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ORIGINAL ARTICLE

Soil amoebae rapidly change bacterial community composition in the rhizosphere of *Arabidopsis thaliana*

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We constructed an experimental model system to study the effects of grazing by a common soil amoeba, *Acanthamoeba castellanii,* on the composition of bacterial communities in the rhizosphere of *Arabidopsis thaliana*. Amoebae showed distinct grazing preferences for specific bacterial taxa, which were rapidly replaced by grazing tolerant taxa in a highly reproducible way. The relative proportion of active bacteria increased although bacterial abundance was strongly decreased by amoebae. Specific bacterial taxa had disappeared already two days after inoculation of amoebae. The decrease in numbers was most pronounced in *Betaproteobacteria* and *Firmicutes.* In contrast, *Actinobacteria, Nitrospira, Verrucomicrobia* and *Planctomycetes* increased. Although other groups, such as betaproteobacterial ammonia oxidizers and *Gammaproteobacteria* did not change in abundance, denaturing gradient gel electrophoresis with specific primers for pseudomonads (*Gammaproteobacteria*) revealed both specific changes in community composition as well as shifts in functional genes (*gacA*) involved in bacterial defence responses. The resulting positive feedback on plant growth in the amoeba treatment confirms that bacterial grazers play a dominant role in structuring bacteria–plant interactions. This is the first detailed study documenting how rapidly protozoan grazers induce shifts in rhizosphere bacterial community composition.

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Introduction

Protozoa and bacteria form one of the oldest predator–prey systems on earth, but apart from reports on phenotypic changes (Jürgens and Matz, 2002; Pernthaler, 2005) surprisingly little is known on the factors driving grazing resistance (Matz and Kjelleberg, 2005) and on the identity of bacterial groups that are consumed and those that survive protozoan grazing in the rhizosphere and soils (Griffiths *et al.*, 1999; Rønn *et al.*, 2002; Kreuzer *et al.*, 2006; Murase *et al.*, 2006). Roughly estimated, 1 g of grassland soil may contain up to 10^9 bacteria and 100 000 protozoa (Finlay *et al.*, 2000). Several studies on the fate of bacterial inocula demonstrate a strong coupling between the densities of bacteria and protozoa in soil. The numbers of bacteria have been shown to decline in presence of protozoa until a dynamic equilibrium with bacterial densities of 10^5–$10^7\,g^{-1}$ is reached (Danso and Alexander 1975; Habte and Alexander, 1975; Acea *et al.*, 1988; Clarholm 1981, 1989). However, the different bacterial taxa that constitute the rhizosphere bacterial community strongly differ in their food quality for protozoa (Bjørnlund *et al.*, 2006; Jousset *et al.*, 2006), suggesting taxon-specific differences in bacterial survival. In fact, studies in freshwater ecosystems uncovered a number of adaptations of bacteria against protozoan grazing, such as changes in motility, size, filament formation, surface masking or toxin production to prevent ingestion, or resistance to digestion by protozoa (Jürgens and Matz, 2002; Pernthaler, 2005). In terrestrial

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ecosystems investigations on bacteria–protozoa interactions are much more difficult because direct observations of shifts in morphology or abundance of bacteria are hampered by the opaqueness and autofluorescence of the soil substrate. Consequently, almost nothing is known on the identity of the bacteria that are consumed and those that survive predation.

Soil protozoa are known to promote plant growth (Bonkowski, 2004) and recent investigations indicate that plant growth promotion by microfaunal predators, such as protozoa and nematodes, may be based on grazing induced changes in rhizosphere bacterial community composition and subsequent favouring of plant growth-promoting bacteria (Bonkowski and Brandt, 2002; Kreuzer *et al.*, 2006; Mao *et al.*, 2006). Among Gram-negative bacteria, pseudomonads are a particular important group of plant growth-promoting rhizobacteria. Pseudomonads may promote plant growth by enhancing root growth (De Leij *et al.*, 2002), or via effects on root pathogens, for example, by inducing systemic plant resistance and by producing antibiotics against soil microbes such as pathogenic fungi (Lugtenberg *et al.*, 2002). Soil amoebae have been shown to graze preferentially on Gram-negative bacteria (Foster and Dormaar, 1991; Andersen and Winding, 2004). Not surprising, the *gacA* regulated antibiotic production of pseudomonads has been found to play also a significant role in bacterial defence against protozoan predators (Jousset *et al.*, 2006, 2008).

Our aim in this study was to monitor shifts in community composition of soil bacteria as a result of protozoan grazing in the early stages of plant development. Soil bacteria community composition was assessed with denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH). DGGE fingerprints yield semiquantitative information, but the sequencing of the bands enables a precise identification of the bacterial species that appear or vanish upon protozoan grazing. The FISH technique gives quantitative data on changes in bacterial taxa and we used it to monitor shifts in the main bacterial phyla present in soil.

Materials and methods

Magenta system

Magenta vessels (Sigma-Aldrich, St Louis, MO, USA) were filled with 220 g dry weight of sand (grain size 1–1.2 mm) and amended with 0.5 g dry weight of a fine powder of dried and milled *Lolium perenne* shoot material (45% C and 4% N), to support bacterial growth. Sand and grass powder were thoroughly mixed and moistened by adding 6 ml sterile, deionised water. The Magenta vessels were autoclaved three times with intermediate incubation periods of 48 h at room temperature to kill sporulating bacteria and fungi. The Magenta vessels were checked for sterility by plating sand on nutrient broth agar (NB; Merck, Darmstadt, Germany). The vessels were inoculated with a protozoa-free filtrate of a natural bacterial suspension. The bacterial filtrate was obtained by suspending 20 g fresh weight of recently collected rhizosphere soil from a meadow (campus of the Faculty of Biology, Darmstadt University of Technology) in 200 ml tap water and filtering the soil slurry through paper filters (Schleicher & Schuell, Dassel, Germany). Protozoa were excluded by subsequent filtering through 5.0 and 1.2 μm Isopore filters (Millipore, Schwalbach, Germany), respectively. To check for protozoan contaminations, the filtrate was cultured for 3 days in sterile NB (Merck) with Neff's modified amoebae saline (NMAS) at 1:9 v/v (NB-NMAS) before use (Page, 1976). For inoculation, 1.5 ml of the protozoa-free inoculum was thoroughly mixed with the sand, and 0.5 ml of an axenic amoeba culture of *Acanthamoeba castellanii* washed in half-strength Hoagland (Sigma-Aldrich) were added to the amoeba treatments, resulting in a final density of approximately 1×10^3 amoebae g^{-1} sand dry weight. Each bacterial treatment received 0.5 ml sterile half-strength Hoagland solution instead. Two days later, *Arabidopsis thaliana* seedlings were transplanted to the Magenta vessels in presence of bacteria, or bacteria plus axenic *A. castellanii* with 10 replicates each (that is, 0 days past inoculation; dpi). Plants were watered every second day with 1 ml modified Gambourg B5-N containing 0.350 mg l^{-1} of ammonium nitrate as described by Zhang and Forde (1998).

