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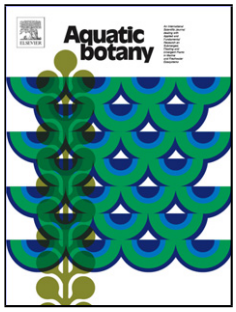

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Nitrogen-fixing bacteria in Mediterranean seagrass (*Posidonia oceanica*) roots

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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.

Keywords: diazotrophy, nitrogen fixation, seagrass-bacteria interaction, endophytes.

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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 (∂^{15}N) in seagrass tissues is an indicator of the nitrogen source and, therefore, of the main nitrogen acquisition process. Low ∂^{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.

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Cyanobacterial mats, salt marsh rhizosphere, seagrass rhizosphere; Zehr et al., 1995; Piceno et al., 1999; Bagwell et al., 2002).

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

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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 sec) 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 60ºC 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, ‰),

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

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

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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' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCC-CCGCCCG(G/C)ACGATGTAGATPTCCTG 3'. Each 20 µl PCR reaction contained template DNA plus (final concentrations) 2mM 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* (DSM 1978) and *Vibrio diazotrophicus* (DSM 2605) provided by DSMZ, Germany). The PCR protocol, slightly modified from Piceno et al. (1999), consisted of an initial denaturing step at 94ºC for 5 min, followed by 20 touchdown cycles (94ºC for 1 min, 58ºC for 1 min, decreased by 0.5ºC $cycle^{-1}$, and 72ºC for 1 min); and 10 cycles of standard amplification (94ºC for 1 min, 48ºC for 1 min and 72ºC for 1 min) with a final elongation step of 72ºC for 7 min. The PCR products were checked by electrophoresis on 1.5% agarose gels. For each positive sample, the products of

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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 48º 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 -20ºC for further processing.

2.5. Sequencing of bands detected in DGGE

The re-amplification of the excised bands (OTUs) was conducted using the same pair of primers used before. Several PCRs were done using the DNA of the excised band as template. The amplicons were pooled together, concentrated and purified from primers and dNTPs using MSB Spin PCRapace kit (Stratec, Germany). Finally, 150 ng of the amplified product was used for the sequencing reaction with the forward primer (not containing the GC-clamp). The sequencing was performed on an ABI 3730 sequencer (Applied Biosystems, CA, USA), using the chemistry BigDye ® Terminator v3.1. The sequences obtained in this study have been deposited in GenBank under the accession numbers JN987872 and JN987873.

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2.6. Sequence analysis

The sequences (~400 bp) were checked for existence of chimeras using the Bellerophon tool available at http://greengenes.lbl.gov and the primer sequences were removed. The sequences were imported into an ARB (Ludwig et al., 2004) database including all *nifH* sequences available in GenBank until April 2009 (16,989 sequences), compiled and constructed by Gaby and Buckley (2011). The sequences were aligned with the guide of a PT-server constructed from all the *nifH* sequences already aligned in the database. The resulting alignment was checked manually and the edges were manually re-aligned when necessary. The aligned sequences were added into the existent tree of *nifH* sequences provided with the database in order to assess their phylogenetic identity. A maximum likelihood tree was constructed in ARB for the sequences obtained in this study, selecting the closest sequence and those sequences taxonomically identified for reference. An archaeal sequence (*Methanosarcina acetivorans*, AE010299) was used as out-group and the bootstrap probabilities were calculated from 10,000 pseudo-replicates.

3. Results and discussion

We detected the presence of nitrogen-fixing bacteria by amplification of the *nifH* gene in 13 samples of *P. oceanica* roots (out of a total of 78 samples) corresponding to 9 meadows (out of 26 meadows, Fig. 1). The fact that *nifH* gene could not be amplified in all samples of the same location suggests that diazotrophs may not be ubiquitous or that their distribution is not uniform along roots and, therefore, might escape to our detection. The presence of diazotrophs in seagrass tissues suggests the possibility of symbiotic bacteria that might play an important role in nitrogen acquisition, particularly in nutrient poor environments where they form extensive meadows. Diazotrophic endophytes have been

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described in roots of salt marsh grasses (McClung and Patriquin, 1980). Similarly, the existence of nitrogen-fixing endophytes has been widely described for many terrestrial plants. For instance, crop species as rice contain diazotrophic endophytes (Ebeltagy et al., 2001; Govinderajan et al., 2008) as components of a highly diverse bacterial community (Ueda et al., 1995).

