How elevated pCO_2 modifies total and metabolically active bacterial communities in the rhizosphere of two perennial grasses grown under field conditions

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Abstract

The response of total (DNA-based analysis) and active (RNA-based analysis) bacterial communities to a pCO_2 increase under field conditions was assessed using two perennial grasses: the nitrophilic *Lolium perenne* and the oligonitrophilic *Molinia coerulea*. PCR- and reverse transcriptase-PCR denaturing gradient gel electrophoresis analysis of 16S rRNA genes generated contrasting profiles. The pCO_2 increase influenced mainly the active and root-associated component of the bacterial community. Bacterial groups responsive to the pCO_2 increase were identified by sequencing of corresponding denaturing gradient gel electrophoresis bands. About 50% of retrieved sequences were affiliated to *Proteobacteria*. Our data suggest that *Actinobacteria* in soil and Myxococcales (*Deltaproteobacteria*) in root are stimulated under elevated pCO_2.

Introduction

Since the beginning of the industrial revolution, the atmospheric CO_2 concentration (pCO_2) has been rapidly increasing, affecting the global climate and the functioning of oceanic and terrestrial ecosystems (Bazzaz & Sombroek, 1999; Fuhrer, 2003). Much research has focused on the consequences of elevated pCO_2 on plant physiology and growth, as well as on vegetation structure. Elevated pCO_2 enhances the net photosynthesis, the shoot and root biomass, and the litter input relative to ambient pCO_2 condition (Sowerby *et al.*, 2000; Zak *et al.*, 2000; Ainsworth *et al.*, 2003), particularly in C3 plants (Long *et al.*, 2004).

Under current ambient atmospheric conditions, up to 50% of the assimilated carbon is translocated to the belowground (Kuzyakov & Domanski, 2000) providing carbon and energy sources easily available for soil biota. Under elevated pCO_2, greater input (Darrah, 1996) and qualitative changes (Hodge *et al.*, 1998) in carbon released into the rhizosphere are likely to impact the soil microflora (Jones *et al.*, 1998). For instance, the effects of a pCO_2 increase were described on arbuscular mycorrhizal fungi (Gamper *et al.*, 2004), on relative frequency (Marilley *et al.*, 1999) and on phenotypic structure of *Pseudomonas* (Roussel-Delif *et al.*, 2005; Tarnawski *et al.*, in press). Firstly, CO_2-induced alterations in carbon supply could modify microbial processes that are directly dependant on carbon input, particularly decomposition and nutrient cycling (Hu *et al.*, 1999). Secondly, elevated pCO_2 could alter the structure of the microbial community due to qualitative changes in carbon supply under these conditions. In turn, the selection or counterselection of plant-deleterious (Chakraborty *et al.*, 2000) or plant-beneficial microorganisms (Gamper *et al.*, 2004; Tarnawski *et al.*, in press) would have feedback effects on plant growth and physiology, because of a shift in microbial balance. In particular, this might enhance plant growth by increasing nutrient acquisition from previously unavailable pools (Hu *et al.*, 1999).

In order to understand how soil–plant systems respond to elevated pCO_2, the response of the microbial community has to be characterized and the populations involved in this response have to be identified. As most microbes are in an 'inactive' state in soils (Hu *et al.*, 1999), whole community parameters (i.e. DNA- and fatty acid-based analyses) are probably less sensitive than those measuring some

component of the active microflora. Recently developed molecular approaches such as stable isotope probing (Radajewski *et al.*, 2000) or RNA-based analysis (Felske & Akkermans, 1998; [19]Koizumi *et al.*, 2003) may be appropriate.

The development of molecular techniques in microbial ecology, including those based on the small subunit ribosomal RNA gene sequence as molecular marker, provides a significant advantage in studying microbial communities in terms of richness and structure, allowing monitoring of changes in microbial communities in a large number of samples (Muyzer *et al.*, 1993; [20]Fromin *et al.*, 2002). Molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) generate snapshots of the bacterial community, displayed as patterns related to the presence of dominant populations. Moreover 16S rRNA transcripts can be targeted. As the ribosome content of cells depends on their activity level, profiles obtained after reverse transcriptase (RT)-PCR on environmental 16S rRNA are therefore weighted according to the actual activity of the related populations (Wagner, 1994). By comparing DNA- and RNA-based profiles, it is then possible to highlight the dominant active members of the community (Felske *et al.*, 1998). These fingerprinting analyses generate a large amount of data, which should benefit from the development of numerical ecology (Legendre & Legendre, 1998; Fromin *et al.*, 2002). Ordination methods can be used to compare DGGE patterns with each other as multivariate responses to environmental variables (Ter Braak, 1986; Borcard *et al.*, 1992).

The aims of the present study were to investigate whether elevated pCO_2 influence the bacterial community and to highlight and identify the most affected populations and their potential metabolic role in these soils. The response of soil and root-associated microflora to high atmospheric pCO_2 content was assessed by molecular fingerprinting of total and active bacterial communities. Two hemicryptophytic perennial grasses were used as model plants: *Lolium perenne* and *Molinia coerulea*. These plants have different trophic requirements (nitrophilic and oligonitrophilic Vazquez de Aldana & Berendse, 1997), allowing testing of the importance of the functional type regarding the plant response to an elevation of atmospheric pCO_2 (Lüscher *et al.*, 1998). A shift in the bacterial community structure under high pCO_2 was revealed by DGGE profiling after direct PCR (total) and RT-PCR (active community) amplification of 16S rRNA genes from soil and root samples. Multivariate statistical analyses were used to highlight responsive bacterial groups.

