MYCOPHAGOUS SOIL BACTERIA

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"Imagination is more important than knowledge"
- Albert Einstein -

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ABSTRACT

Soil microorganisms evolved several strategies to compete for limited nutrients in soil. Bacteria of the genus *Collimonas* developed a way to exploit fungi as a source of organic nutrients. This strategy has been termed "mycophagy". In this thesis, research is presented with a focus on two aspects of bacterial mycophagy: 1) Investigation of strategies and traits that are important for *Collimonas* bacteria to enable a mycophagous lifestyle, 2) Investigation of occurrence of mycophagy among other soil bacteria.

Focusing on *Collimonas* bacteria, we find that several traits related to the mycophagous interaction with the fungal hosts, such as production of fungal inhibitors, are phylogenetically conserved. This implies that differentiation in lifestyles of *Collimonas* strains, is corresponding with phylogenetic distance. Furthermore, we show that collimonads are very motile in a soil-like matrix, especially when being confronted with low nutrient concentrations. This high motility can be used in order to effectively move towards oxalic acid (a metabolite exuded by a range of fungi for different purposes) in a concentration depended manner. Our results suggest that directed motility is an important trait, characterizing the mycophagous lifestyle of collimonads.

In order to screen for other mycophagous bacteria besides collimonads, two baiting approaches (long- and short-term) were developed. With both approaches, we find fungal hyphae to be commonly colonized by specific communities of rhizosphere mycophagous bacteria. Furthermore, mycophagous colonizers show clear feeding preferences for fungal hosts. Interestingly, a surprisingly high amount of mycophagous bacteria belong to genera well known to harbor plant pathogenic strains. Considering the importance of mycophagous bacteria in the rhizosphere, we finally propose the "Sapro-Rhizosphere" concept. This concept states that a substantial amount of plant derived carbon that is channeled through rhizosphere fungi (primary consumers) might be finally consumed by mycophagous bacteria (secondary consumers).

Taken together, by using molecular biological as well as microbiological methods, this thesis further extends our knowledge on the ecology of mycophagous *Collimonas* bacteria and highlights the importance of mycophagous bacteria in the rhizosphere.

INTRODUCTION

COLLIMONADS AND OTHER MYCOPHAGOUS BACTERIA

MICROBIAL SOIL ECOLOGY

Soil is a very complex and heterogeneous environment. Abiotic factors like pH, moisture and physical characteristics vary substantially between soils and can also rapidly change locally, thus creating a variety of possibilities for survival and growth of microbial species with different niches. It has been argued that the high number of potential microbial habitats is a major contributor to below-ground microbial species diversity, ranging from 10^3-10^7 different OTUs (Operational Taxonomical Units) per 10g of soil (Schloss & Handelsman 2006; Roesch *et al.* 2007; Timonen & Bomberg 2009; Uroz *et al.* 2010). The microscopic heterogeneity of soil with different pore sizes, distribution of water films and gradients of organic and inorganic nutrients, offers potential for microbial niche adaptation. Since soil microstructure and environmental conditions vary a lot with fluctuations in soil pH, water content and temperature gradients, adaption to different soil habitats is a frequent mechanism in the evolutionary shaping of soil microbial communities (Crawford *et al.* 2005).
Microorganisms fulfill a variety of functions in soil. They are capable of catalyzing all steps in soil nutrient cycling, being responsible for mineralization and decomposition for example. Breakdown of easily accessible compounds is mainly performed by bacteria that are able to exert quick growth when nutrient conditions are optimal. Saprotrophic fungi are the dominant microorganisms when it comes to degradation of recalcitrant substances like the plant secondary structural cell wall component lignin (de Boer *et al.* 2005). Fungi and bacteria colonize new soil habitats in different ways. Microbial dispersal in soil is strongly controlled by soil moisture. Low moisture content leads to less connectivity between water filled soil pores. This restricts passive (diffusion) and active bacterial movement (flagella), finally influencing bacterial abilities to colonize new microhabitats (Vos *et al.* 2013). Fungi grow with prolongation of their hyphal system, and are therefore able to bypass air filled soil pores. It has also been shown that bacteria are able to move along fungal hyphae in order to cross these air filled gaps (Kohlmeier *et al.* 2005).

THE RHIZOSPHERE AND ITS INHABITANTS

Despite the fact that soils offer an immense amount of microhabitats, the majority of the accessible internal soil surfaces and pores are deserted because of a lack of nutrients. In fact, the vegetated first centimeters of the soil is the place where most microbial life concentrates. Plants release carbon via exudation through their roots, thus creating an oasis for microbial soil life around their roots, a zone called the rhizosphere. Here, most of the interactions between different microorganisms, and also between microorganisms and higher organisms (e.g. plants or nematodes) occur. In the following lines I want to focus on interactions between plants and their direct microbial environment, descriptions of interactions of other higher organisms with microorganisms can be found elsewhere (Bonkowski 2004; Davies 2005; Zientz *et al.* 2005; Curry & Schmidt 2006; Kaneda & Kaneko 2008).
Plant roots interact via exudates with the surrounding microbes (Bais *et al.* 2006; de Boer *et al.* 2006; Haichar *et al.* 2008; Bonfante & Anca 2009; Buee *et al.* 2009; Dennis *et al.* 2010; Uroz *et al.* 2010). Different plant species harbor a microbial community that is well distinguishable and steered by species-specific root exudate composition (Berg & Smalla 2009). Root exudation is in turn affected by the biotic and abiotic environment and by plant developmental stage

(Hartmann *et al.* 2009; Bezemer *et al.* 2010; Uroz *et al.* 2010; Chaparro *et al.* 2013; Chaparro *et al.* 2014). By providing a generally carbon-limited environment with easily accessible energy sources plants attract a diverse community of bacteria and fungi which developed various strategies to acquire plant derived organic nutrients.

One of those strategies is to invade the plant root. Some bacteria and fungi manage to overcome or modify the physical and chemical plant defenses, enabling them to internally colonize the plant root and other organs. These endophytes avoid competition in the rhizosphere by completely living inside the host or by physically "tapping" the source even before it releases nutrients into the rhizosphere. Some endophytes establish a connection with the plant root, but still extend into the rhizosphere with most of their tissue. The mycorrhizal fungi for example colonize the plant root internally but still extend far into the rhizosphere. Those endophytic fungi do not only take carbon from the plant host, they also provide the plant with "goods" such as phosphorus, thus creating a situation with benefits for both partners. Other, parasitic or pathogenic fungi like *Rhizoctonia solani* e.g. colonize the plant root and cause diseases with detrimental effects for plant performance.

FUNGAL-BACTERIAL INTERACTIONS IN THE RHIZOSPHERE

Bacteria and fungi unable to grow endophytically follow other strategies to get access to plant derived exudates. One way is to provide a service to the plant. Plant beneficial microbes are able to directly support plant growth, e.g. by producing phytohormones (Rengel & Marschner 2005; Calvaruso *et al.* 2006). Others can indirectly stimulate plant growth by suppressing plant pathogenic fungi and bacteria (Berg *et al.* 2006; van Overbeek & van Elsas 2008; Vinale *et al.* 2008).

The intensity of competition between bacteria and fungi varies between soils with different parameters, the main selectors being pH, soil moisture, complexity of the carbon substrates and the level of disturbance of the soil matrix since this disrupts hyphal growth of soil fungi (de Boer *et al.* 2006; van der Heijden 2008). Organic nutrients that enter the rhizosphere are generally labile, easily degradable compounds. Traditionally, due to their higher growth rates and metabolic versatility, bacteria have been thought to be the main degrader of exudates. This view however has been recently challenged by the discovery that in several cases plant derived carbon first enters the fungal, rather than the bacterial channel, possibly due to the fact that many saprotrophic fungi are able to grow endophytically as well (Buee *et al.* 2009; Hannula *et al.* 2012). The fact that nutrients can be channeled through fungal hyphae as well as through plant roots creates opportunities for organisms that thrive on fungus rather than on plant derived nutrients.

The occurrence of bacteria living in the mycorrhizosphere (the space influenced by mycorrhizal hyphae) is well known. It has been shown that mycorrhizal fungi are able to select for specific bacterial strains that associate with their hyphae (de Boer *et al.* 2005; Timonen & Marschner 2006; Frey-Klett *et al.* 2007) and positively affect the fungus. The mycorrhizal helper bacteria (MHB) for example positively influence the symbiosis by either a) supporting the formation of the association of the plant with the fungus or by b) positively influencing the function of the already established interaction (Deveau *et al.* 2007; Pivato *et al.* 2009). Only limited research on the effect of the fungus on the MHB has been done until now but there is some evidence that e.g. trehalose, exuded by the fungus at certain "nutrient hotspots" supports bacterial biofilm formation (Frey-Klett *et al.* 2007). It is tempting to speculate that there is a

relationship between bacteria and mycorrhizal fungi of which both partners profit. The fungus could actively provide the bacteria with exudates whereas the bacteria could support the fungus with other benefits. For example when acting together with different MHB, mycorrhizae have been reported to release plants from different kinds of environmental stress, like drought (Vivas *et al.* 2003b), pathogens (Reimann *et al.* 2008) or metal contamination (Vivas *et al.* 2003a; Vivas *et al.* 2005; Vivas *et al.* 2006). There is, however, also evidence for bacteria able to stimulate fungal exudation or exploit fungal tissue as a carbon source. Those "mycophagous" bacteria may appear to rely on the consumption of fungus derived carbon under nutrient limited conditions.

MYCOPHAGY

Microbes evolved many different strategies to cope with carbon limitation in soil have. One of those is the ability to exploit living fungal hyphae as a source of carbon and energy. This "lifestyle" is called mycophagy. The overall objective of the research described in this thesis was to unravel the importance and the evolution of mycophagy.

COLLIMONADS - THE FUNGUS EATERS

Mycophagy is a nutritional strategy that bacteria from the genus *Collimonas* (de Boer *et al.* 2004) have specialized on. Until now, more than 100 *Collimonas* strains are known (Mannisto & Haggblom 2006; Uroz *et al.* 2007; Hoppener-Ogawa *et al.* 2008; Hakvåg *et al.* 2009; Nissinen *et al.* 2012) and 3 species have been formally described. These are *C. fungivorans* (de Boer *et al.* 2004), *C. arenae* and *C. pratensis* (Hoppener-Ogawa *et al.* 2008). The genus *Collimonas* belongs to the *β-Proteobacteria*, order *Burkholderiales*, family *Oxalobacteraceae*, with its closest relatives in the genera *Herbaspirillum* and *Janthinobacterium*.
All strains that have been found so far, exhibit different levels of mycophagous activity but it has also been speculated that non-mycophagous strains may exist (Hoppener-Ogawa *et al.* 2007). The type strains of all three species have been isolated from the same site, a Dutch island in the Wadden Sea named "Terschelling". Isolation of *Collimonas* strains have also been reported for other regions in the world. Soil types and zones differed, but most sites share some characteristics: mild acidity, presence of fungi, low nutrient availability and limited human disturbance (Leveau *et al.* 2010). Low nutrient availability together with the presence of fungi seems to be preferred by collimonads (Hoppener-Ogawa *et al.* 2007; Uroz *et al.* 2009b). Yet, *Collimonas* bacteria are quantitatively not a very important part of the soil microbial community, with an abundance of 10^3 to10^5 cells per gram of soil in different environments (Leveau *et al.* 2010). Next to mycophagy, they possess other ecologically interesting traits. Those are the subject of the following lines.

MINERAL WEATHERING AND SIDEROPHORE PRODUCTION

All described *Collimonas* species have mineral weathering abilities (Uroz *et al.* 2009b). They are able to mobilize inorganic nutrients that are bound e.g. in compounds like biotite or iron phosphate. Those are frequently occurring minerals in soils, but not easy to access by most microorganisms (Kooijman *et al.* 1998; Leveau *et al.* 2010). Weathering by collimonads has been shown to depend on the available carbon source (Uroz *et al.* 2007; Uroz *et al.* 2009b) and since

different fungi also represent specific carbon sources, it has been suggested that besides mycophagy, weathering might represent an adaptation to the "fungiphilic" lifestyle of collimonads. Acidification of the environment or complexation of nutrients by the secretion of chelating compounds are the two main weathering mechanisms that have been suggested until now (Uroz *et al.* 2009a). Production and secretion of chelating compounds (siderophores) that bind Fe^{3+} can significantly improve iron uptake. On the one hand, the possible profit of such compounds in the hyphal surrounding would be an advantage for an association of the fungus with certain bacteria (Frey-Klett *et al.* 2005). On the other hand, the increased production of chelating compounds could also be regarded as an antifungal activity that inhibits fungi by withdrawal of essential nutrients.

OXALIC ACID

Like plant roots, also fungal hyphae exude a variety of substances. It has been suggested that the fungi might select for certain bacterial species by exuding specific compounds like L-arabinose, m-inositol or D-trehalose (Warmink *et al.* 2009). Chemo-attraction and growth mediation by trehalose was shown for bacteria isolated from the vicinity of *Scleroderma* hyphae (Uroz *et al.* 2007) and for *Pseudomonas* bacteria interacting with *Laccaria bicolor* (Tarkka *et al.* 2009). Oxalic acid is a common fungal exudate. It is a mediator in fungal lignin degradation and also thought to be the main weathering agent secreted by a variety of fungi.
For *Collimonas* it has been indicated that oxalic acid might play an important role in the interaction with the fungal host. In a transcriptome study of the interaction of *Collimonas fungivorans* Ter331 and the fungus *Aspergillus niger*, oxalic acid transporters were up-regulated (Mela *et al.* 2011). This up-regulation, and the fact that collimonads could not be cultured on oxalic acid as the sole carbon source gave rise to the idea that the bacteria might be able to use oxalic acid as a signal molecule.

MYCOPHAGY & FUNGAL INHIBITION

Interestingly, mycophagous collimonads do not affect all fungi to the same extent. There is evidence that fungi have different sensitivity or resistance to the bacterial "attack". When tested in controlled experiments (De Boer *et al.* 1998; Hoppener-Ogawa *et al.* 2009a) collimonads did not, or only slightly, reduce fungal biomass, but were able to change fungal community composition. This observation is interesting because it indicates that selective feeding of a low abundant soil bacterium could influence the fungal community, possibly changing ecosystem functions provided by that group of organisms.
For the mycophagous behavior of *Collimonas* the attachment to fungal hyphae seems to be important (De Boer *et al.* 2001; Kamilova *et al.* 2007; Hoppener-Ogawa *et al.* 2009b). In addition, different antimicrobial compounds are thought to be important for mycophagous growth. Collimonads produce chitinases that are able to hydrolyse glycosidic bonds of chitin, a major structural component of the fungal cell wall. However, so far no clear relationship has been found between chitinase production and the ability to grow on fungal hyphae (Leveau *et al.* 2010). Collimonads are also bad competitors for chitinous compounds in soil (De Boer *et al.* 1999). Sequence data of the *Collimonas fungivorans* genome suggests that the acquisition of chitinase genes took place at an evolutionary early time point, before niche differentiation into a mycophagous lifestyle (Fritsche *et al.* 2008). The closely related genus *Janthinobacterium* harbors

strains with good abilities to degrade crystalline chitin (Kielak *et al.* 2013), whereas *Collimonas* can only degrade colloidal chitin. After successful establishment of a mycophagous lifestyle, parts of the chitinolytic genes allowing degradation of cross-polymerized chitin may have gotten lost, leaving mostly activity against native chitin-polymers which are present in the growing zone (hyphal tip) of fungi (Fritsche *et al.* 2008; Leveau *et al.* 2010).
The molecular basics and mechanisms of mycophagy are still unknown. It seems likely that there are several different antifungal compounds involved, and that a complex metabolic "cross-talk" between fungus and bacteria is triggered. One of the compounds produced by some *Collimonas* strains is collimomycin, an antibiotic that is excreted upon confrontation with the fungus (Mela *et al.* 2011). Investigations on the genetic basis of collimomycin production indicated that gene cluster K of *Collimonas fungivorans* Ter331 is needed to invoke the inhibitory phenotype. The cluster probably codes for an antifungal polyyne that has, however, structurally not yet been completely resolved (Fritsche *et al.* 2014).
When confronted with a fungus besides producing antifungal compounds *Collimonas* starts to excrete slime. The production of this compound may facilitate physical attachment and/or be a strategy to absorb nutrients that possibly leak out of the hyphae while feeding on it. The use of the EPS layer for nutrient storage would then prevent other "cheating" bacteria to compete for the nutrients that have been freed by *Collimonas* (Leveau & Preston 2008). Slime production could also be part of an acidic stress response against the low environmental pH around fungal hyphae (Mela *et al.* 2011).

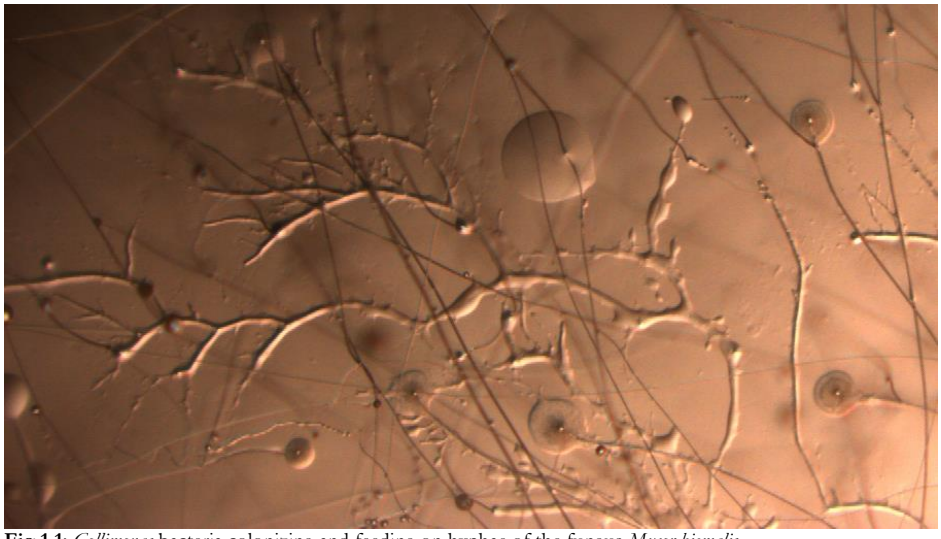

Fig 1.1: *Collimonas* bacteria colonizing and feeding on hyphae of the fungus *Mucor hiemalis*.

OBJECTIVES, RESEARCH QUESTIONS AND THESIS OUTLINE

The major goal of this thesis was to shed light onto the ecology and evolution of mycophagous bacteria. Before this thesis work was started, the knowledge on mycophagous bacteria was mainly restricted to collimonads. An essential part of this thesis was to evaluate the importance

of other mycophagous bacteria besides collimonads. From previous *Collimonas* research there was already ample knowledge on the molecular basis of mycophagy (Mela *et al.* 2008; Leveau *et al.* 2010) but insights on the behavior of mycophagous collimonads and the importance of bacterial mycophagy in natural soil environments were rather limited. A fundamental understanding of the ecology of collimonads and other mycophagous soil bacteria would also enable us to exploit those bacteria more efficiently for the production of antimicrobial compound.

Research questions related to the ecological importance of mycophagy, niche differentiation and evolution of mycophagous bacteria and the role of chemotaxis and movement for mycophagous and soil bacteria in general are separately addressed in distinct chapters of this thesis.

The main research questions of this thesis are as follows:

Research question (1): What is the functional role of oxalic acid in the interaction of collimonads with the fungal host?

The chemotactic behavior of collimonads upon confrontation with different fungus derived compounds is addressed in the *second* chapter. Besides chemotaxis, movement in sand microcosms and the effect of oxalic acid on motility is a central subject of this chapter. Here, I finally propose a conceptual model on how collimonads find their fungal host, move towards it and start feeding.

Research question (2): How important is bacterial movement for colonization of new soil habitats and on which factors does it depend?

Inspired by the findings of the remarkable movement abilities of collimonads, reported in chapter two, the *third* chapter focuses on movement abilities of soil bacteria in general. Here, we identify the most motile soil bacterial taxa, relate their ability to colonize soil microhabitats with nutrient availabilities and moisture, and finally discuss the findings in a broader context.

Research question (3): Did different groups of collimonads evolve different traits and can those be related to phylogeny?

The approach to group taxa based on their traits is a very common one in (plant) ecology (Darwin 1871) and has recently been re-introduced in microbial ecology (Krause *et al.* 2014). In chapter *four*, we experimentally assess *Collimonas'* traits and relate them to phylogeny, finding phylogenetic clusters with common trait investment (phylogenetic signal).

The following two chapters *five* and *six* explore the diversity (and function) of mycophagous bacteria that do not belong to the genus *Collimonas*. In those chapters I present two innovative microbiological methods to efficiently culture the community of mycophagous bacteria associated with different fungal hosts.

Research question (4): What is the importance of mycophagy in the plant rhizosphere?

In chapter *five* we show that a diverse bacterial community is able to quickly attach and selectively feed on distinct host fungi. We introduce the "*Sapro-Rhizosphere Concept"* and discuss the importance of a carbon flow from plants via saprotrophic fungi to associated mycophagous bacteria for below ground food web dynamics.

Research question (5): How can mycophagous bacteria be isolated and which factors influence the isolated bacterial taxa?

Chapter *six* compares two different methods to enrich and isolate mycophagous bacteria. Here, I discuss factors that influence the isolation of different mycophagous bacterial genera. The intriguing fact that fungal hyphae accumulate potentially pathogenic bacteria is discussed as well.
In chapter *seven*, I finally discuss the results of this thesis in the light of what was already known on mycophagy before I started writing my thesis. I indicate gaps in our knowledge on the ecology of mycophagous bacteria and point out possible lanes for future research. I close this chapter with a critical view on progress in microbial ecology.

CHAPTER TWO

OXALIC ACID: A SIGNAL MOLECULE FOR FUNGUS-FEEDING BACTERIA OF THE GENUS COLLIMONAS?

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Revised manuscript (submitted)

SUMMARY:

Mycophagous (=fungus feeding) soil bacteria of the genus *Collimonas* have been shown to colonize and grow on hyphae of different fungal hosts as the only source of energy and carbon. The ability to exploit fungal nutrient resources might require a strategy for collimonads to sense fungi in the soil matrix. Oxalic acid is ubiquitously secreted by soil fungi, serving different purposes. In this study, we investigated the possibility that collimonads might use fungal oxalic acid secretion to localize a host fungus and move towards it. We confirmed earlier indications that collimonads have a very limited ability to use oxalic acid as growth substrate. Using different assays, we show that oxalic acid triggers bacterial movement in such a way that accumulation of cells can be expected at micro-sites with high oxalic acid concentrations.
Based on these observations we propose that oxalic acid functions as a signal molecule to guide collimonads to hyphal tips, the mycelial zones that are most sensitive for mycophagous bacterial attack.

INTRODUCTION:

Exudation of oxalic acid is widespread among all major fungal phyla (*Ascomycota*, *Zygomycota* and *Basidiomycota*), and it serves several different purposes (Dutton & Evans 1996).
In the process of wood decomposition by brown- or white rot fungi, oxalic acid is released and acts as mediator in the degradation of lignin (Shimada *et al.* 1997; Hastrup *et al.* 2012). Plant associated, ecto-mycorrhizal fungi, but also saprotrophic soil fungi (Sullivan *et al.* 2012) secrete citric and oxalic acid to release inorganic nutrients and scavenge metals, possibly by chelation and acidification (Crompton *et al.* 2008; Adeleke *et al.* 2012). Fast growing, saprotrophic fungi like *Penicillium* or *Aspergillus* are also able to secrete oxalic acid, in order to mineralize inorganic phosphorus (Dutton & Evans 1996). Interestingly, in plant-associated mycorrhizal-, as well as in wood and litter decomposing fungi, oxalic acid is secreted and forms oxalate crystals at the hyphal tips (Dutton & Evans 1996; Crompton *et al.* 2008; Heller & Witt-Geiges 2013). Once released, it complexes with metal ions or dissolved organic matter (Bhatti *et al.* 1998; Harrold & Tabatabai 2006), making it very stable and persistent. Among fungal exudates, oxalic acid is the major one, being exuded in concentrations up to 20mM (Guggiari *et al.* 2011; Sullivan *et al.* 2012).
Some bacterial groups have specialized to use oxalic acid and oxalate complexes as a carbon source. These so called "oxalotrophic" bacteria have been found in a variety of different habitats that share high oxalic acid levels (Sahin 2003). One of those habitats is the "hyphosphere", the zone influenced by the presence of fungal hyphae (Bravo *et al.* 2013). For bacteria that live in close association with fungal hyphae, oxalic acid might not only function as nutrient source but also as signal molecule, helping bacteria to find the fungal host. High exudation levels make oxalic acid a good signal that could indicate the presence of a fungal host.
The genus *Collimonas* consists of such host associated, "fungiphilic" bacteria. Collimonads are free-living, gram-negative β-proteobacteria, belonging to the family *Oxalobacteraceae*. *Collimonas* bacteria have shown to be mycophagous (able to feed on living fungal tissue), a lifestyle that requires the presence of a fungal host (de Boer *et al.* 2004). Despite high microbial densities on

basis of soil weight, most of the surface of soil particles is unoccupied by microbes (Young *et al.* 2008). Therefore, collimonads may possess strategies, enabling them to localize fungi and move towards them, in order to feed on fungus derived nutrients. It has already been shown that *Collimonas'* genes involved in motility as well as in uptake and metabolism of oxalic acid were up-regulated during the confrontation with the fungus *Aspergillus niger* (Mela *et al.* 2011). The results of this gene expression study and the fact that oxalic acid seems to be ubiquitously secreted by fungi stimulated the current investigation, focusing on the role of oxalic acid in the interaction between a mycophagous bacterium and its fungal host. Here, we test the hypothesis that *Collimonas* bacteria can use oxalic acid to sense the source of its secretion and to move towards it. We assessed influences of oxalic acid on bacterial movement (swarming- , chemotaxis- and sand accumulation assays) as well as on growth on semi-solid medium and finally discuss its possible role as a signal molecule in the fungal-bacterial interaction.

RESULTS & DISCUSSION:

In a first step to assess whether oxalic acid can act as a signal in the interaction between mycophagous collimonads and fungal hosts, we examined the motility response to different concentrations of the compound. Using a semi-solid, agar-based medium, we tested the influence of oxalic acid on bacterial swarming. We found a reduction of swarming motility with increasing oxalic acid concentrations (Fig 2.1). Spreading of *Collimonas* on agar without oxalic acid showed a typically "wrinkled" swarming pattern, covering the whole plate. The presence of low concentrations of oxalic acid (50 µM) revealed the same pattern but less intense. At high oxalic acid concentrations (500 µM), swarming decreased drastically in intensity and the "wrinkled" swarming morphology did not appear.

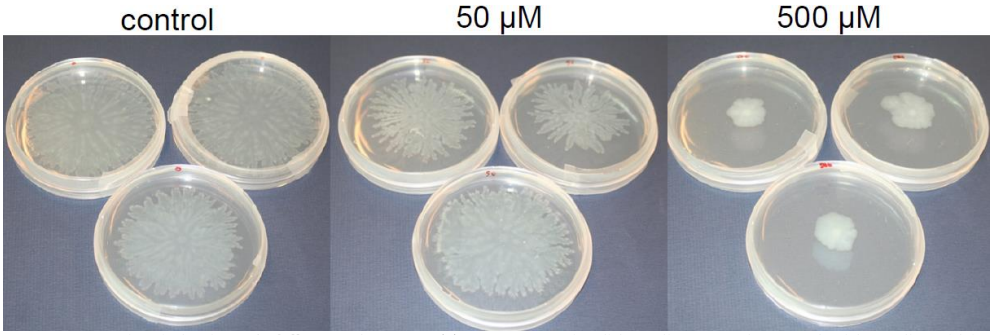

Fig2.1: Swarming behavior of *Collimonas fungivorans* Ter331 on semi-solid medium supplemented with oxalic acid in concentrations of 0 µM, 50 µM or 500 µM. The swarming assay was performed on mineral medium (M9), supplemented with 0.5% agar, as described in Xavier *et al.* (2011). The assay was incubated for 2 days at 20 °C, in triplicates. The pH of different media was measured before pouring the plates in order to exclude a possible pH effect: 0 µM: pH 6.65, 50 µM: pH 6.65, 500 µM: pH 6.60.

From this experiment, we concluded that *Collimonas fungivorans* Ter331 is a very motile bacterium that is able to exert high rates of explorative movement, even if no source of oxalic acid is present. When concentrations of oxalic acid were raised to 500 µM, explorative movement was stopped, which is line with the idea that the bacteria should stop moving when the oxalic acid producing source (= fungus) is found.

In order to gain knowledge on the ability to sense and move towards oxalic acid in a natural system, a sand microcosm approach was used. Here, inocula of collimonads were uniformly distributed in a Petri dish containing acid-washed sea sand. A plug of phytagel, enriched with oxalic acid was added in the center on top of the sand, allowing oxalic acid to diffuse. We observed a significant accumulation of bacteria underneath the plug for all treatments, including the plugs that did not contain oxalic acid ("plug" versus "rim" $F(1, 23) = 74.95$, $p < 0.001$). We also found a significant main effect of the concentration of oxalic acid ($F(2, 23) = 5.79$, $p = 0.00921$). The interaction between the location of sampling ("plug" vs. "rim") and the oxalic acid concentration was also significant ($F(2, 23) = 4.53$, $p = 0.02195$), indicating that the highest oxalic acid concentration (500 µM) in the plug resulted in the highest aggregation of bacteria near the plug (Fig 2.2). Bacterial numbers at the "rim" were not significantly affected by different concentrations of oxalic acid in the phytagel plug.

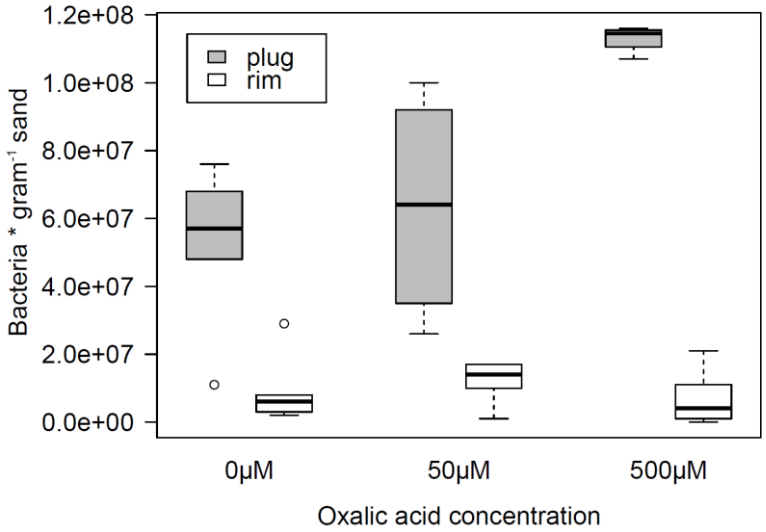

Fig2.2: Impact of a local oxalic acid source on spatial distribution of *Collimonas fungivorans* Ter331 in sand. The local oxalic acid source consisted of a phytagel plug with varying concentrations (0 µM, 50 µM or 500 µM) of oxalic acid. Box whisker plots show the median (black line) and the varying range of the response (boxes). Whiskers indicate the 1.5 interquartile range of the lower and the upper quartile of the data. Dots depict outliers. *C. fungivorans* Ter331 was grown overnight in liquid TSB, washed in MES (morpholineethanesulfonic acid) buffer (pH 5.5), containing 1 gL^{-1} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$ and mixed with dry, acid washed sea sand (particle diameter 0.1 – 0.5 mm; Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany) in order to establish a uniform concentration of 10^5 cells g^{-1} sand. Moisture was adjusted to 10% (w/w) and petri dishes (60 mm x 15 mm) were filled with approximately 20 g of moist sand. Plugs that contained oxalic acid in concentrations of 0 µM, 50 µM and 500 µM were placed on top of the sand, in the center of the petri dish. Next to oxalic acid, plugs contained 4 gL^{-1} phytagel (Sigma-Aldrich) and 0.74 gL^{-1} $MgSO_4$ and had a diameter of 1cm. After 6 days of incubation, samples (0.3g) were taken underneath the plugs, and at the "rim" of the petri dish, by pressing the wide end of 1ml blue pipette tip into the sand. Samples were mixed with 1ml MES buffer (see above) and vortexed for 5 seconds. Finally 50 µL of the suspension was plated on TSA and developing colonies were counted after incubation at 20 °C for 4 days.

This experiment indicated that oxalic acid influences *Collimonas*' movement in soil-like systems. We did not find a difference in attraction towards plugs with 0 µM and 50 µM oxalic acid. In

these treatments, growth of collimonads under the plugs may be mainly the cause of accumulation. Although the release of soluble organic compounds from phytagel is limited it has already been shown that gels consisting of phytagel only can stimulate the growth of some bacteria, including collimonads, to a small extent (chapter *five*, supplementary material).
After having established that oxalic acid was attracting collimonads, we investigated if this coincided with a growth response. Mela *et al.* (2011) reported that *C. fungivorans* Ter331 is not able to grow on oxalic acid in liquid cultures. It has, however, been shown that metabolic properties of *Collimonas* bacteria in liquid media can differ from those on solid media (Fritsche *et al.* 2014). Since our experiments involve movement (and metabolic activities) on (semi) solid surfaces, we decided to confirm the inability of *C. fungivorans* Ter331 to use oxalic acid as a carbon source on semi-solid medium (Fig 2.3).

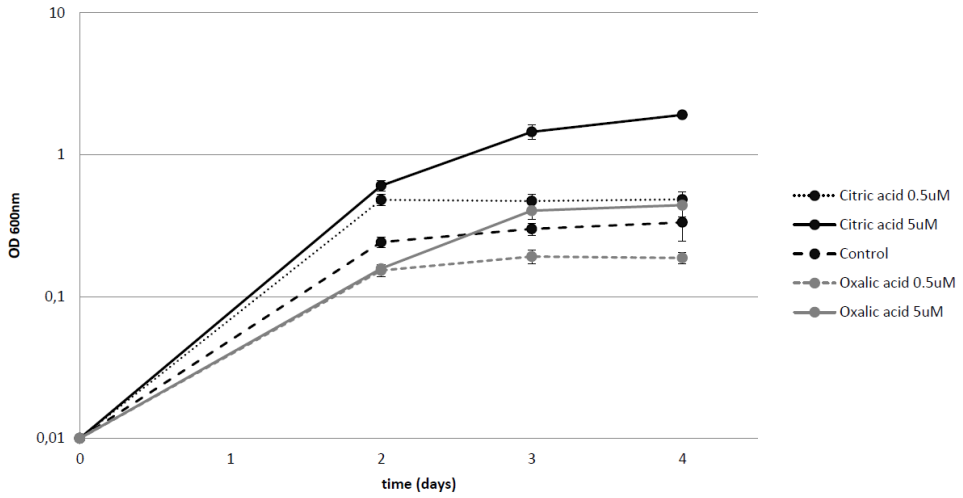

Fig2.3: Growth of *Collimonas fungivorans* strain Ter331 on water-yeast agar (WYA) plates, supplemented with either oxalic acid or citric acid as a carbon source at concentrations of 0.5 mM and 5 mM. Bacterial inocula (50µL of suspended cells in 1 gL^{-1} NaCl solution, OD_{600} = 0.015) consisted of bacteria, pre-grown on TSB plates. Plates were incubated at 20 °C and optical density was measured at 2, 3 and 4 days after adding 2ml of $1g*L^{-1}$ NaCl to the plates, swirling and collecting the suspended bacteria.

Relative to the control (water-yeast agar) without extra added carbon, we observed up to 5.7x and 2x increase of bacterial cells in treatments with citric acid as the added carbon source (5 mM and 0.5 mM, respectively). With 0.5 mM and 5 mM oxalic acid concentrations we found a decrease (0.6x) and very small increase (1.3x) in cell density, respectively. All cell densities at each time point were significantly ($p \leq 0.05$) different from one another, except for the two oxalic acid concentrations (0.5 mM versus 5mM) at day 2, and 0.5 mM citric versus 5 mM oxalic acid at day 4 (Supplementary table S1). Although we observed only a very small 1.3 fold increase in bacterial cell density with 5 mM oxalic acid and a nearly 6-fold increase in the comparable citric acid treatment, we cannot rule out that collimonads are able to produce small amounts of biomass when being confronted with high concentrations of oxalic acid. Interestingly, collimonads do not seem to be able to use small amounts of oxalic acid (0.5mM)

for growth. Thus, from this experiment we conclude that similarly to liquid medium, collimonads are very restricted in their abilities to use oxalic acid as energy source, on semi-solid medium.

In a final experiment, we tested the attraction towards oxalic acid and other fungus- or root-derived compounds (sugars, amino acids etc.) in a chemotaxis assay. This was done in order to evaluate the specificity of the response towards oxalic acid. The assay was based on the "classical" chemotaxis assay, introduced by (Adler & Templeton 1967) which tests the motility of bacteria towards a chemoattractant in a glass capillary. Instead of glass capillaries, the current assay involves 1 ml plastic syringes together with 0.8 mm diameter needles. Next to a significant attraction towards oxalic acid (50 μM and 500 μM oxalic acid ($t(44.05) = 3.83$, $p = 0.0004$ and ($t(38.06) = 2.66$, $p = 0.0114$, respectively), various compounds applied at concentrations of 50 μM were attracting collimonads (Fig 2.4).

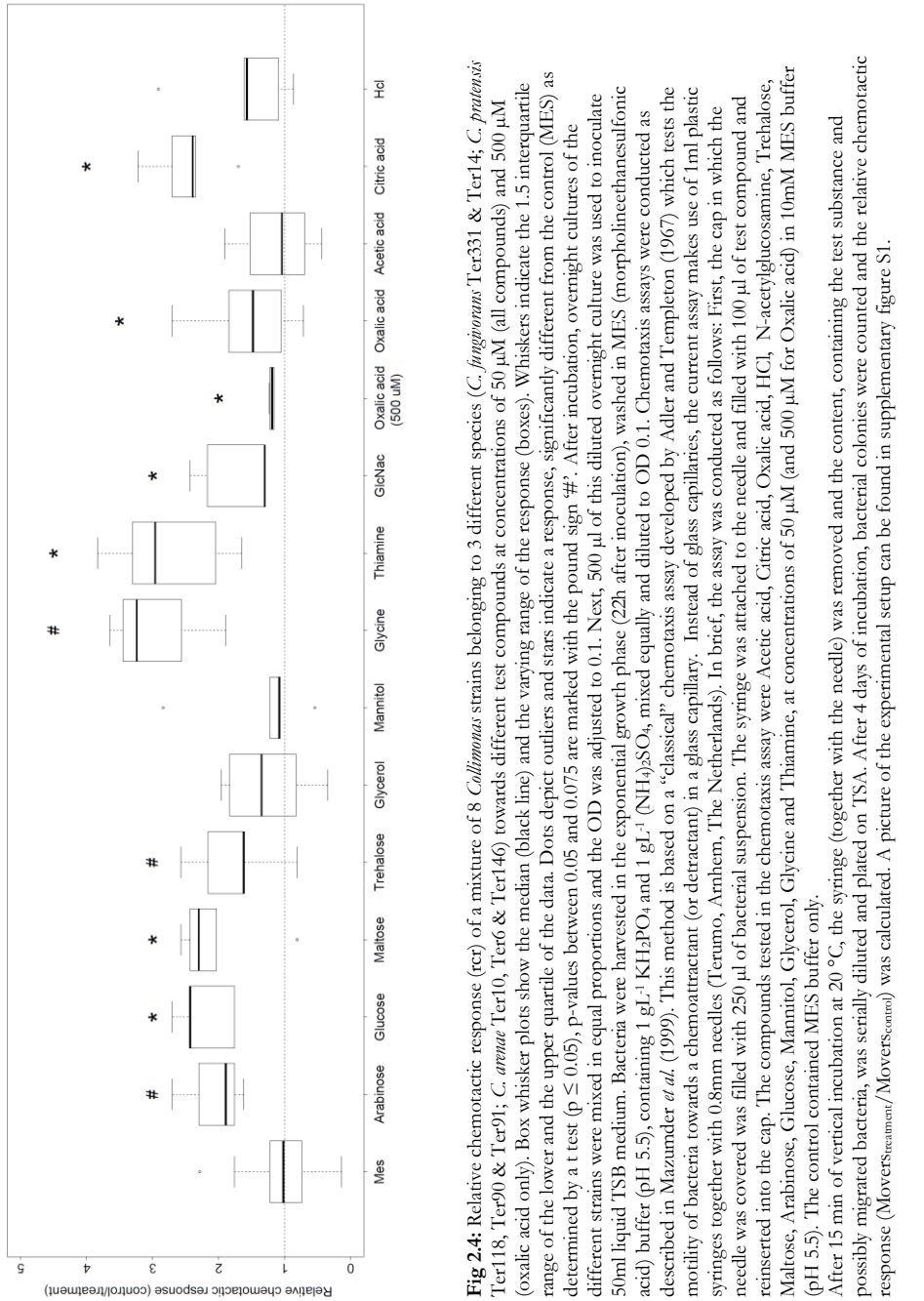

Fig 2.4: Relative chemotactic response (rcr) of a mixture of 8 *Collimonas* strains belonging to 3 different species (*C. fungivorans* Ter331 & Ter14; *C. pratensis* Ter118, Ter90 & Ter91; *C. arenae* Ter10, Ter6 & Ter146) towards different test compounds at concentrations of 50 µM (all compounds) and 500 µM (oxalic acid only). Box whisker plots show the median (black line) and the varying range of the response (boxes). Whiskers indicate the 1.5 interquartile range of the lower and the upper quartile of the data. Dots depict outliers and stars indicate a response, significantly different from the control (MES) as determined by a t test ($p \leq 0.05$), p-values between 0.05 and 0.075 are marked with the pound sign '#'. After incubation, overnight cultures of the different strains were mixed in equal proportions and the OD was adjusted to 0.1. Next, 500 µl of this diluted overnight culture was used to inoculate 50ml liquid TSB medium. Bacteria were harvested in the exponential growth phase (22h after inoculation), washed in MES (morpholinoethanesulfonic acid) buffer (pH 5.5), containing 1 gL^{-1} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$, mixed equally and diluted to OD 0.1. Chemotaxis assays were conducted as described in Mazumder *et al.* (1999). This method is based on a "classical" chemotaxis assay developed by Adler and Templeton (1967) which tests the motility of bacteria towards a chemoattractant (or detractant) in a glass capillary. Instead of glass capillaries, the current assay makes use of 1ml plastic syringes together with 0.8mm needles (Terumo, Arnhem, The Netherlands). In brief, the assay was conducted as follows: First, the cap in which the needle was covered was filled with 250 µl of bacterial suspension. The syringe was attached to the needle and filled with 100 µl of test compound and reinserted into the cap. The compounds tested in the chemotaxis assay were Acetic acid, Citric acid, Oxalic acid, HCl, N-acetylglucosamine, Trehalose, Maltose, Arabinose, Glucose, Mannitol, Glycerol, Glycine and Thiamine, at concentrations of 50 µM (and 500 µM for Oxalic acid) in 10mM MES buffer (pH 5.5). The control contained MES buffer only.
After 15 min of vertical incubation at 20 °C, the syringe (together with the needle) was removed and the content, containing the test substance and possibly migrated bacteria, was serially diluted and plated on TSA. After 4 days of incubation, bacterial colonies were counted and the relative chemotactic response ($Movers_{treatment}/Movers_{control}$) was calculated. A picture of the experimental setup can be found in supplementary figure S1.

