Bacterial N$_2$-fixation in mangrove ecosystems: insights from a diazotroph–mangrove interaction

Gabriela Alfaro-Espinoza* and Matthias S. Ullrich

Molecular Life Science Research Center, Jacobs University Bremen, Bremen, Germany

Mangrove forests are highly productive ecosystems but represent low nutrient environments. Nitrogen availability is one of the main factors limiting mangrove growth. Diazotrophs have been identified as key organisms that provide nitrogen to these environments. N$_2$-fixation by such organisms was found to be higher in the mangrove roots than in surrounding rhizosphere. Moreover, previous studies showed that mangroves grew better in the presence of N$_2$-fixers indicating a potentially mutualistic relationship. However, the molecular signals and mechanisms that govern these interactions are still poorly understood. Here we present novel insights in the interaction of a diazotroph with a mangrove species to improve our understanding of the molecular and ecophysiological relationship between these two organisms under controlled conditions. Our results showed that Marinobacterium mangrovicola is a versatile organism capable of competing with other organisms to survive for long periods in mangrove soils. N$_2$-fixation by this bacterium was up-regulated in the presence of mangrove roots, indicating a possible beneficial interaction. The increase in N$_2$-fixation was limited to cells of the exponential growth phase suggesting that N$_2$-fixation differs over the bacterial growth cycle. Bacterial transformants harboring a transcriptional nifH::gusA fusion showed that M. mangrovicola successfully colonized mangrove roots and simultaneously conducted N$_2$-fixation. The colonization process was stimulated by the lack of an external carbon source suggesting a possible mutualistic relationship. M. mangrovicola represents an interesting genetically accessible diazotroph, which colonize mangrove roots and exhibit higher N$_2$-fixation in the presence of mangrove roots. Consequently, we propose this microorganism as a tool to study molecular interactions between N$_2$-fixers and mangrove plants and to better understand how changes in the environment could impact these important and relatively unknown interactions.

Keywords: nitrogen fixation, mangroves, diazotrophs, nitrogen cycle, bacteria–plant interaction, root colonization

Introduction

Mangrove ecosystems are wetlands located along tropical and subtropical coastlines. The term mangroves can also refer to a characteristic group of shrubs and woody tress that grow on brackish intertidal environments (Feller et al., 2010). These highly productive environments provide many ecosystem functions such as breeding and feeding grounds to diverse marine species, host a variety of organisms such as birds, mammals, and invertebrates (Holguin et al., 2001), acting as...
a barrier against tidal currents and tsunamis (Dahdouh-Guebas et al., 2005), and serve as carbon sinks, therefore, playing a key role in the global carbon cycle (Bouillon et al., 2008; Feller et al., 2010; Donato et al., 2011).

Although mangroves ecosystems are highly productive and rich in carbon, they are considered low nutrient environments. Nitrogen and phosphorus have been highlighted as nutrients limiting mangrove growth (Bashan and Holguin, 2002; Feller et al., 2002; Reef et al., 2010). Major factors contributing to nitrogen loss in these ecosystems are tidal export of nitrogen, denitrification, and the soil type (Boto and Robertson, 1990; Reef et al., 2010). In contrast, N$_2$-fixation by diazotrophs is a major input of nitrogen to the ecosystem (Sen Gupta and Chaudhuri, 1991; Woitchik et al., 1997; Reef et al., 2010). N$_2$-fixation in mangrove ecosystems has been detected in sediments, rhizosphere, decomposing leaves, tree bark, nematophores, and roots (Gotto and Taylor, 1976; Zubерer and Silver, 1978; Uchino et al., 1984; Hicks and Silvester, 1985; Holguin et al., 1992; Pelegri et al., 1997). Moreover, N$_2$-fixation rates were higher in mangrove roots as compared to the rhizosphere soil (Zuberer and Silver, 1978; Sen Gupta and Chaudhuri, 1991; Holguin et al., 1992; Holguin et al., 1992; Ravikumar et al., 2004; Alfaro-Espinoza and Ullrich, 2014). In addition, phylogenetic studies on nifH, the gene encoding for the Fe protein of the nitrogenase enzyme complex and commonly used as a marker for N$_2$-fixation (Zehr and McReynolds, 1989; Zehr et al., 2003) showed that members of the genera Azotobacter, Derxia, Desulfuromonas, Sphingomonas, Pseudomonas, and Vibrio, among others, could also play a role as N$_2$-fixing organisms in mangrove ecosystems (Flores-Mireles et al., 2007; Zhang et al., 2008).

Although the description and isolation of microorganisms responsible for providing nitrogen to nitrogen-poor environments has been done extensively, very little advance has been made on understanding the effects that changing environmental conditions have on the cellular and ecological relationships between microbes and their mangrove environment. Previous studies indicated that some interactions between N$_2$-fixers and mangrove plants are mutualistic (Bashan et al., 1998; Ravikumar et al., 2004). However, molecular signals and cellular mechanisms that govern these interactions are poorly understood. Postulating mechanisms similar to those extensively studied in legume crop plants might be impossible since the considered ecosystems and their contextual geochemical parameters differ significantly. Therefore, a genetically accessible N$_2$-fixing bacterium and a mangrove plant species could strongly facilitate future studies and help us to better understand the importance of this potentially mutualistic interaction for the ecosystem and how future climatic changes could impact this organismal interplay.