Materials and methods

Study site and plant material

Lolium perenne and *Molinia coerulea* were grown under field conditions and current ambient (36 Pa, C for control) vs. elevated pCO_2 (60 Pa, T for treated) in the Free Air CO_2 Enrichment (FACE) facilities at Eschikon, Switzerland (Hebeisen *et al.*, 1997). The atmosphere of treated plots was enriched with CO_2 during the growing season and daytime only. *Lolium perenne* cv Bastion (L.) was grown as a monoculture on three control (LC) and three CO_2-treated (LT) replicate plots from May 1993. The plants were grown on the autochthonous soil, a fertile Eutric Cambisol (FAO classification). The shoots were harvested four times a year. The LC and LT plots received $14\,g\,m^{-2}\,year^{-1}$ N as NH_4NO_3, at the beginning of the season, and then after each cut, except the last (this amount was demonstrated to be limiting for plant growth during the FACE experiment; Daepp *et al.*, 2000). *Molinia coerulea* plants (M) originated from a littoral meadow on the south shore of Lake Neuchâtel (Cudrefin, Switzerland). The local soil, a Gleysol, Typic Haplaquoll, contained about 4.7% clay, 9.5% silt and 85.8% sand, with a pH[H_2O] value of 8.4 (Hamelin *et al.*, 2002). Plants with undisturbed root systems were taken with their surrounding and underlying soil, and transferred to the FACE facilities in September 1999. About $0.7\,m^2$ of littoral meadow with reconstructed soil profile below the root horizon (total depth: 35 cm) was installed in one control plot (MC) and one CO_2-treated plot (MT). The plants were neither cut nor fertilized.

Sampling

Sampling was performed at three sampling dates (21 June 2001, 7 May 2002 and 15 July 2002). Two subsamples were collected, one for DNA extraction and another for RNA extraction (RNase-free sampling conditions). For *L. perenne*, one to three control (C) and CO_2-treated (T) plots were sampled at each date (Table 1). For *M. coerulea*, only technical replicates could be sampled as only one plot per pCO_2 condition was available. Sampling of *L. perenne* was always performed just before a cut. For each sampling, three soil cores (about 3 cm diameter, 10–12 cm depth) including root systems, were taken and pooled for analysis. Two fractions were recovered: the non-adhering soil, obtained by shaking roots (S) and the root itself (R, for rhizoplane-endorhizosphere) after thorough washing of root-adhering soil. Soil and root samples were immediately placed in FastRNA™ matrix tubes for RNA and FastDNA™ matrix tubes for DNA (Bio101, QBiogene, Inc., Basel, Switzerland) and instantaneously frozen in liquid nitrogen.

DNA extraction and purification

DNA extraction and purification were performed on about 0.5 g of fresh root or soil material. A bead-beating apparatus (FP120 FastPrep™ cell disruptor, Savant Instruments, Inc.,

Table 1. DNA and RNA yields ($\mu g\, g^{-1}$ soil or root fresh weight) obtained from soil and root samples of *Lolium perenne* and *Molinia coerulea* growing under ambient or elevated pCO_2 content

				Average yield ± SD (no. replicates)	
Sampling date	pCO_2 treatment	Type of sample	Nucleic acid type	*Lolium perenne*	*Molinia coerulea*
21 June 2001	Control	Soil	DNA	2.5	6.2
			RNA	14.1	9.2
		Root	DNA	3.8	2.9
			RNA	7.8 ± 0.5 (3)	8.8
	Treated	Soil	DNA	6.7	4.0
			RNA	6.5	4.7
		Root	DNA	5.9	7.5
			RNA	8.5 ± 0.2 (3)	7.4
7 May 2002	Control	Soil	DNA	10.3	4.4
			RNA	8.0 ± 0.8 (2)	9.8 ± 5.7 (2)
		Root	DNA	12.3	8.4
			RNA	29.2	10.5 ± 0.7 (2)
	Treated	Soil	DNA	10.8	5.1
			RNA	8.9 ± 7.5 (2)	7.9 ± 2.4 (2)
		Root	DNA	4.2	10.2
			RNA	28.8 ± 3.2 (2)	9.8 ± 1.5 (2)
15 July 2002	Control	Soil	DNA	6.2 ± 1.0 (3)	5.8 ± 1.2 (2)
			RNA	8.3 ± 1.5 (3)	7.2 ± 0.2 (2)
		Root	DNA	6.3 ± 2.8 (3)	7.6 ± 1.0 (2)
			RNA	9.3 ± 1.4 (3)	8.9 ± 0.2 (2)
	Treated	Soil	DNA	3.3 ± 0.8 (3)	6.6 ± 0.4 (2)
			RNA	6.9 ± 3.0 (3)	6.1 ± 2.3 (2)
		Root	DNA	7.7 ± 1.4 (3)	8.2 ± 1.8 (2)
			RNA	5.6 ± 1.2 (3)	11.7 ± 2.1 (2)

Standard deviation (± SD) is indicated when replications were performed. The number of replicates is indicated in parentheses.

Hotbrook, NY) was used in combination with the FastDNA Spin Kit for Soil (Bio101) according to Borneman *et al.* (1996), except that 500 μL of DNA lysate were purified using 500 μL of Binding Matrix (Bio101). The final DNA extracts were quantified using GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Cambridge, UK) and stored at −20 °C before use.

RNA extraction and purification

From sampling until cDNA synthesis, all RNA handling was performed under RNase-free conditions. Aqueous solutions were treated with 0.1% diethyl pyrocarbonate (DEPC). Glassware was heated to 200 °C overnight and plastic material soaked overnight in a 0.1 N NaOH/1 mM EDTA solution, before rinsing with RNase-free water. The working area and materials reserved for RNA handling were treated with RNase-AWAY solution (Molecular BioProducts Inc., San Diego, CA).

Total RNA was extracted and purified using a combination of FastRNA™ tubes with Green Caps (Bio101) and RNeasy® Plant Kit (Qiagen AG, Basel, Switzerland). The samples were put on ice between the extraction steps. In each FastRNA™ tube containing about 150–500 mg of frozen sample, 450 μL of RLT Buffer (Qiagen) were added. The mixture was shaken for 10 s at $6\, m\, s^{-1}$ using the FastPrep™ cell disruptor. This step was repeated once after cooling tubes for 5 min on ice. Borneman & Triplett (1997) found this 20-s period of bead beating to be optimal for maximum cell lysis and minimum RNA shearing. The tubes were then centrifuged for 5 min at 13000 ***g*** and the supernatant was loaded on QIAshredder Spin Columns (Qiagen) and then processed as recommended by the manufacturer. DNA was removed using DNase (Qiagen) according to the manufacturer's protocol. The final RNA extracts were eluted in 100 μL 10-mM Tris pH 7.0, quantified using GeneQuant (Amersham Pharmacia), and stored at −80 °C before use. PCR amplification and DGGE were performed directly on each RNA extract to detect DNA contamination. In a few cases the presence of DNA was detected in the RNA extract, in which case the corresponding band positions were then discarded for further analysis.

