Impact of *Collimonas* bacteria on community composition of soil fungi

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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. Develop-
ment of fungal communities was stimulated by addi-
tion 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 intro-
duction 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 result
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 colli-
monads had an effect on the composition of fungi
invading these bags in the ex-arable site but not in the
forest site.**

Introduction

Mycophagy, i.e. the feeding on living fungi, is widely dis-
tributed among soil-inhabiting organisms, e.g. fungivo-
rous collembola, nematodes, amoebae and mycoparasitic
fungi (cf. Leveau and Preston, 2008). However, little is
known on the occurrence of mycophagy among soil bac-
teria (Leveau and Preston, 2008). This is remarkable as
fungi and bacteria coexist in most soils, where they have
different interactions based on the acquisition of organic
energy sources (De Boer *et al.*, 2005). The availability of
degradable carbon compounds is the most common li-
miting factor for bacterial growth in soils (Demoling *et al.*,
2007). Therefore, it is to be expected that selection has
also favoured bacteria exploiting fungal hyphae as
organic energy source.

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 (De Boer *et al.*, 2004;
Höppener-Ogawa *et al.*, 2009; Leveau and Preston,
2008). Höppener-Ogawa and colleagues (2007) showed
that collimonads are widely spread in terrestrial environ-
ments 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, suggest that
their influence on total fungal biomass turnover is of minor
importance (De Boer *et al.*, 2001). This is supported by
the fact that no significant negative effect of collimonads
on fungal biomass production was observed in sand
microcosms (Höppener-Ogawa *et al.*, 2009).

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 (Allen *et al.*, 1995;
Deacon *et al.*, 2006). 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 (De Boer
et al., 2004; Kamilova *et al.*, 2007).

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, (i) cellulose- and sugar-degrading fungi after enrichment of a soil with cellulose and (ii) 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 1.0×10^6 to $1.6 \times 10^5 \pm 2.3 \times 10^4$ collimonads g^{-1} dry soil and the numbers were under the detection limit (1.5×10^4 collimonads g^{-1} dry soil) after 8 weeks of incubation.

PCR-denaturing gradient gel electrophoresis (DGGE) patterns of fungal 18S rRNA-gene fragments amplified from total DNA differed between the controls and the *Collimonas*-inoculated soil replicates (Fig. 1, lanes 10–18). These differences were confirmed by distance-based redundancy analysis (db-RDA) ($P < 0.001$). This indicates a significant effect of the presence of colli-monads 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. 1) that were present in the controls were much weaker in the soil inoculated with collimonads. In contrast, five distinct bands (F, G, H, I and J) were
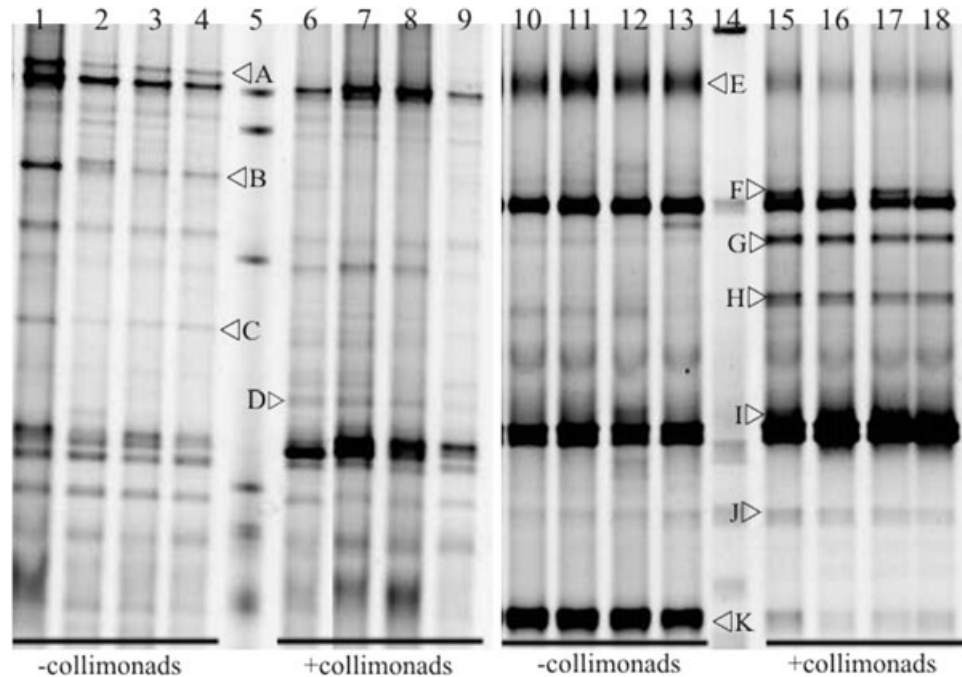

