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Communautés bactériennes dans la rhizosphère de *Lolium perenne* et *Trifolium repens*: dénombrements, biomasse, effet rhizosphérique et influence d'une augmentation en CO_2 atmosphérique.

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Influence d'une augmentation en CO_2 atmosphérique sur les populations bactériennes dans la rhizosphère de *Lolium perenne* et *Trifolium repens*

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UNIVERSITÉ DE NEUCHÂTEL

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Diversité bactérienne du sol et de la rhizosphère et effet d'une augmentation en CO_2 atmosphérique

Soil and rhizosphere bacterial diversity and effect of the atmospheric CO_2 content

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Introduction

Le sol ne constitue pas un environnement homogène, mais une mosaïque d'habitats avec pour chacun des populations bactériennes propre. La rhizosphère représente un compartiment d'intérêt majeur. Une part significative du CO_2 fixé par photosynthèse dans les organes aériens des plantes est relâchée dans le sol sous forme de rhizodépositions. Ces composés servent de substrats de croissance pour les micro-organismes. Ils influencent également les caractéristiques physico-chimiques du sol environnant: son acidité, son potentiel redox, sa teneur en eau, sa conductivité électrique (Lynch, 1990) et le taux d'oxygène libre disponible (Höjberg & Sorensen, 1993). La rhizodéposition conditionne donc le développement de la microflore rhizosphérique. Mieux comprendre l'influence des exsudats racinaires sur le développement de la microflore racinaire permet de mesurer l'influence des changements environnementaux sur les populations bactériennes et finalement sur le fonctionnement de l'écosystème sol.

Actuellement, la concentration en CO_2 atmosphérique est en constante augmentation. Il est prévu qu'elle atteigne le double de sa valeur actuelle au milieu du siècle prochain. Le système FACE (*Free-Air-CO_2-Enrichment*) (Allen, 1992) permet une simulation à l'air libre de cette augmentation. Les parcelles d'étude sont donc soumises aux mêmes conditions (évapotranspiration et vent) que les parcelles témoins. Par le fait de la respiration des racines et des micro-organismes, la teneur en CO_2 du sol est supérieure à celle de l'atmosphère. Il faut donc s'attendre à détecter une influence de l'augmentation en CO_2 atmosphérique par l'intermédiaire des racines des plantes. En effet, il a été mesuré sous une atmosphère enrichie en CO_2 une augmentation de la biomasse racinaire (Rogers & Runion, 1994). Les rhizodépositions en découlant s'en trouveront donc modifiées, soit qualitativement, soit quantitativement. Ces modifications entraîneront donc des changements dans les communautés bactériennes associées à la rhizosphère.

Dans cette étude, nos objectifs sont d'analyser sous des conditions naturelles les changements de la diversité bactérienne en fonction de la proximité de la racine pour des monocultures de *Trifolium repens* (trèfle) et *Lolium perenne* (ray-grass). Nous avons donc

partitionné le sol en trois fractions: le sol lointain, le sol adhérant aux racines et l'endorhizosphère-rhizoplan. On estime à 0.3%-20% la proportion de bactéries cultivables dans un échantillon de sol (Alexander, 1977; Torsvik et al., 1990). Dans la rhizosphère, ce pourcentage est compris entre 1 et 10% (Campbell & Greaves, 1990). Par conséquent, nous avons opté pour une approche basée sur une extraction d'ADN du sol, suivi par une amplification par PCR des gènes codant pour l'ARNr 16S, permettant ainsi de contourner les problèmes liés à la cultivabilité des micro-organismes. Après clonage du produit d'amplification, nous avons effectué une analyse de restriction des ADNr 16S. Les profils de restriction identiques sont groupés en unités taxonomiques opérationnelles (OTUs). Le nombre d'OTUs ainsi que le nombre de clones par OTU permettent de calculer un index de diversité. Les clones dominants ont été séquencés afin de déterminer leur position phylogénétique.

Matériel et méthodes

Site d'étude. Le site d'étude est localisé à Eschikon, près de Zürich, en Suisse. Les parcelles sont des monocultures de *Trifolium repens* cv. Milkanova et de *Lolium perenne* cv. Bastion. Elles sont fertilisées avec du P_2O_5 (12 g/m^2/an), du K_2O (29 g/m^2/an), du Mg (1.6 g/m^2/an), et du N (14 g/m^2/an). Les plantes sont fauchées quatre fois par année. L'enrichissement en CO_2 est effectué par un système FACE (Allen, 1992), permettant de passer de la teneur ambiante de 350 ppm à une teneur de 600 ppm.

Récolte et partitionnement des échantillons. La récolte et le partitionnement des échantillons ont été effectués selon Marilley et al. (1998). Une carotte par échantillon (6 cm de diamètre et 15 cm de long) a été récoltée. Nous avons défini après partitionnement trois fractions de proximité racinaire différente: le sol lointain (BS), obtenu en séparant les racines de la carotte de sol; le sol rhizosphérique (RS), obtenu par lavage du sol adhérant aux racines et le rhizoplan-endorhizosphère, obtenu par broyage des racines lavées deux fois. Les deux premières étapes de partitionnement ont été suivies à la loupe binoculaire afin de s'assurer de la séparation des fractions.

Construction d'une bibliothèque d'ADNr 16S. La méthode a été précédemment décrite par Marilley et al. (1998). L'ADN est extrait selon la procédure de Lee et al. (1996) par une méthode d'extraction directe basée sur une digestion au lysozyme, puis par une lyse au SDS et des chocs thermiques. L'ADN extrait a ensuite été purifié par un traitement au CTAB, suivi d'une extraction au chloroforme et une centrifugation dans du polyéthylène-glycol. Après resuspension du culot dans du Tris-EDTA, de l'acétate d'ammonium est ajouté, puis l'extrait est centrifugé. L'ADN est finalement précipité à l'isopropanol.

L'ADNr 16S a été amplifié par PCR en utilisant des amorces universelles. Les produits PCR sont ensuite excisés d'un gel d'agarose, ligué au vecteur pGEM-T et les cellules compétentes transformées par le produit de la ligation. Les plasmides extraits à partir de 29 clones par fraction ont servi d'ADN-cible pour une seconde amplification par PCR de l'ADNr 16S. 4 µl de produit PCR est ensuite digéré dans un volume de réaction de 20 µl à l'aide de deux unités des enzymes de restriction *Hae*III et *Rsa*I, séparément. Les profils de restriction correspondant sont séparés par électrophorèse sur gel d'agarose.

Calcul des index de diversité. Les clones montrant des profils de restriction identiques sont groupés par unité taxonomique opérationnelle (OTU). Le nombre d'OTUs de même que le nombre de clones par OTU ont servi au calcul de l'index de diversité de Shannon (Shannon & Weaver, 1949).

Séquençage partiel des ADNr 16S et construction d'un arbre phylogénétique. Les séquences d'ADNr 16S sont obtenues selon des protocoles standards à l'aide d'un séquenceur automatique. Les séquences sont ensuite alignées à l'aide du programme Clustal W (Thompson et al., 1994). La matrice de distance est calculée par l'algorithme de Jukes & Cantor (1969) et l'arbre construit selon la méthode UPGMA (Sneath & Sokal, 1973) par le progiciel Phylip.

Résultats et discussion

Les observations à la loupe binoculaire ont montré que le sol rhizosphérique adhère aux racines après la séparation sol-racines. Le sol consiste en une gaine entourant les racines pour *L. perenne* et en des particules de sol lâches pour les racines de *T. repens*. Ces observations confirment que cette fraction est placée sous l'influence directe des exsudats racinaires. Nous avons également pu observer que les apex demeurent intacts après ce lavage.

Le groupement des clones en OTUs a été basé sur l'utilisation combinée de deux enzymes de restriction. Moyer et al. (1996) ont déterminé à l'aide d'une simulation assistée par ordinateur le nombre de taxons identifiables par différentes combinaisons d'enzymes de restriction, à partir de 106 séquences d'ADNr 16S déposées dans les banques de données disponibles. Deux enzymes combinées permettent de discriminer plus de 96% des taxons. Parmi les taxons non-déterminés, la moyenne des homologies de séquence vaut 95.6%. En guise de référence, il est important de savoir qu'habituellement les micro-organismes ayant plus de 97% d'homologie de séquence ont des valeurs de réassociation ADN:ADN supérieure à 70% (Stackebrandt & Goebel, 1994). Cette valeur de 70% est un critère-clef pour la détermination d'une espèce. Même si l'utilisation de deux enzymes de restriction ne permet pas une détermination exhaustive de la diversité, elle permet cependant une estimation assez précise.

Des OTUs dominantes (désignées par les lettres L et M) apparaissent clairement dans les fractions RE et RS de *Lolium perenne* et *Trifolium repens* (fig. 1.1). L'OTU L est dominant dans les fractions RS et RE de *Lolium perenne* et dans la fraction RS de *T. repens*. Dans la fraction RE du trèfle nous observons l'apparition d'une OTU dominante supplémentaire: l'OTU M, reléguant l'OTU L en deuxième position en termes de dominance. Les valeurs du tableau 1, tirées de la figure 1, montrent clairement que la diversité diminue en fonction du rapprochement à la racine: l'index de diversité de Shannon diminue lorsque l'influence de la racine augmente.

Dans la fraction BS de *T. repens* et de *L. perenne*, la valeur de l'index de diversité de Shannon vaut respectivement 1.44 et 1.42 (tab. 1). La valeur maximale de l'index de diversité de Shannon pour le nombre de clones choisis étant 1.46, nous constatons que la diversité est très importante dans cette fraction. La dominance d'un OTU dans la fraction RS peut s'expliquer par l'influence de la rhizodéposition. Selon différentes études, on estime qu'une part significative du carbone fixé par photosynthèse est libéré dans le sol rhizosphérique sous forme de rhizodéposition (Lynch & Whipps, 1990). La composition de ces rhizodépositions n'est pas bien connue, mais on sait que certains de ces composés agissent comme des molécules attractives à très basse concentration. Dans la rhizosphère, malgré l'important flux de carbone dérivant des racines, les micro-organismes sont soumis à des facteurs sélectifs ou électifs qui réduisent leur diversité. Dans la fraction RE, cette diversité est plus faible que dans la fraction RS. Cette fraction n'est en fait pas un échantillon de sol, mais elle se compose de racines broyées. La colonisation de cet habitat particulier est soumise à des contraintes supplémentaires liées à la capacité des bactéries à adhérer et/ou à pénétrer les tissus racinaires.

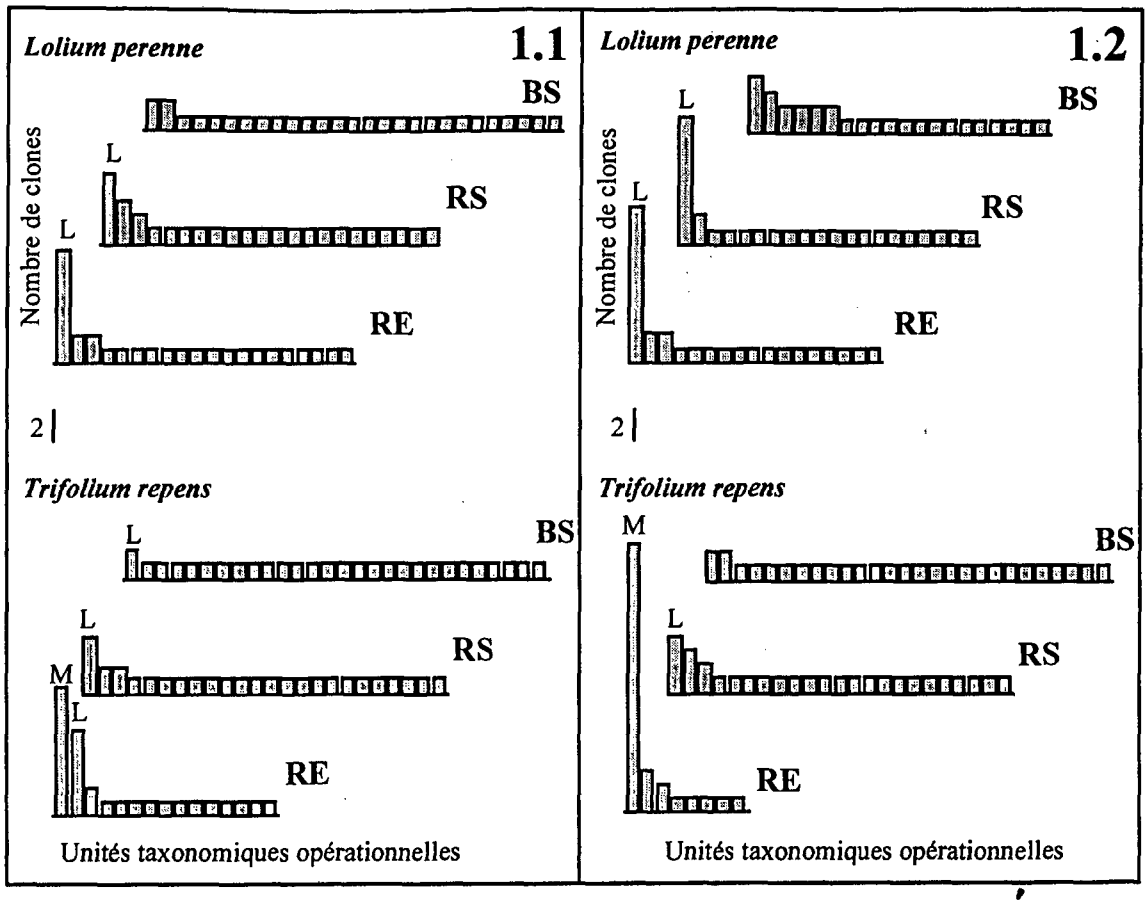

Figure 1. Unités taxonomiques opérationnelles par ordre du nombre de clones après digestion par *Hae*III et *Rsa*I. BS, sol lointain; RS, sol rhizosphérique; RE, rhizoplan et endorhizosphère. 1.1, contrôle (350 ppm CO_2). 1.2, enrichi (600 ppm CO_2).