These were the sugars glucose ($t(5.15) = 5.92$, $p = 0.0018$) and maltose ($t(4.41) = 3.16$, $p = 0.0298$), but also the vitamin thiamine ($t(4.25) = 4.3$, $p = 0.011$). *Collimonas* bacteria were also attracted by N-acetylglucosamine ($t(4.68) = 2.71$, $p = 0.0455$), a major component of the fungal cell wall, and citric acid ($t(4.68) = 5.71$, $p = 0.0029$). Attraction by the sugars arabinose ($t(2.2) = 3.22$, $p = 0.0746$) and trehalose ($t(4.47) = 2.48$, $p = 0.0617$), and the amino acid glycine ($t(2.07) = 3.59$, $p = 0.066$) was close to significance. The results of the chemotaxis assay indicate that oxalic acid is not the only compound that attracts *Collimonas* bacteria. It is, however, unlikely that the other attractants might also mainly serve as signal molecules since collimonads can use those compounds for growth (de Boer *et al.* 2004).Taken all results together, we present strong evidence suggestion that the primary function of oxalic acid for collimonads is a signal molecule and not a nutrient source. Oxalic acid appears to stimulate bacterial accumulation via at least two different ways: (i) the directed movement of bacteria towards oxalic acid (chemotaxis test; sand accumulation assay) and (ii) the oxalic acid concentration dependent regulation of motility.

Especially for bacteria that focus on fungi in order to meet their nutritional demands, a signal molecule that is widespread in the "fungal world" is of high value. It would help collimonads to locate their fungal host and to stop moving once they are close to it. Interestingly, oxalic acid is secreted at the hyphal tips. This is the part of the fungus that is actively growing and most vulnerable to bacterial attack because of a weak, developing cell wall. Indeed, it has already been shown that collimonads prefer to feed at this region of the fungus (De Boer *et al.* 2001; Leveau *et al.* 2010). In addition, it has been demonstrated that collimonads are able to move along fungal hyphae. This trait would be valuable in combination with sensing the host via oxalic acid. First, the bacteria would sense oxalic acid then move towards the point of secretion (either along fungal hyphae or through the soil). Collimonads have been shown to be able to produce homoserine lactones (HSL) (Leveau *et al.* 2010), quorum sensing molecules that are widely used for communication and density dependent coordinated behavior among gram negative bacteria. It is plausible that mycophagous feeding behavior is quorum sensing regulated since collimonads would require high densities in order to be able to efficiently withdraw nutrients from fungi. We propose that attracted by oxalic acid, collimonads aggregate at the hyphal tip until high cell densities have been reached. Finally a quorum sensing mediated, coordinated attack on the fungal exterior is triggered (Fig 2.5).

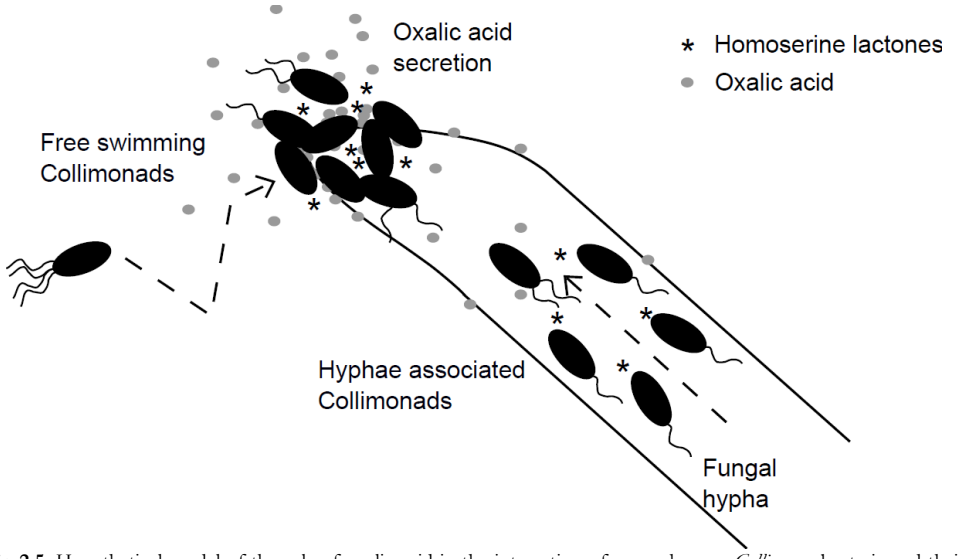

Fig 2.5: Hypothetical model of the role of oxalic acid in the interaction of mycophagous *Collimonas* bacteria and their fungal host. Bacteria are attracted by oxalic acid, move towards the hyphal tip (either through the soil or along fungal hyphae), and finally accumulate at the tip which is the spot where oxalic acid concentrations are highest. Once high cell densities are reached they start feeding on the fungus, possibly mediated by quorum sensing. Also movement of bacteria in groups (swarming) might be coordinated by quorum sensing.

DATA ANALYSIS / STATISTICS:

Welch's two sample t test was used to compare the mean chemotactic responses (rcr) of attractants vs. control and to compare growth on different substrates. In the sand accumulation assay, main effects (oxalic acid concentration and location) were tested using a two-way analysis of variance. In order to make sure that residuals were normally distributed which is a prerequisite for performing ANOVA, the response variable (bacterial cell number) was square root transformed. The ANOVA table can be found in Supplementary table S2. Posthoc pairwise comparisons between all treatment combinations were done with Tukey's HSD test. Statistics and graphs were done in "R" (R Core Team, 2014) and Excel (Microsoft Corp.).

ACKNOWLEDGEMENTS:

This research was supported by a grant from the Netherlands Organization for Scientific Research, division Earth and Life Sciences (NWO-ALW), grant number 819.01.016.

SUPPLEMENTARY MATERIAL:

Fig S2.1: Experimental setup for chemotaxis assays.

Table S2.1 a) p-values, b) degrees of freedom, c) t-values of the t-tests for the growth of *C. fungivorans* Ter331 on water yeast agar, supplemented with different concentrations of oxalic acid.

a) Day 2 p-values

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	0.001429	-	-	-
Control	3.11E-06	3.11E-06	-	-
Oxalic acid 0.5 mM	8.75E-07	1.55E-06	8.77E-06	-
Oxalic acid 5 mM	5.41E-06	4.83E-06	7.79E-05	**0.529**

Day 3

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	1.34E-05	-	-	-
Control	0.0001171	1.11E-05	-	-
Oxalic acid 0.5 mM	1.01E-05	8.64E-06	4.99E-05	-
Oxalic acid 5 mM	0.04853	8.70E-06	0.003496	6.82E-05

Day 4

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	6.74E-12	-	-	-
Control	0.007837	2.35E-11	-	-
Oxalic acid 0.5 mM	5.42E-05	6.01E-09	0.008752	-
Oxalic acid 5 mM	**0.3062**	9.07E-12	0.04693	0.0002953

b) Day 2 degrees of freedom (df)

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	9.296	-	-	-
Control	7.211	6.302	-	-
Oxalic acid 0.5 mM	6.428	5.823	9.497	-
Oxalic acid 5 mM	5.161	5.091	5.686	6.088

Day 3

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	5.891	-	-	-
Control	7.989	5.298	-	-
Oxalic acid 0.5 mM	6.484	5.136	8.783	-
Oxalic acid 5 mM	9.995	5.932	7.879	6.42

Day 4

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	9.929	-	-	-
Control	9.231	9.58	-	-
Oxalic acid 0.5 mM	5.655	5.553	5.362	-
Oxalic acid 5 mM	9.813	9.97	9.76	5.497

Table S2.1 continued

c) Day 2 t-values

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	-4.4725	-	-	-
Control	12.8615	15.3817	-	-
Oxalic acid 0.5 mM	18.2847	19.5632	8.5954	-
Oxalic acid 5 mM	19.1511	20.0642	10.0626	-0.6673

Day 3

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	-13.209	-	-	-
Control	6.968	15.9631	-	-
Oxalic acid 0.5 mM	12.2466	17.6086	7.3372	-
Oxalic acid 5 mM	2.2459	14.1041	-4.1115	-9.069

Day 4

	Citric acid 0.5 mM	Citric acid 5 mM	Control	Oxalic acid 0.5 mM
Citric acid 5 mM	-36.3849	-	-	-
Control	3.3801	34.343	-	-
Oxalic acid 0.5 mM	10.828	58.0926	4.022	-
Oxalic acid 5 mM	1.0794	35.0148	-2.2732	-8.0967

Table S2.2 Analysis of Variance Table for the "sand accumulation" experiment

	Df	Sum Sq	Mean Sq	F value	P value
Oxalic acid concentration	2	35560165	17780083	5.787	0.00921
Treatment (plug or rim)	1	230281002	230281002	74.952	1.08E-08
Oxalic acid concentration * treatment	2	27834076	13917038	4.53	0.02195
Residuals	23	70665064	3072394		

CHAPTER THREE

EARLY COLONIZERS OF NEW HABITATS REPRESENT A MINORITY OF THE SOIL BACTERIAL COMMUNITY

MAX-BERNHARD RUDNICK, ALEXANDRA B. WOLF, WIETSE DE BOER AND GEORGE A. KOWALCHUK

Revised manuscript (submitted)

ABSTRACT

In order to understand (re-)colonization of microhabitats and bacterial succession in soil, it is important to understand which members of soil bacterial communities are most motile in the porous soil matrix. To address this issue, we carried out a series of experiments in sterilized soil microcosms. Using two different model strains, *Pseudomonas fluorescens* and *Collimonas fungivorans*, we first determined the influence of nutrient availability on bacterial expansion rates. Based on these results, we then conducted similar microcosm experiments to examine microbial mobility within natural soil bacterial communities under a single nutrient regime. The expansion of bacterial populations within the community was assayed by quantitative PCR and pyrosequencing of 16S rRNA gene fragments. We observed that only a relatively small subset of the total community was able to expand to an appreciable distance (more than 2 cm) within 48 hours, with the genera *Undibacterium*, *Pseudomonas*, and *Massilia* and especially the family Enterobacteriaceae dominating the communities more distant from the point of inoculation. These results suggest that (re-)colonization of open habitats in soil may be dominated by a few rapidly moving species, which may have important consequences for microbial succession.

INTRODUCTION

The soil environment is highly heterogeneous with sporadic availability of easily degradable energy resources for the soil inhabiting microbial community. The ability to access these spatially distributed resources may contribute to the success of microbial species within the soil environment. Some bacterial species are able to actively move towards energy resources and have evolved a variety of different motility mechanisms, often relying on flagellar movement, while others rely on passive dispersal via water flow or passing invertebrates. Soil hydration status is a major factor determining bacterial colonization of new habitats, as both passive and active motility depend on the presence of water-filled pores or water films covering the surfaces of solid particles (Abuashour *et al.* 1994; Jiang *et al.* 2006; Dechesne *et al.* 2010a).

Despite the obvious importance of soil structure and water content for bacterial movement, most studies that have examined the motility of soil-borne bacteria have not taken these factors into account. Active bacterial motility is typically investigated on agar plates (Harshey 2003; Wang *et al.* 2004; Caiazza *et al.* 2005) or on sterile, porous ceramic surfaces, either by experimental (Dechesne *et al.* 2010b) or using modeling approaches (Long & Or 2009). Although such studies have provided valuable insight into the mechanisms of bacterial movement, they are highly artificial and do not mimic the *in situ* conditions of the soil environment. Studies that follow the fate of specific bacterial populations (e.g. genetically modified or pathogens) in soil (Trevors *et al.* 1990; van Elsas *et al.* 1991; Huysman & Verstraete 1993; Abuashour *et al.* 1994) are generally conducted under the assumption that bacteria are passively distributed, without consideration of active motility. Furthermore, previous studies have focused solely on tracking individual strains, without taking community processes such as microbial interactions into account. Nevertheless, it is known that microbial interactions can have pivotal influences on bacterial motility phenotypes (Garbeva & de Boer 2009). Following the movement of indigenous microorganisms in natural soils is very challenging because unlike introduced microorganism, they cannot be tracked by tagging.

To avoid the limitations of gel assays, the current study utilizes a quartz sand microcosm system that mimics important soil features. This system is well-defined, easy to manipulate and

allows to investigate bacterial colonization potential in a community context (Wolf *et al.* 2013). In the present study, we first examined the movement of two individual model soil bacterial strains, *Pseudomonas fluorescens* and *Collimonas fungivorans*, in the sand microcosms (see Table S3.1 for particle size distribution). These initial experiments were performed in order to examine the influence of substrate availability on bacterial expansion and to determine suitable conditions for subsequent inoculation of microcosms with a complex bacterial community. In the complex community experiment, total bacterial community expansion over time was followed by qPCR, and bacterial community structure was determined as a function of distance from point of inoculation by high-throughput pyrosequencing of bacterial 16S rRNA gene fragments. Using this approach, we could identified bacterial taxa most successful in colonizing new (micro-)habitats, thereby gaining insight into patterns of microbial habitat (re-) colonization.

METHODS

SINGLE-STRAIN INOCULATION EXPERIMENTS

In order to examine the impact of substrate availability on bacterial expansion and to determine suitable conditions for the community experiment, we tested the expansion of two single soil bacterial strains*, Pseudomonas fluorescens* Pf0-1 (Compeau *et al.* 1988) and *Collimonas fungivorans strain* Ter331 (de Boer *et al.* 2004), under different nutrient levels. Strains were inoculated in the center of sand microcosms and sampling was conducted at different distances from the inoculation point at different time points (see below). These strains were chosen as representatives of bacterial genera well known to be able to colonize roots and/or fungal hyphae in a soil environment (Lugtenberg & Dekkers 2001; de Boer *et al.* 2004; Kamilova *et al.* 2007). Both strains were pre-grown overnight, individually, in liquid 10% tryptic soy broth (TSB), washed in 10 mM MES (morpholineethanesulfonic acid) buffer (pH 5.8) containing 1gL^{-1} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$. Microcosms were established in glass Petri dishes (diameter 9 cm) containing 50 g acid-washed sea sand (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany) sterilized by autoclaving and oven-drying. The moisture content was adjusted to 7.5% (w/w), which corresponds to 30% of the water holding capacity, by adding the appropriate volume of liquid growth medium (either 10% or 1%TSB, pH 5.8). Microcosms were inoculated with 5 µL bacterial suspension at the center of the petri dish, sealed with parafilm, incubated at 20°C and sampled after 7, 24 and 48 hours with a multi-pronged sampling device as described by (Wolf *et al.* 2013), which provides samples at 2 mm intervals.

EXPERIMENTAL DESIGN AND SAMPLING FOR COMPLEX SOIL COMMUNITY EXPERIMENTS

Microcosms were established as described in the previous section, adjusted to 7.5% moisture (w/w) with 1% TSB and inoculated at the center of the petri dish with 5 µL soil suspension (4 replicates per time point). The soil suspension inoculum was prepared by dispersing 50 g field wet soil collected from a former arable field site located near Ede, the Netherlands (52°04'N, 5°45'E; see (van der Putten *et al.* 2000) for a detailed description of the soil characteristics) in 450 mL 10 mM phosphate buffer (pH 5.8) by shaking for 30 min and sonicating (Branson 5210 ultrasonic bath) twice for 1 min. The suspension was filtered sequentially through filters with successively smaller pore diameter (11, 8, 6 and 3 µm; Whatman filter papers 1 Cat No

1001-150, 102-150, 1003-150, and Whatman Cellulose Nitrate Membrane Filters 7193-002) to exclude most eukaryotic organisms. After inoculation, the microcosms were sealed with parafilm to prevent moisture loss and incubated at 20°C in the dark. Samples were taken after 24 and 48 h by pushing the wide-end of a sterilized 1 mL pipet tip (inner diameter: 8 mm, outer diameter: 10 mm) at appropriate distances into the sand, thereby sampling at different, defined, distances from the inoculation zone (Fig S3.1). We destructively harvested 5 samples (distances 1-5, with 1 being closest and 5 furthest to the inoculation zone) along the radius of the sand microcosm, at 24 and 48 h. 24 and 48 h samples were taken from different microcosms. Each sample contained approximately 0.3 g sand and was stored at -20°C for further isolation of DNA and bacterial strains.

DNA ISOLATION, QUANTITATIVE PCR AND HIGH-THROUGHPUT PYROSEQUENCING

For each sand sample (about 0.3 g; 2 time points x 5 distances x 4 replicates), total DNA was extracted using the MOBIO PowerSoil DNA isolation kit following the manufacturer's protocol with the modification of heating the sample to 60°C for 10 min after the addition of solution C1, and the adding of 100 µL each of solutions C2 and C3 simultaneously.
To estimate bacterial density after 24 and 48 h across the sampling transect, we determined 16S rRNA gene copy numbers as a proxy of cell numbers via a quantitative real-time PCR (qPCR) approach. Briefly, 5 µL DNA template was added to a master mix consisting of 12.5 µl SYBR green mix (GC Biotech), 2.5 µl BSA (4mg/mL) and 2.3 µl milliQ water. To this, 1.25 µl (5 pmol/µL) each of the Eub338 (forward) and Eub518 (reverse) primers were added (Lane 1991). qPCR calibration curves (gene copy number versus the cycle number at which the fluorescence intensity reached the set threshold cycle value) were obtained using serial dilutions of pure-culture genomic DNA carrying a single 16S rRNA gene sequence (8 calibration points ranging from 1 to 4,171,775 copies/µL). All reactions were performed in duplicate. The qPCR was carried out in a Rotor-Gene Q (Qiagen, Venlo, the Netherlands). The PCR cycling conditions included 45 cycles of 5 seconds at 95°C, 10 seconds at 53°C, and 20 seconds at 72°C. Fluorescence data were recorded at the end of each 72°C step. DNA dissociation profiles were subsequently run from 72°C to 95°C with a ramp of 1°C/5 seconds to confirm product integrity.
For pyrosequencing, the V4 region of the 16S rRNA gene was amplified from the extracted DNA using composite forward and reverse primers, consisting of primer A from 454 Life Sciences, a 10 base sample-specific barcode, a linker sequence GT and primer 515f and primer B from 454 Life Sciences, a 10 base sample specific barcode, linker sequence GG and the primer 806r (Vos *et al.* 2012). Each sample and replicate received a unique barcode sequence. PCR amplifications were performed using 2.5 µl PCR buffer, 2.5 µl dNTP (2 mM), 0.2 µl Fast start DNA polymerase, 1 µL forward primer (5 uM), 1 µL reverse primer (5 uM), 1µL DNA template, and PCR grade H_2O to a total volume of 25 µL. Thermal cycling (C1000 Touch™ Thermal Cycler, Bio-Rad) conditions were as follows: 5 min at 95°C, 30 cycles of 30 sec at 95°C, 1 min at 53°C, and 1 min at 72°C, followed by 10 min at 72°C). The PCR products were verified by 1.5 % agarose gel electrophoresis and then purified with the Qiaquick PCR purification kit (Qiagen, Venlo, the Netherlands). Equal amounts of amplicon from each sample were mixed together and subjected to 454 sequencing on a GS FLX Titanium 454 pyrosequencing platform (Macrogen Europe, Amsterdam, the Netherlands).

BIOINFORMATIC AND STATISTICAL ANALYSES

Sequence data and quality information was transferred to the Galaxy interface (Goecks *et al.* 2010) using the SFF converter tool. Sequences were then de-multiplexed and further analyzed with the QIIME pipeline version 1.6 (Caporaso *et al.* 2010b). In the first step, sequences with a maximum of 6 ambiguous bases, 6 homopolymer runs, zero primer mismatches, a maximum of 1.5 errors in the barcode sequence and passed a quality score window of 50 were binned according to sample id and the barcodes were removed. Further, the DENOISER algorithm version 1.6.0 was used to correct for sequencing errors, and chimeras were removed by USEARCH (Edgar 2010). Sequences were then aligned by PyNAST (Caporaso *et al.* 2010a) and UCLUST (Edgar 2010) and assigned to OTUs (Operational Taxonomical Units), using a minimum sequence identity cutoff of 97%. From all OTU clusters, the most abundant sequence was selected as a representative for taxonomy assignment by using the SILVA database (release 108 SSU) with a minimum identity value of 75%. The relative abundance of different bacterial groups was calculated in each sample by comparing the number of sequences classified as belonging to the specific bacterial groups versus the number of classified bacterial sequences per sample. The Shannon Wiener index was used to calculate diversity in the different samples. Final graphs generated using the program MEGAN (Huson *et al.* 2007). Pyrosequencing data have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB6159.
All mentioned significant differences are the result of a t-test with appropriate variance distributions as determined by an f-test. Tests for significance were performed in Excel (Microsoft Corp.).

IDENTIFICATION OF DOMINANT COLONY FORM IN EXPANSION ZONES

In order to isolate the most abundant OTU from the motility zones of the microcosms, we suspended 0.3 g sand samples (see above) of distances 1 and 2 in 1 ml MES-buffer and plated 50 µL of these suspensions in a dilution series from 1:10 to 1:1000 on 10 % TSB agar plates. At the highest dilutions (1:100 and 1:1000) only one colony type was found. This colony form possessed the typical yellow color and morphology of *Pantoea* bacteria. After picking and streaking to ensure purity, examples were subjected to colony PCR using the primers 27f and 1492r (Weissburg et al 1991) with the following reagents & settings: 18.14 µl H2O, 2.5 µl 10x PCR-buffer containing 2 mM MgCl2 (Roche Scientific, Woerden, the Netherlands), 0.2 mM of each dNTP (Roche Scientific, Woerden, the Netherlands) and 0.4 µM of each Primer, 1 U Fast Start High Fidelity Polymerase (Roche Scientific, Woerden, the Netherlands) and 1 µl template. Cycling conditions consisted of a pre-denaturation step of 10 min at 95°C to break the cells open, an initial denaturation of 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 90 sec with a 1 sec increment per cycle and a final elongation step at 72°C for 10 min. The PCR product was examined by a standard (1.5 %) agarose electrophoresis and subsequently Sanger-sequenced with primer 1492r by Macrogen (Amsterdam, the Netherlands) and aligned with the corresponding OTU in order to confirm movement abilities of the respective OTU.

RESULTS

SINGLE-STRAIN INOCULATION EXPERIMENTS

After 7 hours, *Collimonas fungivorans* Ter331 had moved a distance of 14 mm in sand microcosms at both nutrient levels (1% and 10% TSB). After 24 and 48 hours, we could observe significant differences ($P < 0.05$) in movement between 1% TSB (average ~28 mm and ~40 mm, respectively) and 10% TSB (average ~12 mm and ~24 mm, respectively) (Fig 3.1). An opposite pattern was observed for *Pseudomonas fluorescens*, which moved faster at higher nutrient levels (Fig 3.1). *Pseudomonas fluorescens* had already colonized almost the entire microcosm at 7h at 10% TSB, but not at 1% TSB. The lower nutrient level was therefore chosen for subsequent experiments, because it provided the appropriate range of expansion in the microcosm setup and was more representative of the nutrient poor conditions that are typical for most soils. We chose sampling times of 24 and 48h, as this provided information on the rate of colonization during the period required for full expansion throughout the microcosm.

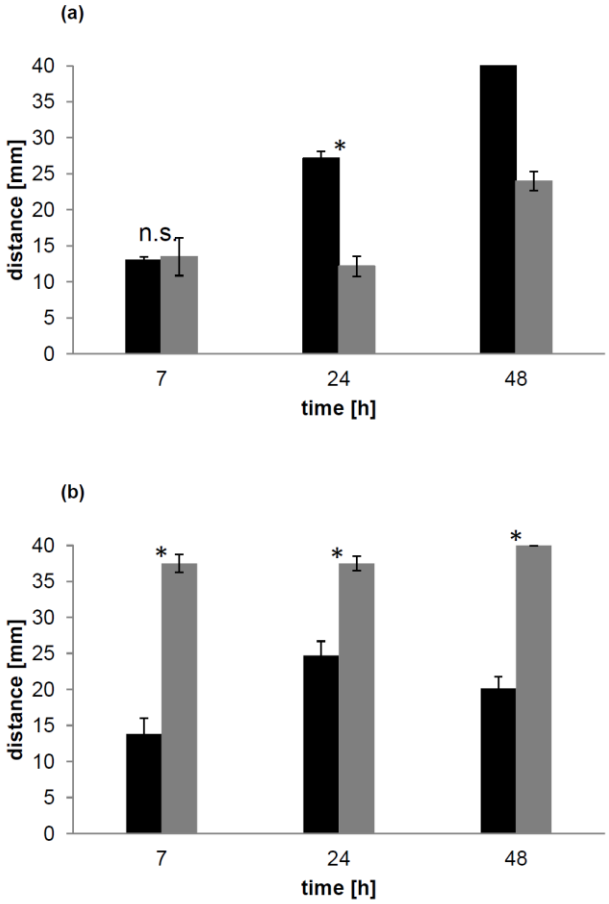

Fig 3.1 Expansion of (a) *Collimonas fungivorans* strain Ter331 and (b) *Pseudomonas fluorescens* strain Pf0-1 in sand microcosms under different nutrient concentrations at 7, 24 and 48 h after inoculation. Error bars depict the standard errors. * indicates statistically significant differences ($P < 0.05$). Black bars = 1% TSB, grey bars = 10% TSB.

TRACKING TOTAL BACTERIAL COMMUNITY EXPANSION BY QPCR

After 24 h, we found on average $7.9*10^3$ 16S rRNA gene copies per gram sand at distance 1 (0.5 - 1.5 cm from the microcosm center) and $2.8*10^3$ at distance 2 (1.5 - 2.5 cm from the microcosm center). At distances 3-5 (2.5 - 5.5 cm from the microcosm center), bacterial gene copy numbers were below the level of detection. At 48 h, there were on average $5.7*10^6$ bacterial ribosomal gene copies at distance 1, $4.4*10^6$ at distance 2, $1.6*10^6$ at distance 3, $2.5*10^5$ at distance 4, and $4.8*10^3$ at distance 5 (Fig 3.2). Thus, the expansion of bacteria was about 0.5 - 1.5 cm after 24 h, whereas after 48 h, nearly the whole sand microcosm was colonized.

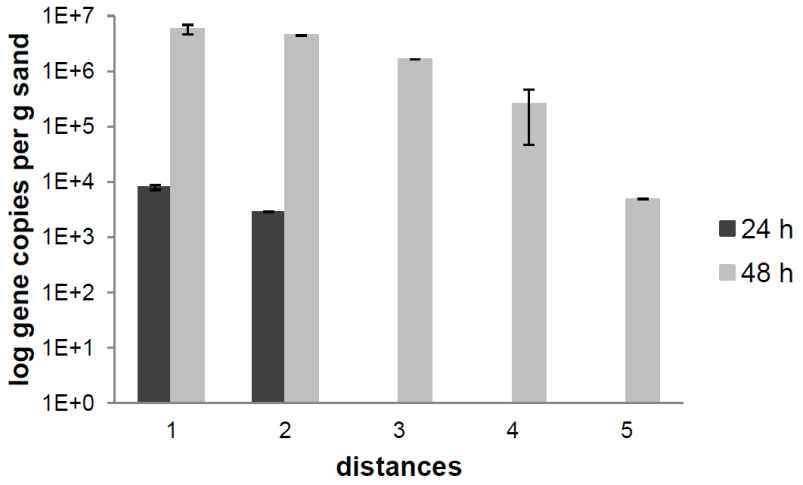

Fig 3.2: Copy numbers of 16S rRNA genes at different distances from the bacterial community inoculation center in sand microcosms at 24 and 48 h as determined by qPCR. Error bars depict the standard deviation, and * symbols indicate statistically significant differences (P< 0.05). Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

TRACKING COMMUNITY EXPANSION VIA 16S RRNA GENE PYROSEQUENCING

Pyrosequencing of the V4 region of bacterial small subunit (16S) ribosomal RNA genes was performed for samples taken at 48 h, where bacteria were found to be present at all sample distances from the inoculation center. Pyrosequencing yielded 112,198 reads that could be classified to the kingdom bacteria. The obtained reads were grouped into a total of 199 Operational Taxonomical Units (OTUs). Read distribution varied substantially among samples (Table 3.1 and S2), and since samples belonging to replicate F only yielded between 0 and 108 reads in total, we decided to exclude all samples from this replicate from further analyses. Distance 5 from replicate H was also excluded because we could not obtain replicated data for that distance.

Table 3.1. Overview of number of reads, Shannon Wiener diversity index and species richness in the samples taken at 24 and 48 h at the different distances from the inoculation zone. Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

type	distance	replicate	timepoint (h)	reads (total n)	shannon diversity (H)
inoculum	0	1	0	3637	3,27
inoculum	0	2	0	8759	2,50
sample	1	A	24	2255	2,54
sample	1	B	24	3979	1,99
sample	1	C	24	8729	0,84
sample	2	C	24	9876	0,41
sample	1	D	24	2184	1,71
sample	2	D	24	782	2,56
sample	1	E	48	1245	0,53
sample	2	E	48	1424	0,41
sample	3	E	48	8448	0,20
sample	4	E	48	992	0,02
sample	1	F	48	108	1,41
sample	2	F	48	0	0,00
sample	3	F	48	24	0,98
sample	4	F	48	62	2,53
sample	1	G	48	3836	0,90
sample	2	G	48	3201	0,62
sample	3	G	48	19580	0,00
sample	4	G	48	469	0,13
sample	1	H	48	703	0,82
sample	2	H	48	11501	0,62
sample	3	H	48	12604	0,00
sample	4	H	48	2873	0,06
sample	5	H	48	4927	0,39

The spatial distribution of the four most abundant bacterial OTUs that had expanded from the center inoculation spot during 48 h is given in Fig 3.3 (average of all microcosms) and in Fig S3.2 (individual microcosms). A single OTU that could be classified within the Enterobacteriaceae increased strongly in relative abundance with increasing distance from the point of inoculation (from 3.5% relative abundance in the inoculum to, 73%, 81%, 99% and 99% at distances 1 to 4, respectively).

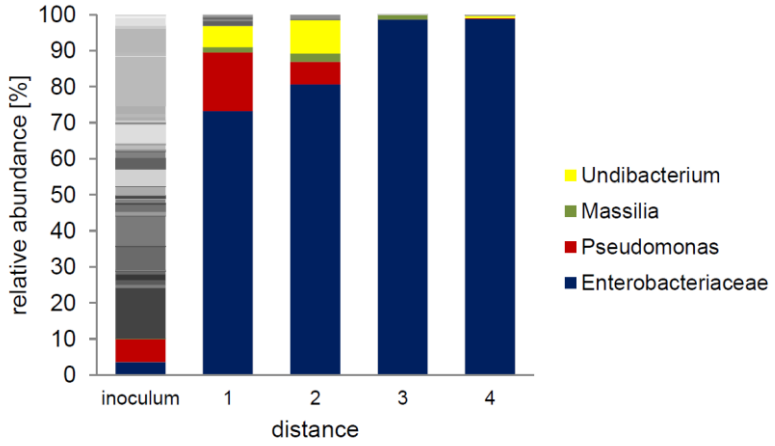

Fig 3.3: Relative abundance of the bacterial phyla at distances 1-4 at 48 h and in the inoculum. The 4 most abundant phyla are displayed in the colors indicated in the legend, and other phyla in various shades of grey (see Table 3.1). Each bar depicts the average of 3 replicate samples. Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

At distances 1 and 2, other bacteria were detected in variable abundances in addition to the Enterobacteriaceae, and these include members of the genera *Pseudomonas*, *Massilia* and *Undibacterium* (Fig S3.2). At distances further away from the point of inoculation (3 and 4), the bacterial community was consistently dominated by apparently fast moving Enterobacteriaceae in all replicates (98.6% and 98.8% relative abundance on average, respectively). Based upon isolation and Sanger-sequencing of 16S rDNA, this dominant expanding population could be tentatively identified as *Pantoea agglomerans*, and this strain was indeed highly motile (not shown).

Bacterial diversity calculated using the Shannon Wiener index was found to be much greater in the microbial inoculum spot ($H = 2.9 \pm 0.54$) than in the zones occupied by colonizing bacteria ($P \leq 0.05$) (Table 3.1). Diversity indices significantly decreased with increasing distance from the central inoculum spot (Fig 3.4 and Table 3.1).

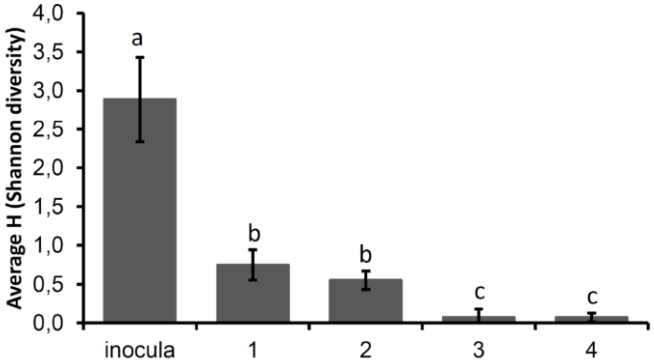

Fig 3.4: Comparison of bacterial diversity between the different sampling points as calculated by the Shannon Wiener diversity index (H). Error bars depict the standard deviation. Different letters indicate significant differences ($P<0.05$). Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

DISCUSSION

In this study, we examined the expansion of two model bacterial strains in sand under different nutrient conditions and identified the bacterial taxa from soil communities that were most successful in colonizing new soil habitats in a short time of 48 hours. In single-strain inoculation experiments, we observed contrasting patterns of expansion in response to nutrient loads. *Collimonas fungivorans* exhibited greater expansion at lower nutrient levels, whereas *Pseudomonas fluorescens* moved faster at higher nutrient levels. Thus, the impact of nutrient levels on bacterial motility in our microcosm setup was strain dependent.

Results of the complex community experiment showed that relatively few bacterial cells had moved after 24 h and only a small radius around the inoculation zone was initially colonized (Fig 3.1). At 48 h, the whole dimension of the microcosm was colonized, with cell densities declining with increasing distance from the inoculation zone (Fig 3.1). Not only did cell densities decrease with increasing distance from the inoculation zone, bacterial diversity was also highest close to the inoculation zone and decreased with distance from that point. To our knowledge, this is the first study that examines the movement of bacteria within a complex natural community under conditions that resemble natural soil environments (*i.e.* a soil-like matrix with low nutrient conditions and relatively low moisture content). The highly selective nature of bacterial expansion and the non-linear nature of expansion (far fewer bacterial cells after 24h than 48h, Fig 3.2) makes it probable that we observed active movement in combination with growth, rather than mere passive diffusion as may occur under higher moisture conditions over shorter distances (Wertz *et al.* 2007). However, future studies are needed to test our findings for different soil types and community composition of the inoculum.

Based upon sequence recovery by high-throughput pyrosequencing, bacteria belonging to the genera *Undibacterium*, *Pseudomonas*, and *Massilia*, and especially the family of Enterobacteriaceae, were most successful in expanding through the sandy microcosm habitat (Figs 3.3 & S3.2). Relative recovery of sequences from these bacteria increased sharply with distance from the inoculation zone, whereas many other taxa found in the original inoculum were no longer

detected in samples more distant from the point of inoculation. The bacterial genera that were most frequently detected at the more distant sample locations are all known to possess flagella and are often abundant in the rhizosphere and on plant roots (Lugtenberg & Dekkers 2001; Chunga *et al.* 2005; Ofek *et al.* 2012).

Interestingly, the by far the most dominant member of the mobile community was an OTU belonging to the family *Enterobacteriaceae* (*γ-Proteobacteria*), tentatively identified as *Pantoea agglomerans*. As the abundance of *Enterobacteriaceae* in soils is generally low, our findings suggest that motility might provide an initial competitive advantage for exploration of new soil habitats, but other microbial groups may take over rapidly during microbial succession. The family of *Enterobacteriaceae* is commonly associated with eukaryotic hosts and motility has indeed been suggested as an important factor explaining the abundance of *Enterobacteriaceae* in bovine feedlot soil.

Bacterial motility is of importance to re-colonization of soils. For instance, strong and sudden disturbances may result in a drastic reduction of biomass (Postma *et al.* 1989) and even sterilization, e.g. in the event of a forest fire (Prieto-Fernandez *et al.* 1998; Neary *et al.* 1999). Motile microorganisms, obviously, have an advantage in re-colonizing disturbed soils or soils with low biomass, especially in the early stages of re-colonization, as they are the first to reach these habitats. This may be of particular importance in microbial succession, given the fact that priority effects are often important in determining the success of bacterial populations when attempting to invade new territories (Remus-Emsermann *et al.* 2013). Microbial re-colonization might be an essential mechanism that helps to stabilize functional redundancy, and therefore an important parameter when considering the restoration of disturbed (microbial) soil systems (Bodelier 2011). Identifying the (most) motile strains thus holds potential to predict and control re-colonization succession of sterilized soils or soils of reduced microbial biomass.

Studies on bacterial movement in soil-like systems are scarce. Wertz *et al.* (2007) investigated bacterial movement from soil into nearly water-saturated sterile soil clods over relatively long time periods (2, 8 and 14 days). Unfortunately, because of the high moisture, the authors could not distinguish between active and passive movement. Additionally, the bacteria that we able to disperse over the shortest time period (2 days) were not identified and movement distance was only monitored over 2 cm. Nevertheless, the authors detected a drastic community shift and a reduction of diversity when comparing the bacterial community that had moved after 2 days to the community at 8/14 days. Among the dominant colonizers at the latter time point, they reported *Collimonas fungivorans* and bacteria from the genus *Burkholderia*. Interestingly, these motile bacteria (Leveau *et al.* 2010) are related to the expanding *Oxalobacteriaceae* detected in our study. When comparing both studies it becomes apparent that Oxalobacteriaceae might be "followers" of the quickly moving *Enterobacteraceae*.

Any assertions with respect to mechanisms of community succession remain premature, but such patterns would be expected to involve both facilitation and inhibition. Future studies will be necessary to examine the rules and mechanisms that govern the succession of microbial communities in the colonization of soil habitats. Further additional research priorities would include examination of how other biotic and abiotic soil features, such as pH, soil texture and disturbance, impact relative mobility, and the presented microcosm system might be ideal for such future examinations (Wolf *et al.* 2013).

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Table S3.1. Sand particle size distribution in sand microcosms indicating which percentage of sand particles belongs to the respective particle size class.

particle size [µm]	particle size distribution [%]
53-106	1.27
106-212	35.77
212-425	62.96

Table S3.2 Relative abundance of OTUs assigned to different taxa in the soil bacterial inoculum that was added to the center of sand microcosms (distance=0) and in sampling spots at distances 1 to 4, that were colonized after 48h. Two replicates for the inoculum (1 and 2) and 3 replicates (E,G and H) for the further distances are shown. Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

sample	ino 1	ino 2	replicate E				replicate G				replicate H			
distance	0	0	1	2	3	4	1	2	3	4	1	2	3	4
taxa														
Enterobacteriaceae	**5,61**	**1,46**	**88,11**	**89,82**	**95,88**	**99,80**	**56,78**	**80,01**	**99,96**	**97,44**	**74,82**	**72,08**	**99,96**	**99,13**
Pseudomonas	**6,49**	**6,35**	**5,30**	**2,74**	**0,24**	**0,00**	**38,27**	**15,96**	**0,00**	**0,00**	**5,26**	**0,12**	**0,02**	**0,49**
Massilia	**0,00**	**0,01**	**4,42**	**6,95**	**3,49**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
Undibacterium	**0,00**	**0,01**	**0,56**	**0,21**	**0,18**	**0,00**	**0,13**	**0,00**	**0,01**	**1,92**	**16,79**	**27,54**	**0,01**	**0,10**
Luteibacter	0,03	0,00	0,56	0,07	0,02	0,00	3,08	1,03	0,00	0,00	0,57	0,01	0,00	0,10
Paenibacillus	0,00	0,00	0,00	0,00	0,00	0,00	0,83	3,00	0,01	0,00	0,00	0,00	0,00	0,07
Actinobacteria	11,69	16,60	0,32	0,00	0,07	0,00	0,03	0,00	0,00	0,00	0,14	0,00	0,00	0,03
Phenylobacterium	1,54	0,00	0,32	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingomonas	0,03	2,44	0,16	0,14	0,01	0,00	0,44	0,00	0,00	0,00	0,28	0,00	0,00	0,00
Neisseriaceae	3,44	0,00	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Caulobacter	1,68	0,00	0,08	0,00	0,00	0,00	0,36	0,00	0,00	0,00	0,00	0,00	0,01	0,03
Prevotella	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00
Pontibacter	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Chlamydiae	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Opitutus	0,00	0,24	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Spartobacteria	2,83	10,45	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Verrucomicrobiaceae	0,33	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Chloroflexi	3,02	13,68	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cyanobacteria	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Deinococcus	0,00	0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Elusimicrobia	0,85	1,43	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Holophagae	3,66	0,26	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Fibrobacteraceae	0,66	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Tumebacillus	1,04	1,30	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacillaceae	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brochothrix	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Granulicatella	1,43	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Leuconostoc	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Weissella	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Streptococcus	4,95	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Erysipelotrichaceae	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Fusobacteria	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Gemmatimonadetes	2,17	7,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Nitrospira	4,87	1,44	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brevundimonas	2,69	0,87	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Beijerinckiaceae	0,30	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Nitrobacter	0,52	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Rhodoplanes	0,00	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Methylobacterium	1,65	0,64	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Rhodobium	0,00	1,39	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pseudolabrys	6,13	4,17	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acetobacteraceae	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Dongia	0,44	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Skermanella	0,71	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Candidatus Captivus	0,14	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Holospora	0,52	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobium	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Alcaligenaceae	1,73	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Burkholderia	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	1,85	0,00	0,00	0,03
Cupriavidus	0,77	0,80	0,00	0,07	0,01	0,00	0,00	0,00	0,00	0,00	0,28	0,25	0,00	0,00
Comamonadaceae	3,63	1,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,64	0,00	0,00	0,00	0,00
Nitrosomonadaceae	9,82	17,46	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Rhodocyclaceae	0,30	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Desulfobacterales	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Nannocystineae	2,23	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Aquicella	3,52	9,38	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Legionella	1,79	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pseudospirillum	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pasteurellaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Acinetobacter	3,66	0,07	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Enhydrobacter	0,00	0,13	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Dokdonella	1,04	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Dyella	0,00	0,03	0,00	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Crenarchaeota	0,00	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halobacteriaceae	0,63	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

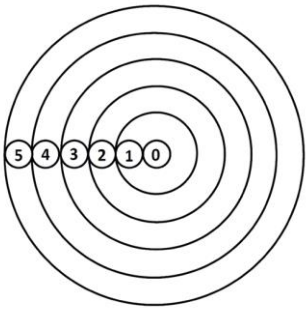

Fig S3.1 Schematic overview of the sampling points in the microcosms. The microcosms were inoculated in the center (distance 0) and samples were taken with sterilized pipet tips (~1cm diameter) at distances 1-5 (approximately 0.5 - 1.5; 1.5 - 2.5; 2.5 - 3.5; 3.5 - 4.5; 4.5 - 5.5 cm away from the center, respectively).

