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Jérôme HAMELIN

Influence d'une augmentation en pCO_2 atmosphérique sur les communautés bactériennes associées à *Molinia coerulea*

Jury:

Michel Aragno	Professeur à l'Université de Neuchâtel (Suisse) et Directeur de thèse
René Bally	Directeur de recherche à l'Université Claude Bernard Lyon1 (France)
Josef Noesberger	Professeur à l'Ecole Polytechnique Fédérale de Zürick (Suisse)
Philippe Küpfer	Professeur à l'Université de Neuchâtel (Suisse)
Franco Widmer	Docteur à la Station Fédérale de Recherche en Agroécologie et Agriculture (Suisse)

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IMPRIMATUR POUR LA THESE

Influence d'une augmentation en pCO_2 atmosphérique sur les communautés bactériennes associées à *Molinia coerulea*

de M. Jérôme Hamelin

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de
Neuchâtel, sur le rapport des membres du jury

MM. M. Aragno (directeur de thèse), P. Küpfer, J. Noesberger (ETH Zürich),
F. Widmer (Reckenholz) et R. Bally (Lyon)

autorise l'impression de la présente thèse.

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Le doyen:

François Zwahlen

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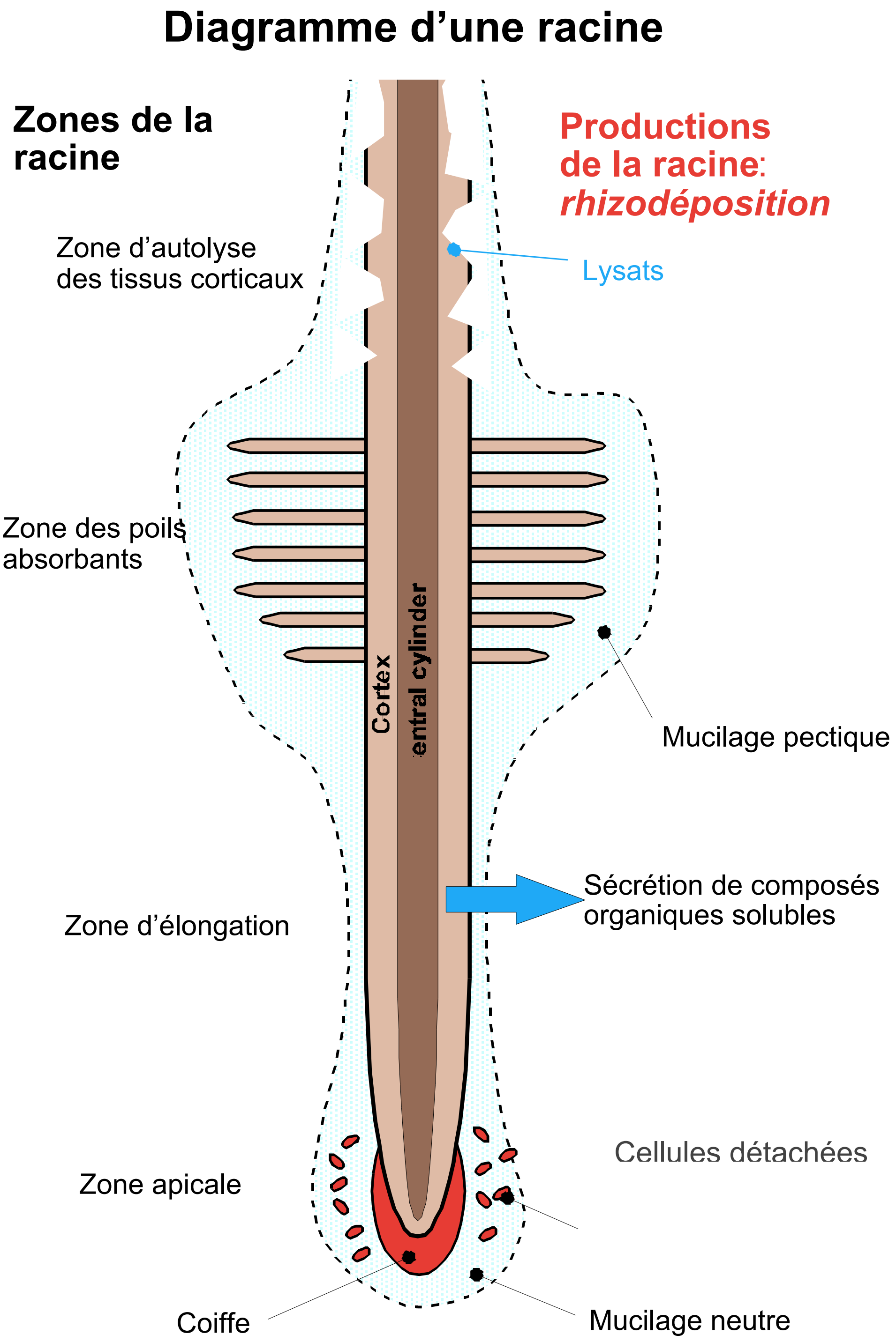


Figure 1.1.A Morphologie racinaire et type de rhizodéposition en fonction de la structure racinaire

Chapitre 1
Analyse bibliographique, contexte de l'étude

1.1 Interactions Sol-Plante: La rhizosphère

Lors de la conférence intitulée '*Interactions in the root environment – An integrated approach*' qui s'est déroulée à IACR-Rothamsted, Harpenden, UK du 10 au 12 Avril 2000, j'ai pu me rendre compte combien les physiologistes végétaux, les microbiologistes et les pédologues ont tout à gagner à travailler ensemble pour améliorer les connaissances sur la rhizosphère. Une phrase de Léonard de Vinci (1452-1519) servant de leitmotiv lors de ces journées m'a beaucoup marqué: '*on connaît mieux le mouvement des étoiles que le sol sous nos pieds*'. Bien que cette phrase soit encore valable de nos jours, l'interface entre le sol et la plante fait l'objet actuellement de nombreuses études.

1.1.1 Définition

Les plantes, et en particulier les plantes pérennes qui poussent d'une année sur l'autre au même endroit influencent physiquement, chimiquement et biologiquement le sol dans lequel elles poussent. L'interface entre les racines et le sol, nommée rhizosphère, constitue une zone privilégiée pour l'étude de cette interaction. Ce terme de rhizosphère a été défini il y a bientôt un siècle par Hiltner (1904) pour décrire la stimulation de la biomasse microbienne autour des racines. D'une manière générale, la rhizosphère indique le volume de sol soumis à l'influence de l'activité racinaire (Darrah 1991). Chaque sol possède des communautés bactériennes propres et certaines populations seulement vont profiter ou au contraire être inhibées par l'arrivée d'une racine. La rhizosphère présente une activité métabolique intense et de nombreux processus biochimiques s'y déroulent en raison de la stimulation de la microflore par le relargage par les racines d'une partie des photosynthétats (Rovira 1965).

1.1.2 L'exsudation racinaire

La composition exacte des exsudats racinaires est largement méconnue car une fraction importante des substrats organiques est rapidement dégradée au fur et à mesure de leur production. Selon Lynch & Whipps (1990) et Grayston et coll. (1996), la rhizodéposition englobe cinq catégories de composés: (1) Les exsudats hydrosolubles qui sont les plus abondants quantitativement (sucres, acides organiques, acides aminés, hormones et vitamines), (2) les enzymes sécrétées, (3) les lysats cellulaires et les exopolysaccharides, (4) les cellules de la coiffe qui se détachent et enfin (5) les gaz (CO_2, HCN et ethylène). Les graminées exsudent entre 10 et 50 % des photosynthétats par les racines (Liljeroth et coll. 1990; Swinnen et coll. 1995; Paterson et coll. 1997). D'une manière générale, les plantes pérennes allouent plus de photosynthétats au sol que les plantes annuelles, probablement pour survivre aux nombreux stress endurés par les plantes pérennes durant l'année entière (Harris et coll. 1980, cité par Grayston et coll. 1996). L'exsudation peut varier de manière importante qualitativement et quantitativement selon la zone racinaire considérée (figure 1.1.A; Darrah 1991), en fonction du stade de croissance de la plante (Rovira 1965), de la présence de bactéries en contact avec les racines (Meharg & Killham 1995), de l'humidité et de la température du sol (Soerensen 1997) et en réponse à une augmentation de CO_2 dans l'atmosphère (cf. § 1.3).

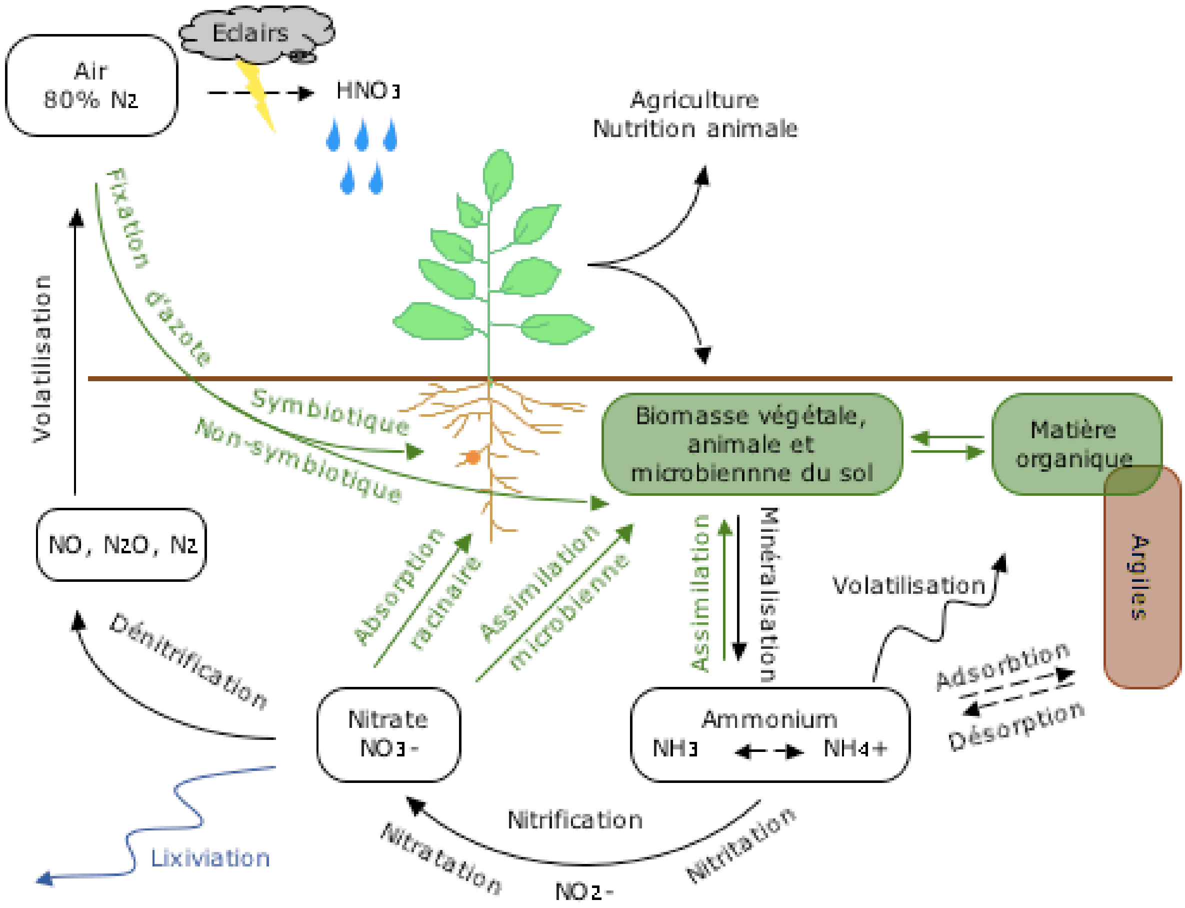


Figure 1.2.A Transformations naturelles impliquées dans le cycle de l'azote. L'azote de l'air constitue le réservoir le plus important mais le moins réactif. L'azote contenu dans la biomasse est impliqué dans de nombreuses réactions. Les formes inorganiques d'azote sont les plus labiles

Les bactéries rhizosphériques

Pour les microbiologistes du sol, la rhizosphère constitue un 'oasis dans un désert énergétique' (Hamelin et coll. 2001). L'exsudation de composés organiques par les racines induit une forte stimulation de certaines populations bactériennes indigènes, appellées rhizobactéries. La plupart sont opportunistes, délétères et d'autres favorisent la croissance des plantes (*Plant Growth Promoting Rhizobacteria, PGPR*). Les bactéries du genre *Pseudomonas* sont particulièrement bien adaptées à la vie rhizosphérique, et sont prises comme modèle de rhizobactérie (cf. chapitre 3)

La respiration des cellules racinaires, en association avec la respiration des communautés bactériennes alentour entraîne une diminution de la pression partielle en oxygène près des racines comparée au sol environnant (Hojberg & Sorensen 1993). Les processus bactériens anaérobies seront favorisés dans cette zone. Pour prendre l'exemple de fonctions impliquées dans le cycle de l'azote, la respiration du nitrate par les bactéries dénitrifiantes sera favorisée à mesure que l'on s'approche de la racine (cf. § 1.2.2; Linne von Berg & Bothe 1992; Ghiglione et coll. 2000). La fixation d'azote atmosphérique bénéficie à la fois d'une source de carbone et d'énergie abondante et de conditions appauvries en oxygène (cf. § 1.2.3; Poly 2000). Certains auteurs suggèrent que la fixation d'azote non-symbiotique puisse être assimilée à un caractère PGPR par l'apport d'azote dans la rhizosphère. L'apport réel à la nutrition azotée des plantes reste encore à prouver, et demeure une source de débat à l'heure actuelle (discussions animées lors du '*9th International Symposium on Nitrogen Fixation with Non-Legumes*' qui s'est tenu à Leuven en Belgique du 1 au 5 Septembre 2002).

1.1.3 Interface d'échange avec le sol

Vue sous l'angle du pédologue, la rhizosphère peut être un lieu d'acidification du sol par libération d'ions H^+ concomitante à l'absorption de NH_4^+ pour la nutrition azotée des plantes (Marschner et coll. 1987), d'altération des minéraux (mobilisation du phosphate inorganique et du fer, échanges d'ions à la surface des argiles) et de formation des aggrégats (mucilage racinaire et polysaccharides bactériens associés aux argiles) (Forster 1990).

Le volume de sol soumis à l'influence des racines varie avec le type de plante. 90 % des plantes sont aptes à être mycorhizées, dont *Molinia coerulea* (cf. § 1.4.1; Jefferies 1916). La symbiose mycorrhizique permet d'explorer un volume de sol bien plus étendu que le réseau racinaire, et le champignon possède une machinerie cellulaire très efficace pour prélever les éléments minéraux du sol (Jakobsen et coll. 2002). D'autres plantes ont développé des structures racinaires, appellées racines protéoïdes, qui sont adaptées à l'extraction des métaux, comme le phosphate inorganique du sol (Neumann et coll. 2000). Les racines protéoïdes excrètent des acides organiques présentant de fortes constantes de stabilité avec les métaux (citrate, malate). Certaines plantes ont aussi la capacité de moduler la densité de leurs racines en fonction de la disponibilité en azote, phosphore ou potassium (Forde & Lorenzo 2001; Robinson 2001).

1.2 Cycle de l'azote

L'azote (N) est un constituant des protéines et des acides nucléiques. Le recyclage de l'azote présent dans la matière organique joue un rôle essentiel dans la vie sur terre. Le cycle de l'azote en figure 1.2.A présente l'ensemble des transformations possibles de l'azote dans la nature. En règle générale, les transformations des composés organiques et inorganiques du sol sont effectuées en grande partie par des microorganismes. Dans le recyclage de l'azote, certaines fonctions essentielles sont contrôlées exclusivement par

Figure 1.2.B L'alternance de conditions oxiques et anoxiques favorise l'alternance de nitrification et de dénitrification. Les ions ammonium sont transformés en nitrate par nitrification puis les nitrates partent en gaz lors de la dénitrification. Cela revient à épuiser le sol en azote. Ces conditions sont retrouvées dans les sols à nappe battante (phénomène décrit avec humour par Ptiluc dans Ni Dieu Ni Bête)

des microorganismes, comme la nitrification (cf. § 1.2.1), la dénitrification (cf. § 1.2.2) ou la fixation biologique (cf. § 1.2.3). Les formes solubles (NH_4^+ et NO_3^-) sont réincorporées dans la biomasse par la nutrition azotée et les formes gazeuses (NO, N_2O et N_2) produites par le processus de dénitrification (cf. § 1.2.2) retournent dans l'atmosphère et sont perdues pour l'écosystème (au moins à court terme).

1.2.1 La nitrification

La nitrification est un processus aérobie responsable de la transformation de l'ammonium (NH_4^+) en nitrate (NO_3^-). Elle est réalisée en deux étapes successives par deux types de bactéries différentes. La nitritation correspond à la transformation de l'ammonium (NH_4^+) en nitrite (NO_2^-) et est réalisée par les bactéries nitreuses (genres *Nitrosococcus*, *Nitrosospira*, *Nitrosomonas*, *Nitrosolobus*, *Nitrosovibrio*). La nitratation correspond à la transformation du nitrite (NO_2^-) en nitrate (NO_3^-) et est réalisée par les bactéries nitriques (genres *Nitrobacter*, *Nitrospina*, *Nitrospira*, *Nitrococcus*).

Les bactéries nitrifiantes sont donc groupées en deux groupes phylogénétiquement homogènes et sont toutes aérobies strictes. Elles ont un métabolisme très lent et craignent l'acidité. Pour toutes ces raisons, les bactéries nitrifiantes sont plus adaptées à des sols basiques, par exemple dans la prairie littorale de la rive sud du lac de Neuchâtel où *Molinia coerulea* prolifère (cf. § 1.4.2).

1.2.2 La dénitrification

La dénitrification correspond à la respiration anaérobie du NO_3^- en composés gazeux (NO, N_2O et N_2). Ce processus entre en compétition avec la nutrition azotée des plantes et des microorganismes pour l'utilisation des nitrates. Les bactéries dénitrifiantes ont généralement un métabolisme chimio-organotrophe et aérobie-anaérobie facultatif. Ces bactéries n'expriment les gènes impliqués dans la dénitrification que lorsqu'il y a carence en oxygène, et substituent des composés azotés oxydés à l'O_2 comme accepteur final de la chaîne respiratoire. Deux étapes clefs de la dénitrification sont très sensibles à l'O_2. La réduction du NO_3^- en NO_2^- par la nitrate réductase codée par les gènes *nar* (cf. § 3.3; Philippot & Højberg 1999), et la réduction du NO_2^- en N_2O par la nitrite réductase codée par les gènes *nos* (Philippot 2002). Il est possible de réaliser des mesures d'activité potentielle en fournissant une quantité non limitante de substrat (NO_3^-), et une source de carbone dans des conditions anaérobies (cf. § 1.1.2; Lensi et coll. 1995). Dans le sol, la dénitrification est favorisée par l'engorgement, une faible porosité, à l'intérieur des aggrégats (Parry et coll. 1999) et dans la rhizosphère (von Rheinbaden & Trolldenier 1984; Linne von Berg & Bothe 1992; Clays-Josserand et coll. 1995; Ghiglione et coll. 2000).

Un sol à nappe battante subit une alternance de conditions oxiques et anoxiques, provoquant une alternance de nitrification et de dénitrification. Dans ces conditions, l'azote minéral disponible tend à disparaître (Figure 1.2.B).

1.2.3 La fixation biologique de l'azote atmosphérique (N_2)

Bien que l'azote moléculaire (N_2) représente près de 80 % de l'atmosphère du globe, seuls quelques organismes procaryotes (*Bacteria* et *Archaea*) sont capables de l'incorporer dans des molécules organiques selon un processus appelé fixation d'azote. Cette transformation requiert de l'énergie (15 moles d'ATP par mole de N_2 en théorie selon Postgate (1982) et beaucoup plus dans la pratique) et des électrons. 50 à 400 mg de carbone sont nécessaire pour fixer 1 mg de N_2. C'est la seule voie biologique d'incorporation d'azote dans les écosystèmes (figure 1.2.A).

La nitrogénase

Le complexe enzymatique responsable de la réduction du N_2 en NH_3 est la nitrogénase. Elle possède une activité très versatile, lui permettant de catalyser la réduction d'autres substances possédant des triples liaisons, par exemple de transformer l'acétylène C_2H_2 en éthylène C_2H_4. On utilise cette propriété pour quantifier la fixation d'azote sur des échantillons biologiques en mesurant le taux de production de C_2H_4 après l'ajout de C_2H_2 dans un flacon scellé. Cette technique couramment utilisée est appellée *Acetylene Reduction Assay* (ARA) (Dilworth 1966; Schöllhorn & Burris 1967; Hardy et coll. 1968).

Le complexe enzymatique de la nitrogénase, ainsi que la transcription des gènes codant pour cette enzyme, sont inhibés par de très faible teneurs en O_2. Les microorganismes fixateurs d'azote sont soit anaérobies stricts (genre *Clostridium*), soit microaérophiles dans le cas des organismes aérobies stricts sans mécanisme de protection de la nitrogénase vis-à-vis de l'O_2 (*Xanthobacter autotrophicus*), soit enfin insensibles à l'O_2 grace à des mécanismes de protection. Par exemple, certaines cyanobactéries (*Nostoc*, *Anabaena*, *Calothrix*, *Fischerella*...) produisent des hétérocystes à parois épaissies limitant la diffusion de l'oxygène. En plus, ces cellules sont dépourvues de photosystème II et ne produisent pas d'oxygène moléculaire. Les bactéries filamenteuses du genre *Frankia* cloisonnent la nitrogénase dans des vésicules à forte teneur en hopanoïdes, des lipides spéciaux qui complexent l'oxygène (Berry et coll. 1993). Enfin, les bactéries symbiotiques des légumineuses (genres *Rhizobium*, *Azorhizobium*, *Sinorhizobium*, *Bradyrhizobium*,...) produisent, en synergie avec la plante au sein des nodosités, de la leghémoglobine ayant un rôle et une structure proche de l'hémoglobine.

Vu la quantité importante d'énergie nécessaire pour transformer le N_2 en NH_3, le processus de fixation d'azote est très sévèrement réprimé par toute forme d'azote inorganique. Les bactéries fixatrices d'azote utiliseront la possibilité d'acquérir l'azote nécessaire à leur croissance par fixation d'azote atmosphérique uniquement lors de carence en azote combiné dans le milieu (NH_3, NO_3^-, substances organiques azotées). Ces conditions sont réunies dans la rhizosphère. Cet environnement est donc favorable à l'établissement de la fixation d'azote.

Diversité du gène nifH

Les bactéries fixatrices d'azote sont phylogénétiquement très diverses (Young 1992). Certaines études moléculaires ciblant les gènes impliqués dans la fixation d'azote utilisent l'intergène *nifD-K* au sein d'un genre bactérien (Simonet et coll. 1999), mais la plupart des études de diversité sont entreprises sur le gène *nifH* qui est plus conservé. Le gène *nifH* code pour une sous-unité clé de la nitrogénase réductase, et semble évoluer de façon congruente vis à vis du gène d'ADNr 16S (Hennecke et coll. 1985; Young 1992; Ben-Porath & Zehr 1994; Achouak et coll. 1999), qualifié d'horloge moléculaire.

Des études de diversité des bactéries fixatrices d'azote, basée sur la diversité du gène *nifH*, ont déjà été réalisées dans de nombreux environnements: les océans (Zehr & McReynolds 1989; Braun et coll. 1999; Zehr et coll. 1998; Mausmi et coll. 2003), les eaux douces (Zani et coll. 2000; Affourtit et coll. 2001; McGregor et coll. 2001), les tapis de cyanobactéries (Zehr et coll. 1995; Steppe et coll. 1996), le tube digestif des termites (Ohkuma et coll. 1996; 1999), le sol (Poly et coll. 2001a; 2001b & 2002), les rhizosphères du pin douglas (Widmer et coll. 1999; Shaffer et coll. 2000), de *Spartina alterniflora* (Chelius & Lepo 1999; Bagwell & Lovell 2000; Lovell et coll. 2000; Piceno & Lovell 2000a; 2000b; Lovell et coll. 2001; Bagwell et coll. 2002; Burke et coll. 2002; Brown et coll. 2003) et de roseau (Burke et coll. 2002), du riz sauvage ou cultivé (Ueda et coll. 1995; Engelhard et coll. 2000).

À ce jour, peu d'études portant sur la diversité du gène *nifH* associé à des plantes perennes non aquatiques ont été entreprises (Poly 2000). La structure des communautés de bactéries fixatrices d'azote actives *in situ* (par caractérisation des ARNm *nifH*) commence à être abordée (cf. § 4.4; Zani et coll. 2000; Burke et coll. 2002; Brown et coll. 2003).

1.3 Influence d'une augmentation en pCO_2 atmosphérique

Les plantes et les microorganismes sont les acteurs principaux qui affectent les cycles du carbone et de l'azote au niveau planétaire (séquestration et émission dans l'atmosphère). Par contre, l'augmentation de la teneur en CO_2 dans l'air est largement imputable aux activités humaines (combustion de matériaux d'origine fossile, agriculture) (Vitousek et coll. 1997). Cependant, environ un quart du CO_2 émis par l'homme ne se retrouve ni dans les océans, ni dans l'atmosphère. De nombreux auteurs expliquent ce phénomène par la séquestration du carbone par les sols et la végétation (Cao & Woodward 1998; Gill et coll. 2002). Il y a actuellement trois fois plus de carbone contenu dans la matière organique des sols que dans l'atmosphère (Schimel 1995). Tout accroissement de la quantité accumulée par les plantes et dans le sol pourrait constituer un moyen de restauration de la qualité de l'atmosphère.

1.3.1 Effet attendu sur les graminées

Dans la problématique de l'augmentation du CO_2 atmosphérique, il est important de différencier les plantes ayant un métabolisme en C4 (la majorité des plantes tropicales, dont le maïs) des plantes en C3 (la grande majorité des plantes des régions tempérées, dont *Molinia coerulea*). L'activité de la ribulose 1,5 biphosphate carboxylase/oxygénase (rubisco), enzyme responsable de la fixation de CO_2 lors de la photosynthèse, est inhibée compétitivement par l'oxygène de l'air (Bazzaz 1990). Seule l'intensité de la photosynthèse chez les plantes en C3 est limitée par la disponibilité en CO_2 (Taiz & Zeiger 1998). De nombreuses études ont déjà montré qu'avec l'accroissement de la teneur en CO_2 dans l'air, les graminées auraient un meilleur rendement de photosynthèse et produiront donc plus de biomasse qu'aujourd'hui (Billes et coll. 1993; Zak et coll. 1993; Paterson et coll. 1996; Ross et coll. 1996; van Ginkel et coll. 1997). Cependant, ce potentiel de croissance supplémentaire va être limité par la disponibilité en eau et en éléments minéraux de la solution du sol (en particulier l'azote et le phosphore). Le frein à l'accroissement de biomasse par manque d'azote a déjà été illustré dans plusieurs publications (Martens 1990; Hebeisen et coll. 1997; Zanetti et coll. 1997; van der Krift et coll. 2001). On sait par ailleurs que les plantes allouent d'autant plus de carbone aux racines que le sol est pauvre en nutriments (Jones & Darrah 1995; Grayston et coll. 1996). Celà favoriserait d'autant le transfert de photosynthétats vers les racines, et donc dans le sol.

1.3.2 Effet attendu sur les bactéries rhizosphériques via la rhizodéposition

Les bactéries telluriques évoluent dans un environnement où les pressions partielles en CO_2 (> 1000 ppm) sont bien supérieures à celles attendues dans l'atmosphère en 2050 (650 ppm) (van Veen et coll. 1991). Il n'y a donc pas de raison d'attendre d'effet direct d'une aumentation de la teneur en CO_2 atmosphérique sur les bactéries telluriques. Par contre, l'augmentation de la température moyenne du globe de 1,5 à 2 °C (effet de serre) aura tendance à se traduire par une augmentation de la vitesse de minéralisation de la matière organique, et donc à appauvrir les sols en carbone (Houghton et coll. 1990). De plus, les climatologues ont prédit des précipitations localement plus abondantes. L'humidité favorisera aussi la minéralisation du carbone et de l'azote des sols.

Comme on l'a vu dans le paragraphe 1.1.2, une grande partie de la production primaire des graminées est incorporée au sol par le processus d'exsudation racinaire. On peut s'attendre à ce qu'une augmentation de la teneur en CO_2 atmosphérique induise prioritairement des augmentations de biomasse (Diaz et coll. 1993; Zak et coll. 1993; Williams et coll. 2000) et d'activité respiratoire (Williams et coll. 2000) des bactéries hétérotrophes rhizosphériques, même si certaines études montrent le contraire (O'Neill 1994; Rodgers et coll. 1994; Paterson et coll. 1996). D'autres auteurs ont montré par ailleurs que l'augmentation de la biomasse bactérienne dans la rhizosphère pouvait être limitée par de faibles teneurs en azote du sol (Liljeroth et coll. 1990; van Ginkel et coll. 1997; Hu et coll. 2001).

Une microflore plus abondante aura tendance à décomposer plus vite la matière organique du sol (Williams et coll. 2000) et à mobiliser plus efficacement les éléments minéraux du sol (en particulier l'azote et le phosphore) en synergie avec une humidité et une température accrue. Celà rendra les éléments minéraux disponibles pour la croissance végétale (Zak et coll 1993), mais risque d'autre part d'appauvrir en nutriements la solution du sol par initiation de la décomposition - effet de *priming* - (Lekkerkerk et coll. 1990; van Veen et coll. 1991; Kuzyakov 2002) et... de limiter la croissance des plantes (Diaz et coll. 1993).

Aucun effet d'une augmentation de la teneur en CO_2 atmosphérique n'a été noté au niveau de la structure génétique des communautés microbiennes associées à la rhizosphère de *Lolium perenne* telle qu'elle apparaît par mesure du %G+C (Griffiths et coll. 1998). Par contre, une étude basée sur le clonage et le séquençage du gène ribosomique d'ADNr 16S avait montré que les populations rhizosphériques de *Pseudomonas* étaient favorisées par une augmentation de la teneur en CO_2 atmosphérique pour cette même plante (Marilley et coll. 1999). La méthodologie utilisée pour mesurer l'impact d'une élévation du pCO_2 atmosphérique sur la microflore est donc déterminante.

1.4 Nos systèmes d'étude modèle

1.4.1 *Molinia coerulea*

Molinia coerulea, ou molinie, est une graminée pérenne hémicryptophyte. Cela indique que le système racinaire (Figure 1.4.A) survit durant l'hiver.

Jefferies (1916) avait déjà remarqué que les racines principales de *Molinia coerulea* étaient fonctionnelles durant trois années consécutives et continuaient à produire de nouvelles racines latérales durant ces trois années. Lors de l'examen du système racinaire de cette plante, il apparait clairement qu'il y a coexistence de racines vivantes et de racines mortes nécrosées (Sheikh & Rutter 1969).

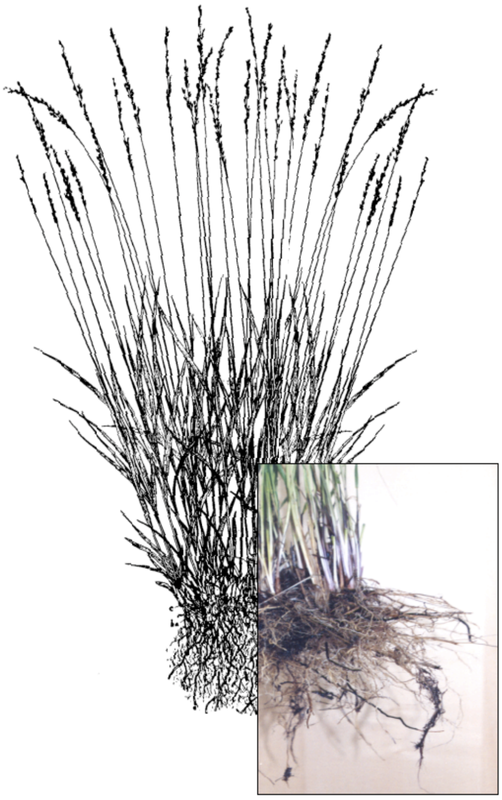


Figure 1.4.A Morphologie de *Molinia coerulea*. Vue générale schématisée (d'après Jefferies 1916) et photographie du système racinaire (plante issue de la rive sud du lac de Neuchâtel). La base des tiges est légèrement violette. Les racines noires correspondent aux veilles racines en décomposition

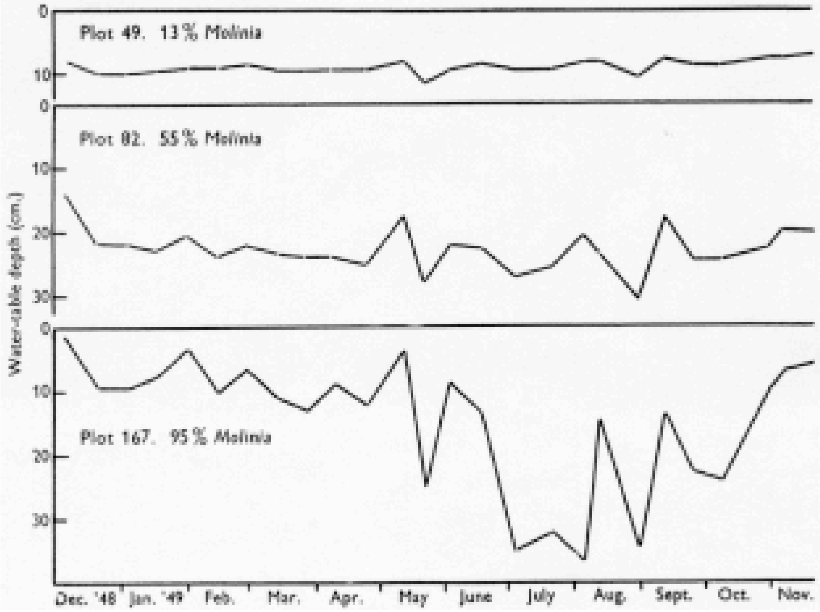


Figure 1.4.B Fluctuations saisonniaires de la hauteur de la nappe phréatique de trois prairies naturelles représentatives. La proportion de *Molinia coerulea* est indiquée pour les 3 parcelles (d'après Rutter 1955)

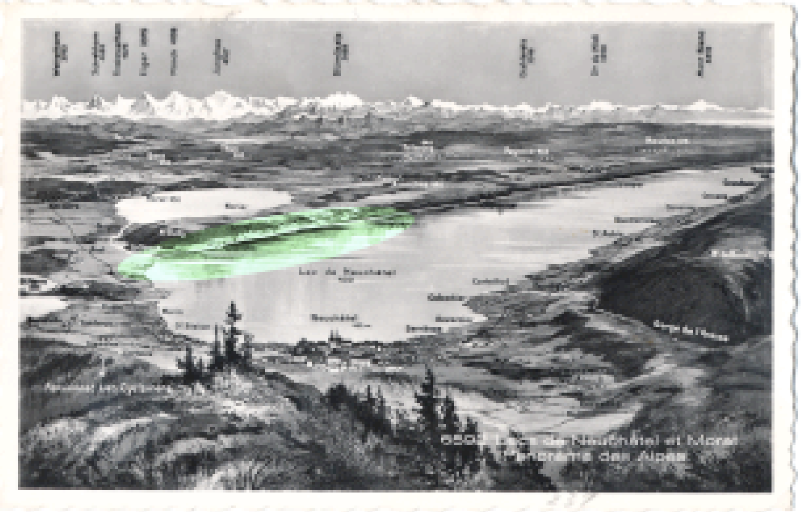


Figure 1.4.C Vue d'ensemble de la rive sud du lac de Neuchâtel. La zone cerclée indique l'emplacement de la prairie littorale correspondant au site d'étude

Molinia coerulea possède un système racinaire souvent mycorhizé (Jefferies 1916) avec de nombreuses racines fines très fragiles (Sheikh & Rutter 1969).

Elle forme des mottes très denses de 8 à 25 cm de diamètre (Jefferies 1915). Sur quatre localités possédant des sols à régimes hydriques différents, Sheikh & Rutter (1969) ont observé un maximum de densité racinaire entre 11 et 19 cm de profondeur, mais les racines de *Molinia coerulea* peuvent descendre jusqu'à 1 mètre de profondeur (Rutter 1955).

Molinia coerulea est bien adaptée aux sols ayant de fortes variations de hauteur de la nappe phréatique (figure 1.4.B; Jefferies 1915; Rutter 1955). Ces auteurs pensent qu'une nappe batante favoriserait l'évacuation de composés toxiques pour la plante (CO_2, H_2S) lors du retrait de l'eau d'une part, et le renouvellement continu de nutriments (bases solubles) lors de la remontée de l'eau d'autre part. Les racines principales possèdent des aérenchymes constitués par des espaces intra-cellulaires dans les cellules corticales amenant ainsi l'O_2 nécessaire à la respiration racinaire lors de l'engorgement du sol (Jefferies 1915; Rutter 1955; Webster 1962).

On retrouve *Molinia coerulea* en prairies denses (quasi monospécifiques) sur des sols à pH variables, aussi bien sur des sols tourbeux acides (pH entre 3,5 et 4,5) que sur des sols basiques (cf. § 1.4.2; Jefferies 1915; Buttler 1987). Elle pousse aussi naturellement à des altitudes différentes (Gore 1963).

Molinia coerulea est caractéristique des sols pauvres en azote disponible. Comparativement à d'autres graminées perennes qui préfèrent les sols riches (*Lolium perenne*), elle assimile plus lentement l'azote disponible et perd moins de composés azotés (exsudation, décomposition racinaire). En revanche, elle possède un système efficace de recyclage de l'azote et cet élément se concentre davantage dans les racines avant que ne meurent les parties aériennes en automne (Vazquez de Aldana et coll. 1996; Vazquez de Aldana & Berendse 1997).

C'est l'ensemble de ces particularités qui a attiré notre attention, en tant que microbiologiste sur le fonctionnement microbiologique de la rhizosphère de cette plante (cf. § 1.5).

1.4.2 Les prairies naturelles

Nos sites de prélèvement se situent dans des prairies à molinie. Deux sites principaux ayant des sols à pH et altitudes contrastés ont été étudiés.

Le site principal de notre étude est situé dans la réserve naturelle de la Grande Cariçaie, en rive sud du lac de Neuchâtel (figure 1.4.C). Entre 1868 et 1891, des travaux de détournement du cours de l'Aar et de drainage ont permis d'assécher 400 km^2 de marécages dans la région du Seeland pour permettre l'agriculture. Le niveau des lacs de Neuchâtel, Bienne et Morat s'est abaissé de 2,7 mètres. Pour la petite histoire, l'île St Pierre situé dans le lac de Bienne, où Rousseau a écrit ses confessions, s'est transformé en... presqu'île! (comme quoi, les idées restent, pas les paysages).

Sur la rive sud du lac de Neuchâtel, un haut-fond s'est formé par érosion des falaises de molasse. Une langue de sable de 20 km^2 a été exondée par l'abaissement du niveau du lac. En quelques années, les grèves se sont couvertes de végétation. Notre site de prélèvement est situé à 431 mètres d'altitude sur un léger dôme dans l'ancien delta d'alluvionnement de l'Aar, sur la commune de Cudrefin (VD, Suisse). Le sol, le régime hydrique et la végétation ont été bien caractérisés (Buttler 1987). Le battement de la nappe phréatique crée une alternance de conditions oxiques et anoxiques, induisant un cycle de nitrification et dénitrification apauvrissant le sol en azote (Figure 1.2.B). Un sol jamais inondé avec variation du niveau de nappe phréatique et pauvre en azote disponible offre des conditions propices à l'établissement d'un Molinion (Annexe 1). Cette prairie à Molinie a retenu toute notre attention car le peuplement est homogène.

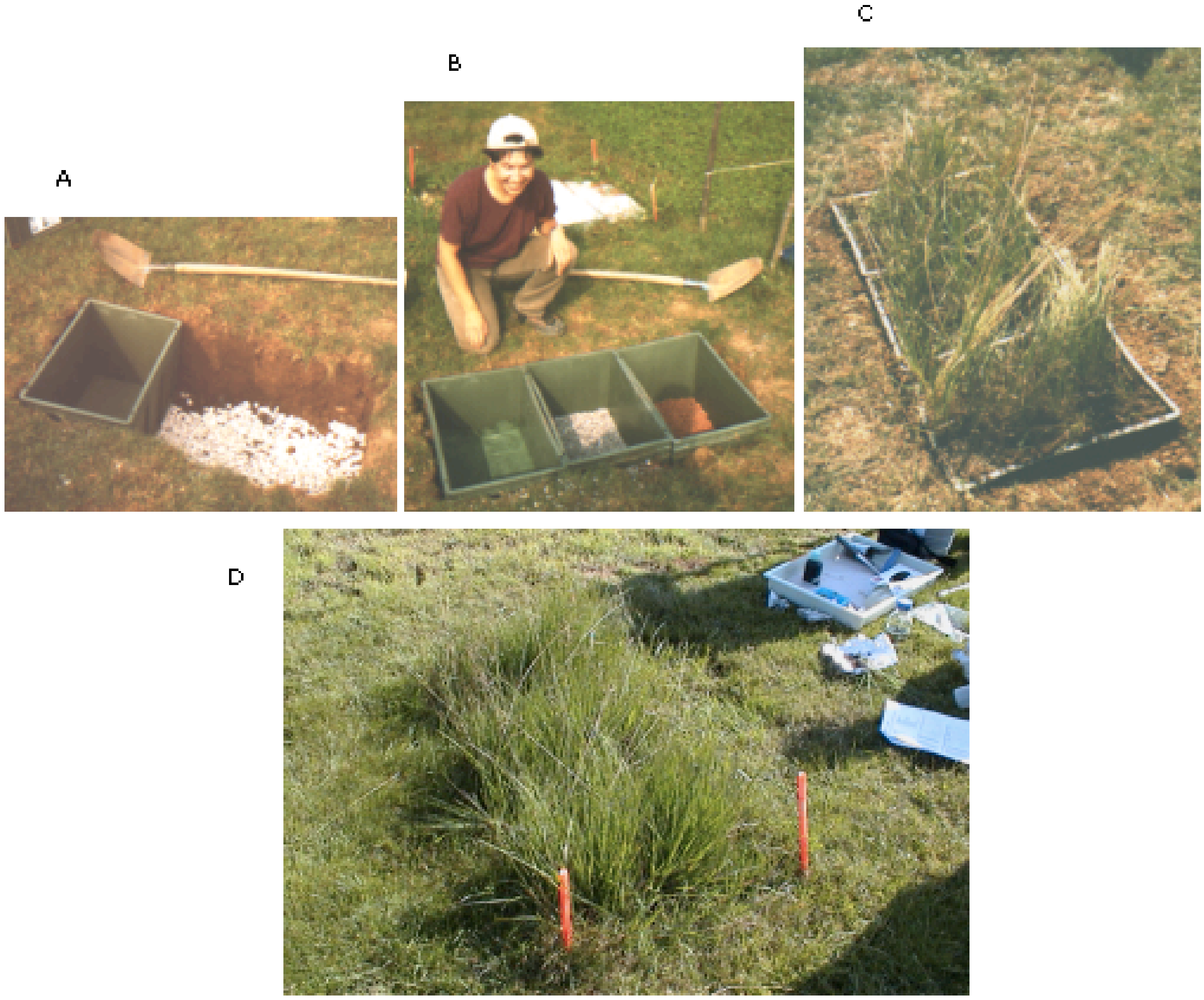


Figure 1.4.D (A) Mise en place des caisses de *Molinia coerulea*, transférées du lac de Neuchâtel aux installations FACE à Eschikon en septembre 1999. (B) Des billes d'argile, du gravier, puis une toile de verre ont été disposées avant le sol. (C) Le profil de sol original est reconstitué sur 35 cm de profondeur. (D) Les molinies en juillet 2002, deux ans après le transfert

Le deuxième site est situé à Chapelle-des-Bois (Doubs, France) à 1090 mètres d'altitude où les molinies poussent au milieu de tourbières de haut-marais (sol à pH=5)(Annexe 2). Ce site nous a surtout permis de confirmer les résultats obtenu sur notre site d'étude principal.

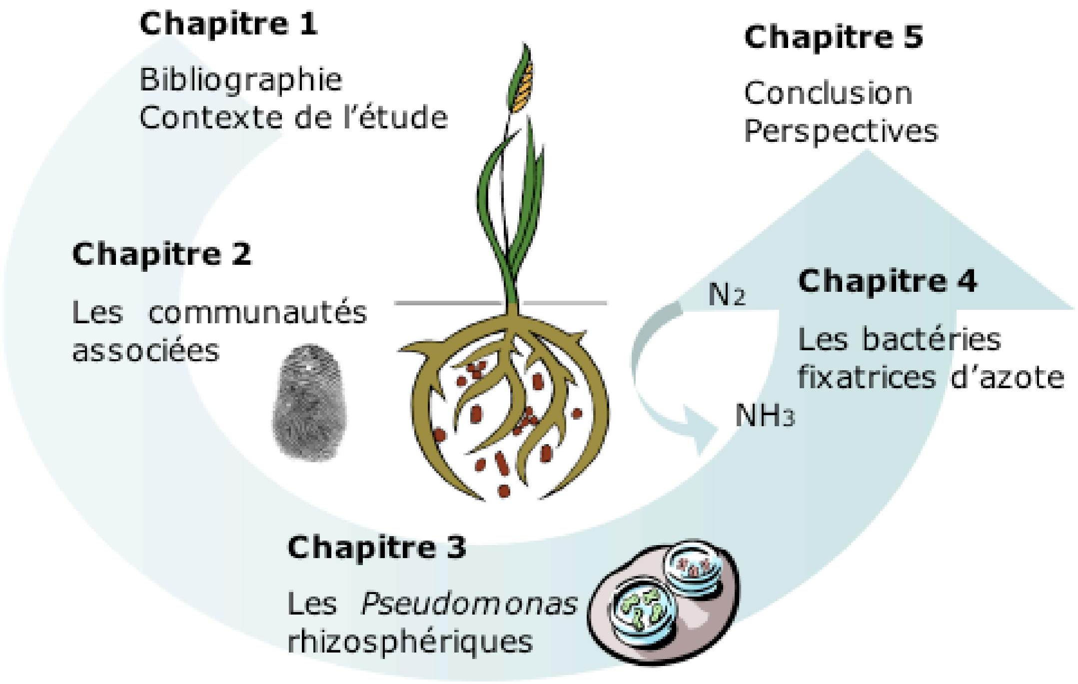
1.4.3 Le système expérimental *Free Air CO_2 Enrichment* (FACE)

Des plants de molinie de la prairie littorale, avec 35 cm de profil de sol reconstitué, ont été tranférés dans des installations FACE en septembre 1999. Les molinies ont été placées dans des caisses en plastique perforées au fond sur une couche de gravillons, une toile de verre, puis le profil de sol ramené de la prairie (Figure 1.4.D).