Fig. 1. DGGE patterns of partial fungal 18S RNA genes from two experiments. Lanes 1–4: Rhizosphere soil of *Plantago lanceolata* grown in soil without addition of collimonads; lanes 6–9: Rhizosphere soil of *Plantago lanceolata* grown in soil with addition of collimonads; lanes 10–13: Cellulose-enriched 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-week 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 1.

Table 1. Sequence analysis of total fungal and arbuscular mycorrhizal PCR-DGGE bands. Letters indicate the coding of the bands as indicated in Figs 1 and 2.

DGGE bands	Closest relatives		
	Order	Phylum affiliations	Accession No., similarity (%)
AM1[a]	–	–	–
AM2[b]	*Glomerales*	*Glomeromycota*	AY842575, 95%
AM3[b]	*Glomerales*	*Glomeromycota*	AJ854582, 98%
AM4[a]	*Glomerales*	*Glomeromycota*	AF389021, 91%
A[a]	*Mucorales*	*Mucoromycotina**	AF157140, 94%
B[a]	*Endogonales*	*Mucoromycotina*	DQ322628, 93%
C[a]	*Agaricales*	*Basidiomycota*	AY916691, 92%
D[b]	*Phyllachorales*	*Ascomycota*	AF222522, 95%
E[c]	–	–	–
F[b]	–	–	–
G[b]	*Lecanorales*	*Ascomycota*	AY584675, 86%
H[b]	*Malasseziales*	*Basidiomycota*	AY083223, 86%
I[b]	*Sordariales*	*Ascomycota*	EU263613, 99%
J[b]	*Cantharellales*	*Basidiomycota*	DQ915460, 92%
K[c]	*Hypocreales*	*Ascomycota*	DQ838790, 100%

a. Band appeared in treatment without collimonads.
b. Band appeared in treatment with collimonads.
c. Band intensity increased in treatment without collimonads.
***Mucoromycotina* is a subphylum, whereas the others are phyla (Hibbett *et al.*, 2007)
The symbol '–' denotes bands that could not be cloned successfully.

detected in the *Collimonas*-inoculated soil. Sequence analysis of the 18S rRNA fragments revealed two high matches (> 98%), namely, one as a taxon within the order Hypocreales (band K, without the addition of collimonads) and one as a taxon within the order Sordariales (band I, with the addition of collimonads).

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. 1, lanes 1–4, 6–9), 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 but were not clearly distinguishable in the presence of collimonads. Sequence analysis revealed that two of these bands (A and B) belonged to the subphylum *Mucoromycotina* (Table 1). One band (D) that was only distinguishable in the soil with collimonads represented an unidentified ascomycete (Table 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. 2). Bands AM1 and AM4 were not distinguishable when collimonads were present, whereas bands AM2 and AM3 were only clearly visible in the presence of collimonads. The results of sequence analysis of these bands are shown in Table 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.

The addition of collimonads to the soils did not affect the AM fungal colonization of *Plantago* roots (Table 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 2).