Plants

A. thaliana seeds were sterilized in 5% $Ca(ClO)_2$ solution (VWR, Darmstadt, Germany) containing 0.1% Tween 80 (VWR) for 10 min, followed by 5 min in 70% ethanol and 5 min in 5% NaOCl (VWR) containing 0.1% Tween 80 (VWR) and were subsequently washed three times with sterile deionised water. Seeds were dried on sterile filter disks and transferred to square Petri dishes (VWR) with Gambourg medium (3.2 g l^{-1} Gambourg plus vitamins, 0.5% sucrose, 1% plant agar; Duchefa, Haarlem, The Netherlands). An agar strip of 3 cm was removed and the Petri dishes were positioned upright. Ten seeds were equally spaced on the small cutting edge of the agar for germination. For vernalization of seeds, the agar plates were incubated at 4 °C for 4 days in darkness. After germination the plants were kept for 3 weeks on the agar plates in upright position before being planted into Magenta vessels. The plants were kept in a growth chamber at 24 °C with a photoperiod of 10 h of light (150 μmol m^{-2} s^{-1}) during their entire growth period.

Plant performance

The plants consistently had produced three leaf pairs at the start of the experiment; cotyledons were

not considered. Plant rosette diameters were monitored at 0, 3 and 6 dpi, respectively. The mean rosette diameter of each plant was calculated from the average of three different vectors from tip to tip of opposite leaves. The values at 0 dpi were subtracted in statistical analyses to give growth increments. Shoots and roots were dried (at 70 °C for 3 days) for biomass determination.

Establishment of an axenic culture of Acanthamoeba castellanii

A. castellanii isolated from woodland soil (Göttinger Wald, Lower Saxony, Germany), were cultured with a natural bacterial community in culture flasks (Nunc A/C, Roskilde, Denmark) in NB-NMAS at room temperature. An axenic culture was established by using PGY medium (1% peptone, 1% glucose, 0.5% yeast-extract) containing the antibiotics streptomycin (10 μg ml^{-1} final concentration) and gentamycin (15 μg ml^{-1} final concentration) as described by Schuster (2002). The *A. castellanii* culture was repeatedly diluted with PGY antibiotic solution every day for 1 week and subsequently incubated 1 further week in PGY gentamycin solution until the cultures were bacteria free. The axenic cultures were kept in PGY medium. Before the addition to the sand system amoebae were washed twice in 0.5 Hoagland solution (Sigma-Aldrich).

Enumeration of protozoa

Amoebae were enumerated with a modified most probable number method (Darbyshire *et al.*, 1974). Briefly, 5 g fresh weight sand were suspended in 20 ml sterile NB-NMAS and gently shaken for 20 min on a vertical shaker. Threefold dilution series with NB-NMAS were prepared in 96-well microtiter plates (VWR) in quadruplicates. The plates were incubated at 15 °C in darkness and the wells were inspected for presence of protozoa using an inverted microscope ($\times$100 to $\times$320 magnification; Leitz, Wetzlar, Germany) after 3 and 5 days, respectively. Densities of amoebae were calculated using an automated analysis software (Hurley and Roscoe, 1983).

Fluorescence in situ *hybridization*

FISH was performed according to Bertaux *et al.* (2007) with modifications listed below. Three days after transferring *A. thaliana* to Magenta vessels, the whole root systems were collected and immersed in 2 ml 3% paraformaldehyde (Merck) buffered with 1$\times$ phosphate-buffered saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.3). The root systems were vortexed to detach the ectorhizosphere sand substrate. After removing the roots, the tubes containing the ectorhizosphere sand substrate were vortexed and incubated at 4 °C overnight for fixation. The tubes were kept horizontal and the sand substrate spread over the whole length of the tube in a thin layer to ensure good penetration of the fixative. A Nycodenz centrifugation step was performed to separate sand and litter particles from the bacterial community (Bertaux *et al.*, 2007). The bacteria were subsequently immobilized on white Isopore GTTP membranes (pore size 0.2 μm, ∅ 47 mm; Millipore). Subsequent hybridization, confocal imaging and semiautomated bacteria enumeration were performed as in Bertaux *et al.* (2007), but analysing five images per probe per replicate. The probes used for hybridization, labelled with cy3, cy5 or fluorescein are listed in Table 1. DAPI (4,6- diamidino-2-phenylindoldihydrochloride) labelling was applied to count all bacteria, including dead and inactive ones, whereas the FISH-probe EUB I,II,III showed all the FISH-detectable bacteria, that is, live and presumably physiologically active ones. To check for unspecific hybridizations, negative controls were performed for each fluorochrome with the probes Apis2A-cy3, T-fluo and U-cy5 specific for aphid endosymbionts, but not for soil bacteria.

DNA extraction from sand

A combined DNA extraction protocol was applied according to the lysis protocol of Lueders *et al.* (2004) but using Lysing Matrix D (MP Biomedicals, Heidelberg, Germany) and bead beating steps for 20 s and 6 m s^{-1} (Jossi *et al.*, 2006). Aliquots were checked for the presence and quality of DNA on agarose gels stained with ethidiumbromide.

