as biofertilisers or pollutant degrading bacteria.

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9. Bibliography

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

Adesina MF, Lembke A, Costa R, Speksnijder A, Smalla K (2007). Screening of bacterial isolates from various European soils for in vitro antagonistic activity towards Rhizoctonia solani and Fusarium oxysporum: Site-dependent composition and diversity revealed. *Soil Biology & Biochemistry* **39:** 2818-2828.

Agrawal AA (2007). Macroevolution of plant defense strategies. *Trends in Ecology & Evolution* **22:** 103-109.

Alfieri SC, Correia CEB, Motegi SA, Pral EMF (2000). Proteinase activities in total extracts and in medium conditioned by *Acanthamoeba polyphaga* trophozoites. *Journal of Parasitology* **86:** 220-227.

Andersen KS, Winding A (2004). Non-target effect of bacterial biological control agents on soil protozoa. *Biology and fertility of soils* **40:** 230-236.

Anderson IJ, Watkins RF, Samuelson J, Spencer DF, Majoros WH, Gray MW *et al* (2005). Gene discovery in the *Acanthamoeba castellanii* genome. *Protist* **156:** 203-214.

Anderson JPE, Domsch KH (1978). Physiological method for quantitative measurement of microbial biomass in soils. *Soil Biology & Biochemistry* **10:** 215-221.

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

Baade J. (2001). Friedrich-Schiller-Universität: Jena.

Baehler E, Bottiglieri M, Pechy-Tarr M, Maurhofer M, Keel C (2005). Use of green fluorescent protein-based reporters to monitor balanced production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens* CHA0. *Journal of Applied Microbiology* **99:** 24-38.

Baehler E, De Werra P, Wick LY, Pechy-Tarr M, Mathys S, Maurhofer M *et al* (2006). Two novel Mvat-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions* **19:** 313-329.

Bai GH, Shaner G (2004). Management and resistance in wheat and barley to Fusarium head blight. *Annual Review of Phytopathology* **42:** 135-161.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006). The role of root exudates in rhizosphere interations with plants and other organisms. *Annual Review of Plant Biology* **57:** 233-266.

Bamforth SS (1988). Interactions between protozoa and other organisms. *Agriculture, Ecosystems and Environment* **24:** 229-234.

Bao Y, Lies DP, Fu H, Roberts GP (1991). An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* **109:** 167-168.

Barazani O, Friedman J (1999). Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *Journal of Chemical Ecology* **25:** 2397-2406.

Beck T, Joergensen RG, Kandeler E, Makeschin F, Nuss E, Oberholzer HR *et al* (1997). An inter-laboratory comparison of ten different ways of measuring soil microbial biomass C. *Soil Biology & Biochemistry* **29:** 1023-1032.

Begon M, Harper JL, Townsend CR (1996). *Ecology: Individuals, populations and communities*, third edn. Blackwell: Malden.

Bennett AJ, Whipps JM (2008). Beneficial microorganism survival on seed, roots and in rhizosphere soil following application to seed during drum priming. *Biological Control* **44:** 349.

Berg G, Opelt K, Zachow C, Lottmann J, Gotz M, Costa R *et al* (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus Verticillium differs depending on plant species and site. *FEMS Microbiology Ecology* **56:** 250-261.

Bergkvist J, Selander E, Pavia H (2008). Induction of toxin production in dinoflagellates: the grazer makes a difference. *Oecologia* **156:** 147-154.

Bergsma-Vlami M, Prins ME, Raaijmakers JM (2005). Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. *FEMS Microbiology Ecology* **52:** 59-69.

Bianciotto V, Andreotti S, Balestrini R, Bonfante P, Perotto S (2001). Mucoid mutants of the biocontrol strain *Pseudomonas fluorescens* CHA0 show increased ability in biofilm formation on mycorrhizal and nonmycorrhizal carrot roots. *Molecular Plant-Microbe Interactions* **14:** 255-260.

Blanc C, Sy M, Djigal D, Brauman A, Normand P, Villenave C (2006). Nutrition on bacteria by bacterial-feeding nematodes and consequences on the structure of soil bacterial community. *European Journal of Soil Biology* **42:** S70-S78.

Blumer C, Haas D (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Archives Of Microbiology* **173:** 170-177.

de Boer W, Folman LB, Summerbell RC, Boddy L (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews* **29:** 795-811.

Boldt TS, Sorensen J, Karlson U, Molin S, Ramos C (2004). Combined use of Different GFP Reporters for monitoring single-cell activities of a genetically modified PCB degrader in the rhizosphere of Alfalfa. *FEMS Microbiology Ecology* **48:** 139-148.

Bonkowski M (2002). Protozoa and plant growth: trophic links and mutualism. *European Journal of Protistology* **37:** 363-365.

Bonkowski M (2004). Protozoa and plant growth: the microbial loop in soil revisited. *New Phytologist* **162:** 617-631.

Bonkowski M, Brandt F (2002). Do soil protozoa enhance plant growth by hormonal effects? *Soil Biology & Biochemistry* **34:** 1709-1715.

Bonkowski M, Cheng WX, Griffiths BS, Alphei G, Scheu S (2000). Microbial-faunal interactions in the rhizosphere and effects on plant growth. *European Journal of Soil Biology* **36:** 135-147.

Bottiglieri M, Keel C (2006). Characterization of PhlG, a hydrolase that specifically degrades the antifungal compound 2,4-diacetylphloroglucinol in the biocontrol agent Pseudomonas fluorescens CHA0. *Applied And Environmental Microbiology* **72:** 418-427.

Brazelton JN, Pfeufer EE, Sweat TA, Gardener BBM, Coenen C (2008). 2,4-diacetylphloroglucinol alters plant root development. *Molecular Plant-Microbe Interactions* **21:** 1349-1358.

Briones AM, Okabe S, Umemiya Y, Ramsing NB, Reichardt W, Okuyama H (2003). Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars. *Plant and Soil* **250:** 335-348.

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

van den Broek D, Chin-a-Woeng TFC, Bloemberg GV, Lugtenberg BJJ (2005b). Molecular nature of spontaneous modifications in GacS which cause colony phase variation in *Pseudomonas* sp. Strain PCL1171. *Journal of Bacteriology* **187:** 593-600.

van den Broek D, Chin-a-Woeng TFC, Eijkemans K, Mulders IHM, Bloemberg GV, Lugtenberg BJJ (2003). Biocontrol traits of *Pseudomonas spp.* are regulated by phase variation. *Molecular Plant-Microbe Interactions* **16:** 1003-1012.

Bull CT, Duffy B, Voisard C, Defago G, Keel C, Haas D (2001). Characterization of spontaneous gacS and gacA regulatory mutants of Pseudomonas fluorescens biocontrol strain CHAO. *Antonie Van Leeuwenhoek* **79:** 327-36.

Burdman S, Okon Y, Jurkevitch E (2000). Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. *Critical Reviews in Microbiology* **26:** 91-110.

Carlone N, Scannerini S (1974). Scanning and transmission electron microscopy evidence of the cytological effect of pyrrolnitrin on *Microsporon audouinii* Gruby CBS 313-54 'in vitro'. *Mycopathologia* **53:** 111-123.