Reverse transcription of total RNA

Reverse transcription reactions were performed using Im-Prom-II™ Reverse Transcription System (Promega Corp.,

Madison, WI) with random hexamer primers in a thermocycler model PTC-200 (MJ Research Inc., Watertown, MA). A total of 3.5 μL of RNA extract (55–70 ng depending on the sample) was mixed with 1 μL of primers (10 mM), and 0.5 μL of RNasin® Ribonuclease Inhibitor. This mixture was incubated at 70 °C for 5 min for an optimal contact between RNA and primers, and chilled on ice until the reverse transcription mix was added. This mix was then combined with (final concentrations) 1× ImProm-II™ Reaction Buffer, 0.05 U μL^{-1} RNasin, 6 mM $MgCl_2$, 0.5 mM each dNTP, 5% (v/v) ImProm-II™ Reverse Transcriptase and DEPC-treated nanopure water in a final volume of 20 μL. The reaction consisted of annealing at 25 °C for 5 min, extension at 42 °C for 1 h and inactivation of reverse transcriptase at 70 °C for 15 min. The resulting cDNA was used immediately for PCR or stored at −20 °C. Positive and negative control reactions were performed as recommended by the manufacturer.

PCR amplification

PCR amplification of the V3 region of 16S rRNA gene was performed in two steps. The whole 16S rRNA gene was first amplified using the forward GM3f (5′-AGAGTTTGATCMTGGC-3′) and the reverse GM4r (5′-TACCTTGTTACGACTT-3′) *Bacteria* primers (Muyzer & Ramsing, 1995). The PCR reaction mix contained (final concentrations) 1X Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 μM of each primer (MWG Biotech AG, Ebersberg, Germany), and 0.05 U μL^{-1} of Taq DNA polymerase (Promega). A total of 2 μL of DNA or cDNA extract were added as template for the PCR. The final reaction volume was adjusted to 20 μL. The reaction mixtures were subjected to 26 amplification cycles in a thermo-cycler. The first heat denaturation step was performed at 94 °C for 4 min 30 s. Cycles consisted of heat denaturation at 94 °C for 1 min, primer annealing at 56 °C for 30 s with a touchdown of 1 °C every 2 cycles for a total of ten cycles, and extension at 74 °C for 1 min. The mixture was maintained at 74 °C for 10 min for the final extension. The forward 338f (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse 520r (5′-ATTACCGCGGCTGCTGG-3′) universal primers (Ovreas *et al.*, 1997) were used for nested amplification of the V3 region of the 16S rRNA gene to increase the amplification yield and to obtain a fragment size suitable for DGGE analysis. A 40-bp GC-clamp (Muyzer *et al.*, 1993) was added on the forward primer for DGGE analysis. The nested-PCR mix was prepared as for the first PCR except 5 μL of PCR-amplified 16S rRNA were added as template and the final volume was adjusted to 50 μL. The first heat denaturation step was performed at 94 °C for 5 min. The reaction mixtures were then subjected to 31 amplification cycles. Cycles consisted of heat denaturation at 94 °C for 1 min, primer annealing at 65 °C for 30 s with a touchdown of 1 °C per cycle for ten cycles, and extension at 74 °C for 1 min. The mixture was maintained at 74 °C for 10 min for the final extension. The PCR products were checked for size and yield on 1% agarose gels in comparison to the Low DNA Mass Ladder (Invitrogen).

DGGE analysis

Denaturing gradient gel electrophoresis analysis of 16S rRNA genes and cDNA amplicons was performed using the D-code electrophoresis system (Bio-Rad Inc., Hercules, CA). About 600–800 ng of PCR products were loaded directly on a 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide 37.5 : 1) with a linear gradient from 30% to 60% denaturants (100% correspond to 40% formamide plus 7 M urea). The strains used to build the reference DGGE pattern were ordered as follows after migration (Fig. 1): *Pseudomonas fluorescens* ATCC 27663, *Bacillus subtilis* ATCC 14893, *Flavobacterium capsulatum* DSM 30196, *Rhizobium meliloti* DSM 1981, *Arthrobacter globiformis* DSM 20124 and *Thermus filiformis* NCIMB 12588. The gels were run at 60 °C and 150 V for 5 h in 1× TAE buffer. They were stained with

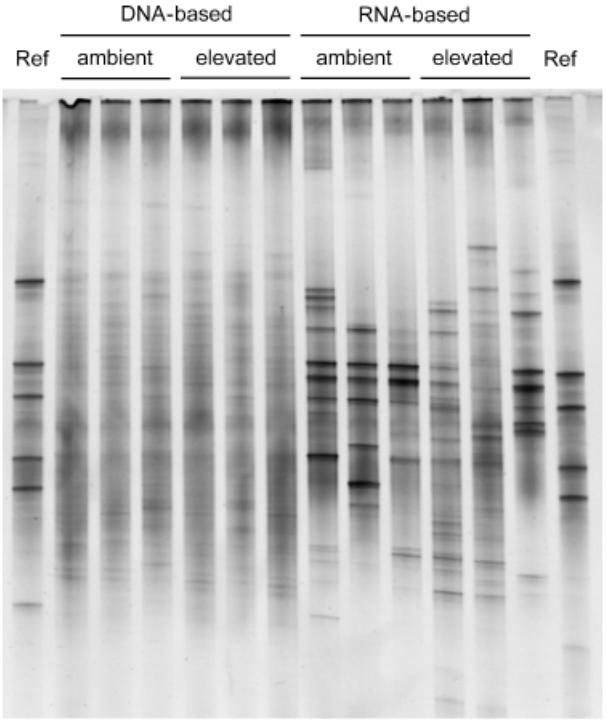

Fig. 1. Example of 16S rRNA gene-based denaturing gradient gel electrophoresis profiles obtained from soil of *Lolium perenne* plots cultivated under ambient and elevated pCO_2. The first six patterns are rDNA-based profiles and the last six are rRNA-based profiles from three replicate plots for each pCO_2 condition for the third sampling date. Ref stands for the reference pattern.