Herein, we established the diazotroph _M. mangrovicola_ Gal22 as an organism that interacts with the mangrove species _Rhizophora mangle_. For this we tested (a) the ecological competence and survival of _M. mangrovicola_ in the mangrove's rhizosphere and roots; (b) the influence of nutrient availability on the root colonization process; and (c) the genetic accessibility of the selected diazotroph. Corresponding results will provide the basis for further research on the symbiosis between diazotrophs and mangroves in these important tropical and subtropical ecosystems.

### Materials and Methods

#### Mangrove Plants, Bacterial Strains, Plasmids, and Primers Used

Bacterial strains used in this study are listed in Table 1. All plasmids and primers used are listed in Table 2. _M. mangrovicola_ Gal22 cells were cultivated overnight at 28°C on HGB agar plates (supplemented with 0.1% yeast extract; Holguin et al., 1992). _Escherichia coli_ DH5α cells were grown overnight at 37°C on Luria–Bertani (LB) agar plates. _E. coli_ ST18 was grown on LB agar.

**Table 1 | Bacterial strains used.**

| Bacterial strains | Characteristic | Reference |
|-------------------|---------------|-----------|
| *Escherichia coli* |               |           |
| DH5α              | supE44 ΔlacU169 (F80 lacZD515) hsdR17 recA1 endA1 gyrA96  | Hanahan (1985) |
| ST18              | thr-1 r441     | Thoma and Schobert (2009) |
| Marinobacterium mangrovicola | Wild type containing nifH::gusA fusion cloned in pBBR1MCS-3 | Alfaro-Espinoza and Ullrich (2014) |
| Gal22             | Wild type     | This study |
| Gal22ΔnifH        | nifH deletion mutant, Crn$^R$ | This study |
| Gal22ΔnifH::pBBR1MCS.GA.gusA | nifH deletion mutant containing gusA, Crn$^R$ | This study |
| Gal22ΔnifH::pBBR1MCS-3.GA.gusA | nifH deletion mutant containing gusA, Tc$^R$ | This study |

_Cm_, Chlomarphenicol; _Tc_, tetracycline; _R_, resistance.
TABLE 2 | Plasmids and primers used.

| Plasmids/Primers | Characteristic | Reference |
|------------------|---------------|-----------|
| **Plasmids**     |               |           |
| pUC119           | pMB1 origin, Ap<sup>R</sup> | Vieira and Messing (1991) |
| pSU23            | p15A origin, Cm<sup>R</sup> | Bartolomé et al. (1991) |
| pKT210           | IncQ, Cm<sup>R</sup>, Sm<sup>R</sup> | Bagdasarian et al. (1981) |
| pBBRMCS          | Broad-host-range cloning vector, Cm<sup>R</sup> | Kovach et al. (1994) |
| pBBRMCS-3        | Broad-host-range cloning vector, Tc<sup>R</sup> | Kovach et al. (1995) |
| pBluescript SK<sup>(+)</sup> | CoE1 origin, Ap<sup>R</sup> | Stratagene |
| pJET1.2          | Replicon from pMB1 vector, eco47IR, Ap<sup>R</sup> | Thermo Fisher Scientific |
| pJETnif<sup>up/down</sup> | 450-bp deletion of nifH. First and last part of nifH (fragment 1 and 2) cloned in pJET1.2 | This study |
| pFCMI            | Cm<sup>R</sup> flanked by FRT sequences | Hoang et al. (1998) |
| pEX18TC          | onT<sup>+</sup> sacB<sup>R</sup>, Tc<sup>R</sup>, MCS from pUC18 | Hoang et al. (1998) |
| pEX.GA.nifH      | Mutagenic construct. 2.7-kb HindIII fragment containing Cm<sup>R</sup> flanked by the first and last part of nifH gene cloned in pEX18Tc | This study |
| pCAM140          | Promoterless gusA, Ap<sup>R</sup> | Wilson et al. (1995) |
| pJETnif          | 1.5-kb fragment containing nifH plus upstream regulatory region | This study |
| pJetgus          | 1.9-kb fragment containing promoterless gusA | This study |
| pJETnif.gus      | 3.4-kb fragment containing nifH-gusA fusion | This study |
| pBBRMCS.G.A.nifH::gusA | 3.4-kb HindIII fragment containing a transcriptional fusion of nifH gene plus upstream regulatory region with promoterless gusA. Cloned in opposite orientation with respect to lac promoter, Cm<sup>R</sup> | This study |
| pKW117           | 2.2-kb fragment containing gusA plus tac promoter and trpA transcriptional terminator, Ap<sup>R</sup> | Wilson et al. (1995) |
| pBBR1MC S. G.A.gusA | 2.2-kb fragment containing gusA plus tac promoter cloned in pBBR1MCS, Cm<sup>R</sup> | This study |
| pBBRMCS-3.GA.gusA | 2.2-kb fragment containing gusA plus tac promoter cloned in pBBR1MCS-3, Tc<sup>R</sup> | This study |
| **Primers**      | **Sequence (5′-3′)** | **Reference** |
| Zf                | GCTGAGCTCAARCGCNGA | Zehr and McReynolds (1989) |
| Zr                | ADNGCCATCTATYTNCC | Zehr and McReynolds (1989) |
| RTnifH            | ACCAGACGTGCAAGCCTTAA | This study |
| RTnifHr           | CGAAGCTGCAAGAAAGTCGA | This study |
| RT16Sf            | ACCACACACCTTTCCTCAAC | This study |
| RT16Sr            | CCAGGCGGAGCTTCTTATAAGC | This study |
| nif-M<sup>+</sup>-f | CCACGTGCGGAGCATACGATGTA | This study |
| nif-M<sup>+</sup>-r | CCACGAAACCTGAGTAGTGCCTTGGC | This study |
| Fw.NotI.HindIII.f2 | GCGGCGCGGAGCTTGGGACTGGAAGACACTGQTG | This study |
| Rv.BamHI.f2       | GGATCTACGCGCTCGCAGCAGAAA | This study |
| Fw.BamHI.flt      | GGATCTACGCGCTCGCAGCAGCAGC | This study |
| Rv.HindIII.fl     | AAAGCCTAGGAGATAGCCTGCTGCTG | This study |
| Mut.nifH.Fw       | TGCGCAGAATTTGCAATACC | This study |
| Mut.nifH.Rv       | CCCAGAACCACATCGAAGA | This study |
| F.nifH.reg        | AAAGTTCTGTTTTCTCCATCATCGAGCAGC | This study |
| R.nifH.reg        | GCATGCGAATTACCTCTCAGCAGCAGC | Egener et al. (1999) |
| F.gus             | GGATAGCACCGATTTTGAAGTAGAGAAGAGACC | This study |
| R.gus             | GCTAGAAAGCAGCTTGTGATTCAGTGGTTTGG | Egener et al. (1999) |