PCR amplification

A nested PCR approach was used to amplify gene fragments with primer pairs as described by Milling *et al.* (2004). First, universal PCR amplifications of the 16S rDNA were carried out with the primer pair 616 V/630R. The PCR reaction contained 5 μl DNA (1:5 dilution from the original genomic DNA) and 45 μl PCR Mix consisting of 1$\times$ Taq buffer with KCl, 0.25 mM dNTP Mix, 2% DMSO, 1.2 μg BSA, 50 pM of each primer, 3.5 mM $MgCl_2$ and 0.5 μl Taq (Fermentas, St Leon-Roth, Germany). The thermal cycling program contained an initial denaturating step at 94 °C for 2 min, subsequently followed by 29 cycles at 94 °C for 1 min, at 50 °C for 45 s, and at 72 °C for 90 s (at 72 °C for 10 min for the last extension). Different phylogenetic groups were amplified in a second PCR step using the primer-system described by Milling *et al.* (2004). The 16S rDNA V3-region, *Alphaproteobacteria*, *Betaproteobacteria* and Pseudomonads were amplified with specific primers using the Hot Start Mastermix (Qiagen, Hilden, Germany). Briefly, 2.5 μl of the purified 16S fragments were added to 12.5 μl Hot Start Mastermix, 1.5 mM $MgCl_2$, 3.125 pM of each primer with a final volume of 25 μl. Thermal cycling

Table 1 rRNA-targeted oligonucleotide probes used for hybridization

Probe name	*Position*[a]	*Sequence (5′-3′)*	*Theoretical stringency (% formamide)*	*Specificity*	*Target*	*Reference*
EUB I[b]	338–355	gctgcctcccgtaggagt	35	Eubacteria	16S rRNA	Amann *et al.* (1990)
EUB II[b]	338–355	gcagccacccgtaggtgt	35	*Planctomycetales*	16S rRNA	Daims *et al.* (1999)
EUB III[b]	338–355	gcagccacccgtaggtgt	35	*Verrucomicrobiales*	16S rRNA	Daims *et al.* (1999)
LGC354A[c]	354–371	tggaagattccctactgc	35	*Firmicutes* (low GC content Gram$^+$ bacteria)	16S rRNA	Meier *et al.* (1999)
LGC354B[c]	354–371	cggaagattccctactgc	35	*Firmicutes* (low GC content Gram$^+$ bacteria)	16S rRNA	Meier *et al.* (1999)
LGC354C[c]	354–371	ccgaagattccctactgc	35	*Firmicutes* (low GC content Gram$^+$ bacteria)	16S rRNA	Meier *et al.* (1999)
HGC69a[d]	1901–1918	tatagttaccaccgccgt	25	*Actinobacteria* (high GC content Gram$^+$ bacteria)	23S rRNA	Roller *et al.* (1994)
ALF1b	19–35	cgttcgytctgagccag	20	*Alphaproteobacteria*, several members of *Deltaproteobacteria*, most spirochetes	16S rRNA	Manz *et al.* (1992)
BET42a[d]	1027–1043	gccttcccacttcgttt	35	*Betaproteobacteria*	23S rRNA	Manz *et al.* (1992)
GAM42a[d]	1027–1043	gccttcccacatcgttt	35	*Gammaproteobacteria*	23S rRNA	Manz *et al.* (1992)
CFB560	560–575	wccctttaaacccart	40	*Cytophaga- Flexibacter-Bacteroides*	16S rRNA	O'Sullivan *et al.* (2002)
Ntspa712[d]	712–732	cgccttcgccaccggccttcc	50	Mostly *Nitrospirae*	16S rRNA	Daims *et al.* (2001)
Nso1225	1224–1243	cgccattgtattacgtgtga	35	Betaproteobacterial ammonia-oxidizing bacteria	16S rRNA	Mobarry *et al.* (1996)
Apis2a	ND	cctctttgggtagatcc	35	*Buchnera aphidicola* endosymbiont	16S rRNA	Moran *et al.* (2005)
T16	ND	gccgacatgaactcagtaaa	35	T-type endosymbiont	16S rRNA	Moran *et al.* (2005)
U16	ND	gtagcaagctactccccgat	35	U-type endosymbiont	16S rRNA	Moran *et al.* (2005)

[a]According to Brosius *et al.* (1981).
[b]Used in equimolar mixture.
[c]Used in equimolar mixture.
[d]Used with the appropriate oligocompetitor.

started with an initial denaturation step of 15 min, followed by 29 cycles of amplification (at 94 °C for 1 min, for 30 s at different annealing temperatures as shown in Milling *et al.* (2004), at 72 °C for 1 min) and a final extension step (at 72 °C for 10 min). The functional diversity of Pseudomonads was characterized with *gacA*-specific primers as described by Costa *et al.* (2006) but using the Hot Start Mastermix (Qiagen) with an initial denaturation step of 15 min followed by 10 cycles of amplification (at 94 °C for 1 min, at 65 °C for 30 s with a touchdown of 1.0 °C every cycle), 20 cycles of amplification (at 94 °C for 1 min, at 55 °C for 30 s, at 72 °C for 1 min) and a final extension step (at 72 °C for 10 min). PCR products were checked for fragment length on ethidiumbromide-stained agarose gels.

DGGE

DGGE analysis of the 16S rDNA was conducted using the DCode system (Bio-Rad, Hercules, CA, USA). PCR products (3 µl) were loaded on a 6% polyacrylamide gel with a linear gradient from 45% to 65% denaturant (100% denaturant is defined as 7 M urea and 40% formamide). Gels were run at 60 °C and 40 V overnight in 1 × TAE-buffer and stained in 0.01% Sybr Green I (Sigma-Aldrich) in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at room temperature. Images created with BDadig compact (Biometra, Göttingen, Germany) were analysed with the BioOne software package (Bio-Rad). DGGE analysis of eubacterial 16S rDNA fragments amplified from the sand were compared by running 5 (0 dpi) or 6 (3 and 6 dpi) replicates of each treatment with or without amoeba.

