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Réponse des populations de *Pseudomonas* à une augmentation de la concentration en CO_2 atmosphérique dans la rhizosphère de *Lolium perenne* et *Molinia coerulea*

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IMPRIMATUR POUR LA THESE

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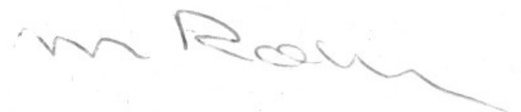

Prof. M. Rahier

« Il est un espace entre l'imagination de l'homme et ce qu'il réalise qui ne peut être franchi que par son ardeur »

Khalil Gibran
Le sable et l'écume

« La sagesse exige l'investigation de nombreuses choses »

Héraclite

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PLAN de la THESE

REPONSE DES POPULATIONS DE *PSEUDOMONAS* A UNE AUGMENTATION DE LA CONCENTRATION EN CO_2 ATMOSPHERIQUE DANS LA RHIZOSPHERE DE *LOLIUM PERENNE* ET *MOLINIA COERULEA*

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CHAPITRE 1

Synthèse bibliographique – Contexte de l’étude

CHAPITRE 1

SYNTHESE BIBLIOGRAPHIQUE - CONTEXTE DE L'ETUDE

INTRODUCTION

Les données paléo-atmosphériques permettent de dater l'augmentation spectaculaire des concentrations en CO_2 atmosphérique (pCO_2) à partir de l'ère industrielle (Figure 1.1). La concentration de pCO_2 est passée de 280 ppm en 1750 à 367 ppm en 1999 (soit une augmentation de 31%). La concentration actuelle de pCO_2 n'a jamais été dépassée depuis les 420 000 dernières années et ne l'a probablement pas été durant les 20 derniers millions d'années (Figure 1.1). L'importante augmentation actuelle du pCO_2 est reliée aux activités humaines (Vitousek et coll. 1997), principalement à l'oxydation du carbone organique lors de la combustion des énergies fossiles et du déboisement. La part d'émission naturelle et anthropique de CO_2 dans l'atmosphère, et la part d'absorption par les terres émergées et les océans durant les 20 dernières années ont été calculées. Les terres émergées présentent un puits potentiel certain, -1.4 Pg de C/an dans les années 90 (Tableau 1.1). Le renforcement de la croissance des végétaux dû à la plus forte teneur en CO_2 atmosphérique et au dépôt d'azote anthropique contribuerait sensiblement à l'absorption du CO_2 et à la séquestration d'une partie du carbone dans les terres émergées (GEICGT, 2001). Cependant, les surplus d'émissions de CO_2 atmosphérique et d'autres gaz à effet de serre (N_2O, CH_4) devraient entraîner une augmentation moyenne de la température de 1.5 à 2°C et des précipitations locales abondantes. De telles conditions favoriseraient aussi la minéralisation de carbone et d'azote du sol.
L'étude présentée ici s'intéresse plus particulièrement à l'impact de l'augmentation en pCO_2 sur la microflore du sol des écosystèmes prairiaux des régions tempérées où le CO_2 est le facteur limitant majeur de l'activité photosynthétique. Dans ces régions, peuplées majoritairement de plante en C3, la pression partielle en CO_2 qui permettrait à la photosynthèse d'atteindre la moitié de sa vitesse maximale est voisine de 450 ppm : une variation de cette pression de 350 à 600 ppm aurait donc un effet important sur la production végétale brute (Aragno et Tarnawski, 2002a).

1.1. INFLUENCE D'UN ENRICHISSEMENT EN pCO_2 SUR LE SYSTEME SOL/PLANTE *LOLIUM PERENNE*

Pour cette étude, des expérimentations sur pâturages prairiaux ont été réalisées dans le système Free Air CO_2 Enrichment (FACE) à Eschikon (ZH, Suisse). Les objectifs étaient de mesurer les effets d'une augmentation de la concentration en CO_2 atmosphérique (pCO_2) dans les différents processus qui affectent la croissance de la plante, le sol, la faune et microflore associée à cet écosystème. L'essentiel des expérimentations porte sur *Trifolium repens* L. et *Lolium perenne* L. (Cf. 1.4.1) cultivés en monoculture et en culture mixte sous des pressions partielles en CO_2 ambiante et augmentée à 600 ppm, ceci pendant 9 années (de fin mai 1993 à fin 2002). Les effets de la fréquence de récolte des parties aériennes (coupes) et de la fertilisation azotée sur la réponse des plantes à l'augmentation de la pCO_2 ont été abordés. Nous présenterons ici les principaux résultats obtenus dans l'expérimentation FACE, concernant la réponse du système prairial *L. perenne* enrichi en pCO_2 pour les paramètres de photosynthèse, biomasse, azote et carbone.

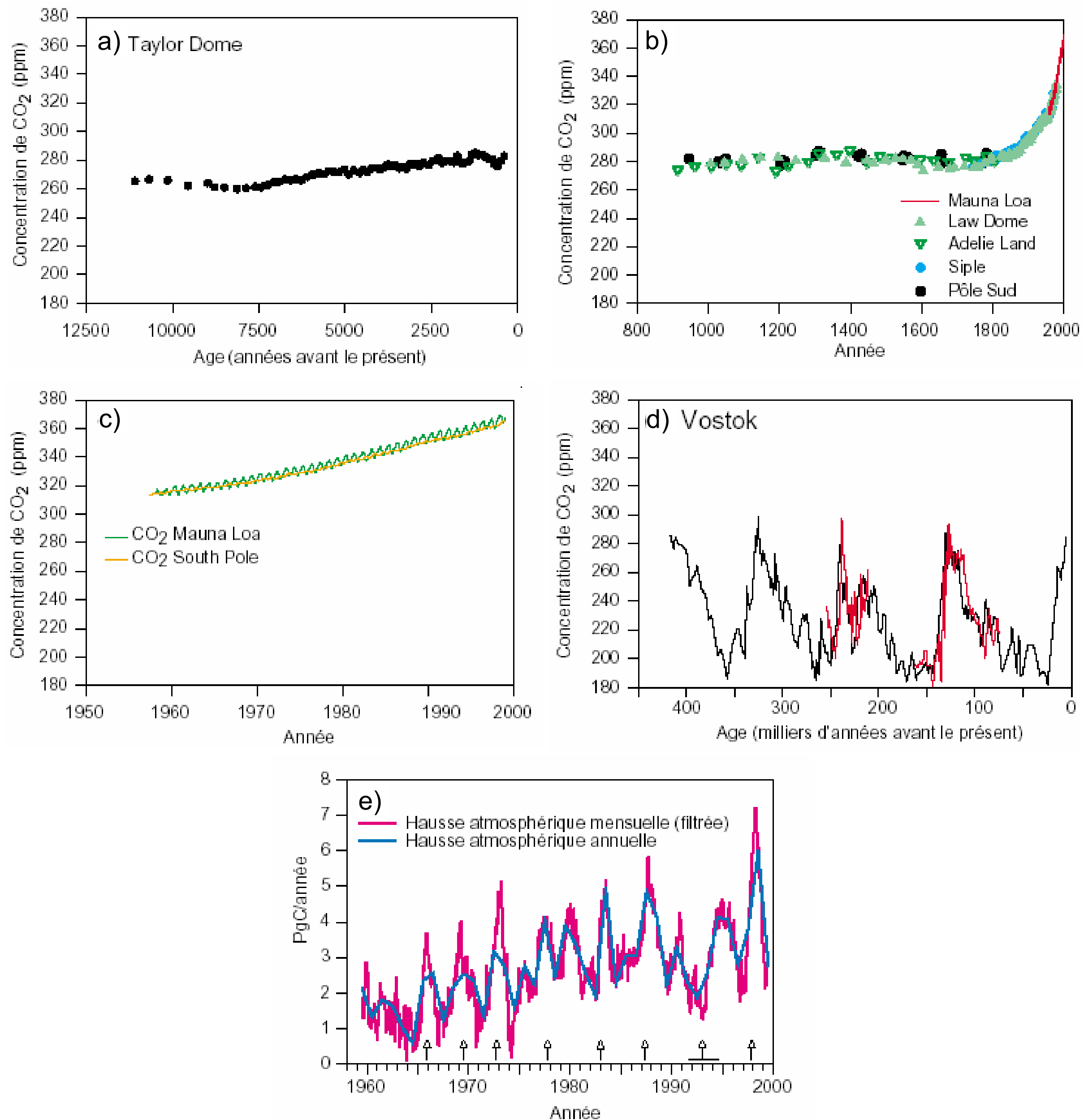

Figure 1.1. Variations de la teneur atmosphérique en CO_2 sur différentes échelles de temps.
a) Teneur en CO_2 des carottes glaciaires antarctiques du Taylor Dome. b) Teneur en CO_2 des carottes glaciaires antarctiques pour le dernier millénaire. Des mesures atmosphériques récentes (Mauna Loa) sont indiquées pour comparaison. c) Mesures directes du CO_2 dans l'atmosphère. *d*) Teneur en CO_2 des carottes glaciaires antarctiques de Vostok (les résultats des différentes études sont représentés par différentes couleurs). e) Hausses annuelles de la teneur atmosphérique en CO_2. Les hausses mensuelles ont été filtrées pour éliminer le cycle saisonnier. Les flèches verticales représentent les phases *El Niño*. La ligne horizontale définit la phase étendue *El Niño* de 1991 à 1994. (Source : GEICGT, Bilan des changements climatiques 2001)

	1980 - 1989	1990 - 1999
Augmentation dans l'atmosphère	+3.2 ± 0.1	+3.2 ± 0.1
Emissions (combustible fossile, ciment)	+5.4 ± 0.3	+6.3 ± 0.4
Flux océan-atmosphère	-1.9 ± 0.6	-1.7 ± 0.5
Flux terres émergées-atmosphère	-0.2 ± 0.7	-1.4 ± 0.7

(Source : GEICGT, Bilan des changements climatiques 2001)

Tableau 1.1. Bilans mondiaux du CO_2 (en PgC/an) **fondés sur des mesures de la concentration de CO_2 et d'O_2 dans l'atmosphère.**
+: flux vers l'atmosphère ;
- : absorption de ces gaz présents dans l'atmosphère.

1.1.1. La photosynthese

Pour *L. perenne*, après 9 années de fumigation, le taux moyen de photosynthèse net en lumière saturante était augmenté de 43% sous pCO_2 élevée comparé à une pCO_2 ambiante, et l'assimilation en CO_2 journalière des feuilles de 36% (Ainsworth et coll. 2003). A faible amendement en azote et dans les 20 premiers jours qui suivaient une coupe, le pourcentage de carbone assimilé photosynthétiquement augmentait de 45% entre les conditions 350 et 600 ppm de CO_2. Cette stimulation était près de deux fois plus importante que celle mesurée plus tard (avant une coupe) pendant le cycle de croissance des plantes (23%) (Rogers et coll. 1998, Isopp et coll. 2000). Ce phénomène d'acclimatation de la photosynthèse a souvent été évoqué. Dans la plante, la disponibilité en azote et les carbohydrates dérivés de la photosynthèse permettent le développement de nouveaux puits. Si l'azote devient limitant, la plante ne peut plus développer de puits supplémentaires, les photoassimilats s'accumulent dans les feuilles, le ratio source/puits augmente, et le taux de photosynthèse diminue en retour. Ce phénomène d'acclimatation n'a pu être observé que dans des prairies aux sols faiblement fertilisés (Bryant et coll. 1998, Farage et coll. 1998). Sur *L. perenne* sous pCO_2 élevée et faible fertilisation azotée, l'acclimatation n'est effectivement pas une conséquence direct de la pCO_2 élevée, mais plutôt un effet indirect d'un faible amendement en azote qui limite le développement de puits, et provoque une accumulation de carbohydrates dans les feuilles déclenchant le phénomène d'acclimatation (Fischer et coll. 1997, Rogers et coll. 1998, Isopp et coll. 2000). Ce phénomène pouvait se chiffrer par une réduction de 25% du contenu en Rubisco des feuilles (Rogers et coll. 1998). Ces résultats suggèrent néanmoins, que le métabolisme des carbohydrates chez *L. perenne* représente un potentiel de croissance qu'une disponibilité adéquate en azote rendrait possible. Après la récolte des parties aériennes, l'acclimatation de la photosynthèse n'est plus observée. En effet, le ratio source/puits pour les photoassimilats diminue après la coupe. Les plantes exposées à une pCO_2 élevée redirigent une plus grande quantité d'azote dans les feuilles et la photosynthèse reprend une forte activité pour la production de nouvelles feuilles.
Pour résumer, un enrichissement en pCO_2 induisait une stimulation maximale de la photosynthèse chez *L. perenne* après la récolte, aux périodes les plus chaudes de l'année et sous fort amendement en azote, ceci durant 10 années. Il semble donc que la stimulation de la photosynthèse sous pCO_2 élevée ne soit pas qu'un phénomène transitoire.

1.1.2. Le rendement

Le rendement de la monoculture de *L. perenne* augmentait de seulement 10 % en moyenne après six ans sous pCO_2 élevée, et était différent suivant l'amendement en azote. Le rendement était négatif à faible amendement en azote au début de l'expérimentation, mais augmentait année après année pour atteindre un accroissement de +9% en 1998. Sous fort amendement en azote, la réponse au pCO_2 du rendement de *L. perenne* commençait à +7 % en 1993 et augmentait régulièrement jusqu'à 25% en 1998 (Daepp et coll. 2000 ; Suter et coll. 2001). Ces lents changements dans la réponse au pCO_2 de *L. perenne* pourraient indiquer une adaptation de l'écosystème à croître sous pCO_2 élevée (Daepp et coll. 2001 ; Hebeisen et coll. 1997a,b). Le ratio des rendements en biomasse des parties racinaires/parties aériennes de *L. perenne* augmentait sous pCO_2 élevée, à faible amendement en azote, et coupe fréquente. L'augmentation de biomasse racinaire chez *L. perenne* serait un symptôme relié à la limitation en azote (Zanetti et coll. 1997), car à fort amendement en azote (112 $g.\ m^{-2}.\ an^{-1}$ N), la relative vigueur de croissance racinaire de *L. perenne* disparaissait sous fort pCO_2. Par ailleurs la pCO_2 élevée augmentait le gain en azote de l'écosystème *L. perenne* à fort amendement en azote (Hartwig et coll. 2000).
En conclusion, les prairies à monoculture de *L. perenne* faiblement amendée en azote montraient un symptôme marqué de déficience en azote par un accroissement important de la croissance racinaire et une réduction très significative de la concentration en azote de la biomasse. Néanmoins, l'augmentation de la pCO_2 entraînait une stimulation du taux de photosynthèse net et de la production de biomasse même dans les sols pauvres en azote (Hartwig et coll. 2002 ; Cannell 1998).

1.1.3. L'AZOTE

Dans des études de marquage du système au ^{15}N ($^{15}NH_4$), 7 années d'exposition à une pCO_2 élevée n'affectaient pas significativement la minéralisation globale, la consommation de NH_4^+, et l'immobilisation de l'azote dans les prairies de *L. perenne* comparé à une pCO_2 ambiante (Richter et coll. 2003). De plus, la taille du pool d'azote microbien n'était pas affectée sous atmosphère enrichie en pCO_2. En d'autre terme, le turnover de l'azote dans le sol ainsi que la disponibilité en azote ne semblait pas modifiés par un exposition à long terme à une pCO_2 élevée. Cependant la dynamique saisonnière de l'azote n'est pas reflétée par ce bilan. En effet, comme on l'a vu précédemment (Cf. 1.1.1 et 1.1.2), sous pCO_2 élevée *L. perenne* constitue un puits d'azote important, variant au cours de la période de croissance (Zanetti et coll. 1997). Par ailleurs, la dénitrification (réduction biologique du NO_3^- en N_2) et les émissions de N_2O augmentaient substantiellement sous pCO_2 élevée (Baggs et coll. 2003 ; Ineson et coll. 1998). Ce processus était identifié comme un puits d'azote supplémentaire participant à la dynamique de l'azote dans le système (Cf. Chapitre 3.2).

1.1.4. LE CARBONE

L'enrichissement en pCO_2 entraîne généralement un taux de photosynthèse plus important, et stimule la production de carbohydrates non-structuraux servant à la production de biomasse supplémentaire (tant que la balance C/N de la plante reste équilibrée). Invariablement cette stimulation de croissance semble entraîner un meilleur transfert du carbone dans le sol, *via* les racines, par l'exsudation racinaire (Weihong et coll. 2000) et la sénescence du matériel végétal (Cotrufo et Gorrisen, 1997).
Cependant, l'augmentation en pCO_2 réduisait de 13 à 14% les taux de décomposition du matériel végétal sénescent présent dans les sols pour *L. perenne* (van Ginkel et Gorissen, 1998 ; van Ginkel et coll. 2000 ; Gorissen et Cotrufo, 2000). Ce taux de décomposition restait significativement diminué (-12%) même quand la température du sol augmentait de +2°C. Par ailleurs, le sol des parcelles de *L. perenne* enrichies en pCO_2, contenait 32% et 96% de carbone en plus, respectivement à faible et fort amendement en azote, sans effet significatif d'une augmentation de la température sur le contenu en C du sol (Casella et Sousanna 1997). En fait, dans une expérience ultérieure, Williams et coll. (2000) suggère que la décomposition était effectivement augmentée sous fort pCO_2 (du fait d'une stimulation de l'activité microbienne, cf. § 1.1.3), mais que, les entrées de carbone étant plus importantes que le taux de décomposition, ceci entraînait une accumulation de C dans le sol.
L'étude de marquage au $^{14}CO_2$ du système montre que sous pCO_2 élevée, la matière sèche de la plante augmentait en moyenne de 20%. Sur la quantité de carbone fixée par la plante sous fort pCO_2, 90% restait dans la plante et les 10% restants étaient retrouvés dans le sol (Cotrufo et Gorrisen, 1997). De plus, la pCO_2 élevée augmentait le contenu en ^{14}C des racine de *L. perenne* de 41%, la concentration en ^{14}C dans la rhizosphère de 19% (Paterson et coll. 1996), la concentration en ^{14}C dans la solution du sol de 30%, la concentration en ^{14}C dans la biomasse microbienne de 46% et celle des résidus carbonés du sol de 53% (Cotrufo et Gorrisen 1997, confirmé par van Ginkel et coll. 2000).
Une étude explorant l'effet du niveau de fertilisation azotée et de la récolte des parties aériennes (coupe) sur la gestion du carbone chez *L. perenne* cultivé en microcosme, montrait qu'en condition de faible amendement en azote la matière sèche du système racinaire augmentait et que la morphologie des racines était altérée (développement d'un système de racines fines). Par ailleurs, en conditions de faible amendement en azote et de coupe fréquente, l'exsudation de composés carbonés par les racines était augmentée (Paterson et Sim, 1999). En fait, il est connu qu'une stimulation de l'exsudation racinaire peut augmenter l'acquisition de nutriments par la plante dans les sols pauvres (Dakora, 2002). Cet effet est important pour comprendre le couplage entre la productivité de la plante et le cycle des nutriments dans le sol en réponse à différentes disponibilités en azote.
Pour résumer, une séquestration plus importante de carbone dans le sol était observée pour *L. perenne* cultivé sous pCO_2 élevée. L'augmentation du flux carbone vers le sol et dans la biomasse microbienne (pour ce qui nous intéresse) semble néanmoins très sensible à la fertilisation azotée.

En conclusion, l'expérience FACE en Suisse a démontré que l'enrichissement en pCO_2 entraînait de multiples changements dans un écosystème prairial. Ces changements pourraient accroître la séquestration d'azote et de carbone dans l'écosystème. Cependant, les mécanismes moteurs/conducteurs sont peu compris.

1.2. INFLUENCE D'UN ENRICHISSEMENT EN pCO_2 SUR LA MICROFLORE DU SOL

La concentration en CO_2 dans le sol est de plus de 1000 ppm alors que dans l'atmosphère elle est d'environ 360 ppm. Il n'y a donc aucune raison pour que l'enrichissement en pCO_2 atmosphérique ait un effet direct sur le sol et les bactéries telluriques. L'effet d'un traitement en pCO_2 de 600 ppm ne pourrait s'exercer dans le sol que de manière indirecte par l'intermédiaire des racines de la plante. L'enrichissement en pCO_2 atmosphérique stimule la croissance et l'activité racinaire de la plus part des plantes (Curtis et coll, 1990 ; Norby, 1994 ; Prior et coll. 1995 ; King et coll, 1996), et accroît l'exsudation, *via* les racines, de composés carbonés dans le sol (Rogers et coll. 1994, Wheihong et coll. 2000). De tels apports, représentent une source de carbone supplémentaire facilement assimilable et utilisable pour le développement et la croissance des microorganismes du sol (Cf. 1.3.4). En d'autres termes, cette augmentation de l'allocation de carbone dans sol est susceptible d'avoir un impact sur les communautés bactériennes associées. Dans ce paragraphe nous aborderons les différents résultats observés concernant la réponse de la microflore du sol à un enrichissement en pCO_2, dans le système FACE de Suisse mais aussi dans d'autres études.

1.2.1. BIOMASSE ET ACTIVITES MICROBIENNES

Des études à court et moyen terme montraient un impact significatif d'un enrichissement en pCO_2 sur les microorganismes du sol. La biomasse bactérienne associé à *L. perenne* accroissait de 42% après trois mois d'exposition à une pCO_2 élevée comparée à celle observait pour le pCO_2 ambiante (Van Ginkel et coll. 2000 ; Van Ginkel et Gorissen, 1998), et de 15% dans des champs agricoles soumis à 2 années de rotation blé-soja (Islam et coll. 2000). Dans des pâturages fertiles gréseux soumis à 5 années d'enrichissement en pCO_2, la biomasse microbienne du sol augmentait simultanément à un accroissement de l'assimilation d'azote par la plante. L'effet net de ce phénomène diminuait l'azote disponible pour les microorganismes, diminuait la respiration microbienne et la décomposition microbienne (Hu et coll. 2001, Cf. Chapitre 3 § 3.3). Par conséquent, ces écosystèmes présentaient une augmentation nette de l'accumulation de carbone dans le sol sous fort pCO_2. De même, le carbone de la biomasse microbienne augmentait de 4% dans des hauts pâturages après 5 années de fumigation de pCO_2, ce qui contribuait à une augmentation du carbone total dans le sol de 8% (Williams et coll. 2000). D'autre part, le nombre de bactéries cultivables augmentait sous pCO_2 élevée alors que le nombre total diminuait par rapport au pCO_2 ambiant (Hodge et coll. 1998), et le nombre de bactéries augmentait significativement dans la rhizosphère associée aux monocultures de *L. perenne* et *T. repens* enrichies pendant 2 années en pCO_2, mais restait inchangé dans le sol distant (Marilley et coll. 1999). Finalement, l'activité des bactéries, des protozoaires, des champignons et des arthropodes était stimulée sous pCO_2 élevée (Arnone et Bohlen, 1998 ; Weihong et coll. 2000 ; Hungate et coll. 2000 ; Lussenhops et coll. 1998 ; Williams 2000), entraînant des modifications de la structuration du sol (Rillig et Allen 1998 ; Rillig 1999).
A l'inverse, sur des expérimentations d'enrichissement en pCO_2 à court terme, des résultats montraient qu'une pCO_2 élevée n'avait pas d'impact significatif sur les microorganismes du sol. L'enrichissement en pCO_2 pendant 2.5 années, d'une plantation de *Populus tremuloides* fortement ou faiblement fertilisée en azote, ne modifiait pas la biomasse bactérienne du sol, ni le taux de minéralisation de l'azote microbien, ni la demande en azote inorganique des microbes. A fort amendement en azote, la biomasse microbienne augmentait de 5 fois par rapport au faible niveau de fertilisation, sans influence de la pCO_2 élevée (Zak et coll. 2000). La proportion de carbone photosynthétique allouée à la biomasse microbienne n'était pas modifiée sous pCO_2 élevée alors que la quantité de carbone non microbien de la rhizosphère augmentait de plus de 2.6 fois (Griffiths et coll. 1998).

1.2.2. STRUCTURE DES POPULATIONS MICROBIENNES

Des études sur *L. perenne* dans le FACE (Griffiths et coll. 1998) et un écosystème tropical artificiel (Insam et coll. 1999) ne montraient aucun changement de la structure des communautés microbiennes associées sous pCO_2 élevée. A l'inverse, d'autres études montraient que la pCO_2 élevée favorisait les populations bactériennes affiliées au genre *Rhizobium* dans les sols associés à la légumineuse *T. repens* (Marilley et coll. 1999). De plus, la structure génétique des populations de *Rhizobium* était modifiée, c'est-à-dire que des espèces différentes étaient favorisées sous pCO_2 élevée par rapport au pCO_2 ambiant, et permettaient la formation de 17% de nodules supplémentaires (Montealegre et coll. 2000). A la différence de *T. repens,* dans les sols associés à *L. perenne* en monoculture, les populations bactériennes affiliées au genre *Pseudomonas* étaient favorisées sous pCO_2 élevée (Marilley et coll. 1999).
Les bactéries du genre *Rhizobium* améliorent la disponibilité en azote pour la plante, *via* la fixation symbiotique d'azote atmosphérique (N_2); de la même manière certaines espèces de *Pseudomonas* sont connues comme promotrices de la nutrition et la croissance des plantes par des mécanismes autres que la fixation d'azote (Cf. Chapitre 3.1). Ainsi les populations bactériennes impliquées dans l'amélioration de croissance de la plante semblaient favorisées sous pCO_2 élevée.

En résumer, l'augmentation du flux de carbone vers le sol en relation avec la disponibilité en azote, pourrait modifier la biomasse, les activités, le nombre et la structure des populations microbiennes dépendantes des sources carbonées dérivées de la plante pour leur existence. Une microflore plus abondante et active pourrait ainsi mobiliser plus efficacement les éléments minéraux du sol, les rendant rapidement disponibles pour la plante (Zak et al 1993 ; Wheihong et coll. 2000).

1.3. LA RHIZOSPHERE

1.3.1 DEFINITION

La rhizosphère est décrite à travers les travaux du début du 20ième siècle comme la région de « la matrice du sol » où la microflore (biomasse et activité microbienne) est stimulée par les racines de la plante (Hiltner 1904 ; Rovira et Davey 1971). La progression des recherches en écologie a montré que la rhizosphère inclut davantage que les seules interactions microbiennes avec la plante, et peut être définie plus justement comme le volume de sol influencé par l'activité racinaire (Hinsinger 1998 ; Darrah 1991). Par exemple, les racines de la plante sont actives dans l'absorption de l'eau et des ions, et engendrent de forts gradients de potentiel hydrique, et des changements dans l'acidité ou l'alcalinité de la rhizosphère, ceci affectant directement la microflore à son voisinage (Darrah 1991). Dans cette région, les nutriments solubles comme le nitrate et les composés volatiles sont présents à quelques millimètres de la surface de la racine, et les nutriments non mobiles comme le phosphate à moins d'un millimètre. L'extension spatiale de la rhizosphère est variable, elle dépend de la structuration, de la taille des particules, du contenu en eau, et de la capacité tampon du sol (Darrah 1993 ; Nye 1984). Ainsi, suivant le type de plante et de sol, la rhizosphère peut clairement se matérialiser comme un manchon de sol adhérent à la racine (Figure 1.2). Dans la rhizosphère *sensus lato* on distingue: l'endorhizosphère (intérieur de la racine), le rhizoplan (surface racinaire), l'exorhizosphère ou sol rhizosphérique (sol lié à la racine par opposition au sol distant) (Gobat et coll. 2003).

1.3.2. ZONE D'INTERACTION ENTRE LA PLANTE ET LE SOL

Les caractéristiques chimiques et biologiques de la rhizosphère diffèrent fortement de celles du sol non adhérent. Elle constitue un milieu complexe où sol, plantes, et microorganismes interagissent en permanence. Une propriété importante des plantes est leur capacité à modifier la rhizosphère au cours de leur croissance. Une plante peut modifier sa rhizosphère

par l'assimilation de certains ions et par le relargage d'autres ions et de photosynthétats qui stimulent la microflore (Rovira 1965 ; Cf. 1.3.4). L'activité racinaire change donc les propriétés physico-chimiques de la rhizosphère de différentes manières résumés dans la Figure 1.3:

- L'absorption de l'eau, des minéraux et nutriments du sol entraîne la modification du pH et du potentiel redox (capacité tampon) (Marschner et coll. 1987).
- La respiration racinaire et microbienne fait varier les teneurs en pression partielle d'O_2 et CO_2 dans la rhizosphère par rapport au sol nu (Højberg et Sørensen, 1993), les processus bactériens anaérobies, comme la dénitrification, y seront favorisés (Ghiglione et coll. 2000).
- La qualité/composition et la quantité des dépôts racinaires influencent notamment la qualité de la microflore (Darrah 1991) et des polysaccharides bactériens impliqués dans la structuration du sol au voisinage des racines (humine microbienne, agrégats ; Forster 1990). La structure du sol, est elle-même impliquée dans la circulation de l'eau (de la solution du sol) et les échanges ioniques entre le sol et la plante (Dakora et coll. 2002).

Figure 1.2. Définition physique « expérimentale » de la rhizosphère de *Lolium perenne*. a) système racinaire avec son sol rhizosphérique, b) système racinaire débarrassé de son sol rhizosphérique. (Source : Jossi M.)

1.3.3. RHIZODEPOSITION

La rhizodéposition est définie comme les composés libérés par les racines (Whipps et Lynch, 1990; Marschner 1995). Ces derniers sont classés en différents groupes suivant leur mode d'excrétion (Lynch et Whipps, 1990; Kuzyakov 2002). On distingue généralement (Figure 1.4):

1. *les exsudats* : composés solubles dans l'eau, de faible poids moléculaire (comme les sucres/photoassimilats, les acides aminés, les acides organiques, les phytosidérophores, les flavonoïdes, les hormones, les vitamines) qui sortent de la racine sans faire intervenir le métabolisme énergétique en suivant le gradient de concentration entre le cytosol de la racine et la solution du sol. La part des exsudats est la plus importante dans la rhizodéposition (Kuzyakov et Demin, 1998 ; Gobat et coll. 2003).
2. *les substances sécrétées* : composés de poids moléculaire le plus souvent élevé (comme le mucilage, les polymères de carbohydrates et les enzymes), ils dépendent de processus métaboliques (contre les gradients de potentiel électrochimique et chimique) pour leur libération dans le sol.
3. *les lysats* : libérés quand les cellules s'autolysent, ils incluent les cellules desquamées de la coiffe et les membranes cellulaires.
4. *les gaz* : comme l'éthylène et le CO_2, et autres composés volatiles.

La rhizodéposition représente une part importante de l'assimilation photosynthétique de la plante (Lynch et Whipps, 1990). 30 à 60 % du carbone net fixé par la plante sont transférés aux racines (Darrah 1996), dont 4% à 70% peuvent être relarguer dans la rhizosphère (Lynch et Whipps, 1990). Les plantes pérennes investissent davantage de leur productivité dans le matériel racinaire que les plantes annuelles. Ceci peut être relié à leur besoin de supporter des

périodes prolongées de stress (El-Shatnawi et coll. 2001). Les graminées investissent entre 10 et 50 % des photosynthétats dans l'exsudation racinaire (Paterson et coll. 1997), et le transfert de carbone dans le sol par *L. perenne* (graminée perenne) est chiffré entre 8% et 28% du carbone assimilé (Kuzyakov et coll. 1999). La rhizodéposition peut varier de manière importante tant au niveau qualitatif que quantitatif selon la zone racinaire considérée (Darrah 1991 ; Figure 1.4). Elle génère des habitats et des ressources hétérogènes pour les microorganismes du sol (Stanton 1988 ; Kuzyakov 2002). Ces rhizodépots sont plus ou moins disponibles pour les microorganismes en tant que source de C au voisinage de la racine. Les exudats (groupe 1) sont utilisés par les microorganismes en quelques heures (Kuzyakov et Demin 1998). Leur solubilité dans l'eau, leur mobilité, ainsi que leur incorporation rapide dans les cellules microbiennes impliquent un effet qualitatif et quantitatif des exudats sur la microflore de la rhizosphère (Schenk 1976), et par conséquent sur le changement du taux de décomposition de la matière organique du sol dans l'environnement direct de la racine (« rhizosphere priming effect », Kuzyakov 2002). Les rhizodépots du groupe (2) et (3) et plus particulièrement les parois cellulaires et les molécules de poids moléculaire élevés, sont assimilés et utilisés moins rapidement par les microorganismes.

1.3.4. Interaction entre la plante et les microorganismes

Les racines de la plante, *via* la rhizodéposition, sont la source clef d'énergie et de nourriture pour les microorganismes présents dans la rhizosphère (Whipps 2001). Ainsi la rhizosphère peut être vue comme un « oasis dans le désert » : jusqu'à 10^9 bactéries sont dénombrées par gramme de racine, ce qui représente des populations de bactéries 5 à 15 fois plus élevées dans la rhizosphère que dans le sol non adhérent. La présence de certaines espèces bactériennes peut être stimulée à hauteur de 100 fois ou plus dans le sol rhizosphérique comparé au sol non rhizosphérique. De même, le taux de réplication de certaines bactéries peut être augmenté. Cependant, le fonctionnement de la rhizosphère reste mal connu en dépit de son importance écologique (implication dans les cycles géo-biochimiques du carbone, de l'azote et du phosphore, et sa répercussion sur la croissance et la santé de la plante). Ainsi, seuls quelques pourcent des microorganismes associés aux plantes sont cultivables et identifiés, cette fraction connue de la microflore végétale représentant tout de même plusieurs milliers d'espèces bactériennes cultivables.
Les populations microbiennes présentent dans la rhizosphère profitent des l'exsudats racinaires et colonisent les racines (Azcon et Ocampo, 1981 ; Same et coll. 1983 ; Goddard et coll. 2001). Les microbes qui interviennent dans l'environnement de la rhizosphère pourraient aussi utiliser les composés mineurs des rhizodépots (hormones, flavonoïdes, composés phénoliques) comme signaux qui guident leurs mouvements vers la surface racinaire et élicitent les changements dans l'expression de gènes appropriés à cet environnement (Bauer et Caetano-Anolles 1990).

Les bactéries particulièrement bien adaptées à la vie dans la rhizosphère sont appelées rhizobactéries. Les rhizobactéries qui colonisent les racines peuvent être :

- saprophytes
- délétères (DRB, deleterious rhizobacteria), c'est le cas des bactéries pathogènes (Suslow et Schroth, 1982).
- bénéfiques, c'est le cas des bactéries qui favorisent la croissance de la plante (PGPR, pour Plant Growth Promoting Rhizobacteria) (Cf. Chapitre 3.1).

Les PGPR peuvent affecter la nutrition de la plante par la fixation d'azote atmosphérique, par la solubilisation de minéraux comme le P, par la production de sidérophores qui solubilisent et séquestrent le fer (Glick 1995 ; Chapitre 3.1), ou encore par la structuration du sol rhizosphérique (Alami et coll. 1999). Elles peuvent aussi modifier la morphologie et la physiologie racinaire par la production de régulateurs de croissance à de très faibles concentrations, comme les hormones (Arshad et Frankenberg, 1998 ; Persello-Cartieaux et coll. 2002). La modification des équilibres hormonaux de la plante peut également améliorer la nutrition de la plante (par exemple l'auxine stimule la production de racines secondaires et de poils absorbants). Les PGPR peuvent également agir dans la protection de la plante par la production de métabolites secondaires (antibiotiques, HCN, sidérophores).