*Underline are the restriction sites incorporated into the primer sequence: GCGGCGGC, NotI; AAGCTT, HindIII; GATC, BamHI; GCATG, PaeI; TCTAGA, XbaI. **In bold, nifH RBS from Gal22; in italics, start codon. Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Sm, streptomycin; R<sup>+</sup>, resistance.

Plates containing 50 μg/ml 5-aminolevulinic acid. M. mangrovicola and E. coli transformants were maintained on HGB and LB medium supplemented with the appropriate antibiotic, respectively. Rhizophora mangle was selected as a host plant because it is a representative mangrove species that has a broad geographic distribution range, is easy to cultivate in the laboratory, and has seedlings which are commercially available in Europe.
**Isolation and Selection of the Bacterial Strain**

N2-fixing bacteria were isolated and identified using the 16S rRNA gene as described in Alfaro-Espinoza and Ullrich (2014). The bacterial model strain was selected according to the following criteria: (a) the capability of the strain to fix N2, (b) its genetic accessibility and, (c) its ability to survive in R. mangle rhizosphere in a so-called fitness test when inoculated together with a group of other mangrove root-derived or sediment-borne indigenous bacterial strains.

N2-fixation was tested in vitro by the acetylene reduction assay (ARA) and confirmed by PCR amplification of the nifH gene with the degenerate primers Zr and Zf (Zehr and McReynolds, 1989; Table 2). Genetic accessibility was tested by transformation of bacterial strains using electroporation (Hamashitna et al., 1995; Wang and Griffiths, 2009) and conjugation, respectively. For this, the following vectors were used: pBBR1MCS (Kovach et al., 1994), pUC119 (Vieira and Messing, 1991), pSU23 (Bartolomé et al., 1995), and pKT210 (Bagdasarian et al., 1981; Table 2). The Fitness test was performed in an open water tank filled with artificial sea water [Tropic Marin (Dr. Biener GmbH, Wartenberg, Germany)]. R. mangle seedlings were cultured in pots filled with threefold autoclaved quartz sand (Carl Roth, Karlsruhe, Germany) mixed with 1/10 of sand from mangrove natural ecosystems [mangrove mud special (Marek Mangroven, Vienna, Austria)], and introduced into the sea water tank. After 6 months, the rhizosphere of the mangrove plants was inoculated with the isolated bacterial strains by mixing the sediment with bacterial inoculum without disturbing the seedling and its root system. The plants were kept at 25°C with a photoperiod of 12 h. After 1 month of incubation, bacteria were re-isolated from the rhizosphere and mangrove roots by first (a) shaking the rhizosphere soil in liquid HGB medium for 30 min, and (b) macerating the mangrove roots. Then, the rhizosphere soil and homogenized roots were subjected to serial dilution and plated on HGB medium. Finally, single colonies were restreaked, and taxonomically identified by sequencing the 16S rRNA gene as described in Alfaro-Espinoza and Ullrich (2014). This experiment was repeated three times with two replicates per sample.

**Colonization of R. mangle Roots by M. mangrovicola Gal22**

**Generation of M. mangrovicola Mutant Gal22ΔnifH**

All restriction enzymes used in this study were obtained from Thermo Fisher Scientific, Schwerte, Germany. A genomic DNA library was constructed to obtain the flanking regions of the nifH gene sequence from M. mangrovicola Gal22. For this, genomic DNA was treated with the endonuclease XhoI thereby generating DNA fragments of different sizes. The XhoI-treated DNA was randomly cloned in XhoI-treated pBluescript SK(+) and ligation products were introduced to E. coli DH5α. The following specific primers were used to pre-screen for the presence of the nifH gene of Gal22, nif-M-f, and nif-M-r (Table 2). A colony giving a PCR signal of 400-bp corresponding to the Gal22 nifH gene was selected. The plasmid of this transformant was isolated and its insert DNA was subsequently sequenced. A total sequence of 2.65-kb was obtained containing the 873-bp nifH gene and its flanking regions.

