Microbial Ecology

Community Structure of Actively Growing Bacterial Populations in Plant Pathogen Suppressive Soil

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Abstract

The bacterial community in soil was screened by using various molecular approaches for bacterial populations that were activated upon addition of different supplements. *Plasmodiophora brassicae* spores, chitin, sodium acetate, and cabbage plants were added to activate specific bacterial populations as an aid in screening for novel antagonists to plant pathogens. DNA from growing bacteria was specifically extracted from the soil by bromodeoxyuridine immunocapture. The captured DNA was fingerprinted by terminal restriction fragment length polymorphism (T-RFLP). The composition of the dominant bacterial community was also analyzed directly by T-RFLP and by denaturing gradient gel electrophoresis (DGGE). After chitin addition to the soil, some bacterial populations increased dramatically and became dominant both in the total and in the actively growing community. Some of the emerging bands on DGGE gels from chitin-amended soil were sequenced and found to be similar to known chitin-degrading genera such as *Oerskovia*, *Kitasatospora*, and *Streptomyces* species. Some of these sequences could be matched to specific terminal restriction fragments on the T-RFLP output. After addition of *Plasmodiophora* spores, an increase in specific Pseudomonads could be observed with *Pseudomonas*-specific primers for DGGE. These results demonstrate the utility of microbiomics, or a combination of molecular approaches, for investigating the composition of complex microbial communities in soil.

Introduction

The soil environment contains a tremendous diversity of microbial life. However, it has been difficult to assess the large majority of microorganisms in soil, due to deficiencies in common isolation techniques. Therefore, there is interest in developing other methods to identify environmental strains of interest, without reliance on cultivation. For example, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments directly amplified from extracted DNA are useful molecular tools for obtaining fingerprints of microbial communities [22, 29]. T-RFLP is particularly useful for distinguishing between different samples according to time or treatment, and for quantification of the relative abundances of individual ribotypes, detected as terminal restriction fragments (TRFs) in the communities. On the other hand, DGGE allows the possibility to obtain information on the identity of individual ribotypes in the community by cloning and sequencing of selected bands from the gel. The analysis of particular taxonomic groups can be achieved by using primers targeting the 16S rRNA genes of specific taxa during amplification from community DNA [4, 5]. This more targeted approach also enables less abundant populations to be analyzed in the often less complex taxon-specific fingerprints [38]. Both T-RFLP and DGGE methods are polymerase chain reaction (PCR)-based and subject to PCR bias, and it is not possible to determine from the fingerprinting patterns whether the DNA is derived from active or inactive cell populations.

Recently, other approaches have been developed to specifically address the active fraction of microbial communities. For example, stable isotope probing has been demonstrated to selectively label active cells by incorporation of radioactivity during metabolism of labeled substrates [34]. The cells that have incorporated the radioactive label can be identified by DGGE, cloning, and sequencing. Recently, we demonstrated the use of bromodeoxyuridine (BrdU) immunocapture as an effec-

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tive approach to selectively identify specific bacteria in soil that associate with mycorrhizal hyphae [2]. Bromodeoxyuridine is a thymidine analog that is incorporated into the DNA of growing cells. This method has been demonstrated to be useful for study of active populations in complex microbial communities such as soil and seawater [6, 13, 32, 41, 47]. The active bacterial fractions can in turn be studied and identified in combination with different fingerprinting tools such as cloning and sequencing [2], or T-RFLP [1].

The aim of our study was to explore the potential of a combination of molecular tools—BrdU immunocapture, T-RFLP, DGGE, and cloning and sequencing—to identify active bacterial populations in soil. The Swedish soil used in this study was previously found to suppress clubroot disease symptoms caused by *Plasmodiophora brassicae* [46] and is one of four European soils chosen as targets for a metagenome sequencing project (EU-Metacontrol Project). The Swedish soil was also recently found to contain a high number of antagonists to *Rhizoctonia solani* (Smalla, unpublished results). Our aim was to activate bacterial populations in the soil that could be antagonistic to plant pathogens, such as *Plasmodiophora*. Toward this aim, *Plasmodiophora* spores, chitin, or sodium acetate were added as stimulants to activate specific bacterial populations in the soil. Chitin was chosen because it is a common component of fungal cell walls and *Plasmodiophora* spores [27], and chitinolytic activity has previously been described as one mechanism for biocontrol of plant fungal pathogens [8, 9, 19]. Sodium acetate was added as a general carbon source for comparison to the other treatments and *Plasmodiophora* spores were added with the aim to increase bacterial populations that are specifically activated in the presence of this pathogen. We also investigated the impact of the presence of cabbage plants in combination with *Plasmodiophora* spores on the soil microbiota because *Plasmodiophora* is an obligate intracellular plant parasite, thus more accurately mimicking the natural disease situation. Finally, the impact of cabbage plants alone was studied as a control. The resulting information could be useful for screening, identification, and eventual isolation of strains for potential biocontrol of clubroot disease.

Materials and Methods

Soil Sampling and Preincubation. Soil (clay loam, pH 6.9, organic carbon content 1.48%) was obtained from a cultivated field (most recently planted with oats) near Uppsala, Sweden. The soil was previously characterized as suppressive to clubroot disease of cabbage caused by the disease agent, *P. brassicae* [46]. Eight soil samples were randomly collected from the top 20 cm of soil and mixed. The soil was sieved through a 4-mm mesh and stored at +4°C under plastic cover for approximately 4 months before initiation of the experiments. Preliminary experiments showed no major changes in the dominant members of the bacterial community by T-RFLP analysis after 6 months storage at 4°C (Hjort, unpublished data).

Approximately 5 kg of soil was preincubated in a plastic container covered with plastic film at approximately 25°C with ambient light and intermittent watering to maintain a relatively constant soil moisture level for 3 weeks in the laboratory.

Soil Supplementation. Twenty-five-gram portions of the preincubated soil were added to 50 mL Falcon tubes and treated with different supplements as follows. For treatment 1, sodium acetate was dissolved in 500 µL sterile distilled water and added to the soil to a final concentration of 1.5 mg/g soil. For treatment 2, colloidal chitin (Crabshell chitinpoly-(1→4)-β-*N*-acetyl glucoseamine; Sigma-Aldrich, St. Louis, MO, USA) was prepared according to the procedure described by Inglis and Kawchuk [17] and added to the soil to a final concentration of 2 mg/g soil. For treatment 3, *Plasmodiophora* spores were separated from Chinese cabbage roots (*Brassica pekinensis*, Bröderna Nelson, Sweden) infected with *P. brassicae* according to the method described by Narisawa and Hashiba [31], except that the purification by stepwise density gradient centrifugation was omitted. A spore suspension of 5.2×10^7 spores/mL was obtained and added to the soil to a final concentration of 2.1×10^5 spores/g of soil. In treatment 4, controls consisted of 500 µL sterile distilled water added to the soil. All four treatments were incubated for 3 days under ambient light conditions (in the laboratory) under plastic cover until sampling and DNA extraction as described below.

Plant-based Study. Cabbage plants were pregrown in plastic pots containing 150 g preincubated soil (above) and incubated in a greenhouse (18°C night, 22°C day) with watering every third day for 3 weeks. Three treatments were set up with two experimental replicates per treatment in Falcon tubes cut to the 20 mL level as follows: (1) soil alone (incubated as above, but without cabbage), (2) one pregrown cabbage plant together with 25 g root associated soil, and (3) one pregrown cabbage plant together with 25 g root associated soil plus 2.1×10^5 *P. brassicae* spores/g soil. The treatments were incubated for an additional 3 days before the addition of BrdU (Sigma-Aldrich) as described below.

Bromodeoxyuridine Immunocapture. Triplicate 2-g soil samples were taken from each of the soil treatments (chitin, sodium acetate, *Plasmodiophora* spores, and water control), 200 µL of 200 mM BrdU in

water were added as droplets from a 200-μL pipette to the soil and the soil was mixed by stirring with the pipette tip. An additional triplicate series of 2-g samples were taken from each treatment and used as controls for comparison to the BrdU-treated samples, with water added to substitute the volume of BrdU. The soil samples were incubated in the dark for 48 h at room temperature. A similar procedure was used for the plant-based study, except that 2.5 mL of 200 mM BrdU was added as droplets from a pipette directly to the 25 g of soil in Falcon tubes to half of the samples, and the rest were used as controls without BrdU. Incubation was continued in the dark under greenhouse conditions for an additional 48 h.