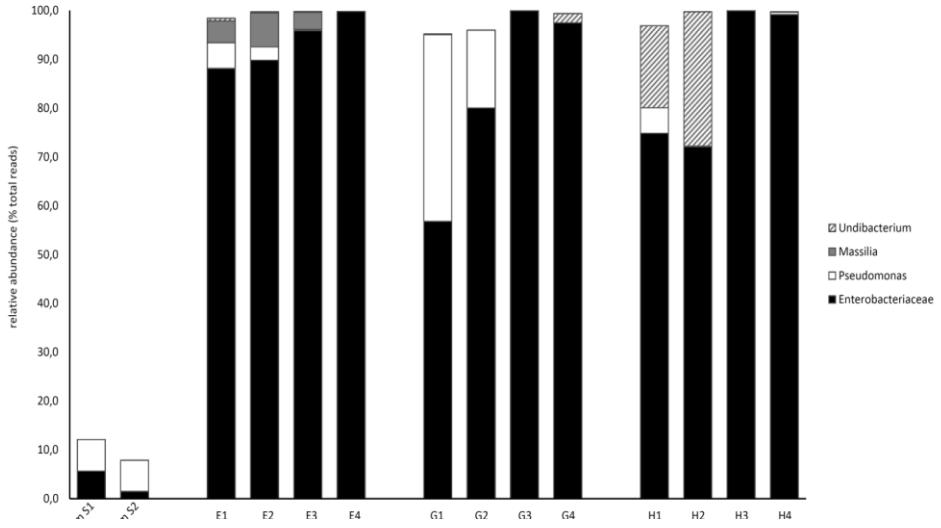

Fig S3.2 Comparison of bacterial community composition between the inoculum and the different sampling distances. Stacked column graph represent the relative distribution of the four most abundant phyla in the different samples. Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig S3.1.

CHAPTER FOUR

TRAIT DIFFERENTIATION AMONG MYCOPHAGOUS COLLIMONAS BACTERIA

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To be submitted

ABSTRACT:

The genus *Collimonas* consists of bacteria that are known for their ability to feed on living fungi (mycophagy). To date, 3 species (*C. fungivorans, C. pratensis and C. arenae*) have been formally described and over 100 strains have been isolated from different habitats. *Collimonas* bacteria interact with soil fungi through functional traits that mostly negatively (e.g. fungal inhibition, mycophagy) but in some cases also positively (e.g. mineral weathering) affect their host. Collimonads show high variability in the quantitative expression of such traits, despite belonging to one genus. In this study, we investigate a) whether phylogenetically related *Collimonas* strains possess similar traits, b) how far phylogenetic resolution influences the detection of phylogenetic signal (possession of similar traits by related strains) and c) how the possession of traits is co-related. We measured genetically encoded (e.g. nifH genes, collimomycin gene cluster) as well as phenotypically expressed traits (violacein, chitinase- and siderophore production, fungal inhibition, feeding on fungi, swarming abilities and others) and relate those to a high-resolution phylogeny (MLSA), constructed by sequencing the housekeeping genes *gyrB* and *rpoB* and concatenation with partial 16S ribosomal sequences. Additionally, high-resolution and 16S derived phylogenies were compared. We show that a multi-housekeeping gene derived phylogeny (MLSA) is superior to a 16S derived phylogeny when analyzing trait distribution and relating it to phylogeny (detecting phylogenetic signal) at a fine taxonomic resolution (a single bacterial genus). We observe that several traits involved in the interaction of collimonads and their host fungus (e.g. fungal inhibition) carry phylogenetic signal. Furthermore, *Collimonas* trait possession is compared with sister genera like *Herbaspirillum* and *Janthinobacterium*.

INTRODUCTION:

The limited availability of energy resources is probably the main driver of bacterial diversification in soil (Hibbing *et al.* 2010). Through constant adaptation, the struggle for nutrients leads to the evolution of high bacterial and fungal diversity, sometimes forcing species to interact in order to efficiently acquire carbon (Folse & Allison 2012). Looking at fungal-bacterial interactions in the soil, the bacterial genus *Collimonas* is of particular interest because of its common association with fungi. Collimonads are able to colonize fungal hyphae and to exploit them as their sole source of carbon and energy, a specialized strategy of nutrient acquisition that has been termed "mycophagy" (Leveau *et al.* 2010). Antifungal traits, such as the production of fungal-inhibiting secondary metabolites, are thought to play an important role in fungal nutrient acquisition by collimonads (Leveau *et al.* 2010; Mela *et al.* 2011).
Introduction of *Collimonas* strains in a soil with low abundance of indigenous collimonads resulted in shifts in fungal community composition without significantly reducing general fungal abundances (Hoppener-Ogawa *et al.* 2009a). This indicates that at least some collimonads have specific feeding preferences for distinct fungal species which might change the outcome of competitive interactions between fungal species. Next to traits which appear to be negative for fungi, some isolates have been shown to carry out functions like mineral weathering that can provide indirect benefits for the fungal host. In the weathering process, *Collimonas* bacteria make inorganically bound minerals available which could not only be of benefit for themselves but also for the fungal hosts. In this respect, it is interesting to note that

the amount of fungal biomass that is converted into *Collimonas* biomass appears to be small (Hoppener-Ogawa *et al.* 2009b). In addition, the presence of *Collimonas* on fungal mycelia can result in increased hyphal branching (Deveau *et al.* 2007; Hoppener-Ogawa *et al.* 2009b). Hence, it seems likely that fungi can function, despite the loss of part of the nutrients to mycophagous collimonads, perhaps comparable with plants that continue growing while losing part of their biomass to small herbivores like aphids. Therefore, the mineral weathering ability of collimonads may be of benefit for fungi and compensate for the loss of biomass due to mycophagous growth.

So far, all studied *Collimonas* strains appear to have the mycophagous ability albeit that none of them is obligatory dependent on fungal nutrition. However, the description of three species within the genus *Collimonas* does indicate diversification within the genus which could be related to differentiation in the relationships with fungi, e.g. specialization for feeding on certain groups of fungi.

In this study, we elaborate on the diversification within the genus *Collimonas* using a "trait-based approach" in order to understand how different *Collimonas* strains interact with their fungal hosts. Trait based approaches try to capture the functional capabilities of taxa by measuring a set of characteristics that the organisms possess or are able to carry out (Munkemuller *et al.* 2012). Trait-based approaches have been used in plant ecology for a long time and functional traits have not only been used to group organisms in functional categories but also to assess their contributions to the provision of ecosystem services (Diaz *et al.* 2013). The interest in functional traits as a tool to study diversification in microbial taxa has become popular among microbial ecologists as well. Most trait-based studies in microbial ecology did however only investigate trait dispersal on higher taxonomic ranks (Martiny et al., 2013).

The aim of this study was to find out if high-resolution phylogenetic grouping of collimonads is coinciding with fine-scale trait distribution. To accomplish this, we first conducted a Multi Locus Sequence Analysis (MLSA), based on concatenated partial sequences of the small subunit of the bacterial ribosome (16S rDNA), DNA gyrase subunit B (*gyrB*) and RNA polymerase β-subunit (*rpoB*). In a second step we relate trait investment (the possession or (for quantitative traits) the strength of a trait) to phylogeny by measuring the phylogenetic signal and testing it against a random trait evolution model. We use different methods, relying on gene presence/absence as well as physiological assays to collect trait data since for some traits like fungal inhibition or mycophagy the underlying genes are not or only partly known making those traits impossible to follow on a molecular basis but easily measureable as phenotype.

Part of the traits that we measured are directly focused on the interaction between collimonads and their fungal host, such as the possession of *in vitro* antifungal activities against a range of fungi and the ability to feed (mycophagy) on living fungi. Antifungal activity and mycophagous growth had already been investigated for a limited set of collimonads (de Boer *et al.* 2004; Hoppener-Ogawa *et al.* 2008). More specifically, we also included data on the production of collimomycin, a secondary metabolite involved in fungal inhibition (Fritsche *et al.* 2008; Fritsche *et al.* 2014). In addition, we determined the ability to digest chitin (chitinases), an important structural component of fungal cell walls. Chitinases have been shown to be commonly produced by collimonads (Fritsche *et al.* 2008) and also by a *Collimonas* sister genus, *Janthinobacterium*. Furthermore, we performed an assay to evaluate the ability to produce iron chelators (siderophores) a trait that has been suggested to be important in *Collimonas*' iron acquisition (Leveau *et al.* 2010), and could possibly be involved in weakening of fungi due to iron depletion (Leong 1986).

Next to the potential antagonistic characteristics, traits were included that could be of benefit for the fungal host. This was the ability to weather inorganic nutrients (mineral weathering) which had already been shown to be an important functional trait for certain collimonads (Uroz *et al.* 2009b). Bacteria in the sister genus of *Collimonas, Herbasprillum,* are known to be able to fix atmospheric nitrogen (N_2) (Schmid *et al.* 2006). Therefore, we examined whether an essential gene for nitrogen fixation (*nifH*) is present in *Collimonas* strains, possibly derived from the common ancestor of the two genera. The bacterial nitrogen fixation could be a potential source of nitrogen for the fungal host, possibly providing a benefit for both, the fungus and the colonizing collimonads.
Finally, we also tested for traits that have not yet been shown to have a functional role in the interaction of collimonads with their fungal host. We visually (purple pigmentation) and genetically (*VioA* and *VioB*) inspected collimonads for the production of violacein, a compound known to have broad spectrum anti-microbial activity (Lichstein & Vandesand 1945). Violacein production is well known for the genus *Janthinobacterium*, a sister genus of *Collimonas* (Pantanella *et al.* 2007). Since the water-insoluble violacein is stored intracellularly it might be used for defensive rather than aggressive matters, for example as a protection against bacterial predators. Lastly, *Collimonas'* swarming movement was assessed. Swarming intensity (space covered by a swarming colony) and shape of the swarming colony was monitored. Collimonads have recently been shown to be excellent movers in liquid and sand systems (*chapter two and three*). We assumed that the ability to swarm would have important implications for mycophagous collimonads, with respect to the ability to locate host fungi in the soil matrix (*chapter two*).
We show that different phylogenetic groups of collimonads possess distinct traits. Furthermore, we show that fine-resolution trait dispersal and phylogeny are required to obtain detailed information on phylogenetic signal at fine taxonomic levels.

MATERIAL AND METHODS:

COLLIMONAS STRAINS:

We included *Collimonas* isolates from France, Finland, and the Netherlands in this study. The habitats from which the bacteria have been isolated are described in Table S4.1. Isolates were assigned to the genus *Collimonas* on basis of sequence analysis of the 16S rDNA gene or its digestion with the restriction enzyme *BstBI.* This enzyme cleaves around position 1000 of the16S rDNA at a site (5'-TTCGAA-3') that is unique for the genus *Collimonas* and therefore allows identification of *Collimonas* bacteria (Leveau *et al.* 2010). Experiments were conducted with a set of 88 different collimonads, except for mycophagy assays, weathering - and collimomycin datasets, which included 35 strains, each. We only tested a subset of the strains for mycophagy because the assay is laborious. Data on weathering and the presence of collimomycin gene clusters were retrieved from Uroz *et al.* (2009b) and (Fritsche *et al.* 2014), respectively.

FUNGI:

The fungi that were used in inhibition and mycophagy assays were acquired from the following sources: *Trichoderma harzianum* CECT 2413 was purchased from the Spanish type culture collection CECT (University of Valencia, Spain), *Mucor hiemalis* and *Fusarium culmorum* were originally isolated by De Rooij-Van der Goes *et al.* (1995) from coastal foredunes in the Netherlands. *Pythium ultimum* P17 (an oomycete) was obtained from WUR Applied Plant Research- PPO, Lisse, the Netherlands.
Fusarium oxysporum (CBS619.87), *Aspergillus niger* N400 (CBS120.49) and *Phoma exigua* var exigua (CBS833.84) were purchased from the Fungal Biodiversity Center (CBS-KNAW, Utrecht, The Netherlands). *Rhizoctonia solani* Ag 2-2 IIIb was isolated by IRS, Bergen op Zoom, the Netherlands. *Fusarium culmorum* was cultured on Synthetic Nutrient Agar (SNA), pH 6.8 (KH_2PO_4 1 gL^{-1}, KNO_3 1 gL^{-1}, $MgSO_47H_2O$ 0.5 gL^{-1}, KCl 0.5 gL^{-1}, Glucose 0.2 gL^{-1}, Sacharose 0.2 gL^{-1} and Agar15 gL^{-1}), all other fungi on Potato Dextrose Agar (PDA), pH 6.8 (Potato Dextrose Agar, 9.75 gL^{-1}; Agar 3.75 gL^{-1}).

MYCOPHAGY ASSAY:

The mycophagy assay was conducted for 35 isolates (Table S4.1) and each isolate was tested against the two fungi *R. solani* and *M. hiemalis*. The assay was conducted as described in *chapter five*. In short, collimonads were inoculated on a petri dish, containing phytagel medium. Use of phytagel enables to create a semi-solid medium that is very nutrient-poor (no release of degradable organic compounds). Then, the fungus is introduced on a nutrient plug, in the middle of the petri dish, separated by a metal disc from the inoculated bacteria. While expanding, the fungus is confronted with the surrounding bacteria. Bacteria increase in biomass if they are able to grow on living fungal tissue or fungal exudates as their sole source of carbon. After incubation, the bacteria are washed of the microcosm and the optical density (OD_{600}) of the bacterial suspension is determined and compared with the bacteria only control or the fungus only control. Mycophagy ratios ($OD_{treatment}/OD_{control}$) were calculated with the higher of the two controls, averaged over 3 replicates. If a significant positive difference between the fungal treatment and the control was detected the respective bacterium was considered to be mycophagous.

SWARMING AND SIDEROPHORE ASSAY:

The swarming assay was done on M9 medium with 0.5% Agar, as described in Xavier *et al.* (2011), scored after 48 hours of incubation at 20°C, subsequently overlaid with CAS medium (Pérez-Miranda et al 2007) and scored for siderophore production after 5 hours. The program ImageJ (Schneider *et al.* 2012) was used to measure the extension of the typical yellow margin around the colonies indicating siderophore production (average of 5 different spots).

FUNGUS INHIBITION ASSAYS:

Assays were conducted on Water Yeast Agar (WYA) (KH_2PO_4 1 gL^{-1}, NaCl 5 gL^{-1}, Yeast extract 0.05 gL^{-1} and Agar 20 gL^{-1}, pH 6.8) using standard size petri dishes. *Collimonas* bacteria were pre-cultured on Tryptic Soy Agar (TSA) (KH_2PO_4 0.5 gL^{-1}, NaCl 2.5 gL^{-1}, Yeast extract 0.05 gL^{-1}, Tryptone 1.5 gL^{-1} and Agar 10 gL^{-1} , pH 6.8) and inoculated on a 2 cm x 6 cm square, 4 days before the fungi/oomycete were introduced as a plug from the margin of an actively growing colony (Fig S4.1). Assays were conducted at 20°C and scored depending on the growth speed of the fungus, after 4 days for *P. ultimum*; after 7 days for *R. solani, M. hiemalis, F. culmorum and T. harzianum;* after 14 days for *F. oxysporum* and *P. exigua*; and after 21 days for *A. niger*.

CHITINASE ASSAY:

Assays to check isolates for chitinolytic activity were conducted on chitin agar (CYA) (De Boer *et al.* 2001) and scored for halo formation around the colonies after 9 days of incubation at 20 °C. Before transfer to CYA, collimonads were pre-grown on TSA.

CHOICE OF HOUSEKEEPING GENES:

Housekeeping genes coding for DNA gyrase subunit B and RNA polymerase β-subunit were chosen because of their ubiquity in bacteria. They code for essential cell metabolic functions, are conserved and mostly change due to insertions, deletions or point mutations. *RpoB* and *gyrB* are not located adjacent to genes that encode for outer surface proteins or hypothetical proteins and are single copy genes. This is important to consider when designing an MLST scheme, since multiple gene copy numbers and/or locations next to elements that are subjected to higher selective evolutionary pressures might distort phylogenetic grouping of the isolates (Maiden 2006).

PRIMER DESIGN:

Primers for the amplification of the two housekeeping genes *rpoB* & *gyrB* were designed based on the corresponding sequences in the genome of *Collimonas fungivorans* Ter331 and related bacteria that belong to the family *Oxalobacteraceae*. We decided to design primers for the family *Oxalobacteraceae* rather than the genus *Collimonas* to ensure amplification from all *Collimonas* isolates. The pipeline Primer Prospector (Walters *et al.* 2011) was used for primer design & specificity testing. The main criterion for primer selection was that *de novo* primers had to match with *Collimonas fungivorans* Ter331. Parameters were then adjusted to include as many related strains as possible while not having more than 10% degeneracies in the primers. Finally primers were sorted and pairs selected based on similar GC-content and annealing temperatures. Information on primers used in this study can be found in Table 4.1.

Gene	Primer name	Sequence (5'-3')	Reference
gyrB	gyrBr	TCYTTGCCGGTGCGCTGGTC	*This study*
gyrB	gyrBf	GGCCTGGAAGCGGTRCGCAA	*This study*
rpoB	rpoBr	TCGGCGATCACGTCRTGCTT	*This study*
rpoB	rpoBf	AACRCCKGAAGGCCCGAACA	*This study*
nifH	IKG3	GCIWTHTAYGGIAARGGIGGIATHGGIAA	*Ando et al 2005*
nifH	DVV	ATIGCRAAICCICCRCAIACIACRTC	*Ando et al 2005*
vioA	VPA3	CCRCAGCTSCAYCCGCATTTCCAG	*Hakvåg et al 2009*
vioA	VPA4	CAGGCYGCCCTCCATCCAGCCRCA	*Hakvåg et al 2009*
vioB	VPB1	CTGTTCAATATGTCGACGCCGC	*Hakvåg et al 2009*
vioB	VPB2	GCGGATCGCACATCTGCCACATC	*Hakvåg et al 2009*

Table 4.1 Primers used in this study.

CULTURING, PCR AMPLIFICATION & SEQUENCING:

Strains were either grown on 10 % TSA plates or in liquid TSB. Single colonies were resuspended in 100 µl water, heated 10 min at 98°C and used directly for PCR amplification (ColonyPCR). Alternatively, DNA was isolated using Phenol/Chloroform, as described in Moore *et al.* (2004). PCR reactions for 16S rDNA were conducted as described in *chapter five*. Nitrogenase (*nifH*) and violacein genes (*VioA* & *VioB*) were amplified as described in Ando *et al.* (2005) and Hakvåg *et al.* (2009), respectively, and fragment size was examined on standard (1.5 w/v) agarose gels. PCR reactions to amplify *rpoB* and *gyrB* genes were carried out in 25 µl reactions, containing: 18.14 µl H_2O, 2.5 µl 10x PCR-buffer incl. 2mM $MgCl_2$ (Roche Scientific, Woerden, The Netherlands), 0.2 mM of each dNTP (Roche Scientific, Woerden, The Netherlands), 0.4 µM of each Primer, 1U Fast Start High Fidelity Polymerase (Roche Scientific, Woerden, The Netherlands) and 1 µl template DNA. Cycling conditions consisted of an initial denaturation of 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec with a final elongation step at 72°C for 7min. PCR products were examined by a standard (1.5 w/v) agarose electrophoresis and cleaned with 20% PEG8000 (SigmaAldrich) before being sent to Macrogen (Amsterdam, The Netherlands) for sequencing (in case of 16S, *rpoB* and *gyrB*).

PHYLOGENETIC ANALYSIS:

Quality check and editing of the obtained sequences was done using the software SequenceScanner (Applied Biosystems) and BioEdit (Hall 1999). Alignment and curation of sequences was done with the program MEGA 5.2 (Tamura *et al.* 2011) and ClustalW. Sequences were concatenated with DAMBE 5.3.9 (Xia & Xie 2001) and MEGA 5.2 was used to construct a Neighbor-Joining Tree with standard settings. The pairwise deletion option was used to treat missing data and gaps; finally the tree was tested with 100 bootstrap replicates.

PHYLOGENETIC SIGNAL:

Phylogenetic signal describes how well phylogeny predicts the trait distribution on a phylogenetic tree (Munkemuller *et al.* 2012).We used different methods to statistically test for the existence of such signal, depending on the nature of the trait data. All methods assume that random trait evolution is best described by a random walk of Brownian motion (BM) along the branches of the phylogenetic tree (Fritz & Purvis 2010; Munkemuller *et al.* 2012).They give an indication of the strength of the signal and test whether it significantly differs from one derived from random trait distribution. For continuous trait data we used Blomberg's K (Blomberg *et al.* 2003) ($K = 1$ indicates trait evolution following BM, $K < 1$ less trait divergence than BM and $K > 1$ clustering of traits which is higher than expected under BM), for discrete traits Pagel's λ (Pagel 1999) ($\lambda = 0$ equals BM trait evolution, $\lambda = 1$ equals trait clustering higher than to be expected on basis of BM), and for binary traits Fritz' and Purvis' D (Fritz & Purvis 2010) ($D \leq 0$ characterizes a higher trait clumping than to be expected under BM, $D = 1$ suggests a random trait evolution). Statistically the signal calculation differs, due to the continuous, discrete or binary nature of the trait data. For details on phylogenetic signal calculation of different trait data we refer to the review by Munkemuller *et al.* (2012). Phylogenetic signal was calculated using the software "R" and the packages "phytools"," geiger" and "caper". Since negative or zero branch lengths interfered with the phylogenetic signal calculation, the smallest branch length (in case of no negative branches but zero branch lengths) or the branch length that would equal a negative branch to zero plus 0.00000001 was added to all branches of the tree.

STATISTICS:

Correlations between traits were calculated using different methods, depending on the nature of the variables. For relationships between continuous variables such as weathering and mycophagy ratios, we used Pearson's correlation coefficient or linear regression. Relations between continuous and 2 level categorical (binary) data like purple pigmentation (violacein) and mycophagy ratios e.g. were investigated using t-tests. When binary data were tested against binary data, Pearsons chi-squared test was used. This was for example the case for fungal inhibition and purple pigmentation. With categorical data that had more levels, we performed analyses of variance against continuous data. This was for example the case for siderophore production and swarming intensity. After the ANOVA we performed Turkey's HSD test to assign significance. Categorical data with more levels were tested against one another or against binary categorical data using logistic regression models. This was for example the case for the relationship between different habitat types and swarming intensity. A summary of all calculations can be found in the supplementary material. All statistics were done using the program R.

EXTERNAL DATA:

Collimomycin data were taken from (Fritsche *et al.* 2014), weathering data from (Uroz *et al.* 2009b) and habitat data from Uroz *et al.* (2007), Hoppener-Ogawa *et al.* (2007), Mannisto and Haggblom (2006) and Nissinen *et al.* (2012).

RESULTS:

PHYLOGENETIC TREE COMPARISON:

Overall, the MLSA tree provides a higher taxonomical resolution than the phylogeny that is based on 16S rDNA sequences only (Fig 4.2). Still, some *Collimonas* strains could not be differentiated from one another even with the MLSA phylogeny. Reasons for this can either be that they are isolates of the same strain or the fact that we used too few housekeeping genes to be able to effectively differentiate all strains. The higher resolution of the MLSA also becomes apparent when comparing it with the grouping of strains into already described species (*C. fungivorans*, *C. pratensis* and *C. arenae*) and clusters (A, B, C and D) (De Boer et al., 2004; Hoppener-Ogawa et al., 2008): a) Cluster B is split into 2 separate clusters, b) The pre-defined clusters are diverse, especially cluster D seems to harbor strains that form subgroups within the cluster, c) We find more evidence for strains that form clusters which have not been described, yet. The latter strains are RA1BR1, RAJ3R3, M1V16, M1V1, J41_1, P1, P2, K2X3 and SO92 and also the group consisting of CPML32, EPMY119, CPML38, EPMY118, CPML37, SO96, J55, R5TW1, AD076, S21 and S5T5. The two groups split from the branch forming cluster A with reasonable bootstrap support (79 and 69, respectively).

MYCOPHAGY ASSAY:

Average mycophagy ratios varied between isolates and were significantly different between the two test fungi (*M. hiemalis:* 64.7, *R. solani:* 5.7, $P \leq 0.0001$ (t-test)). In the case of *M. hiemalis*, 3 out of 36 strains (SO122, AD076, and Ter6) did not show significant mycophagous growth. *R. solani* did not significantly support growth of 13 strains (M133, K2X, SO92, J55, Ter118, SO113, Ter331, CPML32, S33, SO132, AD076, Ter6, and S5T5). We decided to exclude the non-significant strains from the subset of bacteria that were tested for mycophagy as those strains could potentially be mycophagous but the variation between replicates was too high to allow conclusions which could bias follow-up data analyses.

SWARMING ASSAY:

We observed differences in swarming abilities among collimonads. Approximately one third (37 of 90) isolates were not able to swarm at all. The group of swarmers could be divided into slow swarmers which did not colonize the whole petri dish (94 mm dia, 16 mm height) in 48 hours (41 isolates), moderate swarmers which colonized the whole petri dish in 48 hours (12 isolates) and fast swarmers that colonized the whole petri dish in less than 48 hours.

Interestingly, collimonads from cluster D (*C. pratensis)*, earlier described to produce smaller colonies than other collimonads, were also found to be less capable of swarming. Furthermore, swarming isolates could be grouped into different swarming shapes (for the description of shapes we refer to Table S4.2). Congruent shapes were given the same color in Fig 4.1.

SIDEROPHORE ASSAY:

All isolates that were scored siderophore positive, showed orange/yellow halos around the colonies, typically indicating the presence of hydroxamate-type siderophores (Perez-Miranda *et al.* 2007). The ability to produce siderophores differed between the tested isolates. The majority (70 isolates) produced siderophores at the colony margin and underneath the colony. For 3 isolates, the amount of siderophores was not measureable since the colony already covered the whole plate when the assay was conducted. 10 strains produced siderophores only underneath the colony, 3 isolates only at the center of the colony, and 4 isolates did not show siderophore production at all. Siderophore production, indicated by an orange/yellow circle around the colony margin, varied from 0 to 89 mm in diameter, ranging from no siderophore production to a halo that nearly covered the whole petri dish.

INHIBITION ASSAYS:

Fungal inhibition was either scored negative (no difference in fungal growth with or without presence of a *Collimonas* strain) or positive (fungal growth was clearly slowed down/stopped by the bacterial strain in comparison to the fungus only control). For the fungi *R.solani*, *M. hiemalis*, *F. oxysporum*, *F. culmorum*, *A. niger*, *P. exigua*, *T. harzianum* and the oomycete *P. ultimum,* 43, 88, 85, 42, 47, 41, 88, 42 strains had no effect on mycelial extension and 47, 0, 5, 48, 43, 49,0 and 48 strains delayed or stopped mycelial extension, respectively. Generally, we found little variation in the inhibition pattern of the fungi by *Collimonas* bacteria. Most collimonads were either able to inhibit the growth of a whole range of fungi or they were not able to inhibit at all.

CHITINASE ASSAY:

All but 5 strains were scored positive for chitinase production on CYA. The five strains showing no clearing of colloidal chitin were SO95, CPML(37), M1R1, S33 and S18_2.

GENETIC ANALYSES:

All tested strains scored negative for the presence of *nifH*, a key gene involved in nitrogen fixation. Production of violacein could be confirmed by detection of PCR amplicons of partial *VioA* & *VioB* genes (typical sizes being about 1000bp and 900bp, respectively (Hakvåg, et al. 2009)) in the purple colored isolates SO195, SO109, P2, P3, SO112, S5T5, RA1BR1, RAJ3R3, M1U16, J41_1, J40_1, S21, M1U1 and S56_2. Wrong size PCR amplicons were obtained from strains AD076, J8_1 and J23 with *VioA* primers and in strains SO06, SO08, SO09, SO95, Ter14, Ter266, Ter299, Ter6, SO30, Ter330, Ter331, AD064, Ter300, K2X3, Myc51, Ter146,

Ter228, SO147, Myc49, Ter10, Myc52, M1J3, SO115, Myc17, Ter166, Myc46, Ter165, CPML34, SO92, Myc44, SO114, Ter90, Ter282, Ter252, SO132 and SO122 with *VioB* primers.
In Table S4.1, an overview is given of phylogenetic-, functional -, and habitat-related information gathered for all strains. For an overview on taxa and associated traits,all trait data are plotted next to the MLSA phylogeny in Fig 4.1.

CORRELATIONS BETWEEN TRAITS:

We found that several of the studied traits were significantly correlated. Significant positive correlation ($p \leq 0.05$) was found between swarming and weathering, between siderophore production and fungal inhibition (all fungi except for *M. hiemalis*, *T. harzianum* and *F. oxysporum*), between siderophore production and mycophagy on *R. solani*, between inhibition (all fungi except for *M. hiemalis*, *T. harzianum* and *F. oxysporum*) and purple pigmentation and between soil pH and purple pigmentation. Inhibition of all fungi except for *M. hiemalis*, *T. harzianum* and *F. oxysporum* was positively correlated with mycophagy on both fungi with a p value close to significance ($p = 0.052$ and $p = 0.053$, respectively for mycophagy on *M. hiemalis and R. solani*). Significant negative correlations ($p \leq 0.05$) were seen between swarming and siderophore production, between swarming and fungal inhibition (all fungi except for *M. hiemalis*, *T. harzianum* and *F. oxysporum*), between swarming and mycophagy (for *R. solani* only), between weathering and siderophore production, between weathering and mycophagy (for *M. hiemalis* only), between soil pH and mycophagy (for *R. solani* only) and between mycophagy (for *M. hiemalis* only) and purple pigmentation. Environments harboring significantly ($p \leq 0.05$) less inhibitory collimonads concerning all fungi except for *M. hiemalis*, *T. harzianum* and *F. oxysporum* were "dune grassland", unfertilized grassland" and "former agricultural field". The habitats "EMF soil" and "former agricultural field" also had significantly ($p \leq 0.05$) more motile bacteria, characterized by higher swarming abilities. Habitats also differed concerning their pH values, for details we refer to Table S4.2.

PHYLOGENETIC SIGNAL:

Phylogenetic signal calculations for concatenated and 16S rDNA phylogenies can be found in Table 4.2. Briefly, with the housekeeping gene phylogeny we found significant phylogenetic signal for purple pigmentation, *VioA*, *VioB*, collimomycin production, inhibition of all fungi except for *F. oxysporum* (and *M. hiemalis* and *T.harzianum* since those fungi were not inhibited at all), chitinase production, siderophore production, swarming intensity and swarming shape, habitat and soil pH. The phylogenetic signal for mycophagy on *M. hiemalis* was nearly significant ($p = 0.066$). For the 16S phylogeny, significant phylogenetic signal was found for purple pigmentation, *VioA*, *VioB*, collimomycin production, inhibition of all fungi except for *F. oxysporum* (and *M. hiemalis* and *T.harzianum,* see above), chitinase production, swarming intensity and swarming shape. Soil pH was nearly significant ($p = 0.073$). For Blomberg's K, Pagel's ƛ and Fritz' and Purvis' D values we refer to Table 4.2.

DISCUSSION:

Combining the constructed high-resolution phylogeny with the measurement of a set of functional traits, we found evidence for phylogenetically conserved trait divergence. As confirmed by measurement of significant phylogenetic signal, several traits potentially involved in interactions between collimonads and fungi, are not randomly distributed but tend to group with phylogeny (Table 4.2, Fig 4.1). These traits are the inhibition of a diverse set of fungi, namely *R. solani, P. ultimum, F. culmorum, A. niger* and *P. exigua*, the production of violacein and collimomycin, mycophagous growth on *M. hiemalis*, the production of chitinases, swarming ability and swarming shape.
The aforementioned traits do not (or to a lesser extent) carry phylogenetic signal when being examined on basis of a 16S rDNA phylogeny only (Fig 4.2).

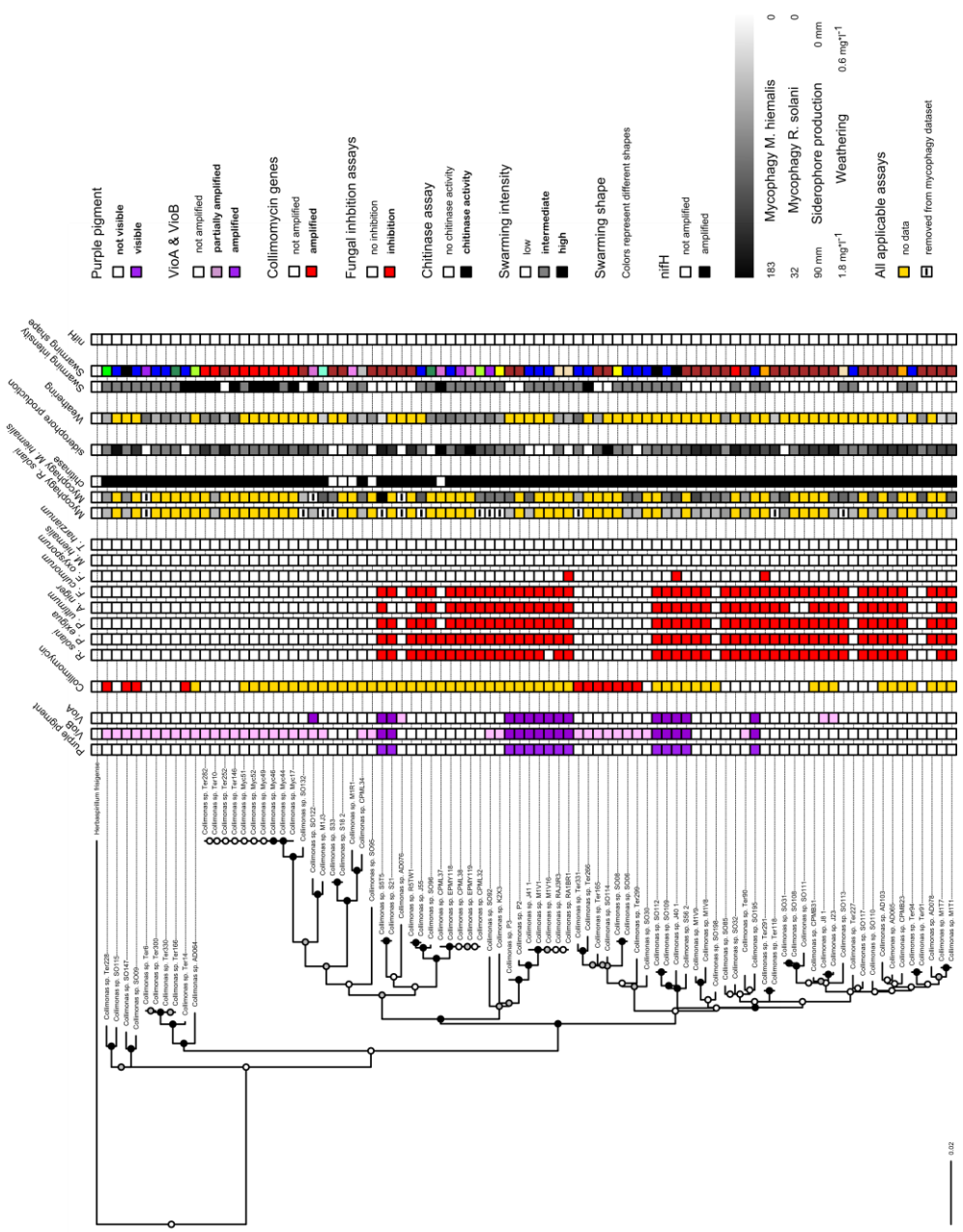

Purple pigment
not visible
visible
VioA & VioB
not amplified
partially amplified
amplified
Collimomycin genes
not amplified
amplified
Fungal inhibition assays
no inhibition
inhibition
Chitinase assay
no chitinase activity
chitinase activity
Swarming intensity
low
intermediate
high
Swarming shape
Colors represent different shapes
nifH
not amplified
amplified
183 Mycophagy M. hiemalis 0
32 Mycophagy R. solani 0
90 mm Siderophore production 0 mm
1.8 mg*g-1 Weathering 0.6 mg*g-1
All applicable assays
no data
removed from mycophagy dataset
Purple pigment
VioB
VioA
Collimomycin
R. solani
P. expansa
P. ultimum
A. rigor
F. oxysporum
M. hiemalis
T. harzianum
Mycophagy R. solani
Mycophagy M. hiemalis
Chitinase
siderophore production
Weathering
Swarming
Swarming intensity
Swarming shape
nifH
Herbaspirillum frisingense
0.02

Fig 4.1 (previous page) Overview on grouping of collected trait data with the concatenated housekeeping gene phylogeny (rpoB, gyrB, 16S). The tree was tested with 100 bootstraps, nodes with white circles represent bootstrap support values lower than 50, grey higher than 50 and black higher than 75.