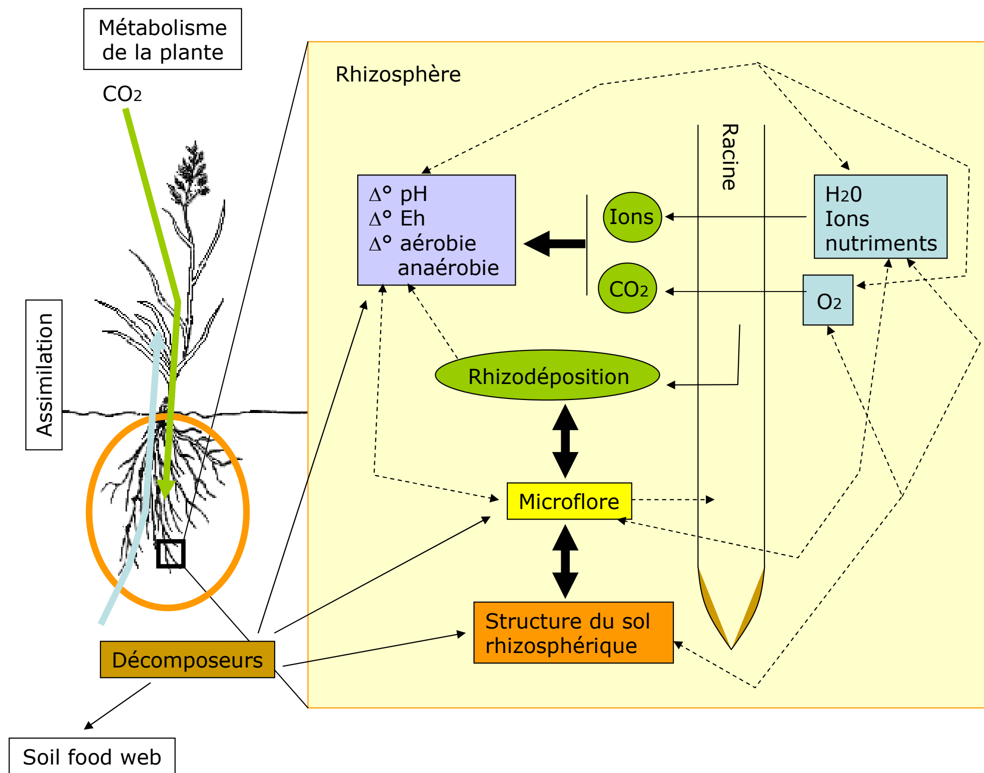

Figure 1.3. Schéma simplifié des interactions physico-chimiques et biologiques dans la rhizosphère

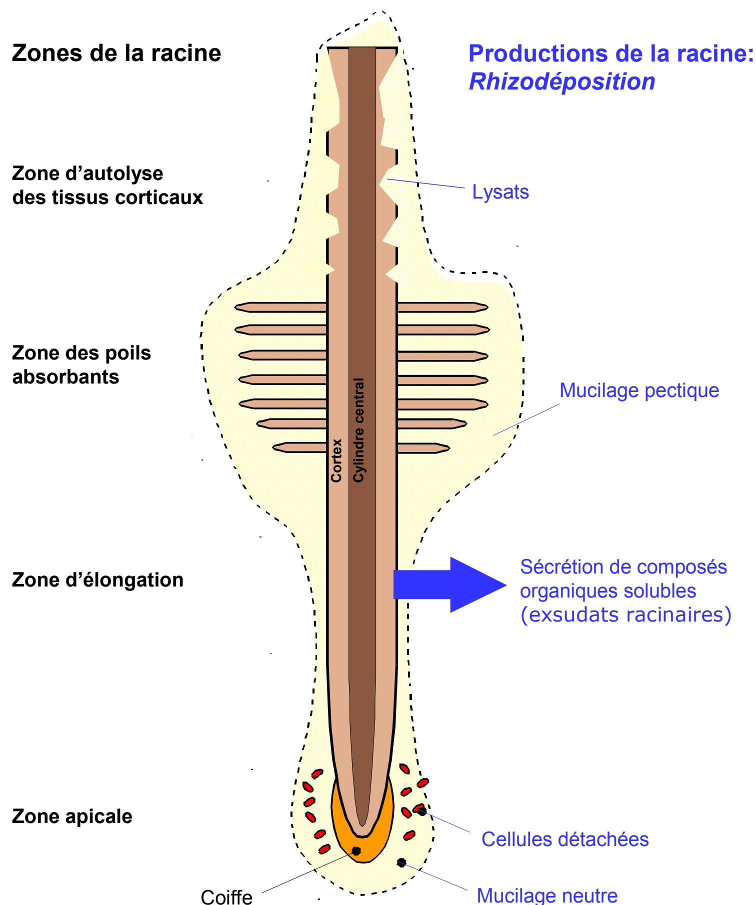

Figure 1.4. Diagramme simplifié d'une racine fine et des rhizodépots en fonction de la structure racinaire. (Source : Aragno et Tarnawski, 2002b)

Certaines rhizobactéries affectent la croissance de la plante par plus d'un de ces mécanismes, et utilisent leurs différentes capacités à promouvoir la croissance à différents stades du cycle de développement de la plante (Bowen et Rovira, 1999). Ainsi, les microorganismes de la rhizosphère peuvent augmenter l'assimilation des nutriments et la translocation dans les plantes jeunes, mais ils peuvent aussi entrer en compétition pour les nutriments avec des plantes plus âgées (Bowen et Rovira, 1999, Hu et coll. 2001, Chapitre 3.3).
Les bactéries du genre *Pseudomonas* sont particulièrement bien adaptées à la rhizosphère (Rainey, 1999) et sont souvent utilisées comme modèle de rhizobactérie (Sorensen 2001). Les *Pseudomonas* spp. constituent une part importante (jusqu'à 10%) de la microflore rhizosphérique cultivable (Kragelund et coll. 1996 ; Chapitre 2.2). Enfin, de nombreuses activité PGP impliquées dans la compétence racinaire sont décrites chez certaines souches (Voisard et coll. 1989 ; Blumer et Haas, 2000 ; Glick 1995).
Par ailleurs, des études de microscopie électronique classiques aussi bien que l'utilisation de souches marquées ont montré une distribution non uniforme des bactéries sur la racine. Lugtenberg et coll. (1999) ont montré que la souche de *Pseudomonas* WCS365 peuplait densément les espaces de jonctions entre les cellules épidermiques racinaires, certaines parties de la surface épidermique, et les sites d'apparition de racines, c'est-à-dire des sites présumés de l'exsudation.

En résumé, la rhizosphère est une zone privilégiée d'interaction entre les microorganismes, la plante et le sol. Elle est le siège d'une activité importante qui a des répercutions sur la diversité, le turn-over et l'activité des populations bactériennes résidentes. Celles-ci, en retour modifient l'environnement racinaire et l'équilibre entre la plante et le sol.

1.4. L'ECOLOGIE MICROBIENNE AU LABORATOIRE DE MICROBIOLOGIE DE L'UNIVERSITE DE NEUCHATEL

1.4.1. LES PLANTES MODELES

Nous avons étudié comme modèles deux graminées pérennes hémicryptophytes de la famille des Poacées : *Lolium perenne* L. (ivraie), et *Molinia coerulea* (canche bleue) (Figures 1.5 et 1.6). Toutes deux possèdent un système racinaire souvent mycorhizé avec de nombreuses racines fines.
On retrouve *M. coerulea* naturellement en prairies dense *quasi* monospécifique sur des sols à pH variables et à des altitudes différentes. *M. coerulea* est bien adaptée au milieu ayant de fortes variations de la nappe phréatique. Elle est caractéristique des sols pauvres en azote disponible, elle assimile lentement l'azote et possède un système efficace de recyclage de l'azote avant la saison hivernale (Vàsquez de Aldana et Berendse, 1997). L'ivraie quand à elle est une espèce vivace fourragère. Exigeante en azote, elle se développe sur des sols riches ou fertilisés dont le pH est proche de la neutralité. On la retrouve dans les prairies et pâturages. Elle ne supporte pas la sécheresse ni les submersions prolongées. *M. coerulea* et *L. perenne* présentent donc des caractéristiques écologiques bien différentes, notamment vis-à-vis de l'azote, ceci suggérant des rhizosphères associées différentes.

1.4.2. LES SYSTEMES EXPERIMENTAUX

1.4.2.1. LA ROSELIERE DE CUDREFIN

Le site d'étude de *M. coerulea* est une prairie à molinie (Photographie 1.1) située dans la réserve naturelle de la Grande Cariçaie, en rive sud du lac de Neuchâtel. Cet écosystème s'est installé après les travaux de détournement de l'Aar et de drainage qui ont permis d'assécher 400 km^2 de marécages dans la région du Seeland entre 1868 et 1891. Le 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 un Gleysol Typique Haplaquoll, le régime hydrique et la végétation ont été caractérisés (Buttler 1987).

DESCRIPTION

Hauteur : 20-70 cm
Base du limbe gén. auriculée
Ligule longue d'environ 1 mm
Epi long de 4-20 cm, axe lisse et flexueux
Epillets longs de 7-15 mm, comprimés, sessiles, appliqués par le coté étroit à l'axe de l'épi

ECOLOGIE

L'ivraie vivace est une espèce fourragère exigeante en azote qui se développe sur des sols riches dont le pH est proche de la neutralité. Elle ne tolère pas la sécheresse ni la submersion prolongée. On la trouve dans les prairies et pâturages

Carl Axel Magnus Lindman :
Bilder ur Nordens Flora (1901-1905)

Figure 1.5. ***Lolium perenne***

DESCRIPTION

Hauteur : 30-100 cm
Tige à un seul nœud
Ligule remplacée par une couronne de poils
Epillets longs de 4-6 mm, verts à violet foncés
Etamines violet noirâtres

ECOLOGIE

La molinie bleue est une espèce indicatrice de conditions oligotrophes et présente une large amplitude écologique. On la trouve dans les marais, tourbières, prairies marécageuses, forêts claires….

Carl Axel Magnus Lindman :
Bilder ur Nordens Flora (1901-1905)

Figure 1.6. ***Molinia coerulea***

L'alternance de conditions oxiques et anoxiques, induites par le battement de la nappe phréatique, engendre un cycle de nitrification et dénitrification intense appauvrissant le sol en azote et permettant un peuplement de molinie très homogène (Buttler 1987).

Photographie 1.1. Prairie naturelle, Grande Cariçaie, rive sud du lac de Neuchâtel

1.4.2.2. LE SITE EXPERIMENTAL FREE AIR CO_2 ENRICHMENT (FACE) D'ESCHIKON

Le site expérimental FACE est localisé à Eschikon (ZH, suisse) près de Zurich, à une altitude de 550 mètres. Le système FACE est une installation en plein air qui permet d'enrichir l'atmosphère en CO_2. Il a permis de suivre l'influence du CO_2 sur la végétation de 1993 à 2002.

Le dispositif FACE est constitué de deux types de parcelles cultivées :

- 3 parcelles contrôles à 350 ppm de pCO_2, soit les conditions ambiantes (C1, C2, C3)
- 3 parcelles enrichies à 600 ppm de pCO_2 (T1, T2, T3)

Chaque parcelle circulaire est entourée de buses à CO_2 (Figure 1.7). Le CO_2 provenant d'un réservoir de stockage est mélangé à de l'air insufflé à travers des conduits souterrains. Ces conduits sont reliés à des tuyaux verticaux contenant des valves. La direction et la vitesse du vent sont mesurées et enregistrées par un ordinateur pour contrôler l'ouverture des valves. Si la vitesse du vent est supérieure à 0.4 m/s, les valves qui sont dans sa direction s'ouvrent. En cas de vent faible, les valves s'ouvrent sur tout le pourtour de la parcelle. La concentration en CO_2 est mesurée au centre et sert à contrôler le débit de CO_2 insufflé.
Les parcelles étudiées consistent en des monocultures de *L. perenne* cv. Bastion, mesurant 2.8 m X 1.9 m. Le sol est un Cambisol Eutrophe, il présente des valeur de pH comprises entre 6.5 et 7.6 et contient 28% d'argile, 33% de limon , 36% de sable et 2.9% à 5.1% de matière organique (Hebeisen et coll. 1997). Les parcelles sont fertilisées avec P_2O_5 (12 g. $m^{-2}.an^{-1}$), K_2O (29 g. $m^{-2}.an^{-1}$), Mg (1.6 g. $m^{-2}.an^{-1}$) et NH_4NO_3 (10 et 42 g. m^{-2}. an^{-1} N en 1993 ; 14 et 56 g. m^{-2}. an^{-1} N depuis 1994). En 1995 les parcelles étaient fauchées 4 fois, et à partir de 1996: 5 fois. Pour compenser les différences de disponibilité en nutriment, la quantité d'engrais (P K Mg) a été augmentée de 35% dans les parcelles C3 et T3 (Marilley 1998).

Des mottes de molinie de la prairie littorale (1.4.2.1), constituées de la plante avec ses racines et son sol, ainsi que 35 cm de profil de sol reconstitué, ont été transférés, par J. Hamelin et N. Fromin, dans les parcelles C3 (contrôle) et T3 (enrichie) des installations FACE en septembre 1999. Pour chacune des parcelles, les molinies ont été placées dans 3 caisses (~60 cm X 50 cm) en plastique perforées au fonds, sur une couche de gravillons, une toile de verre, puis le

profil de sol ramené de la prairie (Photographie 1.2). Durant toute l'étude, les molinies n'étaient ni fauchées ni fertilisées.

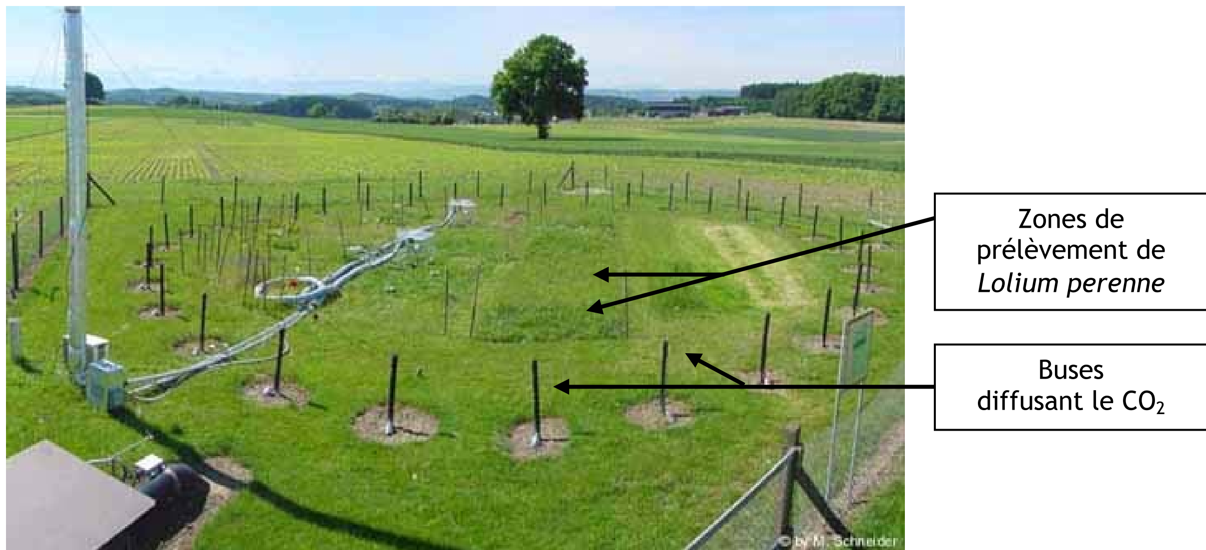

Figure 1.7. Parcelle enrichie en pCO_2 du système FACE. (Source : M. Schneider, ETHZ)

Photographie 1.2. Carré de molinies transféré dans le système FACE à Eschikon. (Source : LAMUN)

Cette «transplantation» nous donnait l'occasion unique de comparer, sous atmosphère enrichie en CO_2, *M. coerulea* (oligonitrophile) dans son sol naturel, et *L. perenne* (nitrophile) en gestion fourragère traditionnelle. Etant données leur écologie bien distincte, leur microflore propre pourrait être affectée différemment en réponse à l'enrichissement de la pCO_2 atmosphérique.

Le système FACE simule une augmentation brusque de la pCO_2 dans l'atmosphère (360 à 600 ppm). A la différence d'autres systèmes expérimentaux tel que les « open-top chambers », le système FACE est applicable pour des études au champ, et qui plus est à long terme. Le suivi de l'influence d'une pCO_2 élevée sur la végétation se fait avec un contrôle précis de la concentration en CO_2, sans interférer avec le vent, l'évapotranspiration, le rayonnement solaire, l'humidité relative, la température et les paramètres édaphiques naturels de l'écosystème.

1.4.3. LES COMMUNAUTES BACTERIENNES ASSOCIEES À *LOLIUM PERENNE* ET *MOLINIA COERULEA*

Suite aux travaux de thèse de L. Marilley (1999), la thématique « influence de l'enrichissement en CO_2 atmosphérique sur la microflore associée aux plantes pérennes dans le système expérimental FACE » a donné lieu à la formation d'un groupe de travail dynamique au sein du laboratoire de Microbiologie. Les études ont été menées en parallèle selon 3 axes de recherche complémentaires. Je les présenterai dans ce paragraphe en développent plus particulièrement

les points qui ont apporté des réponses sur l'influence d'un enrichissement en pCO_2 sur la microflore associée à *L. perenne* et *M. coerulea* (8 et 3 années d'expérimentation).

1.4.3.1. Dynamique du groupe «FACE » au Laboratoire de Microbiologie

Coordinatrice du groupe « FACE » : Nathalie Fromin

Etudes globales des communautés bactériennes associées à la rhizosphère de plantes pérennes, et réponse à une augmentation en pCO_2 (Thèse de Jérôme Hamelin, Travail de diplôme de Maryline Jossi)

- Analyse statistique des approches par fingerprinting (DGE, Denaturing Gradient Electrophoresis) pour la description et le suivi des communautés bactériennes (Fromin et coll. 2002 –Annexe 1)
- Recherche d'indices spécifiques pour l'analyse des profils génétiques de communautés (Hamelin et coll. en préparation)
- Comparaison des communautés bactériennes totales, présentes et actives, associées à *L. perenne* et *M. coerulea* cultivées sous différentes pCO_2 (Jossi et coll. en préparation)
- Modification des profils métaboliques (BIOLOG) des communautés bactériennes associées à *M. coerulea* cultivée sous différentes pCO_2 (Hamelin et coll. en préparation)

Etude fonctionnelle - Diversité des bactéries non-symbiotiques fixatrices d'azote de la rhizosphère de *Molinia coerulea* (Thèse de J. Hamelin 2003)

- Diversité des gènes *nifH* des bactéries associées à la rhizosphère de *M. coerulea* (Hamelin et coll. 2002 – Annexe 2)
- Mise au point de nouvelles amorces pour la détection d'un pool de gènes *nifH* particulier spécifiquement exprimés dans les racines de *M. coerulea* (Hamelin, Thèse)

Etude d'un genre bactérien modèle - Caractérisation des populations de *Pseudomonas* spp. associées à la rhizosphère de plantes pérennes, et réponse à une augmentation en pCO_2 (Thèse de Sonia Tarnawski ; Travaux de diplôme de Laurent Locatelli et Ludovic Roussel-Delif)
Cet aspect fait l'objet du présent travail (cf. 1.5), et sera donc détaillé au cours du manuscrit

1.4.3.1. Principaux résultats

Dans l'étude des communautés bactériennes présentes (basée sur l'analyse du gène d'ARNr 16S) et actives (basée sur l'analyse des transcrits d'ARNr 16S) associées à *L. perenne* et *M. coerulea*, nous avons noté que les profils de communautés bactériennes révélés par approche de fingerprinting restaient très influencés par le plot et la date de prélèvement des échantillons. Néanmoins, les communautés bactériennes des plantes cultivées sous pCO_2 ambiante étaient significativement différentes de celles des plantes cultivées sous pCO_2 élevée. Ces différences étaient généralement plus marquées pour les communautés actives comparées aux communautés présentes (et abondantes), et également plus marquées pour la communauté de la rhizosphère que pour celle du sol. Ces résultats indiquent que la plante a une forte influence sur les communautés qu'elle héberge dans sa rhizosphère, et que l'influence de la pCO_2 se faisait *via* la plante. Par ailleurs, une majorité des populations répondant particulièrement bien à la proximité de la racine et à la pCO_2 élevée étaient affiliées aux δ-*protéobactéria*. Dans le sol, les actinobactéries paraissaient les plus actives.
L'étude des profils métaboliques des communautés bactériennes associées à *M. coerulea,* cultivée sous pCO_2 élevée et ambiante, n'a révélé aucun changement substantiel d'utilisation des substrats BIOLOG pour les communautés bactériennes du sol. Par contre, celles associées aux racines sous pCO_2 élevée tendaient à mieux métaboliser certaines sources de carbones, et plus spécialement les carbohydrates (dans la proportion est augmentée dans les exsudats racinaires sous pCO_2 élevée). Finalement l'analyse fingerprint des communautés bactériennes des cultures d'enrichissement BIOLOG a mis en évidence, pour un même substrat, la présence de plusieurs guildes. Cette tendance à la redondance métabolique était observée pour l'utilisation de la plupart des substrats.

En résumé, il semble que la microflore associée aux racines des plantes, à la différence de celle du sol, répond particulièrement bien à un enrichissement en pCO_2, tant au niveau de la structure des populations que de leur activité. Ces études ont toutes deux confirmées notre hypothèse de travail (Cf. 1.2), suggérée par Marilley et coll. (1999), selon laquelle une pCO_2 élevée devrait influencer en premier lieu les communautés bactériennes proches de la racine, l'effet de l'augmentation en pCO_2 dans l'atmosphère sur la microflore se faisant indirectement par l'intermédiaire de la rhizodéposition.

1.5. SYNTHESE ET OBJECTIFS DU TRAVAIL DE THESE

Il ressort plusieurs points important de cette étude bibliographique :

- Sous pCO_2 élevée, **la photosynthèse est stimulée** ainsi que la production de biomasse de la plante. Le ratio des rendements en biomasse des parties racinaires/parties aériennes de *L. perenne* est accru surtout quand les conditions de carence en azote sont fortes.
- **Le flux de carbone dans le sol est également stimulé** ainsi que le taux de carbone incorporé dans la biomasse microbienne des prairies de *L. perenne*. Ce flux de carbone semblait lié à la disponibilité en azote et à la défoliation.

  → Dans tous les cas une augmentation de la pCO_2 semble modifier l'écosystème en liaison étroite avec la disponibilité en azote pour *L. perenne*. Qu'en est-il pour *M. coerulea* qui est moins dépendante de la disponibilité en azote du sol ?

- Les processus de gestion de l'azote dans le système sol/plante *L. perenne* est mal compris. Néanmoins **les émissions de N_2O mesurées dans la prairie sont augmentées sous pCO_2 élevée**, alors que rien n'est connu pour *M. coerulea*.
- Les **deux plantes étudiées présentent des écologies bien différentes**. L'une est oligonitrophile en prairie naturelle (*M. coerulea*), et l'autre nitrophile en prairie fourragère (*L. perenne*).
- Graminées pérennes en C3, ces plantes investissent *a priori* davantage de leur productivité dans un système racinaire qui reste en place d'une saison de croissance sur l'autre.

  → Elles présentent donc une gestion de l'azote caractéristique, et une rhizosphère tout à fait intéressante et bien différente de celle des plantes annuelles le plus souvent étudiées (blé, maïs, soja…).

- **La microflore du sol répondrait le plus fortement, à un enrichissement de la pCO_2, à proximité des racines de la plante.** La biomasse et l'activité de la microflore étaient augmentées, la structure des communautés bactériennes était modifiée, et **le genre *Pseudomonas* était particulièrement stimulé dans la rhizosphère de *L. perenne***. En effet, au début de l'expérience FACE (1994-98), L. Marilley a mis en évidence une augmentation des populations de *Pseudomonas* dans la rhizosphère de *L. perenne* sous pCO_2 élevée. En est-il de même après 8 années d'enrichissement en pCO_2 ? Qu'en est-il pour *M. coerulea* dont la microflore nous était complètement inconnue à ce jour ?
- **Les organismes de ce genre sont bien adaptés à la rhizosphère, et certains sont impliqués dans la promotion de la croissance de la plante (PGPR).** Les caractères PGP potentiels des populations de *Pseudomonas* spp. présents dans la rhizosphère de ces plantes sont-ils altérés sous pCO_2 élevée ? Est-ce une «guilde» susceptible de participer aux émission d'N_2O (processus de dénitrification) dont le bilan est augmenté sous pCO_2 élevée ? Si oui, quelle est la diversité des espèces de *Pseudomonas* qui y participent ? Cette activité est-elle une fonction répandue parmi les différentes espèces de *Pseudomonas* ?

Dans ce contexte, pour appréhender la microflore *de M. coerulea* (en parallèle des études citées en 1.4.3.1), la diversité des *Pseudomonas* spp. associés à sa rhizosphère a été étudiée dans la prairie naturelle de Cudrefin. Un des objectifs sous-jacent était de développer des outils méthodologiques (applicables aux cultivables et non cultivables) pour la détection et l'étude de la diversité des *Pseudomonas* spp. dans le sol et la rhizosphère (Chapitre 2).

Une seconde étude plus conséquente, reprend la thématique du forçage en pCO_2. Nous avons étudié la structure phénotypique des *Pseudomonas* spp. de la rhizosphère de *L. perenne* et de *M. coerulea* dans les installations FACE, et détaillé plus spécifiquement la diversité des *Pseudomonas* réducteurs de nitrate et dénitrifiants (Chapitre 3).

CHAPITRE 2

Diversité des *Pseudomonas* spp. associés à la rhizosphère de *Molinia coerulea*

Méthodologie appliquée à l'écologie des *Pseudomonas* du sol et de la rhizosphère

CHAPITRE 2

DIVERSITE DES *PSEUDOMONAS* SPP. ASSOCIES A LA RHIZOSPHERE DE *MOLINIA COERULEA*
Méthodologie appliquée à l'écologie des *Pseudomonas*
du sol et de la rhizosphère

INTRODUCTION

Dans l'objectif d'étudier la diversité des *Pseudomonas* associés à la rhizosphère de *M. coerulea*, plusieurs aspects, résumés dans le diagramme 2.1, ont été abordés.

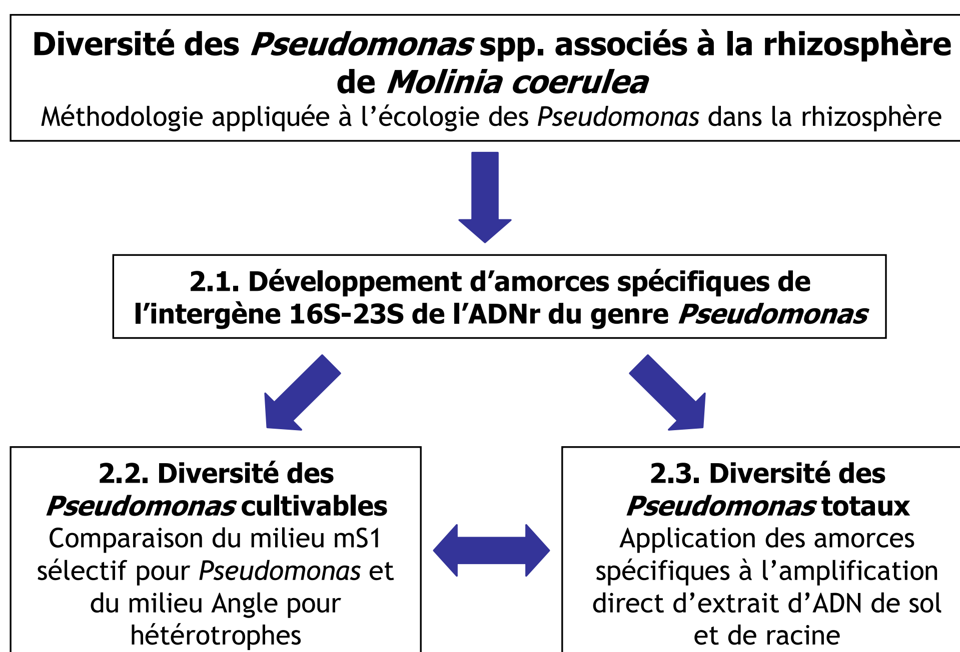

Diagramme 2.1. Vue synthétique de l'organisation des paragraphes du chapitre 2.
L'étude de la diversité des *Pseudomonas* de la rhizosphère de *M. coerulea* en est la ligne directive.

Traditionnellement, les études de la diversité des *Pseudomonas* sont réalisées par isolement sur milieux de culture, et caractérisations génotypiques et/ou phénotypiques des isolats. Cette démarche reste un travail fastidieux et de longue haleine. Nous souhaitions alors développer un nouvel outil pour le suivie des populations de *Pseudomonas* dans l'environnement. Notre attention s'est naturellement porté sur la région de l'intergène 16S-23S de l'ADNr (ITS1). Cette région est plus variable que l'ADNr 16S, et permet une différentiation fine des espèces voir des sous espèces par PCR-RFLP (Cho et Tiedje, 2000 ; Manceau et Horvais, 1997). Des amorces permettant d'amplifier cette région de l'ADNr spécifiquement chez *Pseudomonas* ont étaient définies, et le protocole d'amplification mis au point. Cet aspect sera détaillé dans le paragraphe 2.1.

Pour décrire la diversité des *Pseudomonas* de la rhizosphère de *M. coerulea*, cet outil a été appliqué d'une part à une collection d'isolats de la rhizosphère de *M. coerulea*, d'autre part à l'amplification directe de la séquence ITS1 à partir d'extrait d'ADN total de sol et de racine. Ces deux points seront développés respectivement dans les paragraphes 2.2 et 2.3.
Dans l'étude de la diversité des *Pseudomonas* associés à la rhizosphère de *M. coerulea*, nous nous sommes posés plusieurs questions:
Quelle image de la diversité des *Pseudomonas* est mise en évidence par méthodes culturales classiques ? A *priori*, l'image de diversité obtenue par une approche culturale serait dépendante de la physiologie et du métabolisme des organismes capables de croître sur milieu de culture. Cette hypothèse est testée dans le paragraphe 2.2, en comparant la diversité des populations de *Pseudomonas* décrite par isolement sur un milieu sélectif, et sur un milieu pour hétérotrophes peu sélectif.
Que pouvons-nous attendre d'une approche moléculaire indépendante de la mise en culture ? Etant donné la faible proportion d'organismes cultivables (<1%), une image de diversité plus grande pourrait être décrite avec une approche moléculaire par rapport à l'approche culturale. Cette seconde hypothèse est en partie abordée dans le paragraphe 2.3.

2.1. DETECTION SPECIFIQUE DES *PSEUDOMONAS* PAR AMPLIFICATION DE LA SEQUENCE 16S-ITS1 DE L'ADNr.

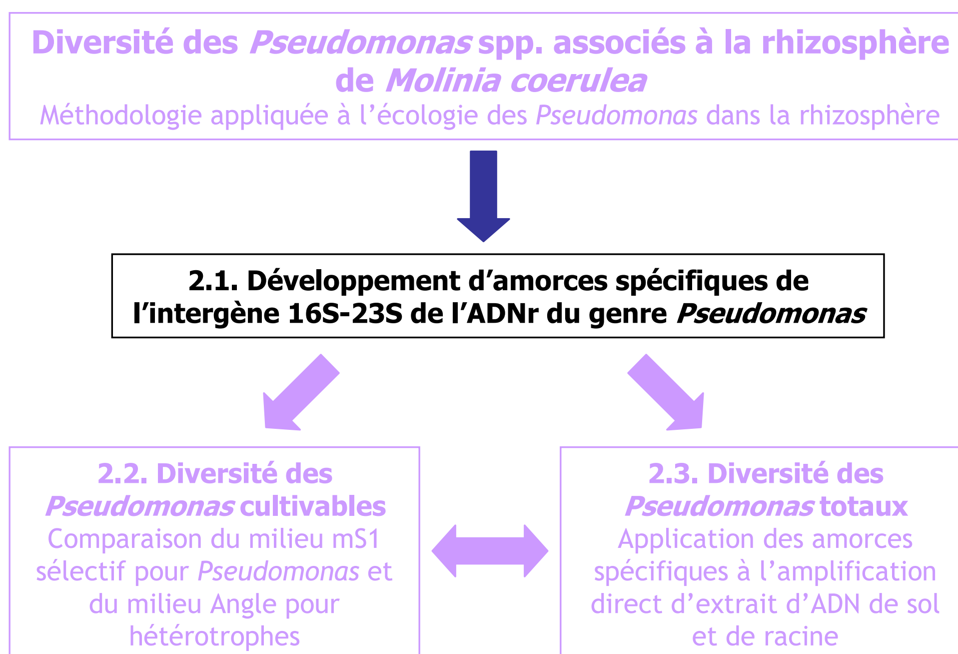

Diagramme 2.2. Vue synthétique de l'organisation des paragraphes du chapitre 2.
L'étude de la diversité des *Pseudomonas* de la rhizosphère de *M. coerulea* en est la ligne directive.

INTRODUCTION

L'étude de la diversité des *Pseudomonas* dans divers environnements nécessite des outils précis et adéquats pour leur détection, et leur caractérisation. Actuellement la plupart des méthodes de détection ciblent l'ADN ribosomique (ADNr). Le plus souvent, la séquence 16S de l'ADNr (codant pour la petite sous-unité des ribosomes) est recherchée par hybridation avec des sondes spécifiques du genre *Pseudomonas*, ou par amplification spécifique. Cette région présente par ailleurs une valeur taxonomique intéressante. Cependant la différentiation, d'organismes très proches, par analyse de restriction de la séquence 16S de l'ADNr, montre ses limites. Le genre *Pseudomonas* présente une grande diversité d'espèces (plus de 100), et la différentiation de souches appartenant à 10 espèces de *Pseudomonas fluorescens* nécessite l'utilisation de 13 enzymes de restriction différentes (Laguerre et coll. 1994), ou un séquençage systématique de la totalité du 16S (soit ~1500 pb). Ceci est peu envisageable dans le cadre d'une étude de diversité et de suivi des populations. Nous nous sommes donc orientés vers la séquence plus variable qu'est l'intergène 16S-23S de l'ADNr (nommé ITS1). Cette séquence a évolué plus vite que celle des ADNr 16S et 23S car elle n'a pas de fonction codante. Elle permettrait, en analyse de restriction, une différenciation d'organismes proches en utilisant peu d'enzymes (Cho et Tiedje, 2000).
Dans ce paragraphe, nous allons donc aborder la mise au point d'amorce spécifique au genre *Pseudomonas*, pour l'amplification de la totalité de l'ITS1 ainsi que la moitié terminale de la séquence 16S de l'ADNr (16S-ITS1).

Les résultats présentés dans ce paragraphe sont issus du travail de diplôme de Laurent Locatelli, réalisé sous la direction de Sonia Tarnawski et Nathalie Fromin.

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Specific PCR Amplification for the Genus *Pseudomonas* Targeting the 3′ Half of 16S rDNA and the Whole 16S–23S rDNA Spacer

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Summary

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.

Key words: *Pseudomonas* – detection – specific primers – 16S–23S rDNA Intergenic Transcribed Spacer – ITS1 – colony hybridisation – soil.

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 (LYCZAC 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 and RAINEY, 1991; RAINEY and TRAVISANO, 1998). Furthermore, studies on environmental isolates revealed the limits of culture media selective for *Pseudomonas* (KRAGELUND et al., 1996; JOHNSEN and 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

Abbreviations:
ITS1 – 16S–23S rDNA Intergenic Transcribed Spacer
RDP – Ribosomal Database Project

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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 and 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.

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 2) were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). 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 and 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 Table 1. They comprise 33 strains of numerous species and subspecies of *Pseudomonas* (Table 1a) as well as 17 strains of non-*Pseudomonas* (Table 1b).

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 (13.000 g for 15 min), 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.

PCR amplification of ITS1

Two primer sets were used for PCR amplification of the ITS1 region (see Fig. 1): 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). For the *Pseudomonas*-specific PCR, the reaction mix contained (final concentrations): 1× 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 · μl^{-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).

Cloning and sequencing of 16S rDNA fragments

16S rDNA genes were 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 vec-

tor (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 and RUSSELL, 2001). The identification of the corresponding organisms was achieved by using a BLAST analysis on the retrieved sequences.

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 (Fig. 1) at locations 756–779 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 γ-proteobacteria 16S rDNA sequences (*Oceanospirillum* group including *Halomonas* and *Marinomonas* genus, *Moraxellaceae*, sulfur-oxidizing symbionts and unidentified γ-proteobacteria). Less than 10% were retrieved from other bacterial groups: 26 sequences from *Rickettsiaceae* (α-proteobacteria) and 22 sequences from δ-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,

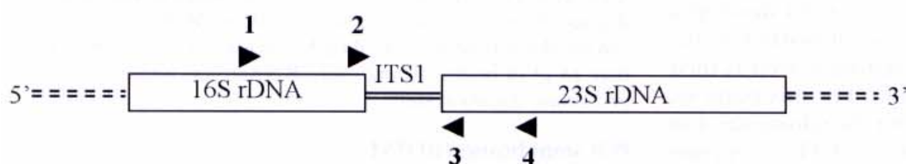

Fig. 1. Primers used for PCR-amplification of ITS1.
1* primer fPs16S (*Pseudomonas*, forward) position 756–779;
2**primer FGPS1472–900 (*Bacteria*, forward) position 1472–1490;
3* primer rPs23S (*Pseudomonas*, reverse) position 1–25;
4** primer FGPL132'38 (*Bacteria*, reverse) position 118–135;
* present study; ** NORMAND et al., 1996.
Positions are given according to *E. coli* numbering on 16S and 23S rDNA respectively.

	1	11	21	31		41
rPs23S (coding sequence)		**GGTCAAGTGA**	**AGAAGCGCAT**	**ACGGT**		
P. fluorescens (AF134704)	....	....	.....GGATG	CCTTG	GCAGT	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

Fig. 2. Multiple nucleotide alignment of the available 5′ end of 23S rDNA sequences of *Pseudomonas* and fPs16S-matching non-*Pseudomonas* species. 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.

Table 1a. *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 9446^T	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 (Switzerland) [1]
Pseudomonas sp.	TM1A3 and TM1A4	tomato (Switzerland) [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 and Johri, 1999.
CFBP – Collection Française de Bactéries Phytopathogènes
type strains are indicated by a ^{T} after the collection number

Table 1b. Non-*Pseudomonas* γ-proteobacterial strains used for primer set validation.

Taxon	Collection number	Family
Azotobacter chroococcum	DSM 374	*Pseudomonadaceae*
Azotobacter chroococcum	DSM 2286	*Pseudomonadaceae*
Azotobacter sp.	DSM 1721, DSM 1722, DSM 1723	*Pseudomonadaceae*
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	*Enterobacteriaceae*
Escherichia coli	NEU 1006*	*Enterobacteriaceae*
Klebsiella oxytoca	NEU 30*	*Enterobacteriaceae*
Proteus vulgaris	NEU 1049*	*Enterobacteriaceae*
Providencia alcalifaciens	NEU 84*	*Enterobacteriaceae*
Salmonella panama	NEU 1065*	*Enterobacteriaceae*
Serratia marescens	NEU 1024*	*Enterobacteriaceae*

* NEU : bacterial collection of the University of Neuchâtel
type strains are indicated by a ^{T} after the collection number
[1] Moebus (1992)

Table 2. 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 (respectively) hybridization with PSM_G probe.
PCR+/PCR–: isolates displaying a positive/negative (respectively) amplification with fPs16S and rPs23S primers.