To develop a nifH gene deletion mutant, primers Fw.BamH1.f1 and Rv.HindIII.f1 (Table 2) were used to PCR amplify a 829-bp fragment (fragment 1) carrying the 5′-end and 558-bp of the upstream sequence of nifH. Primers Fw.NotI.HindIII.f2 and Rv.BamH1.f2 (Table 2) were used to amplify a 750-bp fragment (fragment 2) containing the 3′-end and a 597-bp downstream sequence of nifH. Next, both fragments were cloned separately in vector pJE1T2.1 (Thermo Fisher Scientific) resulting in plasmids pJETnifHup and pJETnifHdown, respectively. In a second cloning step, fragment 1 was cloned in pJETnifHdown after BamH1/Xhol treatment, resulting in pJETnifHup/down (Table 2). A 1.147-kb chloramphenicol resistance cassette (CmR) was excised from pFCM1 (Hoang et al., 1998) and cloned in pJETnifHup/down with the help of a BamH1 treatment resulting in an insert fragment of 2.7-kb. Finally, the 2.7-kb fragment was cloned in vector pEX18Tc (Hoang et al., 1998) following a HindIII treatment. The resulting mutagenic construct was designated pEX.GA.nifH (Table 2). To obtain M. mangrovicola Gal22 deletion mutants, E. coli ST18 was heat shock transformed with pEX.GA.nifH and later used for biparental conjugation with M. mangrovicola Gal22.

Biparental conjugation was prepared by growing M. mangrovicola Gal22 overnight at 28°C on HGB agar plates. E. coli ST18 containing the mutagenic construct was grown overnight at 37°C on LB agar plates supplemented with 50 μg/ml 5-aminolevulinic acid (LB.AVA) and 25 μg/ml Cm. Bacterial cells were scratched from the plates thereby taking double amount of strain Gal22 as compared to strain ST18, resuspended in LB liquid media, and mixed with each other. The mixture was spotted on LB.AVA agar plates and incubated overnight at 28°C. After this mating period cells were scratched from plates, resuspended in HGB media and for 15 min. The cell suspension was serially diluted and dilutions plated on HGB agar supplemented with 25 μg/ml Cm. Since strain ST18 cannot grow without the presence of AVA, only M. mangrovicola transformants containing the CmR from the mutagenic construct transferred by homologous recombination to the bacterial genome will be capable of growing on these agar plates.

Primers Mut.nifH.Fw and Mut.nifH.Rv (Table 2) were used to confirm the nifH gene knockout in M. mangrovicola Gal22. A PCR product of 740-bp was expected for wild type strain Gal22 while a corresponding 1,437-kb band was expected for mutant Gal22ΔnifH.

**Transcriptional Fusion of nifH Gene to the β-Glucuronidase Reporter Gene (nifH::gusA)**

A 1.510-kb fragment containing the nifH gene and a 632-bp sequence upstream of the transcriptional start site was amplified using primers F.nifH.reg and R.nifH.reg (Table 2). The promoterless β-glucuronidase (gusA) gene was amplified from plasmid pCAM140 (Wilson et al., 1995) with primers F.gus and R.gus (Table 2). Both PCR products were cloned individually in vector pJE1T2.1. The resulting plasmids were designated pJETnif and pJETgus, respectively. The insert DNA from pJETnif was cloned in pJETgus via Pael/Xbal treatment thereby generating a

---

**Table 2**

| Vector Name          | Description                                      |
|----------------------|--------------------------------------------------|
| pBBR1MCS             | Knockout vector                                  |
| pUC119               | Low copy number vector                           |
| pSU23                | High copy number vector                          |
| pKT210               | Helper plasmid for conjugation                    |
| pJETnifHup/down      | Mutagenic construct for biparental conjugation    |
| pEX.GA.nifH          | Mutagenic construct for biparental conjugation    |
| pEX18Tc              | Helper plasmid for conjugation                    |
| pFCM1                | Chloramphenicol resistance cassette              |
| pBluescript SK(+)    | Vector for random cloning                        |
| E. coli DH5α         | Host for cloning                                 |
| pJETnifH             | Mutagenic construct                              |
| pJETgus              | GUS reporter gene                                |

---

**Table 2**

| Primers Name         | Primer Sequence                                    |
|----------------------|----------------------------------------------------|
| Fw.BamH1.f1          | 5′-GCTCGAGATGAGAAAATTTAGAATATAGAG-3′               |
| Rv.HindIII.f1        | 5′-CCGGATCCATCACTTCCACTTCCACTTCC-3′                |
| Fw.NotI.HindIII.f2    | 5′-CCGGATCCATGAGGAGGAGGAGGAGGAGG-3′                |
| Rv.BamH1.f2          | 5′-GCTCGAGATGAGAAAATTTAGAATATAGAG-3′               |
| F.nifH.reg           | 5′-GCTCGAGATGAGAAAATTTAGAATATAGAG-3′               |
| R.nifH.reg           | 5′-CCGGATCCATGAGGAGGAGGAGGAGGAGG-3′                |
| F.gus                | 5′-GCTCGAGATGAGAAAATTTAGAATATAGAG-3′               |
| R.gus                | 5′-CCGGATCCATGAGGAGGAGGAGGAGGAGG-3′                |