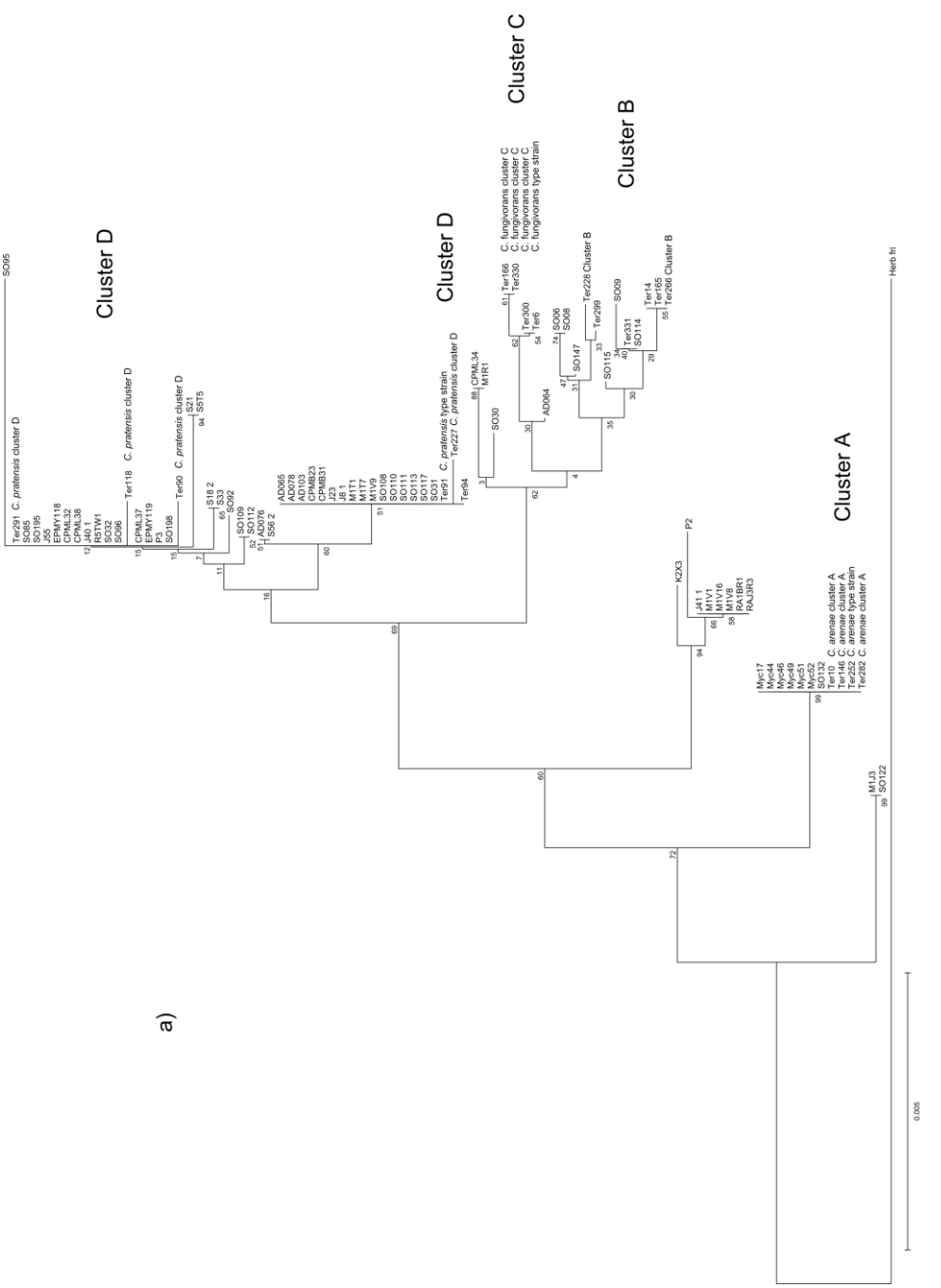
Table 4.2 Trait phylogenetic signal derived from a) a concatenated gene phylogeny (rpoB, gyrB, 16S) and b) 16S only phylogeny. Type of variable (continuous, discrete and binary) and respective phylogenetic signal measurements (Blombergs' K, Pagels λ and Fritz' and Purvis' D) as well as their significance is indicated.

a)

Trait category	Trait	Variable type	Bloombergs K	Pagels λ	Fritz & Purvis D	P-value
Violacein production						
	Purple pigment	binary			-0,27	< 0,0001
	VioB	discrete		1,00		< 0,0001
	VioA	discrete		1,00		< 0,0001
Collimomycin genes						
	Collimomycin	binary			-0,48	< 0,0001
Fungal inhibition						
	R. solani	binary			-0,21	< 0,0001
	P. ultimum	binary			-0,30	< 0,0001
	F. oxysporum	binary			1,02	0,5010
	F. culumorum	binary			-0,31	< 0,0001
	A. niger	binary			-0,06	< 0,0001
	P. exigua	binary			-0,32	< 0,0001
Mycophagy						
	M. hiemalis	continuous	0,55			0,0660
	R. solani	continuous	0,29			0,5140
Chitinase production						
	Chitinase	binary			0,13	0,0020
Siderophore production						
	Siderophores	continuous	0,03			0,0070
Weathering						
	Weathering	continuous	0,10			0,1560
Swarming abilities						
	Intensity	discrete		0,69		0,0001
	Shape	discrete		0,63		0,0020
Environmental data						
	Species	discrete		0,75		0,0009
	Habitat	discrete		0,98		< 0,0001
	Soil pH	continuous	0,06			0,0010

b)

Trait category	Trait	Variable type	Bloombergs K	Pagels λ	Fritz & Purvis D	P-value
Violacein production						
	Purple pigment	binary			0,39	< 0,0001
	VioB	discrete		0,82		< 0,0001
	VioA	discrete		0,85		< 0,0001
Collimomycin genes						
	Collimomycin	binary			-0,33	< 0,0001
Fungal inhibition						
	R. solani	binary			0,06	< 0,0001
	P. ultimum	binary			0,01	< 0,0001
	F. oxysporum	binary			1,24	0,7770
	F. culumorum	binary			0,00	< 0,0001
	A. niger	binary			0,25	< 0,0001
	P. exigua	binary			-0,06	< 0,0001
Mycophagy						
	M. hiemalis	continuous	0,12			0,1070
	R. solani	continuous	0,05			0,7890
Chitinase production						
	Chitinase	binary			0,28	0,0010
Siderophore production						
	Siderophores	continuous	0,00			0,1380
Weathering						
	Weathering	continuous	0,00			0,6610
Swarming abilities						
	Intensity	discrete		0,56		< 0,0001
	Shape	discrete		0,46		0,0029
Environmental data						
	Species	discrete		0,29		0,2578
	Habitat	discrete		0,25		1,0000
	Soil pH	continuous	0,00			0,0730


a)
Cluster D
Cluster D
Cluster C
Cluster B
Cluster A
C. pratensis cluster D
C. pratensis type strain
C. fungivorans cluster C
C. fungivorans type strain
Cluster B
C. arenae cluster A
C. arenae type strain
Herb fri
0.005

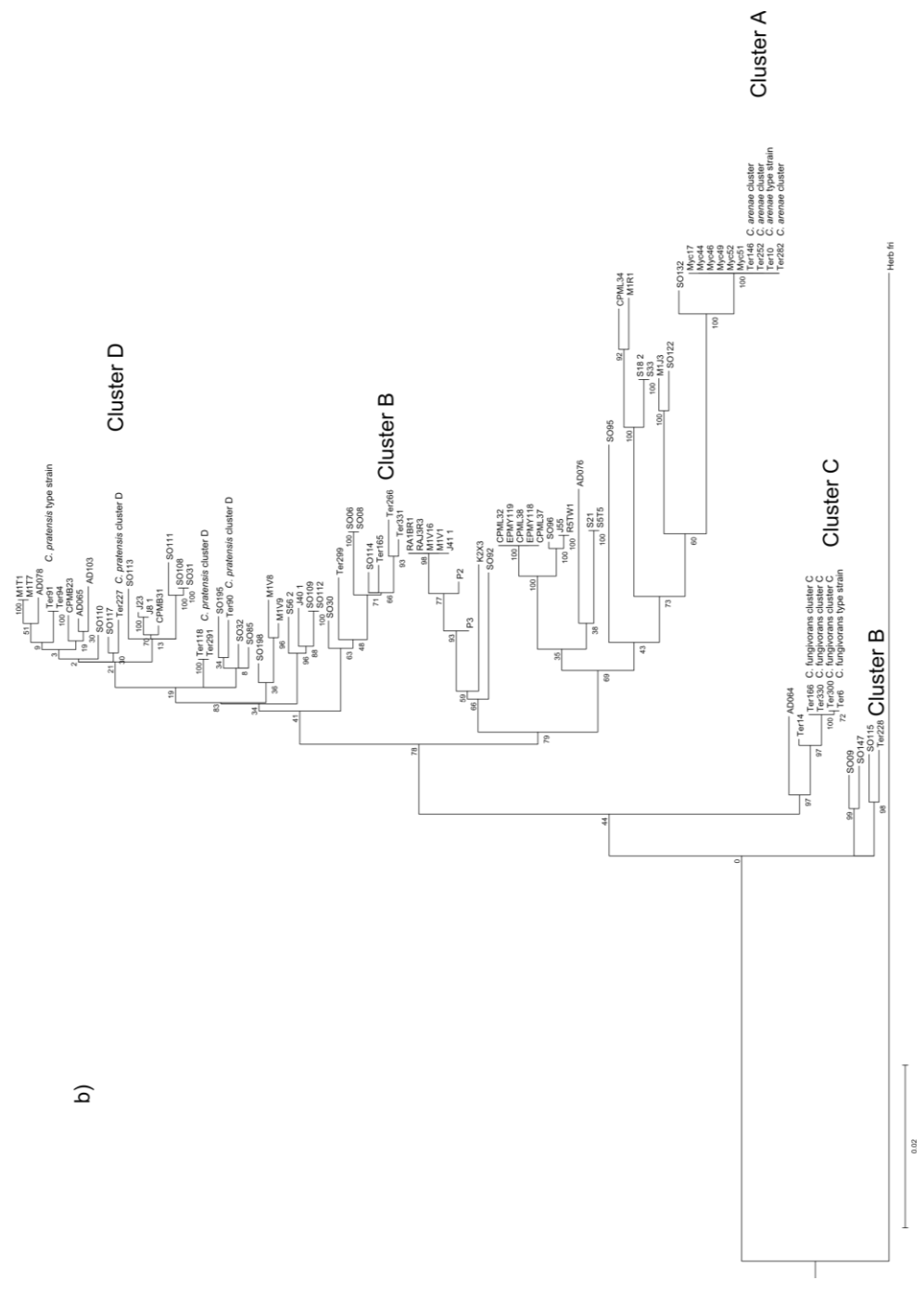

b)
Cluster D
Cluster B
Cluster A
Cluster C
Cluster B
C. pratensis type strain
C. pratensis cluster D
C. pratensis cluster D
C. pratensis cluster D
C. arenae cluster
C. arenae cluster
C. arenae type strain
C. arenae cluster
C. fungivorans cluster C
C. fungivorans cluster C
C. fungivorans cluster C
C. fungivorans type strain
Herb fri
0.02

Fig 4.2 (previous 2 pages) Comparison of concatenated gene (a)derived phylogeny (16S, *rpoB* and *gyrB*) and single gene derived (b) phylogeny (16S). Bacterial strain assignment to clusters as suggested by Hoppener-Ogawa et al (2008) is indicated. The trees were tested with 100 bootstraps and the respective support values are indicated at the tree nodes.

This indicates that with a lower taxonomic resolution, the phylogenetic signal gets "diluted" and it gets harder to assign specific traits to clusters of phylogenetically related bacteria. It has been shown that many genetically complex traits are phylogenetically not widely distributed but rather conserved and deeply rooted in the phylogenetic tree, indicating that many traits might be associated with very fine-scale diversity (Martiny *et al.* 2013).Compared to Martiny *et al.* (2013), spanning the whole kingdom of Bacteria (and also Archeae), we assessed very fine-level trait dispersal in the bacterial genus *Collimonas*. Our study confirms the suggestion by Martiny et al (2013) that fine-resolution trait dispersal and phylogeny are required to obtain detailed information on phylogenetic signal at fine taxonomic levels.
Despite the fact that we find related strains to possess similar traits (phylogenetic signal) we observe that the strength of the phylogenetic signal (dispersal over the phylogenetic tree) differs (Table 1, Fig 4.1). Traits like violacein and chitinase production are deeply rooted in the tree, whereas fungal inhibition is not. An explanation for this could be that although violacein has antifungal activity, its primary function is not that of a diffusible fungal inhibitor. It rather evolved at an evolutionary earlier time point, probably before the development of mycophagy and could have served in the inhibition of bacterivorous predators. Violacein is produced and stored intracellularly and for *Janthinobacterium* bacteria it has been shown to be toxic to bacterivorous nematodes, upon ingestion *(Hornung et al. 2013)*. Furthermore, the compound has not been proven to be actively used as an antifungal compound by collimonads. Indeed, our study revealed that many *Collimonas* strains that were not scored positive for violacein production had similar antifungal inhibition patterns as violacein-producing strains (Fig. 4.1). Hence, antifungal activity of violacein containing *Collimonas* bacteria in *in vitro* assays is probably due to other (diffusible) secondary metabolites. The same could hold for the acquisition of chitinase genes, which might have taken place at an evolutionary earlier time point, the original purpose being degradation of chitin, originating from various sources like invertebrate exoskeletons, decaying fungal remainders etc. (Bai *et al.* 2014). The closely related genus *Janthinobacterium* harbors very efficient degraders of chitin (Kielak *et al.* 2013). However, *Collimonas* bacteria are poor degraders of crystallized chitin. Therefore, they may have lost accompanying enzymes that are needed to degrade crystallized and cross-polymerized chitin and use their chitinases especially in attack of hyphal tips of fungi, where chitin polymers are in their native form and are most vulnerable for lytic enzymes (Leveau *et al.* 2010; Bai *et al.* 2014).

The observed differences in the depth of rooting and the dispersal of the traits on the phylogenetic tree (phylogenetic signal) do not indicate the existence of distinct *Collimonas* "ecotypes" that could possibly be grouped by functional traits only (Fig 4.1). A trait like fungal inhibition could have evolved several times in different *Collimonas* strains, followed by further investment in strain specific traits. Other traits like purple pigmentation for example could be relicts from the sister genus *Janthinobacterium* that provided benefits to some collimonads and were therefore partially maintained (how far those traits could still contribute to the formation of possible ecotypes is, however, unclear). It would be necessary to sequence violacein genes of known violacein producing *Collimonas* sister genera in order to trace the evolution of violacein

production in the *Oxalobacteraceae*. Interestingly, we did not find evidence for nitrogenase genes (*nifH*) in collimonads. As opposed to the results for violacein, these genes seem to have gotten lost upon adaptation to the mycophagous lifestyle.
The distribution of functional traits indicated potential trade-offs between mineral weathering and swarming as well as between fungal inhibition, mycophagy and siderophore production (Fig 4.3).

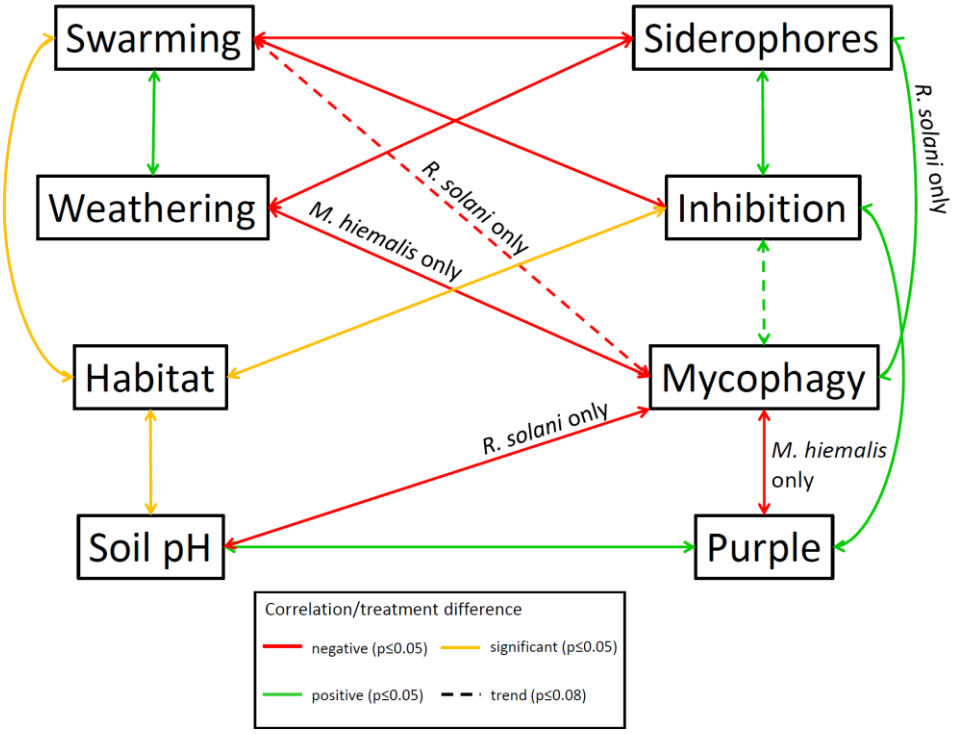


Fig 4.3 Graphical summary of trait correlations. Significant ($p \leq 0.05$) negative correlations are indicated in red, positive correlations in green, correlations close to significance ($p \leq 0.08$) are indicated with dashed lines. Significant differences between treatments of categorical variables are indicated in yellow. Indication of *R. solani* only and *M. hiemalis* only implies that the correlation of a trait with mycophagy is only for mycophagous growth of collimonads on the indicated fungus.

Indications for a possible trade-off between swarming and siderophore production has been shown before by Cheng *et al.* (2013). Here the authors could show that the *Pseudomonas fluorescens* SBW25 *gacS* mutant, impaired in swarming, produced a higher amount of siderophores than the corresponding wildtype and *vice versa*. Tremblay and Deziel (2010) show that actively swarming cells of *Pseudomonas aeruginosa* have generally down-regulated iron acquisition genes as compared to cells in the center of the colony that do not swarm. Also for collimonads, this trade-off would make sense. Siderophores are molecules which are most useful when being produced at high local concentration. Their production requires energy which would be optimally invested when combined with low bacterial movement.

We also found negative correlations (possible trade-offs) between swarming and mycophagy and swarming and fungal inhibition (Fig 4.3). Like for the production of siderophores, the production of antifungal compounds, a pre-requisite for both, would only pay off when they would be produced locally and in high concentrations. Collimonads may, however use motility in order to locate a potential host fungus and produce antifungal metabolites, once the host is located.

Our study also indicated biogeography effects, meaning that certain *Collimonas* genotypes are more prone to be found in certain habitat types than in others. Unfertilized grasslands, abandoned agricultural fields and dune grasslands appear to contain different collimonads than arctic habitats, forests, river dunes e.g. (Fig 4.3, Table S4.2). This differentiation is only linked with two traits, namely less motility in terms of swarming intensity and less fungal inhibition for strains isolated from "former agricultural fields". Therefore, it may indicate that collimonads interact with fungi in a different way or with other fungal hosts, depending on the habitat. We don't know, however, whether the presence or absence of fungal hosts, the competition with other bacteria or the abiotic conditions, traits and factors that we did not measure, further influence the distribution of collimonads. River dunes and dune grasslands are very similar habitats but yet seem to harbor different collimonads. We found differences between the soil pH of different *Collimonas* habitats (Fig 4.3). The dune grassland soil had a higher pH than the river dune grassland the difference was however only close to significant ($p = 0.07$). Thus we cannot exclude a possible pH effect on the distribution of *Collimonas* strains.

In this study, we used different methods to measure phylogenetic signal, depending on the nature of the trait. For Pagels Ʌ and Fritz' and Purvis' D, used for discrete and binary trait data, respectively, the measured values for phylogenetic signal were well in the range of values detected in other studies (Mouquet *et al.* 2012). While still being significant, Bloomberg's K values were below 1, by definition indicating less trait evolution than expected under Brownian motion. There is another study that reports the same extraordinarily low but significantly different Bloomberg's K values for bacteria. Here, the authors found that pH, an environmental parameter that is known to profoundly shape bacterial lifestyle (Lauber *et al.* 2008; Sharp *et al.* 2014), shows phylogenetic signal for methanotrophic bacteria in a similar range as we measured in our study. It remains to be seen if this tendency is common for the evolution of microbial traits.

It is important to note that the observed changes in trait investment represent static data, based on the presence and absence of genes or on the conduction of phenotypic assays under specific conditions. There are two reasons why the results of such studies should be carefully interpreted: a) It has been shown that traits can get lost upon cultivation. Eydallin *et al.* (2014) could for example show that *E. coli* strains experienced changes in morphotype, metabolism and fitness after a few days of cultivation, b) We did not assess the adaptive potential of *Collimonas* strains. It is, however, highly probable that the expression of bacterial functional bacterial traits depends on the abiotic and biotic context. In previous experiments with collimonads, it has been shown that movement and swarming is dependent on the availability of fungal signal compounds, having an inversely proportional effect on bacterial movement (*chapter two*).

One of the main drivers of the *Collimonas* grouping is the ability to inhibit fungi, to feed on them and to produce antimicrobial compounds like collimomycin. Production of antifungal compounds and inhibition of fungi is highly dependent on culture-conditions (*chapter two*, Fritsche, et al., 2014) and collimonads cultured in liquid have shown to express antifungal traits to a lesser extent compared to cultures on solid medium. We pre-cultured collimonads on solid, rather than liquid medium. This has been shown to trigger adaptations in *E.coli* and might explain why the fungal inhibition results for specific strains, e.g. Ter331, obtained in this study differ from previous ones (De Boer *et al.* 1998). The fact that we still found a positive relationship between fungal inhibition and mycophagy supports earlier suggestions that antifungal compounds are an important factor for the mycophagous growth of collimonads (Leveau *et al.* 2010). Yet, our study indicates that fungal inhibition carries phylogenetic signal, whereas mycophagy does not. Thus, suggesting that many different factors contribute to mycophagy, inhibitory compounds probably being only a part of such.

To summarize, our study shows that MLSA enables a more detailed description of phylogenetic relationships within the genus *Collimonas* than 16S rDNA sequences. Using this multi-gene, high-resolution phylogeny we show that a set of traits that are possibly involved in interaction of collimonads and their host fungi are phylogenetically conserved. We find indications for trade-offs for *Collimonas'* trait possession that might be a pre-requisite for the colonization of certain habitat

SUPPLEMENTARY MATERIAL:

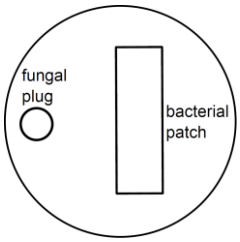


Fig S4.1 Experimental set-up for fungal inhibition assays

Supplementary Table S1: Overview on trait data, habitat data, species affiliation and reference. "Strain" indicates the name of the Collimonas isolate. Collimomycin genes (=Comy) (cluster K, see Fritsche et al 2014) were either not determined (0), not present (1) or present (2). Mineral weathering (free Fe released) was either quantified in mgL^{-1}, see Uroz et al 2009) or not determined (NA). Siderophores (=Sid) were quantified by a CAS-overlay assay (mm diameter of colony sourrounding halo), NA indicates not determined. Chitinase activity (=Chi) was quantified by halo formation on Chitin agar, no degradation (0) and degradation (1) are indicated. In fungal inhibition assays (RS=*R. solani, MH=M. hiemalis, PU=P. ultimum, FO=. oxysporum, FC=F. culmorum, AN=A.niger, PE=P. exigua* and TH=*T. harzianum*, "1" indicates inhibition of the respective fungus and "0" indicates no inhibition of the respective fungus. Purple = "1" indicates a purple pigmentation of the respective strain, purple = "0" no purple pigmentation. VioA/B = "0" means that the respective genes, VioA and VioB, could not be amplified (Hakvåg, et al. 2009), VioA/B = "1" means that fragments could be amplified but they do not have the right size, VioA/B = 2 means that right size fragments were amplified. Mycophagy ratios were determined for possible growth on *M. hiemalis* and *T. harzianum* (NA indicates no measurement and "0" indicates that the data has been deleted from the dataset because of too much variation between replicates. Soil pH indicates the pH of the soil from which the respective strain was isolated (NA = not determined). Country stands for the country of isolation ("1" = The Netherlands, "2" = France, "3" = Finland). Species is either not determined (0), *C. fungivorans* (1), *C. arenae* (2) or *C. pratensis* (3). Habitat indicates the habitat from which the strain was isolated, either being "no_data" = no data available, "emf_soil" = hypha of ectomycorrhizal fungus, "arctic_bulk" = arctic bulk soil, "arctic_forest" = arctic forest soil, "arctic_tundra" = arctic tundra soil, "endophyte" = the inside of a plant root, "former_agr_field" = abandoned agricultural field site, "beech_forest" = beech forest soil, "unfert_grassland" = unfertilized grassland soil, "coastal_outer_dunes" = coastal outer dune soil, "marsh" = marshland soil, "riverdune_grassland" = riverdune grassland soil, "heathland" = heathland soil, "dune_grassland"= dune grassland soil. Swarming behaviour was characterized in terms of intensity ("0" = no swarming, "1" = low, "2" = moderate, "3" = strong) and shape. Shapes were variable and descriptive names were given. Reference indicates the reference for the isolation of the respective strains.

					Fungal inhibition								*Violacein production*			*Mycophagy*						*swarming*		
Strain	Comy	Weathering	Sid	Chi	RS	MH	PU	FO	FC	AN	PE	TH	Purple	VioB	VioA	*M. hiemalis*	*R. solani*	Soil ph	Country	Species	Habitat	Intensity	Shape	Reference
AD064	0	NA	0,5	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,7	1	0	no_data	2	finger_edge_hands	De-Ridder Duine et al., 2005
AD065	0	NA	68,1	1	1	0	1	0	1	1	1	0	0	0	0	75,41	15,17	5,5	1	0	no_data	0	no_swarming	De-Ridder Duine et al., 2005
AD076	0	NA	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	5,1	1	0	no_data	0	no_swarming	De-Ridder Duine et al., 2005
AD078	0	NA	37,7	1	0	0	1	0	1	0	1	0	0	0	0	NA	NA	4,3	1	0	no_data	0	no_swarming	De-Ridder Duine et al., 2005
AD103	0	NA	66	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	1	0	no_data	0	no_swarming	De-Ridder Duine et al., 2005
CPMB23	0	0,8	64	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	2	0	emf_soil	1	runner	Uroz et al., 2007
CPMB31	0	NA	67,3	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	2	0	emf_soil	0	no_swarming	Uroz et al., 2007
CPML32	0	1,3	43,9	1	1	0	1	0	1	1	1	0	0	0	0	62,48	0	4	2	0	emf_soil	1	finger_edge_hands	Uroz et al., 2007
CPML34	0	1	34,8	1	0	0	0	0	0	0	0	0	0	1	0	79,62	8,92	4	2	0	emf_soil	1	diffuse	Uroz et al., 2007
CPML37	0	1,6	NA	0	1	0	0	0	0	0	1	0	0	0	0	NA	NA	4	2	0	emf_soil	3	full_plate	Uroz et al., 2007
CPML38	0	1,5	61,4	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	2	0	emf_soil	1	meteor	Uroz et al., 2007
EPMY118	0	1,7	3,1	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	2	0	emf_soil	1	finger_edge	Uroz et al., 2007
EPMY119	0	1,4	NA	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4	2	0	emf_soil	1	shell	Uroz et al., 2007
J23	0	NA	80	1	1	0	1	0	1	1	1	0	0	0	1	62,33	1,94	6,2	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
J40_1	0	NA	13	1	1	0	1	1	1	1	1	0	1	2	2	99,38	0,68	6,2	3	0	arctic_bulk	1	ring	Nissinen et al., 2012
J41_1	0	NA	0	1	1	0	1	0	1	1	1	0	1	2	2	83,69	0,68	6,2	3	0	arctic_bulk	1	finger_edge	Nissinen et al., 2012
J55	0	NA	14,6	1	1	0	1	0	1	1	1	0	0	0	0	93,43	0	6,2	3	0	arctic_bulk	1	finger_edge	Nissinen et al., 2012
J8_1	0	NA	60,2	1	1	0	1	0	1	1	1	0	0	0	1	NA	NA	6,2	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
K2X3	0	1,2	0,6	1	1	0	1	0	1	1	1	0	0	1	0	67,27	0	4,9	3	0	arctic_forest	1	diffuse_finger_edge	Männistö and Häggblom, 2006
M1J3	0	0,8	13,9	1	0	0	0	0	0	0	0	0	0	1	0	94,44	0	5,3	3	0	arctic_tundra	1	splash	Männistö and Häggblom, 2006
M1R1	0	1,3	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	NA	5,3	3	0	arctic_tundra	1	shell	Männistö and Häggblom, 2006
M1T1	0	0,9	55,4	1	1	0	1	0	1	1	1	0	0	0	0	89,65	1,56	5,3	3	0	arctic_tundra	0	no_swarming	Männistö and Häggblom, 2006
M1T7	0	0,7	52,9	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	5,3	3	0	arctic_tundra	0	no_swarming	Männistö and Häggblom, 2006
M1V1	0	NA	0	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	5,3	3	0	endophyte	1	finger_edge	Nissinen et al., 2012
M1V16	0	NA	0	1	0	0	1	0	1	1	1	0	1	2	2	NA	NA	5,3	3	0	endophyte	1	finger_edge	Nissinen et al., 2012
M1V8	0	NA	71,8	1	1	0	1	0	1	1	1	0	0	0	0	54,05	9,25	5,3	3	0	endophyte	0	no_swarming	Nissinen et al., 2012
M1V9	0	NA	77,1	1	1	0	1	0	1	1	1	0	0	0	0	121,91	14,62	5,3	3	0	endophyte	0	no_swarming	Nissinen et al., 2012
Myc17	0	NA	17,6	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	2	star	this study
Myc44	0	NA	51,2	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	1	star	this study
Myc46	0	NA	23,1	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	2	star	this study
Myc49	0	NA	1,7	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	2	star	this study

					Fungal inhibition								*Violacein production*			*Mycophagy*					*swarming*		
Strain	Comy	Weathering	Sid	Chi	RS	MH	PU	FO	FC	AN	PE	TH	Purple	VioB	VioA	*M. hiemalis*	*R. solani*	Soil ph	Country	Species	Habitat	Intensity	Shape	Reference
Myc51	0	NA	0,7	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	1	star	this study
Myc52	0	NA	13,8	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,6	1	0	former_agr_field	2	star	this study
P2	0	NA	2,5	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	NA	1	0	beech_forest	0	no_swarming	this study
P3	0	NA	2,9	1	1	0	1	0	1	1	1	0	1	2	2	70,18	3	NA	1	0	beech_forest	0	no_swarming	this study
R5TW1	0	NA	73,6	1	1	0	1	0	1	0	1	0	0	0	0	NA	NA	5	3	0	arctic_forest	0	no_swarming	Männistö and Häggblom, 2006
RA1BR1	0	1,1	1	1	1	0	1	1	1	1	1	0	1	2	2	NA	NA	5	3	0	arctic_forest	1	finger_edge_hands2	Männistö and Häggblom, 2006
RAJ3R3	0	1,2	0	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	5	3	0	arctic_forest	1	finger_edge_hands2	Männistö and Häggblom, 2006
S18_2	0	NA	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	NA	6,1	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
S1E1	0	1,3	60,1	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	5,3	3	0	arctic_tundra	0	no_swarming	Männistö and Häggblom, 2006
S21	0	NA	60,1	1	1	0	1	0	1	0	1	0	1	2	2	NA	NA	6,1	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
S33	0	NA	0	0	0	0	0	0	0	0	0	0	0	0	0	64,37	0	6,1	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
S56_2	0	NA	47,3	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	6,1	3	0	arctic_bulk	0	no_swarming	Nissinen et al., 2012
S5T5	0	0,6	80,4	1	1	0	1	0	1	1	1	0	1	2	2	182,64	0	5,3	3	0	arctic_tundra	0	no_swarming	Männistö and Häggblom, 2006
SO06	2	NA	0	1	0	0	0	0	0	0	0	0	0	1	0	79,48	5,83	5,3	1	0	unfert_grassland	1	finger_edge	Höppener-Ogawa et al., 2007
SO08	2	NA	0	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,3	1	0	unfert_grassland	1	diffuse_finger_edge	Höppener-Ogawa et al., 2007
SO09	2	NA	0	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,3	1	0	unfert_grassland	1	finger_edge	Höppener-Ogawa et al., 2007
SO108	1	NA	7,7	1	1	0	1	0	1	0	1	0	0	0	0	NA	NA	5,3	1	2	coastal_outer_dunes	0	no_swarming	Höppener-Ogawa et al., 2007
SO109	0	NA	3,9	1	1	0	1	0	1	1	1	0	1	2	2	61,9	1,27	5,3	1	0	coastal_outer_dunes	1	finger_edge	Höppener-Ogawa et al., 2007
SO110	1	NA	68,3	1	1	0	1	0	1	1	1	0	0	0	0	77,74	19,86	5,3	1	2	coastal_outer_dunes	0	no_swarming	Höppener-Ogawa et al., 2007
SO111	1	NA	7,2	1	1	0	1	0	1	0	1	0	0	0	0	NA	NA	5,3	1	2	coastal_outer_dunes	0	no_swarming	Höppener-Ogawa et al., 2007
SO112	0	NA	19,7	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	5,3	1	0	coastal_outer_dunes	1	ring	Höppener-Ogawa et al., 2007
SO113	1	NA	44,6	1	1	0	1	0	1	1	1	0	0	0	0	82,38	0	5,3	1	2	coastal_outer_dunes	1	ring2	Höppener-Ogawa et al., 2007
SO114	2	NA	56,3	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,1	1	0	former_agr_field	0	no_swarming	Höppener-Ogawa et al., 2007
SO115	1	NA	14,8	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	5,1	1	0	former_agr_field	1	finger_edge	Höppener-Ogawa et al., 2007
SO117	1	NA	0,8	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	5,1	1	2	former_agr_field	0	no_swarming	Höppener-Ogawa et al., 2007
SO122	0	NA	NA	1	0	0	0	0	0	0	0	0	0	1	2	0	1,99	5,8	1	0	unfert_grassland	3	full_plate	Höppener-Ogawa et al., 2007
SO132	0	NA	89,5	1	0	0	0	0	0	0	0	0	0	1	0	9,88	0	4,7	1	3	marsh	0	no_swarming	Höppener-Ogawa et al., 2007
SO147	2	NA	1,2	1	0	0	0	0	0	0	0	0	0	1	0	41,85	6,55	5,6	1	0	former_agr_field	1	ring	Höppener-Ogawa et al., 2007
SO195	1	NA	27,6	1	1	0	1	0	1	1	1	0	1	2	2	NA	NA	5,1	1	2	unfert_grassland	1	finger_edge	Höppener-Ogawa et al., 2007
SO198	0	NA	58,4	1	0	0	0	0	0	0	0	0	0	0	0	77,01	3,76	5,1	1	2	unfert_grassland	0	no_swarming	Höppener-Ogawa et al., 2007
SO30	1	NA	0,3	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	0	river_dune_grassland	1	finger_edge	Höppener-Ogawa et al., 2007

					Fungal inhibition								*Violacein production*			*Mycophagy*						*swarming*		
Strain	Comy	Weathering	Sid	Chi	RS	MH	PU	FO	FC	AN	PE	TH	Purple	VioB	VioA	*M. hiemalis*	*R. solani*	Soil ph	Country	Species	Habitat	Intensity	Shape	Reference
SO31	1	NA	75,2	1	1	0	1	0	1	1	1	0	0	0	0	94,01	32,28	5,3	1	2	coastal_outer_dunes	0	no_swarming	Höppener-Ogawa et al., 2007
SO32	1	NA	64,8	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	5,3	1	2	coastal_outer_dunes	1	star	Höppener-Ogawa et al., 2007
SO85	1	1,3	80,6	1	1	0	1	0	1	1	1	0	0	0	0	50,13	30,25	4,1	1	2	river_dune_grassland	0	no_swarming	Höppener-Ogawa et al., 2007
SO92	0	1,6	35,8	1	1	0	1	0	1	1	1	0	0	1	0	73,67	0	4,9	1	0	heathland	1	meteor	Höppener-Ogawa et al., 2007
SO95	0	1,4	0	0	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	0	heathland	0	no_swarming	Höppener-Ogawa et al., 2007
SO96	0	1,5	0	1	1	0	1	0	1	1	1	0	0	0	0	NA	NA	4,9	1	0	heathland	1	coliflower	Höppener-Ogawa et al., 2007
Ter10	1	1,7	13	1	0	0	0	0	0	0	0	0	0	1	0	26,44	1,39	6,1	1	3	dune_grassland	2	star	De Boer et al., 2004
Ter113	0	NA	58,9	1	1	0	1	1	1	1	1	0	0	0	0	NA	NA	6,1	1	2	dune_grassland	0	no_swarming	De Boer et al., 2004
Ter118	1	0,9	63,4	1	1	0	1	0	1	1	1	0	0	0	0	47,66	0	6,1	1	2	dune_grassland	0	no_swarming	De Boer et al., 2004
Ter14	2	1,2	0	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	0	coastal_outer_dunes	2	finger_edge	De Boer et al., 2004
Ter146	1	1,8	0,7	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	6,1	1	3	dune_grassland	2	star	De Boer et al., 2004
Ter165	2	1,2	24,7	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	0	coastal_outer_dunes	0	no_swarming	De Boer et al., 2004
Ter166	1	1,3	19,1	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	1	dune_grassland	1	coliflower	De Boer et al., 2004
Ter227	1	NA	7,3	1	0	0	0	0	0	0	0	0	0	0	0	52,89	5,66	4,9	1	2	dune_grassland	1	finger_edge	De Boer et al., 2004
Ter228	2	1,1	0,9	1	0	0	0	0	0	0	0	0	0	1	0	33,56	4,99	4,9	1	0	dune_grassland	1	inside_finger	De Boer et al., 2004
Ter252	1	1	88,8	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	6,1	1	3	dune_grassland	0	no_swarming	De Boer et al., 2004
Ter266	2	NA	0	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	0	dune_grassland	2	finger_edge	De Boer et al., 2004
Ter282	1	1,5	66,9	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	6,1	1	3	dune_grassland	2	star	De Boer et al., 2004
Ter291	1	NA	44,6	1	1	0	1	1	1	1	1	0	0	0	0	NA	NA	6,1	1	2	dune_grassland	1	runner	De Boer et al., 2004
Ter299	2	NA	0	1	0	0	0	0	0	0	0	0	0	1	0	71,78	4,31	4,9	1	0	coastal_outer_dunes	1	finger_edge	De Boer et al., 2004
Ter300	1	1,1	0,5	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	1	dune_grassland	1	finger_edge	De Boer et al., 2004
Ter330	1	1,5	0,4	1	0	0	0	0	0	0	0	0	0	1	0	NA	NA	4,9	1	1	dune_grassland	1	finger_edge	De Boer et al., 2004
Ter331	2	1,8	0,4	1	0	0	0	0	0	0	0	0	0	1	0	21,5	0	4,9	1	0	coastal_outer_dunes	1	finger_edge	De Boer et al., 2004
Ter6	1	1,7	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	4,9	1	1	dune_grassland	1	meteor	De Boer et al., 2004
Ter72	0	NA	6,2	1	1	0	1	1	1	1	1	0	0	0	0	NA	NA	6,1	1	0	coastal_outer_dunes	0	no_swarming	De Boer et al., 2004
Ter90	1	1,4	62	1	1	0	1	0	1	1	1	0	0	1	0	34,44	25,23	4,9	1	2	dune_grassland	0	no_swarming	De Boer et al., 2004
Ter91	1	1,4	8,2	1	0	0	0	0	0	0	0	0	0	0	0	92,26	5,49	4,9	1	2	dune_grassland	0	no_swarming	De Boer et al., 2004
Ter94	0	NA	59,8	1	0	0	0	0	0	0	0	0	0	0	0	NA	NA	4,9	1	0	coastal_outer_dunes	1	finger_edge	De Boer et al., 2004

Supplementary Table S2: Abbreveations: sqrt = sqare root transformation of the response variable, df = degrees of freedom, p = p-value, F = F-value, t= t-value, leveneTest= result of Levenes Test, sum sq = sum of squares, means sq = mean sum of squares, diff indicates the difference in means in Tukeys HSD, lwr and upr confidence intervals in Tukeys HSD test, z= z-value, muc_ratio = Mycophagy ratio *M. hiemalis*, rhiz_ratio = Mycophagy ratio *R. solani*. Fungal inhibition abbreviations: Pult = *P. ultimum*, Anig = *A. niger*, Rsol = *R. solani*, Mhie = *M. hiemalis*, Foxy = *F. oxysporum*, Fcul = *F. culmorum*, Phoma = *P. exigua*, Thar = *T. harzianum*. collimomycin = collimomycin genes (cluster K), weathering = freed iron through mineral weathering, siderophores = siderophore production on CAS medium, swarming = swarming intensity, purple = purple pigmentation, Habitat describes the habitat of isolation and is either "no_data" = no data available, "emf_soil" = hypha of ectomycorrhizal fungus, "arctic_bulk" = arctic bulk soil, "arctic_forest" = arctic forest soil, "arctic_tundra" = arctic tundra soil, "endophyte" = the inside of a plant root, "former_agr_field" = abandonned agricultural field site, "beech_forest" = beech forest soil, "unfert_grassland" = unfertilized grassland soil, "coastal_outer_dunes" = coastal outer dune soil, "marsh" = marshland soil, "riverdune_grassland" = riverdune grassland soil, "heathland" = heathland soil or "dune_grassland"= dune grassland soil. Correlations between traits were either tested with a) linear models, b) Pearsons correlation, c) t-test d) Pearsons chi-squared test e) Analysis of Variance, followed by posthoc tests for treatment level differences (Tukeys Honest SignificantDifference (HSD)) or f) logistic regression models

a)

model	explanatory	response	resp transf	p	R^2	df	F
linear	weathering	muc_ratio	sqrt	0,01	0,45	12	11,66
linear	weathering	rhiz_ratio	sqrt	0,85	-0,19	5	0,04
linear	soil ph	rhiz_ratio	sqrt	0,01	0,25	20	4,46
linear	soil ph	muc_ratio	sqrt	0,38	-0,01	30	0,04
linear	soil ph	siderophores	none	0,66	0,00	83	0,20

b)

model	explanatory	response	p	t	df
pearson	soil ph	weathering	0,63	-0,48	33
pearson	siderophores	rhiz_ratio	0,00	3,75	20
pearson	siderophores	muc_ratio	0,37	0,91	31
pearson	rhiz_ratio	muc_ratio	0,98	0,03	20
pearson	weathering	siderophores	0,01	-2,64	31

c)

model	explanatory	response	leveneTest	p	df	t
t-test	weathering	purple		0,72	0,07	33	1,87
t-test	siderophores	purple		0,11	0,17	86	1,37
t-test	weathering	Pult		0,89	0,15	33	1,49
t-test	weathering	Anig		0,89	0,15	33	1,49
t-test	weathering	Rsol		0,83	0,27	33	1,12
t-test	weathering	Mhie		-	-	-	-
t-test	weathering	Foxy		0,21	0,57	33	0,58
t-test	weathering	Fcul		0,89	0,15	33	1,49
t-test	weathering	Phoma		0,83	0,27	33	1,12
t-test	weathering	Thar		-	-	-	-
t-test	muc_ratio	Pult		0,70	0,05	31,00	-2,02
t-test	muc_ratio	Anig		0,70	0,05	31,00	-2,02
t-test	muc_ratio	Fcul		0,70	0,05	31,00	-2,02
t-test	muc_ratio	Foxy		0,34	0,37	31,00	-0,92
t-test	muc_ratio	Mhie	-	-	-	-
t-test	muc_ratio	Phoma		0,70	0,05	31,00	-2,02
t-test	muc_ratio	Rsol		0,70	0,05	31,00	-2,02
t-test	muc_ratio	Thar	-	-	-	-
t-test	rhiz_ratio	Anig		0,00	0,05	13,06	-2,12
t-test	rhiz_ratio	Fcul		0,00	0,05	13,06	-2,12
t-test	rhiz_ratio	Foxy		0,43	0,41	21,00	0,88
t-test	rhiz_ratio	Mhie	-	-	-	-
t-test	rhiz_ratio	Phoma		0,00	0,05	13,06	-2,12
t-test	rhiz_ratio	Pult		0,00	0,05	13,06	-2,12
t-test	rhiz_ratio	Rsol		0,00	0,05	13,06	-2,12
t-test	rhiz_ratio	Thar	-	-	-	-
t-test	rhiz_ratio	purple		0,16	0,08	21,00	1,82
t-test	muc_ratio	purple		0,35	0,02	31,00	-2,36
t-test	soil_ph	purple		0,27	0,08	86	-1,79

d)

model	explanatory	response	χ^2	p	df
Pearson χ^2	Pult	swarming		18,54	0,00	3
Pearson χ^2	Anig	swarming		12,74	0,01	3
Pearson χ^2	Fcul	swarming		18,54	0,00	3
Pearson χ^2	Foxy	swarming		0,96	0,81	3
Pearson χ^2	Mhie	swarming	-	-	3
Pearson χ^2	Phoma	swarming		16,32	0,00	3
Pearson χ^2	Rsol	swarming		15,10	0,00	3
Pearson χ^2	Thar	swarming	-	-	3
Pearson χ^2	Pult	purple		12,67	0,00	1
Pearson χ^2	Anig	purple		11,73	0,00	1
Pearson χ^2	Fcul	purple		12,67	0,00	1
Pearson χ^2	Foxy	purple		0,87	0,35	1
Pearson χ^2	Mhie	purple	-	-	1
Pearson χ^2	Phoma	purple		12,07	0,00	1
Pearson χ^2	Rsol	purple		9,39	0,00	1
Pearson χ^2	Thar	purple	-	-	1

e)

