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Höppener-Ogawa, S.

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CONTENTS

1

INTRODUCTION

Mycophagy is defined as 'feeding on fungi' and it is widely distributed over all kingdoms. Several mammals, including man, some birds, many arthropods and nematodes have been reported to feed, either partially or fully on fungi [2-4]. Fungi feeding on other fungi, mostly referred to as mycoparasitic fungi, are also known [5, 6]. Until recently, however, no clear information was available on the occurrence of prokaryotic mycophagy. In 2004, a new bacterial genus, *Collimonas*, was described which had been shown to grow on living fungal hyphae [7]. The discovery of this soil bacterium was the starting point for a research project that was aimed to obtain more information on the importance of bacterial mycophagy in soils. This thesis describes the results of this research project that focused on the distribution, diversity and ecology of *Collimonas* bacteria in soil.

BACTERIA-FUNGI INTERACTIONS

Life on earth originated in the aquatic environment. In this environment bacteria ruled the two most important processes in organic matter cycling, namely primary production (autotrophy) and decomposition (heterotrophy). Nowadays although eukaryotes, and in particular algae, are major contributors to primary

production, bacteria are still by far the most dominant decomposers in water and sediments.

Life migrated from the ocean to the land. The colonization of land by plants, being the dominant autotrophs, created a new habitat for heterotrophic decomposers, both eukaryotic and prokaryotic. Over evolutionary time, fungi have been able to occupy several terrestrial niches of which the decomposition of recalcitrant organic matter is perhaps the most remarkable one. This implies that, in contrast to the decomposition of aquatic organic matter, bacteria have not been able to monopolize decomposition processes in terrestrial ecosystems. The emergence of fungi in terrestrial ecosystems must have had a strong impact on the evolution of terrestrial bacteria [8]. On the one hand, potential decomposition niches, e.g. lignin degradation, have been lost for bacteria, whereas on the other hand the presence of fungi has itself created new niches for bacteria. This has led to the development of a wide range of interactions between bacteria and fungi can be recognized ranging from competition, amensalism, predation and parasitism to mutualism.

BACTERIAL MYCOPHAGY

Bacterial mycophagy is defined in this thesis as the ability of bacteria to grow at the expense of living fungal hyphae [8, 9]. The term may be used in a broad perspective to cover all fungus-related nutrition of bacteria. The strategies used by bacteria to obtain nutrients from fungal tissue can be subdivided into the following three main categories [10].

(1) *Extracellular necrotrophy* - Bacteria cause host cell death by extracellular induction of cell wall loss or membrane integrity, the inhibition of essential metabolic processes, or the induction of programmed cell death. They do so via the production of proteins or low molecular weight toxins that permeabilize and lyse fungal hyphae, or inhibit fungal metabolism, thereby killing fungal cells

and releasing nutrients for bacterial growth. Bacterial lysis of fungal hyphae *in vitro* has been observed in a wide range of taxonomically distinct bacteria including actinomycetes, β-proteobacteria, bacilli and myxobacteria though, in most cases, it is not clear if this is also occurring under natural conditions [8]. The clearest example of bacterial induced release of nutrients via induced fungal cell death has been reported for pathogenic bacteria of mushrooms, in particular *Pseudomonas tolaasii* the causal agent of brown blotch disease [11].

(2) *Extracellular biotrophy* – Bacteria live in close proximity of fungal propagules, often colonizing surfaces and using nutrients exuded from living fungal cells. *Pseudomonas* and *Burkholderia* spp. are among the most dominant culturable extracellular biotrophs [8]. Biotrophs are able to tolerate or suppress the production of anti-bacterial metabolites by fungal cells, and may be able to modulate fungal metabolisms to promote nutrient release.

Extracellular biotrophic interactions can be beneficial or detrimental to the fungal host. Mycorrhiza helper bacteria (MHB) are an example of beneficial extracellular biotrophs. Many bacterial strains have been reported to be able to promote the establishment of the symbiosis between root and either arbuscular or ectomycorrhizal fungi [12]. MHB promote the symbiosis by stimulating mycelial extension, thereby increasing root-fungus contacts. In addition, MHB may reduce the impact of adverse environmental conditions or pathogens [13]. Thus it has been suggested that the presence of MHB is advantageous to the fungi. At the same time, it is expected that the fungus has a positive effect on MHB. However, this aspect has received little attention so far, since many studies focused primarily on the positive effect of MHB on fungal behavior and on the assessment of the mechanisms of the helper effect.

(3) *Endocellular biotrophy* - Bacteria grow inside fungi and are entirely dependent on their fungal hosts for nutrients. Endocellular biotrophs multiply inside living fungal cells, absorbing nutrients directly from the fungal cytoplasm. Endocellular biotrophs were first discovered in the cytoplasm of AM fungi [14].

Bacterial mycophagy as defined here is supposed to be a combination of the aforementioned feeding types. However the mechanisms of bacterial mycophagy are not known for the details and needs to be elucidated. Mycophagous growth, previously mentioned mycoparasitic growth, was based on the observation that *Collimonas* bacteria increased in numbers in purified sand upon invasion of the sand by growing fungal hyphae [7]. Two important aspects lead to the conclusion of mycophagous growth namely (1) the absence of other nutrients for the bacteria and (2) the lack of response by other soil bacteria examined.

These results pointed at an active role of the *Collimonas* bacteria in obtaining nutrients from fungi. This active role is the basis for the term mycophagy as it will be used in this thesis. Hence, passive bacterial consumption of nutrients released from intact or damaged fungi is not considered as mycophagy. In addition, the ability of some bacteria to lyse fungal hyphae is not necessarily part of mycophagous process when there is no evidence that such bacteria are using the fungus-derived nutrients to multiply or when the lysis is only possible because of the supply of other nutrients.

THE GENUS *COLLIMONAS*

So far, *Collimonas* is the only bacterial genus for which mycophagous growth in soil-like systems has been shown. Originally the first isolates of what appeared to be *Collimonas* bacteria were screened for chitinolytic properties, namely the degradation of colloidal chitin on agar [15]. Several chitinolytic bacteria were isolated and the dominant non-filamentous bacteria appeared to belong to the genus *Pseudomonas*, as based on whole cell fatty acid profiles [15]. These strains were tested for their ability to degrade chitin particles in sand. Most of the unicellular bacteria that were capable of chitin degradation appeared to be poor degraders as compared to filamentous fungi, actinomycetes and gliding bacteria [16]. Further studies revealed that these isolates were able to grow at the expense

of intact, living hyphae of several fungi in sand [7]. 16S rRNA sequence analysis revealed that the isolates were not pseudomonads but were affiliated to the β-*Proteobacteria* [7]. Polyphasic taxonomic characterization of these isolates was conducted and it was proposed that the 22 isolates represent a novel genus, that was named *Collimonas* [7, 17]. Genomic fingerprinting (BOX-PCR), sequencing of 16S rRNA genes and physiological characterization indicated the presence of four clusters of strains [17]. One cluster had been formally classified as a novel species *Collimonas fungivorans*.

Potential mechanisms of Collimonas mycophagy

Chitin is a main component of fungal cell walls. So, in line with the studies on chitin degradation that led to the discovery of *Collimonas* spp, the first concepts on the mechanisms of bacterial mycophagy included the degradation of the chitin polymers of the fungal cell walls. Chtin, the β-1,4-linked polymer of *N*-acetyl-D-glucosamine, is an insoluble linear polymer. After cellulose, it is the second most abundant structural polymer in nature [18]. The main sources of chitin in soil are arthropod exoskeletons and fungal cell walls [19]. In the fungal cell wall, chitin polymers are cross linked and form also links with other polymers e.g. polyglucan. Because of this complex structure, the chitin in the cell wall is not easy accessible and degradable. However, in hyphal tips newly formed chitin polymers are not cross-linked. Therefore, the chitin in hyphal tips is probably most sensitive for chitinases produced by soil microbes.

To check if chitinolysis is important for *Collimonas* mycophagy, the chitinase inhibitor allosamidin was used to see if it represses mycophagous growth of *Collimonas* bacteria in sand. Allosamidin is a powerful inhibitor of endochitinases [20]. Allosamidin did significantly reduce the growth of *Collimonas* bacteria on 2 fungi, namely *Chaetomium globosum* and *Fusarium culmorum,* indicating that chitinases may be involved in mycophagy. However, the incomplete inhibition of growth of *Collimonas* bacteria by allosamidin indicated

that chitinase activity alone could not explain the mechanism of *Collimonas* mycophagy [7].

To date, two distinct loci i.e. *chi* locus A and B were found on the genome of *C. fungivorans* Ter 331 coding for chitinolytic ability [21]. However, strains that were mutated in these loci had still antifungal activity. Hence, the antifungal activities of *Collimonas* involved other agents than chitinases, most likely antibiotics [21]. In addition to chitinases, these antibiotics may be an essential component of mycophagous attack.

Quorum sensing describes the ability of certain bacteria to monitor their own population density and modulate gene expression accordingly [22]. Operons, which are not expressed when cells are free living at low density, are initiated to expression when cells reach a critical concentration (the quorum). Cell population density appears to be monitored via extracellular signaling molecules. One of the best characterized quorum-sensing signaling molecules is exclusive to gram-negative bacteria and relies on acylated homoserine lactones (acyl-HSLs) [23]. The first bacterium for which this quorum sensing was observed is *Vibrio fisheri* for which bioluminescence is controlled by quorum sensing. The quorum sensing dependent genes are often involved in the interaction between bacteria and eukaryotic hosts. Analogously, we hypothesize that quorum sensing regulation may play an important role in *Collimonas* mycophagy. It is hypothesized that *Collimonas* will keep the production of 'mycophagous compounds' at a minimum at low densities. However, activation of mycophagous compounds will proceed when a sufficient population density is reached near a fungus. This ensures a rapid and concerted attack of the host. *C. fungivorans* has been found to be positive for the production of acyl-HSLs (Chernin, unpublished results). However, the role of acyl-HSLs for mycophagous growth of *Collimonas* bacteria has still to be established. *Collimonas* bacteria are motile, possessing flagellae and pili. Signaling may also be involved in chemotactic growth of *Collimonas* bacteria towards host fungi. This is an area that has not yet been explored.

APPLICATIONS OF BACTERIAL MYCOPHAGY

Due to their specific characteristics mycophagous bacteria are likely agents for controlling fungal pathogens of man, animals and plants. In particular, the control of fungal plant pathogens has been called a potentially valuable practical application of *Collimonas* bacteria.

Plant diseases are major yield-limiting factors in the production of food crops and ornamentals. Chemical pesticides are widely used to prevent and control crop diseases, but may have adverse effects on human health and the environment. Therefore, the use of many chemical pesticides has become restricted and there is an increasing need for new, environmentally friendly approaches to control plant diseases. In this context, biological control of plant diseases by application of beneficial microorganisms to soil, seeds or other planting materials has received considerable attention. Yet, there are relatively few successful applications. Available biocontrol agents have only activity against a limited number of plant pathogens and do not always provide the level of control expected by the growers.

Application of the mycophagous fungus *Trichoderma* is one of most successful biocontrol measures. The ability of *Trichoderma* spp. to attack and grow on several phytopathogenic fungi has been used for protecting plants against soil borne plant diseases. *Trichoderma* containing formulations are commercially marketed as biopesticides, biofertilizers and soil amendments.

Collimonas bacteria are also able to grow at the expense of living fungi and do, therefore, also have the potentiality to be biocontrol agent of soil-borne fungal pathogens. The potential for biocontrol has already been tested *in vitro*. In these tests *C. fungivorans* was shown to be able to act as an efficient biocontrol agents towards *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causative agent of tomato foot and root rot [24]. However, it is unclear whether this involved mycophagous activity.

Potentially, mycophagous biocontrol might be superior to other mechanisms of the biological control of plant pathogens, as it leads to the proliferation of the

agents involved and thus, to the growth of the biocontrolling population. This is not the case when the biocontrol activity is due to the production of an anti-pathogen compound, such as antibiotics. Yet, the latter mechanism is the basis of the biocontrol activity of most present commercially available products except for *Trichoderma* based products. In order to exploit the full biocontrol potential of *Collimonas* bacteria, we need to know more about the behavior of the bacteria *in-situ*, their distribution in natural soils, the relevance of mycophagous nutrition for growth and survival, and the feeding preference. In other words, we need to know more about the ecology of the genus.

ECOLOGY OF COLLIMONAS BACTERIA

Prior to the present study described here, several taxonomic and ecological aspects of *Collimonas* bacteria were already known. Cells are strictly aerobic, straight or slightly curved, Gram-negative rods: 0.3-0.5 x 1.0-2.0 μm. They occur unicellular and possess flagella (mostly 1 to 3 polar but in some cases several lateral) and pili when cultured in liquid media. The major cellular fatty acids are: 16:0 and 16:1ω7cis. DNA base composition varies between 57 and 62 mol% G+C. The genus *Collimonas* belongs to the order *Burkholderiales* of the β-subclass of the Proteobacteria. .

Its oxidase activity is positive, catalase activity is negative or weakly positive. The maximum growth rate is observed between 20 and 30 ºC, without a sharp optimum. Growth does also occur al low temperatures (4 ˚C), maximum temperature supporting growth is approximately 35 ºC. The pH-range at which *Collimonas* bacteria can grow is from 5 to 8.

Collimonas bacteria are heterotrophic bacteria that can grow on a wide range of sugars, alcohols, organic acids and amino acids. This may be an important characteristic in order to be able to degrade the cytoplasmic compounds of fungi. However, it may also indicate that *Collimonas* bacteria are usually growing on soil

organic compounds and root exudates and not so much on fungi. Maximum growth rates of *Collimonas* bacteria are relatively low as compared to many other heterotrophic soil bacteria. Hence, their competitive ability for easily degradable compounds may be low, but more information is needed.

The chitinolytic activity of *Collimonas* bacteria is not expressed when other nutrients are available, e.g. glucose or tryptic soy broth. This catabolic repression of chitinolytic activity may indicate that mycophagous behavior is only occurring in the absence of other nutrients. It is, however, possible that the catabolic repression of the chitinolytic activity can be overruled by specific fungal inducers.

AIM AND RESEARCH QUESTIONS

Aim

The main objective of this study was to obtain basic knowledge on the ecology, distribution and relevance of *Collimonas* bacteria in soil ecosystems. Another objective was to extend the phylogenetic characterization of the genus *Collimonas.* The information obtained is needed to assess the potential of *Collimonas* as an antifungal control agent.

In order to be able to perform both *in vitro* and *in vivo* studies, which are described in this thesis, I developed specific methodologies for detection and cultivation dependent and cultivation independent quantification.

Research questions

Five research questions were addressed:

1. What is the geographical distribution of *Collimonas* bacteria? Is the occurrence of *Collimonas* bacteria restricted to fungal-rich soils?
2. Is mycophagous growth of *Collimonas* bacteria restricted to artificial environments or does it also occur in natural soils?
3. What is the effect of *Collimonas* bacteria on fungal biomass turnover?
4. What is the impact of *Collimonas* bacteria on the fungal community structure?
5. Is there interference between fungal and bacterial mycophagy?

Outline of the thesis

This study was started with the development of a molecular method to allow cultivation-independent quantification of *Collimonas* bacteria in environmental samples. Initially, a primer and probe set specific for the genus *Collimonas* were developed (based on the 16S rRNA gene nucleotide sequence) and tested *in silico* (databases) and *in vivo* (with DNA from pure cultures). The set of primers and probe was used for a real-time PCR assay, which was used to investigate the abundance of *Collimonas* bacteria in different soils in the Netherlands. Soil characteristics and vegetation types were also examined in order to determine the factors explaining the abundance of *Collimonas* bacteria. In parallel, a culture-dependent approach in combination with *Collimonas*-specific restriction fragment length polymorphism analysis was used to screen for the presence of culturable *Collimonas* bacteria. (Chapter 2)

A polyphasic taxonomic study to investigate the composition of the genus *Collimonas* was performed with the culturable *Collimonas* bacteria that were available. Two new species were described namely *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov. (Chapter 3)

The occurrence of mycophagous growth by *Collimonas* in natural soils was examined by following the growth response of indigenous soil *Collimonas* bacteria upon invasion of the soil by fungal mycelium. In the same study, I also examined the growth responses of mycophagous fungi (*Trichoderma* spp.). (Chapter 4) The impact of mycophagous growth of *Collimonas* bacteria on fungal biomass production was examined under controlled conditions (soil-like microcosms). A similar set-up was used to examine interactions between mycophagous *Collimonas* bacteria and mycophagous fungi (*Trichoderma harzianum*). (Chapter 4)

Chapter 5 describes the impact of *Collimonas* mycophagy on the community composition of different functional groups of fungi. Sequence analysis

was performed to assess which fungi were influenced by the presence of *Collimonas* bacteria. (Chapter 5)

A general discussion and evaluation of the study is presented in Chapter 6.

2

SPECIFIC DETECTION AND REAL-TIME PCR QUANTIFICATION OF POTENTIALLY MYCOPHAGOUS BACTERIA BELONGING TO THE GENUS *COLLIMONAS* IN DIFFERENT SOIL ECOSYSTEMS

Sachie Höppener-Ogawa, Johan H. J. Leveau, Wiecher Smant, Johannes A. van Veen and Wietse de Boer

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ABSTRACT

The bacterial genus *Collimonas* has the remarkable characteristic to grow at the expense of living fungal hyphae, under laboratory conditions. Here, we report the first field inventory on the occurrence and abundance of *Collimonas* in soils (n = 45) with naturally different fungal densities, in order to test the null-hypothesis that there exists a relationship between the presence of *Collimonas* and fungal biomass. Estimates of fungal densities were based on ergosterol measurements. Each soil was also characterized in terms of its physical and chemical properties and vegetation/management types. Culturable *Collimonas* were identified in plate-spread soil samples by their ability to clear colloidal chitin, in combination with *Collimonas*-specific restriction fragment length polymorphism analysis of 16S rRNA PCR-amplified from individual colonies. Using this approach, we found culturable collimonads only in (semi-) natural grasslands. A real-time PCR assay for the specific quantification of *Collimonas* 16S rRNA in total soil DNA was developed. *Collimonas* were detectable in 80 % of the soil samples, with densities up to 10^5 cells g^{-1} dry weight soil. The numbers of *Collimonas* per gram of soil were consistently lowest in fungal-poor arable soils but surprisingly, also in fungal-rich organic layers of forest soils. When all soils were included, no significant correlation was observed between the number of *Collimonas* and ergosterol-based soil fungal biomass. Based on this, we must reject our null hypothesis and possible explanations for this were addressed.

INTRODUCTION

All described strains of the genus *Collimonas* are soil bacteria that have the interesting capacity of growing at the expense of intact, living fungal hyphae [7]. This property, termed mycophagy [8, 9, 25], has not been well examined for soil bacteria [8]. In contrast, fungal mycophagy, which is better known as mycoparasitism, has been studied extensively [6, 26]. This is especially the case for those mycoparasitic fungi e.g. *Trichoderma* spp. that are applied as biocontrol agents of plant-pathogenic soil fungi [5, 27]. Although the mechanisms of mycophagous growth by collimonads have yet to be elucidated, it is known that these bacteria share some properties with mycoparasitic fungi such as the production of chitinases [7, 15, 17, 25], which are thought to be involved in the destabilization of the fungal cell wall [18, 28, 29]. However, De Boer *et al.* reported that for collimonads, chitinase activity alone could not explain mycophagous growth [7] and other factors should be involved, for example other lytic enzymes and antibiotics [7, 30, 31].

Until now, collimonads have been quantified only in the acidic dune grassland soils from which they were originally isolated [17]. In these soils, numbers of collimonads ranged from 10^3 to 10^5 colony forming units (CFUs) per g dry soil. Enumeration was based on plate counts of chitin-degrading colonies on agar plates containing colloidal chitin. On such plates, *Collimonas* strains can be recognized as halo-producing bacteria due to clearing of chitin, with a concomitant production of translucent biomass. However, identification of collimonads on the basis of colony morphology can not be conclusive without a more specific identification method. For this purpose, we developed and describe here a *Collimonas*-specific RFLP assay based on the restriction analysis of PCR-amplified 16S rRNA.

The enumeration on chitin-agar plates provides an indication of the abundance of collimonads. However, collimonads are relatively slow-growing bacteria and, therefore, their presence can remain undetected when other fast-

growing, chitinolytic bacteria are present as well. Additionally, the method fails to detect potentially non-culturable collimonads. As an alternative to plate enumeration, we developed and applied a culture-independent real-time quantitative PCR assay [32, 33] for the quantification of collimonads in soil. Real-time PCR has been successfully applied to detect and quantify bacterial cell numbers in various environmental samples [33-37], including soils [32, 38-42]. In the current study, the presence of soil collimonads was examined for a wide range of soils (40 sites) using both the plate count/RFLP method and real-time PCR assay. In order to identify the possible factors that determine soil population sizes of collimonads, presence/absence (culturable collimonads) and real time PCR-based numbers (total number of collimonads) were compared to fungal density, based on ergosterol measurements, as well as other soil properties, vegetation composition, and management practices .

MATERIALS AND METHODS

Soils, sampling procedure and soil analyses. In total, 40 sites in the Netherlands were selected on the basis of differences in vegetation (grassland, forest, agricultural crops, heathland and shrub), management practices (agricultural sites, abandoned arable sites and nature reserves) as well as physical and chemical soil characteristics (particle size, pH, moisture content, organic matter, total phosphorus, carbon, nitrogen, C:N ratio and chloride) (Appendix table A). At each sampling site, soils were collected from at least 30 points that were selected randomly in a 50 x 50 m plot using a corer of 3.5 cm diameter and pooled into a composite sample. For most sites, only the upper 10 cm layer was sampled but in 5 forest sites with a well-developed organic horizon, separate samples were taken from the organic layer and the upper 10 cm of the mineral soil. Hence, the total number of samples was 45. The composite samples were sieved (mesh size < 4 mm) and stored at 4 ºC for no more than one week until the analyses were started. Physical and chemical characteristics of soil were analyzed as described elsewhere [43, 44].

Estimates of soil fungal biomass were based on measurements of soil ergosterol content (mg per kg soil). Ergosterol is the major sterol in the membrane of most fungi and is not common outside the fungal kingdom [45, 46]. Soil ergosterol was extracted using an alkaline-extraction procedure and analyzed by high performance liquid chromatography (HPLC) as described elsewhere [47].

Total DNA from soil samples was extracted using the power soil™ DNA isolation kit (MOBIO Laboratories; Solana Beach, CA), according to the manufacturer's instructions except that 2 × 30 s bead-beating by Mixer Mill MM301 (Retsch, Haan, Germany) substituted vortex mixing. In addition, DNA was eluted in a final volume of 50 µl instead of 100 µl. The DNA extract was diluted 10 times before being used as template for real-time PCR quantification.