DGGE supported clone library

To obtain pure DNA sequences from DGGE bands of interest with a fragment length larger than 500 bp, a mixture of 16S rDNA fragments were cloned and sequenced. PCR products with the primer pair F948β/R1492 of five replicates per treatment were mixed and cloned into pGEM T easy vector as recommended by the manufacturer. For transformation 2 µl of the ligation mix were assorted with 50 µl of thawed JM109 competent cells (Promega, Mannheim, Germany). Transformant cells (100 µl) were plated on $LB_{amp/IPTG/x-Gal}$ and incubated overnight at 37 °C. The resulting white colonies were PCR-amplified (with GC clamp) as described above and loaded on DG gels. Their melting behaviours were compared to those of bands present in the original DG gel.

Sequence analysis
PCR products from matched bands were selected for sequencing at Macrogene (Seoul, Korea) with the standard primer M13r (5′ CAG GAA ACA GCT ATG AC ′3) and M13f (5′ GTA AAA CGA CGG CCA G ′3). The nucleotide-nucleotide BLAST search tool (BLASTN) of the National Center for Biotechnology Information (NCBI, USA) was used for all sequences.

Statistical analyses
Statistical analyses of plant rosette diameters and amoebae abundance were performed with a three factor ANOVA (SAS 9.1, Cary, FL, USA); means were compared using Tukey tests at $P<0.05$.

DGGE data (band intensity, lane number and band type) were imported into Excel Software (Microsoft Corp.) for each day separately. Matrices generated for PCA were structured with band intensities in columns and replicates as rows and analysed with CANOCO for windows (Version 4.5 Microcomputer Power; Ithaca, NY, USA). The grazing effect of amoebae on bacterial communities was analysed with a two level factor discriminant function analysis (DFA) via multidimensional scaling.

Statistical analyses of FISH cell counts were performed with STATISTICA 7 (Statsoft, Hamburg, Germany). The experiment consisted of two treatments (plus/minus *A. castellanii*) with five replicates each. For each replicate the number of DAPI and FISH/DAPI labelled bacteria were summed up for five images. Proportions of FISH/DAPI labelled bacteria were calculated as a reference to the total number of DAPI labelled bacteria. To correct for artificial unspecific hybridizations, the proportion of objects detected in the negative controls was subtracted from the numbers obtained. Before ANOVAs, homogeneity of variances was checked by Levene's test and data were log or Poisson transformed if necessary.

Results

Five dpi, the numbers of amoebae had increased about 18-fold to 1.8×10^4 amoebae g^{-1} sand dry weight, suggesting a significant consumption of bacteria. No protozoa were detected in control treatments.

Fluorescence in situ *hybridization*
Compared to the control treatment, amoebae reduced the total numbers of bacteria (DAPI) 3 dpi by 61% ($F_{1,8}=22.44$, $P<0.01$) and the numbers of active bacteria (EUB I,II,III) by 46% ($F_{1,8}=11.22$, $P=0.01$; Figure 1). Despite these reductions the relative proportion of active bacteria increased by 24% in presence of amoebae ($F_{1,8}=37.55$, $P<0.01$).

Among the dominant bacterial groups, *Betaproteobacteria* ($F_{1,8}=6.01$, $P=0.04$) and *Alphaproteobacteria* ($F_{1,8}=4.27$, $P=0.07$) decreased by 40% and 50% in presence of amoebae, respectively, albeit the latter group with marginal significance, whereas numbers of *Gammaproteobacteria* remained constant ($F_{1,8}=0.48$, $P=0.50$; Figure 2). Despite strong reductions in *Betaproteobacteria,* betaproteobacterial ammonia-oxidizers ($F_{1,8}=0.62$, $P=0.45$) were not affected by amoebae. Also, *Firmicutes* ($F_{1,8}=5.27$, $P=0.05$) decreased by half whereas the relative abundance of *Verrucomicrobia* ($F_{1,8}=13.07$, $P<0.01$), Nitrospira ($F_{1,8}=18.93$ $P<0.01$) and *Actinobacteria* ($F_{1,8}=23.38$, $P<0.01$) increased by factor of 19, 7 and 6, respectively. *Planctomycetes* ($F_{1,8}=9.03$, $P=0.02$) were only detected in treatments with amoebae (Figure 2). Filamentous bacterial phenotypes occurred only in the amoebae treatment and belonged to *Verrucomicrobia, Planctomycetes* and *Actinobacteria*. The *Cytophaga–Flexibacter–Bacteroides* group was not affected by amoebae ($F_{1,8}=0.51$, $P=0.50$).

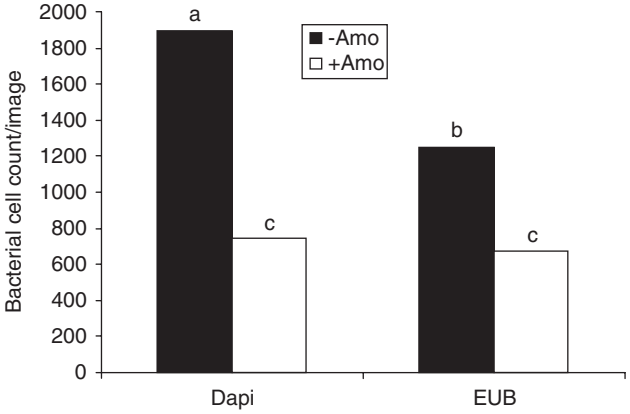

Figure 1 Changes in absolute cell numbers in treatments without amoebae (−Amo) and with amoebae (+Amo) of bacterial populations labelled with DAPI (total bacteria), compared to labelling with EUB I,II,III mix (active bacterial cells) recorded by fluorescence *in situ* hybridization (FISH). Bars with different letters are significantly different (Tukey's Honestly Significant Difference, $P<0.05$).