DNA was extracted from each of three replicate 0.5-g soil samples per treatment by bead beating (Settings; 30 s at speed 5.5 in a Fast prep FP120 Bio101 Savant; Qbiogene, Carlsbad, CA, USA) using a Soil DNA Extraction Kit, BIO101 (Qbiogene). The extracted DNA was suspended in 50 μL sterile distilled water and the concentration was estimated by analysis on 1% agarose gels. Immunochemical purification of BrdU-labeled DNA was performed by a modification of the method described by Urbach *et al.* [41]. Monoclonal anti-BrdU antibodies (3 μL at a concentration of 50 μg/500 μL; Roche, Basel, Switzerland) were mixed at a 1:9 ratio with herring sperm DNA [0.63 mg/mL in phosphate-buffered saline (PBS); Promega, Madison, WI, USA], that had been denatured at 100°C for 5 min, quickly transferred to ice, and kept there for 5 min. The mixture was incubated for 1 h at room temperature. Magnetic beads (Dynabeads; Dynal, Oslo, Norway) coated with goat antimouse immunoglobulin G (Dynal) were washed once with 1 mg/mL acetylated BSA in PBS buffer (PBS–BSA) by using a magnetic particle concentrator (Dynal) and resuspended in PBS–BSA to the original concentration. A 25-μL portion of denatured soil DNA (denatured for 5 min at 100°C, quickly transferred to ice, and kept there for 5 min) was supplemented with 6 μL PBS, mixed with 30 μL of the herring sperm DNA antibody mixture and incubated for hour in the dark at room temperature with constant, gentle agitation (approximately 75 rpm on a rotary table). The samples were mixed with 10-μL portions of Dynabeads and the incubation was continued for an additional hour. Subsequently, the beads were washed three times with 0.5 mL of PBS–BSA. To elute the BrdU-containing DNA fraction, 30 μL of 1.7 mM BrdU (in PBS–BSA) were added, and the samples were incubated for 1 h in the dark at room temperature with constant agitation. The beads were separated from the DNA in solution by using the magnetic particle concentrator (Dynal). The DNA was precipitated by addition of 1/10 volume of 5 M sodium acetate and 2.5 volume 99% ethanol, followed by 15 min centrifugation at 13,000 rpm, and dissolved in 20 μL sterile distilled water. To control the BrdU-trapping efficiency, the above steps were also carried out with triplicate samples of control DNA from each soil treatment, but without any BrdU addition.

Terminal-restriction Fragment Length Polymorphism. For T-RFLP analysis of 16S rRNA gene fragments, the bacterial forward primer fD1-FAM (5′-AGA GTT TGA TCC TGG CTC AG-3′) beginning at nucleotide position 8 (*Escherichia coli* numbering) [43], 5′ end-labeled with phosphoramidite fluorochrome 5-carboxy-fluorescein (5′6-FAM) and reverse primer 926r (5′-CCG TCA ATT CCT TTR AGT TT-3′) [30] were used. All primers were synthesized by Invitrogen (Carlsbad, CA, USA). PCR amplification conditions were as previously described [1]. For each sample, duplicate fluorescently labeled PCR products were pooled. Each pooled PCR product was digested with three different restriction enzymes (*Hae*III, *Hha*I, and *Msp*I; Amersham Biosciences, Piscataway, NJ, USA) in separate 2-h reactions at 37°C. The digested DNA (2 μL) was verified by electrophoresis in 1% agarose gels (containing 0.02% ethidium bromide) in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3). Fluorescently labeled TRFs were separated and detected using an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA, USA). The sizes of the TRFs were determined by comparison with the internal GS ROX-500 size standard (Applied Biosystems). Before injection, 1.4 μL of the DNA sample was denatured in the presence of 10 μL Hi-Di™ formamide and 0.04 μL GS 500 ROX size standard (Applied Biosystems) at 95°C for 5 min. Injection was performed electrokinetically at 10 kV for 50 s, and electrophoresis was run at 7.5 kV for 80 min.

All community profiles presented in the figures were based on T-RFLP analyses from the *Hha*I restriction digestions. Relative peak areas of each TRF were determined by dividing the area of the peak of interest by the total area of peaks within the following threshold values: lower threshold, 60 bp; upper threshold, 500 bp; and a fluorescent threshold of 50. A threshold for the relative abundance was applied at 0.2% and only TRFs with higher relative abundances were included in the remaining analyses. The relative abundances of the remaining peaks were then recalculated. TRFs represented by only one out of three replicates were excluded from further analysis. TRFs differing by ±1 bp were grouped together and rounded to the nearest whole numbers. The possible species identities, based on the fragment lengths of TRFs for three restriction digestions (*Hha*I, *Msp*I, and *Hae*III) from three replicate DNA extractions for a total of nine T-RFLP analyses per treatment, were used for assignment of possible species identities using Fragsort 4.0 (http://www.oardc.ohio-state.edu/trflpfragsort/) and T-RFLP on-line analysis (TAP) in

the Ribosomal Database Project (RDP) database (http://www.cme.msu.edu/RDP) [7]. During database searches, a range of ±3 bp was applied to most TRFs and ±5 bp for those over 400 bp in length.

Statistical Analysis. Principal component analysis (PCA) plots were generated using SIMCA-P (Umetrics, Umeå, Sweden). The statistical significance of clustering patterns in the PCA plots were determined by one-way analysis of variance followed by nonparametric Tukey [40] multiple comparison test using the GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

Denaturing Gradient Gel Electrophoresis. For DGGE analysis of bacterial 16S rRNA gene fragments, the soil DNA extracts obtained above were amplified by PCR using the primer system F984GC and R1378 according to the procedure described by Heuer *et al.* [14]. For the amplification of Actinobacteria- and *Pseudomonas*-16S rRNA gene fragments, a nested PCR approach was used. The PCR consisted of a group-specific amplification of 16S rRNA gene fragments followed by a F984GC/R1378 PCR. Specific actinobacterial 16S rRNA gene fragments were amplified as described by Heuer *et al.* [15] and *Pseudomonas*-specific 16S rRNA gene fragments according to Milling *et al.* [26].

DGGE analysis was essentially performed as described by Heuer *et al.* [16], with a denaturing gradient of 26–58% of the denaturant (urea and formamide) and an acrylamide gradient of 6–9% [11]. PCR products amplified from three replicates per treatment were loaded in blocks on the gel. Gels were silver-stained according to the procedure described by Heuer *et al.* [16]. A mixture of the DGGE-PCR products from 11 bacterial species was applied as a marker to check the electrophoresis run and to compare fragment migration between gels. These species were (in order of the migration distance): *Clostridium pasteurianum* DSM 525, *Erwinia carotovora* DSM 30168, *Agrobacterium tumefaciens* DSM 30205, *Pseudomonas fluorescens* R2f, *Pantoea agglomerans*, *Nocardia asteroides* N3, *Rhizobium leguminosarum* DSM 30132, *Actinomadura viridis* DSM 43462, *Kineosporia aurantiaca* JCM 3230, *Nocardiopsis atra* ATCC 31511, and *Actinoplanes philippiensis* JCM 3001.

Computer-assisted Analysis of DGGE Fingerprints. The DGGE gels were scanned and analyzed with the GelCompar 4.0 program (Applied Maths, Ghent, Belgium), as described by Rademaker *et al.* [33] with the modifications of settings as described by Smalla *et al.* [39]. After normalizing the gel image and background subtraction, the Pearson correlation index (r) for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints, and the clustering of patterns was calculated using the unweighted pair group method using average linkages (UPGMA). As all amplicons to be compared were loaded on the same gels, normalization between gels was not necessary.

Extraction and Cloning of DGGE Bands. DNA was eluted from dominant bands as described by Gomes *et al.* [11]. Two microliters of the resulting solution was used to reamplify the 16S rRNA gene fragment using the conditions described above. After confirming the enrichment of the excised band by DGGE, the PCR fragments (without GC clamp) were cloned using the Promega pGEM-T vector system according to the manufacturer's instructions and amplified using bacterial DGGE primers. Clones were screened by DGGE to select those matching the corresponding DGGE band. Amplified ribosomal DNA restriction analysis (ARDRA) was performed to compare restriction profiles among inserts with the identical electrophoretic mobility as the original band. Inserts were amplified with the universal primers SP6 and T7 (Promega) and digested using *Alu*I and *Msp*I. Inserts showing different ARDRA profiles were submitted to sequencing of the V6–V8 region of the 16S rRNA gene (approximately 450 bp).