Les résultats obtenus à partir des fractions provenant des parcelles situées sous enrichissement en CO_2 sont présentés dans la figure 1.2. Cette figure et les index de diversité de Shannon correspondants (tab. 1) confirment la baisse de diversité due à l'environnement racinaire. Les OTUs dominantes sont à nouveau L et M. L'OTU L ne se retrouve par contre pas dans la fraction RE de *T. repens*. Par comparaison avec les parcelles contrôles, ces résultats montrent que la diversité est plus faible dans les fractions provenant des parcelles enrichies en CO_2. Ceci se mesure pour la fraction RE de *T. repens* et pour les fractions RE et RS de *L. perenne*.

Tableau 1. Index de diversité de Shannon

Fractions	*Lolium perenne*		*Trifolium repens*	
	Contrôle	Enrichi	Contrôle	Enrichi
Sol lointain BS	1.42	1.25	1.44	1.42
Sol rhizosphérique RS	1.27	1.14	1.34	1.31
Rhizoplan et endorhizosphère RE	1.17	1.03	0.98	0.55

Cette baisse de diversité peut s'expliquer par le fait que le taux net photosynthétique est limité par la concentration en CO_2 atmosphérique. Sous enrichissement en CO_2, ce taux augmente, induisant une modification qualitative et/ou quantitative des exsudats racinaires. Le caractère sélectif ou électif de la rhizosphère est par conséquent plus important, modifiant la composition des populations bactériennes présentes dans cette dernière. L'activité microbienne sera donc changée, avec en retour des conséquences pour la plante qu'il s'agit encore de déterminer.

Le séquençage partiel des OTUs dominantes a permis de construire un arbre phylogénétique présenté dans la figure 2. Les OTUs dominantes font partie du groupe des Protéobactéries. Nous constatons que les clones classés dans l'OTU L se situent dans le groupe des *Pseudomonas* spp. et que les clones classés dans l'OTU M présentent une homologie élevée avec *Rhizobium leguminosarum*. Ces résultats confirment que la méthode de biologie moléculaire choisie est bien adaptée à l'étude du sol. En effet *R. leguminosarum* est décrit comme un endosymbionte des racines de *T. repens* et *Pseudomonas* spp. a souvent été décrit comme une bactérie de la rhizosphère.

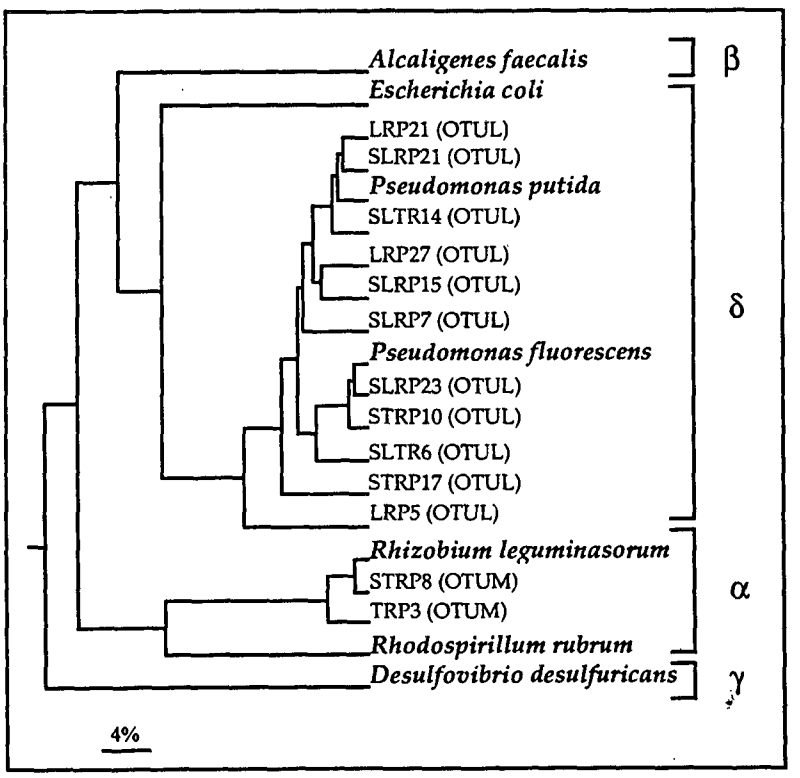

Figure 2. Relations phylogénétiques des séquences partielles des ADNr 16S des OTUs dominantes L et M avec des membres des *Proteobacteria*. La matrice de distance et l'arbre phylogénétique ont été calculés respectivement avec l'algorithme de Jukes et Cantor et la méthode UPGMA. La barre désigne le nombre de substitutions pour 100 nucléotides.

Les méthodes de biologie moléculaire offrent de nouveaux outils pour l'investigation des communautés bactériennes. Bien que les limites de la méthode soient multiples (différences dans les rendements d'extractabilité et d'amplificabilité par PCR des ADN, biais lors de la ligation, etc...), nos résultats montrent que la diversité varie en fonction de la proximité à la

racine. L'augmentation de la teneur en CO_2 atmosphérique accentue la sélectivité ou l'électivité de la rhizosphère. Le séquençage de la bibliothèque d'ADNr 16S est actuellement en cours, ce qui permettra d'analyser plus précisément les remaniements des communautés au sein des différents groupes phylogénétiques.

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Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots

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Abstract

The rhizosphere of *Trifolium repens* and *Lolium perenne* was divided into three fractions: the bulk soil; the soil adhering to the roots; and the washed roots (rhizoplane and endorhizosphere). After isolation and purification of DNA from these fractions, 16S rDNA was amplified by PCR and cloned to obtain a collection of 16S rRNA genes representative of the bacterial communities of these three fractions. The cloned 16S rRNA genes were then partially sequenced and analysed by a molecular phylogenetic approach. Our data show a high diversity in the bulk soil, which is dominated by clones related to Gram-positive, *Cytophaga–Flexibacter–Bacteroides*, *Proteobacteria*, and *Holophaga–Acidobacterium* groups. The ubiquity of members of the *Holophaga–Acidobacterium* group, which is composed of sequences of yet-uncultivated microorganisms, is confirmed. The plant roots have a selective effect towards the gamma *Proteobacteria*, to the detriment of the Gram-positive and the *Holophaga–Acidobacterium* bacteria, leading to a dominance of *Pseudomonas*. This work shows by a culture independent approach that the phylogenetic diversity decreases in the proximity of plant roots.

Keywords: Microbial molecular ecology; Rhizobacteria; Soil bacterial diversity; Rhizosphere; 16S rDNA phylogenetic diversity

1. Introduction

Defining the diversity of soil microbial communities aims at a better understanding of soil bacterial processes. Environmental perturbations may lead to changes in soil microbial populations and therefore in soil functioning (Atlas et al., 1991; Runion et al., 1994; Diaz-Ravina and Bååth, 1996; Smart et al., 1997). Soil cannot be considered as a homogeneous environment. Among other factors, the organic matter under decomposition, the low rate of mixing and soil microfaunal activities determine habitats in soil. Plant roots release organic compounds mainly composed of polysaccharides, soluble secretions and cell lysates (Whipps, 1990). These roots exudates contribute to the soil heterogeneity by creating new habitats. Lemanceau et al. (1995) and Latour et al. (1996) have shown that plants influence the diversity of soil-borne populations of fluorescent *Pseudomonas*. Marilley et al. (1998) found that bacterial diversity decreased as the proximity to the roots increased. The metabolic and adaptative responses to variable supplies of water, oxygen, and nutrients differ from rhizosphere to soil

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2.3. Detection of plant chloroplastic DNA as contamination

Since the RE fraction comprised a significant portion of plant material, contamination of PCR amplification product by plant chloroplastic DNA was a major concern. Amplification of a 1.6 kbp region specific to the gene encoding the large subunit of ribulose bisphosphate carboxylase (*rbc*L) was used as control. The sequence of the primers and the PCR conditions are described by Malik et al. (1994). The PCR cycling parameters were modified as follows: instead of 40, 35 cycles were performed and the final extension step was omitted. Two nanograms of template DNA from soybean and spinach (obtained from A. Spielmann, University of Neuchâtel) served as positive controls.

2.4. Construction and partial sequencing of 16S rRNA gene library

Soil 16S rDNA PCR products were excised from low-melting agarose and then cloned in pGEM-T linear vector and *Escherichia coli* XL1 competent cells. To improve the differentiation of the clones between operational taxonomic units by the PCR restriction profile analysis performed by Marilley et al. (1998), a third tetrameric restriction enzyme (*Hae*III) was used, according to the previously described conditions. Clones showing different restriction patterns were sequenced.

Recombinant plasmids from transformants were purified from overnight cultures by using plasmid miniprep columns (Qiagen GmbH, Hilden, Germany). Plasmid DNA concentrations were estimated spectrophotometrically at 260 nm. The sequences were determined either by Microsynth (Balgach, Switzerland) following standard protocols, or by ourselves. The sequencing reactions were done with a Sequenase (T7) sequencing kit (Amersham, Slough, England), by using T7 or SP6 primers corresponding to vector DNA flanking the cloned insert. Between 200 and 500 ng of template DNA were combined with 0.24 pmol of infra-red dye 41 (MWG-Biotech, Ebersberg, Germany) fluorescently labelled primer, 0.12 μl of DMSO, and 1 μl of reaction mix (A, C, G, or T; Amersham) to a final volume of 4 μl. The cycling parameters were 95°C for 2 min, followed by 30 cycles of 30 s at 56°C, and 1 min at 70°C. After the addition of 3 μl of formamide loading dye (Amersham), the reactions were run on a 4000L (Li-Cor, Lincoln, Neb.) automated laser fluorescence sequencer. Since 16S rRNA genes were bidirectionally cloned inside pGEM-T vector, all sequences showing the reverse PCR primer were repeated using the second sequencing primer.

2.5. Sequence analysis

Partial sequences of 16S rRNA genes were compared with those in the EMBL database using FASTA 3.0 (Pearson and Lipman, 1988; Pearson, 1990). The determined sequences were aligned using Clustal W (Thompson et al., 1994). The distance matrices and the phylogenetic trees were calculated by the Jukes and Cantor (1969) and neighbor-joining (Saitou and Nei, 1987) algorithms, respectively, using the Felsenstein Phylogeny Inference Package (Phylip 3.5 [Felsenstein, 1989]).

2.6. Nucleotide sequence accession numbers

The nucleotide sequences of the soil and rhizosphere 16S rDNA clones were deposited in the EMBL database under accession numbers AJ005871 to AJ005878, AJ005988 to AJ005997, AJ006008 to AJ006014, AJ006027, AJ006028, AJ006087 to AJ006094, and AJ232784 to AJ232884.

3. Results

3.1. DNA isolation

The recovery and purity of DNA from the different fractions are displayed in Table 1. Since the DNA recovery from the RS and RE fractions of *L. perenne* was too low, DNA estimation was performed by comparison with the DNA size ladder on agarose gel. The CTAB, $CHCl_3$ and ammonium acetate steps were repeated once for fractions BS of both plants. The DNA recovery in fractions RS and RE of *L. perenne* was significantly lower than in fractions RS and RE of *T. repens*. The presence of nodules in the roots of *T. repens* explained the difference between RE fractions. The RS fraction consisted of a sheath sur-

microbial communities (Campbell and Greaves, 1990; Sorensen, 1997).

Until recently, the determination of microbial diversity was based on genetic and phenotypic characterisation of isolated and cultivated strains. The limitations of this approach are linked to the low ratio of cultivable bacteria in soil and rhizosphere (Alexander, 1977; Campbell and Greaves, 1990; Torsvik et al., 1990). Molecular techniques provides a powerful alternative. In situ hybridisation using fluorescently labelled probes or antibodies has been successfully used for detecting rhizosphere micro-organisms (Assmus et al., 1997). Torsvik et al. (1990) have used DNA : DNA reassociation measurements to assess soil bacterial diversity. Muyzer et al. (1993) introduced the denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) to study the structural diversity of microbial communities. Bacterial diversity was also assessed by analysing 16S rDNA clone libraries retrieved from environmental DNA. The diversity was measured either by PCR restriction analysis (Moyer et al., 1994; Marilley et al., 1998), or by phylogenetic assignments of 16S rDNA sequences (Giovannoni et al., 1990; Stackebrandt et al., 1993; Ueda et al., 1995; Borneman et al., 1996; Kuske et al., 1997; Felske et al., 1998).