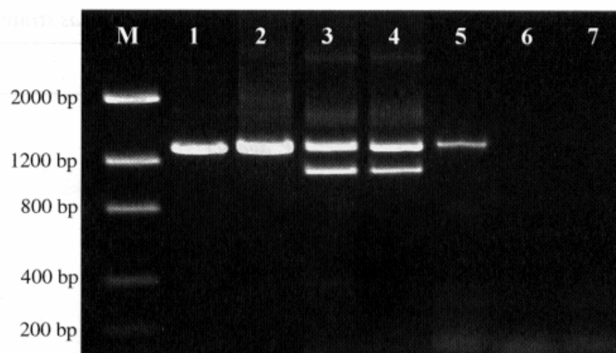

Fig. 3. PCR amplification of collection strain DNA with *Pseudomonas* primers. PCR was performed with fPs16S and rPs23S primers.
M – Low DNA Mass Ladder (Gibco), **1** – *P. asplenii* (LMG 2173), **2** – *P. fluorescens* (ATCC 17513), **3** – *P. fluorescens* (CFBP 2022), **4** – *P. marginalis* pv.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

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 2 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 1a) 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 (Fig. 3). Multiple band patterns of these strains were confirmed by the use of ITS1 *Bacteria* primers. None of the 17 non-*Pseudomonas* collection strains (Table 1b) 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 2. 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).

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., 2000), 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 and HORVAIS, 1997; CHO and 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.

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CONCLUSIONS

Ce travail nous a permis de mettre au point un outil de détection spécifique du genre *Pseudomonas*. Il est adapté à l'amplification d'ADN d'isolats de *Pseudomonas*, mais aussi à l'amplification d'extrait d'ADN totaux de sol et de racine (cf. 2.3). L'analyse de restriction des séquences 16S-ITS1 obtenues sur des échantillons d'isolats ou d'extraits d'ADN totaux, nous a permis d'étudier la diversité de cette population dans les fractions de sol et de racine. Ce point est développé dans les paragraphes qui suivent (2.2 et 2.3).

2.2. DIVERSITE DES *PSEUDOMONAS* CULTIVABLES PRESENTS DANS L'ENVIRONNEMENT RACINAIRE DE *M. COERULEA* DANS LA PRAIRIE NATURELLE.

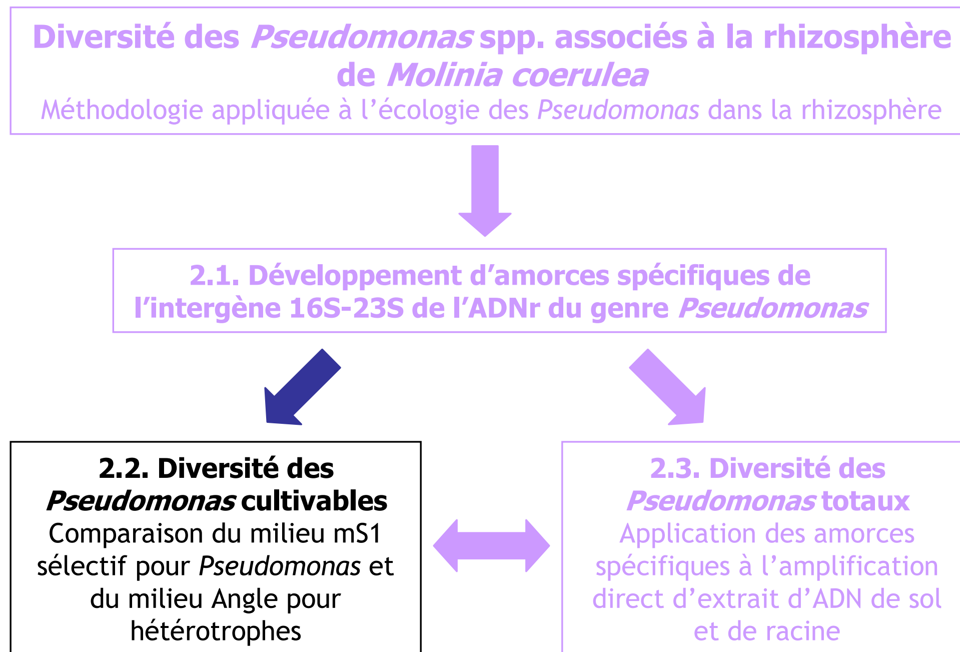

Diagramme 2.3. Vue synthétique de l'organisation des paragraphes du chapitre 2.
L'étude de la diversité des *Pseudomonas* de la rhizosphère de *M. coerulea* en est la ligne directive.

INTRODUCTION

La rhizosphère est une zone privilégiée d'interactions entre la plante et les microorganismes, et les bactéries du genre *Pseudomonas* y sont particulièrement bien adaptées (cf. 1.3.4). Les *Pseudomonas* sont important dans la rhizosphère de *L. perenne* (Marilley et coll. 1999), par contre nous ne connaissons pas son importance dans la rhizosphère de *M. coerulea* (notre seconde plante pérenne modèle, cf. 1.4.1). Dans ce paragraphe, nous allons aborder l'étude des *Pseudomonas* associés aux fractions de sol et de racine de *M. coerulea*, dans la prairies naturelle de Cudrefin (1.4.2.1, cf. aussi 1.4.3 pour les autres études). Les objectifs y sont d'appréhender l'abondance et la diversité de ce groupe bactérien. Une approche culturale a été adoptée pour isoler et estimer la densité des *Pseudomonas*. La diversité des isolats a été décrite par PCR-RFLP (Restriction Fragment Length Polymorphism) en utilisant les amorces spécifiques définies précédemment (2.1). En outre, un regard critique est posé sur l'utilisation de milieux de culture plus ou moins sélectifs pour isoler et décrire la diversité des *Pseudomonas* dans les environnements bien différents que sont le sol et la racine.

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

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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's medium, or on Gould's modified S1 (mS1) *Pseudomonas*-selective medium. Their diversity was assessed by restriction analysis of PCR (polymerase chain reaction)-amplified 16S–23S rDNA internal transcript spacer sequences. The comparison of mS1- and Angle-recovered populations showed that the use of mS1 selective medium led to an underestimation of both *Pseudomonas* counts and diversity, especially in the soil environment.

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Keywords: *Pseudomonas*; Isolation medium; Genetic diversity; 16S–23S ribosomal spacer DNA; PCR–RFLP; Rhizosphere; Perennial grass

1. Introduction

The genus *Pseudomonas* includes several species of general interest, such as human [1] and plant [2] pathogens, xenobiotic degraders [3], plant growth promoters [4] and biocontrol strains [5]. 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 [6], plant-derived compounds released by the roots (rhizodeposition) provide abundant carbon and energy sources [7] 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 [8]. *Pseudomonas* are a significant component of the rhizosphere microflora [9–10], denoting their fitness in the rhizopheric environment [11–13].

Our perception of culturable *Pseudomonas* diversity depends strongly on the isolation procedure used [14–15]. 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* [15–18] 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* [19–21]. The selectivity of Gould's S1 medium is based on an iron limitation, and 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

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high recovery of fluorescent *Pseudomonas* phenotypes from soil samples. Fromin et al. [12] proposed Gould's modified S1 (mS1) medium, without thrimethoprim to allow the growth of non-fluorescent *Pseudomonas*.

Further studies have revealed limitations of these selective media. For instance, Kragelund et al. [9] has shown that *Pseudomonas* counts from barley rhizosphere soil were higher on a non-selective medium (10-fold-diluted tryptic soy agar) than on either Gould's S1 or King's B medium. Comparative investigations using Gould's S1 and King's B media have presented contrasting results. The use of Gould's S1 medium sometimes gave either higher [22] or lower [19] counts of fluorescent *Pseudomonas* than the use of 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 [19]. Furthermore, Aagot et al. [15] 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 [23].

In this study, we investigated the culturable *Pseudomonas* populations in soil and root environments, in a natural meadow dominated by the perennial oligonitrophilic grass *Molinia coerulea*. We compared the influence of selective mS1 medium and non-selective Angle's medium [24] on both quantitative (colony-forming unit (CFU) numbers) and qualitative (genotypic diversity) recovery of *Pseudomonas*.

2. Materials and methods

2.1. Culture media

The *Pseudomonas* selective mS1 medium was similar to S1 medium [18], except that trimethoprim was omitted to allow the growth of non-fluorescent *Pseudomonas*, and that sucrose content was doubled to 20 g l^{-1} [12]. Total heterotrophic aerobic bacteria were enumerated on Angle's medium [24]. This medium was designed to have an ionic strength similar to that found in most non-saline soils. Ion concentrations of Angle's medium were (in mM): 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 carbon source.

2.2. Sampling and isolation

The study site consisted of an oligotrophic littoral meadow dominated by a genetically homogenous population of the perennial grass *M. coerulea* in a Gleysol, Typic Haplaquoll (4.7% clay, 9.5% silt, 85.8% sand, $pH_{[H2O]}$ 8.4) [25]. It is located in the Cudrefin preserved natural area on the southern shore of Lake Neuchâtel (Switzerland), at an elevation of 430 m above mean sea level. In July 2000, three undisturbed soil cores, 2–3 m apart, were collected from the upper 20 cm of soil and immediately analysed. These cores were pooled and then divided into two fractions: soil fraction constituted by soil devoid of root material. The root fraction, corresponding to the 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. Ten-fold 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. CFU, at a proper dilution (20–200 colonies per Petri dish), were enumerated after 72 h of incubation at 24°C.

2.3. Strain handling and cultivation

From mS1 medium, colonies (noted as **S**) were randomly picked from the eight plates for soil and root fractions. From Angle's medium, all soil and root colonies (noted as **A**) from the eight plates were isolated. Angle and mS1 strains were streaked twice on Angle's 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 $\mathbf{AS^+}$ or $\mathbf{AS^-}$ when able or unable to grow on mS1 respectively.

2.4. 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 [26], as previously described [10]. 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 as **AH** (including $\mathbf{AS^+H}$ and $\mathbf{AS^-H}$), and **SH**.

2.5. Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA

AH and **SH** strains were submitted to genomic DNA extraction with a Wizard Genomic DNA Purification Kit

(Promega, Madison, WI, USA) according to the manufacturer's protocol, except that only 400 µl of Nuclei Lysis solution were 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 [27]. 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× at 3.5 V cm^{-1} for 1.5 h and visualised by staining with ethidium bromide. The ΦX174/*Hae*III Fragment Ladder (Gibco) was used as the size ladder. Isolates displaying identical restriction profiles with *Hae*III and *Taq*I enzymes were grouped in the same operational taxonomic unit (OTU) [10].

2.6. Cloning and sequencing of 16S rDNA fragments

The 3′ end of the 16S rDNA gene sequence was amplified with 907f and GM4r primers [28]. 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 [29]. Transformants with an expected size insert were processed with Nucleo-Spin 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 the retrieved sequences [30]. As non-ambiguous affiliation of strains could not always be achieved, they were classified into clusters according to Anzai et al. [31]. These sequences were registered in the EMBL database under the accession numbers AJ512378 to AJ512408, and AJ517396 to AJ517410 (Table 3).

2.7. Data analysis

CFU counts were compared statistically using the Student's *t*-test. Proportions of **H** strains were compared using a χ^2-test. Shannon diversity index was calculated, based on OTUs, as $H' = -\Sigma p_i \log_2 p_i$, where p_i is the number of isolates in the OTU being analysed. Evenness 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's *t*-test, according to Magurran [32]. 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, WA, USA).

3. Results and discussion

3.1. Quantitative evaluation of root and soil Pseudomonas using mS1 and Angle's media

CFU counts on Angle's and mS1 media are presented in Fig. 1A. The total heterotroph A counts were significantly higher (about one log) than the **S** and $\mathbf{AS^+}$ counts for both soil and root fractions (*t*-test, $P < 0.001$). For the soil fraction, $\mathbf{AS^+}$ and **S** counts were not significantly different. But for the root fraction, $\mathbf{AS^+}$ 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's 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 1). About 23% of mS1 soil strains ($\mathbf{AS^+}$ 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's medium (**AH**) were unable to grow after transfer on mS1 medium ($\mathbf{AS^-H}$). They represented 42.4% and 6.7% of **AH** strains for soil and root strains respectively. Proportions of *Pseudomonas* isolated on Angle's medium (**AH**) were statistically equivalent in both soil (9.6%) and root (11.1%) fractions (Table 1). However, the proportion of mS1-growing *Pseudomonas* ($\mathbf{AS^+H}$) among total Angle isolates (**A**) was lower in the soil fraction (5.5%) compared to the root

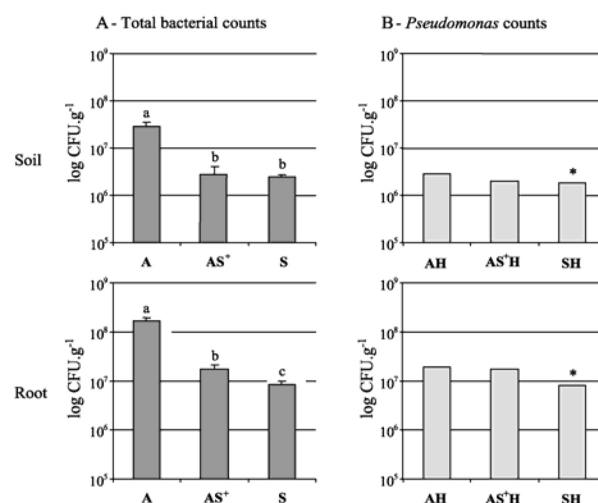


Fig. 1. Number of CFU g $^{-1}$ dry weight for soil and root. A: Total counts. B: *Pseudomonas* counts (calculated using **H** proportions, see Table 1). **A**: on Angle's non-selective medium, **S**: on mS1 *Pseudomonas* selective medium, and $\mathbf{AS^+}$: after the two consecutive non-selective 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, $P < 0.01$).

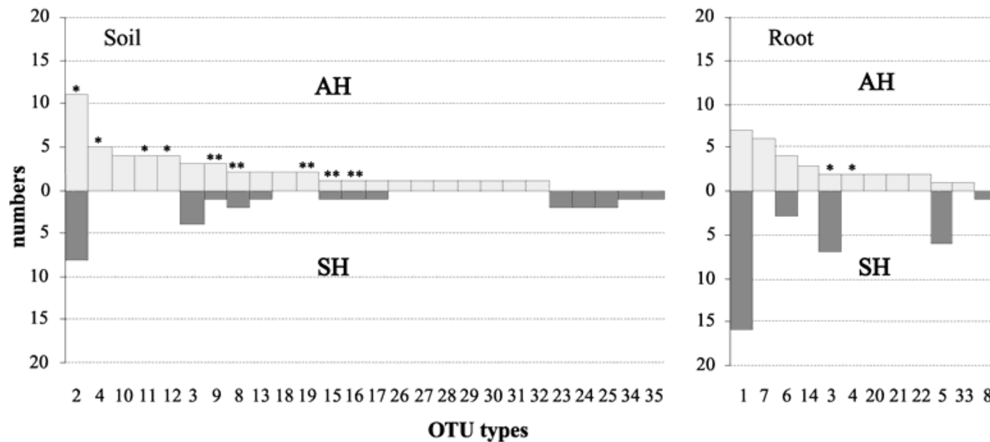


Fig. 2. Distribution of **AH** (isolated on Angle) and **SH** (isolated on mS1 medium) *Pseudomonas* strains among the different OTUs, for soil and root fractions. The name of each OTU is noted at the bottom. * indicates that Angle OTUs contained strains both capable (**AS$^+$H**) and incapable (**AS$^-$H**) 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.

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 [9,15]. This was particularly the case in the soil fraction, due to the occurrence of a large proportion of **AS$^-$H** strains. Furthermore, using both isolation media, *Pseudomonas* was a major component of the rhizosphere microflora, as already described for other grasses [9–10].

Using the proportion of **H** strains (Table 1), we could estimate the abundance of *Pseudomonas* in root and soil fractions from **A**, **AS$^+$** and **S** CFU counts (Fig. 1B). For the soil fraction, mS1-growing *Pseudomonas* counts were 30% lower than the **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 the same ability to grow on mS1.

There are two possible reasons for the differential recovery of *Pseudomonas* from mS1 and Angle's media. It is possibly because some *Pseudomonas* strains are inhibited by mS1 medium, even if transferred from fresh, active cultures on Angle's medium. 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.

3.2. PCR–RFLP analysis

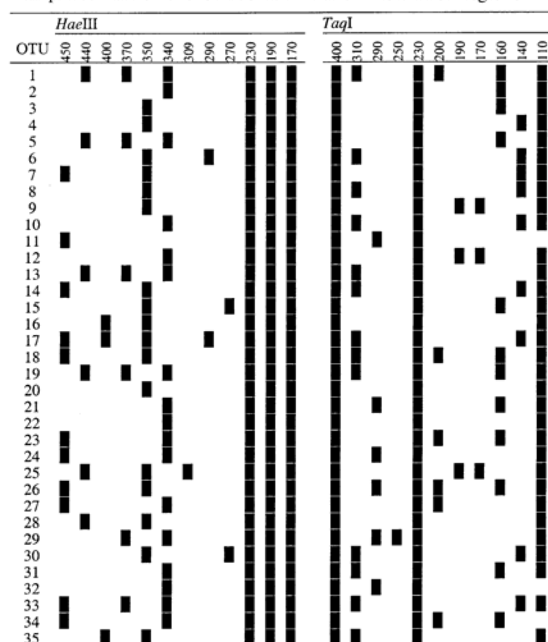
We then compared the genetic diversity of Angle and mS1 *Pseudomonas* strains for soil and root fractions. About 30% of all *Pseudomonas* strains were submitted to

Table 1
Numbers (**H** (*n*)) and proportions (**H** (%)) of root and soil *Pseudomonas* strains isolated on mS1 and Angle's media

		Isolates (*n*)	**H** (*n*)	**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's 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.

Table 2
Schematic OTU pattern restriction profiles obtained by digestion of the PCR-amplified half 3′ end of 16S rDNA and whole ITS1 regions



OTUs were defined on the combined restriction patterns of *Hae*III and *Taq*I. The size of each band is given in base pairs.

PCR–RFLP analysis of 16S-ITS1 rDNA fragments. Fifty-two 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* [27]. For 29% of the strains, two or three PCR fragment sizes were generated (1050–1300 bp), as previously shown [33]. 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 [34]. 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 and Tiedje reported similar results and suggested the existence of at least two types of ITS1 regions in a PCR product of the same size [35].

Pseudomonas strains were grouped using restriction analysis of their PCR products. The corresponding restriction profiles are presented in Table 2. 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 OTUs (Table 2). The clustering of strains into the different OTUs was not influenced by the isolation plate they were retrieved from (data not shown). Fig. 2 presents the repartition of **AH** and **SH** strains for soil and root fractions among the 35 OTUs.

3.3. 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). Twice as many *Pseudomonas* OTUs were described in the soil fraction compared to the 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 the root fraction (Table 3). Previous studies for other plants [12,36–37] 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 (Fig. 2). 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).

3.4. Comparison of Angle and mS1 OTU distribution

The Shannon diversity index H' [32] and evenness J were calculated for root and soil *Pseudomonas* recovered on Angle and mS1 (Table 4). The comparison of Shannon index suggested that 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 was different using Angle's 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 were grouped into 16 OTUs (Table 3). Eleven OTUs were retrieved on both Angle's 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 (Fig. 2). Nineteen OTUs were only retrieved on Angle's medium. These included some abundant OTUs, such as the OTU4 (common to soil and root), OTU7 (19% of root strains), and OTU10, 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 (Fig. 2). Consequently, the ability of strains to grow on mS1 was not related to their clustering using PCR-digestion of the rDNA sequences. Among the 30 OTUs found on Angle, 25 contained **AS⁺H** strains, i.e. they could grow on mS1. Four OTUs comprised **AS⁻H** strains, though these were

Table 3
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

OTU	Global			Angle			mS1			*Pseudomonas*-related group*	Accession numbers
	n	soil	root	*n*	soil	root	*n*	soil	root	(*n*, category of sequenced strains)	
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**)	AJ517399-AJ517400-
										putida/graminis (1, **AS$^+$H**)	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
Strains (*n*)	144	79	65	84	52	32	60	27	33		
OTUs (*n*)	35	26	12	30	21	11	16	13	5		

**Pseudomonas* groups are defined according to Anzai et al. [31].

also retrieved on mS1 medium (OTU8, 9, 15 and 16; Fig. 2). More generally, OTUs including **AS$^-$H** were more frequent in soil (nine among 21 OTUs) than in root (two 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 recovered from soil as well as from 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.

Angle's medium attempts to approximate the composition of the soil solution [24]. 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.

Table 4
Diversity (Shannon index *H′*) and evenness (*J*) indexes among OTUs isolated from soil and/or root fractions, as obtained using Angle's or mS1 medium 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

[15] 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 [38]. Some aspects of plant–microbe environment could also affect the differential recovery using the two media. When comparing mS1 and Angle 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 [39] or adaptation to high nutrient levels [15], and their relative abundance may be overestimated on mS1 isolation medium. As suggested by Wilson and Lindow [40], 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.

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Conclusions

Cette étude nous a permis d'observer que les *Pseudomonas* sont des populations importantes dans la rhizosphère de *M. coerulea*. Ils représentaient environ 10% des bactéries cultivables sur milieu non-selectif adapté aux bactéries telluriques (Angle) dans les fractions de sol et de racine. Au niveau de la diversité, les fractions de sol et de racine présentaient des structures de populations différentes, et le sol une richesse plus importante que la fraction racinaire. Cependant selon le milieu d'isolement, une diversité différente était mise en évidence pour une même fraction. Nos résultats suggèrent que certaines souches sont dans un état physiologique particulier qui ne leur permet pas de former une colonie/de se multiplier directement sur milieu riche (mS1). Toutefois, elles auraient cette potentialité après « revitalisation » sur milieu « pauvre » (Angle). Le niveau d'activités de telles souches dans l'environnement serait probablement très ralenti, mais leur comportement de « revitalisation » (observé surtout pour les cellules provenant du sol) nous interroge sur l'implication de l'existence de ce statut physiologique dans la dynamique des populations de *Pseudomonas* en réponse aux variations environnementales. Ainsi chercher à décrire une diversité la plus exhaustive possible garde un intérêt non négligeable pour comprendre le fonctionnement des populations de *Pseudomonas* et bactériennes en générale. En effet de telles cellules pourraient être soit mourantes soit dans une état de latence avec un potentiel de réactivité en attendant que les conditions environnementales deviennent favorables.

2.3. DIVERSITE DES POPULATIONS DE *PSEUDOMONAS* DANS LA RHIZOSPHERE DE *MOLINIA COERULEA* : APPROCHE CULTURALE *VERSUS* APPROCHE MOLECULAIRE

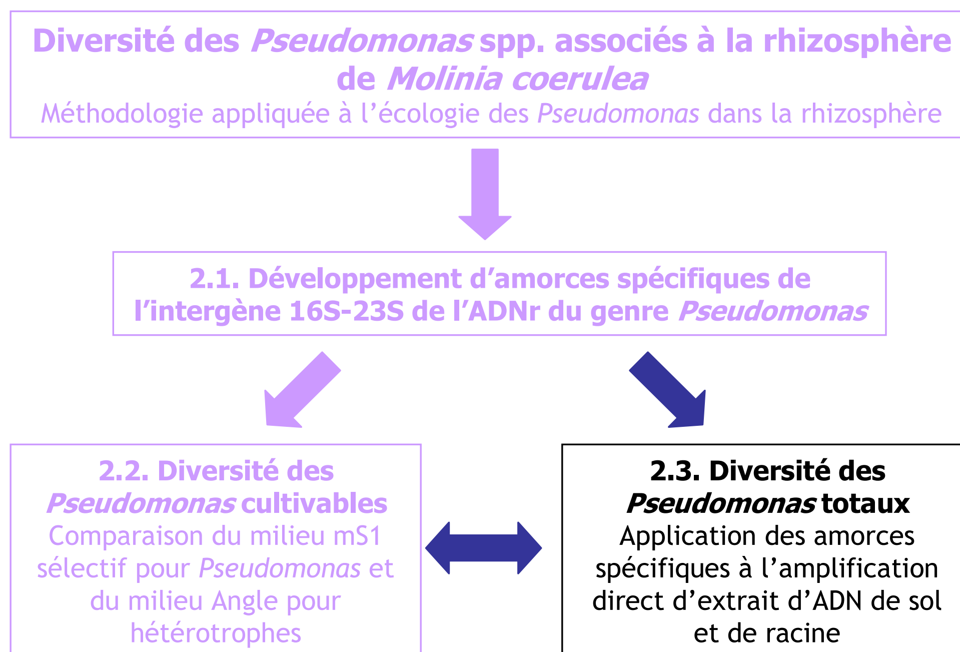


Diagramme 2.4. Vue synthétique de l'organisation des paragraphes du chapitre 2.
L'étude de la diversité des *Pseudomonas* de la rhizosphère de *M. coerulea* en est la ligne directive

INTRODUCTION

Les études de la diversité des *Pseudomonas* sont généralement basées sur l'isolement de souches et leur caractérisation par des approches génotypiques ou phénotypiques (de Vos et coll. 1998 ; Ellis et coll. 1999 ; Johnsen et Nielsen, 1999 ; Aagot et coll. 2001 ; Cho et Tiedje, 2000 ; cf. 2.2). Cependant, plusieurs études mettent en avant le biais des approches culturales, en particulier dans le cas de l'utilisation de milieux de culture sélectifs (Johnsen et Nielsen, 1999 ; Aagot et coll., 2001 ; Tarnawski et coll. 2003). Par ailleurs l'isolement, l'entretien, et la caractérisation d'une grande quantité d'isolats demande beaucoup de temps. Dans ce contexte, les approches moléculaires offrent une alternative prometteuse comme cela a déjà été montré pour d'autres groupes bactériens (Ovreas et coll.1998 ; Grundmann et Gourbière, 1999 ; Hamelin et coll. 2002). Des approches moléculaires basées à la fois sur le clonage (Marilley et coll. 1999 ; Mc Caig et coll. 1999) et sur les profils de communautés (Duineveld et coll. 1998 ; Yang et Crowley, 2000 ; McCaig et coll. 2001 ; Smalla et coll. 2001) ont permis un accès direct à la diversité bactérienne en évitant l'étape de mise en culture.
La correspondance entre la diversité bactérienne décrite par des méthodes culturales et des approches moléculaires directes, a été étudiée le plus souvent au niveau des communautés bactériennes totales (Gonzalez et coll. 1996 ; Smit et coll. 1999 ; Mc Caig et coll. 2001). Généralement, une divergence de résultats entre les deux approches était observée, avec une plus grande diversité décrite par les approches moléculaires. Ceci était probablement dû au faible pourcentage d'organismes cultivables (<1%) dans les environnement naturels (Ward et coll. 1990). *Pseudomonas* est considéré comme facilement cultivable. Il peut représenter jusqu'à 10% des organismes cultivables dans la rhizosphère (Kragelund et coll. 1996 ; Tarnawski et coll. 2003). Toutefois, l'existence de souches « viables mais non cultivables » a été également décrite (Binnerup et coll. 1993 ; Marscher et coll. 2000 ; Edwards 2000).
Dans l'objectif d'étude de la diversité des *Pseudomonas* associé à la rhizosphère de *M. coerulea*, des amorces spécifiques du genre *Pseudomonas* ont été développées pour

l'amplification de la moitié terminale de l'ADNr 16S et l'intégralité de l'intergène 16S-23S de l'ADNr (16S-ITS1) (Paragraphe 2.1). Par analyse de restriction des séquences ADNr 16S-ITS1, la diversité des *Pseudomonas* cultivables du sol et associés à la racine de *M. coerulea* a été étudiée avec succès. Cette étude a mis en évidence l'importance du milieu de culture (sélectif *versus* peu sélectif) dans l'image de la diversité obtenue, ceci tant au niveau de la richesse que de la structure des populations de *Pseudomonas* décrites. Les deux milieux de culture utilisés ont néanmoins permis d'observer une différence de diversité entre les populations de *Pseudomonas* du sol et celles associées à la racine de *M. coerulea* (étude 2.2). En parallèle de cette approche culturale, la diversité des *Pseudomonas* a été étudiée en adoptant cette fois une approche purement moléculaire. Les ADN totaux des fractions de sol et de racine, des même échantillons que pour l'étude 2.2, ont été extraits, et les séquences 16S-ITS1 de *Pseudomonas* directement amplifiées à partir de ces extraits (avec le protocole défini en 2.1). Les résultats de cette étude sont détaillés dans ce paragraphe, et l'image de diversité obtenue est comparée avec celle de l'approche culturale. Cette étude est en cours, je présenterai ici uniquement les résultats mis à jour avec certitude. Ils sont issus d'un travail de collaboration entre Nathalie Fromin pour la partie moléculaire, et Sonia Tarnawski pour la partie cultivable.

2.3.1. MATERIEL ET METHODES

Le protocole schématisé de la démarche expérimentale est présenté en figure 2.1 et 2.2.

Site d'étude, préparation des échantillons, et milieu d'isolement

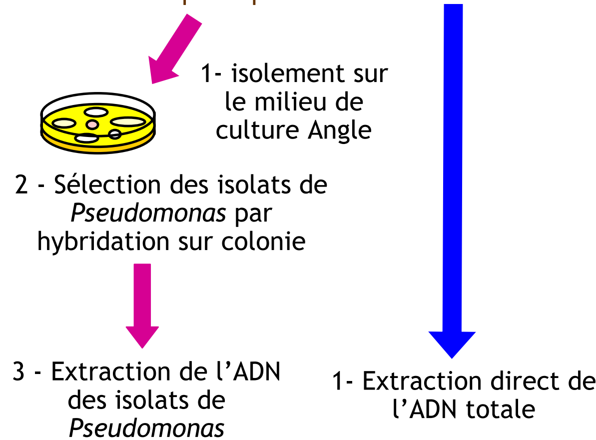
Le site d'étude, et l'échantillonnage des fractions de sol et de racine sont décrits en détails dans le paragraphe 2.2. L'étude culturale prise en compte dans les résultats se réfère aux souches de *Pseudomonas* isolées du milieu de culture Angle (cf. 2.2).

Extraction d'ADN

L'extraction d'ADN des souches environnementales était obtenue en utilisant le Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) (cf. 2.2). Les extractions d'ADN des échantillons de sol et de racine étaient réalisées en utilisant le Fast DNA Spin Kit for Soil (Bio 101 Inc., La Jola, CA) comme décrit précédemment (Borneman et coll. 1996), excepté que (i) 450 µl du surnageant (à la place de 250 µl) étaient utilisés après la précipitation des protéines pour la suite de la purification (ii) un volume (450 µl) de Binding Matrix était utilisée à la place de deux volumes dans le protocole de Borneman et (iii) l'ADN purifié était finalement élué dans 200 µl de TE (pH 8). La quantité et l'intégrité des extraits d'ADN étaient contrôlées par électrophorèse sur gel d'agarose par comparaison aux fragments du λDNA/*Hind*III (Gibco). Les échantillons d'ADN sont stockés à -80°C.

Amplification PCR de la région ITS1

Différents couples d'amorces étaient utilisés pour l'amplification des séquences ITS1 des *Pseudomonas*. Les séquences 16S-ITS1 des ADN d'isolats de *Pseudomonas* étaient amplifiées spécifiquement avec les amorces fPs16S et rPs23S. Les fragments 16S-ITS1 des extraits d'ADN des fractions de sol et de racine, dilués 10 fois, étaient également amplifiés en utilisant ces mêmes amorces pour s'assurer d'une amplification spécifique à *Pseudomonas*. Pour les extraits d'ADN total de sol et de racine, cette première PCR était suivie d'une seconde PCR nichée en utilisant les amorces FGLP50'-73 (Normand et coll. 1996) et rPs23S. Les amplicons résultants correspondaient uniquement à la région ITS1, sans la fin du 16S (également amplifié avec l'amorce fPs16S). Pour les deux couples d'amorces le mélange réactionnel de PCR et le programme PCR étaient les mêmes que ceux du protocole définit en 2.2 pour les amorces rPs16S-fPs23S. Les produits de la PCR nichée étaient contrôlés en électrophorèse sur gel à 1% de Standard Agarose (Eurobio, Les Ullis, France) à 10V/cm pendant 1 heure, en utilisant le Low DNA Mass Ladder comme référence de taille. Les produits PCR étaient ensuite visualisés par coloration au bromure d'éthidium, et les bandes de taille attendue étaient excisées du gel avec un scalpel. L'ADN des bandes excisées était extraite et purifiée en utilisant le MinElute™ Gel Extraction Kit (Qiagen). Les produits de cette purification étaient insérés dans le vecteur pGEM-T (Promega) et clonés dans les cellules compétentes de *E. coli* XL1 (Sambrook et Russel, 2001) pour analyse des séquences individuelles.



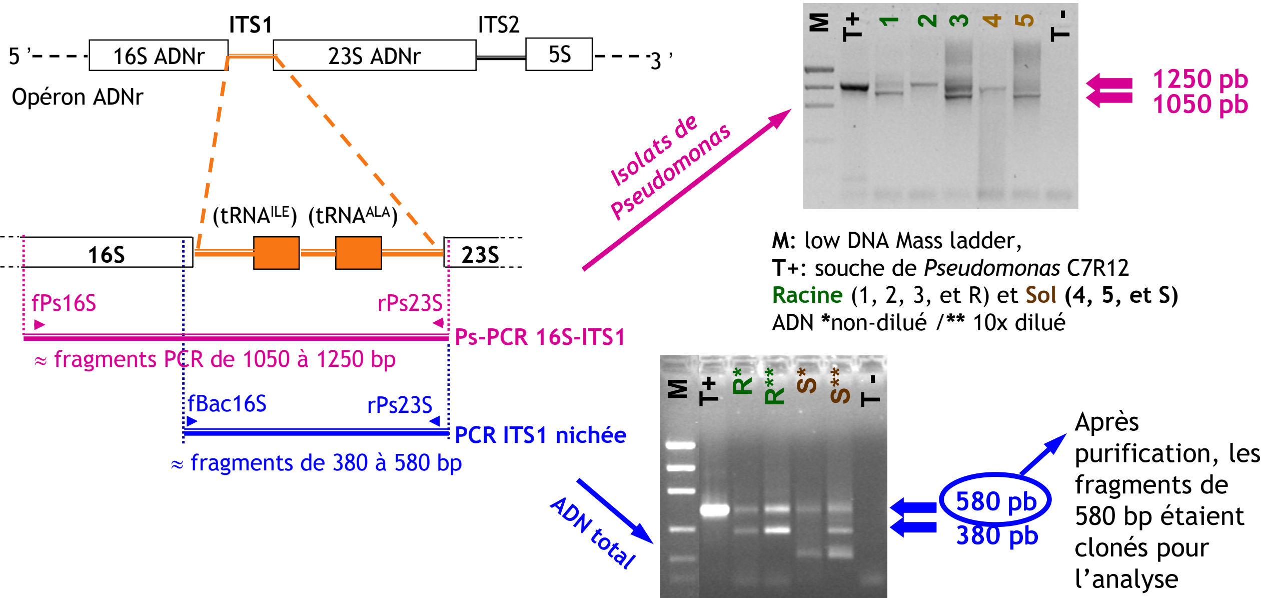


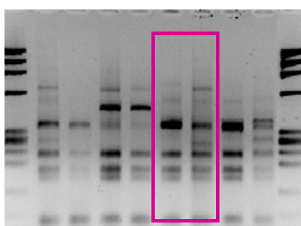
Figure 2.1. Protocoles schématisés de préparation des ADN de souches de *Pseudomonas* et des extraits d'ADN total de sol et de racine de *Molinia coerulea*, des amplifications PCR et de caractérisation des fragments ITS1.

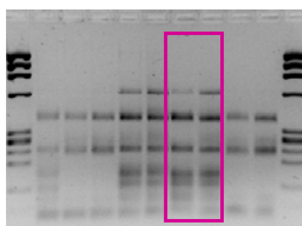
Analyse de restriction des séquences ITS1

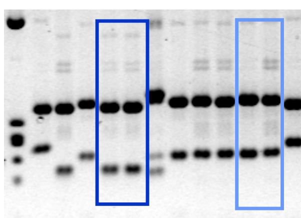
Les fragments PCR 16S-ITS1 des isolats ainsi que les séquences ITS1 des clones (insert FGPL1490-72/rPs23S amplifié avec les amorces T7/SP6), étaient soumis à l'analyse de restriction avec les endonucléases *Hae*III et *Taq*I comme décrit en 2.2. Les séquences des isolats et des clones présentant des profils similaires avec les deux enzymes de restrictions étaient groupées dans une même Unité Taxonomique Opérationnelle (OTU), notée cultOTU pour les isolats et totOTU pour les clones. Les indices de Shannon *H'* de la diversité décrite avec les deux approches étaient calculés comme décrit en 2.2.