Le site expérimental est localisé à Eschikon, près de Zürich (Suisse), à une altitude de 550 m. Le système FACE est une installation en plein air qui permet d'enrichir l'atmosphère en CO_2. Le procédé est simple mais demande une grosse infrastructure. Un détecteur placé au centre des parcelles de 18 mètres de diamètre mesure en permanence la concentration en CO_2. De l'air enrichi en CO_2 (600 ppm) est insufflé constamment par des tuyaux répartis tout autour (Figure 1.4.E). La direction et la vitesse du vent sont mesurées et enregistrées par un ordinateur programmé pour ouvrir uniquement les valves sous le vent si sa vitesse est supérieure à 0,4 m/s. Pour de plus amples informations sur le fonctionnement du FACE, se référer aux travaux de Lewin et coll. (1994).

Figure 1.4.E Chaque parcelle enrichie en CO_2 est constituée d'un cercle de 18 m de diamètre. Une mini-station météo contrôle la teneur en CO_2 (en bas à gauche) et des tuyaux répartis autour permettent d'insuffler de l'air enrichi en fonction de la direction et de la vitesse du vent. Le système fonctionne durant la journée pendant toute la saison de végétation

Le système FACE est applicable pour des études au champ ainsi qu'à long terme, pour suivre l'influence du CO_2 sur la végétation durant plusieurs saisons (Allen 1992). Il a l'immense avantage par rapport aux systèmes expérimentaux classiques (*open-top field chambers*) de permettre un contrôle précis de la composition de l'atmosphère sans interférer avec les autres paramètres naturels: vents, évapotranspiration, qualité et intensité du rayonnement solaire, humidité relative, température et paramètres édaphiques.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NH_3
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Les installations FACE permettent de mimer au mieux les modifications atmosphériques attendues pour ces prochaines années, mais ne permettent de contrôler ni la température ni les précipitations qui vont pourtant être affectées par l'augmentation de la teneur en CO_2 de l'air. De plus, elle se réalise progressivement dans la nature et non pas brutalement comme c'est le cas dans les installations FACE.

1.5 Objectifs de l'étude, approches utilisées

Les graminées pérennes investissent une grande part des composés carbonés issus de la photosynthèse dans le sol. La microflore rhizosphérique serait d'autant plus influencée (comparée aux plantes annuelles) par des changements dans l'exsudation de ces plantes. Le but de ce travail était d'étudier la microflore associée à la rhizosphère de *Molinia coerulea*, une graminée pérenne poussant depuis un siècle dans des sols naturellement oligotrophes. Pour accentuer cette limitation en azote disponible, nous avons transféré des plants de *Molinia coerulea* sous une atmosphère enrichie en pCO_2 en préservant des conditions de culture '*les plus naturelles possibles*'. Une étude antérieure dans le laboratoire (Marilley 1999) portant sur une plante nitrophile, *Lolium perenne*, avait montré un impact des changements de la teneur en CO_2 atmosphérique sur les populations rhizosphériques de *Pseudomonas*. Nous avons formulé l'hypothèse que la rhizosphère de *Molinia coerulea* abritait des bactéries préadaptées à la limitation en azote qui seraient prêtes à réagir aux changements globaux.

Cette étude a été abordée sous différents angles:

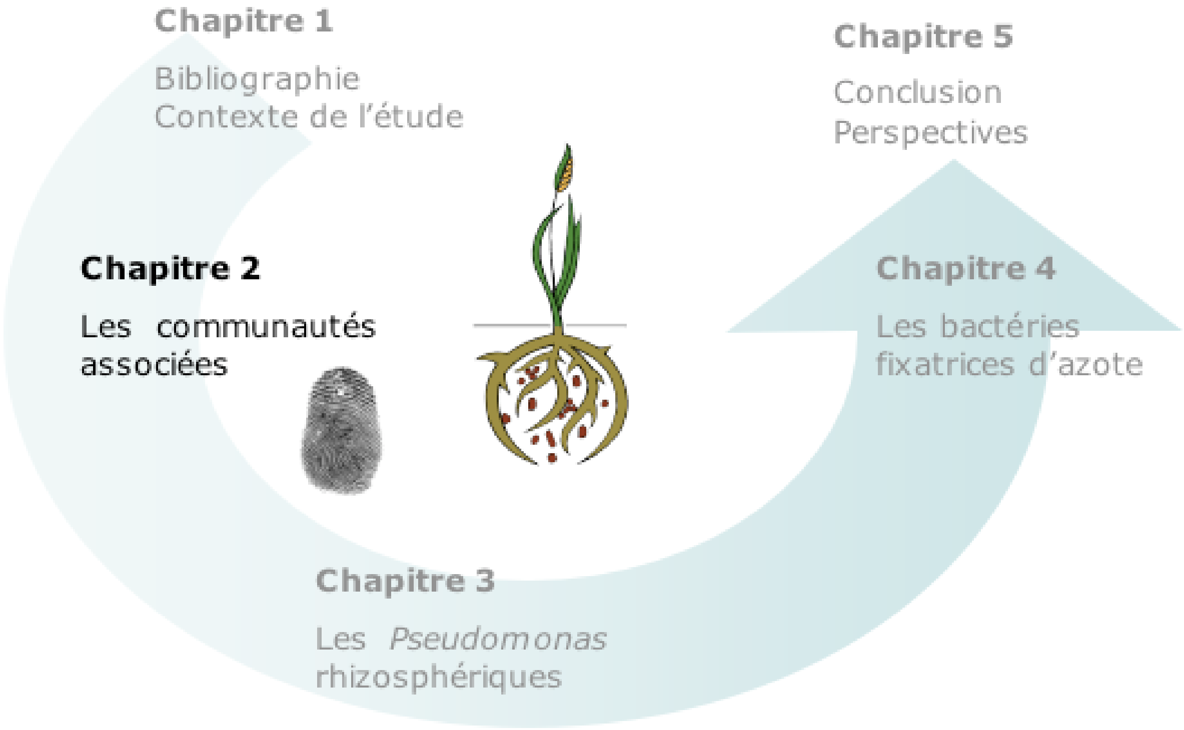
Nous avons d'abord entrepris une description globale des communautés, présenté dans le chapitre 2 et reposant sur la réalisation d'empreintes génétiques d'une part (DGGE) et de profils métaboliques d'autre part (BIOLOG).

Nous avons ensuite ciblé les populations de *Pseudomonas* adaptées à la vie rhizosphérique et potentiellement réactives vis-à-vis d'une modification de la teneur en CO_2 de l'atmosphère (Chapitre 3).

Parallèlement à ces approches, nous avons évalué la diversité et l'activité des bactéries fixatrices d'azote (Chapitre 4).

Chapitre 2

Les communautés bactériennes associées à *Molinia coerulea*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Chapitre 2

Les communautés bactériennes associées à *Molinia coerulea*

D'une façon générale, une communauté bactérienne correspond à l'ensemble des bactéries présentes (toutes espèces confondues) dans un habitat donné. Cet ensemble d'espèces va pouvoir réagir à des modifications environnementales par ajustement des proportions entre espèces (plasticité génétique) ou par modification des propriétés physiologiques (plasticité phénotypique).

Dans ce chapitre, nous avons étudié les communautés bactériennes associées à l'environnement rhizosphérique de *Molinia coerulea* en utilisant deux approches, génétique *vs* physiologique, donnant accès à différentes informations. Les images obtenues par l'une ou l'autre de ces approches peuvent être apparentées à des empreintes génétiques ('*fingerprint*' en anglais) ou métaboliques des communautés étudiées.

Dans la partie 2.1, nous avons caractérisé génétiquement les communautés bactériennes par l'analyse de la variété et de l'abondance relative des espèces présentes et actives dans l'environnement rhizosphérique de *Molinia coerulea* (PCR-DGGE sur le gène d'ADNr 16S). Afin de tirer profit au mieux des *fingerprint* génétiques, nous avons inventorié les différentes approches statistiques permettant l'interprétation des profils (cf. § 2.1.1) d'une part et nous avons développé des indices de diversité adaptés aux fingerprints (cf. § 2.1.2) d'autre part. Enfin, les images des communautés bactériennes dominantes ou celles qui sont les plus actives ont pu être mise en relation avec les paramètres environnementaux (cf. § 2.1.3).

Dans la partie 2.2, les modifications physiologiques d'utilisation de 31 sources de carbone par les communautés bactériennes associées à la rhizosphère de *Molinia coerulea* ont été évaluées (Profils Ecoplate BIOLOG©). La plasticité phénotypique des communautés en réponse à une augmentation de la teneur en CO_2 de l'atmosphère a été mesurée sur les communautés provenant des racines et du sol environnant. L'originalité de ce travail a été de caractériser génétiquement chacune des guildes utilisant ces différentes sources nutritives.

2.1 Empreintes génétiques des communautés microbiennes

Les communautés bactériennes du sol et de la rhizosphère sont très diverses. Torsvik et coll. (1990; 1994) ont évalué entre 4000 et 13000 le nombre probable d'espèces bactériennes présentes dans un gramme de sol. Ces bactéries telluriques sont en général peu actives et seulement de 0,1 à 10 % des cellules du sol et de la rhizosphère sont actuellement cultivables par les milieux de culture traditionnels (Amann et coll. 1995). Afin de prendre en compte ces bactéries non cultivables pour la caractérisation des communautés microbiennes, nous avons choisi d'utiliser des outils de la biologie moléculaire. L'approche utilisée consiste à extraire les acides nucléiques (ADN ou ARN) directement à partir des échantillons environnementaux, puis d'évaluer la variabilité des séquences d'une partie plus ou moins conservée du génome présente chez toutes les bactéries (région V3 de l'ADNr 16S). La technique utilisée dans ce travail, '*Polymerase Chain Reaction & Denaturing Gradient Gel Electrophoresis*' (PCR-DGGE), a été utilisé pour la première fois en écologie microbienne par Muyzer et coll. en 1993. D'après ces auteurs, la DGGE permet d'appréhender toutes les populations bactériennes représentant au moins 1 % des effectifs totaux. À défaut de décrire la diversité bactérienne de façon exhaustive, l'utilisation de la DGGE pour l'analyse de la diversité génétique des communautés permet de suivre la distribution et l'abondance relative des espèces dominantes. L'analyse numérique des données obtenues par DGGE est inspirée des méthodes précédemment développées par les écologistes travaillant sur les communautés végétales et animales.

Le paragraphe 2.1.1 fait le point sur les approches statistiques existantes dans l'analyse des profils génétiques ('*fingerprints*'), en utilisant les données collectées au laboratoire comme illustration de notre propos.

Le Paragraphe 2.1.2 propose de nouveaux indices spécialement conçus pour synthétiser le maximum d'information qu'il est possible de retirer des fingerprints génétiques.

Le paragraphe 2.1.3 expose les résultats obtenus pour les communautés associées à *Molinia coerulea*, qui sont soit les plus abondantes soit les plus actives.

2.1.1 Statistical analysis of Denaturing Gel Electrophoresis (DGE) fingerprinting patterns

Ce paragraphe a fait l'objet d'un article de N. Fromin[1], **J. Hamelin[1]**, S. Tarnawski,[1] D. Roesti[1], K. Jourdain-Miserez[1], N. Forestier[1], S. Teyssier-Cuvelle[1], F. Gillet[2], M. Aragno[1] and P. Rossi[1]. Cet article a été publié dans Environmental Microbiology (Fromin et coll. 2002).

[1] Microbiology Laboratory, University of Neuchâtel, P.O. Box 2, CH-2007 Neuchâtel

[2] Plant Ecology Laboratory, University of Neuchâtel, P.O. Box 2, CH-2007 Neuchâtel

Abstract

Technical developments in molecular biology have found extensive applications in the field of microbial ecology. Among these techniques, fingerprinting methods such as Denaturing Gel Electrophoresis (DGE, including the three options: DGGE, TGGE and TTGE) has been applied to environmental samples over this last decade. Microbial ecologist took advantage of this technique, originally developed for the detection of single mutations, for the analysis of whole bacterial communities. However, the results of these high quality-fingerprinting patterns were until recently restricted to a visual interpretation, neglecting the analytical potential of the method in terms of statistical significance and ecological interpretation. A brief recall is presented here about the principles and limitations of DGE fingerprinting analysis, with an emphasis on the need of standardization of the whole analytical process. The main content focuses on statistical

strategies for analyzing the gel patterns, from single band examination to the analysis of whole fingerprinting profiles. Applying statistical method make the DGE fingerprinting technique a promising tool. Numerous samples can be analyzed simultaneously, permitting the monitoring of microbial communities or simply bacterial groups for which occurrence and relative frequency are affected by any environmental parameter. As previously applied in the fields of plant and animal ecology, the use of statistics provides a significant advantage for the non-ambiguous interpretation of the spatial and temporal functioning of microbial communities.

2.1.1.1 Fingerprinting techniques applied to microbial communities

Molecular approaches in microbial ecology

A major challenge in the field of microbial ecology is to assess the diversity of the microbial cells present in a defined habitat. Assessing the diversity of microbial communities (in terms of richness and structure) is a way to address how they evolve in their environment. In a more general way, it is a possible mean to address the question of the modulation of microbial communities by environmental factors. Phylogenetically meaningful macromolecules, particularly 16S rDNA, directly amplified from environmental DNA, are now widely used for such purposes (Ranjard *et al.* 2000c; O'Donnell *et al.* 2001; Schäfer & Muyzer 2001).

However, information collected by these molecular tools quickly revealed the unsuspected complexity of whole bacterial communities (Ward *et al.* 1990). They were shown in turn to be limited in a practical way (O'Donnell *et al.* 2001). The amount of time and resources needed for the now classical 'cloning-sequencing' technique (which potentially supply an exhaustive description of microbial communities), coupled with the impracticability of complete counts of organisms at present (Dunbar *et al.* 2002), led to the development of alternative solutions. An original way was to separate PCR-amplified fragment pools produced from whole microbial communities by electrophoresis techniques (Table 2.1.1.A). These fingerprinting methods are now widely adopted in the field of bacterial ecology and permit the simultaneous analysis of numerous samples (Ferrari & Hollibaugh 1999).

Table 2.1.1.A Fingerprinting methods used for the characterization of microbial communities, with recent publications in the related field

Amplified Ribosomal DNA Restriction Analysis (ARDRA)	Smit *et al.* 1997; Tiedje *et al.* 1999
Denaturing Gel Electrophoresis (DGE)	Muyzer & Smalla 1998
Ribosomal Intergenic Spacer Analysis (RISA)	Fisher & Triplett 1999; Ranjard *et al*. 2000b
Single Strand Conformation Polymorphism (SSCP)	Schwieger & Tebbe 1998; Dabert *et al.* 2001
Terminal Restriction Fragment Length Polymorphism (T-RFLP)	Moeseneder *et al.* 1999; Dollhopf *et al.* 2001

DGE fingerprinting of microbial communities

In 1993, Muyzer *et al*. first applied Denaturing Gel Electrophoresis (DGE) techniques for the analysis of whole bacterial communities. DGE allows the separation of small PCR products, commonly up to 400 bp. The separation of the DNA fragments is achieved as a function of their different G+C content and distribution. Consequently, the fingerprinting pattern is built according to the melting behavior of the sequences along a linear denaturing gradient (Myers *et al.* 1985). Such a gradient is obtained using either denaturing chemicals for Denaturing Gradient Gel Electrophoresis (DGGE) or heat for Temperature Gradient Gel Electrophoresis (TGGE) and Temporal Temperature Gradient Electrophoresis (TTGE).

The DGE techniques were applied using 16S rDNA fragments to the analysis of bacterial communities in numerous habitats such as soil and rhizosphere (Bruns *et al.* 1999; Yang & Crowley 2000; Duineveld *et al.* 2001; Ibekwe *et al.* 2001; McCaig *et al.* 2001; Smalla *et al.* 2001) and aquatic environments (Murray *et al.* 1996, 1998; Ferrari & Hollibaugh 1999; Moeseneder *et al.* 1999; Schäfer *et al.* 2001; Schäfer & Muyzer 2001; van der Gucht *et al.* 2001). Interestingly, an increasing number of studies based on DGE are carried out on archaeal (Murray *et al.* 1998; Rölling *et al.* 2001) or eukaryal communities (van Hannen *et al.* 1999; van Elsas *et al.* 2000; Diez *et al.* 2001; Mohlenhoff *et al.* 2001).

The sensitivity of DGE analysis can be refined with the targeting of precise (and even non-dominant) taxonomic groups, by using specific PCR primers (Heuer *et al.* 1997; Nübel *et al.* 1997; Heilig *et al.* 2002) or by identifying community members by hybridization of blotted DGE gels with group-specific oligonucleotidic probes (Heuer *et al.* 1999). Other developments were based on the use of 16S rRNA as a target (Felske & Akkermans 1998; Kowalchuk *et al.* 1999; Duineveld *et al.* 2001; Schäfer *et al.* 2001) to highlight metabolically active populations only. Functional genes (Watanabe *et al.* 1998; Bruns *et al.* 1999; Lovell *et al.* 2000; Fjellbirkeland *et al.* 2001) or even their transcripts (Wawer *et al.* 1997) were also analyzed, which heralds very interesting prospects in clarifying the functioning of microbial communities.

Guidelines for the interpretation of DGE fingerprinting patterns

Some features of the fingerprinting techniques have to be considered before applying statistics for the analysis of DGE profiles.

In DGE analysis, the generated banding pattern is considered as an 'image' of the whole bacterial community. An individual discrete band refers to a unique 'sequence type' or phylotype (Muyzer *et al.* 1995; van Hannen *et al.* 1999), which is treated in turn as a discrete bacterial population. The term population classically refers to a group of bacterial cells present in a specified habitat and belonging to the same species. We are expecting that PCR fragments generated from a single population to display identical electrophoretic mobility in the analysis. This was confirmed by Kowalchuk *et al*. (1997) who showed that co-migrating bands generally corresponded to identical sequences. However, it was shown that rDNA fragments of closely related bacteria are not necessarily resolved (Buchholz-Cleven *et al.* 1997) or may produce separated bands (Jackson *et al.* 2001). Moreover, non-related sequences might co-migrate at an identical position (Vallaeys *et al.* 1997), especially when treating complex community patterns (Kowalchuk *et al.* 1997; Ben Omar & Ampe 2000). In this case, the question of the resolution of the gel needs to be addressed. Crowding of the gel has been discussed already and algorithms to assess it were proposed by Nübel *et al.* (1999a). Degenerated primers should be avoided also as one single bacterial strain, or even a single clone, may generate a multiple band pattern (Kowalchuk *et al.* 1997; Piceno *et al.* 1999). Some authors have also detected artificial bands when analyzing complex DNA templates, probably induced by heteroduplex molecules (Ferris & Ward 1997). Consequently, care should be taken in assigning a single band to a single bacterial population.

Another assumption for DGE fingerprinting interpretation is that the band intensity is directly related to the density of corresponding bacterial phylotypes within the sample. Results obtained by Murray *et al*. (1996) suggested a relationship between band intensity and relative abundance of the corresponding phylotype in the template DNA mixture. Such an assumption implies that no bias was obtained during the whole extraction-amplification procedure of the bacterial genomes (Muyzer *et al.* 1993; Wang & Wang 1997; Garcia-Pichel *et al.* 2001). DGE analysis should be restricted probably to samples treated using identical methods, in which DNA extraction and amplification biases are supposed to occur homogeneously. Moreover, it is commonly accepted that the main populations only (those representing more than 0.1 - 1 percent of the target organisms in terms of relative proportion) are displayed in the profiles (Muyzer *et al.* 1993; Murray *et al.* 1996). As a result, all populations present within a habitat do not appear on DGE banding patterns. When assessing the above considerations, the image of the communities which is provided by DGE fingerprinting patterns probably relates more to its structure, i.e. to the relative abundance of the main bacterial populations, than to its total richness (Muyzer & Smalla 1998). These features and restrictions are nevertheless common to all PCR-based approaches (Lee *et al.* 1996; Fisher & Triplett 1999; Schäfer & Muyzer 2001).

The last consideration about this analytical technique is about the reproducibility of the DGE analysis. Reproducibility of sample analysis depends on the upstream analytical steps (from the sampling to the DNA extraction and amplification procedures) as well as the care brought to the DGE gels themselves. A thorough standardization at each level of the experiments results in very high reproducibility. The use of reference patterns, the loading of precise amounts of PCR-amplified fragments and the precision of gel staining are required. As a consequence, identical samples loaded on a single gel display identical patterns (Simpson *et al*. 1999; Schäfer *et al*. 2001; Yang *et al.* 2001) and patterns from different gels can be compared with a high degree of confidence. The analysis of large numbers of samples can be exploited for the characterization of the intrinsic variability of the bacterial community structures. This large amount of data can be analyzed in turn with statistical tools, which provide a significant advantage for the non-ambiguous interpretation of the observed variability (Morris *et al.* 2002).

2.1.1.2 Analysis and comparison of DGE community profiles

DGE techniques have been extensively used to monitor bacterial communities in space and time (Ferris & Ward 1997; Murray *et al.* 1998; Nübel *et al.* 1999b; van der Gucht *et al.* 2001) or to evaluate the impact of environmental disturbances (Ibekwe *et al.* 2001; Müller *et al.* 2001). The variations between DGE profiles were classically described visually on a single DGE gel by the disappearance, the appearance or the changes in the intensity of selected bands. However, an increasing number of studies propose statistical investigations of DGE banding patterns, which undoubtedly lead to refined results. These advanced analyses are based on a computer-assisted characterization of the banding patterns and the subsequent treatment of the data using a statistical approach.

An example of computer-assisted guideline for the analysis of fingerprinting profiles was proposed by Rademaker *et al*. (1999) using the GelCompar software package (Applied Maths, Kortrijk, Belgium). Briefly, banding patterns were first standardized with a reference pattern included in all gels. Each band was described by its position (Y, in pixel on the image file) and its relative intensity in the profile (P_i), which could be calculated by the relative surface of the peak in the profile ($P_i = n_i / N$, where n_i is the surface of the peak i, and N is the sum of the surfaces for all the peaks within the profile). Using these data, various statistical methods can be applied, based either on single band or on whole DGE profile analysis.

Analysis of DGE profiles based on single bands

One way to analyze DGE fingerprinting patterns is to observe the possible changes in the presence/absence or in the variation of intensity of a single band (Murray *et al.* 1996). Putative indicator bands highlighted in this way can be excised from the gels and their sequences analyzed using a cloning-sequencing procedure (Kowalchuk *et al.* 1997; Watanabe *et al.* 1998; Ibekwe *et al.* 2001).

The variation in band presence or intensity can be exploited in two different ways. First of all, the relevance of indicator bands can be evaluated by testing their occurrence in relation with various biological and physico-chemical parameters (Widmer *et al.* 2001) as well as with the presence or absence of other bands in the patterns. In the example shown in table 2.1.1.B, 16S rDNA TTGE banding patterns of 30 raw milk samples were analyzed in this way. The occurrence of each TTGE band was tested against qualitative descriptors using a Fisher's exact test and bands found at the positions *Y*=230 and *Y*=300 were positively correlated to the cleaning frequency of the milking device and to the hygienic status of the cow tits, respectively.

Table 2.1.1.B Significant correlation ($p<0.05$, Fisher's exact test) between the presence of a selected band within a gel pattern and a qualitative descriptor. The bands were identified using a cloning-sequencing procedure (Rossi unpublished)

Position of the band (in pixels on Y axis)	Descriptors Frequency of cleaning of the milking device	 Hygienic status of the cow tits before milking	Identification of 16S rDNA fragment (% identity)
Y = 230	p = 0.0001	no correlation	*Bacillus* sp. (> 95%)
Y = 300	no correlation	p = 0.004	*Pseudomonas* sp. (> 95%)

Secondly, single band analysis can also be used for computing a regression between band intensity (dependent quantitative variable) and an environmental descriptor (independent quantitative variable). In the example given above, the TTGE patterns were analyzed by plotting the relative intensities (P_i) of each band *versus* various physical parameters measured from the same samples. A positive correlation (r^2 = 0.69) was found between the relative intensity of the band *Y*=300 (identified as *Pseudomonas* sp.*;* Table 2.1.1.B) and the percent of fat measured in the raw milk (Figure 2.1.1.C).

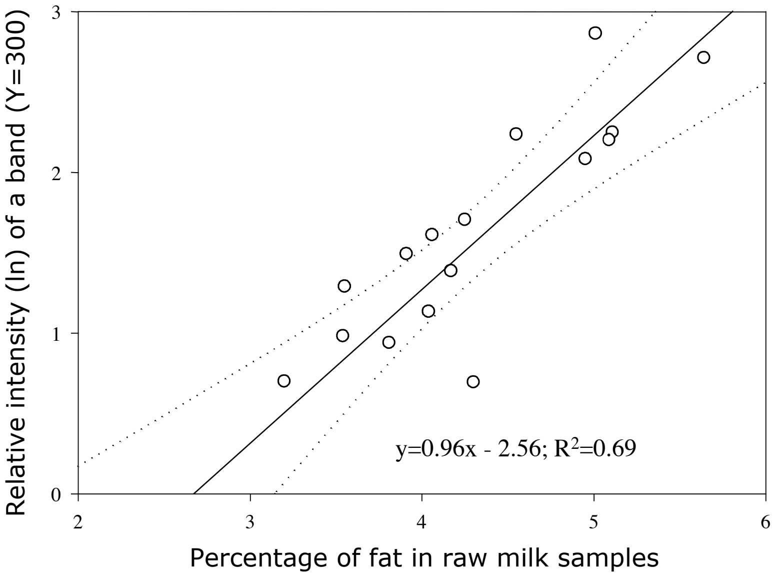

Figure 2.1.1.C Regression analysis between the relative intensity (ln) of the band at the *Y*=300 position and the percentage of fat found in the corresponding raw milk samples (Rossi unpublished)

Whole profile analysis

The second approach for a comparative analysis of DGE patterns is based on the whole set of bands present within the profiles. The total number of bands (called sometimes "band richness") in each sample pattern is related to the number of dominant phylotypes, and can be used for comparison purposes (Müller *et al.* 2001; van der Gucht *et al.* 2001). Comparison of profiles can be refined by taking into account the relative intensity of each band (P_i). Thus, diversity indices, such as Shannon-Weaver and Evenness indices (Nübel *et al.* 1999a; Simpson *et al.* 1999; McCaig *et al.* 2001; Kocherginskaya *et al.* 2001; Ogino *et al.* 2001), can be calculated to describe possible changes in the dominance among phylotypes. An interesting feature is to combine these indices with other sets of environmental data. For instance, Nübel *et al.* (1999a) found a positive linear correlation between Shannon-Weaver indices calculated from both DGE patterns and carotenoid types in oxygenic-phototrophic microbial communities.

Computation of similarity matrix

When considering the presence/absence of the bands, similarities between banding patterns, taken in pairs, can be expressed as a percentage value of a similarity coefficient such as Jaccard (Diez *et al.* 2001) or Dice (van der Gucht *et al.* 2001) coefficient, or a distance coefficient such as Euclidean measure (McSpadden Gardener & Lilley 1997). Other coefficients, such as the Steinhaus coefficient (Figure 2.1.1.D) or the product moment, also named Pearson correlation coefficient (Rölling *et al.* 2001; Smalla *et al.* 2001), allows to take into consideration the relative intensity (P_i) of each band (Legendre & Legendre 1998; Rademaker *et al.* 1999).

As noticed by Murray *et al.* (1998), the use of these similarity coefficients for the calculation of pair-wise levels of similarity between patterns does not require a one-to-one correspondence between the number of bands and the number of sequence types. Similarity or distance matrices can be displayed graphically as a dendrogram, but also give way to clustering and ordination methods.

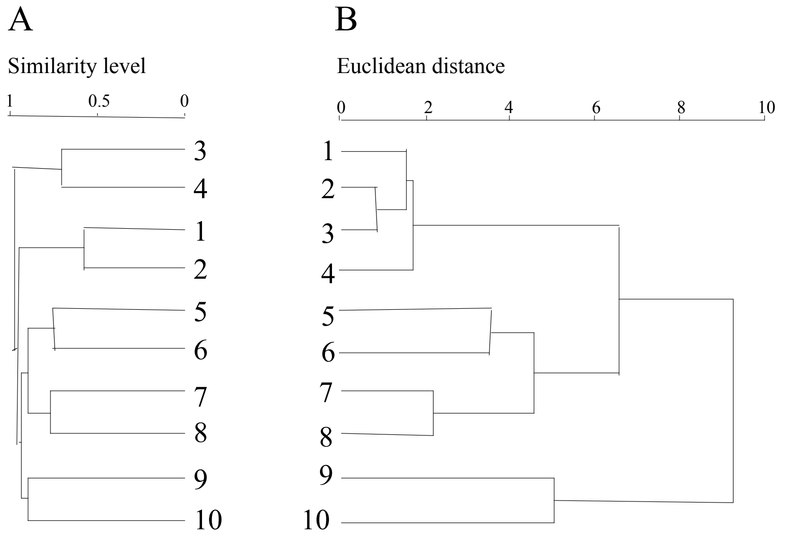

Figure 2.1.1.D UPGMA clustering of 10 samples taken along a vertical gradient form the small eutrophic Lake Loclat (Neuchâtel, Switzerland). Samples are ranked by depth: 1 corresponds to the surface and 10 to the bottom of the lake (8.7 m). A: clustering according to DGGE data; 135 band positions (using Steinhaus coefficient). B: clustering according to 23 physical and chemical variables using Euclidean distance (Forestier *et al.* 2002). Linkage levels were computed using the R package (Casgrain & Legendre 2001)

Clustering techniques

Clustering techniques, such as the Unweighted Pair Group Method using arithmetic Averages (UPGMA), are applied to the DGE profiling with the aim of identifying the samples which generate similar patterns (Yang *et al.* 2001; Ibekwe *et al.* 2001; Boon *et al.* 2002). One advantage of this presentation is that the coherence of the fingerprinting patterns can be assessed rapidly.

In the example given above (Forestier *et al.* 2002), ten samples were taken from a holomictic eutrophic lake along a vertical gradient and were analyzed for major ions, organic content, physical parameters and DGGE analysis of 16S rDNA fragment genes. Computation of the DGGE and environmental parameters matrices was carried out using the Euclidean distance and Steinhaus coefficient respectively and UPGMA was selected as a clustering method for the presentation of the results. The resulting dendrograms (Figure 2.1.1.D) showed that the samples were clustered according to the depth of their sampling, in agreement with measured physical and chemical parameters.

Ordination methods

Another way of analyzing DGE profiles is to bring out major tendencies of the variance of the samples for the whole set of descriptors using multivariate ordination methods. Legendre & Legendre (1998) provide a excellent review of these methods which are commonly used in the field of ecology. These methods are used for the integration of complex sets of data (i.e. bands in the DGE patterns) into new mathematical variables, which can be projected into a few-dimension perspective (reduced space). The major advantage of these methods is to display the whole set of samples on a simple scheme, and to highlight the possible descriptors which are governing their dispersion (ter Braak *et al.* 1995). Of course, true correlation can only be deduced when sufficient amounts of data are provided: the results of proposed statistical analysis should be considered with care, as coincidence or convergence mechanisms cannot be excluded.

Common ordination methods include Non-metric MultiDimensional Scaling (NMDS), Principal Component Analysis (PCA), Correspondence Analysis (CA), Canonical Variate Analysis (CVA), and Canonical Correspondence Analysis (CCA). Several complementary statistical procedures can be applied to analyse DGE data (Yang *et al.* 2001). Details on the specific underlying theory of each of these methods can be found elsewhere (McSpadden Gardener & Lilley 1997; Legendre & Legendre 1998).

NMDS is an ordination method which reduces complex DGE patterns to a point in a two-dimension space. By connecting the consecutive points, the relative changes in the bacterial community can be visualized. van Hannen *et al*. (1999) proposed to calculate Nei-Li distances from the binary data resulting from DGE profile analysis and to represent these distances using this ordination method. The authors showed that bacterial communities that developed on two distinct detritus substrates differed significantly: the distances calculated between communities from different substrates were greater ($p<0.05$) than the distances calculated between the replicates for a given substrate. NMDS was used elsewhere for the interpretation of DGE data (Diez *et al.* 2001; Schäfer *et al.* 2001). The advantage of NMDS is to represents the objects in two or three dimensions, with dissimilar objects far apart and similar objects close to one another in the ordination space.

PCA generates new variables, called principal components (linear components of the original variables), which explain the highest dispersion of the samples. This method was often used for the interpretation of DGE community fingerprinting analysis (Müller *et al.* 2001; Ogino *et al.* 2001; van der Gucht *et al.* 2001; Yang *et al.* 2001). As an example, Müller *et al*. (2001) used PCA to compare 16S rDNA DGGE profiles for bacterial communities present in mercury-contaminated soils. Their investigations showed that the DGGE approach generated more distinctive results than colony morphotyping and substrate utilization. Van der Gucht *et al*. (2001) showed that the composition of bacterioplanktonic communities differed between two lakes and during seasons using a PCA applied to presence/absence of bands within 16S rDNA DGGE patterns. Using

Spearman's rank correlation, the observed seasonal variations were found to be positively correlated with environmental variables such as temperature, nitrate concentration or microbial biomass. However, PCA is probably not the most suitable statistical approach for analyzing DGE patterns, as its underlying model assumes that biological populations have a linear response curve along the axes of ecological variation. Niche theory tells us that populations have ecological preferences. An unimodal (i.e. bell-shaped) response distribution of the different bacterial populations present in a community is probably closer to reality, with more individuals near some optimal environmental values.

CA may be applied to any data table that is dimensionally homogenous. ter Braak (1985) showed that the underlying model was adapted to presence/absence or abundance data tables and consequently, that the analysis was well suited for populations with unimodal distribution along environmental gradients. Using this statistical analysis, Jourdain-Miserez *et al*. (2001) analyzed 16S rDNA gene fragments issued from milk samples on TTGE gels. The results clearly showed different community structures between organic and conventional farming practices (Figure 2.1.1.E). CA was also used elsewhere for similar approaches (Ibekwe *et al.* 2001; Yang *et al.* 2001).

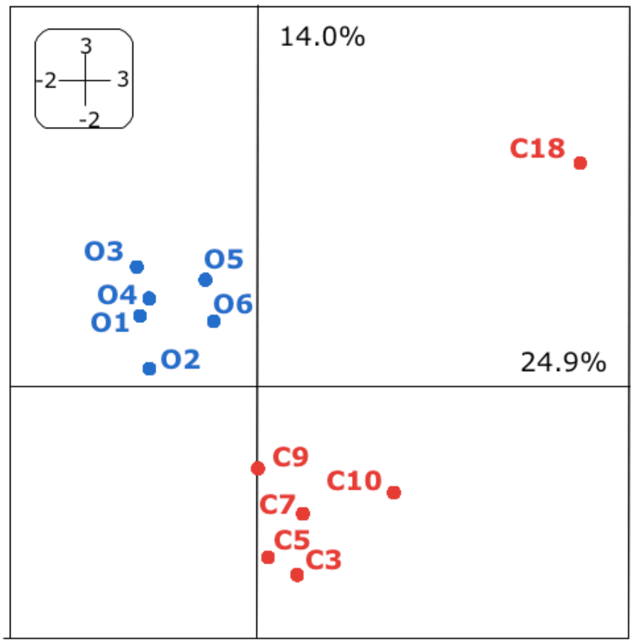

Figure 2.1.1.E Correspondence analysis of TTGE community profiles from milk samples from 12 farms (Switzerland). TTGE data of five samples taken on five consecutive days were pooled before being analyzed (sum of unconstrained eigenvalues: 1.74). **C**: conventional and **O**: organic farming practices. Numbers refer to the different farms (Jourdain-Miserez *et al.* 2001)

Interpretation of DGE patterns with environmental variables

From our point of view, the greatest opportunity of the statistical analysis of DGE patterns is offered when the community profiles are combined in a joint analysis with environmental data sets. The relevant question here is to know whether the variations observed between different banding patterns could be associated with the variations of measured environmental variables.

McCaig *et al*. (2001) applied multivariate analysis to reduce the original data for grassland DGGE community patterns into six principal components. They showed clear differences between improved and non-improved grassland communities using CVA. This method requires an *a priori* definition of groups and finds linear combinations of variables that maximize the ratio between-group variation to within-group variation.

The 'community matrix' obtained from DGE profiles can be tested also against a second matrix obtained from environmental data sets measured on the same samples. CCA is a powerful canonical ordination technique (multivariate direct gradient analysis) allowing the explanation of the structure of a 'species' data table by quantitative environmental descriptors and assuming unimodal distributions of species (ter Braak 1986). Using this technique, Yang & Crowley (2000) compared bacterial rhizospheric communities associated to barley plants under iron-limiting and iron-sufficient growth conditions. As a result, they showed that about 40 % of the variation between microbial communities could be attributed to plant iron nutritional status. The Figure 2.1.1.F presents a CCA of a DGGE analysis carried out on samples taken from the water column of Lake Loclat (Forestier *et al.* 2002). In this case, five environmental variables were selected according to their high probability of correlation with the samples ($p<0.05$) using Mantel tests (Mantel 1967).

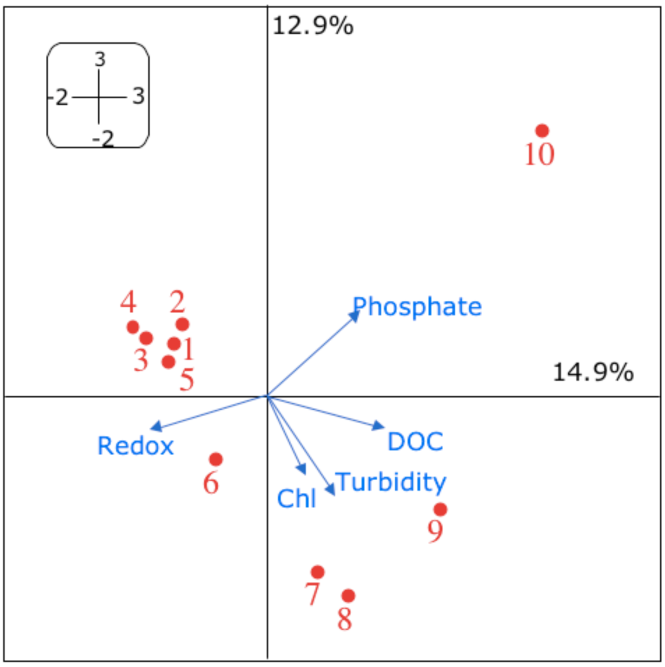

Figure 2.1.1.F Canonical Correspondence Analysis of microbial community patterns generated by 16S rDNA DGGE analysis for ten water samples (Lake Loclat) ranked by depth (1 for surface to 10 at 8.7 m depth). The total inertia of the matrix was 4.45 and the selected variables explained 59 % of the variance of the DGGE data set (sum of canonical eigenvalues: 2.62) (Forestier *et al.* 2002)

This test is based on the linear correlation between two distance or similarity matrices obtained from independent data. As shown in the Figure 2.1.1.F, the redox and the dissolved organic carbon were the variables that influenced mostly the dispersion of the samples. The first five samples taken from the aerobic zone (samples 1 to 5) are closely related, defining a homogenous bacterial structure. The samples taken from the anaerobic section of the water column (points 6 to 10) were displayed according to depth indicating a continuum of different bacterial communities.

2.1.1.3 Conclusions

DGE fingerprinting techniques are very effective methods for the characterization of bacterial community structures. These techniques are convenient for the simultaneous analysis of numerous samples. They are consequently well suited for the monitoring of whole communities, focusing on phylotypes for which the occurrence and/or the relative frequency are affected by any environmental change. As shown above, emphasis should be brought to the standardization of the whole analytical procedure as a means for increasing the reliability of the method and the reproducibility of the patterns. For instance computer-assisted analysis of the profiles should be generalized, escaping the merely qualitative reading of the fingerprinting patterns.

The exploratory aspect of the statistical techniques applied to DGE patterns that we present here is the consequence of statistical developments brought to the field of plant and animal ecology. It is now possible to approach causality in microbial ecology with statistical methods using experimental designs which were impossible to conceive a few years ago, principally because of the time and resources needed for the analysis of high number of samples. Examples provided above showed that it is possible to apply statistical tools to DGE data sets efficiently. The first result is in the validation of the interpretation of the patterns, such as shifts in the microbial community structure or the identification of key-populations which may be affected by changing conditions. Moreover, whole pattern data generated by the DGE analysis can be directly tested for correlation analysis against any single or combination of environmental sets of variables.

However, care should be taken in the choice of the statistical analytical procedure. As shown above, the underlying theoretical model should be carefully assessed before any attempt of application. Some analysis used up to now were probably not well suited to this type of data sets. On the contrary, CA is particularly well suited for abundance data sets, and PCA (normalized using correlation) is perfectly adapted for the analysis of environmental data sets (standardized descriptors).

The complementation of DGE analysis with a statistical approach leads to the definition of new hypotheses and to new prospects in terms of spatial or temporal functioning of microbial systems. Statistical methods reveal putative correlations between different sets of variables. They do not permit however to draw conclusions regarding the causality of these correlations. Therefore, statistical analyses should not be considered alone, but in a dialectic relationship with an ecological hypothesis. Automated pattern recognition and mechanistic dynamic modeling (combined with field and lab experiments) will probably very soon be the future steps in this field. In this sense, it will be conceivable to describe more precisely the relations between the observed diversity of the organisms and their ecological niches, leading to the development of the promising concept of ‘bacterial sociology’.

Acknowledgement

The authors would like to thank Camilla Rusca and Simona Casati, Noémie Matile and Muriel Meier for their technical assistance, Jacqueline Moret and Florian Kohler for their advises in statistics and Patrick Guerin for the English correction of the manuscript.

En dépit de certaines différences d'appréciation entre chercheurs sur le choix des techniques d'ordination à utiliser, un certain consensus se dégage sur le fait que l'utilisation des méthodes d'ordination a contribué à l'amélioration sensible de l'interprétation des données de fingerprint en écologie microbienne moléculaire ces dernières années.

2.1.2 A la recherche d'indices spécifiques pour l'analyse des profils de communautés

Notre analyse de la littérature (§ 2.1.1) a fait apparaître que l'utilisation judicieuse d'outils statistiques permettait non seulement de décrire la structure des communautés avec pertinence, mais aussi d'expliquer, en partie au moins, les modifications des communautés observées. La décennie 1990-2000 a vu l'émergence de l'écologie microbienne moléculaire. Elle sera peut-être considérée dans quelques années comme l'époque de naissance d'une nouvelle discipline, la 'bactério-sociologie'.

En plus d'informations sur le nombre d'espèces présentes et sur leur importance relative, les fingerprints contiennent de l'information sur la disparité génétique entre espèces. Ce dernier aspect intéresse beaucoup les écologistes microbiens qui surveillent des modifications intervenues entre deux communautés de microorganismes. Dans ce paragraphe, nous allons utiliser ces informations sur la disparité génétique pour calculer des indices de diversité qui extraient une information différente de celle utilisée jusqu'ici (Shannon & Weaver 1963; Simpson 1949).

Afin de parler de l'adéquation de l'indice de Shannon aux fingerprints de communautés (parfois appellé indice de Shannon-Wiener, Shannon-Weiner, Shannon-Weaver ou encore indice de Shannon-Weaner!!), je retiens une citation d'Oscar Wilde (Wilde 1893), reprise par Hill et coll. (2003):

> '*to lose valuable information by relying upon an enigmatic and perhaps inappropriate index may be regarded as a misfortune, but to lose its name as well looks like carelessness*'

From microbial community barcode to genetic heterogeneity indices

Ce paragraphe a fait l'objet d'un article de **J. Hamelin[1]**, F. Poly[1], O. Besson[2], S. Fall[3], A. Brauman[3], S. Tarnawski[1], M. Aragno[1] and N. Fromin[1]. Cet article a été soumis à Molecular Ecology.

[1] Laboratoire de Microbiologie, Université de Neuchâtel, CP 2, CH-2007 Neuchâtel

[2] Institut de Mathématiques, Université de Neuchâtel, CP 2, CH-2007 Neuchâtel

[3] Laboratoire d'Ecologie Microbienne des Sols Tropicaux, IRD-ISRA, BP 1386, Dakar (Sénégal).

Abstract

Electrophoretic profiles of the PCR amplification products from environmental DNA samples, such as DGGE, RISA, SSCP, T-RFLP, provide fingerprinting patterns expressing snapshots of microbial communities. These patterns directly highlight the structure of the dominant populations. In this sense, community fingerprinting has been extensively used to follow changes in single populations, or in the community structure. Shannon or Simpson indices are frequently used to compare fingerprinting profiles. However, these classical diversity indices overlook information about distance heterogeneity of bands observed in community fingerprints. Consequently, relevant indices to compare fingerprinting patterns are often lacking while ordination methods already take the information about the band disparity into account. In this article, we have developed two diversity indices especially designed for fingerprinting comparison, based on the distribution of bands. These not only account for the intensity of bands, but also the distance of migration of bands in the whole profile. The Average Migration Index (*AMI*) represents the gravity center of the profile, while the Spatial Gathering Index (*SGI*) describes the disparity of bands distribution. When used in combination or in association with classical tools, these indices reflect the shifts of microbial community stuctures and the relatedness of fingerprinting profiles observed by visual inspection.