Based on 16S rDNA clone libraries retrieved from soil, several studies of phylogenetic microbial diversity have been performed (Stackebrandt et al., 1993; Ueda et al., 1995; Borneman et al., 1996; Rheims et al., 1996; Kuske et al., 1997; Ludwig et al., 1997; Felske et al., 1998). These studies described the main bacterial groups involved in soil processes and indicated the presence of rRNA sequences related to yet-uncultivated species. None of the above-mentioned studies dealt with changes in the bacterial community structure due to the rhizosphere effect. Our objective was to analyse, under field conditions, how the phylogenetic bacterial diversity varied as a function of root influence in plantations of *Lolium perenne* and *Trifolium repens*. To do this, we divided the soil into three fractions; the bulk soil; the soil adhering to the roots; and the washed roots (rhizoplane and endorhizosphere). Our methodology is based on the sequence analysis of the 16S rDNA clone library retrieved by Marilley et al. (1998) from these different soil fractions. Phylogenetic assignments were performed by comparison with sequences in the EMBL database.

2. Materials and methods

2.1. Sampling site

The sampling site consisted of monocultures of *Lolium perenne* cv Bastion and *Trifolium repens* cv Milkanova fertilised with P (5.2 g m^{-2} $year^{-1}$), K (24 g m^{-2} $year^{-1}$), Mg (1.6 g m^{-2} $year^{-1}$), and N (14 g m^{-2} $year^{-1}$). The crops were harvested four times a year. The soil was a fertile, eutric cambisol (clay loam), adequate for intensively managed grassland. The plots were located at 550 m asl in Eschikon, which is near Zürich (Switzerland).

2.2. Soil sampling and partitioning, DNA extraction and 16S rDNA amplification

Sampling of soil cores, partitioning of soil fractions, DNA extraction and purification, and 16S rDNA amplification by PCR have been described elsewhere (Marilley et al., 1998). One soil core for DNA extraction was collected in October 1995 from each monoculture. Sampling was performed in October to obtain results representative of the bacterial populations established during the growing season. Both cores were separated into three fractions: the bulk soil (BS); the rhizospheric soil (RS); and the rhizoplane-endorhizosphere (RE).

Soil DNA was extracted and purified by the procedure of Ogram (1998). Nucleic acids were extracted by a direct method using lysozyme digestion, SDS treatment and heat shocks. DNA was purified by a CTAB treatment, a chloroform-isoamyl alcohol (24 : 1) extraction, a centrifugation in polyethylene glycol, and an ammonium acetate precipitation. Since *Taq* DNA polymerase may be inhibited by humic compounds (Steffan et al., 1988; Tsai and Olson, 1991; Tebbe and Vahjen, 1993), the CTAB, $CHCl_3$ and ammonium acetate steps were repeated once for extracts which still showed a dark brown colour. The 16S rDNA was selectivity amplified from the purified genomic DNA by PCR, using oligonucleotide primers designed to anneal to conserved positions in the bacterial 16S rDNA.

Table 1
Recovery and purity of DNA. Samples from bulk soil (BS), rhizospheric soil (RS), and the rhizoplane-endorhizosphere (RE)

	L. perenne			*T. repens*		
	BS	RS	RE	BS	RS	RE
Spectrophotometric measurement (μg)	73.4	nd[a]	nd[a]	48.2	15.2	25.2
$A_{260\ nm}/A_{280\ nm}$ ratio	1.71	nd[a]	nd[a]	1.75	1.38	1.88
Gel estimation (μg)	nd[a]	1.2	1.2	nd[a]	nd[a]	nd[a]

[a] Not determined.

rounding the roots in *L. perenne* and of discrete soil particles adhering to the roots in *T. repens*. DNA recovered from RE fraction of *T. repens* was of high purity.

PCR amplification of the *rbc*L subunit was used as control for plant DNA contamination (Fig. 1). No PCR product was detected in the BS, RS and RE fractions, contrary to the 16S rDNA PCR amplification. Positive *rbc*L PCR products were obtained from spinach and soybean template DNA and from 5 ng of BS DNA from *L. perenne* and *T. repens* supplemented with 0.5 ng of spinach DNA.

3.2. Partial 16S rDNA sequence analysis

Among the 143 clones partially sequenced, four were discarded in regard to the high number of undetermined nucleotides. The lengths of the sequences varied between 430 and 780 bp with the Li-Cor sequencer and between 420 and 620 bp with the Microsynth system. Forty-four clones were sequenced by using both sequencing primers. They were used to control the formation of chimerae, which can occur during the PCR amplification of 16S rDNA from mixed bacterial genomes (Liesack et al., 1991; Kopczynski et al., 1994). Depending on the sequencing primer used, two clones (clone 4 and clone 11 from fractions RS and BS of *T. repens*, respectively) were associated to different micro-organisms by FASTA program, and therefore considered a chimerae.

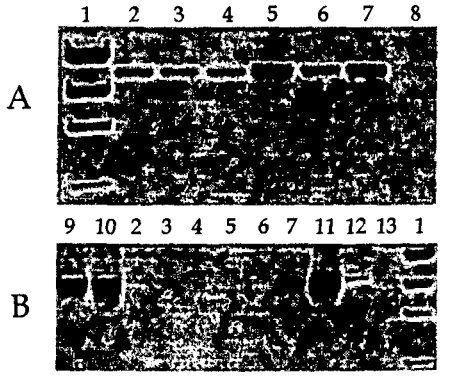

Fig. 1. (A) 16S rDNA and (B) *rbc*L PCR amplifications of BS, RS, and RE fractions of *T. repens* and *L. perenne* monocultures. Lane 1, molecular weight marker DNA mass ladder (Gibco BRL, Eggenstein, Germany); Lanes 2, 4, and 6; BS, RS, and RE fractions of *L. perenne*, respectively; Lanes 3, 5, and 7; BS, RS, and RE fractions of *T. repens*, respectively; Lanes 8 and 13, negative controls; Lanes 9 and 10, BS fraction of *L. perenne* and *T. repens*, respectively, supplemented with spinach genomic DNA; Lane 11, spinach genomic DNA; Lane 12, soybean genomic DNA.

3.3. Phylogenetic assignments and molecular diversity

16S rDNA sequences were aligned from the forward 16S rDNA PCR primer. Phylogenetic relationships among the clones from each fraction are illustrated in Fig. 2. FASTA program determined the most closely related sequence in the EMBL database. In the rhizosphere fractions, the phylogenetic affiliation to reference sequences was more accurate and the number of clones related to cultivated bacteria increased dramatically. Distribution of 16S rDNA clones within the major taxa is shown in Table 2.

Gamma *Proteobacteria* dominated in all soil fractions, except in the BS fraction of *L. perenne*. This dominance was more pronounced as the proximity to the roots increased. *Pseudomonas* represented the major members of this phylogenetic group. More than half of gamma *Proteobacteria* were related to *Pseudomonas* in T. repens fractions and in the RE fraction of *L. perenne*. The nodules present in the clover roots led to a dominance of alpha *Proteobacteria* affiliated to *Rhizobium leguminosarum* in the RE fraction of *T. repens*. Members of the *Cytophaga–Flexibacter–*

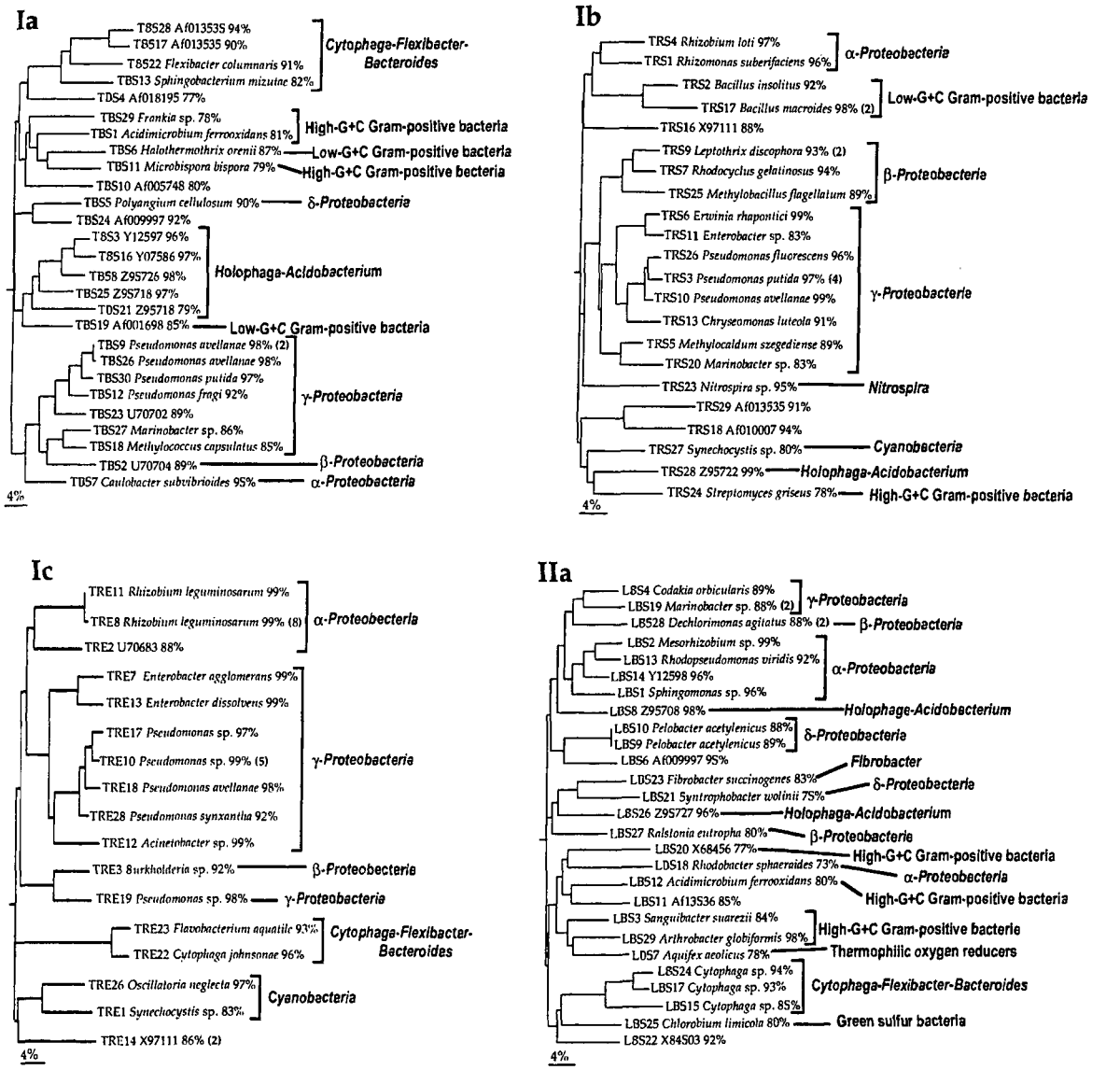

Fig. 2. Phylogenetic relationships of partial 16S rRNA genes from (a) BS, (b) RS, and (c) RE fractions of (I) *T. repens* and (II) *L. perenne* monocultures. The sequences were aligned by Clustal W and the trees calculated by the Jukes and Cantor and neighbor-joining algorithms, respectively. Clones are represented by their name followed by the most closely related sequence obtained from the EMBL database and by the corresponding homology value, as determined by FASTA. The EMBL accession number is given for clones related to yet-uncultured bacteria. The number of clones showing identical PCR restriction patterns after digestion with three restriction enzymes is shown in brackets. Bare scale infers the number of nucleotide substitution.

Bacteroides phylum occurred in all fractions except in the RS fraction of *T. repens*. The clones affiliated to Gram-positive bacteria occurred mainly in the BS and RS fractions of both plants. Sequences related to the high-G+C Gram-positive bacteria ranked among the main phylogenetic groups in the BS and RS fractions of *L. perenne* and in the BS fraction of *T. repens*. The results also showed the presence of clones affiliated to the newly described *Holophaga–Acidobacterium* phylum (Ludwig et al., 1997). These clones were detected mainly in the RS fractions of *L. perenne* and in the BS fraction of *T. repens*.