0.01% SYBR Green (Molecular Probes, Leiden, the Netherlands) in 1× TAE at 4 °C in the dark for 20 min, then UV photographed with the Multi-Analyst package (Bio-Rad).

Data handling and statistical analysis of DGGE patterns

The gel images were normalized regarding the band positions of the reference patterns for each gel, then the sample patterns were compared using GelCompar software (Applied Maths, Kortrijk, Belgium). The fingerprints obtained were codified in terms of migration length and relative intensity (p_i) of each band (i) within the profile (total intensity of the profile $\Sigma p_i = 1$). Two datasets were obtained: (1) DNA data matrix, containing relative intensity of bands for DNA-based profiles and (2) RNA data matrix containing relative intensity of bands for RNA-based profiles. For both plants, co-amplification of amyloplastic DNA and RNA was often observed for root samples (the position of plant 16S rRNA fragment after DGGE analysis was checked using plant axenic DNA extract as PCR template). Corresponding bands were discarded for further analyses.

The percentage of similarity between DNA- and RNA-based profiles obtained from both pCO_2 conditions (ambient/treated) was calculated using the Steinhaus coefficient. It was computed from the relative intensity of DGGE bands (set between 0 to 1), by the sum of the lower frequency observed for each pair of common bands (Legendre & Legendre, 1998). It gives more weight to intense common bands than to weak common bands. All calculations were done using the R 1.9.0 package (R Development Core Team, 2004).

The data matrices were then transformed for ordination analyses. Bands appearing only in one DNA- or RNA-based profile were discarded from dataset due to their low representation within profiles to avoid a rare species effect. Band intensities from the remaining data (64 bands for RNA matrix and 55 bands for DNA matrix) were normalized using the log-transformation: $p_i' = \ln(p_i + 1)$.

Canonical Correspondence Analysis (CCA) was applied on both transformed data matrices to represent the influence of explanatory variables on the one hand, and to evidence the most 'impacted' bands on the other hand. Data were initially submitted to variation partitioning analysis (Borcard *et al.*, 1992) using a series of partial CCA to display variability of the patterns constrained by factors of interest. Three sets of explanatory variables were employed: (1) sampling date (environmental conditions at sampling time) and replication; (2) plant–soil system (*M. coerulea*/*L. perenne*); and (3) root presence combined with pCO_2 treatment (ambient/elevated). The significance of the results was tested with the Monte Carlo permutation test. The whole process was based on computations made with R 1.9.1 (R Development Core Team, 2004). In a second step, in order to focus on the effects of pCO_2 and roots on DGGE profile variability, part of the variability explained by sampling date and replication was subtracted by using these as co-variables for partial CCA. Data obtained from *L. perenne* and from *M. coerulea* were separated to distinguish key DGGE bands for each plant–soil system.

Selection of indicative bands

Various bands were selected according to the number of profiles in which they were detected (frequent bands were preferred to rare ones) and their position on CCA plots (bands distant from the graphic axes origin were preferred to others). Among these bands, we considered that those which were close to the centroïds of the explanatory variables corresponded to indicative populations that were most influenced by these factors. Five indicative bands (corresponding to position 225 for *L. perenne* samples and 151, 224, 246 and 249 for *M. coerulea* samples; Table 2) were excised from different DGGE patterns to check the corresponding sequence homogeneity.

Identification of the responsive populations

Selected DGGE bands were excised. Corresponding DNA was eluted overnight at 4 °C with 15 µL nuclease-free water and the electrophoresis – excision – elution cycle was repeated to ensure that the recovered DNA corresponded to a given DGGE band. Then the recovered DNA was amplified with a 338f – 520r primer set using 15 amplification cycles (heat denaturation at 94 °C for 1 min, primer annealing at 65 °C for 30 s and extension at 74 °C for 1 min). PCR products were purified and cloned into pGEM-T and introduced into *Escherichia coli* XL1 by electroporation. The sequence homogeneity within a single DGGE band was checked on obtained clones with *Hae*III restriction patterns of T7-SP6 PCR amplicons. For each band, one clone was sequenced for each restriction profile type. The phylogenetic affiliation of corresponding organisms was achieved by BLAST analysis (Altschul *et al.*, 1997). Sequences were deposited at EMBL under the accession numbers AJ851090 to AJ851151.

Results

Extraction yields and extracts purity

The average DNA and RNA extraction yields were respectively 6.0 (SD ± 2.5) and 8.1 (SD ± 2.4) µg g^{-1} fresh weight for soil and 7.1 (SD ± 2.7) and 12.2 (SD ± 8) µg g^{-1} fresh weight for root samples (Table 1). The quality and quantity of the extracts were always sufficient for PCR and RT-PCR reactions, irrespective of the plant-soil system and the pCO_2 treatment.

Table 2. Characterisation of bands obtained from 20 and 11 DNA- and 39 and 27 RNA-based denaturing gradient gel electrophoresis (DGGE) profiles for *Lolium perenne* and *Myxococcales coerulea* respectively. The bands were selected for their variation in intensity within profiles according to the origin of the sample, to the pCO_2 content and to the nucleic acid type. The total length of the DGGE gel corresponds to 500 pixels. The representativity of a given band was expressed as the percentage of DNA- or RNA-based profiles in which the band was detected. Changes in band intensities induced by pCO_2 elevation are expressed as increase (+%) or decrease (−%) in band intensity. For each band, several clones were checked, the number of clone affiliated to the same restriction type is mentioned in parentheses. One clone of each type has been sequenced, accession numbers of the sequences are indicated after the affiliation group. An asterisk was added next to the DGGE band name when the sequences was retrieved from RNA profile.