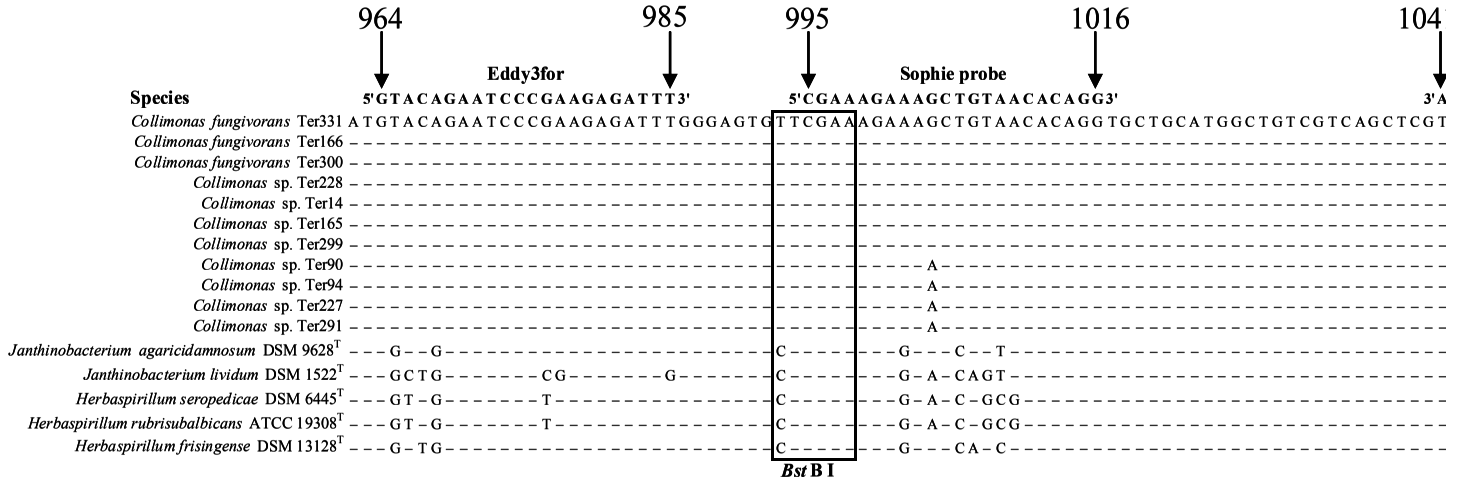

FIG 2.1: ***Alignment of partial 16S rRNA sequences from previously described collimonads and closely related species.*** *The numbering positions correspond to the 16S rRNA sequence of C. fungivorans Ter331 (AJ310395). Dashes indicate nucleotides identical to those in the Ter331 sequence. Indicated are the relative locations of primers Eddy3for and Eddy3rev as well as the dual-labeled probe Sophie. The boxed nucleotides indicate the BstBI restriction site unique to collimonads. Accession numbers for the 16S rRNA sequences of strains, Ter166, Ter300, Ter228, Ter14, Ter165, Ter299, Ter90, Ter94, Ter227, Ter291, DSM 9628^T, DSM 1522^T, DSM 6445^T, ATCC 19308^T and DSM 13128^T are AY281140, AY281145, AY281148, AY281135, AY281139, AY281144, AY281136, AY281138, AJ496445, AY281143, Y08845, Y08846, Y10146, AB021424 and AJ238358, respectively.*

Detection and identification of culturable collimonads by RFLP analysis of 16S rRNA. Soil suspensions were prepared as described elsewhere [15]. Fifty microliters of ten-fold dilutions were plated on chitin/yeast-extract agar with the same composition as described previously [17] but with extra addition of 0.1 g per liter of the fungal inhibitor delvocid (DSM, Delft, NL) [17]. The plates were sealed with parafilm, incubated at 20 ºC for 2 weeks and inspected regularly for halo-forming (= chitinolytic) bacterial colonies. Among the chitinolytic bacterial colonies, we screened for *Collimonas*-like isolates according to the morphology described by de Boer *et al*. [17]. In addition, other chitinolytic colony types were sampled as well. In total, 205 chitinolytic isolates were identified. All isolates were streaked on 1/10 strength tryptone soy broth (TSB; Oxoid) agar containing chitin [17]. This TSB-chitin agar was used to see if the chitinolytic ability of the strains was repressed by the presence of TSB, as such catabolic repression of chitinase production has been reported for all described *Collimonas* strains so far [17]. Plates were screened for chitinolytic activity after 2 weeks of incubation at 20 ºC.

Total DNA of the 205 chitinolytic isolates was extracted from 3 ml King's broth cultures (King's B), incubated at 27 ºC on a rotary shaker (200 rpm) for 48 h, using the same procedure as the DNA extraction from soil samples.

Near-complete fragments of 16S rRNA genes were amplified from isolated genomic DNA using the universal bacterial primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GRTACCTTGTTACGACTT-3') [1]. Bacterial DNA (1 µl of 10 × diluted genomic DNA) was added to a final volume of 25 µl containing 0.6 µM of each primer, 200 µM dNTPs, 2.5 µl 10 x buffer (Promega, Leiden, NL) and Taq polymerase (0.056 U per reaction) (Promega). The PCR was performed using a touchdown program in which the annealing temperature initially decreased from 65 to 55 °C by 2 °C per cycle, followed by 12 cycles at 55 °C, each for 1 min. The denaturing step was 30 sec at 92 °C, and the extension was 2 min at 68 °C.

The restriction enzyme *Bst*BI (BioLabs, New England, MA) was used to digest the PCR amplicons. Based on the current information on RDP (Ribosomal

Database Project II, http://rdp.cme.msu.edu/), we determined that collimonads are the only members of the family *Oxalobacteraceae* which share a *Bst*BI restriction site in their 16S rRNA gene on the position corresponding to nucleotides 993 to 998 of the 16S rRNA gene of *C. fungivorans* Ter331 (accession number AJ310395). Restriction fragments were examined on 1.8 % agarose gels in 0.5 x TSB buffer [17], using O' GeneRuler™ 1kb DNA Ladder Plus, ready-to-use (Fermentas, St.Leon-Rot, Germany).

The specificity of the RFLP assay towards collimonads was tested using five representative species of genera closely related to *Collimonas*, namely three *Herbaspirillum* type strains (*H. seropedicae* DSM 6445^T, *H. rubrisubalbicans* ATCC 19308^T and *H. frisingense* DSM 13128^T) and two *Janthinobacterium* type strains (*J. agaricidamnosum* DSM 9268^T and *J. lividum* DSM 1522^T) [17]. *C. fungivorans* Ter331 was used as a positive control [17].

PCR products that were identified by RFLP analysis as from *Collimonas* as well as 38 PCR products that were identified as not originating from *Collimonas* were sequenced (Greenomics^{TM}, Wageningen, NL) using the universal primer U1115R (5'-TCCCGCAACGAGCGCAACC-3') [48] as sequencing primer. DNA sequences, up to 650 bp in length, were compared with those available in Genbank (http://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project II (http://rdp.cme.msu.edu/). The sequences were aligned and compared using Clustal-W in the Lasergene DNA and protein analysis software (DNASTAR, Madison, WI).

Development, validation and application of a Collimonas-specific real-time quantitative PCR assay. In this dual-labeled probe assay, the primers used for real-time PCR quantification of collimonads were Eddy3for and Eddy3rev. The forward primer Eddy3for (5'-GTACAGAATCCCGAAGAGATTTGG-3') was based on a previously reported FISH probe specific to *Collimonas* species [17]. In combination with the non-specific reverse primer Eddy3rev (5'-ACTTAACCCAACATCTCACGACA-3'), it yields an amplicon of 100 bp.

To achieve specificity of the assay, we designed a *Collimonas*-specific probe Sophie (5`- 6-Fam-CGAAAGA+AA+GC+TG+TA+ACACAGG-BHQ1-3`) (FIG 2.1), which contains FAM as the fluorophore, BHQ as a quencher, and several locked nucleic acids (LNA; + symbol denotes the LNA base). LNA is a modified nucleic acid with increased binding affinity for complementary DNA sequences [49]. Primers and probe were synthesized by Biolegio BV (Nijmegen, NL) and Sigma-Proligo (Boulder, CO, U.S.A.), respectively.

Real-time PCR was carried out using a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia). Primer and probe concentrations were optimized according to the manufacturer's guide.

ABsolute qPCR mix (ABgene) was used at a 1x final concentration in the real-time reaction. Primers were added to a final concentration of 100 and 70 nM for Eddy3for and Eddy3rev, respectively. The final concentration of the Sophie probe was 50 nM. The reaction mixture was adjusted to 20 µl using nuclease-free water. The reaction mixture contained 400 ng of bovine serum albumin (BSA) per µl to minimize soil PCR inhibition [42, 50]. After addition of 5 µl DNA extracts from soil or control DNA (see below), amplifications were performed using the following conditions: 15 min at 95 ºC followed by 45 cycles of 15 sec for 95 ºC and 45 sec for 66 ºC. Each DNA extract was tested in duplicate. In all cases, negative controls containing nuclease-free water instead of DNA extracts were included.

For quantification of collimonads, a standard curve was generated using serial dilutions of genomic DNA isolated from *C. fungivorans* Ter331. Standard curves were generated by plotting threshold cycles (Ct) versus genome equivalents of strain Ter331. The undiluted genomic DNA extract contained 40 µg/mL genomic DNA as measured spectrophotometrically. The size of genomic DNA of *C. fungivorans* Ter331 has been estimated at 5.1 Mbp (unpublished data). Considering that 1 kb of double-stranded DNA is equivalent to 6.5×10^5 Daltons (1 dalton = 1.65×10^{-24} g), one genome of *C. fungivorans* Ter331 weighs 5.5×10^{-15} g. Thus, the undiluted DNA extract contained 7.3×10^6 genomic DNA

equivalents per 1 μL. We considered one genome as being equivalent to one cell.

Ct values indicate the minimum number of PCR-cycles needed to obtain fluorescence signals that significantly rise above the background of the exponential phase of the PCR amplification. The Rotor-Gene 6 software (Corbett Research, Sidney, Australia) was used to establish Ct values for each sample.

Statistics. The Chi-square test was applied to compare the patterns of distribution of culturable collimonads over arable-, forest- and (semi-) natural grassland soils. For this test, sites cropped with maize and fertilized grassland were categorized as arable land, non-fertilized grassland as well as ex-arable lands that were abandoned > 10 years were categorized as (semi-) natural grasslands and recently abandoned arable sites (< 10 years) were removed from all the analyses on land management since they represent a transient stage from arable lands to natural areas.

Data on real-time PCR-based (log-transformed) numbers of *Collimonas* and soil ergosterol contents were analyzed by one-way ANOVA with different land management practices (arable, forest and (semi-) natural grassland) and different layers (mineral and organic) of forest soil as treatments. Differences between groups were tested for significance with modified Tukey's honest significant difference test at $P < 0.05$. Possible relationships between real-time PCR-based numbers of *Collimonas* and physical and chemical characteristics of soil were examined using correlation analyses. Principal component analysis (PCA) was performed to ordinate the soils on basis of soil characteristics. Scaling was focused on inter-sample distances. Species scores, in this case soil characteristics, were divided by standard deviation and used for centering. The analysis was performed using CANOCO 4.5 [51]. All other statistical analyses were performed using STATISTICA (Statsoft Inc. Tulsa, OK).

RESULTS

Soil characteristics and soil fungal biomass. The 45 soil samples used in this study covered a wide range of physical and chemical properties as well as different vegetation characteristics and land management practices (Appendix table A). The concentration of ergosterol, which we used as an indicator of fungal biomass, in mineral soil ranged from 0.1 to 4.0 mg kg^{-1} dry soil in grasslands, from 0.6 to 7.3 mg kg^{-1} dry soil in forests and from 0.5 to 2.6 mg kg^{-1} dry soil in arable lands, respectively. The average amount of ergosterol in mineral soils was highest in the forest sites and lowest in the arable sites (FIG 2.2a). The organic layers of forest soils were particular rich in ergosterol (19.7 to 34.0 mg kg^{-1} dry forest-organic soil) indicating a high fungal biomass (FIG 2.2b).

Identification of culturable* Collimonas *by RFLP and 16S rRNA sequence analysis. In 4 forest soils (site: 1, 2, 13 and 37), we were not able to detect the possible presence of culturable *Collimonas* strains due to the rapid expansion of fast-growing gliding bacteria over the whole agar plate. From the remaining 41 soil samples, a total of 205 chitinolytic isolates was obtained. Sixty-nine of these isolates showed colony morphologies similar to those of the described *Collimonas* strains [17]. 16S rRNA of all 205 isolates was amplified by PCR and used for RFLP analysis using *Bst*BI. Twenty-six of the amplified 16S rRNA fragments showed the same banding pattern (i.e. one fragment of 1 kb and one of 0.5 kb) as the reference strain *C. fungivorans* Ter331 (FIG 2.3). The other isolates showed either a single, undigested PCR product or two bands with sizes that were clearly different from those of *Collimonas* (FIG 2.3).

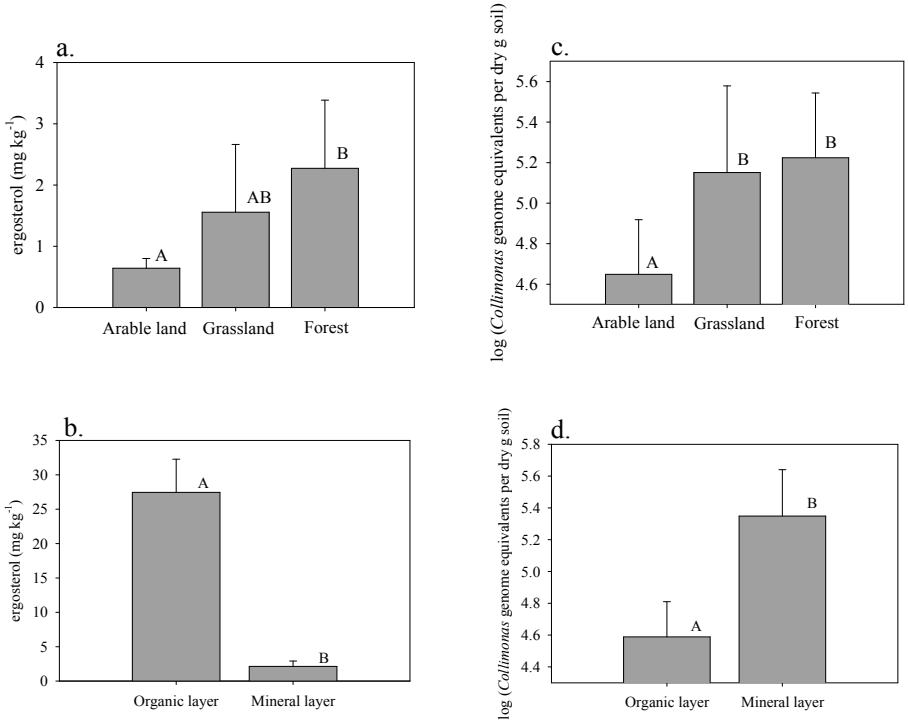

FIG 2.2: Average ergosterol contents (mg kg^{-1} dry weight soil) in the 0-10 cm upper layer of the mineral soil in arable -, forest - and (semi-) natural grassland sites (Fig. 2a), and in organic and mineral layers of 5 forest sites (Fig. 2b). *Averages of log-transformed Collimonas genome equivalents per dry g soil in the 0-10 cm upper layer of the mineral soil in arable -, forest - and (semi-) natural grassland sites (Fig. 2c), and in organic and mineral layers of 5 forest sites (Fig. 2d). Different letters are used to indicate significant differences at the 0.05% level (Modified Tukey's honest significant difference).*

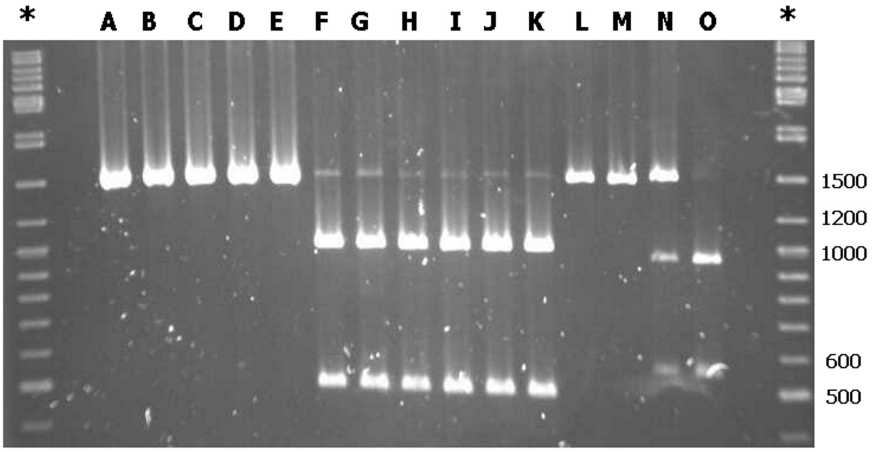

FIG 2.3: RFLP analysis of BstBI-digested 16S rRNA genes amplified to near completion from*: A. J. agaricidamnosum (DSM 9628^T), B. J. lividum (DSM 1522^T), C. H. rubrisubalbicans (ATCC 19308^T), D. H. seropedicae (DSM 6445^T), E. H. frisingense (DSM 13128^T), F. C. fungivorans Ter331, G-H. Collimonas sp. from soil sample 19, I. Collimonas sp. from soil sample 26, J. Collimonas sp. from soil sample 15, K. Collimonas sp. from soil sample 26, L. Isolate 142 from soil sample 16 = Flavobacterium sp., M. Isolate 134 from soil sample 27 = Burkholderia sp., N., Isolate 186 from soil sample 36 = Rhodanobacter sp., O. Isolate 88 from soil sample 9 = Pedobacter sp., *. 1 Kb Marker. Strains A-E were obtained from DSMZ (Braunschweig, Germany). Strains G-O were chitinolytic isolates from our inventory study.*

The 26 strains that were identified as collimonads by RFLP as well as 38 randomly selected other chitinolytic isolates, including a sub-set of strains resembling *Collimonas* colony morphology, were subjected to partial (0.4 - 0.6 kb) sequence analysis of the 16S rRNA gene. The results revealed that the isolates identified as *Collimonas* sp. by RFLP had also sequence similarity of > 98 % with published *Collimonas* sequences and as such were classified as confirmed collimonads. The other 38, RFLP-negative strains were clearly not *Collimonas* species, since their 16S rDNA showed highest sequence similarities with species

belonging to the families *Burkholderiaceae* and *Xanthomonadaceae.* This confirms the accuracy of our RFLP assay in establishing *Collimonas* identity.

The 26 confirmed collimonads originated from 9 different sampling sites, i.e. site 4, 5, 6, 15, 18, 19, 22, 26 and 27. These were 8 (semi-) natural grasslands and 1 heathland soil (Table 2.1). All 26 *Collimonas* isolates showed a colony morphology that resembled that of the colony morphologies described for collimonads [17]. However, 2 isolates obtained from grassland site 26 produced a purple pigment, which was previously described for species from the related genus *Janthinobacterium* [17, 52, 53]. The purple color was not clear on the original counting plates. However, when colonies were re-streaked on both chitin/yeast-extract agar and TSB-chitin plates for purity, the purple color became apparent. Production of purple pigments by collimonads has been reported before [54].
Isolates were also studied for their ability to produce haloes on chitin agar containing TSB since it was reported that the chitinolytic ability of collimonads was completely repressed by TSB [17]. This appeared to be only the case for half (13 strains) of the new isolates. These results show that colony morphology, colony color and catabolic repression of chitinase production are not reliable characteristics for the identification of *Collimonas* sp. as was suggested previously [17].

Table 2.1: ***Numbers of arable sites, (semi-) natural grasslands and forests in which culturable collimonads were present or absent***

	Total number of soil* samples	*Collimonas* present	*Collimonas* absent
arable land	8	0	8
grassland	18	8	10
forest	9	8	9
total	35	8	27

The distribution is significant at the P<0.05 level (Chi-squre test).

**Organic layer of forest soil, heathland and ex-arable land were excluded from the analysis.*

Development and validation of a Collimonas-specific dual-labeled probe assay. Although the aforementioned combination of plate counts and RFLP-based identification gave us an indication of the distribution of collimonads in different soil types, there were still many limitations, e.g. the presence of non-culturable collimonads and the apparent absence of collimonads due to presence of fast-growing chitinolytic gliding bacteria. Therefore, we developed a 16S rRNA gene-based real-time PCR assay. A *Collimonas*-specific primers/probe set was designed by alignment of 16S rRNA gene sequences of 12 representative collimonads [17] and the 22 most closely related bacterial species from the family '*Oxalobacteraceae*' (accession numbers: AY167838, Y08845, Y08846, AF174648, AY247410, Y10146, AB021424, AJ238358, AB074524, U49757, PSJ001384, AF543312, AB008506, AB024305, AY133107, AF529336, AF154097, AY177773, AF529095, AF358019, AY214204 and AF407411).

The probe which was designed based on a 22-bp stretch only shared by collimonads had a single mismatch (G or A in the 10^{th} position) (FIG 2.1). The single base pair mismatch of the probe did not influence the Ct value (data not shown) indicating that the probe was specific for real-time PCR analysis of

collimonads.

The specificity of the dual-labeled probe assays was tested empirically using genomic DNA of representative species of genera closely related to *Collimonas* (i.e. *H. seropedicae* DSM 6445^T, *H. rubrisubalbicans* ATCC 19308^T, *H. frisingense* DSM 13128^T, *J. agaricidamnosum* DSM 9268^T and *J. lividum* DSM 1522^T). The dual-labeled probe assay did not give a signal during a 40 cycles of the reaction (results not shown).

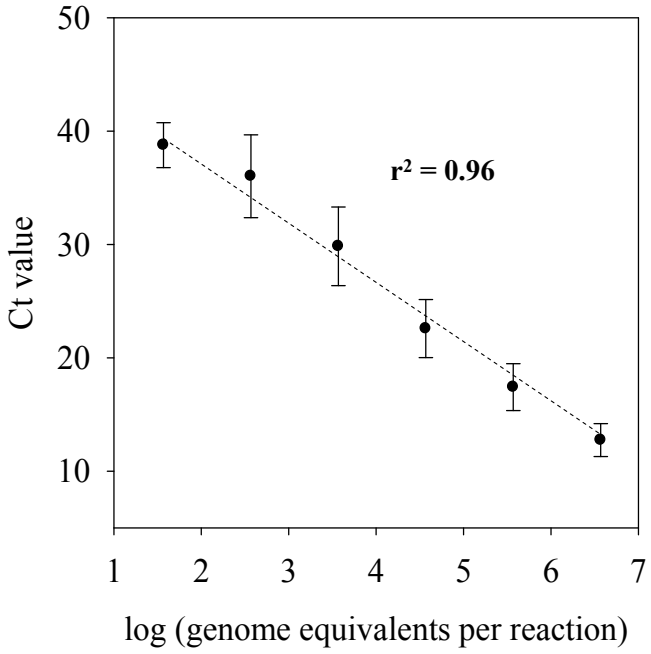

FIG 2.4: ***Standard curve for the Collimonas-specific dual-labeled probe PCR assays.*** *This curve was created using known amounts of DNA ranging from 3.7 ×10^1 to 3.7 × 10^6 genome equivalents of C. fungivorans Ter331 genomic DNA per reaction mixture. The values shown are representative results derived from multiple independent assays (n = 5).*

Direct detection and quantification of Collimonas in soils using the dual-labeled probe PCR assay. FIG 2.4 shows a composite standard curve that was derived using Ct values from five independent runs of PCR assays. The standard curve was obtained in the range of 3.7 x 10^1 to 3.7 x 10^6 genome equivalents per reaction with R^2 = 0.96 and CV (%) = 10.3 ± 2.58. Amplification efficiencies, calculated using the methods described by Pfaffl [55], was 1.54 ± 0.07. The lower limit of quantification was 3.7 × 10^1 genome equivalents per reaction, which corresponds to a mean Ct value of 38 whereas the negative controls did not give a signal during the 40 cycles of the reaction. The lower limit of 3.7 × 10^1 genome equivalents per reaction equals 3.7 × 10^1 genome equivalents per 5 µl of 10 × diluted DNA extract, i.e. 3.7 × 10^3 genome equivalents per 50 µl of the undiluted DNA extract. This 50 µl volume was extracted from 0.25 dry g soil, which implies that the quantification limit of our real-time PCR assay was 1.5 × 10^4 *Collimonas* genome equivalents (cells) g^{-1} dry weight soil. The constructed standard curve was used to estimate the number of genome equivalents or cells of *Collimonas* in all soil samples.

