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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_2 treatments: the NAR form was more abundant close to the root surface and for plants grown under elevated pCO_2. Putative denitrifiers harbored 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.

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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 microbiota. Several studies demonstrated that plants influence soil-inhabiting nitrate-dissimilating bacterial microbiota [12, 31, 44]. Indeed, nitrate dissimilation should be favored 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 nonrhizospheric 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 because of root and microbial respiration [21]. In contrast, 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 nonrhizospheric 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 oxide 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 metalloenzymes 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 [7–9, 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 microbiota 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 were 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 Web site (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 three control (C) and three 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, 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 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 (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, three soil cores (~5 cm diameter, 10–12 cm depth), including dense root systems, were taken and mixed for analysis. For *L. perenne*, three fractions were recovered: nonrhizospheric soil (NRS) for soil devoid of roots, rhizospheric soil (RS) for root-adhering soil (recovered by washing the roots twice in 20 mL, 0.1 M sodium phosphate buffer, pH 7.0 (SPB), under agitation for 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 analyzed.

Pseudomonas Isolation. Isolation of *Pseudomonas* strains was performed as previously described [52]. Briefly, 1 g of fresh weight root or soil was crushed in 10

mL SPB. The root and soil suspensions were 10-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), 12 (July, October 2000 and July 2001), and 15 bacterial colonies (December 2001) were randomly picked from appropriate dilution plates (between 20 and 200 colonies per Petri dish) and checked for purity on 10-fold diluted Tryptic Soy Agar (Difco). The affiliation of mS1 isolates to the genus *Pseudomonas* was checked by hybridization 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 (seven for *L. perenne* and one for *M. coerulea)*, the corresponding proportions were not further considered.

The proportions of NR and D *Pseudomonas* in the different treatments were analyzed 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 generalized 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, WA).

Co-Amplification of narG and napA Fragment Genes. Genomic DNA of all nitrate-dissimilating strains for three sampling dates (June 2000, July 2001, and December 2001) was extracted using Wizard Genomic DNA Purification kit (Promega, 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* (the 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) 1× Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.2 mM each dNTPs (Promega), 0.25 μM each primers DMHPF, DNDWI, Fnap, and Rnap (MWG Biotech, AG, Ebersberg, Switzerland), 0.1 μg μL^{-1} T4 Gene 32 Protein (QBiogene), 0.05 U μL^{-1} *Taq* DNA Polymerase (Promega), and 0.1 μL μL^{-1} pure or 10-fold diluted DNA extract. The reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., MA). 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 Uls, 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 an unexpected size were obtained, the DNA extract was resubmitted to PCR amplification with *narG* and *napA* primer sets separately.

RFLP Analysis of narG and napA Fragment Genes. Each PCR product (~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

Table 1. PCR primers used for the amplification of *narG* and *napA* gene fragments

Primers	*Target genes*	*Position 5′–3′*[a]	*5′–3′ sequences*[b]
DMHPF[c]	*narG*	2413–2426	GAYATGCAYCCGTT
DNDWI[c]	*narG*	3412–3425	AYCCARTCRTTRTC
Fnap[d]	*napA*	245–269	TTYYTNHSNAARATHATGTAYGG
Rnap[d]	*napA*	1102–1123	TGYTGRTTRAANCCCATNGTCCA

[a]Position on *Pseudomonas aeruginosa* strain A01 *narG* (accession number AE004804) and *napA* (NC_002516) genes, respectively.
[b]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.
[c]Delorme, S (2001) PhD Thesis, Université de Bourgogne, France.
[d]Adapted from [18].

computer simulated restriction analysis of some available *napA* sequences), according to the enzyme manufacturer's (Promega) instructions. Digestion products were analyzed after electrophoresis in 2% STG agarose gel (Eurobio) for 2 h 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 Discussion

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 fertilization amounts [14]. Differences in NR and D according to the sampling dates could result from the evolution of the microbiota 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