Sequencing of the Clones. PCR fragments were purified with a MiniElute PCR Purification Kit (Qiagen, Hilden, Germany). Both strands were sequenced with an ABI Prism automatic sequencer (Greenomics, Plant Research International, Wageningen, The Netherlands) by using SP6 and T7 primers. Sequence identities were determined by Seqmatch at the RDP-II website [7].

Sequence accession numbers were as follows: Clone 1.1, DQ144423; Clone 2.15, DQ144424; Clone 3.14, DQ144425; Clone 4.35, DQ144426; Clone 5.26, DQ144427; Clone 6.10, DQ144428; Clone 6.15, DQ144429; Clone 6.30, DQ144430.

Results

Impact of Soil Supplements on Total Community

T-RFLP Results. T-RFLP was used to assess the bacterial community structure in soil that had been supplemented with chitin, sodium acetate, or *Plasmodiophora* spores, and in untreated soil. Although only the dominant bacterial populations are represented in the T-RFLP analyses, we refer to these data as representing the "total bacterial community structure" to distinguish from the "active bacterial community structure" that will be discussed below. In all treatments, a large number (approximately 80) of TRFs was found (Fig. 1A).

Four days after chitin addition, a substantial change in the T-RFLP pattern was observed as shown by differences in relative abundances of several TRFs (Fig. 1A) and

the corresponding PCA plot of the T-RFLP data (Fig. 1B). Five very dominant TRFs, together constituting approximately 46% of the total relative abundance, emerged in the community profiles (Fig. 1A). Two of these were novel TRFs (440 and 466), whereas the other three (TRFs 87, 207, and 364) increased in relative abundance. Taken together, these differences in total community composition upon chitin addition were highly significant ($p < 0.001$) (Fig. 1B).

The other two treatments had a less pronounced effect on the composition of the total bacterial community and the changes were not significant (Fig. 1). Upon sodium acetate addition, no novel peaks could be detected but the relative abundances of a few peaks increased two- to threefold compared to untreated soil (Fig. 1A). The T-RFLP community pattern of soil inoculated with *Plasmodiophora* spores resembled that of the control soil (Fig. 1), although some minor novel TRFs (approx. 8) with a relative abundance below 0.4% could be detected, and some peaks increased slightly in relative abundance compared to the control soil (Fig. 1A).

DGGE Results. The PCR amplicons obtained from three DNA replicates per treatment were loaded on DGGE gels in blocks (Fig. 2A). Similar to the T-RFLP results noted above, the bacterial patterns of all treatments were highly complex—indicating a high number of equally abundant ribotypes—and there were clear differences in the banding patterns according to treatment. The number of bands

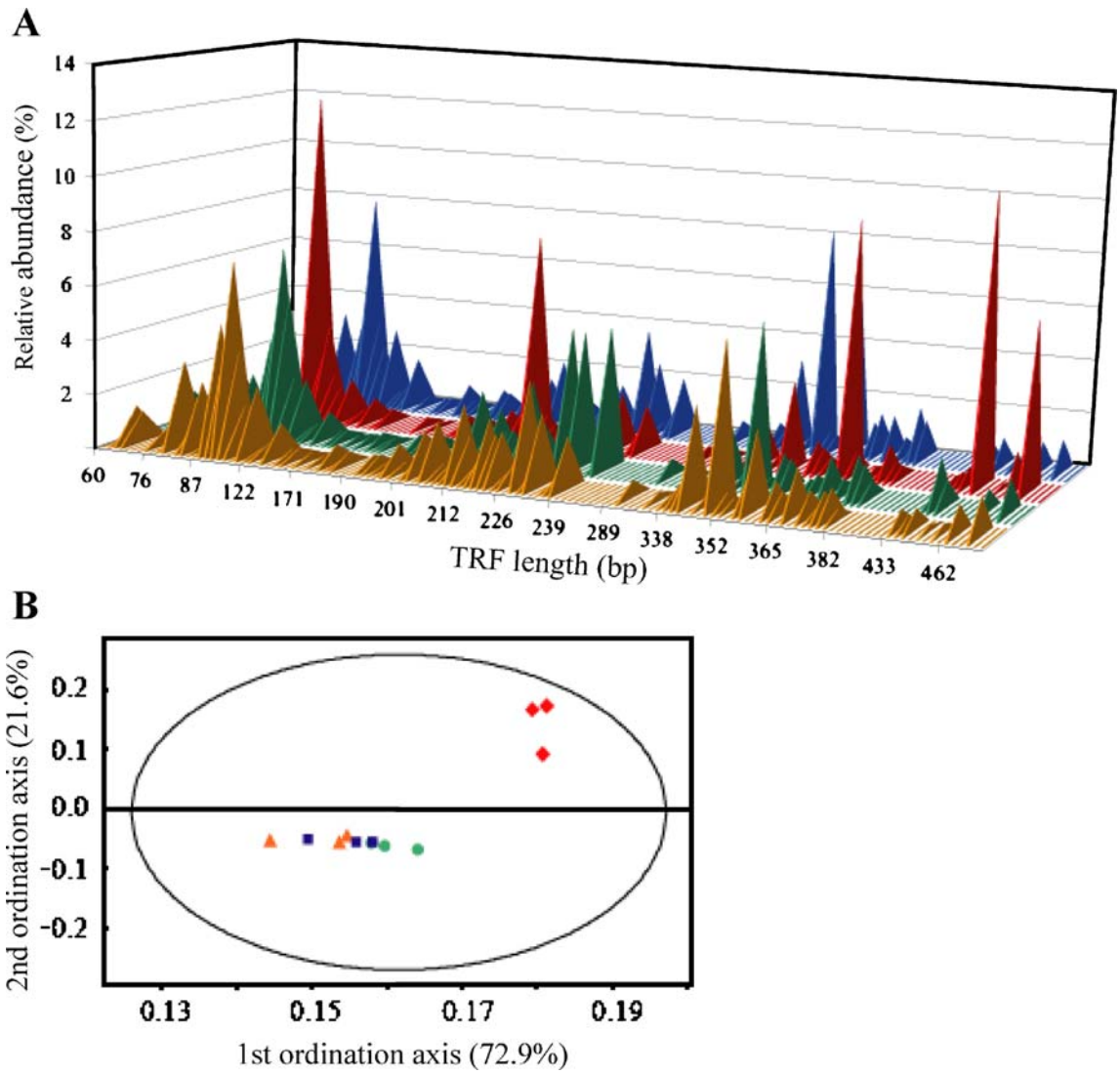

Figure 1. Impact of soil supplements on the total soil bacterial community assessed by T-RFLP. (A) Average relative abundances of TRFs (bp) obtained from soil samples supplemented with 2 mg chitin/g soil (red), 1.5 mg sodium acetate/g soil (green), 2.1×10^5 *Plasmodiophora* spores/g soil (orange) or untreated soil (blue) and incubated for 3 days. All results presented were generated from DNA digested with *Hha*I. Only TRFs represented in at least two replicates are shown. (B) Corresponding PCA plot of the T-RFLP data; with the same colors corresponding to treatments.

in the patterns of the control soil seemed to be higher than for the soils that were supplemented. Most remarkable was the emergence of two dominant bands (5 and 6) in the bacterial DGGE patterns after chitin treatment (Fig. 2A). These differentiating bands were excised, reamplified, and cloned. A total of 30 clones each from bands 5 and 6 (Fig. 2A) were screened by DGGE to identify clones having an identical electrophoretic mobility as the excised band. The four clones that had the same electrophoretic mobility as band 5 shared the same ARDRA patterns, whereas the two clones that resembled band 6 had two different ARDRA patterns. Four clones (clones 5.26, 5.28 comigrating with band 5; clones 6.15 and 6.30 comigrating with band 6) were sequenced and their sequences were compared to 16S rRNA gene sequences deposited in databases. The sequences of clones 5.26 and 5.28 showed the highest sequence similarity to some *Oerskovia* species (Table 1). For band 6, one clone (6.30) showed the highest similarity to a *Lentzea* species, whereas the other clone (6.15) was most similar to an uncultured *Betaproteobacterium*.