Real-time PCR showed that 36 soil samples contained collimonads above the quantification limit, up to 10^5 cells g^{-1} dry weight soil. The soils that did not show detectable level of collimonads were 4 arable soils, 3 forest soils and 2 natural grassland soils. For all soils, the extent of inhibition of real-time PCR was examined. This was done by adding 10^3 or 10^4 genome equivalents of *C. fungivorans* Ter331 to soil DNA dilutions. The degree of PCR inhibition was calculated by measuring the recovery of added DNA. The recovery of genome equivalents of *C. fungivorans* Ter331 added to soil DNA was complete for most soils (n = 37) and higher than 90 % for the rest (n = 8), indicating that there was no significant underestimation of native collimonads numbers in these soils due to PCR inhibition. The average numbers of collimonads per gram soil were significantly different ($P < 0.05$) between land management practices (FIG 2.2c). Numbers of collimonads were higher in grassland and forest soil than in arable land. The same trend was found for ergosterol-based fungal biomass (FIG 2.2a). In forest soils, the number of collimonads per gram soil was significantly higher (P <

0.05) in the mineral layer than in the organic layer (FIG 2.2d). This is in contrast to the fungal biomass that was much higher in the organic layer than in the mineral layer (FIG 2.2b). Correlation analysis did not show a significant relationship between the number of collimonads and any soil characteristics, including ergosterol. Furthermore, principal component analysis did not point to factors contributing effectively to the presence or absence of *Collimonas* (data not shown).

DISCUSSION

The *Collimonas*-specific RFLP assay and real-time PCR protocol described in this study were successfully used for specific detection of collimonads among culturable chitin-degrading soil bacteria and in total soil DNA, respectively. The RFLP analysis of 16S rRNA fragments proved to be a reliable method for genus-specific identification of culturable collimonads, whereas a combination of the developed primers and probe allowed for highly specific real-time PCR quantification of *Collimonas* 16S rRNA genes in soil.

Real-time PCR has the advantage to quantify both culturable and non-culturable bacteria in the soil microbial community [32, 38, 39]. The discrepancy between real-time PCR detection and the results from the isolation experiments (Table 2.1) could be explained by the inability of some *Collimonas* strains to grow on the enumeration plates (chitin/yeast-extract agar). Another explanation could be that fast-growing antagonistic or gliding bacteria prevented or masked the (slow) development of *Collimonas* colonies.

In theory, the quantification limit of the plate enumeration is 2.0×10^2 *Collimonas* cells g^{-1} dry weight soil. However, in practice the detection limit will be higher due to suppression of colony development by fast-growing antagonistic and gliding chitinolytic bacteria. The quantification limit of our real-time PCR assay was 1.5×10^4 *Collimonas* cells g^{-1} dry weight soil. Our qPCR results indicate that the population of collimonads was about 10^5 g^{-1} dry soil in 60 % of soils that we tested. In the other soils containing *Collimonas* (20 %), the numbers were close to the quantification limit. The presence of 10^5 cells g^{-1} soil corresponds to maximally 0.01 % of the total number of soil bacteria (based on DAPI counts, result not shown). In the nine soils where they were detected, culturable collimonads amounted to 0.1 % of total culturable chitinolytic bacteria (data not shown). Hence, it seems that the abundance of collimonads is generally low when averaged over the whole soil. This does not point at a strong competitive ability in soil, even though it has been shown that collimonads are metabolically versatile

[17]. *Collimonas* was originally isolated in search of chitinolytic bacteria. However, it is known that collimonads are weak chitin degraders as compared to fungi and actinomycetes [16]. Hence, their growth on chitin sources, mainly dead fungal hyphae and exoskeletons of arthpods, in soil is probably insignificant.

As another approach to find an indication of the abundance of collimonads, we used BLAST to screen for the presence of *Collimonas* sequences in published gene clone libraries of bacteria. Only 3 studies had collimonads in 16S rRNA gene clone libraries of soils, supporting our results that they do not occur in high numbers in soil. In one of these studies, collimonads were found in the lichen-dominated surface of forest soils and the authors suggest that they may play a role in the decay of the lichen biomass which consists for a major part of fungi [54]. Another study showed that collimonads were found in a forest soil-based microbial biofilm exposed to 2,4-dichlorophenoxyacetic acid [56]. Opelt *et al*. found collimonads in the bacterial community associated with bryophytes living in the Baltic sea coast area [57].

Real-time PCR results indicated that collimonads were widely distributed, but their numbers were significantly higher in (semi-) natural ecosystems than in arable soils. In addition, culturable *Collimonas* were only detected in natural grasslands. The relatively low numbers of collimonads in arable sites and production grasslands are in line with mycophagous growth, as these sites are fungal-poor due to agricultural management practices [43]. The low numbers are also compatible with a parasitic or predatory growth strategy [58]. For example, *Bdellovibrio* species, which are bacteria predating other bacteria, also occur in low numbers ($10^2 – 10^4$ CFU per g^{-1}) in soils [58].

Despite the apparent preference for the more fungal-rich natural ecosystems, numbers of collimonads were not pair-wise correlated with ergosterol-based soil fungal biomass. The lack of a statistically significant correlation between the fungal biomass and the numbers of collimonads could imply that mycophagy is not essential as a growth strategy for collimonads under natural conditions. Alternatively, enumeration of collimonads by qPCR may also include as yet

unknown strains that are not mycophagous. Furthermore, preferential feeding of *Collimonas* strains on specific taxonomic or functional fungal groups may interfere with a correlation between ergosterol-based soil fungal biomass and numbers of collimonads. Indeed, under lab conditions preferential growth of *Collimonas* strains was demonstrated: Proliferation of collimonads on the zygomycete *Mucor hiemalis* was much higher than on the ascomycetes *Chaetomium globosum* and *Fusarium culmorum* [7].

Surprisingly, within the forest soil profile, collimonads were present in relatively low numbers in the fungal-rich forest organic layers. If mycophagy is an important *in situ* growth strategy for collimonads, this must indicate that the environmental conditions or the type of fungal species in the organic layer of forest soils is not favorable to collimonads. Recently, Lindahl *et al*. (2006) reported spatial separation in vertical distribution of different functional groups of fungi in the organic layer of boreal forest soils. It was observed that saprotrofic fungi were primarily confined to the top organic layer (litter), whereas ectomycorrhizal fungi dominated the lower layers [59, 60]. Hence, it may be that higher numbers of collimonads in the mineral layer are caused by a preference for hyphae of ectomycoorhizal fungi.

A functional group of fungi with short turn-over time of hyphae are arbuscular mycorrhizal (AM) fungi [61]. If these fungi are an important source of food for collimonads, than this will not become apparent from ergosterol measurements as AM fungi do not contain ergosterol [62].

Obviously, other approaches are needed to indicate whether or not mycophagy is important for collimonads under natural growth conditions. One possible strategy is to introduce fungal hyphae as bait in soil samples that naturally contain collimonads. Application of the real-time PCR assay we developed here would allow us to follow changes in *Collimonas* numbers and assess whether the mycophagous property is essential for *in situ* growth. Future studies will also need to be aimed at increasing our knowledge on the types of fungal species that are preferably targeted by collimonads. This will enable re-assessing a possible link

between the abundance of collimonads and the presence of fungi by focusing more specifically on the presence of fungal species that are actually susceptible to collimonads. Depending on their specificity towards different fungi, collimonads could fulfill different functions in a soil environment, ranging from being deleterious, e.g. due to disturbance of symbiosis between plants and AM fungi, to being beneficial to plants by targeting plant pathogenic fungi.

Appendix Table A. Vegetation, soil characteristics and detection and quantification results of collimonads in field site samples used in this study

Field site sample	Ecosystem / Land management (Year of abandonment)	pH (H_2O)	C (g kg^{-1})	C:N ratio	Tot P (mg kg^{-1})	Cl (mg kg^{-1})	Particles >53 mm (%)	moisture % (w/w)	Ergosterol (mg kg^{-1})	NH_4 (mg kg^{-1})	NO_3 (mg kg^{-1})	P-CaCl (mg kg^{-1})	qPCR based *Collimonas* numbers (genome equivalents per reaction)	Detection of culturable *Collimonas*
1	forest	3.1	100.6	15.6	1328	13.4	74.9	58.6	7.3	15.5	45.5	43.0	0.0E+00	no
2	forest	3.5	36.1	26.8	262	1.2	94.9	6.9	2.8	12.7	2.8	9.4	5.2E+05	no
3	forest-organic	3.4	383.3	23.5	495	n.a.[a]	n.a.	80.3	22.9	n.a.	n.a.	n.a.	8.2E+04	no
4	grasses/herbs	4.9	9.6	11.9	169	0.0	97.8	2.5	1.0	2.5	1.8	1.7	1.5E+05	yes
5	grasses/herbs	5.3	77.3	10.2	1312	3.1	52.2	57.7	2.5	6.0	37.9	1.1	2.5E+05	yes
6	grasses/herbs	5.1	15.9	9.8	858	0.6	87.3	20.5	0.6	5.4	6.0	1.6	4.2E+05	yes
7	forest	5.8	49.7	12.1	881	9.3	57.3	39.3	3.0	3.1	45.2	0.7	1.2E+05	no
8	ex-agricultural (2002)	5.8	25.0	21.4	1003	1.6	91.6	14.3	0.7	3.2	3.8	9.4	1.1E+05	no
9	forest	3.8	41.1	25.3	219	0.6	87.0	17.8	2.8	8.0	3.1	0.7	1.6E+05	no
10	forest-organic	3.6	304.9	23.5	492	n.a.	n.a.	128.9	28.0	n.a.	n.a.	n.a.	2.7E+04	no
11	ex-agricultural (1999)	6.0	18.4	13.7	1105	0.6	90.1	13.9	1.5	4.5	8.0	8.9	0.0E+00	no
12	forest	3.6	48.4	27.7	158	0.6	86.0	27.3	3.2	8.9	2.9	1.1	0.0E+00	no
13	forest-organic	3.3	312.0	21.2	454	n.a.	n.a.	160.1	19.7	n.a.	n.a.	n.a.	0.0E+00	no
14	ex-agricultural (1985)	5.2	25.1	16.6	597	1.3	89.4	17.8	2.0	4.8	10.9	2.4	1.0E+05	no
15	heathland	4.1	77.5	32.8	245	0.6	85.2	29.2	5.5	19.0	1.8	0.5	1.2E+00	yes
16	ex-agricultural (2003)	5.4	30.4	25.4	727	1.3	85.8	15.5	1.0	2.9	9.1	1.9	2.1E+05	no
17	arable land	5.6	27.3	20.3	926	0.6	81.7	16.2	0.5	2.0	5.0	1.7	1.0E+05	no
18	ex-agricultural (1995)	5.1	19.0	13.2	713	1.3	77.2	12.9	0.9	5.5	9.5	3.1	7.2E+05	yes
19	grasses/herbs	5.8	34.8	12.1	682	4.7	26.9	38.2	2.6	5.5	3.5	0.4	1.9E+05	no
20	production grassland	6.1	37.2	10.9	1133	7.8	17.9	29.2	2.6	4.3	37.1	4.3	0.0E+00	no
21	forest	4.9	47.3	13.6	1207	6.2	19.1	37.6	3.7	5.9	33.6	3.1	2.3E+05	no
22	ex-agricultural (1992)	5.6	31.1	19.2	412	2.5	85.1	15.3	2.7	5.3	7.6	0.5	1.5E+05	yes
23	grasses/herbs	7.9	1.0	18.7	72	40.1	99.3	4.2	0.1	0.1	0.0	0.3	1.2E+05	no
24	grasses/herbs	8.0	3.3	19.4	90	7.6	98.9	3.5	0.8	1.8	0.4	0.6	8.7E+05	no
25	grasses/herbs	7.2	6.3	14.9	82	0.6	98.0	4.3	1.1	1.8	0.4	1.0	1.2E+05	no
26	grasses/herbs	5.3	6.1	17.4	52	0.0	97.4	5.9	0.9	1.5	0.0	0.5	1.1E+05	yes
27	grasses/herbs	4.7	35.1	26.1	70	46.6	94.4	86.3	4.0	3.5	0.1	0.4	7.9E+04	yes
28	forest	4.4	7.3	29.2	38	0.0	98.3	5.0	1.3	1.7	0.0	0.6	1.1E+05	no
29	forest-organic	3.6	244.1	49.0	213	n.a.	n.a.	69.4	34.0	n.a.	n.a.	n.a.	3.3E+04	no
30	production grassland	5.1	84.8	12.5	918	24.8	73.4	76.7	1.7	4.6	29.2	2.3	0.0E+00	no
31	grasses/herbs	7.8	14.1	26.8	331	4.5	91.2	9.3	0.6	1.0	4.6	1.1	0.0E+00	no
32	grasses/herbs	7.3	63.5	15.5	1933	66.9	22.0	45.0	2.1	4.8	32.6	3.1	0.0E+00	no
33	pioneer plant	7.5	58.5	15.5	1115	14.0	31.7	37.7	1.2	8.9	19.6	0.5	2.2E+04	no
34	forest	7.7	66.5	30.6	1550	42.6	44.2	41.7	0.6	2.8	63.0	1.0	5.2E+04	no
35	grasses/herbs	4.5	26.5	15.7	166	1.9	95.1	12.5	2.7	3.5	1.4	1.0	2.8E+04	no
36	forest	4.0	14.8	19.0	179	0.6	96.1	8.6	1.6	11.3	2.1	3.1	2.8E+05	no
37	forest-organic	3.6	175.1	34.5	224	n.a.	n.a.	60.8	24.9	n.a.	n.a.	n.a.	3.1E+04	no
38	grasses/herbs	7.5	17.6	13.7	401	8519.5	80.0	55.2	0.7	5.5	1.7	1.6	3.7E+04	no
39	grasses/herbs	8.5	3.7	15.3	123	3.8	96.1	3.9	0.6	0.3	0.7	0.5	4.9E+04	no
40	shrub	7.8	17.2	13.6	190	3.2	94.4	8.8	3.4	3.6	10.1	2.0	1.3E+05	no
41	production grassland	6.5	20.2	12.6	401	3.8	91.7	22.4	0.7	6.0	12.7	4.1	0.0E+00	no
42	production grassland	6.2	21.7	15.3	1009	1.3	87.4	18.1	0.9	4.4	11.8	5.5	2.2E+04	no
43	arable land	5.4	28.4	15.9	788	3.2	89.1	20.1	0.6	5.7	23.9	3.0	3.2E+04	no
44	production grassland	6.4	27.9	26.9	396	5.7	91.5	13.8	0.6	1.1	4.8	2.1	3.5E+04	no
45	production grassland	6.4	25.9	22.6	369	1.9	90.8	14.3	0.6	1.1	6.2	1.2	6.9E+04	no

[a] n.a. = not analyzed

3

COLLIMONAS ARENAE SP. NOV. AND *COLLIMONAS PRATENSIS* SP. NOV., ISOLATED FROM (SEMI-) NATURAL GRASSLAND SOILS

S. Höppener-Ogawa, W. de Boer, J. H. J. Leveau, J. A. van Veen, E. de Brandt, E. Vanlaere, H. Sutton, D. J. Dare and P. Vandamme

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Summary

A polyphasic taxonomic study was performed to compare 26 novel bacterial isolates obtained from (semi-) natural grassland soils and a heathland soil in the Netherlands with 16 strains that had been assigned to the genus *Collimonas* [17]. Genomic fingerprinting (BOX-PCR), whole-cell protein electrophoresis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry of intact cells, and physiological characterization (Biolog) of the isolates confirmed the existence of different strain clusters (A-D) within the genus *Collimonas* [17]. Until now, only cluster C strains have been formally classified as *Collimonas fungivorans*. In this study DNA-DNA hybridizations were performed with a selection of strains representing the four clusters. The results showed that cluster B strains also belong to *C. fungivorans* and that strains of clusters A and D represent two novel species within the genus *Collimonas.* The latter novel species could be differentiated by means of phenotypic and genotypic characteristics and are classified as *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov., with strains LMG 23964^T (= CCUG 54727^T = Ter10^T) and LMG 23965^T (=CCUG 54728^T = Ter91^T), respectively, as the type strains.

The genus *Collimonas* was described on basis of 22 isolates that were obtained from slightly acidic dune soils from the Wadden island Terschelling, the Netherlands [7, 17]. *Collimonas* strains have the interesting ability to grow at the expense of living fungal hyphae (mycophagy) albeit under laboratory conditions [7, 17]. The taxonomy of these bacteria was examined using genomic fingerprinting (BOX-PCR), sequencing of 16S rRNA genes and physiological characterization which revealed four clusters of strains [17]. So far, only cluster C strains have been formally classified as the novel species *Collimonas fungivorans*. The present investigation was designed to establish the taxonomic position of the three other clusters and of 26 new *Collimonas* isolates from different types of soils in the Netherlands (Table 3.1).

The new *Collimonas* isolates (n = 26) were taken from chitin agar enumeration plates of soil samples of 8 (semi-) natural grasslands and a heathland in the Netherlands as described previously [63] (Table 3.1). Based on colony morphology in combination with *Collimonas*-specific restriction fragment length polymorphism analysis of 16S rRNA genes, these 26 isolates were identified as *Collimonas* isolates [63]. All isolates were stored at -80 °C and maintained on 10× diluted tryptone soy broth (TSB) agar for routine culturing. The 10× diluted TSB agar contained l^{-1} water: 1 g KH_2PO_4, 5 g NaCl, 3 g TSB (Oxoid) and 20 g agar. Media were adjusted to pH 6.5 with 1 M NaOH before autoclaving.

Repetitive sequence-based PCR profiles of the isolates were determined using the BOX-A1R primer as described by Rademaker *et al*. (1997). Colony PCR was performed using fresh colonies that were taken from 10× diluted TSB agar after 24 hours of incubation [63]. Visual comparison of the banding profiles and UPGMA clustering of strains using Pearson's product moment correlation coefficients in the BIONUMERICS version 3.5 software package revealed that the majority (n = 18) of the new isolates fell within the clusters B and D

Table 3.1: *Strains used in this study and their origin*

Strain	Strain name [17]	GenBank accession no. 16S rRNA	Phylogenetic position†	Origin§	Reference
LMG 23964^T	TER10^T	AY281146	Cluster A	Dune grassland	De Boer *et al.* (2004)
LMG 23966	TER282	AY281142	Cluster A	Dune grassland	De Boer *et al.* (2004)
LMG 23967			Cluster A	Flooded dune grassland, 27	Höppener-Ogawa *et al.* (2007)
R-35550	TER252	AY281149	Cluster A	Dune grassland	De Boer *et al.* (2004)
R-35551	TER146	AY281147	Cluster A	Dune grassland	De Boer *et al.* (2004)
LMG 23971	TER266	AY281141	Cluster B	Dune grassland	De Boer *et al.* (2004)
LMG 23972	TER228	AY281148	Cluster B	Dune grassland	De Boer *et al.* (2004)
LMG 23973			Cluster B	Ex-agricultural land, 22	Höppener-Ogawa *et al.* (2007)
R-35505			Cluster B	Unfertilized grassland, 5	Höppener-Ogawa *et al.* (2007)
R-35506			Cluster B	Unfertilized grassland, 5	Höppener-Ogawa *et al.* (2007)
R-35507			Cluster B	Unfertilized grassland, 5	Höppener-Ogawa *et al.* (2007)
R-35508			Cluster B	Unfertilized grassland, 5	Höppener-Ogawa *et al.* (2007)
R-35509			Cluster B	Unfertilized grassland, 4	Höppener-Ogawa *et al.* (2007)
R-35522			Cluster B	Ex-agricultural land, 18	Höppener-Ogawa *et al.* (2007)
R-35523			Cluster B	Ex-agricultural land, 18	Höppener-Ogawa *et al.* (2007)
LMG 21973^T	TER6^T	AJ310394	Cluster C	Dune grassland	De Boer *et al.* (2004)
R-35554	TER300	AY281145	Cluster C	Dune grassland	De Boer *et al.* (2004)
R-35555	TER330	AY281150	Cluster C	Dune grassland	De Boer *et al.* (2004)
R-35556	TER166	AY281140	Cluster C	Dune grassland	De Boer *et al.* (2004)
LMG 23965^T	TER91^T	AY281137	Cluster D	Dune grassland	De Boer *et al.* (2004)
LMG 23968			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
LMG 23969	TER90	AY281136	Cluster D	Dune grassland	De Boer *et al.* (2004)
LMG 23970	TER291	AY281143	Cluster D	Dune grassland	De Boer *et al.* (2004)
R-22726	TER227	AJ496445	Cluster D	Dune grassland	De Boer *et al.* (2004)
R-35510			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35511			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35512			Cluster D	Unfertilized grassland, 4	Höppener-Ogawa *et al.* (2007)
R-35516			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35518			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35521			Cluster D	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35524			Cluster D	Ex-agricultural land, 18	Höppener-Ogawa *et al.* (2007)
R-35529			Cluster D	Unfertilized grassland, 6	Höppener-Ogawa *et al.* (2007)
R-35530			Cluster D	Unfertilized grassland, 6	Höppener-Ogawa *et al.* (2007)
R-35552	TER113	AJ496444	Cluster D	Dune grassland	De Boer *et al.* (2004)
R-35553	TER118	AJ496444	Cluster D	Dune grassland	De Boer *et al.* (2004)
R-35513			Out of the established four clusters	Heathland, 15	Höppener-Ogawa *et al.* (2007)
R-35514			Out of the established four clusters	Heathland, 15	Höppener-Ogawa *et al.* (2007)
R-35515			Out of the established four clusters	Heathland, 15	Höppener-Ogawa *et al.* (2007)
R-35517			Out of the established four clusters	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35520			Out of the established four clusters	Dune grassland, 26	Höppener-Ogawa *et al.* (2007)
R-35525			Out of the established four clusters	Unfertilized grassland, 19	Höppener-Ogawa *et al.* (2007)
R-35526			Out of the established four clusters	Unfertilized grassland, 19	Höppener-Ogawa *et al.* (2007)

† *based on grouping by [17].*

§ *Sites from which TER strains were isolated are described by [15]; Numbers refer to sites described by [63].*

described previously (De Boer *et al*., 2004). Only one of the new isolates fell in cluster A. The remaining 7 isolates occupied distinct positions in the dendrogram (FIG 3.1).