The finding of nitrogen-fixing bacteria was in agreement with the averaged low isotopic nitrogen content ($\delta^{15}N$) of *P. oceanica* tissues in the region (4.07‰ ± 0.29 in leaves and 3.9‰ ± 0.3 in rhizomes), ranging from 2.29 to 7.57‰ in leaves and from 0.69 to 7.62‰ in rhizomes (Fig. 1). The nitrogen isotopic dataset is reported in Garcias-Bonet et al. (2016). This isotopic signature evidences incorporation of atmospheric nitrogen into plant tissues, when compared with values reported for *P. oceanica* growing in other Mediterranean regions (Papadimitriou et al., 2005). However, the averaged $\delta^{15}N$ did not differ significantly between meadows where *nifH* was detected from those meadows where *nifH* was not detected (Students' *t* test; Leaves, p=0.8; Rhizomes, p=0.7). This can be due to the fraction of nitrogen fixed by bacteria inhabiting the rhizosphere and the epiphytic community in leaves that contribute to the global pool of available nitrogen that has been incorporated to the seagrass tissues. Moreover, the clonal architecture of seagrasses allows mobilization and sharing of nutrients acquired at different regions of the clone with their neighbors. Specifically, for *P. oceanica* clones, a translocation experiment using labeled nitrogen isotopes, demonstrated that acquired nitrogen could travel within the clone at spatial scales of decimeters in both directions (i.e. towards apex edge and towards older parts) from the shoot incorporating the tracer (Marbà et al., 2002). This clonal connectivity

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suggests that even if nitrogen-fixing bacteria were only present in some of the roots, the whole clone would benefit from the fixed nitrogen.

We identified a clear and homogenous DGGE band profile among all samples, with 2 different bands, which were never found simultaneously in the same root sample. Sequencing all the detected DGGE bands yielded two different *nifH* sequences that we named as Band A (P.o_root_A_nifH, JN987872) and Band B (P.o_root_B_nifH, JN987873). The identification of only two different *nifH* sequences in all the 11 *P. oceanica* surface-sterilized roots suggests a very species-specific diazotroph-seagrass relationship. This contrasts with rhizosphere diazotrophic communities, which have been reported to be highly diverse. For instance, in the rhizosphere of the salt marsh cord grass *Spartina alterniflora* the analysis of diazotrophic community by DGGE of the amplified *nifH* gene yielded 58 different *nifH* sequences (Lovell et al., 2008). Similarly, 67 different *nifH* sequences were recovered by DGGE analysis in the sediments colonized by mixed seagrasses meadows (*Thalassia testudinum* and *Syringodium filiforme*) (Bagwell et al., 2002).

According to our ARB analysis, the sequence obtained from Band A (P.o_root_A_nifH, JN987872) fell into cluster I (Fig. 2), which contains *nifH* genes from most Proteobacteria, all Cyanobacteria and some Firmicutes and Actinobacteria (Gaby and Buckley, 2011). The closest relative, with 87.24% of similarity, was an unknown *nifH* sequence (DQ402931) amplified from a salt marsh cord grass (*Spartina alterniflora*) rhizosphere (Lovell et al., 2008) that was very similar to the marine nitrogen-fixing *Vibrio diazotrophicus* (AF111110). The other closest relatives were unknown diazotrophs isolated from diverse

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environments, as seawater, soil and mangrove sediments, although with lower similarity (from 79.5% to 87%).

The sequence obtained from Band B (P.o_root_B_nifH, JN987873) fell into cluster III (Fig. 2), which contains *nifH* genes from anaerobic diazotrophs (Gaby and Buckley, 2011). However, its phylogenetic affiliation cannot be more precisely elucidated, as the closest relatives derived from ARB analysis were *nifH* sequences of unknown diazotrophs (AY091864 and DQ402844, with 83.16% and 82.62% of similarity, respectively) from the rhizosphere of salt marsh cord grass (*Spartina alterniflora*; Lovell et al., 2008). The other closest relatives (with 82% of similarity) were also unknown diazotrophs isolated from *S. alterniflora* rhizosphere and non-sterilized mangrove roots (Flores-Mireles et al., 2007).