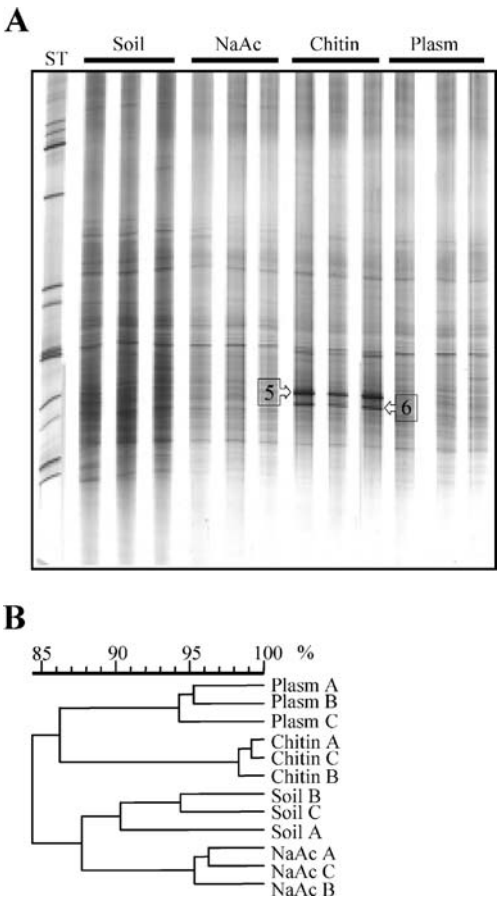

Figure 2. Impact of soil supplements on the total soil bacterial community assessed by DGGE. (A) DGGE fingerprint of soil supplementation experiment. ST: standard; 5 and 6: enriched/differentiating marked bands were cut out, cloned, and sequenced (Table 1). (B) Dendrogram generated by cluster analysis using UPGMA representing the similarity between the different treatments of the soil supplementation experiment. See Fig. 1 legend for details of treatments: soil, untreated control; NaAc, sodium acetate addition; Chitin, chitin addition; Plasm, Plasmodiophora spore addition. A, B and C refer to replicates.

To reduce the complexity of the DGGE banding patterns and to analyze specific taxonomic groups that are known to be frequently involved in antagonistic activity, *Pseudomonas* and actinobacterial patterns were generated. The actinobacterial banding patterns were still rather complex with up to five dominant bands (data not shown). Although UPGMA based on Pearson similarity indices indicated that the soil patterns formed a separate cluster, the similarity between all patterns was very high, reflected by 70% similarity in the cluster analysis (data not shown). Except for the enrichment of one band (corresponding to clone 4.35) in the chitin treatment, no effect of soil supplementation on the relative abundance of actinobacterial ribotypes was detected (data not shown). The sequence of clone 4.35 showed a rather low similarity with other sequences in the database (*Kitasatospora*: 91.2%; *Streptomyces*: 90.1%; Table 1).

In contrast to the actinobacterial fingerprints, the *Pseudomonas* patterns (Fig. 3A) were unique for each treatment. This finding was also supported by UPGMA analysis (Fig. 3B): the patterns of soils treated with chitin or *Plasmodiophora* both formed tight clusters that were clearly separated from the cluster of the control soil and two replicates of the sodium acetate-treated soil. The number of bands in the chitin-treated soil was reduced because of the increased abundance of three bands that were fainter or not observed in the patterns of the other treatments (Fig. 3A). The relative abundance of one ribotype seemed to be drastically increased upon chitin treatment (band 1). The two clones obtained with the same electrophoretic mobility as band 1 displayed identical ARDRA patterns. The partial 16S rRNA gene sequence showed the highest similarity to *Pseudomonas lutea* (Table 1). Band 2 (Fig. 3A) occurred in all DNA replicates of soil that received *Plasmodiophora* spores. Only three of the 15 clones screened by DGGE had the same electrophoretic mobility as this DGGE band and two ARDRA types were observed. One of the ARDRA types was sequenced, and it showed the highest sequence similarity to *Pseudomonas synxantha*, *P. rhodesiae*, *P. marginalis*, and *P. veronii* (Table 1).

Total Community in Plant-based Study

T-RFLP Results. The soil incubated in the greenhouse showed similar T-RFLP patterns for the total bacterial community as the soil incubated in the laboratory, regarding the number of TRFs (approx. 80) and a similar set of dominant TRFs (Figs. 1A and 4A). Some TRFs (60, 332, and 356) were unique to soil that was planted with

Table 1. Comparison of information obtained by DGGE and by T-RFLP

Treatment	DGGE primer (DGGE band/clone no.)	Identification based on clone sequence	Identity[a]	HhaI		MspI		HaeIII	
				In silico[b]	TRF (RA)	In silico[b]	TRF (RA)	In silico[b]	TRF (RA)
Chitin	Pseudomonas (1/1.1)	*Pseudomonas lutea*	0.995	208	207 (7.7%)	491	490 (2%)	40	o.r.
		Pseudomonas sp. A3YdB	0.995	209	207 (7.7%)	492	490 (2%)	39	o.r.
		Pseudomonas sp. SE7#1	0.995	207	207 (7.7%)	490	490 (2%)	39	o.r.
Plasmodiophora	Pseudomonas (2/2.15)[c]	*Pseudomonas synxantha*	0.960	207	207 (7.7%)	475	n.m.	39	o.r.
		Pseudomonas rhodesiae	0.960	207	207 (7.7%)	490	490 (2%)	39	o.r.
		Pseudomonas veronii	0.960	208	207 (7.7%)	491	490 (2%)	39	o.r.
		Pseudomonas marginalis	0.960	207	207 (7.7%)	490	490 (2%)	39	o.r.
Plasmodiophora and cabbage	Pseudomonas (3/3.14)[c]	*Pseudomonas synxantha*	0.995	207	207 (7.7%)	475	n.m.	39	o.r.
		Pseudomonas rhodesiae	0.995	207	207 (7.7%)	490	490 (2%)	39	o.r.
		Pseudomonas veronii	0.995	208	207 (7.7%)	491	490 (2%)	39	o.r.
		Pseudomonas marginalis	0.995	207	207 (7.7%)	490	490 (2%)	39	o.r.
Chitin	Actinobacteria (4/4.35)	*Kitasatospora phosalacinea*	0.912	440	440 (10.7%)	159	160 (3.1%)	224	223 (2.4%)
		Streptomyces sp. 65	0.901	467	466 (6.2%)	159	160 (3.1%)	224	223 (2.4%)
Chitin	Bacteria (5/5.26)	*Oerskovia enterophila*	0.998	370	n.m.	280	280 (3.7%)	473	n.m.
		Oerskovia jenensis	0.981	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
		Oerskovia paurometabola	0.981	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
		Oerskovia turbata	0.981	445	440 (10.7%)	132	130 (7.1%)	473	n.m.
		Cellulomonas sp.	0.981	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
Chitin	Bacteria (5/5.28)	*Oerskovia enterophila*	0.848	370	n.m.	280	280 (3.7%)	473	n.m.
		Oerskovia turbata	0.834	445	440 (10.7%)	132	130 (7.1%)	473	n.m.
		Oerskovia jenensis	0.834	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
		Oerskovia paurometabola	0.834	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
		Cellulomonas sp.	0.834	445	440 (10.7%)	132	130 (7.1%)	229	227 (4.4%)
Chitin	Bacteria (6/6.15)	Uncultured β-proteobacterium[d]	0.955	67	n.m.	141	141 (1.4%)	202	201 (1.4%)
		Burkholderia sp.	0.710	207	207 (7.7%)	141	141 (1.4%)	219	219 (3.5%)
Chitin	Bacteria (6/6.30)	*Lentzea albidocapillata*	0.909	440	440 (10.7%)	159	160 (3.1%)	67	n.m.
		Lentzea flaviverrucosa	0.909	n.s.		n.s.		n.s.	
		Lentzea sp. YM-11	0.909	n.s.		n.s.		n.s.	

RA: relative abundance of TRF; o.r.: out of range; n.m.: no good match; n.s.: no sequence or matching region in 16SrRNA for primer available.
[a]Similarity of cloned 16S rRNA gene sequence to nearest matching organism in database based on BLAST search.
[b]*In silico* digestions of sequenced clones from DGGE bands.
[c]Bands 2 and 3 from DGGE gels result from different treatments, but represent the same species.
[d]The 16S rRNA gene sequence from the NCBI database did not contain the T-RFLP primer region.