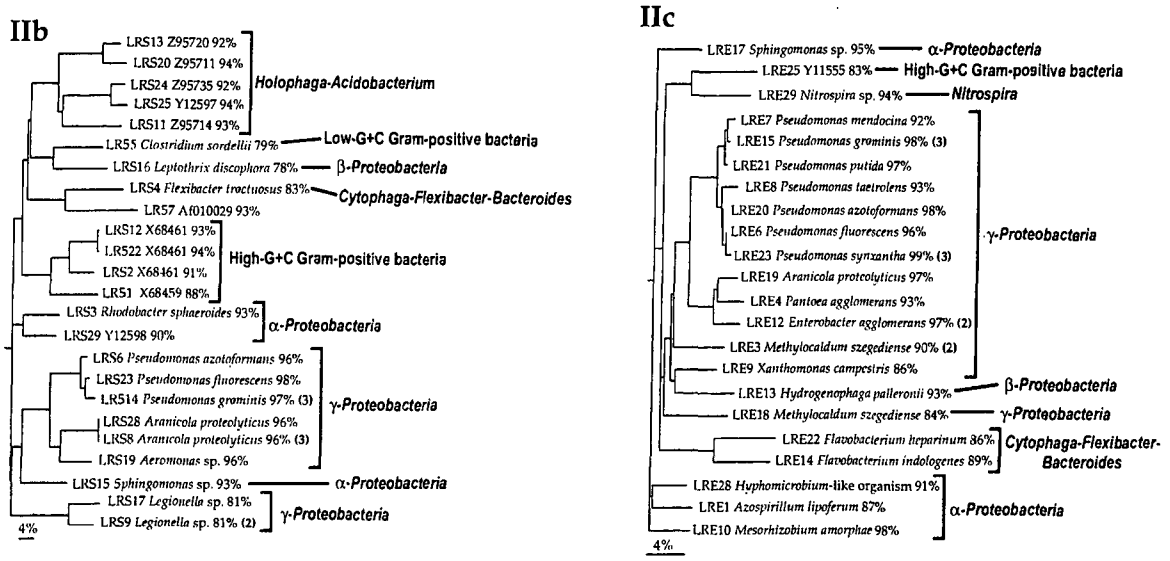

Fig. 2 (*Continued*).

Table 2
Distribution of 16S rDNA clones within the major taxa

Major taxa and groups	Number of clones					
	L. perenne			*T. repens*		
	BS	RS	RE	BS	RS	RE
Green sulfur	1	0	0	0	0	0
Cyanobacteria	0	0	0	0	1	2
Low-G+C Gram-positive	0	1	0	2	3	0
High-G+C Gram-positive	4	4	1	3	1	0
Cytophaga–Flexibacter–Bacteroides	3	1	2	4	0	2
α-*Proteobacteria*	5	3	4	1	2	10
β-*Proteobacteria*	3	1	1	1	4	1
γ-*Proteobacteria*	3	13	19	8	11	12
Pseudomonas	0	5	11	5	6	9
δ-*Proteobacteria*	3	0	0	1	0	0
Nitrospira	0	0	1	0	1	0
Thermophilic oxygen reducers	1	0	0	0	0	0
Fibrobacter	1	0	0	0	0	0
Holophaga–Acidobacterium	2	5	0	5	1	0
Unclustered	3	1	0	3	3	2

4. Discussion

Two approaches have been used to extract DNA from the environment: cell extraction followed by cell lysis (Steffan et al., 1988; Torsvik et al., 1990; Jacobsen and Rasmussen, 1992) or direct lysis of bacterial cells and DNA purification (Ogram et al., 1987; Tsai and Olson, 1991; Malik et al., 1994). To avoid the bias linked to the efficiency of the separation of microorganisms from soil particles, we used a direct lysis

method, which yields significantly more DNA than indirect approaches (Steffan et al., 1988; Leff et al., 1995). The positive 16S rDNA amplification products showed that the DNA extracted and purified from BS, RS, and RE fractions was pure, even though direct lysis methods may lead to DNA of lower purity, so that amplification by PCR can be inhibited by humic compounds that are copurified with the nucleic acids (Steffan et al., 1988; Tsai and Olson, 1991; Tebbe and Vahjen, 1993; Chandler et al., 1997).

Direct lysis methods may lead to DNA extracted from non-bacterial sources. Since the RE fraction consisted mainly of pounded plant material, *rbc*L PCR amplification served as control for chloroplastic plant DNA contamination. However, this control should be used for samples with low number of prokaryotic photosynthesizers. Indeed, the *rbc*L gene, which encodes the large subunit of ribulose bisphosphate carboxylase (involved in the fixation of CO_2 during photosynthesis), is highly conserved and common to a high number of plants (Malik et al., 1994), as well as prokaryotic photosynthesizers (Ogram, 1998). The negative *rbc*L PCR amplification products from BS, RS, and RE DNA templates showed that the DNA extraction method did not lead to a contamination by chloroplastic plant DNA (Fig. 1). The positive *rbc*L amplification products obtained from BS DNA supplemented with plant DNA ascertained that the *rbc*L PCR amplification was not inhibited by copurified compounds.

Recovery of soil DNA is affected by the differences in the lysis efficiency between soil micro-organisms. The procedure used in this work, based on lysozyme digestion of the peptidoglycan cell wall, on cell lysis by a SDS treatment and on freezing-thawing cycles is gentle enough to avoid DNA recovery from endospores, but may lead to an underestimation of difficult-to-lyse bacteria, such as Gram-positive bacteria (Ogram, 1998). Gram-positive bacteria accounted for 6% (Stackebrandt et al., 1993), 28% (Borneman et al., 1996), 15% (Rheims et al., 1996), or 7% (Kuske et al., 1997) of soil clones retrieved from different geographical locations. All the procedures used in these studies were based on direct cell lysis and on mechanical disruption with glass beads, except Rheims et al. (1996), who omitted the glass bead treatment. In our work, between 14% and 18% of clones from soil fractions (BS and RS) were related to Gram-positive bacteria, which is in agreement with the studies cited above.

The procedure of DNA extraction is thought to influence the formation of chimeric DNA. The advantage of gentle DNA extraction methods, which are not based on mechanical disruption of bacterial cells, is that they minimise DNA shearing, which may lead to a high frequency of chimeric amplicons (Ogram, 1998). Studies based on environmental 16S rDNA sequence analysis have detected <3.3% of chimeras (Liesack and Stackebrandt, 1992; Moyer et al., 1994; Borneman et al., 1996; Felske et al., 1998; Kuske et al., 1997; Rappé et al., 1998). Nevertheless, chimerae may artificially increase the bacterial diversity by generating new sequences, but the occurrence remains low.

The abundance of sequences related to the phylum of Gram-positive bacteria was significant in the BS and RS fractions and had the same magnitude as in other soil 16S rDNA clone library analyses (Stackebrandt et al., 1993; Borneman et al., 1996; Rheims et al., 1996). Half of the clones obtained from an acidic Drentse A grassland soil were affiliated to *Bacillus*, but this could be explained by the selectivity of this soil (Felske et al., 1998). As in our fractions, clones related to the *Cytophaga–Flexibacter–Bateroides* phylum were commonly found in an agricultural soil in Wisconsin (Borneman et al., 1996) and in an arid soil in Southwestern United States (Kuske et al., 1997). The presence in BS and RS fractions of clones falling into the *Holophaga–Acidobacterium* phylum confirms the ubiquity of this monophyletic group, which was defined by Ludwig et al. (1997) on the basis of 16S rDNA sequences retrieved from many geographical locations (Stackebrandt et al., 1993; Ueda et al., 1995; Kuske et al., 1997; Ludwig et al., 1997; Felske et al., 1998). The analysis of the sequences of this phylum indicated the existence of yet-uncultivated organisms phylogenetically related to *Acidobacterium capsulatum*, a chemo-organotrophic bacterium isolated from an acidic environment by Hiraishi et al. (1995). Member of this new line of descent are thought to rank among the most important metabolizers in soil (Felske et al., 1998). Clones clustered in the *Proteobacteria* were commonly found (Stackebrandt et al., 1993; Borneman et al., 1996; Felske et al., 1998), but no evidence for a dominance of gamma *Proteobacteria* was underlined by these authors.

Our work showed for both plants studied a clear selective effect of the roots towards the gamma *Proteobacteria*, to the detriment of the Gram-positive and *Holophaga–Acidobacterium* bacteria (Fig. 2, Table 2). If we neglect the occurrence of clones related to *Rhizobium leguminosarum* in RE fraction of *T. repens*, due to the well-known nodule symbiosis, the rhizoplane of both plants is dominated by *Pseudomonas*. Pseudomonads can play a role in the rhizosphere as plant growth promoting rhizobacteria (PGPR). They prevent the development of phytopathogens by secreting antibiotics, hydrogen cyanide, and enzymes that can lyse fungal cells, by competing with other micro-organisms for root colonisation, and by secreting siderophores which decrease iron available to other micro-organisms while improving plant iron assimilation. They also improve plant growth by synthesising phytohormones and by contributing to the plant mineral uptake (for a review, see Glick, 1995).

In this work, bacterial diversity assessment is based on sequence analysis of a 16S rDNA clone library. We are aware that DNA does not necessarily indicate the activity or the viability of organisms, contrary to the ribosomes, which appear to be useful markers of the microbial activity (Wagner, 1994). Felske et al. (1998) studied the phylogeny of the most active soil bacterial cells. In their work, the main group of 16S rDNA sequences, obtained after soil DNA extraction, cloning and sequencing, corresponded to the most active micro-organisms.

5. Conclusions

Soil bacterial communities are very complex. Of course, a complete description of these communities by the analysis of a 16S rRNA gene library of a few hundred clones is not possible. Nevertheless, soil microbial ecologists have successfully assessed diversity in studies devoted to restricted goals, such as the determination of new lines of descent (Rheims et al., 1996; Kuske et al., 1997; Ludwig et al., 1997), the most active physiological groups (Felske et al., 1998), or the influence of roots on the main bacterial clusters (Marilley et al., 1998). Although the limits of the method are numerous (due, for example, to sample handling (Rochelle et al., 1994) and to preferential amplification of specific templates, due either to PCR primer choice (Suzuki and Giovanonni, 1996), or to rRNA gene copy number (Farrelly et al., 1995)), the redundancy of the same main bacterial groups within soils of different geographical locations shows the accuracy of this approach. Our results confirm the presence of main soil bacterial groups and show by a culture independent approach that the plant roots decrease the microbial phylogenetic diversity by selecting micro-organisms related to *Pseudomonas*.

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Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA

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Abstract

The rhizosphere of *Trifolium repens* and *Lolium perenne* was divided into three fractions: the bulk soil, the soil adhering to the roots and the washed roots (rhizoplane and endorhizosphere). After isolation and purification of DNA from these fractions, 16S rDNA was amplified by PCR and cloned to obtain a collection of 16S rRNA genes representative of the bacterial communities of these three fractions. The genes were then characterized by PCR restriction analysis. Each different profile was used to define an operational taxonomic unit (OTU). The numbers of OTUs and the numbers of clones among these OTUs allowed to calculate a diversity index. The number of OTUs decreased as root proximity increased and a few OTUs became dominant, resulting in a lower diversity index. In the root fraction of *T. repens*, the restriction profile of the dominant OTU matched the theoretical profile of the 16S rRNA gene of *Rhizobium leguminosarum*. This study showed that plant roots create a selective environment for microbial populations.

Abbreviations: BS – bulk soil, OTU – operational taxonomic unit, RE – rhizoplane-endorhizosphere, RS – rhizospheric soil

Introduction

In soils, it is estimated that only 0.3% to 20% of the bacteria are culturable (Alexander, 1977; Torsvik et al., 1990). In the rhizosphere, this percentage is comprised between 1 and 10% (Campbell and Greaves, 1990). Consequently, any approach which avoids the cultivation of the microorganisms of a microbial population to determine their identity and diversity, will have the potential to become a powerful new tool for research in microbial ecology. A soil is not a homogenous environment, but rather a mixture of several habitats, each with its own trophic characteristics. This determines a mosaic of ecological niches with their specific microbial populations. A compartment of major interest is the rhizosphere, defined as the part of soil under the direct influence of plant roots. A significant part of the organic material photosynthesized in the aerial organs of the plant is released by the living roots into the soil, a process known as rhizodeposition. The matter deposited comprises, among others, freed cap cells, polysaccharides, soluble secretions and lysates (Whipps, 1990). Not only can these materials serve as substrates to nearby microorganisms, but they will also induce changes in the physicochemical characteristics of the surrounding soil, such as its acidity, moisture, electrical conductivity, redox potential (Lynch, 1990) and oxygen availability (Hojberg and Sorensen, 1993). These features will certainly influence the diversity of the soil microorganisms. Obtaining a better understanding of the bacterial communities in soil habitats (including the rhizosphere) is of crucial importance to assess the possible effects of environmental changes such as the release of ge-

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netically engineered microorganisms, soil pollution, climatic disturbances, and intensive farming.

Our objective in this study was to analyze, under field conditions, how the microbial diversity varied as a function of the root influence in plantations of *Lolium perenne* and *Trifolium repens*. To do this, we divided the soil into three fractions: the bulk soil, the soil adhering to the roots, and the washed roots (rhizoplane and endorhizosphere). Soil bacterial diversity has already been investigated with molecular methods (Bornemann et al., 1996; Liesack and Stackebrandt, 1992), but root proximity was not considered in these studies. Our methodology is based on the PCR restriction analysis (PRA) of a clone library of 16S rRNA genes from each fraction of the soil, without taking into account the phylogeny of the clones. Each new restriction profile is used to define a new operational taxonomic unit (OTU). The number of OTUs and the number of clones in each OTU allow the calculation of the Shannon diversity index H' (Shannon and Weaver, 1949).