DGGE and cloning
High molecular weight DNA was recovered from all treatments. The DGGE fingerprints (Figure 3) demonstrated good reproducibility. Clear differences between the treatments with and without amoebae were detectable by visual comparison of the lanes (Figure 3). Amoebae rapidly changed the composition of the bacterial community since some bands already disappeared at 0 dpi. The pattern consisted of 16 main bands compared to 19 bands at 0 and 3 dpi, respectively. At 0 dpi, the banding pattern consisted of five stronger bands and a large number of less intense bands, indicating that few bacterial populations dominated whereas many populations were less abundant. At 3 dpi, the

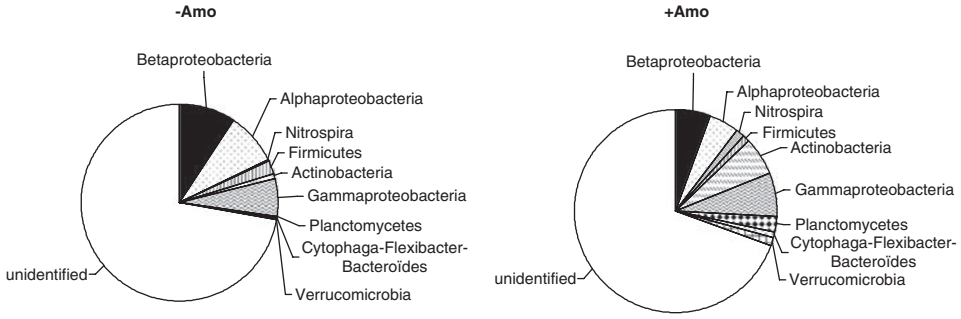

Figure 2 Shifts in relative abundance of bacterial phyla after 3 dpi in treatments without amoebae (−Amo) and with amoebae (+Amo) recorded by fluorescence *in situ* hybridization (FISH).

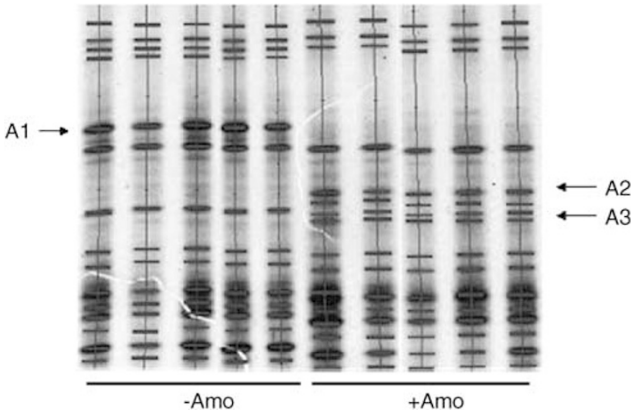

Figure 3 16S rDNA gene fragments specific denaturing gradient gel electrophoresis (DGGE) fingerprints at 3 dpi of treatments without amoebae (−Amo) and with amoebae (+Amo), bands were edited with BioOne software (Biometra); A1, A2 and A3 represent cloned and sequenced bands: A1 *Variovorax* sp.; A2 *Herbaspirillum* sp.; A3 uncultured bacterium.

number of strong bands had decreased, instead a higher number of weaker bands indicated a more equal abundance of ribotypes (Figure 3). At both sampling times, 3 dpi and 6 dpi, the DGGE banding pattern in treatments without and with inoculation of *A. castellanii* were clearly separated in a PCA ordination plot (Figure 4a and b). The separation occurred mainly along the first axis representing 61% and 69% of the overall variation in the dataset of 3 and 6 dpi, respectively. Similarly, the DFA clearly separated the grazed from the ungrazed treatments at all time points (Table 2).

In amoeba treatments, some bands disappeared whereas others appeared instead in comparison to control treatments at both sampling dates, 3 and 6 dpi, respectively. After cloning and sequencing different bands at 3 dpi, bands A1 (disappearing), A2 and A3 (both appearing) in amoebae treatments (Figure 3) showed the highest similarity to *Variovorax* sp. KS2D-23 (99%, member of *Comamonadaceae*), *Herbaspirillum* sp. SE1 (99%) and an uncultured bacterium (95%), respectively.

Group-specific primers

To reduce the complexity of the banding pattern, specific primer for *Alpha-, Betaproteobacteria* and Pseudomonads were used to analyse bacterial communities in samples without or with amoebae. The pattern obtained with *Betaproteobacteria*-specific primer was similar to that obtained with the universal proteobacterial 16S rDNA-based DGGE gel. In contrast, with primers for the *Alphaproteobacteria* fewer bands with three strong and up to eight weak bands were obtained. The pattern for Pseudomonads consisted of 4 strong and 14 weak bands. The betaproteobacterial pattern differed strikingly between grazed and ungrazed treatments. However, the analyses for Pseudomonads and *Alphaproteobacteria* also showed distinct and repeatable changes in the community composition, which were clearly separated into two different clusters without and with amoebae by UPGMA cluster analysis (data not shown).

Diversity of gacA *functional genes*

The richness of bands in the *gacA* compared to the Pseudomonads pattern decreased with up to 15 bands in the Pseudomonads specific gels to three stronger bands in the *gacA* genes (Figure 5a–c). Despite no changes in the number of bands were observed in the Pseudomonads specific pattern, the pattern of the functional *gacA* gene changed strikingly due to protozoan grazing (Figure 5). After checking the melting behaviour of 48 *gacA* clones obtained from gacA2/gacA-1F amplified DNA from 0, 3 and 6 dpi, four clones were selected for sequencing, which showed the same migration behaviour in DGGE as the bands G1 to G4 (Figure 3). The gene sequence of G1, G2 and G3 showed similarity to *Pseudomonas fluorescens* PFO-1 (85%, 87% and 84%), G4 shared 86% similarity with *P. fluorescens* Pf-5.