DGGE band description							Corresponding populations	
			Representativity among profiles (%)		Effect of pCO_2 elevation on band relative intensities (%)		Closest phylogenetic group (affiliation based on Blast)	
Name	Position (pixels)	Origin	DNA	RNA	Root	Soil	Restriction type A	Restriction type B
Lolium perenne								
L1*	101	Soil	0	13	−100	+100	*Gammaprot.*/Pseudomonadales (4/8); AJ851090	Actinobacteria (4/8); AJ851091
L2*	112	Root	5	18	−100	U	unaffiliated (3/3); AJ851092	
L3	128	Root	20	28	−11	+100	Bacteroidetes (7/7); AJ851093	
L4	133	Root	40	41	+82	–15	*Betaprot.*/Burkholderiales (1/4); AJ851094	*Gammaprot.*/Enterobacteriales (3/4); AJ851095
L5*	164	Root	20	15	+81	+444	*Betaprot.*/Burkholderiales (3/8); AJ851096	*Deltaprot.*/Myxococcales (5/8); AJ851097
L6*	168	Root	20	36	+35	−3	*Gammaprot.*/Enterobacteriales (1/8); AJ851098	*Deltaprot.*/Myxococcales (7/8); AJ851099
L7	179	Soil	20	18	−13	+12	Bacteroidetes (2/7); AJ851100	Bacteroidetes (5/7); AJ851101
L8*	190	Root	25	26	−86	+48	*Alphaprot.*/Rhizobiales (2/5); AJ851102	*Deltaprot.*/Myxococcales (3/5); AJ851103
L9	225	Soil	50	62	+136	+1021	unaffiliated (4/4); AJ851104	
L10*	225	Root	50	62	+136	+1021	unaffiliated (8/8); AJ851105	
L11*	225	Soil	50	62	+136	+1021	Actinobacteria (6/6); AJ851106	
L12*	225	Root	50	62	+136	+1021	*Alphaprot.* (8/8); AJ851107	
L13*	231	Root	15	26	+1264	−1	*Deltaprot.*/Myxococcales (5/5); AJ851108	
L14*	237	Soil	25	33	+197	+100	Actinobacteria (1/4); AJ851109	unaffiliated (3/4); AJ851110
L15*	290	Root	25	36	−54	+4	*Deltaprot.*/Desulfurellales (3/6); AJ851111	*Deltaprot.*/Myxococcales (3/6); AJ851112
L16	293	Soil	30	13	+74	–58	unaffiliated (1/8); AJ851113	Actinobacteria (7/8); AJ851114
L17	298	Soil	5	8	U	–56	unaffiliated (1/3); AJ851115	*Alphaprot.* (2/3); AJ851116
Myxococcales coerulea								
M1*	98	Soil	0	11	U	+96	Bacteroidetes (3/8); AJ851117	unaffiliated (5/8); AJ851118
M2	140	Root	46	11	+82	+317	unaffiliated (4/4); AJ851119	
M3	145	Root	36	44	+61	+32	unaffiliated (1/8); AJ851120	Actinobacteria (7/8); AJ851121
M4	151	Root	73	37	+105	−66	*Gammaprot.*/Enterobacteriales (1/5); AJ851122	Firmicute/Bacillales (4/5); AJ851123
M5*	151	Root	73	37	+105	−66	*Gammaprot.*/Enterobacteriales (2/3); AJ851125	*Deltaprot.*/Myxococcales (1/3); AJ851124
M6*	172	Soil	55	44	+121	+297	unaffiliated (3/8); AJ851126	*Betaprot.*/Burkholderiales (5/8); AJ851127
M7*	207	Soil	9	26	−100	−38	*Betaprot.*/Burkholderiales (3/5); AJ851128	*Betaprot.*/Burkholderiales (2/5); AJ851129
M8*	224	Soil	55	41	+635	−70	*Betaprot.*/Burkholderiales (3/8); AJ851130	Actinobacteria (5/8); AJ851131
M9*	224	Root	55	41	+635	−70	Bacteroidetes (6/8); AJ851132	unaffiliated (2/8); AJ851133
M10*	233	Soil	27	41	+4	−53	Firmicutes (1/3); AJ851134	*Deltaprot.*/Myxococcales (2/3) ; AJ851135
M11	237	Root	9	7	+100	+100	*Deltaprot.*/Myxococcales (1/6); AJ851136	*Alphaprot.* (5/6); AJ851137
M12	246	Root	18	15	−35	−100	*Deltaprot.*/Myxococcales (3/7); AJ851138	*Deltaprot.*/Myxococcales (4/7); AJ851139
M13*	246	Root	18	15	−35	−100	unaffiliated (1/8); AJ851140	*Deltaprot.*/Myxococcales (7/8); AJ851141
M14	249	Root	27	37	+267	+1740	unaffiliated (1/3); AJ851142	*Alphaprot.*/Rhodospirillales (2/3); AJ851143
M15*	249	Soil	27	37	+267	+1740	unaffiliated (1/4); AJ851144	*Deltaprot.*/Myxococcales (3/4); AJ851145
M16*	265	Root	27	26	−100	−97	Chloroflexi (1/4); AJ851146	*Alphaprot.*/Rhodospirillales (3/4); AJ851147
M17*	303	Soil	18	33	+30	+549	*Alphaprot.*/ Rhodospirillales (2/4); AJ851148	unaffiliated (2/4); AJ851149
M18*	332	Soil	18	19	+100	+241	Chloroflexi (1/5); AJ851150	Actinobacteria (4/5); AJ85115