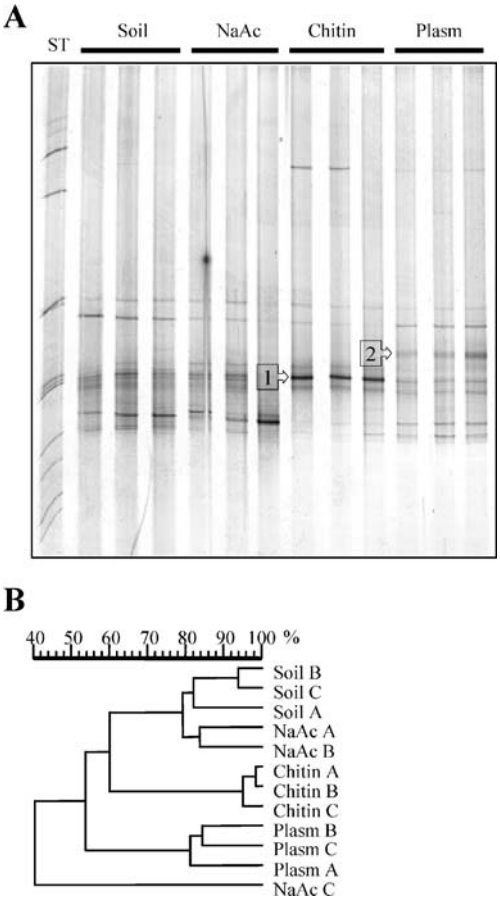

Figure 3. Impact of soil supplements on the *Pseudomonas* community assessed by DGGE. (A) DGGE fingerprint (*Pseudomonas* primers) of soil supplementation experiment. (B) ST: standard; 1 and 2: enriched/differentiating marked bands were cut out, cloned, and sequenced (Table 1). (B) Dendrogram generated by cluster analysis using UPGMA representing the similarity between the different treatments of the soil supplementation experiment. See Fig.1 legend for details of treatments: soil, untreated control; NaAc, sodium acetate addition; Chitin, chitin addition; Plasm, Plasmodiophora spore addition. A, B and C refer to replicates.

cabbage, with or without addition of *Plasmodiophora* spores (Fig. 4A). In addition, two TRFs in the planted soil, 61 and 63, increased 10-fold in their relative abundances compared to unplanted soil.

The total bacterial community structure in the soil planted with cabbage and inoculated with *Plasmodiophora* spores resembled that of the soil planted with cabbage that was not inoculated (Figs. 4A and B). Although several TRFs (approx. 10) were unique to the soil inoculated with *Plasmodiophora* spores, few had relative abundances above 0.5% (Fig. 4A).

DGGE Results. Samples from the greenhouse study were also analyzed by DGGE. Virtually no changes in the banding patterns were observed upon *Plasmodiophora* spore addition to soil planted with cabbage when using the bacterial (Fig. 5A) and actinobacterial (data not shown) primers. Although clusters (Fig. 5B) resembling the different treatments were obtained, overall the similarities between replicates and treatments were high.

In contrast, 4 days after the soil planted with cabbage had received *Plasmodiophora* spores, their *Pseudomonas* banding patterns on DGGE gels exhibited some differences from the controls planted with cabbage that did not receive spores (Fig. 6A). UPGMA analysis indicated that these treatments formed clearly separated clusters that shared only approximately 40% similarity (Fig. 6B). In all three replicates of the *Plasmodiophora*-treated soil planted with cabbage, a strong band (band 3) was observed with the same electrophoretic mobility as band 2 in the previous experiment when the soil was supplemented with *Plasmodiophora* (Figs. 3A and 6A). Again, this band (band 3) was excised, reamplified, and cloned. Fifteen clones were screened by DGGE, and one clone with identical electrophoretic mobility as band 3 (and 2 above) was obtained. The ARDRA pattern of this clone was identical to the clone (2.15) obtained from band 2 (Fig. 3A) and the sequence matched the same Pseudomonads as obtained from band 2 (Table 1).

Impact of Soil Supplements on Active Community. BrdU-labeled and immunocaptured DNA was analyzed by T-RFLP to ascertain the active fraction of the total bacterial community upon addition of different supplements to the soil. There was not sufficient BrdU-incorporated and extracted DNA to also perform DGGE analyses of the active profiles; hence, only T-RFLP data (*Hha*I restriction digestion) is presented. In this experiment, some peaks that were not highly represented in the total community profiles became much more dominant. For example, after chitin addition, TRFs 241, 440, and 466 became the most dominant in the active bacterial community profile (Fig. 7A). Of these, TRF 440 was the most dominant peak with a relative abundance of 31% compared to untreated soil, where it had a relative abundance of less than 1% in the active community. By contrast, TRFs 87, 207, and 364, which dominated the total bacterial community (Fig. 1A), were not present or were present at low relative abundances (less than 0.2%) in the active bacterial community upon chitin addition (Fig. 7A). The large differences in bacterial community structures after chitin addition for both the active and total communities were clearly seen in PCA plots of the T-RFLP data (Figs. 1B and 7B), and these differences were highly significant ($p < 0.001$).

When the soil was treated with sodium acetate, TRFs 241 and 467 increased compared to the control soil and became the most dominant TRFs in the active bacterial community (Fig. 7A). There were also increases in relative abundances of some other TRFs after this treatment, and the change in the active bacterial

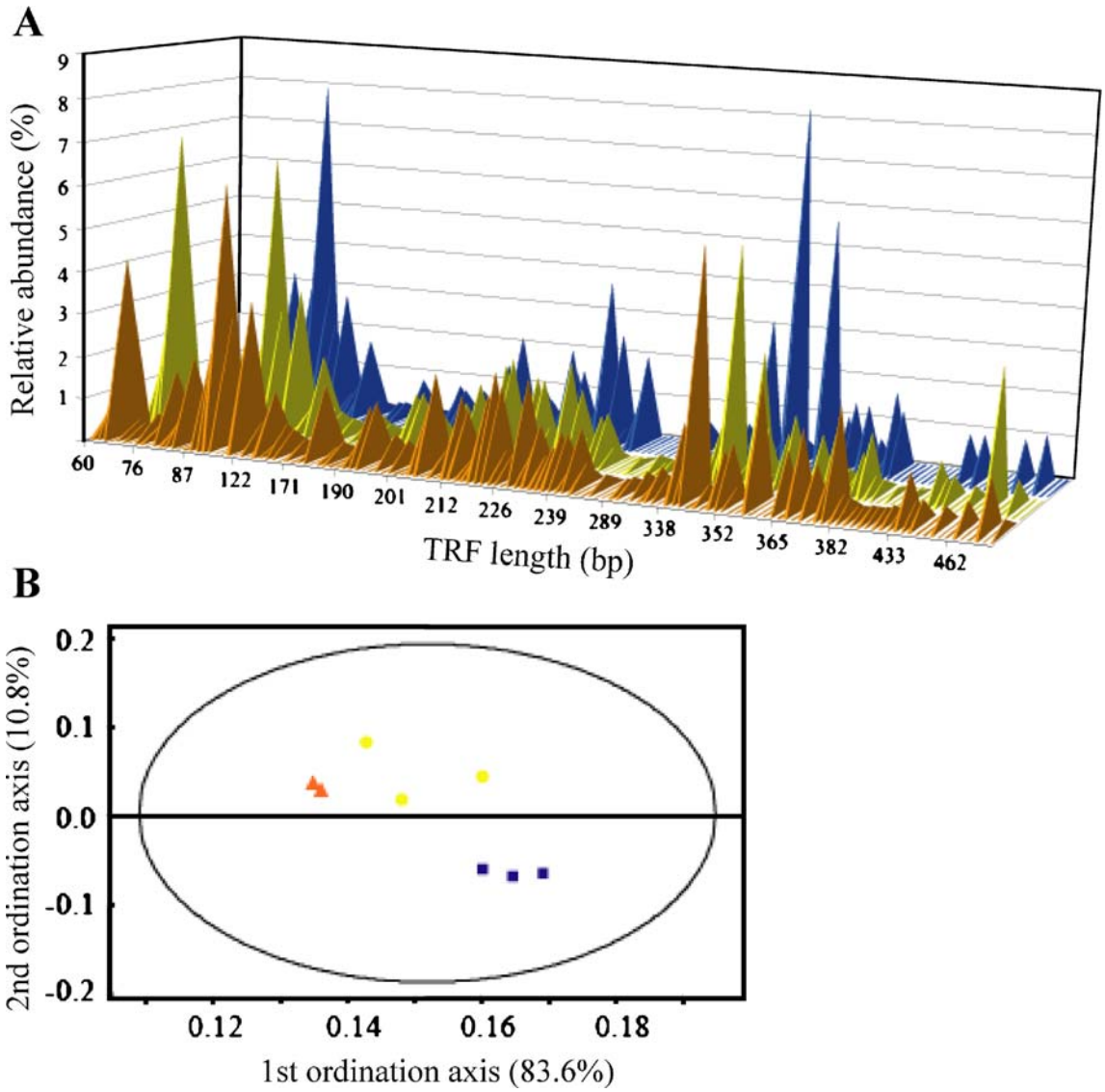

Figure 4. Impact of cabbage ± *Plasmodiophora* spore inoculation on the total soil bacterial community assessed by T-RFLP. (A) Average relative abundances of TRFs (bp) obtained from untreated soil (blue), soil planted with cabbage (yellow), or soil planted with cabbage and inoculated with 2.1×10^5 *Plasmodiophora* spores/g soil (orange). All results presented were generated from DNA digested with *Hha* I. Only TRFs represented in at least two replicates are shown. (B) Corresponding PCA plot of the T-RFLP data; with the same colors corresponding to treatments.

community structure after supplementation with sodium acetate was significant ($p < 0.01$) (Fig. 7B).