2.1.2.1 Introduction

Organisms living in different habitats constitute complex communities. These communities are characterized by their species richness and abundance and by the genetic distance between species. Diversity indices initially designed for the description of the community structure of multicellular, higher organisms, were only based on the number and abundance of distinct populations (Shannon & Weaver 1963; Simpson 1949). Recently, Hill *et al.* (2003) showed that Shannon index is inaccurate when coverage of diversity is low (what is generally observed for cloning sequencing approach) and gives more weight to individuals of rare species than those of common ones. These authors discussed whether rare species need to be highlighted, as they are *a priori* not ecologically important. Nonetheless, such indices were often used in microbial ecology (Nübel *et al*. 1999; Simpson *et al*. 1999; Ampe & Miambi 2000; Kocherginskaya *et al.* 2001; McCaig *et al*. 2001; Ogino *et al*. 2001; Müller *et al*. 2002).

Informative microbial diversity measurements should focus not only on species richness and abundance, but also be complemented with information concerning the heterogeneity of organisms (Clarke & Warwick 1998; Martin 2002). Indeed, the genetic diversity in microbial communities, as revealed by the disparity of sequences, cast a light on the stability or on the functional potential of the environments studied (Finlay *et al*. 1997; Bengtsson 1998; Hunter-Cevera 1998).

The development of community studies, using PCR-based fingerprinting techniques during the 1990's gave access to complex community structure in a single snapshot. PCR fragment heterogeneity is evaluated by G+C% content and distribution of the DNA fragment for Denaturing Gradient Gel Eelectrophoresis (DGGE) (Muyzer *et al.* 1993), by their differential renaturation for Single Strand Conformation Polymorphism (SSCP) (Peters *et al.* 2000), by the length of 16S-23S rDNA sequences for Ribosomal Intergenic Spacer Analysis (RISA) (Ranjard *et al.* 2000b), or by the length of the terminal 16S rDNA restriction fragment for Terminal Restriction Frangment Length Polymorphism (T-RFLP) (Moeseneder *et al.* 1999). These techniques allow the dominant DNA fragments to be displayed as distinct bands along a profile, like barcodes (those representing 0.1 to 1 % of the all sequences (Muyzer *et al*. 1993)). These bands reflect not only the presence and abundance of corresponding populations, but also their genetic heterogeneity in the microbial community. Distant bands within a pattern reflect a taxonomic disparity of

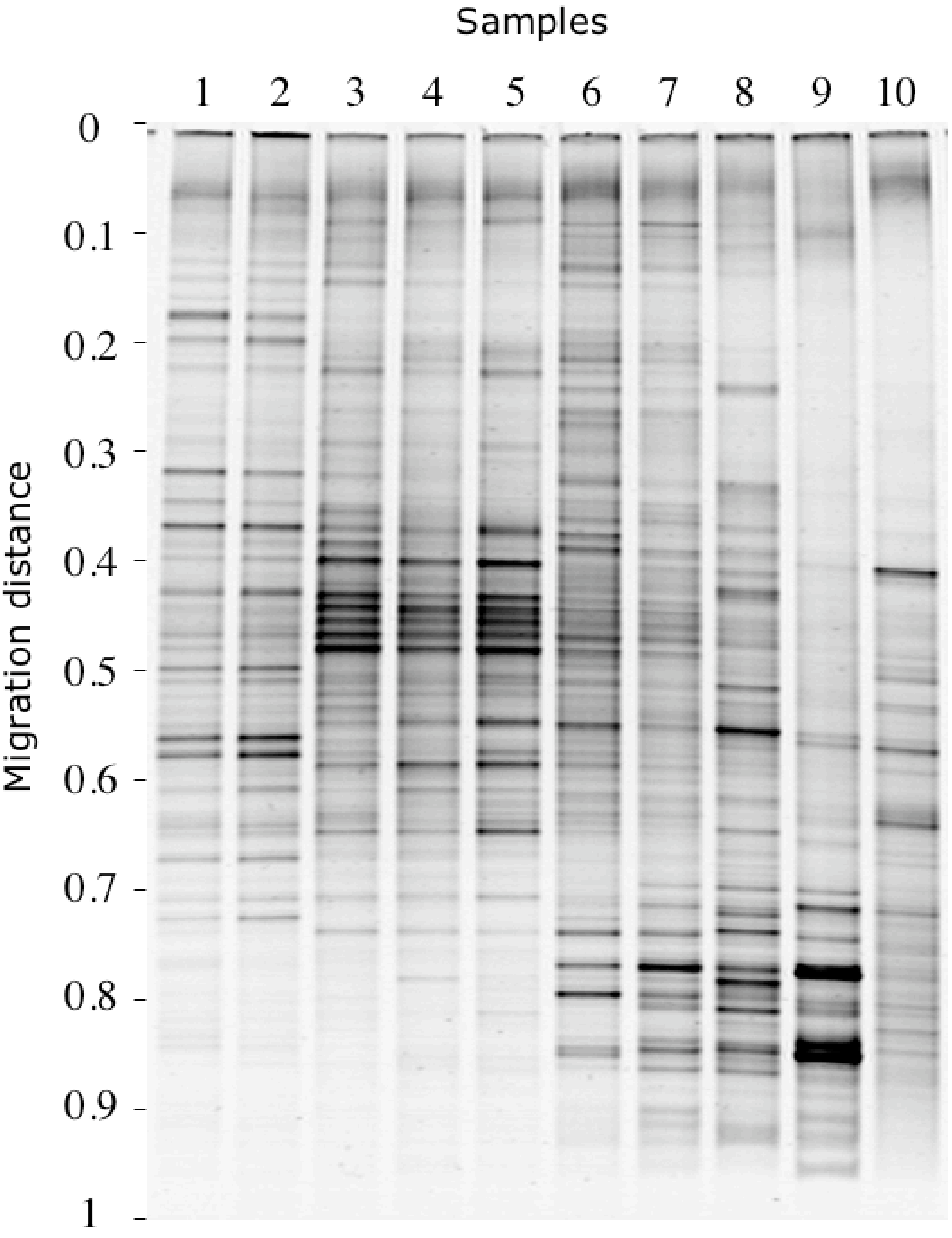

Figure 2.1.2.A DGGE profiles (Fall in prep) corresponding to the bacterial communities along the gastro-intestinal track of the termite *Cubitermes niokoloensis*, its nest, and the surrounding soil. Line 1, mouth; line 2, middle digestive track; line 3, posterior digestive track; lines 4 & 5, whole gastro-intestinal track; line 6, faeces; line 7, galeries; line 8, external wall; line 9, inner wall; line 10, surrounding savanna soil

corresponding organisms, whereas close bands do not necessarily imply that the corresponding organisms are closely related. In molecular fingerprints, single bands are treated as single Operational Taxonomic Unit (OTU) (Fromin *et al.* 2002).

Troussellier & Legendre (1981), Bongers (1990), Heip *et al.* (1998) and Martin *et al.* (2002) advise microbial ecologists to use indices that take into account information about taxonomy, functional evenness, and/or body size of populations. Watve & Gangal (1996) argued that the current definition of bacterial species is unsatisfactory for the appreciation of diversity indices. In that sense, Faith (1995) developed an alternative approach, called 'Phylogenetic Diversity', to estimate organism biodiversity at different levels using information about phylogenetic relationships between studied organisms. Troussellier & Legendre (1981) looked at a functional evenness index based on physiological information adapted to microbial world.

Ecologists have developed diversity indices based on species abundance (Shannon and Simpson) to compare communities but we wonder about such indices whether they 'extract' enough information from the fingerprints to characterize the profiles. In this paper, we explore the possibility of applying new diversity measurements to microbial community fingerprinting patterns with the minimal loss of information, in order to complete the classical diversity indices. We choose two indices describing independently one type of information enclosed in fingerprints. We proposed that the band distribution and the gathering of bands in a profile could be good descriptors of the genetic heterogeneity, and should be added in more integrated indices. The Average Migration Index (*AMI*), which stands for the gravity centre of bands, and the Spatial Gathering Index (*SGI*) developed here complete the description of fingerprinting pattern given by available diversity indices. As compared to ordination methods, *AMI* and *SGI* also quantitatively grasp the visual differences between profiles but make the comparison easier without the need of a reference pattern. We applied these indices on DGGE patterns for demonstration.

2.1.2.2 Materials and Methods

DGGE profiles presented in figure 2.1.2.A (Fall in prep) were obtained with partial 16S rDNA molecules, using the D-code electrophoresis system from Bio-Rad. 600 to 800 ng of PCR products. The molecules were loaded directly on a 8 % (wt/vol) polyacrylamide gel with gradient ranging from 30 % to 60 % denaturants (one hundred percent correspond to 40 % formamide plus 7 M urea). The gels were run at 60 °C and 150 V for 5 hours in 1× TAE buffer, and stained in 0.01 % Sybr Green (Molecular Probes, Leiden, The Netherlands) at 4 °C in dark for 20 min, then UV photographed. The DGGE gel was digitalised with 1000 pixels in Y axis. The number of bands (n), their position in the gel (d_i; distance of migration of the i^{th} band in the gel on a 0 to 1 scale), and the relative intensity (p_i) of each band within the profile (from 0 to 1) were reported using GelCompar software (Applied Maths, Kortrijk, Belgium). The sum of relative intensities of all bands is equal to 1. The program allows the user to define a lower limit for relative intensity of bands ($p_i \geq 0.001$). Each band was treated as unique OTU for statistical analysis (cf. § 2.1.1; Fromin *et al.* 2002). In this study, two bands were considered as different when they differed by at least 0.5 % of the gel length ($d_i \geq 0.005$), corresponding to the minimum value allowed by the analytical procedure.

The band richness (S), corresponding to the number of detected bands, was determined for each banding pattern. The Shannon diversity Index (H') (Shannon & Weaver 1963) is calculated as follows:

$$H' = -\sum_{i=1}^{n} p_i \cdot \ln p_i$$

Table 2.1.2.B Comparison of Richness, Shannon, Simpson, Average Migration, and Spatial Gathering indices obtained with the ten DGGE profiles presented in figure 1. Minimal (min) or maximal (max) values of indices are calculated using the observed richness, the defined lower limit of relative intensity for bands ($p_i \geq 0.001$) and the minimal distance between bands (di ≥ 0.005), as descriebed in the materials and methods section

DGGE	Richness	Shannon (*H'*)			Simpson (*D*)			Average Migration (*AMI*)			Spatial Gathering (*SGI*)		
lines	(*S*)	calc	min	max	calc	min	max	calc	min	max	calc	min	max
1	46	3.218	0.355	3.829	0.055	0.022	0.914	0.421	0.057	0.948	0.261	0.024	2088
2	43	3.177	0.331	3.761	0.054	0.023	0.920	0.433	0.050	0.955	0.621	0.028	2115
3	47	3.233	0.363	3.850	0.053	0.021	0.912	0.433	0.059	0.946	2.016	0.023	2080
4	48	3.017	0.371	3.871	0.069	0.021	0.910	0.481	0.061	0.944	4.004	0.022	2071
5	46	3.031	0.355	3.829	0.060	0.022	0.914	0.480	0.057	0.948	2.265	0.024	2088
6	61	3.547	0.473	4.111	0.036	0.016	0.887	0.524	0.097	0.909	0.220	0.014	1960
7	63	3.574	0.488	4.143	0.036	0.016	0.884	0.577	0.103	0.902	0.341	0.013	1944
8	53	3.323	0.410	3.970	0.046	0.019	0.901	0.632	0.074	0.931	0.534	0.018	2028
9	34	2.577	0.260	3.526	0.105	0.029	0.936	0.688	0.033	0.972	7.060	0.045	2213
10	47	3.276	0.363	3.850	0.047	0.021	0.912	0.682	0.059	0.946	1.033	0.023	2080

The Simpson dominance Index (Simpson 1949) is calculated as follows:

$$D = \sum_{i=1}^{n} p_i^2$$

2.1.2.3 Results and discussion

We designed new indices using both intensity and distance of migration of the bands. The Averaged Migration Index (*AMI*) measures the 'centre of gravity' of the profile, *i.e.* the mean position of the bands taking into account their intensity. *AMI* is calculated as follow:

$$AMI = \sum_{i=1}^{n} d_i \, . \, p_i$$

where p_i represented the relative intensity (on a 0 to 1 scale) and d_i the distance of migration (on a 0 to 1 scale) of band *i* in the profile. *AMI* varies from 0 to 1. The Spatial Gathering Index (*SGI*) also developed in this paper measures the disparity of the major bands in a fingerprinting profile, and is calculated as follows:

$$SGI = \sum_{\substack{i,j=1 \\ i \neq j}}^{n} \left(\frac{p_i \, . \, p_j}{d_{ij}} \right)^2$$

where p_i and p_j expressed the intensity of the i^{th} and j^{th} bands (from 0 to 1), and d_{ij} was the distance between bands *i* and *j* (from 0 to 1). In the formula, the iterations $p_i.p_j/d_{ij}$ for each couple of bands was squared in order to minimize the importance of the bands with a low intensity in the index. The *SGI* emphasises close couple of bands all the more that these bands are intense.

As an illustration, the DGGE gel based on ten bacterial community samples in figure 2.1.2.A was chosen for the calculation of classical diversity indices and new indices based on the distribution of bands along profiles. Table 2.1.2.B summarises the values obtained with Richness (*S*), Shannon (*H'*), Simpson (*D*), Average Migration (*AMI*), and Spatial Gathering (*SGI*) indices. When used in combination or in association with usual tools, these new indices reflected the relatedness of fingerprinting profiles observed by visual inspection (Figure 2.1.2.C).

The band richness varied from 34 to 63 with pattern (Table 2.1.2.B), using the same detection sensitivity. The Shannon Index (*H'*) varied from 2.577 to 3.574. These values fall in the range of 1.5-4.5 usually found in most communities (Magurran 1988). Shannon Index is commonly used to assess diversity. It is highly influenced by the overall number of bands (Table 2.1.2.D) or by a few intense bands, which is often observed in fingerprinting profiles. The Simpson dominance index varied from 0.036 to 0.105 when calculated from the ten patterns of figure 2.1.2.A. The *AMI*, which measures the 'centre of gravity' of bands in the profile, varied gradually from the left (0.421 for line 1) to the right (0.688 for line 9). This variation is in agreement with the visual inspection of the profiles. *AMI* expresses different biological traits depending on the technique of profiling used. In case of DGGE fingerprinting patterns, *AMI* is related to the averaged percentage of G+C in sequences. In case of RISA fingerprinting, *AMI* reflects the average length of 16S-23S rDNA intergenic spacer. On the contrary to DGGE and RISA, we cannot expect any significant biological information for *AMI* calculated from SSCP and T-RFLP profiles.

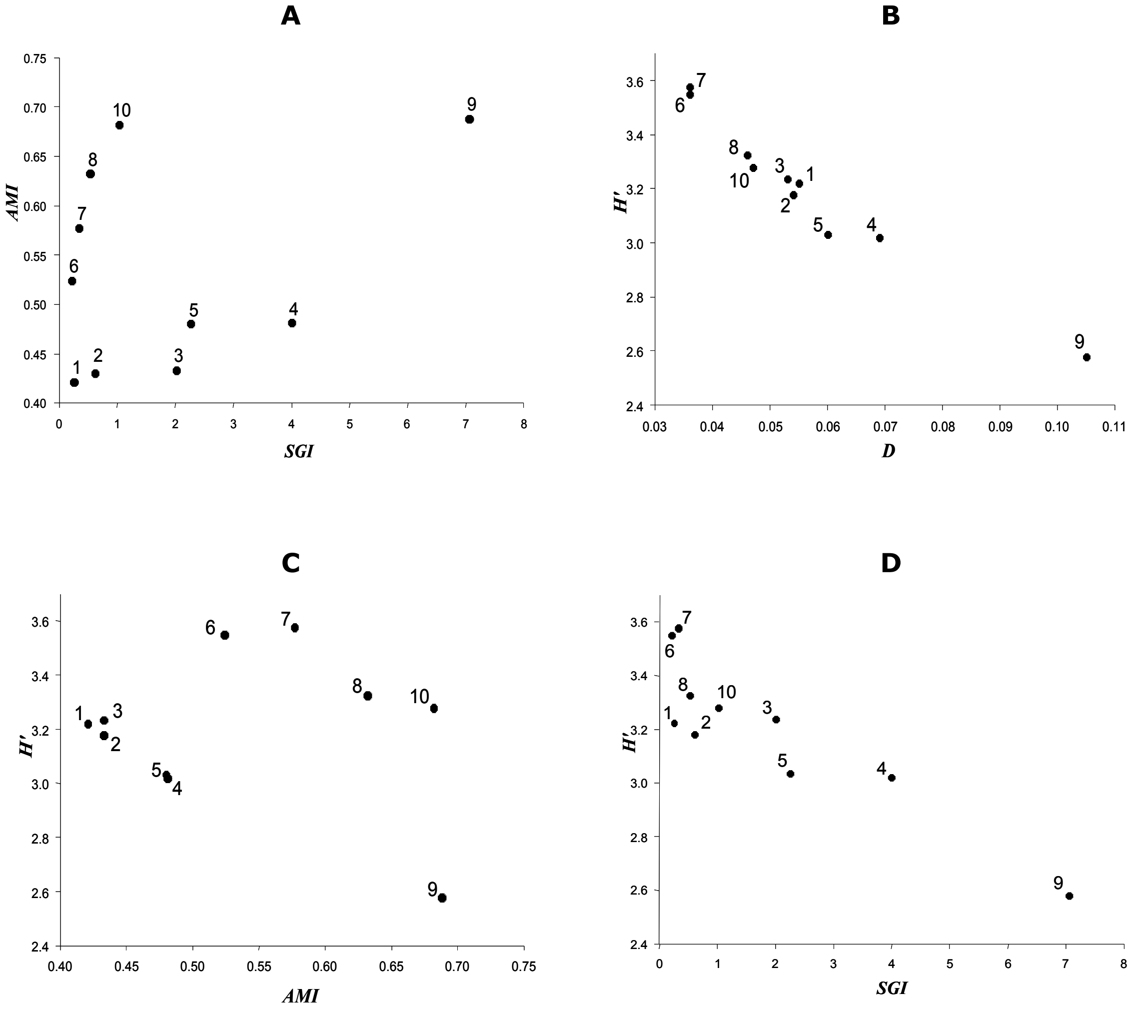

Figure 2.1.2.C Fingerprinting profiles from figure 1 ordered in a two-dimension space, from the values obtained with diversity indices (A) Average Migration -*AMI*- *versus* Spacial Gathering -*SGI*-; (B) Shannon -*H'*- *versus* Simpson -*D*-; (C) *H' versus AMI*; and (D) *H' versus SGI*

The disparity of sequences in a profile is further emphasised by the *SGI*, varying from 0.220 (line 6) to 7.060 (line 9). A profile with a low calculated *SGI* correspond to a fingerprint composed of bands with equal intensity (high Shannon value) and scattered along the whole length of the profile. For example, the profiles 1, 3 and 10 had similar calculated *H'* values (between 3.21 and 3.28) but different *SGI* values (0.261, 2.061 and 1.033 respectively). Line 3 had a high *H'* value due to a large number of bands, but also an high *SGI* value due to the clustering of the most intense bands, this is not the case for both of the other profiles. The diversity indices used highlighted different features (number of bands, dominance of few bands, or genetic dispersion of OTUs) which are enclosed in fingerprints. The figure 2.1.2.C compared the index values obtained in pairs. The Shannon and Simpson indices, based on the relative intensity of bands, are, as expected, inversely correlated. *SGI* and *AMI*, calculated on both intensity and migration of bands, showed more independent changes as they described different types of information which are enclosed in fingerprints. The communities are well dispersed when the Shannon index is represented in association with new indices, especially when *AMI* versus *H'* was considered.

Table 2.1.2.D Summary of differential characteristics for some classical indices (Magurran 1988) and for the indices adapted to fingerprinting pattern analysis

Indices calculated	Designed for fingerprinting	Discriminant ability patterns	Influenced by	Calculation
Species Richness	no	good	richness	simple
Shannon	no	moderate	richness	intermediate
Shannon evenness	no	poor	dominance	simple
Simpson	no	moderate	dominance	intermediate
Average Migration	yes	good	dominance	intermediate
Spatial Gathering	yes	good	dominance	complex

The communities represented in lines 2 and 3 differ visually (Figure 2.1.2.A). A pertinent index should reflect such a difference. Calculated Simpson Index values were almost the same for both lanes (0.054 and 0.053 respectively). This index is highly influenced by few intense bands rather than a large number of bands (Table 2.1.2.D). Shannon index values were similar (3.177 and 3.233 respectively) for these two samples, probably because of a similar number of bands detected for these two DGGE lanes (43 and 47 respectively). For this comparison, the calculated *AMI* is not informative. The average migration of bands was globally similar for both profiles, but with completely different band distribution, as dominant bands are gathered in line 3 as compared to line 2. These indices failed to highlight crucial information, as lines 2 and 3 could not be regarded as identical by visual inspection. The *SGI* differentiated these communities (0.621 for line 2 and 2.016 for line 3), because it considers both gathering and intensities of bands. Same conclusions were noticed for the comparison of lines 8 and 10 (Table 1.2.2.A).

This *SGI* stresses proximal bands all the more these bands are intense. Furthermore *SGI* value provides information about genetic homogeneity in the community. High *SGI* values observed in lines 3, 4, 5 and 9 indicate a high genetic homogeneity of dominant populations in these communities. Whereas lines 1, 2, 6, 7, and 8 harboured a low *SGI* values meaning that in these environments, bacterial communities were characterized by genetically disparate OTUs (bands scattered on the whole length of the gel).

When the proximity of bands within a fingerprint pattern is related to the taxonomic relatedness of corresponding organisms, the *AMI* and *SGI* are useful indices to give information on the genetic heterogeneity of microbial communities. This notion is lacking in the other indices whereas it is important for describing and comparing the diversity of microbial communities (Martin 2002). Both *AMI* and *SGI* parameters correspond to what microbial ecologists are interested in fingerprinting patterns.

The information contained in fingerprinting profiles can by also summarise by using ordination methods. Principal Componant analysis (PCA) or Correspondence Analysis (CA) are powerful tools for highlighting and selecting the populations which are the most discriminative between community fingerprinting profiles (Fromin *et al.* 2002). Moreover, ordination methods allow changes in populations or communities with physico-chemical parameters to be correlated. The use of diversity indices presents an alternative approach, with the major advantage on the ordination methods that it provides a comparison of results obtained separately without the need of a reference pattern. Diversity indices values obtained from the profiles remain unchanged with the addition of new data in the analysis and make the comparison between fingerprinting profiles easier.

The new indices (*SGI* and *AMI*) are not only focussing on the intensity of bands, but also on the migration distance in the whole fingerprinting profile. In association, the diversity indices are powerful tools to summarise all the fingerprinting profile information (especially the genetic disparity), that is not taken into account with classical diversity measurements used in ecology (Shannon and Simpson indices). There is a need to develop new indices to analyse genetic fingerprinting profiles, in particular to correlate diversity indices with environmental variables. The fingerprint-based diversity indices should express a realistic view of communities along ecological parameters such as transects, time scale analysis and seasonal variations.

Acknowledgment

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2.1.3 Comparaison des communautés bactériennes présentes et actives associées à *Molinia coerulea*

Ce travail s'inscrit dans le cadre du diplôme de Maryline Jossi (Jossi 2003), réalisé sous la direction de **J. Hamelin** et de N. Fromin. F. Kohler du Laboratoire d'écologie végétale et de phytosociologie a été d'un conseil précieux pour l'utilisation des méthodes d'ordination.

2.1.3.1 Introduction

Les communautés rhizosphériques associées à la molinie sont sous influence des exsudats racinaires. Dans le § 1.1.2, nous avons vu comment la rhizodéposition pouvait varier en fonction des nombreux facteurs environnementaux. Dans l'étude présentée, nous avons exposé les plantes à une atmosphère enrichie en pCO_2 (système FACE décrit dans le § 1.4.3) de manière à évaluer l'impact potentiel des changements globaux sur les communautés bactériennes associées à la rhizosphère de cette plante pérenne (§ 1.3.2). L'étude a été menée en parallèle sur *Lolium perenne*, une autre graminée perenne, mais je me limiterai ici aux résultats concernant la molinie.

Nous avons utilisé une méthode de fingerprinting moléculaire basée sur le gène ribosomique 16S. L'extraction des ARN totaux, en parallèle à l'extraction classique des ADN à partir du sol et des racines, nous a permis de mettre en évidence à la fois les populations métaboliquement actives et/ou les populations les plus abondantes, respectivement, lors des prélèvements. En effet, le contenu en ribosomes (et donc le nombre de copies de molécules d'ARNr 16S constitutive des ribosomes) est proportionnel au taux d'activité des cellules (Wagner 1994). Les différences observées au niveau des communautés bactériennes actives ou présentes ont été mises en relation avec les paramètres environnementaux (effet du pCO_2 atmosphérique, présence de la plante, date de prélèvement).

2.1.3.2 Matériels et méthodes

Echantillonnage

Toutes les manipulations, depuis le prélèvement jusqu'aux analyses de laboratoire, ont été menées dans des conditions *'RNase free'* avec les précautions d'usage: manipulation avec des gants, solutions aqueuses traitées au DEPC 0.1 %, paillasse nettoyée avec du RNase-away (Promega), pipettes dédiées aux manipulations avec l'ARN, embouts cotonnés provenant de boîtes neuves, verrerie placée à 200 °C pendant une nuit, matériel plastique nettoyé avec une solution de 0.1 N NaOH/1 mM EDTA puis rincé à l'eau traitée et travail dans la glace.

Les prélèvements ont été effectués dans le système FACE (cf. § 1.4.3) les 21 juin 2001 (date n°**1**), 7 mai 2002 (date n°**2**) et 15 juillet 2002 (date n°**3**). Pour chaque prélèvement et pour les deux conditions de culture (**C**, control et **T**, CO_2-treated), des échantillons de sol dépourvu de racines (**BS**, bulk soil) et des racines lavées à l'eau traitée (**RE**, rhizoplane/endorhizosphere) ont été prélevés à partir de deux carottes de sol. 500 mg ont été réverveés aux analyses d'ADN (**D**) et 500 mg aux analyses d'ARN (**R**). Le matériel biologique a été directement stocké dans les tubes utilisés pour l'extraction. Les tubes étaient immédiatement plongés dans de l'azote liquide puis conservé à –80 °C avant extraction des acides nucléiques.

Extractions d'ADN et d'ARN

Nous avons adapté les protocoles d'extraction d'ADN (Borneman et coll. 1996) et d'ARN (Borneman & Triplett 1997), basé sur la technique de *bead-beating*, pour qu'ils soient les plus proches possible l'un de l'autre. Le protocole d'extraction d'ADN de sol utilisé a été décrit précédemment (cf. § 4.2; Hamelin et coll. 2001). L'extraction des ARN totaux de racine et de sol combine le kit d'extraction FastRNA kit Green (Qbiogene) pour la phase de lyse (matrix tube D) et le RNeasy kit (Qiagen) pour la purification.

450 µl de tampon RTL (Qiagen) contenant du guanidine isothiocyanate, additionné de 4.5 µl de ß-mercaptoethanol, ont été ajoutés aux échantillons congelés. Ces deux composés inactivent les RNases et stabilisent les ARN libérés lors de la lyse des cellules. Les tubes ont été agités dans l'amalgameur Fastprep (Bio101) pendant 10 s à 6 $m.s^{-1}$ puis refroidis dans la glace. Cette étape a été répétée afin de lyser le maximum de bactéries. Après 5 min dans la glace, les tubes ont été centrifugés 5 min à 13000 g.

Le surnageant contenant l'extrait brut d'ARN a été transféré dans une colonne de purification QIAshredder (Qiagen) puis a été centrifugé 2 min à 13000 g pour homogénéiser la solution et éliminer les éventuels débris cellulaires. 300 µl d'éluat ont été transférés dans un tube Eppendorf propre et mélangés à 0.5 vol d'ethanol absolu. Le tout a été filtré sur une RNeasy spin column (Qiagen) en centrifugeant 15 sec à 10000 g pour fixer les ARN à la matrice de la colonne. L'éluat contenant les contaminants solubles dans l'alcool ont été écartés. L'élimination des ADN contaminants a été assurée par l'action d'une DNase (Promega) à température ambiante (5 µl de DNaseI, 40 µl de tampon et 5 µl de $MnCl_2$) puis stopée par 200 µl de DNase stop solution au bout de 15 min. La RNeasy spin column a été de nouveau centrifugée 1 min à 10000 g puis l'ARN adsorbé à la colonne a été lavé avec 700 µl de RW1 buffer (Qiagen) et centrifugé 15 sec à 10000 g. Les deux derniers lavages ont été effectués avec 500 µl de RPE buffer (Qiagen) puis centrifugé 15 sec à 10000 g. Enfin, l'extrait purifié d'ARN a été élué dans un tube propre en centrifugeant 50 µl d'eau nuclease-free à travers la colonne pendant

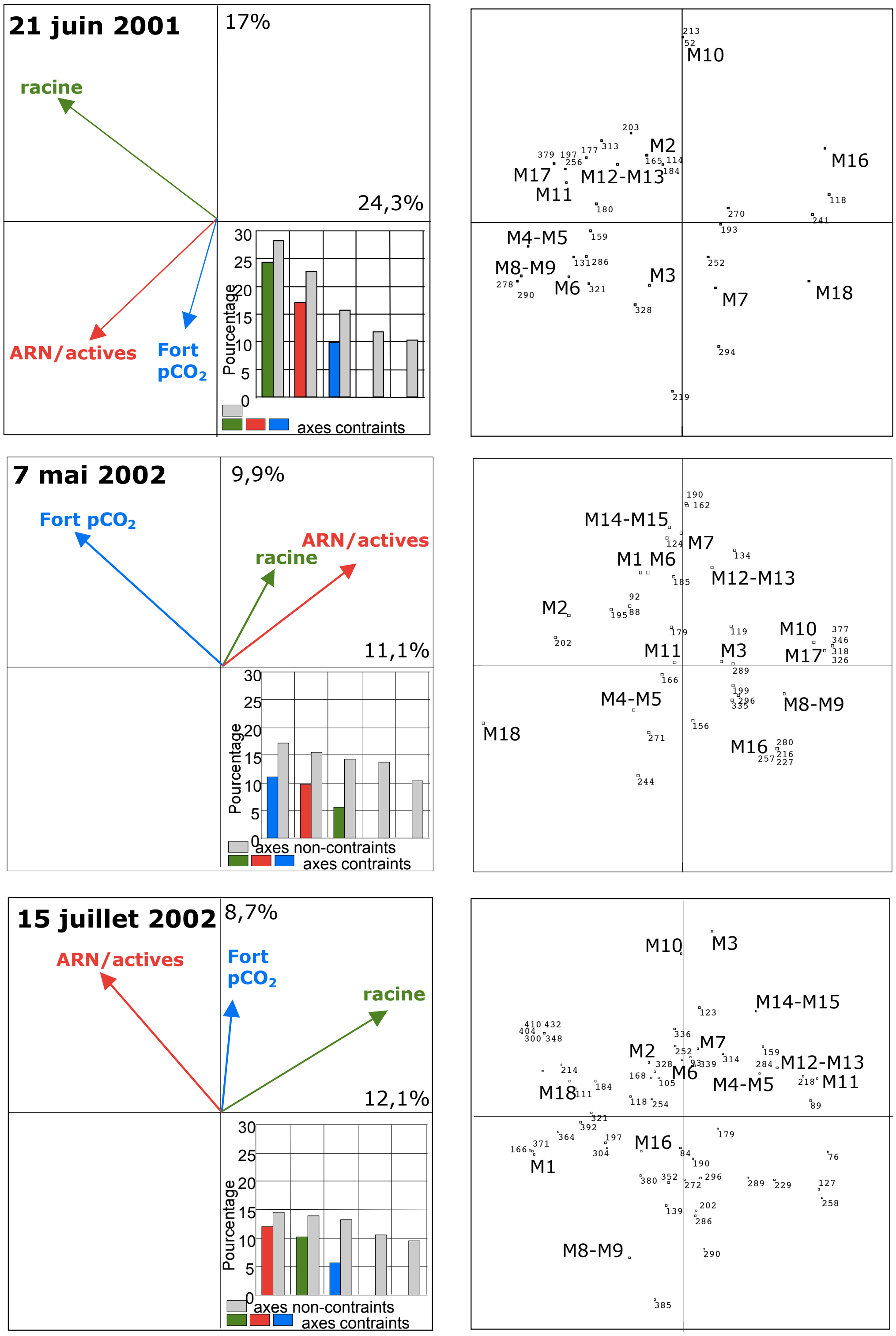


Figure 2.1.3.B Ordination par ACC des profils DGGE pour les trois dates de prélèvement prises séparément. L'inertie des matrices était de 2.64, 4.13 et 3.33 pour chacune des dates. Les graphiques de gauche indiquent l'importance relative des paramètres environnementaux utilisés dans l'ACC pour expliquer la dispersion de nos données. La longueur du vecteur est proportionnelle au pourcentage d'explication et la direction indique sur quel axe se répartissent les profils DGGE pour le paramètre considéré. Les graphiques de droite indiquent la position des bandes DGGE sur le plan d'ordination. Plus les points sont éloignés du centre du graphique, plus les bandes expliquent la variabilité des échantillons. Les bandes M1 à M18 ont été séquencées et les résultats sont présentés en détail dans le tableau 2.1.3.C

1 min à 10000 g. 50 µl de Tris 20 mM ont été ajoutés avant conservation des ARN à –80 °C. Les rendements d'extractions ont été évalués sur gel d'agarose et avec un spectrophotomètre capillaire GeneQuant (Pharmacia).

Denaturing Gradient Gel Electrophoresis (DGGE)

Le protocole de DGGE et la numérisation des gels ont été présentés dans le § 2.1.2. Les gels DGGE ont été numérisés avec une résolution de 500 pixels.

Similarité entre profils DGGE

Les similarités entre les profils de communautés ont été calculées pour les profils obtenus à partir d'échantillons cultivés sous une atmosphère contenant différentes pCO_2 ou à partir des profils obtenus à la fois sur des extraits d'ADN et d'ARN pour un même échantillon. La figure 2.1.3.A exprime les valeurs de similarité obtenues pour le sol en comparaison à la racine. Le coefficient de similarité de Steinhaus a été choisi car il prend en compte les bandes communes en donnant plus de poids dans l'analyse aux bandes intenses.

Analyse canonique des correspondances des gels DGGE

L'utilisation des méthodes d'ordination pour l'analyse des profils DGGE en général, des analyses factorielles de correspondance (AFC) et des analyses canoniques des correspondances (ACC) en particulier, a été expliquée dans le § 2.1.1.

Les gels DGGE ont été numérisés et, à partir des profils, ont été généré des matrices où à chaque position de bande correspondait une espèce bactérienne (cf. § 2.1.1 & § 2.1.2). L'apparition, la disparition ou la variation d'intensité des bandes DGGE a été mise en relation avec les différentes caractéristiques des échantillons définies dans notre plan d'échantillonnage:

√ la date de prélèvement: (**1**) juin 2001, (**2**) mai 2002 et (**3**) juillet 2002
√ la présence de la racine: (**S**) sol et (**R**) racine
√ les conditions de culture: (**C**) *ambient* pCO_2*-control* et (**T**) *elevated* pCO_2*-treated*
√ population présentes (**ADN**) et/ou actives (**ARN**)

La variabilité totale des profils DGGE est donnée par l'inertie totale de l'AFC. La variabilité expliquée par les paramètres retenus correspond à l'addition des valeurs propres de chacun des axes canoniques obtenus en ACC. Ces valeurs sont indiquées sur les graphiques d'ordination (Figure 2.1.3.B).

Choix des bandes DGGE caractéristiques

A partir des graphiques obtenus par ACC (Figure 2.1.3.B), nous avons sélectionné les bandes qui étaient visiblement liées à une ou plusieurs variables explicatives et les plus éloignées du point d'origine, pour au moins deux des trois dates d'échantillonnage. Ces bandes sont notées de M1 à M18. Le tableau 2.1.3.C indique quel critère a été retenu pour chacune des bandes retenues.

Séquençage des bandes DGGE sélectionnées

Les échantillons pour lesquelles nos bandes caractéristiques (M1 à M18) semblaient les plus intenses ont été de nouveau soumises à une électrophorèse (DGGE). Les bandes ont été localisées puis excisées au scalpel. L'ADN a été ensuite élué dans 30 µl d'eau pendant

48 heures à 4 °C. L'ensemble de l'ADN élué a été redéposé en DGGE et la bande a été de nouveau récupérée dans les mêmes conditions afin de s'assurer de leur pureté. Une amplification par PCR de 15 cycles de la région V3 de l'ADNr 16S, sans GC-clamp, a toujours donné une quantité suffisante de produit PCR pour permettre le clonage. Ce protocole est un bon compromis entre l'obtention de bandes pures et la minimalisation des risques de production de chimères ou d'erreurs de lecture lors de l'amplification par PCR. Le clonage a été effectué dans le plasmide pGEM-T (Promega) avec des cellules électro-compétentes préparées au laboratoire. Les clones obtenus après régénération sur des boîtes de LB agar additionné d'ampicilline (1 µl.ml^{-1}), de X-Gal et d'IPTG. Les inserts de 5 clones par bande en moyenne ont été amplifiés avec les primers T7 & SP6 et les amplifiats ont été digérés avec l'enzyme de restriction *Hae*III pour estimer la variabilité. 2 inserts différents pour chaque bande ont été séquencés (Synergene Biotech, Switzerland). L'affiliation phylogénétique présentée dans le tableau 2.1.3.C a été réalisée par une analyse BLAST (Altschul et coll. 1997).

2.1.3.3 Résultats et discussion

Similarités entre profils

Nous avons obtenu des profils de communautés pour les 36 échantillons testés, aussi bien à partir des extractions d'ADN que d'ARN. D'une manière générale, les similarités (coefficient de Steinhaus) entre profils DGGE issus d'extraits d'**ADN** et d'**ARN** étaient inférieures à 45 % entre les échantillons de sol et 39 % entre les échantillons de racine.
Les communautés bactériennes détectées sous atmosphère normale (**C**) différaient de celles obtenues sous atmosphère enrichie en CO_2 (**T**).

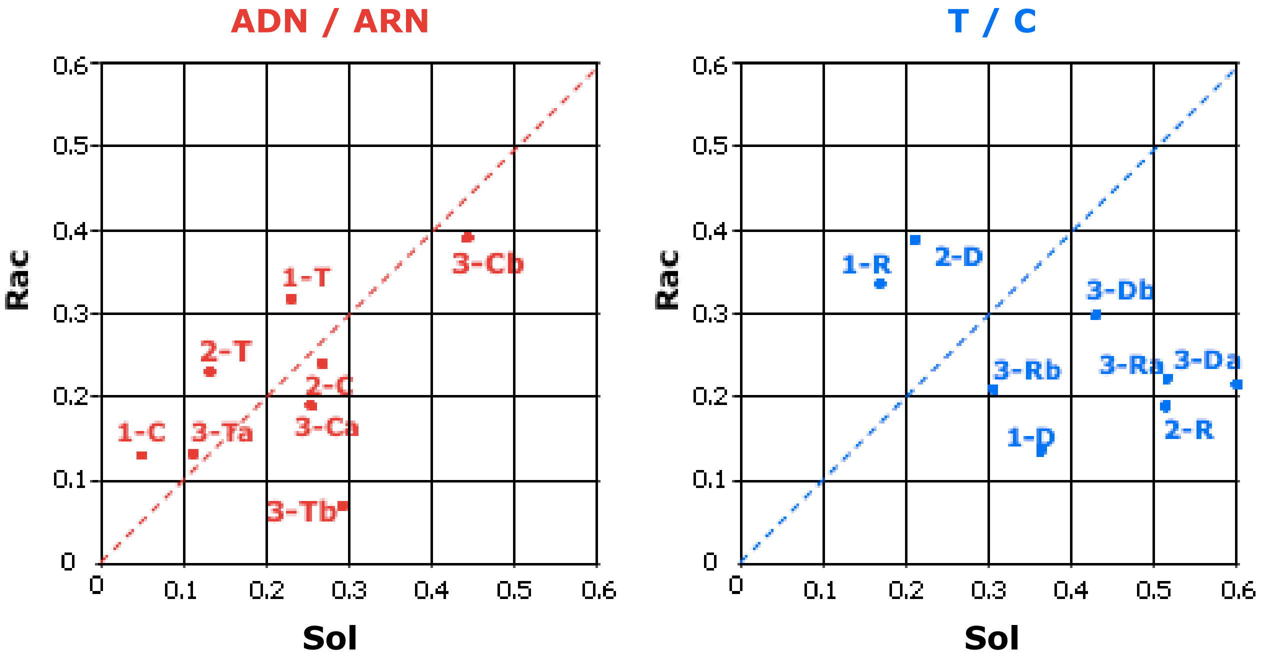


Figure 2.1.3.A Similarités des profils DGGE entre communautés présentes (**ADN**) et actives (**ARN**) pour le sol comparé à la racine (graphique de gauche) et entre les échantillons *p*CO_2*-control* (**C**) et *p*CO_2*-treated* (**T**) pour le sol comparé à la racine (graphique de droite).

Tableau 2.1.3.C Séquences obtenues par séquençage des bandes DGGE M1 à M18. Deux clones par bande ont été séquencés. Les séquences donnant des affiliations phylogénétiques différentes avec les deux clones issus d'une même position de bande ont été indiquées

nom	position gel DGGE	critères retenus pour le choix des bandes	affiliation phylogenetique des séquences proches	origine des séquences proches	homologies (%)	numeros d'accès
M1	97-100	pop. apparaissant uniquement sur des profils issus d'ARN	alpha proteobacteria, Rickettsiales groupe CFB, *Flavobacterium xanthum*	symbionte *Acanthamoeba* souche ATCC 81^T	150/163 (92) 188/193 (97)	AF132134 AF030380
M2	137-142	pop. qui fluctue avec pCO$_2$	alpha proteobacteria, Rickettsiales	symbionte *Acanthamoeba*	150/163 (91)	AF132135
M3	143-146	pop. racinaire active sous ↑ pCO$_2$	Gram$^+$, Actinomycetales	*Rhodococcus erythropolis*	177/178 (99)	AY147846
M4	150-152	populations racinaires souvent présentes	Gram$^+$, Bacillales gamma proteobacteria	*Sporocarcina macmurdoensis* clone, forêt de pin	196/198 (98) 188/195 (96)	AJ514408 AF257858
M5	150-152		gamma proteobacteria delta proteobacteria	clone de sol clone environnemental	192/196 (97) 188/195 (96)	AJ006010 AJ318168
M6	170-173	populations actives sous ↑ pCO$_2$	alpha proteobacteria, Rickettsiales beta proteobacteria, Burkholederiales	symbionte *Acanthamoeba* *Leptotrix* sp.	149/163 (91) 191/196 (97)	AF132135 AB087576
M7	206-208	population active ↑ pCO$_2$	beta proteobacteria, Burkholederiales	*Delftia acidovorans*	197/198 (99)	AF538930
M8	222-226	populations telluriques actives	Gram$^+$, Actinomycetales beta proteobacteria, Burkholderiales	clone environnemental *Ralstonia* sp.	195/199 (97) 198/198 (100)	AY177762 AY191856
M9	222-226		gamma proteobacteria, Chromatiaceae groupe CFB, Bacteoridetes	clone, eau douce clone, eau douce	170/186 (91) 188/192 (97)	AF513943 AY038778
M10	232-235	population active sous ↑ pCO$_2$	delta proteobacteria delta proteobacteria, Myxococcales	clone rhizosphere *Chondromyces lanuginosus*	186/195 (95) 181/195 (92)	AF431398 AJ233939
M11	236-238	populations racinaires qui fluctuent avec pCO$_2$	alpha proteobacteria delta proteobacteria, Myxococcales	clone de rivière *Chondromyces lanuginosus*	180/195 (92) 179/195 (91)	AY038725 AJ233939
M12-13	246-247	population racinaire	delta proteobacteria	clone de sol agricole	191/198 (96)	AY037592
M14	248-250	populations racinaires qui fluctuent avec pCO$_2$	alpha proteobacteria, Rhodospirillales	*Defluvicoccus vanus*	158/182 (86)	AF179678
M15	248-250		delta proteobacteria	clone de biofiltre	185/195 (94)	AJ318168
M16	264-267	population caractéristique de pCO$_2$ ambiant	alpha proteobacteria, Rhodospirillales green nonsulfur bacteria, Chloroflexi	*Defluvicoccus vanus* clone de tapis bactérien	168/173 (97) 156/164 (95)	AF179678 AF423366
M17	302-305	populations actives et peu abondantes	alpha proteobacteria, Rhodospirillales Gram$^+$, lowGC	*Defluvicoccus vanus* clone de sol forestier	167/173 (96) 98/114 (85)	AF179678 AF507708
M18	331-334	populations telluriques	Gram$^+$, Actinomycetales, *Pilimelia terevasa* green nonsulfur bacteria	souche DSM 43040 *Chloroflexi*	161/167 (96) 157/175 (89)	X93190 AB067647

La figure 2.1.3.A représente les similarités **C**/**T** obtenue pour chaque prélèvement avec les échantillons de sols en abscisse et les racines en ordonnée. Les similarités entre les profils **T**/**C** étaient en général plus importantes pour les échantillons de sol donc la majorité des points sont situés plus près de l'abscisse. Celà indique que les communautés telluriques ont moins été perturbées par des modifications de l'atmosphère que les communautés bactériennes racinaires. Ce résultat confirme que l'effet du traitement pCO_2 sur la microflore tellurique se fait principalement par l'intermédiaire de la plante (cf. § 1.3.2). Cette constatation avait déjà été faite par Marilley et coll. (1999) pour les communautés bactériennes associées à la racine d'une autre graminée pérenne, *Lolium perenne*.

Analyse d'ordination des profils DGGE

Le paramètre qui expliquait le plus la dispersion de nos échantillons dans l'analyse d'ordination (ACC) était la date de prélèvement. Cette constatation était aussi valable pour les données récoltées sur *Lolium perenne*. Pour faciliter l'interprétation des données, nous présentons des résultats d'ACC séparément pour chacune des dates (Figure 2.1.3.B). Les deux premiers axes d'ordination expliquaient 41.3, 21.0 et 22.4 % de la variabilité des profils DGGE pour les dates 1, 2 et 3 respectivement. La comparaison des axes non-contraints (issus de l'AFC) et des axes contraints (effet plante, pCO_2 et ADN/ARN) montrent que d'autres facteurs que ceux qui sont utilisés expliquent la dispersion des échantillons. Nous n'avons pas pu dégager de tendance commune pour les trois dates de prélèvement.