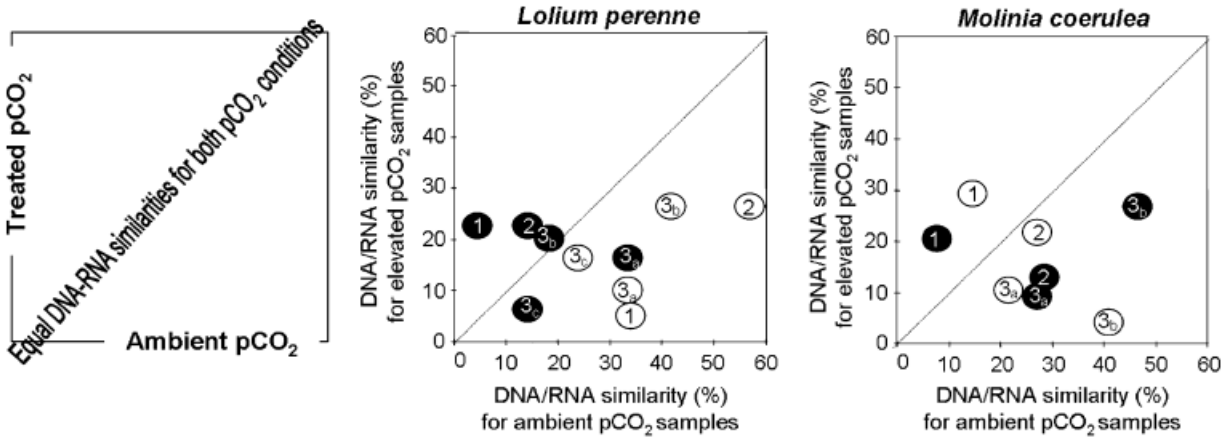

Fig. 2. DNA-/RNA-based profile similarity compared for ambient and elevated pCO_2 conditions (calculated with Steinhaus coefficient). (•) Soil samples; (○) Root samples. Sampling dates are indicated with numbers (1 for June 2001, 2 for May 2001 and 3 for July 2002) and replicates with small letters (a; b; c). Samples scattered on the left upper part of the plot indicate that their DNA and RNA patterns are more similar for treated plots compared to control plots. Samples scattered on the right lower part of the plot indicate that DNA and RNA patterns are more similar for ambient plots than for treated plots.

DGGE pattern description

DNA and RNA extracts obtained from the same sample, after PCR and RT-PCR amplification, generated contrasting DGGE profiles (Fig. 1). Band intensity was more uniform within DNA-based patterns, whereas RNA profiles displayed a clear dominance of a few bands. Soil patterns displayed smeared areas, probably representing clusters of low intensity bands, whereas root profiles often presented sharp bands.

For both plant–soil systems, the similarities between DNA- and RNA-based profiles for a given sample (Fig. 2) were generally below 50%. The DNA and RNA profile similarities were higher for ambient than for elevated pCO_2 plots (eight out of nine for root fraction, and five out of nine for soil fraction), indicating an influence of pCO_2 on community profiles.

Sources of the DGGE profile variability

The variation partitioning analysis (Fig. 3) allowed the relative influence of: (1) replicate plots and sampling date; (2) plant–soil system; and (3) elevated pCO_2 and root proximity on total (DNA-based) and active (RNA-based) bacterial community profiles to be shown. This analysis first revealed the high percentage of unexplained variance (71–79%, Fig. 3). The remaining 21% and 29% of the variance were significantly explained by each of the identified descriptors. The descriptors displaying the highest part of explained profile variability were the sampling date and plots. Globally, 15.1% of DNA- and 11.9% of RNA-based pattern variation were attributed to these descriptors, which integrate numerous environmental conditions (e.g. temperature, soil water content) varying in time and space. The plant–soil system explained 8.8% (DNA-) and 6.3% (RNA-

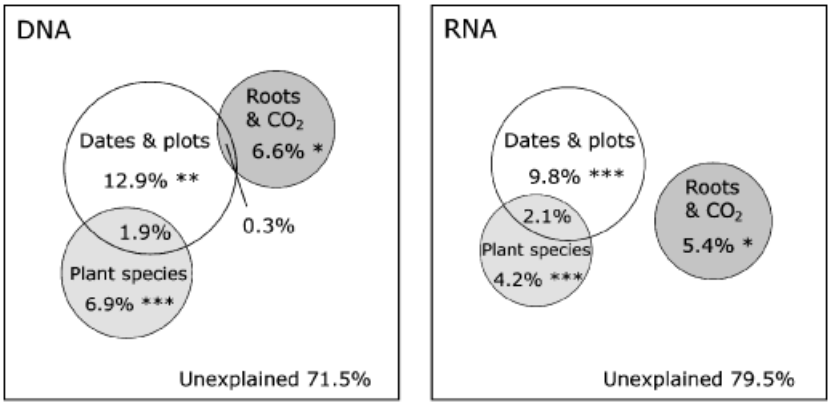

Fig. 3. Variation partitioning for data obtained from DNA- and RNA-based denaturing gradient gel electrophoresis patterns. The variation partitioning was tested for significance with 999 permutations using the Monte Carlo test for each set of descriptors: (1) plant–soil system; (2) pCO_2 treatment and root proximity; and (3) sampling date and replicate plots (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

based patterns) of the variability. The pCO_2 treatment alone did not influence microbial fingerprints; however, the combined influence of root vicinity and pCO_2 treatment significantly accounted for DNA- (6.9%) and for RNA-based (5.4%) profile variation, suggesting that elevated pCO_2 impacts bacterial communities through the roots.

Canonical Correspondence Analysis (Fig. 4) allowed us to ordinate response variables in a single ordination plane, constrained only by root and pCO_2 treatment after removing part of the pattern variability explained by date and plot replication, and by the soil-plant system. Bands influenced by elevated pCO_2 condition were generally associated with the root fraction. This is in agreement with data from similarity coefficients (Fig. 2). Root influence (as shown by strong correlation of the corresponding centroïd to CCA axis 1) was more important than pCO_2 influence (more correlated to CCA axis 2) (Fig. 4). Changes induced by pCO_2 increase were observed for both DNA- ($P = 0.021$) and RNA-based ($P = 0.026$) community profiles for *M. coerulea*. A similar trend was observed for metabolically active communities associated with *L. perenne* ($P = 0.087$).