Plant growth

Rosette diameter 6 dpi of *A. thaliana* had increased from 2.46 ± 0.64 to 3.19 ± 0.79 cm by a factor of 1.3 in

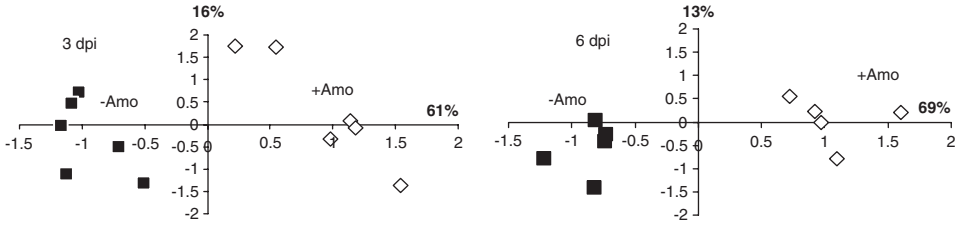

Figure 4 PCA ordination of denaturing gradient gel electrophoresis (DGGE) bands of bacterial communities at 3 and 6 dpi in treatments without amoebae (−Amo) and with amoebae (+Amo), respectively. The explained variation (%) is given for the respective axes: diamonds, without amoeba; squares, with amoeba.

Table 2 Analysis of DGGE gels performed for 16S rDNA using discriminant function analysis (DFA) via multidimensional scaling (MDS) of grazed and ungrazed bacterial communities of three different time points 0, 3 and 6 days after transferring the plants

Time point (dpi)	*df*	*F*	P
0	8.1	245.1	<0.05
3	7.4	268.8	<0.0001
6	8.2	1091.9	<0.001

the presence of amoebae ($F_{1,18}=5.10$; $P<0.05$). Similarly, shoot biomass in presence of amoebae increased from 1.03 ± 0.12 to 1.38 ± 0.12 mg dry weight by a factor of 0.75 ($F_{1,18}=44.62$; $P<0.0001$) and root biomass increased from 0.54 ± 0.06 to 0.65 ± 0.12 mg dry weight by a factor of 0.83 ($F_{1,18}=6.82$; $P<0.05$), respectively.

Discussion

The DGGE profiles demonstrated that our inoculation procedure reestablished a diverse bacterial community, containing all major groups of rhizosphere bacteria (Zul *et al.*, 2007) in our Magenta system. Cloning and matching of the sequences to excised bands proved not only the presence of a diverse range of different phylogenetic groups but even of uncultured bacteria, suggesting a successful establishment of natural microbial communities in our experimental systems.

Our microcosm system allowed a reliable detection of the fast turnover rates of bacteria exposed to protozoan grazing. Acanthamoebae reduced total cell numbers by 61%, confirming their strong impact as bacterial predators in the bulk sand substrate and rhizosphere. However the proportion of active bacteria increased in the amoeba treatment by 24% indicating that the loss in bacterial numbers was partly compensated by increased bacterial activity. A comparable increase in energy metabolism of grazed rhizosphere microbial communities was previously described (Alphei *et al.*, 1996) and is thought to result from removal of senescent bacteria and a relatively higher increase in the contribution of younger individuals within actively dividing populations with higher metabolic activity (Posch *et al.*, 1999; Bonkowski 2004).

The rapidity by which the bacterial communities responded to protozoan grazing was unexpected. DGGE with universal primers 2 days past inoculation of amoebae showed the loss of bands in amoeba treatments, and simultaneously an appearance of new bands, indicating that certain bacterial taxa were consumed whereas others gained competitive advantage in presence of protozoan grazers. Similarly, FISH analyses performed at three days past transferring the plants testified rapid and significant shifts in the relative abundances for 6 out of 10 dominant taxonomic groups of soil bacteria. The repeatable, treatment-specific banding patterns demonstrate grazing preferences of amoebae for distinct bacterial taxa, which were replaced by grazing tolerant taxa in a deterministic way.

Although DGGE rather assessed changes in diversity within chosen phyla, FISH yielded quantitative information on relative abundance of major phyla present in the sand substrate (Janssen, 2006). *A. castellanii* most strongly affected the diversity of *Betaproteobacteria* (DGGE) whereas decreasing their relative abundance (FISH). *Betaproteobacteria* in particular seem less grazing tolerant to protozoa than other soil bacterial groups as corresponding findings by Kreuzer *et al.* (2006) and Murase *et al.* (2006) indicate. For example, *Variovorax* sp., a member of the *Comamonadaceae* (*Betaproteobacteria*) had virtually disappeared 2 days after the addition of the protozoan grazers, demonstrating that not all *Comamonadaceae* are as grazing resistant as reported from aquatic systems (Hahn and Höfle, 1998; Matz and Kjelleberg, 2005). However, not all groups of *Betaproteobacteria* decreased. A number of microcosm studies with different groups of protozoa have shown that bacterial nitrifyers generally strongly increase in presence of protozoa (Griffiths, 1989; Verhagen *et al.*, 1993; Alphei *et al.*, 1996; Bonkowski *et al.*, 2000), potentially due to increased availability of ammonium released by protozoan grazing. However, there was no information available on which taxa of nitrifyers were affected. Our study showed that despite *Betaproteobacteria* decreased by half, the relative contribution of betaproteobacterial ammonia-oxidizers was not

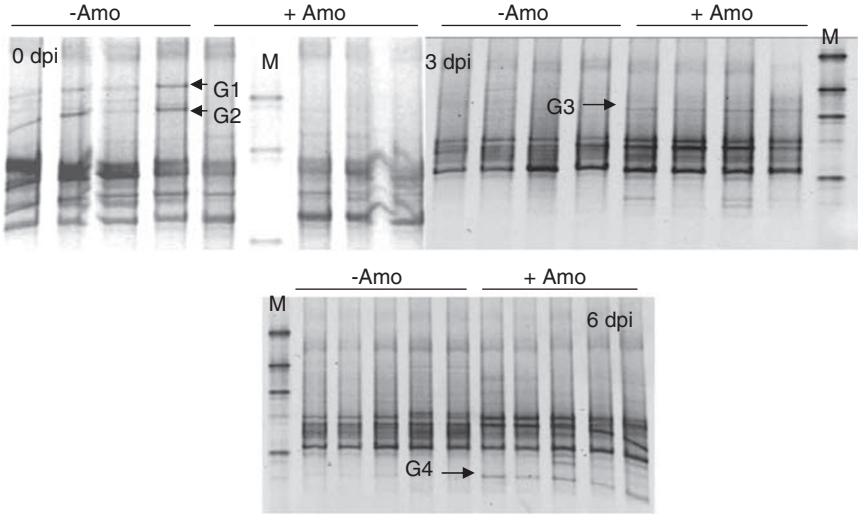

Figure 5 Changes in denaturing gradient gel electrophoresis (DGGE) fingerprints of *gacA* functional genes of pseudomonads at three different sampling dates (0, 3 and 6 dpi) in treatments without amoebae (−Amo) and with amoebae (+Amo), respectively; M bacterial marker; G1, G2 bands disappearing in amoeba treatments; G3, G4 new bands appearing in amoeba treatments.

affected by amoebae. However, most striking was the sevenfold increase of *Nitrospira* in amoeba treatments (Figure 2).