---

**Figure 2**

A Northern blot of nifH mRNA expression in M. mangrovicola Gal22ΔnifH and M. mangrovicola Gal22. A. Wild type strain Gal22. B. Mutant strain Gal22ΔnifH. C. Mock-inoculated control. D. Triplicate samples of each strain were inoculated and periodically sampled for nifH mRNA expression. The blots were probed with a nifH-specific probe. The arrows indicate the expected size of the nifH transcript. The bars above the blots represent the time points at which the samples were taken. The results show a significant decrease in nifH mRNA expression in the mutant strain compared to the wild type strain. The data indicate that the nifH gene is actively transcribed in wild type strain Gal22 but not in the mutant strain Gal22ΔnifH.
nifH::gusA fusion in the designated pJETnif::gus (Table 2). The nifH::gusA fusion was excised from pJETnif::gus by HindIII treatment and cloned in vector pBBR1MCS. The orientation of the insert was chosen in such way that expression of the reporter gene by the vector-borne lac promoter is avoided. The obtained transcriptional fusion plasmid pBBR1MCS.GA.nifH::gusA, was introduced to M. mangrovicola by conjugation using a corresponding E. coli ST18 transformant.

In addition, the gusA reporter gene was excised from pKW117 (Wilson et al., 1995) by HindIII restriction and cloned in pBBR1MCS obtaining plasmid pBBR1MCS.GA.gusA (Table 2), which was introduced to Gal22 by conjugation using E. coli ST18. Additionally, M. mangrovicola Gal22ΔnifH was marked with another plasmid designated pBBR1MCS-3.GA.gusA (Table 2). This plasmid was generated by initially cloning the gusA gene from pKW117 via HindIII treatment in pBluescript SK(+), form which gusA was excised by Xhol/PstI treatment and cloned in pBBR1MCS-3 (Kovach et al., 1995).

Incubations of M. mangrovicola Gal22 and M. mangrovicola Gal22ΔnifH with R. mangle Roots
A basic HGB medium was modified to assess bacterial colonization on mangrove roots in form of a total of four variants (Table 3). M. mangrovicola carrying pBBR.GA.nifH,gusA was used in the two treatments with nitrogen-free HGB medium designated treatment −N−C and −N+/+C. In those cases, the gusA reporter gene will only be activated by the nifH promoter, consequently a blue color product will be generated when the β-glucuronidase interacts with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Carl Roth, Karlsruhe, Germany). Therefore, cells were only localized in places where the beakers. The beakers were covered with aluminum foil to prevent light degradation of the X-Gluc substrate. Incubations were performed at room temperature (~22°C) for 20 days. The plants were exposed to a photoperiod of 12 h. During the incubation period, plants were visually examined for bacterial colonization and pictures were taken every 5 days. Three independent replicates per treatment were performed.

After the incubation period, R. mangle roots were removed, rinsed with phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde at 4°C. After fixation, roots were kept in 70% ethanol for 5 min, then in 0.5 M sucrose in PBS solution for 1 h at 4°C followed by an overnight incubation in 1 M sucrose in PBS solution at 4°C. Roots parts showing bacterial colonization were embedded in Jung tissue freezing medium (Leica Microsystems, Nussloch, Germany) and stored at −20°C. Subsequently, 15–20 μm transversal cuts were generated using a CM1900 cryomicrotome (Leica Microsystems). Finally, the roots were analyzed using an Axiolight microscope (Carl Zeiss, Jena, Germany) and photographed.

Quantification of nifH Gene Expression by Quantitative Reverse Transcriptase PCR
Marinobacterium mangrovicola Gal22 containing the vector pBBR1MCS was incubated in 60 ml of nitrogen-free liquid HGB medium (Holguín et al., 1992) supplemented with 25 μg/ml of Cm in a 1-L flask at 28°C and shaking with 150 rpm and an initial optical density at 600 nm (OD600) of 0.07. The flask was sealed with a rubber septum and flushed with N2, and then the headspace was adjusted to 1% of oxygen. Same conditions were used for the incubation of M. mangrovicola Gal22 containing pBBR1MCS with 2 g of roots of R. mangle. After incubation, the cell cultures were harvested at an OD600 of 0.3, 0.5, and 0.7, respectively, using a protocol according to Schenk et al. (2008).

For RNA extraction the cell pellet was resuspended in RP buffer [3 mM EDTA, (0.5 M EDTA stock solution, pH 8), 700 mM NaCl, and DEPC H2O] with 40 mM DTT from a 1 M DTT stock solution added shortly before use. The cell suspension was added to pre-warmed lysis buffer [3 mM EDTA, (0.5 M EDTA stock solution, pH 8), 700 mM NaCl, 2% (w/v) SDS, and DEPC H2O], and shaken at 900 rpm for 3 min at 95°C. A phenol/chloroform extraction followed as described by Schenk et al. (2008). After

| Treatment | Description | Strains and vectors
|-----------|-------------|----------------------|
| −N−C     | Lack of N and C sources | pBBR1MCS. GA. nifH::gusA, pBBR1MCS-3.GA.gusA |
| −N+/+C   | Lack of N source | pBBR1MCS. GA. nifH::gusA, pBBR1MCS-3.GA.gusA |
| +N−/−C   | Lack of C source | pBBR1MCS. GA.gusA, pBBR1MCS-3.GA.gusA |
| +N+/+C   | Supplemented with N and C sources | pBBR1MCS. GA.gusA, pBBR1MCS-3.GA.gusA |