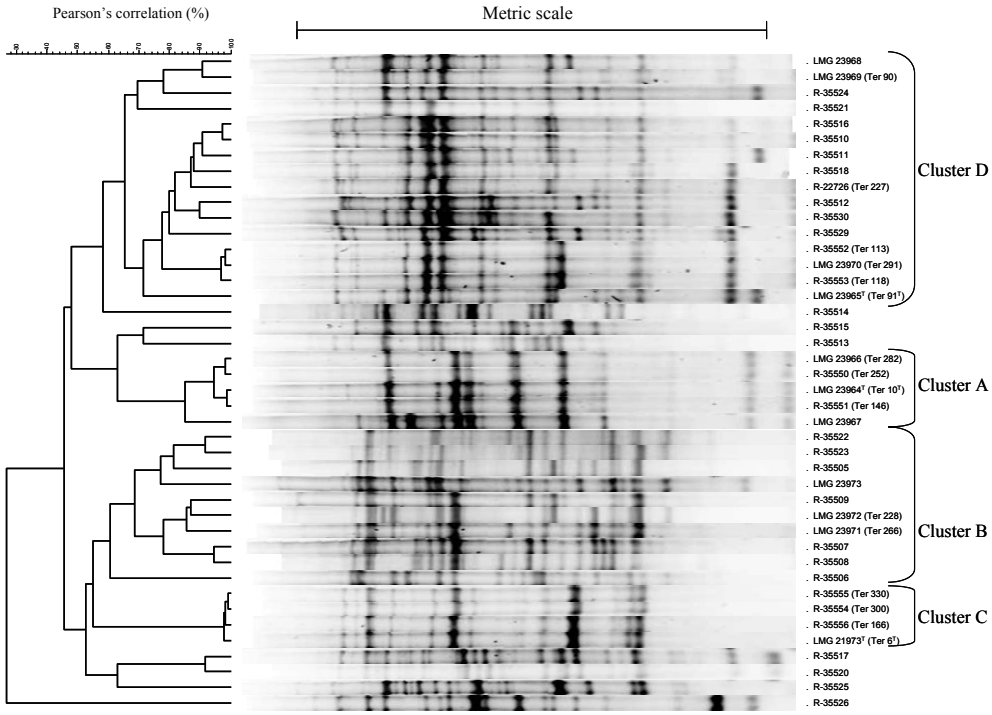

FIG 3.1: ***Grouping of normalized digitized BOX-PCR fingerprint patterns of the 42 Collimonas isolates in a dendrogram based on UPGMA clustering of Pearson's correlation similarity coefficients.***

Isolates grown for 48 hours at 28 °C on phosphate-buffered nutrient agar (pH 6·8) which contained l^{-1} water: 0.45 g KH_2PO_4, 2.39 g $Na_2HPO_4 \cdot 12H_2O$, 2.39 g nutrient agar (Oxoid) and 20 g agar, were subjected to SDS-PAGE analysis of whole-cell proteins. One-dimensional analytical SDS-PAGE was performed with a 12 % separating gel and a 5 % stacking gel using a previously described protocol [37]. Analysis of the whole cell protein patterns supported the clustering result of the BOX-PCR fingerprint patterns (results not shown).

Twenty isolates (FIG 3.2) were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) of intact cells. Bacterial cultures were grown and maintained on Columbia Blood Agar (CBA), containing 5% (v/v) sheep blood. Incubation was standardized to 24 hours and strains were grown aerobically at 28°C. All strains were sub-cultured three times prior to MALDI-TOF analysis. Sample and target plate preparation, data acquisition using a M@LDI Linear TOF Mass Spectrometer (Waters Corporation (Micromass) Ltd., Manchester, UK) and data processing (with the aid of the MassLynx/MicrobeLynx ^{TM} software, Micromass) were performed as described previously [63]. After cluster analysis of the spectral profiles (FIG 3.2), 17 isolates formed four clusters confirming results obtained by BOX-PCR fingerprinting and whole-cell protein electrophoresis (FIG 3.1; data not shown). The mass range m/z 2500-7500 Da contained the most discriminatory peaks, whereas the low (m/z 500-2500Da) and high (m/z 7500-10000 Da) mass ranges were very similar (data not shown). Strain R-35526 which occupied a distinct position in the BOX-PCR analysis (FIG 3.1), and strains LMG 23973 (cluster B) and R-35529 (cluster D) represented a fifth cluster in the numerical analysis of the MALDI-TOF MS profiles.

To analyze whether the different clusters represent distinct species, DNA-DNA hybridization experiments were performed using a modification of the microplate method [63] as described by Willems *et al*. [64]. Genomic DNA was prepared as described by Marmur *et al*. [65]. A hybridization temperature of 45 °C was used. Isolates with different DNA fingerprints (FIG 3.1) were selected as representatives of each cluster and were subsequently hybridized with each other (Appendix A Table 3.3). The cluster B strains (LMG 23971 and LMG 23973) showed DNA-DNA hybridization values of 75 % and 70 %, respectively, towards *C. fungivorans* LMG 21973^{T} (cluster C). The strains LMG 23968 and R-35524 exhibited DNA-DNA hybridization values towards strain LMG 23965^{T} of 75 % and 87 %,

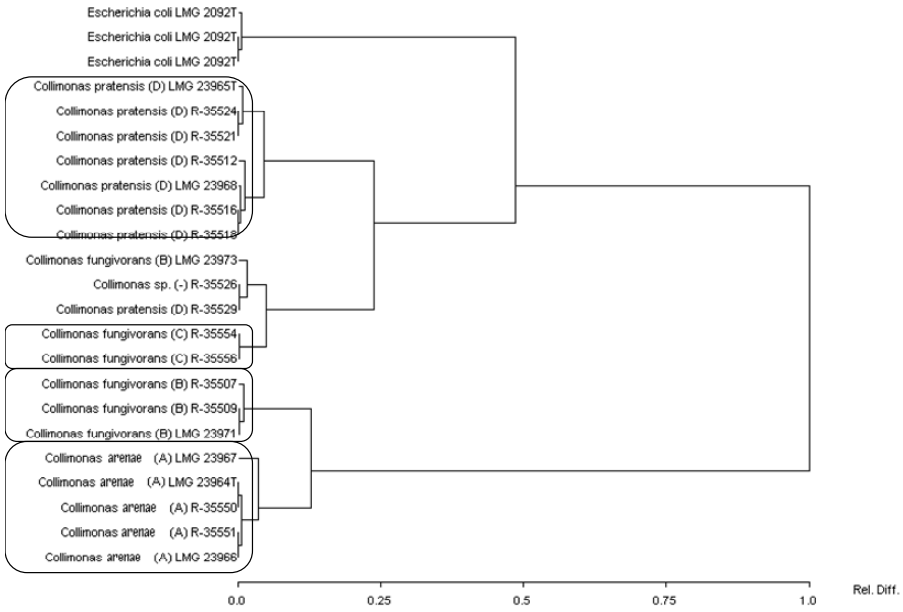

FIG 3.2: Numerical analysis of MALDI-TOF MS spectral patterns generated using the MicrobeLynx^{TM} software package. *Cluster A-D strains are shown in boxes. Three repeat measurements of Escherichia coli LMG 2092^{T} (included as positive control) are shown to illustrate the reproducibility of the profiles.*

respectively, indicating that these three cluster D strains represent a single genospecies. All DNA-DNA hybridizations between strains representing distinct clusters yielded low to intermediate values in the range of 31 to 64% (Appendix A Table 3.3). These data indicate that the cluster B isolates belong to *C. fungivorans* whereas the cluster A and D isolates represent two novel genospecies. The DNA G + C contents of LMG 21973^{T}, LMG 23964^{T}, LMG 23965^{T} and LMG 23971, as determined by HPLC [66], were 59, 57, 59 and 59 mol%, respectively.

Biochemical tests were performed for isolates representing cluster A (LMG 23964^{T}, LMG 23966, LMG 23967, R-35550, and R-35551); cluster B (LMG 23971, LMG 23972, LMG 23973, R-35508 and R-35509); cluster C (LMG 21973^{T}, R-35554, R-35555, and R- 35556 and; cluster D (LMG 23965^{T}, LMG 23968, R-35510, R-35511, R-35512, R-35516, R-35518, R-35524, R-35529 and R-35530).

Strains were examined for catalase and oxidase activity [67]. The ability to oxidize various carbon sources was tested using Biolog GN plates following the manufacture's instructions (Table 3.2). Detection of enzymatic activities was tested using the API 20NE and API ZYM microtest systems (bioMérieux) according to the manufacture's instructions (Table 3.2). The presence of the *nif*H gene was examined as described by [68]. Test results and differential biochemical characteristics are listed in Table 3.2 and in the species descriptions below.

Cellular fatty acid contents reported by De Boer *et al*. [17] could not be used to distinguish clusters within the genus *Collimonas* (results not shown).

In summary, data from the present study demonstrate that cluster B isolates belong to *C. fungivorans* and that cluster A and D isolates represent two novel *Collimonas* species which can be differentiated from each other and from *C. fungivorans* by means of genotypic (FIG 3.1) [17] and phenotypic (Table 3.2) characteristics. We propose to formally classify the cluster A and D strains into the novel species *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov., respectively. The taxonomic status of 7 isolates (Table 3.1) identified as members of the genus *Collimonas* by their ability to clear colloidal chitin and by their *Collimonas*-specific 16S rRNA restriction pattern [63] needs further studies. These strains may represent additional species within the genus *Collimonas*. The data obtained by MALDI-TOF MS indicate that this novel technology is useful for the rapid identification of *Collimonas* strains at the genus and species levels although 2 out of 20 isolates examined clustered aberrantly.

Description of *Collimonas arenae* sp. nov.

Collimonas arenae (L. gen. n. arenae, of sand, isolated from sandy soil)

After 2 days of incubation at 20 °C on 0·1 × TSB agar, colonies are flat, translucent, whitish with a yellowish central part and 3-7 mm in diameter with a granular-structured periphery (colony type II) [17]. Cells exhibit oxidase and weak

catalase activity. The *nif*H gene required for nitrogen-fixation was not detected by PCR-based methods. Carbon source utilization is presented in Table 3.2.

C. arenae can be differentiated from *C. fungivorans* and *C. pratensis* by the inability to assimilate D-trehalose and the lack of β-galactosidase activity.

The type strain is LMG 23964^T (=CCUG 54727^T = Ter10^T). It has a DNA G + C content of 59 mol% and was isolated from (semi-) natural grassland in the Netherlands in 1998.

Description of *Collimonas pratensis* sp. nov.

Collimonas pratensis (pra.ten'sis L. fem. adj. *pratensis* growing in a meadow, isolated from grassland)

After 2 days of incubation at 20 °C on 0·1 × TSB agar, colonies were small, glossy and whitish of 1-3 mm diameter (colony type III) [17]. One isolate (R-35518) produced a purple pigment which deviates from the general genus description [17]. Cells exhibit oxidase but no or weak catalase activity. The *nif*H gene required for nitrogen-fixation was not detected by PCR-based methods. Carbon source utilization and enzyme production are given in Table 3.2 and indicate a strong phenotypic flexibility within the species. *C. pratensis* can be differentiated from *C. fungivorans* by its colony morphology and its pronounced lipase activity. Additionally, the comparison between type strains of *C. pratensis* and *C. fungivorans* showed more differences in use of carbon substrates and production of enzymes (Table 3.2). Differentiation of *C. pratensis* from *C. arenae* is discussed above.

The type strain is LMG 23965^T (=CCUG 54728^T = Ter91^T), which has a DNA G + C content of 59 mol% and was isolated from (semi-) natural grassland in the Netherlands.

Table 3.2: Physiological characterization of C. fungivorans sp. and the novel species. *+, positive; w, weakly positive; -, negative; d, strain-dependent with reaction for type strain in parentheses. Results given by the API 20NE and API ZYM microtest systems were indicated with the symbols "*" or "‖", respectively. Substrates without symbol refer to Biolog tests. All strains characterized in this study were positive for utilization of bromo succinic acid, D-fructose, D-galactose, α-D-glucose*, L-asparagine, D-glucuronic acid, L-glutamic acid, D-mannitol*, β-hydroxy butyric acid and malic acid*. Also all strains were positive for production of alkaline phosphatase‖, leucine arylamidase‖, acid phospadase‖ and naphthol-AS-BI-phosphohydrolase‖. All strains were negative for utilization of i-erythritol, D-melibiose, α-cyclodexitrin, β-methyl D-glucoside, Itaconic acid, D-raffinose, thymidine, gentiobiose, L-rhamnose, L-phenyl alanine, phenyl ethylamine, putrescine, sucrose, α-lactose, 2,3-butanediol, adonitol, α-D-lactose lactulose, maltose, sebacic acid, D,L-camitine, glucose-1-phosphate and γ-hydroxy butyric acid. Also all strains were negative for production of cystine arylamidase‖, β-glucuronidase‖, α-glucosidase‖, α-fucosidase‖, adipic acid*, phenylacetic acid*, indole production*, D-glucose fermentation* and arginine dihydrolase*. All strains were weakly positive for production of esterase‖, esterase lipase‖ and valine arylamidase‖.*

Characteristics	Cluster B and C	Cluster A	Cluster D
Assimilation of:			
Urocanic acid	d (+)	−	d (+)
Succinamic acid	−	d (−)	d (w)
Hydroxy L-proline	−	−	d (−)
Inosine	d (+)	+	d (+)
L-fucose	+	+	d (+)
Uridine	+	+	d (+)
Formic acid	+	+	d (+)
L-omithine	−	d (−)	−
α-keto valeric acid	d (−)	d (−)	−
D-alanine	d (w)	d (−)	−
D-galacturonic acid	−	d (+)	d (−)
L-proline	+	+	d (+)
m-inositol	+	+	d (+)
D-gluoconic acid	+	d (+)	d (+)
Malonic acid	d (−)	−	−
2-amino ethanol	d (+)	−	−
D-trehalose	+	−	+
D-glucosaminic acid	+	d (+)	d (+)
Propionic acid	d (+)	+	d (w)
D-serine	d (w)	−	−
L-aspartic acid	+	+	d (+)
Glycerol	+	+	d (+)
L-arabinose*	d (+)	+	d (+)
Xylitol	d (+)	−	d (+)
α-hydroxy butyric	d (w)	+	d (−)
D-saccharic acid	+	+	d (+)
D, L-α-glycerol	−	d (−)	d (−)
D-arabitol	d (+)	+	+
Cellobiose	d (−)	−	−
Succinic acid	+	+	d (+)

Glucose-6-phosphate	−	d (+)	d (−)
D-mannose*	d (+)	d (w)	d (+)
Potassium gluconate*	d (w)	d (+)	d (w)
Capric acid*	−	d (−)	−
Trisodium citrate*	d (+)	d (−)	+
Production of:			
Lipase‖	W	d (w)	+
Trypsin‖	d (+)	d (w)	d (w)
α-chymotypsin‖	d (+)	d (w)	d (w)
α-galactosidase‖	−	−	d (w)
β-galactosidase‖	+	−	+
α-mannosidase‖	D (w)	−	d (−)
β-glucosidase‖	d (−)	−	d (−)
protease*	D (+)	d (+)	d (w)

Appendix A:

Table 3.3:*DNA-DNA relatedness (%) among the seven tested strains*

Strain	LMG 23964^T	LMG 23973	LMG 23971	LMG 21973^T	LMG 23968	R-35524	LMG 23965^T
Cluster A LMG 23964^T	100						
Cluster B LMG 23973	–	100					
Cluster B LMG 23971	46	–	100				
C. fungivorans LMG 21973^T	44	70	75	100			
Cluster D LMG 23968	–	41	–	31	100		
Cluster D R-35524	56	–	53	64	–	100	
Cluster D LMG 23965^T	47	43	58	46	75	87	100

–, *not done*

4

MYCOPHAGOUS GROWTH OF *COLLIMONAS* BACTERIA IN NATURAL SOILS, IMPACT ON FUNGAL BIOMASS TURN-OVER AND INTERACTIONS WITH MYCOPHAGOUS *TRICHODERMA* FUNGI

Sachie Höppener-Ogawa, Johan H. J. Leveau, Johannes A. van Veen and Wietse de Boer

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Abstract

Bacteria of the genus *Collimonas* are widely distributed in soils albeit at low densities. In the laboratory, they were shown to be mycophagous i.e. they are able to grow at the expense of living hyphae. However, so far the importance of mycophagy for growth and survival of collimonads in natural soil habitats is unknown. Using a *Collimonas*-specific real-time PCR assay, we show here that invasion of field soils by fungal hyphae (*Absidia* sp.) resulted in a short-term, significant increase (average 4-fold) of indigenous collimonads. No such responses were observed for other soil bacteria studied (*Pseudomonas, Burkholderia,* PCR-DGGE patterns of total bacteria and *Burkholderia*). Hence, it appears that the stimulation of growth of *Collimonas* bacteria by fungal hyphae is not common among other soil bacteria. In the same field soils, *Trichoderma*, a fungal genus known for mycophagous (mycoparasitic) growth, increased upon the introduction of *Absidia* hyphae. Hence, mycophagous growth by *Collimonas* and *Trichoderma* can occur in the same soils. However, in controlled experiments (sand microcosms) collimonads appeared to have a negative effect on mycophagous growth of a *Trichoderma* strain. The effect of mycophagous growth of collimonads on fungal biomass dynamics was studied in sand microcosms using the same *Absidia* sp. as a test fungus. The growth of collimonads did not cause a significant reduction of the *Absidia* biomass.

Overall the study indicates that mycophagous nutrition may be important for collimonads in natural soils, but the impact on fungal biomass turn-over is likely to be minor.

INTRODUCTION

Mycophagy, i.e. the feeding on living fungi, has been reported for soil bacteria of the genus *Collimonas* [10, 17]. Mycophagous growth was based on the proliferation of collimonads in gnotobiotic sand microcosms that contained living fungal mycelium as the only source of energy and carbon [7]. However, whereas the nutrient poor conditions in these microcosms were realistic for natural soils, other conditions, e.g. the absence of other (micro) organisms and plant roots, were different from the natural soil environment.

In a field inventory we investigated the distribution of collimonads among different soils [63]. Collimonads appeared to be more abundant in fungal-rich natural grassland and forest soils than in fungal-poor arable soils. Yet, no clear relationship between fungal biomass and abundance of collimonads was observed. Hence, further studies are required to elucidate the importance of mycophagy for *in situ* growth of collimonads, in particular because they can grow on a wide range of organic substrates, i.e. they are facultative mycophagous [15].

Unlike the few studies that have been carried out on bacterial mycophagy, fungal mycophagy, which is better known as mycoparasitism, has been the subject of many studies [5, 27]. Most of these studies deal with *Trichoderma* species [29, 69]. In particular, the application of *Trichoderma* spp. to control plantpathogenic fungi (e.g. *Rhizoctonia spp.* and *, Fusarium spp*) has received much attention [5, 70, 71]. *Trichoderma* spp. are widely distributed in the terrestrial environment [72]. However, as for collimonads, the actual importance of mycophagous growth for *Trichoderma* spp. under natural soil conditions is not known. Like collimonads, *Trichoderma* spp. are facultative mycophagous [15]. Hence for both collimonads and *Trichoderma* spp. other sources of energy, e.g. soil organic matter and root exudates, may be more important for their growth than fungal-derived substrates.

The current study was aimed (1) to find evidence for the importance of mycophagous growth for collimonads in natural soils, (2) to assess the consequence of mycophagous growth of collimonads for fungal biomass

production and (3) to examine possible interactions between collimonads and *Trichoderma* spp. to perform their mycophagous growth.

MATERIAL AND METHODS

Soils. In January 2007, soil samples (upper 10 cm of mineral layer) were collected from sites, where collimonads had been detected earlier [63]. The soils were a forest soil (site 2), 2 grassland soils (site 4 and 5) and a soil from an abandoned arable site (site 22), respectively. Characteristics of these soils (site 2, 4, 5 and 22) were described previously as pH_{water} 3.5, 4.9, 5.3 and 5.6, total organic carbon 36.1, 9.6, 77.3 and 31.1 g kg^{-1} and C/N ratio 26.8, 11.9, 10.2 and 19.2, respectively [63].

Bacterial strains. The strains used in this study were *Collimonas fungivorans* Ter 331 (AJ310395) and Ter 6^T (LMG 21973), *C. arenae* Ter 10^T (LMG 23964) and *C. pratensis* Ter 91^T (LMG 23965), respectively [17, 73]. These 4 strains have been isolated from the same soil [17]. Hence, their co-occurrence is natural. Based on the experimental conditions and feeding-preferences, the mixture of the 4 strains was used to provide the best chances for mycophagous growth in this study (De Boer *et al*., 2004). *Pseudomonas fluorescens* strain AD21 is a soil isolate that has been described before [74]. *Burkholderia* JS is a soil isolate kindly provided by Dr. Drigo (Plant Research International, Wageningen, The Netherlands) which has 98% identity with a *Burkholderia cepacia* strain (AY741358).

Fungal strains. Based on earlier work, it was evident that growth of collimonads can be especially stimulated by zygomycetal fungi [7]. In the current study we used a zygomycetal fungus, isolated from a grassland soil, that was identified as *Absidia* sp. on basis of the sequence analysis of the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit, using primers ITS1-F and ITS4-B [75]. *Absidia* spp. are common saprotrophic soil fungi [76]. *Trichoderma harzianum* CECT 2413 [77] was purchased from the Spanish type culture collection - CECT (University of Valencia, Spain).

Experiment 1: Growth responses of indigenous* Collimonas *spp.,* Pseudomonas *spp.,* Burkholderia *spp. and* Trichoderma *spp. to invasion of field soils by* Absidia *mycelium Soils collected from each sampling site were homogenized and portions (40g) of the soils were transferred to Petri dishes (diameter, 8.5 cm) and spread evenly. A potato dextrose agar (PDA) disk (diameter, 1 cm) from the growing margin of *Absidia* was inverted and placed on an autoclaved metal slide and was centrally placed in the middle of the Petri dish. The metal coin was used to prevent leaching of nutrients from the agar disks into the soil. The Petri dishes were sealed and incubated at 20 °C. After 1 week of incubation, *Absidia* mycelium covered the whole soil surface. Samples were taken by scraping the soil surface after 0, 2 and 3 weeks of incubation. In addition, comparable samples were taken from Petri dishes without invading *Absidia* hyphae. For all soils, treatments (with and without *Absidia*) were done in 6-fold.

Soil DNA extracted from the collected soil samples was used for real-time PCR enumerations of collimonads and fungi belonging to the genus *Trichoderma*. Real-time PCR enumerations of indigenous *Pseudomonas* and *Burkholderia* bacteria as well as PCR-denaturating gradient gel electrophoresis (PCR-DGGE) analysis of the bacterial community structure were performed to evaluate the specificity of the growth increase of collimonads upon introduction of fungal mycelium. DNA was extracted from an amount of soil equivalent to 0.25 g dry soil using the MOBIO kit (MOBIO laboratories, Solana Beach, CA) according to the manufacturer's instruction, except that soil DNA was finally eluded in 50 µl instead of 100 µl.

Experiment 2: Estimation of the mycophagous biomass production by collimonads and evaluation of interactions between collimonads and* T. harzianum *upon mycophagous performance in gnotobiotic sand microcosms. Gnotobiotic sand microcosms were used to study the impact of *Collimonas* spp. on fungal biomass production. *Collimonas* strains were pre-grown on chitin-yeast agar

at 20°C for 14 days as described by de Boer *et al*. [7]. *Absidia* sp. was grown on potato-dextrose agar (PDA) at 20 °C for 4 days.

The 4 different *Collimonas* strains were mixed by adding equal numbers of cells of each strain to P-buffer (KH_2PO_4, 1 g L^{-1} [pH 6.5]). The suspension was mixed into autoclaved, acid-purified beach sand to give a moisture content of 5 % (wt/wt) and a total bacterial density of 10^4 cells g^{-1} of sand (based on microscopic counts), which is a common density of collimonads in field soils [63]. Portions (40 g) of the incubated sand were transferred to Petri dishes (diameter, 8.5 cm) and spread evenly. The Petri dishes were sealed, placed at 20 °C, and pre-incubated for 1 week to allow the micro-organisms to adapt to the prevailing conditions. Next, an agar disk (PDA; diameter, 1 cm) from the growing margins of *Absidia* was inverted and placed on an autoclaved metal slide and this was centrally placed in the middle of Petri dish. The Petri dishes were sealed and incubated at 20 °C. After 3 weeks of incubation, sand was removed from the surface covered by hyphae of *Absidia* and homogenized before measurement of fungal biomass (ergosterol) and abundance of collimonads (real-time PCR).

The same experimental set-up was used to study interactions of collimonads with the mycophagous fungus *Trichoderma harzianum* during growth on *Absidia* hyphae.

Sterile sand with or without collimonads (see above) was inoculated with a spore suspension (10^4 spores g^{-1} sand) of *T. harzianum*. Conidia had been produced on PDA and were collected and suspended in P-buffer. After vortexing, the suspension was centrifuged at 4000 rpm for 1 minute. The supernatant was collected as spore suspension and was mixed into autoclaved, acid-purified beach sand to give a moisture content of 5 % (wt/wt) and a total density of 10^4 spores g^{-1} of sand (based on microscopic counts). All treatments were done in 6-fold. Using the prepared microcosms in the same experimental set-up with the inoculation of *Absidia* (see above), Petri dishes were sealed and incubated at 20 °C. After 3 weeks of incubation, sand was removed from the surface covered by hyphae of

Absidia and homogenized before measurement of abundance of collimonads and *T. harzianum* (real-time PCR).