Caractérisation des bandes DGGE

La plupart des bandes qui permettent le mieux d'expliquer la dispersion des échantillons sur les profils DGGE (les bandes les plus éloignées du centre dans les ACC; Figure 2.1.3.B) étaient présentes dans les profils de communautés lors des trois campagnes de prélèvement. Nous avons découpé, puis séquencé des bandes DGGE lorsqu'elle semblaient spécifiques d'une fraction (M3-5, M8-9, M11-15, M18), lorsqu'elles réagissaient à la modification de la teneur en pCO_2 (M2-3, M6-7, M10-11 et M14-16) ou lorsque l'activité métabolique des populations semblait plus marquée que leur abondance (M1, M3, M6-10, M17). Les affiliations phylogénétiques sont présentées dans le tableau 2.1.3.C. Sur les 36 séquences obtenues à partir des 18 bandes choisies, 73.5 % sont affiliées à des proteobactéries, principalement des alpha- et delta-proteobactéries. Les protéobactéries réagissaient donc plus facilement à la présence de la racine de *Molinia coerulea* ou à la variation de pCO_2 dans l'air.

Certaines bandes migrant à des positions identiques ont été découpées sur des échantillons d'origines différentes (M4-5, M8-9, M12-13, M14-15). La majorité des clones obtenus à partir des bandes M12-13 ou M14-15 semblaient homogènes alors que les séquences obtenues à partir des bandes issues des positions 150-152 (M4-5) et 222-226 (M8-M9) correspondaient à des populations phylogénétiquement éloignées ($Gram^+$lowGC, gamma- et delta-proteobacteria). Il faut garder à l'esprit qu'on ne pourra jamais séparer les 13000 espèces bactériennes théoriquement présentes dans 1 gramme de sol (Torsvik et coll. 1994) sur un gel DGGE.

2.1.3.4 Conclusion

L'amélioration des techniques d'extraction des acides nucléiques du sol (ADN d'abord puis ARN désormais), en parallèle à l'émergence des techniques de fingerprinting des communautés microbiennes, devrait permettre de développer nos connaissances de l'écologie microbienne. Les profils de communautés obtenus après extraction des ADN totaux nous renseignent sur la présence et l'abondance des différentes espèces dominantes alors que des profils obtenus après extraction des ARN totaux donne une image des espèces actives. La synergie entre ces nouvelles techniques analytiques permet désormais d'appréhender les bactéries environnementales en ciblant spécifiquement celles qui sont actives *in situ* et qui jouent donc probablement un rôle dans le fonctionnement de l'écosystème. Il me parait donc nécessaire de multiplier les analyses basées sur l'ADN et l'ARN en parallèle afin de valider l'approche basée sur les ARN.

Je pense que le travail présenté ici n'a pas assez pris en compte les paramètres environnementaux qui auraient pu expliquer les différences entre les communautés pour les différentes dates (teneur en azote et aération du sol, température et ensoleillement lors du prélèvement, stades de croissance de la plante…). Certains résultats importants ressortent tout de même de cette étude.

Certaines bandes DGGE étaient présentes uniquement dans les profils issus des extraits d'ARN (bande M1) ou correspondaient à des populations très actives mais peu abondantes (M3, M6-10 et M17). D'une manière générale, la comparaison des communautés présentes et actives montre une assez grande divergence pour un même prélèvement (similarité des profils DGGE toujours inférieure à 45 %). Cette situation observée dans la rhizosphère de la molinie se retrouve aussi dans l'environnement rhizosphérique de *Lolium perenne,* une autre graminée pérenne (Jossi 2003).

Cette constatation soulève des questions. Les biais d'extractions de l'ADN et de l'ARN seraient-t-ils suffisamment différents pour modifier l'image de la structure des communautés révélées par DGGE? Dans l'affirmative, les différences de rendements ne devraient pas être sélectives pour certaines séquences d'ADN ou d'ARN. En admettant que l'image des communautés obtenue après extraction des acides nucléiques ait subi les mêmes distorsions, comment se fait-t-il que co-existent tant de populations actives peu nombreuses? Soit l'intense activité détectée est transitoire et ne permet pas la croissance et la division cellulaire, soit les populations en question possèdent une activité de traduction intense (de nombreux ribosomes) et participent activement au fonctionnement du sol avec peu d'individus. Nous pouvons aussi imaginer que certaines populations bactériennes soient préférentiellement consommées par des protozoaires, maintenant en permanence de faibles effectifs (Aragno, communication personnelle).

2.2 Modification des profils physiologiques (BIOLOG) des communautés associées à *Molinia coerulea* cultivée sous différentes pCO_2

L'analyse de la structure des communautés microbiennes (cf. § 2.1), en tant que bioindicateur, peut être un bon moyen pour suivre l'effet d'une perturbation, d'un changement ou de la résilience d'un écosystème. Il en existe beaucoup d'autres, parfois plus faciles à mesurer. Dans cette partie, nous avons évalué la plasticité phénotypique des communautés racinaires et du sol environnant de *Molinia coerulea* en réponse à une augmentation du pCO_2. Nous avons choisi de mesurer globalement la capacité des bactéries à oxyder 31 sources de carbone. La structure génétique de chaque guilde, ensemble des populations capables d'utiliser une même source de carbone, a été ensuite caractérisée par DGGE.

Effect of an elevated atmospheric pCO_2 on the microbial community associated with *Molinia coerulea* following a Biolog utilisation

Ce paragraphe fait l'objet d'un article en cours de rédaction de **J. Hamelin[1]**, F. Kohler[2], S. Tarnawski[1], M. Aragno[1] and N. Fromin[1].

[1] Laboratoire de Microbiologie, Université de Neuchâtel, CP 2, CH-2007 Neuchâtel

[2] Laboratoire d'écologie végétale et de phytosociologie, Université de Neuchâtel, CP 2, CH-2007 Neuchâtel

Abstract

The root exudates of perennial grasses provide constant and abundant carbon and energy sources for the surrounding soil bacterial communities. Environmental factors, such as an elevated atmospheric pCO_2, can favour the plant growth and can modify the quantity and the composition of the flow of root exudates. We stressed the metabolic capabilities of bacterial communities to oxidise the 31 substrates of Biolog ECOplates in relation to the roots of *Molinia coerulea* grown under ambient (360 ppm) or elevated (600 ppm) pCO_2 content. The bacterial community associated with the roots of *Molinia* grown under elevated pCO_2 tend to better metabolize some carbon sources, especially carbohydrates. No tremendous effect of elevated pCO_2 could be noticed for the soil bacterial community. Furthermore, we looked at the guilds responsible for the oxidation of substrates by PCR-DGGE of 16S rDNA on Biolog cultures. A trend to a metabolic redundancy (low similarity of DGGE profiles with similar oxidation) was observed for most of substrates, either for root and soil inocula. The reliability of the characterization of environmental communities by Biolog ECOplate using substrate utilisation pattern is discussed.

2.2.1 Introduction

The impact of global changes on microbial communities has been intensively studied in agro systems (Torbert *et al*. 2000) but few reports dealing with natural meadows were proposed (Mayr *et al*. 1999). We focused here on the microbial communities inhabiting the roots of *Molinia coerulea*, an oligotrophic perennial grass and its surrounding soil. We studied the effect of an elevated carbon dioxide concentration in the atmosphere (pCO_2), which will probably be reached in the years 2050 (600 ppm), as compared to the present situation (360 ppm).

As the pCO_2 in the gaseous phase of soils is much higher than in the air (van Veen *et al*. 1991), we hypothesised that the impact of an increase of pCO_2 for the soil bacterial communities will occur through the plant rhizodeposition near the roots. The expected modifications will concern the enhancement of photosynthesis for plants with a C3-metabolism (Taiz & Zeiger 1998), followed by an augmentation of root exudation. It will provide a greater quantity of easily assimilable substrates for the surrounding bacterial communities. In order to test this hypothesis, we compared the contribution of bacterial communities in association with plants grown under ambient and elevated pCO_2 atmosphere to respire the thirty-one relevant substrates of Biolog ECOplate, especially designed for soil bacteria studies.

The significance of changes in use of specific substrates is difficult to interpret without understanding which group of microorganisms within the community are affected. We then evaluated the changes in the bacterial guilds utilising single sources of carbon by PCR-DGGE characterization of the bacteria present in the ECOplate wells after growth. The relationship between substrate utilisation and the bacteria consuming these substrates is discussed.

2.2.2 Materials and Methods

Experimental set-up

Molinia coerulea plants originated from a natural meadow located at the south shore of Lake Neuchâtel (Switzerland) at 46°98 latitude north 7°04 longitude east at an elevation of 430 meters (cf. § 1.4.2). The surface soil (Gleysol, Typic Haplaquoll) texture was 4.7 % clay, 9.5 % silt, 85.8 % sand, and had an high calcareous content (Buttler 1987). pH_{H2O} value was 8.4. Entire plants with surrounding and underlying soil 45 cm deep were transferred in September 1999 in a Free Air CO_2 Enrichment (FACE) site at Eschikon (Zürick, Switzerland) (cf. § 1.4.3). About 0.7 m^2 of plants were grown during two seasons under field conditions, with ambient (360 ppm) or elevated (600 ppm) atmospheric pCO_2.

Sampling

The plants were sampled at the end of the second growing seasons in September 2001, and submitted the same day to analysis. Intact roots and surrounding soil cores were sampled from control plots (**C**) and CO_2 treated plots (**T**). The roots were separated from the soil with permanent shaking during one hour in sodium phosphate buffer 0.1 M, pH 7.0. 1 g of soil and 0.5 g of roots were crushed manually in 5 ml sterile distillated water to obtain the soil (**S**) and the root (**R**) suspensions.

ECOplates inoculation and incubation

Soil and root suspensions were diluted in sterile distilled water to obtain about 10^3 cells per ml, according to Zak *et al.* (1994). The numbers of colony forming units (CFU) in the inocula were assessed on Angle medium plates (Angle *et al*. 1991) after 48 hours incubation at 24 °C (Table 2.2.A). Two ECOplates (BIOLOG, Inc. Hayward, CA) were inoculated per sample with 150 µl of suspension per well, allowing six measurements for each sample. The negative control corresponded to a well without any carbon source. The thirty-one substrates used are listed in Table 2.2.B, and were classified into biochemical categories (polymers, carbohydrates, carboxylic acids, amino acids, amines, aromatic compounds) following that of Insam (1997). Incubation of the ECOplates was carried out in the dark at 24 °C for 48 hours without agitation, and the level of respiratory activity for each well was measured by optical densities at 630 nm (OD_{630}) using an automatic microplate reader (Dynatech MR7000) at 0, 12, 18, 24, 36, 42, and 48 hours of incubation.

DNA extraction and PCR amplification

The six soil or root cultures for each substrate were pooled after 48 hours of incubation prior to DNA extraction. This step allowed averaging the bacterial suspension compositions for DGGE analysis. 900 µl of each pooled sample was subjected to DNA extraction with the Wizard genomic DNA purification Kit (Promega).

The forward 338f (5'–ACTCCTACGGGAGGCAGCAG–3') and the reverse 520r (5'–ATTACCGCGGCTGCTGG–3') universal primers (Ovreas *et al*. 1997; Jensen *et al*. 1998) were used for PCR amplification of the V3 region of the 16S rDNA gene. A 40 bp GC-clamp (Muyzer *et al.* 1993) was added on the 5' end of the forward primer for DGGE analysis. The PCR reaction mix contained 1 X buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs (Gibco), 0.25 mM of each primer, and 1 U of *Taq* DNA polymerase (Promega). 5 µl of ten-fold diluted DNA was added as template for the PCR. The final reaction volume was adjusted to 50 µl. The reaction mixtures were subjected to 31 amplification cycles with thermo-cycler model PTC-200 (MJ Research Inc., Watertown, Massachusetts). The first heat denaturation step was performed at 94 °C for 5 min. Cycles consisted of heat denaturation at 94 °C for 1 min, primer annealing at 65 °C for 30 s with a touchdown of 1 °C per cycle during ten cycles, and extension at 74 °C for 1 min. The mixture was maintained at 74 °C for 10 min for the final extension. The 220 bp PCR products were checked for size and yield on 1.3 % agarose gels in comparison to the Low DNA Mass Ladder (Gibco).

Denaturing gradient gel electrophoresis (DGGE) analysis

The 128 DGGE profiles were performed with the D-code electrophoresis system (Bio-Rad Inc., Hercules, CA). About 600 to 800 ng of PCR product were loaded directly on a 8 % (wt/vol) polyacrylamide gel with a linear gradient ranging from 30 % to 60 % denaturants (one hundred percent correspond to 40 % formamide plus 7 M urea). The strains used to build the reference DGGE pattern are ordered as follow after migration: *Pseudomonas fluorescens* ATCC27663, *Bacillus subtilis* ATCC14893, *Sinorhizobium meliloti* DSM1981, *Flavobacterium capsulatum* DSM30196, *Arthrobacter globiformis*^{T} DSM20124 and *Thermus filiformis* NCIMB12588. The gels were run at 60 °C and 150 V for 5 hours in 1× TAE buffer. They were stained in 0.01 % Sybr Green (Molecular Probes, Leiden, The Netherlands) at 4 °C in dark for 20 min, then UV photographed with Multi-Analyst package (Bio-Rad). The images were normalised and the patterns were compared using GelCompar software (Applied Maths, Kortrijk, Belgium). The fingerprints obtained were codified in term of presence of a band, migration length of each band, and relative intensity of the band within the profile (cf. § 2.1.2). Each band was considered as corresponding to a single bacterial population for statistical analysis (Fromin *et al*. 2002).

Data handling and statistics

For each Biolog Ecoplate, the absorbance value of the control well was subtracted from the well absorption values, yielding a single corrected value, according to manufacturer's instructions. The color developement was estimated using the six measurements during 48 hours by a curve-integration approach, according to Guckert *et al*. (1996). The average well color development (AWCD) among the thirty-one substrates was calculated according to Garland & Mills (1991) at 48 hours of incubation, except for RT sample that reached an AWCD over 0.5 at 36 hours of incubation.

DGGE bacterial community profiles obtained after growth on each carbon source were compared. The Steinhaus coefficient similarities between control and CO_2-treated communities, for both soil and root samples, were computed using progiciel R (Casgrain & Legendre 2001).

2.2.3 Results

This study aimed at differentiating the metabolic capabilities of bacterial communities associated with the roots (**R**) of *Molinia coerulea* and its surrounding soil (**S**), grown under control (**C**) or pCO_2 treated atmosphere (**T**). The inoculum densities in Biolog microplate wells varied from 1.7 10^3 to 6.3 10^3 CFU.ml^{-1} (Table 2.2.A) depending on the sample, but the densities used here did not affect the results, and especially the lag period before Biolog colour development (data not shown).

Table 2.2.A Biolog ECOplates inoculum densities for each sample, evaluated on Angle medium (Angle *et al*. 1991), and the average well color developpement (AWCD) after 48 hours of growth, except for RT sample that reached a value of AWCD over 0.5 after 36 hours of incubation*.

Sample designation	Abbreviation	Inoculum CFU.ml^{-1} (SD)	AWCD OD_{630} (SD)
Soil under ambient atmosphere	**SC**	2260 (940)	0.68 (0.07)
Soil under CO_2 treated atmosphere	**ST**	1700 (780)	0.58 (0.12)
Root under ambient atmosphere	**RC**	4490 (1250)	0.52 (0.09)
Root under CO_2 treated atmosphere	**RT**	6300 (520)	0.65 (0.03)*

Variability of Biolog substrates oxidation

The aerobic growth of bacteria on thirty-one ecologically relevant substrates was monitored independently. During the experiment, the colour developpements were unequal depending on the carbon sources and on the samples (Table 2.2.B). The community level physiological profiles (CLPP) for soil and root bacterial communities sampled under ambient and elevated pCO_2 conditions are represented as boxplots in Figure 2.2.C. As some substrates were oxidized differently among replicated measurements, the median was calculated instead of the mean. The median gives the most probable value of utilisation. For example, utilisation of N-acetyl-D-glucosamine by the **SC** and **ST** communities seemed similar regarding there mean values (19.62 and 20.72 respectively) while the medians were inequal (20.67 and 27.06 respectively). The mean utilisation of N-acetyl-D-glucosamine by **ST** bacterial community was lower than the median because two wells showed much lower activity than others (among the six replicates). The respiratory response was highly variable especially for soil samples, as shown by the size of boxes encompassing half of the variability. Furthermore, 21 outliers were highlighted by this analysis for soil samples and only 10 for the root samples.

Table 2.2.B List of carbon substrates in Biolog ECOplates with their classification into biochemical categories (Garland & Mills 1991; Insam 1997), and the level of aerobic substrate consumption, based on curve-integration approach (Guckert *et al*. 1996)

	ECOplate substrates	Short names	Biochemical categories	Level of respiration for each sample median among six replicates			
				SC	**ST**	**RC**	**RT**
1	pyruvic acid methly ester	Pyru	carboxylic acid	12.70	11.32	12.08	20.07
2	Tween 40	Tw40	polymer	13.23	15.15	18.52	19.17
3	Tween 80	Tw80	polymer	12.92	11.11	14.99	16.21
4	-cyclodextrine	-cyclo	polymer	3.85	1.93	4.33	5.43
5	glycogen	Glyco	polymer	39.15	1.18	4.52	5.82
6	D-cellobiose	D-cell	carbohydrate	20.52	10.93	17.53	28.80
7	-D-lactose	D-lac	carbohydrate	0.21	0.19	0.25	15.71
8	-methyl-D-glucoside	-M-glu	carbohydrate	19.73	24.48	5.21	31.97
9	D-xylose	D-xyl	carbohydrate	4.16	1.94	4.62	13.76
10	i-erythritol	i-eryth	carbohydrate	1.55	1.38	5.19	3.61
11	D-mannitol	D-manni	carbohydrate	21.71	18.03	9.33	32.33
12	N-acetyl-D-glucosamine	N-A-D-glu	carbohydrate	20.67	27.06	25.19	33.78
13	D-glucosaminic acid	Gluco acid	carboxylic acid	2.84	1.74	3.88	2.07
14	glucose-1-phosphate	Glc-1-P	carbohydrate	0.89	1.34	6.44	6.52
15	D,L--glycerol phosphate	DL-gly	carbohydrate	2.70	1.25	0.48	9.13
16	D-galacturonic acid -lactone	D-gal--lac	carboxylic acid	15.36	14.23	11.81	25.84
17	D-galacturonic acid	D-galac	carboxylic acid	22.85	26.99	18.38	27.18
18	2-hydroxyl benzoïc acid	2-h-benzo	carboxylic acid	0.32	0.89	3.21	9.34
19	4-hydroxyl benzoïc acid	4-h-benzo	carboxylic acid	6.60	9.81	7.86	17.67
20	-hydroxybutyric acid	Butyr acid	carboxylic acid	6.17	9.71	5.22	12.31
21	itaconic acid	Itac	carboxylic acid	2.32	5.21	1.73	3.04
22	-ketobutyric acid	-keto	carboxylic acid	1.26	1.07	4.63	4.25
23	D-malic acid	D-mal	carboxylic acid	0.86	7.65	3.66	22.02
24	L-arginine	L-arg	amino acid	12.08	14.21	10.38	19.34
25	L-asparagine	L-asp	amino acid	22.97	19.12	17.55	30.24
26	L-phenylalanine	L-phe	amino acid	2.15	1.29	3.23	4.82
27	L-serine	L-ser	amino acid	12.18	11.76	11.79	14.93
28	L-threonine	L-thr	amino acid	0.54	0.83	1.11	2.07
29	glycyl-L-glutamic acid	L-glu	amino acid	1.4	0.80	1.16	2.81
30	phenyl ethylamine	Ethylamine	amine	5.31	1.32	6.21	11.29
31	putrescine	Putres	amine	10.79	5.18	8.77	17.75
			polymers	16.26	8.68	10.57	11.96
			carboxylic acids	7.62	9.84	8.12	14.36
			carbohydrates	9.75	8.45	8.11	15.36
			amino acids	8.96	8.04	7.39	8.85
			amines	7.53	3.59	8.13	12.83

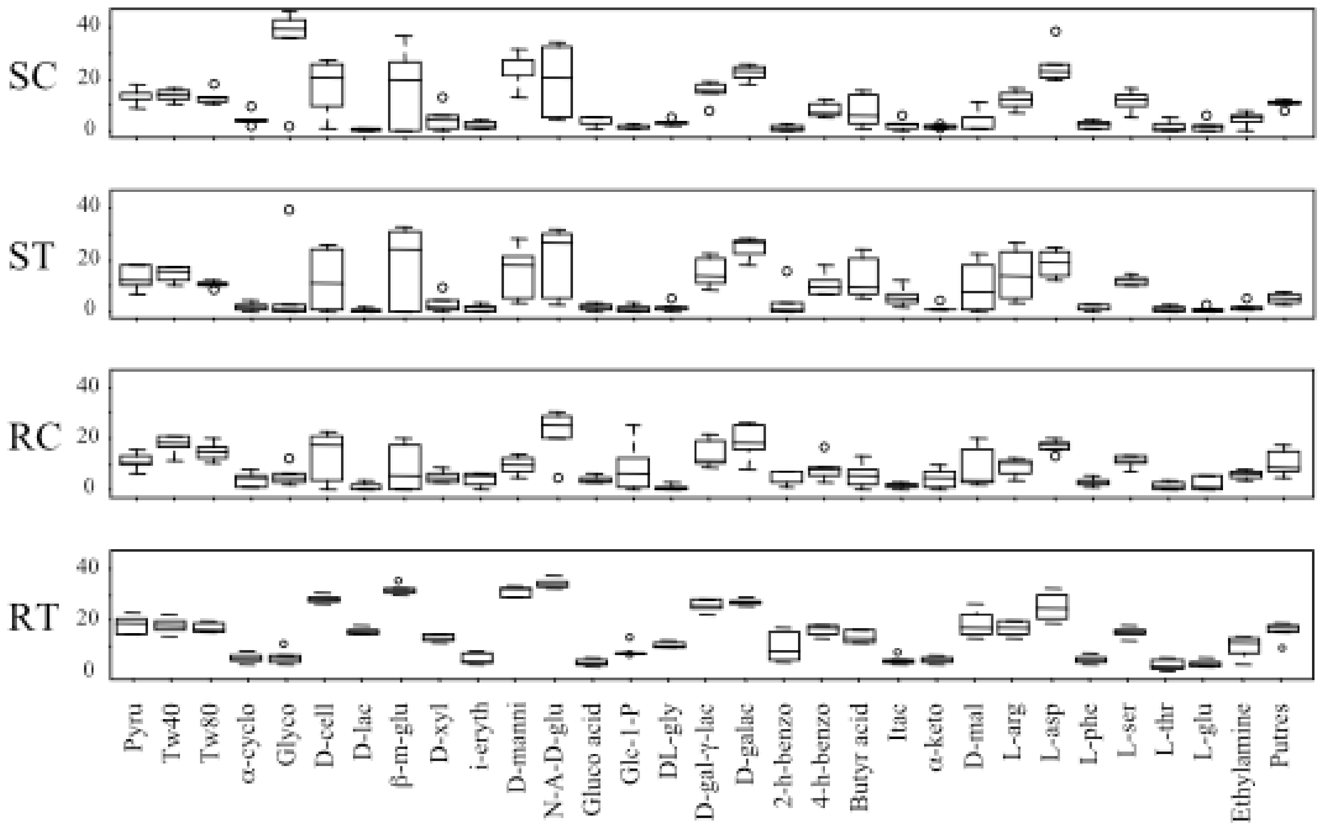


Figure 2.2.C Community level physiological profiling (CLPP) as expressed by the differential respiration of 31 substrates from Biolog Ecoplates, based on a curve-integration approach (Guckert *et al*. 1996) for soil and root samples under both pCO_2-growing conditions. The boxes contained values from the 25th to the 75th percentile. The horizontal bar inside the boxes stands for the median. The outliers are noted with º

Atmospheric pCO_2 effect on Biolog substrate utilisation

The figure 2.2.D highlights the substrates that contribute mostly to the differentiation of CLPP between CO_2 treatments, for soil and root samples. Differentially consumed substrates (i.e. for which boxplots for **C** and **T** communities did not overlap; Figure 2.2.C), were represented as filled symbols. Two carbon sources (glycogen and putrescine) were less oxidized by control soil community than by the treated one, while for the root, one third of the substrates were more efficiently used for CO_2 treated as compared to control community. These ten carbon sources were grouped into different biochemical categories (Table 2.2.B): amino acids (L-arginine), carboxylic acids (D-galacturonic acid -lactone and 4-hydroxyl benzoic acid) and most (7 among 9) of carbohydrates (D-cellobiose, -D-lactose, -methyl-D-glucoside, D-xylose, D-mannitol, N-acetly-D-glucosamine and D,L--glycerol phosphate).

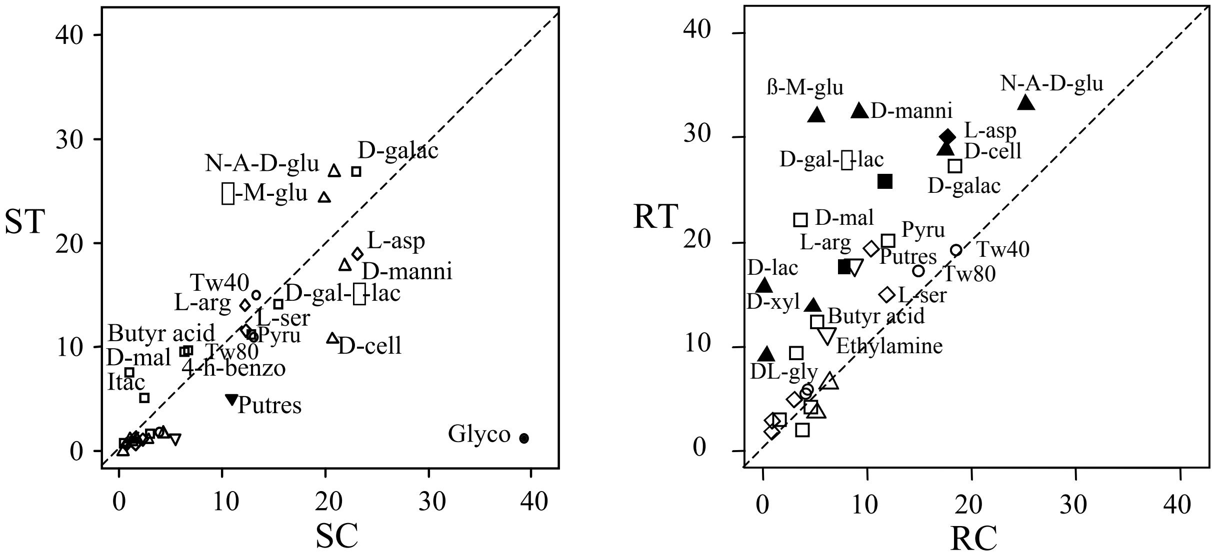


Figure 2.2.D Oxidation level of substrates for control bacterial communities plotted against oxidation level for pCO_2-treated samples communities. Each biochemical category of substrate is represented with a symbol: '○' for polymers, '△' for carbohydrates, '□' for carboxylic acids, '◇' for amino acids and '▽' for amines. Filled symbols corresponded to differentially oxidized carbon sources between control and treated plots

Molecular characterization of single-substrate oxidizing bacterial guilds

The guild oxidizing each of the thirty-one substrates were marked out by PCR-DGGE directly on Biolog enrichment cultures. The bacterial fingerprints obtained allowed to compare the genetic structure of the microbial fraction able to respire the substrate. Among the 128 fingerprinting patterns performed, an average of 19 bacterial populations was detected. No significant change in richness, the number of bands, was measured for any substrate category.

We hypothesized on the one hand that **C** and **T** communities displaying similar guilds should also oxidize a substrate similarly. On the other hand, physiological modifications in Biolog measurements could be attributed to a shift in communities. Figure 2.2.E displays the relatedness of soil and root guilds (similarity between **C** and **T** DGGE patterns) as compared to the differential utilisation of carbon sources in Biolog plates (ratio of **C** and **T** Biolog values). The substrates scattered on the right lower part of the graphs in figure 2.2.E were oxidized by communities with similar structures but had differencial Biolog measures. For exemple, the guilds oxidizing D,L-□-glycerol phosphate, □-methyl-D-glucoside or D-malic acid were close for **RC** and **RT** samples (49.0 %, 68.9 % and 63.7 % of similarity respectively), while the oxidation levels for **RC** sample were especially lower than for **RT** sample (5.0 %, 15.8 % and 16.5 % respectively).

Furthermore 22 substrates for soil and 20 for root tend to have homogeneous substrate utilisation behaviour but a low similarity for their genetic profiles. They were plotted in the left upper part of figure 2.2.E. For exemple, Tween 80 allowed the growth of different kind of bacteria (Figure 2.2.F; 34.8 % of similarity for DGGE patterns for soil and 26.2 % for root) with a similar level of substrate oxidation (86.0 % for soil and 87.9 % for root).

The similarities between the DGGE patterns were always below 73 %, even for bacterial communities grown on a same carbon source. The figure 2.2.F shows representative DGGE patterns for some selected substrates (Tween 80, D,L-□-glycerol phosphate and D-malic acid).

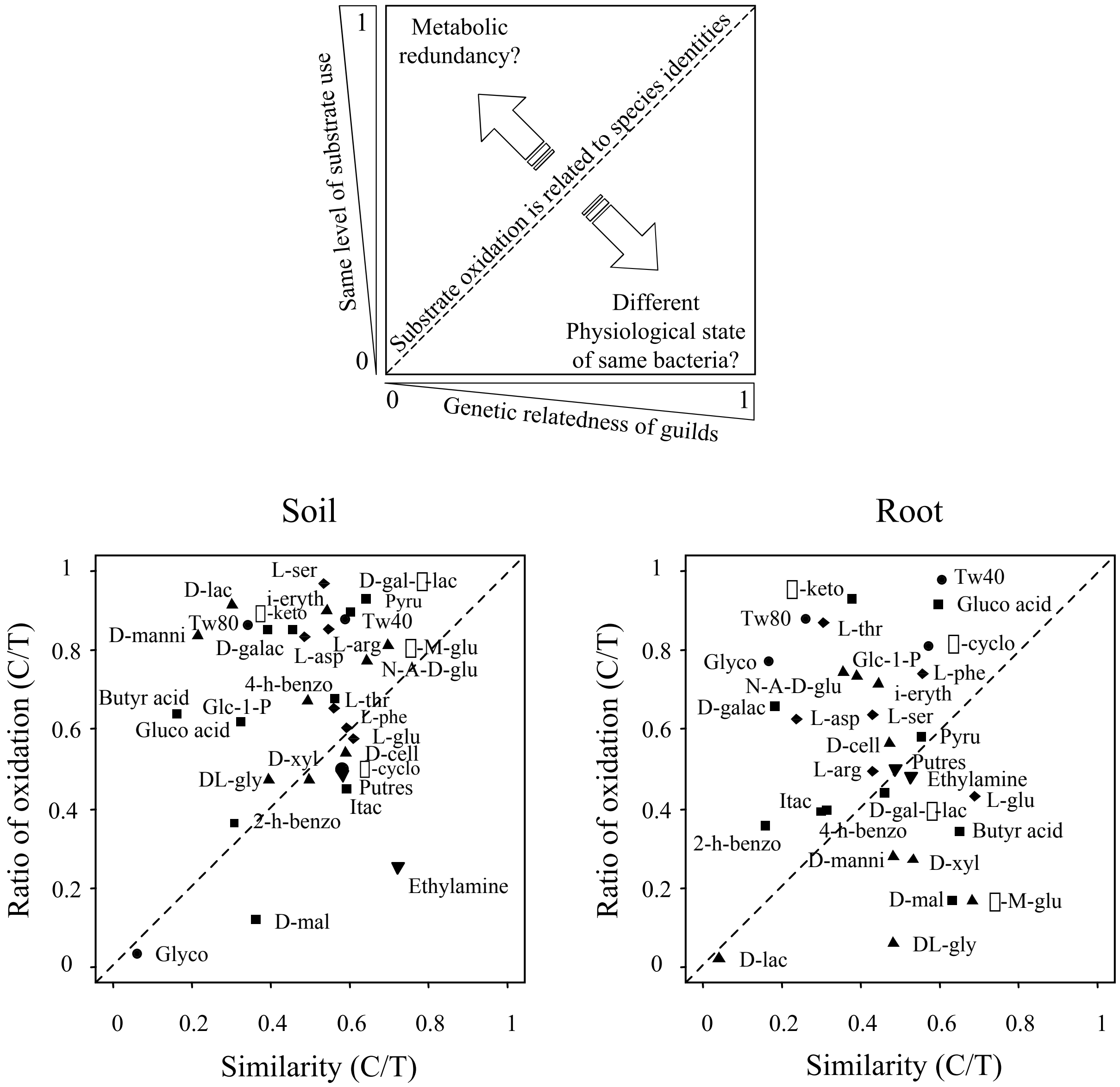


Figure 2.2.E Relation between the similarity of soil (**SC**/**ST**) and root (**RC**/**RT**) guilds (Steinhaus coefficient on DGGE profiles) and the ratio of substrate oxidation in Biolog wells. Substrates scatters on the dotted line indicated a relation between the Biolog measurements and the bacterial community compositions. The left upper part of the graphs scatters the substrates for which different communities have close metabolic capability toward a specific substrate. The right lower part of the graphs scatters the substrates for which similar bacteria displayed differencial capabilities toward the oxidation of a substrate. The name of a carbon source was indicated after the symbol indicating his biochemical category: '○' for polymers, '△' for carbohydrates, '□' for carboxylic acids, '◇' for amino acids and '▽' for amines

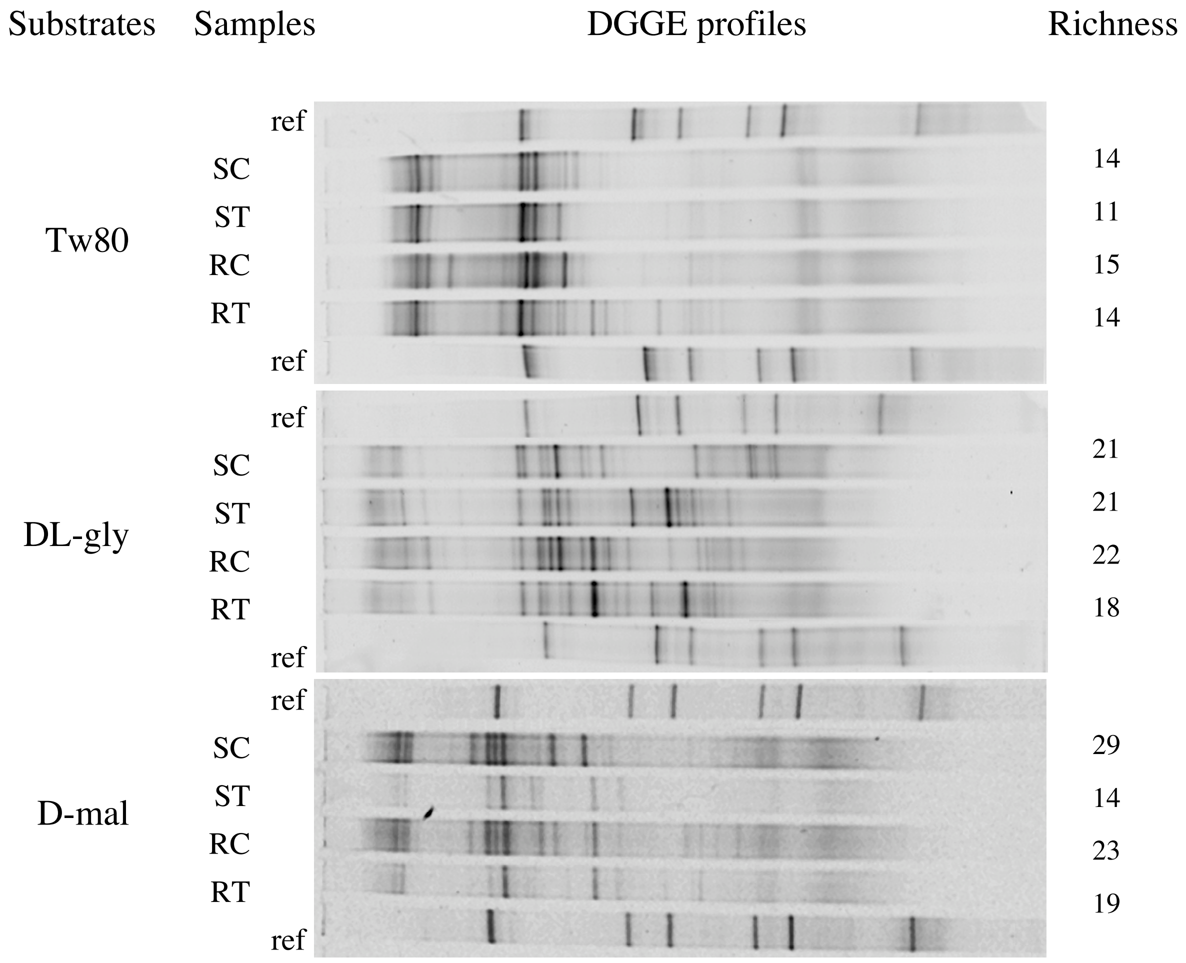


Figure 2.2.F 16S rDNA PCR-DGGE patterns of bacterial communities coming from soil (**SC** and **ST**) or root (**RC** and **RT**) suspensions, after growth on Biolog ECOplates with Tween 80, D,L--glycerol phosphate or D-malic acid as sole carbon source. The conditions of migration and the reference strains used for reference pattern are mentionned in the materials and methods section

2.2.4 Discussion

The question whether the soil and root bacterial communities are affected by an elevated CO_2 concentration is still understudied (Gorissen *et al*. 1995; van Ginkel *et al*. 2000) and contradictory results were obtained, depending on the methodological approach used (Griffiths *et al.* 1998; Marilley *et al.* 1999). Here, we looked at the bacterial guilds having differential ability to oxidize various ecologically relevant carbon sources, within the cultivable fraction of the bacterial communities inhabiting the roots of *Molinia coerulea* or the surrounding soil.

Variability of Biolog values

The inoculum densities in Biolog microplate were about the same as in Zak *et al.* (1994) study. The inoculum size could affect the colour development in wells because of the time required to attain 10^8 CFU.ml^{-1} (Garland & Mills 1991; Haack *et al.* 1995). But the densities used here did not affect the length of the lag period. No tremendous increase in the lag period was detected for **RC** sample as compared to **RT**. The increased oxidation of the carbon sources for **RT** sample reflected a better catabolic rate as compared to **RC** sample, which can indicate the use of different oxidation pathways and therefore different functional diversity of corresponding bacterial community. This hypothesis was assessed by looking at the bacteria responsible for the Biolog measurement values, and is discussed further.

As expected, the variability of respiratory response for soil inocula (**SC** and **ST**) was greater than for the root inocula (**RC** and **RT**), as represented by larger boxes and a greater number of outliers (Figure 2.2.C).

Differential use of substrates in response to pCO$_2$ treatment

Figure 2.2.D highlights ten substrates of Biolog ECOplates that were more oxidized by the root bacterial community from pCO$_2$ treated plot. The oxidation of most of the carbohydrates (7 among 9) was positively influenced by an elevated pCO$_2$. Hodge *et al*. (1998) found no difference in the pattern of carbon source utilisation by the rhizospheric community for *Lolium perenne* grown under ambient and elevated pCO$_2$. Mayr *et al*. (1999) showed an effect of elevated pCO$_2$ in alpin grassland but did not observe responses that were restricted to any particular substrate group. In contrast, Bossio & Scow (1995) observed that carbohydrate utilisation by soil communities were positively related to carbon input (rice straw). Marstorp (1996) also noticed a better respiration of water soluble compounds (sugar, free amino-acids) for *Lolium multiflorum* grass shoot material associated communities, occuring about half a day after carbon source application. Grayston *et al*. (1998) measured significantly greater utilisation by the rhizospheric community for a native C3 Australian grass for all tested carbon sources except those with a low C/N content. The authors attributed this effect to a quantitative increase of root excretion, mostly composed of high C/N compounds, under elevated pCO$_2$ (Grayston *et al*. 1998; Paterson *et al*. 1996). These contrasting results suggest that the rhizosphere bacterial communities of different plants are affected by an elevated pCO$_2$ to different extents.

Interestingly, soil bacterial communities did not respond to pCO$_2$ treatment by substantial changes in Biolog substrates utilisation. This was in agreement with our working hypothesis, which assumes that an elevated pCO$_2$ atmosphere will first influence the bacterial communities in close association with the roots. Such a result was previously shown using a different approach (cloning-sequencing) on the bacterial communities associated with roots of *Lolium perenne* (Marilley *et al*. 1999).

Physiological state of inoculated bacteria

Biolog analysis requires organisms that are metabolically active in culture conditions to be highlighted. It cannot reflect the potential metabolic capability of the entire community in the environment. The bacteria displayed by PCR-DGGE from Biolog wells may have the genetic equipements to oxidize the substrates but the physiological state of some bacteria at the time of inoculation may not allow sufficent growth to initiate development (cf. § 3.2; Haack *et al.* 1995; Tarnawski *et al.* 2003). Hodge *et al*. (1998) showed that the proportion of culturable bacteria increased in the rhizosphere of *Lolium perenne* under elevated pCO$_2$ conditions, and proposed that the increase in C flow from the root stimulated the bacteria to become culturable in response to nutrient addition. Furthermore, higher rate of carbon sources utilisation were observed in our study for root-associated communities exposed to elevated pCO$_2$ (data not shown), as also noticed by Hodge *et al.* (1998).

Metabolic redundancy

A same substrate oxidation value did not always lead to similar bacterial guilds after incubation. This was observed in the same order of magnitude either for soil and root samples (Figure 2.2.E). Large changes of population structure for guilds retrieved from the Biolog wells yielded small changes in Biolog use in the time course of the manipulation. This is a consequence of the metabolic redundancy among bacteria, which means that a loss of some species or a change in population structure may generate same substrate utilisation pattern (Frederickson *et al.* 1991). As previously described (Konopka *et al.* 1998; Smalla *et al*. 1998), the substrate utilisation patterns may be insensitive to changes in population structure and do not necessarily reflect the functional potential of the most abundant populations before incubation. These data suggests that the use of Biolog plates is not sufficient to characterize microbial communities. Their use should be complemented with a genetic analysis of the dominant bacteria responsible for the substrate utilisation. Frederickson *et al.* (1991) reported that 20 % of isolates from subsurface sediments could catabolize 55 % of Biolog GN substrates. This metabolic redundancy lowers the sensitivity of Biolog to detect potential changes in communities. Furthermore, oligotrophic bacteria are generally able to catabolize a greater variety of substrates compared to copiotrophic microbes (Upton & Nedwell 1989). Thus, it is likely that a very diverse microbial community in an oligotrophic environment, such as in soil (Williams 1985), will harbor bacterial communities with a higher functional redundancy, leading to a higher resistance to changes compared to less diverse environments.

2.2.5 Conclusion

In nutrient-poor soils, the consequences of an elevated pCO_2 may be transient in nature (Oechel *et al*. 1994). The present study was done after only two growing season of elevated pCO_2 exposure. It is difficult to draw general conclusions and long-term experiments are needed to assess the effects of global changes on *Molinia coerulea* associated rhizosphere community. Nevertheless, an enhancement of substrate use by root-associated bacteria could lead to a greater turn over of the organic matter near the roots. Some studies demonstrated that a greater mineralisation near the roots comes with a greater mobilisation of the soil organic matter (Houghton *et al*. 1990). The question arises if the greater mineralisation of soil organic carbon by the bacterial community under elevated pCO_2 atmosphere could overcome the benefit of a greater photosynthesis rate, and then limits the response of plants to an increased pCO_2.

2.3 Conclusion générale sur l'analyse des communautés

Les espèces végétales et animales sont définies par des limites génétiques et physiologiques claires, mais les bactéries peuvent être perçues comme un continum physiologique et génétique (Cohan 2002). La vitesse de réponse aux changements de l'environnement différencie les organismes eucaryotes des bactéries. La nutrition bactérienne est principalement réalisée par digestion externe de polymères par des exoenzymes. La limite physique d'influence d'une bactérie sur son environnement immédiat est donc floue. Cette *'bactério-sphère'* dépend de l'humidité, de la diffusion des nutriments et de la capacité d'échange des minéraux du sol.

Du point de vue génétique, le concept d'espèce bactérienne est flou (Watve & Gangal 1996; Cohan 2002) et la para-sexualité bactérienne permet une certaine plasticité génétique entre espèces et l'adaptation rapide à des changements environnementaux. L'ADN des bactéries mortes peut rester présent dans le sol assez longtemps hors des cellules (England et coll. 1997) et des études de communautés basées uniquement sur la présence d'ADN peuvent mener à des conclusions erronées.

L'empreinte moléculaire d'une communauté obtenue par profil génétique reste sujette à contreverse du fait qu'on ne maitrisera jamais avec précision la compartimentation des microcolonies, les interactions entre les organismes lors du prélèvement, l'état physiologique des cellules ou le rôle potentiel des bactéries mises en évidence dans le fonctionnement global du sol. Malgré tout, ces profils génétiques conçus au départ pour décrire un grand nombre de communautés avec le plus de représentativité possible ont permi petit à petit d'expliquer des changements dans les communautés en reliant les modifications de structure observées avec des paramètres environnementaux (cf. § 2.1).

Bien que la plupart des espèces soient rares, Finlay (1997) voit les communautés bactériennes comme un ensemble d'espèces ubiquistes qui sont seulement en train d'attendre *'que le vent tourne'* et qui prospèrent dès que les conditions deviennent favorables. Le concept de redondance fonctionnelle correspondrait au remplacement d'une espèce bactérienne par une autre (n'importe quelle autre espèce?) pour occuper la nouvelle niche microbienne apparue lors d'un changement environnemental. Nous avons abordé ce point dans le § 2.2 en s'intéressant au potentiel métabolique des communautés bactériennes racinaires de *Molinia coerulea* et du sol environnant, sous deux conditions de pCO_2. Nous avons effectivement remarqué que des guildes génétiquement différentes pouvaient avoir une activité métabolique globale similaire lorsqu'elles étaient placées dans des microplaques Biolog (cultures électives avec une seule source de carbone par puits).