FISH analyses further showed a relative decrease of *Alphaproteobacteria* and *Firmicutes*, but an increase of *Actinobacteria.* The results for *Firmicutes* are surprising because Gram-positive bacteria are believed to be less preferred by protozoa due to their protective cell wall and have been shown to benefit from protozoan grazing (Griffiths *et al.*, 1999; Rønn *et al.*, 2002; Murase *et al.*, 2006).

In *Gammaproteobacteria* neither diversity (DGGE) nor the relative abundance (FISH) was affected. However, DGGE with specific primers for pseudomonads documented a strong shift in the diversity among these specific *Gammaproteobacteria*, a result consistent with findings of Rønn *et al.* (2002) who studied effects of protozoa on bacterial communities in soil organic patches. The strong and highly reproducible changes in the *gacA*-banding pattern further revealed a major shift in this master gene controlling antibiotics production of pseudomonads (De Souza *et al.*, 2003). We suggest that pseudomonads quickly upregulated secondary metabolite production in response to protozoan predators, which is in accordance with Jousset *et al.* (2006) who demonstrated that antibiotics of *P. fluorescens* are of particular toxicity to protozoa; and that antibiotic-producing *P. fluorescens* disproportionally benefit from protozoan predation when their bacterial competitors are consumed and nutrients excreted by the protozoan predators (Jousset *et al.*, 2008).

Positive effects of bacteria–protozoa interactions on plant growth are well documented (Bonkowski 2004) and recent findings strongly indicated that grazing induced shifts in bacterial diversity and function are responsible for plant growth promoting effects of bacterial grazers (Bonkowski and Brandt, 2002; Kreuzer *et al.*, 2006; Mao *et al.*, 2007). In fact, shoot and root biomass of *A. thaliana* increased significantly in presence of amoebae and the early growth response of plants was not linked to increased nutrient availability from consumed bacterial biomass (Krome *et al.*, 2009). Our results confirm that grazing-induced changes in bacterial community composition are strongly interlinked with protozoan effects on plant growth. These findings have important implications for the success of applied studies, such as plant inoculations with growth-promoting bacterial strains.

In conclusion, protozoan grazing rapidly and significantly affected the diversity, activity and function of rhizosphere bacteria. Dominant bacterial groups were reduced, marginal groups gained competitive advantage, leading to greater evenness of grazed communities. However, the treatment-specific banding pattern in DGGE gels indicates that distinct mechanisms based on specific feeding preferences and competitive outcomes structured bacterial community composition in a well-defined way, despite bacterial communities were highly diverse. Our model system has been shown to warrant standardized experimental conditions to further investigate the mechanisms responsible for structuring of bacterial communities and its coupling to plant growth promotion by protozoa. Undoubtedly, protozoa need to be considered an important structuring force in investigations on plant–microbial interactions.

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References

Acea MJ, Moore CR, Alexander M. (1988). Survival and growth of bacteria introduced into soil. *Soil Biol Biochem* **20**: 509–515.

Alphei J, Bonkowski M, Scheu S. (1996). Protozoa, Nematoda and Lumbricidae in the rhizosphere of *Hordelymus Europaeus* (*Poaceae*): faunal interactions, response of microorganisms and effects on plant growth. *Oecologia* **106**: 111–126.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Andersen KS, Winding A. (2004). Non-target effects of bacterial biological control agents on soil protozoa. *Biol Fertil Soils* **40**: 230–236.

Bertaux J, Gloger U, Schmidt M, Hartmann A, Scheu S. (2007). Routine fluorescence *in situ* hybridization in soil. *J Microbiol Methods* **69**: 451–460.

Bjørnlund L, Mork S, Vestergard M, Rønn R. (2006). Trophic interactions between rhizosphere bacteria and bacterial feeders influenced by phosphate and aphids in barley. *Biol Fertil Soils* **43**: 1–11.

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

Bonkowski M, Brandt F. (2002). Do soil protozoa enhance plant growth by hormonal effects? *Soil Biol Biochem* **34**: 1709–1715.

Bonkowski M, Cheng WX, Griffiths BS, Alphei J, Scheu S. (2000). Microbial-faunal interactions in the rhizosphere and effects on plant growth. *Eur J Soil Biol* **36**: 135–147.

Brosius J, Dull TL, Sleeter DD, Noller HF. (1981). Gene organization of primary structure of a ribosomal operon from *Escherichia coli*. *J Mol Biol* **148**: 107–127.

Clarholm M. (1981). Protozoan grazing of bacteria in soil—impact and importance. *Microb Ecol* **7**: 343–350.

Clarholm M. (1989). Effects of plant-bacterial-amoebal interactions on plant uptake of nitrogen under field conditions. *Biol Fertil Soils* **8**: 373–378.

Costa R, Salles JF, Berg G, Smalla K. (2006). Cultivation-independent analysis of *Pseudomonas* species in soil and in the rhizosphere of field-grown *Verticillium dahliae* host plants. *Environ Microbiol* **8**: 2136–2149.

Daims H, Brühl A, Amann R, Schleifer KH, Wagner M. (1999). Probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 438–448.