Material and methods

Study site

The study site was located at 550 m above sea level in Eschikon, which is near Zürich, Switzerland. The soil description has already been published (Zanetti et al., 1996). The plots, which measured 2.8×1.9 m, consisted of monocultures of *Trifolium repens* cv. Milkanova and *Lolium perenne* cv Bastion. The crops were cultivated in field and fertilized with P_2O_5 (12 g m^{-2}), K_2O (29 g m^{-2}), Mg (1.6 g m^{-2}), and N (14 g m^{-2} y^{-1}). The swards were harvested four times a year.

Collection and partitioning of soil

Soil samples for DNA extraction were collected in October 1995 by removing for each plant one soil core 6 cm in diameter and 15 cm long from the *L. perenne* and *T. repens* monocultures. The cores were placed in plastic bags and kept at approximately 5 °C for a maximum of three hours before being processed. Once in the laboratory, each core was separated into three fractions. The bulk soil (BS) was the soil remaining after picking out the roots from the core. The rhizospheric soil (RS) fraction was the soil still adhering to the roots after gentle shaking. It was collected in a centrifuge tube containing 20 mL of a sterile physiological solution by vigorous agitation on a vortex mixer at high speed for 20 sec. During these two partitioning steps, the roots were observed under a stereomicroscope to ascertain that the various fractions were properly seperated. The rhizoplane-endorhizosphere (RE) fraction was obtained after washing the roots a second time in 20 mL of sterile physiological solution. The roots were placed on sterile absorbant paper to remove excess of the remaining water and pounded in a sterile mortar containing 20 mL of the physiological solution and a pinch of autoclaved quartz sand.

DNA extraction and purification

Soil DNA was extracted and purified by the procedure of Lee et al. (1996) with the modifications described below. Nucleic acids were extracted by a direct method using lysozyme digestion, SDS treatment and heat shocks. The RS and RE fractions were first centrifuged at 8,000 g for 10 min and the supernatant was discarded. 10 mL of washing buffer (120 m*M* sodium phosphate buffer pH = 8.0) was added to 50 mL centrifuge tubes containing 3 g (wet weight) of BS, or the RS and RE fractions. Subsequently, 8 mL of lysis solutions I (150 m*M* NaCl, 0.1 *M* EDTA pH = 8.0, 10 mg of lysozyme per mL) and II (100 m*M* NaCl, 500 m*M* Tris-HCl pH = 8.0, 10% w/v SDS) were added. The samples were frozen in a −70 °C ice-ethanol bath and thawed in a 65 °C water bath, and this freezing and thawing cycle was repeated three times (Tsai and Olson, 1991). The lysates were finally centrifuged at 7,000 g for 10 min.

For purification of the DNA 2.7 mL of 5 *M* NaCl and 2.1 mL of 10% w/v CTAB in 0.7 *M* NaCl were added to the supernatant of the mixture. The DNA solution was incubated at 65 °C for 10 min before an equal volume of chloroform-isoamyl alcohol (24:1) was added. An equal volume of 13% w/v polyethylene glycol in 1.6 *M* NaCl was added to the upper phase. The solution was kept on ice for 10 min and centrifuged at 25,000 g for 15 min at 4 °C. The pellet was washed with 70% ethanol, dried and dissolved in 750 μL of TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA pH = 8.0). 110 μL of 10 *M* ammonium acetate was then added to the DNA solution, which was placed on ice for 10 min, before being centrifuged at 11,000 g for 15 min. The supernatant was transferred to a clean tube and the DNA precipitated by adding an equal volume of isopropanol. The DNA was pelleted, washed with 70% ethanol and resuspended in

200 μL of TE buffer. Since *Taq* DNA polymerase may be inhibited by humic compounds (Steffan et al., 1988; Tebbe and Vahjen, 1993; Tsai and Olson, 1991), the CTAB, $CHCl_3$ and ammonium acetate steps were repeated once for extracts which still showed a dark brown color. The extracted DNA was checked by electrophoresis on a 0.8% horizontal agarose gel containing ethidium bromide (0.5 μg/mL), in 0.5X TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA pH = 8.3). Furthermore, the concentration and purity of the DNA was determined spectrophotometrically at 280 and 260 nm. If the DNA concentration was low and could not be measured spectrophotometrically (fractions RS and RE from *L. perenne*), the amount of DNA obtained was estimated in gel, by comparison of the subsequent bands with the bands of the DNA ladder. In the fractions where it could be measured (fraction BS from *L. perenne* and fractions BS, RS and RE from *T. repens*), the $A_{260/280}$ ratios ranged between 1.38 and 1.71.

16S rDNA amplification

The 16S rDNA was selectively amplified from the purified genomic DNA by PCR, using oligonucleotide primers designed to anneal to conserved positions in the 3′ and 5′ regions of bacterial 16S rDNA. The forward primer, UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTCA), corresponded to positions 11 to 26 of *Escherichia coli* 16S rRNA, and the reverse primer, UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTGTA), corresponded to the complement of positions 1411 to 1393 of *E. coli* (Brosius et al., 1978). The primers were supplied by Microsynth, Balgach, Switzerland.

The reaction conditions were as follows: 5 ng of template DNA, 5 μL of 10X PCR reaction buffer (750 m*M* Tris-HCl pH = 9.0, 200 m*M* $(NH_4)_2SO_4$, 0.1% Tween 20), 1.5 m*M* $MgCl_2$, 1 U *Taq* DNA polymerase (Eurogentech, Seraing, Belgium), 0.25 μM of each primer and 170 μM each of dATP, dCTP, dGTP, and dTTP were combined in a total reaction volume of 50 μL. To eliminate all risk of contamination by undesirable foreign DNA, the reaction mixture without the template DNA and *Taq* DNA polymerase was UV-irradiated for 30 min (Goldenberger and Altwegg, 1995). Amplification was carried out as follows: a preliminary denaturation step was done at 95 °C for 5 min, followed by 35 cycles of 30 sec at 94 °C (denaturation), 30 sec at 62 °C (except for the three first touchdown cycles, which were successively at 68, 66 and 64 °C), and 1 min at 72 °C (extension). Negative controls, with no addition of template DNA, were systematically included. PCR products were checked by electrophoresis in 1.3% agarose gels as described above. If no PCR product was obtained, which can occur due to inhibition of the *Taq* DNA polymerase by humic acids, the amplification was repeated using 500 or 50 pg of template DNA.

Construction of bacterial 16S rDNA library

PCR products were excised from 2% low melting agarose (Sigma, St-Louis, MO) and the DNA was purified using a Geneclean II kit (Bio 101 Inc., La Jolla, CA). Purified amplicons were then ligated into a pGEM-T vector and competent *E. coli* cells were transformed according to the manufacturer's instructions (Promega corp., Madison, WI).

Plasmid preparations of 35 randomly picked colonies of transformed *E. coli* were performed by using the alkaline lysis method followed by chloroform extraction (Birnboim and Doly, 1979; Sambrook et al., 1989).

PCR restriction analysis of 16S rDNA

The 16S rDNA genes ligated into the pGEM-T vector were amplified by PCR as described above. As controls to ascertain that *E. coli* 16S genomic genes were not amplified instead of the inserts, we used plasmids preparations from *E. coli* colonies, either non-transformed, or transformed by pGEM-T vector without insert. Two tetrameric enzymes were used separately for the 16S rDNA restrictions. Aliquots (4 μL) of the PCR products were digested in 20 μL reaction volumes, either with 2 U *Rsa*I restriction endonuclease (Amersham, Bucks., UK), or with 6 U of *Taq*I (New England Biolabs Inc., Beverly, MA), using the manufacturer's recommended buffers and temperatures. The restriction fragment were then separated by gel electrophoresis in 2.5% agarose gels, as described above. Fragments shorter than 80 bp were not taken into consideration, because they were too near the detection threshold. The more abundant clones were digested as described above with 2 U of an additional endonuclease, *Hha*I (Gibco BRL, Eggenstein, Germany).

Calculation of diversity index

The clones with identical patterns for both restriction profiles were grouped in the same OTU. The number

Table 1. Shannon diversity indexes

Soil fractions	*Lolium perenne*	*Trifolium repens*
Bulk soil	1.42	1.42
Rhizospheric	1.34	1.31
Endorhizosphere and rhizoplane	1.15	0.99

of OTUs and the number of clones in each of them were used to calculate the Shannon diversity index, H' (Shannon and Weaver, 1949).

Results and discussion

Stereomicroscopic observation of the roots throughout the partitioning of the soil fractions showed that the rhizospheric soil still adhered to the roots after the root-soil separation. It consisted of a sheath surrounding the roots in *L. perenne* and of discrete soil particles adhering to the roots in *T. repens*. This demonstrated that this fraction was under the direct influence of the root. We observed that the washing step (agitation with a vortex mixer at high speed) did not break the root ends and that the roots were free of soil from the RS fraction after this treatment.

To construct the bacterial 16S rDNA library, a total of 35 colonies positively transformed were initially chosen for each fraction. However, among the 210 plasmids extracted and purified, 6 were shown to have the same size as the pGEM-T vector when they were run on an agarose gel. They did not contain the insert and had to be discarded. Twenty-nine plasmid preparations from each fraction were then arbitrarily selected (amounting to a total of 174 clones) and used to amplify the 16S rDNA. None of the obtained profiles found in this study resembled the expected *E. coli* 16S rDNA digest. In recent literature, quite different numbers of clones were utilized to assess bacterial diversity in natural habitats, ranging from 12 to 124 (Benlloch et al., 1995; Boivin-Jahns et al., 1996; Borneman et al., 1996; Moyer et al., 1994). As this study focused mainly on a comparison between bacterial diversity from multiple soil fractions, the use of 29 clones per sample appeared reasonable.

For 3 of the 174 clones, the analysis of the 16S rDNA profiles showed that the sum of the band-sizes of the restriction fragments was 2800 bp. These clones were considered double-vector transformants (Weston et al., 1981), discarded and replaced. Among the 6 fractions, a total of 120 OTUs were identified, 62 in

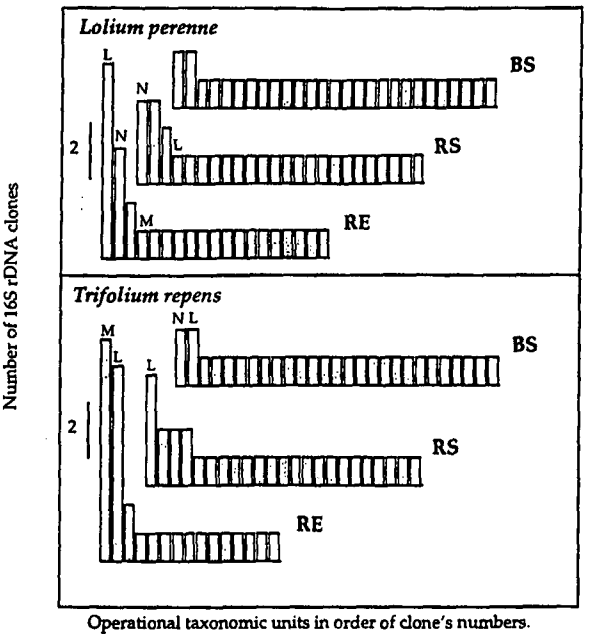

Figure 1. Distribution of OTUs of bacterial 16S rDNA clones from *L. perenne* and *T. repens* plantings. The soils were partitioned in three fractions: rhizoplane and endorhizosphere (RE), rhizospheric soil (RS) and bulk soil (BS). Letters above some of the bars identify dominant OTUs, referred to in the text.

T. repens samples and 66 in *L. perenne* samples. 7 OTUs were common to *L. perenne* and to *T. repens* samples. The distribution of the 174 clones among these OTUs is shown in Figure 1. Clearly dominant OTUs appeared in the RE and RS fractions. These OTUs were called L, M and N. OTU M was dominant in the RE fraction of *T. repens*, OTU L in the RE and RS fractions of *T. repens* and in the RE fraction of *L. perenne*. OTU N was found in the RE and RS fractions of *L. perenne*. The Shannon diversity indexes were then calculated (Table 1). In both plants, they decreased as the proximity to the roots increased.

The clustering of clones into OTUs was based on the use of two tetrameric restriction enzymes. Moyer et al. (1996) determined the number of taxa, that could be differentiated by a computer-simulated restriction profile analysis, among a selection of 106 16S rDNA sequences belonging to the domain of *Bacteria*, and deposited in the Ribosomal Database Project (Maidak et al., 1994). By combining two tetrameric restriction enzymes, it was possible to discriminate >96% of the taxa. Among the undiscriminated taxa, the median sequence similarity was 95.6%. Usually, organisms with more than 97% sequence identity have DNA-DNA reassociation values of 70% or more (Stackebrandt and Goebel, 1994). This value is used as a key criterion in

defining a species. Therefore, the use of two tetrameric enzymes, even if it does not provide an exhaustive discrimination, allows a good appraisal of the diversity. In this work, the occurrence of chimeric rDNA (Kopczynski et al., 1994; Liesack et al., 1991) was not assessed, because the probability of generating biases in this way is less than 2% (Bornemann et al., 1996; Liesack and Stackebrandt, 1992; Moyer et al., 1994).