The active bacterial community structure of soil inoculated with *Plasmodiophora* spores showed several novel and increased TRFs that were not present in the total bacterial community (comparison of Figs. 1A and 7A). The only TRF in common in both the active and the total bacterial communities of soil inoculated with *Plasmodiophora* with a relative abundance over 1.5% was TRF 462, but this TRF was also present in the total community of untreated soil (Fig. 1A). The change in structure of the active bacterial community after addition of *Plasmodiophora* spores was significant ($p < 0.05$; Fig. 7B).

Active Community in Plant-based Study. In the greenhouse study, the most dominant active peak in soil planted with cabbage and inoculated with *Plasmodiophora* was TRF 241, as for all other experimental setups except for soil planted with cabbage alone (Fig. 8A), although this TRF was not dominant in the total bacterial communities from the same treatments (Fig. 4A). According to Fragsort and TAP analysis, this TRF could represent a species belonging to the *Bacillus* and/or *Paenibacillus* genera. In addition, both the total and the active bacterial communities planted with cabbage (with or without *Plasmodiophora*) had increased TRFs around 60 bp in length (Figs. 4A and 8A). Statistical analysis of the

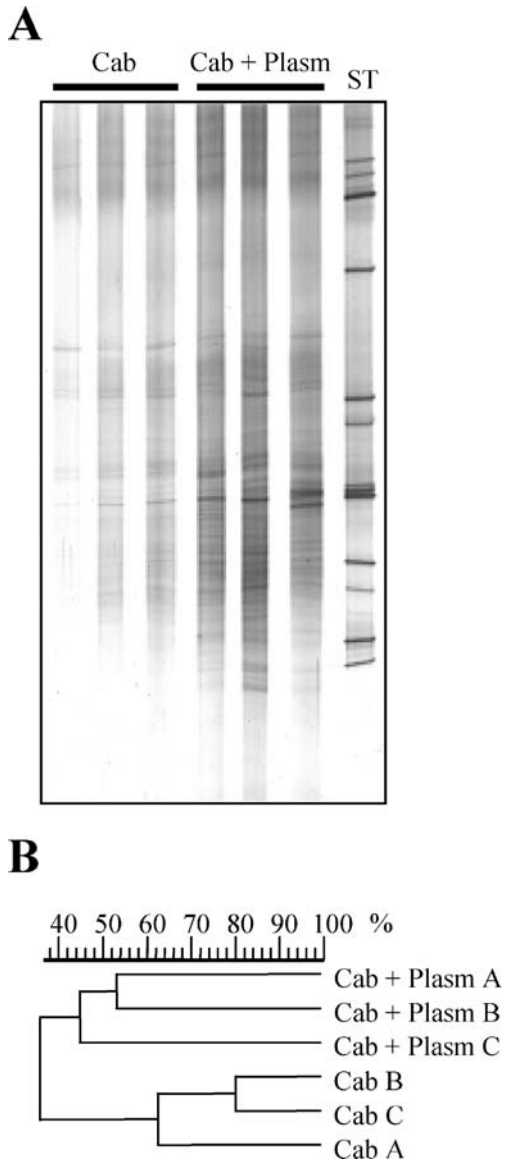

Figure 5. Impact of cabbage ± *Plasmodiophora* spore inoculation on the total bacterial community. (A) DGGE fingerprint of the greenhouse experiment. ST: standard. (B) Dendrogram generated by cluster analysis using UPGMA representing the similarity between the different treatments of the greenhouse experiment. See Fig. 4 legend for details of treatments: Cab, cabbage; Plasm, Plasmodiophora spores; A, B and C refer to replicates.

PCA plots showed that the active bacterial communities in soil planted with cabbage were significantly different ($p < 0.001$) from those in the unplanted soil (Fig. 8B).

After inoculation of *Plasmodiophora* spores to soil planted with cabbage, some novel TRFs emerged and some increased in relative abundance in the active community profile compared to uninoculated soil planted with cabbage (Fig. 8A). The difference in active community structure in soil planted with cabbage due to *Plasmodiophora* inoculation was highly significant ($p < 0.001$) in PCA plots of the T-RFLP data (Fig. 8B). This suggests that some bacteria were actively growing in response to the presence of *Plasmodiophora* spores in the soil in the presence of cabbage.

Coupling of DGGE and T-RFLP Results. Most of the sequenced 16S rRNA genes obtained from cloned DGGE bands could be matched to corresponding TRFs in the T-RFLP analyses. For example, almost identical matches were found in chitin-treated soil for the *Kitasatospora phosalacinea* sequence from a DGGE clone obtained using Actinobacteria primers to TRFs found in all three restriction enzyme profiles of the T-RFLP data

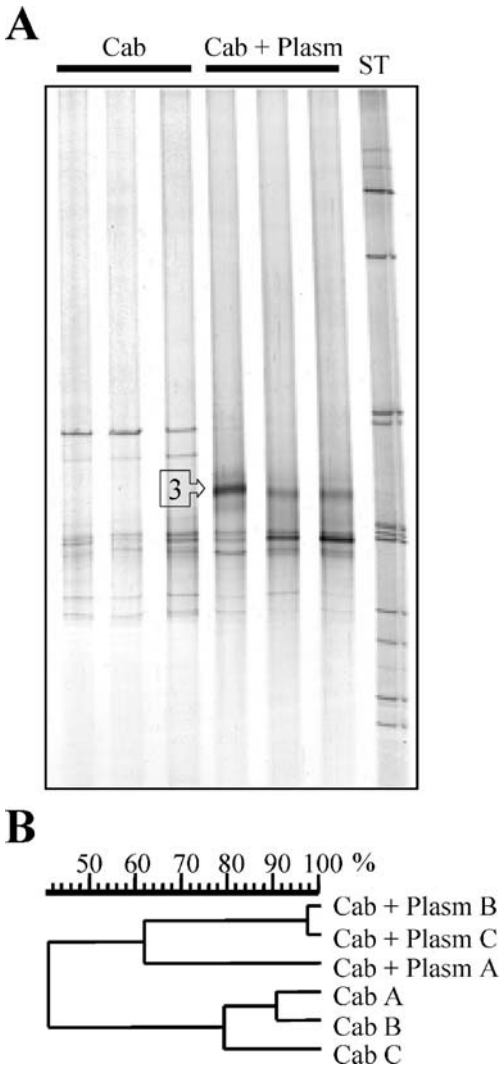

Figure 6. Impact of cabbage ± *Plasmodiophora* spore inoculation on the *Pseudomonas* community assessed by DGGE. (A) DGGE fingerprint (Pseudomonas primers) of the greenhouse experiment. (B) ST: standard; 3: enriched/differentiating marked band was cut out, cloned, and sequenced (Table 1). (B) Dendrogram generated by cluster analysis using UPGMA representing the similarity between the treatments of the greenhouse experiment. See Fig. 4 legend for details of treatments: Cab, cabbage; Plasm, Plasmodiophora spores; A, B and C refer to replicates.