Real-time PCR. Real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia) to reduce variation caused by pipetting errors. Quantification of collimonads in soil DNA samples was performed as described by Höppener-Ogawa *et al.* [78].

For quantification of pseudomonads, *Burkholderia* spp. and *Trichoderma* spp., the Absolute qPCR SYBRGreen mixture (ABgene) was used at a final concentration of 1× for the real-time reaction. The standard curves for *Pseudomonas* and *Burkholderia* were made from genomic DNA extracted from a pure culture of *Pseudomonas fluorescens* strain AD21 and *Burkholderia* JS (see strain description). The *Pseudomonas* specific primer sets used were PSf (5'-GGT CTG AGA GGA TGA TCA GT-3') and PSr (5'-TTA GCT CCA CCT CGC GGC-3')[79]. The *Burkholderia* specific primer sets used were Burk3 (5'- CTG CGA AAG CCG GAT -3') and BurkR (5'- TGC CAT ACT CTA GCY YGC -3')[80].

For quantification of *Trichoderma* spp., we used a modified protocol of Hagn *et al.* [78]. The *Trichoderma* standard curve was made from DNA extracted from a pure culture of *T. harzianum* CECT 2413. PCR-amplification was done using the fungal universal primer sets NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') [81] and TW13 (5'-GGT CCG TGT TTC AAG ACG-3')[82].

For quantification of *Absidia* sp.*,* real-time PCR quantification was done using universal fungal specific primers as described elsewhere [83]. In addition, measurement of the fungal cell membrane component ergosterol via an alkaline extraction protocol was performed to quantify the biomass of *Absidia* sp. [47].

PCR-denaturing gradient gel electrophoresis analysis. All PCR reactions and denaturing gradient gel electrophoresis (DGGE) were carried out as described elsewhere [80, 84]. The primers 968f-GC, 1378r [85] and FR1-GC, FF390r [83]

were used to analyze bacterial and fungal communities, respectively. The nested PCR reactions were performed for *Burkholderia* spp. with primer Burk3, R1378 [80]. The products from the first PCR were diluted 1:1,000 and used as the template in the second PCR with primers Burk3-GC and BurkR [80].

Statistical analyses. The banding patterns of DGGE gels were analyzed using the Image Master 1D program (Amersham Bioscience, Roosendaal, the Netherlands). The resulting binary matrices were exported and used in statistical analysis as "species" presence-absence matrices.

The effect of the introduction of *Absidia* hyphae, sampling site and the interaction of these two factors on the community structure as analyzed by PCR-DGGE was tested by distance-based redundancy analysis [86]. Jaccard's coefficient of similarity was calculated and the resulting similarity matrix was exported to Canoco 4.5 as species data for redundancy analysis (RDA) [51]. Variables to be included in the model were chosen by forward selection at a 0.05 baseline. The significance of canonical model was tested with 999 permutations. To test the effects of each of the two variables (*Absidia* invasion and soil origin), the individual variables were recorded using dummy binary-variables of which one was used in Canoco as the only environmental variable in the model and the other as co-variable. To test the interaction, the only variable entered in the model was the interaction between *Absidia* invasion and soil origin, while both individual factors were included (without interaction) as co-variables. The significances of such models were tested with 999 permutations.

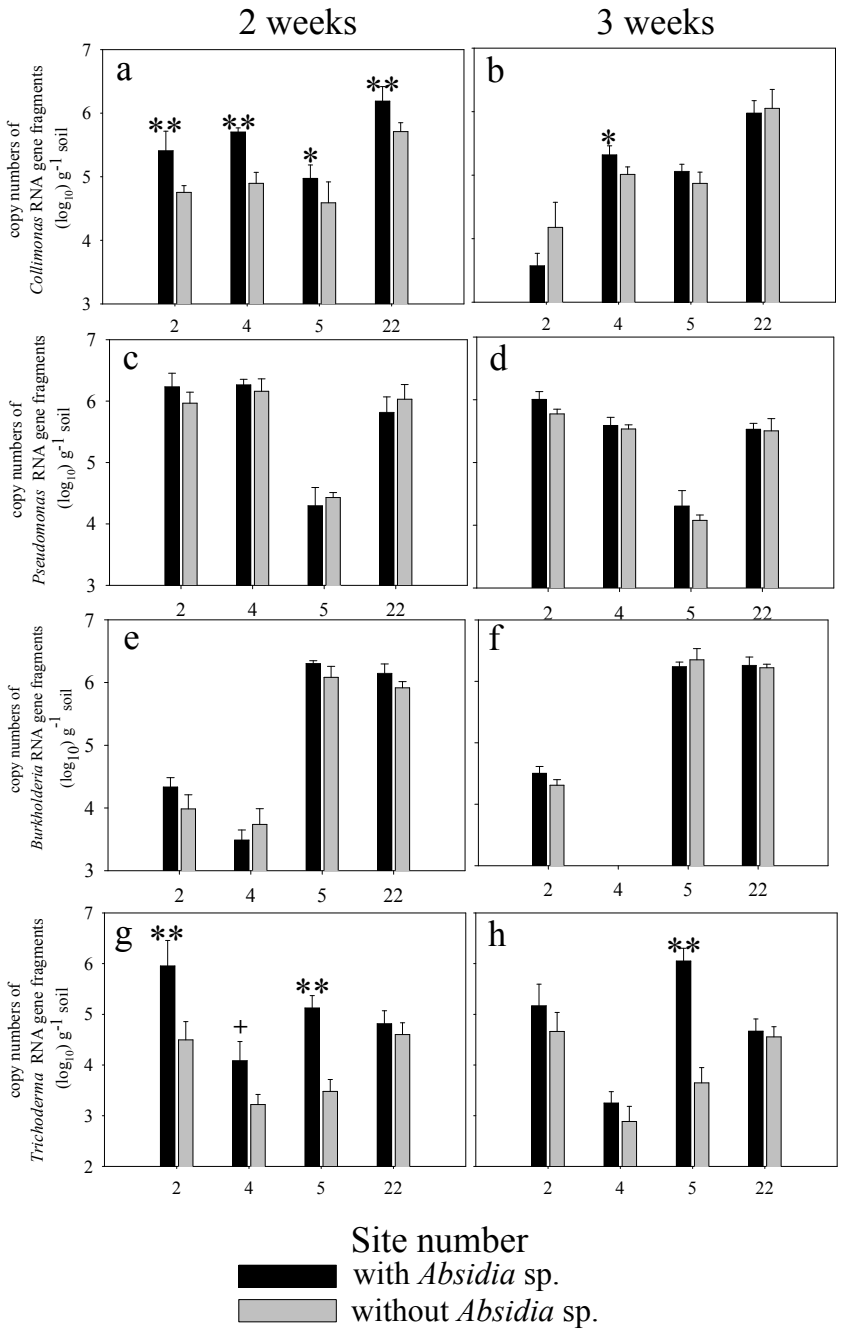
All ANOVAS were performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK). For ANOVA, normal distribution of data was tested with Shapiro-Wilks test and variance homogeneity by Levene's test. When data failed to satisfy one of the tests, an appropriate transformation was applied (log or square root transformation). Tukey's honest significant difference (HSD) method modified for unequal sample size (Unequal N HSD in Statistica) $P < 0.05$ was used.

RESULTS

Growth response of indigenous collimonads and* Trichoderma *spp. to invasion of field soils by* Absidia *mycelium (experiment 1). The effect of introduction of *Absidia* mycelium on the numbers of collimonads is given in FIG 4.1a and 4.1b. The real-time PCR-based numbers of indigenous collimonads in the soils were 5.0 $\times 10^4$ g^{-1} soil for site 2, 4 and 5 and 7.5 $\times 10^5$ g^{-1} soil for site 22. These numbers did not increase during 2 weeks incubation without introduction of fungal hyphae. Numbers of collimonads had increased (4.1 fold on average) in all 4 soils, 2 weeks after invasion by *Absidia* hyphae. However, with the exception of soil 4, the stimulating effect of invasion by *Absidia* hyphae was no longer apparent when the incubation period was one week longer (3 weeks in total).

The growth dynamics of two bacterial genera, *Pseudomonas* and *Burkholderia,* were examined to have an indication of the specificity of the growth response of collimonads upon introduction of *Absidia.* No effects of introduction of *Absidia* on bacterial numbers within these genera were detected (FIG 4.1c to 4.1f). The numbers of *Burkholderia* bacteria in soil 4 were under detection limit (1.0×10^3 copies g^{-1} soil) after 3 weeks incubation.

FIG 4.1: Growth response of indigenous soil bacteria *belonging to the genus Collimonas (1A,1B), Pseudomonas (1C,1D) , Burkholderia (1E,1F) and of indigenous soil fungi belonging to the genus Trichoderma (1G,1H) upon extension of mycelium of the fungus Absidia in 4 field soil microcosms (site 2, 5, 4 and 22). Copy numbers of the 16S rRNA of the bacterial genera and of the ITS region of Trichoderma rRNA were determined by real-time PCR in the mycelial zone of Absidia (black bar) and in a comparable zone of microcosms without introduction of Absidia (gray bar). Real-time PCR based quantifications were done 2 and 3 weeks after introduction of the fungal inoculum. Data represent the means and standard deviations for six replicates that were harvested at the indicated times. Different symbols within a graph indicate significant differences for a particular soil between microscosms with and without introduction of Absidia (+ ; $P \leq 0.1$, * ; $P \leq 0.05$, ** ; $P \leq 0.01$) based on Tukey's HSD test.*


2 weeks
3 weeks
a
b
c
d
e
f
g
h
copy numbers of Collimonas RNA gene fragments (log10) g-1 soil
copy numbers of Pseudomonas RNA gene fragments (log10) g-1 soil
copy numbers of Burkholderia RNA gene fragments (log10) g-1 soil
copy numbers of Trichoderma RNA gene fragments (log10) g-1 soil
2
4
5
22
Site number
with Absidia sp.
without Absidia sp.

Real-time PCR-based numbers of indigenous fungi belonging to the genus *Trichoderma* were significantly increased in 2 soils (soil 2 and 5), two weeks after introduction of *Absidia* (FIG 4.1g and 4.1h). In soil 4, the increase was significant at the level of $P < 0.1$. The average increase in real-time PCR based numbers in these 3 soils was 26.8 fold. After 3 weeks of incubation, a significant stimulation of abundance of *Trichoderma* spp. was only found for soil 5.

Impact of soil colonization by* Absidia *mycelium on bacterial and fungal community structure (experiment 1). The effect of invading *Absidia* mycelium on microbial community structure as assessed by the PCR-DGGE in the different soils was analyzed by db-RDA (Table 4.1)(Appendix FIG 4.4). The bacterial community structure, including *Burkholderia* community structure, appeared to be unchanged whereas the fungal community structure was significantly influenced by introduction of the *Absidia* in all the soils that we tested. For all soils, the band corresponding to *Absidia* was clearly visible in the gel.

Table 4.1: Distance-based redundancy analyses (db-RDA) *of the effect of invasion of hyphae of the fungus Absidia sp. on microbial community structures (presence-absence of PCR-DGGE bands) in microcosms of 4 different field soils.*

Incubation time		Bacteria		Fungi	
	Factors	**F-ratio**	**P-value**	**F-ratio**	**P-value**
2 weeks	*Absidia*	0.900	0.518	3.069	*0.001*
	Soil origin	42.907	*0.001*	3.088	*0.001*
	Absidia × Soil	0.900	0.464	2.629	*0.001*
3 weeks	*Absidia*	0.153	0.967	3.399	*0.007*
	Soil origin	21.666	*0.001*	4.399	*0.001*
	Absidia × Soil	0.071	1.000	2.728	*0.001*

Growth response of* Absidia *to* Collimonas *mycophagy (experiment 2). The introduction of *Absidia* in purified sand containing collimonads resulted in a 5.2 fold increase of the real-time PCR-based number of collimonads in the mycelial zone (FIG 4.2a). The dynamics of the abundance of *Absidia* was assessed by quantification of 18S rRNA gene copies and ergosterol (FIG 4.2b and 4.2c, respectively). Results of real-time PCR indicated that 18S rRNA gene copies of the *Absidia* tended to increase in the presence of collimonads although the differences

between controls and collimonads-containing microscosms were not significant at the 5% level. The quantities of the fungal membrane component ergosterol did not differ significantly between controls and collimonads-containing microscosms.

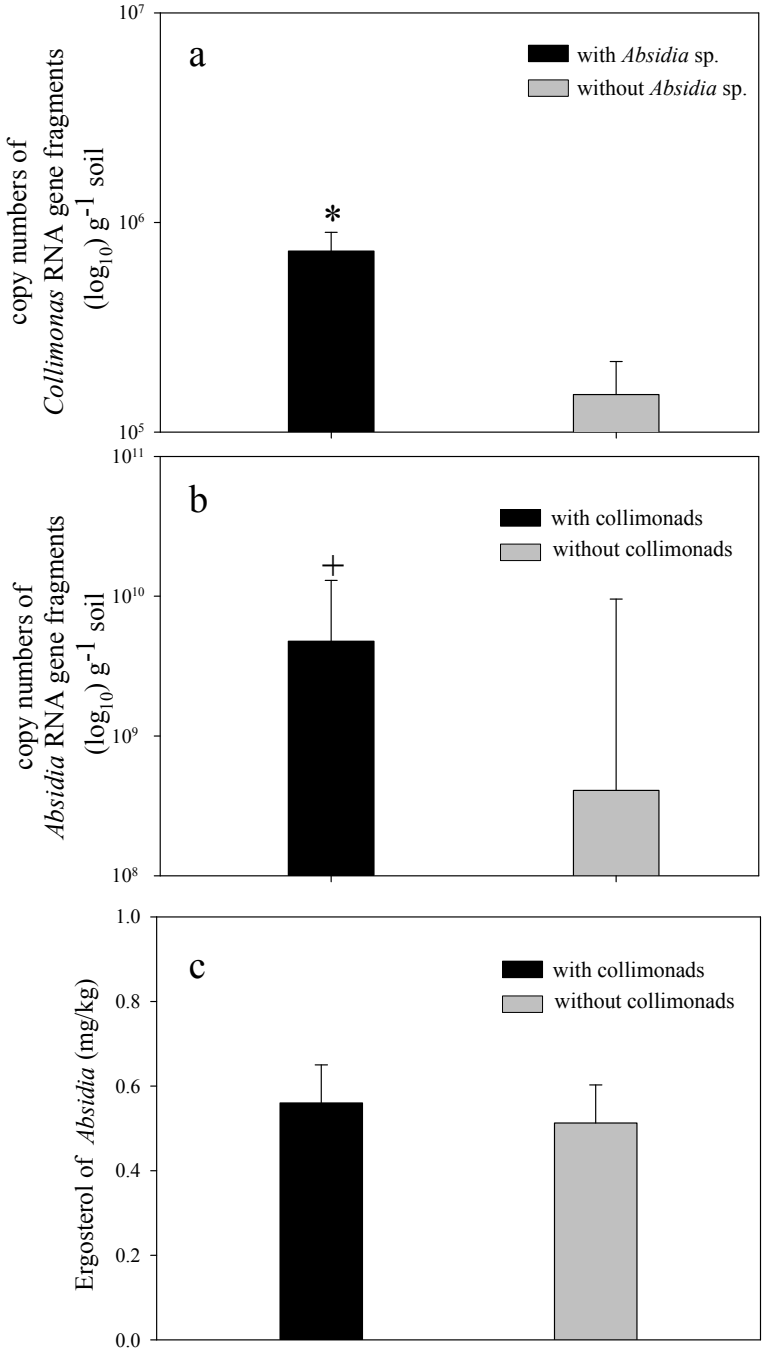

a
with *Absidia* sp.
without *Absidia* sp.
copy numbers of *Collimonas* RNA gene fragments (log_{10}) g^{-1} soil
10^7
10^6
10^5
*
b
with collimonads
without collimonads
copy numbers of *Absidia* RNA gene fragments (log_{10}) g^{-1} soil
10^{11}
10^{10}
10^9
10^8
+
c
with collimonads
without collimonads
Ergosterol of *Absidia* (mg/kg)
1.0
0.8
0.6
0.4
0.2
0.0

FIG 4.2: Growth response of collimonads and the fungus Absidia to each others presence in microcosm containing purified sand. *Collimonads were mixed into the soil and Absidia invaded the soil from a nutrient-rich agar disk. (a) Numbers of 16S rRNA fragments of collimonads, (b) Numbers of 18S rRNA fragments of Absidia, (c) Concentration of the fungal membrane component ergosterol. Data represent the means and standard deviations of six replicates that were harvested 2 and 3 weeks after introduction of the Absidia inoculum. Different symbols within a graph indicate significant differences between single (Absidia or collimonads) and mixed (Absidia and collimonads) treatments. (+ ; $P \leq 0.1$, * ; $P \leq 0.05$, ** ; $P \leq 0.01$) based on Tukey's HSD test.*

Interaction between* Collimonas *and* Trichoderma *mycophagy (experiment 2). The increase of real-time PCR-based numbers of the ITS region of *Trichoderma* rRNA upon introduction of *Absidia* was significantly lower in the presence of collimonads (FIG 4.3). The increase of numbers of collimonads due to invasion of *Absidia* hyphae was not significantly affected by the presence of *T. harzianum*.

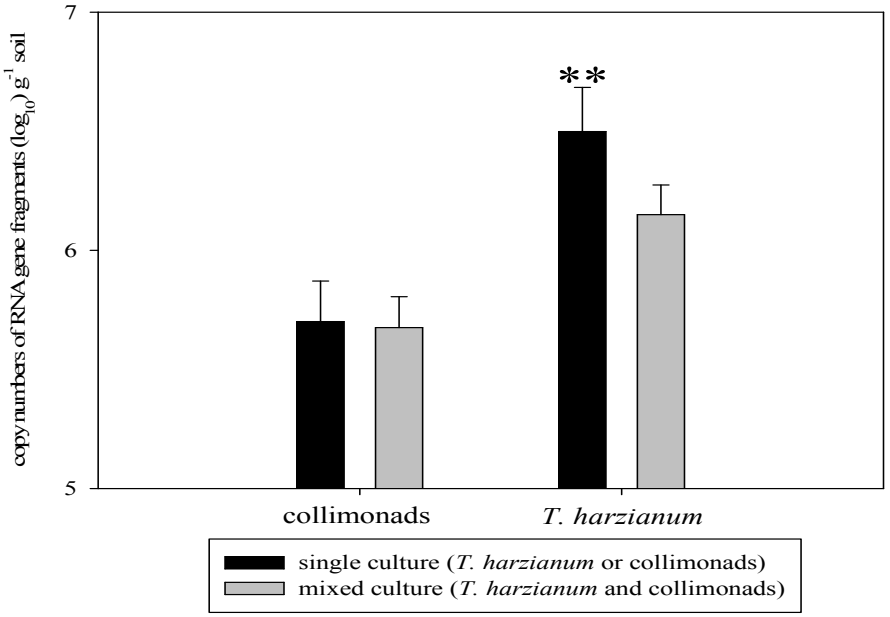


FIG 4.3: Growth response of collimonads and T. harzianum to invading hyphae of the fungus Absidia in microcosms containing purified sand. *Treatments involved the presence of only collimonads, of only T. harzianum or of both collimonads and T. harzianum. Copy numbers of the Collimonas 16S rRNA or Trichoderma ITS region were determined by real-time PCR in the mycelial zone of Absidia 3 weeks after introduction of this fungus. Data represent the means and standard deviations for six replicates that were harvested. Different symbols within a graph indicate significant differences between single (T. harzianum or collimonads) and mixed (T. harzianum and collimonads) treatments. (+ ; $P \leq 0.1$, * ; $P \leq 0.05$, ** ; $P \leq 0.01$) based on Tukey's HSD test.*

DISCUSSION

The increase in numbers of indigenous soil collimonads upon introduction of *Absidia* mycelium indicates that the fungal-induced growth response of collimonads is not restricted to artificial environments (sand microcosms, agar) but can also occur in natural soils. In contrast to collimonads, numbers of bacteria belonging to genera *Pseudomonas* and *Burkholderia* did not increase after the soil had been invaded by *Absidia* hyphae. Several *Pseudomonas* and *Burkholderia* spp. were found to be associated with fungal hyphae, probably growing on fungal exudates [8, 87, 88]. The fact that numbers of bacteria belonging to these genera did not increase upon introduction of *Absidia* hyphae, makes it unlikely that the growth response of collimonads was due to assimilation of exudates. Hence, our investigation points towards an active mycophagous growth by collimonads on *Absidia* hyphae in field soils.

Comparison of bacterial DGGE patterns between control soils and soils subjected to invasion by *Absidia* did not reveal other groups of bacteria that were stimulated by the introduction of the fungus. Hence, it appears that the stimulation of growth of *Collimonas* bacteria by fungal hyphae is not common among other soil bacteria. However, PCR-DGGE analysis does only cover a limited number of dominant bacterial taxa, and the presence of other bacteria with similar growth responses as collimonads can by no means be excluded [89].

Remarkably, the increase of indigenous collimonads upon invasion of *Absidia* hyphae in the field soils was only short-term. Within 3 weeks, numbers had dropped again to the levels of the control for most soils. The same dynamics, i.e. a short-term increase in collimonads numbers, was also found when *Collimonas* strains were inoculated in purified sand and exposed to invading hyphae of different fungal species [7](FIG 4.2a). In the study of De Boer *et al*. (2001) collimonads increased only during the extension of fungal hyphae and shortly thereafter. The subsequent decrease of *Collimonas* CFUs in the study of De Boer *et al* (2001) could have been due to a reduction in cultivability of starving

cells. Such an explanation is unlikely for the current study where the real-time PCR enumeration of collimonads was not dependent on the cultivability of strains. Since the same dynamics of numbers of collimonads, i.e. short-term increase was found for both the indigenous collimonads in field soils and *Collimonas* isolates in pure sand, it is unlikely to be caused by predatory or antagonistic soil microorganisms. The same trend of decrease in real-time PCR based numbers during prolonged incubation was also found for pseudomonads, *Burkholderia* spp. and *Trichoderma* spp. for some of the soils. Increase of inhibition of real-time PCR in the prolonged incubated samples does not offer an explanation as we did not find PCR inhibition in any of our samples (data not shown). Hence, for the time being we cannot explain the apparent decrease of collimonads following the fungal-induced increase.

Whereas there is a significant growth increase of collimonads to invading hyphae in both field soils and pure sand, the absolute amount of bacterial biomass produced at the expense of living fungal hyphae is low : Assuming a cell volume of 0.17 μm^3 and a density of 0.8 g/cm^3 [90], the increase of *Collimonas* biomass in 2 weeks incubation was calculated to be 0.05 μg per g soil. Ergosterol data were used to calculate the fungal biomass. For zygomycetes a conversion factor of 3 mg ergosterol per g biomass can be applied [62]. This implies that the amount of 0.5 μg of ergosterol that was measured per g soil is equivalent to 167 μg *Absidia* biomass. This amount of fungal biomass is 3.3×10^3 times bigger than the 0.05 μg of *Collimonas* biomass produced. So even when we assume a low growth efficiency of the collimonads, the reduction of fungal biomass production by collimonads appears to be negligible. Our observation that biomass production by *Absidia* was not significantly reduced by the presence of collimonads is in line with these calculations. Real-time quantification of 18S rRNA fragments of *Absidia* showed even a trend towards increase in the presence of collimonads, but this trend was not observed with ergosterol measurements. Overall, it can be concluded that the impact of mycophagous collimonads on fungi is small with respect to biomass production but the bacteria may be able to introduce

morphological changes e.g. increased branching. Changes in fungal morphology as a result of the presence of collimonads have been observed by Deveau *et al.*, [91]. They showed that *C. fungivorans* Ter331 decreased the mycelial extension of *L. bicolor* S238N but increased the branching density.