Séquençage et analyse des séquences

L'ADNr 16S et ITS1 d'isolats et ITS1 de clones sélectionnés dans chaque OTUs étaient séquencées (MWG Biotech, Ebersberg, Allemagne) après purification des plasmides comme décrit en 2.2. L'identification de l'organisme correspondant à la séquence obtenue est réalisée par analyse dans le programme BLAST (Altshul et coll. 1991) comme décrit en 2.2.







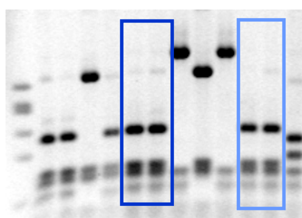


Figure 2.2. Profils de restriction des séquences ITS1 des isolats et des clones avec les endonucléases *Hae*III et *Taq*I.
Les cadres de couleurs différentes désignent des isolats ou des clones présentant des profils identiques avec les deux enzymes.

2.3.2. RESULTATS ET DISCUSSION

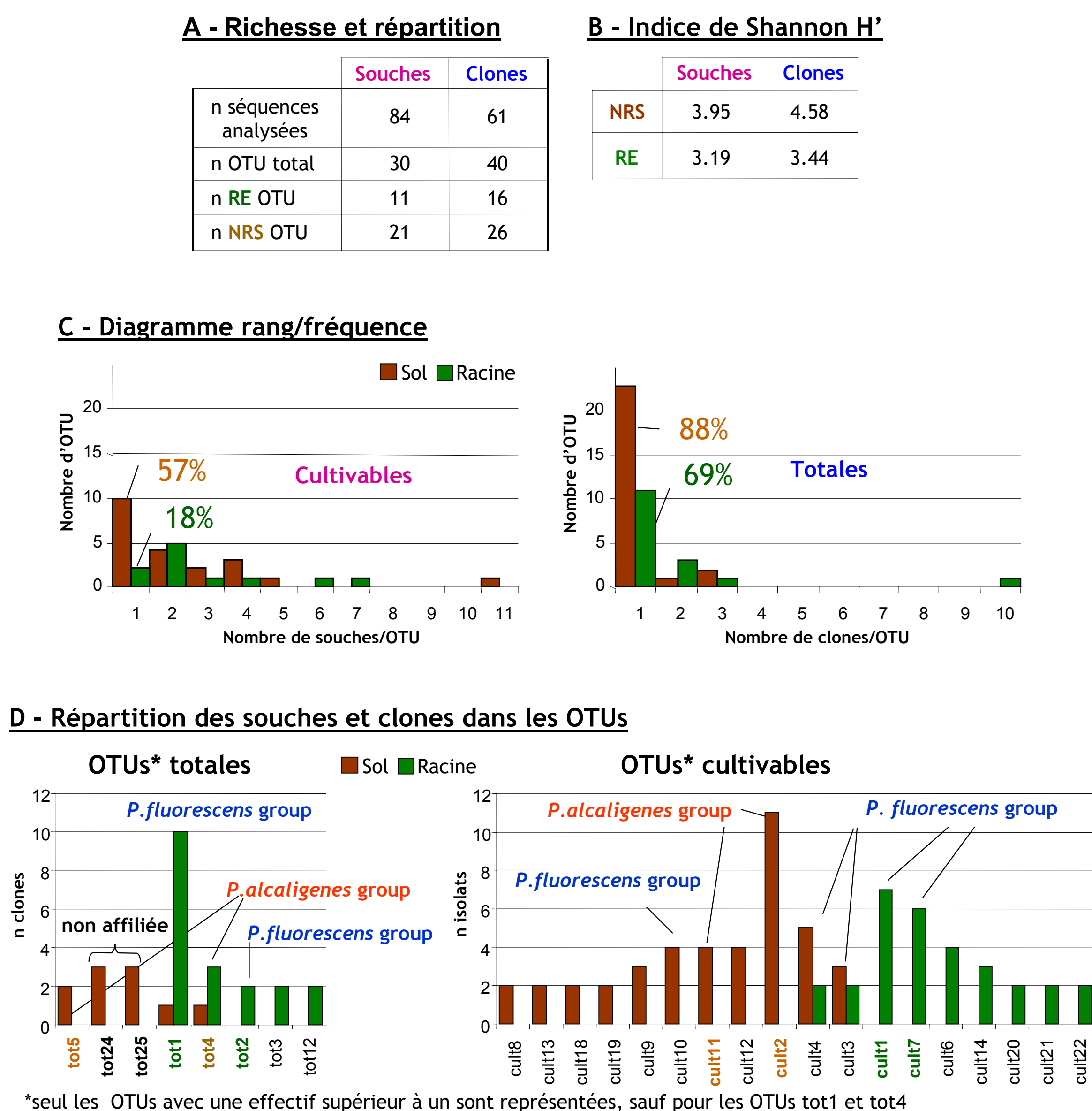
Les résultats comparatifs entre les approches culturale et moléculaire sont présentés dans la Figure 2.3.

2.3.2.1. Amplification PCR des séquences ITS1 pour les extraits d'ADN de sol et de racine

Pour conserver la spécificité de l'approche PCR décrite en 2.1, l'amplification PCR des extraits d'ADN environnementaux était réalisée dans les même conditions de stringence (concentration des amorces, température d'hybridation, etc.) que pour les extraits d'ADN des isolats. De la même manière, Widmer et coll. (1998) ont réalisé une amplification PCR directe avec des amorces spécifiques *Pseudomonas.* Ces amorces étaient effectivement spécifiques de ce genre bactérien lorsqu'elles étaient testées sur des ADN extraits de cultures pures, mais l'amplification directe d'ADN de sol nécessitait de diminuer la température d'hybridation des amorces pour obtenir un produit d'amplification PCR visible. Dans leur étude certains des clones récupérés étaient dérivés de non-*Pseudomonas* ou était des artefacts chimériques de PCR. Dans notre cas, une stratégie de PCR nichée était sélectionnée de manière à conserver la spécificité de la première amplification PCR *Pseudomonas* (Locatelli et coll. 2002 ; Figure 2.1). En utilisant les amorces rPs16S-fPs23S aucun produit d'amplification PCR n'était visualisable pour 5µl de produit PCR déposés sur gel, ceci pour les extraits d'ADN de sol et de racine. Par contre, la PCR nichée, réalisée avec les amorces FGLP50'-73 et rPs23S, générait des amplicons à la taille attendue : deux bandes à environ 580pb et 380 pb, pour le sol une bande additionnelle de 200pb était détectée (Figure 2.1). Des opérons ribosomiques multiples avec des tailles différentes peuvent être présents dans une même souche de *Pseudomonas*, ceci du

fait de la présence ou de l'absence des gènes codant pour l'ARNt^{ILE} et/ou l'ARNt^{ALA} (Jensen et coll. 1993). Par conséquent, l'approche par PCR nichée générait des amplicons de taille multiple comme ce fut également le cas pour les amplifications PCR sur les extraits d'ADN d'isolats (Figure 2.1).

Dans cette étude était analysée uniquement la bande de 580pb du produit PCR obtenu à partir de l'amplification des extraits d'ADN totaux. Cette taille correspondait à la région ITS1 contenant les deux gènes d'ARNt. Néanmoins, il faut garder en mémoire que tous les *Pseudomonas* ne génèrent pas cette taille d'amplicon (Locatelli et coll. 2002). La bande à 580 pb a été excisée du gel, purifiée et clonée pour les analyses de diversité.



A - Richesse et répartition

	Souches	Clones
n séquences analysées	84	61
n OTU total	30	40
n RE OTU	11	16
n NRS OTU	21	26

B - Indice de Shannon H'

	Souches	Clones
NRS	3.95	4.58
RE	3.19	3.44

Figure 2.3. Analyse comparative de la diversité décrite par approche culturale et moléculaire pour les fractions de sol et de racine de *M. coerulea*. n, indique les effectifs ; NRS, et RE, correspondent respectivement aux fractions de Sol non-rhizosphérique, et Racine-endorhizosphère. tot, indique les OTUs décrites à partir des séquences ADNr ITS1 clonées; et cult, indique celles décrites à partir des séquences ADNr 16S-ITS1 des souches. A) Richesse et répartition des OTUs de souches et de clones pour les fractions RE et NRS. B) Indice de diversité de Shannon *H'* (voir Tarnawski et coll. 2003). C) Diagramme rang/fréquence des OTUs cultivables et totales. D) Distribution des souches et des clones des fractions RE et NRS dans différentes OTUs cultivables et totales.

2.3.2.2. Diversité des isolats et des clones de *Pseudomonas* du sol et de la racine basée sur l'analyse de restriction des séquences ITS1 de l'ADNr.

L'analyse de diversité des *Pseudomonas* cultivables isolés de sol et de la racine de *M. coerulea* sur milieu Angle est décrite en détail en 2.2 (voir aussi Figure 2.3). Brièvement, 84 isolats de *Pseudomonas*, dont 52 isolés de la fraction sol et 32 de la fraction racine, étaient soumis aux protocoles d'extraction d'ADN, de PCR spécifique *Pseudomonas* et à l'analyse de restriction avec les endonucleases *Hae*III et *Taq*I. En combinant les différents profils obtenus, les 84 isolats étaient groupés en 30 OTUs numérotées et notées cult1 à cult21, dont 11 OTUs pour la racine et 21 pour le sol (Figure 2.3 A). Seul les OTUs cult3 et cult4 étaient communes au sol et à la racine (Figure 2.3 D). 57% des cultOTUs de la fraction du sol, et 18% pour la fraction racinaire, comprenaient 1 seul représentant (Figure 2.3 C). Les populations de *Pseudomonas* du sol et de la racine présentaient une diversité différente : la fraction du sol présentait un indice de diversité de Shannon plus élevé (*H'*= 3.95) que celui de la fraction racine (H'=3.19) (Figure 2.3 B).

Après l'amplification directe des séquences ITS1 à partir des extraits d'ADN de sol et de racine puis clonage, 31 clones de sol, et 30 clones de racine étaient prélevés aléatoirement. L'insert correspondant était amplifié avec les amorces T7/SP6 et soumis au même protocole de restriction que les amplicons d'isolats. Ces 61 clones généraient 12 et 23 profils types différents avec *Hae*III et *Taq*I respectivement. En regroupant ces résultats, les 61 clones étaient distribués dans 40 totOTUs numérotées et notées tot1 à tot40 (Figure 2.3 A). Les 31 clones du sol étaient regroupés dans 26 totOTUs (incluant 23 totOTUs constituées d'un seul clone, soit 88% des totOTUs) alors que les 30 clones de la racine étaient regroupé dans 16 totOTUs (incluant 11 totOTUs constituées d'un seul clone, soit 69% des totOTUs) (Figure 2.3 C). Deux OTUs seulement, tot1 et tot4, était commune au sol et à la racine (Figure 2.3 D). Les populations de *Pseudomonas* du sol et de la racine présentaient une diversité différente encore plus marqué que par approche culturale: la fraction sol présentait un indice de diversité de Shannon plus élevé (*H'*= 4.58) que celui de la fraction racine (*H'*=3.44) (Figure 2.3 B).

Ainsi, avec les deux approches expérimentales, les populations de *Pseudomonas* du sol et de la racine présentaient des richesses et des structures bien différentes (Figure 2.3). En effet, une plus faible diversité (en terme de richesse en OTUs) était mise en évidence dans la racine par rapport au le sol (11 versus 21 OTUs pour les isolats, et 16 versus 26 OTUs pour les clones ; Figure 2.3 A). De même les indices de Shannon *H'* révélaient une diversité plus faible dans la racine que dans le sol, cette différence étant d'autant plus marquée dans l'approche moléculaire (Figure 2.3 B). Un tel résultat était en accord avec d'autres études sur la diversité des *Pseudomonas fluorescens* dans la rhizosphère (Lemanceau et coll. 1995, Latour et coll. 1996). La plante semble en effet exercer une sélection/une pression de sélection, *via* la rhizodeposition, à l'encontre des populations résidentes dans le sol.

Par contre, le nombre de totOTUs (40 OTUs pour 60 clones) était plus important comparé au nombre de cultOTUs (30 OTUs pour 84 isolats analysés). Ceci suggère qu'une plus grande diversité de types d'OTUs pourrait être décrite en utilisant l'approche moléculaire. Cependant au niveau expérimental, les amplicons ITS1 de l'ADN total peuvent s'insérer dans le plasmide dans deux sens différents (5'-3' ou 3'-5'), ce qui générerait des profils de restriction différents avec l'enzyme *Taq*I (dont le site de restriction est T/CGA). Théoriquement le nombre de profils décris avec *Taq*I, pourrait être deux fois plus important pour des séquences clonées que pour des séquences non-clonés. Dans notre cas, nous obtenons 23 profils *Taq*I pour 61 clones et 16 profils pour les 84 souches. Le nombre de profils *Hae*III obtenus pour les souches et les clones était similaire, 14 et 12 profils *Hae*III respectivement. Le site de restriction de *Hae*III (GG/CC) est palindromique, et le sens d'insertion de la séquence n'influence pas le profil de restriction. Par conséquent le nombre plus important d'OTUs obtenues par la méthode moléculaire pourrait être un biais dû à l'étape de clonage. Le séquençage systématique des fragments ITS1 pourrait lever cette ambiguïté.
Par ailleurs, Stach et coll. (2001) par une approche PCR-SSCP avec des amorces spécifiques de la séquence 16S de l'ADNr des *Pseudomonas* (Widmer et coll. 1998), comparaient les séquences des populations de *Pseudomonas* cultivables avec celles obtenues par clonage après amplification direct d'extrait d'ADN de sol. Les auteurs concluaient que le nombre d'OTUs

affiliées au genre *Pseudomonas* dans la banque de clones était similaire au nombre d'OTUs obtenues sur le milieu de culture. Cependant ils n'ont pas comparé les séquences pour les OTUs de clones et de cultivables.
Mc Caig et coll. (2001) décrivaient qu'une banque de clones d'ADNr 16S affiliés à *Pseudomonas*, incluait seulement une séquence qui était similaire à celle d'une souche isolée dans le même échantillon de sol.
Dans notre cas, les profils de restriction des séquences ITS1 des clones et des séquences 16S-ITS1 des isolats de *Pseudomonas* ne pouvaient être comparés directement. Les fragments PCR analysés en restriction n'étaient pas de taille identique dans les deux approches (Figure 2.1). Ainsi les séquences ITS1 des clones et des isolats pour les OTUs majoritaires (effectif ≥ 2) ont été séquencées pour permettre l'analyse comparative des populations de *Pseudomonas* retrouvées avec les deux approches. Il apparaît que les OTUs majoritaires de la fraction racinaire sont affiliées au groupe *Pseudomonas fluorescens*, qu'elles soient mises en évidence par approche culturale (cult1 et cult7) ou moléculaire (tot1) (Figure 2.3 D). Pour la fraction de sol, les isolats des OTUs majoritaires cult2, cult11 et cult12 étaient affiliés au groupe *Pseudomonas alcaligenes*, et l'OTU cult4 au groupe des *Pseudomonas fluorescens*. Avec l'approche moléculaire les OTUs tot4 et tot5 sont également affiliées au groupe des *Pseudomonas alcaligenes*. Cependant pour les OTUs majoritaires tot24 et tot25, il n'existait pas dans les bases de données de séquences ITS1, d'organismes connus ou non, similaires à celles de ces OTUs. Il pourrait s'agir de *Pseudomonas* non-cultivables, ou des séquences d'un autre genre non-cultivable qui seraient amplifiées avec le protocole PCR. L'alignement des séquences ITS1 des isolats, des clones, de souches de collection (*Pseudomonas* ou non *Pseudomonas*), et de celles retrouvées dans les bases de données GenBank et RDP nous permettrait de construire un dendrogramme de similarités, et éventuellement d'affilier au mieux les séquences des OTUs tot24 et tot25.

CONCLUSION

A la vue des résultats déjà obtenus, le protocole PCR établi en 2.1 semble efficace pour l'amplification spécifique de l'ITS1 de *Pseudomonas* directement sur des extraits d'ADN de sol et de racine. Les deux approches, culturale et moléculaire, ont permis de mettre en évidence une différence de diversité entre le sol et la racine de *M. coerulea*. Cependant l'image de la diversité décrite (richesse et structure) différait entre les deux approches, surtout pour la fraction de sol où sont mises en évidence deux populations majoritaires sans affiliation. Ce point est actuellement examiné.

CONCLUSIONS DU CHAPITRE 2

Dans ce chapitre l'objectif principal était de développer des outils méthodologiques pour appréhender la diversité des *Pseudomonas* dans l'environnement et d'appliquer ces outils pour évaluer la diversité des *Pseudomonas* associés à la rhizosphère de *M. coerulea*.
Nous avons mis en évidence que le genre *Pseudomonas* représente également une part importante de la microflore cultivable de *M. coerulea*, comme précédemment montré pour *L. perenne* (Marilley et coll. 1999).
Du point de vue méthodologique, l'utilisation de l'outil PCR mis au point en 2.1, en combinaison avec une approche culturale (2.2) et moléculaire (2.3) a montré que *M. coerulea* influençait la diversité des populations présentes dans le sol à proximité de la racine. En effet les populations de *Pseudomonas* dans la rhizosphère de *M. coerulea* étaient bien différentes de celles du sol (groupe *P. fluorescens* dans la racine *vs*. groupe *P. alcaligenes* dans le sol), et la diversité était plus faible dans la racine.
La diversité plus grande attendue avec l'approche moléculaire, semble effective dans l'étude 2.3, mais doit être encore vérifiée. *A priori*, dans la racine, les populations de *Pseudomonas* majoritaires étaient affiliées au groupe des *Pseudomonas fluorescens* dans les deux approches (cultivable et moléculaire), mais s'agit il des même espèces ? Dans le sol, deux types ITS1 majoritaires non identifiés étaient mis en évidence. Si ils représentent effectivement des populations de *Pseudomonas*, alors la diversité décrite par amplification directe des séquences ITS1 de *Pseudomonas* sur des extraits de sol serait bien différente de celle mise en évidence par isolement sur milieu de culture. Néanmoins, travailler sur des bactéries cultivables permet de caractériser leurs fonctions (comme la denitrification potentielle) ou de tester leurs caractères physiologiques (AIA, siderophore, HCN…).
Le protocole PCR mis au point en 2.1 a permis l'étude de la diversité des *Pseudomonas* cultivables et totaux de la rhizosphère de *M. coerulea.* Nous avons mis en évidence par étude comparative que le choix du milieu d'isolement avait un effet sur la diversité des populations de *Pseudomonas* décrite, celle-ci serait d'autant plus biaisée que le milieu utilisé est sélectif. Cependant la comparaison, relative à un milieu d'isolement, de la diversité de deux environnements différents amenait les mêmes conclusions, dans notre cas que la diversité des *Pseudomonas* de la fraction racinaire était différente et plus faible que celle du sol. Par ailleurs la mise en évidence de souches capables d'être réactivées sur milieu pauvre (2.2) suggère des populations tout à fait intéressantes de par leur comportement métabolique. Sont-elles des cellules mourantes et fragilisées ? Ou pourrions-nous les relier au stade « viable mais non-cultivable » parfois décrit dans les sols en conditions limitantes ? (Normander et coll. 1999 ; Haas et coll. 2000 ; Edward 2000).
Ces résultats illustrent bien le fait que la rhizosphère est une zone de stimulation de la microflore *via* la rhizodéposition (cf. 1.3). Les *Pseudomonas* sont particulièrement bien adaptés à cet environnement car ils sont capables de métaboliser rapidement un grand nombre de substrats carbonés organiques (Latour et Lemanceau, 1997). Cependant les sources carbonées de la rhizosphère sont également à disposition des autres microorganismes capables de les utiliser, et il s'établirait dans la rhizosphère un équilibre entre les différents groupes bactériens. La plante sélectionnerait les populations (voire les souches) les plus adaptées à l'environnement racinaire. Certaines souches de *Pseudomonas* sont caractérisées par des caractères de compétence rhizosphérique et de promotion de la croissance de la plante (PGP, cf. 1.3.4), ce type d'organismes se trouverait plus particulièrement à la proximité de la racine. Ce point sera en partie abordé dans le chapitre suivant.

CHAPITRE 3

Effet de l'augmentation en pCO_2 atmosphérique sur les populations de *Pseudomonas* cultivables associés à la rhizosphère de *Lolium perenne* et *Molinia coerulea*

CHAPITRE 3

EFFET DE L'AUGMENTATION EN pCO_2 ATMOSPHERIQUE SUR LES POPULATIONS DE *PSEUDOMONAS* CULTIVABLES ASSOCIEES A LA RHIZOSPHERE DE *LOLIUM PERENNE* ET *MOLINIA COERULEA*

INTRODUCTION

Dans le chapitre précédent, nous avons étudié l'importance et la diversité des *Pseudomonas* associés à la rhizosphère de *M. coerulea* dans la prairie naturelle de Cudrefin. A présent, nous allons évaluer l'influence du traitement en pCO_2 (360 ppm *vs.* 600 ppm) sur les *Pseudomonas* associés à la rhizosphère de *Molinia coerulea* et *Lolium perenne* cultivées dans les installations FACE à Eschikon. Plusieurs aspects, résumés dans le diagramme 3.1 ont été abordés sous cette problématique. Chacune des études présentées dans ce chapitre s'articule autour de deux axes principaux (1) effet de l'augmentation de la pCO_2 atmosphérique (2) influence du système racinaire sur les populations de *Pseudomonas* du sol.

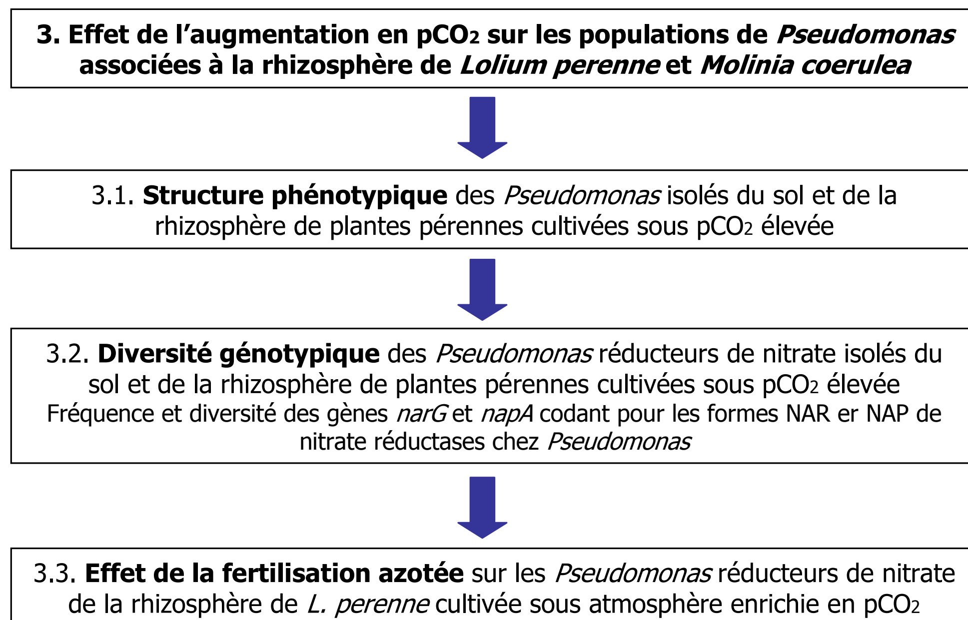


Diagramme 3.1. Vue synthétique de l'organisation des paragraphes du chapitre 3.

Rappels: influence d'une augmentation de la pCO_2 sur le système sol-plante

L'enrichissement en pCO_2 influençait à plusieurs niveaux la prairie fourragère de *L. perenne* en culture dans le système FACE à Eschikon (cf. Chapitre 1). Brièvement, la photosynthèse, le rendement, et le ratio du rendement des parties racinaires/aériennes étaient augmentés sous pCO_2 élevée. Il en était de même du flux de carbone dans le sol (augmentation de la translocation de carbohydrates des feuilles vers les racines et de leur exsudation dans le sol). Par ailleurs, la biomasse et l'activité microbienne rhizosphérique étaient souvent stimulées sous pCO_2 élevée. Ce dernier point répondait à l'hypothèse formulée

au début de ce projet concernant l'effet d'une augmentation en pCO_2 sur la microflore (Marilley, 1998): l'augmentation en pCO_2 influencerait en premier lieu la microflore du sol dans l'environnement proche de la racine, c'est-à-dire dans la rhizosphère, l'effet du pCO_2 se faisant indirectement *via* des modifications quantitatives et qualitatives de la rhizodéposition (Darrah, 1996).

Gestion de l'azote des plantes modèles *L. perenne* et *M. coerulea*, et pCO_2 élevée

Pour *M. coerulea* aucune mesure physiologique de biomasse ou de rendement sous fort pCO_2 n'est disponible. Néanmoins sa gestion de l'azote, tout à fait différente de celle de l'ivraie (*L. perenne* est nitrophile et *M. coerulea* est oligonitrophile), pourrait lui conférer un avantage adaptatif face à l'augmentation en pCO_2. En effet, un déséquilibre entre la demande en azote de la plante et la disponibilité en azote du sol est souvent observé sous pCO_2 élevée, comme c'est le cas pour *L. perenne* à faible amendement en azote (cf. 1.1.1, 1.1.2 et 1.1.4). Les rhizosphères des ces deux plantes seraient bien différentes, et la microflore associée propre à chaque système sol/plante (*L. perenne* dans le sol d'Eschikon et *M. coerulea* dans son sol naturelle de la prairie de Cudrefin, cf. 1.4.2.2).

Réponse du genre *Pseudomonas* à l'augmentation de la pCO_2

Dans le système FACE d'Eschikon après 3 années de fumigation, la fréquence des clones ADNr 16S affiliés au genre *Pseudomonas* augmentait sous atmosphère enrichie en CO_2, en particulier pour les fractions rhizosphériques de *L. perenne* (Marilley et coll. 1999). Les *Pseudomonas* représentent également une part importante de la microflore cultivable de *M. coerulea* (~10%), et leur proportion semble augmenter avec la proximité à la racine (cf. 2.2). Ce groupe bactérien est-il également stimulé dans la rhizosphère de *M. coerulea* cultivée sous pCO_2 élevée ? Cette stimulation est-elle toujours vraie pour *L. perenne* après 8 années d'enrichissement en pCO_2 ? (cf. 3.1 et 3.3).
La structure des communautés bactériennes globales était également modifiée sous pCO_2 élevée (cf. 1.2.2, et 1.4.3.1). En est-il de même pour les populations de *Pseudomonas* ? Les bactéries appartenant à ce genre ont une croissance rapide, et sont capables d'utiliser des substrats carbonés variés comme source d'énergie (Latour et Lemanceau, 1997). Certaines souches présentent également des caractères phénotypiques importants dans la promotion de la croissance des plantes et dans la compétence racinaire (colonisation et maintien) (Glick 1995, cf. 1.4.3.1). Ce dernier point leurs confèrerait un avantage adaptatif dans la rhizosphère par rapport à d'autres groupes bactériens (cf. 1.3.4). Dans ce contexte la modification de la rhizodéposition sous pCO_2 élevée aurait un impact majeur sur les populations *Pseudomonas* dans la rhizosphère. Cette hypothèse sera discutée à différents niveaux dans ce chapitre.

Etude des populations de *Pseudomonas* de la rhizosphère et augmentation de la pCO_2

Tout abord, la réponse à l'augmentation en pCO_2 des populations de *Pseudomonas* isolés du sol et de la rhizosphère de *L. perenne* et *M. coerulea*, en terme de structure phénotypique, a été évaluée. Les isolats de *Pseudomonas* ont été caractérisés pour l'expression potentielle de caractères PGP et/ou impliqués dans la compétence rhizosphérique. Ces points seront détaillés dans le paragraphe 3.1.
Ensuite, nous avons restreint notre étude aux populations de *Pseudomonas* réducteurs de nitrate. Ces populations réalisaient au moins une étape du processus biologique de réduction du nitrate (NO_3^-) en azote gazeux (N_2), c'est-à-dire de la dénitrification. L'effet du pCO_2 sur la fréquence des gènes codant pour les deux nitrate réductases (périplasmique NAP, et membranaire NAR) a été évalué parmi les *Pseudomonas* réducteurs de nitrate provenant du sol et de la racine de *L. perenne* et *M. coerulea*. La diversité des populations de *Pseudomonas* dissimilant le nitrate a été considérée par analyse des profils de restriction des gènes des nitrates réductases *narG* (membranaire) et *napA* (périplasmique), et de la séquence 16S-ITS1 (cf. 2.2). Ces points seront détaillés dans le paragraphe 3.2.
Finalement la disponibilité en azote du sol pourrait modifier la réponse à un enrichissement en pCO_2 des *Pseudomonas* dissimilant le nitrate dans la rhizosphère. L'activité de réduction des nitrates représentant une perte d'azote pour le système (dégagement de N_2O et N_2), elle pourrait rapidement entrer en compétition pour le nitrate avec la nutrition de la plante. Cette hypothèse sera développée dans le paragraphe 3.3. Nous avons évalué l'influence du niveau de fertilisation azotée et de pCO_2 sur les *Pseudomonas* réducteurs de nitrate isolés du sol et de la rhizosphère de *L. perenne*.

3.1. STRUCTURE PHENOTYPIQUE DES *PSEUDOMONAS* SPP. ASSOCIES AU SOL ET A LA RHIZOSPHERE DE *LOLIUM PERENNE* ET *MOLINIA COERULEA* CULTIVEES SOUS ATMOSPHERE ENRICHIE EN pCO_2

INTRODUCTION

Pour comprendre le fonctionnement de la rhizosphère, et plus particulièrement dans notre cas, l'interaction entre les populations de *Pseudomonas* et les racines de la plante, il est important de connaître les protagonistes qui entrent en jeux. Le genre bactérien *Pseudomonas* a été décrit pour la première fois par Migula en 1894. Actuellement ce genre *sensu stricto* comprend plus de 50 à 100 espèces (Anzaï et coll. 2000 ; GeneBank - http://www.ncbi.nlm.nih.gov/ Taxonomy/Browser/wwwtax.cgi), et présente une grande amplitude écologique. Composé d'organismes au métabolisme versatile, il regroupe, entre autre, des espèces délétères ou bénéfiques pour la croissance de la plante (cf. 1.4.3.1, Suslow et Schroth 1982, Glick 1995). Ce dernier point est particulièrement bien étudié en lutte biologique, mais souvent peu considéré dans les populations bactériennes naturelles de la rhizosphère. Dans cette étude, nous examinons deux rhizosphères modèles, celles de *L. perenne* et *M. coerulea*, deux plantes aux besoins trophiques bien différents notamment vis-à-vis de l'azote. Chacun de ces systèmes sol/plante présenterait une rhizosphère et une microflore associées qui leur est propre. Dans le paragraphe 2.2, nous avons montré que le groupe des *P. fluorescens* était dominant dans la rhizosphère de *M. coerulea*, alors que dans le sol, le groupe des *P. alcaligenes* était plus fréquemment détecté (Tarnawski et coll. 2003). Les *Pseudomonas* fluorescents produisent des sidérophores qui chélatent le fer Fe^{3+} dans la rhizosphère, le rendant ainsi indisponible pour d'autres microorganismes. Cette activité leur donne un avantage dans la colonisation de la rhizosphère. D'autres caractères phénotypiques impliqués dans la compétence rhizosphérique et la promotion de la croissance de la plante ont été décrits chez *Pseudomonas* (cf. 1.4.3.1, Glick 1995). Ceci leur confèreraient un rôle majeur dans le fonctionnement de la rhizosphère.

Sous atmosphère enrichie en pCO_2, la pression partielle en oxygène serait diminuée dans la rhizosphère du fait de la stimulation de la respiration racinaire et de la microflore (cf. 1.3.2). Par ailleurs, le flux de carbone dans le sol était stimulé pour *L. perenne* (Suter et coll. 2002) et la composition des exsudats probablement modifiés (Darrah 1996). Ces changements dans l'environnement rhizosphérique en réponse à l'augmentation en pCO_2 pourraient se traduire par une altération de la structure phénotypique des populations de *Pseudomonas*. Pour appréhender cette hypothèse nous avons caractérisé des isolats de *Pseudomonas* associés au sol et à la rhizosphère de *M. coerulea* et *L. perenne* pour leur capacité potentielle à produire de l'auxine, des sidérophores, du cyanure d'hydrogène, et à réduire les nitrates.

Phenotypic structure of *Pseudomonas* populations is altered under elevated pCO_2 in the rhizosphere of perennial grasses

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Abstract

The increasing atmospheric CO_2 content (pCO_2) is likely to modify the ecosystem functioning including rhizosphere bacteria that are directly dependent on rhizodeposition. This may include alteration of *Pseudomonas* populations that display phenotypic traits in relation with plant fitness. In the present study, 1228 *Pseudomonas* strains were isolated from the non-rhizosphere soil, rhizosphere soil and root fractions of perennial grassland systems: *Lolium perenne* and *Molinia coerulea*. Both plants were grown under ambient (360 ppm) and elevated (600 ppm) pCO_2 in the Swiss Free Air CO_2 Enrichment (FACE) system. *Pseudomonas* spp. were tested for their ability to produce auxin, siderophores and hydrogen cyanide, and to dissimilate nitrate. No effect of root proximity and elevated pCO_2 was observed on the proportions of auxin producers. For *L. perenne* and *M. coerulea*, siderophore and hydrogen cyanide *Pseudomonas* producers were stimulated in root fraction. In contrast lower frequencies of nitrate reducers were observed in root fraction compared to non-rhizosphere soil. The frequencies of siderophore producers and nitrate dissimilating strains were higher, and those of hydrogen cyanide producers lower, under elevated pCO_2 for *L. perenne*. This alteration of the phenotypic structure of *Pseudomonas* guild in the root fraction is discussed in relation with the physico-biochemical modifications of the rhizosphere condition *via* rhizodeposition and environmental changes occurring under elevated pCO_2.

1. Introduction

Since the beginning of the industrial revolution, the atmospheric CO_2 content (pCO_2) is increasing significantly, changing the global climate and ecosystems functioning (Fuhrer, 2003). For instance, the rate of net photosynthesis (Isopp et al., 2000) and carbon allocation to the roots (Suter et al., 2002) are enhanced under elevated pCO_2. A significant part of this carbon is released by plants roots (rhizodeposition) into the rhizosphere, the region of soil in close proximity and under the influence of living plant roots (Lynch and Whipps, 1990). In the case of meadow plants, up to 30-50% of total assimilated carbon can be translocated to the soil (Kuzyakov, 2001; Gobat et al., 2004). Consequently, plants modify their surrounding soil and play a major role in the structuration of rhizosphere microflora (Lynch and Whipps, 1990; Lemanceau et al., 1995; Paterson, 2003). Under elevated pCO_2, the carbon flow into the soil increases (Darrah, 1996; Suter et al., 2002), and its composition may be altered (Hodge et al., 1998). This might stimulate the growth and activities of soil microbiota feeding on plant-derived carbon sources (Weihong et al., 2000). Due to their metabolic versatility and high growth rate, bacteria belonging to the genus *Pseudomonas* are well adapted to the rhizosphere (Lemanceau et al. 1995; Lugtenberg and Dekkers, 1999; Rainey, 1999). Because of their competitivity for nutrients and for suitable niches on the root surface, they are able to respond promptly to environmental modifications

(Glick, 1995; Sørensen et al., 2001; Stres et al., 2004). Indeed, the dominance of 16S rDNA clones affiliated to the genus *Pseudomonas* was shown to increase in the rhizosphere of perennial ryegrass after 3 years under elevated pCO_2 (Marilley et al., 1999). Moreover, numerous *Pseudomonas* strains display plant growth promoting activities. Plant growth promoting rhizobacteria can act either directly (e.g. by enhancement of plant nutrient uptake or by production of phytohormones) or indirectly (e.g. by biological control of root pathogens and alteration of microbial populations balance) (Glick, 1995).
During the last decade, questioning about the response of plant-microorganism interactions to a pCO_2 increase has raised, and changes in diversity and structure of microbial populations were described for plants grown under elevated pCO_2 using either metabolic (Grayston et al., 1998; Hodge et al., 1998) or molecular approaches (Griffiths et al., 1998; Kandeler et al., 1998; Marilley et al., 1999; Montealegre et al., 2002). However, few is known about the stimulation or repression by elevated pCO_2 of bacterial activities that interfere with plant fitness in the rhizosphere, an oxygen- and iron-depleted environment (Lugtenberg and Dekkers, 1999). We hypothesized that environmental modifications induced by elevated pCO_2 in the rhizosphere *via* the plant could alter the structure of *Pseudomonas* populations with particular traits related to plant fitness. Two perennial grasses with different trophic requirements (Vázquez de Aldana and Berendse, 1997) were used: the oligonitrophilic *Molinia coerulea*, and the nitrophilic *Lolium perenne*. Culturable *Pseudomonas* strains were isolated during two growing seasons and tested for their ability to produce auxin, siderophore and hydrogen cyanide and to dissimilate nitrate.