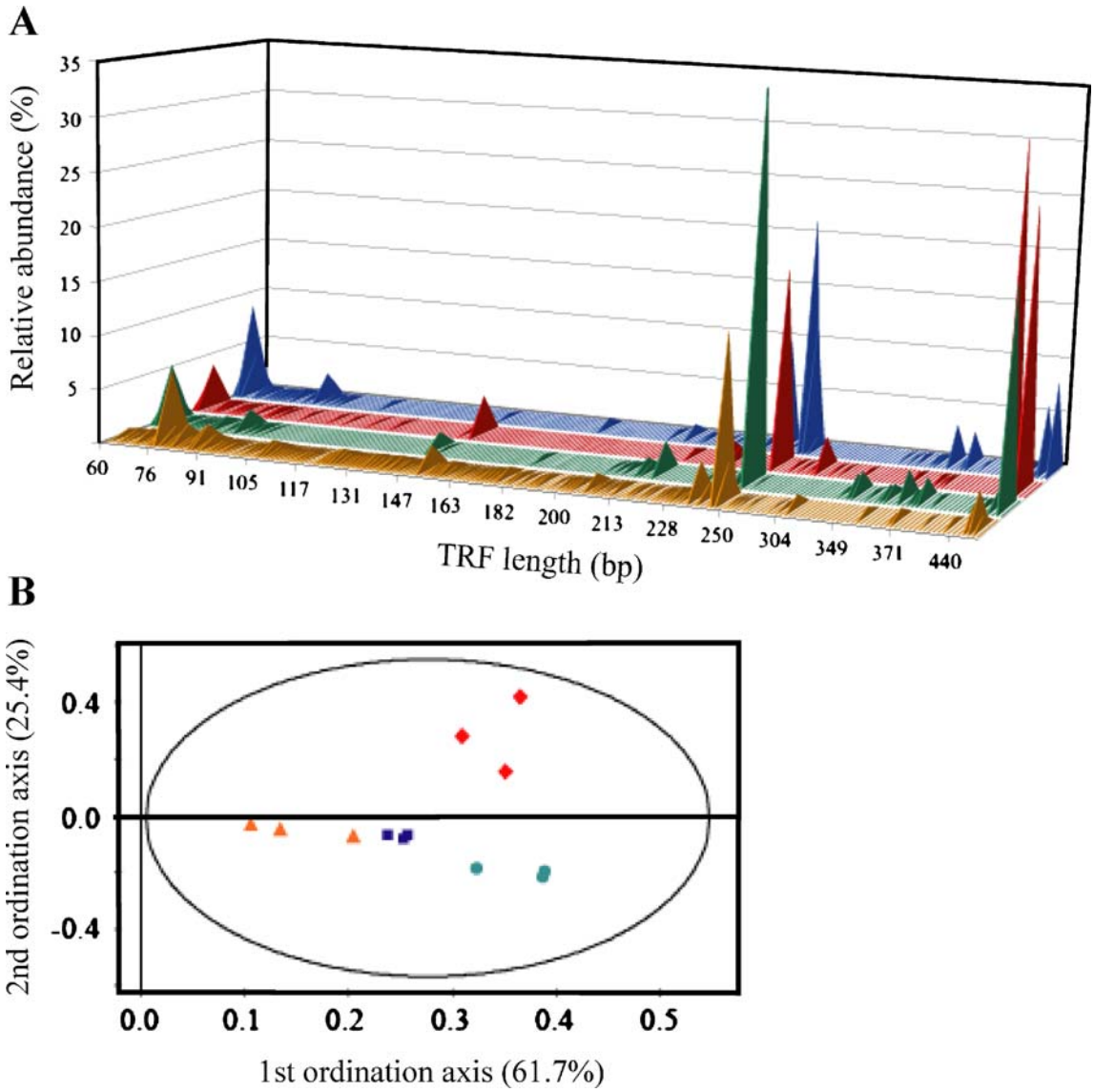

Figure 7. Impact of soil supplements on the active soil bacterial community assessed by BrdU immunocapture in combination with T-RFLP. (A) Average relative abundances of TRFs (bp) obtained from triplicate soil samples supplemented with 2 mg chitin/g soil (red), 1.5 mg sodium acetate/g soil (green), 2.1×10^5 *Plasmodiophora* spores/g soil (orange) or untreated soil (blue), and incubated for 3 days; followed by incubation with BrdU for 2 additional days. All results presented were generated from DNA digested with *Hha* I. Only TRFs represented in at least two replicates are shown. (B) Corresponding PCA plot of the BrdU-T-RFLP data; with the same colors corresponding to treatments.

(Table 1). In addition, the sequence of the clone corresponding to DGGE band 5 (Fig. 2A) from chitin-treated soil had the highest sequence similarity to species in the genus *Oerskovia*, and when digested *in silico* it could also be matched to dominant TRFs (±2 to 5 bp) for all three restriction enzymes used (Table 1). The sequence of clone 6.30 (closest similarity to *Lentzea* genus) had close matches *in silico* to TRFs from two of the three T-RFLP restriction digestions. However, *Lentzea albidocapillata* was the only *Lentzea* species of the three putative matches in the database that had a long enough 16S rRNA gene sequence deposited to analyze the fragmentation pattern from all restriction enzyme digestions *in silico*. The sequence of clone 6.15 matched several betaproteobacteria species in the database. Of these, a very good match to the T-RFLP data was obtained for an organism from the order *Burkholderiales* (Table 1).

We also attempted to match the sequences resulting from bands 1, 2, and 3 (*Pseudomonas* primers) to specific TRFs. By *in silico* digestions of the sequence data, corresponding TRFs could be found in the T-RFLP analyses typical of Pseudomonads (Table 1). Because of the location of primers for Pseudomonads within a well-conserved region of the 16S rRNA gene, it is not possible to separate the group of Pseudomonads by T-RFLP by using the most common restriction enzymes. In addition,