The growth dynamics of the mycophagous *Trichoderma* spp. were examined in a similar way as those of collimonads. Except for site 22, the results of the real-time PCR analyses indicated that copy numbers of *Trichoderma* ITS fragments had increased significantly after 2 weeks of exposure to invasion by *Absidia* hyphae. Thus, the increase of indigenous mycophagous collimonads did not prevent an increased abundance of mycophagous *Trichoderma* spp. and *vice versa*. Yet, the experiments in the gnotobiotic microcosms containing both collimonads and *T. harzianum* indicated that the presence of collimonads can have a negative effect on mycophagous growth of *Trichoderma* spp. (FIG 4.3). This negative effect is most likely caused by antibiosis as resource competition, i.e. competition for *Absidia* hyphae, is not likely given the fact that collimonads do not reduce the fungal biomass of *Absidia* (FIG 4.2). Another explanation might be competition for the preferential zones to attack *Absidia* hyphae. However, whereas the attack by collimonads is probably limited to hyphal tips [7], this is not the case for *Trichoderma* spp. which can penetrate mature hyphae via appressorium formation [92]. There is also the possibility that collimonads grew at the expense of *T. harzianum* hyphae, but tests on water-agar indicated that this is not likely (data not shown). Production of antibiotics by collimonads has been indicated as an important factor in mycophagous behavior but it may also result in inhibition of biomass formation of *Trichoderma* [63].

In conclusion, we showed that indigenous soil collimonads respond to the introduction of *Absidia* hyphae into soil. This supports the importance of mycophagy as a life history characteristic of collimonads. The mycophagous growth of collimonads is of minor importance for fungal biomass dynamics but may affect fungal growth patterns and also community dynamics. In addition, we

showed that collimonads can have a negative effect on the mycophagous growth of *T. harzianum*.

Appendix

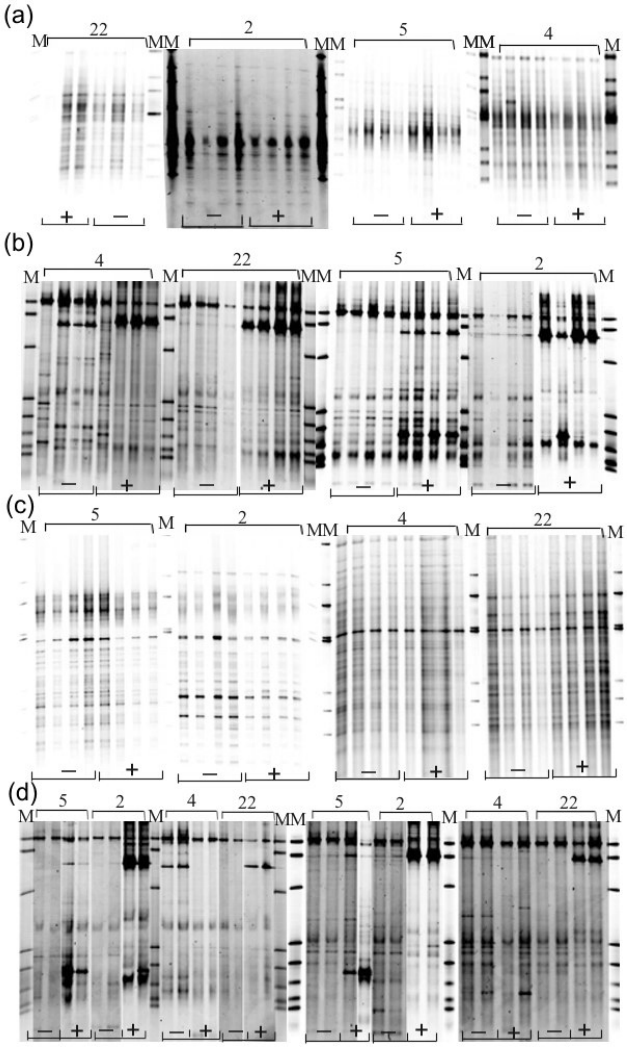


FIG 4.4: *DGGE gels of PCR-amplified fragments of 16S-RNA genes (bacteria) and 18S-RNA genes (fungi) of 4 soils with and without invading hyphae of the fungus Absidia: (a) bacterial PCR-DGGE after 2 weeks (b) fungal PCR-DGGE after 2 weeks (c) bacterial PCR-DGGE after 3 weeks (d) fungal PCR-DGGE after 3 weeks (e) Burkholderia PCR-DGGE after 2 weeks (d) Burkholderia PCR-DGGE after 3 weeks. Sign; + denote samples with invading hyphae of the fungus Absidia. Sign; – denote samples without invading hyphae of the fungus Absidia. Sign; M denote a molecular marker made of an artificial mix of different bacterial/fungal species. Numbers are corresponding to site numbers of 4 soils (site: 2, 4, 5 and 22).*

5

IMPACT OF *COLLIMONAS* BACTERIA ON COMMUNITY COMPOSITION OF SOIL FUNGI

Sachie Höppener-Ogawa, Johan H. J. Leveau, Maria P.J. Hundscheid, Johannes A. van Veen and Wietse de Boer

Submitted.

Summary

The genus *Collimonas* consists of soil bacteria that have the potential to grow at the expense of living fungal hyphae. However, the consequences of this mycophagous ability for soil fungi are unknown. Here we report on the development of fungal communities after introduction of collimonads in a soil that had a low abundance of indigenous collimonads. Development of fungal communities was stimulated by addition of cellulose or by introducing plants (*Plantago lanceolata*). Community composition of total fungi in soil and rhizosphere and of arbuscular mycorrhizal fungi in roots was examined by PCR-DGGE. The introduction of collimonads altered the composition of all fungal communities studied but had no effects on fungal biomass increase, cellulose degrading activity or plant performance. The most likely explanation for these results is that differences in sensitivity of fungal species to the presence of collimonads results in competitive replacement of species. The lab and greenhouse experiments were complemented with a field experiment. Mesh bags containing sterile sand with or without collimonads were buried in an ex-arable field and a forest. The presence of collimonads had an effect on the composition of fungi invading these bags in the ex-arable site but not in the forest site.

Introduction

Niche formation of bacteria in terrestrial environments may have been strongly influenced by the presence of fungi [8]. A niche that is clearly related to the presence of fungi is mycophagy, i.e. the feeding on living fungi. However, mycophagous growth of soil bacteria has, so far, received little attention [10].

Soil bacteria of the genus *Collimonas* have been shown to grow at the expense of living fungal hyphae in soil-like microcosms and natural soils [10, 17, 93]. Höppener-Ogawa *et al.* [63] showed that collimonads are widely spread in terrestrial environments but that their abundance is rather low (up to 8.7×10^5 cells g^{-1} dry weight soil). The low densities of collimonads in soil as well as the moderate increase (from 10^4 up to 10^6 cells g^{-1} dry soil) upon invasion of fungal hyphae in soils or soil-like systems suggests that their influence on total fungal biomass turnover is of minor importance [7]. This is supported by the fact that no significant negative effect of collimonads on fungal biomass production was observed in sand microcosms [93].

A minor effect of collimonads on fungal biomass turnover does, however, not exclude the possibility that collimonads have an effect on soil fungal community composition. As for bacteria, functional redundancy among fungi is high and so competition for the same resources will be severe. This holds true for both saprotrophic and mycorrhizal fungi [94, 95]. Collimonads may have an impact on the outcome of competition between fungi in case there are differences in sensitivity of fungal species towards mycophagous attack. Indeed, there are indications that such differences between fungal species exist [17, 24].

The aim of the current study was to test the effect of the presence of collimonads on the mycelial accumulation and community composition for different functional groups of soil fungi namely (1) cellulose- and sugar-degrading fungi after enrichment of a soil with cellulose and (2) root-exudate consuming fungi and arbuscular mycorrhizal fungi after introducing a plant (*Plantago*

lanceolata). In addition, the effect of the presence of collimonads on composition of fungi invading a sterile sand patch in natural soils was examined.

RESULTS

Effect of collimonads on the community composition of fungi in cellulose-enriched soil. Two weeks after the addition of cellulose to an ex-arable soil, the soil fungal biomass indicator ergosterol had increased from 1.0 ± 0.4 mg kg^{-1} dry soil to 8.7 ± 1.0 mg kg^{-1} dry soil in the controls and to 9.6 ± 0.7 mg kg^{-1} dry soil in the soil inoculated with collimonads, respectively. The stronger increase of ergosterol content in the presence of collimonads was nearly significant (P = 0.09). After prolonged incubation (8 weeks after the addition of cellulose) ergosterol contents had decreased to 3.9 ± 0.5 and 4.4 ± 0.2 mg kg^{-1} dry soil in the controls and in the *Collimonas*-inoculated soil, respectively. The difference between the controls and the *Collimonas*-inoculated soil was again nearly significant (P = 0.06). After 2 weeks of incubation, cellulase activity in the control samples (89.0 ± 37.6 nmol RBB release 24 h^{-1} g^{-1} dry soil) did not significantly differ from the *Collimonas*-inoculated soil 134.6 ± 50.7 nmol RBB release 24 h^{-1} g^{-1} dry soil).

Two weeks after the inoculation of collimonads, qPCR-based numbers of collimonads had dropped from 1.0 $\times 10^6$ to 1.6 $\times 10^5$ ± 2.3 $\times 10^4$ collimonads per g dry soil after 2 weeks incubation and the numbers were under the detection limit (1.5 $\times 10^4$ collimonads per g dry soil) after 8 weeks incubation.

PCR-DGGE patterns of fungal 18S rRNA-gene fragments amplified from total DNA differed between the controls and the *Collimonas*-inoculated soil replicates (FIG 5.1, lanes 10-18). These differences were confirmed by db-RDA analysis (P <0.001). This indicates a significant effect of the presence of collimonads on the development of the fungal community in the cellulose-enriched soil. No further changes in cellulolytic fungal composition became apparent during prolonged incubation time (from 2 to 8 weeks). Two major bands (E and K, FIG 5.1.) that were present in the controls were not distinguishable in the soil inoculated with collimonads. In contrast, five bands (F, G, H, I, and J) distinct bands were detected in the *Collimonas*-inoculated soil. Sequence analysis of the 18S rRNA fragments revealed 2 high matches (> 98%), namely 1 with the genus

Cordyceps (band K, without the addition of collimonads) and 1 with the genus *Trichocladium* (band I, with the addition of collimonads).

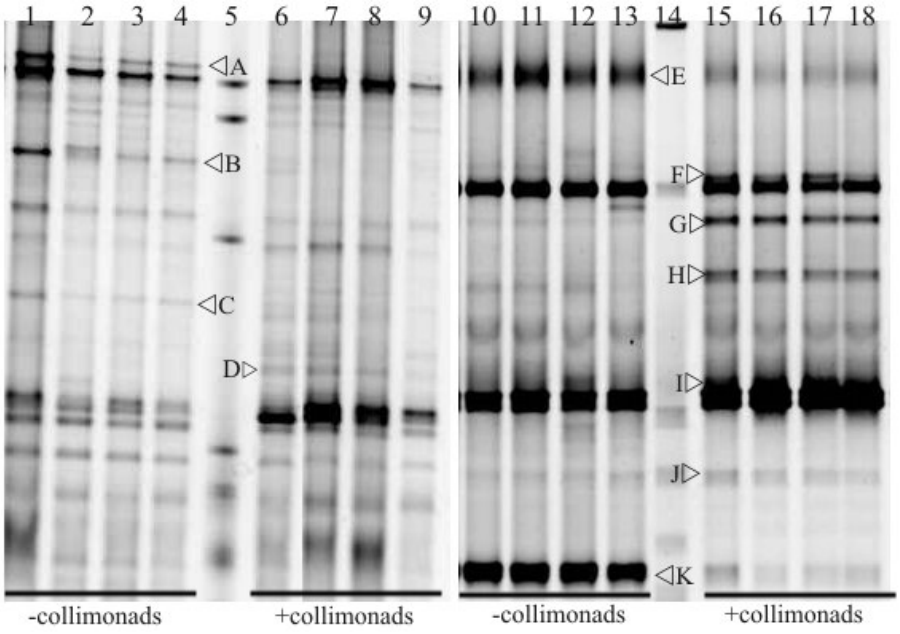


FIG 5.1: ***DGGE patterns of partial fungal 18S RNA genes from 2 experiments.*** *Lanes 1-4: Rhizosphere soil of Plantago lanceolata grown in soil without addition of collimonads; lanes 6 – 9: Rhizosphere soil of Plantago lanceolata grown in soil with addition of collimonads; lanes 10- 13: Cellulose-enriched soil without addition of collimonads; lanes 15 -18: Cellulose-enriched soil with addition of collimonads. The patterns of the cellulose-enriched soil are from the 8 weeks incubation. Fungal markers, made of an artificial mix of different fungal species, are presented in lanes 5 and 14. Coding of bands refers to Table 5.1.*

Effect of collimonads on the community composition of rhizosphere fungi and arbuscular mycorrhizal fungi associated with roots of Plantago lanceolata. The ergosterol content in the rhizosphere soil of *P. lanceolata* was 1.14 ± 0.39 and 0.98 ± 0.43 mg kg^{-1} dry soil with and without the addition of collimonads in soil, respectively. The difference in ergosterol content between the *Collimonas*-inoculated soils and the controls was not significant (P = 0.48).

The presence of collimonads had a significant effect on the fungal community structure in the rhizosphere of *P. lanceolata* (FIG 5.1, lanes 1-8), as shown by db-RDA analysis (P<0.004).

Three distinct bands (A, B and C) were detected in the rhizosphere of the control treatment. Sequence analysis revealed that two of these bands (A and B) belonged to the subphylum *Mucoromycotina* (Table 5.1). One band (D) that was only distinguishable in the soil with collimonads had the highest match (95%) with the genus *Plectosphaerella*, which belongs to the phylum *Ascomycota* (Table 5.1).

The presence of collimonads was accompanied by a significant change (db-RDA analysis; P<0.004) in AM fungal community structure inside the root (FIG 5.2). Band AM 1 and AM 4 were not distinguishable when collimonads were present, whereas bands AM 2 and AM 3 were only visible in the presence of collimonads. The results of sequence analysis of these bands are shown in Table 5.1. All sequenced bands, including bands that did not differ between the treatments, had the highest match with the class *Glomeromycota*, confirming the selectivity of the primers specific for arbuscular mycorrhizal fungi.

Table 5.1: *Sequence analysis of total fungal and arbuscular mycorrhizal PCR-DGGE bands. Letters indicate the coding of the bands as indicated in FIG 5.1 and 2.*

	Closest relatives		
DGGE bands	**Species**	**Phylum affiliations**	**Accession no.,**
AM1[a]	–[*]	–	–
AM2[b]	*Glomus intraradices*	Glomeromycota	AY916691, 92%
AM3[b]	*Glomus intraradices,*	Glomeromycota	AF222522, 95%
AM4[a]	*Glomus microaggregatum*	Glomeromycota	–
A[a]	*Hesseltinella vesiculosa*	Mucoromycotina[1]	–
B[a]	*Endogone pisiformis*	Mucoromycotina	AY584675, 86%
C[a]	*Crinipellis zonata*	Basidiomycota	AY083223, 86%
D[b]	*Plectosphaerella cucumerina*	Ascomycota	EU263613, 99%
E[c]	–	–	DQ915460, 92%
F[b]	–	–	DQ838790, 100%
G[b]	*Lopezaria versicolor*	Ascomycota	
H[b]	*Malassezia furfur*	Basidiomycota	
I[b]	*Trichocladium asperum*	Ascomycota	
J[b]	*Marchandiobasidium aurantiacum*	Basidiomycota	
K[c]	*Cordyceps gunnii*	Ascomycota	

[a] *Band appeared in treatment without collimonads.*
[b] *Band appeared in treatment with collimonads.*
[c] *Band intensity increased in treatment without collimonads.*
[*] *−; denotes bands that could not be cloned successfully.*
[1] *Mucoromycotina is a subphylum, whereas the others are phyla [67]*

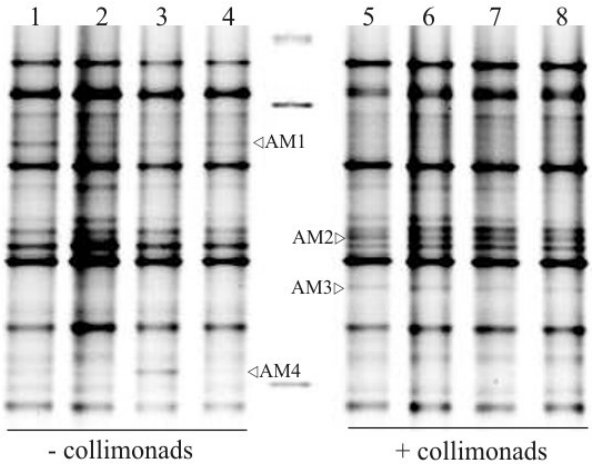


FIG 5.2: ***DGGE patterns of partial 28S RNA genes of AM fungi associated with roots of Plantago lanceolata.*** *Lanes 1-4: plants grown in soil without addition of collimonads; lanes 5-8: plants grown in soil with addition of collimonads. Coding of bands refers to Table 5.1.*

The addition of collimonads to the soils did not affect the AM fungal colonization of *Plantago* roots (Table 5.2). There was also no effect of the addition of collimonads on the production of root and shoot biomass by *P. lanceolata* nor was the nitrogen and phosphorus content of *P. lanceolata* affected by the presence of collimonads (Table 5.2).

Table 5.2: Plant growth parameters of Plantago lanceolata and presence of structures of arbuscular mycorrhizal fungi in Plantago roots in soil with and without addition of collimonads. *Data indicate the means of 6 replicates. Differences between means were tested for significance using one way ANOVA.*

Treatment	Shoot dry weight (g/pot)	Root dry weight (g/pot)	AC (%)*	VC (%)	HC (%)	N (mg g^{-1})	P (mg g^{-1})
Addition of collimonads	0.40^{a**}	3.6^{a}	0.47^{a}	0.37^{a}	0.84^{a}	11.6^{a}	4.6^{a}
Control without adding collimonads	0.58^{a}	3.8^{a}	0.50^{a}	0.31^{a}	0.90^{a}	10.6^{a}	5.0^{a}

** Percentage of intersections containing structures of AM fungi: Arbuscule-like structures (AC), Vesicule-like structures (VC) and hyphal-like structures (HC) were present.*
*** Different letters denote significant differences ($P < 0.05$) between the treatments*

Field experiment: Sand-containing mesh bags. After the 2 month incubation of sand-containing mesh bags in field soils, qPCR-based numbers of collimonads in the sand had dropped from 1.0×10^6 to $2.7 \times 10^5 \pm 5.5 \times 10^4$ collimonads per g dry soil in the ex-arable site and to $5.3 \times 10^5 \pm 2.6 \times 10^5$ collimonads per g dry soil in the forest site.

Quantification of ergosterol contents indicated that the biomass of fungi invading sterile sand in buried mesh bags was low (< 0.5 mg ergostrerol kg^{-1} sand). Despite the low fungal biomass present, fungal community structure in the mesh bags could be assessed by PCR-DGGE (Appendix FIG S5.1). Principal coordinate analysis (PCoA), based on Jaccard similarity, of fungal 18S rDNA-DGGE patterns were shown in FIG 5.3. Samples clustered according to the soil origin. Statistical analysis (db-RDA) also revealed that the composition of the invading fungi was dependent on the soil origin (P=0.001). The presence of collimonads in the sterile

sand had a significant effect on the composition of the invading fungi in the ex-arable site (P = 0.007) but not in the forest site (P = 0.66).

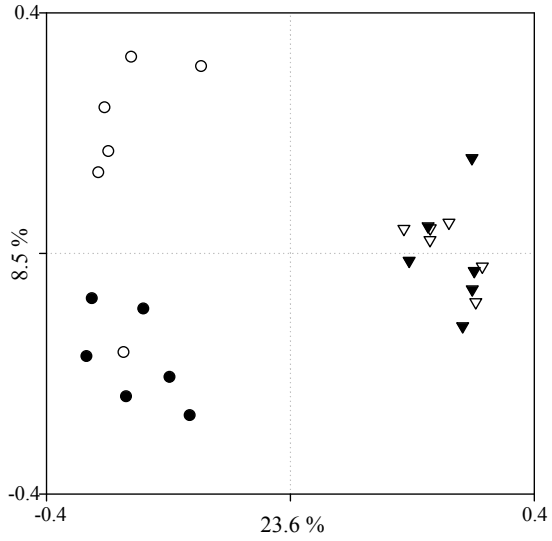


FIG 5.3: ***Principal coordinate analysis (PCoA)****, based on Jaccard similarities, of DGGE patterns obtained from PCR-amplified fungal 18S rRNA genes in acid-purified sand that had been buried for 2 months in nylon mesh bags in an ex-arable field and a forest site. At the start of the field incubation, half of the bags contained sterile sand with the addition of collimonads, whereas the other half contained sterile sand only: ●: Sand with collimonads in ex-arable land, ○: Sand without collimonads in ex-arable land, ▼: Sand with of collimonads in the forest site , ▽: Sand without collimonads in the forest site*

DISCUSSION

Our results clearly indicate that the introduction of collimonads in a soil that was apparently devoid of such bacteria had a significant impact on the composition of fungal communities. The impact on fungal community composition was not accompanied by a reduction in fungal biomass or significant changes in cellulase activity.

The numbers of collimonads that were added to the soil (10^6 g^{-1} dry soil) are similar to the highest densities of collimonads present in natural soils [63]. No further increase of collimonads was observed after introduction of cellulose or *P. lanceolata* plants. The actual dynamics of collimonads in these experiments is, however, not clear as the survival after introduction is not known. Yet, it is obvious that the increase of fungal biomass upon introduction of cellulose or plants did not result in densities of collimonads exceeding the initial density of 10^6 g^{-1} dry soil. In a previous study we observed that a fungal-induced increase from 10^5 to 10^6 collimonads g^{-1} dry soil had no negative effect on fungal biomass production [93].

Therefore, it is not surprising that we did not find a negative effect of collimonads on fungal biomass production in the current study.

Changes in fungal community composition in the presence of collimonads became apparent for all 3 fungal groups studied (cellulolytic fungi, rhizosphere fungi and AM fungi). The most likely explanation is that differences in sensitivity of fungal species or strains to the presence of collimonads changes the competitive relationships between different fungal taxa. Some fungal species that have a selective advantage in the absence of collimonads appear to lose this advantage in their presence and vice versa.

It is tempting to ascribe the changes in fungal community composition to preferential mycophagous growth of collimonads. For instance, the apparent inability of two fungi belonging to the subphylum *Mucormycotina* to colonize the *P. lanceolata* rhizosphere in the presence of collimonads is in line with the

reported strong mycophagous growth of collimonads at the expense of zygomycetes [7, 63]. However, selective effects may also have been due to other antifungal activities of collimonads [24]. Small, short-term effects of antibiotic producing biocontrol bacteria on non-target fungi in the rhizosphere have been reported [68, 96]. Compared with these studies the effects of collimonads on fungal communities appear to be more consistent. However, this may be due to differences in the experimental set-up (field trials *versus* containers; seed inoculation *versus* soil inoculation).