Figure 1 clearly shows the presence of dominant OTUs in the rhizospheric and still more in the rhizoplane-endorhizosphere fractions. To improve the comparison of the restriction profiles of the dominant OTUs L, M and N (Figure 1) with theoretical restriction profiles calculated from 16S rDNA sequences available from the EMBL nucleotide sequence database, we used a third tetrameric restriction enzyme (*HhaI*). The combination of three tetrameric restriction enzymes detects 99% of the taxa restricted by the computer-simulated analysis of Moyer et al. (1996). The restriction profiles of the OTU M matched the theoretical profiles calculated from the 16S rDNA of *R. leguminosarum* (EMBL # X67233). The combination of the three endonucleases used discriminate *R. leguminosarum* between the seven other species of *Rhizobium*, various *Rhizobium* spp., *Bradyrhizobium japonicum* and *Bradyrhizobium* spp., and *Agrobacterium tumefaciens* and *A. rhizogenes* (Laguerre et al., 1994). This confirms the hypothesis that the method is well adapted to the study of soil microbial populations, because the RE fraction of *T. repens* is composed of nodules which are the outcome of the symbiosis with *R. leguminosarum* (for a review, see Van Rhijn and Vanderleyden, 1995).

As shown in Figure 1, the bacterial diversity increased with root distance. The BS diversity is very high. Indeed, with 29 clones, the maximum possible value for the Shannon diversity index is 1.46 (in the case where each clone forms a unique cluster of restriction profiles). We obtained 1.42 for both BS fractions. The dominance of some OTUs in the RS fractions can be explained by the rhizodepositions. It is estimated that 10 to 40% of all the carbon assimilated by plants is released into the soil in the form of root secretions (Van Veen et al., 1991; Whipps, 1990). The composition of these secretions is not well documented, but it is known that several organic compounds act as chemoattractants for rhizosphere bacteria at very low concentrations (Van Overbeek and van Elsas, 1995). In the rhizosphere, bacteria find an important source of nutrients. Several authors have suggested that these growth substrates increase the microbial biodiversity in the rhizosphere (Campbell and Greaves, 1990; Gilbert et al., 1996), but this is not in agreement with our work. The decrease in bacterial diversity was most pronounced between the RS and RE fractions (Figure 1). The RE fraction, in fact, did not consist of soil, but of a mixture of the rhizoplane and root webs. The colonization of this particular habitat by bacteria is subject to many constraints, requiring that the bacteria be capable of adhering to the root surface and penetrating the root webs. The rhizoplane and endorhizosphere are more selective than the rhizospheric soil, where microbial populations react only to trophic and chemotactic stimuli.

Molecular methods offer new tools for the understanding of microbial diversity and distribution. Although the limits of the method are numerous (due, for example, to yet unquantified differences in the extractability of DNA from different bacteria, to differences in their amplificability, to biases during the ligation step), our results showed that microbial diversity clearly varied as a function of plant-root proximity. The sampling was performed in October to analyse the bacterial populations which had set up during the growing season. This approach appears to be a promising tool for monitoring changes in bacterial communities due to external factors. Sequencing of the main clones of this library is now in progress. It will bring about a better understanding of the phylogenetic relations within these microbial communities.

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Influence of an Elevated Atmospheric CO_2 Content on Soil and Rhizosphere Bacterial Communities Beneath *Lolium perenne* and *Trifolium repens* under Field Conditions

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ABSTRACT

The increase in atmospheric CO_2 content alters C_3 plant photosynthetic rate, leading to changes in rhizodeposition and other root activities. This may influence the activity, the biomass, and the structure of soil and rhizosphere microbial communities and therefore the nutrient cycling rates and the plant growth. The present paper focuses on bacterial numbers and on community structure. The rhizospheres of two grassland plants, *Lolium perenne* (ryegrass) and *Trifolium repens* (white clover), were divided into three fractions: the bulk soil, the rhizospheric soil, and the rhizoplane–endorhizosphere. The elevated atmospheric CO_2 content increased the most probable numbers of heterotrophic bacteria in the rhizosphere of *L. perenne*. However, this effect lasted only at the beginning of the vegetation period for *T. repens*. Community structure was assessed after isolation of DNA, PCR amplification, and construction of cloned 16S rDNA libraries. Amplified ribosomal DNA restriction analysis (ARDRA) and colony hybridization with an oligonucleotide probe designed to detect *Pseudomonas* spp. showed under elevated atmospheric CO_2 content an increased dominance of pseudomonads in the rhizosphere of *L. perenne* and a decreased dominance in the rhizosphere of *T. repens*. This work provides evidence for a CO_2-induced alteration in the structure of the rhizosphere bacterial populations, suggesting a possible alteration of the plant-growth-promoting-rhizobacterial (PGPR) effect.

Introduction

An increase in atmospheric CO_2 content alters functioning of soil ecosystems. Nevertheless, the major influence of a doubling of CO_2 atmospheric concentration on soil microbial communities is indirect because CO_2 concentration in soil is greater than 0.1 kPa [66], whereas the current atmospheric content is 0.035 kPa. Consequently, the main influence is thought to occur by the intermediation of the plants. Indeed, because of the competitive inhibition by O_2 of the ribulose-1,5-bisphosphate carboxylase, which is responsible

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for CO_2 fixation during photosynthesis [7], the C_3 plant photosynthetic rate increases under a CO_2-enriched atmosphere [28, 47, 51]. An increased root biomass under CO_2 enrichment has been shown by several authors [25, 30, 47, 52, 53].

Soil bacterial communities probably play a key role in the response to the elevated atmospheric CO_2. Under normal conditions, 12 to 54% of the carbon fixed by photosynthesis is released into the soil by the roots [26, 27, 36, 46]. Lekkerkerk et al. [32] showed that the root-derived, easily biodegradable compounds of wheat roots increased under a CO_2-enriched atmosphere. Van Veen et al. [66] suggested quantitative and qualitative changes in rhizodeposition linked to CO_2 enrichment. Since rhizodeposition consists mainly of organic matter, these changes may influence the biomass, activity, and structure of the bacterial community, leading to changes in soil processes involving plant roots, microorganisms, and soil physico-chemical properties.

Studies on bacterial numbers and biomass in the rhizosphere and under CO_2 enrichment are not well documented. Zak et al. [70] showed an increased bacterial biomass in the soil and the rhizosphere of *Populus grandidentata*. Schortemeyer et al. [57] found in samples taken in the spring a positive effect of the elevated CO_2 on the bacterial numbers in the rhizosphere of ryegrass, but a negative effect in the rhizosphere of *T. repens*. On the other hand, Runion et al. [54] detected no changes in bacterial numbers in the rhizosphere of cotton, and inconsistent results were found by Whipps [68] in *Zea mays* and by O'Neill et al. [43] in *Liriodendron tulipifera* rhizospheres. Studies are still needed to better understand the influence of the increased atmospheric CO_2 on soil bacterial processes, and therefore on plant growth. According to Diaz et al. [17], the elevated CO_2 atmospheric content leads to an increase in substrate release into the rhizosphere. This induces a mineral nutrient sequestration by an expanding microbiota and a consequent limitation of plant growth. According to Cardon [12], the influence is also linked to the nutrient status of the soil. On the other hand, Clarholm et al. [15] and Zak et al. [70] suggested an increased protozoan grazing due to an enhanced carbon availability and therefore an increased mineralization and plant nutrient availability.

Plant growth may also be influenced directly by the rhizobacterial populations through altered plant-growth promoting effects. Even though the recent emergence of molecular methods applied to soil microbial ecology allows better description of soil and rhizosphere microbial communities [4, 9, 18, 35, 37, 38, 50, 60], there are limited data dealing with the effects of elevated CO_2 on the structure of rhizosphere microbial populations. Based on phospholipid fatty acid profile analysis of total extractable bacteria, Zak et al. [71] found no CO_2 influence on the microbial community in soils planted with *Populus grandidentata;* nor did Griffiths et al. [22] find such as influence for *L. perenne* and *Triticum aestivum* by using broad-scale DNA techniques. Other works have focused on particular groups of microorganisms. In the rhizosphere of *T. repens*, the population of *Rhizobium leguminosarum* biovar *trifolii*, determined by MPN plant infection assay of *T. repens* seedlings, was increased twofold under CO_2 enrichment [57]. Zanetti et al. [74] reported an increase in symbiotic nitrogen fixation activity for *T. repens* growth under enriched CO_2 atmosphere. The elevated CO_2 had no effect on population of nitrifiers in the rhizospheres of *L. perenne* and *T. repens* [57] and *Liriodendron tulipifera* [43].

Our main objective in this study was to analyze, with a culture-independent approach and under field conditions, how the bacterial community structure was influenced by an elevated atmospheric CO_2 content in swards of *Lolium perenne* and *Trifolium repens*. To do this, we divided the soil into three fractions: the bulk soil, the soil adhering to the roots, and the washed roots (rhizoplane and endorhizosphere). The first stage of our methodology was based on amplified ribosomal DNA restriction analysis (ARDRA) and on the phylogenetic sequence analysis of cloned 16S rRNA genes retrieved from soil and rhizosphere fractions, allowing us to determine the main bacterial groups involved in the CO_2 response. Colony hybridization with a PSM_G oligonucleotide probe served in a second stage to show the influence of the CO_2 enrichment on the population of *Pseudomonas* spp. Moreover, a cultural approach was used to determine the influence of the CO_2 enrichment on the numbers of heterotrophic bacteria.

Materials and Methods

Study Site and CO_2 Fumigation

The study site was located at 550 m above sea level in Eschikon, canton Zürich (Switzerland). It consisted of three control (C1, C2, C3: ambient pCO_2) and three fumigated (F1, F2, F3: 60 Pa CO_2) open-air rings, situated at least 100 m apart and 18 m in diameter. Free-air-CO_2-enrichment (FACE) technology [33] was used to release CO_2 into the open field rings during the photoperiod. The experiment [25, 74] started at the end of May 1993 and lasted during the entire growing seasons. Monocultures of *Lolium perenne* cv Bastion and *Trifolium repens* cv Milkanova were cultivated in

plots measuring 2.8 × 1.9 m and fertilized with P_2O_5 (12 g m^{-2} yr^{-1}), K_2O (29 g m^{-2} yr^{-1}), Mg (1.6 g m^{-2} yr^{-1}), and N (14 g m^{-2} yr^{-1}). To counterbalance differences in nutrient availability, the amount of P–K–Mg fertilizers was increased by 35% in rings C3 and F3. The swards were harvested four times a year in 1995 and five times in 1996 and 1997. The soil was a fertile, eutric cambisol with pH (H_2O) values between 6.5 and 7.6 and consisted of 28% clay, 33% silt, 36% sand, and between 2.9% and 5.1% organic matter [25].

Soil Sampling and Preparation of Rhizosphere Fractions

Soil sampling and partitioning were performed as described by Marilley et al. [38]. Soil cores were collected from *L. perenne* and *T. repens* monocultures and transported cooled to the laboratory. Within the same day, soil cores were separated into three fractions: the bulk soil (BS), the rhizospheric soil (RS), and the rhizoplane–endorhizosphere (RE) [38]. The fractions were then placed at 4°C for the cultural methods and at −20°C for the DNA extractions.

Enumeration of Heterotrophic Bacteria

Samples for the determination of most probable numbers (MPNs) of cultivable heterotrophic bacteria were collected in 1995 and 1996. Inoculations were performed the day after the soil sampling and partitioning. 10 g of BS fraction and 5 ml of homogenized suspensions of RS and RE fractions were added to 90 ml and 5 ml, respectively, of a sterile 0.9% (w/v) NaCl solution. The dry weight of BS fraction was measured after an overnight incubation in a vacuum oven at 70°C. The values were the mean of two 10 g fresh weight subsamples. The dry weight of fraction RS was determined by drying 10 ml of the rhizospheric soil suspension at 70°C during 2 days. The MPN of heterotrophs from fraction RE was displayed as function of the fresh weight of roots measured before the pounding step [38].

The suspensions were shaken at 150 rpm for 30 min at room temperature. The samples were then tenfold serially diluted in a medium containing ionic concentrations of nutrients similar to those found in the soil solution [2]. This culture medium was modified and the following composition was used: 0.2 g L^{-1} NH_4NO_3, 0.69 g L^{-1} $CaSO_4$, 0.406 g L^{-1} $MgCl_2$ and 2 g L^{-1} yeast extract. To this solution, 1 ml of trace element solution [3], 110 µl of Fe/EDTA solution (1.4 g L^{-1} Na_2EDTA, 5 g L^{-1} $FeSO_4 \cdot 7H_2O$, and 0.05% (v/v) concentrated H_2SO_4), 680 µl of KH_2PO_4 0.1% (w/v), 0.5 ml of 1 M KOH, and 40 ml of 0.5 M Tris-HCl (pH 7.0) were added to a final volume of 1 L. After autoclaving, the medium was supplemented with a filter-sterilized solution of glucose to obtain a final concentration of 1 g L^{-1}. MPN of heterotrophic bacteria obtained on this medium was increased more than fivefold in comparison with the MPN obtained on 4 g L^{-1} nutrient broth. Eight parallel 200 µl microplate wells were filled with each dilution. No growth occurred in the absence of yeast extract and glucose. Wells containing noninoculated medium served as a control of cross-contamination. Microtiter plates were incubated at 15°C for 5 days and wells were scored positive when a bacterial pellet was visible. MPN values were calculated using the program of Schneider [56].

