The moderately halophilic bacterium *Halomonas maura* is a free-living diazotroph

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Abstract

*Halomonas maura* is a moderately halophilic bacterium which lives in saline soils and synthesises an exopolysaccharide known as mauran. Strain S-31 T grew in a nitrogen-free medium under an N₂ atmosphere; the acetylene reduction assay proved positive under specific conditions. We identified the *nifH* gene in this strain by using degenerate oligonucleotides designed from highly preserved gene sequences obtained from the alignment of a large number of *nifH* sequences from different microorganisms. Our results lead us to conclude that *H. maura* is capable of fixing nitrogen under microaerobic conditions.

Keywords: Halophilic bacteria; *Halomonas maura*; Nitrogen fixation; Diazotroph

1. Introduction

Biological nitrogen (N₂) fixation, a process that reduces atmospheric N₂ gas to biologically available ammonium, is ecologically important as an input of fixed nitrogen (N) into many terrestrial and aquatic habitats [1]. Both Archaea and Bacteria are capable of nitrogen fixation [2].

Microorganisms catalyze nitrogen fixation via the enzyme nitrogenase, which has been highly preserved throughout evolution. The nitrogenase complex is composed of two protein components, dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). All N₂ fixers carry the *nif* (nitrogen fixation) genes, which encode the nitrogenase complex. These *nif* genes are repressed in the presence of combined nitrogen in a form such as ammonia, the immediate product of the nitrogenase reaction, and/or oxygen, which oxidises the iron in the Fe protein and thus rapidly and irreversibly inactivates the nitrogenase proteins [3]. The *nif* operon contains, among other genes, the nitrogenase structural gene *nifH*, the cloning and sequencing of which has provided us with a large, rapidly expanding database of sequences from diverse terrestrial and aquatic environments [4].

Some thirty *Halomonas* species, belonging to the γ-proteobacteria, have been described to date [5]. They are capable of growing in salt concentrations of between 1% and 20% w/v and appear to be the commonest moderately halophilic inhabitants of saline environments [6], having been isolated from saline soils and water all over the world.
Halomonas maura is a Gram-negative rod, first isolated from soil samples taken from a solar saltern at Asilah in Morocco [7], and has since been commonly found in saline soils, especially around the rhizosphere of xerophytic plants [8]. It has a chemo-organotrophic respiratory metabolism but can respire anaerobically by using nitrate as alternative terminal electron acceptor [7]. It has a versatile carbon and nitrogen metabolism, which favors its success in the competitive environment of the rhizosphere. It can make use of ammonium, nitrate, nitrite and amino acids as nitrogen sources. H. maura also synthesizes considerable quantities of exopolysaccharide [9], a characteristic common to nitrogen-fixing microorganisms such as Rhizobium, which loses this capacity if it does not synthesise EPS [10]. Preliminary research revealed that strain S-31T of H. maura possesses a cbb3-type cytochrome oxidase (Argandoña, personal communication) present in numerous nitrogen-fixing microorganisms [11,12].

All the above considerations led us to suspect that H. maura might act as a diazotroph. Thus we report here on the nitrogen-fixing capacity of this moderately halophilic bacterium, which we have ascertained via the classical acetylene reduction assay and also at the molecular level by showing the presence of nif genes.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Halomonas maura S-31T [7] was grown at 32 °C in MY medium [13] modified with a balanced mixture of sea salts to a final concentration of 7.5% w/v [14]. Genomic DNA was extracted from strain S-31T in MM minimal medium (0.4 g yeast extract, 2 g peptone No. 3 per litre) supplemented with the same mixture of sea salts (except for MgSO4) to a final concentration of 2% w/v [14] and 14.26 g/l of sodium thioglycolate sterilized by filtration. We designed this medium for standard use in experiments in which we need to minimize the production of exopolysaccharides in Halomonas strains. Megaplasmids were extracted by the method described elsewhere [15]. Nitrogen fixation assays were performed in semi-solid Burk’s N-free medium modified to the appropriate saline concentration of 7.5% w/v (0.64 g K2HPO4, 0.16 g KH2PO4, 58.5 g NaCl, 14.73 g MgSO4·7H2O, 0.4 g CaSO4·2H2O, 8 g glucose, 0.001 g Na2MoO4·2H2O, 0.003 g FeSO4 per litre) (BHM) and supplemented with agarose 0.2% w/v. Azospirillum brasilense Sp7T [16] was cultured at 32 °C in TP medium (4 g peptone, 0.5 g yeast extract, 0.5 g tryptone, 0.2 g MgSO4·7H2O, 0.2 g CaCl2·2H2O per litre). Escherichia coli XL1 Blue was grown at 37 °C in LB medium containing tetracycline (10 μg/ml). The pH values of the media were adjusted to 7.2 with NaOH 1 M. Solid media contained an additional 20 g/l of Bacto-agar (Difco).