Collimonads appeared to affect fungi within all major fungal (sub)phyla that can be found in soils i.e. *Glomeromycota*, *Mucoromycotina*, *Ascomycota* and *Basidiomycota*. For all these (sub)phyla with exception of the *Mucoromycotina*, both appearing and disappearing amplicons (bands) in the PCR-DGGE analysis were found. Several bands could not be reliably identified to the genus level because the match with existing sequences in databases was too low. For the ones that could be identified to the genus level (match in Blast search >98%), interpretation should be considered with care as detection of functional and morphological characteristics is required to confirm the identification.

The highest match (100%) obtained was with the genus *Cordyceps*, namely for band K that was only prominently present in cellulose-enriched soil without the addition of collimonads. This is surprising as *Cordyceps* species are known as parasites of arthropods [97]. However, the existence of a saptrotrophic *Cordyceps* strain that is very closely related to parasitic strains has been reported [98]. Band I, which was only strongly present in the cellulose-enriched soil with collimonads, had a high match (99%) with genera *Trichocladium*. These are cellulolytic fungi that are mostly known from aquatic environments where they are involved in the decomposition of wood and leaves [99]. However, soil isolates of genera *Trichocladium* have also been reported [100]. Although the cellulolytic fungal community was changed due to the presence of collimonads, total cellulase activity in soil samples was unchanged. This is in line with the expected functional redundancy in soil fungi for cellulose degradation [95].

Arbuscular mycorrhizal fungi form associations with many plant species and have an important role in the plant's mineral nutrient acquisition [101]. Earlier studies have indicated that the occurrence of collimonads may be related to the occurrence of AM fungi [102]. Such a relationship could point at preferential mycophagous growth of collimonads on AM fungi with a possible negative impact on plant performance. The current study showed that the presence of collimonads had a clear effect on the community composition of AM fungi inside the roots. However, there were no apparent effects on colonization of roots of *P. lanceolata* by arbuscular mycorrhizal fungi or on plant performance. Hence, different sensitivity of AM fungal species/strains to collimonads appears to result in a shift in AM community composition without affecting the plant nutrient acquisition.

In addition to the laboratory and greenhouse experiment we performed an experiment to see whether collimonads affect fungal community structure under field conditions. Only small amounts of fungi had colonized the sterile sand in the buried mesh bags after 2 months of incubation in the fields. Yet, the effect of the presence of collimonads on the community composition of the invading fungi in the ex-arable soil was apparent. No such effects were seen for the forest soil. Major fungal groups normally present in ex-arable soils consists of fast growing saprotrophic fungi and arbuscular mycorrhizal fungi that have less persistent hyphae than lignocellulolytic basidiomycetes and ecto-mycorrhizal fungi which are typically abundant in forest soils [103]. Fungi with more persistent hyphae may also be less sensitive to mycophagous attack by collimonads.

In conclusion, collimonads had a strong effect on the composition of functional groups of soil fungi whereas the effect on fungal biomass production was small.

Conceptually, these observations are of great interest as it shows that a minor component of the soil bacterial community can have a strong impact on fungal community composition in the soil ecosystem.

Experimental procedures

Study sites. Two field sites (a deciduous forest and an ex-arable field) used in this study are located in the central part (Veluwe) of the Netherlands. The forest site (major tree species beech and oak) which is located near the village Doorwerth was only used for the field experiment. The ex-arable field was referred to as Mossel in the study of van der Wal *et al*. [43]. The soil characteristics of the ex-arable land and forest site (mineral layer) were pH_{water} 5.6 and 3.5, total organic carbon 21 and 35 g/kg and C/N ratio 17.1 and 24.3, respectively. More details on the ex-arable site are given by van der Wal *et al*. [43]. The number of indigenous collimonads in the ex-arable soil was lower than 1.5×10^4 g^{-1} dry weight soil (detection limit) as assessed by *Collimonas*-specific real-time PCR [63]. At the ex-arable site, soil (0 – 10 cm layer) was sampled from at least 30 randomly selected points in a 50 x 50 m plot. Samples were pooled, mixed and sieved (4mm mesh) before use in microcosm experiments 1 and 2 (see below).

Bacterial strains. The strains used in this study were *Collimonas fungivorans* Ter 331 (AJ310395) and Ter 6^T (LMG 21973), *C. arenae* Ter 10^T (LMG 23964) and *C. pratensis* Ter 91^T (LMG 23965) [17, 73].

Effect of collimonads on the community composition of fungi in cellulose-enriched soil. The *Collimonas* strains Ter 6, 10, 91 and 331 were pre-grown on chitin-yeast agar at 20°C for 14 days as described by de Boer *et al*. [7]. Bacteria were suspended in P-buffer (KH_2PO_4, 1 g L^{-1} [pH 6.5]), pelleted by centrifugation (16.000 x g) and again suspended in P-buffer. A mixed suspension of *Collimonas* strains was made by adding equal numbers of cells of each strain (direct microscopic counting) to P-buffer. The bacterial suspension was mixed into autoclaved, acid-purified beach sand to give a moisture content of 14 % (wt/wt). For the control treatment the same amount of P-buffer without collimonads was used. Acid-purified sand with or without collimonads was mixed (1:9) with the

homogenized ex-arable soil. The mixing with collimonads-containing sand resulted in a final density of 10^6 cells g^{-1} dry soil. After inoculation, the soil was pre-incubated at 20 °C for 1 week to allow the bacteria to adapt to conditions in the ex-arable soil. Next, the soil was enriched with α-cellulose (Sigma), to a final concentration of 2 mg C g^{-1} dry soil. $(NH_4)_2SO_4$ was also added to establish an added C:N ratio of 17.1 which is similar to the C:N ratio of the ex-arable soil. Portions (40 g) of soil were transferred to Petri dishes (diameter, 8.5 cm) and spread evenly. The Petri dishes were sealed with Para-film and incubated at 20 °C. Per treatment (i.e. with and without collimonads) and per time interval 6 replicate samples were incubated. After 2 and 8 weeks of incubation, fungal biomass, fungal community composition and numbers of collimonads were determined. Quantification of ergosterol, via an alkaline extraction method, was used as an estimate of fungal biomass [47].

Analysis of cellulase activity was based on the release of remazol brilliant blue from dyed carboxymethyl cellulose as described by van der Wal *et al*. [103].

Fungal community structure was examined using PCR-denaturating gradient gel electrophoresis (PCR-DGGE)(see below). Numbers of collimonads were determined using real-time PCR on extracted soil DNA (see below) [63].

Effect of collimonads on the community composition on rhizosphere fungi and arbuscular mycorrhizal fungi associated with roots of Plantago lanceolata. Seeds of *Plantago lanceolata* (Ribwort Plantain) were surface-sterilized by gently shaking them in 0.4 % hypochlorite solution for 10 min and rinsed in sterile distilled water for 5 min. Sterile seeds were germinated on 1.5 % Bactoagar (Difco Co., Detroit, MI, #0140-01-0) at 20 °C for 7 days. Seedlings free of microbes were used for the experiment.

The ex-arable soil was inoculated with a mixture of collimonads as described in the previous sub-section. Non-inoculated controls were also prepared as described above.

Portions of 1 kg of soil were put in containers (diameter 11.4 cm; height 14.3 cm). Six replicate pots were prepared for treatments with or without the addition of collimonads. Per container 3 seedlings were planted. All containers were placed in the greenhouse at a temperature of 25 ± 1 °C (day) and 22 ± 1 °C (night) with a relative humidity of 70 % and with a photoperiod of 16:8 h (day:night). Natural daylight was supplemented with 400W metal halide bulbs (one per 1.5 m^2). The weight of the containers was checked daily, and if necessary water was added to maintain the moisture content of 14% (wt/wt).

After 1 week, the number of seedlings was reduced to 1 per container. After 8 weeks, plants were harvested. Thirty 1 cm-long sections of thin roots (diameter approximately 2 mm) from each pots were randomly sampled for the determination of colonization by AM fungi using the magnified intersections method [104]. Rhizosphere soil was collected by brushing soil adhering to roots. Next, roots were washed with water. Half of the roots were used for determination of root dry weight, the other half were used for analysis of AM communities. The shoots were used for determination of dry weight, total C, N and P. Total C and N were determined using a FlashEA 1112 Series NC soil analyzer. Total P was determined colorimetrically after acid destruction [105, 106].

Soil DNA was extracted as described below. The extracted DNA was used for the examination of total fungal and arbuscular mycorrhizal fungal community structure using PCR-DGGE analysis (see below).

Composition of AM fungal community inside the roots was also determined using the freeze dried root tissues. DNA was extracted from 2 g of freeze dried material using bead beating for 3 minutes to crush the root tissues. DNA extracted from the freeze-dried roots was analyzed for AM fungal community structure by PCR-DGGE analysis (see below).

Effect of collimonads on invading fungi (*Field experiment).* Fungal in-growth bags with an opening on 1 side were constructed of nylon mesh pieces (mesh size 35 μm; bag size, 10×5×2 cm) by fusing the edges with a thermo-sealer. The mesh

size used allowed the in-growth of fungal hyphae but not of roots [107]. *Collimonas* strains Ter 6, 10, 91 and 331 were grown and mixed with acid-purified sand as described above, except that the moisture content was kept at 5% (wt/wt). Portions (120 g) of sand containing collimonads were transferred into the bags. After sealing the opening of the bags, they were placed at 20 °C, and pre-incubated for 1 week to allow bacteria to adapt to conditions in sand. For the control treatment, bags with sand without collimonads were prepared. After preincubation, the nylon bags were placed horizontally at the interface between the organic horizon (4 cm thick) and the mineral soil in the forest site. In the ex-arable site, the nylon bags were placed at 3 cm below the surface. Bags of the 2 treatments (with and without collimonads) were placed close to each other. In total 6 replicates of both treatments were placed at 6 randomly selected spots within an area of 100 m^2.

The start of the field incubation was 11 September 2007, after 8 weeks of incubation in the field, the bags were collected. Per bag, sand was carefully mixed and samples were taken for fungal biomass (ergosterol) measurements [47] and determination of fungal community structure by PCR-DGGE analysis (see below). Soil DNA was extracted as described in the next sub-section.

PCR-denaturing gradient gel electrophoresis analysis, cloning **and sequencing.** For all experiments, soil DNA was extracted from an amount of moist soil equivalent to 0.25 g dry soils and an amount of 2 g freeze-dried root tissues using the MOBIO kit (MOBIO laboratories, Solana Beach, CA) and the DNeasy plant mini kit (QIAGEN, the Netherlands) according to the manufacture's instruction, except that soil DNA was finally eluded in 50 µl buffer. Table 5.3 summarizes the primers, thermocycling regimes and electrophoresis conditions used to analyze fungal and AM fungal communities. Partial sequences of 18S rRNA genes and 28S rRNA genes were analyzed for total fungi and AM fungi, respectively. All PCR reactions, denaturing gradient gel electrophoresis (DGGE) and cloning were carried out as described elsewhere [103] except that we added 2.5 µl of bovine serum albumin (BSA; 4 mg ml^{-1}) to the PCR reaction mixture to reduce PCR

inhibition. The initial denaturation steps were 94 °C and 93 °C for fungi and AM fungi, respectively. Sequencing of DGGE bands was performed by Macrogen (Seoul, Korea). To identify fungal species, similarities between obtained sequences were compared with nucleotide sequences available in GenBank by using the nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Statistical analyses. The banding patterns of DGGE gels were analyzed using the Image Master 1D program (Amersham Bioscience, Roosendaal, the Netherlands). The resulting binary matrices were exported and used in statistical analysis as "species" presence-absence matrices.

Ordination of samples resulting from image analysis of DGGE profiles were carried out using principle coordinates analysis (PCoA), based on Jaccard's similarity. The use of Jaccard's coefficient is recommended for binary species data like DGGE patterns scored for either presence or absence [86]. The effects of the presence of collimonads (all 3 experiments), the soil type (experiment 3) and interaction of these factors as environmental variables on the microbial structure as analyzed by PCR-DGGE was tested by distance-based redundancy analyses (db-RDA)[86] in Canoco 4.5 for Windows [51]. To test the effects of each of the two variables (the presence of collimonads and the soil type), the individual variables were recorded using dummy binary-variables of which one was used in Canoco as the only environmental variable in the model and the other as co-variable. To test the interaction, the variable entered in the model was the interaction between the presence of collimonads and the soil type, while both individual factors were included (without interaction) as co-variables. The significances of such models were tested with 999 permutations.

All ANOVAS were carried out in Statistica 7.0 (StatSoft Inc., Tulsa, OK). For ANOVA, data normality was tested with Shapiro-Wilks test and variance homogeneity by Levene's test. When data failed to satisfy one of the tests, an appropriate transformation was applied (log or square root transformation).

Turkey's honestly significant difference (HSD) method modified for unequal sample size (Unequal N HSD in Statistica) was used for post-hoc comparison with a 0.05 grouping baseline.

Table 5.3: ***Primers, PCR and denaturing gradient gel electrophoresis (DGGE) conditions used in this study***

Community	Primers	PCR protocol	DGGE gradients	Reference
Fungi	FR1-gc/FF390	Touchdown 55-47°C; 37 cycles	40-55% denaturant	Vanio & Hantula [83]
AM fungi	LR1/FLR2 followed by	58°C; 35 cycles	20-55% denaturant	Trouvelot [108] and van Tuinen [109]
	FLR3/FLR4			Gollotte [110]

Appendix

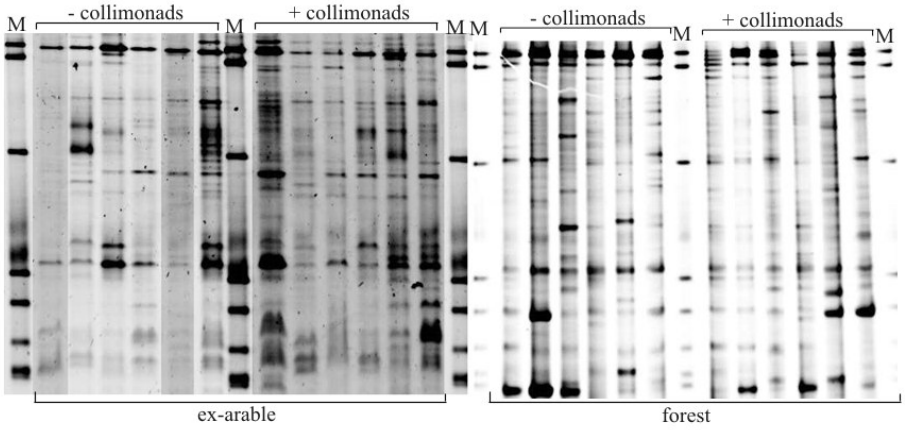


FIG S5.1: *DGGE gels of PCR-amplified fragments of 18S-RNA genes (fungi) extracted from nylon mesh bags containing acid-purified sand that had been buried for 8 weeks in an ex-arable field and a forest site. At the start of the field incubation, half of the bags contained sterile sand with addition of collimonads, whereas the other half contained sterile sand only. M: denotes a molecular marker made of an artificial mix of different fungal species.*

6

GENERAL DISCUSSION

At the start of the Ph.D study, only cultivation techniques were available to study the abundance and population dynamics of collimonads [7]. However, cultivation techniques have several limitations e.g. the need for additional, morphological and physiological identification, repression of slow-growing collimonads by fast-growing soil bacteria and the possibility of missing non-culturable collimonads [33]. Therefore, molecular methods were developed and applied to screen collimonads among isolates and to detect and quantify collimonads directly in natural soil samples. The developed methods were used to address the following issues (1) Description of the natural soil environment in which collimonads occur, (2) *In situ* occurrence of *Collimonas* mycophagy, (3) Phylogeny of *Collimonas* strains, (4) Impact of collimonads on fungal biomass turnover and community composition, (5) Interactions between fungal and bacterial mycophagy.

Identification and quantification techniques specific for *Collimonas* bacteria

A semi-selective medium (chitin-yeast agar) in combination with colony morphology were initially used to distinguish *Collimonas* strains from other chitinolytic isolates [7]. Based on sequence information of 16S rRNA of previously described *Collimonas* strains, restriction fragment length polymorphism analysis (PCR-RFLP analysis) was developed to identify collimonads (Chapter 2). The specific PCR-RFLP based methodology was used for additional confirmation

at the DNA level on top of morphological observations to screen for collimonads among chitinolytic strains [79]. The specificity of PCR-RFLP analysis was based on the available sequence information that can be found in NCBI (http://www.ncbi.nlm.nih.gov/). Thus, in future the specificity of this approach should be checked again when more sequence data will be available in NCBI.

Similarly the sequence information of 16S rRNA of previously described *Collimonas* strains was used to design *Collimona*s-specific primers and probes to be used in real-time PCR assays for the quantification of *Collimonas* 16S rRNA in total soil DNA. With the use of real-time PCR, now it is possible to quantify unculturable collimonads as well. Using both the specific plate count/identification technique and the specific real-time PCR assay, enumeration of both fractions of culturable and non-culturable collimonads can be assessed adequately in soil samples (Chapter 2).

Distribution of *Collimonas* bacteria among different soil types

Using the developed detection methods, I investigated the macro-distribution of collimonads in different soil and vegetation types (Chapter 2). Special attention was given to the relationship between numbers of collimonads and the amount of soil fungal biomass. I hypothesized that a positive correlation would be in line with predominant mycophagous nutrition of collimonads. The results of the field inventory revealed that collimonads are wide-spread in terrestrial environments at densities ranging from 10^4-10^6 cells per gram of soil. The numbers of collimonads were not significantly correlated with any of the soil characteristics that we measured. Although the numbers of collimonads were lowest in fungal-poor, intensively managed agricultural soil, no pair-wise correlation with soil fungal biomass was found when all soils were taken into account. There are several possible explanations form this observation. One explanation is that collimonads have a certain feeding preference for specific taxonomic or functional groups of fungi. In the study of de Boer *et al*. [7] we showed that the highest increase of

Collimonas cells was found when *Mucor hiemalis* which belongs to the phylum Zygomycota was inoculated as a bait fungus in comparison with *Fusarium culmorum* and *Chaetomium globosum* as baits. Fungi in the phylum zygomycota have less persistent hyphae as compared to lignocellulolytic basidiomycetes and ecto-mycorrhizal fungi which are typically abundant in forest soils [103]. Fungi with more persistent hyphae may also be less sensitive to mycophagous attack by collimonads. If this is the case, it explains why more collimonads were found in grassland soils rather than in fungal rich forest soils because forest soils are rich in lignocellulolytic basidiomycetes and ecto-mycorrhizal fungi. In chapter 5, I also report on changes in fungal community structure due to the presence of collimonads. The results described there indicated that the sensitivity of each fungus may differ towards *Collimonas* mycophagy. Another explanation could be that the lack of correlation between the numbers of collimonads and fungal biomass is due to the presence in soil of non-mycophagous collimonads or the growth of collimonads on other resources than fungi. Collimonads are heterotrophic bacteria and nutrient versatile (Chapter 3). Thus they can grow without feeding on fungi if other nutrients (easy degradable substrates such as root exudates) are available in soil. So, the question arises why collimonads possess the unique mycophagous property though they are nutrient versatile. One likely explanation might be that collimonads are weak competitors for which the mycophagous property is an additional mechanism for survival in soil. Their slow growing behavior on agar plates is an indication of their weak competitive ability for obtaining nutrients (de Boer *et al*. unpublished data). In future experiments, more attention should be paid to the (weak) competitive ability of collimonads for other nutrients than fungal hyphae in order to address properly the question why collimonads possess the mycophagous property which is unique among soil bacteria, and when it will be used under natural soil conditions.

Mycophagous growth of *Collimonas* bacteria in natural soils

Originally, mycophagous growth of collimonads was determined in autoclaved, acid-purified beach sand with invading fungal hyphae [7]. The advantage of the sand microcosm system was that (1) except for fungal hyphae there were no other nutrients available for bacterial growth (2) enumeration of collimonads could be performed by plating without interference of other bacteria present. Similar experiments have been performed for other chitinolytic and non-chitinolytic bacteria and it appeared that the growth at the expense of living fungi was unique for collimonads, at least with respect to the other bacteria examined. However, it was also clear that the sand microcosm system was artificial and could not be used to support the importance of mycophagous growth of collimonads in natural soils. To indicate *in situ* occurrence of *Collimonas* mycophagy in field soils, new experiments were prepared replacing the sand by different field soils that contained indigenous collimonads. An increase in numbers of indigenous collimonads upon invasion of the soil by a bait-fungus was observed supporting the importance of mycophagy for *Collimonas* bacteria in natural soils. To make sure that the growth of the collimonads was not caused by a consumption of fungal exudates, real-time PCR quantification of bacteria of the genera *Pseudomonas* and *Burkholderia* was performed as well. Several strains of these genera have been reported to be associated with the hyphal surface of fungi and are probably growing on fungal exudates. However, no growth of these bacteria was observed, giving extra support to active mycophagous growth of collimonads (Chapter 4).

In both microcosm experiments including either sand or natural soil, the growth response of collimonads appeared to be a short-term effect. One explanation for this phenomenon is that collimonads can grow only on young hyphae as we observed earlier that they colonized preferentially the tip of fungal hyphae [7]. Thus it is possible that the bait fungus ceased the production of new young hyphae shortly after inoculation since the agar disk was the only nutrient source for the bait fungus to grow in the microcosm. Another reason could be that the constant incubation temperature at 20 °C was an artificial suboptimal growth

condition for collimonads. It is possible that collimonads could not adjust to the experimental conditions and did not survive for a long period in the microcosms (Toby Kiers, personal communication). Another explanation would be a defense reaction by the bait-fungus which would result in a decrease in *Collimonas* numbers. At this moment, there is not sufficient information to provide an adequate explanation for these observations.

Collimonas arenosa sp. nov. and *Collimonas pratensis* sp. nov.

Twenty-six *Collimonas* strains, that were newly isolated, were polyphasically studied together with 16 strains that had previously been assigned to the genus *Collimonas*. This taxonomy study of the strains resulted in the description of two novel species: *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov.

The BOX-PCR results indicated that our culture collection include more potentially new species of collimonads. Further sequence analysis of the isolates is necessary to describe and confirm their taxonomical position (Chapter 3). In another related project on the genomic structure of collimonads we have carried out a comparative genomic study using the genomic information of the three species. This study revealed several striking differences and similarities among the genomic structure of the *Collimonas* species (Mela *et al*, personal communication). All three described species of *Collimonas* possess mycophagous capabilities. However some physiological characteristics are different between the species, in particular for their metabolic capacities.

Impact of *Collimonas* bacteria on soil fungal biomass turn over and soil fungal community composition

Results of both chapters 4 and 5 indicate that mycophagous growth of collimonads had little effect on fungal biomass production. This result seems to be logic since

calculations showed that the amount of fungal carbon cycled via collimonads is rather low. Hence, based on these results we may conclude that bacterial mycophagy does not have a strong impact on fungal carbon turn-over.

As for bacteria, functional redundancy among fungi is high implying that competition for the same substrates and niches will be severe. This holds true for both saprotrophic and mycorrhizal fungi [94, 95]. Even a minor reduction in growth rate or biomass as caused by mycophagous attack of collimonads may change the competitive relationships between fungal species [68]. In chapter 5 it was shown that the addition of collimonads to a soil with low numbers of indigenous collimonads clearly altered the composition of all fungal communities studied but that it had no effects on fungal biomass, plant performance (nitrogen and phosphorus uptake) or cellulase activities in soil samples. The most likely explanation for these results is that there are differences in sensitivity of functionally equivalent fungal species to the presence of collimonads.

The presence of collimonads had also an impact on the community structure of AM fungi but did not affect AM colonization of the host plant or plant performance. This is an important observation from the point of view of biocontrol perspective. A negative effect of collimonad on AM infection and plant performance would strongly reduce the chances to introduce collimonads as biocontrol agents.