2. Materials and methods

2.1. Study site and plant material
L. perenne and *M. coerulea* were grown under ambient (360 ppm, control plots) and elevated pCO_2 (600 ppm, treated plots) in the Free Air CO_2 Enrichment (FACE) facility, Eschikon, Switzerland (Hebeisen et al., 1997). The treated plots were enriched with CO_2 during daylight between March and December. *L. perenne* cv *Bastion* was grown as a monoculture on 3 control and 3 CO_2-treated replicate plots since May 1993. The plants were cultivated in the local autochthonous fertile, Eutric Cambisol. Shoots were harvested four times a year. The control and treated plots received 14 g m^{-2} y^{-1} N as NH_4NO_3 (at the beginning of the season, and then after each cut, except the last). *M. coerulea* plants originated from a littoral meadow located at the southern shore of Lake Neuchâtel (Cudrefin, Switzerland). The local soil is a Gleysol, typic Haplaquoll (Hamelin et al., 2002) with low N content (Buttler, 1987 - thesis manuscript). Plants with undisturbed root systems were taken with their surrounding and underlying soil and transferred to the FACE facility in September 1999. About 0.7 m^2 of littoral meadow with reconstructed soil profile below the root horizon (total depth: 35 cm) was installed in one control plot and one CO_2-treated plot. *M. coerulea* plants were neither cut nor fertilized.

2.2. Sampling plan
For *L. perenne*, the three replicates for both control and treated plots were sampled in June, July, and October 2000, as well as in July and December 2001. During the growth season, sampling of *L. perenne* was always performed before a cut in order to avoid the effect of cut on plant N demand (Daepp et al, 2000). For *M. coerulea*, both the control and the treated plots were sampled in June 2000, then in July and December 2001. From each sampled plot, 3 soil cores (about 3 cm diameter, 10-12 cm depth), including dense root systems, were taken and pooled for analysis. Three fractions were recovered: non-rhizosphere soil (NRS) for soil devoid of roots, rhizosphere soil (RS) for root-adhering soil (recovered by washing the roots with its adhering soil twice in 20 ml sodium phosphate buffer 0.1 M pH 7.0 (SPB) under agitation during 30 min), and rhizoplane-endorhizosphere (RE) for thoroughly SPB washed roots. For *M. coerulea*, as the rhizosphere fraction is not easy to recover, only the two fractions NRS and RE were analysed.

2.3. Pseudomonas *counts and isolation*
Counting and isolation of *Pseudomonas* strains was performed as previously described (Tarnawski et al, 2003). Briefly, approximately 1 g of fresh weight root or soil was crushed in 10 ml SPB. The root and soil suspensions were ten-fold serially diluted in SPB and spread on mS1 medium, which is selective for *Pseudomonas* (Tarnawski et al, 2003). Colony forming units (CFU) were determined after 72h of incubation at 24°C. Twenty (June 2000), twelve (July, October 2000 and July 2001), and fifteen bacterial colonies (December 2001) were randomly picked from appropriate dilution plates (between 20 to 200 colonies per Petri dish) and checked for purity on ten-fold diluted Tryptic Soy Agar (Difco). The

affiliation of mS1 isolates to the genus *Pseudomonas* was ensured by hybridisation with PSM_G probe (Marilley et al., 1999). This method was previously applied to screen soil *Pseudomonas* from *M. coerulea* (Locatelli et al., 2002).

2.4. Auxin production assay

For indole-3-acetic acid (IAA) production assay, fresh cultures were inoculated in Nuclon microtitre plates (Nagel Nunc International, NY, USA) containing 100 µl Angle liquid medium per well (Angle et al., 1991) supplemented with L-tryptophane (final concentration 5 mM), a metabolic precursor of auxin (Bric et al., 1991). After 72h incubation at 24°C in dark, the supernatant of each culture was transferred in a new microtitre plate. The presence of IAA in the supernatant was detected by a colorimetric technique specific for the detection of indolic compounds (Bric et al., 1991; Glickmann and Dessaux, 1995). 100 µl of Salkowski reagent (2 % 0.5 M $FeCl_3$ in 35% perchloric acid) were added to each well, and the absorbance at 550 nm was measured after 30 min at room temperature. The accuracy of the test was checked by including on each plate the IAA-producing *Pseudomonas fluorescens* strain CHA0 and the non-producing *P. fluorescens* strain ATCC 17400 as controls. Addition of the Salkowski reagent result in a faint pink coloration when auxin compounds are produced in the medium, and no visually detectable colour reaction is produced with non-auxin-producing strains. Strains positive for auxin production were noted *iaa+*.

2.5. Siderophores production assay

The ability of *Pseudomonas* strains to synthesize and excrete siderophores when grown under Fe^{3+}-limiting conditions was evaluated during a plate assay, as described by Schwyn and Neilands (1987). Fresh cultures were plated onto blue-agar CAS plates, using Angle medium (Angle et al., 1991), buffered with 0.1 M piperazin-1,4-bisethanesulfonic acid (PIPES) (Acros Organics, Geel, Belgium), and added with 10 µM $FeCl_3$, $6H_2O$ (Fluka, Buchs, Switzerland) complexed to 10 µM chrome azurol S (CAS, Fluka) in the presence of 0.2 mM hexadecyltrimethylammonium bromide (HDTMA, Merck AG, Dietikon, Switzerland). When the Fe^{3+} is removed from the chrome azurol S complex by high-affinity siderophores, its colour changes from blue to orange. The occurrence of orange halos around colonies was determined after 48h at 24°C and strains were noted *sid+* when positive. *Pseudomonas fluorescens* strains CHA0 and *Enterococcus cloacae* strain NEU 1027 (bacterial strain collection, University of Neuchâtel) were used as positive and as negative references respectively.

2.6. Hydrogen cyanide production assay

Each *Pseudomonas* strain was tested for the production of hydrogen cyanide (HCN) as described by Castric and Castric (1983) in 96 wells Nuclon microtitre trays. Each well was filled with 100 µl of a synthetic medium (Castric, 1977) consisted in (final concentrations): L-glutamate (20 mM), glycine (12.5 mM), DL-methionine (5 mM), K_2HPO_4 (5 mM), NaH_2PO_4 (5mM), $MgSO_4.H_2O$ (2 mM), ferric citrate (0.02 mM), tris(hydroxymethyl)aminomethane (50 mM), and 13 $g.l^{-1}$ agar. Glutamate, glycine and methionine were added as precursors of hydrogen cyanide (Castric, 1983). The pH of the medium was adjusted to 7.5. Wells were inoculated and covered with an indicator paper consisting of Whatman 3MM paper (Amersham Pharmacia Biotech, Buckinghamshire, England) soaked in 5 $mg.ml^{-1}$ copper(II)ethylacetoacetate (Strem chemicals, Newburyport, MA) and 5 $mg.ml^{-1}$ 4,4-methylenebis-*N,N*-dimethylaniline (Fluka) in chloroform, and air-dried. Production of cyanide caused the indicator on the paper to turn blue. *Pseudomonas fluorescens* strains CHA0 and *Enterococcus cloacae* strain NEU 1027 were used as positive and negative references respectively. After 48h at 24°C, strains positive for hydrogen cyanide production were noted *hcn+*.

2.7. Nitrate reduction assay

Nitrate-dissimilative activity was tested as described by Roussel-Delif et al. (2004). Briefly each strain was cultivated in tubes containing 6 ml Nutrient Broth (devoid of fermentable substrate) (Merck) supplemented with 2 $g.l^{-1}$ KNO_3. A small inverted Durham tube was added to each tube in order to detect gas formation. The cultures 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), reagent B (N,N dimethyl-1-naphthylamine 0.6% v/v) and powdered zinc to reduce the remaining nitrate to nitrite. Strains that were able to reduce nitrate, were noted *nr+* and included strains reducing nitrate to nitrite or to gaseous compounds (putative denitrifiers).

2.8. Statistical analysis
The frequencies of *iaa*+, *sid*+, *hcn*+ and *nr*+ strains were analysed using a generalised linear model (*glm*) with a logistic regression model. This analysis was used to compute the probabilities corresponding to the effects of the descriptors: pCO_2 treatment, fractions, sampling dates, and plots, on the frequencies of *iaa*+, *sid*+, *hcn*+ and *nr*+ strains (i.e. *glm* analysis can show whether the variability of a frequency is significantly explained by one or more descriptors). The probability that the *iaa*+, *sid*+, *hcn*+ and *nr*+ *Pseudomonas* frequencies are different between fractions for each pCO_2 conditions or between pCO_2 conditions for a given fraction was computed using multiple comparison tests. Tukey multiple comparison test (for *L. perenne* data) or Fisher LSD exact test (for *M. coerulea* data) were used. Data were analyzed after grouping the different sampling dates and plots, taking into account the variability due to the sampling dates and plots evidenced by *glm* analysis. For all statistical analyses, the null hypothesis (similar frequencies) was rejected, and the observed differences were considered as either significant for $p \leq 0.05$ or highly significant for $p \leq 0.01$. The tests were performed using S-plus 6 Statistical Software (Insightful Corporation, Seattle, Washington).
Links between characters were displayed using an unconstrained ordination method: Correspondence analysis (CA) was used to plot the four *iaa+, sid+, hcn+* and *nr+* characters regarding the proportions of the combined sixteen possible phenotypes among the isolated *Pseudomonas* strains. Calculations were done with R 1.9.1 (R Development Core Team, 2004).

3. Results

Pseudomonas densities in the rhizosphere of *L. perenne* and *M. coerulea* grown under both ambient and elevated pCO_2 were assessed by CFU counting on the *Pseudomonas*-selective mS1 medium. *Pseudomonas* densities were highly variable and no consistent trend was observed regarding the influence of sampling date (data not shown), root proximity, or pCO_2 treatment (Fig. 1). After checking their affiliation, 1228 *Pseudomonas* isolates were analysed, and the frequencies of strains producing siderophore (*sid+*), hydrogen cyanide (*hcn+*), auxin (*iaa+*) or dissimilating nitrate (*nr+*) were compared regarding the fraction they originated (soil: NRS, rhizosphere soil: RS, and root: RE) and the pCO_2 conditions (360 *vs.* 600 ppm) (Fig. 2).

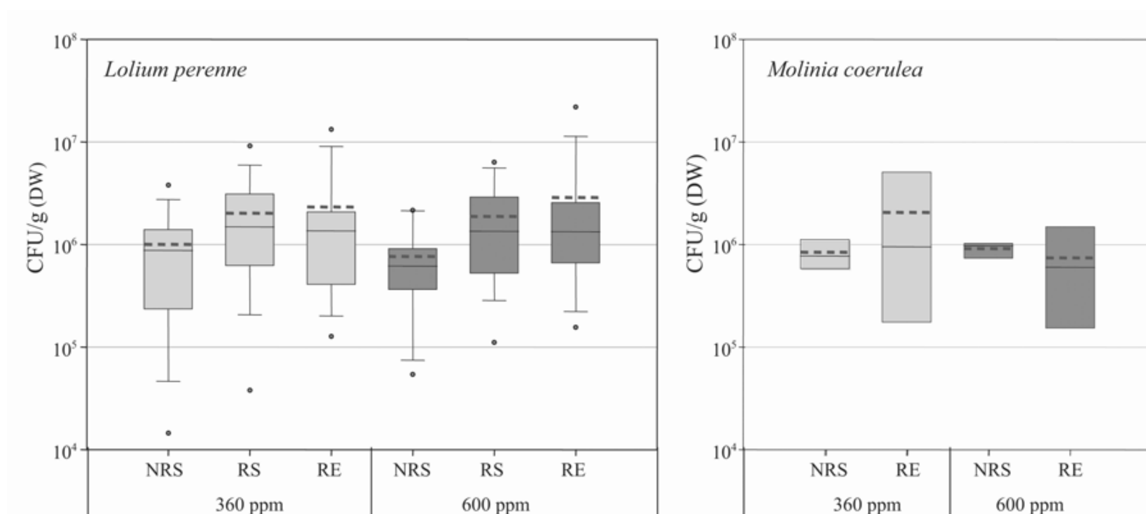

Fig. 1. Variation of colony forming units (CFU) per gram of dry weight counted on mS1 *Pseudomonas* selective medium for NRS: non-rhizosphere soil, RS: rhizosphere soil, RE: root fraction; from data of the 5 and 3 sampling dates for *Lolium perenne* and *Molinia coerulea* respectively. Dash line indicates the mean; full line indicates the median; each box represents dispersion of the values between 25 and 75 percentiles; circle indicates outlier points.

3.1. Frequency of auxin producers

26.8 % of the strains associated with *L. perenne* as well as 25.4 % of the strains associated with *M. coerulea* were *iaa*+. For both plants, global *glm* analysis allowed to evidence only an effect of sampling date on *iaa*+ frequencies ($p < 0.001$, data not shown). No significant effect of fraction or pCO_2 condition was observed (Fig. 2).

3.2. Frequency of siderophore producers

70.3 % of the *Pseudomonas* strains associated with *L. perenne* were *sid*+ compared to 44.4% only for *M. coerulea*. For *L. perenne*, the frequencies of *sid*+ strains were different according to the sampling date ($p < 0.01$), the fraction ($p < 0.001$), and the pCO_2 condition ($p < 0.001$). Higher *sid*+ frequencies were retrieved in root (RE) compared to soil (RS and NRS) fractions for both ambient and elevated pCO_2 (Fig. 2): these frequencies increased significantly from RS to RE fractions by 17 % and 22 % under ambient and elevated pCO_2 respectively. Moreover, *sid*+ frequencies were significantly higher under elevated pCO_2 compared to ambient pCO_2 (+ 16 % and + 8 % for NRS and RE fractions respectively, Fig. 2). For *M. coerulea*, the *sid*+ frequencies varied according to the fractions ($p < 0.01$) and the pCO_2 conditions ($p < 0.05$). In contrast with *L. perenne* results, *sid*+ frequencies were significantly lower under elevated than under ambient pCO_2 for the NRS fraction (Fig. 2). Finally, as for *L. perenne*, higher frequencies of *sid*+ strains were retrieved in RE (53 %) compared to NRS (20 %) under elevated pCO_2 only ($p < 0.001$).

3.3. Frequency of hydrogen cyanide Pseudomonas

23.0 % and 28.5 % of the *Pseudomonas* strains isolated from *L. perenne* and *M. coerulea* respectively were *hcn*+ respectively. The frequencies of *hcn*+ *Pseudomonas* differed according to plots ($p < 0.05$), to sampling dates ($p < 0.01$), and to root proximity ($p < 0.001$). For both plants and pCO_2 conditions, significantly higher proportions of *hcn*+ were retrieved in root compared to soil fractions, *hcn*+ frequencies being similar for *L. perenne* in RS and NRS fractions. From NRS to RE fractions, significantly higher frequencies (+42 % and +18 % for *L. perenne* and +41 % and +49 % for *M. coerulea*) were observed for ambient and elevated pCO_2 respectively (Fig. 2).
Regarding the influence of pCO_2, *hcn*+ frequencies were 20 % lower in RE fraction for *L. perenne* ($p < 0.001$) grown under elevated pCO_2, and tended to be higher under elevated pCO_2 for *M. coerulea* ($p < 0.05$ in June 2000).

3.4. Frequency of nitrate-dissimilating Pseudomonas

The data related to nitrate-dissimilating *Pseudomonas* were detailed in Roussel-Delif *et al.* (2004), and are summarised in Fig. 2. About 52 % of *Pseudomonas* strains from *L. perenne* plots were able to reduce nitrate, compared to 32 % from *M. coerulea*. For *L. perenne*, the frequencies of *nr*+ *Pseudomonas* were influenced by the fractions ($p < 0.001$), the pCO_2 content ($p < 0.01$), the plots ($p< 0.001$) and the sampling dates ($p < 0.001$). For ambient and elevated pCO_2, significantly higher proportions of *nr*+ were retrieved in the soil fraction (NRS) compared to the root fraction (RS and RE) (Fig. 2), *nr*+ proportions being similar in RS and RE fractions under elevated pCO_2. The pCO_2 influence was observed only in RE fractions (higher *nr*+ frequencies under elevated pCO_2). For *M. coerulea*, proportions of *nr*+ *Pseudomonas* were neither significantly influenced by elevated pCO_2 nor by root proximity.

3.5. Link between phenotypic characters

The link between *iaa*+, *sid*+, *hcn*+ and *nr*+ phenotypic in *Pseudomonas* strains is displayed in correspondence analysis, Figure 3. The *nr*+ and *hcn*+ traits were negatively related and the (*hcn*+ *nr*+) phenotype was present only in 4.3% of the tested strains. The opposition between *nr*+ and *hcn*+ is displayed along the CA1 axis which account for 42.8 %. *iaa*+ trait behaved independently of *nr*+ and *hcn*+ traits.

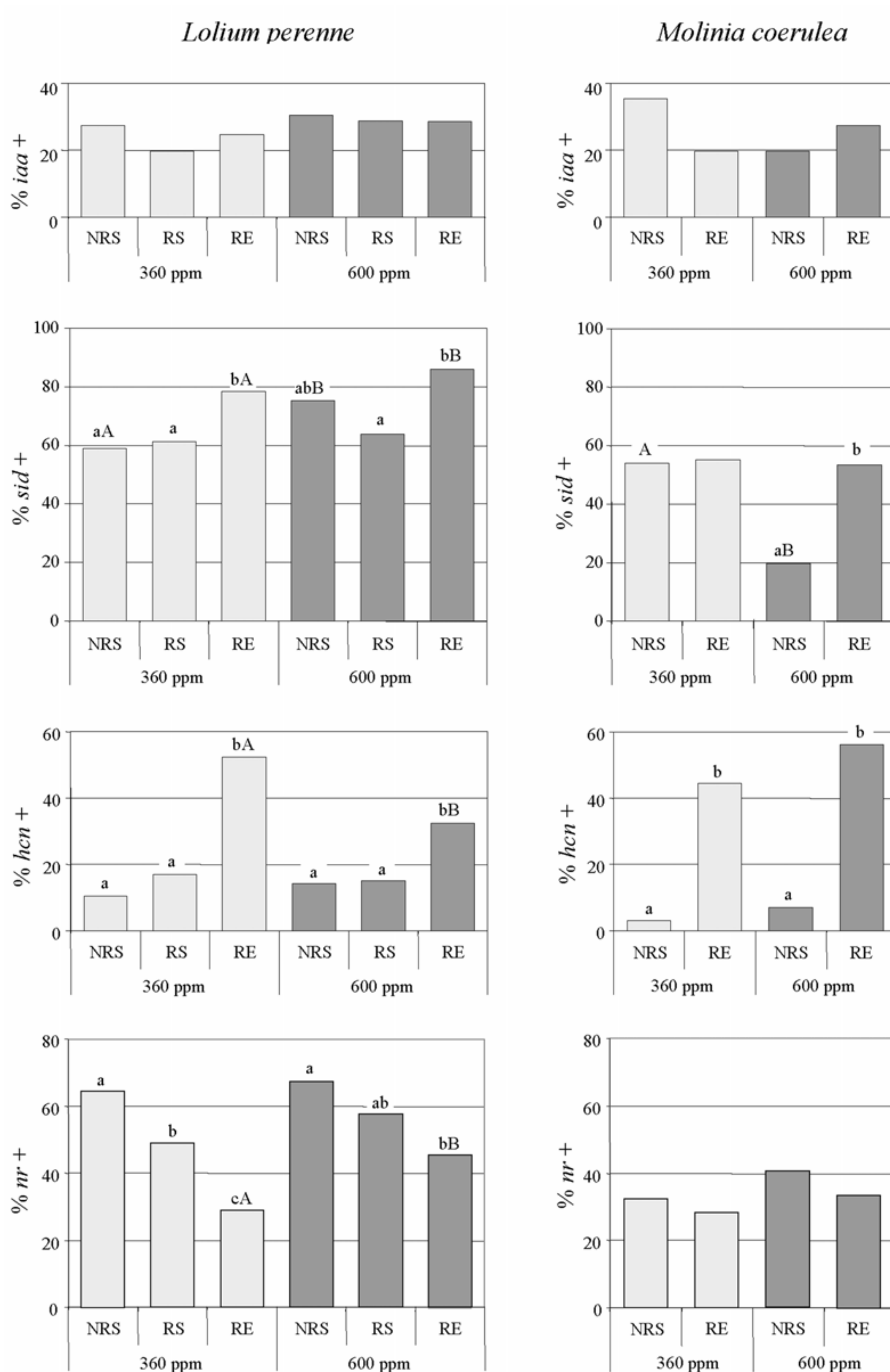

Fig. 2. Frequencies of auxin (*iaa*+), siderophore (*sid*+) hydrogen cyanide (*hcn*+) producing and nitrate reducing strains (*nr*+) associated with *L. perenne* and *M. coerulea* under ambient (360 ppm) and elevated (600 ppm) pCO_2 growing conditions; result for 5 (*L. perenne*) and 3 (*M. coerulea*) sampling dates. NRS: non-rhizosphere soil; RS: rhizosphere soil; RE: root fraction. A, B: statistical differences between pCO_2 conditions within a fraction; a, b, c: statistical differences between fractions within a pCO_2 condition. Similar values are not indicated.

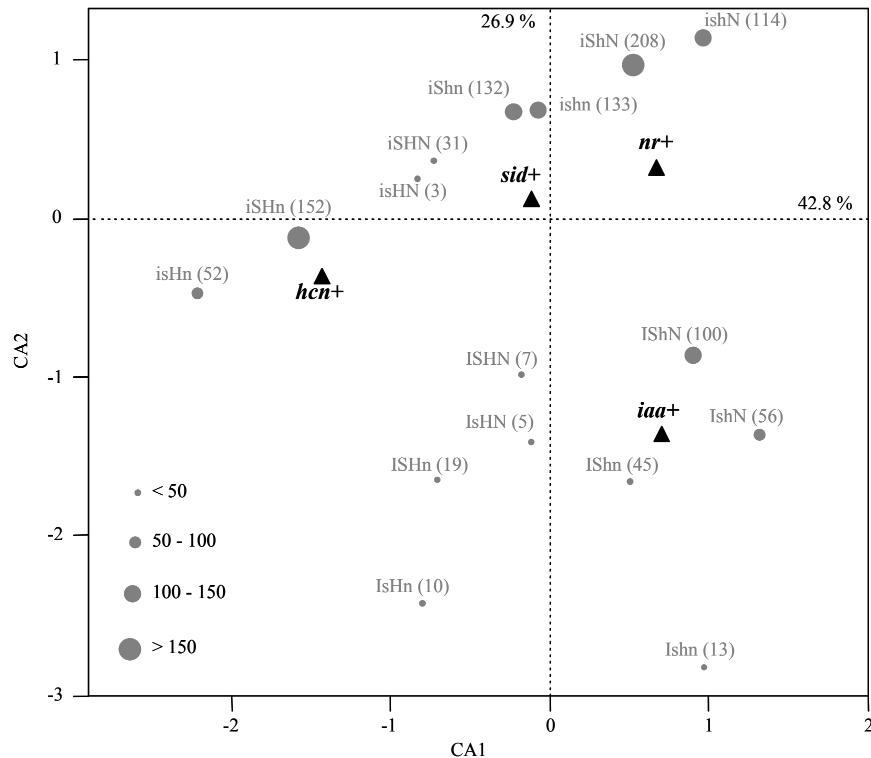

Fig. 3. Correspondence analysis (CA) based on the physiological test *iaa+, sid+, hcn+* and *nr+* data. The four characters (triangles) were plotted according to the proportions of the sixteen different phenotypes (circles) obtained by testing 1080 strains isolated from *Lolium perenne* and *Molinia coerulea*. The phenotype notation used is: I/i, for *iaa+*/*iaa-*; S/s, for *sid+*/*sid-*; H/h, for *hcn+*/*hcn-*; N/n, for *nr+*/*nr-*. Numbers of strains within phenotypes were indicated between brackets, and circle size is proportional to this number. Total inertia of the matrix was 0.7817. Opposite direction of triangles, according to the origin of the graph, corresponded to negatively linked characters.

4. Discussion

4.1. Response of Pseudomonas *populations to elevated* pCO_2

Culturable *Pseudomonas* associated with the soil and the rhizosphere of *L. perenne* and *M. coerulea* grown under ambient and elevated pCO_2 content were investigated. *Pseudomonas* densities were not consistently influenced by pCO_2 treatment after 8 years of fumigation (Fig. 1). In contrast, with a cloning sequencing approach, Marilley et al. (1999) showed a stimulation of *Pseudomonas* in the rhizosphere of *L. perenne* after 3 years of elevated pCO_2. Such a difference could be explained by different methodological approach (molecular *vs*. cultural). A possible resilience of *Pseudomonas* populations during long term pCO_2 enrichment should be also evoked. There is a lack of information on microbial responses to CO_2-induced changes in natural mature ecosystems, as most of the published results have been obtained from short-term or from studies using high-nutrient experiments (Hu et al. 1999).

1228 isolated strains were tested *in vitro* for their potential ability to produce auxin (*iaa*+), hydrogen cyanide (*hcn*+), siderophore (*sid*+) and to dissimilate nitrate (*nr*+). The results do not presume the actual *in situ* activity in the rhizosphere, but give insight about the potential functionality of *Pseudomonas* spp. in the rhizosphere.

Gross results showed that elevated pCO_2 influenced the phenotypic structure (*sid*+, *hcn*+, and *nr*+ frequencies) of *Pseudomonas* associated mainly with the root fraction of *L. perenne*.

4.2. Elevated pCO_2 *did not influenced IAA producing* Pseudomonas

IAA production is a widespread trait among rhizobacteria (Forlani et al., 1995; Persello-Cartieaux et al., 2003). Bacterial synthesis of IAA in the rhizosphere may affect the development of root system either positively (production of root hairs and lateral roots) at very low concentrations (Arshad and

Frankenberg, 1991) or negatively at higher concentrations. For the plant, it can result in an increase of ion uptake (Persello-Cartieaux et al., 2003). A stimulation of root hair and lateral root production would be all the more important under elevated pCO_2 where plants need more nutrients for their increased growth (Daepp et al., 2000). In return, the advantage for root-associated bacteria would be the access to a rich supply of nutrients due to rhizodeposition (Patten and Glick, 2002). 25% of the *Pseudomonas* isolates were auxin producers whatever the sampling date (data not shown), the soil fraction, or the pCO_2 content for both soil-plant systems considered. Other studies showed that the auxin producing strain frequencies were influenced by root proximity (Lebuhn et al., 1997) or sampling season (Halda-Alija, 2003). Nevertheless information about auxin production level by strains could be important (Khalid et al., 2004) as it would reveal differences in potential auxin level that can not be reflected by *iaa+* frequencies.

4.3. Siderophore producing Pseudomonas *responded differently to elevated* pCO_2 *for* L. perenne *and* M. coerulea

Siderophore production confers a competitive advantage in rhizosphere competence and root colonization (Lugtenberg and Dekkers, 1999; Delorme, 2001 - thesis manuscript). The ecological significance of siderophore production by *Pseudomonas* strains in root colonization and plant growth promotion but also in phyto-pathogenesis is well documented (Kloepper, 1980; O'sullivan and O'Gara, 1992; Glick, 1995; Lugtenberg and Dekkers, 1999; Rainey, 1999; Sørensen et al., 2001). In the rhizosphere, a carbon rich and iron limited environment, production of siderophores that chelate the ferric iron may result in growth inhibition of others microorganisms whose affinity for iron is lower (Elad and Baker, 1985; Lemanceau et al, 1993). Competition for iron would be strengthened under elevated pCO_2 condition because of the higher carbon input into the soil and the mineral limitation due to increased plant growth. In our experiment, both perennial grassland systems responded differently to the pCO_2 increase regarding the frequency of siderophores producing *Pseudomonas* (decrease for *M. coerulea*, increase for *L. perenne*). Anyway for both plants, the siderophore producing *Pseudomonas* populations benefited generally from the root influence.

4.4. HCN producing Pseudomonas *were mainly influenced by root proximity*

HCN is a potent inhibitor of cytochrome c oxidase and of several other metalloenzymes. No role is known for HCN in primary bacterial metabolism, and it is generally considered as a secondary metabolite (Blumer and Haas, 2000). HCN producing bacteria can help plants in their defence against fungal pathogens (Voisard et al., 1989; Blumer and Haas, 2000). This property was predominantly described among *Pseudomonas* strains (Kremer et al., 2001). In the present study, about 50% of root associated *Pseudomonas* isolates were potential HCN producers for both *L. perenne* and *M. coerulea*, as previously shown in potato and wheat rhizosphere (Bakker and Schippers, 1987). These frequencies were higher in root compare to soil fractions for both plants (Fig. 2). The physico-chemical conditions prevailing in the rhizosphere could explain this stimulation of HCN producing bacteria. Indeed, Castric (1983) showed that the bacterial ability to produce hydrogen cyanide was enhanced under microoxic conditions. Oxygen consumption by root and respiration of rhizosphere microbial populations creates a decreasing gradient of oxygen from the soil to the root (Højberg et al., 1999). This might provide favourable conditions for HCN producing bacteria, as shown in this study.

4.5. Root proximity and elevated pCO_2 *influenced nitrate dissimilating* Pseudomonas

Denitrification is an anaerobic nitrate respiration process in soil environments (Gamble et al., 1977). The ability to use nitrate as alternative electron acceptor could be a competitive advantage for bacteria in the rhizosphere where carbon sources are abundant and oxygen pressure is low (Ghiglione et al., 2000). However, our results showed lower frequencies of nitrate dissimilating *Pseudomonas* in the root compared to soil fractions for both plants (Roussel-Delif et al., 2004). This result contrast with those of several studies showing that nitrate-dissimilating heterotrophic bacteria (i.e. Linne von Berg and Bothe, 1992) among which *Pseudomonas* (Clays-Josserand et al., 1999) were more frequently detected in root-associated habitats than in non-rhizosphere soil. In the plant–soil systems studied, being for *L. perenne* cultivated with low N supply (Daepp et al., 2000), or for *M. coerulea* growing on its native oligotrophic soil (Hamelin et al., 2002), the nitrogen availability is clearly limiting. A low availability of nitrate could explain the lower proportions of nitrate-dissimilating strains in root-associated fractions, where N depletion is increased by plant uptake (Fromin et al., in prep).

4.6. Rhizosphere environment under elevated pCO_2 *altered* Pseudomonas *populations*

Hydrogen cyanide production and nitrate reduction are two physiological activities energetically dependent on organic carbon sources availability and low oxygen pressure; such conditions are prevailing in the rhizosphere. Interestingly, the frequencies of hydrogen cyanide producers and nitrate reducers responded differently to root proximity (increase for *hcn*+ and decrease for *nr*+, Fig. 2). Correspondence analysis of statistical link between these two characters confirmed that *nr*+ strains were generally not *hcn*+. These results suggest that phenotypically different *Pseudomonas* guilds inhabited soil and root environments, and that *nr*+ and *hcn*+ characters may be involved in selection or counter-selection of *Pseudomonas* strains in the rhizosphere.

Root respiration and rhizodeposition (consisting mostly in low molecular weight organic carbon compounds, Darrah, 1996) increased under elevated pCO_2 for *L. perenne* (Suter et al., 2002). Oxygen limitation as well as availability in organic energy source would be strengthened near the roots and under elevated pCO_2. Consequently, we expected both nitrate reducing and HCN producing bacteria to be favoured under elevated pCO_2. This was the case for nitrate reducing *Pseudomonas*, which were generally more frequent under elevated pCO_2 for both plants (see also Roussel-Delif et al., 2004). However the *hcn*+ frequencies were lower under elevated pCO_2 in root fraction for *L. perenne*. The significance of this result is unclear, but it could indicate an alteration of rhizodeposit composition under elevated pCO_2 (Hodge et al,, 1998; Darrah, 1996), as the type of amino-acid present can influence the HCN biosynthesis (Castric, 1983).

Conclusions

This study showed that *Pseudomonas* associated with different plant/soil systems may respond differently to elevated pCO_2. This differential response could be explained by the contrasting ecology of the two plants used as model in this study (Vàsquez de Aldana and Berendse, 1997) or by the different exposure time to elevated pCO_2 (2-3 years for *M. coerulea* and 7-8 years for *L. perenne*). As pCO_2 influence was mostly visible for *L. perenne* associated *Pseudomonas*, a possible long term selection of the best adapted strains to the plant rhizosphere under raising pCO_2 could be evoked. This modification of *Pseudomonas* guilds under elevated pCO_2 should in term exert a feedback effect on plant fitness.

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CONCLUSION

Cette étude a montré que les populations de *Pseudomonas* productrices de sidérophores, de cyanure d'hydrogène et réductrices de nitrate étaient modifiées sous atmosphère enrichie en pCO$_2$, surtout dans la rhizosphère de *L. perenne* et *M. coerulea*. Sur la base des quatre caractères phénotypiques testés, il n'était pas possible de donner le profil type d'un *Pseudomonas* de la rhizosphère. Cependant, la fréquence importante des producteurs de sidérophores dans la rhizosphère y suggérait une dominance du groupe des *Pseudomonas* spp. fluorescents (cf. 3.2.2). Les souches capables de produire de l'HCN étaient rarement capables de réduire également le nitrate. Par ailleurs, la fréquence des producteurs d'HCN était stimulée dans la rhizosphère et celle des réducteurs de nitrate diminuée. Ces résultats suggèrent que les *Pseudomonas* spp. producteurs de cyanure d'hydrogène et réducteurs de nitrates forment deux groupes distincts, l'un étant sélectionnée et l'autre contre sélectionnée dans les rhizosphères de *L. perenne* et de *M. coerulea*. Finalement, cette étude a également montré que les producteurs de sidérophores et les réducteurs de nitrate étaient stimulés sous pCO$_2$ élevée dans la rhizosphère. Ces changements dans les populations de *Pseudomonas* en réponse à l'augmentation en pCO$_2$ pourraient être reflétés également au niveau de leur structure génotypique. La réponse inhabituelle des *Pseudomonas* réducteurs de nitrate dans la rhizosphère (diminution dans les fractions racinaires) et leur stimulation sous pCO$_2$ élevée, en fait une population intéressante à étudier plus précisément du point de vue de leur diversité, et de la disponibilité en nitrate dans le sol. Ces deux aspects seront abordés respectivement dans les paragraphes 3.2 et 3.3.

3.2. DIVERSITE DES *PSEUDOMONAS* REDUCTEURS DE NITRATE ASSOCIES A LA RHIZOSPHERE DE *LOLIUM PERENNE* ET *MOLINIA COERULEA* SOUS pCO$_2$ ELEVEE

INTRODUCTION

Dans la thématique de l'augmentation de la concentration atmosphérique en CO_2, la disponibilité en azote joue un rôle important dans l'équilibre C/N du système. Pour les prairies de *Lolium perenne* cultivées avec un faible amendement en azote, une diminution de la disponibilité en azote était mise en évidence au cours du cycle de croissance de la plante (cf. 1.1.1). Par ailleurs, une stimulation des émissions de N_2O était observée sous fort pCO$_2$ lorsque la fertilisation en azote était non-limitante, et suggérait une stimulation des organismes dénitrifiants dans ces conditions (Baggs et coll. 2003). Nous venons de voir en 3 .1, que la fréquence des *Pseudomonas* réducteurs de nitrate était augmentée à proximité de la racine sous pCO$_2$ élevé, ceci à la fois pour *L. perenne* (faible fertilisation azotée) et *M. coerulea*. Plus généralement, *Pseudomonas* a été décrit comme l'un des principaux genres bactériens cultivables dénitrifiants dans le sol (Gamble et coll. 1977). Dans ce contexte, il nous paraissait important d'examiner comment la proximité de la plante et la pCO$_2$ élevée pouvaient influencer la diversité des populations de *Pseudomonas* dissimilant le nitrate.
Actuellement deux formes de nitrate réductases sont connues chez *Pseudomonas*, une forme membranaire (NAR) et une forme périplasmique (NAP), codées respectivement par les gènes *nar* et *nap*. Les fréquences de l'une ou l'autre forme, ou encore des deux formes, ont été étudiées parmi les *Pseudomonas* spp. réducteurs de nitrate isolés du sol et de rhizosphère de *L. perenne* et *M. coerulea* cultivées sous pCO$_2$ élevée et ambiante. La diversité des *Pseudomonas* a également été évaluée par analyse PCR-RFLP, en ciblant les gènes *narG* et *napA* et la séquence 16S-ITS1 (cf. 2.1 et 2.2). Ces aspects seront détaillés dans les paragraphes 3.2.1 et 3.2.2. Le lien possible entre la taxonomie des *Pseudomonas* réducteurs de nitrate et le type de nitrate réductase qu'ils présentent a été examiné et sera également abordé en 3.2.2.

3.2.1. FREQUENCE ET DIVERSITE DES GENES DE NITRATES REDUCTASES DES *PSEUDOMONAS* REDUCTEURS DE NITRATE ASSOCIES A LA RHIZOSPHERE DE PLANTES PERENNES

Les résultats présentés dans ce paragraphe ont été obtenus lors du travail de diplôme de Ludovic Roussel-Delif, réalisé sous la direction de Sonia Tarnawski et Nathalie Fromin.

Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions

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ABSTRACT

A total of 1246 *Pseudomonas* strains were isolated from the rhizosphere of two perennial grasses (*Lolium perenne* and *Molinia coerulea*) with different nitrogen requirements. The plants were grown in their native soil under ambient and elevated atmospheric CO_2 content (pCO_2) at the Swiss FACE (Free Air CO_2 Enrichment) facility. Root-, rhizosphere-, and non-rhizospheric soil- associated strains were characterized in terms of their ability to reduce nitrate during an *in vitro* assay and with respect to the genes encoding the membrane-bound (named NAR) and periplasmic (NAP) nitrate reductases so far described in the genus *Pseudomonas*. The diversity of corresponding genes was assessed by PCR-RFLP on *narG* and *napA* genes, which encode the catalytic subunit of nitrate reductases. The frequency of nitrate-dissimilating strains decreased with root proximity for both plants and was enhanced under elevated pCO_2 in the rhizosphere of *L. perenne*. NAR (54% of strains) as well as NAP (49%) forms were present in nitrate-reducing strains, 15.5% of the 439 strains tested harbouring both genes. The relative proportions of *narG* and *napA* detected in *Pseudomonas* strains were different according to root proximity and for both pCO_2treatments: the NAR form was more abundant close to the root surface and for plants grown under elevated pCO_2. Putative denitrifiers harboured mainly the membrane-bound (NAR) form of nitrate reductase. Finally, both *narG* and *napA* sequences displayed a high level of diversity. Anyway, this diversity was correlated neither with the root proximity nor with the pCO_2 treatment.