Daims H, Nielsen JL, Nielsen PH, Schleifer K-H, Wagner M. (2001). *In situ* characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. *Appl Environ Microbiol* **67**: 5273–5284.

Danso SKA, Alexander M. (1975). Regulation of predation by prey density-protozoan-rhizobium relationship. *Appl Microbiol* **29**: 515–521.

Darbyshire JF, Wheatley RE, Greaves MP, Inkson RHE. (1974). Rapid micromethod for estimating bacterial and protozoan populations in soil. *Rev Ecol Biol Sol* **11**: 465–475.

De Leij FAAM, Dixon-Hardy JE, Lynch JM. (2002). Effect of 2,4-diacetylphloroglucinol-producing and non-producing strains of *Pseudomonas fluorescens* on root development of pea seedlings in three different soil types and its effect on nodulation by *Rhizobium*. *Biol Fertil Soils* **35**: 114–121.

De Souza JT, Mazzola M, Raaijmakers JM. (2003). Conservation of the response regulator gene *gacA* in *Pseudomonas* species. *Environ Microbiol* **5**: 1328–1340.

Finlay BF, Black HIJ, Brown S, Clarke KJ, Esteban GF, Hindle RM *et al.* (2000). Estimating the growth potential of the soil protozoan community. *Protist* **151**: 69–80; Erratum Protist 151: 367.

Foster RC, Dormaar JF. (1991). Bacteria-grazing amoebas *in situ* in the rhizosphere. *Biol Fertil Soils* **11**: 83–87.

Griffiths BS. (1989). Enhanced nitrification in the presence of bacteriophagous protozoa. *Soil Biol Biochem* **21**: 1045–1051.

Griffiths BS, Bonkowski M, Dobson G, Caul S. (1999). Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa. *Pedobiologia* **43**: 297–304.

Habte M, Alexander M. (1975). Protozoa as agents responsible for decline of *Xanthomonas campestris* in soil. *Appl Microbiol* **29**: 159–164.

Hahn MW, Höfle MG. (1998). Grazing pressure by a bacterivorous flagellate reverses the relative abundance of *Comamonas acidovorans* Px54 and *Vibrio* Strain Cb5 in chemostat co-cultures. *Appl Environ Microbiol* **64**: 1910–1918.

Hurley MA, Roscoe ME. (1983). Automated statistical-analysis of microbial enumeration by dilution series. *J Appl Bacteriol* **55**: 159–164.

Janssen PH. (2006). Identifying the dominant soil bacterial taxa in libraries of 16 s rRNA and 16 s rRNA genes. *Appl Environ Microbiol* **72**: 1719–1728.

Jossi M, Fromin N, Tarnawski S, Kohler F, Gillet F, Aragno M *et al.* (2006). How elevated pCO_2 modifies total and metabolically active bacterial communities in the rhizosphere of two perennial grasses grown under field conditions. *FEMS Microbiol Ecol* **55**: 339–350.

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

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

Jürgens K, Matz C. (2002). Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie Van Leeuwenhoek* **81**: 413–434.

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

Krome K, Rosenberg K, Bonkowski M, Scheu S. (2009). Grazing of protozoa on rhizosphere bacteria alters

growth and reproduction of *Arabidopsis thaliana*. *New Phytol* (in press).

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

Lugtenberg BJJ, Chin-a-Woeng TFC, Bloemberg GV. (2002). Microbe-plant interactions: principles and mechanisms. *Antonie Van Leeuwenhoek* **81**: 373–383.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

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

Mao XF, Hu F, Griffiths B, Li H. (2006). Bacterial-feeding nematodes enhance root growth of tomato seedlings. *Soil Biol Biochem* **38**: 1615–1622.

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

Meier H, Amann R, Ludwig W, Schleifer KH. (1999). Specific oligonucleotide probes for *in situ* detection of a major group of Gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* **22**: 186–196.

Milling A, Smalla K, Maidl FX, Schloter M, Munch JC. (2004). Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. *Plant Soil* **266**: 23–39.

Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl Environ Microbiol* **62**: 2156–2162. Erratum Appl Environ Microbiol 63: 815.

Moran NA, Russell JA, Koga R, Fukatsu T. (2005). Evolutionary relationships of three new species of *Enterobacteriaceae* living as symbionts of aphids and other insects. *Appl Environ Microbiol* **71**: 3302–3310.

Murase J, Noll M, Frenzel P. (2006). Impact of protists on the activity and structure of the bacterial community in a rice field soil. *Appl Environ Microbiol* **72**: 5436–5444.

O'Sullivan LA, Weightman AJ, Fry JC. (2002). New degenerate Cytophaga-Flexibacter-Bacteroides-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. *Appl Environ Microbiol* **68**: 201–210.

Page FC. (1976). An illustrated key to freshwater and soil amoebae. *Freshwater Biological Assoc* **34**: 11.

Pernthaler J. (2005). Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Microbiol* **3**: 537–546.

Posch T, Simek K, Vrba J, Pernthaler S, Nedoma J, Sattler B *et al.* (1999). Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. *Aquat Microb Ecol* **18**: 235–246.

Roller C, Wagner M, Amann R, Ludwig W, Schleifer K-H. (1994). *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**: 2849–2858.

Rønn R, McCaig AE, Griffiths BS, Prosser JI. (2002). Impact of protozoan grazing on bacterial community structure in soil microcosms. *Appl Environ Microbiol* **68**: 6094–6105.

Schuster FL. (2002). Cultivation of pathogenic and opportunistic free-living amoebas. *Clin Microbiol Rev* **15**: 342–354.

Verhagen FJM, Duyts H, Laanbroek HJ. (1993). Effects of grazing by flagellates on competition for ammonium between nitrifying and heterotrophic bacteria in soil columns. *Appl Environ Microbiol* **59**: 2099–2106.

Zhang HM, Forde BG. (1998). An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279**: 407–409.

Zul D, Denzel S, Kotz A, Overmann J. (2007). Effects on plant biomass, plant diversity, and water content on bacterial communities in soil lysimeters: implications for the determinants of bacterial diversity. *Appl Environ Microbiol* **73**: 6916–6929.