Analysis of Variance Table siderophores vs swarming

	df	sum sq	mean sq	f value	p value
swarming	2	18900	9450,10	13,55	0,00
Residuals	85	59284	697,50		

TukeyHSD

	diff	lwr	upr	p
slow - no	-29,06	-43,33	-14,78	0,00
moderate - no	-31,52	-53,91	-9,13	0,00
moderate - slow	-2,47	-24,74	19,81	0,96

Analysis of Variance Table sqrt_rhiz_ratio vs swarming

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
swarming	3,00	13,96	4,65	2,62	0,08
Residuals	19,00	33,71	1,77		

TukeyHSD

	diff	lwr	upr	p
slow - no	-1,40	-3,06	0,25	0,11
moderate - no	-2,16	-6,06	1,74	0,42
strong - no	-1,93	-5,83	1,97	0,52
moderate - slow	-0,76	-4,71	3,19	0,95
strong - slow	-0,53	-4,47	3,42	0,98
strong - moderate	0,23	-5,07	5,53	1,00

Analysis of Variance Table sqrt_muc_ratio vs swarming

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
swarming	2,00	9,94	4,97	1,35	0,27
Residuals	30,00	110,35	3,68		

Analysis of Variance Table weathering vs swarming

	df	sum sq	mean sq	f value	p value
swarming	3	0,77	0,26	3,07	0,04
Residuals	31	2,59	0,08		

TukeyHSD

	diff	lwr	upr	p
slow - no	0,21	-0,09	0,51	0,24
moderate - no	0,45	-0,01	0,91	0,06
strong - no	0,50	-0,32	1,32	0,36
moderate - slow	0,24	-0,19	0,67	0,45
strong - slow	0,29	-0,52	1,09	0,76
strong - moderate	0,05	-0,83	0,93	1,00

Analysis of Variance Table soil ph vs swarming

	df	sum sq	mean sq	f value	p value
swarming	3	3,05	1,02	2,78	0,05
Residuals	84	30,78	0,37		

TukeyHSD

	diff	lwr	upr	p
slow - no	-0,24	-0,61	0,12	0,31
moderate - no	0,31	-0,26	0,88	0,48
strong - no	-0,41	-1,56	0,74	0,79
moderate - slow	0,55	-0,01	1,11	0,05
strong - slow	-0,17	-1,31	0,98	0,98
strong - moderate	-0,72	-1,95	0,51	0,42

Analysis of Variance Table soil ph vs Habitat name

	df	sum sq	mean sq	f value	p value
Habitat name	12	23,41	1,95	14,04	0,00
Residuals	75	10,42	0,14		

TukeyHSD

	diff	lwr	upr	p
arctic forest - arctic bulk	-1,18	-1,95	-0,41	0,00
arctic tundra - arctic bulk	-0,86	-1,53	-0,18	0,00
coastal outer dunes - arctic bulk	-0,94	-1,49	-0,40	0,00
dune grassland - arctic bulk	-0,73	-1,26	-0,20	0,00
emf soil - arctic bulk	-2,16	-2,78	-1,54	0,00
endophyte - arctic bulk	-0,86	-1,62	-0,09	0,02
former agr field - arctic bulk	-0,71	-1,29	-0,12	0,01
heathland - arctic bulk	-1,26	-2,11	-0,41	0,00
marsh - arctic bulk	-1,46	-2,80	-0,11	0,02
no data - arctic bulk	-1,24	-1,95	-0,52	0,00
river dune grassland - arctic bulk	-1,66	-2,65	-0,66	0,00
unfert grassland - arctic bulk	-0,84	-1,51	-0,17	0,00
arctic tundra - arctic forest	0,33	-0,50	1,15	0,98
coastal outer dunes - arctic forest	0,24	-0,48	0,96	1,00
dune grassland - arctic forest	0,45	-0,26	1,16	0,62
emf soil - arctic forest	-0,98	-1,76	-0,19	0,00
endophyte - arctic forest	0,33	-0,58	1,23	0,99
former agr field - arctic forest	0,48	-0,28	1,23	0,63
heathland - arctic forest	-0,08	-1,05	0,90	1,00
marsh - arctic forest	-0,28	-1,70	1,15	1,00
no data - arctic forest	-0,06	-0,91	0,80	1,00
river dune grassland - arctic forest	-0,48	-1,58	0,63	0,96
unfert grassland - arctic forest	0,34	-0,48	1,16	0,97
coastal outer dunes - arctic tundra	-0,09	-0,71	0,54	1,00
dune grassland - arctic tundra	0,13	-0,49	0,74	1,00
emf soil - arctic tundra	-1,30	-1,99	-0,61	0,00
endophyte - arctic tundra	0,00	-0,82	0,82	1,00
former agr field - arctic tundra	0,15	-0,51	0,81	1,00
heathland - arctic tundra	-0,40	-1,30	0,50	0,95
marsh - arctic tundra	-0,60	-1,98	0,78	0,95
no data - arctic tundra	-0,38	-1,15	0,39	0,89
river dune grassland - arctic tundra	-0,80	-1,84	0,24	0,31
unfert grassland - arctic tundra	0,02	-0,72	0,75	1,00
dune grassland - coastal outer dunes	0,21	-0,26	0,68	0,94
emf soil - coastal outer dunes	-1,21	-1,78	-0,65	0,00
endophyte - coastal outer dunes	0,09	-0,64	0,81	1,00
former agr field - coastal outer dunes	0,24	-0,29	0,76	0,95
heathland - coastal outer dunes	-0,31	-1,13	0,50	0,98
marsh - coastal outer dunes	-0,51	-1,83	0,81	0,98
no data - coastal outer dunes	-0,29	-0,96	0,37	0,95
river dune grassland - coastal outer dunes	-0,71	-1,68	0,25	0,37
unfert grassland - coastal outer dunes	0,10	-0,52	0,72	1,00
emf soil - dune grassland	-1,43	-1,98	-0,87	0,00
endophyte - dune grassland	-0,13	-0,84	0,59	1,00
former agr field - dune grassland	0,03	-0,49	0,54	1,00
heathland - dune grassland	-0,53	-1,33	0,28	0,57
marsh - dune grassland	-0,73	-2,04	0,59	0,80
no data - dune grassland	-0,51	-1,16	0,15	0,30
river dune grassland - dune grassland	-0,93	-1,88	0,03	0,07
unfert grassland - dune grassland	-0,11	-0,72	0,50	1,00
endophyte - emf soil	1,30	0,52	2,08	0,00
former agr field - emf soil	1,45	0,85	2,05	0,00
heathland - emf soil	0,90	0,04	1,76	0,03
marsh - emf soil	0,70	-0,65	2,05	0,86
no data - emf soil	0,92	0,19	1,65	0,00
river dune grassland - emf soil	0,50	-0,51	1,51	0,89
unfert grassland - emf soil	1,32	0,63	2,01	0,00
former agr field - endophyte	0,15	-0,60	0,90	1,00
heathland - endophyte	-0,40	-1,37	0,57	0,97
marsh - endophyte	-0,60	-2,03	0,83	0,96
no data - endophyte	-0,38	-1,24	0,48	0,95
river dune grassland - endophyte	-0,80	-1,90	0,30	0,40
unfert grassland - endophyte	0,02	-0,81	0,84	1,00
heathland - former agr field	-0,55	-1,39	0,29	0,57
marsh - former agr field	-0,75	-2,09	0,59	0,78
no data - former agr field	-0,53	-1,23	0,17	0,33
river dune grassland - former agr field	-0,95	-1,94	0,04	0,07
unfert grassland - former agr field	-0,13	-0,79	0,53	1,00
marsh - heathland	-0,20	-1,67	1,27	1,00
no data - heathland	0,02	-0,91	0,95	1,00
river dune grassland - heathland	-0,40	-1,56	0,76	0,99
unfert grassland - heathland	0,42	-0,49	1,32	0,93
no data - marsh	0,22	-1,18	1,62	1,00
river dune grassland - marsh	-0,20	-1,76	1,36	1,00
unfert grassland - marsh	0,62	-0,76	1,99	0,94
river dune grassland - no data	-0,42	-1,49	0,65	0,98
unfert grassland - no data	0,40	-0,38	1,17	0,86
unfert grassland - river dune grassland	0,82	-0,22	1,86	0,28

Analysis of Variance Table weathering vs Habitat name

	df	sum sq	mean sq	f value	p value
Habitat name	6	1,06	0,18	2,14	0,08
Residuals	28	2,30	0,08		

TukeyHSD

	diff	lwr	upr	p
arctic tundra - arctic forest	-0,23	-0,88	0,41	0,91
coastal outer dunes - arctic forest	0,23	-0,51	0,98	0,95
dune grassland - arctic forest	0,20	-0,39	0,79	0,93
emf soil - arctic forest	0,16	-0,47	0,79	0,98
heathland - arctic forest	0,33	-0,41	1,08	0,78
river dune grassland - arctic forest	0,13	-0,92	1,18	1,00
coastal outer dunes - arctic tundra	0,47	-0,18	1,11	0,28
dune grassland - arctic tundra	0,43	-0,02	0,89	0,07
emf soil - arctic tundra	0,40	-0,11	0,90	0,21
heathland - arctic tundra	0,57	-0,08	1,21	0,11
river dune grassland - arctic tundra	0,37	-0,62	1,35	0,89
dune grassland - coastal outer dunes	-0,03	-0,62	0,55	1,00
emf soil - coastal outer dunes	-0,07	-0,70	0,56	1,00
heathland - coastal outer dunes	0,10	-0,64	0,84	1,00
river dune grassland - coastal outer dunes	-0,10	-1,15	0,95	1,00
emf soil - dune grassland	-0,04	-0,47	0,39	1,00
heathland - dune grassland	0,13	-0,45	0,72	0,99
river dune grassland - dune grassland	-0,07	-1,01	0,88	1,00
heathland - emf soil	0,17	-0,46	0,80	0,97
river dune grassland - emf soil	-0,03	-1,00	0,94	1,00
river dune grassland - heathland	-0,20	-1,25	0,85	1,00

Analysis of Variance Table sqrt_rhiz_ratio vs Habitat name

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Habitat_name	10,00	25,29	2,53	1,36	0,30
Residuals	12,00	22,38	1,87		

Analysis of Variance Table sqrt_muc_ratio vs Habitat name

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Habitat_name	13,00	70,18	5,40	2,05	0,08
Residuals	19,00	50,10	2,64		

Tukey HSD

	diff	lwr	upr	p
arctic_forest-arctic_bulk	-0,73839532	-7,519693	6,0429019	1,00
arctic_tundra-arctic_bulk	2	-3	6	0,91
beech_forest-arctic_bulk	-1	-7	6,2184232	1,00
coastal_outer_dunes-arctic_bulk	-0,84588778	-4,594391	2,9026153	1,00
dune_grassland-arctic_bulk	-2,17605372	-5,924557	1,5724494	0,63
emf_soil-arctic_bulk	-0,52650783	-5,705809	4,6527935	1,00
endophyte-arctic_bulk	0,25635395	-4,922947	5,4356552	1,00
former_agr_field-arctic_bulk	-2,47106679	-9,252364	4,3102304	0,97
heathland-arctic_bulk	-0,35710141	-7,138399	6,4241958	1,00
marsh-arctic_bulk	-5,79697766	-12,578275	0,9843196	0,14
no_data-arctic_bulk	-0,25633125	-7,037628	6,524966	1,00
river_dune_grassland-arctic_bulk	-1,85997016	-8,641267	4,9213271	1,00
unfert_grassland-arctic_bulk	-0,09487948	-5,274181	5,0844218	1,00
arctic_tundra-arctic_forest	2,69844747	-4,449667	9,8465624	0,96
beech_forest-arctic_forest	0,17552135	-8,579096	8,9301384	1,00
coastal_outer_dunes-arctic_forest	-0,10749246	-6,793942	6,5789568	1,00
dune_grassland-arctic_forest	-1,4376584	-8,124108	5,2487908	1,00
emf_soil-arctic_forest	0,21188749	-7,369833	7,7936083	1,00
endophyte-arctic_forest	0,99474927	-6,586972	8,57647	1,00
former_agr_field-arctic_forest	-1,73267147	-10,487289	7,0219456	1,00
heathland-arctic_forest	0,38129391	-8,373323	9,135911	1,00
marsh-arctic_forest	-5,05858234	-13,813199	3,6960347	0,63
no_data-arctic_forest	0,48206407	-8,272553	9,2366811	1,00
river_dune_grassland-arctic_forest	-1,12157483	-9,876192	7,6330422	1,00
unfert_grassland-arctic_forest	0,64351584	-6,938205	8,2252366	1,00
beech_forest-arctic_tundra	-2,52292612	-9,671041	4,6251888	0,98
coastal_outer_dunes-arctic_tundra	-2,80593993	-7,183248	1,5713686	0,49
dune_grassland-arctic_tundra	-4,13610587	-8,513414	0,2412027	0,08
emf_soil-arctic_tundra	-2,48655998	-8,137641	3,164521	0,90
endophyte-arctic_tundra	-1,7036982	-7,354779	3,9473828	0,99
former_agr_field-arctic_tundra	-4,43111894	-11,579234	2,716996	0,53
heathland-arctic_tundra	-2,31715356	-9,465268	4,8309613	0,99
marsh-arctic_tundra	-7,75702981	-14,905145	-0,6089149	0,03
no_data-arctic_tundra	-2,2163834	-9,364498	4,9317315	0,99
river_dune_grassland-arctic_tundra	-3,8200223	-10,968137	3,3280926	0,73
unfert_grassland-arctic_tundra	-2,05493163	-7,706013	3,5961494	0,97
coastal_outer_dunes-beech_forest	-0,28301381	-6,969463	6,4034354	1,00
dune_grassland-beech_forest	-1,61317975	-8,299629	5,0732695	1,00
emf_soil-beech_forest	0,03636614	-7,545355	7,6180869	1,00
endophyte-beech_forest	0,81922792	-6,762493	8,4009487	1,00
former_agr_field-beech_forest	-1,90819282	-10,66281	6,8464242	1,00
heathland-beech_forest	0,20577256	-8,548845	8,9603896	1,00
marsh-beech_forest	-5,23410369	-13,988721	3,5205134	0,59
no_data-beech_forest	0,30654272	-8,448074	9,0611598	1,00
river_dune_grassland-beech_forest	-1,29709618	-10,051713	7,4575209	1,00
unfert_grassland-beech_forest	0,46799449	-7,113726	8,0497153	1,00
dune_grassland-coastal_outer_dunes	-1,33016594	-4,904223	2,2438915	0,97
emf_soil-coastal_outer_dunes	0,31937995	-4,735101	5,3738605	1,00
endophyte-coastal_outer_dunes	1,10224173	-3,952239	6,1567222	1,00
former_agr_field-coastal_outer_dunes	-1,62517901	-8,311628	5,0612702	1,00
heathland-coastal_outer_dunes	0,48878637	-6,197663	7,1752356	1,00
marsh-coastal_outer_dunes	-4,95108988	-11,637539	1,7353594	0,29
no_data-coastal_outer_dunes	0,58955653	-6,096893	7,2760058	1,00
river_dune_grassland-coastal_outer_dunes	-1,01408238	-7,700532	5,6723669	1,00
unfert_grassland-coastal_outer_dunes	0,7510083	-4,303472	5,8054888	1,00
emf_soil-dune_grassland	1,64954589	-3,404935	6,7040264	0,99
endophyte-dune_grassland	2,43240767	-2,622073	7,4868882	0,84
former_agr_field-dune_grassland	-0,29501307	-6,981462	6,3914362	1,00
heathland-dune_grassland	1,81895231	-4,867497	8,5054015	1,00
marsh-dune_grassland	-3,62092394	-10,307373	3,0655253	0,71
no_data-dune_grassland	1,91972247	-4,766727	8,6061717	1,00
river_dune_grassland-dune_grassland	0,31608357	-6,370366	7,0025328	1,00
unfert_grassland-dune_grassland	2,08117424	-2,973306	7,1356548	0,93
endophyte-emf_soil	0,78286178	-5,407587	6,9733109	1,00
former_agr_field-emf_soil	-1,94455896	-9,52628	5,6371618	1,00
heathland-emf_soil	0,16940642	-7,412314	7,7511272	1,00
marsh-emf_soil	-5,27046983	-12,852191	2,311251	0,37
no_data-emf_soil	0,27017658	-7,311544	7,8518974	1,00
river_dune_grassland-emf_soil	-1,33346233	-8,915183	6,2482585	1,00
unfert_grassland-emf_soil	0,43162835	-5,758821	6,6220774	1,00
former_agr_field-endophyte	-2,72742074	-10,309142	4,8543	0,97
heathland-endophyte	-0,61345536	-8,195176	6,9682654	1,00
marsh-endophyte	-6,0533316	-13,635052	1,5283892	0,20
no_data-endophyte	-0,5126852	-8,094406	7,0690356	1,00
river_dune_grassland-endophyte	-2,1163241	-9,698045	5,4653967	1,00
unfert_grassland-endophyte	-0,35123343	-6,541683	5,8392157	1,00
heathland-former_agr_field	2,11396538	-6,640652	10,8685824	1,00
marsh-former_agr_field	-3,32591087	-12,080528	5,4287062	0,96
no_data-former_agr_field	2,21473554	-6,539882	10,9693526	1,00
river_dune_grassland-former_agr_field	0,61109664	-8,14352	9,3657137	1,00

	diff	lwr	upr	p
unfert_grassland-former_agr_field	2,37618731	-5,205533	9,9579081	0,99
marsh-heathland	-5,43987625	-14,194493	3,3147408	0,53
no_data-heathland	0,10077016	-8,653847	8,8553872	1,00
river_dune_grassland-heathland	-1,50286874	-10,257486	7,2517483	1,00
unfert_grassland-heathland	0,26222193	-7,319499	7,8439427	1,00
no_data-marsh	5,54064641	-3,213971	14,2952635	0,51
river_dune_grassland-marsh	3,9370075	-4,81761	12,6916246	0,89
unfert_grassland-marsh	5,70209817	-1,879623	13,283819	0,27
river_dune_grassland-no_data	-1,6036389	-10,358256	7,1509782	1,00
unfert_grassland-no_data	0,16145177	-7,420269	7,7431725	1,00
unfert_grassland-river_dune_grassland	1,76509067	-5,81663	9,3468115	1,00

Analysis of Variance Table siderophores vs Habitat name

	df	sum sq	mean sq	f value	p value
Habitat name	13	11832	910,13	1,02	0,45
Residuals	74	66352	896,65		

Analysis of Variance Table siderophores vs Pult

	df	sum sq	mean sq	f value	p value
Pult	1	11349	11348,70	14,60	0,00
Residuals	86	66835	777,20		

Analysis of Variance Table siderophores vs Anig

	df	sum sq	mean sq	f value	p value
Anig	1	9410	9410,10	11,77	0,00
Residuals	86	68774	799,70		

Analysis of Variance Table siderophores vs Fcul

	df	sum sq	mean sq	f value	p value
Fcul	1	11349	11348,70	14,60	0,00
Residuals	86	66835	777,20		

Analysis of Variance Table siderophores vs Foxy

	df	sum sq	mean sq	f value	p value
Foxy	1	74	73,50	0,08	0,78
Residuals	86	78111	908,26		

Analysis of Variance Table siderophores vs Mhie

	df	sum sq	mean sq	f value	p value
Mhie	1	-	-	-	-
Residuals	86	-	-	-	-

Analysis of Variance Table siderophores vs Phoma

	df	sum sq	mean sq	f value	p value
Phoma	1	11349	11348,70	14,60	0,00
Residuals	86	66835	777,20		

Analysis of Variance Table siderophores vs Rsol

	df	sum sq	mean sq	f value	p value
Rsol	1	12190	12190,40	15,89	0,00
Residuals	86	65994	767,40		

Analysis of Variance Table siderophores vs Thar

	df	sum sq	mean sq	f value	p value
Thar	1	-	-	-	-
Residuals	86	-	-	-	-

f)

Logistic regression table siderophores vs swarming

	df	z	p
swarming no	1	1,01	0,31
swarming moderate	1	-0,01	0,99
swarming strong	1	0,00	1,00
Residuals	87		

Logistic regression table swarming vs habitat

	df	z	p
Habitat_namearctic_forest	1	1,32	0,19
Habitat_namearctic_tundra	1	0,00	1,00
Habitat_namebeech_forest	1	-0,01	0,99
Habitat_namecoastal_outer_dunes	1	1,10	0,27
Habitat_namedune_grassland	1	1,67	0,10
Habitat_nameemf_soil	1	2,06	0,04
Habitat_nameendophyte	1	0,57	0,57
Habitat_nameformer_agr_field	1	1,96	0,05
Habitat_nameheathland	1	0,98	0,33
Habitat_namemarsh	1	-0,01	0,99
Habitat_nameno_data	1	-0,70	0,48
Habitat_nameriver_dune_grassland	1	0,44	0,66
Habitat_nameunfert_grassland	1	1,77	0,08
Residuals	77		

Logistic regression table purple vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,19	0,85
Habitat_namearctic_tundra	1	-1,08	0,28
Habitat_namebeech_forest	1	0,00	1,00
Habitat_namecoastal_outer_dunes	1	-1,54	0,12
Habitat_namedune_grassland	1	-0,01	1,00
Habitat_nameemf_soil	1	0,00	1,00
Habitat_nameendophyte	1	0,19	0,85
Habitat_nameformer_agr_field	1	0,00	1,00
Habitat_nameheathland	1	0,00	1,00
Habitat_namemarsh	1	0,00	1,00
Habitat_nameno_data	1	0,00	1,00
Habitat_nameriver_dune_grassland	1	0,00	1,00
Habitat_nameunfert_grassland	1	-1,08	0,28
Residuals	77		

Logistic regression table pult vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,01	0,99
Habitat_namearctic_tundra	1	-0,47	0,64
Habitat_namebeech_forest	1	0,01	1,00
Habitat_namecoastal_outer_dunes	1	-0,68	0,50
Habitat_namedune_grassland	1	-2,38	0,02
Habitat_nameemf_soil	1	-0,14	0,89
Habitat_nameendophyte	1	0,01	0,99
Habitat_nameformer_agr_field	1	-2,61	0,01
Habitat_nameheathland	1	-0,38	0,70
Habitat_namemarsh	1	-0,01	1,00
Habitat_nameno_data	1	-1,10	0,27
Habitat_nameriver_dune_grassland	1	-0,77	0,44
Habitat_nameunfert_grassland	1	-2,11	0,04
Residuals	77		

Logistic regression table anig vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,30	0,76
Habitat_namearctic_tundra	1	0,00	1,00
Habitat_namebeech_forest	1	0,01	1,00
Habitat_namecoastal_outer_dunes	1	-0,78	0,43
Habitat_namedune_grassland	1	-1,96	0,05
Habitat_nameemf_soil	1	0,38	0,71
Habitat_nameendophyte	1	0,01	0,99
Habitat_nameformer_agr_field	1	-2,28	0,02
Habitat_nameheathland	1	0,00	1,00
Habitat_namemarsh	1	-0,01	1,00
Habitat_nameno_data	1	-1,24	0,22
Habitat_nameriver_dune_grassland	1	-0,44	0,66
Habitat_nameunfert_grassland	1	-1,77	0,08
Residuals	77		

Logistic regression table Fcul vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,01	0,99
Habitat_namearctic_tundra	1	-0,47	0,64
Habitat_namebeech_forest	1	0,01	1,00
Habitat_namecoastal_outer_dunes	1	-0,68	0,50
Habitat_namedune_grassland	1	-2,38	0,02
Habitat_nameemf_soil	1	-0,14	0,89
Habitat_nameendophyte	1	0,01	0,99
Habitat_nameformer_agr_field	1	-2,61	0,01
Habitat_nameheathland	1	-0,38	0,70
Habitat_namemarsh	1	-0,01	1,00
Habitat_nameno_data	1	-1,10	0,27
Habitat_nameriver_dune_grassland	1	-0,77	0,44
Habitat_nameunfert_grassland	1	-2,11	0,04
Residuals	77		

Logistic regression table Foxy vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,63	0,53
Habitat_namearctic_tundra	1	0,00	1,00
Habitat_namebeech_forest	1	0,00	1,00
Habitat_namecoastal_outer_dunes	1	-0,33	0,74
Habitat_namedune_grassland	1	0,10	0,92
Habitat_nameemf_soil	1	0,00	1,00
Habitat_nameendophyte	1	0,00	1,00
Habitat_nameformer_agr_field	1	0,00	1,00
Habitat_nameheathland	1	0,00	1,00
Habitat_namemarsh	1	0,00	1,00
Habitat_nameno_data	1	0,00	1,00
Habitat_nameriver_dune_grassland	1	0,00	1,00
Habitat_nameunfert_grassland	1	0,00	1,00
Residuals	77		

Logistic regression table Mhie vs habitat

	df	z	p
Habitat_namearctic_forest	1	-	-
Habitat_namearctic_tundra	1	-	-
Habitat_namebeech_forest	1	-	-
Habitat_namecoastal_outer_dunes	1	-	-
Habitat_namedune_grassland	1	-	-
Habitat_nameemf_soil	1	-	-
Habitat_nameendophyte	1	-	-
Habitat_nameformer_agr_field	1	-	-
Habitat_nameheathland	1	-	-
Habitat_namemarsh	1	-	-
Habitat_nameno_data	1	-	-
Habitat_nameriver_dune_grassland	1	-	-
Habitat_nameunfert_grassland	1	-	-
Residuals	77		

Logistic regression table Phoma vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,01	1,00
Habitat_namearctic_tundra	1	-0,47	0,64
Habitat_namebeech_forest	1	0,00	1,00
Habitat_namecoastal_outer_dunes	1	-0,68	0,50
Habitat_namedune_grassland	1	-2,38	0,02
Habitat_nameemf_soil	1	0,52	0,60
Habitat_nameendophyte	1	0,01	1,00
Habitat_nameformer_agr_field	1	-2,61	0,01
Habitat_nameheathland	1	-0,38	0,70
Habitat_namemarsh	1	0,00	1,00
Habitat_nameno_data	1	-1,10	0,27
Habitat_nameriver_dune_grassland	1	-0,77	0,44
Habitat_nameunfert_grassland	1	-2,11	0,04
Residuals	77		

Logistic regression table Rsol vs habitat

	df	z	p
Habitat_namearctic_forest	1	0,01	0,99
Habitat_namearctic_tundra	1	-0,47	0,64
Habitat_namebeech_forest	1	0,01	1,00
Habitat_namecoastal_outer_dunes	1	-0,68	0,50
Habitat_namedune_grassland	1	-2,38	0,02
Habitat_nameemf_soil	1	0,52	0,60
Habitat_nameendophyte	1	-0,11	0,91
Habitat_nameformer_agr_field	1	-2,61	0,01
Habitat_nameheathland	1	-0,38	0,70
Habitat_namemarsh	1	-0,01	1,00
Habitat_nameno_data	1	-1,65	0,10
Habitat_nameriver_dune_grassland	1	-0,77	0,44
Habitat_nameunfert_grassland	1	-2,11	0,04
Residuals	77		

Logistic regression table Thar vs habitat

	df	z	p
Habitat_namearctic_forest	1	-	-
Habitat_namearctic_tundra	1	-	-
Habitat_namebeech_forest	1	-	-
Habitat_namecoastal_outer_dunes	1	-	-
Habitat_namedune_grassland	1	-	-
Habitat_nameemf_soil	1	-	-
Habitat_nameendophyte	1	-	-
Habitat_nameformer_agr_field	1	-	-
Habitat_nameheathland	1	-	-
Habitat_namemarsh	1	-	-
Habitat_nameno_data	1	-	-
Habitat_nameriver_dune_grassland	1	-	-
Habitat_nameunfert_grassland	1	-	-
Residuals	77		

CHAPTER FIVE

A SAPROTROPHIC EXTENSION OF THE MYCORRHIZOSPHERE: MYCOPHAGOUS RHIZOBACTERIA RECOVERED FROM FUNGUS INCUBATION-BAITING ASSAYS

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ABSTRACT:

Fungi and bacteria are primary consumers of plant-derived organic compounds and therefore considered to be at the basis of soil food webs. Trophic interactions among these microorganisms could however induce shifts in food web energy flows. Given increasing evidence for a prominent role of saprotrophic fungi as primary consumers of root-derived carbon, we propose that fungus-derived carbon may be an important resource for rhizosphere bacteria. Two common saprotrophic, rhizosphere-inhabiting fungi, *Trichoderma harzianum* and *Mucor hiemalis,* were confronted in a microcosm system with bacterial communities extracted from two plant rhizospheres (*Carex arenaria* and *Festuca rubra*). We observed that the ability to adhere to and obtain nutrients from living saprotrophic fungal hyphae was widespread among rhizosphere bacterial species. Fungus rather than plant identity affected the composition of these mycophagous bacteria. Based on our results, we suggest that food web models should take the possibility of bacterial secondary consumption into account as this has important consequences for carbon fluxes with more carbon dioxide released by microbes and less microbial carbon available for the soil animal food web. Finally, we argue that consumption of fungal-derived nutrients by bacteria may affect ecosystem functions such as the natural control of pathogenic fungi.

INTRODUCTION:

The soil around plant roots (the rhizosphere) harbors an active and diverse community of microbiota. Plants release 20 to 50% of their photosynthetically obtained carbon into the soil via their roots either directly or via associations with mycorrhizal fungi (mycorrhizosphere) (Kuzyakov & Domanski 2000). Those rhizodeposits are composed of passively sloughed off root cells and actively released substances such as mucilage, volatiles, and exudates (Dennis *et al.* 2010). Rhizosphere microbial communities are structured by the amount and composition of rhizodeposition which is determined by plant species and - age, but also by biotic and abiotic soil characteristics (Berg & Smalla 2009). Rhizodeposits are degraded by the rhizosphere microbial community. Yet, bacteria are thought to be the main decomposers of simple soluble compounds, like root exudates, whereas fungi are assumed to be mainly degrading solid recalcitrant polymers (de Boer *et al.* 2006). However, it is becoming increasingly recognized that the role of fungi is not restricted to this "recalcitrant carbon" niche (van der Wal *et al.* 2013). Several studies indicate that saprotrophic fungi can form an abundant fraction of the active microorganisms in the rhizosphere that metabolize root exudates (Denef *et al.* 2007; Buee *et al.* 2009). In a recent stable isotope labeling study, Hannula and colleagues (2012) followed a pulse of labeled $^{13}CO_2$ through potato plants (*Solanum tuberosum*) and from the plant roots into the root associated microbiota. They showed that rhizosphere fungi belonging to the phylum *Ascomycota* were rapidly incorporating recently fixed plant carbon and indicated that this may be due to the penetration of plant roots by hyphae of saptrotrophic rhizosphere fungi. Indeed, it has been shown that not only the obligatory symbiotic arbuscular mycorrhizal fungi and plant-pathogenic fungi but also saprotrophic fungi such as *Trichoderma harzianum* are able to enter plant roots (Harman *et al.* 2004). Hence, for such fungi, the hyphae extending into the rhizosphere (external phase) can be supplied with nutrients by hyphae that have entered the plant root (internal phase). Another explanation for the rapid uptake of recently fixed plant

carbon by saprotrophic fungi is that their competitive ability for certain root exudates might under some circumstances be higher than that of bacteria. This is not immediately expected for the most common root exudates, namely sugars, organic acids and amino acids (Paterson *et al.* 2007). However, small aromatic compounds are increasingly released at older parts of the root and by older plants in general and these compounds have been shown to trigger fungal activity (Waldrop & Firestone 2004; Badri & Vivanco 2009).

Taken together, these studies suggest that several situations exist where saprotrophic fungi can dominate the degradation of carbon compounds in the rhizosphere. In such situations, successful rhizosphere bacteria may exploit other niches. A niche for bacteria that cannot directly access root exudates would be to feed on fungal resources (tissue or fungal exudates) and to meet their energy demands as a secondary consumer. The existence of bacteria that are able to feed on living fungal tissue or fungal exudates has been described (Leveau and Preston, 2008). This so-called mycophagous nutrition has been extensively studied for soil bacteria of the genus *Collimonas* which use a combination of antibiotics and enzymes to get access to organic nutrients present in living fungal hyphae (Leveau *et al.* 2010). According to definition proposed by Leveau and Preston (2010) only bacteria that are actively involved in getting access to fungal nutrients, e.g. by causing leakage of fungal membranes, are being considered as mycophagous. So far, the documentation on the occurrence of mycophagous nutrition in other soil bacteria is limited. Growth on fungal exudates of bacteria that are associated with ectomycorrhizal fungi has been indicated (Frey-Klett *et al.* 2007). However, since it is not clear if these bacteria have an effect on the exudate efflux they can only be considered as potential mycophagous. The possible importance of fungus-derived nutrition in rhizosphere bacterial communities is not known.

Given the aforementioned ability of saprotrophic fungi to rapidly incorporate root exudates we hypothesize that a substantial part of the bacteria that colonize the rhizosphere is able (i) to adhere to hyphae of saprotrophic fungi and (ii) to feed on fungal nutrients as the only source of carbon and energy.

Saprotrophic fungi inhabiting the rhizosphere are known for the three major phyla/divisions of terrestrial fungi, namely, the *Zygomycota*, the *Ascomycota* and the *Basidiomycota*. Different fungal species can also vary in cell wall composition (Bartnick 1968), and since physical attachment is needed for hyphal colonization, this could impose a selection on possible adhering bacteria. We, therefore, further hypothesize that (iii) phylogenetically different fungi are colonized by different bacterial communities.

We tested our hypotheses in an experimental microcosm system where we confronted the fungi *Trichoderma harzanium* (*Ascomycota*) and *Mucor hiemalis* (*Zygomycota*) with bacterial inocula extracted from the rhizosphere of two wild plants, namely the sedge *Carex arenaria* and the grass *Festuca rubra*. Both fungal species have been found to be dominant among saprotrophic fungi colonizing the rhizosphere of these two plants (De Rooij-Van der Goes *et al.* 1995). Bacterial community DNA was isolated from fungal hyphae and subjected to 454 pyrosequencing. Simultaneously, hyphae-adhering bacteria were isolated and further screened for their ability to obtain nutrients from fungal hyphae. We found that a substantial amount of rhizosphere bacteria were able to feed on fungal derived nutrients as their only source of energy and carbon.

MATERIAL AND METHODS:

PLANT SPECIES AND SOILS:

Samples were taken at an inland river dune in Bergharen, The Netherlands (51°10'N, 05°40'E),. The sampling area is characterized by slightly acidic sandy soils (pH 5.5) that are low in organic matter, colonized by early succession plant species. More detailed information on the location and soil characteristics is given by de Boer *et al.* (2008). *Carex arenaria* L. (sand sedge) and *Festuca rubra* L. (red fescue) plants were dug up to collect rhizosphere soil samples in December 2011. Both plant species were dominant early colonizers of the sand dunes. *C. arenaria* is non-mycorrhizal whereas *F. rubra* associates with arbuscular mycorrhizal fungi. We defined rhizosphere soil as the soil that was still adhering to roots after vigorous shaking.

PREPARATION OF BACTERIAL RHIZOSPHERE INOCULA:

Bacterial inocula from both rhizosphere soils were prepared using the following protocol: 1 g of soil was added to 10 ml of 10 mM MES (morpholineethanesulfonic acid) buffer (pH 5.5) containing 1 gL^{-1} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$ and was shaken for 90 min (200 rev min^{-1}, 20 °C). The soil suspensions were then subjected to sonification for 2 min at 47 kHz followed by another 30 min of shaking. Finally, the suspensions were passed through several different mesh size filters down to a 3 μm cellulose-acetate filter (Whatman Netherlands BV, Den Bosch, Netherlands), to have mainly bacteria. . The absence of fungi in these inocula was confirmed by plating 50 μl of the inoculum on PDA (Potato Dextrose Agar, 9.75 gL^{-1} ; Agar 3.75 gL^{-1}) containing the bacterial antibiotics oxy-tetracycline (50 mgL^{-1}) and streptomycine (100 mgL^{-1}). The cells in 4 ml of the filtered suspension (either *C. arenaria* or *F. rubra*) were spun down (3000rpm, 10min) and re-suspended in 100 μL liquid M-Medium (see below) without glucose.

FUNGAL HOSTS:

Two fungi, namely *Trichoderma harzianum* and *Mucor hiemalis*, were used as fungal host strains in the experiments. *Trichoderma harzianum* CECT 2413 was purchased from the Spanish type culture collection (CECT, University of Valencia, Spain) and *Mucor hiemalis* was originally isolated by De Rooij-Van der Goes *et al.* (1995) from coastal foredunes of the island Terschelling, the Netherlands.
The two fungal species are dominant members of the rhizosphere fungal communities of both plants (De Rooij-Van der Goes *et al.* 1995; de Boer *et al.* 2008). *Trichoderma harzianum* (phylum *Ascomycota)* is a saprotrophic fungus that is also able to feed on other fungi, a mode of feeding known as mycoparasitism or mycophagy, but it has also been reported to be able to penetrate the outer cell wall of living plant roots (Harman et al., 2004). *Mucor hiemalis* is a saprotrophic soil fungus of the phylum *Zygomycota,* common in the rhizosphere of plants growing in sandy dune soils (De Rooij-Van der Goes *et al.* 1995). In order to make sure that no bacterial contamination was introduced together with the fungi, both fungi were pre-cultured on PDA containing oxy-tetracycline (50 mgL^{-1}) and streptomycine (100 mgL^{-1}). Fungi were also checked for endophytic bacteria using PCR with Primers 27f and 1492r on fungal DNA extracts and found to be free of bacteria (see below for PCR conditions).

MICROCOSM SYSTEM WITH HOST FUNGI:

Microcosms were set up in two-compartment Petri dishes (Fig 5.1), similar to the Petri dish system established by (Scheublin *et al.* 2010). Fungi were inoculated in the first compartment on autoclaved, modified M-Medium (Becard & Fortin 1988) amended with 1% glucose and 0.3% phytagel (Sigma-Aldrich, St. Louis, MO, USA.). Phytagel is a very pure agar and resistant to enzymatic breakdown by many digestive enzymes (Sutherland & Kennedy 1996). Compared to the original medium, we replaced sucrose by glucose and the pH was adjusted to 5.5 before autoclaving. The medium in the second compartment was free of glucose. After the fungus crossed the barrier to the second compartment and colonized it halfway, a 10 cm^2 plug of phytagel was cut out near the growth zone of the fungus. This "incubation-slot" was filled with 4 ml liquid M-Medium without glucose and the liquid was left to be colonized by the fungus. The colonization of the petri dish, including the "incubation-slot", took 20 to 22 days. Next, the "incubation-slots" were inoculated with concentrated rhizosphere bacterial suspensions (100 µl/incubation slot). Microcosms were then incubated in triplicate at 20°C for 24 hours.

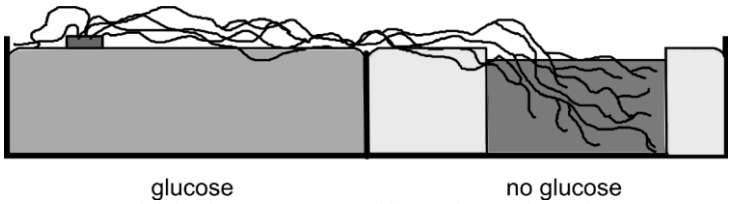


Fig 5.1 Two-compartment petri dish microcosm system. While growing on glucose in one compartment, the fungus colonizes the other compartment and finally the "incubation-slot" where rhizosphere bacteria are inoculated. The two compartments are separated by a plastic barrier in order to prevent glucose diffusion.

BACTERIAL SAMPLING:

After 24 hours of incubation the liquid in the "incubation-slots" and the hyphae that colonized the "incubation-slots" were sampled. A pipette was used to carefully collect 1 ml samples of the "incubation-slot" liquid avoiding the collection of hyphal material. Samples were centrifuged 5 min at 10.000 rpm (Sartorius Sigma Microcentrifuge 1-15P, Nieuwegein, the Netherlands) and the pellets were frozen at -20°C. Hyphae colonizing the "incubation-slot" were harvested with sterile, disposable inoculation loops and washed in 1 ml 10 mM MES buffer (pH 5.5) by vortexing for 5 sec before they were again transferred with sterile, disposable inoculation loops to new, sterile Eppendorf cups. The washing procedure of the hyphal fragments was done to avoid extraction of bacteria that were not firmly attached to the hyphae. With this step, we selected for bacteria that rapidly attached to the fungal hyphae, which we considered as an important strategy for feeding on fungal carbon. Hyphal samples were either frozen at -20 °C for later DNA extraction, or directly used for isolating attached bacteria.

DNA EXTRACTION:

DNA from frozen samples (hyphae with attached bacteria and bacterial pellets obtained by centrifuging "incubation-slot" liquid) was extracted following a protocol based on (Griffiths *et al.* 2000) with the following modifications: After CTAB addition, samples were incubated at 65

°C for 5 min and cell lysis was performed with bead beating, using 200 mg of 0.17 - 0.18 mm diameter glass beads for 15 sec at a speed of 4 m s^{-1} in a MP FastPrep-24 machine (M.P. Biomedicals, Noordwijkerhout, the Netherlands).