Construction of 16S rDNA Libraries

Soil and rhizosphere DNA was extracted and purified following the method described by Ogram [45]. Microorganisms were lysed by a direct procedure based on a lysozyme digestion, SDS treatment, and heat shocks. DNA was then purified by CTAB treatment, extraction with chloroform, centrifugation in polyethylene glycol, and ammonium acetate precipitation. Since *Taq* DNA polymerase may be inhibited by humic compounds [61, 62], the CTAB, $CHCl_3$, and ammonium acetate steps were repeated for extracts that still showed a dark brown color and for those that were difficult to amplify by PCR.

The amplification by PCR of 16S rDNA and the construction of cloned 16S rRNA gene libraries have been already described by Marilley et al. [38]. The amplicons were purified from low-melting agarose gels and cloned in pGEM-T linear vector (Promega Corp., Madison, WI) and *Escherichia coli* XL1 competent cells.

Amplified Ribosomal DNA Restriction Analysis

After construction of 16S rDNA libraries, plasmids were extracted from overnight cultures of transformed *E. coli* by using the alkaline lysis method followed by chloroform extraction [55]. Twenty-nine cloned 16S rRNA genes per fraction were then amplified by PCR and digested as described by Marilley et al. [38] using 2 U of the restriction enzymes *Hae*III and *Rsa*I, and 6 U of *Taq*I. The clones with identical restriction profiles with the three endonucleases were clustered in the same operational taxonomic unit (OTU).

Colony Hybridization

The construction of the clone libraries was performed as described above, except for slight modifications: four to eight 20 µl PCR products were pooled, precipitated with ethanol following standard protocols [55], and resuspended in 20 µl of autoclaved TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). PCR products were then purified using a QiaexII Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. The purified amplicons were resuspended in 20 µl of sterile distilled water before being ligated in the pGEM-T vector (Promega).

About 90 transformed *E. coli* XL1 colonies were randomly picked and inoculated with sterile toothpicks on LB agar [55] supplemented with 150 µg ml^{-1} ampicillin. To control the size of the ligated inserts, 24 plasmid preparations per sample were performed and separated by electrophoresis on a 0.8% (w/v) agarose gel. Transfer of bacterial colonies to Hybond-N^+ nylon membranes (Amersham, Buckinghamshire, UK) was performed by standard methods [55]. Filters were then air-dried and the DNA fixed by an UV exposure of 70 J cm^{-2} at 254 nm. To remove bacterial cell debris from colony blots, membranes were washed for 1 h at 42°C in a solution containing 2× SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, and 2.5 mM EDTA [pH 7.4]), 0.1% (w/v) SDS, and 100 µg ml^{-1} pronase, followed by an incubation for 30 min at 65°C in 2× SSPE and 0.1% (w/v) SDS.

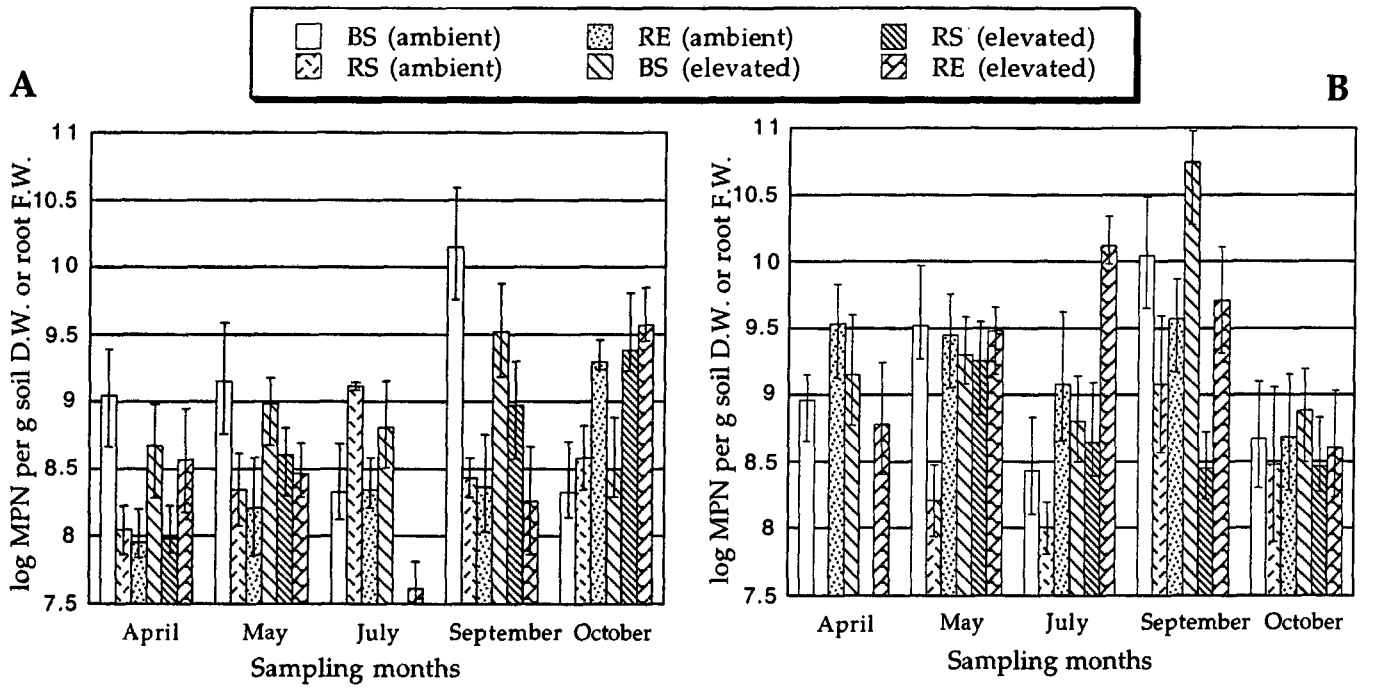

Fig. 1. Most probable numbers (MPNs) of heterotrophic bacteria in BS, RS, and RE fractions of (A) *Lolium perenne* and (B) *Trifolium repens* monocultures under ambient and elevated atmospheric CO_2. MPNs in CO_2-enriched RS fraction of *L. perenne* (July) and in fractions RS of *T. repens* (April) were not determined. BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere. Error bars are confidence intervals (0.05) with 10,000 bootstraps.

The oligonucleotide probe used was PSM_G (5'-CCTTCCTCC-CAACTT-3'; *E. coli* positions 440 to 454), which is complementary to a region of the 16S rDNA conserved in the genus *Pseudomonas* and a few other bacteria [1, 10]. It was synthesized and HPLC purified by Microsynth (Balgach, Switzerland) following standard protocols. The oligonucleotide was labeled using the DIG Oligonucleotide 3'-End Labelling Kit (Boehringer Mannheim, Penzberg, Germany), according to the manufacturer's instructions.

Membranes were prehybridized for 1 h at the hybridization temperature (36°C) in 5× SSC (750 mM NaCl and 75 mM sodium citrate [pH 7.0]), 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosin, and 1% (w/v) blocking reagent (Boehringer). Hybridization was performed overnight in the solution described above supplemented with 1 pmol ml^{-1} of the oligonucleotide probe. After two low-stringency washes for 5 min at room temperature in 2× SSC and 0.1% (w/v) SDS, membranes were washed twice for 15 min at the hybridization temperature in 5× SSC and 0.1% (w/v) SDS. The hybridized probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase (Fab fragments, Boehringer) and visualized with the colorimetric substrates NBT and BCIP, as described in the manufacturer's protocol (Boehringer).

Results

Enumeration of Heterotrophic Bacteria

Elevated atmospheric CO_2 did not influence bacterial numbers in the BS fractions of *L. perenne* and *T. repens* (Fig. 1). Numbers of heterotrophs in the RS fraction of *L. perenne* were increased under CO_2 fumigation, reaching a sixfold increase in October. In the RS fraction of *T. repens*, the results showed an increase in samples retrieved in May and July, but not at the end of the growing season, in September and October. Elevated CO_2 had no influence on heterotrophic bacteria in the RE fraction of *L. perenne*, in which differences between control and enriched samples were inconsistent. Heterotrophic bacteria were fivefold less numerous under enrichment in RE fraction of *T. repens* (April) but were tenfold increased in July; MPNs performed in September and October did not show any CO_2 influence.

Community Structure

To construct the 16S rDNA libraries, 29 clones for ARDRA (sampling in 1995) and approximately 90 clones for colony hybridization (sampling in 1997) were arbitrarily selected for each fraction. Clones retrieved from ring C1 (ambient pCO_2) in 1995 were partially sequenced and phylogenetically assigned to the closest 16S rDNA sequence in the EMBL, allowing calculation of a frequency of chimera formation of 4.5% [37], which is in the same order of magnitude as those given in other works [9, 18, 41, 49]. The ligation and trans-

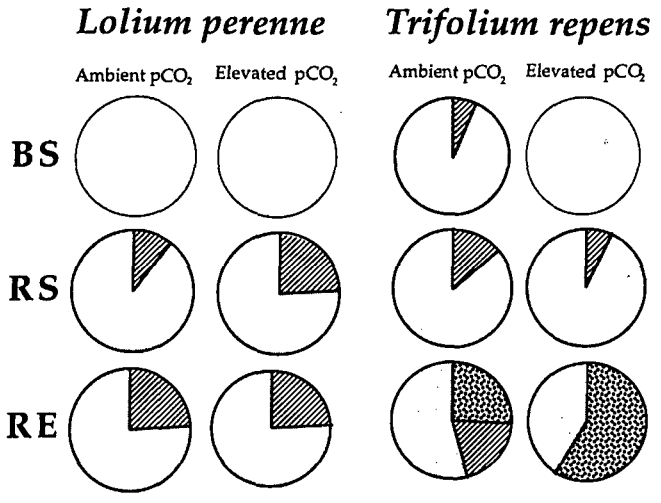

Fig. 2. Distribution of 16S rDNA operational taxonomic units (OTUs) retrieved from *Lolium perenne* and *Trifolium repens* monocultures based on restriction profile analysis with *Hae*III, *Rsa*I and *Taq*I. (▨), clones phylogenetically related to *Pseudomonas* spp. (▩), clones phylogenetically related to *Rhizobium leguminosarum*. BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere.

formation steps performed for the colony hybridization were controlled by agarose gel electrophoresis of 24 plasmids per fraction. Of the analyzed clones, 6.3% were shown to have an unexpected size.

Soil samples for ARDRA were collected in October 1995 from rings C1 and F1. Clones showing identical restriction profiles with the three restriction enzymes used served to define OTUs. The frequency of clones phylogenetically related to *Pseudomonas* spp. increased as the proximity to the roots of *L. perenne* and of *T. repens* (ambient pCO_2) increased (Fig. 2). The RE fractions of *T. repens* were dominated by clones related to *Rhizobium leguminosarum*. The elevated atmospheric CO_2 content led to a greater dominance of *Pseudomonas* spp. in the RS and RE fractions of *L. perenne*, while their dominance decreased under CO_2 enrichment in the RS and RE fractions of *T. repens*. The presence of clones related to *R. leguminosarum* increased under CO_2 enrichment in the RS and in the RE fractions of *T. repens*.

Samples for detection of *Pseudomonas* spp. were collected in October 1997 from rings C1, C2, C3, F1, F2, and F3. Their frequency was assessed by colony hybridization of the 16S rDNA clone libraries with the oligonucleotide probe PSM_G (Fig. 3). The specificity of the hybridization was controlled on the 174 16S rDNA clones retrieved in 1995 from ring C1. The phylogenetic position of the above-mentioned OTUs was determined after partial sequencing [37]. Among these 174 clones, 34 had restriction profiles matching those of clones showing more than 92% of homology with fluorescent pseudomonad 16S rDNA sequences. The hybridization signals were positive with these 34 clones, and negative with all the others. Moreover, *Pseudomonas synxantha* (LMG 2190), *Pseudomonas synringae* (LMG 5066), *Pseudomonas putida* (LMG 2257), and *Pseudomonas fluorescens* (DSM 2005) positively hybridized, contrarily to *Escherichia coli* XL1.

The results of the detection of *Pseudomonas* spp. by colony hybridization showed a selective rhizosphere effect toward pseudomonads in both plants, except for the RE fraction of *T. repens*, where their frequency decreased. The CO_2 enrichment led to an increased presence of *Pseudomonas* spp. in *L. perenne* fractions. In fractions of *T. repens*, their frequency decreased as the CO_2 fumigation was applied.

Discussion

In this study, we focused on *L. perenne* and *T. repens* mainly to compare our data with other studies performed on the FACE field experiment in Eschikon (Switzerland) [23, 25, 29, 30, 42, 57, 72, 74]. Moreover, the plants have different rhizosphere structures. *L. perenne*, a grass, lacks symbiotic nitrogen fixation but establishes a rhizospheric sheath surrounding its roots, whereas rhizospheric soil of *T. repens* consists of discrete soil particles adhering to the roots.