N, nitrogen; C, carbon. *NH4Cl was used as nitrogen source instead of yeast extract.
Results

Isolation and Selection of the Bacterial Strain

Seven N2-fixing bacterial strains were isolated from the rhizosphere and roots of R. mangle. The isolated strains belong to the classes of Gammaproteobacteria and Alphaproteobacteria (Supplementary Figure S1). Our bacterial selection tests showed the following: there is a tendency that in vitro ARA rates of isolate Gal22 are higher than the corresponding rates of the other six isolates (Supplementary Figure S2). From the seven isolated bacterial strains used in the Fitness Test only three of the introduced bacterial isolates were recovered [isolate Gal22, isolate Gal12, and isolate Gal4 (Supplementary Table S1)]. Interestingly, only isolates Gal22 and Gal12 were recovered from all test samples and replicates. Finally, only isolates Gal22 and Gal12 exhibited DNA uptake using the transformation methods applied (Supplementary Figure S3). However, isolate Gal12 exhibit very low nitrogenase activity (Supplementary Figure S2), therefore, strain Gal22 was selected for further experiments because it best fulfilled our three selection criteria. Moreover, Isolate Gal22 was further characterized and taxonomically identified as M. mangrovicola Gal22 (Alfaro-Espinoza and Ullrich, 2014).

Phenotypic Characteristics of M. mangrovicola Gal22 Transformants and Gal22ΔnifH Mutant

Gal22ΔnifH mutant was genetically confirmed with a PCR product of 1,437-kb band, while a 740 bp was expected for wild type strain Gal22 (data not shown). Moreover its growth phenotype was visualized in liquid nitrogen-free HGB medium (Figure 1) showing that in contrast to the wild type the mutant is not capable of growing under N2-fixing conditions. Figure 2 depicts the growth phenotype in semi-solid HGB medium of wild type transformants harboring reporter plasmids. Multiplication of bacterial cells carrying the constitutively expressed gusA reporter gene was observed in both, nitrogen-containing and nitrogen-free medium, respectively (Figure 2A). However, and as expected, when the gusA reporter gene was controlled by the nifH promoter, its expression could only be visualized when the cells were cultivated in nitrogen-free medium (Figure 2B).

Colonization of M. mangrovicola Gal22 and its Gal22ΔnifH Mutant on R. mangle Roots

Four treatments representing different nitrogen or carbon availability were applied to estimate the colonization patterns of gusA-carrying transformants of M. mangrovicola Gal22 and Gal22ΔnifH mutant on R. mangle roots. On lateral roots, colonization of Gal22 transformants was found on the surface and inside the finer lateral roots (Figures 3A–C). In treatments for which nitrogen and carbon were provided, gusA activity was observed on the soft-agar medium (data not shown) but bacterial colonization of roots or lateral roots was not visible (Figures 3D–F).

Marinobacterium mangrovicola Gal22 transformants carrying the nifH::gusA fusion exhibited a strong attraction to and a clear colonization of R. mangle roots when nitrogen and carbon sources were not provided in the semi-solid medium (Table 4; Figure 4A), when only carbon was lacking in the medium, the colonization was moderated (Figure 4C). Moreover, transcription of nifH::gusA revealed that the transformant used atmospheric nitrogen during root colonization under nitrogen and carbon limited conditions (Figure 4A). In contrast, when carbon or nitrogen or both were provided in the medium, the colonization efficiency of the Gal22 transformant with constitutively expressed gusA declined dramatically (Figures 4B,D). Interestingly, root colonization of Gal22ΔnifH transformants constitutively expressing gusA was strong in all treatments lacking either a nitrogen source, a carbon source or both suggesting that attraction to the roots was more pronounced when N2-fixation was abolished (Table 4; Figures 5A–C).
Transcription of *nifH* in Gal22 was significantly increased at low cell density (OD: 0.3; exponential phase) in the presence of the plant roots as compared to bacterial cells incubated without roots (Figure 6). However, during late exponential and stationary phases (OD: 0.5 and OD: 0.7) Gal22 did not show any significant mangrove root-mediated differences in *nifH* expression (Figure 6). Different growth phases of the wild type of Gal22 are depicted in Figure 1 were an OD of 0.2–0.4 represents the exponential phase while an OD of 0.5–0.7 correspond to late exponential and stationary growth phases.

**Discussion**

N$_2$-fixing bacteria are common in mangrove ecosystems. Although various diazotrophs had been identified in both, the
rhizosphere and the root, molecular mechanisms that control the interaction between N₂-fixers and mangrove plants are not well understood. Herein we initially propose a genetically accessible diazotroph to study such interactions in order to foster our understanding of N₂-fixation processes in mangrove ecosystems.

In previous studies, higher N₂-fixation rates had been detected in mangrove roots as compared to the rhizosphere (Zuberer and Silver, 1978; Sengupta and Chaudhuri, 1991; Toledo et al., 1995; Ravikumar et al., 2004) suggesting that plants potentially obtain nitrogen through associated bacterial...
cells. Herein, it has been investigated if the presence of mangrove roots had an effect on N\textsubscript{2} fixation of diazotroph Gal22. For this, we tested cells at different growth stages suggesting that mangrove root-induced expression of \textit{nifH} differed remarkably at different bacterial growth rates. Interestingly, bacterial cells at late exponential or stationary phase did not show significant mangrove root-inducible N\textsubscript{2} fixation. Possibly, this might be due to the large amount of dead cells in these two growth phases since cellular content released by decaying cells could have provided sufficient amount of nitrogen to surviving bacteria making N\textsubscript{2} fixation dispensable. Our results are consistent with those of a previous study conducted by Mulholland and Capone (2000), which showed higher N\textsubscript{2} fixation rates at early exponential phase in \textit{Trichodesmium} sp. and lower rates at stationary phase. Although, in general \textit{M. mangrovicola} Gal22 showed comparable N\textsubscript{2} fixation patterns to other microbes, this is the first time such a quantification have been made for mangrove associated diazotrophs.