INTRODUCTION

The dissimilatory reduction of nitrate to nitrite by bacteria can be followed by a reduction of nitrite to either ammonium during nitrate ammonification, or to gaseous nitrogen compounds (NO, N_2O and N_2) during the respiratory denitrification process. Denitrification was described as the main process of nitrate dissimilation in soil environments [20, 53]. It depends on the presence and availability of electron donors, mostly organic compounds, and of the electron acceptors (nitrogen oxides, oxygen). All these proximate

factors could be modified by plant (exudation of organic compounds, nitrate assimilation) as well as by associated microflora. Several studies demonstrated that plants influence soil-inhabiting nitrate-dissimilating bacterial microflora [12, 31, 44]. Indeed, nitrate dissimilation should be favoured in the rhizosphere, where rhizodeposition provides organic substrates [46]. Several authors showed that heterotrophic bacteria [29] or *Pseudomonas* isolates [11, 12, 16] displaying nitrogen-dissimilating activity were more frequently detected in root-associated habitats than in non-rhizospheric soil. Moreover, the ability to use nitrate as alternative electron acceptor could be a competitive advantage for bacteria in the rhizosphere, where oxygen is limiting due to root and microbial respiration [21]. On the contrary, Nijburg and co-workers [37, 38] reported a lower proportion of nitrate-reducing bacterial isolates in the rhizosphere of the aerenchymatous wetland plant *Glyceria maxima* compared to non-rhizospheric soil, suggesting that the availability of nitrate is also crucial for nitrate dissimilation. Various studies showed that denitrification in soil is also influenced by soil properties and agricultural practices [9, 12, 17, 40, 54].

Among the factors that might influence denitrification in the soil plant systems, the increasing atmospheric CO_2 content (pCO_2), due to increasing fossil fuel burning and land use conversion [56] is of main interest. Elevated pCO_2 induces alteration of both rhizodeposition [15] and nitrogen transformations [34, 48, 57]. Activity measurements have shown that both potential and actual denitrification rates were higher in grassland soils under elevated pCO_2 [6, 49], resulting in increased nitrous oxides emission [1, 28].

The complete denitrification pathway is a modular process comprising four sequential enzymatic reductions (involving nitrate-, nitrite-, nitric oxide- and nitrous oxide- reductases) [59]. The dissimilative reduction of nitrate to nitrite is the most commonly occurring step and the corresponding genes were used as molecular markers for studying the nitrate dissimilating communities [10, 18, 23, 24, 39, 44]. So far, two metallo-enzymes involved in the dissimilatory reduction of nitrate have been described in bacteria: a membrane-bound nitrate reductase (termed NAR), which is expressed under anaerobic conditions, and a periplasmic enzyme (NAP), whose regulation varies in different organisms [42, 47, 59]. NAP was described as being involved in aerobic or anaerobic nitrate dissimilation, in the transition from aerobic to anaerobic metabolism [3, 47], in dissipating the excess of photosynthetic reducing power [36], or finally in nitrate scavenging [45]. Both forms of nitrate reductases can be present simultaneously in a single strain [3, 7, 18, 24, 41].

Numerous investigations suggested that bacteria affiliated to the genus *Pseudomonas* are the predominant culturable denitrifiers in soil [7, 8, 9, 20]. Moreover, Marilley and co-workers [33] showed that the proportion of *Pseudomonas* was enhanced in the rhizosphere of *L. perenne* grown under elevated pCO_2. The nitrate-dissimilating *Pseudomonas* populations (size and composition) are therefore very likely to be altered in the rhizosphere of plants grown under elevated pCO_2. The aim of this work was to assess the influence of the plant and elevated pCO_2 treatment on the structure of nitrate-dissimilating *Pseudomonas*. Two perennial grasses with different nitrogen requirements [55] were used as model plants: the nitrophilic *Lolium perenne* and the oligonitrophilic *Molinia coerulea*. Bacteria affiliated to the genus *Pseudomonas* were previously shown to account for a significant part of the microflora associated to the rhizosphere of these two plants [33, 52]. A large collection of nitrate-dissimilating *Pseudomonas* strains isolated from the root, rhizosphere and surrounding soil of these plants was characterized regarding their frequency and their nitrate reductase genes. The nature of nitrate reductase(s) (periplasmic *versus* membrane-bound) and the diversity of the corresponding *narG* and *napA* genes was assessed by PCR and restriction fragment length polymorphism.

METHODS

Study site and plant material.

Lolium perenne and *Molinia coerulea* were grown under ambient (360 ppm, noted C for control) and elevated pCO_2 (600 ppm, noted T for treated) at the FACE (Free Air CO_2 Enrichment) facility, Eschikon, Switzerland [26]. The meteorological data during the period of experiment (rainfall, temperature and radiation) can be found on the Swiss FACE website (http://www.fb.ipw.agrl.ethz.ch/FACE.html). The treated plots have been enriched with atmospheric CO_2 during daylight between March and December. *L. perenne cv Bastion* (noted L) was grown as a monoculture on 3 control (C) and 3 pCO_2-treated (T) replicate plots since May 1993. The plants were grown in the local autochthonous fertile, eutric cambisol. Shoots were harvested four times a year. The C and T plots received 14 g m^{-2} y^{-1} N, NH_4NO_3, (at the

beginning of the season, and then after each cut, except for the last). This amount is limiting for plant growth [14]. *M. coerulea* plants (noted M) originated from a littoral meadow located at the southern shore of Lake Neuchâtel (Cudrefin, Switzerland). The local soil is a gleysol, typic haplaquoll, and contained 4.7% clay, 9.5% silt, 85.8% sand, and had a $pH_{[H2O]}$ value of 8.4 [25]. Plants with undisturbed root systems were taken with their surrounding and underlying soil and transferred to FACE facility in September 1999. About 0.7 m^2 of littoral meadow with reconstructed soil profile below the root horizon (total depth: 35 cm) was installed in one control plot (C) and one pCO_2-treated plot (T). *M. coerulea* plants were neither cut nor fertilized.

Sampling plan.
For *L. perenne*, the three replicates for both LC and LT plots were sampled in June, July, and October 2000, as well as in July and December of the following year. During the growth season, sampling of *L. perenne* was always performed immediately prior to a cut. For *M. coerulea*, both C and T plots were sampled in June 2000, then in 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. For *L. perenne*, 3 fractions were recovered: non-rhizospheric soil (NRS) for soil devoid of roots, rhizospheric soil (RS) for root-adhering soil (recovered by washing the roots twice in 20 ml sodium phosphate buffer 0.1M pH 7.0 (SPB) under agitation during 30 min), and rhizoplane-endorhizosphere (RE) for thoroughly washed roots. For *M. coerulea*, the rhizosphere fraction is not easy to define. Thus only two fractions (NRS and RE) were analysed.

***Pseudomonas* isolation.**
Isolation of *Pseudomonas* strains was performed as previously described [52]. Briefly, approximately 1 g of fresh weight root or soil was crushed in 10 ml SPB. The root and soil suspensions were ten-fold serially diluted in SPB and spread on mS1 medium, which is selective for *Pseudomonas* [19, 52]. Colony forming units (cfu) were determined after 72 h of incubation at 24°C. Twenty (June 2000), twelve (July, October 2000 and July 2001), and fifteen bacterial colonies (December 2001) were randomly picked from appropriate dilution plates (between 20 to 200 colonies per Petri dish) and checked for purity on ten-fold diluted Tryptic Soy Agar (Difco). The affiliation of mS1 isolates to the genus *Pseudomonas* was checked by hybridisation with PSM_G probe [5], and confirmed by PCR amplification of 16S-23S rDNA spacer region with *Pseudomonas*-specific primers [30].

Nitrate-reduction and denitrification activity assays.
Nitrate-dissimilative activities were tested as follows. Each strain was cultivated in duplicate tubes containing 6 ml Nutrient Broth (devoid of fermentable substrate) (Merck) supplemented with 2 g l^{-1} KNO_3. A small inverted Durham tube was added to each tube in order to detect gas formation. The cultures 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), reagent B (N,N dimethyl-1-naphthylamine 0.6% v/v) and powdered zinc to reduce the remaining nitrate to nitrite. If nitrate was not reduced and no gas formation was observed, the bacteria were scored as non-dissimilative (NDi). Strains that were able to reduce nitrate, were scored as dissimilative, which included nitrate reducers (NR) and putative denitrifiers (D) [32]. 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 term 'denitrifiers' refers to all bacteria that produced gas under the experimental conditions used. The exact gas composition was not determined. In case nitrate removal occurred but neither nitrite nor gas was formed, the bacteria were considered as nitrate-ammonifiers or nitrate assimilators. As very few strains did fall in these categories (7 for *L. perenne* and 1 for *M. coerulea*), the corresponding proportions were not further considered.

The proportions of NR and D *Pseudomonas* in the different treatments were analysed using either Tukey multiple comparison test (proportions of NR for *Lolium*) or Fisher LSD exact test (D proportions for *Lolium*, NR and D proportions for *Molinia*). The whole data for *L. perenne* were also analysed using a generalised linear model with a logistic regression model, which is adapted for binary data. This analysis was used to compute the probabilities corresponding to the effects of the parameters (sampling date, C/T treatment, and plot in C/T treatments) on the NR and D proportions. For all analyses, the null hypothesis (similar proportions) was rejected and the observed differences were considered as either significant for $P \leq 0.05$ or highly significant for $P \leq 0.01$. The tests were performed using S-plus 6 Statistical Software (Insightful Corporation, Seattle, Washington).

Co-amplification of *narG* and *napA* fragment genes.
Genomic DNA of all nitrate-dissimilating strains for 3 sampling dates (June 2000, July 2001 and December 2001) was extracted 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 exopolysaccharides. *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 1) were used to amplify a 1008 bp fragment, according to Delorme (Delorme S 2001 PhD Thesis, Université de Bourgogne, France). Fnap and Rnap primers (Table 1) were used to amplify an 890 bp fragment of *napA*. They corresponded to previously described V66 and V17 primers [18], which were slightly shortened in order to get annealing temperatures compatible with those of *narG* primers. The primer sets were designed for the amplification of *narG* from *Pseudomonas* and of *napA* for a large range of bacteria. 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 $\mu g\ \mu l^{-1}$ T4 Gene 32 Protein (QBiogene), 0.05 $U\ \mu l^{-1}$ Taq DNA Polymerase (Promega) and 0.1 $\mu l\ \mu l^{-1}$ pure or ten-fold diluted DNA extract. The reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachussetts). 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 1: PCR primers used for the amplification of *narG* and *napA* gene fragments.

Primers	Target genes	Position 5'-3'(1)	5' - 3' Sequences
DMHPF*	*narG*	2413-2426	GAYATGCAYCCGTT
DNDWI*	*narG*	3412-3425	AYCCARTCRTTRTC
Fnap**	*napA*	245-269	TTYYTNHSNAARATHATGTAYGG
Rnap**	*napA*	1102-1123	TGYTGRTTRAANCCCATNGTCCA

(1) position on *Pseudomonas aeruginosa* strain A01 *narG* (accession number AE004804) and *napA* (NC_002516) 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.
*Delorme S 2001 PhD Thesis, Université de Bourgogne, France
** adapted from [18]

RFLP analysis of *narG* and *napA* fragment genes.
Each PCR product (about 100 ng) was submitted to enzymatic restriction with *Alu*I for *narG* [44] and with *Hae*III for *napA* fragments (*Hae*III was selected 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 restriction profile were clustered in *narG* and *napA* operational taxonomic units (OTUs) noted nar1 to nar37, and nap1 to nap49 respectively.

RESULTS AND DISCUSSIONS

The proportions of nitrate-reducing *Pseudomonas* as well as the frequency and diversity of membrane-bound (*narG*) and periplasmic (*napA*) nitrate reductase genes were investigated for a large collection of *Pseudomonas* strains isolated at different sampling dates from the root (RE), rhizosphere (RS) and surrounding soil (NRS) of two perennial grasses: the nitrogen-demanding *L. perenne* and the oligonitrophilic *M. coerulea*, for plants grown under ambient and elevated pCO_2.

Nitrate reducing and denitrifying activities in mS1-isolated *Pseudomonas*.
A total of 960 and 286 strains isolated from the root (RE) and associated soil (RS, NRS) of *L. perenne* and *M. coerulea* respectively were typified as *Pseudomonas* and retained for further analyses. These 1246 *Pseudomonas* strains were classified as nitrate-reducers (NR), putative denitrifiers (D), or non-dissimilators (NDi). For *L. perenne*, about 52% of the 960 *Pseudomonas* strains were able to reduce nitrate to nitrite (501 NR and D strains), including 14% up to gaseous compounds (135 D strains). For *M. coerulea*, these proportions were 32% (94 NR and D strains) and 13% (38 D strains) respectively.

For *L. perenne*, logistic regression analysis showed that NR frequencies were sometimes statistically different between the different sampling dates and the replicate plots in C and T treatments (especially for plot number 3 under ambient pCO_2). Interestingly, this plot was described as having lower nutrient availability and received increased fertilisation amounts [14]. Differences in NR and D according to the sampling dates could result from the evolution of the microflora in relationship to soil water content before the time of sampling. Indeed, soil water content controls air diffusion, which in turn, along with aerobic respiratory activity, determines the soil oxygen content [17, 53, 54]. Low water content may also limit denitrification process because of higher redox potential and nitrate diffusion [51, 54].

Proportions of nitrate reducing and denitrifying Pseudomonas were compared between NRS, RS and RE fractions and between control and elevated pCO_2. The results are presented in Table 2 (each sampling date separately) and Fig. 1 (gathering of all sampling dates). Lower frequencies of NR Pseudomonas were generally detected in the root fraction (RE,) compared to soil (NRS) fraction, for both plants (Fig. 1), with statistically significant differences for some sampling dates (Table 2). For rhizospheric soil (RS), intermediate NR frequencies were generally observed. The proportions of D strains also decreased in RE compared to NRS fraction for L. perenne. However, putative denitrifying Pseudomonas were more frequently detected in root fraction for M. coerulea, especially in December 2001.

In the plant–soil systems studied, being for *L. perenne* cultivated with low N supply [14], or for M. coerulea growing on its native oligotrophic soil [25], the nitrogen availability is clearly limiting. The availability of N is dependant on the N supply (fertilisation), N uptake by the plants and the N transformations, which can imply N losses from the system (i.e. denitrification, leaching). A low availability of nitrate could explain the lower proportions of nitrate-dissimilating strains in root-associated fractions, where N depletion is increased by plant uptakes. Corre and co-workers [13] suggested a high competition for available N between microorganisms and plant. Moreover, Nijburg and co-workers [37, 38] noticed that addition of nitrate in the rhizosphere of G. maxima resulted in an increased number of NR strains. In order to test the role of nitrate availability, a similar experiment was conduced on L. perenne grown with low and high (non-limitative) nitrogen supply, during which the role of nitrate availability was confirmed (L. Roussel-Delif, S. Tarnawski, J. Hamelin, EM Baggs, M. Aragno, and N. Fromin, in prep).

Nitrate-dissimilating *Pseudomonas* frequency under elevated pCO_2 treatment
With respect to the effect of pCO_2 treatment, higher proportions of nitrate reducing and putative denitrifying *Pseudomonas* were generally observed in the rhizosphere of *L. perenne* grown in pCO_2-treated plots compared to control plots (Fig. 1). For *M. coerulea*, no relation between pCO_2 treatment and proportions of nitrate dissimilating *Pseudomonas* was observed.

Under elevated pCO_2, the rhizodeposition rate increases due to higher net photosynthesis rate [15]. This possibly enhances microbial activities that are dependent on C supply, such as nitrogen transformations [57]. Indeed, higher N_2O emissions were measured in high N-fertilised *L. perenne* monoculture under elevated pCO_2 [1, 28]. Anyway, long-term CO_2 enrichment experiments have shown that N may become a limiting factor for the reaction of plants (including grasses) to elevated pCO_2 [14, 58]. In the studied (N limited) plots, experimental results suggested that pCO_2 treatment had no significant effect on apparent N uptake and transformations [22, 48]. Anyway, the nitrate-dissimilating

Pseudomonas, which were more frequent under elevated pCO$_2$, seemed to benefit from the higher C entry in the system.

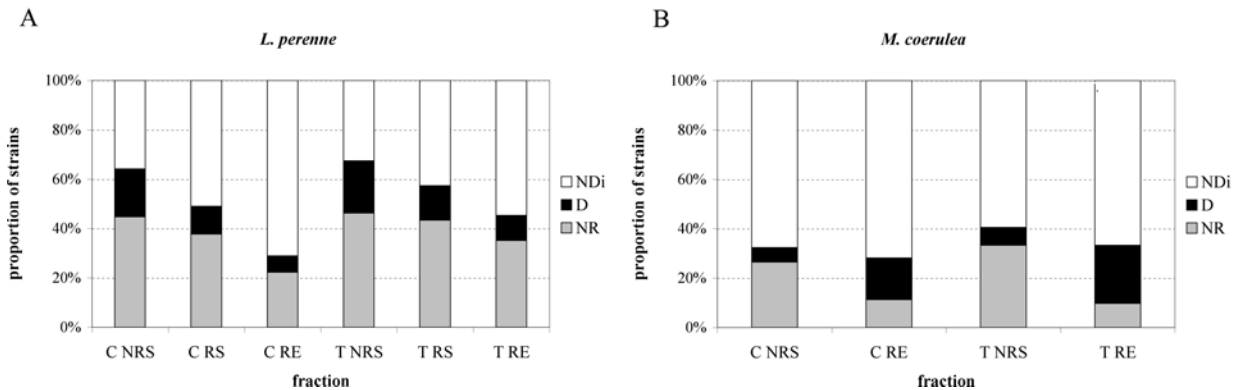

Fig. 1. Proportions of nitrate-reducing (NR), denitrifying (D) and non-dissimilating (NDi) *Pseudomonas* associated with A: *Lolium perenne* (n = 960 strains) and B: *Molinia coerulea* (n = 286 strains): total for all sampling dates. C: control plots (ambient pCO$_2$); T: pCO$_2$-treated plots; NRS: non-rhizospheric soil; RS: rhizospheric soil; RE: root fraction.

Type of nitrate reductase genes (*narG vs. napA*) among nitrate-dissimilating *Pseudomonas*

The presence of NAR, NAP, or both forms of nitrate reductases was checked by a *narG*-*nap*A multiplex PCR protocol for the nitrate reducing and denitrifying *Pseudomonas* strains isolated from 3 sampling dates. The amplification with crossed pairs of primers for strains harbouring both genes did generate additional minor, faint nonspecific PCR products (Fig. 2), which did not interfere with the further restriction analysis. A total of 344 NR or D *Pseudomonas* isolated from *L. perenne*, and 95 from *M. coerulea* were analysed (Fig. 3). There was a good correspondence between the nitrate reductase activity and the presence of *narG* or/and *napA* as 91.6% of the 439 NR or D strains generated a *narG* and / or *napA* amplification product of expected size. Nine strains yielded a nonspecific amplification product. For the remaining 38 strains (neither *narG* nor *napA* detected), the nitrate reductase activity could be related either to a nitrate reductase encoded by *narG* or *napA* genes that could not be amplified (quality of the DNA extract, sequence mismatch) or to another form of nitrate reductase.

The NAP as well as the NAR forms were detected in nitrate-dissimilating *Pseudomonas* strains, as previously suggested [3]. A total of 180 strains (56%) had the *narG* gene only, 154 strains (51%) had the *napA* gene and 68 strains (15.5%) possessed both nitrate reductase genes. Strains displaying amplification for both genes were more frequently recovered from *M. coerulea* (30.5% of strains) than from *L. perenne* (11.3%). Carter *et al*. [7] noticed that soil isolates displaying a periplasmic nitrate reductase (NAP) were as abundant as those displaying the membrane-bound (NAR) form. A significant proportion of bacterial isolates displaying both forms was also observed from a freshwater sediment [24].

Most of the putative denitrifying (gas producers) *Pseudomonas* had the NAR form, alone or together with NAP (65 among the 69 D strains analysed for *L. perenne* and 27 among the 29 D strains for *M. coerulea*). A few D strains had only the NAP form (4 for *L. perenne* and 2 for *M. coerulea*). In several D strains, including those having the NAP form only, the denitrifying trait was confirmed by the amplification of *nosZ* gene (encoding the nitrous oxide reductase) fragment (data not shown).

For *L. perenne* a higher proportion of *narG* (alone or together with *napA*) was observed in root compared to soil fraction, for both control (LC) and treated (LT) plots, for the three sampling dates (statistically significant when grouping the data from the 3 sampling dates). An increasing occurrence of *narG* form (alone or together with *napA*) was also detected under elevated pCO$_2$ treatment as compared to control plots, for both NRS and RE fractions (Fig. 3). Such an increase was observed at the 3 sampling dates for root fraction, and in June 2000 and December 2001 for soil fraction (data not shown). Regarding this distribution of nitrate reductase forms, the *Pseudomonas* isolated from RS fraction were intermediate and the effects of pCO$_2$ treatment was less straightforward. For *M. coerulea*, higher proportions of nitrate-reducing *Pseudomonas* strains having *narG* (alone or together with *napA*) were also noticed in root compared to soil fraction and under elevated pCO$_2$ content for both root and soil fraction (Fig. 3).

Table 2: Proportions of nitrate-reducing (NR) and denitrifying (D) *Pseudomonas* associated with *Lolium perenne* (total: 960 *Pseudomonas* strains) and *Molinia coerulea* (total: 286 *Pseudomonas* strains).

L. perenne	Control plots			Treated plots		
	NRS	RS	RE	NRS	RS	RE
June 2000	n = 49	n = 35	n = 37	n = 57	n = 48	n = 37
NR (%)	55 [a]	51	24 [bA]	47 [a]	73 [b]	59 [B]
D (%)	8	3	0	21	13	3
July 2000	n = 35	n = 23	n = 28	n = 22	n = 18	n = 22
NR (%)	37	56.5	11	68	50	14
D (%)	46 [aA]	26	7 [b]	18 [B]	6	0
Oct 2000	n = 32	n = 19	n = 25	n = 24	n = 20	n = 18
NR (%)	41	26	12	29	30	6
D (%)	9	5	12	29	5	22
July 2001	n = 34	n = 29	n = 34	n = 27	n = 22	n = 13
NR (%)	44	17	41	48	18	31
D (%)	12	17	6	15	14	8
Dec 2001	n = 43	n = 45	n = 45	n = 45	n = 36	n = 38
NR (%)	40	36	18 [A]	42	22	39 [B]
D (%)	23	9	9	22	25	18

M. ceorulea	Control plot		Treated plot	
	NRS	RE	NRS	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 [a]	27 [b]	5 [a]	43 [b]

n, number of strains tested for the corresponding fraction. Different letters indicate NR or D proportions that are statistically different: [a,b] for differences between fractions NRS/RS/RE for a given treatment, and [A, B] for differences between control (C) and elevated pCO$_2$-treated (T) plots for a given fraction. Similar proportions were not indicated. For *L. perenne*, the proportions were calculated using the 3 replicate plots.

These results taken as a whole suggests that the NAR and NAP forms have probably different functions within the corresponding organisms, despite the fact that they catalyse a similar reaction. The periplasmic nitrate reductase (NAP) is probably a functionally versatile enzyme according to the organisms [42, 47]. In some organisms, NAP clearly has a role in anaerobic denitrification [3, 47, 50]. For some others, it may provide the biochemical apparatus for aerobic nitrate respiration [7, 35], including for *Pseudomonas* strains [18]. As noted by some authors [9, 20], nitrate-reducing bacteria were abundant in soil (66% of the 368 *Pseudomonas* in soil fraction for *L. perenne*), a prevailing oxic environment, except in case of poorly drained soils. An aerobic nitrate respiration would be useful on organic carbon substrates or under micro-oxic conditions [4], such conditions being frequent in soil and rhizosphere microhabitats. In that sense, Carter *et al*. [7] suggested that the ability to co-respire oxygen and nitrate may be particularly important in environments that are rich in organic carbon or subjected to limiting or fluctuating oxygen availability, such as in soil aggregates [40]. The membrane-bound nitrate reductase (NAR) is expressed under low oxygen partial pressure [2, 43]. A predominance of this form was observed among *Pseudomonas* isolated from root environment, where oxygen content is low. We also provided evidence of a higher proportion of NAR form among *Pseudomonas* strains isolated from plants grown

under elevated pCO_2. The significance of this result is unclear. However, a predominance of the NAR form was expected under elevated pCO_2, where higher amounts of free sugars are released in the rhizosphere, leading to increased oxygen consumption by microbial cell respiration (G. Bachmann, T. Röhrich, K. Fertinger, and F. Thomas, pers. comm.).

Diversity of *narG* and *napA* genes.

The diversity of *narG* and *napA* genes among nitrate-reducing and putative denitrifying *Pseudomonas* was then evaluated by restriction analysis of PCR products. A total of 287 strains generated an interpretable restriction profile. Both genes displayed a high level of diversity: the 188 *narG*-harbouring strains were clustered into 37 *narG* (noted nar1 to nar37) and the 205 *napA*-harbouring strains in 49 *napA* (noted nap1 to nap49) OTUs. The most abundant *narG* and *napA* OTUs were identified among strains isolated at several sampling dates (data not shown) confirming the good reproducibility of the results over time.

Forty-three *Pseudomonas* strains harbouring both *narG* and *napA* genes generated usable restriction profiles for both genes. The strains fell in 11 *narG* OTUs and 26 *napA* OTUs, and no relationship between the type of *narG* and that of *napA* could be demonstrated. As mentioned above, most of the putative denitrifying strains harboured the *narG* gene. They were clustered into 14 of the 37 *narG* OTUs, including the three most abundant OTUs. For instance, 77% of the strains belonging to the most abundant *narG* OTU were putative denitrifiers. The 35 denitrifying strains harbouring the *napA* gene (alone or together with *narG*) were distributed among 19 of the 49 *napA* OTUs. In both cases, none of the major *narG* or *napA* OTU was composed only of D strains or of NR strains.

For *L. perenne*, the allocation of strains into the different OTUs was considered according to the root proximity and the pCO_2 (Fig. 4). The 141 *narG*-strains grouped into 31 *narG* OTUs (noted nar1 to nar31). Twelve *narG* OTUs were represented by a single isolate. The 167 *napA*-harbouring strains were clustered into 38 different *napA* OTUs, noted nap1 to nap38, of which seventeen were represented by a single strain. The most abundant *narG* (nar1 to nar5) and *napA* (nap1 to nap5) OTUs as well as many others were recovered from soil and root-associated fractions, and from both control and pCO_2-treated plots. Interestingly, nap4 was quite abundant among rhizosphere strains, whereas it was poorly represented in other fractions, and two *napA* OTUs (nap9 and nap13) were detected among root strains for control plots only.

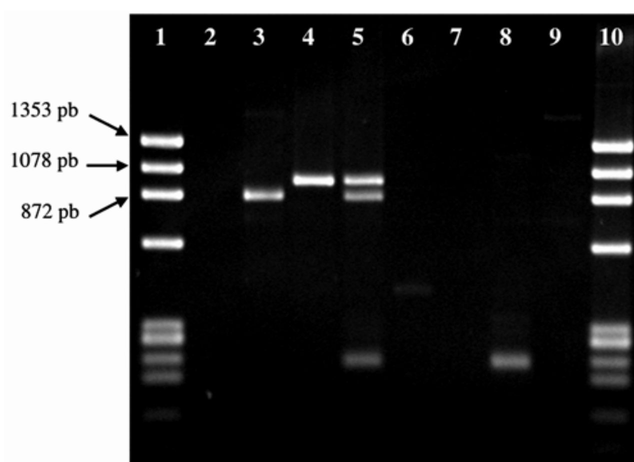

Fig. 2. PCR co-amplification of *narG* and *napA* gene fragments from strain ELC3RE3. Lanes 1 and 10: ΦX 174 DNA/ *Hae*III molecular weight marker (Promega); lane 2: no DNA template; lane 3: Fnap and Rnap; lane 4: DMHPF and DNDWI; lane5: Fnap, Rnap, DMHPF and DNDWI; lane 6: Fnap and DMHPF; lane 7: Fnap and DNDWI; lane 8: Rnap and DMHPF; lane 9: Rnap and DNDWI.

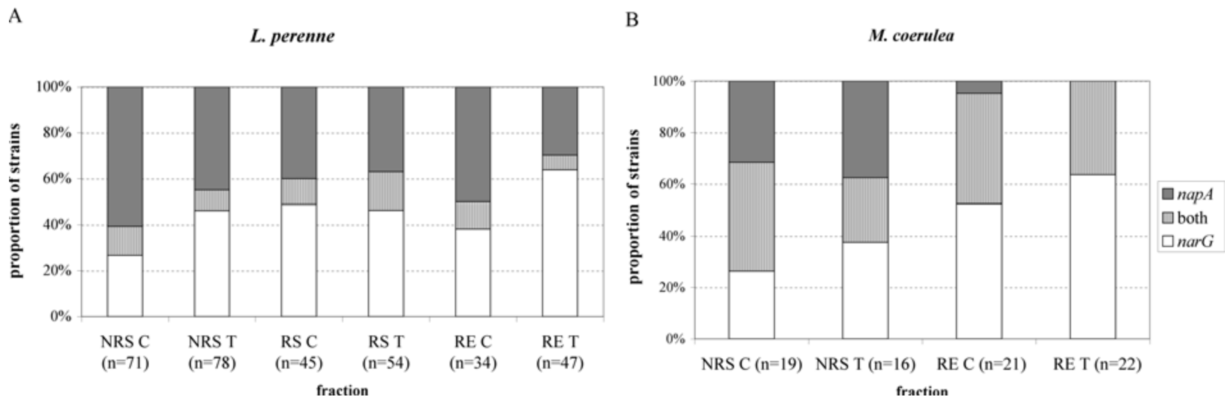

Fig. 3. Proportions of strains having *narG* and/or *napA* genes among NR *Pseudomonas* associated with A: *Lolium perenne* and B: *Molinia coerulea*. NRS: non-rhizospheric soil, RS: rhizosphere fraction, RE: root fraction; C: control plots, T: pCO_2-treated plots; n= number of strains analysed (NR strains giving no amplification product or a product with unexpected size were not considered).

Several *narG* (nar1, nar2, nar3, nar11 and nar19) and *napA* OTUs (nap5, nap11, nap22 and nap23) were detected associated with both plants (data not shown). OTUs nar1, nar2 and nar3 were detected as the most abundant OTUs for *L. perenne* as well as for *M. coerulea*: they represented 34 of the 47 strains isolated from *M. coerulea*. However, six additional *narG* OTUs (with n ≥ 2) were detected among strains from *M. coerulea* only

These data suggest that root proximity and pCO_2 treatment had no major influence on the diversity of these genes, and consequently of the corresponding organisms. A study dealing with the diversity of *narG* sequences (Kathrin Deiglmayr, unpublished data) reached similar conclusions (no relation between pCO_2 treatment and the structure of the total nitrate-reducing community for *L. perenne* grown in the FACE, but a different structure within the replicate plots). Moreover, growth of perennial grasses at the same location for several years may lead to the enrichment of the most adapted bacterial populations in both fractions. This was previously observed for dinitrogen-fixing bacteria using a molecular inventory of *nifH* sequences in a natural meadow [25].

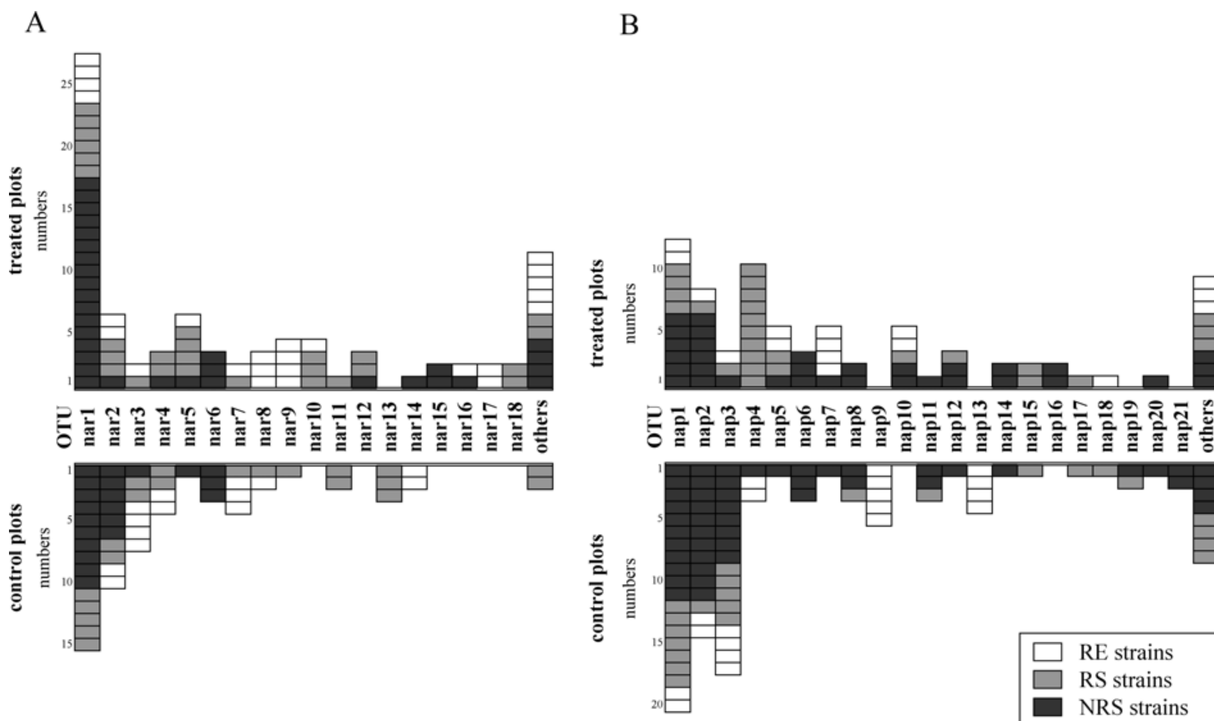

Fig. 4. Distribution of nitrate-dissimilating *Pseudomonas* strains among *narG* (A) and *napA* (B) OTUs for *Lolium perenne*. Each box represents a strain. OTUs harbouring a single strain (n=1) were grouped as 'others'.

In conclusion, the bacteria belonging to the genus *Pseudomonas* were shown to be responsive to the "rhizosphere effect" as well as to elevated pCO_2, as their abundance increased with root proximity, and in the rhizosphere of *L. perenne* grown under elevated pCO_2 [33]. As shown in the present study, the *Pseudomonas* responded to these factors also in terms of population structure (proportions of nitrate dissimilating strains as well as the type of nitrate reductase), even if no effect on the diversity of the corresponding genes was observed. Some authors suggested that shifts in the structure of denitrifying bacteria in soil could influence the *in situ* denitrification rate [8, 27]. Moreover, the nitrate dissimilating *Pseudomonas* guilds associated with *L. perenne* and *M. coerulea* responded in a similar way to the plant and to the pCO_2-treatment, despite the different ability of these plants to use soil nitrogen [55]. Such a response may affect the *in situ* nitrate dissimilation activity, with probable consequences on the nitrogen transformations and budget of these soils.

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3.2.2. Diversite des sequences d'ADNr 16S-ITS1 des *Pseudomonas* reducteurs de nitrate associes au sol et a la racine de *L. perenne* cultivee sous pCO_2 elevee et ambiante

Introduction

Dans l'étude précédente, nous n'avons pas observé de différences dans la diversité des gènes *narG* et *napA* pour les souches de *Pseudomonas* dissimilant les nitrates isolées des fractions de racine et de sol, et dans les différentes conditions de pCO_2 pour *L. perenne* et *M. coerulea*. Dans ce paragraphe, la diversité des *Pseudomonas* réducteurs de nitrate est abordée par analyse des séquences 16S-ITS1 de l'ADNr (cf. 2.2, Tarnawski et coll. 2003). Nous avons également évalué les relations entre les types ITS1 et les types *narG* et *napA* obtenus précédemment.

Materiel et methodes

La méthode PCR-RFLP de la région 16S-ITS1 de l'ADNr, détaillée dans le chapitre 2 paragraphes 2.1 et 2.2, a été appliquée aux isolats de *Pseudomonas* réducteurs de nitrate de l'étude présentée en 3.2.1. Les séquences 16-ITS1 étaient amplifiées à partir de l'ADN des *Pseudomonas* isolés du sol et de la racine de *L. perenne* cultivée sous atmosphère enrichie en pCO_2 et ambiante. Leur diversité était évaluée par analyse des profils de restriction obtenus après digestion avec l'enzyme *Taq*I des séquences ADNr 16S-ITS1. Les souches présentant des profils de restriction identiques étaient classées dans une même OTU its (Operational Taxonomic Unit). La séquence 16S partielle de l'ADNr a été amplifiée avec les amorces Ps16S (position 756-779, Locatelli et coll. 2002) et GM4r (position 1492-1507, Muyzer et coll. 1995), pour 47 souches provenant de différentes OTUs its, et a été clonée (Tarnawski et coll. 2003) et séquencée (Macrogen, Korea). Les séquences ADNr 16S obtenues ont été alignées avec les séquences les plus proches récupérées dans la banque de données Genbank en utilisant le programme BLAST (Altshul et coll. 1991). Un dendrogramme de similarité a été construit avec les logiciels ClustalX et NJplot en utilisant la méthode de Neighbour Joining.