454 PYROSEQUENCING:

The V4 region of the small subunit of the bacterial ribosomal gene was amplified with the forward primer 515f (5'GCCTTGCCAGCCCGCTCAGGTGTGCCAGCMGCCGCGGTAA' 3) and the reverse primer 806r (5'GCCTCCCTCGCGCCATCAGGGGGACTACVSGGGTA TCTAAT'3)(Vos *et al.* 2012). The forward primer consisted of primer A from 454 Life Sciences followed by a 10 base sample specific barcode, the 2-base linker sequence GT, and the conserved bacterial primer sequence 515F. The reverse primer consisted of 454 Life Sciences primer B, a 10 base long sample specific barcode, the 2-base linker sequence GG, and the conserved bacterial primer 806R. Each of the 26 bacterial community samples (2 rhizosphere bacterial inocula (*C. arenaria, F. rubra*), 2 host fungi (*T. harzanium, M. hiemalis*), 2 bacterial community subsets (attached / suspended), 3 replicates plus 2 initial inocula) received a unique barcode sequence.
PCRs were carried out in 4 separate 25 µL reactions. Detailed description of cycling parameters and PCR reagents can be found in Vos *et al.* (2012). PCRs were finally pooled and cleaned with the Qiaquick PCR purification kit (Qiagen, Venlo, the Netherlands). Nucleic acids were then sequenced on a GS FLX Titanium 454 pyrosequencing platform (Macrogen, Amsterdam).

SEQUENCE ANALYSIS:

In the first step, the sequences and the corresponding quality information was extracted from the Standard Flowgram Format (SFF) files. This was done using the SFF converter tool in the Galaxy interface (Goecks *et al.* 2010). Sequences were de-multiplexed and the quality was controlled using several scripts of the QIIME pipeline version 1.6. First, sequences that had a maximum of 6 ambiguous bases, 6 homopolymer runs, zero mismatches with the Primer, passed a quality score window of 50 and a maximum of 1.5 errors in the barcode sequence were binned according to sample id and the barcode sequences were trimmed. In a second step, the sequence data were corrected for sequencing errors by using the DENOISER algorithm version 1.6.0 and chimeras were removed from the dataset using USEARCH (Edgar 2010). The sequences that passed the quality filtering were aligned using PyNAST (Caporaso *et al.* 2010a) and UCLUST (Edgar 2010) and assigned to Operational Taxonomical Units (OTUs), using a minimum sequence identity cutoff of 97%. From all sequences that clustered together as one OTU the most abundant one was selected as representative for taxonomy assignment. The taxonomy was assigned to the OTUs by comparing OTUs with sequences in the SILVA database (release 108 SSU) with a minimum identity cutoff of 75%. Finally, OTU tables were produced and an archeal outgroup sequence was added to the alignment of all OTUs to construct a Lowest Common Ancestor (LCA) rooted tree by using the fasttree program (Price *et al.* 2009). Statistical and graphical analyses were done using the package phyloseq (McMurdie & Holmes 2011) in the program R (R Development Core Team, 2013). Bacterial community compositions were analyzed using non-metric MultiDimensional Scaling (nMDS)on Bray-Curtis distances. The program "PAST" (Hammer *et al.* 2001) was used to test for differences in bacterial community composition between by non-parametric multivariate

analysis of variance (NPMANOVA), using 9999 permutations (Anderson 2001). In order to be able to compare relative abundances of OTUs, absolute abundance data were transformed into relative abundance data for the analyses. An exception was made for the calculation of the richness. Here, non-transformed, absolute abundance data were used since the Chao richness estimator has been shown to be sensitive to transformations (Chao & Shen 2003). Since a part of the ten most abundant OTUs could not be identified to the genus level using the aforementioned workflow, we further matched those against the rdp database (see below) in order to find the most related strain.

ISOLATION, CULTURING AND SANGER SEQUENCING:

Hyphal fragments collected from the "incubation-slots" were plated on 10% TSB agar containing fungal inhibitors (100 mgL^{-1} cycloheximide & 50 mgL^{-1} delvocide). Growing colonies were picked randomly and dilution streaked until contamination-free. Since it is known that mycophagous *Collimonas* strains inhibit growth of several fungi on nutrient-poor agar (Leveau *et al.* 2010), we conducted a growth inhibition assay on water-yeast agar as a first indication of mycophagy. For the fungus-inhibitory bacteria colony-PCR with the primers27f and 1492r (Weissburg et al 1991) were run with reagents & settings: 18.14 µl H_2O, 2.5 µl 10x PCR-buffer containing 2 mM $MgCl_2$ (Roche Scientific, Woerden, the Netherlands), 0.2 mM of each dNTP (Roche Scientific, Woerden, the Netherlands) and 0.4 µM of each primer, 1 U Fast Start High Fidelity Polymerase (Roche Scientific, Woerden, the Netherlands) and 1 µl template. Thermal cycling conditions consisted of a pre-denaturation step of 10 min at 95°C to break the cells open, an initial denaturation of 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 90 sec with a 1 sec increment per cycle and a final elongation step at 72°C for 10 min. PCR products were examined by a standard (1.5 %) agarose electrophoresis and cleaned with 20 % PEG8000 (Sigma-Aldrich). The gene encoding the small subunit of the bacterial ribosome (16S) was sequenced unidirectional, using primer 1492r at Macrogen (Amsterdam, the Netherlands), yielding sequences ≥700bp after quality filtering and trimming (DNA BASER, Heracle BioSoft). 'Preliminary taxonomic assignments were made via BLASTn, which were also used to decided which isolates to test on mycophagy. Afterwards, distance trees were calculated using Neighbor-Joining in the software MEGA version 5.2.1. Taxonomic assignments were made using Seqmatch (rdp classifier) (Wang *et al.* 2007) and alignment with the ClustalW algorithm and finally tested with 100 bootstraps and exported using the software MEGA 5.2.1. (Tamura *et al.* 2011). The results of the rdp assignment represent the closest matches in the database. Partial sequences of 16S ribosomal DNA of isolated bacteria have been deposited at Genbank under the accession numbers KJ396109 - KJ396179.

POTENTIAL MYCOPHAGY ASSAY:

The ability of the isolated, antagonistic bacteria to grow on a living fungus as the only organic energy source was tested in a Phytagel based microcosm system. This system was developed and tested using the known mycophagous bacterium *Collimonas fungivorans* Ter331 (see Fig S5.1 in Supporting Information). Bacteria were grown overnight in liquid 10 % TSB at 20 °C, washed twice in 10 mM MES buffer, and 100 µL of cell suspension (10^4 cells/ml) was spread on Petri dishes containing Phytagel. Subsequently plugs from the colony margin of *T.*

harzanium and *M. hiemalis*, pre-grown on PDA and were introduced in the middle of the Petri dish on sterilized plastic- and metal discs, respectively, in order to prevent nutrient leakage from the PDA plugs into the Phytagel. During the following 14 days of incubation at 20 °C the growing fungal hyphae encountered the bacterial isolates. Living fungal tissue or compounds exuded by the fungus represented the only source of carbon for bacterial growth. Microcosms without fungi were used to control for possible traces of metabolizable carbon, introduced with the phytagel. To harvest bacterial cells, the fungal plug and plastic/metal disc were removed; 2 ml 10 mM MES buffer was added & uniformly spread over the Petri dish. The microcosms were incubated for 30 min at room temperature without shaking. Next, the buffer was swirled, collected, and finally the OD of the suspension was measured at 600 nm. Mycophagy was quantified by dividing the average OD_{600} of each treatment by the average of the highest OD_{600} of the controls (either bacteria only or fungus only) to account for OD_{600} increases by hyphal fragments or bacterial growth on the Phytagel medium. Experiments were done in triplicates. Data of isolates that were taxonomically classified as being the same were pooled. Treatment and control OD were compared by a one tailed t-test, with either homogeneously or heterogeneously distributed variance (F-test) in Excel (Microsoft Corp.). Mycophagy ratios were plotted in Excel, imported in the program Inkscape (open source, http://www.inkscape.org) and mapped on the phylogenetic trees.

LINKING BACTERIAL ISOLATE IDENTITIES TO COMMUNITY MEMBERS:

The sequences of the bacterial isolate collection were searched by similarity with the 454 sequence reads to determine correspondences between the cultivated and uncultivated bacteria. The 16SrDNA data from the isolates (Sanger sequencing) was uploaded to the galaxy platform and treated as a database against which obtained 454 reads were compared (blastn algorithm). Matches between Sanger sequencing reads and OTUs could then be compared by similarity values.

RESULTS:

FUNGAL-ASSOCIATED BACTERIAL COMMUNITIES:

Pyrosequencing of the V4 region of the bacterial 16S ribosomal genes of the 26 bacterial community samples of "hyphae-adhering" bacteria, "liquid-phase" bacteria and rhizosphere bacterial inocula yielded 156.666 high-quality sequence reads(minimum sequence length 231bp, maximum sequence length 441bp, average sequence length 335bp) that could be classified to the kingdom of bacteria. Those sequences could be assigned to 425 OTUs at a cutoff level of 97%. Number of sequences varied between samples, with on average less sequences being generated from the hyphal samples than from the "incubation-slot" and inocula (Table S5.3 in Supporting Information). Pyrosequencing data have been deposited at ENA (European Nucleotide Archive) under accession number PRJEB5862.

OTU RICHNESS:

OTU richness of the rhizosphere inocula was highest, with on average 343 taxa, followed by 125 taxa for the non-attached bacterial communities in the "incubations-slots" and 73 taxa for

bacterial communities attached to hyphae. Bacterial communities originating from *C. arenaria* and *F. rubra* rhizospheres showed similar trends for both fungi with the "incubation-slot" communities always having a higher number of taxa than the respective attached communities (Fig S5.2 in Supporting Information). All mentioned differences are significant (t-test, $p<0.05$).

BACTERIAL COMMUNITY STRUCTURE:

Ordination analysis of the 454 pyrosequencing dataset revealed the clustering of communities in groups composed of bacteria associated with either *M. hiemalis* or *T. harzianum* (Fig 5.2). Those groups were significantly different from each other ($p < 0.0001$) and from the rhizosphere inocula ($p = 0.0121$ and $p = 0.009$, respectively), which formed a third group. For *T. harzianum* associated bacteria, two different subgroups ($p = 0.002$) became apparent, namely bacteria attached to hyphae and bacteria present in the "incubation-slot". This difference could not be observed for the other host fungus, *M. hiemalis*. Grouping of attached or "incubation-slot" fractions was not affected by the origin of the bacterial inoculum (*C. arenaria- versus F. rubra* rhizosphere).

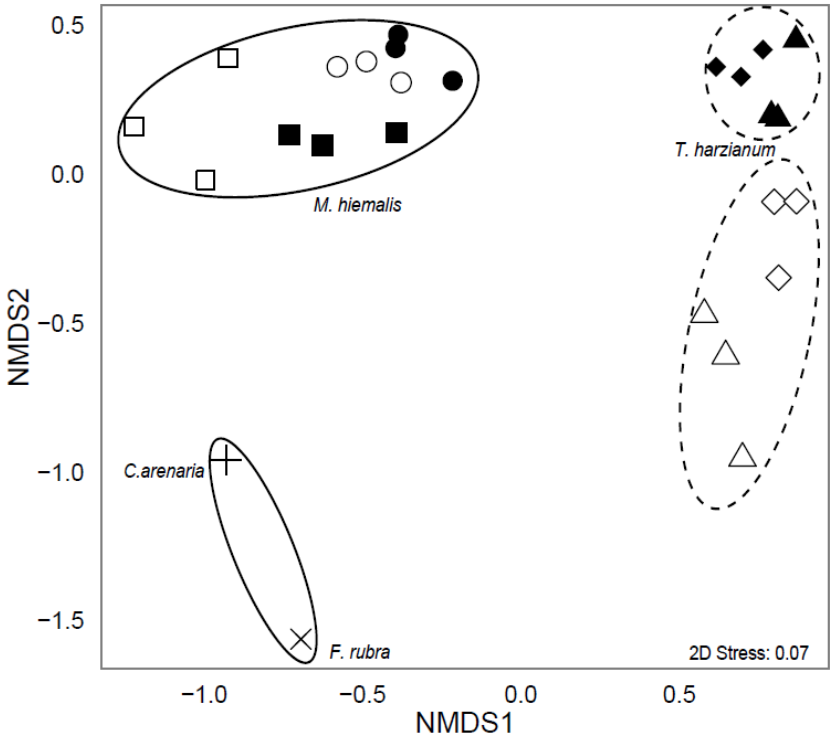


Fig 5.2 nMDS ordination of Bray-Curtis similarities of bacterial community compositions. Crosses = rhizosphere inoculum communities, open symbols = hyphae-attached communities, filled symbols = "incubation-slot" communities, triangles = host fungus *Trichoderma harzianum* with *Festuca rubra* inoculum, diamonds = host fungus *T. harzianum* with *Carex arenaria* inoculum, squares = host fungus *Mucor hiemalis* with *F. rubra* inoculum, circles = host fungus *M.hiemalis* with *C. arenaria* inoculum.

In general, the liquid phase as well as hyphal surfaces were colonized by a complex microbial community with a few dominant OTUs and many OTUs being present at relatively low abundances. Bacteria adhering to hyphae of *T. harzianum* were dominated (> 90 % of total OTUs) by four OTUs that had relatively high similarities with the following taxa: OTU 1(class: *β-Proteobacteria*), 8 (family: *Oxalobacteraceae*), 7 (genus: *Pseudomonas*), and 9 (class: *γ-Proteobacteria*). The hyphae of *M. hiemalis* were dominated by the OTUs 3 (class: *β-Proteobacteria*), 1 (class: *β-Proteobacteria*), 22 (class: *Actinobacteria*), 23 (family: *Spingomonadaceae*) which accounted for about 61% of all attaching OTUs (Table 5.1). Several OTUs significantly increased in relative abundance on fungal hyphae as compared to the "incubation-slot". In the case of *M. hiemalis*, these were OTU 22 (*Actinobacteria*), 23 (*a-Proteobacteria*), and 26 (*β-Proteobacteria*). For *T. harzianum* these were OTU 8 (*β-Proteobacteria*), 7 and 9 (both *γ-Proteobacteria*). These taxa seem to be very efficient hyphal colonizers. Although many hyphal-adhering OTUs were not present in high abundances, these rare hyphae associates clearly grouped with sample origin in a consistent way.

	Rdp database		
OTU	Closest match	Similarity	Accession nr.
3	*Burkholderia phenazinium (T)*	0.980	U96936
1	*Janthinobacterium sp.*	0.986	AB196254
22	*Streptacidiphilus neutrinimicus*	0.986	AF074410
23	*Sphingomonas sp.*	0.983	AB076396
12	*Dyella sp.*	0.983	HM748080
26	*Leptothrix sp.*	0.980	AB480710
162	*Burkholderia glathei*	0.983	AY154370
325	*Streptomyces aureofaciens*	0.986	Y15504
81	*Novosphingobium sp.*	0.959	JF958133
28	*Luteibacter rhizovicinus*	0.983	AJ580497
1	*Janthinobacterium sp.*	0.986	AB196254
8	*Zoogloea ramigera*	0.983	X74914
7	*Pseudomonas sp.*	0.986	AF511508
9	*Pseudomonas meliae*	0.990	AB021382
25	*Telluria mixta*	0.989	DQ005909
3	*Burkholderia phenazinium (T)*	0.980	U96936
15	*Buttiauxella sp.*	0.983	FJ587224
23	*Sphingomonas sp.*	0.983	AB076396
28	*Luteibacter rhizovicinus*	0.983	AJ580497
12	*Dyella sp.*	0.983	HM748080

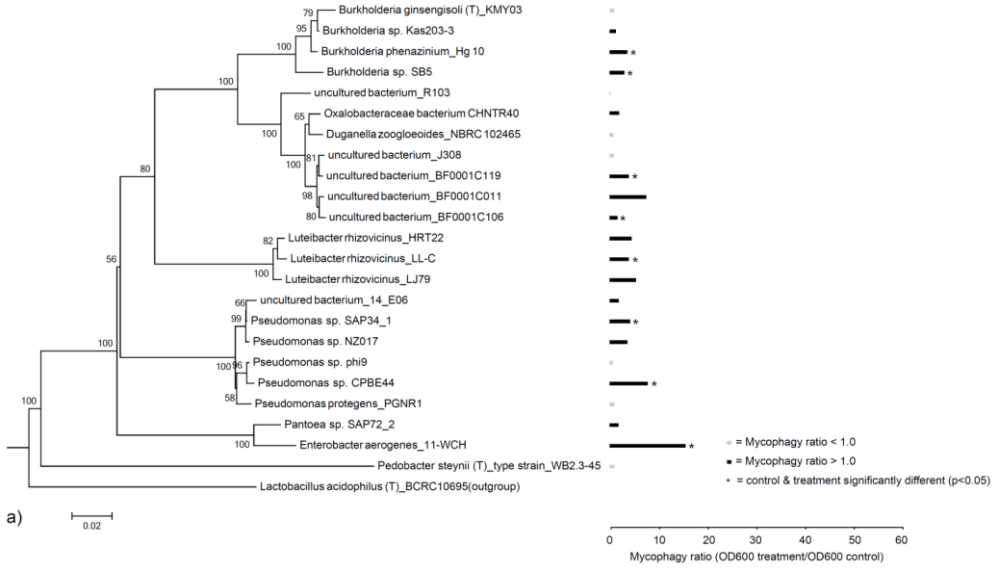
Table 5.1 Taxonomic classification of most abundant bacterial operational taxonomical units (OTUs) present in the "incubation slots" as hyphae-attached (A) and ,liquid-phase (L), and inoculum (I) bacteria. OTUs are ordered based on their relative abundance in the attached fraction for M. hiemalis (MH) and T. harzianum (TH), respectively. P-values as a result of a t-tests between relative abundances of fractions are shown. Bold values indicate signifiance at p-level ≤ 0.05. Stdev indicates the standard deviation of the mean. Closest matches of OTUs with the Rdp database are given, including similarity score and accession number.

		Attached		Suspension		Inoculum		Significance							
OTU	Host	Rel. ab	Stdev	Rel. ab	Stdev	Rel. ab	Stdev	A vs L	A vs I	L vs I	Phylum	Class	Order	Family	Genus
3	MH	44,40	6,71	47,57	5,89	7,92	6,42	0,20	**0,00**	**0,00**	Proteobacteria	β-Proteobacteria	Unknown	Unknown	*Unknown*
1	MH	16,12	10,49	23,82	7,54	1,87	2,04	0,09	0,06	**0,00**	Proteobacteria	β-Proteobacteria	Unknown	Unknown	*Unknown*
22	MH	7,49	6,05	0,12	0,07	0,33	0,32	**0,02**	0,08	0,27	Actinobacteria	Actinobacteria	Unknown	Unknown	*Unknown*
23	MH	6,36	5,16	0,38	0,12	0,21	0,03	**0,02**	**0,02**	**0,05**	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	*Unknown*
12	MH	3,93	2,13	5,44	3,05	0,85	0,75	0,17	**0,05**	**0,05**	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Dyella*
26	MH	2,72	1,32	0,54	0,62	1,14	0,24	**0,00**	0,08	0,12	Proteobacteria	β-Proteobacteria	Burkholderiales	Unknown	*Unknown*
162	MH	2,30	0,50	3,32	2,51	1,71	1,76	0,19	0,36	0,22	Proteobacteria	β-Proteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*
325	MH	1,66	4,00	0,07	0,06	0,18	0,19	0,19	0,32	0,10	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Unknown*
81	MH	0,97	1,34	0,08	0,09	0,16	0,18	0,08	0,22	0,19	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium*
28	MH	0,96	1,11	0,77	1,03	0,62	0,15	0,38	0,35	0,43	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteibacter*
1	TH	41,48	18,15	80,74	6,52	1,87	2,04	**0,00**	**0,01**	**0,00**	Proteobacteria	β-Proteobacteria	Unknown	Unknown	*Unknown*
8	TH	32,04	14,72	2,64	2,17	0,19	0,13	**0,00**	**0,00**	0,09	Proteobacteria	β-Proteobacteria	Burkholderiales	Oxalobacteraceae	*Unknown*
7	TH	10,45	4,40	2,28	1,98	3,10	0,47	**0,00**	**0,03**	0,30	Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
9	TH	6,86	2,69	1,31	0,84	3,27	2,00	**0,00**	0,07	**0,04**	Proteobacteria	γ-Proteobacteria	Unknown	Unknown	*Unknown*
25	TH	1,68	2,01	0,24	0,15	0,05	0,05	0,07	**0,05**	0,07	Proteobacteria	β-Proteobacteria	Burkholderiales	Oxalobacteraceae	*Unknown*
3	TH	1,07	0,60	2,43	1,61	7,92	6,42	**0,05**	0,19	0,22	Proteobacteria	β-Proteobacteria	Unknown	Unknown	*Unknown*
15	TH	0,80	1,01	1,50	1,85	0,43	0,28	0,22	0,32	0,23	Proteobacteria	Unknown	Unknown	Unknown	*Unknown*
23	TH	0,39	0,55	0,05	0,02	0,21	0,03	0,09	0,34	**0,00**	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	*Unknown*
28	TH	0,36	0,45	0,33	0,16	0,62	0,15	0,44	0,23	**0,03**	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteibacter*
12	TH	0,16	0,23	3,13	2,68	0,85	0,75	**0,02**	0,20	0,15	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Dyella*

Table 5.1 (continued)

PHYLOGENY AND POTENTIAL MYCOPHAGY OF ISOLATED BACTERIA:

In total, 390 isolates (cultivable on TSA) were retrieved from hyphae-adhering communities. More than 50% (203) of these isolates inhibited fungal growth or altered fungal hyphal morphology on nutrient-poor agar. About 65% (n=132) of the fungal-inhibiting bacteria originated from *M. hiemalis*, 35% (n=71) from T. *harzanium*.
Bacteria with antagonistic effects on fungal growth of both fungal species were well represented among the *γ*- and *β-Proteobacteria*. Antagonistic bacterial isolates belonging to the phylum *Actinobacteria* were only obtained from hyphae of *M. hiemalis*. Differences in abundance and taxonomic classification of cultivable antagonists clearly separated the two bacterial communities between the fungal species (Fig 5.3).



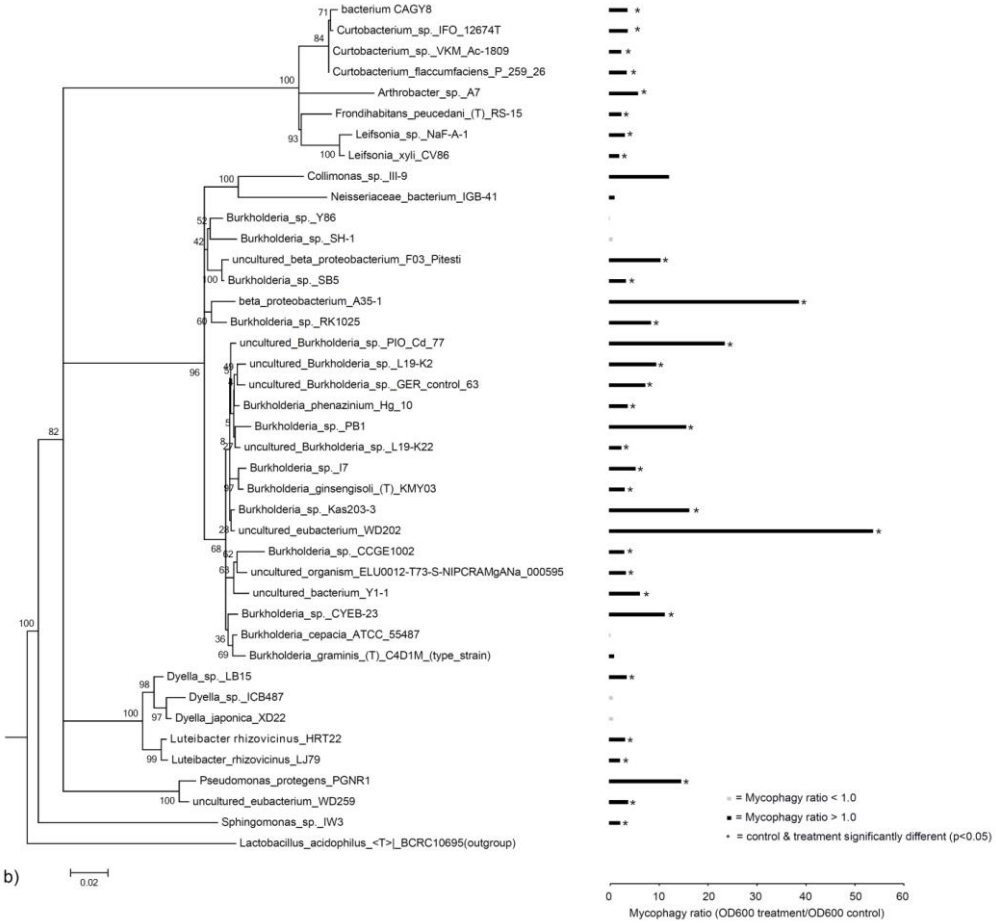


Fig 5.3 Phylograms of hyphae-associated bacterial taxa of a) *Trichoderma harzianum* and b) *Mucor hiemalis* and their degree of mycophagy (growth on living fungal hyphae). Black bars show mycophagy ratios (mean treatment OD_{600}/mean control OD_{600}) > 1.0, grey bars show mycophagy ratios < 1.0. Significant differences ($p \leq 0.05$) between bacterial growth in the presence of fungal hyphae and in controls (without fungi) are marked with an asterisk.. Phylograms were calculated using the Neighbor-Joining algorithm. Branch support values are based on a bootstrap analysis based on 100 pseudoreplicated datasets. The scale bars indicate evolutionary distances (nucleotide substitutions per site).

Most of the antagonistic isolates (59% for *M. hiemalis* and 91% for *T. harzianum*) had representatives among the 10 most abundant OTUs in the pyrosequencing dataset with high sequence similarities (Fig.4, Table S5.1 and S5.2 in Supporting Information). With Sanger sequencing, larger DNA stretches were sequenced (≥700bp) as compared to 454 pyrosequencing (~300bp). That is why several OTUs frequently grouped with more than one isolate (Table S5.1 & S5.2).

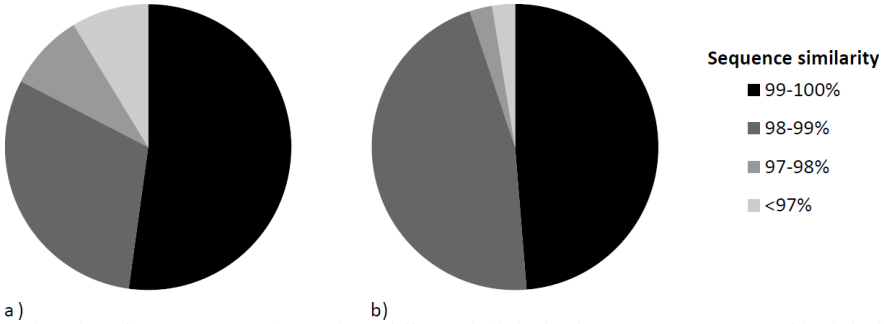


Fig 5.4 Clustering of 454 Pyrosequencing reads (at different similarity levels) to Sanger sequences of hyphal-adhering bacterial isolates with antifungal properties for a) *Trichoderma harzanium* & b) *Mucor hiemalis*.

The percentage of hyphal-adhering-, antagonistic bacteria that were able to grow with living hyphae as the only source of nutrients was higher for *M. hiemalis* (80 %) than for *T. harzianum* (35%). The relative increase in bacterial biomass (average "mycophagy ratio") was also considerably higher for *M. hiemalis* (9.1) than for *T. harzianum* (5.5) (Fig 5.3). For *M. hiemalis* the most efficient potential mycophagous colonizers belonged to the genus *Burkholderia* (average "mycophagy ratio" 10) and the genera *Curtobacterium* and *Leifsonia* (class *Actinobacteria*) (average "mycophagy" ratio 3.4). In the case of *T. harzianum*, we found that the genus *Burkholderia* was also an important representative of potential mycophagous bacteria, but we discovered the same number of potential mycophagous isolates for the class *γ-Proteobacteria* (genera *Luteibacter* and *Pseudomonas*). Isolate names, Accession numbers and taxonomic classification are listed in Table S5.4.

DISCUSSION:

Studies tracking the fate of fixed $^{13}CO_2$ in plants have indicated that saprotrophic fungi can be the dominant primary consumers of root derived carbon (Denef *et al.* 2007; Buee *et al.* 2009; Hannula *et al.* 2012). Therefore, the flow of energy from roots via saprotrophic fungi to fungal-feeding bacteria may be an important process. Indeed, Hannula et al (2012) and also (Drigo *et al.* 2010) showed an increase in ^{13}C-labeled bacteria after the initial ^{13}C enrichment of saprotrophic or mycorrhizal fungi in the rhizosphere. In a follow-up study, it was shown that the bacteria labeled in the experiment of Hannula et al (2012) consisted of genera such as *Burkholderia* and *Pseudomonas* which harbor potential mycophagous bacteria as shown in our research (Dias et al., 2013). Yet, they could not prove that these bacteria were feeding on fungi, as ^{13}C-labeled plant resources were also still present. We want to highlight that differentiation between bacterial-, fungal- and plant derived carbon in such a system is extremely difficult.

Therefore, we chose a growth assay-based approach to find support for the existence of bacteria acting as secondary consumers of fungus-derived nutrients.

In the current study we revealed that part of the rhizosphere bacteria of a grass and sedge has the ability to rapidly form tight associations with hyphae of saprotrophic fungi and are able to grow with no other energy resources than the hyphae. Therefore, we propose that for those bacteria, fungal hyphae might be an important source of organic nutrients in the rhizosphere and we suggest the term "sapro-rhizosphere" to describe this phenomenon (Fig. 5.5). This term is deduced from the term "mycorrhizosphere", that has been used to describe the zone in the rhizosphere where bacteria are influenced by mycorrhizal fungi (Timonen & Marschner 2006).

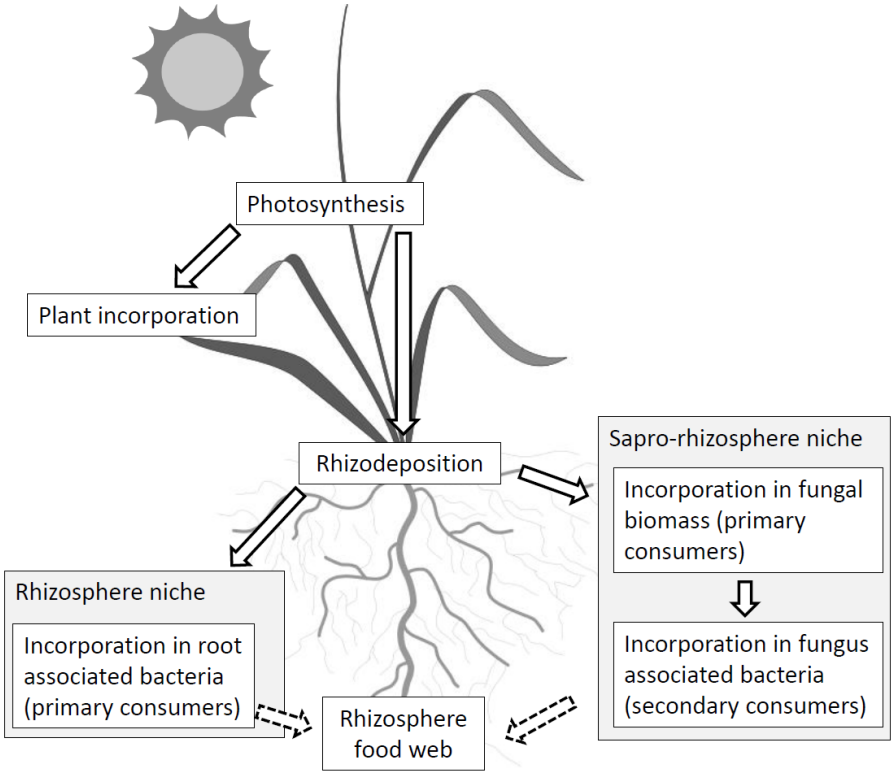


Fig 5.5 Proposed main energy fluxes to bacteria in the "sapro-rhizosphere" and rhizosphere.

Since we tested hyphal-adhering bacteria that showed *in vitro* antifungal activity, antifungal secondary metabolites may have had a role in getting access to fungal nutreients e.g. by destroying the exterior of fungal hyphae or by stimulate increased exudation of nutrients. Such a feeding habit where bacteria are actively involved in obtaining fungal nutrients has been termed bacterial mycophagy (Leveau & Preston 2008). However, since we did not study the interactions between bacteria and fungi in detail we cannot exclude that bacteria may only have

been feeding on fungal exudates without being actively involved in getting access to those nutrients. Therefore, we refer to the bacteria having a positive growth response when encountering a host fungus as potential mycophagous bacteria. Our results indicate that many rhizosphere bacteria were able to colonize fungal hyphae and to convert fungal nutrients into bacterial biomass. Bacteria belonging to the order *Burkholderiales,* which are common members of rhizosphere bacterial communities**,** were overall well represented on fungal hyphae and also efficient feeders on *M. hiemalis* (high mycophagy ratio). Most of the mycophagous bacteria that we isolated belonged to *Burkholderia*, a genus of the order *Burkholderiales* that is already known to be able to colonize fungal hyphae of a variety of fungi (Frey-Klett *et al.* 2011). Despite the known association with fungi, there is only little knowledge about possible mycophagous abilities of *Burkholderia* (Cuong *et al.* 2011). However, it has been indicated that a *Burkholderia terrae* strain can stimulate the fungal exudation of glycerol, a compound that is efficiently used by this strain (Nazir et al., 2012). Scheublin *et al.* (2010) showed that the family *Oxalobacteraceae* which also belongs to the order *Burkholderiales* was an abundant colonizer of hyphae of arbuscular mycorrhizal fungi. We showed that members of *Oxalobacteraceae* were able to colonize and feed on *T. harzianum*.

Interestingly, bacteria of the genera *Burkholderia, Pseudomonas, Sphingomonas* and *Dyella* have been shown to migrate or co-migrate along fungal hyphae (Kohlmeier *et al.* 2005; Warmink *et al.* 2011). In our study, these genera were well represented among the potential mycophagous colonizers as well. It has already been hypothesized that motility could be an important trait for mycophagous bacteria to find sites along the fungal mycelium where energy resources are most accessible (Leveau & Preston 2008). For instance, it has been shown that the hyphal tips, the actively growing parts of mycelia, are most vulnerable to the attack by mycophagous bacteria of the genus *Collimonas* (Leveau *et al.* 2010).

The ability of bacteria to colonize hyphae of arbuscular and ecto-mycorrhizal fungi is well known (Scheublin *et al.* 2010; Voronina *et al.* 2011). However, mycophagous abilities of those bacteria have mostly not been quantitatively addressed or are based on indirect measurements, i.e. by showing the potential to grow on substrates that are mainly of fungal origin such as trehalose (Frey-Klett *et al.* 2007). Based on the taxonomic similarity of mycophagous rhizosphere bacteria found in this study with those of fungal-adhering bacteria in other studies, it seems likely that many of the latter do also have the potential to obtain energy resources from hyphae.

The fungus associated bacteria in our study represent a subset of the diverse rhizosphere bacterial community. Hence, a part of the rhizosphere bacteria is able to attach, colonize and feed on saprotrophic fungal hyphae. This is in line with research by Frey-Klett *et al.* (2007), showing that ecto-mycorrhizal fungi exert a selective effect on the associated bacteria. On top of that, in our study, rhizosphere fungi of different phyla were colonized by distinct bacterial species. Differences in "mycophagy ratios" of the colonizers but also in the percentage of potential mycophagous bacteria associated, showed that overall *M. hiemalis* was colonized by a higher number as well as more aggressive bacteria (higher "mycophagy ratios") than *T. harzianum*.

The phylogenetic differentiation between bacterial colonizers of *M. hiemalis* and *T. harzianum* could be a result of selective effects that fungi impose on bacteria via cell wall/hyphal surface properties, composition of fungal exudates and production of bactericidal compounds (Bartnick 1968; Keller *et al.* 2005). Here, another notable result was the exclusive association of *Actinobacteria* with hyphae of *M. hiemalis* (Fig.3). The presence of *Actinobacteria* in hyphospheres and the ability to feed on arbusular mycorrhizal fungi has been reported.(Lecomte *et al.* 2011; Schrey *et al.* 2012). *Actinobacteria* are known to be potent producers of antimicrobial substances. The mycophagous *Collimonas* bacteria were also shown to produce a variety of metabolites that have antifungal properties (Leveau *et al.* 2010; Fritsche *et al.* 2014). Since the production of antimicrobials by mycophagous bacteria plays an important role in the interaction with the fungus we suggest that mycophagous bacteria could be a promising source for the discovery of novel antibiotic compounds. The selective association of *Actinobacteria* with *M.hiemalis* suggests that antibiotic mining of specific bacterial groups could be realized by using different fungal "baits".

Interactions between host-associated microbiota can range from mutualistic to antagonistic, including pathogenicity, and only small changes in the genome or the uptake of mobile genetic elements can make the difference (Freeman & Rodriguez 1993). Several of the bacteria isolated from hyphae are putative plant-pathogens like *Curtobacterium flaccumfaciens, Enterobacter aerogenes, Leifsonia xyli* and *Burkholderia cepacia* but also potential plant beneficial bacteria such as *Pseudomonas protegens* or *Luteibacter rhizovicinus*. It has already been shown that bacteria from those genera associate with fungal or other eukaryotic hosts (Warmink & van Elsas 2009; Cuong *et al.* 2011; Aylward *et al.* 2012; Kamei *et al.* 2012; Hoffman *et al.* 2013). The ability to exploit living fungi could be an important trait for both functional groups (mutualists and parasites) to survive in the soil environment.

We observed attachment to fungal hyphae within 24 hours after the introduction of the bacterial community inoculum. Such quick attachment suggests that for mycophagous bacteria the association with the fungal hypha is of importance. The fungus would provide a stimulus, guiding mycophagous bacteria to the hyphae by secreting exudates or possibly quorum sensing molecules (Frey-Klett *et al.* 2011) that might serve as signal molecules for directed bacterial movement towards the fungal host.

Our study indicates that many rhizosphere bacteria can act as fungal-feeding secondary consumers. Given the prominent role of saprotrophic fungi as primary decomposers of root-derived organic carbon that has been indicated in several studies, the flow of carbon from plants via fungi to fungal-feeding bacteria may be substantial. This probably has strong impacts on the functioning of rhizosphere food webs. For instance, the partitioning of root-derived C into carbon dioxide and soil microbial biomass is based on primary microbial, mostly bacterial, consumers and their growth efficiencies (Farrar *et al.* 2003). Secondary microbial consumption will change these carbon fluxes with more carbon dioxide released by microbes and less microbial carbon available for the soil animal food web. Soil animal food webs rely strongly on the input of root-derived carbon (Bonkowski *et al.* 2009). Hence, to have a better understanding of the functioning of the soil animal food webs, it is necessary to understand how carbon and inorganic nutrients are cycled in rhizosphere microbial communities before they enter the soil animal food chain.

CONCLUSIONS:

Our results show that saprotrophic rhizosphere fungi can form the only source of energy for many members of the rhizosphere bacterial community. This could indicate that secondary consumption of saprotrophic fungi by bacteria is an important process in the rhizosphere. So far, the flow of root-derived organic nutrients via saprotrophic fungi to bacteria has not been considered, but it may be substantial, given the accumulating evidence that saprotrophic fungi can rapidly assimilate root exudates. This would, however, change our view on the functioning of rhizosphere bacterial communities and their relationship with the functioning of plants. Therefore, we encourage further studies that elucidate the trophic relationship between plants, rhizosphere inhabiting saprotrophic fungi and bacteria and test the "sapro-rhizosphere concept" (possibly by ^{13}C isotope tracing).

ACKNOWLEDGEMENTS:

We thank Marlies van den Berg and Maria Hundscheid for technical assistance. We are grateful to Almudena Gonzales-Mula for technical assistance in conducting pilot studies. This research was supported by a grant from the Netherlands Organization for Scientific Research, division Earth and Life Sciences (NWO-ALW), grant number 819.01.016.

SUPPLEMENTARY MATERIAL:

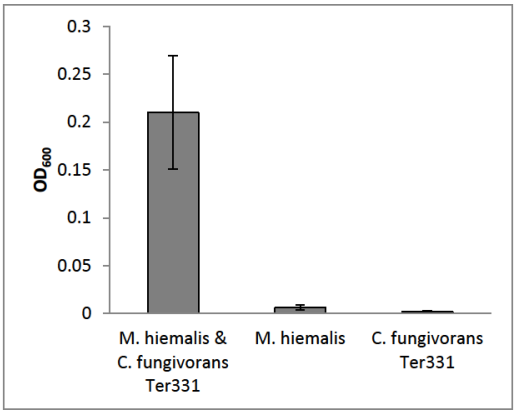


Fig S5.1 Testing of the mycophagy assay with a known mycophagous bacterium (Collimonas fungivorans). Given are means and standard deviations of mycophagy assay optical density (OD_{600}) measurements. Details on harvesting the bacteria for OD_{600} measurements are given in Material & Methods.