Cavalier-Smith T (2009). Predation and eukaryote cell origins: A coevolutionary perspective. *International Journal Of Biochemistry & Cell Biology* **41:** 307-322.

Chancey ST, Wood DW, Pierson EA, Pierson LSI (2002). Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl. Environ. Microbiol.* **68:** 3308-3314.

Chen CY, Nace GW, Irwin PL (2003). A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocytogenes, and Escherichia coli. *Journal of Microbiological Methods* **55:** 475-479.

Chernin L, Brandis A, Ismailov Z, Chet I (1996). Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. *Current Microbiology* **32:** 208.

Christensen S, Bjornlund L, Vestergard M (2007). Decomposer biomass in the rhizosphere to assess rhizodeposition. *Oikos* **116:** 65-74.

Clardy J, Fischbach MA, Walsh CT (2006). New antibiotics from bacterial natural products. *Nature Biotechnology* **24:** 1541-1550.

Clarholm M (1981). Protozoan grazing of bacteria in soil—impact and importance. *Microbial Ecology* **7:** 343.

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* **71:** 4951-4959.

Conrath U, Beckers GJM, Flors V, Garcia-Agustin P, Jakab G, Mauch F *et al* (2006). Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* **19:** 1062-1071.

Coombs JT, Michelsen PP, Franco CMM (2004). Evaluation of endophytic actinobacteria as antagonists of Gaeumannomyces graminis var. tritici in wheat. *Biological Control* **29:** 359-366.

Corno G, Jurgens K (2006). Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Applied and Environmental Microbiology* **72:** 78-86.

Costa R, van Aarle IM, Mendes R, van Elsas JD (2009). Genomics of pyrrolnitrin biosynthetic loci: evidence for conservation and whole-operon mobility within Gram-negative bacteria. *Environmental Microbiology* **11:** 159-175.

Costa R, Gotz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* **56:** 236-249.

Costacurta A, Vanderleyden J (1995). Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* **21:** 1-18.

Coûteaux M-M, Darbyshire JF (1998). Functional diversity amongst soil protozoa. *Applied Soil Ecology* **10:** 229-237.

Cronin D, MoenneLoccoz Y, Fenton A, Dunne C, Dowling DN, Ogara F (1997). Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode Globodera rostochiensis. *Applied and Environmental Microbiology* **63:** 1357-1361.

Czaban J, Gajda A, Wroblewska B (2007). The motility of bacteria from rhizosphere and different zones of winter wheat roots. *Polish Journal of Environmental Studies* **16:** 301-308.

Danso SKA, Alexander M (1975). Regulation of predation by prey density: the protozoan-*Rhizobium* relationship. *Applied Microbiology* **29:** 515-521.

Darbyshire JF, Wheatley RE, Greaves MP, Inkson RHE (1974). Rapid micromethod for estimating bacterial and protozoan populations in soil. *Revue d' Ecologie et de Biologie du Sol* **11:** 465-475.

De Cock A, Levesque CA, Melero-Vara JM, Serrano Y, Guirado ML, Gomez J (2008). *Pythium solare* sp nov., a new pathogen of green beans in Spain. *Mycological Research* **112:** 1115-1121.

De Ley J (1970). Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. *Journal of Bacteriology* **101:** 738-754.

Declerck P, Behets J, De Keersmaecker B, Ollevier F (2007). Receptor-mediated uptake of *Legionella pneumophila* by *Acanthamoeba castellanii* and *Naegleria lovaniensis*. *Journal of Applied Microbiology* **103:** 2697-2703.

Degrassi G, Devescovi G, Solis R, Steindler L, Venturi V (2007). *Oryza sativa* rice plants contain molecules that activate different quorum-sensing N-acyl homoserine lactone biosensors and are sensitive to the specific Aiia Lactonase. *FEMS Microbiology Letters* **269:** 213-220.

Denison RF, Bledsoe C, Kahn M, O'Gara F, Simms EL, Thomashow LS (2003). Cooperation in the rhizosphere and the "free rider" problem. *Ecology* **84:** 838-845.

Dobbelaere S, Vanderleyden J, Okon Y (2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences* **22:** 107-149.

Dong YH, Zhang LH (2005). Quorum sensing and quorum-quenching enzymes. *Journal of Microbiology* **43:** 101-109.

van Donk E (2007). Chemical information transfer in freshwater plankton. *Ecological Informatics* **2:** 112-120.

Dubuis C, Haas D (2007). Cross-species GacA-controlled induction of antibiosis in pseudomonads. *Applied And Environmental Microbiology* **73:** 650-654.

Dubuis C, Keel C, Haas D (2007). Dialogues of root-colonizing biocontrol pseudomonads. *European Journal of Plant Pathology* **119:** 311-328.

Dubuis C, Rolli J, Lutz M, Defago G, Haas D (2006). Thiamine-auxotrophic mutants of Pseudomonas fluorescens CHA0 are defective in cell-cell signaling and biocontrol factor expression. *Applied And Environmental Microbiology* **72:** 2606-2613.

Duffy B, Keel C, Defago G (2004). Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. *Applied and Environmental Microbiology* **70:** 1836-1842.

Duffy BK, Defago G (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by Pseudomonas fluorescens biocontrol strains. *Applied And Environmental Microbiology* **65:** 2429-2438.

Duffy BK, Defago G (2000). Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Applied and Environmental Microbiology* **66:** 3142-3150.

Duijff BJ, Pouhair D, Olivain C, Alabouvette C, Lemanceau P (1998). Implication of systemic induced resistance in the suppression of fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417R and by nonpathogenic *Fusarium oxysporum* FO47. *European Journal of Plant Pathology* **104:** 903-910.

Dunne C, Moenne-Loccoz Y, McCarthy J, Higgins P, Powell J, Dowling DN *et al* (1998). Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of Pythium-mediated damping-off of sugar beet. *Plant Pathology* **47:** 299-307.

Edwards SW, Lloyd D (1978). Properties of mitochondria isolated from cyanide-sensitive and cyanide-stimulated cultures of *Acanthamoeba castellanii*. *Biochemical Journal* **174:** 203-211.

Ekelund F, Frederiksen HB, Ronn R (2002). Population dynamics of active and total ciliate populations in arable soil amended with wheat. *Applied and Environmental Microbiology* **68:** 1096-1101.

Ekelund F, Ronn R (1994). Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. *FEMS Microbiology Reviews* **15:** 321-353.

El-Tarabily KA, Sivasithamparam K (2006). Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biology & Biochemistry* **38:** 1505-1520.

Euringer K, Lueders T (2008). An optimised PCR/T-RFLP fingerprinting approach for the investigation of protistan communities in groundwater environments. *Journal Of Microbiological Methods* **75:** 262-268.

Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a Pseudomonas strain. *Appl Environ Microbiol* **58:** 3873-8.

Ferris H, Venette RC, Scow KM (2004). Soil management to enhance bacterivore and fungivore nematode populations and their nitrogen mineralisation function. *Applied Soil Ecology* **25:** 19-35.

Foissner W (1999). Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. *Agriculture, Ecosystems and Environment* **74:** 95-112.