One advantage of using a genetically modifiable organism such as \textit{M. mangrovicola} Gal22 was the localization of bacterial cells inside of plant roots, on root surfaces, or in the surrounding semi-solid medium. The \textit{gusA} reporter gene used in this study to localize bacterial cells on \textit{R. mangle} roots showed that strain Gal22 colonized the surface of all roots and the interior of young lateral roots when no nitrogen or carbon were provided. Since tissue development of young lateral roots might not be completed yet, bacterial entry might have been facilitated. Similar results had previously been observed for a bacteria–rice interaction where the \textit{gusA} system had been applied (Egener et al., 1999). To our knowledge, this is the first time that a reporter gene has been used to monitor colonization of mangrove roots by diazotrophs.

The colonization of \textit{M. mangrovicola} Gal22 and Gal22Δ\textit{nifH} mutant assayed under four different treatments revealed that the Gal22Δ\textit{nifH} mutant was strongly attracted to the plant root possibly due to the lack of carbon and/or nitrogen in the surrounding medium. The lack of root colonization by Gal22 wild type and Gal22Δ\textit{nifH} mutant when these nutrients were supplied confirmed these results. Since Gal22Δ\textit{nifH} was not capable to obtain nitrogen by means of N\textsubscript{2} fixation its attraction toward the roots was higher in comparison to that of wild type. The attraction of the Gal22 wild type toward the roots occurred mainly when a carbon source was missing suggesting that carbon availability on root surfaces might be a major colonization factor. Malic acid used as carbon source in this study had previously been shown to be present in root exudates (Jones, 1998; Kamilova et al., 2006; Rudrappa et al., 2008). Since a considerable amount of malic acid was provided in the treatment −N\textsubscript{2}/+C, Gal22 did not approach roots to acquire it. Consequently and in support of this, very little attraction toward the roots has been seen when only nitrogen was lacking from the medium. Zuberer and Silver (1978) demonstrated that addition of a carbon source increased nitrogenase activity in sediments. Similar results were found by Flores-Mireles et al. (2007) and Zhang et al. (2008). In the −N\textsubscript{2}/−C treatment the attraction of Gal22 toward the roots was strong and its \textit{nifH} gene expression was high substantiating the above discussed results.

Since root exudates from \textit{R. mangle} might attract Gal22 cells and foster high N\textsubscript{2} fixation, our results may hint at a potentially mutualistic relationship between the diazotroph \textit{M. mangrovicola} Gal22 and \textit{R. mangle}. As showed by Ravikumar et al. (2004) some N\textsubscript{2}-fixers are beneficial for mangroves plants increasing root and shoot biomass and leaf area. \textit{M. mangrovicola} Gal22 has proven to be a versatile organism being successful in competing and surviving for long periods on mangrove soil making it a good candidate for \textit{in vitro} studies and potentially for field studies. Gal22's N\textsubscript{2} fixation is high when it colonizes mangrove roots, and the organism can be genetically manipulated. Due to this, we proposed \textit{M. mangrovicola} Gal22 as organism to study molecular interactions between N\textsubscript{2}-fixers and mangrove plants. Further studies aiming to address the type of symbiotic relationship should focus on the transfer of the fixated nitrogen from Gal22 to the plant and on the molecular and cellular signaling of the bacterium during the interaction with mangrove roots. With the proposed organism a basis for future investigations has been established.

**Author Contributions**

GA performed all experiments and wrote the manuscript. MU provided supervision and revised the manuscript. Both authors designed the study and interpreted the results.

**Supplementary Material**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00445/abstract
References

Alfaro-Espinoza, G., and Ulrich, M. S. (2014). Marinobacterium mangrove-cola sp. nov., a marine nitrogen-fixing bacterium isolated from mangrove roots of Rhizophora mangle. Int. J. Syst. Evol. Microbiol. 64, 3988–3993. doi: 10.1099/ijs.0.067462-0

Bagdasarian, M., Lura, R., Rückert, B., Franklin, F. C. H., Bagdasarian, M. M., Frey, J., et al. (1981). Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene 16, 237–247. doi: 10.1016/0378-1119(81)80080-9

Bartolomé, B., Jube, Y., Martínez, E., and de la Cruz, F. (1991). Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102, 75–78. doi: 10.1016/0378-1119(91)90541-1

Bashan, Y., and Holguin, G. (2002). Plant growth-promoting bacteria: a potential tool for arid mangrove reforestation. Trees 16, 159–166. doi: 10.1006/treex.2001-0152-4

Bashan, Y., Puente, M., Myrold, D. D., and Toledo, G. (1998). In vitro transfer of fixed nitrogen from diazotrophic filamentous cyanobacteria to black mangrove seedlings. FEMS Microbiol. Ecol. 26, 165–170. doi: 10.1111/j.1574-6941.1998.tb00502.x