Resultats et discussion

Diversité des *Pseudomonas* réducteurs de nitrate - PCR-RFLP de l'ADNr 16S-ITS1

La diversité des *Pseudomonas* réducteurs de nitrate est présentée dans la figure 3.2.2.1 pour les deux conditions de pCO_2 (360 *vs.* 600 ppm), et les différentes fractions sol et racine de *L. perenne*. 259 séquences ADNr 16S-ITS1 (sur 287 souches) amplifiées avec les amorces Ps16S-Ps23s (751 pb) donnaient un profil de restriction interprétable. La richesse et les indices de Shannon correspondant à la distribution des souches de *Pseudomonas* dans les différentes OTUs its, sont présentés dans le tableau 3.2.2.1, avec un rappel à titre indicatif, de quelques données sur la diversité des séquences *napA* et *narG* (cf. 3.2).

Les souches de *Pseudomonas* dissimilant le nitrate présentaient un niveau élevé de diversité, elles se groupaient en 39 OTUs its différentes. L'OTU its1 était constituée de 24% des souches, et 11 OTUs ne contenaient qu'une seule souche. La richesse en OTUs et les indices de Shannon étaient équivalents à 600 ppm et 360 ppm de CO_2 (Tableau 3.2.2.1). Les OTUs majoritaires, its1, 2 , 3, 4 et 5 (regroupant 59% des souches) étaient constituées à la fois de souches NRS, RS et RE, et détectées sous pCO_2 élevée et ambiante (Figure 3.2.2.1). On note également, que l'OTU its3 était majoritaire sous pCO_2 ambiante comparée au pCO_2 élevée, inversement pour les OTUs its5 et 6. Néanmoins, ces résultats semblaient montrer, comme l'analyse de restriction des gènes *narG* et *napA*, que la proximité de la racine et le traitement en pCO_2 n'avaient pas d'influence majeure sur la diversité des séquences ADNr 16S-ITS1, et par conséquent sur la structure génotypique des populations de *Pseudomonas* réducteurs de nitrate correspondantes.

Diversité et analyse phylogénétique des *Pseudomonas* réducteurs de nitrate

Le dendrogramme de similarités des séquences d'ADNr 16S des souches des OTUs its est présenté dans la figure 3.2.2.2. Les 47 séquences étaient affiliées au groupe des *Pseudomonas fluorescens* (défini par Anzai et coll. (2002) et regroupant des espèces proches de *Pseudomonas fluorescens*). Ce résultat, en accord avec l'étude de Clays-Josserand et coll. (1999), montrait que la réduction du nitrate est une activité répandue parmi les différentes espèces de P*seudomonas* fluorescents.

Les séquences d'une même OTU its étaient parfois affiliées aux séquences d'espèces différentes de *Pseudomonas*. Par exemple, les 9 séquences ADNr 16S de l'OTU its1 étaient proches de celles de *P. fluorescens*, *P. jessenii*, et *P. lini*. De ce fait, les OTUs définies sur la base des profils de restriction des séquences ADNr 16S-ITS1 n'étaient pas complètement représentatives des groupements en espèces de la taxonomie actuelle (voir aussi Tarnawski et coll. 2003, § 2.2). Finalement, aucune séquence n'était affiliée aux séquences des groupes *P. aeruginosa* et P. *stutzeri* (Anzai et coll. 2000). Ces espèces sont décrites comme réductrices de nitrate, mais le plus souvent dans des environnements aquatiques (Liu et coll, 2003 ; Jayakumar et coll. 2004).

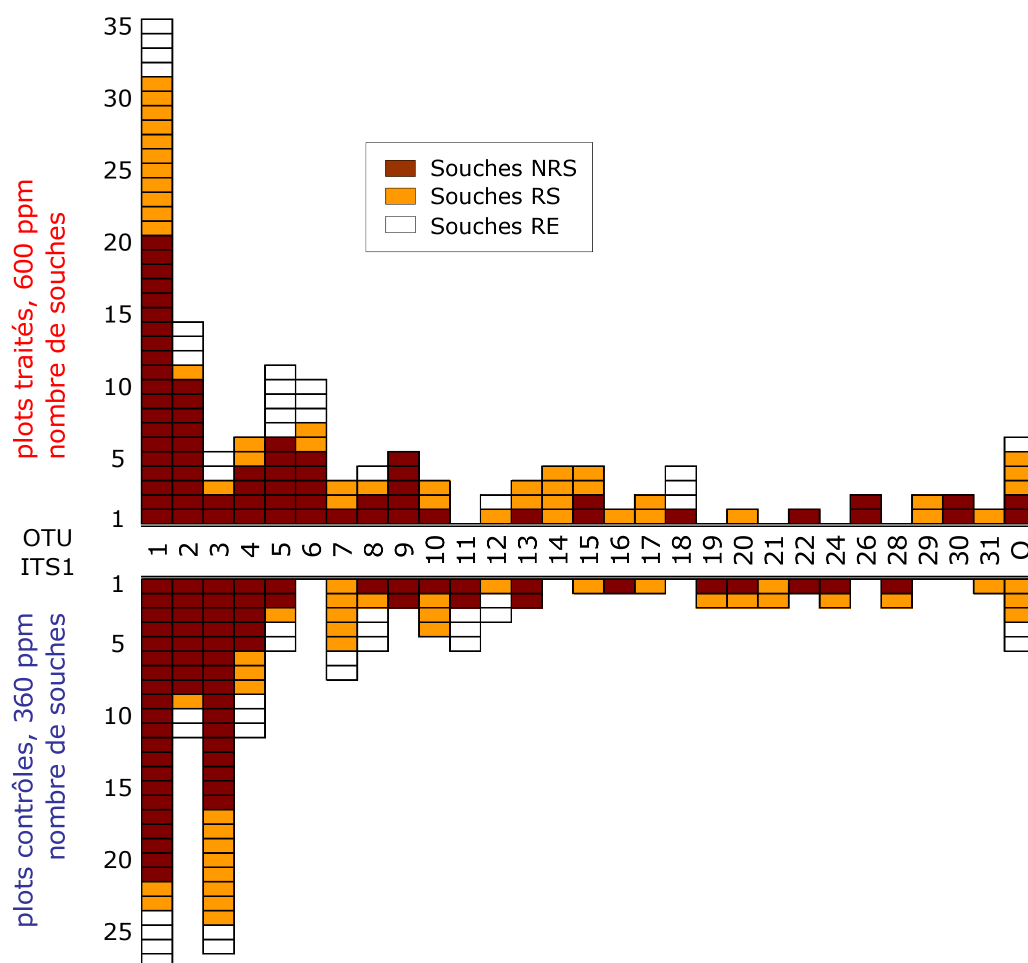

Figure 3.2.2.1. Distribution des souches de *Pseudomonas* dissimilant les nitrates associés à *Lolium perenne* parmi les OTUs its. Les OTUs sont définies par analyse de restriction des séquences 16S-ITS1 avec l'enzyme *Taq*I. Chaque « brique » représente une souche. Les OTUs constituées d'une seule souche (n=1) sont regroupées dans la catégorie OTU ITS1 O pour « others ». NRS: Sol non rhizosphérique, RS: Sol rhizosphérique, RE: Racine et endorhizosphère.

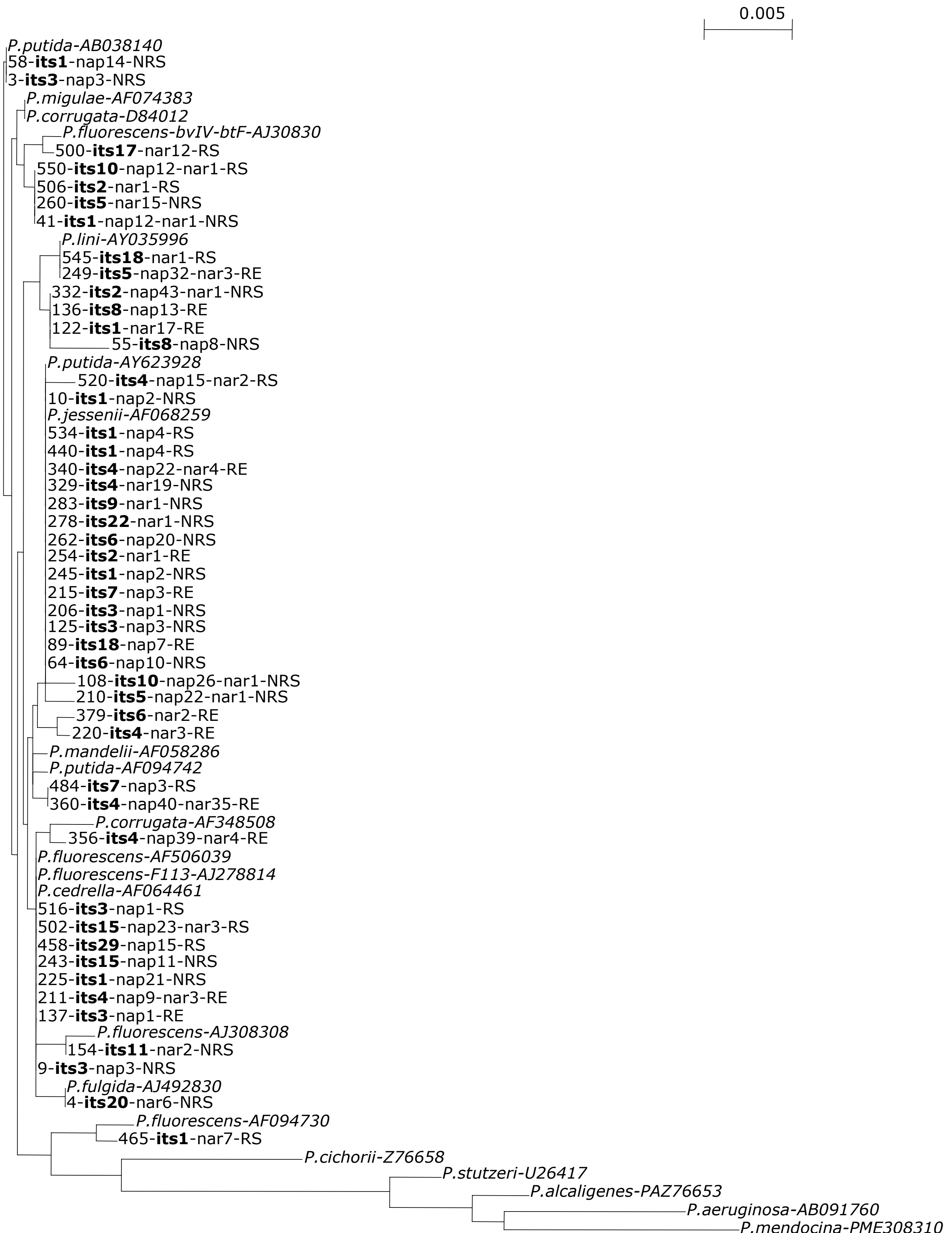

Figure 3.2.2.2. Dendrogramme de similarités des séquences d'ADNr 16S des souches de *Pseudomonas* réductrices de nitrate. Les OTUs its correspondantes à la figure 3.2.2.1 apparaissent en gras. L'origine, et les types nap et nar correspondants à la souche analysée sont présentés à titre indicatif. NRS : Sol non rhizosphérique, RS: Sol rhizosphérique, RE: racine-endorhizosphère.

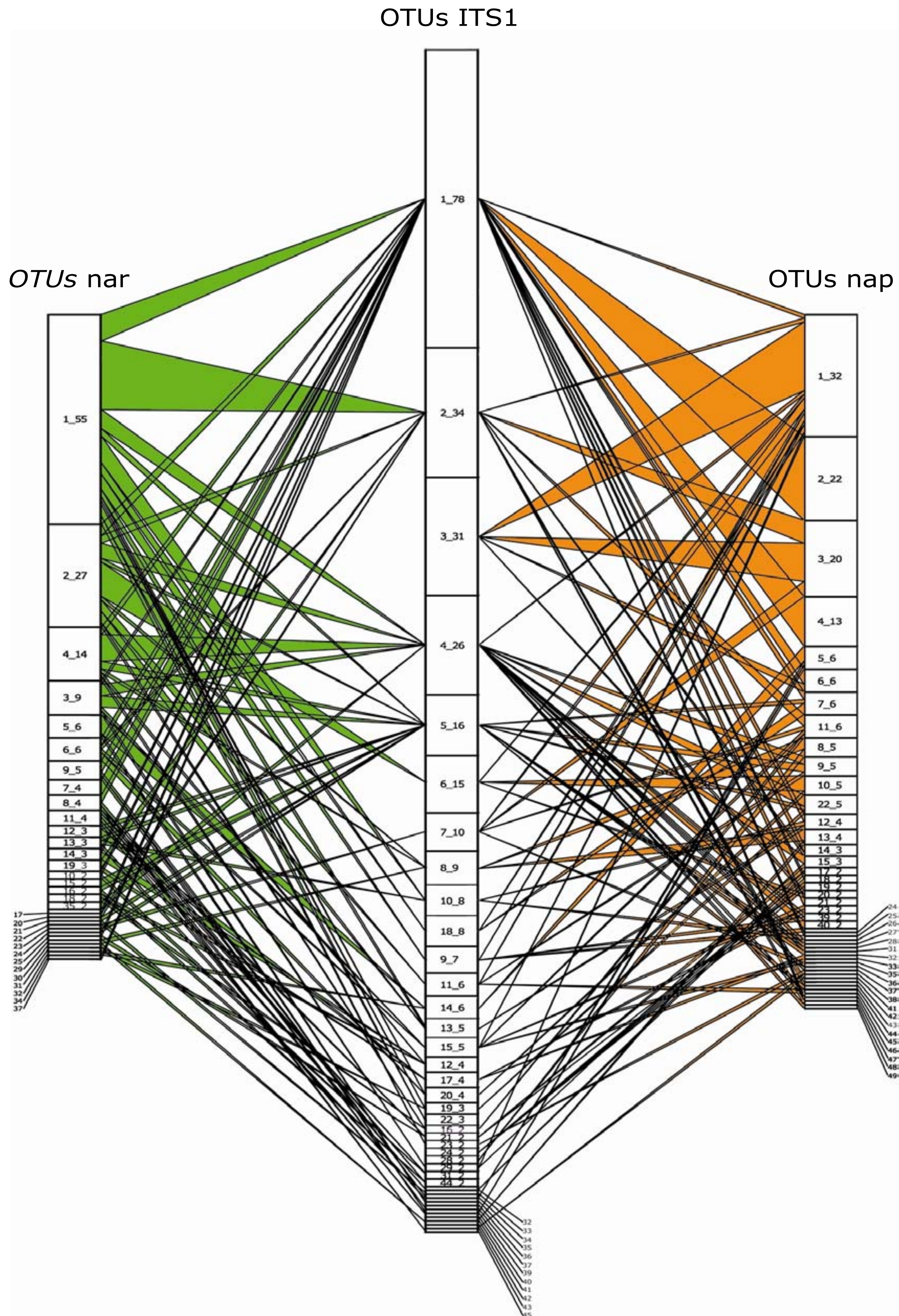

Figure 3.2.2.3. Correspondance entre les OTUs ITS1 et les OTUs nar et nap. Chaque rectangle représente une OTU. La taille des rectangles est proportionnelle au nombre de souches de *Pseudomonas* réducteurs de nitrate qui les constituent. Dans chaque rectangle, la notation X_Y correspond à: nom de l'OTU _ effectif de l'OTU. Les souches qui présentent des profils de restriction de la séquence 16S-ITS1 et du gène *narG* identiques sont reliées par des triangles verts. Les souches qui présentent des profils de restriction de la séquence 16S-ITS1 et du gène *napA* identiques sont reliées par des triangles oranges. La largeur du fuseau coloré est proportionnelle au nombre de souches de *Pseudomonas* réducteurs de nitrate qui constitue le lien.

A) Its

	600 ppm	360 ppm
H'	2,76	2,74
n OTU	29	27
n tot	131	128

	NRS	RS	RE
H'	2.41	2.98	2.47
n OTU	24	28	14
n tot	133	78	48

B) nar

	600 ppm	360 ppm
H'	2.71	2.26
n OTU	28	14
n tot	83	56

	NRS	RS	RE
H'	1.78	2.56	2.64
n OTU	14	17	18
n tot	53	48	39

C) nap

	600 ppm	360 ppm
H'	2,86	2,64
n OTU	27	26
n tot	75	90

	NRS	RS	RE
H'	2,63	2,51	2,41
n OTU	23	20	13
n tot	83	49	35

Tableau 3.2.2.1. Indices de Shannon *H'* calculés sur la base de la distribution des souches de *Pseudomonas* réducteurs de nitrate en OTUs its (A), nar (B), et nap (C) (voir figure 3.2.2.1 et figure 4 du paragraphe 3.2.1), pour les conditions en pCO_2 élevée (600 ppm) et ambiante (360 ppm), et les fractions de sol non rhizospherique (NRS), de sol rhizosphérique (RS) et de racine et endorhizosphère (RE). n OTU, indique la richesse en OTUs de l'échantillon considéré. n tot, indique le nombre de souches testées.

Relation ITS1 - *narG* - *napA*

En première approche, les souches d'une même OTU its pouvaient avoir soit la forme NAR soit la forme NAP soit les deux, excepté l'OTU its3 qui était exclusivement constituée de souches possédant la forme NAP de nitrate réductase.
Les liens possibles entre les types ITS1 et les types *nar*G et *nap*A ont été évalués. Pour cela, les relations existantes entre les OTUs its et les OTUs nap et nar ont été analysées. Une représentation graphique de ces relations est proposée dans la figure 3.2.2.3. Les souches appartenant à une même OTU its étaient réparties dans plusieurs OTUs nap et/ou nar, et inversement les souches d'une même OTUs nar et/ou nap se distribuaient dans des OTUs its différentes. Cependant parmi les OTUs majoritaires, trois font exception: les OTUs its3 (voir ci-dessus), nap2 et nap4. Les OTUs nap2 et nap4 étaient constituées de souches appartenant exclusivement à l'OTU its1, et représentaient 90% des souches de l'OTU its1 (n=52). 85% des souches de l'OTU its2 était de type nar1, mais les souches de l'OTU nar1 se distribuaient dans plus de 10 OTU its.
L'utilisation du gène *narG* comme marqueur moléculaire pour l'analyse des communautés bactériennes totales réductrices de nitrate était proposé par Philippot (2002). Cependant la phylogénie dérivée des séquences *narG* n'était pas entièrement en concordance avec celle basée sur les séquences d'ADNr 16S (Gregory et coll. 2003). Pour le genre *Pseudomonas*, aucune relation claire n'était mise en évidence entre les différents types ITS1 et *narG* d'une part, et entre les types ITS1 et *napA* d'autre part. De la même manière, Delorme et coll. (2003) observaient un manque de congruence entre la phylogénie des séquences *narG* et d'ADNr 16S chez les *Pseudomonas* spp. fluorescents. Ces résultats suggèrent une évolution indépendante des séquences d'ADNr et des séquences *napA* et *narG* au sein du genre *Pseudomonas*.

CONCLUSIONS

Cette étude a démontré une réponse des populations de *Pseudomonas* réducteurs de nitrate cultivables avec la proximité de la racine, et l'augmentation en pCO_2. Cette modification était mise en évidence lors de l'analyse des proportions entre les formes NAR et NAP de nitrate réductase présentes parmi les populations de *Pseudomonas* des différentes fractions et traitement en pCO_2 (3.2.1). Toutefois, aucun effet sur la diversité génotypique des gènes *narG* et *napA* ou encore des séquences 16S-ITS1 n'était observé (3.2.1 et 3.2.2). La forme NAR était la plus détectée dans la fraction racinaire et sous pCO_2 élevée par rapport à la forme NAP. La signification de la présence de ces deux formes de nitrate réductase parmi les *Pseudomonas*, voir dans le même organisme, n'est pas clairement élucidée. La forme NAR serait principalement fonctionnelle en respiration anaérobie. Ceci semble confirmé par la présence de la forme NAR dans la majorité des souches de *Pseudomonas* dénitrifiantes *sensu stricto* de cette étude. L'expression de cette forme de nitrate réductase parmi les souches

isolées de la fraction racinaire sous pCO_2 élevée était envisageable étant donné les conditions plutôt anoxiques et les sources énergétiques importantes dans la rhizosphère (cf. 1.3), et *a priori* renforcées sous pCO_2 élevée. Dans ces conditions, une respiration des nitrates est une voie alternative avantageuse pour les microorganismes de la rhizosphère bien qu'elle suggère également une compétition pour les nitrates avec la plante (cf. 3.3). La présence de la forme NAP est beaucoup plus équivoque. Cette forme était mise en évidence dans des organismes différents utilisant le nitrate tant sous conditions oxiques qu'anoxiques ou microoxiques. Dans cette étude, la forme NAP était majoritairement détectée dans les souches isolées du sol (environnement considéré comme moins anoxique que la rhizosphère). Ainsi, chez *Pseudomonas* elle pourrait être impliquée dans la respiration en condition oxique des nitrates. Cette versatilité de fonctionnement de la forme NAP d'un organisme à un autre suggèrerait un avantage adaptatif dans un environnement où les conditions physico-chimiques peuvent varier rapidement et fréquemment, comme dans le sol et la rhizosphère (Richardson, 2000). Etant donné l'importance du souchier réalisé lors de cette étude, il serait intéressant d'analyser plus spécifiquement ces mécanismes de régulation. En première approche par des cultures continues de nos souches, sous différentes concentrations en oxygène, et en suivant l'expression des gène *narG* et/ou *napA* par RT-PCR. Des analyses similaires sur sols cultivés apporteraient une image tenant compte des interactions existantes entre microorganismes, plante, sol, et des conditions environnementales générales du système.
Finalement, aucune relation entre l'affiliation taxonomique des souches *Pseudomonas*, sur la base des séquences ribosomiques, et le type de nitrate réductase (NAR ou NAP) ou le type de gènes *narG* et *napA* n'était observable. L'approfondissement de cette analyse est actuellement en cours au laboratoire avec le séquençage des gènes *narG* et *napA* des souches dont la séquence ADNr 16S est déjà connue (cf. 3.2.2). Dans le contexte de la réponse au pCO_2 et à la proximité racinaire, l'analyse des séquences en acide aminés de ces gènes en relation avec l'origine des souches correspondantes (NRS/RS/RE et 300/600 ppm), pourrait mettre en lumière certains aspects non révélés par la grande diversité des séquences nucléotidiques.

3.3. INFLUENCE DU NIVEAU DE FERTILISATION AZOTEE SUR LES POPULATIONS DE *PSEUDOMONAS* REDUCTEURS DE NITRATE DE LA RHIZOSPHERE DE *LOLIUM PERENNE* CULTIVEE SOUS ATMOSPHERE AMBIANTE ET ENRICHIE EN pCO_2

INTRODUCTION

Dans les paragraphes 3.1 et 3.2 nous avons vu que la fréquence des *Pseudomonas* réducteurs de nitrate diminuait avec la proximité de la racine que ce soit sous pCO_2 ambiante ou élevée en condition de faible fertilisation azotée. Des études portant sur les *Pseudomonas* réducteurs de nitrate montraient à l'inverse que ces populations étaient stimulées à proximité des racines (Clays-Josserand et coll. 1995 et 1999). La différence majeure entre ces études et la notre pourrait être la disponibilité en nitrate dans la rhizosphère. En effet, pour les prairies de *L. perenne* à Eschikon, faiblement fertilisée, la disponibilité en azote était limitante pour permettre une croissance supplémentaire de la plante sous pCO_2 élevé (Daep et coll. 2000). Par ailleurs, les émissions de N_2O mesurées n'étaient pas significativement différentes sous pCO_2 élevée et ambiante (Baggs et coll. 2003). En fait, la dénitrification représente une perte d'azote supplémentaire pour le système, et une telle activité pourrait entrer en compétition pour le nitrate avec la plante (demandeuse d'azote pour sa croissance). Ceci expliquerait la diminution de la fréquence des *Pseudomonas* réducteurs observée dans la rhizosphère à faible amendement en azote. Dans le paragraphe 3.1, nous avons également observé que la fréquence des populations de *Pseudomonas* dissimilant le nitrate augmentait sous pCO_2 élevée dans la fraction racinaire et à faible amendement en azote. A fort amendement en azote, les conditions étaient *a priori* non limitantes pour la croissance de *L. perenne* sous pCO_2 élevée (Daepp et coll. 2000), et les émissions de N_2O étaient stimulées (Baggs et coll. 2003). Ainsi, une disponibilité en azote plus grande dans le système profiterait aussi bien à la plante qu'aux populations microbiennes réductrices de nitrate associées. Pour tester ces hypothèses, les fréquences de *Pseudomonas* réducteurs de nitrate associés au sol et à la rhizosphère de *L. perenne* cultivée sous pCO_2 ambiante et élevée ont été examinées à faible et fort amendement en azote (14 g m^{-2} an^{-1} et 56 g m^{-2} an^{-1} NH_4NO_3 respectivement).

Les résultats présentés dans ce paragraphe sont le fruit d'un travail collaboratif entre Nathalie Fromin, Ludovic Roussel-Delif et Sonia Tarnawski.

SHORT COMMUNICATION

Nitrogen fertilisation level alters the response of nitrate-dissimilating Pseudomonas spp. in the rhizosphere of Lolium perenne grown under elevated pCO_2 (Swiss FACE)

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Abstract

The effect of elevated pCO_2 (60 Pa) on the frequency of nitrate-dissimilating Pseudomonas (NDP) was investigated in the rhizosphere of fertilised Lolium perenne swards in the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. Numbers of cultivable root-associated Pseudomonas were greater under elevated (60 Pa) than under ambient (36 Pa) pCO_2 in both high and low N-fertilised swards. For both pCO_2 conditions, the NDP frequency decreased with closer root proximity to L. perenne roots in low fertilised swards. In high N swards the NDP frequency was similar in root and soil fractions. Thus, N availability may be a major factor influencing NDP populations under elevated pCO_2, most likely due to increased competition between plant and nitrate-dissimilating bacteria.

There is uncertainty about the effects of increasing atmospheric concentrations of CO_2 (pCO_2) on denitrification in terrestrial ecosystems. Various studies have shown that potential denitrifying enzyme activity may be stable or decrease (Hungate et al., 1997; Barnard et al., 2004), whereas N_2O emissions may increase (Arnone and Bohlen, 1998; Baggs et al., 2003a, 2003b) for herbaceous systems under elevated pCO_2. Little is known about the response of microorganisms involved in denitrification (Roussel-Delif et al., in press; Deiglmayr et al., 2004), partly because of the widespread ability to denitrify, and functional differences among denitrifying microorganisms make it difficult to relate denitrification activity to soil microbial populations (Cavigelli and Robertson, 2001). Roussel-Delif et al. (in press) recently showed that the frequency of root-associated nitrate-dissimilating Pseudomonas (NDP) in low fertilised (14 g N m^{-2} y^{-1}) L. perenne swards was increased under elevated pCO_2, suggesting that increased pCO_2 provided favourable conditions for the dissimilation of nitrate and related organisms. However, this is likely to be dependant on N fertilisation and competition with roots for available soil N.

Here we report an investigation of the frequency of NDP in the rhizosphere of L. perenne swards receiving different N fertilisation levels at the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. The hypothesis was that increased belowground C allocation under elevated pCO_2 would increase the number and frequency of nitrate-dissimilating Pseudomonas and that the extent of this response would vary with N application rate and proximity to L. perenne roots, due to competition with plants for available soil nitrate. The FACE experiment at Eschikon (8°41'E, 47°27'N; 550 masl), Switzerland, consists of three elevated (60 Pa) and three ambient (36 Pa) CO_2 rings, established in an open field situation in 1993 (Hebeisen et al., 1993). The soil is a clay loam (sand 36%, silt 33%, clay

28%, organic matter 2.9-5.1%, pH 6.5-7.6) classified as an Eutric Cambisol (FAO classification). L. perenne L. (cv Bastion) swards were fertilised with NH_4NO_3 in solution at rates of 14 (low N) and 56 g N m^{-2} y^{-1} (high N) split between five swards regrowth periods. Three undisturbed plant-soil cores (20 cm depth) were sampled from each replicate plot on July 15 2002, three weeks after application of 2.8 g N m^{-2} (low N) and 11.2 g N m^{-2} (high N), one week before the third cut to avoid any effect of defoliation on N availability (Gloser et al., 2000). Soil mineral N was determined in July 2002 from soil sampled (0-25 cm) from each plot (Baggs and Blum, 2004) (Table 1).

Table 1. Soil mineral N (mg N kg^{-1} dry soil) averaged over the 5 week re-growth period in low (14 g N m^{-2} y^{-1}) and high (56 g N m^{-2} y^{-1}) N-fertilised L. perenne swards under ambient (36 Pa) and elevated (60 Pa) pCO_2. Values in parentheses are ± 1 SEM. After Baggs and Blum (2004).

	Low N		High N	
	36 Pa	60 Pa	36 Pa	60 Pa
Available NH_4^+ (mg NH_4^+-N kg dry $soil^{-1}$)	39.9 (± 11.6)	24.7 (± 6.1)	30.8 (± 7.6)	46.5 (± 6.1)
Available NO_3^- (mg NO_3^--N kg dry $soil^{-1}$)	35.9 (± 12.5)	51.3 (± 15.6)	190.6 (± 27.6)	135.1 (± 20.5)

Non-rhizosphere soil (NRS), rhizosphere soil (RS) and root (noted RE for rhizosphere-endorhizoplane) crushed samples were diluted in phosphate sodium buffer 0.1 M, pH 7.0, and plated on selective mS1 medium. Thirty bacterial isolates were randomly selected for each sample and their affiliation to the Pseudomonas genus was checked by specific hybridization (Tarnawski et al., 2003). Pseudomonas counts were deduced from the proportion of hybridizing mS1 isolates. The ability of 680 Pseudomonas strains to dissimilate nitrate to nitrite and to gaseous compounds was tested during an *in vitro* assay, according to Roussel-Delif et al. (in press) and the strains were scored as non-dissimilators, nitrate reducers, or denitrifiers. The frequencies of nitrate-dissimilating (nitrate-reducing and denitrifying) strains were analysed using a generalised linear model (glm) with a logistic regression model.

1. *Pseudomonas* numbers

Nitrogen application rate had no consistent effect on Pseudomonas counts. Pseudomonas counts in both low and high-fertilised swards were higher in root-associated fractions under elevated than under ambient pCO_2 (Figure 1), confirming previous results obtained at this site using a 16S rDNA-based approach (Marilley et al., 1999). This was attributed to greater belowground C allocation associated with increased root biomass, turnover and exudation under elevated pCO_2 (van Kessel et al., 2000) favouring Pseudomonas populations that are known to be good heterotrophic competitors for C sources, irrespective of their denitrifying abilities (Tiedje, 1988). Pseudomonas can metabolize a wide range of organic compounds and use electron acceptors other than oxygen, which confers a competitive advantage to these organisms in nutrient-rich environments such as the rhizosphere (Latour and Lemanceau, 1997). In the same FACE experiment, Pseudomonas abundance varied temporally but was always higher than 10^5 cfu g^{-1} root or soil dry weight (Tarnawski, 2004).

2. Frequency of nitrate dissimilating *Pseudomonas*

NDP frequency was primarily influenced by the rhizosphere fraction ($P < 0.05$) and by the N application rate ($P < 0.01$), rather than by elevated pCO_2 ($P = 0.2$) suggesting that N availability had a greater influence on NDP than pCO_2 treatment. In high N swards, the annual application of 56 g N m^{-2} y^{-1} exceeded L. perenne requirements (29 g N m^{-2} y^{-1}) (Richter, 2003), resulting in high availability of mineral N for nitrification and denitrification (Table 1). Accordingly, the NDP frequency did not decrease at root proximity.

In low N swards (14 g N m^{-2} y^{-1}), nitrate was probably limiting for nitrate-dissimilating bacteria in close proximity to roots because of plant uptake, and so competition would have arisen between NDP and

Lolium plants for available N. Additionally, greater plant N demand (Daepp et al., 2000) or microbial immobilisation (Hungate et al., 1997) under elevated pCO_2 would have further reduced nitrate availability for NDP as indicated by low available soil nitrate in the high N swards (Table 1).

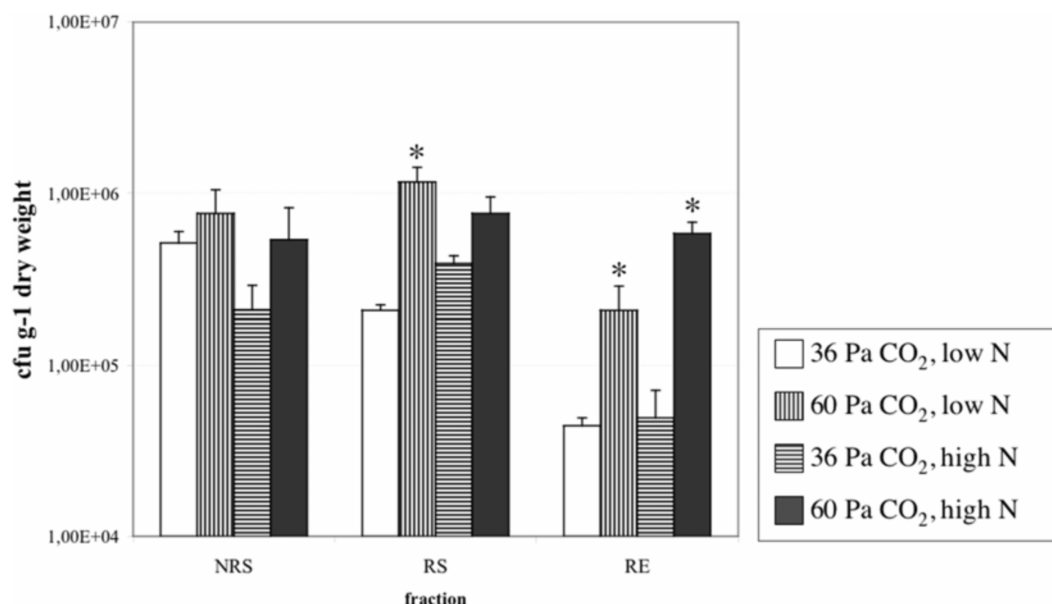

Fig. 1- Pseudomonas colony forming units (cfu) counts
Cultivable Pseudomonas cfu counts were obtained by applying the proportion of PSM_G-hybridizing colonies to the total number of bacterial colonies growing on the Pseudomonas-selective mS1 medium. NRS: non-rhizosphere soil; RS: rhizosphere soil; RE: rhizoplane-endorhizosphere (root). * indicates a statistically significant difference ($P < 0.05$) in cfu counts between control (36 Pa) and CO_2-treated (60 Pa) plots for a given fraction.

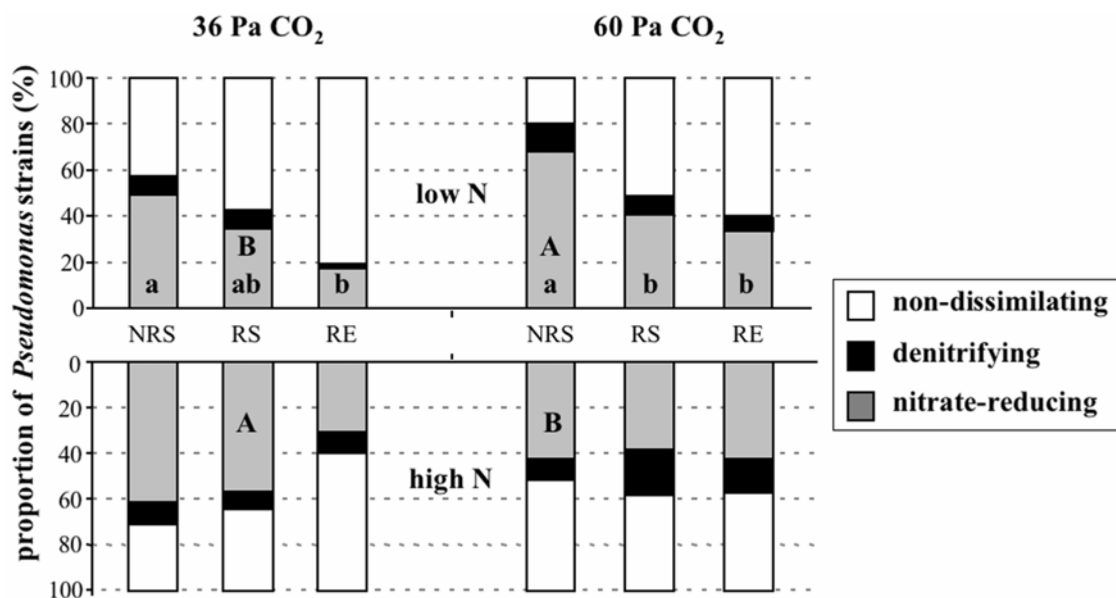

Fig. 2- Frequencies of nitrate-reducing, denitrifying and non-dissimilating Pseudomonas strains in the rhizosphere of low and high fertilised L. perenne under ambient and elevated pCO_2
Tukey multiple comparison test (comparison between fractions), or Fisher LSD exact test (comparisons between treatments) were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, Washington). Different letters indicate NDP frequencies that are statistically different ($P < 0.05$): [a,b] for

differences between fractions for a given pCO$_2$ and N fertilisation treatment; [A, B] indicates differences between low and high N treatments for a given fraction and pCO$_2$ condition.