Folman LB, Postma J, Veen JA (2001). Ecophysiological characterization of rhizosphere bacterial communities at different root locations and plant developmental stages of cucumber grown on rockwool. *Microbial Ecology* **42:** 586-597.

Fravel DR (2005). Commercialization and implementation of biocontrol. *Annual Review of Phytopathology* **43:** 337-359.

Frias-Lopez J, Thompson A, Waldbauer J, Chisholm SW (2009). Use of stable isotope-labelled cells to identify active grazers of picocyanobacteria in ocean surface waters. *Environmental Microbiology* **11:** 512-525.

Friman VP, Hiltunen T, Laakso J, Kaitala V (2008). Availability of prey resources drives evolution of predator-prey interaction. *Proceedings Of The Royal Society B-Biological Sciences* **275:** 1625-1633.

de la Fuente L, Mavrodi DV, Landa BB, Thomashow LS, Weller DM (2006). *phlD*-based genetic diversity and detection of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. *FEMS Microbiology Ecology* **56:** 64-78.

de la Fuente L, Mavrodi DV, Thomashow LS, Weller DM (2007). Utilization of trehalose, benzoate, valerate, and seed and root exudates by genotypes of 2,4-diacetylphloroglucinol producing *Pseudomonas fluorescens*. *Soil Biology & Biochemistry* **39:** 2712-2722.

Fyda J, Warren A, Wolinska J (2005). An investigation of predator-induced defence responses in ciliated protozoa. *Journal Of Natural History* **39:** 1431-1442.

Gallagher LA, Manoil C (2001). Pseudomonas aeruginosa PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *Journal Of Bacteriology* **183:** 6207-6214.

Gantner S, Schmid M, Durr C, Schuhegger R, Steidle A, Hutzler P *et al* (2006). In situ quantitation of the spatial scale of calling distances and population density-independent N-acylhomoserine lactone-mediated communication by rhizobacteria colonized on plant roots. *FEMS Microbiology Ecology* **56:** 188-194.

Girlanda M, Perotto S, Moenne-Loccoz Y, Bergero R, Lazzari A, Défago G *et al* (2001). Impact of biocontrol *Pseudomonas fluorescens* CHA0 and a genetically modified derivative on the diversity of culturable fungi in the cucumber rhizosphere. *Applied and Environmental Microbiology* **67:** 1851–1864.

Gonzalez JE, Keshavan ND (2006). Messing with bacterial quorum sensing. *Microbiology and Molecular Biology Reviews* **70:** 859-+.

Gonzalez JM, Sherr EB, Sherr BF (1993). Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. *Marine Ecology-Progress Series* **102:** 257-267.

Goswami RS, Kistler HC (2004). Heading for disaster: Fusarium graminearum on cereal crops. *Molecular Plant Pathology* **5:** 515-525.

Gray EJ, Smith DL (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil Biology & Biochemistry* **37:** 395-412.

Greig D, Travisano M (2004). The Prisoner's dilemma and polymorphism in yeast SUC genes. *Proceedings of the royal society of London, series B: Biological sciences* **271:** S25-S26.

Griffiths BS (1994). Soil nutrient flow. In: Darbyshire JF (ed). *Soil Protozoa*. CAB International Willingford, UK. pp 123-146.

Griffiths RI, Bailey MJ, Mcnamara NP, Whiteley AS (2006). The functions and components of the Sourhope soil microbiota. *Applied Soil Ecology* **33:** 114-126.

Haas D, Defago G (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* **3:** 307-319.

Haas D, Keel C (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Reviews of Phytopathology* **41:** 117-153.

Haas D, Keel C, Reimmann C (2002). Signal transduction in plant-beneficial rhizobacteria with biocontrol properties. *Antonie van Leeuwenhoek* **81:** 385-395.

Hahn MW, Lunsdorf H, Janke L (2004). Exopolymer Production and Microcolony Formation by Planktonic Freshwater Bacteria: Defence Against Protistan Grazing. *Aquatic Microbial Ecology* **35:** 297-308.

Hahn MW, Moore ERB, Hofle MG (1999). Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. *Applied and Environmental Microbiology* **65:** 25-35.

Haichar FE, Marol C, Berge O, Rangel-Castro JI, Prosser JI, Balesdent J *et al* (2008). Plant host habitat and root exudates shape soil bacterial community structure. *Isme Journal* **2:** 1221-1230.

Harrison F, Buckling A (2005). Hypermutability impedes cooperation in pathogenic bacteria. *Current Biology* **15:** 1968-1971.

Heeb S, Haas D (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Molecular Plant-Microbe Interactions* **14:** 1351-1363.

Henkes G, Jousset A, Bonkowski M, Thorpe MR, Scheu S, Schurr U *et al* (submitted). Modification of carbon delivery to roots by *Fusarium graminearum* and its systemic repression by *Pseudomonas fluorescens* in barley. *New Phytologist*.

Henkes GJ, Thorpe MR, Minchin PEH, Schurr U, Rose USR (2008). Jasmonic acid treatment to part of the root system is consistent with simulated leaf herbivory, diverting recently assimilated carbon towards untreated roots within an hour. *Plant Cell And Environment* **31:** 1229-1236.

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR *et al* (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148:** 87-102.

Hewitt E (1966). *Sand and water culture methods used in the study of plant nutrition*. Commonwealth Agricultural Bureau: Farnham.

Hilbi H, Weber SS, Ragaz C, Nyfeler Y, Urwyler S (2007). Environmental predators as models for bacterial pathogenesis. *Environmental Microbiology* **9:** 563-575.

Hirsch AM, Bauer WD, Bird DM, Cullimore J, Tyler B, Yoder JI (2003). Molecular signals and receptors: Controlling rhizosphere interactions between plants and other organisms. *Ecology* **84:** 858-868.

Horn M, Wagner M (2004). Bacterial endosymbionts of free-living amoebae. *Journal of Eukaryotic Microbiology* **51:** 509-514.

Hurek T, Reinholdhurek B, Vanmontagu M, Kellenberger E (1994). Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *Journal of Bacteriology* **176:** 1913-1923.

Hutchinson GE (1957). Population Studies - Animal Ecology And Demography - Concluding Remarks. *Cold Spring Harbor Symposia On Quantitative Biology* **22:** 415-427.

Iavicoli A, Boutet E, Buchala A, Metraux JP (2003a). Induced systemic resistance in Arabidopsis thaliana in response to root inoculation with Pseudomonas fluorescens CHA0. *Mol Plant Microbe Interact* **16:** 851-8.

Iavicoli A, Boutet E, Buchala A, Metraux JP (2003b). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions* **16:** 851-858.

Jeschke JM (2006). Density-dependent effects of prey defenses and predator offenses. *Journal Of Theoretical Biology* **242:** 900-907.

Jezbera J, Hornia K, Siimek K (2005). Food selection by bacterivorous protists: insight from the analysis of the food vacuole content by means of .uorescence in situ hybridization. *FEMS Microbiology Ecology* **52:** 351–363.

Jezbera J, Hornak K, Simek K (2006). Prey selectivity of bacterivorous protists in different size fractions of reservoir water amended with nutrients. *Environmental Microbiology* **8:** 1330-1339.