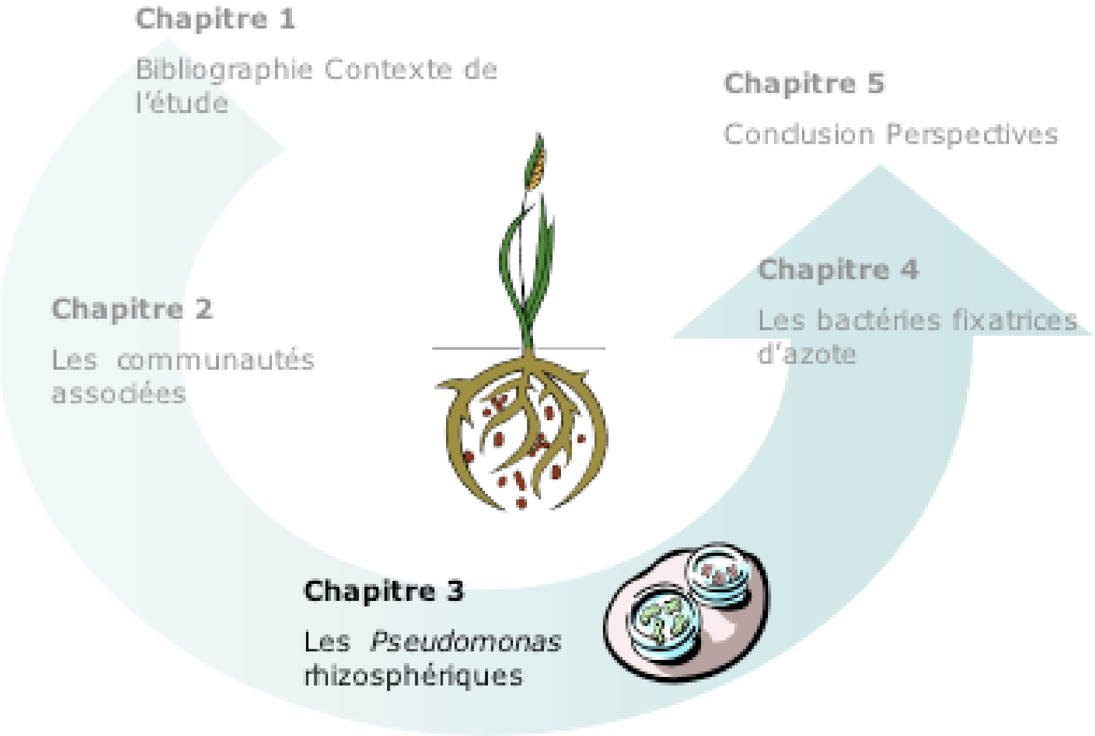
On peut interpréter les différences entre les profils de communautés obtenus à partir d'ADN et d'ARN de différentes façons. On peut imaginer que certaines populations très actives ne deviennent jamais dominates numériquement car elles se feraient consommer préférentiellement par les protozoaires du sol (Aragno, communication personnelle). Ces différences de profils génétiques obtenus à partir d'ADN et d'ARN peuvent aussi s'expliquer par l'hétérogénéité du sol. Notre échantillon analysé correspond à un échantillon moyen de 0,5 gramme de sol. Des études de fractionnement du sol sur les communautés bactériennes totales (Ranjard et coll. 2000; Fall et coll. sous presse) ou sur les bactéries des genres *Nitrobacter* (Grundmann & Normand 2000) et *Agrobacterium* (Vogel et coll. 2003) ont souligné l'hétérogénéité des micro-organismes dont on ne pouvait pas voir dans l'analyse globale d'un échantillon. On aborde là un problème crucial en écologie microbienne du sol. Existe-t-il des bactéries spécialisées (très probablement non-cultivables pour l'instant) occupant de minuscules niches qui seraient indispensables au bon fonctionnement du sol? Comment expliquer que certains groupes phylogénétiques soient métaboliquement les plus actifs dans les sols (Felske & Akkermans 1998) alors qu'aucun représentant n'est cultivable? Cette perspective ouvre des voies pour de futures recherches.

Une limitation des méthodes de fingerprinting moléculaire réside dans la sensibilité de la mesure. Il faut qu'il y ait des changements réellement importants pour qu'ils soient visibles au niveau des communautés. Heuer et coll. (1997), Nübel et coll. (1997) ou Heilig et coll. (2002) ont en partie contourné ce point en ciblant des groupes phylogénétiques précis susceptibles de réagir à une modification du milieu. Par rapport aux bactéries impliquées dans les transformations de l'azote (cf. § 1.2), les communautés de bactéries nitrifiantes ont été étudiées par fingerprint sur la base du gène d'ADNr 16S (Kowalchuk et coll. 1999) et sur la base du gène fonctionnel *amoA* (Bruns et coll. 1999). La guilde des bactéries fixatrices d'azote a aussi été suivie par DGGE en se basant sur le gène fonctionnel *nifH* (Piceno et coll. 1999; Lovell et coll. 2000 & 2001; Bagwell et coll. 2002). Cette approche fonctionnelle du compartiment microbien de l'écosystème est prometteuse car elle permet réellement de relier la structure des communautés à une fonction microbienne clé qui participe au recyclage des éléments.

L'utilisation des fingerprints bactériens a souvent pour but de sélectionner la ou les populations qui sembent bio-indicatrices d'une situation particulière. Cela revient à choisir des populations intéressantes parmi l'ensemble celles qui sont présentes. Nous avons trouvé (cf. § 2.1.3) que près de trois quarts des populations qui étaient influencées par la teneur en CO_2 dans l'atmosphère ou qui modifiaient notablement leur activité métabolique étaient des protéobactéries. Dans le chapitre suivant (Chapitre 3), nous avons directement ciblé les populations de *Pseudomonas* (□-proteobacteria) cultivables associés à la racine, sachant que les bactéries de ce genre bactérien sont considérées comme adaptées à la vie rhizosphérique (Rainey 1999).

Chapitre 3

Caractérisation des populations de *Pseudomonas* associées à la rhizosphère de *Molinia coerulea*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Bibliographie Contexte de l'étude
Chapitre 5
Conclusion Perspectives
Chapitre 4
Les bactéries fixatrices d'azote
Chapitre 2
Les communautés associées
Chapitre 3
Les *Pseudomonas* rhizosphériques

Chapitre 3

Caractérisation des populations de *Pseudomonas* associées à la rhizosphère de *Molinia coerulea*

Introduction

En parallèle à l'étude des communautés, nous avons pris en compte les fluctuations de populations de *Pseudomonas* (Figure 3.A). Le choix des *Pseudomonas* comme populations modèles n'a pas été fait au hasard. D'une part Marilley et coll. (1999) ont montré que les espèces de *Pseudomonas* adaptées à la vie rhizosphérique de *Lolium perenne* réagissaient particulièrement bien à des variations atmosphériques de pCO_2. D'autre part, de nombreux caractères phénotypiques pouvant aider la croissance des plantes (PGPR) sont étudiés en utilisant des bactéries du genre *Pseudomonas* comme modèle.

Tous les résultats obtenus sur les populations de *Pseudomonas* ont été mis en oeuvre par Sonia Tarnawski au cours de sa thèse et par Nathalie Fromin comme coordinatrice du projet. Deux graminées perennes ont été étudiées en parallèle: *Lolium perenne* et *Molinia coerulea*. Je me limiterai ici aux résultats obtenus sur *Molinia coerulea*, mais je ferai référence à *Lolium perenne* à titre de comparaison.

Le paragraphe 3.1 présente un nouvel outil basé sur l'amplification spécifique de l'intergène 16S-23S de l'ADNr ciblant les bactéries du genre *Pseudomonas*.

La différenciation des espèces par PCR-RFLP est plus facile sur la base de l'ITS1 que le gène 16S, car cette région est plus variable.

Le Paragraphe 3.2 donne un aperçu de la diversité des *Pseudomonas* cultivables présents dans l'environnement racinaire de *Molinia coerulea* dans la prairie littorale.

Cette étude a permis de mettre en avant l'importance du milieu d'isolement dans l'image de la diversité obtenue.

Dans le paragraphe 3.3, les *Pseudomonas* réducteurs de nitrate et dénitrifiants ont été étudiés plus particulièrement.

Les critères d'éloignement à la racine de *Molinia coerulea* et de teneur en CO_2 dans l'air ont été pris en compte dans l'analyse.

3.1 Détection par PCR spécifique des *Pseudomonas* cultivables dans la prairie naturelle

Les résultats de ce paragraphe ont été en partie obtenus lors du travail de diplôme de Laurent Locatelli (Locatelli 2001). Laurent a reçu le Prix Louis-Paris (1000 CHF) pour la qualité de son travail lors de la cérémonie de remise des diplômes du 23 novembre 2001.

Ce paragraphe a aussi fait l'objet d'un article de L. Locatelli, S. Tarnawski, **J. Hamelin**, P. Rossi, M. Aragno et N. Fromin. Cet article a été publié dans Systematic and Applied Microbiology (Locatelli et coll. 2002).

Le développement méthodologique présenté ici avait pour but la caractérisation fine des *Pseudomonas* du point de vue moléculaire sur la base de l'intergène 16S-23S de l'ADN ribosomique, une région plus variable que les genes d'ADNr 16S et d'ADNr 23S. Le développement d'un nouvel outil peut paraître superflu de prime abord, mais les fluctuations des populations de *Pseudomonas* dans la rhizosphère des plantes perennes nécessite l'utilisation d'outils de caractérisation précis. L'originalité du protocole PCR présenté ici est d'amplifier à la fois une partie d'un gène conservé à valeur taxonomique associé à une région variable, l'intergène 16S-23S de l'ADNr.

Le polymorphisme de l'intergène (mis en évidence par des endonucléases de restriction) permet de différencier facilement les *Pseudomonas* entre eux, et le séquençage du produit d'amplification permet de connaître l'identité des bactéries correspondantes.

Specific PCR amplification for the genus *Pseudomonas* targeting the 3’ half of 16S rDNA and the whole 16S-23S rDNA spacer

Abstract

A PCR protocol was developed for the selective amplification of a segment of the ribosomal RNA operon in *Pseudomonas* strains. Two specific conserved sequences suitable for PCR priming were identified in the middle of the 16S rDNA and at the very beginning of the 23S rDNA respectively. As a result, amplified region includes the 3’ half of the 16S rDNA with the whole 16S-23S rRNA Internal Transcripted Spacer (ITS1) sequence. The specificity of the primer set was checked on sequence databases and validated on collection strains and on one hundred soil bacterial isolates. Our results showed that both collection, soil-inhabiting *Pseudomonas* and some Pseudomonas-related *Azotobacter* DNAs could be amplified. This specific PCR for the detection of *Pseudomonas* strains was in good agreement with colony hybridisation using a *Pseudomonas*-specific probe. The targeted segment is relevant for a characterisation at the species (16S rDNA) as well as at the infraspecific (ITS1) levels. This PCR-based approach offers promising potential for the characterisation of environmental *Pseudomonas* populations.

3.1.1 Introduction

The genus *Pseudomonas* was originally described by Migula in 1894. This genus was redefined recently and its phylogenetic relationships with related groups or previously misnamed *Pseudomonas* spp. were elucidated using 16S rDNA sequences (Kersters *et al*. 1996). The genuine *Pseudomonas* currently comprise more than 50 species (Anzai *et al*. 2000). Bacteria belonging to the genus *Pseudomonas* are widely dispersed in soil and water environments. The genus *Pseudomonas* also contains important pathogens for plants, fungi, animals and even human (Lyczack *et al*. 2000), including opportunistic pathogens that have become more and more problematic in term of public health. Nevertheless, some strains showed promising properties for the promotion of plant growth, the inhibition of plant pathogens (Walsh *et al*. 2001) or the degradation of xenobiotic compounds (Daane *et al*. 2001). Understanding the role and evolution of *Pseudomonas* populations requires a comprehension of their diversity. Reliable tools for the detection of the members of the genus *Pseudomonas* are therefore needed.

Historically, the identification of *Pseudomonas* strains relied only on morphological and physiological features. However, such phenotypic traits have proved to be variable at infraspecific level. Intraclonal phenotypic variation was also described in numerous *Pseudomonas* species (Grewal & Rainey 1991; Rainey & Travisano 1998). Furthermore, studies on environmental isolates revealed the limits of culture media selective for *Pseudomonas* (Kragelund *et al*. 1996; Johnsen & Nielsen 1999; Aagot *et al*. 2001).

Molecular approaches based on oligonucleotide probing or selective PCR amplification were proposed for the detection and the identification of *Pseudomonas* spp. in both environmental and clinical samples. Group-specific probes targeted against *Pseudomonas*-specific region for universal genes (Braun-Howland *et al*. 1993; Ludwig *et al*. 1994; Amann *et al*. 1996) permitted differentiation of members of the genuine *Pseudomonas* from related genera. Recently, Widmer *et al*. (1998) proposed a primer pair, which could be used for the amplification of a 16S rDNA gene fragment in *Pseudomonas* from environmental DNA extract. Similarly, De Vos *et al*. (1997) developed primer sets targeting the functional *oprL* and *oprI* outer membrane genes for a specific detection of *P. aeruginosa* and fluorescent *Pseudomonas* spp. respectively.

Most of the oligonucleotides developed to date targeted the 16S rDNA sequence. The 16S–23S rDNA Internal Transcribed Spacer (named ITS1) sequence has evolved faster than the sequence of rDNA genes, because of its non-coding function. The ITS1 comprises conserved regions (generally corresponding to tRNA genes) as well as regions highly variable in length and sequence (Gürtler & Stanisich 1996; García-Martínez *et al*. 1999). Therefore, one can expect this sequence to express differences between closely related organisms, especially at the infraspecific level. The corresponding DNA fragments can be amplified, taking advantage of conserved nucleotide regions in the flanking 16S and 23S rDNA sequences. Moreover, the target region may include a more or less large part of the 16S rDNA sequence, being suitable for a reliable taxonomic characterisation at higher level.

The objective of the present work was to develop a method allowing a rapid identification of isolates to the genus *Pseudomonas* during routine testing as well as their subsequent genotypic characterisation. For this purpose, a new set of PCR primers specific for *Pseudomonas* was developed, allowing the amplification of ITS1 together with a phylogenetically significant part of 16S rDNA. The validity of this PCR protocol was assessed on collection strains as well as environmental soil isolates.

3.1.2 Material and methods

All sequence positions presented in the current work are in accordance with positions corresponding to *Escherichia coli* gene (16S rDNA, 23S rDNA) numbering.

Design of Pseudomonas-specific primers

Complete 16S rDNA sequences for 57 *Pseudomonas* species *sensus stricto* (Anzai *et al*. 2000) and *E. coli* (accession number J01859), and 10 sequences available for the very beginning of the 23S rDNA gene for some *Pseudomonas* spp. and for *E. coli* (Figure 3.1.E) were retrieved from GenBank database. The 16S and 23S rDNA sequences respectively were aligned with GeneBase program (Applied Maths, Kortrijk, Belgium). This alignment was used to determine conserved regions for PCR primer design.

Theoretical evaluation of Pseudomonas-specific primers

For each region, the most relevant oligonucleotidic sequence (named fPs16S for the forward primer on the 16S rDNA gene and rPs23S for the reverse one on the 23S rDNA gene) was selected for its conservation and specificity to *Pseudomonas* sequences.

Candidate sequences were 5′ - ACTGACACTGAGGTGCGAAAGCG – 3′ for fPs16S (position 756-779) and 5′ - ACCGTATGCGCTTCTTCACTTGACC – 3′ for rPs23S (position 1-25).

These two sequences revealed compatible melting temperatures (61.3 °C and 61.2 °C respectively, using the G/C rule, Sambrook & Russell 2001) and could be used as primers for PCR amplification using stringent conditions to ensure the specificity of the annealing.

The theoretical matchings of both sequences were assessed in the RDP database using CheckProbe analysis version 2.1r3 (Maidak *et al*. 2001), and in the GenBank database using the BLAST program (Altschul *et al*. 1997).

Collection strains and environmental isolates

The collection strains used in this study and their source of isolation are listed in Tables. They comprise 33 strains of numerous species and subspecies of *Pseudomonas* (Table 3.1.A) as well as 17 strains of non-*Pseudomonas* (Table 3.1.B).

Environmental isolates originated from a natural soil under a littoral meadow (at south shore of lake Neuchâtel, Switzerland). Total cultivable heterotrophic aerobic bacteria were recovered by tenfold serial dilutions of a soil suspension and plating on non-selective Angle medium (Angle *et al*. 1991). About one hundred Gram-negative isolates were randomly selected.

DNA extraction

Overnight cultures of collection and environmental strains on Nutrient Agar were collected in sterile microtubes and washed by shaking for 4 min in 0.4 M NaOH. The bacterial cells were pelleted by centrifugation, washed in TE buffer pH 8.0, and re-centrifuged in the same conditions. Bacterial cells were submitted to DNA extraction following the procedure described by Moore *et al*. (1999), except that 15 µl of boiled 10 mg.ml^{-1} RNAse was added during the proteinase digestion step.

Table 3.1.A *Pseudomonas* strains used in this study

Taxon	Collection number	Original source
P. aeruginosa	ATCC 10145	unknown
P. agarici	ATCC 25941^T	*Agaricus bisporus*
P. asplenii	LMG 2173^T	*Asplenium nidus*
P. chlororaphis	ATCC 17415	soil
P. chlororaphis	DSM 6698^T	clay in kerosene
P. blatchfordae	ATCC 9446^T	*Phaseolus vulgaris*
P. caricapapayae	ATCC 33615^T	*Carica papaya*
P. chlororaphis	ATCC 17415	plate contaminant
P. cichorii	ATCC 10857^T	*Cichorum endiva*
P. corrugata	ATCC 29736^T	*Lycopersicon esculentum*
P. fluorescens	ATCC 27663	soil
P. fluorescens	CFBP 2022	*Allium sativum*
P. fluorescens bv IV	ATCC 12983	soil
P. fluorescens bv IV	ATCC 17513	water
P. fluorescens bv III	ATCC 17400	hen egg
P. fluorescens bv II	ATCC 17482	unknown
P. fluorescens bv I	ATCC 13525^T	water reservoir
P. fluorescens bv I	ATCC 17397	tap water
P. fluorescens bv VI	ATCC 17552	water
'Pseudomonas gingeri'	LMG 5327	*Agaricus bisporus*
P. marginalis pv marginalis	ATCC 17819	pleural fluid
P. putida biotype C	ATCC 17386	water
P. putida biotype A	ATCC 12633^T	soil
P. putida biotype B	ATCC 17430	unknown
P. tolaasii	ATCC 33618^T	*Agaricus bisporus*
P. stutzeri	ATCC 17588^T	unknown
P. fluorescens	CHA0	Tobacco (CH) (1)
Pseudomonas sp.	TM1A3 and TM1A4	Tomato (CH) (1)
Pseudomonas sp.	PGNL1 and PGNR1	Tomato (Ghana) (1)
Pseudomonas sp.	FL3	rhizoplane soybean (2)
Pseudomonas sp.	FL9	rhizosphere of pea (2)

(1) Keel *et al*. 1996 (2) Rao & Johri 1999.

CFBP: Collection Française de Bactéries Phytopathogènes

type strains are indicated by a ^{T} after the collection number

Table 3.1.B non-*Pseudomonas* γ-proteobacterial strains used for primer set validation

Taxon	Collection number	Family
Azotobacter chroococcum	DSM 374	*Pseudomonadaceae*
Azotobacter chroococcum	DSM 2286	"
Azotobacter sp.	DSM 1721, DSM 1722, DSM 1723	"
Azomonas agilis	DSM 375^{T}	*Pseudomonadaceae*
Pseudoalteromonas gracilis(1)	H40	*Alteromonadaceae*
Vibrio fischeri	DSM 507	*Vibrionaceae*
Enterobacter cloacae	NEU 1027	*Enterobacteriaceae*
Enterobacter aerogenes	DSM 30053	"
Escherichia coli	NEU 1006*	"
Klebsiella oxytoca	NEU 30*	"
Proteus vulgaris	NEU 1049*	"
Providencia alcalifaciens	NEU 84*	"
Salmonella panama	NEU 1065*	"
Serratia marescens	NEU 1024*	"

* NEU : bacterial collection of the University of Neuchâtel

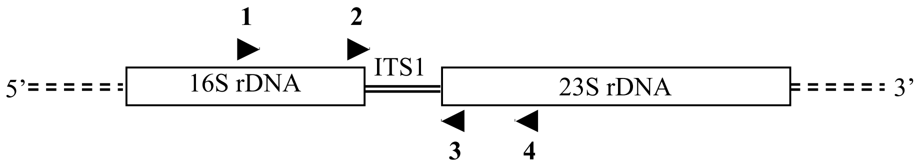
type strains are indicated by a ^{T} after the collection number

(1) Moebus 1992

PCR amplification of ITS1

Two primer sets were used for PCR amplification of the ITS1 region (Figure 3.1.C): fPs16S / rPs23S (*Pseudomonas* primers defined in the present study) and S-D-Bact-1522-b-S-20 / L-D-Bact-132-a-A-18 (*Bacteria* primers; Normand *et al*. 1996).

Figure 3.1.C Primers used for PCR-amplification of ITS1



1* : primer fPs16S (*Pseudomonas*, forward) position 756-779

2** : primer S-D-Bact-1522-b-S-20 (*Bacteria*, forward) position 1472-1490

3* : primer rPs23S (*Pseudomonas*, reverse) position 1-25

4** : primer L-D-Bact-132-a-A-18 (*Bacteria*, reverse) position 118-135

* present study

****** Normand *et al.* 1996

For the *Pseudomonas*-specific PCR, the reaction mix contained (final concentrations): 1x Thermophilic DNA Buffer (Promega), 3 mM $MgCl_2$, 0.2 mM each dNTPs (Gibco), 0.25 µM each primer (Microsynth, Balgach, Switzerland), 0.05 $U.\mu l^{-1}$ Taq DNA polymerase (Promega), and 10 % (vol:vol) of ten-fold diluted template DNA. The reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachussets) with an initial denaturation of 3 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 74 °C, and a final extension step at 74 °C for 5 min. PCR products were analysed by electrophoresis in 1.3 % agarose gel (Eurobio, Les Ullis, France) and visualised after staining with ethidium bromide. PCR amplification with *Bacteria* primers was used in order to check the quality of DNA extract and the size of ITS1 region. It was carried out as previously described (Normand *et al*. 1996).

Hybridisation with PSM_G probe

Bacterial colonies were tested for their hybridisation with PSM_G probe (target position: 440-456 on 16S rDNA) specific for *Pseudomonas* spp. (Braun-Howland *et al*. 1993) as previously described (Marilley *et al*. 1999).

Table 3.1.D Amplification results with fPs16S – rPs23S primers and PSM_G hybridization of soil bacterial isolates

	PCR +	**PCR -**	**total**
H+	35	2	**37**
H-	0	64	**64**
total	**35**	**66**	**101**

H+ / H- : isolates diplaying a positive / negative hybridization.

PCR+ / PCR- : isolates displaying a positive / negative amplification.

Cloning and sequencing of 16S rDNA fragments

A 16S rDNA fragment (position 907-1498) was amplified with Gm3f and GM4r primers (Muyzer *et al*. 1995). PCR products were purified using Nucleotrap extraction kit for nucleic acids (Macherey-Nagel GmbH, Düren, Germany), and cloned into pGEM-T vector (Promega) and *E. coli* competent cells. Transformants were processed with NucleoSpin Plasmid kit (Macherey-Nagel) for plasmid extraction, as recommended by the manufacturer. The corresponding inserts were sequenced using T7 labelled primers (Sambrook & Russell 2001). The identification of the corresponding organisms was achieved by using a BLAST analysis on the retrieved sequences.

3.1.3 Results

Design of specific primers

The definition of *Pseudomonas*-specific primers was based on the sequences available in GenBank database. Target regions conserved and specific for the genus *Pseudomonas* (relatively to the available sequence data) were identified (Figure 3.1.C) at locations 756-

Figure 3.1.E Multiple nucleotide alignment of the available 5' end of 23S rDNA sequences of *Pseudomonas* and fPs16S-matching non-*Pseudomonas* species

	1	11	21	31	41
rPs23S (coding sequence)		**GGTCAAGTGA**	**AGAAGCGCAT**	**ACGGT**	
P. fluorescens (AF134704)	....	....	.....GGATG	CCTTGGCAGT	CAGAGGCGA
P. mendocina (L28159, L28160, L28161)	....	....	....	.......RR.	..S......
P. aeruginosa (AE004502, Y00432)	....	....	....	....	....
P. aeruginosa (L28148, L28149, L28150)	....	....	....	.......RR.	..S......
P. stutzeri (U65012, X87289)	....	....	....	....	....
P. perfectomarina (L03788)	....	....	....	....	....
Azotobacter vinelandii (AF079809)	....	....	....	....	....
Stenotrophomonas maltophilia (L28166)	.......C..	.T......C	....	.......RR.	..S......
Halomonas pacifica (AJ306882)	.......C..	TT........	....	C	....
Moraxella catarrhalis (A27628)	........A.	T....T....	.T........	....	....
Vibrio vulnificus (X87293)	...T......	CT.....T.C	....	....	....
Escherichia coli (V00348)	...T...C..	CT.....T..	....	....	....
Acinetobacter calcoaceticus (X87280)	A.......A.	TT...T....	GT........	....	....
Buchnera aphidicola (U09230)	...T...CA.	.T.....T.C	.T....A...	....	....
Ehrlichia chaffeensis (AF000721)	.....GA...	..GTATCA.A	T.CTGAATCC	ATAG..GTT.	G.AGC.AAC
***Coxiella burnetii* (X79704)**	**...AG..GAG**	**C.TTCT.T.A**	**C.T...A.G.**	**TGA.C.AGAG**	**GTTT.CT.G**

Nucleotides are identified for mispairings only. Nucleotides identical to the rPs23S sequence are indicated by dots.

The positions are given according to *E. coli* 23S rDNA numbering. R: A/G ; S: G/C

779 for 16S rDNA sequences (fPs16S), and 1-25 for 23S rDNA sequences (rPs23S). fPs16S and rPs23S sequences were conserved in the fifty seven 16S rDNA and the ten 23S rDNA *Pseudomonas* available sequences respectively. They displayed 2 and 5 mismatches with the 16S and 23S rDNA *E. coli* corresponding sequences, respectively.

Theoretical evaluation of Pseudomonas *primers*

The fPs16S sequence displayed a perfect match with 551 sequences among 7322 16S rDNA prokaryotic sequences available in RDP database. We considered these matching sequences for their phylogenetic affiliation. Briefly, 66 % of these sequences corresponded to *Pseudomonas* or uncultured and unidentified *Pseudomonas*-related sequences. Four sequences (less than 1 %) were related to *Azotobacter* spp. (*Pseudomonadaceae*). About 19 % corresponded to other □-proteobacteria 16S rDNA sequences (*Oceanospirillum* group including *Halomonas* and *Marinomonas* genus, *Moraxellaceae*, sulfur-oxidizing symbionts and unidentified □-proteobacteria). Less than 10 % were retrieved from other bacterial groups: 26 sequences from *Rickettsiaceae* (□-proteobacteria) and 22 sequences from □-proteobacteria (*Desulforhabdus*, *Desulfovibrio*, *Lawsonia* spp.). Four other sequences matching with fPs16S (Y13327, AF112477, Z29622, D88521) corresponded to misnamed organisms and were actually closely related to *Pseudomonas* spp. (identity > 98 %).

Only two 23S rDNA sequences related to *Pseudomonas* (among 1400 sequences from eukaryotic, prokaryotic and mitochondrial origin) were available in the large ribosomal subunit RDP database. We assessed the target sequences of rPs23S using GenBank database. A BLAST analysis on rPs23S sequence displayed a perfect match for only 23 sequences in GenBank database. These included sequences from 16 identified *Pseudomonas* spp., one unidentified sugarcane isolate (AF251157) related to *Pseudomonas* (94 % identity with *P. tolaasii*), two Chlamydia-associated clinical sample related to *P. aeruginosa* (99 % of identity), three environmental clones (AF422501, AF4224999 and AF422492, related to *Pseudomonas* ITS1 sequences) and one *Azotobacter vinelandii* (AF079809). Figure 3.1.E presents the alignment of available 23S rDNA sequences with particular interest for *Pseudomonas* and organisms for which 16S rDNA sequences displayed a perfect match with fPs16S.

When grouping sequences matching with both primers, all available non-*Pseudomonas* sequences in RDP and GenBank databases displayed at least 1 mismatch for at least one of the specific primers, except *Azotobacter vinelandii* (sequences L40329 for 16S rDNA and AF079809 for 23S rDNA).

Experimental validation of fPs16S and rPs23S specific primers

Pseudomonas and non-*Pseudomonas* DNA extracts were tested for amplification using the fPs16S - rPs23S primer set. All the 33 *Pseudomonas* strains (Table 3.1.A) were positively amplified using these primers and generated usually about 1300 bp PCR products. Some *Pseudomonas* strains generated 2 or 3 discrete bands after PCR amplification, whose size ranged from about 1100 to 1300 bp (Figure 3.1.F).

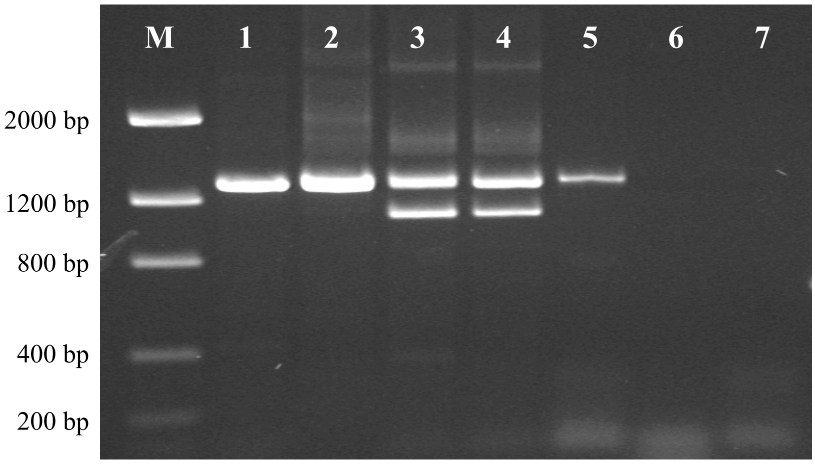


Figure 3.1.F PCR amplification of collection strain DNA with *Pseudomonas* primers. PCR was performed with fPs16S and rPs23S primers. M: Low DNA Mass Ladder (Gibco), 1: *P. asplenii* (LMG 2173), 2: *P. fluorescens* (ATCC 17513), 3: *P. fluorescens* (CFBP 2022), 4: *P. marginalis* pv. *marginalis* ATCC 17819, 5: *A. chroococcum* DSM 374, 6: *E. coli*, 7: control (no DNA template).

Multiple band patterns of these strains were confirmed by the use of ITS1 *Bacteria* primers. None of the 17 non-*Pseudomonas* collection strains (Table 3.1.B) generated an amplicon with fPs16S and rPs23S primers, except *Azotobacter chroococcum* DSM374, for which a PCR product, at the expected size (about 1300 bp), was obtained. The four other *Azotobacter* strains were recorded as negative for PCR amplification with specific primers, although their DNA could be amplified with Bacteria primers.

One hundred and one Gram negative soil isolates were tested for amplification with fPs16S and rPs23S. The strains giving an amplification were recorded as positive (noted PCR+). When no amplicon was obtained, the strains were recorded as negative (noted PCR-). Thirty-five isolates were PCR+ and generated an amplicon at the expected size (1 to 3 fragments ranging from about 1100 to 1300 bp). Eight of these isolates were submitted to 16S rDNA sequencing and their affiliation to *Pseudomonas* genus was confirmed (identity > 94 % using BLAST analysis). Three of the retrieved sequences were closely related to the 16S rDNA of *P. alcaligenes* (> 99 % identity). One additional isolate, which generated unusual profiles after restriction analysis of the amplicon (data not shown) was also submitted to partial 16S rDNA sequencing and could be affiliated to *Stenotrophomonas maltophilia (Xanthomonas* group, γ-proteobacteria). Nonetheless, the 16S (i.e. AB008509) and 23S (L28166) rDNA sequences available in databases displayed several mismatches with fPs16S and rPs23S.

The reliability of fPs16S – rPs23S primers was assessed using hybridisation with the *Pseudomonas*-specific PSM_G probe. The soil isolates were recorded as positive (H+) when hybridisation generated a strong signal and as negative (H-) when no signal or a faint signal was recorded. The comparison between specific amplification and PSM_G hybridisation results is presented in Table 3.1.C. All PCR+ strains were also H+ and all the H- strains were PCR-. Two bacterial strains were recorded H+ but were negative for amplification with fPs16S – rPs23S. Partial 16S rDNA sequencing revealed that these strains were related to *Pantoea agglomerans* and *Pantoea ananatis* (100 % identity on 755 bp and 99 % identity on 735 bp respectively for BLAST analysis). When considering their 16S rDNA sequences, as well as *S. maltophilia*-related sequence, we could not retrieve the complete 15 bp putative hybridisation site for PSM_G probe (only 9 to 10 bp length sequences displayed a match with PSM_G).

3.1.4 Discussion

Limited data were available for the definition of a consensus oligonucleotide for Pseudomonas 23S rDNA. Such sequences were retrieved from sequences of the ITS1, which included the beginning of 23S rDNA (Gill *et al*. 1994; Tyler *et al*. 1995; Guasp *et al*. 2000), or from the few complete 23S rDNA gene sequences available for *Pseudomonas* (for instance Ludwig *et al*. 1994). On the contrary, recent work on *Pseudomonas* taxonomy (Moore *et al*. 1996; Anzai *et al*. 2000) has given access, for the very first time, to a representative collection of 16S rDNA target sequences for this genus. A target region specific for *Pseudomonas* genus (fPs16S) was identified in the middle of the 16S rDNA sequence, allowing to include a phylogenetically meaningful part of the 16S rDNA gene in the amplified region. fPs16S and rPs23S, when combined, formed a primer set which was conserved for all available *Pseudomonas* sequences.

Theoretical matching with sequences in GenBank and RDP databases, as well as experimental testing on collection and environmental strains, have confirmed the specificity and efficiency of fPs16S and rPs23S for the specific amplification of 1100 to 1300 bp fragment(s) of the ribosomal operon in *Pseudomonas* spp. This primer set also revealed matches for *Azotobacter* spp. According to De Vos *et al*. (1985), the genus *Azotobacter* is closely related to the genus *Pseudomonas*. Moreover, in the taxonomic hierarchy proposed by the RDP, *Azotobacter* spp. are included in the "*Pseudomonas* and relatives" cluster. Consequently, it was not surprising that some *Azotobacter* DNAs could be amplified with our primer set. As suggested by Anzai *et al*. (2000), we recommend an extensive study for a definite conclusion on the taxonomy of this genus and its phylogenetic relationships with *Pseudomonas*.

In our study, some of the collection and environmental *Pseudomonas* strains yielded multiple size PCR products (up to 3), in agreement with previously published data (Bennasar *et al*. 1998). For a given strain, the number of PCR products was identical with *Pseudomonas* and with *Bacteria* primers, confirming that the region targeted by our primer set included the ITS1. The ITS1 length varies both between species and between multiple operons in a given organism. Four rDNA transcriptional units have been described in *P. stutzeri* (Ginard *et al*. 1997) and *P. aeruginosa* (Römling *et al*. 1989). Variations in the length of ITS1 are due, in part, to the number and type of tRNA genes that it may contain (for most of proteobacteria, including *Pseudomonas* spp.: $tRNA^{ALA}$ and $tRNA^{ILE}$) (Jensen *et al*. 1993). The size of generated PCR fragments, which include about 750 bp of 16S rDNA sequence, is in good agreement with previously published data about the size of ITS1 for *Pseudomonas*: this may range from 515 to 548 bp (Gill *et al*. 1994; Sawada *et al*. 1999; Guasp *et al*. 2000), when including both tRNA genes. In our study, limited length polymorphism was also detected for ITS1 sequences from a large collection of *Pseudomonas*.

Specific PCR amplification with fPs16S and rPs23S was in very good agreement with PSM_G probing on soil isolates, as all the PCR+ strains were also H+. Nonetheless, a few H+ PCR- strains were not related to the genus *Pseudomonas*. These results suggested that the experimental (stringency) conditions were not optimal regarding the specificity of the probe.

The probing approach provides the information on the presence and numbers of bacteria affiliated to *Pseudomonas* genus (Braun-Howland *et al*. 1993; Marilley *et al*. 1999). On the other hand, specific PCR amplification provides a reliable tool for (i) detecting *Pseudomonas* strains, (ii) confirming their affiliation to this genus and (iii) assessing their genotypic diversity, using the sequence variability of the generated PCR products.

The proposed approach could take advantage of the 16S rDNA fragment of the amplicon. This one displays a limited level of sequence variation or restriction polymorphism (Brosch *et al*. 1996; Achouak *et al*. 2001), and generates restriction fragments that are specific for some species or groups (Laguerre *et al*. 1994). Moreover, the ITS1 sequences were shown to be perfectly identical or very slightly different from strains belonging to a same biovar or genomovar, whereas they displayed frequent insertion or deletion events between strains from different subspecies (Sawada *et al*. 1999; Guasp *et al*. 2000). The ITS1 region was also used for the definition of strain- or group-specific probes (Gill *et al*. 1994 ; Tyler *et al*. 1995) or PCR primers (Yang *et al*. 2000) in *Pseudomonas* genus, which can be useful for identification and monitoring purposes. Moreover, restriction analysis of ITS1 was shown to be valuable for the differentiation of *Pseudomonas* strains at an infra-specific level (Manceau & Horvais 1997; Cho & Tiedje 2000; Jeng *et al*. 2001).

Finally, preliminary results showed that the amplification of ITS1 fragments with fPs16S and rPs23S could be directly applied on environmental DNA extract. This feature seems particularly relevant for avoiding the bias of cultivability. Such an approach would be suitable for the monitoring of *Pseudomonas* populations in environmental samples, offering new promise in understanding the ecology of *Pseudomonas* organisms.

Acknowledgement

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3.2 Diversité des *Pseudomonas* cultivables dans la prairie naturelle évaluée par PCR-RFLP

Ce paragraphe a fait l'objet d'un article de S. Tarnawski, L. Locatelli, **J. Hamelin**, M. Aragno et N. Fromin. Cet article a été publié dans la revue FEMS Microbiology Ecology (Tarnawski et coll. 2003).

Examination of Gould's modified S1 (mS1) selective medium and Angle's non-selective medium for collecting diversity of *Pseudomonas* spp. in soil and root environments

Abstract

Studies on the diversity of environmental culturable *Pseudomonas* populations are dependent on the isolation procedure. This procedure includes the use of selective media which may influence the recovery of strains and thus the diversity described. In this study, we assessed the use of two agar isolation media for describing the diversity of soil and root inhabiting *Pseudomonas* associated with the perennial grass *Molinia coerulea*. A total of 382 *Pseudomonas* strains were recovered on either non-selective Angle medium, or on modified Gould S1 (mS1) *Pseudomonas*-selective medium. Their diversity was assessed by restriction analysis of PCR-amplified 16S-23S rDNA internal transcript spacer (ITS1) sequences. The comparison of mS1- and Angle-recovered populations showed that the use of mS1 selective medium lead to an underestimation of both *Pseudomonas* counts and diversity, especially in the soil environment.

3.2.1 Introduction

The genus *Pseudomonas* includes several species of general interest, such as human (Davies 2002) and plant (Kimura *et al*. 2001) pathogens, xenobiotic degraders (Saadoun 2002), plant growth promoters (Glick 1995) and biocontrol strains (Mazzola 2002). Because of these multiple roles and its wide distribution in the environment, this genus has become one of the best-studied bacterial taxa.

Whereas the soil is considered as an oligotrophic environment (Darrah 1991), plant-derived compounds released by the roots (rhizodeposition) provide abundant carbon and energy sources (Whipps 2001) as well as selective compounds for rhizosphere living-microorganisms. Consequently, the rhizosphere is altogether an elective and selective environment, favouring specific populations, which best fit these conditions (Ramos *et al*. 2000). *Pseudomonas* are a significant component of the rhizosphere microflora (Kragelund *et al*. 1996; Marilley *et al*. 1999), denoting their fitness in rhizophere environment (Lugtenberg & Dekkers 1999; Elasri *et al*. 2001; Fromin *et al*. 2001).

Our perception of culturable *Pseudomonas* diversity depends strongly on the isolation procedure used (Johnsen *et al*. 1999; Aagot *et al*. 2001). Consequently, one requirement for such investigations is the development of culture media which permit the recovery of the largest (even exhaustive) diversity of culturable *Pseudomonas* and which are selective for this genus. The cultivation of microorganisms depends on their physiological and metabolic properties. The various culture media proposed for the selective isolation of *Pseudomonas* (King *et al*. 1954; Gould *et al*. 1985; Sands & Rovira 1970; Aagot *et al*. 2001) are usually iron-deficient, thus enhancing siderophore production. Among them, King's B and Gould's S1 media are often used for the isolation, enumeration and diversity evaluation of fluorescent *Pseudomonas* (Johnsen & Nielsen 1999; Sigler *et al*. 2001). The selectivity of Gould's S1 medium is based on an iron limitation, an high glycerol and sucrose contents. It is also based on other compounds such as sodium lauroyl sarcosine (which inhibits the growth of Gram positive bacteria) and trimethoprim (an antibiotic limiting the growth of non-fluorescent *Pseudomonas*). This medium allowed a high recovery of fluorescent *Pseudomonas* phenotypes from soil samples. Fromin *et al.* (2001) proposed the mS1 medium (for Gould's modified S1 medium), without thrimethoprim to allow the growth of non-fluorescent *Pseudomonas*.

Further studies have revealed limits of these selective media. For instance, Kragelund *et al*. (1996) has shown that *Pseudomonas* counts from barley rhizospheric soil were higher on a non-selective medium (tenfold diluted tryptic soy agar) than on either Gould's S1 or King's B media. Comparative investigations using Gould's S1 and King's B media presented contrasting results. The use of Gould's S1 medium sometimes gave either higher (Elliott & Des Jardin 1999) or lower (Johnsen & Nielsen 1999) counts of fluorescent *Pseudomonas* than using King's B medium. Nonetheless, a wider genotypic variety of *Pseudomonas*, of both fluorescent and non-fluorescent types, could be recovered using S1 compared to King's B (Johnsen & Nielsen 1999). Futhermore, Aagot *et al*. (2001) recovered different counts and diversity of *Pseudomonas* from the same soil sample by varying the concentration of casamino acids in NAA *Pseudomonas*-selective medium.

Most of these studies were performed only on soil environments and did not test the validity of these selective media for *Pseudomonas* populations inhabiting environments with contrasting properties, such as rhizosphere habitats. Moreover, studies on *Pseudomonas* diversity in the rhizosphere usually deal with annual crops, while studies on perennial plants are rare. These latter plants might induce a long-term selection of the most adapted populations due to continuous exchanges between soil and roots (Hamelin *et al*. 2002).

In this study, we investigated the culturable *Pseudomonas* populations in soil and root environments, in a natural meadow dominated by the perennial oligotrophic grass *Molinia coerulea*. We compared the influence of selective mS1 and non-selective Angle (Angle *et al*. 1991) media on both quantitative (CFU numbers) and qualitative (genotypic diversity) recovery of *Pseudomonas.*

3.2.2 Materials and methods

Culture media

The *Pseudomonas* selective mS1 medium was similar to S1 medium (Gould *et al*. 1985), except that trimethoprim was omitted to allow the growth of non-fluorescent *Pseudomonas*, and that sucrose content was doubled to 20 $g.l^{-1}$ (Fromin *et al*. 2001). Total heterotrophic aerobic bacteria were enumerated on Angle medium (Angle *et al*. 1991). This medium was designed to have a ionic strength similar to that found in most non saline soils. Ion concentrations of Angle medium were (in millimolar): 2.5 NO_3^-, 2.5 NH_4^+, 0.05 HPO_4^{2-}, 2.5 Na^+, 4.0 Ca^+, 2.0 Mg^{2+}, 0.503 K^+, 4.0 Cl^-, 5.0 SO_4^{2-}, and 0.02 Fe^{2+}. Glucose (1 $g.L^{-1}$) was used as carbone source.

Sampling and isolation

The study site consisted of a oligotrophic littoral meadow dominated by a genetically homogenous population of the perennial grass *Molinia coerulea* in a Gleysol, Typic Haplaquoll (4.7 % clay, 9.5 % silt, 85.8 % sand, and $pH_{[H2O]}$ 8.4) (Buttler 1987). It is located in the Cudrefin preserved natural area on the southern shore of Lake Neuchâtel (Switzerland), at an elevation of 430 meters above mean sea-level. In July 2000, three undisturbed soil cores, two to three meters apart, were collected from the upper 20 cm of soil and immediately analysed. These cores were pooled and then divided in two fractions: soil fraction constituted by soil devoid of root material. The root fraction, corresponding to root of M. coerulea, was washed twice in sodium phosphate buffer 0.1 M pH 7.0 (SPB). For each fraction, 1 g of fresh material was finely crushed in 10 ml of SPB. Tenfold serial dilutions of root and soil suspensions were prepared and 100 µl aliquots from the appropriate dilutions were spread out on eight mS1 and eight Angle plates. The same suspension was used to inoculate both media. Colony forming units (CFU), at a proper dilution (20-200 colonies per Petri dish), were enumerated after 72 hours of incubation at 24°C.

Strains handling and cultivation

From mS1 medium, colonies (noted **S**) were randomly picked from the 8 plates for soil and root fractions. From Angle medium, all soil and root colonies (noted **A**) from the 8 plates were isolated. Angle and mS1 strains were streaked twice on Angle medium for purity check and conserved on Angle plates at 4 °C. Strains isolated on Angle were tested on mS1, and noted to be either **AS$^+$** or **AS$^-$** when able or unable to grow on mS1 respectively.

