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Short communication

Nitrogen-fixing bacteria in Mediterranean seagrass (*Posidonia oceanica*) roots

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ABSTRACT

Biological nitrogen fixation by diazotrophic bacteria in seagrass rhizosphere and leaf epiphytic community is an important source of nitrogen required for plant growth. However, the presence of endophytic diazotrophs remains unclear in seagrass tissues. Here, we assess the presence, diversity and taxonomy of nitrogen-fixing bacteria within surface-sterilized roots of *Posidonia oceanica*. Moreover, we analyze the nitrogen isotopic signature of seagrass tissues in order to notice atmospheric nitrogen fixation. We detected nitrogen-fixing bacteria by *nifH* gene amplification in 13 out of the 78 roots sampled, corresponding to 9 locations out of 26 meadows. We detected two different types of bacterial *nifH* sequences associated with *P. oceanica* roots, which were closely related to sequences previously isolated from the rhizosphere of a salt marsh cord grass and a putative anaerobe. Nitrogen content of seagrass tissues showed low isotopic signatures in all the sampled meadows, pointing out the atmospheric origin of the assimilated nitrogen by seagrasses. However, this was not related with the presence of endophytic nitrogen fixers, suggesting the nitrogen fixation occurring in rhizosphere and in the epiphytic community could be an important source of nitrogen for *P. oceanica*. The low diversity of nitrogen-fixing bacteria reported here suggests species-specific relationships between diazotrophs and *P. oceanica*, revealing possible symbiotic interactions that could play a major role in nitrogen acquisition by seagrasses in oligotrophic environments where they form lush meadows.

1. Introduction

Seagrasses form highly productive meadows in often nutrient-poor coastal areas (Hemminga and Duarte, 2000), where nutrients can limit their growth (e.g. Powell et al., 1989; Lee et al., 2007). Seagrasses acquire inorganic (e.g. Lee et al., 2007) and organic (Vonk et al., 2008) nutrients through leaves and roots. These nutrients can be supplied from land (e.g. run off, riverine, agriculture, groundwater and sewage discharges) and from sediment organic matter mineralization. Moreover, fixation of atmospheric nitrogen into ammonia by diazotrophic bacteria is an important additional source of nitrogen covering the nutrient requirements of seagrasses (Patriquin and Knowles, 1972; O'Donohue et al., 1991; Welsh, 2000). The isotopic composition of the nitrogen content ($\delta^{15}N$) in seagrass tissues is an indicator of the nitrogen source and, therefore, of the main nitrogen acquisition process. Low $\delta^{15}N$ values indicate that atmospheric nitrogen fixation process is involved (Bedard-Haughn et al., 2003).

The main bacterial protein involved in the nitrogen fixation is dinitrogenase. This enzyme is highly regulated by transcriptional and post-transcriptional controls. The genes coding for dinitrogenase (*nifHDK* genes) are highly conserved and show a high degree of similarity among organisms. Particularly, the sequence variability of the *nifH* gene shows good correspondence with the taxonomic affiliation of diazotrophic bacteria (Zehr and Capone, 1996). Thus, sequences of an amplified fragment of *nifH* gene can be used to determine the taxonomic affiliation of uncultured and unknown environmental nitrogen-fixing bacteria (Zehr et al., 1995; Ueda et al., 1995). The detection of the nitrogenase gene has been widely used in biological samples as an indicator of nitrogen-fixing capabilities and also to estimate the diversity of diazotrophic communities in many environments (e.g. Cyanobacterial mats, salt marsh rhizosphere, seagrass rhizosphere; Zehr et al., 1995; Piceno et al., 1999; Bagwell et al., 2002).

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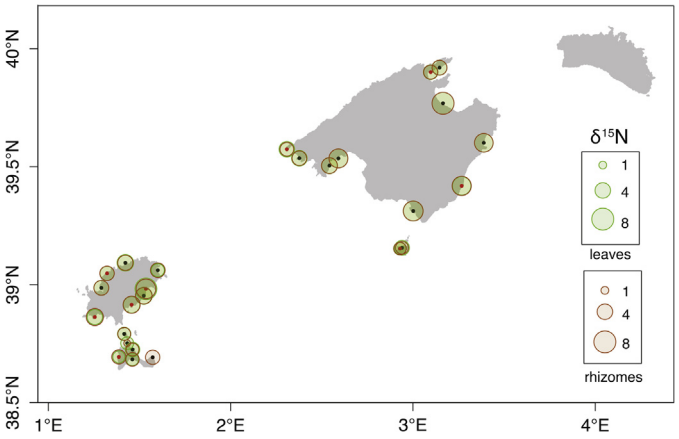

Fig. 1. Detection of nitrogen fixers in surface-sterilized roots and nitrogen isotopic signature ($\delta^{15}N$) of tissues in the sampled *P. oceanica* meadows. Red points indicate locations where *nifH* genes were amplified from surface-sterilized roots; black points indicate no *nifH* genes detected at that location. Colored circles represent the $\delta^{15}N$ values (‰) measured in leaves (green) and rhizomes (brown).