Johansen A, Knudsen IMB, Binnerup SJ, Winding A, Johansen JE, Jensen LE *et al* (2005). Non-target effects of the microbial control agents *Pseudomonas fluorescens* DR54 and *Clonostachys rosea* IK726 in soils cropped with barley followed by sugar beet: a greenhouse assessment. *Soil Biology & Biochemistry* **37:** 2225-2239.

Johansen JE, Binnerup SJ, Lejbolle KB, Mascher F, Sorensen J, Keel C (2002). Impact of biocontrol strain *Pseudomonas fluorescens* CHA0 on rhizosphere bacteria isolated from barley (*Hordeum vulgare* L.) with special reference to *Cytophaga*-like bacteria. *Journal of Applied Microbiology* **93:** 1065-1074.

Jousset A, Lara E, Wall LG, Valverde C (2006). Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape predation. *Applied and Environmental Microbiology* **72:** 7083-7090.

Jousset A, Rochat L, Keel C, Pechy-Tarr M, Scheu S, Bonkowski M (2009). Predators promote toxicity of rhizosphere bacterial communities by selective feeding on non-toxic cheaters. *ISME Journal* **accepted**.

Jousset A, Scheu S, Bonkowski M (2008). Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. *Functional Ecology* **22:** 714-719.

Kamjunke N, Henrichs T, Gaedke U (2007). Phosphorus gain by bacterivory promotes the mixotrophic flagellate *Dinobryon* spp. during re-oligotrophication. *Journal of Planktonic Research* **29:** 39-46.

Kay E, Dubuis C, Haas D (2005). Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0 . *Proceedings of the National Academy of Sciences of the United States of America* **102:** 17136-17141.

Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U *et al* (1992a). Suppression of root diseases by *Pseudomonas fluorescens* CHA0 - Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Molecular Plant-Microbe Interactions* **5:** 4-13.

Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U *et al* (1992b). Suppression of root diseases by Pseudomonas fluorescens CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Molecular Plant-Microbe Interactions* **5:** 4-13.

Keel C, Ucurum Z, Michaux P, Adrian M, Haas D (2002). Deleterious impact of a virulent bacteriophage on survival and biocontrol activity of Pseudomonas fluorescens strain CHA0 in natural soil. *Molecular Plant-Microbe Interactions* **15:** 567-576.

Keller L, Surette MG (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nature Reviews Microbiology* **4:** 249-258.

Khan NA (2006). *Acanthamoeba*: biology and increasing importance in human health. *Fems Microbiology Reviews* **30:** 564-595.

Kiers ET, Denison RF (2008). Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology, Evolution, and Systematics* **39:** 215-236.

Koch B, Jensen L, Nybroe O (2001). A panel of Tn7-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. *Journal of Microbiological Methods* **45:** 187-195.

Koutsoudis MD, Tsaltas D, Minogue TD, von Bodman SB (2006). Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 5983-5988.

Kowalchuk G, Buma D, de Boer W, Klinkhamer P, van Veen J (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie van Leeuwenhoek* **81:** 509.

Kreuzer K, Adamczyk J, Iijima M, Wagner M, Scheu S, Bonkowski M (2006). Grazing of a common species of soil protozoa (*Acanthamoeba castellanii*) affects rhizosphere bacterial community composition and root architecture of rice (*Oryza sativa* L.). *Soil Biology & Biochemistry* **38:** 1665-1672.

Kuikman PJ, Jansen AG, Vanveen JA (1991). 15N-nitrogen mineralization from bacteria by protozoan grazing at different soil-moisture regimes. *Soil Biology & Biochemistry* **23:** 193-200.

Kumar R, Bhatia R, Kukreja K, Behl RK, Dudeja SS, Narula N (2007). Establishment of *Azotobacter* on plant roots: chemotactic response, development and analysis of root exudates of cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.). *Journal of Basic Microbiology* **47:** 436-439.

Kusch J (1999). Self-recognition as the original function of an amoeban defense-inducing kairomone. *Ecology* **80:** 715-720.

Kuzyakov Y (2002). Review: factors affecting rhizosphere priming effects. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **165:** 382-396.

Lambert C, Morehouse KA, Chang CY, Sockett RE (2006). Bdellovibrio: Growth and Development During the Predatory Cycle. *Current Opinion in Microbiology* **9:** 639-644.

Lambertsen L, Sternberg C, Molin S (2004). Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environmental Microbiology* **6:** 726-732.

Lanoué A, Burlat V, Henkes GJ, Koch I, Schurr U, Röse USR (submitted). De novo biosynthesis of defense root exudates during Fusarium attack in barley. *Plant Physiology*.

Lapouge K, Schubert M, Allain FHT, Haas D (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Molecular Microbiology* **67:** 241-253.

Lapouge K, Sineva E, Lindell M, Starke K, Baker CS, Babitzke P *et al* (2007). Mechanism of hcnA mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*. *Molecular Microbiology* **66:** 341-356.

Levrat P, Pussard M, Steinberg C, Alabouvette C (1991). Regulation of *Fusarium oxysporum* populations introduced into soil - the amoebal predation hypothesis. *FEMS Microbiology Ecology* **86:** 123-129.

Liu XY, Shi M, Liao YH, Gao Y, Zhang ZK, Wen DH *et al* (2006). Feeding characteristics of an amoeba (Lobosea : Naegleria) grazing upon cyanobacteria: food selection, ingestion and digestion progress. *Microbial Ecology* **51:** 315-325.

Van Loon LC, Bakker P, Pieterse CMJ (1998). Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36:** 453-483.

Loper J, Gross H (2007). Genomic analysis of antifungal metabolite production by Pseudomonas fluorescens Pf-5. **119:** 265-278.

Lueders T, Kindler R, Miltner A, Friedrich MW, Kaestner M (2006). Identification of bacterial micropredators distinctively active in a soil microbial food web. *Applied and Environmental Microbiology* **72:** 5342-5348.

Lueders T, Manefield M, Friedrich MW (2004a). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environmental Microbiology* **6:** 73-78.

Lueders T, Wagner B, Claus P, Friedrich MW (2004b). Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. *Environmental Microbiology* **6:** 60-72.

Lugtenberg BJJ, Chin-A-Woeng TFC, Bloemberg G (2002). Microbe-plant interactions, principles and mechanisms. *Antonie van Leeuwenhoek* **81:** 373-383.

Lugtenberg BJJ, Dekkers L, Bloemberg G (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Reviews of Phytopathology* **39:** 461-490.

Mahenthiralingam E, Urban TA, Goldberg JB (2005). The multifarious, multireplicon Burkholderia cepacia complex. *Nature Reviews Microbiology* **3:** 144-156.

Manefield M, Whiteley AS, Griffiths RI, Bailey MJ (2002). RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. *Applied and Environmental Microbiology* **68:** 5367-5373.

Mao XF, Hu F, Griffiths B, Chen XY, Liu MQ, Li HX (2007). Do bacterial-feeding nematodes stimulate root proliferation through hormonal effects? *Soil Biology & Biochemistry* **39:** 1816-1819.

Mark GL, Dow JM, Kiely PD, Higgins H, Haynes J, Baysse C *et al* (2005). Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 17454-17459.

Marschner P, Crowley DE, Higashi RM (1997). Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L). *Plant and Soil* **189:** 11-20.