The frequency of root-associated NDP tended to be higher under elevated pCO$_2$ (Figure 2), as previously shown at this site (Roussel-Delif et al., in press), although this was not significant (95% confidence limit). This tendency towards higher NDP frequency under elevated pCO$_2$ may reflect favourable conditions provided in the rhizosphere under elevated pCO$_2$ for these bacteria, *i.e.* increased belowground C flow (van Ginkel et al., 2000) or higher soil water content (Morison, 1985) that results in lower pO$_2$, two of the main determinants of denitrification (Tiedje, 1988; Arnone and Bohlen, 1994). In accordance with this, Baggs et al. (2003a) measured increased emissions of denitrified N_2O from high-fertilised Lolium swards under elevated pCO$_2$ at this site, which were positively correlated with soil organic C (Baggs and Blum, 2004). The response of NDP can not be directly related to the denitrification activity measurements made in the field because other organisms could have been responsible for nitrate transformations, and also because N_2O emission can result from other processes (Baggs et al., 2003b; Müller et al., 2004). The influence of elevated pCO$_2$ on NDP frequency was not as strong as hypothesised. It is possible that the nine year exposure to elevated pCO$_2$ resulted in an acclimation of the soil-plant system to function under these conditions (Rogers et al., 1998). Previous experiments have shown that the type of nitrate reductase enzyme carried by the NDP differed under ambient and elevated pCO$_2$ (Roussel-Delif et al., in press), suggesting that NDP populations may respond to elevated pCO$_2$ by altering both the enzyme type, and probably its activity.

Acknowledgments

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CONCLUSION

En conditions non-limitantes de fertilisation azotée, la fréquence des réducteurs de nitrate ne diminuait pas avec la proximité de la racine, comparé aux conditions limitantes. Par ailleurs, la fréquence des réducteurs de nitrate n'était pas davantage augmentée à proximité de la racine ou sous pCO_2 élevé, malgré la disponibilité en azote plus importante. Ces résultats montrent que les populations de *Pseudomonas* réducteurs de nitrate étaient plus influencées par la disponibilité en nitrate dans la rhizosphère de *L. perenne* que par l'enrichissement en pCO_2, et n'auraient qu'une participation mineure dans les émissions de N_2O mesurées sous pCO_2 élevé. La contre sélection de ces populations dans la rhizosphère de *L. perenne* cultivée dans les conditions du FACE semble se confirmer. Cependant les *Pseudomonas* réducteurs de nitrate restent influencés par la proximité de la racine surtout en condition limitante en azote (cf. 3.2).

CONCLUSIONS GENERALES

CONCLUSIONS GENERALES DE L'ETUDE ET PERSPECTIVES

L'ensemble de cette étude a porté sur l'écologie des *Pseudomonas* de la rhizosphère de plantes pérennes et en particulier sur la réponse à une pCO_2 élevée des *Pseudomonas* associés à la rhizosphère de *L. perenne* et *M. coerulea.* C'est en abordant tant la diversité que la physiologie de ce genre bactérien que nous avons pu répondre à plusieurs des objectifs fixés en 1.5.

En rappel, L. Marilley (1999) avait observé (par approche moléculaire) une stimulation des *Pseudomonas* dans la rhizosphère de *L. perenne* sous pCO_2 élevée après 4 années de fumigation. Pour *L. perenne* (après 4 années de fumigation supplémentaires) comme pour *M. coerulea* (3 années seulement), nous n'avons observé, sur la base des dénombrements sur le milieu sélectif mS1 à différentes dates d'échantillonnage, aucune stimulation significative de la densité des *Pseudomonas* sous pCO_2 élevée. La grande variabilité de ces résultats aux différentes dates d'échantillonnage ne nous permettait pas de conclure quand à une modification de l'abondance des *Pseudomonas* dans la rhizosphère de *L. perenne* et *M. coerulea* en réponse à l'augmentation en pCO_2. Néanmoins, les comptages sur milieu sélectif mS1 (cf. 3.1.) confirmaient que les *Pseudomonas* représentaient une population bactérienne importante de la rhizosphère des deux plantes cultivées dans les expérimentations FACE. Dans l'étude menée en parallèle sur les communautés bactériennes totales et actives de la rhizosphère de *L. perenne* et *M. coerulea* (Jossi et coll. *in prep*), les *Pseudomonas* (γ-protéobactéria) ne ressortaient pas non plus comme des populations influencées par l'augmentation du pCO_2 (en terme d'abondance de gènes d'ARNr 16S ou de transcrits d'ARNr 16S). Les populations de *Pseudomonas* de la rhizosphère seraient donc d'abondance équivalente et stable aux deux concentrations de pCO_2.

Dans la suite de l'étude, nous avons observé une altération de la structure phénotypique des populations de *Pseudomonas* sous l'effet de l'augmentation en pCO_2 et de la proximité de la racine, notamment pour *L. perenne* (cf. 3.1, Tarnawski et coll. *in prep*). Dans cette étude de la structure phénotypique des populations de *Pseudomonas*, la discussion de la sélection ou contre-sélection de caractères phénotypiques dans la rhizosphère de *L. perenne* ou *M. coerulea* a été mise en relation avec les modifications des conditions physico-chimiques du sol qu'impliquent la présence de la plante (rhizosphère) et le fort CO_2. Dans ce cadre, je retiendrais en particulier la guilde des *Pseudomonas* dissimilant le nitrate. Celle-ci était stimulée dans la fraction racinaire de *L. perenne* sous pCO_2 élevée (3.1). Cependant, les *Pseudomonas* réducteurs de nitrate semblaient être contre-sélectionnés dans la rhizosphère de *L. perenne*, et d'autant plus en condition d'azote limitant dans le sol (cf. 3.3, Fromin et coll., *in prep*). Les bactéries réductrices de nitrate, bien que favorisées par l'augmentation de la rhizodéposition sous fort pCO_2, entraient probablement en compétition avec la plante pour les nitrates (Hu et coll. 2001). Ainsi la disponibilité en azote influençait la réponse à un enrichissement en pCO_2 des populations de réducteurs de nitrate dans la rhizosphère. Plus généralement, Freeman et coll. (1998), montraient également que l'augmentation de la pCO_2 favorisait la plante plutôt que la microflore dans la compétition pour les nutriments inorganiques du sol. La valeur première pour la plante de la présence de microorganismes dans le sol est qu'ils agissent sur les cycles des nutriments. Si l'activité des microorganismes est altérée ou réduite sous pCO_2 élevée, le taux de décomposition du pool d'azote organique sera également ralentie et le taux de nutriments inorganiques relargués dans le sol sera plus faible pour les croissances ultérieure de la plante. Ainsi la plante pourrait paraître en carence nutritive même si elle pousse sur un sol *a priori* plutôt fertile, comme on l'observe pour *L. perenne* à faible amendement en azote. Ceci suggère un effet en retour négatif sur la croissance de la plante.
En parallèle du changement de biodisponibilité des éléments minéraux dans le sol sous fort CO_2 (gestion des pools d'azote dans le sol), et de l'augmentation de la rhizodéposition, la

modification qualitative probable des rhizodépots sous pCO_2 élevée expliquerait davantage les changements de la microflore que nous avons observé dans la rhizosphère. Malheureusement, les exsudats sont difficiles à caractériser *in situ*, et les modifications de la rhizodéposition sous pCO_2 élevée sont encore peu étudiées en terme de type de composés exsudés. Néanmoins, une augmentation de l'exsudation de composés carbonés est généralement observé sous pCO_2 élevée, notamment de carbohydrates non-structuraux (Hodge et coll. 1998, Darrah 1996). Par ailleurs, les résultats obtenus sur les profils métaboliques des communautés associés à la rhizosphère de *M. coerulea* (Biolog) montrent que les communautés associées aux racines tendaient à mieux métaboliser certaines sources de carbones, et plus spécialement les carbohydrates sous pCO_2 élevée (Hamelin et coll. *in prep*, cf. 1.4.3.1). La composition des exsudats apparaît donc comme force motrice de la sélection ou contre-sélection de certaines populations microbiennes dans la rhizosphère (Benizri et coll., 2002) sous fort pCO_2. Au niveau des rhizodépots azotés, aucune modification majeure de la quantité d'acides aminés libres et de protéines totales dans les exsudats n'était décrite sous pCO_2 élevée (en condition axénique, Hodge et coll. 1998), la nature et le ratio des composés azotés exsudés restant inconnue. Pourtant les acides aminés sont souvent des précurseurs de voix métaboliques secondaires bactériennes (comme celle de la synthèse d'HCN, d'AIA, d'antibiotiques…) dont les produits sont impliqués dans l'interaction de la microflore avec la plante (PGP). Je pense qu'il serait important de définir davantage le type de composé azoté présent dans la rhizosphère. Un moyen simple d'aborder cette lacune consisterait à tester (à la manière des Biolog) un grand nombre de substrats azotés et de comparer leurs niveaux de métabolisation par différentes communautés bactériennes provenant du sol et de la rhizosphère sous pCO_2 ambiante et élevée. Les substrats les mieux métabolisés dans un temps court pourraient être représentatifs du type d'exsudat présent dans la rhizosphère, et influençant les communautés microbiennes.

Ce travail a abouti à la production d'un important souchier de *Pseudomonas* de la rhizosphère de *L. perenne* et *M. coerulea*. Comme nous l'avons vu, certaines espèces de *Pseudomonas* améliorent la croissance et la santé des plantes, d'autres sont capables de réduire les oxydes d'azote et de dégrader des composés xénobiotiques. Il serait à présent intéressant de poursuivre la caractérisation de ces souches en réalisant d'autres tests physiologiques et métaboliques (solubilisation des phosphates, résistance aux antibiotiques, production d'exopolysaccharides ou d'autres polymères, d'homosérine lactone, de protéases…). Serait-il possible de définir le « portrait-robot » d'une bactérie *Pseudomonas* de la rhizosphère de plantes pérennes ? Une première piste était donnée dans l'article de Latour et coll. (2003), où les auteurs montraient que les *Pseudomonas* spp. fluorescents adaptés à la rhizosphère présentent un métabolisme spécifique caractérisé par un système efficace d'acquisition du fer et une aptitude à réduire les oxydes d'azote. Dans notre étude, la capacité à réduire les nitrates ne semble pas être un caractère rhizosphérique spécifique, même en condition d'azote non-limitant. L'association type de sol/plante semble ainsi influencer la sélectivité de la plante parmi les bactéries telluriques du sol. C'est l'avancement de la compréhension de ces types de « communication » entre les acteurs de la rhizosphère (Bais et coll. 2004; Singh et coll. 2004) qui apportera une compréhension nouvelle du fonctionnement du sol, mais aussi le développement d'engrais biologiques efficaces (Benizri et coll., 2001).
Ce souchier constitue également une base environnementale comparative importante pour d'autres études de la microflore de *M. coerulea* ou *L. perenne* qui seraient réalisées en conditions contrôlées. Le travail de thèse de M. Jossi (commencé en 2003) en tire déjà avantage dans sa volonté de caractériser et d'identifier les populations microbiennes impliquées dans la colonisation racinaire de différents cultivars de *L. perenne* cultivés en pots.

Dans le Chapitre 3.2.2., nous avons observé que la fonction de réduction des nitrates semblait répandue uniquement parmi les espèces de *Pseudomonas* du groupe *fluorescens*. La diversité génotypique des *Pseudomonas* spp. réducteurs de nitrate décrite sur la base des séquences *narG*, *napA* et d'ADNr 16S était importante, mais ne semblait pas modifiée par le traitement pCO_2 ou la proximité à la racine pour *L. perenne* et *M. coerulea* (diversité *narG* et *napA* seulement pour *M. coerulea*) (cf. 3.2). La diversité génétique des *Pseudomonas* réducteurs de nitrates observée dans cette étude semblait ainsi présenter une grande inertie quelles que soient les conditions environnementales. Par contre nous avons observé une influence de l'enrichissement en pCO_2 sur les proportions du type de nitrate réductases (NAR/NAP) présentes dans les souches de *Pseudomonas.* Le rôle de ces différentes enzymes

en relation avec l'origine des organismes correspondants (fractions /traitement en CO_2) nous a permis d'appréhender la diversité fonctionnelle de la rhizosphère (cf. 3.2.1), et nous fournit des informations intéressantes concernant le fonctionnement de l'écosystème (Cavigelli et Robertson, 2001). En effet, la forme NAR impliquée dans la respiration anaérobie des nitrates était retrouvée plus fréquemment dans les souches d'origine rhizosphérique sous fort pCO_2 relativement à la forme NAP qui était plus souvent détectée dans les souches isolées du sol, un environnement plus oxique que la rhizosphère. Par ailleurs environ 10% des souches possédaient à la fois les gènes de la forme NAR et NAP. Ces résultats amènent un questionnement sur la coexistence de ces souches de *Pseudomonas* réducteurs de nitrate, possédant la forme NAR et/ou NAP, dans le même environnement qu'est la rhizosphère. Ils pourraient indiquer une différenciation de niches en rapport avec les ressources à disposition et les conditions physico-chimiques du milieu (Gray et coll. 2004). A présent, il me semble important de faire le pas entre la présence des ces gènes et leur expression, leur fonctionnement effectif dans l'environnement. Il semble que la disponibilité en oxygène du milieu soit un facteur déterminant (en plus de celle du nitrate) dans ce fonctionnement. Ainsi comme proposé en conclusion du point 3.2., la régulation de l'expression des gènes *narG* et *napA* pourrait être suivie par RT-PCR pour différentes souches (NAR/NAP, NAR, et NAP) en culture continue avec différentes concentrations en oxygène (et en nitrate) ou dans des sols cultivés. Ce type d'étude nous permettrait d'élucider un peu plus le rôle de la forme NAP chez *Pseudomonas*.

Finalement, les études DGGE menées sur les transcrits d'ARNr 16S de communautés bactériennes totales tentent de faire le lien complexe entre la diversité des populations résidantes, leur structure, et le fonctionnement du sol. L'analyse des transcrits d'ARNr 16S projettent effectivement une image de l'activité de traduction des populations à interpréter en terme d'activité en relation avec leur environnement. Cependant le lien entre la diversité microbienne et le fonctionnement du sol n'en devient pas pour autant évident (Griffiths et coll. 2000, Nannipieri et coll. 2003). Je pense que l'analyse des ARNm totaux ou de gènes fonctionnels particuliers permettrait de mieux identifier les fonctions écologiques importantes dans la rhizosphère et d'estimer l'implication des microorganismes dans ces fonctions (Neher 1999). Ce type de problématique pourrait tirer avantage des nouvelles techniques mise au point en écologie microbienne comme l'hybridation *in situ* combinant la mRNA-FISH et la rRNA-Fish (Pernhaler et Amann 2004), ou encore le Stable Isotope Probing (Mandefield et coll. 2002). Par ce biais, il devient possible d'identifier l'organisme ou les populations qui expriment un gène particulier dans un milieu (en équilibre ou non). Dans notre cas cibler les ARNm des gènes *narG* et *napA* ainsi que ceux d'autre gènes du cycle de l'azote, dans différente fractions du sol et de la rhizosphère, nous permettrait de faire le lien entre les approches holistiques de la dynamique de l'azote dans le sol dans un système tel que le FACE, et celles de l'écologiste microbien dans sa démarche d'analyse de la diversité des communautés.
Dans un avenir plus lointain, nous pouvons espérer que la nouvelle compréhension du fonctionnement de la rhizosphère apporté par ce type d'études permettra d'identifier plus précisément les effets feedback sur la plante induits par l'activité de la microflore rhizosphérique.

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ANNEXES

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Minireview

Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns

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Summary

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 ecologists took advantage of this technique, originally developed for the detection of single mutations, for the analysis of whole bacterial communities. However, until recently, the results of these high quality fingerprinting patterns were 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 analysing 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 analysed 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.

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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 means 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.,* 2000a; O'Donnell *et al.,* 2001; Schäfer and 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 polymerase chain reaction (PCR)-amplified fragment pools produced from whole microbial communities by electrophoresis techniques (Table 1). These fingerprinting methods are now widely adopted in the field of bacterial ecology and permit the simultaneous analysis of numerous samples (Ferrari and Hollibaugh, 1999).

DGE fingerprinting of microbial communities

Muyzer *et al.* (1993) first applied denaturing gel electrophoresis (DGE) techniques for the analysis of whole bacterial communities. Denaturing gel electrophoresis allows

Table 1. 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 and Smalla (1998)
Ribosomal intergenic spacer analysis (RISA)	Fisher and Triplett (1999); Ranjard *et al.* (2000b)
Single strand conformation polymorphism (SSCP)	Schwieger and Tebbe (1998); Dabert *et al.* (2001)
Terminal restriction fragment length polymorphism (T-RFLP)	Moeseneder *et al.*, (1999); Dollhopf *et al.* (2001)

the separation of small polymerase chain reaction 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 behaviour 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 and 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 and Hollibaugh, 1999; Moeseneder *et al.*, 1999; van der Gucht *et al.*, 2001; Schäfer and Muyzer, 2001; Schäfer *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 oligonucleotide probes (Heuer *et al.*, 1999). Other developments were based on the use of 16S rRNA as a target (Felske and 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 analysed, 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 an 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 and 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 analysing complex DNA templates, probably induced by heteroduplex molecules (Ferris and 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 and Wang, 1997; Garcia-Pichel *et al.*, 2001). The DGE analysis should probably be restricted 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% 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 and Smalla, 1998). These features and restrictions are nevertheless common to all PCR-based approaches (Lee *et al.*, 1996; Fisher and Triplett, 1999; Schäfer and 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 analysed in turn with statistical tools, which provide a significant advantage for the non-ambiguous interpretation of the observed variability (Morris *et al.*, 2002).

Analysis and comparison of DGE community profiles

Denaturing gel electrophoresis techniques have been extensively used to monitor bacterial communities in space and time (Ferris and 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 analyse 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 analysed 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 physicochemical 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 16S rDNA TTGE banding patterns of 30 raw milk samples were analysed 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.

Second, 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

Table 2. 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 (P. Rossi, unpublished data).

	Descriptors		
Position of the band (in pixels on Y axis)	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%)

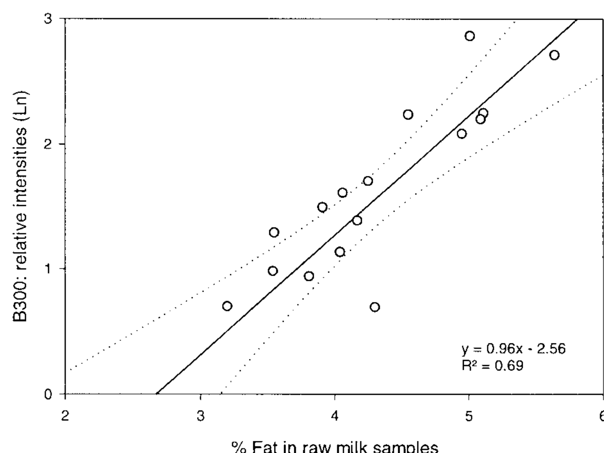

Fig. 1. Regression analysis between the relative intensity (ln) of the band at the $Y = 300$ position and the percentage of fat found in the corresponding raw milk samples (P. Rossi, unpublished data).

given above, the TTGE patterns were analysed 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) and the per cent of fat measured in the raw milk (Fig. 1).

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; Kocherginskaya *et al.*, 2001; McCaig *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 correlations 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 and Lilley, 1997). Other coefficients, such as the Steinhaus coefficient (Fig. 2) or the product moment, also named Pearson correlation coefficient (Rölling *et al.*, 2001; Smalla *et al.*, 2001), allow to take into consideration the relative intensity (P_i) of each band (Legendre and 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

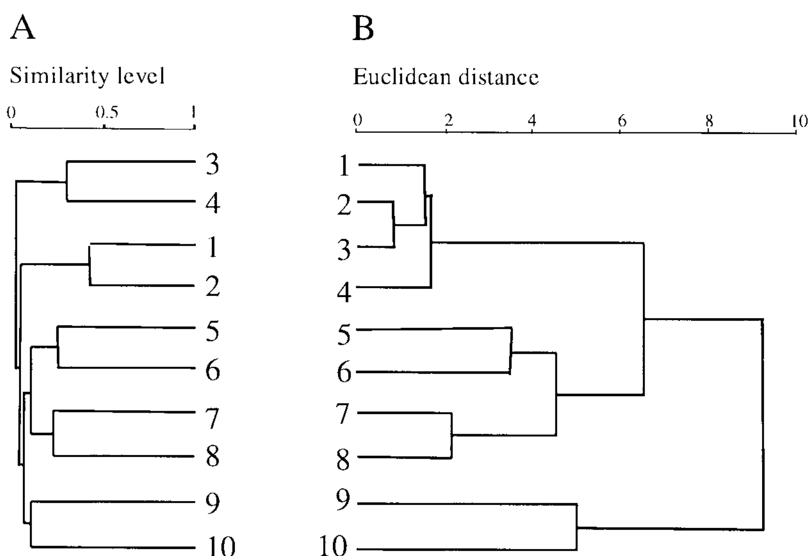

Fig. 2. UPGMA clustering of 10 samples taken along a vertical gradient form the small eutrophic Lake Loclat (Neuchâtel, Switzerland). Samples are ranked by depth: 1, corresponds to the surface; 10, to the bottom of the lake (8.7 m).
A. Clustering according to DGGE data (using Steinhaus coefficient).
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 and Legendre, 2001).

sequence types. Similarity or distance matrices can be displayed graphically as a dendrogram, but also give way to clustering and ordination methods.

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 (Ibekwe *et al.*, 2001; Yang *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), 10 samples were taken from a holomictic eutrophic lake along a vertical gradient and were analysed for major ions, organic content, physical parameters and DGGE analysis of 16SrDNA 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 (Fig. 2) 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 analysing 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 and 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 and Lilley, 1997; Legendre and Legendre, 1998).

Non-metric multidimensional scaling is an ordination method which reduces complex DGE patterns to a point in a two-dimensional 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. Non-metric multidimensional scaling 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.

Principal component analysis 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 (van der Gucht *et al.*, 2001; Müller *et al.*, 2001; Ogino *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 analysing 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.

Correspondence analysis 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) analysed 16S rDNA gene fragments issued from milk samples on TTGE gels. The results clearly showed different community structures between organic and conventional farming practices (Fig. 3). Correspondence analysis was also used elsewhere for similar approaches (Ibekwe *et al.*, 2001; Yang *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. Canonical correspondence analysis 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 and 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. Figure 4 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). This test is based on the linear correlation between two distance or similarity matrices obtained from independent data. As shown in the Fig. 4, the redox and the dissolved organic carbon were the variables which influenced mostly the dispersion of the samples. The first five samples taken from the aerobic zone (samples 1–5) are closely related, defining a homogenous bacterial structure. The samples taken from the anaerobic section of the water column (points 6–10) were displayed according to depth indicating a continuum of different bacterial communities.

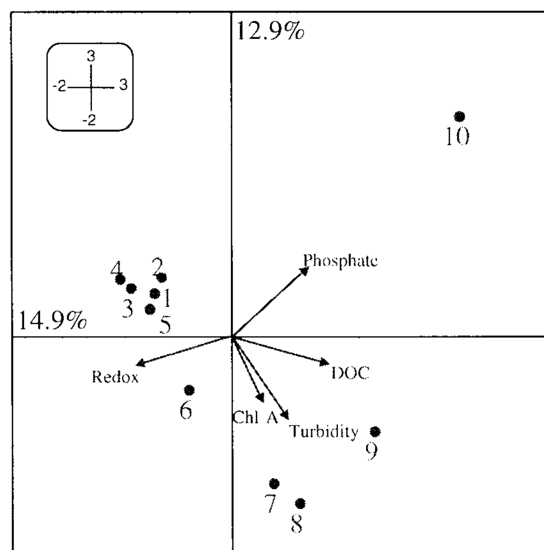

Fig. 4. Canonical correspondence analysis of microbial community patterns generated by 16S rDNA DGGE analysis for 10 water samples (Lake Loclat) ranked by depth (1 for surface to 10 at 8.7 m depth). The total inertia of the matrix was 4.45 and the selected variables explained 59% of the variance of the DGGE data set (sum of canonical eigenvalues: 2.62) (Forestier *et al.*, 2002).

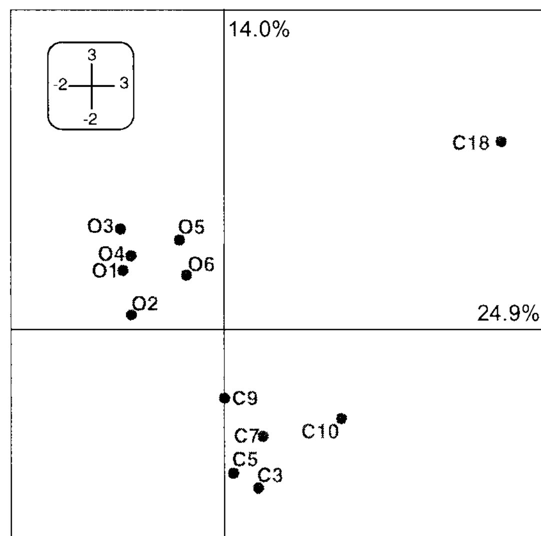

Fig. 3. Correspondence analysis of TTGE community profiles from milk samples from 12 farms (Switzerland). TTGE data of five samples taken on five consecutive days were pooled before being analysed (sum of unconstrained eigen values: 1.74). C, conventional; O, organic farming practices. Numbers refer to the different farms (Jourdain-Miserez *et al.*, 2001).

Conclusions

Denaturing gel electrophoresis 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, conclusions to be drawn 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 laboratory 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'.

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Brief report

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

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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*, a hemicryptophytic perennial grass naturally occurring in contrasted oligonitrophilic soils. The diversity of the dinitrogen-fixing bacteria associated with 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. The predominance of few NifH sequence types in the roots and the rhizosphere of *Molinia coerulea* indicate that the plant environment mediates a favourable niche for such dinitrogen-fixing bacteria.

Introduction

The biological dinitrogen-fixation process provides the major biological source of nitrogen in natural ecosystems. Most studies on associative nitrogen fixation have focused on crops of agronomic interest such as rice or sugar cane (Ueda *et al.*, 1995; Engelhard *et al.*, 2000; Steenhoudt and Vanderleyden, 2000), where fertiliser is required for crop growth. Few studies have aimed to understand the role of the associative dinitrogen fixation in nitrogen-limited natural ecosystems (Piceno *et al.*, 1999; Bagwell and Lovell, 2000; Piceno and Lovell, 2000a,b). *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 its 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.

As 0.1–10% 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) (Zehr and 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 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 to the perennial grass environment.

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 the pH_{H2O} value was 8.4. The sampled population of *Molinia coerulea* consisted of genetically homogeneous diploïd individuals.

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Preliminary acetylene reduction activity (ARA) measurements on soil cores indicated the presence of active NFB in association with *Molinia coerulea* rhizosphere (Hamelin *et al*., 2002). The proximity of the root creates an environment favourable for nitrogen fixer 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 × 20 × 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 samples (0.5 g) of fresh root and soil material were subjected to DNA extraction and purification using the 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 1 represents the phylogenetic position of the detected partial NifH sequences compared to sequences published for other NFB. Sequences with a 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* or *Azoarcus*.

Sequences from α-, β- and γ-*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 α-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 β-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 and 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 1). 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 (20–100 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 δ-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 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 1). 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 characterized 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, nor any information about its physiology. Other studies revealed environmen-

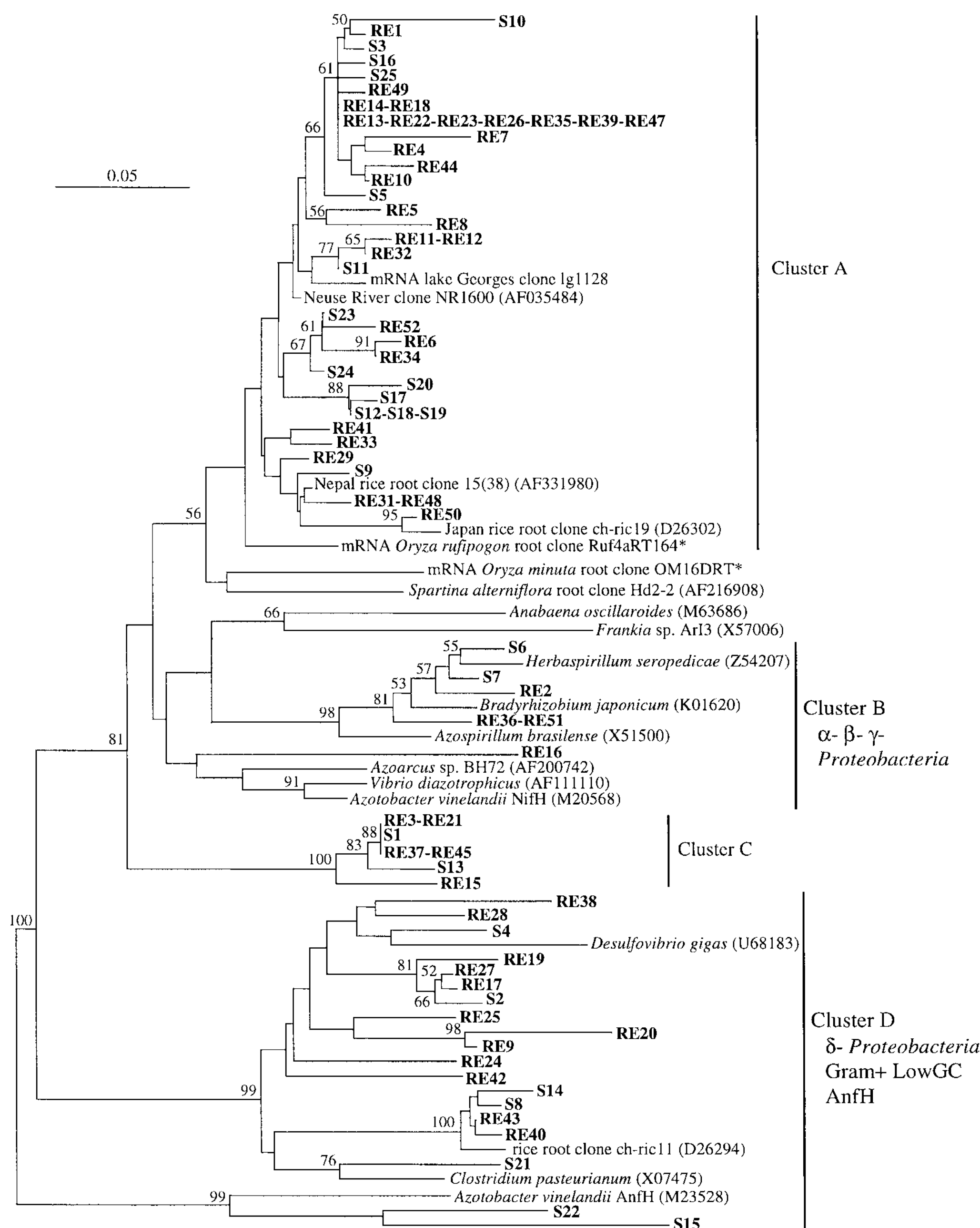

Fig. 1. Phylogenetic tree showing the position of the NifH sequences based on the alignment (ClustalX; Thompson *et al.* 1997) of 112 amino-acid residues corresponding to the positions 44–154 in the *Azotobacter vinelandii* NifH protein. The tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) with NJplot (Perrière and Gouy, 1996). 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 T. Hurek.

tal 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 (Zani *et al.*, 2000; T. 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

Table 1. Distribution of partial NifH sequences retrieved in the rhizosphere of *Molinia coerulea*.

	Cluster A		Cluster B		Cluster C		Cluster D		
Fractions	nb	(%)	nb	(%)	nb	(%)	nb	(%)	Total nb of analysed sequences
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

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 1).

Several analyses on environmental *nifH* genes have been performed for 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 and 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.

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« La journée avait été longue et Daniel était très fatigué. Il ne chercherait sûrement pas à savoir avant le lendemain ce que représentaient ces lumières. L'important pour lui était de bien dormir et de vivre une formidable séance de surf à la première heure le jour suivant. »

Sergio Bambaren
Le dauphin qui rêvait

Résumé

Cette thèse a pour objectif d'évaluer la réponse des *Pseudomonas* à une augmentation de la concentration en CO_2 atmosphérique (pCO_2) dans la rhizosphère de deux graminées pérennes: *Lolium perenne* et *Molinia coerulea.*

La première partie de ce travail s'intéresse au développement d'outils méthodologiques pour l'étude de la diversité des *Pseudomonas* dans le sol et la rhizosphère. Nous avons mis au point un protocole d'amplification d'une partie de l'ADNr 16S et de l'intergène 16S-23S de l'ADNr (séquences 16S-ITS1) spécifique au genre *Pseudomonas*. L'analyse PCR-RFLP des séquences d'ADNr 16S-ITS1 a ensuite été utilisée pour la caractérisation des populations de *Pseudomonas* associées à la rhizosphère de *M. coerulea* par approche culturale et amplification directe des séquences 16S-ITS1. Les *Pseudomonas* représentaient jusqu'à 10% de la microflore cultivable du sol et de la rhizosphère, et leur diversité était plus faible dans la fraction racinaire que dans le sol. Les *Pseudomonas* fluorescents dominaient dans la fraction racinaire alors que des organismes proche de *P. alcaligenes* étaient plus fréquemment retrouvés dans le sol.

La seconde partie de cette étude est consacrée à l'influence d'une augmentation du pCO_2 sur les populations de *Pseudomonas* associés à la rhizosphère des graminées pérennes. L'analyse de caractères en relation avec la plante (production d'auxine, sidérophores, de cyanure d'hydrogène, et réduction des nitrates) de 1228 isolats montrait que la structure phénotypique des populations de *Pseudomonas* était altérée sous fort pCO_2 dans la rhizosphère, les deux plantes influençant différemment leur microflore. La fréquence des *Pseudomonas* réducteurs de nitrate était stimulée sous pCO_2 élevée avec la proximité de la racine. La diversité génotypique des *Pseudomonas* dissimilant les nitrates, basée sur l'analyse des séquences ITS1, et des gènes de nitrate réductase *narG* et *napA* n'était pas significativement modifiée sous pCO_2 élevée ou à proximité de la racine. Les *Pseudomonas* réducteurs de nitrates semblaient contre-sélectionnés dans la rhizosphère de *L. perenne*, et limités dans leur réponse au pCO_2 élevée par la disponibilité en azote. L'étude des formes de nitrate réductases présentes dans les souches correspondantes, a révélé une modification de la diversité fonctionnelle parmi les souches de *Pseudomonas* associés à la rhizosphère sous pCO_2 élevée. Ce changement dans les populations de *Pseudomonas* est discuté en relation avec les possibles modifications des conditions physico-biochimiques de la rhizosphère sous fort pCO_2.

Abstract

The objective of this thesis was to assess the response of *Pseudomonas* to an increase atmospheric CO_2 concentration (pCO_2) in the rhizosphere of the two perennial grasses: *Lolium perenne* and *Molinia coerulea*.

The first part of this work was interested in the development of methodological tools for studying *Pseudomonas* diversity in the soil and in the rhizosphere. We proposed a protocol for the specific *Pseudomonas* amplification of the partial 16S rDNA and 16S-23S rDNA spacer (16S-ITS1 sequences). PCR-RFLP analysis of 16S-ITS1 rDNA sequences was used for the characterization of *Pseudomonas* associated with the rhizosphere of *M. coerulea* by culture approach and direct amplification of the 16S-ITS1 sequences. *Pseudomonas* accounted for up to 10% of the cultivable microflora in soil and rhizosphere, and its diversity was lower in the root fraction. Fluorescent *Pseudomonas* dominated in the rhizosphere whereas organisms close to *P. alcaligenes* were more frequently found in the soil.

The second part this study was devoted to the influence of an increase in pCO_2 on *Pseudomonas* populations associated with the rhizosphere of the perennial grasses. Analysis of characters in relation with the plant (auxin, siderophores, hydrogen cyanide production and nitrate reduction) among 1228 isolates showed that the phenotypic structure of *Pseudomonas* populations was altered under elevated pCO_2 in the rhizosphere, the two plants influencing their microflora differently. Nitrate reducers *Pseudomonas* were stimulated under elevated pCO_2 at the root proximity. The genotypic diversity of nitrate dissimilating *Pseudomonas*, based on 16S-ITS1 rDNA sequences and *narG* and *napA* nitrate reductase genes analysis, was not significantly modified by plant or by elevated pCO_2. Nitrate reducing *Pseudomonas* seemed to be counter-selected in the rhizosphere of *L. perenne*, and limited in their response to elevated pCO_2 by nitrogen availability. The study of nitrate reductase forms present in the corresponding strains revealed a modification of functional diversity among rhizospheric *Pseudomonas* under elevated pCO_2. This change among *Pseudomonas* populations is discussed in relation with possible modifications of the rhizosphere physico-biochemical conditions under elevated pCO_2.