In conclusion, collimonads had a strong effect on soil fungal community dynamics whereas the effects on fungal biomass production were small. Conceptually, these observations are of great interest for a better understanding of the role of minor groups in shaping microbial communities in a complex system of the soil with its huge microbial diversity and redundancy (Chapter 5).

Interactions between bacterial and fungal mycophagy in field soils

The invasion of field soils by a bait-fungus did not only stimulate growth of indigenous collimonads but also of fungi belonging to the genus *Trichoderma*, a genus known for mycophagous growth. In contrast to bacterial mycophagy fungal mycophagy has been intensively studied with *Trichoderma* strains as model organisms. Yet, the ecology of *Trichoderma* is not known well except that they are wide-spread in terrestrial environments. In the current Ph.D study, a start has been made with examining the interactions between fungal and bacterial mycophagy. Mycophagous growth of *Trichoderma* was significantly inhibited by the presence of collimonads whereas no impact of *Trichoderma* was found on mycophagous growth of collimonads. The negative effect of collimonads on *Trichoderma* cannot be due to the exploitation of the hyphae of the host fungus by collimonads, as the number of collimonads did not increase in the presence of *Trichoderma*. Possibly, collimonads induce a defense system in the host fungus that has a negative effect on the mycopahgous behavior of *Trichoderma.* This is a hypothesis that is currently under investigation

Perspectives for practical applications

As mentioned before, the mycophagous behavior of collimonads offers excellent opportunities for practical applications. The present study has provided essential information as to the distribution and the ecology of the mycophagous bacteria. Most interestingly for further exploitation of collimonads is their ability to alter the community structure of fungi in soil. (Chapter 5) This indicates that some fungi are more sensitive to the presence of collimonads than others are. For the application of collimonads as biocontrol agents, the relative sensitivity of target pathogens needs to be established. The fact that collimonads do not appear to influence root infection by AM fungi and plant performance, is important. Negative effects of

collimonads on AM-related plant nutrition are not to be expected. Effect of collimonads as biocontrol agents towards soil plant pathogenic fungal community composition needs to be further studied in green house experiment.

This study has shown that mycophagy could occur in natural soil. Thus it is rather likely, that there are other mycophagous bacteria. At the moment, there is on-going research to find out which genes are expressed when collimonads are confronted with the bait-fungus. These studies will reveal the important genes involved in *Collimonas* mycophagy. Once mycophagous genes are found, oligonucleotides specific for those gene can be designed to screen for other mycophagous bacteria among soil isolates and to detect and quantify mycophagy in soil.

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SUMMARY

Mycophagous nutrition, i.e. the feeding on fungi, is not well known for soil bacteria despite the fact that fungi and bacteria share many soil habitats. In 2004, the genus *Collimonas* was described, a genus consisting of soil bacteria that can grow at expense of living fungal hyphae. The discovery of collimonads opens the possibility to study the distribution, diversity and ecology of mycophagous bacteria in soil as well as the importance of mycophagy as a nutritional strategy and for food web relationships. In this thesis the following research questions were addressed (1) What is the abundance of collimonads in different types of soils and is there a relationship with fungal density? (2) How many *Collimonas* species are there? (3) Can mycophagous growth of collimonads be demonstrated in natural soils? (4) How does bacterial mycophagy interact with fungal mycophagy/mycoparasitism (5) What is the impact of bacterial mycophagy on fungal biomass turnover? (6) What is the impact of bacterial mycophagy on the structure of the fungal community in soil?

In order to address these questions, it was necessary to develop a *Collimonas*-specific detection and quantification method. Collimonads could be identified among other bacterial isolates using restriction fragment length polymorphism analysis (PCR-RFLP). The analysis is based on digestion of nucleotide sequences of 16S ribosomal genes with restriction enzyme *Bst*BI. It produces two fragments of the same size which is specific for *Collimonas* strains. Nucleotide sequences of 16S ribosomal genes of *Collimonas* strains were also used to design *Collimonas*-specific primers and probes. These primers and probes were used in real-time PCR assays in order to quantify *Collimonas* 16S ribosomal genes in total extracted soil DNA. (Chapter 2)

Using the developed identification and quantification method, the distribution of *Collimonas* bacteria in different soils and vegetation types was studied to obtain information on field site preferences. Specific attention was given to the relationship between numbers of *Collimonas* bacteria and soil fungal biomass to test the hypothesis that mycophagous *Collimonas* bacteria will be most abundant in fungal-rich soils. The results of a field inventory revealed that *Collimonas* bacteria are wide-spread in terrestrial environments at densities ranging from 10^4 to 10^6 cells per gram of soil. The numbers of *Collimonas* bacteria were not significantly correlated with any of the soil characteristics that we measured. Although the numbers of collimonads were lowest in fungal-poor, intensively managed agricultural soil, no correlation with soil fungal biomass was found when all soils were taken into account.

Twenty-six *Collimonas* strains, that were newly isolated, were studied polyphasically, together with 16 strains that had previously been assigned to the genus *Collimonas*. Analyses of 16S rRNA, genomic fingerprinting, and whole-cell protein electrophoresis were used to select candidate strains for different species. DNA-DNA hybridization of the candidates was performed as a final confirmation of speciation. This taxonomical study of the strains resulted in the description of two novel species: *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov. (Chapter 2 & Chapter 3)

Originally, mycophagous growth of *Collimonas* bacteria was assessed by introducing them as a suspension into autoclaved, acid-purified beach sand. The increase in numbers of *Collimonas* bacteria upon inoculation with a bait-fungus was considered as an indicator of mycophagous growth. In chapter 4, it was shown that a similar fungal-induced increase was observed for the numbers of indigenous *Collimonas* bacteria in different natural field soils based on real-time PCR measurements. In order to ensure that the growth of the *Collimonas* bacteria was due to mycophagous activity and not to growth on fungal exudates, real-time PCR quantification of other groups of indigenous soil bacteria (*Pseudomonas* and *Burkholderia* bacteria) was performed as well. These groups are known for growth

on fungal exudates. No growth of these bacteria was observed, which suggests that the response of the indigenous *Collimonas* bacteria to invading hyphae was probably due to their attack of the fungal hyphae.

In the same microcosm experiments, we also found a positive response of indigenous mycophagous (mycoparasitic) fungi (*Trichoderma* species) to the fungal bait. Hence, bacterial and fungal mycophagy appeared to co-exist. The interaction between bacterial and fungal mycophagy was investigated in more detail using sand microcosms with *Collimonas* bacteria and the fungus *Trichoderma harzianum* as model organisms. Mycophagous growth of *Trichoderma* was significantly inhibited by the presence of *Collimonas* bacteria whereas no impact of *Trichoderma* was found on mycophagous growth of *Collimonas* bacteria. The mechanism of inhibition of *T. harzianum* by collimonads is, as yet, unknown as agar tests revealed that mycophagous growth of collimonads on *T. harzianum* is unlikely.

The possible impact of mycophagous growth of *Collimonas* bacteria on fungal biomass turn-over was investigated in both sand microcosms and field soils. The increase of numbers of collimonads had no negative effect on fungal biomass production. This observation is in agreement with calculations of microbial C budgets, which suggests that only a minor fraction of fungal carbon was used by collimonads.

The absence of a significant effect of collimonads mycophagy on fungal biomass turn-over does not imply that there is neither effect on the structure of the fungal community. Since competition for the same niche may be severe among soil fungi, even a minor reduction in growth rate or biomass caused by mycophagous attack of *Collimonas* bacteria may alter the balance in competitive relationships between fungal species. Inoculation of collimonads into a field soil that appeared to lack these bacteria had strong effects on the community development of fungi of different functional groups: cellulose-degraders, rhizosphere fungi and arbuscular mycorrhizal fungi. The functions carried out by these functional groups, e.g. cellulose decomposition and plant nutrient acquisition, were not changed by the

presence of collimonads. Hence, it seems that the presence of collimonads results in shifts in the abundances of functionally equivalent fungal species. So, *Collimonas* bacteria had a strong effect on soil fungal community dynamics whereas the effects on fungal biomass production were small. Conceptually, these observations are of great interest for a better understanding of the role of minor groups of soil bacteria in shaping microbial communities in a complex system of the soil with its huge microbial diversity and redundancy. (Chapter 5)

Although the research described in this thesis is fundamental in nature, the results may contribute to the development of new approaches using mycophagous bacteria as biocontrol agents for the control of soil-borne plantpathogenic fungi.

SAMENVATTING

Mycofagie is het eten van paddenstoelen. Paddenstoelen zijn de vruchtlichamen van schimmels dus mycofagie kan ook worden opgevat als het eten van schimmels. Deze ruimere definitie wordt gevolgd in proefschrift.

Over het voorkomen van mycofagie bij bodembacteriën is heel weinig bekend ondanks het feit dat schimmels en bacteriën in veel bodems naast elkaar leven. In 2004, werd een nieuw bacterieel genus, *Collimonas*, beschreven, dat bestaat uit bodembacteriën die kunnen groeien ten koste van levende schimmelhyfen. Door de ontdekking van collimonaden ontstond de mogelijkheid onderzoek te doen naar de verspreiding, de diversiteit en de ecologie van mycofage bodembacteriën alsmede naar het effect van mycofage bacteriën op bodemschimmels. De volgende onderzoeksvragen vormden de leidraad van het onderzoek dat is beschreven in dit proefschrift: (1) Is er een relatie tussen het voorkomen van collimonaden in de bodem en de hoeveelheid bodemschimmels? (2) Hoeveel verschillende soorten kunnen er binnen het genus *Collimonas* worden onderscheiden? 3) Zijn er aanwijzingen dat mycofagie door collimonaden daadwerkelijk in natuurlijke bodems plaatsvindt? (4) Heeft bacteriële mycofagie effect op schimmel mycofagie (mycoparasitisme)? (5) Wat is het effect van bacteriële mycofagie op de omzetting van schimmelbiomassa? (6) Wat is het effect van bacteriële mycofagie op de structuur van de schimmelgemeenschap in de bodem?

Om deze vragen te kunnen beantwoorden was het noodzakelijk een specifieke detectie- en kwantificeringsmethode voor het genus *Collimonas* te ontwikkelen. Collimonaden konden van andere, verwante bacteriën worden onderscheiden met een zogenaamde 'restriction fragment length polymorphism' analyse (PCR-RFLP). Deze analyse is gebaseerd op het knippen van 16S

ribosomale genen door het restrictie-enzym *Bst* BI. Hierbij worden 2 fragmenten geproduceerd met een lengte die specifiek is voor *Collimonas* stammen. Op basis van de nucleotide-volgorde van de 16S ribosomale genen van collimonaden konden ook *Collimonas*-specifieke 'primers' en 'probes' worden ontwikkeld. Deze 'primers'and 'probes' werden gebruikt in een kwantitatieve PCR-analyse (qPCR of real-time PCR) waarbij de hoeveelheid *Collimonas* 16S ribosomale genen kon worden bepaald in DNA dat is geëxtraheerd uit de bodem. (Hoofdstuk 2)

Met behulp van de ontwikkelde methoden werd aangetoond dat collimonaden wijdverspreid voorkomen in Nederlandse bodems maar in relatief lage aantallen: 10^4 tot 10^6 cellen per gram grond. De aantallen collimonaden waren het laagst in intensief-bewerkte, schimmel-arme landbouwbodems. Maar op basis van de gegevens van alle onderzochte bodems was er geen significante correlatie tussen schimmelbiomassa en aantallen collimonaden.

Van 26 *Collimonas* stammen die bij de veldinventarisatie waren geïsoleerd werden verschillende kenmerken gemeten en vergeleken met de 16 stammen waarop de beschrijving van het genus *Collimonas* is gebaseerd. Dit taxonomisch onderzoek resulteerde in de beschrijving van 2 nieuwe soorten: *Collimonas arenae* sp. nov. en *Collimonas pratensis* sp. nov. (Hoofdstuk 3)

De oorspronkelijke vaststelling van mycofage groei van collimonaden is gebaseerd op toename van collimonaden in steriel zand dat werd gekoloniseerd door hyfen van een test-schimmel. Deze condities zijn anders dan in een natuurlijke bodem waar collimonaden samen voorkomen met vele andere bacteriesoorten en waar ook andere substraten dan schimmels kunnen bijdragen aan de groei van collimonaden. Op basis van de kwantitatieve PCR-analyse kon worden aangetoond dat collimonaden die van nature aanwezig zijn in een bodem ook toenemen na kolonisatie door schimmelhyfen (Hoofdstuk 4). Dit was niet het geval voor 2 belangrijke groepen van bodembacteriën (*Pseudomonas* en *Burkholderia*) die bekend staan om hun vermogen op exudaten van wortels en schimmels te groeien. Dit wijst erop dat de groei van collimonaden op schimmelhyfen een aktief proces is; dat wil zeggen dat de hyfen door de

collimonaden worden aangevallen of worden aangezet om voedingsstoffen uit te scheiden.

De kolonisatie van natuurlijke bodem door een schimmel resulteerde niet alleen in de groei van collimonaden maar ook van *Trichoderma* schimmels die bekend staan om mycofage (mycoparasitaire) groei. Dus mycofage groei van schimmels en bacteriën lijkt naast elkaar voor te kunnen komen in de bodem. In model-experimenten bleek de mycofage groei van *Trichoderma* echter wel te worden beperkt door aanwezigheid van collimonaden. Het tegenovergestelde, dus een beperking in de mate van mycofage groei van collimonaden in aanwezigheid van *Trichoderma* schimmels, werd niet gevonden. Het mechanisme van de negatieve interactie tussen collimonaden en *Trichoderma* is niet bekend; op basis van andere experimenten is groei van collimonaden op *Trichoderma* schimmels onwaarschijnlijk en vormt dus geen goede verklaring.

Het effect van mycofage groei van collimonaden op schimmelbiomassa-omzetting werd onderzocht in modelbodems en natuurlijke bodems. Toename van collimonaden had geen significant negatief effect op de netto productie van schimmelbiomassa. Dit is in overeenstemming met berekeningen van koolstofbudgetten, die aangeven dat voor de gemeten toename van collimonaden slechts een geringe fractie van de schimmelbiomassa hoeft te worden omgezet.

Hoewel collimomaden dus een gering effect hadden op de schimmelbiomassa-productie was er een sterk effect van de aanwezigheid van collimonaden op de samenstelling van bodemschimmelgemeenschappen. Toevoeging van collimonaden aan een bodem die arm was aan deze bacteriën (gebaseerd op kwantitatieve PCR) had sterke effecten op de ontwikkeling van verschillende functionele groepen schimmels: cellulose-afbrekers, rhizosfeerschimmels en arbusculaire mycorrhiza. (Hoofdstuk 5) De functies die met de schimmelgroepen zijn verbonden, o.a. cellulose-afbraak en fosfaatvoorziening van de plant, werden niet gewijzigd door aanwezigheid van collimonaden. Het lijkt er dus op dat de door collimonaden veroorzaakte veranderingen in de samenstelling van de schimmelgemeenschappen geen effect

heeft op de schimmel-gerelateerde ecosysteemfuncties. Aangezien de dichtheden van collimonaden in de bodems laag zijn, wordt met dit onderzoek duidelijk gemaakt dat ook niet-dominante bodembacteriën een sterk effect kunnen hebben op de samenstelling van de bodemmicrobiële gemeenschap.

Het onderzoek zoals beschreven in dit proefschrift is fundamenteel gericht. De resultaten kunnen echter een ondersteuning vormen voor de toepassing van collimonaden als biologisch bestrijdingsmiddel van plantenziekteverwekkende bodemschimmels.

要旨

土壌中において多くのハビタットを共有している菌と細菌に関する研究はこれまで盛んに行われてきた。それにもかかわらず、土壌菌食性細菌の存在は2004年になって初めて報告され、糸状菌の生菌糸を摂取して増殖する細菌としてコリモナス新属に分類された。 コリモナス細菌の発見により、土壌菌食性細菌の分布、及び種多様性、また、栄養獲得の戦略上における菌食の重要性、さらに、細菌－菌の間の食物網の研究等に挙げられる生態学に関する研究を行う事が可能になった。

本論文では、菌食性細菌の生態学に関する研究結果について述べる。具体的には、（1）コリモナス細菌がどのような種類の土壌に多く生息しているか。また、コリモナス細菌数と菌量に有意な相関関係がみられるか（2）コリモナス属内に何種の新種が見つかるか（3）野外土壌中においても、コリモナス細菌は菌食性を発揮し増殖できるのか（4）菌食性細菌と菌食性菌の間にはどのような相互作用があるか（5）菌食は菌バイオマス量の変化に影響を与えるか（6）菌食が土壌菌類群集構造に影響を与えるか、に挙げた項目に焦点を当て実験的研究を行った。

はじめに、これまでの方法的制約を打開すべく、コリモナス細菌に特異な検出・計測方法を新たに開発した。始めに、既に分離されたコリモナス細菌株の16SrRNA遺伝子の配列をもとにPCR法によって16SrDNAを増幅し、*Bst*BI制限酵素で切断した際に得られるバンドパターンの比較によってコリモナス細菌を容易に同定できることを報告した (PCR-RFLP

法)。さらに、16SrRNA 遺伝子の配列をもとにコリモナス属に特異なプライマーとプローブを設計し、リアルタイム PCR 法による土壌中のコリモナス細菌数の測定を可能にした。（第 2 章）

開発された検出・計測法を用い、コリモナスの野外における地理的分布を調査した。植生や土壌環境因子とコリモナスの細菌数の相関を検討し、植生や土壌環境因子が土壌中の菌食性の発生に与える影響を評価した。さらに、コリモナス細菌数と土壌菌バイオマス量の相関を検討し、餌となる菌が多い環境中にコリモナス細菌が多く繁殖するという仮説を検証した。その結果、コリモナス細菌は土壌環境中に広く分布するが、その細菌数は 10^4-10^6 個/グラムであり、低密度の個体群として土壌微生物の中に存在することが明らかにされた。土壌環境因子とコリモナス細菌数の間に有意な相関関係は見つからなかった。植生間の比較では、農地におけるコリモナス細菌数が低いことが分かった。これは、耕起、播種、除草などの作業が行われる農地では、森林や草原と比較して菌バイオマス量が少ないことに起因すると考えられる。菌バイオマス量とコリモナス細菌数の間に有意な相関関係は認められず、野外における細菌にとっての菌食性の重要性を示唆することはできなかった。(第 2 章)

新規に分離されたコリモナス 26 株はこれまでに分離されていた 16 株と合わせて系統分類学的に解析された。16SrRNA 系統解析、DNA フィンガープリント法、また whole-cell protein 分析により候補となる株が選ばれ、DNA-DNA 分子交雑法により新種であることを確定した。これにより、コリモナス属の 2 新種（*C. arenae* sp. nov.、*C. pratensis* sp. nov.）が発見された。(第 3 章)

従来、コリモナス細菌の菌食性の発現は、コリモナス細菌を混ぜた砂培地上での餌糸状菌接種よるコリモナス細菌の増加を指標として確認してきた。第 4 章では、餌糸状菌の接種によるコリモナス細菌の増加が、野外から得た野外コリモナス細菌と他の野外微生物が含まれる土壌ベースの培地内でも確認されたことを示した。次に、コリモナス細菌の増殖が菌食性に特異であることを確認するために、菌食性を持たない細菌（シュードモナス細菌・バークホルデリア細菌）の細菌数も測定した。餌糸状菌接種による非菌食性細菌の増殖は確認されなかったため、コリモナス細菌の増殖が餌糸状菌の菌食に特異である事が示唆された。

上記の土壌ベースの培地内では、餌糸状菌接種による野外菌食性菌であるトリコデルマ菌の増殖も確認された。これにより、細菌による菌食と菌による菌食の発生はお互いに抑制されず、同じ土壌中で同時に起こることが確認された。そこで、コリモナス細菌とトリコデルマ菌をモデルとし、滅菌済み砂培地上で細菌または菌の菌食性の発生がお互いに与える影響を調査した。この結果、コリモナス細菌の菌食による増殖はトリコデルマ菌の存在に影響されなかったが、トリコデルマ菌の菌食による増殖はコリモナス細菌の存在により有意に抑制された。寒天培地上における、トリコデルマ菌を餌糸状菌とした対峙実験では、コリモナス細菌の増殖が確認されなかった為、コリモナス細菌がトリコデルマ菌を菌食し、抑制している可能性は低く、この抑制に関わるメカニズムに関しては現在明確になっていない。(第 4 章)

コリモナス細菌の菌食が菌のバイオマス量に与える影響について、滅菌済み砂培地と野外土壌を用いて調査した。細菌の増殖が確認された培地内で、菌バイオマスの減少は確認できなかった。この結果は、増殖したコリモナス細菌のバイオマスから炭素量を計算すると、同量の炭素量にあたる菌体バイオマス量は微量であるという結果と一致する。(第 4 章)

コリモナス細菌の菌食が菌バイオマス量に与える影響は大きくなかったが、菌の群集構造に与える影響の可能性は否定できない。なぜならば、土壌環境中では栄養や生存空間をめぐる競争が厳しいと推測されるので、コリモナス細菌が菌食により特定の菌の成長速度やバイオマスを例え微小であれ減少させることにより、菌類間における競争の平衡関係を乱し、菌の群集構造に影響を与えることが予想されるからである。この仮説を検討するため、野外群集を用いた操作実験を行った。野外から採取したコリモナス細菌を含まない土壌にコリモナス細菌を接種すると、異なる機能群（セルロース分解菌、根圏菌　またはアーバスキュラー根圏菌）の菌類群集構造形成に大きな変化が確認された。コリモナス細菌の存在によりこれらの菌群の機能（土壌セルラーゼ酵素活性、植物の栄養収量）が変化しなかったので、コリモナス細菌の存在は異なる種が属する機能群内の種組成を変化させたと考察された。よって、コリモナス細菌の菌食が菌バイオマス量に与える影響は小さいが、その菌類群集構造に与える影響は大きい事が示された。低密度で存在する土壌微生物が、豊かな多様性や冗長性を有す複雑なシステムである土壌微生物群集に与える役割を理解する上で、これらの発見は概念的に非常に重要である。(第 5 章)

本論文では基礎的研究が行われたが、得られた結果は、植物病原菌の制御を目的とした菌食性細菌の生物的防御資材としての利用の発展に貢献される事が期待できる。

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Sachie

Utrecht, October 2008

CURRICULUM VITAE

Sachie Höppener-Ogawa was born on 9 March 1976 in Tokyo, Japan. In 2002 she obtained the MSc degree in Food Science, specializing in Applied Microbiology, at Tokyo University of Fisheries. During a year of her MSc she received a scholarship to work at the Department of Food Science, University of Massachusetts at Amherst, USA. She carried out a research on Biofilm formation of *Listeria monocytogenes*. In 2002, she started a second MSc, which dealt with the biological filtration with use of biofilm for drinking water treatment to remove biological threat agents. This project was supervised Dr. Jin Li and Dr. Sandra Mclellan, financed by the NIEHS Center for Marine and biomedical science. The research was conducted at University of Wisconsin at Milwaukee and Great lake water institute. In July 2004 she started her PhD project at the Department of Terrestrial Microbial Ecology of The Netherlands Institute of Ecology (NIOO) in Heteren, under the supervision of Dr. Wietse de Boer, Dr. Johan Leveau and Prof. Hans van Veen. This project was financed by NWO and the research resulted in the thesis presented here. During her PhD project, she visited and collaborated with Prof. Peter Vandamme at Gent University, Belgium. From July 2008 she started as a post-doc in the project on "The role of indole acetic acid in the interactions between plants, nematodes and bacteria" at NIOO in Heteren.

PUBLICATIONS

Sachie Höppener-Ogawa, Johan H. J. Leveau, M.P.J. Hundscheid, Johannes A. van Veen and Wietse de Boer. Impact of *Collimonas* bacteria on community composition of soil fungi. *In revision.*

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