Marschner P, Neumann G, Kania A, Weiskopf L, Lieberei R (2002). Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus Albus* L.). *Plant and Soil* **246:** 167-174.

Martin FN, Loper JE (1999). Soilborne Plant Diseases Caused by Pythium Spp: Ecology, Epidemiology, and Prospects for Biological Control. *Critical Reviews in Plant Sciences* **18:** 111-181.

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

Mathesius U, Mulders S, Gao M, Teplitski M, G C-As, Rolfe B *et al* (2003). Extensive and specific responses of a Eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sci. USA* **100:** 1444-1449.

Matz C, Bergfeld T, Rice SA, Kjelleberg S (2004a). Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environmental Microbiology* **6:** 218-226.

Matz C, Deines P, Boenigk J, Arndt H, Eberl L, Kjelleberg S *et al* (2004b). Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. *Applied and Environmental Microbiology* **70:** 1593-1599.

Matz C, Kjelleberg S (2005). Off the hook – how bacteria survive protozoan grazing. *Trends in Microbiology* **13:** 302-307.

Matz C, Moreno AM, Alhede M, Manefield M, Hauser AR, Givskov M *et al* (2008a). Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. *Isme Journal* **2:** 843-852.

Matz C, Webb JS, Schupp PJ, Phang SY, Penesyan A, Egan S *et al* (2008b). Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *PLoS ONE* **3:** e2744.

Mazzola M (2002). Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81:** 557-564.

Mazzola M, Funnell DL, Raaijmakers JM (2004). Wheat cultivar-specific selection of 2,4-diacetylphloroglucinol-producing fluorescent Pseudomonas species from resident soil populations. *Microbial Ecology* **48:** 338-348.

McMullen M, Halley S, Schatz B, Meyer S, Jordahl J, Ransom J (2008). Integrated strategies for Fusarium head blight management in the United States. *Cereal Research Communications* **36:** 563-568.

McMullen M, Jones R, Gallenberg D (1997). Scab of wheat and barley: A re-emerging disease of devastating impact. *Plant Disease* **81:** 1340-1348.

Meyer JR, Kassen R (2007). The effects of competition and predation on diversification in a model adaptive radiation. *Nature* **446:** 432-435.

Mishra RPN, Singh RK, Jaiswal HK, Kumar V, Maurya S (2006). Rhizobium-mediated induction of phenolics and plant growth promotion in rice (Oryza sativa L.). *Current Microbiology* **52:** 383-389.

Moore JC, McCann K, Setälä H, De Ruiter PC (2003). Top-down is bottom-up: Does predation in the rhizosphere regulate aboveground dynamics? *Ecology* **84:** 846-857.

Muller J, Kuttler C, Hense BA, Rothballer M, Hartmann A (2006). Cell-cell communication by quorum sensing and dimension-reduction. *Journal of Mathematical Biology* **53:** 672-702.

Murase J, Frenzel P (2007). A methane-driven microbial food web in a wetland rice soil. *Environmental Microbiology* **9:** 3025-3034.

Natsch A, Keel C, Pfirter HA, Haas D, Defago G (1994). Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. *Applied and Environmental Microbiology* **60:** 2553-2560.

Neidig N, Jousset A, Paul R, Scheu S (in prep). Bacterial toxins from the biocontrol bacterium *Pseudomonas fluorescens* CHA0 act nematostatic and repellant against *Caenorhabditis elegans*.

Nguyen C (2003). Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* **23:** 375-396.

Normander B, Hendriksen NB, Nybroe O (1999). Green Fluorescent Protein-Marked *Pseudomonas fluorescens*: Localization, Viability, and Activity in the Natural Barley Rhizosphere. *Applied and Environmental Microbiology* **65:** 4646–4651.

Notz R, Maurhofer M, Dubach H, Haas D, Defago G (2002a). Fusaric acid-producing strains of Fusarium oxysporum alter 2,4-diacetylphloroglucinol biosynthetic gene expression in Pseudomonas fluorescens CHA0 in vitro and in the rhizosphere of wheat. *Appl Environ Microbiol* **68:** 2229-35.

Notz R, Maurhofer M, Dubach H, Haas D, Defago G (2002b). Fusaric acid-producing strains of Fusarium oxysporum alter 2,4-diacetylphloroglucinol biosynthetic gene expression in Pseudomonas fluorescens CHA0 in vitro and in the rhizosphere of wheat. *Applied And Environmental Microbiology* **68:** 2229-2235.

Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D, Defago G (2001). Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene phlA in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. *Phytopathology* **91:** 873-881.

Ongena M, Jacques P (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends In Microbiology* **16:** 115-125.

Ownley BH, Duffy BK, Weller DM (2003). Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. *Applied and Environmental Microbiology* **69:** 3333-3343.

Page FC (1988). *A new key to freshwater and soil gymnaboeae*. Freshwater Biological Association: Ambleside.

Parry JD (2004). Protozoan grazing of freshwater biofilms. pp 167-196.

Paterson E, Sim A, Standing D, Dorward M, McDonald AJ (2006). Root exudation from Hordeum vulgare in response to localized nitrate supply. *J Exp Bot* **57:** 2413-20.

Pechy-Tarr M, Bottiglieri M, Mathys S, Lejbolle KB, Schnider-Keel U, Maurhofer M *et al* (2005). RpoN (sigma(54)) controls production of antifungal compounds and biocontrol activity in Pseudomonas fluorescens CHA0. *Molecular Plant-Microbe Interactions* **18:** 260-272.

Pechy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Vogne C, Henkels MD *et al* (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of Pseudomonas fluorescens. *Environmental Microbiology* **10:** 2368-2386.

Pedersen A, Nybroe O, Winding A, Ekelund F, Bjørnlund L (2009). Bacterial Feeders, the Nematode Caenorhabditis elegans and the Flagellate Cercomonas longicauda, have different Effects on Outcome of Competition among the Pseudomonas Biocontrol Strains CHA0 and DSS73. *Microbial Ecology*.

Pernthaler J (2005). Predation on prokaryotes in the water column and its ecological implications. *Nature Reviews Microbiology* **3:** 537-546.

Phillips DA, Ferris H, Cook DR, Strong DR (2003). Molecular control points in rhizosphere food webs. *Ecology* **84:** 816-826.

Phillips DA, Fox TC, King MD, Bhuvaneswari TV, Teuber LR (2004). Microbial products trigger amino acid exudation from plant roots. *Plant Physiology* **136:** 2887-2894.

Pickup ZL, Pickup R, Parry JD (2007). Effects of bacterial prey species and their concentration on growth of the amoebae *Acanthamoeba castellanii* and *Hartmannella vermiformis*. *Applied and Environmental Microbiology* **73:** 2631-2634.

Pieterse CMJ, Van Pelt JA, Verhagen BWM, Ton J, Van Wees SCM, Leon-Kloosterziel KM *et al* (2003). Induced systemic resistance by plant growth-promoting rhizobacteria. *Symbiosis* **35:** 39-54.

Pohnert G, Steinke M, Tollrian R (2007). Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. *Trends in Ecology & Evolution* **22:** 198.