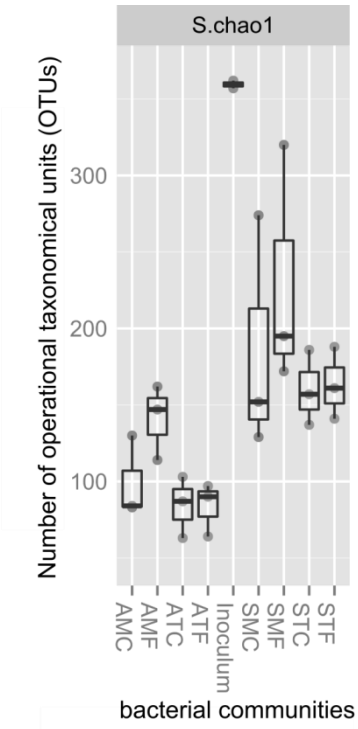


Fig S5.2 Box whisker plots of Chao estimates of the total number of operational taxonomical units (OTUs) in the different bacterial communities. A= hyphae-attached communities, S= "Incubation-slot" communities, M= host fungus *M. hiemalis*, T= host fungus *T. harzianum*, C= *C. arenaria* inoculum, F= *F. rubra* inoculum. Box whisker plots are based on 3 biological replicates. The median is indicated by black lines and the varying range of the estimate by the boxes. Whiskers indicate the 1.5 interquartile range of the lower and the upper quartile of the data.

most similar strain	Accession number (rdp)	sequence similarity with OTUs 99-100%	98-99%	97-98%	top10 taxonomy match (OTU)
Arthrobacter sp. A7	JX010952	74			no match
Curtobacterium sp. IFO 12674T	AB046364	105	71	20	no match
β- proteobacterium A35-1	AY049942	216	**162**	153, 360, 137	*Burkholderia*
Burkholderia cepacia ATCC 55487	AY741358		216, **3**, **162**	360, 137	*Burkholderia*
Burkholderia ginsengisoli (T) KMY03	AB201286				no match
Burkholderia graminis (T) C4D1M (type strain)	U96939		216, **3**, **162**	360, 137	*Burkholderia*
Burkholderia phenazinium Hg 10	AY154373		**3**, 216	**162**, 360	unknown (class: β-Proteobacteria)
Burkholderia sp. CCGE1002	CP002013		221		no match
Burkholderia sp. CYEB-23	FJ422385		216, **3**, **162**	360, 137	*Burkholderia*
Burkholderia sp. I7	JF763858		216	162, **3**	*Burkholderia*
Burkholderia sp. Kas203-3	AB114270		216, **3**, **162**	360, 137	*Burkholderia*
Burkholderia sp. PB1	AJ971346	**3**	216	360	unknown (class: β-Proteobacteria)
Burkholderia sp. RK1025	AB591835		153	216	no match
Burkholderia sp. SB5	AJ971354	216, **162**	137	**3**	*Burkholderia*
Burkholderia sp. SH-1	DQ472169	31			no match
Burkholderia sp. Y86	FJ772045	216, **162**	137	**3**	*Burkholderia*
Collimonas sp. III-9	AB531409		460	436	no match
Curtobacterium flaccumfaciens P 259/26	AJ310414	105	71	431	no match
Curtobacterium sp. IFO 12674T	AB046364	105	71	20	no match
Curtobacterium sp. VKM Ac-1809	AB042088		431	105	no match
Dyella japonica XD22	AB110497		**12**	362, 254	*Dyella*
Dyella sp. ICB487	HM748080	**12**			*Dyella*
Dyella sp. LB15	JQ864383			362, 254, **12**	*Dyella*
Frondihabitans peucedani (T) RS-15	FM998017	431			no match
Leifsonia sp. NaF-A-1	FJ872398	20		105, 71	no match
Leifsonia xyli CV86	AJ717351	20		105, 71	no match
Luteibacter rhizovicinus LJ79	AJ580497	**28**			*Luteibacter*
Neisseriaceae bacterium IGB-41	FN994890		58		no match
Pseudomonas protegens PGNR1	AJ417071		9	7	no match
Sphingomonas sp. IW3	AB076396	**23**			*Sphingomonas*
uncultured bacterium IYF62	DQ984599	**28**			*Luteibacter*
uncultured bacterium Y1-1	JF766461		**3**, 216	**162**, 360	unknown (class: β-Proteobacteria)
uncultured *β- proteobacterium* F03_Pitesti	DQ378169	216, **162**	137	**3**	*Burkholderia*
uncultured *Burkholderia sp.* GER_control_63	KC112031		**3**, 216	**162**, 360	unknown (class: β-Proteobacteria)
uncultured *Burkholderia sp.* L19-K22	EU477807		**3**, 216	**162**, 360	unknown (class: β-Proteobacteria)
uncultured *Burkholderia sp.* PIO_Cd_77	KC112057		**3**, 216	**162**, 360	unknown (class: β-Proteobacteria)
uncultured eubacterium WD202	AJ292637	216	**162**, **3**	360	*Burkholderia*
uncultured eubacterium WD259	AJ292672	9		7, 88	no match
uncultured organism ELU0012-T73-S-NIPCRAMgANa_000595	HQ742320		216, **162**	**3**, 137	*Burkholderia*

Table S5.2 Sequence similarity between isolated bacteria and 454 pyrosequencing OTUs attached to *T. harzianum*, bold numbers depict top10 OTUs.

most similar strain	Accession number (rdp)	sequence similarity with OTUs 99-100%	98-99%	97-98%	top10 taxonomy match (OTU)
Burkholderia ginsengisoli (T) KMY03	AB201286				*no match*
Burkholderia phenazinium Hg 10	AY154373		**3**	162, 360	unknown (class: β-Proteobacteria)
Burkholderia sp . Kas203-3	AB114270		216, **3**, 162	360, 137	unknown (class: β-Proteobacteria)
Burkholderia sp. SB5	AJ971354	216, 162	137	**3**	unknown (class: β-Proteobacteria)
Duganella zoogloeoides NBRC 102465	AB681807				*no match*
Enterobacter aerogenes 11-WCH	FJ811872		**15**		unknown (phylum: Proteobacteria)
Luteibacter rhizovicinus HRT22	JF778702	**28**			*Luteibacter*
Luteibacter rhizovicinus LJ79	AJ580497	**28**			*Luteibacter*
Luteibacter rhizovicinus LL-C	EU022023			254, **28**	*Luteibacter*
Oxalobacteraceae bacterium CHNTR40	DQ337591	**8**			Oxalobacteraceae
Pantoea sp. SAP72_2	JN872531			349, **15**, 68	unknown (phylum: Proteobacteria)
Pedobacter steynii (T) type strain: WB2.3-45	AM491372	136			*no match*
Pseudomonas protegens PGNR1	AJ417071		**9**	**7**	unknown (class: γ-Proteobacteria)
Pseudomonas sp. CPBE44	DQ840551	214	287	**9**	unknown (class: γ-Proteobacteria)
Pseudomonas sp. NZ017	AY014805	**7**, 88	**9**		*Pseudomonas*
Pseudomonas sp. phi9	GU233953		214, 287	**9**	unknown (class: γ-Proteobacteria)
Pseudomonas sp. SAP34_1	JN872537	9	**7**, 88		unknown (class: γ-Proteobacteria)
uncultured bacterium 14_E06	FN421658		**9**	**7**, 88	unknown (class: γ-Proteobacteria)
uncultured bacterium BF0001C011	AM697170	**8**			Oxalobacteraceae
uncultured bacterium BF0001C106	AM697265	**8**			Oxalobacteraceae
uncultured bacterium BF0001C119	AM697278	**8**			Oxalobacteraceae
uncultured bacterium J308	GQ451298		**8**		Oxalobacteraceae
uncultured bacterium R103	HM069038	**1**			unknown (class: β-Proteobacteria)

Table S5.2 (continued)

Table S5.3 Average number and standard deviation of sequence reads, grouped by the origin of the communities

Association	Host fungus	Rhizosphere	Average reads	Stdev
Attached	*M. hiemalis*	*F. rubra*	1736	639,93
Suspension	*M. hiemalis*	*F. rubra*	5351	4209,67
Attached	*M. hiemalis*	*C. arenaria*	2890	1099,16
Suspension	*M. hiemalis*	*C. arenaria*	6566	2829,95
Attached	*T. harzanium*	*F. rubra*	6287	1027,31
Suspension	*T. harzanium*	*F. rubra*	7906	3579,25
Attached	*T. harzanium*	*C. arenaria*	8054	2365,73
Suspension	*T. harzanium*	*C. arenaria*	8905	1349,12
Inoculum	na	*F. rubra*	7418	*
Inoculum	na	*C. arenaria*	6165	*

Table S5.4 Genbank accession numbers, isolate numbers, fungal association and respective rdp classification.

M. hiemalis	*T. harzanium*	Isolate	Genbank accession nr	Strain name	RDP accession nr
x	x	CR5_1	KJ396109	*Burkholderia sp.* SB5	AJ971354
x	x	CR7_6	KJ396110	*Pseudomonas protegens* PGNR1	AJ417071
x	x	FR1_11	KJ396111	*Luteibacter rhizovicinus* LJ79	AJ580497
x		FR3_1	KJ396113	*Curtobacterium flaccumfaciens* P 259/26	AJ310414
x		MC1_1	KJ396114	*Dyella sp.* ICB487	HM748080
x	x	MC1_10	KJ396115	*Burkholderia sp.* SB5	AJ971354
x		MC1_11	KJ396116	uncultured bacterium Y1-1	JF766461
x		MC1_12	KJ396117	uncultured *Burkholderia sp.* PIO_Cd_77	KC112057
x	x	MC1_13	KJ396118	*Burkholderia ginsengisoli* (T) KMY03	AB201286
x		MC1_16	KJ396119	uncultured organism ELU0012-T73-S-NIPCRAMgANa_000595	HQ742320
x		MC1_18	KJ396120	*Leifsonia xyli* CV86	AJ717351
x		MC1_2	KJ396121	uncultured eubacterium WD259	AJ292672
x		MC1_21	KJ396122	*Curtobacterium sp.* IFO 12674T	AB046364
x		MC1_22	KJ396123	uncultured beta proteobacterium F03_Pitesti	DQ378169
x		MC1_23	KJ396124	*Burkholderia sp.* SH-1	DQ472169
x		MC1_24	KJ396125	uncultured *Burkholderia sp.* PIO_Cd_77	KC112057
x		MC1_26	KJ396126	uncultured organism ELU0012-T73-S-NIPCRAMgANa_000595	HQ742320
x		MC1_27	KJ396127	*Burkholderia sp.* Y86	FJ772045
x		MC1_7	KJ396128	bacterium CAGY8	AF538747
x		MC1_8	KJ396129	*Burkholderia sp.* RK1025	AB591835
x	x	MC2_16	KJ396130	*Burkholderia sp.* SB5	AJ971354
x		MC2_18	KJ396131	*Burkholderia sp.* CCGE1002	CP002013
x	x	MC2_26	KJ396132	*Burkholderia sp.* Kas203-3	AB114270
x	x	MC2_27	KJ396133	*Burkholderia sp.* Kas203-3	AB114270
x		MC2_29	KJ396134	*Burkholderia graminis* (T) C4D1M (type strain)	U96939
x		MC2_3	KJ396135	*Burkholderia cepacia* ATCC 55487	AY741358
x		MC3_11	KJ396136	uncultured bacterium Y1-1	JF766461
x		MC3_12	KJ396137	*Arthrobacter sp.* A7	JX010952

Table S5.4 continued

M. hiemalis	*T. harzanium*	Isolate	Genbank accession nr	Strain name	RDP accession nr
x		MC3_13	KJ396138	*Frondihabitans peucedani* (T) RS-15	FM998017
x		MC3_14	KJ396139	*Curtobacterium flaccumfaciens* P 259/26	AJ310414
x		MC3_18	KJ396140	*Leifsonia sp.* NaF-A-1	FJ872398
x		MC3_23	KJ396141	*Neisseriaceae* bacterium IGB-41	FN994890
x	x	MF1_1	KJ396142	*Luteibacter rhizovicinus* HRT22	JF778702
x		MF1_19	KJ396143	*Curtobacterium sp.* IFO 12674T	AB046364
x		MF1_21	KJ396144	uncultured eubacterium WD202	AJ292637
x	x	MF1_22	KJ396145	*Burkholderia sp.* Kas203-3	AB114270
x	x	MF2_10	KJ396146	*Burkholderia phenazinium* Hg 10	AY154373
x		MF2_13	KJ396147	*Dyella japonica* XD22	AB110497
x		MF2_15	KJ396148	*Sphingomonas sp.* IW3	AB076396
x		MF2_17	KJ396149	*Dyella sp.* LB15	JQ864383
x		MF2_18	KJ396150	uncultured *Burkholderia sp.* GER_control_63	KC112031
x		MF2_21	KJ396151	beta proteobacterium A35-1	AY049942
x		MF2_24	KJ396152	*Burkholderia sp.* CYEB-23	FJ422385
x	x	MF2_25	KJ396153	*Burkholderia phenazinium* Hg 10	AY154373
x		MF2_27	KJ396154	uncultured *Burkholderia sp.* L19-K22	EU477807
x		MF2_3	KJ396155	*Burkholderia sp.* I7	JF763858
x		MF2_4	KJ396156	*Dyella sp.* LB15	JQ864383
x		MF2_5	KJ396157	*Burkholderia sp.* PB1	AJ971346
x		MF3_1	KJ396158	*Collimonas sp.* III-9	AB531409
x		MF3_15	KJ396159	*Frondihabitans peucedani* (T) RS-15	FM998017
x	x	MF3_24	KJ396160	*Burkholderia sp.* Kas203-3	AB114270
x		MF3_26	KJ396161	uncultured *Burkholderia sp.* L19-K2	EU477797
x		MF3_27	KJ396162	*Curtobacterium sp.* VKM Ac-1809	AB042088
	x	FR1_16	KJ396112	*Pedobacter steynii* (T) type strain: WB2.3-45	AM491372
	x	TC1_13	KJ396163	*Luteibacter rhizovicinus* LL-C	EU022023
	x	TC1_29	KJ396164	uncultured bacterium R103	HM069038
	x	TC1_4	KJ396165	uncultured bacterium J308	GQ451298
	x	TC2_12	KJ396166	uncultured bacterium 14_E06	FN421658
	x	TC2_13	KJ396167	uncultured bacterium BF0001C011	AM697170
	x	TC2_19	KJ396168	*Pseudomonas sp.* SAP34_1	JN872537
	x	TC2_22	KJ396169	*Pseudomonas sp.* SAP34_1	JN872537
	x	TC2_9	KJ396170	*Pseudomonas sp.* phi9	GU233953
	x	TC3_1	KJ396171	*Pantoea sp.* SAP72_2	JN872531
	x	TC3_11	KJ396172	*Enterobacter aerogenes* 11-WCH	FJ811872
	x	TC3_26	KJ396173	*Pseudomonas sp.* CPBE44	DQ840551
	x	TC3_29	KJ396174	uncultured bacterium BF0001C011	AM697170
	x	TF4_16	KJ396175	uncultured bacterium BF0001C106	AM697265
	x	TF4_17	KJ396176	uncultured bacterium BF0001C119	AM697278
	x	TF5_11	KJ396177	*Oxalobacteraceae* bacterium CHNTR40	DQ337591
	x	TF5_15	KJ396178	*Duganella zoogloeoides* NBRC 102465	AB681807
	x	TF5_25	KJ396179	*Pseudomonas sp.* NZ017	AY014805

CHAPTER SIX

BAITING AND ENRICHING FUNGUS FEEDING (MYCOPHAGOUS) BACTERIA

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To be submitted

ABSTRACT:

Mycophagous soil bacteria have the potential to feed on living fungal hyphae. However, knowledge on the importance of this feeding strategy in soil bacterial communities is limited as most studies have focused on the genus *Collimonas*. In this study, we compared two different approaches, based on baiting or enriching bacteria on fungal hyphae, to obtain mycophagous bacteria from rhizosphere soil. We test the isolated bacteria for their ability to inhibit fungal growth and to feed on fungi as the sole source of carbon. Both methods yielded a range of potential mycophagous bacterial isolates with little overlap between the methods. Another important factor contributing to the differentiation in these fungus-feeding isolates was the identity of the fungus used for baiting. Surprisingly, there were several potential plant pathogenic bacteria among the mycophagous isolates. Furthermore, we discuss perspectives for applications of mycophagous bacteria.

INTRODUCTION:

Bacteria and fungi commonly co-occur in a variety of habitats (Frey-Klett *et al.* 2011). The habitat containing the highest diversity of both groups is the soil. In soil, available nutrients are scarce and thus microorganisms have to compete for them. Soil bacteria have developed different strategies to cope with limited resources, such as the production of toxic secondary metabolites that suppress competitors or the ability to adapt to the use of specific energy resources (Hibbing *et al.* 2010).The soil bacteria of the genus *Collimonas* have developed the ability to exploit living fungi to obtain energy for growth, a strategy that has been termed mycophagy (Leveau & Preston 2008). *Collimonas* bacteria use a combination of antibiotics and enzymes to get access to organic nutrients present in living fungal hyphae (Leveau *et al.* 2010). They are especially abundant in (semi-)natural, acidic grassland soils where they can have an impact on the composition of fungal communities, probably due to selective feeding on particular fungal species (Hoppener-Ogawa *et al.* 2007; Hoppener-Ogawa *et al.* 2009a). Besides this information, very little is known on the importance of bacterial mycophagy in soil ecosystems. However, given the frequently recorded association of bacteria with hyphae of a wide range of fungi, covering all important functional fungal groups, mycophagous feeding by bacteria is likely to be much more common than currently appreciated (Leveau & Preston 2008; Nazir *et al.* 2010; Frey-Klett *et al.* 2011).

Other aspects that warrant further study on the occurrence of bacterial mycophagy among soil bacterial species are the perspectives to use this feeding strategy for biocontrol of soil-borne pathogenic fungi or for the discovery of novel fungal-inhibiting antibiotics (Kamilova *et al.* 2007; Mela *et al.* 2011; Fritsche *et al.* 2014).

An indication for mycophagous abilities of bacteria can only be obtained when it is certain that intact living fungi form the only source of nutrients that can sustain bacterial growth. In an earlier study, mycophagous growth of *Collimonas* bacteria was shown in microcosms where fungal hyphae invading pure sand formed the only source of nutrients (De Boer *et al.* 2001). However, this sand microcosm approach is not suitable to screen a high number of bacterial isolates. Therefore, we developed another method where phytagel, a very pure agar substitute, was used as the nutrient-free environment in which growing fungal hyphae encounter bacteria (*chapter five*). In that study, we used the phytagel- assay to screen for the mycophagous potential

of bacterial isolates that were rapidly adhering (24 h) to fungal hyphae growing in a nutrient-free liquid environment ("liquid hyphal baiting") that was inoculated with bacteria extracted from the rhizosphere of a grass and sedge. This rapid baiting method combined with the phytagel-mycophagy assay revealed that the potential to feed on common saprotrophic rhizosphere fungi is taxonomically widespread among rhizosphere bacteria. Besides *Collimonas*-related *β-Proteobacteria*, the community of hyphae colonizing rhizosphere bacteria also comprised of *Actinobacteria*, *a-* and *γ-Proteobacteria*.
The short-term baiting method might, however, bias the recovery of mycophagous bacteria to quickly attaching ones. To further explore the mycophagous potential among soil bacteria the current study was aimed to determine whether a long term baiting "transfer-enrichment" method, based on repeated transfer of hyphal-adhering bacteria, will yield other mycophagous soil bacteria than the already established short-time baiting method. Our comparison of long- and short-term baiting methods could form a basis for future screenings and applications of associations between mycophagous bacteria and fungi. Both methods allow the isolation of distinct bacterial groups with specific feeding preferences.

METHODS:

SOIL INOCULA AND HOST FUNGI:

We used filtered soil suspensions from the rhizosphere of the sedge *Carex arenaria* and the grass *Festuca rubra* to inoculate both microcosm systems. These plant species co-occur in nutrient-poor sandy soils with low organic matter content, such as dune soils. For detailed description of both, the location where rhizosphere samples were taken and the soil inoculum preparation, we refer to *chapter five*. In short, rhizosphere soil was suspended in a diluted salt solution, shaken, sonificated and filtered repeatedly, until the filtrate mainly consisted of bacteria. The plant-pathogen *Rhizoctonia solani* and the saprotrophs *Mucor hiemalis* and *Trichoderma harzianum* were used as host fungi. Details on the origin of these fungi are given in *chapter five*. Before performing the experiments, all fungi were pre-cultured on Potato Dextrose Agar supplemented with the bactericidal antibiotics oxy-tetracycline (50 mgL^{-1}) and streptomycine (100 mgL^{-1}) and subsequently controlled and found free of bacterial contamination by DNA isolation and PCR.

SHORT-TERM HYPHAL-BAITING METHOD

This system was already described in detail in *chapter five* as "liquid hyphal baiting" method. Briefly, the fungi were inoculated in a two-compartment Petri-dish in modified M-medium containing glucose (Becard & Fortin 1988). This medium was solidified with phytagel (Sigma-Aldrich, St Louis, MO, USA). Phytagel is an agar substitute composed of interlinked D-sugars. The availability of Phytagel-derived carbon resources for microbial growth is extremely low (Sutherland & Kennedy 1996). During growth, fungi crossed the plastic barrier that separated the two compartments and colonized the second compartment that contained the same medium but without degradable carbon resources. Finally, a plug was cut out of the phytagel in the second compartment and the slot was filled with liquid medium of the same composition (i.e. free of carbon sources) but without phytagel as the gelling agent. Fungi were left to colonize the liquid medium (the incubation-slot) which was subsequently inoculated with the

filtered rhizosphere bacterial suspension. Hyphal-adhering bacteria were sampled after 24 hours. In this chapter we present the results of short-term liquid baiting for the the plant-pathogenic fungus *R. solani*, whereas results for the saprotropic fungi *M. hiemalis* and *T.harzianum* have been described in *chapter five*.

LONG-TERM HYPHAL-BAITING METHOD ("TRANSFER-ENRICHMENT"):

The microcosm system that was developed for the long-term enrichment of mycophagous bacteria consisted of a petri dish (94 mm dia, 16 mm height), filled with 4 % wV^{-1} Phytagel (supplemented with 0.74 gL^{-1} $MgSO_4$). In the middle of the microcosm a sterile Eppendorf cup lid was placed and filled with ~200 µl hot Malt Extract Agar (MEA) (15 gL^{-1} agar, 3 gL^{-1} peptone and 20 gL^{-1} malt extract). Fungal cultures were inoculated onto MEA by introducing small plugs (~2-3 mm^2) from fungal MEA pre-cultures. MEA provides a nutrient source for the fungus from which it further colonizes the surrounding nutrient free Phytagel medium. The rim of the Eppendorf Cup lid can easily be overgrown by fungal hyphae, but prevents diffusion of nutrients into the Phytagel. Hence, growing fungal hyphae form the only source of nutrients for bacteria on the Phytagel (See Fig.S6.1).

Microcosms were left to be colonized by the 3 different fungi. Colonization speed differed among fungi: *M. hiemalis* colonized the microcosm in 10 to 14 days, *T. harzianum* and *R. solani* in 5 to 7 days. After fungal colonization was completed, microcosms were inoculated with filtered rhizosphere bacterial suspensions (see above). A total amount of 400 µl suspension was carefully distributed over the whole plate, using a pipette. Inoculation was done in a full factorial design, using 3 replicates. After 14 days of incubation, the developing bacteria were transferred by stamping to a new microcosm that had already been colonized by the same host fungus. Stamping refers to the technique of replica plating (Lederberg & Lederberg 1952) which has originally been developed to screen for antibiotic resistant bacteria. We used a sterile velvet cloth for consecutive transfer of bacteria that were associated with a fungal species. We repeated this enrichment procedure 6 times every 14 days and finally washed the enriched hyphal-adhering colonies from the Petri dishes, using 2 ml of Mes buffer. In the remainder of the article we refer to this approach as "transfer-enrichment".

BACTERIAL ISOLATION, SEQUENCING, CONFRONTATION- AND MYCOPHAGY ASSAY:

A detailed description of isolation and identification on basis of sequencing of 16S rDNA fragments can be found in *chapter five*. Briefly, hyphal fragments collected from both systems were dilution plated on Tryptic Soy Agar (TSA) containing fungicides (100 mgL^{-1} cycloheximide & 50 mgL^{-1} delvocide). Bacterial colonies were randomly picked and transferred to fresh TSA plates until they were considered pure. Subsequently, isolated bacteria were scored for the ability to inhibit their host fungus in a confrontation assay on water-yeast agar (for details see *chapter five*), since it has been indicated that antifungal activity is an important factor for mycophagous growth of collimonads (Leveau *et al.* 2010; Mela *et al.* 2011). The identities of the inhibitory bacteria were determined by Sanger sequencing of 16S rDNA fragments and in case of identical sequences, representative isolates were chosen for subsequent testing of mycophagous abilities of the inhibitory isolates via the mycophagy assay as described in *chapter five*. Briefly, bacteria were inoculated on a Petri-dish, containing phytagel

medium. Subsequently, the host fungus was introduced on a nutrient-rich patch in the middle of a petri dish. A metal disc separated the nutrient patch from the content of the Petri-dish, thus preventing diffusion of nutrients into the phytagel. Natural fungal colony expansion forced the encounter of potentially mycophagous bacteria and the fungal host. After incubation, bacterial cells were washed of the Petri-dish and optical density at 600 nm wavelength (OD_{600}) was measured and mycophagy ratios were calculated (OD_{600} treatment/ OD_{600} control). We performed a "fungus only" and a "bacteria only" control and chose the higher OD_{600} value of the two as control for the calculation. All measurements were done in triplicates.

PHYLOGENY AND DATA ANALYSIS:

Partial 16S rDNA sequences were aligned and manually curated. Phylogenetic trees were constructed using the neighbor joining method with standard settings in MEGA 6 (Tamura *et al.* 2011). Missing nucleotide data were treated as a complete deletion in the alignment and trees were tested with 100 bootstraps. Other graphs, tables and statistics (t-tests) were done in Excel (Microsoft Corp.).

RESULTS:

Like the short-term hyphal baiting method, the long-term transfer-enrichment method was successful in obtaining a phylogenetically diverse group of antifungal bacteria. Only a subset of those anti-fungal bacteria showed a significant increase in numbers when confronted with fungi (Fig 6.1 and Fig 6.2).

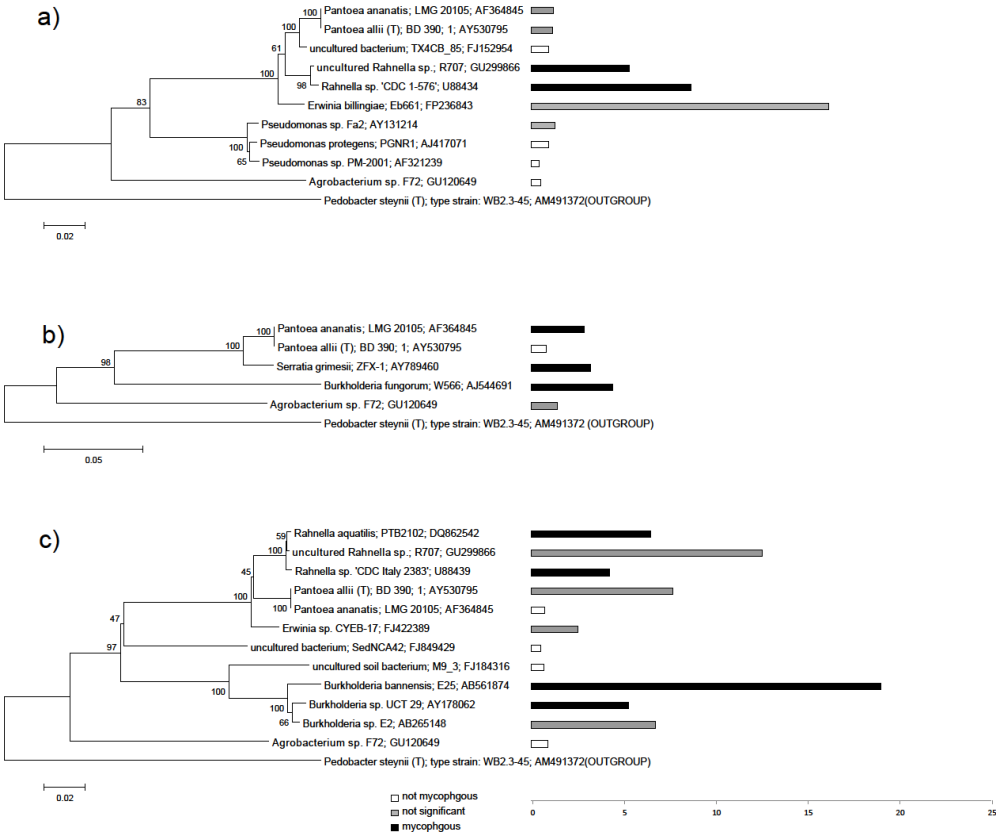

Fig 6.1: Phylogeny of antifungal, hyphal-adhering bacteria retrieved with the "transfer-enrichment" approach. Separate trees are presented for bacteria associated with a) *T. harzianum* b) *M. hiemalis* and c) *R. solani*. Bars indicate measured mycophagy ratios: with black bars representing bacteria demonstrating significant mycophagous growth (ratio > 1; P < 0.05), grey bars representing bacteria with possible mycophagous growth (ratio > 1; P > 0.05), and white bars represent bacteria with no mycophagous growth (ratio ≤ 1)).

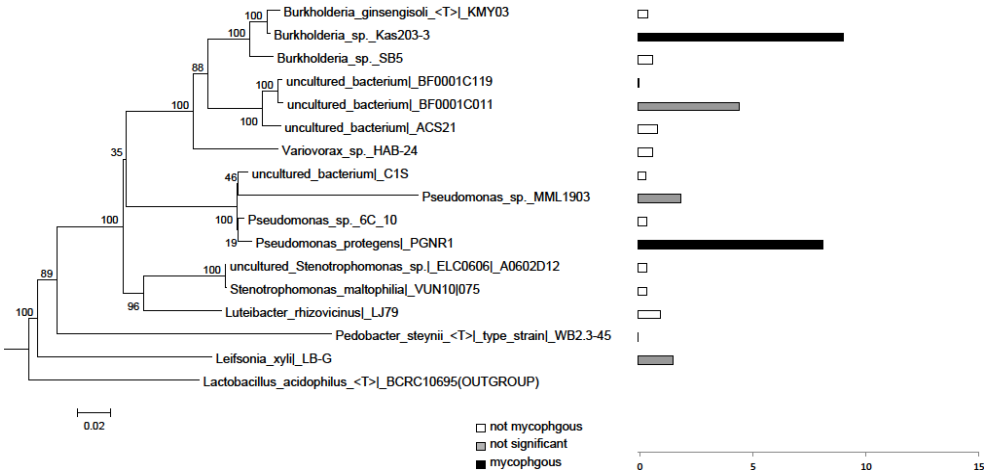

Fig 6.2: Phylogeny of antifungal, hyphal-adhering bacteria retrieved with the short-term "liquid hyphal-baiting" approach, associated with *R. solani*. Bars indicate measured mycophagy ratios with black bars representing bacteria demonstrating significant mycophagous growth (ratio > 1; $P < 0.05$), grey bars representing bacteria with possible mycophagous growth (ratio > 1; $P > 0.05$), and white bars represent bacteria with no mycophagous growth (ratio ≤ 1)).

With the "liquid hyphal-baiting" method, we obtained 78 isolates of bacteria adhering to hyphae of *R. solani*. Of these isolates 51 (65%) showed *in vitro* inhibitory activity against the host fungus. The "transfer-enrichment" method yielded 540 isolates in total (180 from each host fungus), of which 43 (24 %), 58 (32 %) and 47 (26 %) isolates were able to inhibit the growth of their respective host fungus (*M. hiemalis, T. harzianum, R. solani*). In a few cases bacterial isolates obtained by both methods could be assigned to the same species (*Pseudomonas protegens*) or the same genus (*Burkholderia, Pantoea* and *Pseudomonas*) (Fig 6.3 and Table 6.1).

Table 6.1: Mycophagy characteristics of sequenced, hyphae-adhering, anti-fungal bacterial strains, grouped by fungal host and isolation method. Given are average mycophagy ratios, percentage of bacteria with significant mycophagous growth (myc), - with possible mycophagous growth (n.s.) and - without mycophagous growth (not myc).

fungus	experiment	reference	total	myc(%)	not myc (%)	n.s.(%)
M. hiemalis	liquid hyphal-baiting	Rudnick *et al.* 2014	40	80	12,5	7,5
T. harzianum	liquid hyphal-baiting	Rudnick *et al.* 2014	23	34,8	30,4	34,8
R. solani	liquid hyphal-baiting	this study	16	12,5	68,8	18,8
Overall	**liquid hyphal-baiting**	**this study**	**79**	**53,2**	**29,1**	**17,7**
M. hiemalis	transfer-enrichment	this study	5	60	20	20
T. harzianum	transfer-enrichment	this study	10	20	40	40
R. solani	transfer-enrichment	this study	12	33,3	33,3	33,3
Overall	**transfer-enrichment**	**this study**	**27**	**33,3**	**33,3**	**33,3**

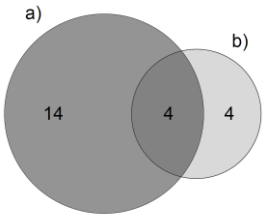

Fig 6.3: Venn diagram showing numbers of separated and shared bacterial genera for which hyphal-adhering, antifungal isolates were retrieved with a) "liquid hyphal-baiting" and b) "transfer-enrichment" methods.

Both the baiting method and the fungal host species had a strong effect on the composition of the anti-fungal bacteria. Bacteria assigned to the genera *Rahnella, Pseudomonas, Pedobacter, Pantoea, Luteibacter, Leifsonia, Erwinia, Agrobacterium and Burkholderia* were isolated from more than one fungal host. Strains assigned to the genera *Agrobacterium, Erwinia* and *Rahnella* were only isolated with the "transfer-enrichment", whereas strains assigned to the genera *Luteibacter, Leifsonia* and *Pedobacter* were only obtained with the "liquid hyphal-baiting" method. Bacteria that showed highest sequence similarity with hitherto uncultured bacteria were isolated with both methods. A number of genera were only represented by bacterial strains isolated from one of the 3 fungal species with either the "liquid hyphal-baiting"– or "transfer-enrichment" method. With both baiting methods, we found specific as well as generalist anti-fungal bacteria (Fig 6.4, Fig 6.5 and Fig 6.6).

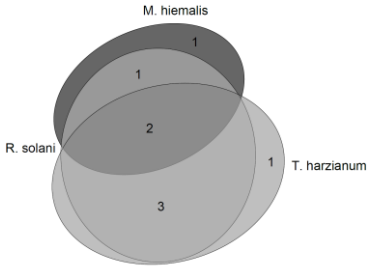

Fig 6.4: Venn diagram showing overlap between antifungal, hyphal-adhering bacterial genera isolated with the "transfer-enrichment" method, associated to *M. hiemalis, R. solani* and *T. harzianum.*

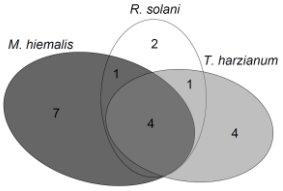

Fig 6.5: Venn diagram showing overlap between antifungal, hyphal-adhering bacterial genera isolated with the "liquid hyphal-baiting" method, associated to *M. hiemalis*, *R. solani* and *T. harzianum*. Data on isolates from *M. hiemalis* and *T. harzianum* have been taken from chapter five.

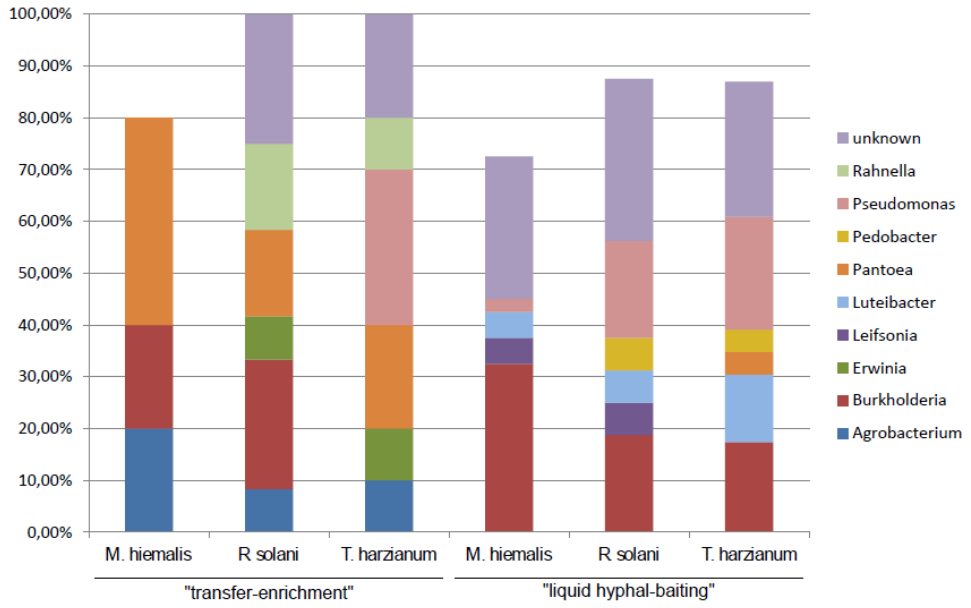

Fig 6.6: Relative amount of antifungal, hyphal-adhering bacterial isolates grouped by bacterial genera, the three host fungi (*M. hiemalis*, *R. solani*,*T. harzianum*) and the two isolation methods (long-term baiting "transfer-enrichment" and short-term "liquid hyphal-baiting". Only genera are displayed of which isolates were obtained that colonized more than one fungal species or that were isolated with both methods.

MYCOPHAGOUS BACTERIA:

The percentage of anti-fungal bacteria that was able to grow when encountering their host fungus was overall highest for the "liquid hyphal-baiting" method. Here, 53 % of the anti-fungal bacteria showed a positive growth response, compared to 33 % of the antifungal isolates obtained by the "transfer-enrichment" method. In both methods, *M. hiemalis* was by far the most " attractive" fungus, stimulating growth of 80 % ("liquid hyphal-baiting") and 60 % ("transfer-enrichment") of all its anti-fungal colonizers, followed by *T. harzianum* (34.8 % and 20 %, respectively) and *R. solani* (12.5 % and 33.3 %, respectively) (Table 6.1). The actual number of antifungal bacteria with the ability to feed on fungi may have been higher, as for several isolates the variation between replicates was considerable resulting in a non-significant, positive mycophagy ratio (Fig 6.1 and Fig 6.2).

While many mycophagous isolates at the level of bacterial genera appeared to have a broad host range in terms of colonization, a few strains isolated by the same method, could colonize and feed on more than one fungus (Table 6.2).

Table 6. 2 Mycophagous feeding patterns of anti-fungal bacterial isolates that were found to be colonizing hyphae of different fungal species: a) short-term "liquid hyphal-baiting" and b) long-term hyphal baiting ("transfer-enrichment"). Identification of the isolates is based on the closest match with the 16S rDNA sequences of strains or environmental sequences that are indicated in the table. Black background indicates high, grey intermediate and white low mycophagy ratios. Missing data indicate that the bacterium was not isolated from the respective fungus, and therefore not tested; "n.s." stands for none significant feeding because of variation between the replicates (average mycophagy ratios given in brackets). Data on strains obtained by "liquid-hyphal- baiting" from hyphae of *M. hiemalis* and *T. harzianum* were retrieved from *chapter five* (also see Table 6.1).

a) short-term "liquid hyphal-baiting"

strain	*R. solani*	*M. hiemalis*	*T. harzianum*
Pseudomonas protegens PGNR1	8,2	14,8	0,6
Burkholderia sp. Kas203-3	9,1	16,4	1,2
Burkholderia sp . SB5	0,7	3,4	3,0
Burkholderia phenazinium Hg 10		3,8	3,6
Burkholderia ginsengisoli (T) KMY03	0,4	3,2	0,9
Luteibacter rhizovicinus LJ79	1,0	2,2	n.s.(5,4)
uncultured bacterium BF0001C119	0,0		3,9
uncultured bacterium BF0001C011	n.s.(4,5)		n.s.(7,5)
Pedobacter steynii (T) WB2.3-45	0,0		0,9

b) long-term "transfer-enrichment"

strain	*R. solani*	*M. hiemalis*	*T. harzianum*
Pantoea allii (T) BD 390	7,7	0,8	1,2
Pantoea ananatis LMG 20105	0,8	2,9	1,2
uncultured *Rahnella sp* . R707	n.s.(12,6)		5,4
Agrobacterium sp. F72	0,9	1,4	0,6

With the "liquid hyphal-baiting" method, we isolated strains with closest match to *Pseudomonas protegens* PGNR1 and *Burkholderia sp. Kas203-3* from all three fungi, but the bacteria did only significantly increase in biomass on *M. hiemalis* and *R. solani*. Other isolates, like strains with closest match to *Burkholderia sp.* SB5 and *Burkholderia phenazinium* Hg 10 only grew on *M. hiemalis* and *T.harzianum* whereas they were not found to colonize *R. solani,* respectively. Other examples of selective growth are strains assigned to the species *Burkholderia ginsengisoli* and *Luteibacter rhizovicinus* as well as a strain with closest match to an uncultured bacterium strain BF0001C119 (*Oxalobacteraceae)*. Finally, some bacteria exhibited selectively colonization but were not able to grow on the colonized fungi. Those were isolates closely related to *Pedobacter steynii* WB2.3-45 and the uncultured bacterium BF0001C011 (*Oxalobacteraceae)*. We also observed selective feeding for closely related "transfer-enrichment" isolates. Strains that had high similarity to *Pantoea allii* BD 390*, Pantoea ananatis* LMG 20105 and *Agrobacterium sp.* F72 colonized all three fungi but only grew on *R. solani, M. hiemalis* or on none of the three, respectively (see also Table 6.2).

DISCUSSION:

The aim of this study was to compare two different methods to bait for soil bacteria with fungus-feeding abilities. Using short-term and long-term fungal baiting incubations, we isolated hyphae adhering bacteria from grass and sedge rhizosphere soils and evaluated the mycophagous potential of those bacteria that showed *in vitro* antifungal activity. The short-term

baiting method (referred to as "liquid hyphal-baiting") is selecting for fast attaching bacteria of which several can use fungal compounds as the only source of energy. In the long-term "transfer-enrichment" approach, the most successfully growing mycophagous bacteria are transferred to a new microcosm, getting a head start in colonizing a "fresh" fungal host. Here, the bacteria that successfully compete with co-colonizers are enriched. The "liquid hyphal-baiting" method had already successfully been used in a previous study (*chapter five*).

FUNGAL INHIBITION

Since antifungal activity appears to be an important factor for mycophagous growth of *Collimonas* bacteria, all hyphae colonizing bacteria were screened for inhibition of the host fungus. Antifungal bacterial strains assigned to the genera *Rahnella, Pseudomonas, Pedobacter, Pantoea, Luteibacter, Leifsonia, Erwinia, Agrobacterium* and *Burkholderia* were obtained from more than one fungal species. Notably, those generalists were also the most abundant ones, making up for up to 80 % of the antifungal, hyphal-adhering isolates. Species of the genera *Rahnella, Pseudomonas* and *Burkholderia* have already been described as „universal fungiphilic", based on their ability to adhere to hyphae of a range of fungi (Warmink *et al.* 2009). Our study indicates that those bacteria are not only able to colonize a range of fungi but that they are also able to inhibit their growth. Specialist colonizers, i.e. bacterial species only found with one fungus, were generally not very abundant.