Colony hybridisation

All the isolated strains were tested for their affiliation to the genus *Pseudomonas* by colony hybridisation with the *Pseudomonas* specific 16S rDNA PSM_G probe (Braun-Howland *et al*. 1993) as previously described (Marilley *et al*. 1999). The specificity of the hybridisation protocol to the genus *Pseudomonas* was confirmed by testing *Pseudomonas putida* (ATCC 17430) and *Pseudomonas fluorescens* (ATCC 17397) as positive controls, and *Escherichia coli* (DSM 2840) and *Enteroccocus faecalis* (ATCC 29212) as negative controls on each membrane. Strains giving a positive hybridisation signal were noted **AH** (including **AS$^+$H** and **AS$^-$H**), and **SH**.

PCR-RFLP analysis of the ribosomal DNA

AH and **SH** strains were submitted to genomic DNA extraction with a Wizard Genomic DNA Purification Kit (Promega corp., Madison, WI) according to the manufacturer's protocol, except that only 400 µl of Nuclei Lysis Solution was used. A 1100-1300 bp fragment of the rDNA operon (named 16S-ITS1 rDNA), including the 3' half of 16S-rDNA and the whole 16S-23S rDNA internal transcript spacer sequence (ITS1), was amplified using a *Pseudomonas* specific PCR protocol (Locatelli *et al*. 2002). The PCR products were checked for size and yield on 0.8 % Standard Agarose gel (Eurobio, Les Ullis, France) using Low DNA Mass Ladder (Gibco). Two aliquots of eight µl of the PCR product were digested using *Hae*III endonuclease on one hand, and *Taq*I on the other hand (Macherey-Nagel GmbH, Düren, Germany). The digested products were resolved by electrophoresis in 2 % STG agarose (Eurobio) in TBE 0.5 X at 3.5 $V.cm^{-1}$ for 1 hours and visualised by staining with ethidium bromide. The ΦX174/*Hae*III Fragment Ladder (Gibco) was used as size ladder. Isolates displaying identical restriction profiles with *Hae*III and *Taq*I enzymes were grouped in a same operational taxonomic unit (OTU) (Marilley *et al*. 1999).

Cloning and sequencing of 16S rDNA fragments

The 3′ end of the 16S rDNA gene sequence was amplified with 907f and GM4r primers (Muyzer *et al*. 1995). PCR products were purified using a Nucleotrap Extraction Kit for Nucleic Acids (Macherey-Nagel), and cloned into pGEM-T vector (Promega) and *E. coli* competent cells (Sambrook & Russell 2001). Transformants with an expected size insert were processed with NucleoSpin Plasmid kit (Macherey-Nagel) for plasmid extraction. The corresponding inserts were sequenced (MWG Biotech, Ebersberg, Germany). The identification of the corresponding organisms was achieved using a BLAST analysis of on the retrieved sequences (Altschul *et al*. 1997). As non-ambiguous affiliation of strains could not always be achieved, they were classified into clusters according to Anzai *et al.* (2000). These sequences were registered in the EMBL database under the accession number AJ512378 to AJ512408, and AJ517396 to AJ517410.

Data analysis

CFU counts were compared statistically using the Student *t*-test. Proportions of **H** strains were compared using a χ^2 test. Shannon diversity index was calculated, based on OTUs, as $H' = -\sum(p_i \log_2 p_i)$ where p_i is the number of isolates in the OTU being analysed. Eveness was calculated as $J = H'/H'_{max}$, where H'_{max} is the value of H' when all isolates are evenly distributed among the OTUs. Differences between Shannon index-values for both media and for both fractions were evaluated using Student *t*-test, according to Magurran (1988). Differences in OTU distribution on both media, and in both fractions were evaluated using Fischer's exact test. The statistical analyses were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, Washington).

3.2.3 Results and discussion

Quantitative evaluation of root and soil Pseudomonas *using mS1 and Angle*

CFU counts on Angle and mS1 media are presented in figure 3.2.A. The total heterotroph **A** counts were significantly higher (about one log) than the **S** and **AS$^+$** counts for both soil and root fractions (*t*-test, $p < 0.001$). For the soil fraction, **AS$^+$** and **S** counts were not significantly different. But for the root fraction, **AS$^+$** count was significantly higher than **S** count (*t*-test $p < 0.001$). This result showed that more root bacteria could grow on mS1 when they were first isolated on Angle medium.

From Angle plates, 1770 colonies were isolated and tested for growth on mS1 medium. From mS1 plates, 108 soil and 118 root colonies were randomly picked up. Strains hybridising with the *Pseudomonas*-specific PSMg probe, and consequently affiliated to *Pseudomonas*, were designated with a **H** (Table 3.2.A).

Table 3.2.A Numbers (n **H**) and proportions (% **H**) of root and soil *Pseudomonas* strains isolated on mS1 and Angle media.

		n isolates	n **H**	(% **H**)
Soil	A	689	66	(9.6)
	AS$^+$	51	38	(74.5)
	AS$^-$	638	28	(4.4)
	S	108	83	(76.9)
Root	A	1081	120	(11.1)
	AS$^+$	114	112	(98.2)
	AS$^-$	967	8	(0.8)
	S	118	113	(95.8)

A: total heterotroph bacteria isolated on Angle medium

AS$^+$: Angle isolates growing on *Pseudomonas* selective mS1 medium

AS$^-$: Angle isolates unable to grow on *Pseudomonas* selective mS1 medium

S: strains directly isolated on mS1 selective medium.

About 23 % of mS1 soil strains (**AS$^+$** and **S**) were not affiliated to *Pseudomonas* (4 % only for root strains). Consequently, mS1 medium permitted the growth of non-*Pseudomonas* strains. Some *Pseudomonas* strains isolated on Angle medium (**AH**) were unable to grow after transfer on mS1 medium (**AS$^-$H**). They represented 42.4 % and 6.7 % of **AH** strains for soil and root strains respectively. Proportions of *Pseudomonas* isolated on Angle medium (**AH**) were statistically equivalent in both soil (9.6 %) and root (11.1 %) fractions (Table 3.2.A). However, the proportion of mS1-growing *Pseudomonas* (**AS$^+$H**) among total Angle isolates (**A**) was lower in the soil fraction (5.5 %) compared to the root fraction (10.4 %) ($p < 0.01$). Our experimental set-up does not allow us to draw definite conclusions. Anyway, these results suggested that using the mS1 selective medium led to an underestimation of *Pseudomonas* proportions, as shown in previous studies (Kragelund *et al*. 1996; Aagot *et al*. 2001). This was particularly the case in the soil fraction, due to the occurrence of a large proportion of **AS$^-$H** strains. Futhermore, using both isolation media, *Pseudomonas* was a major component of the rhizosphere microflora, as already described for other grasses (Kragelund *et al*. 1996; Marilley *et al*. 1999).

Using the proportion of **H** strains (Table 3.2.A), we could estimate the abundance of *Pseudomonas* in root and soil fractions from **A**, **AS$^+$** and **S** CFU counts (Figure 3.2.B). For the soil fraction, mS1-growing *Pseudomonas* counts were 30 % lower than **AH** count, using either direct isolation (**SH**) or prior cultivation on Angle (**AS$^+$H**). For the root fraction, the **SH** count was 57 % lower than from **AH**, whereas the counts estimated from **AH** and **AS$^+$H** were similar. This result confirmed that isolation on mS1 medium led to an underestimation of *Pseudomonas* counts. Preliminary isolation on Angle improved the recovery of *Pseudomonas* strains capable of growing on mS1, mainly in the root fraction. Consequently root and soil-inhabiting *Pseudomonas* strains did not have same ability to grow on mS1.

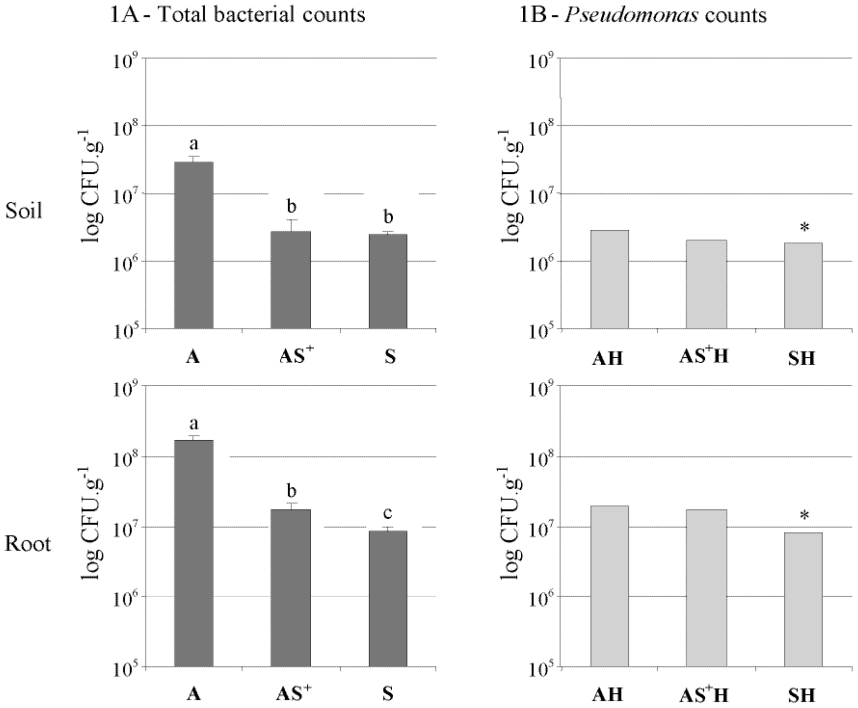

Figure 3.2.B Number of colony forming units (CFU) per gram of dry weight for soil and root. 1A, total counts. 1B, *Pseudomonas* counts (calculated using **H** proportions, see Table 3.2.A). **A**: on Angle non-selective medium, **S**: on mS1 *Pseudomonas* selective medium, and **AS$^+$**: after the two consecutive non-selective and selective media. *: Indicates an estimation from one hundred randomly picked colonies from mS1 medium. a, b, c: Different letters correspond to a highly significant statistical differences (*t*-test, p < 0,01).

There are two possible reasons for the differential recovery of *Pseudomonas* from mS1 and Angle media. This may be because some *Pseudomonas* strains are inhibited by mS1 medium, even if transferred from fresh, active cultures on Angle. This should be the main feature in soil. Alternatively, our results suggest that the physiological state of some cells do not allow them to form a colony by direct plating on mS1 at the time of isolation.

PCR-RFLP analysis

We then compared the genetic diversity of Angle and mS1 *Pseudomonas* strains for soil and root fractions. About 30 % of all *Pseudomonas* strains were submitted to PCR-RFLP analysis of 16S-ITS1 rDNA fragments. 52 soil strains and 32 root strains from Angle (**AH**) and 27 soil strains and 33 root strains from mS1 (**SH**) were analysed. The 16S-ITS1 rDNA sequences of these 144 strains could be amplified with the *Pseudomonas*-specific PCR protocol, confirming their affiliation to the genus *Pseudomonas* (cf. § 3.1; Locatelli *et al*. 2002). For 29 % of the strains, 2 or 3 PCR fragment sizes were generated (1050 to 1300 bp), as previously shown (Dawson *et al*. 2002). The amplicons of different sizes were due to the presence or absence of tRNA genes within the ITS1 region of different ribosomal operons within the same genome (Guasp *et al*. 2000). When ITS1 PCR products were digested, the sum of restriction fragment sizes was higher than undigested ITS1 fragments, even for a single sized PCR product. Cho & Tiedje (2000) reported

Table 3.2.C Schematic OTU pattern restriction profiles obtained by digestion of the PCR-amplified half 3′ end 16S rDNA and whole ITS1 regions. OTUs were defined on the combined restriction patterns of *Hae*III and *Taq*I. Size of each band is given in base pairs

	*Hae*III												*Taq*I										
OTU	450	440	400	370	350	340	309	290	270	230	190	170	400	310	290	250	230	200	190	170	160	140	110
1		–		–		–				–	–	–	–	–			–	–			–		–
2						–				–	–	–	–				–				–		–
3					–					–	–	–	–				–				–		–
4					–					–	–	–	–				–					–	–
5		–		–		–				–	–	–	–				–				–		–
6					–			–		–	–	–	–	–			–					–	–
7	–				–					–	–	–	–				–					–	–
8					–					–	–	–	–	–			–					–	–
9					–					–	–	–	–				–		–	–			–
10						–				–	–	–	–	–			–					–	–
11	–									–	–	–	–		–		–						
12						–				–	–	–	–				–		–	–			–
13		–		–		–				–	–	–	–	–			–						–
14	–				–					–	–	–	–	–			–					–	–
15					–				–	–	–	–	–				–				–		–
16			–		–					–	–	–	–				–						–
17	–		–		–			–		–	–	–	–	–			–					–	–
18	–				–					–	–	–	–	–			–	–			–		–
19		–		–		–				–	–	–	–	–			–				–		–
20					–					–	–	–	–				–						–
21						–				–	–	–	–		–		–				–		–
22						–				–	–	–	–				–						–
23	–					–				–	–	–	–				–	–			–		–
24	–					–				–	–	–	–		–		–						–
25		–			–		–			–	–	–	–				–		–	–			–
26	–				–					–	–	–	–		–		–	–			–		–
27	–					–				–	–	–	–				–	–					–
28		–			–					–	–	–	–				–						–
29				–		–				–	–	–	–		–	–	–						–
30					–				–	–	–	–	–	–			–					–	–
31						–				–	–	–	–	–			–				–		–
32						–				–	–	–	–		–		–						
33	–			–		–				–	–	–	–	–			–					–	–
34	–					–				–	–	–	–				–	–			–		
35			–		–					–	–	–	–	–			–						–

Table 3.2.D Isolate numbers in each OTU, spread out between media (Angle and mS1) and fractions (soil and root), and phylogenetic affiliation based on partial 16S rDNA sequence. *, *Pseudomonas* groups are defined according to Anzai *et al.* (2000)

	Global			Angle			mS1			*Pseudomonas* related group *	Accession
OTU	n	soil	root	n	soil	root	n	soil	root	(n, category of sequenced strains)	numbers
1	23	.	23	7	.	7	16	.	16	*fluorescens/aeruginosa* (2, **AS^{+}H-AS^{+}H**)	AJ512378-AJ512379
2	19	19	.	11	11	.	8	8	.	*aeruginosa/putida* (2, **AS^{-}H-AS^{+}H**)	AJ512380-AJ512381
3	16	7	9	5	3	2	11	4	7	*fluorescens* (2, **AS^{+}H**-**AS^{-}H**) *putida/graminis* (1, **AS^{+}H**)	AJ517399-AJ517400-AJ512382
4	7	5	2	7	5	2	.	.	.	*fluorescens* (2, **AS^{+}H**-**AS^{-}H**)	AJ512383-AJ512384
5	7	.	7	1	.	1	6	.	6	*fluorescens* (2, **AS^{+}H**-**SH**)	AJ517398-AJ517397
6	7	.	7	4	.	4	3	.	3	*chlororaphis/fluorescens* (1, **AS^{+}H**)	AJ517396
7	6	.	6	6	.	6	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512385
8	5	4	1	2	2	.	3	2	1	*fluorescens* (2, **AS^{+}H**-**SH**)	AJ512386-AJ512387
9	4	4	.	3	3	.	1	1	.	*aeruginosa*(2, **AS^{-}H**-**SH**)	AJ517403-AJ517404
10	4	4	.	4	4	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512388
11	4	4	.	4	4	.	.	.	.	*aeruginosa* (2, **AS^{+}H**-**AS^{-}H**)	AJ512389-AJ517410
12	4	4	.	4	4	.	.	.	.	*aeruginosa* (2, **AS^{+}H**-**AS^{-}H**)	AJ512390-AJ517408
13	3	3	.	2	2	.	1	1	.		
14	3	.	3	3	.	3	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512391
15	2	2	.	1	1	.	1	1	.	*aeruginosa/fluorescens* (2, **AS^{-}H**-**SH**)	AJ517401-AJ517402
16	2	2	.	1	1	.	1	1	.	*aeruginosa* (2, **AS^{-}H**-**SH**)	AJ517405-AJ517406
17	2	2		1	1	.	1	1	.	*putida* (1, **AS^{+}H**)	AJ517409
18	2	2	.	2	2	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512392
19	2	2	.	2	2	.	.	.	.	*fluorescens* (1, **AS^{-}H**)	AJ512393
20	2	.	2	2	.	2	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512394
21	2	.	2	2	.	2	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512395
22	2	.	2	2	.	2	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512396
23	2	2	.	.	.	.	2	2	.	*fluorescens* (1, **SH**)	AJ512397
24	2	2	.	.	.	.	2	2	.	*fluorescens* (1, **SH**)	AJ512398
25	2	2	.	.	.	.	2	2	.	*fluorescens* (1, **SH**)	AJ512399
26	1	1	.	1	1	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512400
27	1	1	.	1	1	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512401
28	1	1	.	1	1	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512402
29	1	1	.	1	1	.	.	.	.	*fluorescens* (1, **AS^{+}H**)	AJ512403
30	1	1	.	1	1	.	.	.	.	*putida* (1, **AS^{+}H**)	AJ512404
31	1	1	.	1	1	.	.	.	.	*aeruginosa* (1, **AS^{+}H**)	AJ512405
32	1	1	.	1	1	.	.	.	.	*tolaasi* (1, **AS^{+}H**)	AJ517407
33	1	.	1	1	.	1	.	.	.	*syringae* (1, **AS^{+}H**)	AJ512406
34	1	1	.	.	.	.	1	1	.	*fluorescens* (1, **SH**)	AJ512407
35	1	1	.	.	.	.	1	1	.	*fluorescens* (1, **SH**)	AJ512408
n strains	144	79	65	84	52	32	60	27	33		
n OTUs	35	26	12	30	21	11	16	13	5		

similar results and suggested the existence of at least two types of ITS1 regions in a PCR product of same size.

Pseudomonas strains were grouped using restriction analysis of their PCR products. The corresponding restriction profiles are presented in Table 3.2.C. The endonucleases were equally discriminating, as 14 and 16 different restriction patterns were obtained with *Hae*III and *Taq*I enzymes respectively. When combining these profiles, the 144 strains were grouped in 35 different Operational Taxonomic Units (OTUs) (Table 3.2.D). The clustering of strains into the different OTUs was not influenced by the isolation plate they were retrieved from (data not shown). Figure 3.2.E presents the repartition of **AH** and **SH** strains for soil and root fractions among the 35 OTUs.

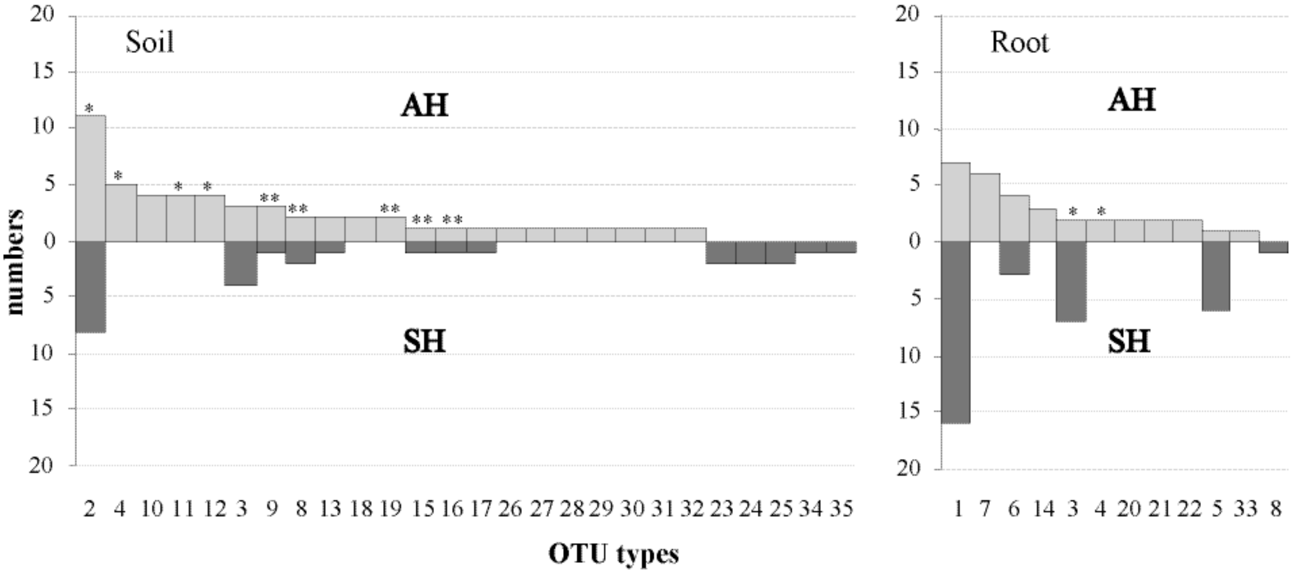

Figure 3.2.E Distribution of **AH** (isolated on Angle) and **SH** (isolated on mS1 medium) *Pseudomonas* strains among the different OTUs, for soil and root fractions. The name of each OTU is noted at the bottom. *: indicates that Angle OTUs contained strains both capable (**AS^{+}H**) and incapable (**AS^{-}H**) of growing on mS1 medium. **: indicates Angle OTUs where all strains were unable to grow on mS1 (**AS^{-}H)**. Other OTUs were composed of **AS^{+}H** strains.

OTU distribution between soil and root

The 79 soil strains were grouped in 26 OTUs. In contrast, the 65 root strains formed only 12 OTUs (Table 3.2.D). Twice many *Pseudomonas* OTUs were described in the soil fraction compared to root fraction for each culture medium. The overall distribution of *Pseudomonas* strains among OTUs was different between soil and root fractions. Such a difference was observed on mS1 ($p < 0.0001$) as well as on Angle ($p < 0.\ 0001$). Twenty three OTUs were recovered from soil alone, while nine OTUs were specifically found in root fraction (Table 3.2.D). Previous studies for other plants (Clays-Josserand *et al*. 1995; Latour *et al*. 1996; Fromin *et al*. 2001) have shown that root environment was selective for some *Pseudomonas* types. Three OTUs (3, 4 and 8) were found among both soil and root strains. Nine soil OTUs and one root OTU were represented by a single isolate. Root and soil fractions harboured different major OTUs (Figure 3.2.E). OTU1 grouped 35 % of root strains exclusively, while OTU2 grouped 24 % of soil strains exclusively. The OTU3 was the second most abundant in both soil and root fractions (9 % and 14 % of strains respectively).

Comparison of Angle and mS1 OTU distribution

The Shannon diversity index *H′* (Magurran 1988) and evenness *J* were calculated for root and soil *Pseudomonas* recovered on Angle and mS1 media (Table 3.2.F).

Table 3.2.F Diversity (Shannon index *H′*) and evenness (*J*) indices among OTUs isolated from soil and/or root fractions, as obtained using Angle's or mS1 media for the isolation of strains.

	Soil		Root	
	Angle	mS1	Angle	mS1
H′	3.95	3.27	3.19	1.90
J	0.69	0.69	0.64	0.38

The comparison of Shannon index suggested that the Angle's medium permitted a higher diversity to be recovered, compared to the mS1 medium, in both soil and root fractions (*t*-test non significant). The evenness values for soil were similar using both media, whereas for root strains, the evenness was lower on mS1, indicating different population structures. The distribution of *Pseudomonas* strains among OTUs were different using Angle and mS1 media, for both soil and root fractions (p = 0.0002 for root). Indeed, the 84 **AH** strains were grouped into 30 OTUs, while the 60 **SH** strains grouped into 16 OTUs (Table 3.2.D). Eleven OTUs were retrieved on both Angle and mS1 media, including the most abundant OTU1 (root), OTU2 (soil) and OTU3 (common). OTU1 and OTU2 displayed the highest strain numbers on both media. Five non-abundant soil types (OTU23, 24, 25, 34 and 35) were retrieved on the mS1 medium alone (Figure 3.2.E). Nineteen OTUs were only retrieved on Angle medium. These included some abundant OTUs, such as the OTU4 (common to soil and root), OTU7 (19 % of root strains), and OTUs 10, 11 and 12 (23 % of soil strains).

Eight OTUs (as OTU3, 4, 11, and 12) contained strains both capable and incapable of growing on mS1 (Figure 3.2.E). Consequently, the ability of strains to grow on mS1 was not related to their clustering using PCR-digestion of the rDNA sequences. Among the thirty OTUs found on Angle, twenty five contained **AS$^+$H** strains, i.e. they could grow on mS1. Four OTUs comprised **AS$^-$H** strains, though these were also retrieved on mS1 medium (OTU8, 9, 15 and 16; Figure 3.2.E). More generally, OTUs including **AS$^-$H** were more frequent in soil (9 among 21 OTUs) than in root (2 among 11) fractions. The soil OTU19 was the only one composed exclusively of **AS$^-$H** strains, i.e. it was never detected on mS1.

Eighteen OTUs were never detected by direct isolation on mS1. This was the case for about 50 % of OTUs, for soil as well as of root fractions. For example, the root OTU7 was abundant among Angle strains and all its representatives were **AS$^+$H**. In contrast, none of these strains were directly isolated on the mS1 medium. This suggested that the physiological state of bacteria at the time of isolation influenced their direct recovery on the mS1 medium.

The Angle medium attempts to approximate the composition of the soil solution (Angle *et al*. 1991). Its use should enhance the recovery of soil-inhabiting bacteria, as shown in this study. In contrast, high nutrient levels in the mS1 medium may affect the capacity of environmental *Pseudomonas* to grow and form colonies. This could explain in part the lower recovery of soil *Pseudomonas* strains on mS1. Aagot *et al.* (2001) also showed that media with high nutrient concentrations can have a negative effect on *Pseudomonas* colony formation in samples collected from soil. They suggested that *Pseudomonas* isolated on nutrient-poor media might occupy different ecological niches to those *Pseudomonas* recovered on traditional nutrient-rich isolation media. More generally, bacteria with different physiological states might be present simultaneously in the rhizosphere (Semenov *et al*. 1999). Some aspects of plant-microbe environment could also affect the differential recovery using the two media. When comparing mS1 and Angle media for root *Pseudomonas* populations, we noticed that OTU3 and OTU5 displayed high numbers on the mS1 medium, whereas these were poorly represented among Angle strains. Such a result was also observed, to a lesser extent, for the major root OTU1. The corresponding strains could display a certain tolerance to high osmotic levels (Miller & Wood 1996) or adaptation to high nutrient levels (Aagot *et al*. 2001), and their relative abundance may be overestimated on mS1 isolation medium. As suggested by Wilson & Lindow (2000), the culturability of environmental bacteria may be affected by components of the medium required for their selective isolation from environmental samples. In conclusion, there are two features which may explain the failure to isolate environmental *Pseudomonas* strains on mS1 medium. This may be due to the intrinsic inability of some strains to grow on mS1 medium. Alternatively it may be due to the inability of other strains to be revived on the mS1 medium even if they are able to grow on mS1 after being transferred from active culture.

Acknowledgement

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3.3 Fréquence des *Pseudomonas* cultivables réducteurs de nitrate et dénitrifiants en réponse à une augmentation du pCO_2

Ces résultats ont été obtenus lors du travail de diplôme de Ludovic Roussel-Delif (Roussel-Delif 2002) dans le cadre du groupe FACE. Cet article est en préparation. Nous en avons extrait les généralités et les résultats exclusivement consacrés à *Molinia coerulea.*

En parallèle à la caractérisation taxonomique des *Pseudomonas* présents dans l'environnement rhizosphérique de *Molinia coerulea* (cf. § 3.1 & 3.2), nous nous sommes intéressé aux bactéries qui avaient la capacité à réduire les nitrates et à dénitrifier. La dénitrification a pour conséquence la perte d'azote pour les écosystèmes car elle résulte en une réduction des nitrates en composés gazeux qui s'échappent dans l'atmosphère (cf. § 1.2.2). Des études antérieures ont montré que la dénitrification semblait plus fréquente chez les bactéries rhizosphériques que chez celles du sol (von Rheinbaden & Trolldenier 1984; Linne von Berg & Bothe 1992; Clays-Josserand et coll. 1995; Ghiglione et coll. 2000). La plupart des études antérieures ont été réalisées sur des plantes cultivées (tomate, lin) dans des sols riches.

Ici, les conditions en azote disponible sont limitantes car *Molinia coerulea* pousse dans un sol naturellement pauvre en azote disponible (cf. § 1.4.1) et la dénitrification pourrait entrer directement en compétition avec les racines pour la nutrition azotée des plantes. La fréquence allélique des deux nitrate réductases, périplasmique et membranaire, a été calculée pour les *Pseudomonas* provenant du sol et de la racine et en fonction de pCO_2.

Ce travail a été réalisé en parallèle sur des plantes de *Lolium perenne* ayant été faiblement fertilisées avec 140 kg $N.ha^{-1}.an^{-1}$. Les conditions de culture de *Lolium perenne* utilisées ici sont limitantes pour sa nutrition azotée. L'influence des deux plantes sur les populations de *Pseudomonas* dénitrifants pourra être comparée.

Diversity of nitrate reductases in culturable rhizospheric *Pseudomonas* of the oligonitrophilic perennial grass *Molinia coerulea* grown under ambient and elevated CO_2 content

L. Roussel-Delif[1], S. Tarnawski[1], **J. Hamelin**[1], L. Philippot[2], M. Aragno[1] and N. Fromin[1]

[1]Laboratoire de Microbiologie, Université de Neuchâtel, rue Emile Argand 11, 2007 Neuchâtel, Switzerland.

[2] Institut National de la Recherche Agronomique, UMRA111, Microbiologie des Sols - Géosols, Laboratoire de Microbiologie des Sols, 21065 Dijon Cedex, France.

Abstract

286 *Pseudomonas* strains were isolated from the root and surrounding soil of *Molinia coerulea*, a perennial grass with low nitrogen trophic requirement. They were characterised regarding their ability to reduce nitrate as well as their genes encoding for nitrate reductases. These data were evaluated in relation to the root (soil and crushed roots) and to the partial pressure of atmospheric CO_2 (pCO_2) in the swiss FACE installations. The diversity of corresponding strains was assessed using PCR-RFLP on *narG* and *napA* genes, encoding for the large subunit of the membrane-bound and periplamsic nitrate reductases respectively. The same study was performed on culturables *Pseudomonas* populations associated with *Lolium perenne* but only results from the *Molinia* environment are presended here. Both proportions of nitrate reducers and nitrate reductase systems (NAR *versus* NAP) among *Pseudomonas* isolates were influenced by both root proximity (soil / root) and CO_2 content (ambient / elevated). NAR and NAP forms were present in nitrate reducing strains (83 and 54 % respectively) and 37 % of strains harboured both genes. Putative denitrifiers had preferentially the membrane-bound (NAR) form, and both *narG* and *napA* genes displayed a high level of sequence diversity.

3.3.1 Introduction

The dissimilation of nitrate by bacteria begins by the reduction of nitrate to nitrite and can carry on by the reduction of nitrite to either ammonium (nitrate ammonification) or N gaseous compounds, mainly N_2O or N_2 (denitrification). Denitrification was described as the main nitrate dissimilation process occurring in soil environments (Gamble *et al*. 1977; Tiedje 1988).

Recent studies suggested that denitrification process in soils may be influenced by soil properties and agricultural practices (Chèneby *et al*. 2000; Drury *et al*. 1991; Clays-Josserand *et al*. 1999; Parry *et* al. 1999; van Oorschot *et al*. 2000; Strong & Fillery 2002) as well as by the presence and type of plant (Mahmood *et al*. 1997; Nijburg *et al*. 1997; Clays-Josserand *et al*. 1999; Philippot *et al*. 2002). Contradictory results were reported regarding the influence of the plant on the nitrate-dissimilating communities. In the rhizosphere of *Glyceria maxima*, Nijburg *et al*. (1997) reported a lower proportion of nitrate-dissimilating bacterial isolates compared to non-rhizospheric soil isolates. However, this proportion was higher among root-associated bacteria than among soil isolates for various terrestrial plants (Linne von Berg & Bothe 1992; Clays-Josserand *et al*. 1995 & 1999).

The nitrogen availability is probably crucial for nitrate reduction and denitrification activities (Nijburg *et al*. 1997; Strong & Fillery 2002). Consequently, the question of the effect of an elevated partial pressure of atmospheric CO_2 (pCO_2) on grassland ecosystems, which is strongly related to N-cycling, seems relevant. Activity measurement showed that potential as well as actual denitrification in grassland soils were higher under elevated pCO_2, leading to an increased nitrous oxide (N_2O) emission in nitrogen non-limiting conditions (Smart *et al*. 1997; Ineson *et al*. 1998; Carnol *et al*. 2002).

The ability to reduce nitrate was described for various bacteria, including the genus *Pseudomonas*. Numerous investigations suggested that *Pseudomonas* are predominant denitrifiers in soil (Gamble *et al*. 1977; Carter *et al*. 1995; Chèneby *et al*. 2000), even if molecular-based approaches of denitrifying microflora tend to minimise their importance (Philippot *et al*. 2002). To date, two metallo-enzymes involved in the dissimilatory reduction of nitrate were described in bacteria: a membrane-bound nitrate reductase (termed NAR) which is only expressed in anaerobic conditions, and a periplasmic enzyme (NAP), which regulation is more versatile regarding the organisms (Zumft 1997; Philippot & Højberg 1999). Indeed, NAP was described to be involved in aerobic, or anaerobic nitrate dissimilation, or finally in the transition from aerobic to anaerobic metabolism (Bedzyk *et al*. 1999). In the genus *Pseudomonas*, both forms of nitrate reductases were described (Carter *et al*. 1995; Bedzyk *et al*. 1999; Flanagan *et al*. 1999; Gregory *et al*. 2000; Philippot 2002). Moreover, both forms can be present simultaneously in a single strain (Gregory *et al*. 2000; Philippot 2002), but the respective roles of these two apparently redundant enzymes remain so far misunderstood.

In this study, we investigated the nitrate dissimilative abilities among soil-inhabiting and root-associated *Pseudomonas* in the rhizosphere of the oligonitrophilic perennial grass *Molinia coerulea*. The influence of root and of elevated pCO_2 on these populations was assessed. The nature of nitrate reductases (periplasmic *versus* membrane-bound) was also determined. Finally, the diversity of corresponding encoding genes was assessed by a PCR-RFLP approach on the corresponding genes.

3.3.2 Material and Methods

Study site and plant material

M. coerulea plants originated from a littoral meadow located in the south shore of Lake Neuchâtel (Cudrefin, Switzerland). Plants with undisturbed root systems were taken and transferred to FACE (Free Air CO_2 Enrichment) facilities at Eschikon, Switzerland

(Hebeisen *et al*. 1997) in september 1999. *Molinia coerulea* plants were installed with their native soil, a Gleysol, Typic Haplaquoll containing 4.7 % clay, 9.5 % silt and 85.8 % sand, with a $pH_{[H2O]}$ value of 8.4 (Buttler 1987). About 0.7 m^2 of littoral meadow, with reconstituted soil profile below the root horizon (total depth: 35 cm) was installed in one control plot (**C**) and one CO_2-treated plot (**T**). The plants were neither cut nor did receive any fertilisation. The treated plots have been treated with CO_2 during daylight from March to December.

Sampling plan

Both C and T plots were sampled in June 2000, July and December 2001. For each sampled plot, 3 soil cores (about 5 cm diameter, 10-12 cm depth), including dense root systems, were taken and mixed for analysis. The rhizosphere fraction was not easy to define, as the soil fraction is strongly influenced by the root system. Two separate fractions were defined. The non-rhizospheric soil (NRS) for soil devoid of roots and the rhizoplane-endorhizosphere (RE) for roots thoroughly washed in 20 ml sodium phosphate buffer 0.1 M pH 7.0 (SPB) under agitation during 30 min.

Pseudomonas *isolation*

Isolation of *Pseudomonas* strains was performed as previously described (cf. § 3.1 and § 3.2; Locatelli *et* al. 2002; Tarnawski *et al*. 2003). Briefly, about 1 g fresh weight root or soil was crushed in 10 ml SPB. The root and soil suspensions were serially ten-fold diluted in SPB and spread on mS1 medium, which is selective for *Pseudomonas* populations (Fromin *et al*. 2001; Tarnawski *et al*. 2003). The colony forming units (cfu) were counted after 72 h of incubation at 24 °C. Twenty (June 2000), twelve (July 2001) and fifteen bacterial colonies (December 2001) were randomly selected for each fraction. Colonies were picked from appropriate dilution plates (between 20 to 200 colonies per Petri dish) and isolated twice on ten-fold diluted Tryptic Soy Agar (TSA/10). The affiliation of mS1 isolates to the genus *Pseudomonas* was checked by either hybridisation with PSM_G probe (Braun-Howland *et al*. 1993) or by PCR amplification of 16S-23S rDNA region with *Pseudomonas*-specific primers as previously described (cf. § 3.1 and § 3.2).

Nitrate-reduction and denitrification activity assays

Nitrate-dissimilative activities were tested as follows. Each strain was cultivated in duplicate tubes containing 10 ml Nutrient Broth (devoid of fermentable substrate) (Merck) supplemented with KNO_3 2 $g.l^{-1}$. A small inverted Durham tube was added in each tube in order to detect gas formation. The tubes were incubated for 7 days at 24 °C. The presence of nitrate and nitrite in tubes after incubation was evaluated using standard Griess reagent A (sulfanilic acid 0.8 % w/v) and reagent B (N,N dimethyl-1-naphthylamine 0.6 % v/v) and powdered zinc to reduce the remaining nitrate to nitrite. When nitrate was not reduced and neither nitrite nor gas formation was recorded, the bacteria were scored as non-dissimilative (**NDi**). Other strains, which were able to reduce nitrate, were scored as dissimilative (**Di**). They included nitrate reducers (**NR**), putative denitrifiers (**D**) (Mahne & Tiedje 1995) and nitro-ammonifyers (**NA**). Isolates were scored as **NR** when no more nitrate was detected and nitrite accumulated in the medium. They were recorded as **D** when no more nitrate was detected and gas accumulated in the inverted tube (in this study, the 'denitrifiers' corresponded to the bacteria producing gas under the experimental conditions used; the exact composition of this gas, N_2O, N_2, was not controlled). In case nitrate reduction occurred but neither nitrite nor gas was detected in the medium, the bacteria were scored as **NA**. The proportions of **NR** and **D** *Pseudomonas* in the different treatments were analysed using Fisher LSD exact test. The null hypothesis (similar proportions) was rejected and the observed differences were considered as significant for $p \leq 0.05$. The tests were performed using Splus package (Insightful Corporation, Seattle, Washington).

Co-amplification of narG *and* napA *fragment genes*

All nitrate dissimilating strains were submitted to genomic DNA extraction using Wizard Genomic DNA Purification kit (Promega corp., Madison, WI) according to the manufacturer instructions, except that cell pellets were washed with 0.1 M NaOH prior to lysis in order to remove exopolysaccharide.

narG (gene encoding the large subunit of membrane-bound nitrate reductase NAR) and *napA* (gene encoding the large subunit of periplasmic nitrate reductase NAP) fragments were detected using a multiplex PCR protocol. For *narG*, DMHPF and DNDWI primers (Table 3.3.A) were used to amplify a 1008 bp fragment, according to Delorme (2001). Fnap and Rnap primers (Table 3.3.A) were used to amplify a 890 bp fragment of *napA*. They corresponded to previously described V66 and V17 primers (Flanagan *et al*. 1999), which were slightly shortened in order to get compatible annealing temperatures with those of *narG* primers.

Table 3.3.A PCR primers used in this study

Primer	Target gene	Position 5'-3'(1)	5' - 3' Sequence	References
DMHPF	*narG*	2413-2426	GAY ATG CAY CCG TT	Delorme
DNDWI	*narG*	3412-3425	AYC CAR TCR TTR TC	Delorme
Fnap	*napA*	245-269	TTY YTN HSN AAR ATH ATG TAY GG	Flanagan
Rnap	*napA*	1102-1124	TGY TGR TTR AAN CCC ATN GTC CA	Flanagan

(1) position on *Pseudomonas aeruginosa* strain A01 *narG* (accession number AE004804) and *napA* (NC002516) genes respectively.

Ambiguity code : N = G, A, T or C; Y = C or T; R = G or A; H = A, C or T; S = G or C.

References: Flanagan *et al*. 1999; Delorme 2001

The primer sets were designed for the amplification of *Pseudomonas narG* and of a large range of bacteria *napA* genes. Each DNA extract was tested with the multiplex PCR protocol including both primer sets. The PCR mix contained (final concentrations) 1x Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.2 mM each dNTPs (Promega), 0.25 µM each primers DMHPF, DNDWI, Fnap and Rnap (MWG Biotech, AG, Ebersberg, Switzerland), 0.1 $µg.µl^{-1}$ T4 Gene 32 Protein (QBiogene), 0.05 $U.µl^{-1}$ Taq DNA Polymerase (Promega) and 0.1 $µl.µl^{-1}$ pure or ten-fold DNA extract. The reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachussets). This mix was submitted to an initial denaturation step at 95 °C for 3 min, 30 cycles including 60 s denaturation at 94 °C, 45 s annealing at 50 °C and 60 s elongation at 74 °C, with a final extension step at 74 °C for 5 min. PCR products were run on 1 % standard agarose (Eurobio, Les Ullis, France) gel electrophoresis to check their number and size(s). For each strain, the presence of *narG* and/or *napA* fragment(s) was recorded. When both PCR fragments were detected, or when PCR amplicons with a non-expected size were obtained, the DNA extract was re-submitted to PCR amplification with *narG* and *napA* primer sets separately.

Table 3.3.B Proportions of nitrate reducing (**NR**) and denitrifying (**D**) *Pseudomonas* associated to *M. coerulea* (total: 286 *Pseudomonas* strains). n represents the number of strains tested for the corresponding fraction. Different letters indicate proportions that are statistically different: ^{a,b} for differences between fractions (**NRS** *vs* **RE**). Nitrate ammonifying (**NA**) strains were included in the **NR** category

	MC NRS	**MC RE**	**MT NRS**	**MT RE**
June 2000	*n = 10*	*n = 17*	*n = 17*	*n = 18*
% **NR**	0	0	**29**	0
% *D*	*10*	***0***	*12*	***0***
July 2001	*n = 14*	*n = 11*	*n = 14*	*n = 17*
% **NR**	**57**	**27**	**43** ^{a}	**6** ^{b}
% *D*	***0***	***0***	*7*	*6*
Dec. 2001	*n = 44*	*n = 44*	*n = 43*	*n = 37*
% **NR**	**23**	**11**	**28**	**16**
% *D*	*7*	*27*	*5* ^{a}	*43* ^{b}
All dates	*n = 68*	*n = 72*	*n = 74*	*n = 72*
% **NR**	**26%**	**11%**	**31%**	**10%**
% *D*	*6%*	*17%*	*7%*	*24%*

RFLP analysis of narG and napA fragment genes

Each PCR product (about 100 ng) was submitted to enzymatic restriction with *Alu*I for *narG* (Delorme 2001) and with *Hae*III for *napA* fragments (*Hae*III was retained after computer simulated restriction analysis of some available *napA* sequences), according to enzyme manufacturer's (Promega) instructions. Digestion products were analysed after electrophoresis in 2 % STG agarose gel (Eurobio) during 2 hours at 3 V.cm^{-1}. The gels were stained with ethidium bromide and photographed under UV light. The strains displaying a similar profile were clustered in *narG* and *napA* RFLP types noted nar1, nar2… (for membrane-bound form) and nap1, nap2… (for periplasmic form) respectively. Reference sequences for *narG* and *napA* genes were obtained from GeneBank database for some organisms. For *narG* gene: *Pseudomonas fluorescens* strain C7R12 (accession number AF197465), *P. fluorescens* strain AK15 (PFU71398), *P. aeruginosa* strain A01 (AE004804), and *Escherichia coli* strain K12 (AE000221). For *napA* gene: *P. aeruginosa* strain A01 (NC002516).

3.3.3 Results

Nitrate Reducing and Denitrifying activities in S1-isolated Pseudomonas

A total of 286 *Pseudomonas* strains (among 309 isolates) were analysed. They were classified as nitrate-reducers (**NR**) and denitrifiers (**D**). Proportions of **NR** and **D** *Pseudomonas* were compared in relationship with their fraction of origin (NRS or RE) and pCO_2. The results are presented in table 3.3.B by sampling date and Figure 3.3.C gathered all sampling dates. 19.5 % of the *Pseudomonas* strains were able to reduce nitrate to nitrite (56 **NR** strains) and 13 % nitrate to gaseous compounds (38 **D** strains). Only one strain was scored as **NA** and was no further considered.

Lower **NR** proportions among *Pseudomonas* were observed in root compared to soil fraction (Figure 3.3.C), with a statistically significant difference in July 2001 for **T** plots (Table 3.3.B). But putative denitrifying *Pseudomonas* were more frequently detected in the root fraction than in the soil fraction for both **C** and **T** plots.

No influence of elevated pCO_2 on the proportions of *Molinia coerulea*-associated **NR** or **D** *Pseudomonas* was evidenced, probably in part because of the low numbers of strains.