Pollierer MM, Langel R, Korner C, Maraun M, Scheu S (2007). The underestimated importance of belowground carbon input for forest soil animal food webs. *Ecology Letters* **10:** 729-736.

Posch T, Jezbera J, Vrba J, Šimek K, Pernthaler J, Andreatta S *et al* (2001). Size Selective Feeding in *Cyclidium glaucoma* (Ciliophora, Scuticociliatida) and Its Effects on Bacterial Community Structure: A Study from a Continuous Cultivation System. *Microbial Ecology* **42:** 217-227.

Postma J, Hok-A-Hin CH, J.A. VV (1990). Role of microniches in protecting introduced *Rhizobium leguminosarum* biovar trifolii against competition and predation in Soil. *Applied and Environmental Microbiology* **56:** 495-502.

Postma J, Schilder MT, Bloem J, van Leeumen-Haagsma WK (2008). Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. *Soil Biology & Biochemistry* **40:** 2394-2406.

Pussard M, Alabouvette C, Levrat P (1994). Protozoan interactions with the soil microflora and possibilities for biocontrol of plant pathogens. In: Darbyshire JF (ed). *Soil Protozoa*. CAB International Willingford, UK. pp 123-146.

van der Putten WH, Mortimer SR, Hedlund K, Van Dijk C, Brown VK, Leps J *et al* (2000). Plant species diversity as a driver of early succession in abandoned fields: a multi-site approach. *Oecologia* **124:** 91-99.

Queck SY, Weitere M, Moreno AM, Rice SA, Kjelleberg S (2006). The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. *Environmental Microbiology* **8:** 1017-1025.

Raaijmakers JM, Vlami M, De Souza JT (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81:** 537-547.

Ramirez C, Alexander M (1980). Evidence Suggesting Protozoan Predation on *Rhizobium* Associated with Germinating Seeds and in the Rhizosphere of Beans (*Phaseolus vulgaris* L.). *Applied and Environmental Microbiology* **40:** 492-499.

Ramos C, Molbak L, Molin S (2000a). Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents and synthesis Rates. *Applied and Environmental Microbiology* **66:** 801-809.

Ramos C, Molbak L, Molin S (2000b). Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents and synthesis rates. *Appl Environ Microbiol* **66:** 801-9.

Van Rhijn P, Vanderleyden J (1995). The *Rhizobium*-plant symbiosis. *Microbiological reviews* **59:** 124-142.

Rochat L, Pechy-Tarr M, baehler E, Maurhofer M, Keel C (in prep). Fluorescent dual-color reporters for simultaneous monitoring of root colonization and antifungal gene expression by the biocontrol agent *Pseudomonas fluorescens* CHA0 with flow cytometry.

Rodriguez-Zaragoza S (1994). Ecology of free-living amebas. *Critical Reviews in Microbiology* **20:** 225-241.

Rodriguez H, Fraga R, Gonzalez T, Bashan Y. (2002). Springer: Salamanca, SPAIN, pp 15-21.

Rønn R, Grunert J, Ekelund F (2001). Protozoan response to addition of the bacteria *Mycobacterium chlorophenolicum* and *Pseudomonas chlororaphis* to soil microcosms. *Biology and fertility of soils* **33:** 126-131.

Rønn R, McCaig A, Griffiths B, Prosser J (2002). Impact of protozoan grazing on bacterial community structure in soil microcosms. *Applied and Environmental Microbiology* **68:** 6094-6105.

Roscher C, Schumacher J, Baade J, Wilcke W, Gleixner G, Weisser WW *et al* (2004). The role of biodiversity for element cycling and trophic interactions: an experimental approach in a grassland community. *Basic and Applied Ecology* **5:** 107-121.

Rosenberg K, Bertaux J, Scheu S, Bonkowski M (2009). Soil amoeba rapidly change bacterial community composition in *Arabidopsis thaliana* rhizosphere. *ISME Journal* **in press**.

Ross-Gillespie A, Gardner A, West SA, Griffin AS (2007). Frequency dependence and cooperation: theory and a test with bacteria. *American Naturalist* **170:** 331-342.

Rudrappa T, Biedrzycki ML, Bais HP (2008). Causes and consequences of plant-associated biofilms. *Fems Microbiology Ecology* **64:** 153-166.

Sacherer P, Defago G, Haas D (1994). Extracellular protease and phospholipase-C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiology Letters* **116:** 155-160.

Sakaguchi M, Murakami H, Suzaki T (2001). Involvement of a 40-kDa glycoprotein in food recognition, prey capture, and induction of phagocytosis in the protozoon Actinophrys sol. *Protist* **152:** 33-41.

Salcher MM, Pernthaler J, Psenner R, Posch T (2005). Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community. *Aquatic Microbial Ecology* **38:** 215-229.

Saleem M, Arshad M, Hussain S, Bhatti AS (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *Journal of Industrial Microbiology & Biotechnology* **34:** 635-648.

Sambrook J, Russell D (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Sanchez-Contreras M, Martín M, Villacieros M, O'Gara F, Bonilla I, Rivilla R (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *Journal of Bacteriology* **184:** 1587-1596.

Sandoz KM, Mitzimberg SM, Schuster M (2007). Social cheating in Pseudomonas aeruginosa quorum sensing. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **104:** 15876-15881.

Scheu S (1992). Automated Measurement of the Respiratory Response of Soil Microcompartments - Active Microbial Biomass in Earthworm Feces. *Soil Biology & Biochemistry* **24:** 1113-1118.

Schmidt-Eisenlohr H, Gast A, Baron C (2003). Inactivation of GacS does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. *Applied and Environmental Microbiology* **69:** 1817-1826.

Schnider-Keel U, Seematter A, Maurhofer M, Blumer C, Duffy B, Gigot-Bonnefoy C (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *Journal of Bacteriology* **182**.

Schnider U, Keel C, Blumer C, Troxler J, Defago G, Haas D (1995). Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. *J. Bacteriol.* **177:** 5387-5392.

Schouten A, van den Berg G, Edel-Hermann V, Steinberg C, Gautheron N, Alabouvette C *et al* (2004). Defense responses of *Fusarium oxysporum* to 2,4-diacetylphloroglucinol, a broad-spectrum antibiotic produced by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions* **17:** 1201-1211.

Schroeder KL, Paulitz TC (2006). Root Diseases of Wheat and Barley During the Transition From Conventional Tillage to Direct Seeding. *Plant Disease* **90:** 1247-1253.

Schuhegger R, Ihring A, Gantner S, Bahnweg G, Knappe C, Vogg G *et al* (2006). Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell and Environment* **29:** 909-918.

Serrano-Luna JD, Cervantes-Sandoval I, Calderon J, Navarro-Garcia F, Tsutsumi V, Shibayama M (2006). Protease activities of *Acanthamoeba polyphaga* and *Acanthamoeba castellanii*. *Canadian Journal of Microbiology* **52:** 16-23.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY (2004). Improved monomeric red, orange and yellow fluorescent proteins derived From *Discosoma sp* red fluorescent protein. *Nature Biotechnology* **22:** 1567-1572.

Shanks RMQ, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA (2006). Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Applied and Environmental Microbiology* **72:** 5027-5036.