Impact of Elevated CO_2 on Soil Heterotrophic Bacteria

Our data did not show any CO_2 influences on the most probable numbers of heterotrophic bacteria in the BS fractions (Fig. 1). In a prairie ecosystem such as the FACE facility in Eschikon, soil carbon input is mainly the result of dead plants and rhizodeposition. Jongen et al. [30] measured in the FACE study site of Eschikon an increased C:N ratio of *L. perenne* roots grown in monoculture at elevated pCO_2, while they detected no changes in the C:N ratio of clover roots. From the perspective of microbial numbers, Zak et al. [70] suggested that an increased carbon input into the soil could be counterbalanced by an increased C:N ratio, leading to unchanged microbial numbers. It is likely that in a grass monoculture under elevated CO_2 the availability of soil mineral nitrogen decreases because of the increased C:N ratio of plant-derived organic matter introduced into the soil [24].

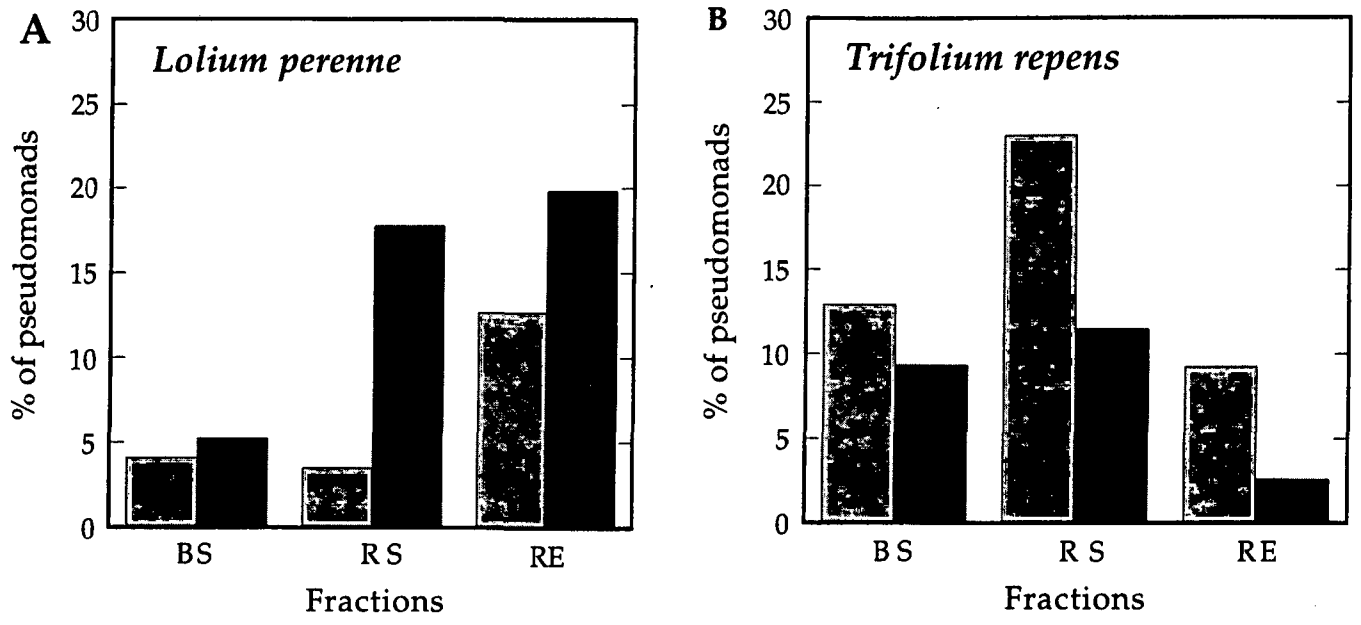

Fig. 3. Frequency of *Pseudomonas* spp., as determined by colony hybridization with the PSM_G oligonucleotide probe of 16S rDNA clones retrieved from *L. perenne* and *T. repens* monocultures. Data presented are the mean of three replica fractions. (▣), ambient pCO_2; (■), elevated; BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere.

Diaz et al. [17] showed a nutritional limitation of plant growth due to CO_2-induced mineral nutrient sequestration by expanding microbiota. However, the symbiotic nitrogen fixation occurring in clover roots could counterbalance the mineral nutrient limitation. Indeed, the relative contribution of symbiotically fixed N to the total plant nitrogen increased with increasing pCO_2 [59, 72, 74].

One may also expect an increased root production under elevated pCO_2 [23], but CO_2-induced changes in the quality of the root material and an increased C sequestration into soil organic matter, as reported for the experiment in Eschikon [29, 42], may lead to unaltered microbial numbers in BS fractions. These CO_2-unaltered MPN values may also suggest that the size of the microbial population is regulated through predation by soil protozoa [70].

Impact of Elevated CO_2 on Heterotrophic Bacteria in the Rhizosphere

In the rhizosphere, bacterial populations are under the direct influence of rhizodeposition, which consists mainly of soluble secretions, cell lysates, polysaccharides, and sloughed-off cells [68]. Rhizosphere bacterial populations are supplied in this particular soil habitat with easily degradable organic compounds. Our data (Fig. 1) showed under CO_2 enrichment a consistent increase in the most probable numbers of heterotrophs inhabiting rhizospheric soil surrounding *L. perenne* roots. This result is explained by an increased amount of CO_2-induced rhizodeposition [47, 52, 66]. Qualitative changes in the composition of rhizodeposition may also be an explanation, but this feature is not well documented. Lekkerkerk et al. [32] showed under CO_2 enrichment an increase in easily digestible organic compounds released from wheat roots. Although results on microbial numbers are scarce and inconsistent [43, 67], Schortemeyer et al. [57] reported in the FACE fields in Eschikon a positive CO_2 effect in the rhizosphere of *L. perenne* sampled in May, but not in November. Our data provide evidence for a CO_2-induced increase in bacterial numbers in the rhizospheric soil, but not in the rhizoplane–endorhizosphere.

MPN values of heterotrophs in the RS fraction of *T. repens* were increased under CO_2 enrichment in samples retrieved in May and July, but not in September and October (Fig. 1). This result is not in agreement with the trends obtained in the Eschikon FACE field experiment by Schortemeyer et al. [57], who found a weakly significant decrease in heterotrophic microbial numbers. However, these authors retrieved rhizospheric soil in 1994. We suggest that *T. repens* root exudation enhanced microbial chemotaxis and growth at the beginning of the plant growing season. Gaworzewska

and Carlile [20] showed a positive chemotaxis of bacteria toward root exudates. We are aware that additional data are needed to confirm this hypothesis.

Since it is likely that the plant requirement for symbiotic nitrogen fixation increases under CO_2 enrichment, the exudation of root-mediated molecular signals, such as flavonoids and isoflavonoids, should also be enhanced under elevated pCO_2 at the beginning of the growing season. These signals act as chemoattractants at the first stage of the establishment of the *Rhizobium*–clover symbiosis [14, 65], explaining the positive CO_2 effect on bacteria numbers in the RS fraction and the tenfold increase in the RE fraction retrieved in July. In this latter fraction, bacterial counts are mainly assigned to *Rhizobium leguminosarum* counts because the *Trifolium–Rhizobium* symbiosis is highly specific [14, 65] and because the bacteroids are present in high numbers in nodules. These results are in agreement with those obtained by Schortemeyer et al. [57]. These authors showed in the rhizosphere of *T. repens* a twofold increase in *R. leguminosarum* counts in samples retrieved in May and in November. Moreover, in the same FACE study site, Zanetti et al. [74] showed that total N yield increased significantly in *T. repens* under elevated CO_2 and was assigned to an increased formation of nodules [73].

Because of the incubation conditions (5°C for 5 days), our results are representative of the soil bacterial fraction that is composed of r-strategists. Their increased presence in the RS fraction of *L. perenne* does not suggest an increase in the numbers of the whole bacterial community, even though the ratio of cultivable bacteria is enhanced in the rhizosphere of plants [5, 58].

Impact of Elevated CO_2 on the Microbial Community Structure

Molecular methods based on 16S rDNA analysis have successfully been used to describe bacterial community structure either after ARDRA of clones retrieved from an active hydrothermal vent system [41], from hypersaline environments [39], and from thermophilic compost piles [8], or after partial sequencing and phylogenetic assignments [9, 18, 60]. This allowed, for example, demonstration of the ubiquity of as-yet only molecularly detected soil bacteria, with DNA sequences related to the *Holophaga/Acidobacterium* phylum [35], or detection of new actinomycete lines of descent [50].

Even though molecular methods have the powerful advantage of bypassing the limitations linked to the low ratio of cultivable bacteria in soil and rhizosphere [11, 63], they are biased by several factors due, for example, to differences in the lysis efficiency between soil microorganisms, to the formation of chimeric DNA during PCR amplification, to the effect of genome size and *rrn* gene copy number, and to differences in PCR amplification between bacteria (for a review, see [69]). From the perspective of this work, molecular biases are discussed in previous papers [37, 38]. Moreover, we deliberately chose not to control all the clones obtained during the construction of the 16S rDNA clone library performed in 1997 because we assume that it is preferable to analyze large clone libraries with a small percentage of misligated clones than a reduced number of clones, all containing the expected insert.

The results obtained after ARDRA and after colony hybridization with the *Pseudomonas* spp. targeted oligonucleotide probe PSM_G clearly showed a CO_2-induced increased and, respectively, decreased dominance of pseudomonads in the rhizosphere fractions of *L. perenne* and *T. repens* retrieved at an interval of two years (Figs. 2 and 3). To our knowledge, this is the first work reporting at the level of the whole bacterial community structural changes linked to an elevation of the atmospheric CO_2 content. Both the study of Zak et al. [71], based on the phospholipid fatty acid profile analysis of total extractable bacteria, and the study of Griffiths et al. [22], based on community DNA hybridization and % G+C base profiling by thermal denaturation, showed that the rhizosphere bacterial community structure of *Populus grandidentata, Lolium perenne,* and *Triticum aestivum* remained unaffected by the elevated pCO_2. These contrasting data show the importance of the molecular-based method in revealing more or less subtle structural influences, as suggested by Paterson et al. [46].

Fluorescent pseudomonads play a key role in the rhizosphere of plants. They enhance plant growth by several mechanisms, including (i) the control of phytopathogens by antibiotic production [16, 48], by competition for mineral nutrients [44], by secretion of enzymes that can lyse fungal cells [34], and by induction of plant resistance [64]; (ii) the contribution to plant mineral nutrition by secretion of plant-utilizable siderophores that bind iron [6] or by solubilizing minerals such as phosphorus [21]; (iii) nitrogen fixation [13]; and (iv) the production of phytohormones (for a review, see [21]). To influence the plant, root colonization by associative rhizobacteria is considered as a factor of primary importance [31]. Among others, bacterial chemotaxis response to root exudation is involved in this process.

Futama et al. [19] showed that soil salinity exerted an effect on root colonization by fluorescent pseudomonads through altered amino acid root exudation and therefore through altered bacterial chemotaxis. In accordance with these considerations, this work brings new insights to the plant response to elevated atmospheric CO_2 content through a possible alteration of the plant growth promoting effect by altered rhizodeposition and consequent altered composition of rhizobacterial populations.

Since the N concentration of *L. perenne* growth in monoculture decreases under elevated atmospheric CO_2 while the N concentration of *T. repens* remains unaffected and since the C:N ratio of *L. perenne* was unaltered by CO_2 fumigation when this latter plant is cultivated with *T. repens* [23], the elevated CO_2 induces an increase in the plant requirement for mineral nitrogen. This is supported by the CO_2-induced increased symbiotic nitrogen fixation [59, 72, 74] and proportion of *T. repens* in mixed cultures [23–25]. In grassland ecosystems, leguminous plants play a key role in the response to elevated CO_2. As a consequence of CO_2 enrichment, plant roots may alter the quality and the quantity of rhizodeposition, leading to a modified selective effect toward bacterial communities. This may explain the observation that *R. leguminosarum* outcompetes pseudomonads in the clover rhizosphere (Figs. 2 and 3). Montealegre et al. [40] have shown under elevated CO_2 changes in the genetic structure of the populations of *R. leguminosarum* isolated in the FACE study site of Eschikon from root nodules. These authors suggest that this CO_2 influence is linked to an alteration of the chemical signals released into the soil by clover roots. In the rhizosphere of *L. perenne*, rhizodeposition could also select populations of fluorescent pseudomonads that are more suitable for the CO_2-induced increased plant requirement for mineral nutrients.

In this work, we applied an experimental strategy targeted mainly to molecular approaches, but also to a cultural method. In soil grown with *L. perenne* and *T. repens*, the 16S rDNA-based approach allowed us to show the selective rhizospheric effect [38], to describe the main phylogenetic bacterial groups inhabiting soil and rhizosphere [37], and ultimately to provide evidence for a CO_2-induced modification of the bacterial community structure in the rhizosphere. We suggest investigation not only of the CO_2 influence on the soil bacterial processes linked to increased plant litter production, altered C:N ratios of plant residues, and rhizodeposition, such as organic matter decomposition and mineralization, but also of possible modifications of the plant-growth-promoting effects that could take place in the rhizosphere. To do this, further work dealing with genotypic and phenotypic analysis of the rhizosphere *Pseudomonas* spp. populations is urgently needed.

Acknowledgments

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