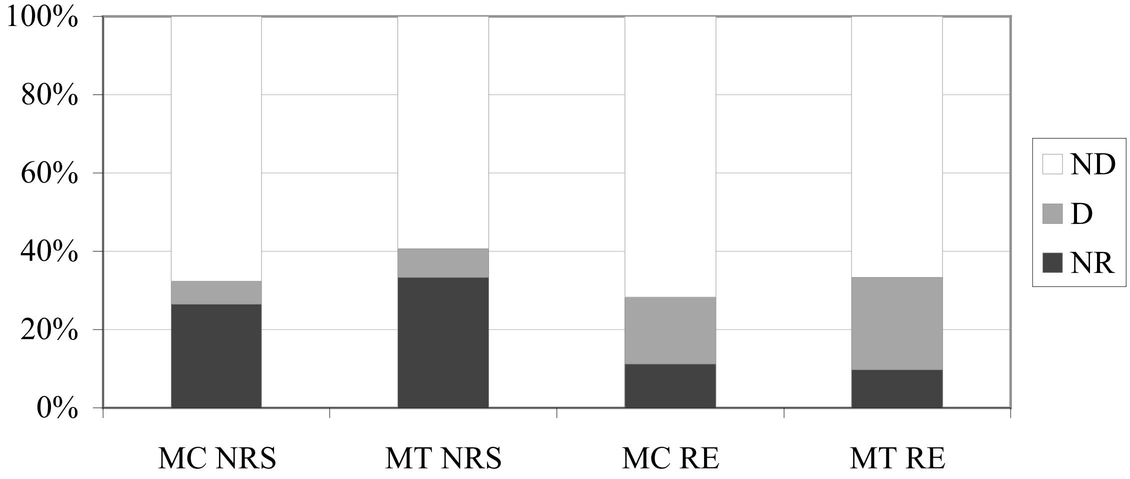


Figure 3.3.C Proportions of the *Pseudomonas* strains able to reduce nitrate to nitrite (**NR**) and able to reduce nitrate to gaseous compounds (**D**). **MC**, *Molinia* grown under normal air; **MT**, *Molinia* grown under pCO_2-treated atmosphere; **NRS**, soil fraction; **RE**, root fraction

Identification of nitrate-reductase narG *and* napA *genes among NR* Pseudomonas

The presence of NAR (membrane-bound form), NAP (periplasmic form) or both forms of nitrate reductases was checked by a *narG-nap*A multiplex PCR protocol for the *Pseudomonas* strains isolated at the three sampling dates (june 2000, july 2001 and december 2001). Figure 3.3.D presents the results of PCR-amplification with the different primer combinations. The use of crossed pairs of primers for strains harbouring both genes did generate minor (faint) aspecific PCR products, which were not annoying for further restriction analyses.

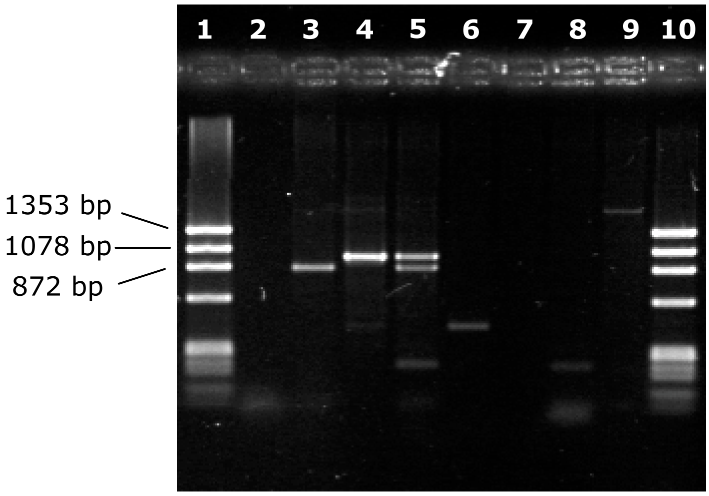


Figure 3.3.D PCR co-amplification of *narG* and *napA* gene fragments from strain ALT3NRS1. Lanes 1 & 10 : φX174/*Hae*III molecular weight marker (Promega); lane 2: no DNA template; lane 3: Fnap and Rnap ; lane 4: DMHPF and DNDWI; lane5: Fnap, Rnap, DMHPF and DNDWI; lane 6: Fnap and DMHPF; lane 7: Fnap and DNDWI; lane 8 : Rnap and DMHPF; lane 9: Rnap and DNDWI

A total of 78 **NR** *Pseudomonas* were analysed (Figure 3.3.E). 37.2 % of **NR** *Pseudomonas* strains isolated from *Molinia coerulea* roots or surrounding soil harboured both nitrate reductase genes (11 % only for *Lolium perenne*, data not shown).

Higher proportions of nitrate-reducing *Pseudomonas* strains having *narG* only were detected in root compared to soil fraction. *narG* was found in 42 among 43 root strains but only in 65.7 % of soil strains.

narG was also more frequently detected among strains isolated from CO_2-treated plots (84 % of NR strains from treated plots displayed *narG* alone or together with *napA*, compared to 57.5 % for strains isolated from control plots).

Most of the putative denitrifying *Pseudomonas* (gas producers) had the Nar form (27 among 39 **D** strains). Only 3 denitrifying strains had only the Nap form and the 9 remaining strains had both forms.

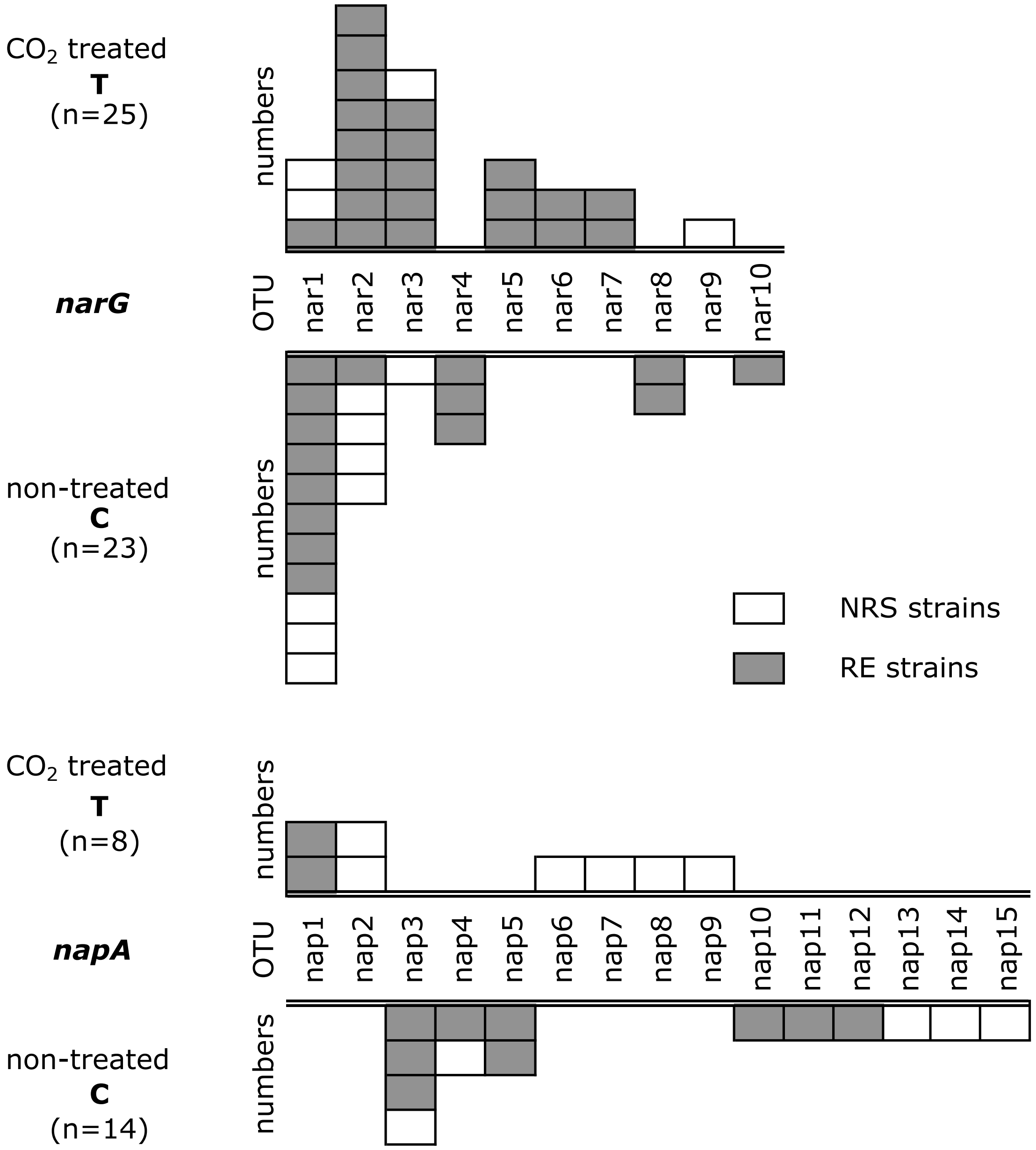


Figure 3.3.F OTU distribution of *Pseudomonas* strains harbouring *narG* and *napA* genes. NRS strains are in bright and RE strains are in dark

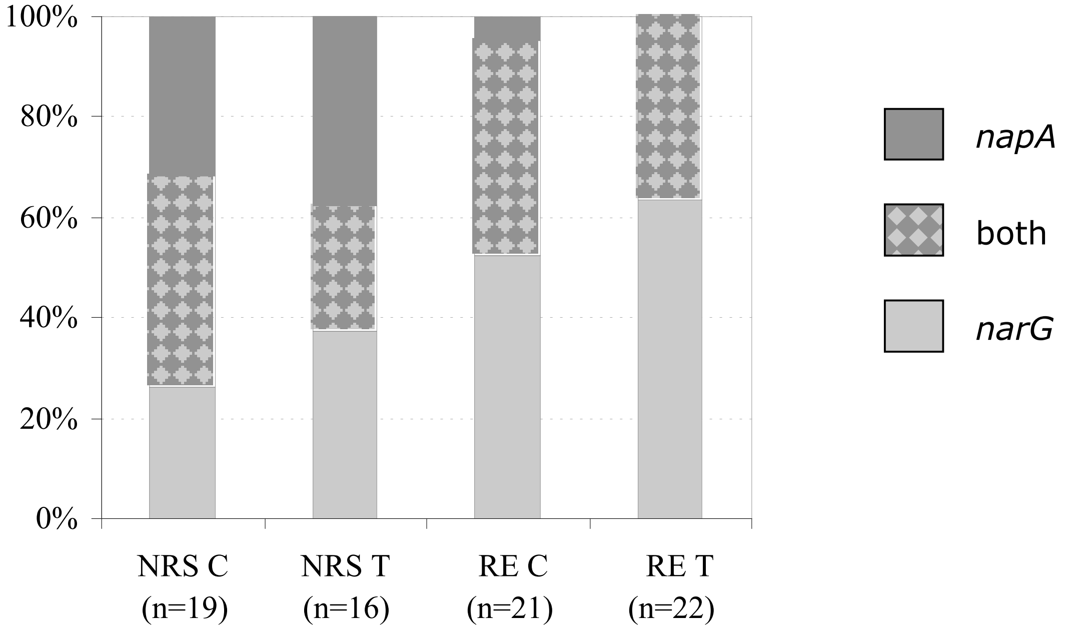


Figure 3.3.E Proportions of **NR** *Pseudomonas* strains having *narG* and/or *napA* genes for the 3 sampling dates. **NRS**, non-rhizospheric soil; **RE**, root fraction; **C**, control plots; **T**, CO_2-treated plots; n, number of strains analysed

Diversity of narG *and* napA *genes*

The diversity of *narG* and *napA* gene fragments was assessed by restriction analysis of generated PCR products. 78 nitrate-dissimilating *Pseudomonas* strains were clustered in nar OTUs or/and nap OTUs.

Among the 60 strains which generated an usable restriction profile, 48 *Pseudomonas* strains harboured *narG*. They grouped into 10 *narG* OTUs (noted nar1 to nar10). The figure 3.3.F presents the repartition of strains into these OTUs according to the fraction (**NRS** & **RE**) and the treatment (**C** & **T**). The three most abundant OTUs (nar1, nar2 and nar3) comprised strains isolated from the roots of *M. coerulea* as well as from its associated soil. They grouped about 71 % of the strains having *narG* gene. These three OTUs as well as two additionnal OTUs (nar4 and nar5) were also detected among strains isolated from the rhizospheric environment of *Lolium perenne* (data not shown).

Despite the low number of strains having *napA*, a greater diversity was observed for this gene than for *narG*. The 22 *Pseudomonas* strains habouring *napA* were clustered into 15 nap OTUs, of which 10 OTUs included a single strain. *napA* OTUs were never found under both control and treated conditions (Figure 3.3.F).

3.3.4 Discussion

NR and D Pseudomonas *populations in the rhizosphere of plants*

As previously mentionned, this study was carried out on *Lolium perenne* (data not shown) as well as on *Molinia coerulea* and major conclusions about the lower proportion of nitrate reducing *Pseudomonas* near the roots compared to soil fraction are the same. However, the denitrifiers were more frequent in the root fraction of *Molinia coerulea*. Previous studies, using different soil-plant systems, showed that heterotrophic bacteria or *Pseudomonas* displaying nitrogen dissimilative activity or genes were also more frequently detected in the root-associated habitats than in non-rhizospheric soil, suggesting that the ability to use nitrate as alternative electron acceptor could be a competitive advantage for bacteria in the rhizosphere (Linne von Berg & Both 1992; Clays-Josserand *et al*. 1995 & 1999; Delorme 2001). Indeed, the use of *Pseudomonas* isogenic (Nar$^-$) mutant showed that this trait is involved in the competitive colonisation of rhizosphere in non-limiting nitrate conditions (Ghiglione *et al*. 2000), but also in saprophytic ability of *Pseudomonas* strains in the soil (Mirleau *et al*. 2001).

In our study, the low availability of nitrates could explain the lower proportions of nitrate-reducing *Pseudomonas* in root-associated fractions, where N depletion is worsened by plant uptakes. In the plant–soil systems studied, being for *L. perenne* cultivated with limiting N supply or for *M. coerulea* growing on its native oligotrophic soil, nitrogen availability is a limiting factor for plant growth and may be also limiting for denitrification (Strong & Fillery 2002). Corre *et al*. (2002) suggested high competition for available N between micro-organisms and plant. Mahmood *et al*. (1997) showed that maize plants can lower N content and outcompeted denitrification process. Vasquez de Aldana & Berendse (1997) measured the nitrogen-use efficency for six grasses and showed that *Molinia coerulea* was especially not competitive for nitrogen uptake, as compared to *Lolium perenne*. Nijburg and collaborators (Nijburg *et al*. 1997; Nijburg & Laanbroek 1997) noticed that addition of nitrate resulted in a increased number of **NR** strains in the rhizosphere of *Glyceria maxima*. In order to test this nitrogen limiting hypothesis, we are currently conducing an experiment on *L. perenne* grown with low and high (non-limitative) nitrogen supply.

Effect of an elevated CO_2 content on the proportion of ***NR*** Pseudomonas

In this study, the proportions of nitrogen dissimilative *Pseudomonas* in the rhizosphere of *M. coerulea* increased for CO_2-treated plots. The results obtained for *M. coerulea* are in agreement with previous studies. Under elevated pCO_2 in the swiss FACE, Hartwig *et al*. (2000) reported that N balance was altered in *L. perenne* monoculture growing in N non-limiting conditions. Higher N_2O emissions were measured in *L. perenne* monoculture under elevated CO_2 for the plots used for our experiments (Ineson *et al*. 1998). Higher potential denitrification activities in soil were noticed under elevated atmospheric CO_2 content for other soil–plant systems (Smart *et al*. 1997; Carnol *et al*. 2002).

Nitrate reductase genes present in the Pseudomonas *strains*

The most abundant *nar* and *nap* OTUs were identified among strains isolated at the different sampling dates on both plant studied, confirming the good reproducibility of the experiment with different plant systems and over time.

In our study, there was a good correspondance between the nitrate reductase activity and the presence of *narG* or/and *napA* detected by PCR amplification. 83.3 % of the tested strains had the *narG* gene and 53.8 % the *napA* form. A significant proportion of nitrate reducing strains (about one third) displayed both forms, suggesting that the NAR and NAP forms have probably different functions within the corresponding organisms, despite the similar biochemical reaction that they catalyse. The membrane-bound nitrate reductase (NAR) is involved in anaerobic nitrate respiration whereas the periplasmic form (NAP) is probably a more functionally versatile enzyme (Philippot & Højberg 1999). Its regulation in *Pseudomonas* is to date misunderstood. It likely provides the biochemical apparatus for aerobic nitrate respiration (Carter *et al*. 1995; McDevitt *et al*. 2000), including for *Pseudomonas* strains (Flanagan *et al*. 1995).

We noticed a predominance of NAR among *Pseudomonas* isolated from root environment, where oxygen tension is low. This result is in agreement with the expression of this nitrate reductase form in more anaerobic conditions (Philippot & Højberg 1999) than NAP form. Carter *et al*. (1995) showed that soil isolates displaying a periplasmic nitrate reductase (Nap) were as abundant as those displaying the membrane-bound form. They suggested that the ability to co-respire oxygen and nitrate may be particularly important in environments that are rich in reduced carbon or subjected to a limiting or fluctuating oxygen availability, such as in soil aggregates (Parry *et al*. 1999) or soils with fluctuating water-table, like those colonized by *Molinia coerulea* (cf. § 1.4.2).

Both NAR and NAP forms were described to date in nitrate-reducing organisms (Bedzyk *et al*. 1999). In our study, most of the putative denitrifying (gas producing) *Pseudomonas* had the Nar form (27 among 39 **D** strains). Only 3 denitrifying strains had only the NAP form and the 9 remaining strains had both forms. In most of these strains, the denitrifying trait was confirmed by the amplification of *nosZ* gene fragment (data not shown). This last result confirmed that the periplasmic nitrate reductase (Nap), as well as the membrane-bound form, can be involved in nitrate dissimilation in denitrifying *Pseudomonas* strains.

Acknowledgement

This work was supported by grants of the Swiss National Science Foundation numbers 3100-055899.98 & 31-68208.02 and by the Swiss National Centre of Competence in Research (NCCR Plant Survival). We would like to thank Marie-Laure Heusler for her technical assistance and J. Moret for her precious help for statistical analysis.

3.4 Conclusion générale sur l'analyse des *Pseudomonas*

Le fait de travailler sur des bactéries cultivables donne acces à la caractérisation des fonctions bactériennes, comme la dénitrification potentielle (cf. § 3.3), ou à la réalisation de tests physiologiques à caractère PGPR (croissance sur précurseur de l'auxine: ACC, production de HCN, production de sidérophores). Ces analyses sont en cours dans le cadre de la thèse de Sonia Tarnawski.

Il faut rappeller ici que l'isolement puis le repiquage régulier des souches obtenues constitue, à eux seuls, de très lourdes tâches. C'est l'une des limitation majeure de cette approche. De plus, les bactéries cultivables ne représentent qu'une faible proportion de la microflore présente et des questions de représentativité des résultats se posent toujours. Le paragraphe 3.2, où est présentée l'étude de la structure génétique des isolats obtenus en fonction du milieu d'isolement utilisé, met bien en relief ce problème.

À la fin du chapitre 2 consacré à l'étude des communautés, nous nous sommes posé des questions sur le rôle des bactéries très actives mais peu nombreuses détectées par les fingerprints de communauté (cf. § 2.1.3; bandes révélées par DGGE à partir d'ARNr mais absentes sur les extraits d'ADNr). Au vue du paragraphe 3.2, on peut s'interroger sur le statut physiologique des *Pseudomonas* incapable d'être isolés sur un milieu sélectif riche (milieu mS1) mais ayant les potentialités de croître après revitalisation sur un milieu plus pauvre (milieu Angle).

Un autre résultat frappant de cette étude portant sur les *Pseudomonas* reside dans la diminution de la proportion de *Pseudomonas* réducteurs de nitrate concomitante à l'augmentation de la proportion de *Pseudomonas* dénitrifiants dans les racines de *Molinia coerulea* par rapport au sol environnant pauvre en azote disponible. On peut imaginer qu'en prélevant du nitrate pour sa croissance, la plante limiterait la prolifération des bactéries réductrices de nitrate mais pas les *Pseudomonas* dénitrifiants car la dénitrification donnerait un avantage compétitif aux *Pseudomonas* pour la colonisation racinaire.

Chapitre 4

Diversité des bactéries fixatrices d'azote associées à *Molinia coerulea*

Chapitre 4

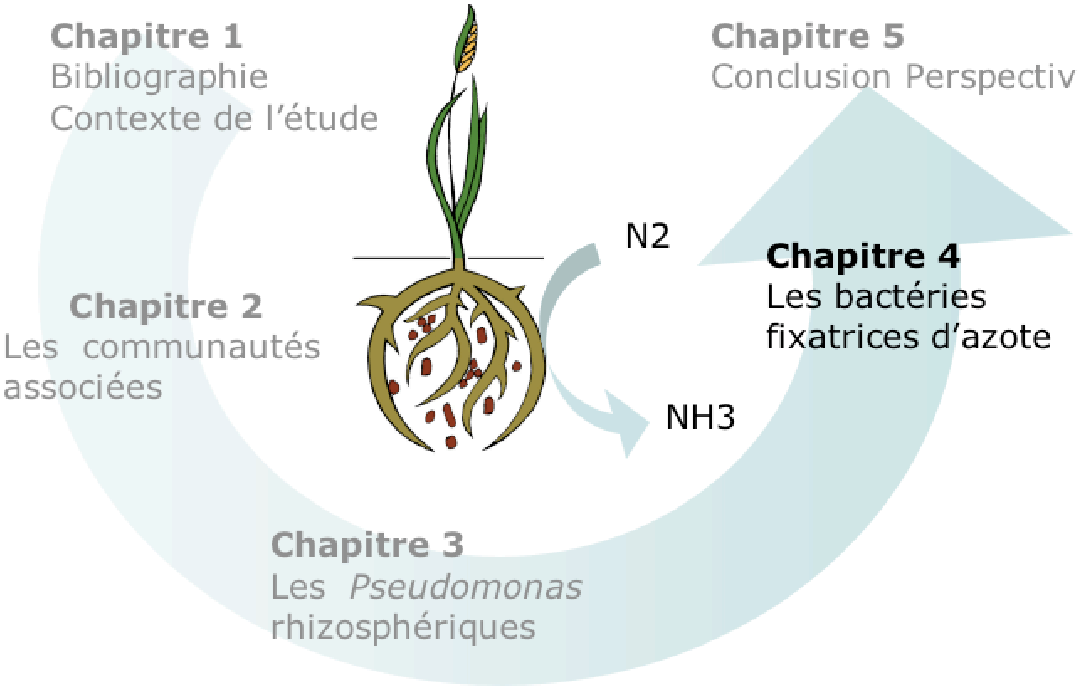
Les bactéries fixatrices d'azote associées à *Molinia coerulea*

Dans une approche globale du fonctionnement microbiologique du système racinaire de *Molinia coerulea*, nous avons d'abord étudié les communautés dans leur ensemble (Chapitre 2), puis des populations spécialisées dans la vie rhizosphérique (Chapitre 3). Dans ce chapitre, nous allons distinguer une composante fonctionnelle des communautés globales et nous intéresser à une fonction microbienne clé, la fixation d'azote atmosphérique (cf. § 1.2.3).

Nous avons adopté une démarche écologique pour étudier la fonction de fixation d'azote. Nous nous sommes intéressés d'une part au potentiel de transformation biochimique réalisé par cette fonction (ici, réduction du N_2 en NH_3) et d'autre part à la diversité des bactéries génétiquement aptes à réaliser cette fonction (la guilde des fixateurs d'azote).

Après nous être assuré que l'environnement racinaire de *Molinia coerulea* était propice à la réalisation de la fixation d'azote atmosphérique (§ 4.1), nous avons évalué la diversité des bactéries fixatrices d'azote associées à la racine et au sol environnant en nous basant sur le gène *nifH* (§ 4.2). Nous avons développé une amplification spécifique d'un groupe particulier de séquences *nifH*, dominant aussi bien dans la racine que dans le sol (§ 4.3).

D'après la littérature, la racine est une niche propice à l'expression de la fixation d'azote atmosphérique par comparaison au sol environnant car tous les facteurs environnementaux régulant cette activité y sont rencontrés (source de carbone abondante, compétition pour l'azote minéral et faible tension partielle en oxygène). Nous avons donc comparé les pools de gènes *nifH* présents et transcrits dans le sol et dans le système racinaire de *Molinia coerulea* (§ 4.3).



4.1 Mise en évidence de l'activité fixatrice d'azote dans la prairie naturelle

Les résultats de ce paragraphe ont été en partie obtenus lors du travail de diplôme de Nahida Syed (Syed 2001). Elle a reçu le Prix Léon Dupasquier (500 CHF) pour la qualité de son travail lors de la cérémonie de remise des diplômes du 23 novembre 2001 de la faculté des sciences de Neuchâtel.

Ce paragraphe a aussi fait l'objet d'un article de **J. Hamelin**, N. Fromin, N. Syed, S. Teyssier-Cuvelle, N. Duvanel et M. Aragno. Cet article a été publié dans le Bulletin de la Société Suisse de Pédologie (Hamelin et coll. 2001).

Nitrogen fixation activity detected in the rhizospheric environment of naturally occuring *Molinia coerulea*

4.1.1 Introduction

The biological reduction of N_2 to NH_3 by the nitrogenase enzyme is an important process which contributes to the recycling of nitrogen into living matter (cf. § 1.2). Support for the occurrence of non-symbiotic nitrogen fixation comes from a variety of non-leguminous and non-actinorhizal plant systems. Long term net N gain or ^{15}N enrichments in roots, stems or leaves were detected with wetland rice (App *et al*. 1984), sugarcane (Ruschel *et al*. 1975), sorghum (Giller *et al*. 1984), kallar grass (Malik *et al*. 1997) and other grasses (Morris *et al*. 1985; Boddey 1987).

Molinia coerulea is an hemicryptophytic perennial grass characteristic from wet and nitrogen poor habitats (cf. § 1.4.2). It grows well in oligonitrophilic conditions where there is little amount of nitrogen available in soil for plant use. We assumed that there is active dinitrogen fixing bacteria populations associated with the roots of the plant, which provide nitrogen to the system. The nitrogenase catalyses the reaction of fixation of molecular nitrogen (N_2) into ammonia (NH_3) but this enzymatic complex has a wide substrate versatility and can also catalyse the reduction of acetylene (C_2H_2) into ethylene (C_2H_4). Ethylene may be detected with great sensitivity so its formation provides a rapid and valuable test for nitrogenase activity (Postgate 1982). We quantified the nitrogen fixation activity on intact grass-soil cores of *Molinia* from two contrasted origins using acetylene reduction assay, named ARA (Dilworth 1966; Schöllhorn & Burris 1967).

4.1.2 Material and methods

Sampling of root-soil cores of Molinia coerulea

Sampling was done the 17 July 2000 in the south shore of Lake Neuchâtel (Switzerland) and the 19 July 2000 in a peat bog of Chapelle-des-bois in Jura mountains in France. Both locations were already described in paragraph 1.4.2. 30 cm thick and 15 cm deep soil cores were removed and transferred to the laboratory. The humidity of the soil was calculated on the samples after each measurement. The soil from littoral meadow had 50 % of humidity and the peat bog sample contained 87 % of water. A 30.4 g dry weight soil-root cores from the littoral meadow and 9.9 g dry weight core from the peat bog were analysed.

Nitrogen fixation activity measurement

Samples were put into 250 ml serum bottles with headspace filled with N_2. Nitrogenase activity was determined by acetylene reduction assay (ARA) replacing 10 % of N_2 atmosphere with acetylene and 1 % with air. Control serum bottles contained soil-root cores with N_2 only as atmosphere. Soil cores were incubated at 30 °C in water bath with slight rotation. C_2H_4 production from C_2H_2 was assayed by analysis of 500 µl of headspace gaz sample removed from the serum bottles. The gaz samples were injected in a CARLO ERBA gas chromatograph (Fractovap series 4130) equiped with a Porapack N (1,8 m x 3 mm) column (Brechbühler) maintained at 80 °C. N_2 was used as carrier gaz and hydrogen and air maintained the flame in the detector (FID). The retention time after sample injection to obtain a peak was 1.25 min for ethylene and 1.78 min for acetylene with the conditions used. The C_2H_4 peak was plotted on a graph with a Hewlett Packard integrator (HP 3395) and the calculated pic area was compared to a standard curve of C_2H_4 (r^2=0.99) to estimate gaz production. Production of C_2H_4 from C_2H_2 was measured each hour during eight hours and the maximal rate of nitrogenase activity was expressed in nmoles of C_2H_4 produced per gram dry weight per hour (Hamelin *et al.* 2001; Syed 2001).

4.1.3 Results and discussion

The figure 4.1.A shows the comparison of ARA measurements obtained for both Molinia samples (from littoral meadow and peat bog) with those obtained by Poly (2000) during his PhD thesis from a natural prairie spontaneously colonized by *Arrhenatherum elatius*. In this study, same order of magnitude was found for nitrogen fixation activity with planted *Agrostis vulgaris*. The ARA values were similar, despite the fact that the procedure used by Poly (2000) was not exactely the same; he incubated the soil for a ten days period with an addition of 1 mg of C-glucose and 1 mg of C-malate per g of soil at the beginning of experiment. Despite the enrichment culture performed, he obtained similar ARA values.

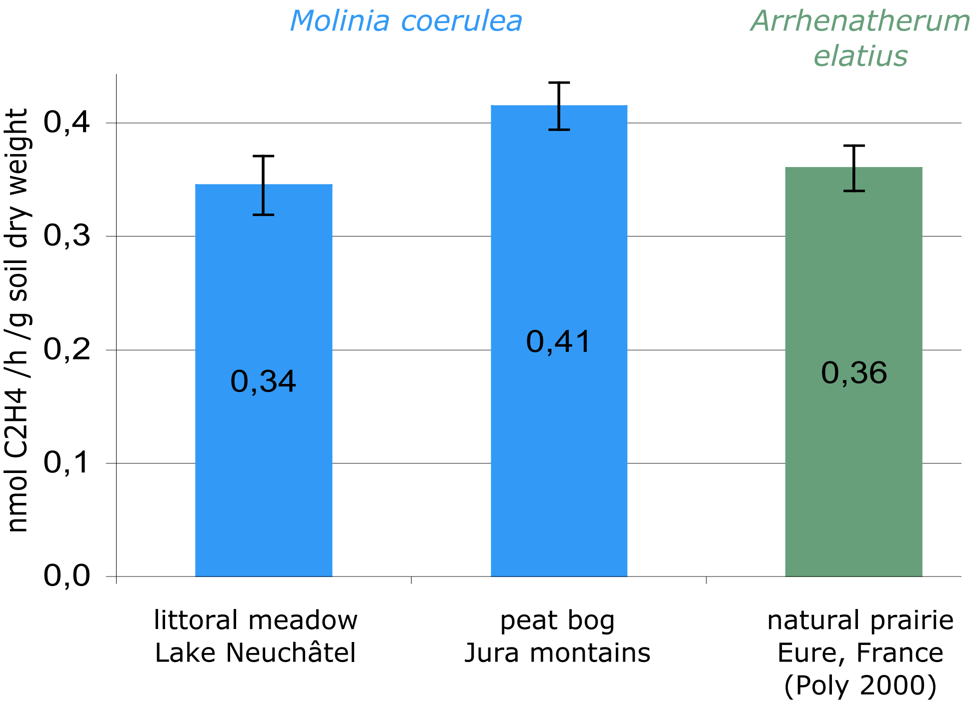


Figure 4.1.A Nitrogen fixation activities measured as acetylene reduction assay (ARA), for three different soil cores containing roots. Values are indicated in the graphs and are expressed in nmol C_2H_4 produced. h^{-1}. g dry weight^{-1}

The ARA measurements may lead to an overestimation of N_2 fixation since the acetylene is not the physiological substrate for nitrogenase (Postgate 1982). Therefore the results presented should be viewed as estimates of the potential N_2 fixation of the diazotrophic populations but do not necessarily correspond to the N_2 fixation occuring *in situ*.

In conclusion, the level of the ARA measured in the rhizospheric environment of *Molinia coerulea* were significant and allowed us to confirm the existence of active nitrogen fixing bacteria in the rhizosphere of *Molinia coerulea* growing on N-poor soils. The biological dinitrogen fixation could help the N-plant nutrition and could partly explain the presence of this plant in oligonitrophilic soils.

4.2 Diversité des bactéries fixatrices d'azote dans la prairie naturelle

Ce paragraphe a fait l'objet d'un article de **J. Hamelin**, N. Fromin, S. Teyssier-Cuvelle, S. Tarnawski et M. Aragno. Cet article a été publié dans Environmental Microbiology (Hamelin et coll. 2002).

nifH gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass

Abstract

Rhizosphere associative dinitrogen fixation could be a valuable source of nitrogen in many nitrogen limited natural ecosystems, such as the rhizosphere of *Molinia coerulea*, an hemicryptophytic perennial grass naturally occurring in contrasted oligonitrophilic soils. The diversity of the dinitrogen fixing bacteria associated to this environment was assessed by a cloning-sequencing approach on the *nifH* gene directly amplified from environmental DNA extracts. Seventy seven randomly picked clones were analysed. One type of NifH sequence was dominant in both roots and surrounding soil, and represented 56 % of all retrieved sequences. This cluster included previously described environmental clones and did not contain any NifH sequences similar to cultivated diazotrophs ones. The predominance of few NifH sequences types in the roots and the rhizosphere of *Molinia coerulea* indicate that the plant environment mediates a favourable niche for such dinitrogen fixing bacteria.

4.2.1 Introduction

The biological dinitrogen fixation process provides the major biological source of nitrogen in natural ecosystems. Most studies on associative nitrogen fixation have focussed on crops of agronomic interest such as rice or sugar cane (Ueda *et al.* 1995; Engelhard *et al.* 2000; Steenhoudt & Vanderleyden 2000), where fertiliser is required for crop growth. Few studies aimed at understanding the role of the associative dinitrogen fixation in nitrogen limited natural ecosystems (Piceno *et al.* 1999; Bagwell & Lovell 2000; Piceno & Lovell 2000a & 2000b). *Molinia coerulea*, a perennial grass, occurs mainly in contrasted oligotrophic environments (e.g. acidic peat bog, slightly basic littoral meadows) (Leps 1999). This plant is hemicryptophytic, the root system ensuring the survival during the cold season. We hypothesized that under such conditions, biological dinitrogen fixation could provide a valuable source of nitrogen for microbial and plant nutrition.

Since 0.1 to 10 % only of bacterial cells in soil are cultivable in currently used media (Amann *et al.* 1995), molecular methods give a more accurate image of the total bacterial diversity. Such approaches may be applied to functional genes, such as dinitrogen fixation genes. The *nifH* gene was widely used to detect Nitrogen Fixing Bacteria (NFB) (cf. § 1.2.3; Zehr & McReynolds 1989; Ueda *et al.* 1995; Ohkuma *et al.* 1999; Piceno *et al.* 1999; Widmer *et al.* 1999; Zani *et al.* 2000; Poly *et al.* 2001). It encodes for the dinitrogenase reductase, a key enzyme in the nitrogen fixation process. Despite the fact that NFB are very diverse, the *nifH* genes have evolved similarly to the 16S rDNA genes and can be used as a molecular evolution marker (Young 1992). Comparison with available *nifH* sequences from databases provides a taxonomical information on the corresponding NFB.

The present work focuses on the diversity of NFB associated to a natural population of *Molinia coerulea* in an oligonitrophilic littoral meadow. We assessed the *nifH* gene pool directly amplified from soil and root DNA. We discuss the relationship between NFB diversity and rhizosphere functioning with particular interest for perennial grass environment.

4.2.2 Results and discussion

Our study site was located in a littoral meadow in the south shore of Lake Neuchâtel (Switzerland), where the surface soil (Gleysol, Typic Haplaquoll) texture was 4.7 % clay, 9.5 % silt, 85.8 % sand (Buttler 1987), and pH_{H2O} value was 8.4. The sampled population of *Molinia coerulea* consisted of genetically homogeneous diploïd individuals.

Preliminary acetylene reduction activity (ARA) measurements on soil cores indicated the presence of active NFB in association with *Molinia coerulea* rhizosphere (Hamelin *et al.* 2001). The proximity of the root creates an environment favourable for nitrogen fixers settlement (Balandreau 1986); root and rhizobacteria cells respiration decreases oxygen partial pressure, whereas rhizodeposition provides an abundant source of energy. Moreover, the soluble nitrogen content in the studied soil (NH_4-N and NO_3-N content of 94.9 and 48.4 $\mu g.g^{-1}$ dry soil respectively; Buttler 1987) was far below the concentrations known to repress nitrogenase genes.

In July 1999, a 20 x 20 x 20 cm core was collected at midday. Mixed growing, mature and decaying roots were taken off and washed in PBS buffer (0.1 M, pH 7.0). Representative 0.5 g samples of fresh root and soil material were submitted to DNA extraction and purification using bead beating technique (Borneman *et al.* 1996). A nested-PCR amplification with consensus primers (Widmer *et al.* 1999) generated a 370 bp *nifH* gene fragment. PCR products were cloned into pGEM-T vector (Promega corp., Madison, WI). The transformants were randomly picked, and named RE1 to RE52 for the root fraction and S1 to S25 for the soil fraction. Sequence analysis was performed on a 4200L DNA sequencer (Li-Cor, Lincoln, Neb.) then corrected manually. All the *nifH* clones tested had an insert related to *nifH* sequence when submitted to BLAST comparison (Altschul *et al.* 1997). The sequences were registered in the EMBL databank under the accession numbers AJ313233 to AJ313309. The nucleotidic sequences were translated into amino acid sequences to allow a better comparison between remote organisms and to enhance similarity within a group of related sequences.

Figure 4.2.B represents the phylogenetic position of the detected partial NifH sequences compared to sequences published for other NFB. Sequences with high level of similarity were grouped into clusters. No cluster was composed of sequences exclusively from one

fraction. About 91 % (70/77) of the obtained sequences had no close relatives in published sequences for cultivable organisms. We did not detect any putative sequence from the NFB belonging to *Archaea*, *Cyanobacteria*, *Frankia*, *Paenibacillus*, *Vibrio* and *Azoarcus*.

Sequences from □□, □□ and □□ *Proteobacteria* gathered in cluster B. As previously observed for *Proteobacteria* NifH sequences (Ohkuma *et al.* 1999), low bootstrap values were obtained for this cluster. High similarities with the □-Proteobacterium *Bradyrhizobium japonicum* NifH for root sequences in this cluster (RE2: 92.9 % and RE36-RE51: 94.9 %) were obtained with ClustalX (Thompson *et al.* 1997). This bacterium traditionally occurs in legume nodules, but its presence as an active nitrogen-fixing endophyte of African wild rice was previously observed (Chaintreuil *et al.* 2000). The S6 and S7 clones were related to the □-Proteobacterium *Herbaspirillum seropedicae* with 96.0 % similarity. *Herbaspirillum* is naturally associated with a wide range of graminaceous plants, but it is not supposed to survive well in soils (Olivares *et al.* 1994). The presence of related *nifH* sequences in the soil fraction suggests a close relationship and frequent exchanges between the root and its surrounding soil. The genera *Azoarcus* sp. (Hurek *et al.* 1994) and *Azospirillum* sp. (Steenhoudt & Vanderleyden 2000) were often described as being associated with grass roots. However, we did not detect any sequence related to these bacteria, even if the *nifH* genes from these organisms should have been amplified with the primers used (Widmer *et al.* 1999).

Cluster D accounts for 27.3 % of all analysed sequences (Table 4.2.A).

Table 4.2.A Distribution of partial NifH sequences retrieved in the rhizosphere of *Molinia coerulea*

Fractions	Cluster A		Cluster B		Cluster C		Cluster D		total nb of analysed sequences
	nb	(%)	nb	(%)	nb	(%)	nb	(%)	
Root (RE)	29	(55.8)	4	(7.7)	5	(9.6)	14	(26.9)	52
Soil (S)	14	(56.0)	2	(8.0)	2	(8.0)	7	(28.0)	25
Total	43	(55.8)	6	(7.8)	7	(9.1)	21	(27.3)	77

Such a high proportion was observed in both fractions. This group comprised sequences from known anaerobic bacteria as well as previously described environmental sequences. It is not a phylogenetically well defined cluster, although such a grouping of NifH sequences from anaerobic organisms has already been proposed (Ohkuma *et al.* 1999; Zani *et al.* 2000; MacGregor *et al.* 2001). Even if the root zone of *Molinia coerulea* is never fully saturated, the proximity of water table (20 to 100 cm below the soil surface during the growth season) and associated capillary fringe, along with the respiratory activity of root cells and rhizospheric microorganisms, should favour anoxic microenvironments. The S4 clone had 88.9 % similarity with the □-Proteobacterium *Desulfovibrio gigas* and the S21 clone grouped with *Clostridium pasteurianum* with 89.9 % similarity.

Cluster C was composed of environmental sequences without close known relatives. The RE37 clone reached 80.8 % similarity with the closest retrieved sequence from cluster A

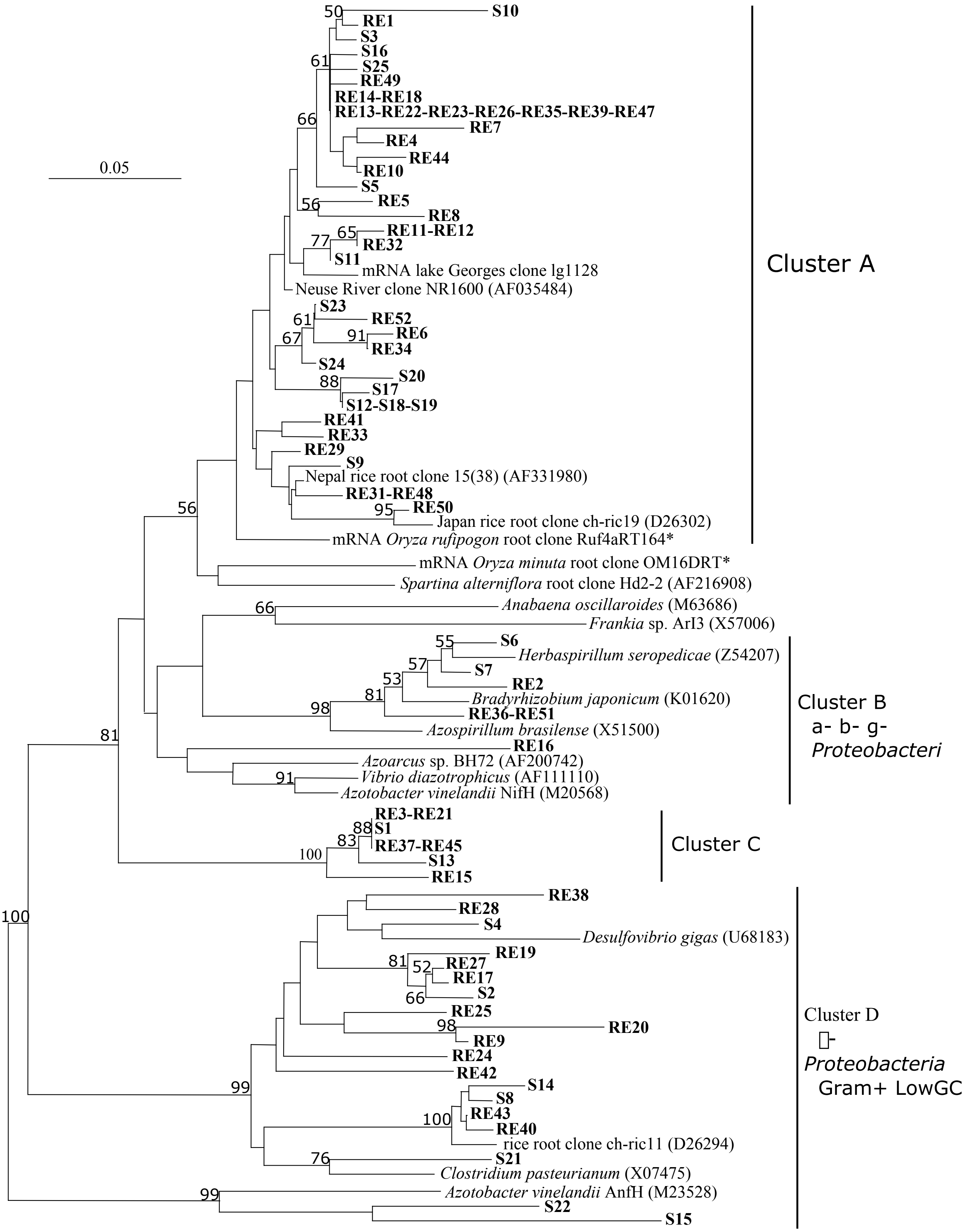


Figure 4.2.B Phylogenetic tree showing the position of the NifH sequences based on the alignment (ClustalX; Thompson *et al.* 1997) of 112 amino-acid residues corresponding to the positions 44-154 in the *Azotobacter vinelandii* NifH protein. The tree was constructed by the neighbor-joining method (Saitou & Nei 1987) with NJplot (Perrière & Gouy 1987). The scale bar denotes 0.05 % of sequence distance. The retrieved sequences, in bold, were grouped into clusters A, B, C, and D. Bootstrap values above 50 % (Felsenstein 1985) are indicated at the branching nodes. Sequences marked with * were kindly provided by Thomas Hurek

and 79.8 % similarity with *Azotobacter vinelandii* NifH sequence, the closest sequence from cluster B.

Cluster A dominated in both root and soil fractions (56 % of all sequences, Table 4.2.A). Because of this dominance, the diversity of *nifH* gene pools was low as compared to other studies (Ueda *et al.* 1995; Widmer *et al.* 1999). No sequence in cluster A was related to so far characterised NifH sequences from cultivated organisms. The NifH sequences of this cluster retrieved in the present study grouped above 86.9 % similarity. The bootstrap resampling value for the cluster A was 56 %. This major 'lineage' was deeply branched to sequences from known cultivated organisms, indicating that the corresponding bacteria might be only distantly related to already cultivated diazotrophs (the closest sequence was *Azotobacter vinelandii* NifH). Consequently, we could not give any phylogenetic affiliation for this group, neither informations about their physiology. Other studies revealed environmental sequences belonging to cluster A in water (Zani *et al.* 2000; Affourtit *et al.* 2001), and rice roots (Ueda *et al.* 1995; Engelhard *et al.* 2000). Some of them were detected using RT-PCR on mRNA (cf. § 4.3; Zani *et al.* 2000; Hurek, personal communication) indicating that cluster A could be an active contributor to nitrogen fixation *in situ*. For these studies, cluster A clones represented less than 10 % of all the retrieved sequences, using different nucleic acid extraction protocols and PCR primer sets.