Shaw LJ, Morris P, Hooker JE (2006). Perception and modification of plant flavonoid signals by rhizosphere microorganisms. *Environmental Microbiology* **8:** 1867-1880.

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

Sorensen M, Lippuner C, Kaiser T, Misslitz A, Aebischer T, Bumann D (2003). Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Letters* **552:** 110-114.

Sorensen SJ, Schyberg T, Ronn R (1999). Predation by protozoa on Escherichia coli K12 in soil and transfer of resistance plasmid RP4 to indigenous bacteria in soil. *Applied Soil Ecology* **11:** 79-90.

de Souza JT, Arnould C, Deulvot C, Lemanceau P, Gianinazzi-Pearson V, Raaijmakers JM (2003). Effect of 2,4-diacetylphloroglucinol on *Pythium*: cellular responses and variation in sensitivity among propagules and species. *Phytopathology* **93:** 966-975.

Stacey G (1995). *Bradyrhizobium japonicum* nodulation genetics. *FEMS Microbiology Letters* **127:** 1-9.

Standing D, Banerjee S, Rangel-Castro JI, Jaspars M, Prosser JI, Killham K (2008). Novel screen for investigating in situ rhizosphere production of the antibiotic 2,4-diacetylphloroglucinol by bacterial inocula. *Communications In Soil Science And Plant Analysis* **39:** 1720-1732.

Steiner UK (2007). Investment in defense and cost of predator-induced defense along a resource gradient. *Oecologia* **152:** 201-210.

Steinkellner S, Lendzemo V, Langer I, Schweiger P, Khaosaad T, Toussaint JP *et al* (2007). Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules* **12:** 1290-1306.

Stephens PM, O'Sullivan M, O'Gara F (1987). Effect of Bacteriophage on Colonization of Sugarbeet Roots by Fluorescent Pseudomonas spp. *Applied and Environmental Microbiology* **53:** 1164–1167.

Strigul NS, Kravchenko LV (2006). Mathematical modeling of PGPR inoculation into the rhizosphere. *Environmental Modelling & Software* **21:** 1158-1171.

Teasdale ME, Liu J, Wallace J, Akhlaghi F, Rowley DC (2009). Secondary metabolites produced by the marine bacterium *Halobacillus salinus* that Inhibit quorum sensing-controlled phenotypes in gram-negative bacteria. *Appl. Environ. Microbiol.* **75:** 567-572.

Tripathi RK, Gottlieb D (1969). Mechanism of action of antifungal antibiotic pyrrolnitrin. *Journal of Bacteriology* **100:** 310-&.

Troxler J, Zala M, Natsch A, Moenneloccoz Y, Defago G (1997). Autecology of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the rhizosphere and inside roots at later stages of plant development. *FEMS Microbiology Ecology* **23:** 119-130.

Valverde C, Heeb S, Keel C, Haas D (2003). RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Molecular Microbiology* **50:** 1361-1379.

Velicer GJ, Kroos L, Lenski RE (2000). Developmental cheating in the social bacterium *Myxococcus xanthus*. *Nature* **404:** 598-601.

Verma R, Naosekpam AS, Kumar S, Prasad R, Shanmugam V (2007). Influence of soil reaction on diversity and antifungal activity of fluorescent pseudomonads in crop rhizospheres. *Bioresource Technology* **98:** 1346-1352.

Vessey JK (2003a). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil* **255:** 571-586.

Vessey JK (2003b). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil* **255:** 571-586.

Vincent MN, Harrison LA, Brackin JM, Kovacevich PA, Mukerji P, Weller DM *et al* (1991). Genetic analysis of the antifungal activity of a soilborne Pseudomonas aureofaciens strain. *Appl Environ Microbiol* **57:** 2928-34.

Voisard C, Bull CT, Keel C, Laville J, Maurhofer M, Schnider M (1994). Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In: O'Gara F, Dowling DN and Boesten B (eds). *Molecular ecology of rhizosphere microorganisms.* VCH Verlagsgesellschaft mbH. pp 67-89.

Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Zeng LH *et al* (2005). Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiology* **139:** 822-835.

Wall LG (2000). The actinorhizal symbiosis. *Journal of Plant Growth Regulation* **19:** 167-182.

Walsh UF, Morrissey JP, O'Gara F (2001). *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Current Opinion in Biotechnology* **12:** 289-295.

Watt M, Hugenholtz P, White R, Vinall K (2006). Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence in situ hybridization (FISH). *Environmental Microbiology* **8:** 871-884.

Weekers PHH, Engelberts AMW, Vogels GD (1995). Bacteriolytic activities of the free-living soil amebas, *Acanthamoeba castellanii, Acanthamoeba polyphaga and Hartmannella vermiformis*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **68:** 237-243.

van Wees SCM, van der Ent S, Pieterse CMJ (2008). Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* **11:** 443-448.

Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S (2005). Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. *Environmental Microbiology* **7:** 1593-1601.

Weller DM (2007). Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* **97:** 250-256.

Weller DM, Landa BB, Mavrodi OV, Schroeder KL, De La Fuente L, Blouin Bankhead S *et al* (2007). Role of 2,4-diacetylphloroglucinol-producing fluorescent Pseudomonas spp. in the defense of plant roots. *Plant Biol (Stuttg)* **9:** 4-20.

Weller DM, Raaijmakers JM (2002). Microbial populations responsible for specific soil suppressiveness to plant patogens. *Annual Review of Phytopathology* **40:** 309-348.

de Werra P, Baehler E, Huser A, Keel C, Maurhofer M (2008). Detection of plant-modulated alterations in antifungal gene expression in *Pseudomonas fluorescens* CHA0 on roots by flow cytometry. *Applied and Environmental Microbiology* **74:** 1339-1349.

Van West P, Morris BM, Reid B, Appiah AA, Osborne MC, Campbell TA *et al* (2002). Oomycete plant pathogens use electric fields to target roots. *Molecular Plant-Microbe Interactions* **15:** 790-798.

Whiteley AS, Manefield M, Lueders T (2006). Unlocking the 'microbial black box' using RNA-based stable isotope probing technologies. *Current Opinion in Biotechnology* **17:** 67-71.

Whiteley AS, Thomson B, Lueders T, Manefield M (2007). RNA stable-isotope probing. *Nature Protocols* **2:** 838-844.

Wieland G, Neumann R, Backhaus H (2001). Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Applied and Environmental Microbiology* **67:** 5849-5854.

Wildschutte H, Wolfe DM, Tamewitz A, Lawrence JG (2004). Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. *Proceedings of the National Academy of Sciences* **101:** 10644-10649.

Willard SS, Devreotes PN (2006). Signaling pathways mediating chemotaxis in the social amoeba, *Dictyostelium discoideum*. *European Journal of Cell Biology* **85:** 897-904.

Williams P (2007). Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* **153:** 3923-3938.

Winding A, Binnerup SJ, Pritchard H (2004). Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. *FEMS Microbiology Ecology* **47:** 129-141.

Wootton EC, Zubkov MV, Jones DH, Jones RH, Martel CM, Thornton CA *et al* (2007). Biochemical prey recognition by planktonic protozoa. *Environmental Microbiology* **9:** 216-222.