5. Conclusions

Seagrass roots contain nitrogen-fixing bacteria that could explain their successful colonization of oligotrophic environments. The diazotrophs detected could be species-specific as derived from the two unique different sequences obtained. These findings reconcile the dominant atmospheric origin of *P. oceanica* nitrogen content with the presence of diazotrophic bacteria in their roots and suggest that only a few specific diazotrophs contribute to endophytic nitrogen fixation in *P. oceanica*. These results provide new insight into the endophytic bacterial community of seagrasses and open a new research topic in the ascertainment of the roles of these bacteria in the ecophysiology of marine plants.

Acknowledgments

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fixing microorganisms in seagrass, *Posidonia oceanica*, roots and nitrogen isotopic signature of seagrass tissues [Dataset] Digital CSIC. URI: http://hdl.handle.net/10261/128883 [the dataset will be published upon acceptance of the manuscript]

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Figure captions

Figure 1. Detection of nitrogen fixers in surface-sterilized roots and nitrogen isotopic signature ($\partial^{15}N$) 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 ∂15N values (‰) measured in leaves (green) and rhizomes (brown).

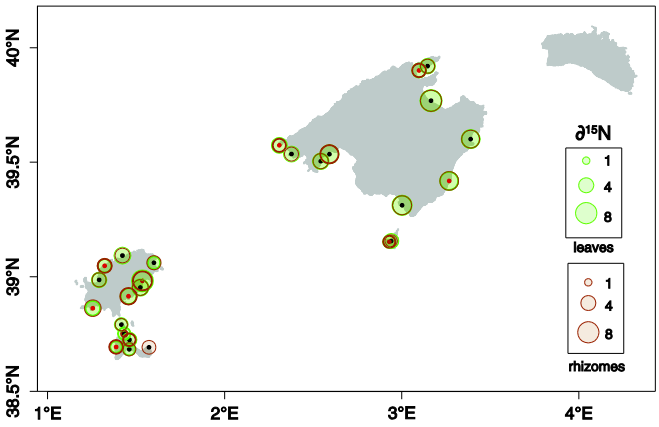
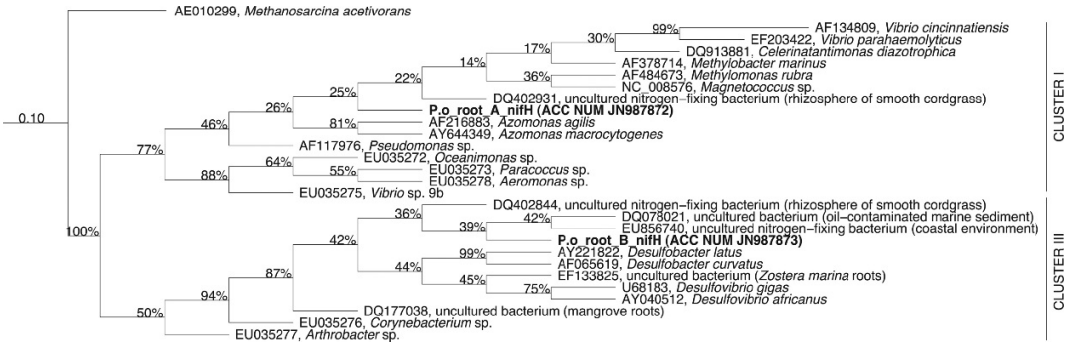


Figure 2. Maximum likelihood tree constructed in ARB for the *nifH* sequences obtained from *P. oceanica* surface-sterilized roots (in bold), showing the closest relatives and those sequences with taxonomic information for reference. An archaeal sequence (*Methanosarcina acetivorans*, AE010299) was used as out-group and the bootstrap probabilities from10,000 pseudo-replicates are shown.



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