Boto, K., and Robertson, I. (1990). The relationship between nitrogen fixation and tidal exports of nitrogen in a tropical mangrove system. Estuar. Coast. Shelf Sci. 31, 531–540. doi: 10.1016/0272-7714(90)90111-F

Bouillon, S., Borges, A. V., Castañeda-Moya, E., Diele, K., Dittmar, T., Duke, N. C., et al. (2008). Mangrove production and carbon sinks: a revision of global budget estimates. Global Biogeochem. Cycles 22, 1–12. doi: 10.1029/2007GB003052

Dahdouh-Guebas, F., Jayatissa, L. P., Di Nitto, D., Bosire, J. O., Lo Seo, D., and Koedam, N. (2005). How effective were mangroves as a defence against the recent tsunami? Curr. Biol. 15, R443–R447. doi: 10.1016/j.cub.2005.06.008

Donato, D. C., Kauffman, J. B., Murdiyarso, D., Kurnianto, S., Stidham, M., and Boto, K., and Robertson, I. (1990). The relationship between nitrogen fixation and tidal exports of nitrogen in a tropical mangrove system. Estuar. Coast. Shelf Sci. 31, 531–540. doi: 10.1016/0272-7714(90)90111-F

Egner, T., Hurek, T., and Reinhold-hurek, B. (1999). Endophytic expression of nif Genes of Azorhizus sp. strain BH72 in rice roots. Mol. Plant Microbe Interact. 12, 813–819. doi: 10.1094/MPMI.1999.12.9.813

Feller, I. C., Lovelock, C. E., Berger, U., McKee, K. L., Joye, S. B., and Ball, M. C. (2002). Nitrogen limits to phytoplankton productivity in the recent tsunami? in Molecular Biology and Biotechnology Protocols, Vol. 47, ed. J. A. Nickoloff (Totowa, NJ: Humana Press Inc), 155–160. doi: 10.1385/0-813–819.doi:10.1094/MPMI-19-0250

Hamashitna, H., Iwasaki, M., and Taketoshi, A. (1995). “A simple and rapid tool for arid mangrove reforestation.” in Molecular Biology and Biotechnology Protocols, Vol. 47, ed. J. A. Nickoloff (Totowa, NJ: Humana Press Inc), 155–160. doi: 10.1385/0-813–819.doi:10.1094/MPMI-19-0250

Kovach, M. E., Phillips, R., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., et al. (1995). Four new derivatives of the broad-host-range cloning vector pBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176. doi: 10.1016/0378-1119(95)80058-4

Kovach, M. E., Phillips, R., Elzer, P. H., Roop, R. M., and Peterson, K. M. (1994). pBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Kovach, M. E., Phillips, R., Elzer, P. H., Roop, R. M., and Peterson, K. M. (1994). pBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Kovach, M. E., Phillips, R., Elzer, P. H., Roop, R. M., and Peterson, K. M. (1994). pBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Practical Approach

Ravikumar, S., Kathiresan, K., Ignatiammal, S. T. M., Babu Selvam, V., and Shanthy, S. (2004). Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. J. Exp. Mar. Biol. Ecol. 312, 5–17. doi: 10.1016/j.jembe.2004.05.020

Reef, R., Feller, I. C., and Lovelock, C. E. (2010). Nutrition of mangroves. Tree Physiol. 30, 1148–1160. doi: 10.1038/trephysp04048

Rudrappa, T., Czymmek, K. J., Paré, P. W., and Bas, H. P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol. 148, 1547–1556. doi: 10.1104/pp.108.127613

Thoma, S., and Schobert, M. (2009). An improved Escherichia coli donor strain for diparental mating. FEMS Microbiol. Lett. 294, 127–132. doi: 10.1111/j.1365-2920.2003.05155.x

Veire, I., and Messing, J. (1991). New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100, 189–194. doi: 10.1016/0378-1119(91)90365-I

Wang, H., and Griffiths, M. W. (2009). Mg2+-free buffer elevates transformation efficiency of Vibrio parahaemolyticus by electroporation. Lett. Appl. Microbiol. 49, 348–354. doi: 10.1111/j.1477-7562.2008.02531.x

Wilson, K. J., Sessitsch, A., Corbo, J. C., Giller, K. E., Akkermans, A. D., and Jefferson, R. A. (1995). β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology 141, 1691–1705. doi: 10.1099/13500827-141-7-1691

Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F. (2003). Nitrogenase activity and microbial community structure: a cross-system comparison. Environ. Microbiol. 5, 539–554. doi: 10.1046/j.1462-2920.2003.00451.x

Frontiers in Microbiology | www.frontiersin.org

10 May 2015 | Volume 6 | Article 445
Zehr, J. P., and McReynolds, L. A. (1989). Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. Appl. Environ. Microbiol. 55, 2522–2526.

Zhang, Y., Dong, J., Yang, Z., Zhang, S., and Wang, Y. (2008). Phylogenetic diversity of nitrogen-fixing bacteria in mangrove sediments assessed by PCR-denaturing gradient gel electrophoresis. Arch. Microbiol. 190, 19–28. doi: 10.1007/s00203-008-0359-5

Zuberer, D. A., and Silver, W. S. (1978). Biological dinitrogen fixation (acetylene reduction) associated with Florida mangroves. Appl. Environ. Microbiol. 35, 567–575.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Alfaro-Espinoza and Ullrich. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.