Yang Z, Kong FX, Shi XL, Cao HS (2006). Morphological response of Microcystis aeruginosa to grazing by different sorts of zooplankton. *Hydrobiologia* **563:** 225-230.

Zangerl AR (2003). Evolution of induced plant responses to herbivores. *Basic and Applied Ecology* **4:** 91-103.

Zuber S, Carruthers F, Keel C, Mattart A, Blumer C, Pessi G *et al* (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions* **16:** 634-644.

Zubkov MV, Sleigh MA (2000). Comparison of growth efficiencies of protozoa growing on bacteria deposited on surfaces and in suspension. *Journal of Eukaryotic Microbiology* **47:** 62-69.

„Oh tiempo tus pirámides“

J. L. Borges

Eidestattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation ohne fremde Hilfe angefertigt und mich keiner anderen als die von mir angegebenen Schriften und Hilfsmittel bedient habe.

Ich habe noch keinen weiteren Promotionsversuch unternommen.

……..
(Alexandre Jousset)

Darmstadt, den 23. März 2009

Curriculum vitae

Alexandre Jousset

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Personal data

Birth Date: April 27th, 1981 in Lausanne, Switzerland
Nationality: French, Swiss
Sex: male

Languages

French: Mother Tongue
German: Fluent (written and spoken)
Spanish: Fluent (written and spoken)
English: Fluent (written and spoken)

Professional Experience

2006-2009 PhD at the Technische Universität Darmstadt, Germany
Thematic:
- Ecological relevance of toxin production by soil bacteria: chemical communication in the rhizosphere, importance in biotic interactions and implications for the use of plant beneficial bacteria in the agriculture

2005-2006 Universidad Nacional de Quilmes, Argentina:
-Interactions between biocontrol bacteria *Pseudomonas fluorescens CHA0* and its potential protozoan predators.

2004 Umweltforschungszentrum (UFZ), Leipzig, Germany: Setting up of an experiment aimed to track the influence of pesticides on the microbial loop and the degradation pathways in an agricultural soil.

Department of Archaeology, University of Lausanne, Switzerland: Study of the holy plants of Hinduism in Nepal, with emphasis on their religious, symbolical and practical signification.

Education

Summary

2003-2004: Diploma in Biology (MSc Degree equivalent) at the microbial ecology group of the ENAC department of the Swiss Federal Institute of Technology, Lausanne, Switzerland. (Supervisor: Dr. Hauke Harms)
1999-2004: Biology studies at the University of Lausanne, Switzerland.
1996-1999: Scientific Maturity at the Gymnase de Chamblandes, Pully, Switzerland.

Specialisations

Botany, Plant physiology, Modelling, Plant genetics, Environmental Microbiology

2004 Degree Project

Key words: Ciliates, Soil Ecology, PAH, Microbial Loop, DGGE, Sequencing
Development of a denaturing gradient gel electrophoresis (DGGE) fingerprinting method to screen ciliate communities in soils and use ciliate community structure as a bioindicator of Polycyclic Aromatic Hydrocarbon (PAH) contamination. This project was part of a research program on the bioremediation of organic pollutants.

2003 Exchange year at the Freie Universität Berlin

Specialisations: Plant genetics, Plant physiology and Eukaryotic Microbiology

2002 Module of Botany

Specialisations: plant determination, modelling of plant distribution using Geographic Information System (GIS).
Semester project: Determination of the biotic and abiotic factors influencing the distribution of the heavy metals hyperaccumulating plant *Thlaspi caerulescens* in the swiss Jura.

Publications

Jousset A, Lara E, Wall L, Valverde C. (2006). Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape protozoan grazing. AEM 72(11): 7083–7090.

Jousset A., Scheu S., and Bonkowski M. (2008) Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. Functional Ecology 22: 714-719.

Jousset A., Rochat L., Keel C., Pechy-Tarr M., Scheu S., and Bonkowski M. (2009) Predators promote toxicity of rhizosphere bacterial communities by selective feeding on non-toxic cheaters. ISME Journal **in press**.

Schebbes N, Vielhaber T, **Jousset A**, Karst U. (submitted) Development of a New Comprehensive Screening Methodology for Proteases. Journal of Chromatography A **in press**.

Henkes G, **Jousset A**, Bonkowski M, Thorpe M, Scheu S, Lanoue A, Schurr U, Rose U. (submitted) Systemic response of barley to the biocontrol bacterium *Pseudomonas fluorescens* and the root pathogen *Fusarium graminearum* induces rapid changes in root carbon allocation.

Manuscripts in preparation

Jousset A., Rochat L., Keel C., Scheu S., and Bonkowski M. *(in prep)* Predator-prey chemical warfare determines the antifungal activity of rhizosphere pseudomonads.

Jousset A, Lara E, Harms H, Chatzinotas A, (*in prep*). Development of a DGGE method to screen ciliate diversity in soils.

Neidig N, **Jousset A**, Paul R, Scheu S (in prep): Mutual toxicity determines the fitness of the nematode *Caenorhabditis elegans* and the amoeba *Acanthamoeba castellanii.*

Neidig N, Paul R, Scheu S, **Jousset A** (in prep): Different repellent and toxicity roles of bacterial toxins against the nematode *Caenorhabditis elegans*.

Contributions to international meetings

Jousset A, Valverde C, 2006. "Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape protozoan grazing" (**oral presentation**). 11th International Symposium on Microbial Ecology (ISME), 20-25 August 2006, Vienna, Austria.

Jousset A, Rochat L, Scheu S, Keel C, Bonkowski M, 2007: "Plant manipulation of the production of antibiotics by rhizosphere biocontrol *Pseudomonas fluorescens*" (**poster**). XIII- IS-MPMI Congress, 21-27 July 2007, Sorrento, Italia

Jousset A, Scheu S, Bonkowski M, 2007. "Secondary metabolite production confers high competitive advantage for Pseudomonas fluorescens CHA0 through grazing resistance against protozoan predators (*Acanthamoeba castellanii*)" (**Oral presentation**).Rhizosphere 2 International conference, 26-31 August 2007, Montpellier, France

Jousset A, *Rochat L, Pechy-Tarr M , Keel C, Scheu S, Bonkowski M, 2008.* "Predators promote cooperative defense and repress cheating by rhizosphere bacteria" (**Oral presentation**). 12th International Symposium on Microbial Ecology (ISME), 17-22 August 2008, Cairns, Australia.

Students supervised

09-10 2009: Ellen Latz, TU Darmstadt. (Research trainee) Interaction of biocontrol bacteria with micro- and mesofaunal invertebrates. Dispersion of *P. fluorescens* CHA0 by nematodes, collemboles and earthworms, bacterial colonization of soil invertebrates.

2008-2009: Nina Neidig, TU Darmstadt. (Diploma work) Sensing of and reaction to bacterial toxins by the nematode *Caenorhabditis elegans.* Investigation on chemotactic behaviour, stress response (DAF pathway) and survival in response to the biocontrol bacterium *P. fluorescens* CHA0 and derivative mutants.

02-03 2007: Nina Neidig, TU Darmstadt. (Research trainee) Mutual inhibition of the soil amoeba *Acanthamoeba castellanii* and the nematode *Caenorhabditis elegans.*