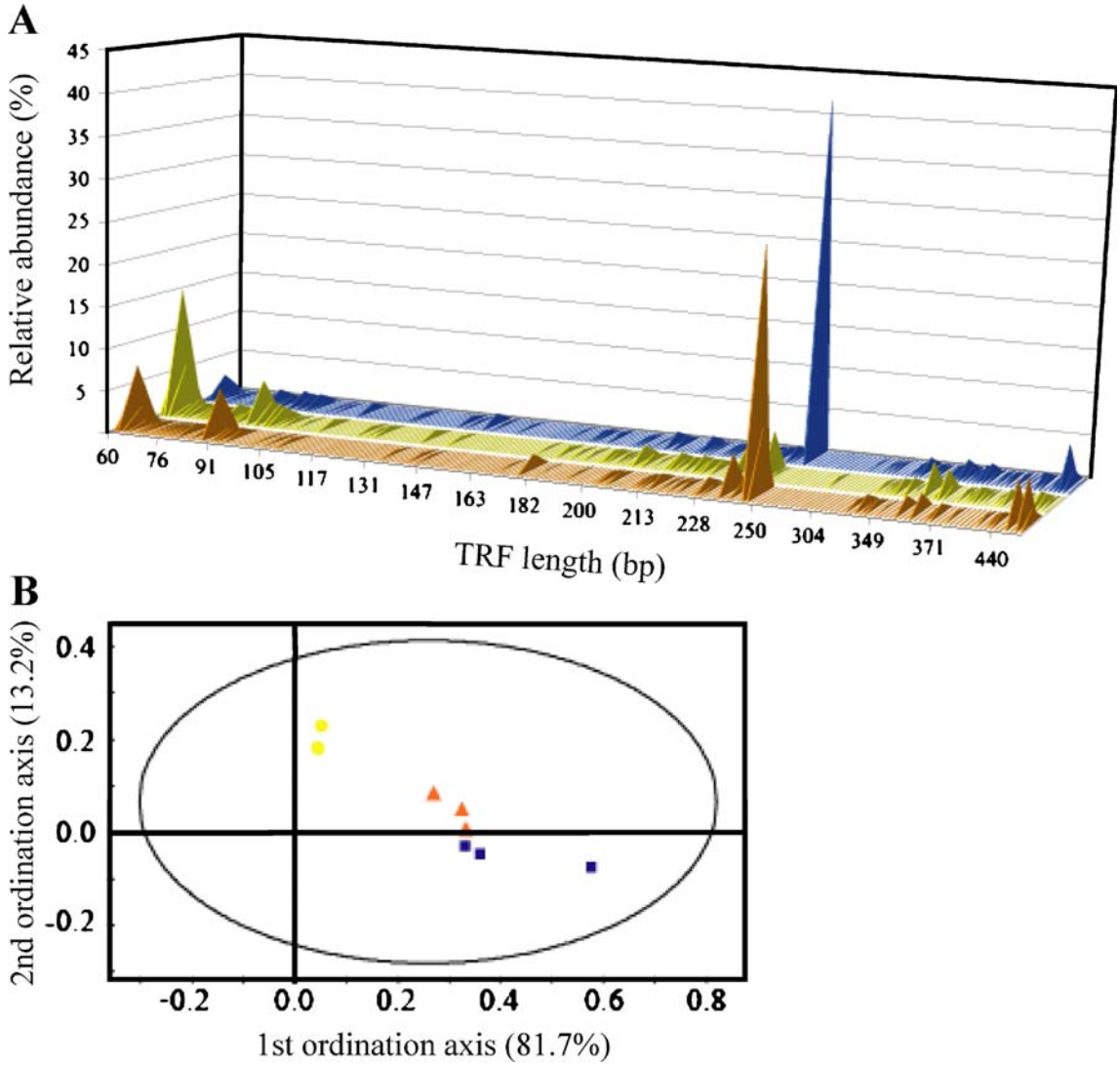

Figure 8. Impact of cabbage ± *Plasmodiophora* spore inoculation on the active soil bacterial community assessed by BrdU immunocapture in combination with T-RFLP. (A) Average relative abundances of TRFs (bp) obtained from triplicate soil samples planted with cabbage (yellow), planted with cabbage, and inoculated with 2.1×10^5 *Plasmodiophora* spores/g soil (orange), or untreated soil (blue). All results presented were generated from DNA digested with *Hha*I. Only TRFs represented in at least two of the replicates are shown. (B) Corresponding PCA plot of the BrdU-T-RFLP data; with the same colors corresponding to treatments.

with *Hae*III the TRF length for Pseudomonads is below the threshold range of T-RFLP with a length of 39 bp.

Based on the relative abundance values of the TRFs shown in Table 1, TRF 440 is more dominant in the *Hha*I restriction digestion when compared to the other two restriction digestions. One possible explanation for this high abundance of TRF 440 (*Hha*I) could be that it represents more than one organism having the same fragment length.

Discussion

We used a combination of molecular tools to investigate the complex structure of bacterial communities and their actively growing members in soil. This microbiomics approach allowed us to screen for bacterial populations in soil that were activated by specific supplements or the presence of a plant. We focused on a Swedish soil that was previously characterized as being suppressive to clubroot disease symptoms normally caused by *P. brassicae*. Although the Swedish soil was not extensively characterized regarding the factors contributing toward disease suppression, there are reports of other soils with a presumed biotic contribution toward suppression of *P. brassicae* [28].

Similar changes in community patterns according to treatment were detected by T-RFLP and DGGE approaches. Most significant changes in the soil bacterial community occurred after the addition of chitin. Populations that were stimulated by chitin addition

may benefit by their ability to use this compound as a carbon and/or a nitrogen source. Numerous bacteria are known to produce chitinases that catalyze the degradation of chitin to oligomers for the assimilation of carbon and nitrogen [12, 45]. Chitinase production has been identified as a mechanism for biocontrol against fungal phytopathogens [8, 9, 19] because chitin is the major component of numerous fungal cell walls. Chitin is also a major component (25%) of the *P. brassicae* spore cell wall [27], and therefore chitinase production could be contributing to suppression of this disease in the soil we sampled; however, other biological or abiotic factors could be the cause as well. In this study, we cannot be sure that chitin degraders are those that are responsible for suppressiveness in the studied soil. However, they may be at least partly responsible for suppression and are a first step toward identification of bacteria that grow in the presence of chitin and that may have chitinase genes.

Some of the increased TRFs and DGGE bands observed after chitin addition correlate to well-known producers of chitinases from the actinobacterial group (*Streptomyces*, *Kitasatospora*, *Oerskovia*, *Cellulomonas*, and *Lentzea* species) (Table 1). In a separate study, we isolated *Streptomyces mutamycini* from this soil, which was shown to have chitinase activity (Smalla, unpublished results). *In silico* digestion of the 16S rRNA gene sequences from this isolate matched TRF 440 that increased with chitin addition in this study. However, when *Streptomyces* sequences deposited in the RDP database were digested *in silico*, we did not find any that produced the 440 TRF fragment length, presumably because *S. mutamycini* sequences have not yet been deposited. In addition, it was difficult to find matches to *Oerskovia*, *Cellulomonas*, and *Lentzea* species in the databases because they are rarely deposited. Although there is very little known about the functional significance of these genera in soil, the approaches used in this study indicate that they respond favorably to the presence of chitin. It has previously been demonstrated that *Cellulomonas* and *Oerskovia* species can degrade chitin [35], and a cell wall degrading enzyme from *Oerskovia* sp. has also been published [23]. Therefore, our hypothesis is that these organisms were activated to grow in the studied soil when chitin was made available as a source of nutrients.

The combined data provide complementary clues that Actinobacteria are stimulated by chitin in our soil. This hypothesis is supported by previous findings that members of the Actinobacteria increased after chitin addition to soil [21, 25]. Chitin addition to soil microcosms and buried litterbags resulted in increasing numbers of actinobacteria, and large differences between the actinobacterial DGGE pattern compared to unamended control soil were found [21]. In another study, Metcalfe *et al.* [25] constructed clone libraries of chitinase genes from chitin containing litterbags in soil and found that actinobacterial-type chitinases were most dominant.

Several Pseudomonads have previously been identified as the causative agents of soil suppressiveness [24, 44], and we therefore also specifically focused on this group by using specific primers by DGGE. When *Pseudomonas*-specific primers were used, increases in some DGGE bands were observed for both the chitin and *Plasmodiophora* spore-treated soils. In addition, we found that some TRFs with closest matches to members of the *Pseudomonas* or *Burkholderia* groups were also dominant after chitin addition when using general primers for T-RFLP. Several Pseudomonads are also well known for chitinase [18, 37, 42] and proteinase production [3, 5, 20], and this could account for their increase in relative abundance when the chitin- and protein-rich *Plasmodiophora* spores were added, as—in addition to 25% chitin—the cell wall of the *Plasmodiophora* spore contains 34% proteins and 17.5% lipids that could also be used as nutrient sources [27]. Naturally, the increase in relative abundance of organism(s) represented by TRFs or DGGE bands could also be attributable to other, or to less specific, reasons as well.

Our results indicate that only a fraction of the total bacterial community members were detected as active cell populations in the different soil treatments. In fact, some of the dominant cell populations in the total community were hardly detectable in the active community. These populations might represent abundant dormant cells, cells with long generation times or cells that are dead but persistent against DNA degradation and/or lysis. There might also be a problem of assimilation of BrdU because not all cell types assimilate this compound at the same rate [41]. However, some recent reports have shown that representatives of a variety of different bacterial domains are at least capable of assimilation of BrdU [1, 13, 32].

Surprisingly, only few of the active bacterial populations matched dominant members of the total soil bacterial communities. For example, TRF 241 (*Bacillus* and/or *Paenibacillus*) was the most dominant TRF, with a relative abundance between 15% and 37%, in the active bacterial communities in soil treated with different supplements, or untreated soil (Figs. 7A and 8A), but this TRF did not display a high relative abundance in the total bacterial community (Fig. 1A). Artursson *et al.* [1] also found that the same TRF length increased in relative abundance in the presence of mycorrhizal spores from a different soil. These findings suggest that the bacteria represented by this TRF are more readily stimulated to be active compared to others in the community, or the cells are more efficient at incorporation of BrdU compared to other bacteria in soil.

By comparison to the total dominant community members, there was a lower diversity of active commu-

nity members, represented by fewer TRFs. This finding is substantiated by other studies that have also found a higher species diversity and richness in the total bacterial community by comparison to the active community members obtained by BrdU immunocapture [1, 47]. Using a different approach, Sessitsch *et al.* [36] found few TRFs by RT–PCR of 16S rRNA when they compared different RNA extractions from soils in combination with T-RFLP. Together, these results suggest that the number of active community members in soil is limited. By contrast, Girvan *et al.* [10] used rRNA/DGGE to detect active cell populations, and rRNA gene/DGGE and rRNA gene/T-RFLP to establish the total bacterial diversity and found only small differences between the total and the active bacterial communities. However, all methods based on RT–PCR from 16S rRNA generate a background of organisms that are not active but still contain ribosomes. Using the BrdU immunocapture approach, only DNA from growing organisms are analyzed, and no background from inactive cells could be detected in our experiments.

In conclusion, we have shown that a combination of DGGE, BrdU immunocapture, and T-RFLP enables detailed analysis of the architecture of soil microbial communities, including information about those members that are growing under specific conditions. This combination of molecular approaches should be applicable to the study of other complex microbial communities as well.

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