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 g^{-1} dry soil in the ex-arable site and to $5.3 \times 10^5 \pm 2.6 \times 10^5$ collimonads g^{-1} 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 (Fig. S1). Principal coordinate analysis (PCoA), based on Jaccard similarity, of fungal 18S rDNA-DGGE patterns
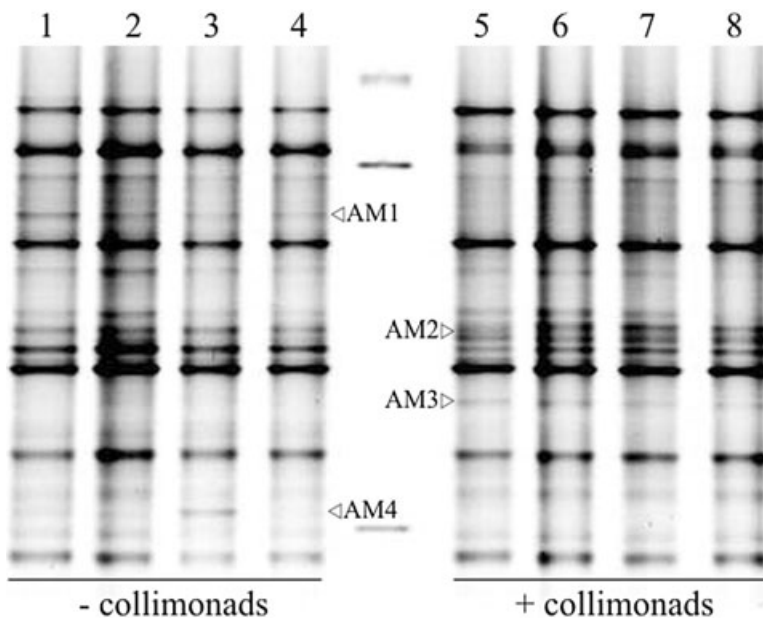
Fig. 2. DGGE patterns of partial 28S RNA genes of AM fungi associated with roots of *Plantago lanceolata*. Lanes 1–4: plants grown in soil without addition of collimonads; lanes 5–8: plants grown in soil with addition of collimonads. Coding of bands refers to Table 1.

was shown in Fig. 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 ($P = 0.66$).

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 (Höppener-Ogawa *et al.*, 2007). 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 (Höppener-Ogawa *et al.*, 2009).

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 three fungal groups studied (cellulolytic fungi, rhizosphere fungi and AM fungi). 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. The most likely explanation is that differences in

Table 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.

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), vesicle-like structures (VC) and hyphal-like structures (HC) were present.
Similar letters denote that there were no significant differences ($P < 0.05$) between the treatments.
Data indicate the means of six replicates. Differences between means were tested for significance using one-way ANOVA.
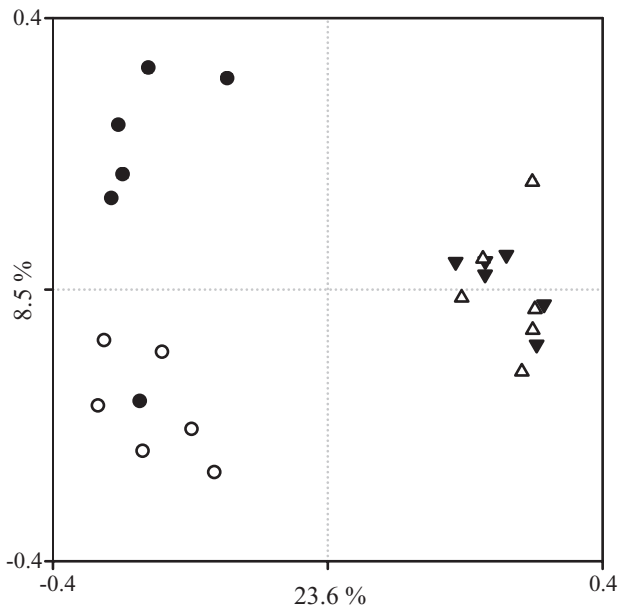
Fig. 3. Principal coordinate analysis (PCoA), based on Jaccard similarities, of DGGE patterns obtained from PCR-amplified fungal 18S rRNA genes in acid-purified sand that had been buried for 2 months in nylon mesh bags in an ex-arable field and a forest site. At the start of the field incubation, half 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.

sensitivity of fungal species or strains to the presence of collimonads change 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 (De Boer *et al.*, 2001; Höppener-Ogawa *et al.*, 2009). However, selective effects may also have been due to other antifungal activities of collimonads (Kamilova *et al.*, 2007). Small, short-term effects of antibiotic producing biocontrol bacteria on non-target fungi in the rhizosphere have been reported (Glandorf *et al.*, 2001; Scherwinski *et al.*, 2008). 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).

The effects of collimonads on fungal community composition did not appear to have an effect on the cellulose-degrading activity, plant biomass production and plant N and P content. Hence, functionality of fungal communities appeared not to be affected by a change in fungal community composition. This is in line with the expected functional redundancy of soil fungi (Deacon *et al.*, 2006).

Arbuscular mycorrhizal fungi form associations with many plant species and have an important role in the plant's mineral nutrient acquisition (Rillig, 2004). Earlier studies have indicated that the occurrence of collimonads may be related to the occurrence of AM fungi (Offre *et al.*, 2007). 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, consist 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 (van der Wal *et al.*, 2006a). Fungi with more persistent hyphae may also be less sensitive to mycophagous attack by collimonads.