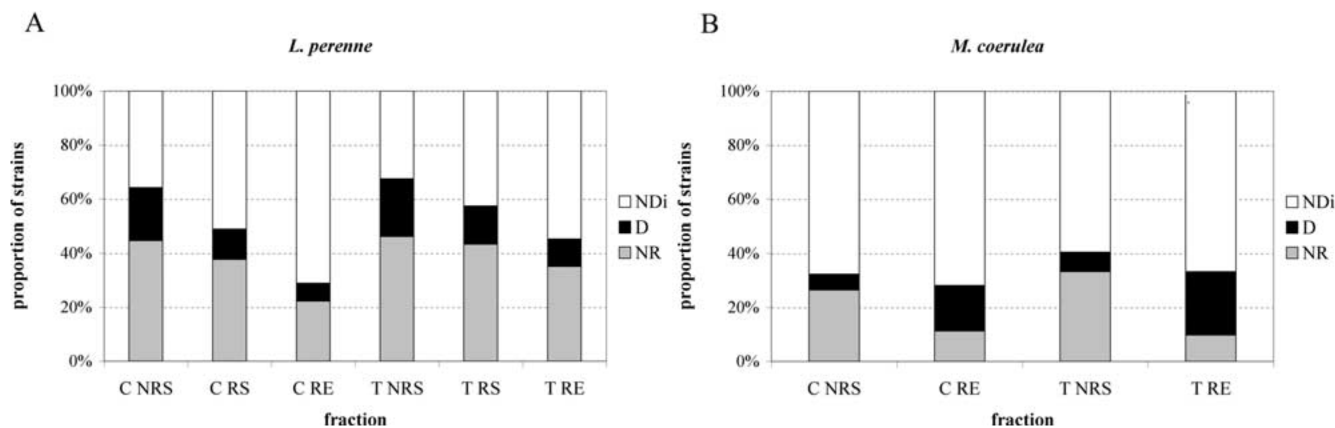

Figure 1. Proportions of nitrate-reducing (NR), denitrifying (D), and non-dissimilating (NDi) *Pseudomonas* associated with (A) *Lolium perenne* (n = 960 strains) and (B) *Molinia coerulea* (n = 286 strains): total for all sampling dates. C: Control plots (ambient pCO_2); T: pCO_2-treated plots; NRS: non-rhizospheric soil; RS: rhizospheric soil; RE: root fraction.

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)

	Control plots			Treated plots		
L. perenne	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

	Control plot		Treated plot	
M. ceorulea	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 three replicate plots.

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 (fertilization), 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 conducted 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, E.M. Baggs, M. Aragno, and N. Fromin, in preparation).

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-fertilized *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.

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–napA* multiplex PCR protocol for the nitrate reducing and denitrifying *Pseudomonas* strains isolated from three sampling dates. The amplification with crossed pairs of primers for strains harboring 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 analyzed (Fig. 3). There was a good correspondence between the nitrate reductase activity and the presence of *narG* and/or *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.

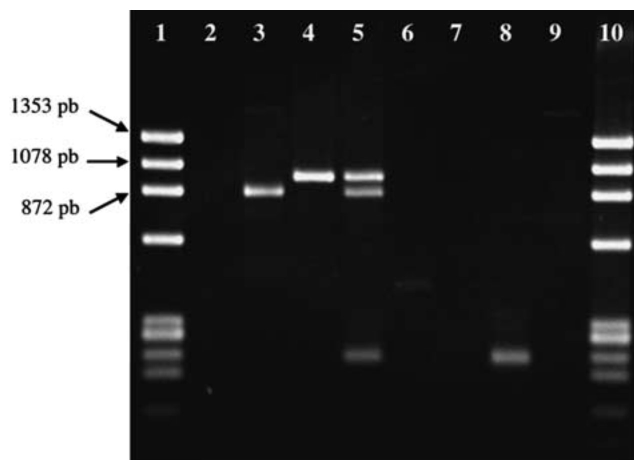

Figure 2. PCR co-amplification of *narG* and *napA* gene fragments from strain ELC3RE3. Lanes 1 and 10: ϕX 174 DNA/*Hae*III molecular weight marker (Promega); lane 2: no DNA template; lane 3: Fnap and Rnap; lane 4: DMHPF and DNDWI; lane 5: Fnap, Rnap, DMHPF, and DNDWI; lane 6: Fnap and DMHPF; lane 7: Fnap and DNDWI; lane 8: Rnap and DMHPF; lane 9: Rnap and DNDWI.

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

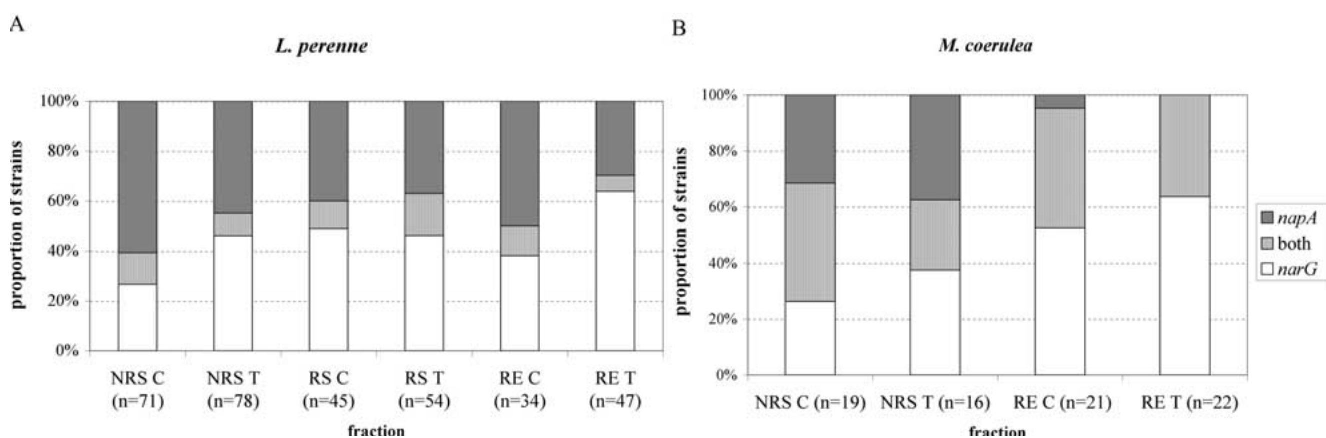

Figure 3. Proportions of strains having *narG* and/or *napA* genes among NR *Pseudomonas* associated with (A) *Lolium perenne* and (B) *Molinia coerulea*. NRS: Non-rhizospheric soil; RS: rhizosphere fraction; RE: root fraction; C: control plots; T: pCO_2-treated plots; *n*: number of strains analyzed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

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 analyzed for *L. perenne* and 27 among the 29 D strains for *M. coerulea*). A few D strains had only the NAP form (four for *L. perenne* and two 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 three 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 three 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).

