

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 diploid 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 (NH₄-N and NO₃-N content of 94.9 and 48.4 µg 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-

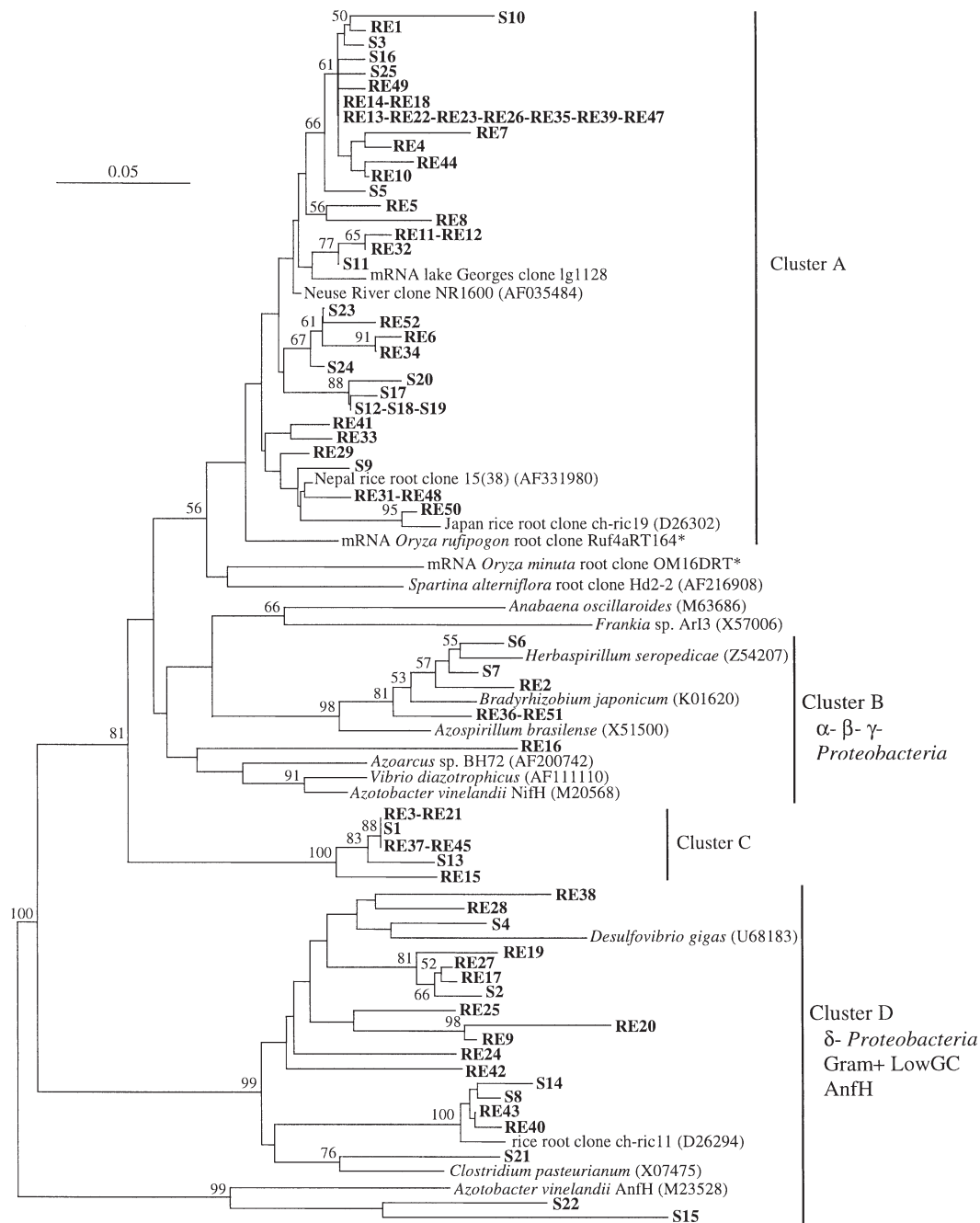


Fig. 1. Phylogenetic tree showing the position of the NifH sequences based on the alignment (ClustalX; Thompson *et al.* 1997) of 112 amino-acid residues corresponding to the positions 44–154 in the *Azotobacter vinelandii* NifH protein. The tree was constructed by the neighbour-joining method (Saitou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

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

bog (data not shown). As a hemicytrophytic 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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