As the two field soils differ in acidity, it may be argued that apparent differences in mycophagous performance are due to different pH conditions. However, this is unlikely as an earlier study showed mycophagous growth of collimonads in soils varying in pH from 3.6 to 5.6, i.e. covering the pH values of the soils used in this study (Höppener-Ogawa *et al.*, 2009).

In conclusion, collimonads had a strong effect on the composition of functional groups of soil fungi, whereas the effect on fungal biomass production and functioning of fungi 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 and colleagues (2006b). 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^{-1}, and C/N ratio 17.1 and 24.3, respectively. More details on the ex-arable site are given by van der Wal and colleagues (2006b). 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 (Höppener-Ogawa *et al.*, 2007). At the ex-arable site, soil (0–10 cm layer) was sampled from at least 30 randomly selected points in a 50×50 m plot. Samples were pooled, mixed and sieved (4 mm 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) (De Boer *et al.*, 2004; Höppener-Ogawa *et al.*, 2008).

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 and colleagues (2001). Bacteria were suspended in P-buffer (KH_2PO_4, 1 g l^{-1}, pH 6.5), pelleted by centrifugation (16 000 *g*) and re-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% (w/w). 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 six 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 (De Ridder-Duine *et al.*, 2006).

Analysis of cellulase activity was based on the release of remazol Brilliant blue from dyed carboxymethyl cellulose as described by van der Wal and colleagues (2006a).

Fungal community structure was examined using PCR-DGGE (see below). Numbers of collimonads were determined using real-time PCR on extracted soil DNA (see below) (Höppener-Ogawa *et al.*, 2007).

Effect of collimonads on the community composition on rhizosphere fungi and arbuscular mycorrhizal fungi associated with roots of Plantago lanceolata

Seeds of *P. 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 (#0140-01-0; Difco, Detroit, MI) 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 subsection. 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 three 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 400 W 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% (w/w).

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 (McGonigle *et al.*, 1990). 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 analyser. Total P was determined colorimetrically after acid destruction (van der Wal *et al.*, 2007).

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 min to crush the root tissues. DNA extracted from the freeze-dried roots was analysed for AM fungal community structure by PCR-DGGE analysis (see below).

Table 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	Vainio and Hantula (2000)
AM fungi	LR1/FLR2 followed by FLR3/FLR4	58°C; 35 cycles	20–55% denaturant	Trouvelot *et al.* (1999) and van Tuinen *et al.* (1998) Gollotte *et al.* (2004)

Effect of collimonads on invading fungi (Field experiment)

Fungal in-growth bags with an opening on one 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 (Wallander *et al.*, 2001). *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% (w/w). 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 pre-incubation, 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 two treatments (with and without collimonads) were placed close to each other. In total, six replicates of both treatments were placed at six randomly selected spots within an area of 100 m².

The start of the field incubation was 11 September 2007, and 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 (De Ridder-Duine *et al.*, 2006) and determination of fungal community structure by PCR-DGGE analysis (see below). Soil DNA was extracted as described in the next subsection.

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 soil 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 manufacturer's instruction, except that soil DNA was finally eluded in 50 μl buffer. Table 3 summarizes the primers, thermocycling regimes and electrophoresis conditions used to analyse fungal and AM fungal communities. Partial sequences of 18S rRNA genes and 28S rRNA genes were analysed for total fungi and AM fungi, respectively. All PCR reactions, DGGE and cloning were carried out as described elsewhere (van der Wal *et al.*, 2006a), 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 roughly identify fungi, 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 analysed 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 was carried out using 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 (Legendre and Anderson, 1999). The effects of the presence of collimonads (all three experiments), the soil type (experiment 3) and interaction of these factors as environmental variables on the microbial structure as analysed by PCR-DGGE were tested by db-RDA (Legendre and Anderson, 1999) in Canoco 4.5 for Windows (Ter Braak and Šmilauer, 2002). 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 covariable. 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 covariables. The significances of such models were tested with 999 permutations.

All ANOVAs were carried out in Statistica 7.0 (StatSoft, 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.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. 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 addi-tion of collimonads, whereas the other half contained sterile sand only. M: denotes a molecular marker made of an artifi-cial mix of different fungal species.

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