Choice of characteristic bands and affiliation of corresponding populations

A total of 17 and 18 characteristic bands were selected for *L. perenne* and *M. coerulea*, respectively, based on the DGGE bands representativity (relative intensity and frequency of occurrence among all DNA- and RNA-based profiles, Table 2) and on CCA graphic representation (Fig. 4). On average, the intensity of all selected bands within a given profile represented 26% of the total intensity (data not shown). Twenty-seven (*L. perenne*) and 35 (*M. coerulea*) sequences were retrieved from these selected bands. Clones obtained

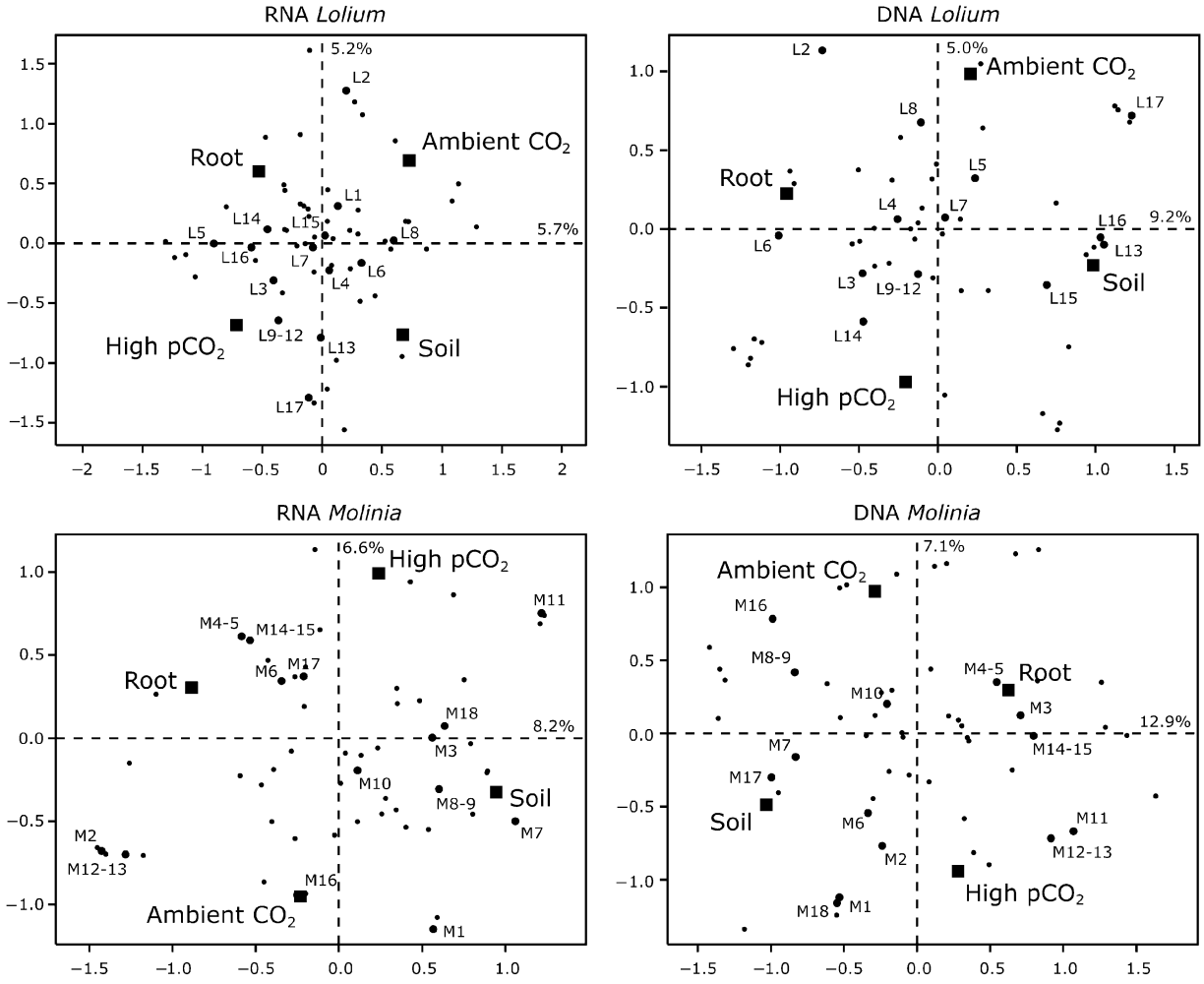

Fig. 4. Biplots of partial Canonical Correspondence Analysis of data obtained from DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE) patterns for *Lolium perenne* and *Molinia coerulea datasets*. Constrained axes 1 and 2 were used for graphic representation. DGGE bands selected for sequencing are indicated with a letter (M or L).

from the same excised band corresponded to one or two restriction types. In most cases, sequences from a single DGGE band were affiliated to the same group or to related groups (e.g. bands L7, M7 and M12, Table 2). However sometimes sequences from a single band were affiliated to different phylogenetic groups (e.g. bands L1 and M8). Some sequences obtained from co-migrating bands for different samples were affiliated to related groups (bands M12 and M13), whereas some others were affiliated differently (bands L11 and L12). Generally, sequences obtained from both plant–soil systems displayed similar affiliations. Among the 27 sequences retrieved for *L. perenne*, 52% were affiliated to *Proteobacteria* (*Alpha-*, *Beta-*, *Gamma-*, *Delta-*), of which 36% were related to Myxococcales (*Deltaproteobacteria*). Other sequences were affiliated to *Actinobacteria* (15%), *Bacteroidetes* (11%), and others were unaffiliated. Similar proportions were observed for the 35 sequences obtained from *M. coerulea*. The average intensity of selected bands doubled under elevated compared to ambient pCO_2 (Table 2). The average intensity of bands corresponding to *Actinobacteria* increased under elevated pCO_2 (root: +77%, soil: +291% for *L. perenne*; root: +265%, soil: +68% for *M. coerulea*). Myxococcales-related bands displayed higher intensities under elevated pCO_2, particularly in root (+248%) compared to soil (+79%) for *L. perenne*, and in soil (+254%) compared to root (+67%) for *M. coerulea*.