In grassland ecosystems, plant species could modulate the composition of NFB guilds to a larger extent than the soil characteristics do (Bardgett *et al.* 1999). We also revealed numerous cluster A related sequences associated to the root of *Molinia coerulea* grown in acidic peat bog (data not shown). As a hemicryptophytic grass, *Molinia coerulea* grows every year at the same location, and root system survival during winter allows plant regeneration. As for other perennial grass meadows, the simultaneous presence of high densities of growing, mature and decaying roots from the same grass species provides a continuous enrichment of adapted bacterial populations between the roots and soil. Historical records suggest that the meadow came into existence following a drop in the level of Lake Neuchâtel in 1888. These conditions strongly contrast with rotating cultures of annual crops, and could explain that repartition of NifH sequences was similar for soil and root fractions (Table 4.2.A).

Several analyses on environmental *nifH* genes have been performed since a decade. However, the role of *nifH* diversity in relation to the ecosystem functioning is not clear. This study revealed the simultaneous presence of *nifH* sequences from bacteria having different ecological niches (aerobic and anaerobic bacteria). Some authors suggested that bacterial functional redundancy could help to maintain bacterial processes after environmental modifications (Kennedy & Smith 1995). In order to assess the role of the observed diversity, we are currently studying the differences in the structure of *nifH* gene pools associated with *Molinia coerulea* growing on different soils.

Acknowledgement

This work was supported by grants # 31-40693.94 and # 31-55899.98 of the Swiss National Science Foundation. We are grateful to François Bretagnolle, Jean-Michel Gobat and Philippe Küpfer for plant characterisation. We thank Noémie Duvanel for her technical assistance. We also wish to express our thanks to Thomas Hurek for providing us *nifH* sequences, and for fruitful discussions.

4.3 Design of new primers for a group-specific amplification of *nifH* gene and *nifH* mRNA expression in the roots of *Molinia coerulea*

Une partie des résultats de ce paragraphe ont été présentés dans un poster réalisé par **J. Hamelin**, M. Jossi, S Tarnawski, M. Aragno et N. Fromin lors du *9th international symposium on nitrogen fixation with non-legumes* qui s'est tenu à Leuven en Belgique du 1 au 5 septembre 2002. L'étude complète sera présentée en conférence lors du *1st FEMS congress of european microbiologists* qui va se tenir à Ljubljana en Slovénie du 29 juin au 3 juillet 2003.

Abstract

The diversity of the dinitrogen fixing bacteria associated with the rhizospheric environment of *Molinia coerulea* was described by a cloning-sequencing approach on the *nifH* gene (cf. § 4.2). One type of NifH sequence, named cluster A, was dominant in both roots and surrounding soil. It comprised only previously described environmental clones and did not contain any NifH sequences similar to cultivated diazotrophs ones (Hamelin *et al*. 2002). A specific primer set for this cluster was designed. *nifH* genes were detected by specific PCR on total DNA extracts in both roots and surrounding soil of this perennial grass. But cluster A *nifH* mRNA, as well as total *nifH* mRNA, were detected in the root fraction only (RT-PCR on RNA extracts). We concluded that the soil environment constitutes a reservoir of diazotrophs and the grass root environment could provide a favourable niche for the expression of the nitrogen fixation.

4.3.1 Introduction

The identification of the diazotrophs which are involved in the biological dinitrogen fixation, and the localisation of their diazotrophitic activity in natural conditions is important for our understanding of ecosystem processes. The diversity and the localization of dinitrogen fixing bacteria in nature is increasingly studied (cf. § 1.2.3), as nitrogen fixation provides the major biological source of nitrogen in natural ecosystems. Most of the studies in this field are focussing on known cultivable bacteria like *Azospirillum*, *Azoarcus* (Hurek *et al*. 2002), *Herbaspirillum* (Olivares *et al*. 1994) or *Rhizobium*. We focussed here on a group of closely related *nifH* sequences amplified from extracted environmental DNA, designed as cluster A. In database so far, sequences similar to cluster A never belonged to cultivated organisms (cf. § 4.2; Hamelin *et al*. 2002). *nifH* encodes for the dinitrogenase reductase, a key enzyme in the nitrogen fixation process. The occurence of cluster A sequences was similar for root and surrounding soil of *Molinia coerulea*, and represented the major group of diazotrophs (56 % of analyzed sequences) present in the littoral meadow.

In order to check the presence of cluster A in the rhizosphere of perennial grasses grown under different pCO_2 conditions (*Molinia coerulea* and *Lolium perenne*), we looked for specific primers. At the same time, the transcription of the nitrogenase gene was checked by reverse transcription on total RNA extracts followed by a *nifH* gene amplification.

Burke *et al*. (2002) compared the overall and the active populations of nitrogen-fixing bacteria in the root environment of *Spartina* in salt marsh sediment. The image of active diazotrophs was stable among the different maturity stages of the plant studied, and was different from the image of actual populations of diazotrophs. Similar approach was developped by Hurek *et al*. (2002) who observed that the roots of rice seemed to represent a niche for dinitrogen fixation activity of *Azoarcus* sp., even if those diazotrophic bacteria are in an unculturable state.

4.3.2 Material and methods

Sampling

Soil and roots of *Molinia coerulea* and *Lolium perenne* growing in FACE installations (cf. § 1.4.3) were sampled in RNase free conditions, as described in paragraph 2.1.3. Two sampling were performed the 7 of May and the 15 of July 2002.

DNA extraction and purification

DNA extraction and purification were performed on 0.5 g of fresh root or soil material. The same bead beating technique was used for both fractions with FastDNA Spin Kit for Soil (Bio101, Qbiogene, Inc.) according to Borneman's conditions (Borneman *et al*. 1996) except that twice more DNA extract (500 µl) were purified with 500 µl of Binding Matrix (Bio101). Extraction and purification were performed within 1 h30 and DNA was pure enough for subsequent PCR amplification. Genomic DNA was stored at -20°C before use.

Extraction and purification of total RNA from soil and root

Soil and root subsamples were immediately placed in FastRNA™ tubes with GREEN Caps (Bio101) (which contains RNase-free matrix) and frozen in liquid nitrogen. Total RNA were extracted and purified using combination of FastRNA™ tubes with GREEN Caps (Bio101) and RNeasy® Plant Kit (Qiagen AG, Basel).

In each FastRNA™ tube containing about 150 to 500 mg of frozen sample, 450 µl of RLT Buffer (Qiagen) were added. The mixture was shaken for 10 s at 6 m.s^{-1} using FP120 FastPrep™ cell disruptor (Savant Instruments, Inc. Hotbrook NY). This step was repeated once after cooling the tubes for 5 min on ice. This 20 s period of beat beating was found to be optimal by Borneman & Triplett (1997) regarding a maximum cell lysis and a minimum RNA shearing. The tubes were then centrifugated for 5 min at 13000 g and the supernatant was loaded on QIAshredder Spin Columns (Qiagen) and then processed as recommended by the manufacturer in the RNeasy Plant Mini Protocol. The final RNA extract was eluted in 100 µl of 10 mM Tris and a DNase treatment was added in order to remove any DNA contamination: 90 µl of RNA extract were combined with 1 U of RQ1 RNase-Free DNase (Promega) and 10 µl of corresponding buffer. This mixture was incubated for 15 min at 37 °C. The reaction was stopped by adding 10 µl RQ1 DNase Stop Reaction and the DNase was finally inactivated by a 10 min incubation at 65 °C. The final RNA extracts were quantified by capillary spectrophotometry using GeneQuant (Pharmacia) and stored at -20 °C before use.

Reverse transcription of total RNA into cDNA

Reverse transcription reactions were performed using ImProm-II™ Reverse Transcription System (Promega) with random hexamer primers (Promega). 3.5 µl of RNA extract (from 55 to 70 ng depending of the sample) were mixed with 1 µl of primers (10 mM), 0.5 µl of RNasin$^{®}$ Ribonuclease Inhibitor (Promega). This mixture was incubated at 70 °C for 5 min for RNA and primers denaturation, and chilled in ice until reverse transcription mix was added. This mix was then combined to (final concentrations) 1 X ImProm-II™ Reaction Buffer, 6.0 mM $MgCl_2$, 0.5 mM each dNTP, 5 % (vol/vol) ImProm-II™ Reverse Transcriptase and diethyl pyrocarbonate-treated water in a final volume of 20 µl. The reaction comprised annealing at 25 °C for 5 min, extension at 42 °C for one hour and inactivation of reverse transcriptase enzyme at 70 °C for 15 min. Positive and negative control reactions were performed as recommanded by the manufacturer to detect contamination during these steps. The resulting cDNAs were used immediatly for PCR or stored at –20 °C.

PCR amplification of *nifH* genes

Total *nifH* gene pools were amplified by a hemi-nested PCR protocol according to Widmer *et al.* (1999). The primers (Table 4.3.A) *nifH*(forA) and *nifH*(rev) were used for the first PCR amplification and the primers *nifH*(forB) and *nifH*(rev) were used for the hemi-nested PCR. The PCR reaction mix contained 1 X buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM of each primer, and 0.1 U.µl^{-1} of *Taq* polymerase (Promega). 2.5 ng.µl^{-1} of T4 Gene 32 protein (Qbiogen) were added to enhance PCR yield (Kreader *et al.* 1996). 2 µl of nucleic acids (DNA or cDNA) were added as template for the first PCR and 2 µl of the first PCR product were used as template for the hemi-nested PCR. The reaction mixtures were subjected to 30 (first PCR) and 35 (hemi-nested PCR) amplification cycles with thermocycler model PTC-200 (MJ Research Inc., Watertown, Massachusetts). The first heat denaturation step was performed at 94 °C for 4 min 30 s. Cycles consisted of heat denaturation at 94 °C for 45 s, primer annealing at 48 °C for 8 s followed by 50 °C for 30 s, and extension at 72 °C for 1 min. The mixture was maintained at 72 °C for 10 min for the final extension. The PCR products were checked for size and yield on agarose gel in comparison to Low DNA Mass Ladder (Gibco).

*Design and validation of cluster A-*nifH *specific primers*

The *nifH* sequences belonging to cluster A group, retrieved from *Molinia coerulea* environment (Hamelin *et al.* 2002) and rice roots (Ueda *et al.* 1995), were aligned and compared to other *nifH* sequences of the Genbank database using GeneBase software (Applied Maths, Kortrijk, Belgium). A consensus sequence (fcluA) with low degeneracies was found to be exclusively present in cluster A-*nifH* sequences. Figure 4.3.B gives the position of the specific primer compared to the other primers used. All the *nifH* sequences in the targeted zone, except cluster A-*nifH* sequences, possess at least one *Sac*I recognition site. The reliability of the cluster A-specific PCR amplification toward cluster A sequences was assessed with *Sac*I digestion of PCR fragments.

4.3.3 Results and discussion

Cluster A-specific nifH *gene amplification*

We designed a specific primer (Table 4.3.A) targeting specifically cluster A-*nifH* genes (fcluA) previously detected in the littoral meadow where *Molinia coerulea* occurs (cf. § 4.2; Hamelin *et al.* 2002). The combination of fcluA and *nifH*(rev) primers gave a single PCR product of 305 bp with soil and root samples, as presented in figure 4.3.B. We wondered whether the presence and the nitrogen fixation activity of cluster A-containing diazotrophs associated with *Molinia coerulea* and *Lolium perenne* were also influenced by different pCO_2 growing conditions.

Table 4.3.A PCR primers used for the *nifH* amplifications. [a] primer designed by Widmer *et al*. (1999). [b] position according to the *Azotobacter vinelandii nifH* gene sequence (GenBank accession number M20568). [c] IUPAC conventions was used: R, A/G; Y, C/T; W, A/T; V, A/C/G; B, C/G/T; N, A/C/G/T and I, Inosine

nifH primers	*nifH* gene target[b]	Sequence (5′ 3′)[c]	Degeneracies
nifH(forA)[a]	19-38	GCIWTITAYGGNAARGGNGG	128
nifH(forB)[a]	112-131	GGITGTGAYCCNAAVGCNGA	48
nifH(rev)[a]	463-482	GCRTAIABNGCCATCATYTC	96
fcluA	177-190	ATGGA**Y**CT**G**GT**K**CG ATGGA**C**CT**A**GT**G**CG	5

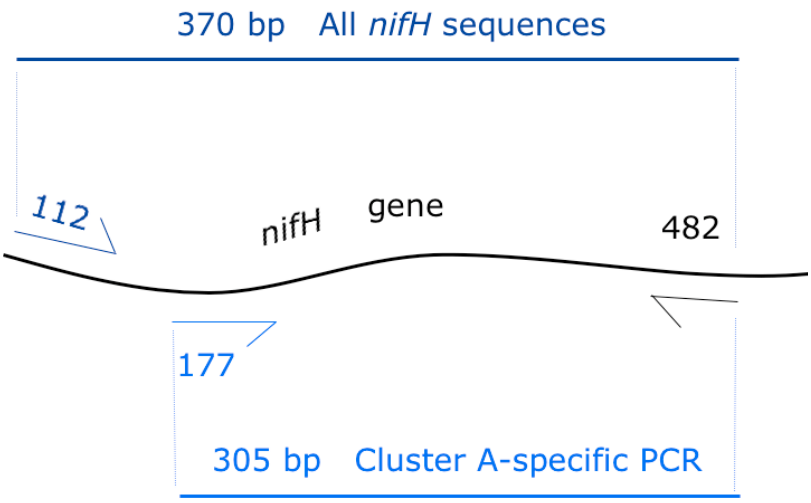


Figure 4.3.B Position of the *nifH* primers and sizes of *nifH* resulting PCR fragments. The 370 pb fragment (dark blue) corresponded to the consensus *nifH* gene amplification and the 305 pb fragment (pale blue) corresponded to the cluster A-specific *nifH* amplification

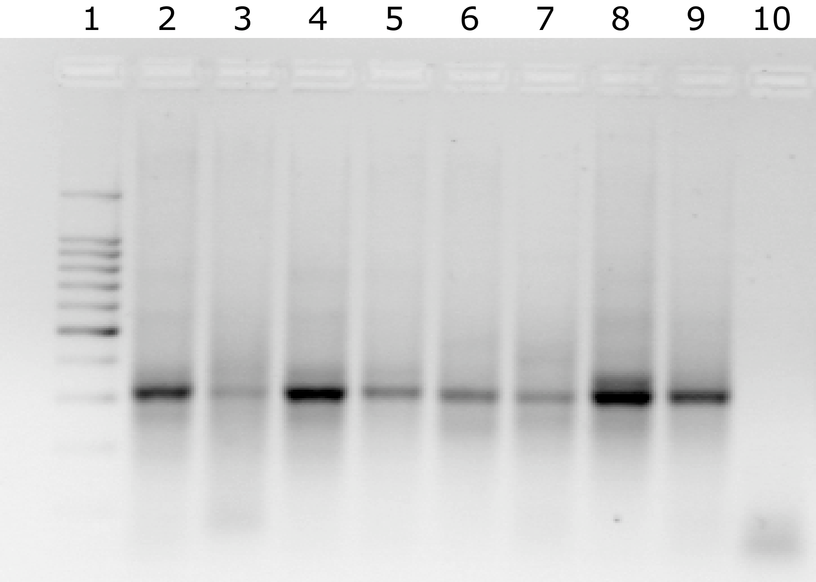


Figure 4.3.C 105 bp PCR fragment of cluster A-specific amplification of *nifH* genes for two perennial grasses. Line 1, 100 bp mass ladder; lines 2 & 3, soil and root of *Molinia coerulea* grown under normal pCO_2 atmosphere; lines 4 & 5, soil and root of *M. coerulea* grown under elevated pCO_2 atmosphere; lines 6 & 7, soil and root of *Lolium perenne* grown under normal pCO_2 atmosphere; lines 8 & 9, soil and root of *L. perenne* grown under elevated pCO_2 atmosphere; line 10, no DNA added

Figure 4.3.C shows the *nifH* gene PCR amplification with the new designed cluster A-specific primer for *Molinia coerulea* and *Lolium perenne* rhizospheric environments. The positive PCR signal indicated that cluster A diazotrophs could be widespread in association with perennial grass root system.

This study is still in progress but needs to be pursued. The *Sac*I digestion could be performed on every total *nifH* amplification (combining *nifH*(forB) and *nifH*(rev) primers), and the proportion of the remaining non-digested PCR fragments will correspond to the initial proportion of cluster A-*nifH* sequences in the samples.

mRNA nifH *transcribed genes*

Total DNA and RNA were extracted and purified from root and soil samples for both grasses (*Molinia coerulea* and *Lolium perenne*) grown under ambient and elevated pCO_2 content. RNA extracts were submitted to reverse transtription. All DNA and cDNA retrieved in this study could be amplified using a 16S rDNA approach (cf. § 2.1.3).

Whereas *nifH* sequences could be amplified either from soil or from root DNA extracts with both primer sets (total and cluster A-specific primers), transcribed *nifH* genes (total as well as cluster A mRNA *nifH*) were only detected in root RNA extracts. We conclued that diazotrophic bacteria, including cluster A, transcribe the iron protein nitrogenase *nifH* gene only in the root environment. The roots of grasses seems to represent a niche for dinitrogen fixation. The rhizodeposition provides an abondant source of carbon and energy for rhizospheric bacteria. Moreover, the rhizosphere is depleted in mineral nitrogen because of plant uptake (cf. § 1.2.2 & § 1.2.3). It is likely that these conditions can explain our results. However, using this global RNA extraction, we cannot exclude a greater lability of RNA in soil than RNA in roots. If so, the quantity of *nifH* mRNA retrieved from soil samples could be too low to be detected. Other mRNA extraction protocols from soil (Bürgmann *et al.* 2003) should be tested on *Molinia coerulea* environment.

We did not measure nitrogen fixation activity *in situ* but the expression of nitrogen fixation genes gives insight about this process. Hurek *et al*. (2002) obtained a good correlation between the level of expression of nitrogen fixation genes and the activity measured. They inoculated the strain BH72 of *Azoarcus* sp., a known diazotrophic bacterium able to colonize many grasses (Hurek *et al*. 1994), in the root system of Kallar grass. *Azoarcus* is a natural endophyte of this grass but lost its cultivability after a stage of 3 months in the rhizospheric environment of Kallar grass. At the same time, *Azoarcus* kept the ability to express the nitrogen fixation genes (RT-PCR on *nifH* mRNA) and to fix nitrogen (^{15}N labelling) in an unculturable state. Abundant *nifH* transcripts were detected also in uninoculated plants taken from the natural environment, from which *Azoarcus* could not be isolated. The *Spartina patens* plants presented also stable populations of active nitrogen fixers in the root during major phenological stages from dormancy to senescence while fluctuating diazotrophic populations was observed (Burke *et al*. 2002). The survey of active (based on mRNA) instead of present (based on DNA) bacteria seems relevant when focussing on a functional gene, like the *nifH* gene.

The survey of a special group of diazotroph (eg. those carrying cluster A-*nifH* sequences) is not possible in natural conditions because we cannot exclude the activity of other diazotrophs inhabiting the roots of grasses. In order to overcome this problem, it would be possible to selectively target cluster A-*nifH* mRNA in the root system and surrounding soil of plants growing in natural conditions using the fluorescent *in situ* hybridization (FISH) on mRNA. With such experiments, we could also verify the presence of active nitrogen fixers among the soil bacteria, or conclude if the soil is only a 'reservoir' for the diazotrophs.

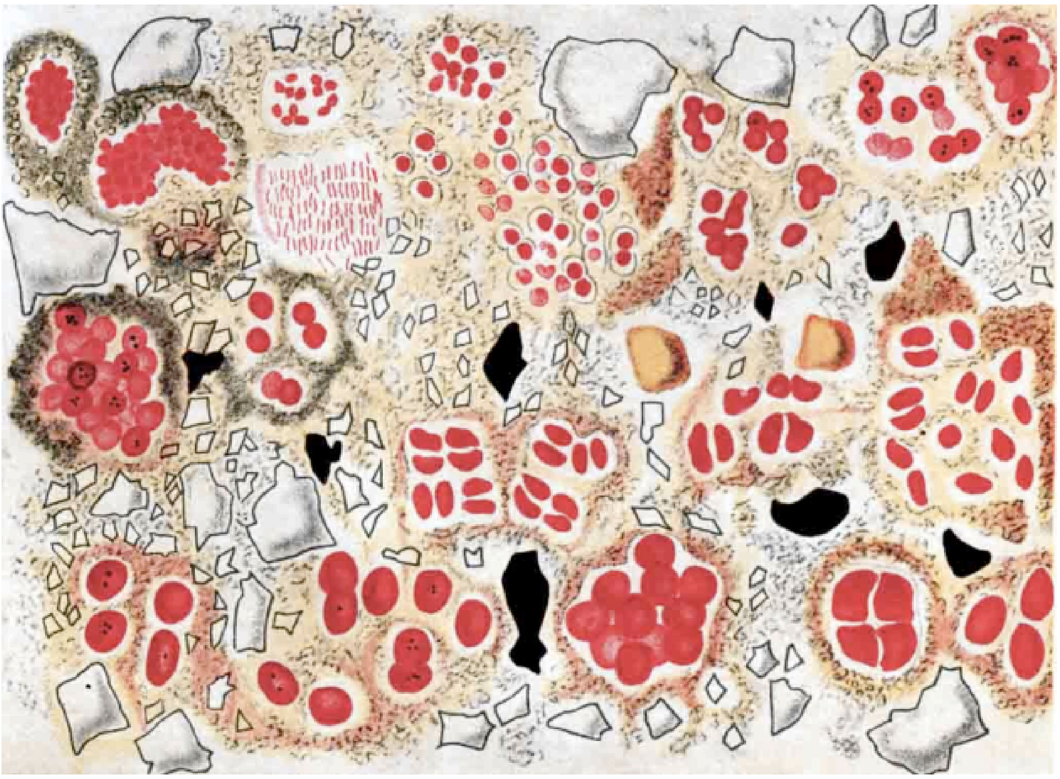


Figure 4.5.A Le sol est constitué d'une multitude de micro-niches qui sont susceptibles d'accueillir autant d'espèces différentes capable d'exploiter ces environnements différents. Sur ce dessin d'un sol observé par Winogradski (1949), plusieurs micro-colonies bactériennes exploitent chacune une ressource différente du sol

4.4 Conclusion générale sur les bactéries fixatrices d'azote

Cultivabilité

Les dénombrements des bactéries fixatrices d'azote cultivables n'ont pas été présentés dans ce document. Le milieu d'isolement utilisé (ABpy), reprend la composition minérale du milieu Schlegel (Aragno & Schlegel 1992) sans source d'azote combiné avec du pyruvate comme source de carbone. L'incubation se faisait sous atmosphère d'azote avec 1 % d'O_2. Après vérification de la présence d'une fixation d'azote potentielle des souches isolées par mesure d'ARA, nous avons montré que très peu étaient des souches véritablement fixatrices. Nous pensons que nous avons isolé principalement des bactéries oligonitrophiles. Dans le cadre du travail de diplôme de Nahida Syed, nous avons testé plusieurs milieux d'isolement, en remplaçant l'agar par un gel de silice pour supprimer les éventuelles impuretés du milieu de culture, ou en faisant varier l'origine des inocula (prairie littorale *vs* tourbière). Cette stratégie s'est avérée infructueuse. Si j'avais un regret à formuler dans cette étude, c'est de ne pas avoir réussi à isoler de représentant du groupe de bactéries fixatrice d'azote possèdant un gène *nifH* de type cluster A (Hamelin et coll. 2002).

Sachant que le sol est constitué d'une multitude de micro-niches (Figure 4.5.A), il faudrait en théorie pouvoir développer autant de milieux de cultures qu'il existe de niches différentes. Une manière d'augmenter la cultivabilité des organismes du sol (et en particulier celle des bactéries diazotrophes) serait de cibler les bactéries anaérobies aérotolérantes ou micro-aérophiles, car la pression partielle en oxygène de l'atmosphère du sol est souvent faible. Lors du 9ème congrès de l'ISME à Amsterdam, un poster a attiré notre attention (Stevenson et coll. 2001). En ajoutant au milieu de culture des enzymes impliquées dans les mécanismes de protection vis-à-vis de l'oxygène (catalase, super-oxide dismutase), les auteurs observaient sept fois plus de colonies bactériennes. Dans l'éventualité où les bactéries nécessitent la présence de la plante pour se développer, l'ajout d'extrait de racine est suceptible d'améliorer la cultivabilité. C'est probablement vers ce type de stratégies qu'il faudrait tendre pour isoler les bactéries fixatrices d'azote *'non-cultivables'* associées à la molinie.

RT-PCR sur les gènes fonctionnels

La taille et la diversité de la guilde fonctionnelle (ensemble des espèces ayant la capacité génétique à réaliser une fonction) nous renseignent directement sur le potentiel métabolique d'un environnement pour cette fonction. Toute modification d'activité potentielle ou de structure de la communauté fonctionnelle peut être directement interprété biologiquement et peut se traduire par une perturbation du fonctionnement de l'écosystème étudié. De ce fait, les études basées sur les communautés fonctionnelles sont souvent très pertinentes par rapport aux études de communautés basées sur le gène ribosomal 16S (cf. chapitre 2). L'inventaire des espèces bactériennes nous renseigne rarement sur le rôle ou les fonctions des organismes détectés.

Nous avons évalué assez précisément la diversité des bactéries génétiquement aptes à réaliser la fixation d'azote (la guilde des fixateurs d'azote) mais nous venons à peine d'entamer l'étude sur l'activité de fixation des bactéries (cf. chapitre 4). La nature exacte de l'association entre les racines de *Molinia coerulea* et les bactéries diazotrophes appartenant au cluster A reste à élucider. Existe-t-il des signaux provenant de la plante qui favorisent l'activité de fixation d'azote? Quelle proportion de l'azote réellement fixé peut être transféré à la plante? L'activité de fixation d'azote est-t-elle strictement circonscrite aux racines?

La génétique reconnaît l'influence de l'environnement sur les caractères. La causalité est diluée entre le gène et l'environnement, mais n'est jamais répartie de manière symétrique entre les composants. Le généticien moderne dira donc que ce qui *détermine* le caractère, c'est le gène. Pour valider cette affirmation, il démontrera effectivement que, *toutes choses égales par ailleurs*, une modification du gène par mutation est nécessaire et suffisante pour modifier le caractère. Mais "toutes autres choses" peuvent constituer autant de causes si elles ne sont pas égales. Et elles sont d'autant moins égales que l'une d'entre elles a varié et que, dans le système étudié, tout est lié. Dans la réalité, *rien n'est jamais égal par ailleurs*. La cause est nécessairement ce que l'expérimentateur a choisi de faire varier. En toute rigueur, les conséquences de la mutation démontrent que le gène *participe* au caractère et non qu'il en est la cause. De plus, il est possible d'observer que, à gène égal, l'environnement est capable de modifier les caractères. Pourquoi cela ne démontre-t-il pas l'hérédité environnementale des caractères? Si tout le monde s'accorde à dire que le gène *et* l'environnement déterminent les caractères, alors pourquoi l'affirmation "les gènes portent les caractères" est-elle plus acceptable que "l'environnement porte les caractères"?

Pierre Sonigo & Isabelle Stengers. L'évolution (2003) Ed. EDP Sciences p. 39

Chapitre 5

Conclusion et Perspectives

Chapitre 5

Conclusion et perspectives

La vision qu'on se fait du monde qui nous entoure dépend de notre façon de le regarder. L'essor de la biologie moléculaire en écologie microbienne depuis une dizaine d'année a permis de prendre en compte la plupart de la diversité microbienne encore méconnue jusqu'alors. Désormais, les communautés bactériennes peuvent être abordées dans leur ensemble. Dans le chapitre 2, nous avons étudié comment les communautés bactériennes associées à la rhizosphère de *Molinia coerulea* réagissaient à des modifications environnementales. En parallèle à cette approche, nous avons ciblé les populations de *Pseudomonas* cultivables (chapitre 3). Enfin, le chapitre 4 a été consacré aux bactéries fixatrices d'azote. Chacune des approches apporte une information particulière et c'est en multipliant les angles de vue qu'on cerne le mieux les communautés bactériennes étudiées.

On dessine toujours les éléphants plus petits que nature, mais les puces toujours plus grandes.
Jonathan Swift (1667-1745), Pensées sur divers sujets

L'étude des communautés bactériennes par des profils génétiques permet d'avoir un aperçu global des populations dominantes à un instant donné. En appliquant aux communautés bactériennes les outils statistiques développés dans les années 1970 pour les communautés végétales, nous pouvons expliquer, en partie du moins, des variations de populations par des facteurs environnementaux (cf. § 2.1.1). Ce travail s'apparente à une nouvelle discipline que l'on pourrait nommer la 'bactério-sociologie' (Fromin et coll. 2002). Cette manière de voir les populations bactériennes comme des organismes multicellulaires est récurrente dans la littérature (Shapiro 1998).

Nous avons par ailleurs développé des indices de diversité adaptés à l'analyse des profils génétiques de communauté (cf. § 2.1.2). Il reste à les valider dans de futures études.

Ensuite, nous avons montré que de nombreuses populations bactériennes étaient peu abondantes mais très actives, aussi bien dans la racine de la molinie que dans le sol environnant (Jossi 2003). Cette constatation nous interroge sur le véritable rôle de ces populations bactériennes dans le fonctionnement biologique du sol, sur l'importance de la prédation par les protozoaires, ou encore sur l'impact de l'hétérogénéité du sol dans les analyses génétiques globales utilisées. Nous avons aussi remarqué que la plupart (75 %) des espèces indicatrices - dont l'abondance ou l'activité variaient avec des changements de la concentration en CO_2 atmosphérique - étaient des protéobactéries (cf. § 2.1.3). La technologie des puces à ADN pourrait avantageusement permettre de suivre l'ensemble de ces populations indicatrices au cours d'une saison de végétation, ou en association avec la molinie poussant naturellement dans des sols différents.

Après avoir étudié les communautés du point de vue génétique, nous nous sommes intéressés au potentialités métaboliques des communautés en réalisant des profils d'utilisation de 31 sources de carbone (cf. § 2.2). La moitié des substrats ont été plus oxydés par les communautés bactériennes racinaires provenant des plantes poussant sous atmosphère enrichie en pCO_2 par rapport à celles poussant sous atmosphère normale. Les communautés telluriques n'ont pas montré de telles différences. Cette constatation était attendue car l'effet présumé d'une augmentation de pCO_2 sur les communautés bactériennes passe par la plante, via la rhizodéposition (cf. § 1.3.2).

L'originalité de ce travail a été de caractériser chaque guilde - les populations bactériennes utilisant chacun des substrats - par DGGE. En moyenne, 19 populations utilisaient les substrats proposés. Nous avons remarqué que le niveau d'utilisation des sources de carbone ne pouvait pas être mis directement en relation avec les guildes correspondantes et qu'il existait une certaine redondance fonctionnelle des populations pour l'utilisation des deux tiers des sources de carbone.

La rhizosphère de *Molinia coerulea* a aussi été étudiée par le suivi des populations cultivables de *Pseudomonas*. Nous avons d'abord développé une amplification PCR spécifique au genre *Pseudomonas* (cf. § 3.1). Cette première étape ouvre des perspectives intéressantes en terme de comparaison des populations cultivables en parallèle à la caractérisation de l'ensemble des *Pseudomonas* présents. Nous aurons ainsi une idée plus précise de la diversité des *Pseudomonas* associés à la rhizosphère de la molinie et qui ne sont pas cultivables sur des milieux de culture traditionnels.

Dans un second temps, nous avons comparé la diversité des *Pseudomonas* cultivables dans la rhizosphère et dans le sol environnant à partir de deux milieux d'isolement différents (cf. § 3.2). Nous avons mis en évidence des populations qui sont physiologiquement incapables de pousser sur le milieu sélectif pour *Pseudomonas* (milieu mS1) même après isolement sur milieu Angle et d'autres populations qui ne peuvent pas être isolées directement sur le milieu sélectif mais ayant les potentialités d'y croître après revitalisation sur un milieu plus pauvre (milieu Angle). Les bactéries dites non cultivables sur un milieu de culture peuvent être classées en deux catégories: celles qui ne sont génétiquement pas aptes à supporter les conditions des milieux de culture et celles qui ont les potentialités métaboliques nécessaires mais qui sont dans un état physiologique tel que le milieu de culture les tuent ou inhibent leur croissance (choc osmotique, …).

Nous avons terminé l'étude des *Pseudomonas* en nous intéressant à la fois aux souches capables de réduire les nitrates en nitrite et à celles qui dénitrifient complètement (cf. § 3.3). Nous avons remarqué que la proportion de réducteurs de nitrate diminuait dans les fractions racinaires alors que la proportion de bactéries dénitrifiantes augmentait. Cette tendance était encore plus marquée lorsque les molinies étaient cultivées sont atmosphère enrichie en CO_2. Nous avons postulé que, dans les conditions oligotrophes étudiées, la compétition pour les nitrates dans la racine était importante et devait limiter la présence des bactéries réductrices de nitrate. Les bactéries qui dénitrifient bénéficient de conditions favorables dans l'environnement racinaire et seraient favorisées. Cette constatation est en accord avec la bibliographie sur le sujet (Linne von Berg & Both 1992; Clays-Josserand et coll. 1995 & 1999; Delorme 2001).

L'étude des *Pseudomonas* rhizosphériques se poursuit actuellement au laboratoire dans le cadre de la thèse de Sonia Tarnawski. Deux graminées pérennes ayant des exigences trophiques distinctes sont comparées: *Lolium perenne* qui nécessite des amendements réguliers en azote pour une croissance normale et *Molinia coerulea* retrouvée naturellement dans des sols oligotrophes.

Du point de vue du microbiologiste du sol, la diversité microbienne du sol est quasi infinie (Torsvik et coll. 1990 & 1994) mais ne garantit pas à elle seule le bon fonctionnement du sol. Par contre, certaines transformations de la matière organique et le recyclage de certains éléments ne sont réalisés que par des micro-organismes. De plus, les études basées sur les communautés fonctionnelles sont souvent très pertinentes et nous renseignent directement sur les fonctions réalisées (Poly 2000).

Le chapitre 4 rassemble les travaux portant sur les bactéries fixatrices d'azote associées à la rhizosphère de la molinie. Nous avons évalué l'activité globale par mesure d'ARA (cf. § 4.1) et la diversité des bactéries fixatrices d'azote sur la base du gène *nifH* comme marqueur moléculaire (cf. § 4.2). Les pools de gènes *nifH* semblent identiques entre les racines et le sol environnant. On peut attribuer cette homogénéité génétique par l'influence permanente des racines de *Molinia coerulea* sur son sol environnant depuis que la prairie naturelle existe (cf. § 1.4.2).

Après avoir évalué la diversité de la guilde des fixateurs d'azote, il nous restait à étudier si ces bactéries étaient actives *in situ* pour la fixation d'azote (cf. § 4.3). Nous avons travaillé sur les transcrits de gènes *nifH* (ARNm) dans le sol et dans la racine de molinie. Cette approche est très novatrice et très prometteuse. Nous avons détecté des gènes *nifH* transcrits uniquement dans la racine et avons postulé que la racine représentait une niche favorable pour le processus de fixation d'azote par rapport au sol environnant, considéré comme un habitat pouvant servir de 'réservoir' pour les diazotrophes. Cette dernière hypothèse reste à confirmer. Afin d'y parvenir, je crois en des techniques de type *In Vivo Expression Technology* (IVET) où des sondes marquées sont appliquées sur des coupes fines provenant d'échantillons biologiques traités de manière à conserver les ARN intacts.

La nature même de la relation qui existe entre les bactéries fixatrices d'azote du groupe "Cluster A" et la molinie reste à explorer. Le décryptage des signaux moléculaires qui induisent la transcription des gènes de fixation d'azote, par l'étude des gènes de régulation du gène de structure *nifH* par exemple, pourrait aider à comprendre l'importance relative de ce groupe bactérien dans l'environnement racinaire de la molinie (Bally, communication personnelle).

Nous avons volontairement basé nos recherches en multipliant les points de vue pour l'étude des communautés bactériennes associées à la molinie (structure des communautés, diversité au sein des *Pseudomonas*, guilde des fixateurs d'azote). Même si il est parfois difficile de mener des recherches dans plusieurs directions en parallèle, j'ai beaucoup aimé cette façon d'étudier la rhizosphère.

Je crois cependant que les études basées sur des gènes fonctionnels apportent des informations directement interprétables dans le fonctionnement des écosystèmes. Par exemple, Taroncher-Oldenburg et coll. (2003) ont utilisé des sondes fixées sur des puces à ADN ciblant les gènes responsables des fonctions clé du cycle de l'azote (*amoA*, *nifH*, *nirK* & *nirS*). Cette manière d'aborder un écosystème me parait très pertinente.

Maintenant que les communautés bactériennes associées à la rhizosphère de la molinie poussant dans la prairie littorale sont mieux connues, il serait intéressant de réaliser des transplantations réciproques de plantes poussant naturellement dans le sol basique avec des molinies retrouvées dans les tourbières à pH acide (Küpfer, communication personnelle). Ceci permettrai de cerner les microorganismes qui sont liés strictement à la plante (mesure indirecte du degré d'endophytisme des bactéries?) par rapport à ceux dont la présence est favorisée par les conditions de l'environnement et/ou par le sol environnant.

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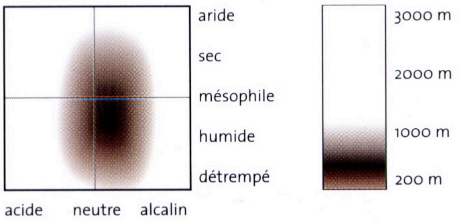
Annexes

Annexe 1 Association végétale correspondant au site de prélèvement de la molinie sur la rive sud du lac de Neuchâtel, information obtenue du Guide des milieux naturels de Suisse (Delarze et coll. 1998)

Prairie à molinie

Physionomie et écologie

Prés à litière où domine la canche bleue (*Molinia caerulea*, parfois aussi *M. arundinacea*), qui atteint environ 1 m de hauteur en été. De nombreuses plantes de plus petite taille occupent des plages de sol nu entre les touffes de cette graminée.
Le sol est souvent calcaire mais parfois acide en surface. La molinaie occupe typiquement des zones où la nappe fluctue à faible profondeur (humidité temporaire). Ce sol est toujours pauvre en nutriments, mais riche en matière organique. Sur sols plus eutrophes, il cède la place au *Filipendulion* ou au *Calthion*.



Relations avec l'homme

Traditionnellement exploitée comme pré à litière non engraissé, la molinaie est un groupement anthropogène qui dépend d'une fauche régulière, vers la fin de l'été ou en automne. Abandonnée, elle évolue rapidement vers la mégaphorbiaie et vers la forêt.

Problèmes d'identification

Molinia caerulea forme des tapis pauvres en espèces dans le sous-bois de certaines forêts (6.3.1, 6.5.1) et dans des stades de dégradation de bas-marais et de marais bombés (2.2.3, 2.4.1, 5.4.1.1).
En comparaison, le véritable *Molinion* est plus riche, souvent très fleuri à la fin de l'été.
Contrairement aux associations de bas-marais (2.2), les cypéracées n'y sont pas dominantes, sauf dans certains stades pionniers à *Carex punctata*.

Valeurs biologiques

La molinaie possède une richesse floristique exceptionnelle: elle abrite des plantes rares spécialistes des sols à humidité variable (*Carex hartmanii**, *Cirsium tuberosum**, *Ophioglossum vulgatum**, *Gentiana pneumonanthe**, *Gladiolus* spp, *Gratiola officinalis**, *Tephroseris (=Senecio) helenitis**, *Thalictrum simplex*, *Trifolium spadiceum**, *Viola canina* ssp. *schultzii**, etc.) et de nombreuses orchidées protégées (*Orchis laxiflora**, *O. coriophora**, *Herminium monorchis**, etc.).
Cette richesse est cependant très variable d'une station à l'autre.

Phytosociologie

Molinion caeruleae W. Koch 26

COR	1991 :	*Molinion* (37.31)
HBZ	1993 :	*Molinion* (69)
ELL	1996 :	*Molinion caeruleae* (5.411)
OBE	1994 :	*Molinion caeruleae*
MGW	1993 :	*Molinion*

Deux alliances voisines, qui n'apparaissent chez nous que sous forme fragmentaire et peu typique, ont été regroupées avec le *Molinion : Cnidion dubii, Juncion acutiflori*.
On classe parfois l'aile acidophile du *Molinion* dans une alliance distincte, le *Junco conglomerati-Molinion*.

Allium angulosum, A. suaveolens*, Anagallis tenella*, Carex tomentosa, Cirsium tuberosum*, Gentiana amarella*, G. asclepiadea, G. pneumonanthe*, Gladiolus imbricatus*, G. palustris*, Gratiola officinalis*, Iris sibirica*, Juncus acutiflorus, Laserpitium prutenicum*, Lathyrus palustris*, Lotus maritimus, Oenanthe lachenalii*, O. peucedanifolia*, Ophioglossum vulgatum,*, Scorzonera humilis*, Selinum carvifolia, Serratula tinctoria*, (Sisyrinchium montanum*), Viola elatior*, V. persicifolia*, V. pumila*.*

Achillea ptarmica, Calamagrostis epigejos, Carex flacca, C. distans, C. punctata, Cirsium tuberosum, Colchicum autumnale, Dactylorhiza maculata, Dianthus superbus, Galium boreale, G. uliginosum, Genista tinctoria, Hieracium caespitosum, Inula salicina, ***Juncus conglomeratus****, Lotus tenuis, Molinia arundinacea,* ***M. caerulea****, Platanthera chlorantha, Polygala amarella, Rhinanthus minor, Sanguisorba officinalis, Silene flos-cuculi, Stachys officinalis, Succisa pratensis, Valeriana dioica, Vicia cracca.*

Coenonympha oedippus (Satyridae), Eurodryas aurinia* (Nymphalidae), Maculinea alcon*, M. nausithous*, M. teleius* (Lycaenidae).*

Haltica pusilla (Chrysomelidae).

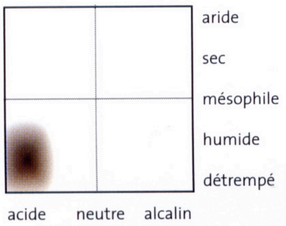
Annexe 2 Association végétale correspondant au site de prélèvement de la molinie à chapelle des bois, information obtenue du Guide des milieux naturels de Suisse (Delarze et coll. 1998)

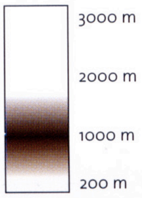
Tourbière à sphaignes

Physionomie et écologie

Tapis spongieux de sphaignes gorgées d'eau, parsemées d'éricacées et de cypéracées à feuilles étroites. Ce groupement est le moteur de la génèse des tourbières bombées; il en occupe la partie centrale et y forme des buttes surélevées qui alternent souvent avec des groupements plus hygrophiles (*Rhynchosporion*, voir 2.2.4).

La très grande pauvreté en azote assimilable favorise les plantes carnivores (*Drosera*) et les espèces mycorhiziques (éricacées).





Relations avec l'homme

La tourbe a été exploitée intensivement dès le début du siècle passé, principalement pour le chauffage et pour l'agriculture. Compte tenu de l'extrême lenteur de la régénération du milieu, celui-ci n'offre pratiquement aucune possibilité d'utilisation durable.

Problèmes d'identification

La végétation des buttes à sphaignes alterne souvent avec des cuvettes (Schlenken) qui abritent des éléments du *Rhynchosporion* (2.2.4).

On rencontre des taches de sphaignes dans certains bas-marais, dans des landes et dans des forêts à sous-bois très acide. Ces espèces, qui supportent un faible apport d'eau minéralisée, sont différentes de celles du haut-marais.

Les landes de dégradation, plus sèches, se distinguent par le recul des sphaignes et l'abondance de la callune (*Calluno-Genistion*, 5.4.1)

Valeurs biologiques

Refuge d'espèces boréo-arctiques rares, la tourbière abrite aussi des formes biologiques particulières (voir 2.4), liées à une situation trophique extrême, d'où son très grand intérêt scientifique.

Les pollens conservés dans les couches de tourbe accumulées au cours de millénaires permettent de reconstituer en détail l'histoire de la végétation et du climat. Il s'agit d'archives irremplaçables.

Phytosociologie

Sphagnion magellanici Kästner et Flössner 33

COR	1991 :	*Sphagnion magellanici* (51.11)
HBZ	1993 :	*Sphagnion fusci* (129)
ELL	1996 :	*Sphagnion magellanici* (1.811)
OBE	1994 :	*Sphagnion magellanici*
MGW	1993 :	*Sphagnion medii*

L'unité comprend des fragments de l'alliance voisine de l'*Ericion tetralicis* (*Erica tetralix* n'est pas indigène en Suisse).

Divers auteurs incluent dans le *Sphagnion* les pineraies de tourbière, à cause de leur affinité floristique et écologique avec les buttes non boisées; s'agissant d'une formation arborée (voir introduction), nous préférons la traiter comme alliance distincte, le *Vaccinio uliginosi-Pinion* (6.5.2).

Andromeda polifolia, Betula nana*,* ***Eriophorum vaginatum,*** *(Sarracenia purpurea), (Vaccinium macrocarpon), V. microcarpum, V. oxycoccos.*

Calypogeia sphagnicola, Cephalozia connivens, Dicranum bergeri, Odontoschisma sphagni*,* ***Sphagnum fuscum*, S. magellanicum****, S. papillosum, S. rubellum*, S. russowii, S. tenellum*.*

Carex pauciflora, Calluna vulgaris, Drosera rotundifolia, Empetrum nigrum*, (Erica tetralix*), Juncus squarrosus*,* ***Vaccinium myrtillus, V. uliginosum****.*

Aulacomnium palustre*, Cephalozia loitlesbergeri*, C. macrostachya*, Gymnocolea inflata, Kurzia pauciflora*, Lophozia capitata ssp. laxa*, Meesia longiseta*, Mylia anomala, Pohlia sphagnicola*, Polytrichum strictum, Sphagnum compactum, S. imbricatum*, S. molle*.*

Galerina sphagnorum, Hypholoma elongatum*, Lyophyllum palustre, Marasmius androsaceus.*

Boloria aquilonaris (Nymphalidae).*

Agonum ericeti (Carabidae), Helodes punctipennis (Scirtidae), Atheta arctica, Lathrobium rufipenne, L. sphagnetorum, Mycetoporus bergrothi, Philonthus nigrita (Staphylinidae).*