ISOLATION OF MYCOPHAGOUS BACTERIA

Both methods were shown to be successful in obtaining mycophagous bacteria, yet, they yielded different sets of isolates. The "liquid hyphal-baiting" method selected for a diverse community of bacterial attachers and a high number of fungus feeders. Especially *M.hiemalis* attracted a diverse community of antifungal bacteria of which 80 % was mycophagous (average mycophagy ratio 9.1). At the genus level we observed overlaps in mycophagous isolates obtained with both methods (Fig 6.3). This was, however, rarely found at the species level (Table 6.2).
The bacterial community, recovered with the "transfer-enrichment" method had a lower diversity (Table 6.1). This was probably due to the nature of the method: the repetitive stamping might have enriched only for the fastest and/or most competitively growing mycophagous bacteria. Only successful hyphal colonizers were enriched and transferred ("stamped") to the next microcosm.

PHYLOGENETIC RANGE OF MYCOPHAGOUS BACTERIA ISOLATED

We isolated a broad range of mycophagous bacteria with the two methods applied. Compared to dilution plating on chitin-yeast agar in combination with a rather laborious sand microcosm assay which has been used to isolate and demonstrate mycophagous growth of collimonads (De Boer *et al.* 2001), baiting mycophagous bacteria with growing fungi as the only source of carbon is a superior, more efficient isolation method. The baiting methods clearly showed that mycophagy is not restricted to collimonads. In fact, we did not succeed to isolate collimonads with the "transfer-enrichment" method. Also the previous study only reported the isolation of one single *Collimonas* strain by the "liquid hyphal-baiting" method. The reason for this might be

the generally very low abundance of collimonads in rhizosphere bacterial communities (Hoppener-Ogawa *et al.* 2007). The two baiting methods allowed for the discovery of mycophagous bacteria, belonging to different taxonomic groups. Our study also shows that the class *Burkholderiales* which is known to be engaged in interactions with eukaryotic hosts harbors many other mycophagous bacteria besides the genus *Collimonas*. It is remarkable that none of the fungus associates isolated belonged to the bacterial phylum of *Firmicutes*. *Bacilli* and especially *Paenibacilli* are prominent genera of the *Firmicutes* which have been reported to be able to colonize hyphae of both, plant pathogenic fungi but also beneficial ectomycorrizal fungi (Dijksterhuis *et al.* 1999; Toljander *et al.* 2006). The absence of *Firmicutes* in our study may be due to the fact that the appropriate hosts were not included.

NON-MYCOPHAGOUS BACTERIA

The ability to metabolize fungal-derived carbon as the only source of energy varied among the "generalist" hyphal colonizers. A range of bacteria seems to be able to attach to fungal hyphae. However, for several bacterial isolates colonization of a fungal host appeared not to be associated with the ability to feed on that fungus, despite the fact that they had inhibitory activity against the host fungus in *in vitro* screenings. This could mean that those bacteria lack the specific molecular machinery to attack the fungus thereby being restricted to feed on energy resources leaking out of the fungus. If these energy resources are leaking out of the fungus as result of the activities of mycophagous bacteria the growing of non-mycophagous attaching bacteria could be considered as cheaters (profit without investment) (Hibbing *et al.* 2010). They could also be using the fungal hyphae for translocation. Several of the isolated genera (*Burkholderia*, *Agrobacterium*, *Pseudomonas* e.g.) have been described to be able to move along "fungal-highways", possibly to reach favorable habitats (Warmink *et al.* 2011; Bravo *et al.* 2013).

MYCOPHAGOUS OR POTENTIAL MYCOPHAGOUS?

According to the definition given by Leveau and Preston (2008) mycophagous bacteria should be actively involved in getting access to fungal nutrients, e.g. by causing leakage of fungal membranes. The mycophagy test on phytagel could also indicate a positive response of isolates that merely grow on fungal exudates without any impact of the bacteria on the efflux of fungal nutrients. To prove that bacterial isolates are real mycophagous detailed studies, including microscopic observation and determination of growth responses of mutants, are needed for each bacterial-fungus combination. Yet, we think that our assay is pointing at real mycophagous bacteria as we focused only on bacteria with antifungal properties. Many compounds with antifungal activity disturb the cell membrane integrity and cause leakage (Ghannoum & Rice 1999). In addition, the fact that many hyphal-adhering isolates did not show a growth response when encountering hyphae on phytagel makes it less likely that the passive efflux of fungal exudates on this nutrient-poor medium was sufficient to sustain bacterial growth.

BIOCONTROL AND HOST FUNGUS EFFECTS

Mycophagous bacteria may be interesting candidates for application as biocontrol agents as they are directly targeted to grow at the expense of fungi. This could make them less vulnerable for problems with rhizosphere establishment as they do not have to compete with indigenous bacteria for root exudates. Establishment of biocontrol strains is thought to be one of the major causes of the inconsistency of biocontrol effects observed (Fravel 2005). For biocontrol purposes, quick and specific colonization of soil-borne fungal pathogens by antifungal, mycophagous bacteria would be desirable. In addition, mycophagous biocontrol bacteria should not harm beneficial fungi such as mycorrhizae.
We found that mycophagous "specialists" that selectively colonize and feed on the pathogenic fungus *R. solani* did only make up a minority of the fungus colonizing bacterial community. Inoculation of rhizosphere soil with such fungus-specific bacteria in high concentrations may help those bacteria to dominantly colonize the hyphae of the target fungi and to exert control on them. An alternative approach could be to use "generalist" mycophagous bacteria that have a preference for feeding on pathogenic fungi. Theoretically, those bacteria would have a better chance to establish themselves as they are not dependent on the presence of specific fungal pathogens. Hence, sustaining of such bacteria on non-pathogenic fungi could create a long term protection against invading pathogenic fungi.

FACTORS INFLUENCING COLONIZATION

An interesting question is how the bacterial colonization may take place and why there is selectivity in the colonization of different fungal species. On the one hand, the fungus could try to avoid bacterial colonization with deterring bacteria actively (e.g. antibacterial metabolite secretion (Mela et al. 2011)) or passively (cell wall/hyphal surface properties e.g. (Free 2013)). On the other hand, bacteria might be able to sense fungal exudate patterns and may use different mechanisms to attach to the hyphal surface and obtain fungal nutrients (Leveau & Preston 2008; Nazir et al. 2010). It has been shown that fungi as well as bacteria actively respond to one another's presence at the transcriptomic level (Mela *et al.* 2011; Deveau *et al.* 2014). Unraveling the mechanisms and compounds involved in cell wall modulation and host recognition remains to be a challenge.

POTENTIAL PLANT PATHOGENS

With the "liquid hyphal-baiting" method, it was already shown that several bacteria colonizing hyphae of *M. hiemalis* and *T. harzianum* belong to genera that harbor plant-pathogenic bacteria (e.g. genera *Curtobacterium, Enterobacter* and *Leifsonia*) (*chapter five*). Using the "transfer-enrichment" method, we found more bacteria belonging to genera with plant-pathogenic representatives (e.g. *Pantoea, Agrobacterium* and *Erwinia*). The repeated isolation of potential plant-pathogenic bacteria with the different baiting methods may indicate that the fungal hyphae provide plant-pathogenic bacteria with a good habitat. The benefit of associating with an alternate eukaryotic host is unclear. Since many saprotrophic and pathogenic fungi are able to colonize plant roots endophytically, the colonization of fungal hyphae in combination with movement along the fungal highway might enable those potential plant pathogens to infect plant roots using the fungus as a vector. This is known for other host-bacterium interactions.

The *Vibrio cholera* infection process is, for example, facilitated by the colonization of a protozoan vector which is subsequently taken up by the human host through contaminated drinking water (Nelson *et al.* 2009). Potential plant pathogens could also benefit from colonizing and feeding on alternate eukaryotic hosts like fungi under circumstances when their preferred plant host is not available. This could, for example, be the case for seasonal crop plants that only represent a favorable host during growing season. Once such crops get decomposed in autumn, the fungal community would serve as carbon source until the next growing season begins.

SUMMARY

We present an innovative transfer-enrichment approach to enrich antifungal, mycophagous bacteria from soil and compared it to another short-term baiting method that we already successfully used for the isolation of mycophagous bacteria. Both methods retrieve distinct, phylogenetically diverse sets of inhibitory, mycophagous bacteria indicating that the potential to grow on fungal resources is widespread among rhizosphere bacteria.

ACKNOWLEDGEMENTS

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CHAPTER SEVEN

GENERAL DISCUSSION

WHAT WE KNEW ABOUT MYCOPHAGY

COLLIMONAS

When I started with my PhD thesis at the NIOO in April 2010, the first *Collimonas* bacteria had already been dug up from coastal sandy dune soils 12 years ago (De Boer *et al.* 1998). They were first considered as pseudomonads before later on three species were formally described by de Boer *et al.* (2004) and Hoppener-Ogawa *et al.* (2008). It was discovered that those dune soil bacteria were able to feed on living fungal hyphae as their only source of energy and carbon, a lifestyle that is called "mycophagy". The existence of bacteria able to feed on fungi had been well perceived by soil scientists who began to describe and isolate *Collimonas* strains all around the world (Mannisto & Haggblom 2006; Uroz *et al.* 2007; Hakvåg *et al.* 2009; Leveau *et al.* 2010).

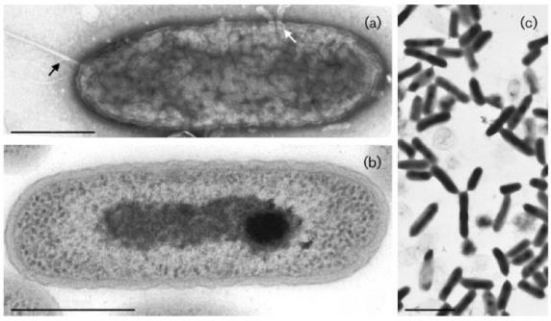

Fig 7.1 Transmission electron micrographs of *Collimonas* isolates. Highlighted are a) flagellum b)Gram negative cell wall structure and c) general morphology (de Boer *et al.* 2004).

MOLECULAR MECHANISMS

Collimonas fungivorans Ter331 quickly became the model organism to study mycophagy. Much effort was put into the understanding of the molecular machinery of mycophagy. It was recognized that not only chitinolytic enzymes (De Boer *et al.* 2001; Fritsche *et al.* 2008) but also antifungal compounds were contributing to the mycophagous activity (Mela *et al.* 2011). Investigations on the antifungal metabolites continued and finally a cluster of genes was described, probably coding for a broad spectrum antifungal polyyne, named "collimomycin" (Fritsche *et al.* 2014).

TRAITS AND ECOLOGY

Next to explorations of the molecular antifungal repertoire, research focused on the understanding of the bacterium-fungus interaction from an ecological perspective. Collimonads were shown to be not only present in sandy dune soils but were also isolated from a variety of other habitats, mostly sharing relatively low pH, high sand content and presence of fungi (Hoppener-Ogawa *et al.* 2007). It was shown that upon introduction of fungi

in natural soils, indigenous collimonads were able to increase in biomass (Hoppener-Ogawa *et al.* 2009b). Moreover, experiments had indicated that not all fungi stimulated growth of collimonads equally (DeBoer et al., 2001, 2004). These feeding preferences might have been the reason for *Collimonas*-induced changes in soil fungal community composition, later on observed by Hoppener-Ogawa *et al.* (2009a). Knowledge on mycophagous growth of collimonads was applied in experiments performed to evaluate the possible biocontrol of phytopathogenic fungi of tomato (Kamilova *et al.* 2007). In France, the group of Pascale Frey-Klett discovered (Uroz *et al.* 2007) and explored (Koele *et al.* 2009; Uroz *et al.* 2009b; Leveau *et al.* 2010) another ecologically very interesting trait of collimonads, namely the remarkable ability to solubilize inorganic nutrients from minerals (mineral weathering). Interestingly, at the same time collimonads were found to be among the first colonizers of sterilized patches of water saturated soils (water potential -1 kPa) (Wertz *et al.* 2007), hinting at the importance of another *Collimonas* trait: motility.

WHAT DID I FIND OUT ABOUT MYCOPHAGY?

When I started my PhD many puzzle pieces that gave us an idea of the ecology and evolution of this obstinate soil bacterium (abundances normally ranging between 10^3 and 10^5 cells/gram of soil (Hoppener-Ogawa *et al.* 2007)) had been collected but important parts were still missing.

TAXONOMY OF MYCOPHAGOUS BACTERIA

Collimonads were the first soil bacteria for which mycophagous growth has been unambiguously demonstrated, namely without possible interference of other available nutrients (De Boer *et al.* 2001). Thus, almost all work on molecular but also ecological aspects of mycophagy has subsequently focused on the genus *Collimonas*. An important part of my PhD project was to find other mycophagous bacteria besides *Collimonas*. Using two innovative methods, one "bait-method" (based on Scheublin *et al.* (2010)) and one "enrichment-method" (based on replica plating originally introduced by Lederberg and Lederberg (1952)), I successfully isolated mycophagous soil bacteria of various genera. Main findings were the existence of generalist and specialist bacteria concerning the attachment as well as the ability to feed on fungal hyphae of different taxonomical origin and the high abundance of potential plant pathogenic bacteria among fungal hyphae colonizers.

RHIZOSPHERE IMPORTANCE

Although the research of Hoppener-Ogawa *et al.* (2009a and 2009b) had already demonstrated that consumption of living fungal tissue by collimonads can be an ecologically relevant process in soil systems we did not have knowledge on the importance of bacterial mycophagy in the rhizosphere. I collected bacterial communities and abundant fungi from the rhizospheres of the sedge *Carex arenaria* and the grass *Festuca rubra*, identified members of these communities that were found to attach to fungal hyphae, and quantified mycophagous abilities with a newly developed assay. My main findings from this work were: a) the ability to grow mycophagous is taxonomically widespread among rhizosphere bacteria, b) there is differentiation in the ability to exploit different fungal species and c) the attachment to the host fungus happens quickly - a diverse community of bacteria was recovered after only 24 hours. Since fungi have repeatedly

been shown to be important primary consumers of root exudates (Denef *et al.* 2007; Buee *et al.* 2009; Hannula *et al.* 2012), these results strongly suggest that mycophagy (essentially representing a way to feed on root derived nutrients as a secondary consumer) could be an important rhizosphere process. Many bacteria seem to have acquired this nutritional strategy, possibly in order to avoid competition in the immediate rhizosphere.

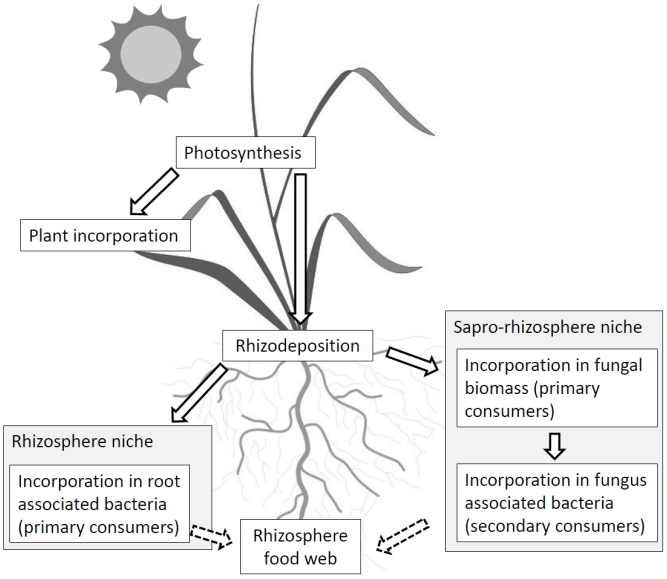

Fig 7.2 Proposed main energy fluxes to bacteria in the "sapro-rhizosphere" and rhizosphere.

THE NATURE AND EVOLUTION OF THE INTERACTION

Although genetic and functional divergence in the genus *Collimonas* was already indicated with the description of 3 species (de Boer *et al.* 2004; Hoppener-Ogawa *et al.* 2008), little attention had been paid to divergence in traits related to the mycophagous phenotype. Therefore, I characterized the interaction between host fungi and *Collimonas* bacteria in order to further elucidate the evolution of mycophagy. The principle results of this project were: a) The construction of a high-resolution phylogeny of collimonads, b) trait investment of collimonads is phylogenetically conserved (carries phylogenetic signal).

LOCALIZATION OF THE FUNGAL HOST

Previous experiments carried out by Mela *et al.* (2011) indicated that during the confrontation with the fungal host, genes of *Collimonas fungivorans* Ter331 related to oxalic acid metabolism as well as genes related to motility are up-regulated. Since oxalic acid is rather ubiquitously secreted by fungi, I had the idea that it might be used by *Collimonas* as a signal to guide collimonads towards fungal hyphae. In a series of experiments I was able to show that oxalic acid had a reverse concentration dependent effect on motility of collimonads. Furthermore, I

could show that oxalic acid can hardly be used as a carbon source, further underlining its role as a signal molecule in the fungal bacterial interaction.

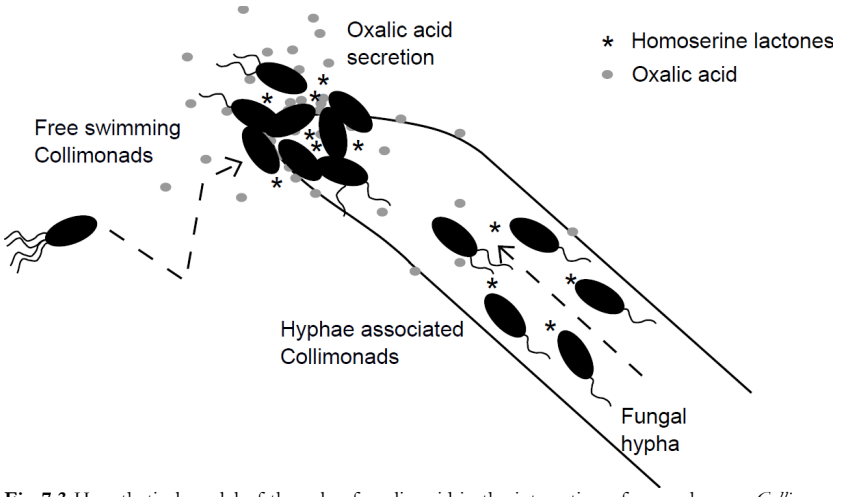

Fig 7.3 Hypothetical model of the role of oxalic acid in the interaction of mycophagous *Collimonas* bacteria and their fungal host. Bacteria are attracted by oxalic acid, move towards the hyphal tip (either through the soil or along fungal hyphae), and finally accumulate at the tip which is the spot where oxalic acid concentrations are highest. Once high cell densities are reached they start feeding on the fungus, possibly mediated by quorum sensing. Also movement of bacteria in groups (swarming) might be coordinated by quorum sensing.

MOTILITY AND COLONIZATION OF NEW HABITATS

While doing motility experiments with collimonads I was intrigued by their ability to quickly move through sterilized, but also living soil and I decided to continue to study the movement of single bacterial strains and of bacterial communities. I investigated bacterial abilities to colonize new soil habitats with using traditional microbiological in combination with next generation sequencing approaches. I could show that also in comparison with other soil bacteria, collimonads were capable of quickly spreading through sterile soil. Interestingly, low rather than high nutrient concentrations stimulated their movement. Looking at movement in a community context, I found that the family *Enterobacteriacea* is rapidly colonizing unoccupied soil patches.

WHAT DO WE STILL NEED TO KNOW ABOUT MYCOPHAGY?

APPLICATION OF MYCOPHAGOUS BACTERIA

Studies on collimonads had already revealed that certain traits of mycophagous bacteria, such as fungal inhibition, mycophagy and mineral weathering, can potentially be exploited for use in agriculture (Kamilova *et al.* 2007; Uroz *et al.* 2009b). There is a clear need for sustainable control of plant pathogenic fungi. In addition, depleting phosphate sources world-wide create the demand for a higher efficiency of the use of soil phosphorous resources by crops. The

application of bacteria that enhance the availability of inorganic soil phosphorus sources for crops is a promising possibility that deserves further exploration.
Although there is already plenty of work on P-solubilization by *Collimonas* bacteria (Collignon *et al.* ; Uroz *et al.* 2007; Koele *et al.* 2009; Uroz *et al.* 2009b; Lapanje *et al.* 2011) there is only limited knowledge on the mechanisms used. It has been suggested that those mechanisms would be siderophore production or acidification but on the molecular level, evidence is missing. Acidification would only make sense in neutral or alkaline environments, whereas most collimonads were isolated from slightly acidic soils. Since collimonads have been shown to efficiently weather inorganic nutrients in laboratory experiments, a further understanding of the involved mechanisms could prove fruitful as a next step to reach application of potent P-solubilizers in the field.
With respect to pathogen control, the results of my thesis show that the ability to colonize and feed on hyphae of different fungi differs between mycophagous isolates. I found indications that mycophagy seems to be an important strategy for the acquisition of plant-derived carbon in the rhizosphere. We have to extend our knowledge on the community composition of sapro-rhizosphere bacteria in order to make a targeted inoculation or stimulation of indigenous soil bacteria for fungal pest control possible. Here, it will be important to screen for mycophagous bacteria that specifically feed on a pathogenic fungus, are not harmful to mutualistic soil fungi and can permanently establish in the rhizosphere.

ASSEMBLY OF FUNGAL HYPHAE ADHERING BACTERIAL COMMUNITIES

Preliminary experiments done for the research described in chapter *five* showed that the diversity (colony morphologies) of chitinolytic bacteria that attach to fungal hyphae of different species peaks after 24 hours of hyphal exposure to a soil bacterial suspension. What became apparent from those experiments is that the community of (chitinolytic) hyphal attachers is quickly changing over time. It would be interesting to look at these dynamics during microbial community assembly processes in more detail. Bacterial communities on fungal hyphae do not seem to be very diverse, comprising around 100 operational taxonomical units at maximum. They are ideally suited to study bacterial community assembly. One could test macroecological concepts like succession during the colonization of new habitats or investigate invasibility of such habitats by pathogenic bacteria (those have been shown to be very abundant colonizers of fungal hyphae in some cases).

MECHANISMS OF RECOGNITION AND SELECTIVITY

In order to successfully apply mycophagous biocontrol bacteria in the field, but also in order to predict bacterial community assembly, it is important to understand the mechanisms of recognition that finally give rise to fungus-specific associated bacterial communities. In this context a number of questions remain to be solved: who imposes selection on whom? Is it the bacteria that select the hyphae that they colonize or is it the fungi that select for the bacteria by which they want to be colonized? If the bacteria are choosing, what is the basis for their choice? There is evidence that fungi produce specific exudates and have distinct cells wall components (Keller *et al.* 2005). I obtained evidence that collimonads might use oxalic acid as a signal molecule (chapter *two*). Other mycophagous bacteria could use similar compounds to recognize their host fungus. Fungi have very different cell wall structures and those may

provide specific surfaces to the bacterial attachers. Hence, if fungi impose selectivity on the bacterial colonizers, this could be realized through composition of the hyphal surface and the secretion of attracting or detracting metabolites.

NATURE OF THE FUNGUS-BACTERIUM INTERACTION

Chapter *four* reports on indications for the evolution of distinct sets of traits of collimonads that could define the interaction with the fungal host. From this study, it became apparent that collimonads differ in mycophagous abilities. Some even seem to be able to carry out functions like mineral weathering that are beneficial for the host. I also found that certain habitats seem to favor collimonads possessing a certain set of traits. It would be interesting to further investigate the relative contribution of macro-habitat (plant species, ecosystem types etc.) or micro-habitat (pore size, water holding capacity, nutrient content) characteristics on influencing the relative abundance of potentially mutualistic or parasitic mycophagous bacteria. Furthermore, the nature of the interaction between the newly discovered mycophagous bacteria (chapters *five* and *six*) should be elucidated. Why do mycophagous bacteria have host preferences? Do those bacteria also restrict benefits like mineral weathering to specific hosts? How fast can a beneficial interaction become a parasitic one (or the other way around)?

THE IMPORTANCE OF (MYCOPHAGOUS) BACTERIAL MOTILITY

Even though soil harbors a great number of bacteria, it is generally not very crowded under our feet. In fact, most soil micro-habitats have been predicted to be empty most of the time. A reason for this is the high structural complexity (huge surface area of soil particles), mainly driven by differences in water filled soil pore space and varying nutrient distribution (Young *et al.* 2008). I found indications that hyphae-associated bacteria are able to quickly move through unoccupied soil habitats (chapter *three*) and that oxalic acid (a major fungal metabolite) could act as a signal molecule for mycophagous collimonads. Other work on movement of fungus-associated soil bacteria shows that those bacteria are able to move along fungal hyphae in order to bridge air-filled soil pore spaces with fungal assistance (Kohlmeier *et al.* 2005). Taken together, these results suggest that (fungus mediated) dispersal of fungus-associated bacteria might be important for the colonization of new soil microhabitats. In the succession of the colonization of soil microhabitat space those bacteria would be the pioneers. This aspect is certainly worth to be investigated further. The colonization of microhabitats could be the first step for microbial community development in empty soil habitats. Understanding of microbial community assembly in soil microhabitats is not only one of the most important aspects in advancing our fundamental understanding of soil microbiology. It is also of very important for the sustainable application and establishment of biocontrol bacteria in their native habitat.

IMPORTANCE OF ENERGY FLOW THROUGH THE SAPRO-RHIZOSPHERE

Although we already found that the sapro-rhizosphere harbors a diverse community of bacteria and we showed that those bacteria are able to exploit the fungus as a carbon and energy source, it is not clear how important this associated bacterial community is when being compared to the rhizosphere effect (microbial community selected in the immediate plant vicinity). It is important to go one step further, to quantify the contribution of the sapro-

rhizosphere to the total rhizosphere carbon flow into to the associated food web. This could be realized by stable-isotope-probing approaches, enabling the researcher to follow the flow of heavy carbon isotopes through the plant, into the (sapro-)rhizosphere.

MECHANISMS OF MYCOPHAGOUS BACTERIAL FEEDING

We know that in the interaction between different strains of mycophagous collimonads and different host fungi, various, sometimes strain specific enzymes, antibiotics, volatiles and many other bacterial metabolites are secreted (Charles & Allan 2002; Fritsche *et al.* 2008; Mela *et al.* 2011; Mela *et al.* 2012; Fritsche *et al.* 2014). We already observed variation of secretion of those antimicrobial compounds among *Collimonas* strains. The role and the timing of secretion of those compounds in the process of feeding on fungi is, however, not very clear. It has recently been shown that *Burkholderia rhizoxinica,* a bacterium related to collimonads, uses the type-2-secretion system in combination with chitinases to invade fungal hyphae (Moebius *et al.* 2014). Those chitinases are expressed by bacteria colonizing the hyphal surface. These findings suggest that for collimonads, chitinases might play an important role in mycophagy, though only after contact with the fungus has been established.
It is also tempting to speculate that with taxonomically more distant species the number of new, undescribed compounds involved in the interaction should arise. Since those compounds could either be used to defend the fungal hypha against competing mycophagous colonizers or to attack the fungal host itself, one would expect the discovery of antibacterial as well as antifungal compounds.

ON PROGRESS IN MICROBIAL ECOLOGY

Microbial Ecology has made big progress in the last years. Methodological developments especially in sequencing technologies made it possible for scientists to identify most dominant bacterial genera in their dogs poo (Hibbing *et al.* 2010), on their keyboards (Munkemuller *et al.* 2014), in space crafts (Matias *et al.* 2014) and many other more or less relevant microbial habitats (actually there are few places that cannot be considered as a microbial habitat). Sometimes the long lists of microbial taxa and their correlations with abiotic or biotic factors reminds one of the birth days of natural history when Lineé and Humboldt set out to systematically describe macroorganisms on this planet (Humboldt however made remarkable contributions to biogeography later on (Humboldt & Bonpland 1807)). On the contrary to the taxonomic catalogization of macroorganisms, there are several aspects that hamper the complete taxonomical inventory of microorganisms and their habitats. Those are for example: a) the lack of a bacterial species definition, b) the fast generation times that together with interspecies horizontal gene transfer dramatically influence the speed of bacterial evolution, c) the fact that we still do not know at what scale we should look at microbiological processes in soil. This also distorts correlations with soil biotic and abiotic factors because those are probably also measured at the wrong scale.
In my opinion, explorative studies that are used for correlations with biotics or abiotics should only be conducted to create hypotheses that can afterwards be tested in controlled experiments (or the other way around). It is a pity that most correlative analyses are the end- rather than the starting point of a study and that those studies are still embraced and readily published by some journals in microbial ecological research. Instead of the description of never-ending

microbial diversity, efforts should be undertaken to understand the formation of diversity. Microbial ecosystems are the perfect model systems for such studies. The number of species that can be used in an experiment, combined with short microbial generation times allow to observe ecology shaping evolution in "realtime". This is a luxury that makes every macroecologist envious.

In order to progress in Microbial Ecology, experimental studies should be designed to test specific hypotheses. Focus should be put on testing macroecological theory with using the appropriate, not necessarily the most high-tech methodology.

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SUMMARY

Fungi and bacteria are two major microbial groups in the soil. They are responsible for elemental cycling in the rhizosphere and therefore provide the basis for plant nutrition. They also form the basis of the soil food web. Most fungal and bacterial species obtain metabolic energy for growth from oxidation of organic compounds. The availability of easily degradable organic compounds is limiting the growth of free-living bacteria and fungi in soils. Several strategies have evolved to compete for the limited organic energy resources. Bacteria of the genus *Collimonas* developed a different way to obtain organic nutrients. Those soil bacteria are able to exploit living fungi as a source of energy. This strategy has been termed "mycophagy". The research described in this thesis is on bacterial mycophagy and involves two major themes: 1) Investigation of strategies and traits that are important for *Collimonas* bacteria to perform a mycophagous lifestyle, 2) Investigation of occurrence of mycophagy among other soil bacteria.
Chapter *two* is zooming in on the mechanisms that enable collimonads to find fungal hosts in soil. Oxalic acid is a common metabolite exuded by a range of fungal species for different purposes, such as lignin degradation and mineral weathering. In this chapter, research on the role of oxalic acid in the interaction between mycophagous *Collimonas* bacteria and fungi is presented. Evidence is provided that oxalic acid is used as a signal molecule by collimonads in order to find a fungal host (the source of secretion,) and move towards it.
In chapter *three* a closer look is taken at the motility of collimonads in a soil-like matrix. It was shown that expansion of *Collimonas* bacteria in sandy microcosms is stimulated by low availability of organic substrates. This indicates (together with the findings from chapter two) that motility is important for *Collimonas* bacteria to explore the soil environment for fungi. Chapter *three* also describes the dynamics of colonization of sterile soil patches by bacteria from natural soil communities. It is shown that the early colonizers consist of a few bacterial taxa, mostly belonging to the *Enterobacteriaceae*.
Chapter *four* deals with the question how characteristics (traits) related to the interaction of collimonads with their fungal host are reflected by their phylogenetic relatedness (carry phylogenetic signal). Multi Locus Sequencing Analysis (MLSA) as well as a screening of functional genes and phenotypes was performed for 88 *Collimonas* isolates obtained from different collections. The main findings of this work are that a) MLSA indicates a fine-scale phylogenetic differentiation of collimonads that was not apparent from 16S rDNA analysis b) certain mycophagy-related functional traits seem to follow the phylogenetic clustering of collimonads.
At the start of this thesis project almost all information on bacterial mycophagy was based on *Collimonas* research. In chapter *five* and *six* results of screenings for other mycophagous bacteria than collimonads are described. In Chapter *five,* a short-term liquid baiting approach that selects for hyphal-adhering, antifungal bacteria within natural communities of rhizosphere bacteria was applied. Subsequent testing of these bacteria for their potential to use living fungi as only energy resource indicated that the potential to grow mycophagous is taxonomically wide-spread among rhizosphere bacteria. By combining these observations with the increasing evidence that saprotrophic fungi have been underestimated as primary consumers of root-derived compounds the "Sapro-Rhizosphere" concept is being proposed which states that a

substantial amount of plant derived carbon that is channeled through rhizosphere fungi (primary consumers) might be finally consumed by mycophagous bacteria (secondary consumers).

Chapter *six* describes the use of a long-term baiting technique for selecting mycophagous rhizosphere bacteria and compares the results with the short-term baiting technique described in Chapter *five*. Although there was some overlap, the two different methods mainly enriched different sub-sets of mycophagous rhizosphere bacteria. Both baiting approaches revealed specialist and generalist mycophagous feeders among the hyphal-adhering bacterial isolates. Interestingly, both methods did also reveal that a surprisingly high amount of mycophagous bacteria seem to belong to genera well known to harbor plant pathogenic strains.

Taken together, with using molecular biological as well as microbiological methods, this thesis further extends our knowledge on the ecology of mycophagous *Collimonas* bacteria and highlights the importance of mycophagous bacteria in the rhizosphere.

SAMENVATTING

In de bodem wordt de afbraak van organische stoffen gedomineerd door twee groepen van micro-organismen: schimmels en bacteriën. De anorganische verbindingen die hierbij vrijkomen vormen belangrijke minerale voedingsstoffen voor planten. Naast het belang van schimmels en bacteriën in de cycli van elementen staan ze ook aan de basis van het bodemvoedselweb. De energie die schimmel- en bacteriesoorten nodig hebben om te kunnen groeien wordt voor verreweg de meeste soorten verkregen uit de oxydatie van organische verbindingen. De beschikbaarheid van deze energiebronnen, en met name die van de makkelijk afbreekbare organische verbindingen, is een echter een beperkende factor voor de groei van de meeste bodemmicro-organismen. Om met deze schaarste van voedingsstoffen om te kunnen gaan hebben zich verschillende voedingsstrategieën ontwikkeld. Een bijzondere aanpassing is gevonden voor bacteriën van het geslacht *Collimonas*. Deze bacteriën zijn in staat om te groeien op voedingsstoffen die ze onttrekken aan levende schimmels; een strategie die mycofagie wordt genoemd. Het onderzoek dat in dit proefschrift is beschreven gaat over bacteriële mycofagie en kent twee hoofd-themas: 1) Onderzoek naar de strategieën en eigenschappen die *Collimonas* bacterien in staat stellen om mycofaag te kunnen groeien, 2) Onderzoek naar het voorkomen van mycofage groei bij andere bodembacteriën.

In Hoofdstuk 2 wordt ingegaan op de mechanismen die *Collimonas* bacteriën in staat stellen om schimmels in de bodem op te sporen. Daarbij wordt met name de rol van oxaalzuur belicht. Oxaalzuur wordt door heel veel soorten schimmels uitgescheiden voor verschillende doeleinden, zoals lignine afbraak en het vrijmaken van minerale voedingsstoffen. In dit hoofdstuk, wordt het onderzoek beschreven dat is gedaan naar de rol die oxaalzuur speelt bij de interacties tussen *Collimonas* bacteriën en schimmels. De resultaten geven aan dat oxaalzuur kan fungeren als signaalstof voor *Collimonas* bacteriën, waardoor ze in staat zijn de schimmel als bron van oxaalzuur te traceren en er naar toe te bewegen.

In Hoofstuk 3 wordt het onderzoek naar bewegelijkheid van *Collimonas* bacteriën in zand beschreven. Het onderzoek toont aan dat de bewegelijkheid van *Collimonas* bacterien in zand groter is bij een laag aanbod aan organische voedingsstoffen. Samen met de resultaten van Hoofdstuk 2 geeft dit aan dat bewegelijkheid een belangrijke factor is bij het "zoeken" van schimmels in de bodem door *Collimonas* bacteriën. In Hoofdstuk 3 wordt ook de kolonisatie van steriel zand vanuit een natuurlijke soortenrijke gemeenschap van bodembacteriën beschreven. De vroegste kolonisten bestaan slechts uit een gering aantal soorten, voornamelijk behorende tot de familie *Enterobacteriaceae*.

Hoofdstuk 4 richt zich op de vraag of variatie in karakteristieken (eigenschappen) die mogelijk van belang bij interacties tussen *Collimonas* bacteriën en schimmels een fylogenetisch signaal hebben, dat wil zeggen of ze gerelateerd zijn aan de fylogenetische verwantschap tussen *Collimonas* stammen. Daartoe werd een Multi Locus Sequencing Analyse (MLSA) gedaan voor 88 *Collimonas* stammen verkregen uit verschillende collecties en deze resulaten werden gekoppeld aan een inventarisatie van fysiologische-, morfologische- en biochemische eigenschappen. De belangrijkste bevindingen zijn dat a) met MLSA een fijnschalige fylogenetische indeling onthuld wordt die niet wordt verkregen met verwantschapsanalyses op

basis van 16s rDNA, en b) dat het voorkomen van een aantal mycofagie gerelateerde functionele eigenschappen de fijnschalige fylogenetische verwantschap lijken te volgen.
Aan het begin van dit promotieonderzoek had vrijwel alle informatie over bacteriële mycofagie betrekking op *Collimonas* bacteriën. In de hoofdstukken 5 en 6 worden de resultaten beschreven van inventarisaties naar het voorkomen van mycofagie bij andere bodembacteriën In hoofdstuk 5 wordt een "mycofagie-screening" geintroduceerd die gebaseerd is op snelle aanhechting van bodembacteriën aan schimmeldraden in een vloeibaar medium. Een deel van de aangehechte bacteriën, namelijk degene met schimmelremmende eigenschappen, werden getest op hun vermogen om te kunnen groeien met alleen levende schimmels als bron van voedingsstoffen. Dit onderzoek toonde aan dat mycofagie wijdverbreid voorkomt bij bacteriesoorten die in de wortelomgeving leven. Op basis van deze observatie en de toenemende aanwijzingen dat saprofytische schimmels belangrijk kunnen zijn als directe consumenten van wortel exudaten is het "Sapro-Rhizosfeer" concept voorgesteld. Dit concept houdt in dat een aanzienlijk deel van de rhizosfeer bacteriën niet rechtstreeks groeien (primaire consumenten) op de door de wortel afgescheiden organische verbindingen maar indirect (secundaire consumenten) via consumptie van rhizosfeer schimmels.
Hoofdstuk 6 beschrijft een techniek waarbij mycofage bacteriën gedurende opeenvolgende incubaties met schimmelhyfen worden opgehoopt. De resultaten van deze techniek zijn vergeleken met die van de "snelle aanhechtingstechniek" beschreven in hoofdstuk 5. De samenstelling van mycofage bacteriesoorten die werden opgehoopt verschilde sterk tussen de gebruikte technieken. Bij beide technieken werden bacteriën gevonden die in meer of mindere mate gespecialiseerd leken wat betreft het vermogen om op verschillende schimmelsoorten te groeien. Beide technieken leverden ook een aantal mycofage bacteriesoorten op die bekend staan als ziekteverwekkers van planten.
Alles bij elkaar genomen heeft het onderzoek beschreven in dit proefschrift meer inzicht verschaft in de ecologie van mycofage *Collimonas* bacteriën en in het belang van mycofagie als voedingsstrategie voor bodembacteriën.

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CURRICULUM VITAE:

Max-Bernhard Rudnick was born on the 11th of April 1983 in Recklinghausen, Germany. After finishing high school in 2002, he did his civil service at a school for physically and mentally handicapped children, in Recklinghausen. Driven by his love for nature and the curiosity to find out how it works, he afterwards decided to study Biology at the University of Osnabrück, Germany. His growing interest in aquatic organisms and marine habitats as well as the urge for more fieldwork made him successfully apply for a DAAD fellowship to study one year at the University of Costa Rica in San José, Costa Rica. After returning from Costa Rica and multiple watery transects later, it became clear that he was not made for studying marine habitats. In his diploma thesis (2009), supervised by Teja Tscharntke (University of Göttingen) and Till Eggers (University of Osnabrück), focusing on agriculturally used soils, he successfully combined his interests in Ecology and Microbiology. In his thesis he studied the effect of the landscape surrounding an agriculturally used field on the microbial community composition and diversity. In the year 2010, Max started with his PhD project at the Netherlands Institute of Ecology, which is presented in this thesis. Max now works as a postdoctoral fellow at the Leibniz Institute of Vegetable and Ornamental Crops in Großbeeren, Germany.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- The importance of mycophagy (feeding on fungi) for the bacterial genus *Collimonas* and other soil bacteria

Writing of project proposal (4.5 ECTS)
- The importance of mycophagy (feeding on fungi) for the bacterial genus *Collimonas* and other soil bacteria

Post-graduate courses (3.9 ECTS)
- Soil ecology; PE&RC (2010)
- EPS Autumschool host microbe interactions; EPS (2011)
- Evolution and ecology of host associated microbiota; Basel University (2012)

Laboratory training and working visits (0.3 ECTS)
- Interactions of Protozoa with the below-ground community; Terrestrische Ökologie, Köln, Germany (2011)

Invited review of (unpublished) journal manuscript (2 ECTS)
- FEMS Microbiology Letters: enhanced rhizosphere colonization of beneficial *Bacillus amyloliquefaciens* SQF by pathogen infection (2013)
- ISME Journal: environmental bacteria promote plant infection by *Phytophthora species* (2014)

Competence strengthening / skills courses (0.6 ECTS)
- Working effectivity and planning; KNAW (2012)
- How to write a world class paper; WUR (2013)
- Experimental design; NIOO-KNAW (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)
- NERN (2011)
- NERN (2012)
- NERN (2013)

Discussion groups / local seminars / other scientific meetings (5.4 ECTS)
- Collimonas group meeting (2010-2011)
- PhD Discussion group meeting (2010-2013)
- Microbial Ecology group meeting (2010-2014)

International symposia, workshops and conferences (9 ECTS)
- Ecology of Soil Microorganisms; Prague (2011)
- Functions and Ecology of the Plant Microbiome; Rhodos (2012)
- FEMS; Leipzig (2013)
- CNRS – Jacques Monod Conference : bacterial-fungal inter actions: a federative field for fundamental and applied microbiology (2013)

Lecturing / supervision of practical's / tutorials (3 ECTS)
- Internship student (2011-2012)
- Internship student (2012)
- Internship student (2012-2013)

Supervision of MSc students (3 ECTS)
- *T. harzannium* as vector for plant pathogenic bacteria

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