Discussion

Comparison between total and active 16S rRNA gene community profiles

Whereas RNA-based profiles highlight active bacterial populations at the time of sampling, DNA-based profiles display the most abundant bacterial populations, independently of their current activity. DNA- and RNA-based profiles for a given sample generally shared less than 50% of pattern similarity (Fig. 2), as previously observed (Muyzer & Smalla, 1998; Kowalchuk *et al.*, 1999; Duineveld *et al.*, 2001).

Field-induced variation

The FACE system currently provides the most realistic way to estimate how plants will respond to elevated pCO_2 in their native environment, avoiding the modification of natural air flow induced by other CO_2 enrichment systems (Long *et al.*, 2004). However, field experiments imply large variations in environmental conditions within time and space (e.g. soil heterogeneity, root distribution, temperature, precipitation). A high unexplained variation was observed, as in most ecological studies (Borcard *et al.*, 1992; Ritz *et al.*, 2004). The largest part of the profiles variability was explained by time of sampling and plots (Fig. 3). Such a high percentage of fingerprint variability between sampling dates is consistent with the short-term response of microbial populations to environmental changes.

Plant–soil system influences

Bacterial communities associated with the ecologically contrasting perennial grasses *L. perenne* and *M. coerulea* (Vazquez de Aldana & Berendse, 1997) were different: 6.3% (RNA) and 8.8% (DNA) of the variability of community profiles could be significantly explained by the plant–soil system (Fig. 3). The soil characteristics are different for *L. perenne* and *M. coerulea* swards: they allow different bacterial communities to settle (Latour *et al.*, 1996).

We expected the response of soil microbial communities to elevated pCO_2 to be dependent on the plant type, through rhizodeposition (Wardle *et al.*, 2004), whereas the same bacterial groups were found to be influenced by pCO_2 in the rhizosphere of both plants. This suggests that bacterial communities associated with the two plants (both being perennial hemicryptophytic grasses) responded similarly to the pCO_2 increase, despite the functional differences between the two host plants (nitrophilic for *L. perenne* vs. oligonitrophilic for *M. coerulea*).

Influence of elevated pCO_2

The direct influence of an atmospheric pCO_2 increase on soil bacterial communities is probably negligible because of the naturally high pCO_2 concentrations in the soil atmosphere (200–3500 Pa) (Gobat *et al.*, 2004). However, bacterial communities were significantly modified by the combined effect of pCO_2 treatment and root vicinity (Fig. 3). The effect of pCO_2 enrichment on soil bacterial communities is likely mediated by the plant through quantitative and qualitative changes in rhizodeposition (Paterson *et al.*, 1996; Hodge *et al.*, 1998).

Changes induced by high pCO_2 were more pronounced on active than on total bacterial communities at root vicinity. Similarities between DNA- and RNA-based profiles were higher under ambient than under elevated pCO_2, except for *L. perenne* soil samples (Fig. 2). This could reflect a more stable state of the bacterial community under ambient pCO_2, whereas low similarity between total and active communities under high pCO_2 would reflect a shifting state of the bacterial community due to fluctuations in the metabolic activity of specific populations (Montealegre *et al.*, 2000), because of root-mediated modification in trophic fluxes due to higher pCO_2 (Hodge *et al.*, 1998).

Total community analysis frequently failed to indicate pCO_2-induced changes (Griffiths *et al.*, 1998; Jones *et al.*, 1998; Insam *et al.*, 1999; Ebersberger *et al.*, 2004) and soil

microbial biomass often seemed unaffected under elevated pCO_2 (Zak *et al.*, 2000). By contrast, pCO_2-induced changes could be observed when targeting specific functional or taxonomic groups (Jones *et al.*, 1998; Ronn *et al.*, 2003; Roussel-Delif *et al.*, 2005; Tarnawski *et al.*, in press). The activity of some functional groups such as fungal cellulose decomposers (Jones *et al.*, 1998) and simple carbohydrate consumers (Hodge *et al.*, 1998) was shown to be enhanced under elevated pCO_2. Belowground responses of microbial communities to global change could generate feedback effects on aboveground biota, such as plant physiology and diversity (Van der Heijden *et al.*, 1998; Jackson *et al.*, 2002). The identification of responsive groups is necessary to understand putative feedbacks on the functioning of soil-plant systems.

Key populations

The bacterial groups responding to root vicinity and pCO_2 increase were highlighted by sequencing selected DGGE bands from total or metabolically active communities. A high proportion (11%) of the selected bands corresponded to sequences affiliated to *Actinobacteria*. They were generally retrieved from the active fraction of the soil bacterial community, regardless of the plant-soil system studied. *Actinobacteria* are known to be soil engineers using soil organic matter as their main carbon source (Ensign, 1992). Their dependence on plant exudates is therefore weak and *Actinobacteria* may be less affected by root-mediated perturbations. The importance of *Actinobacteria* in terms of abundance and activity in soils was demonstrated (Gremion *et al.*, 2003), including under elevated pCO_2 (Billings & Ziegler, 2005).

Myxococcales were identified as the most responsive group to either pCO_2 increase or root influence (19% of retrieved sequences). The relative intensity of corresponding bands increased under elevated pCO_2. Myxococcales are known to be cellulolytic organisms (Reichenbach & Dworkin, 1992). Cellulolytic fungi were shown to be favoured under elevated pCO_2 conditions (Jones *et al.*, 1998), as shown for Myxococcales in the present study. Root growth and exudation are increased under elevated pCO_2 (Zak *et al.*, 2000), leading to a greater availability of cellulose (Robinson *et al.*, 1997), and a stimulation of cellulolytic organisms.

Currently, a major challenge to understand better the role of microbial communities in plant-soil system functioning is to link taxonomic diversity and functions. This requires a prior identification of organisms and their corresponding functions. Some bacterial groups (*Actinobacteria*, Myxococcales) were identified as key organisms in the response of soil-plant systems to elevated pCO_2. This study will be useful further to identify bacterial functions involved in the response of ecosystems to global changes.

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