These results taken as a whole suggest that the NAR and NAP forms probably have different functions within the corresponding organisms, despite the fact that they catalyze 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 corespire 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*-harboring strains were clustered into 37 *narG* (noted nar1 to nar37) and the 205 nap *A*-harboring 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 harboring 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 harbored 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 harboring 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* OTUs 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*-harboring strains were clustered into 38 different *napA* OTUs, noted nap1 to nap38, of which 17 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.

Several *narG* (nar1, nar2, nar3, nar11, and nar19) and *napA* OTUs (nap5, nap11, nap22, and nap23) were

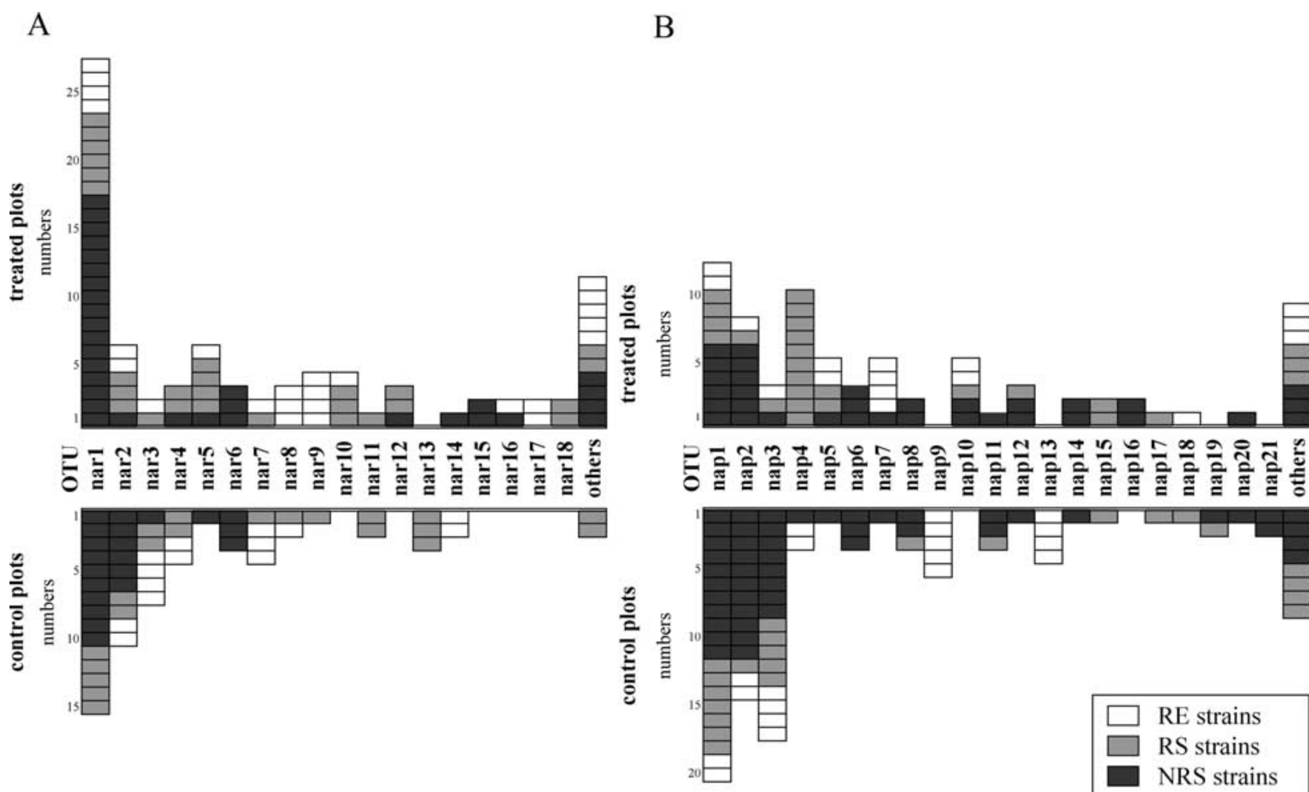

Figure 4. Distribution of nitrate-dissimilating *Pseudomonas* strains among *narG* (A) and *napA* (B) OTUs for *Lolium perenne.* Each box represents a strain. OTUs harboring a single strain ($n = 1$) were grouped as "others".

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 $n \geq 2$) 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].

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 for the nitrogen transformations and budget of these soils.

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