To date, nitrogen fixation in seagrass meadows has only been described in sediment, rhizosphere and leaf epiphytes (Patriquin and Knowles, 1972; Bagwell et al., 2002; Lyimo and Hamisi, 2008). Although there is ample evidence of endophytic nitrogen fixation in terrestrial plants, the presence of nitrogen-fixing endophytes in seagrasses has not been demonstrated yet. Previously, we identified a bacterial sequence (520 bp, Accession number JF292436) highly similar (94%) to a nitrogen-fixing bacteria (*Celerinatantimonas diazotrophica,* DQ913889) in the roots of the Mediterranean seagrass *P. oceanica* (Garcias-Bonet et al., 2012). These findings aimed us to ascertain the presence of nitrogen-fixing bacteria in seagrass roots by detecting the nitrogen-fixing functional gene *nifH*.

Here, we detect nitrogen-fixing bacteria by amplification of *nifH* gene in surface-sterilized roots of *P. oceanica* meadows in the Balearic Islands (Western Mediterranean). We analyze the isotopic composition of the nitrogen content in seagrass tissues, in order to identify atmospheric nitrogen fixation and incorporation into plants. In addition, we analyze the bacterial diversity and taxonomy by DGGE and by comparing the bacterial *nifH* sequences obtained from seagrass roots to a database of *nifH* genes.

2. Materials and methods

2.1. Sampling strategy

Triplicate samples of *P. oceanica* were randomly collected by SCUBA diving at 26 locations along the Balearic Islands (Fig. 1) during the summers of 2005 and 2006. The plants were transported to the laboratory in seawater from the same location and processed immediately. The youngest leaf (free of epiphytes) of three *P. oceanica* shoots and three young rhizome fragments were collected in each meadow to analyze nitrogen isotopic composition. Roots were subjected to a surface-sterilization protocol adapted from Coombs and Franco (2003). Briefly, the protocol consisted in immersing each root in ethanol (99% for 1 min), then in NaOCl (3.125% for 6 min), then in ethanol (99% for 30 s) and finally washing gently with autoclaved seawater. The surface-sterilized roots (2 roots per replicate) were frozen in liquid nitrogen until nucleic acid extraction was performed.

2.2. Isotopic composition of nitrogen content in seagrass tissues

Isotopic analyses were conducted as described by Fourqurean et al. (2007). The samples were dried at 60 °C for 48 h and ground to a fine powder using a motorized agate mortar and pestle. All isotopic analyses were measured using standard elemental analyzer isotope ratio mass spectrometer (EA-IRMS) procedures. The EA was used to combust the organic material and to reduce the formed gas into N_2, which was measured on a Finnigan MAT Delta C IRMS in a continuous flow mode. Isotopic ratios (R) are reported in the standard delta notation (δ, ‰),

$$\delta_{sample} = 1000\left[\left(\boldsymbol{R_{sample}/R_{standard}}\right) - 1\right]$$

where $R = {}^{15}N/{}^{14}N$. These results are presented with respect to the International standard of atmospheric nitrogen (AIR, N_2). Analytical reproducibility of the reported δ values, based on sample replicates, was better than ±0.2‰.

The statistical significance of differences in isotopic signal between the locations where *nifH* gene was detected and those locations where *nifH* gene could not be detected was assessed by Student's *t* test.

2.3. Nucleic acid extraction and amplification of nifH gene

Surface-sterilized roots (100 mg of roots per sample) were ground with the help of a sterilized pestle prior to nucleic acid extraction. Total nucleic acids were extracted using the DNeasy Plant Kit (Qiagen®). The DNA extract, containing plant and endophyte DNA when present, was amplified by PCR with degenerate primers for *nifH* gene sequences containing a GC-clamp for DGGE analysis described previously by Piceno et al. (1999): Forward primer 5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′ and reverse primer 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG(G/C)-ACGATGTAGATPTCCTG-3′. Each 20 μL PCR reaction contained template DNA plus (final concentrations) 2 mM of dNTPs mixture, 20 μM of each primer and 0.5 units of *Taq* Polymerase (Takara) suspended in the buffer provided by the manufacturer of the polymerase. In each batch of PCR reactions, we run additional control reactions: one negative control (no DNA) and two positive controls (DNA from two known diazotrophic bacteria: *Mesorhizobium ciceri* (DSM1978) and *Vibrio diazotrophicus* (DSM2605) provided by DSMZ, Germany). The PCR protocol, slightly modified from Piceno et al. (1999), consisted of an initial denaturing step at 94 °C for 5 min, followed by 20 touchdown cycles (94 °C for 1 min, 58 °C for 1 min, decreased by 0.5 °C cycle^{-1}, and 72 °C for 1 min); and 10 cycles of standard amplification (94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min) with a final elongation step of 72 °C for 7 min. The PCR products were checked by electrophoresis on 1.5% agarose gels. For each positive sample, the products of several replicate reactions (minimum of 2) were pooled prior to DGGE in order to load approximately 1 μg of PCR product per lane on the DGGE gel.

2.4. Denaturing gradient gel electrophoresis (DGGE)

The amplification products of the fragment of the *nifH* gene were resolved by DGGE in a 6.5% polyacrylamide gel containing a gradient of denaturants ranging from 72.5% to 95% (where 100% is 7 M urea and 40% formamide). Gels were run for 9 h at 200 V in 1X TAE (Tris-Acetate-EDTA) buffer at 48° C in a DGGE system (CBS Scientific Co. CA, USA). Following electrophoresis, the gels were stained with SyberGold for 30 min in the dark and photographed using a G:BOX imaging system (Syngene, Cambridge, UK). All the detectable bands were excised with the help of a sterilized scalpel and stored frozen in 20 μL of autoclaved Milli-Q water at −20 °C for further processing.