2.2. Nitrogen fixation determination

The nitrogenase activity of H. maura strain S-31T was calculated by the acetylene (C2H2) reduction assay (ARA) [17]. A 5-ml saturated culture of strain S-31T (O.D600 ~ 2) grown aerobically (120 rpm) overnight at 32 °C in MY broth medium with 7.5% w/v NaCl was diluted to 1:100 in the same medium. The culture was subsequently grown under the same conditions to the exponential phase (optical density of approximately 0.8 at 590 nm). Aliquots of 100, 200 and 500 μl were then transferred into 18-ml tubes containing 10 ml of modified semisolid Burk’s N-free medium plus 7.5% w/v NaCl and sealed with silicone. The tubes were incubated at 32 °C for 8, 12, 15, 19, 21 and 23 h without agitation. Five tubes were used to test each condition. Once each incubation time was reached, approximately 10% of the remaining air in the tubes (8 ml) was replaced with 1 ml of acetylene and they were then incubated for 3 h at 32 °C. The ethylene (C2H4) concentrations in 1 ml headspace samples were determined using a gas chromatograph (Perkin-Elmer 8420, Beaconsfield, Bucks, UK) equipped with a H2-FID detector and Poropak-T (800–100 mesh) column. In the experiments to calculate basal ethylene levels, tubes containing cultures with no added acetylene and others containing uncultured medium injected with acetylene were used as negative controls. Viable cell numbers in H. maura S-31T colony-forming units (cfu) were estimated by standard plate counts in MY with 2% w/v NaCl. Acetylene reduction activity was expressed as nmol C2H4 (107 cfu)−1 h−1.

2.3. Southern blot analysis

Genomic DNA of H. maura S-31T was completely digested with EcoRI, BamHI, and SalI. DNA fragments were separated on 0.7% agarose gel and transferred onto a nylon filter by standard techniques. Fragments containing the nifH gene were located with a digoxigenin-labelled nifH gene probe using a digoxigenin DNA labelling and detection kit (Boehringer, Mannheim, Germany) according to the manufacturer’s instructions. A 400 bp internal fragment from the nifH gene, to be used as a probe, was amplified from the genome of H. maura S-31T chromosomal DNA using the following degenerated oligonucleotides: nif-F (5’-TAC-GGKAAGGGGBGKWATCGG-3’) and nif-R (5’-GATYTCCTGRGCYTTGT-3’), which were designed in our laboratory on the basis of preserved nifH sequences obtained from the alignment of these genes in different nitrogen-fixing bacteria. PCR was performed by using 30 cycles of 30 s at 98 °C, 30 s at 50 °C and 50 s at 72 °C.
DNA from the megaplasmids of *H. maura* 31<sup>T</sup> was separated on 0.7% w/v agarose gel as described by Argandoña et al. [15] and transferred to a nylon filter by standard techniques. The *nifH* gene was located using the digoxigenin DNA labelling kit referred to above. In this case, a 330 bp internal fragment from the *nifH* gene from *H. maura* S-31<sup>T</sup>, obtained as described below, was used as a probe.

### 2.4. Gene cloning and sequencing

The conjectured *nifH* gene was amplified from the genomic DNA of *H. maura* S-31<sup>T</sup> by nested PCR reactions. We used the degenerate primers Pol-F and Pol-R [18] and the degenerate oligonucleotides, *nif*-F and *nif*-R mentioned above. We carried out an initial PCR reaction using the couple of primers located farthest apart in the chromosome: *nif*-F and PolR. The 420 bp PCR product obtained was then used as a second template to amplify an internal fragment of 330 bp, for which we used the primers Pol-F and *nif*-R. PCR entailed 30 cycles of 30 s at 98 °C, 30 s at 59 °C (first PCR: *nif*-F and PolR primers) and 51 °C (second PCR: Pol-F and *nif*-R primers) and 50 s at 72 °C.

The 330 bp DNA fragment was extracted from a 1.5% w/v agarose gel with a GFX<sup>TM</sup> PCR DNA and gel band purification Kit (Amersham), then cloned into pGEM-T plasmid (Promega) and transformed into *E. coli* strain XL1-Blue. Clones were selected in LB agar containing 4 µg/ml of isopropyl β-D-thiogalactopyranoside (IPTG), 40 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal), 10 µg/ml of tetracycline and 100 µg/ml of ampicillin.

DNA sequences were determined by the dideoxynucleotide-chain-termination method of Sanger et al. [19].

### 2.5. Nucleotide sequence Accession Number

The nucleotide sequence reported in this paper has been assigned Accession No. AY827547 by the EMBL data library.

### 3. Results and discussion

*Halomonas maura* is to be found very commonly in saline soils [8], often adhering to the roots of halophytic plants such as *Salicornia* sp. Strain S-31<sup>T</sup> of *H. maura* has a chemo-organotrophic metabolism and is capable of anaerobic respiration in the presence of nitrate, which it uses as final electron acceptor. This bacterium has recently been found to contain a *cbb<sub>3</sub>*-type cytochrome oxidase similar to that which we have found in *H. maura*, such as *A. brasilense* Sp7<sup>T</sup>, *Rhizobium leguminosarum*, *Azorhizobium caulinodans*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*, are capable of fixing atmospheric nitrogen under microaerobic conditions [11,12,20].

The initial test to be made to ascertain whether a microorganism is capable of fixing nitrogen is to see whether it can live in a nitrogen-deficient medium. Thus we first satisfied ourselves that strain S-31<sup>T</sup> of *H. maura* was capable of growing in such a medium in a zone close to the substrate surface (Fig. 1) where the oxygen pressure was suitable both for its growth and for the activation of the genes responsible for nitrogenase synthesis, in the same way that *Azospirillum*, another free-living, aerobic, diazotrophic bacterium does [23]. We also found that *H. maura* did not grow in a stirred nitrogen-free liquid (aerobiosis), which indicates that oxygen inhibits the process and thus it only occurs under microaerobic conditions.

Our next aim was to confirm via the acetylene reduction activity test that our strain fixed nitrogen under microaerobic conditions. We carried out various preliminary qualitative assays under different conditions, altering the glucose concentration (0.5% and 1% w/v), the quantity of inoculate (100, 200 and 500 µl) and the incubation times before adding the acetylene to the test tubes (8, 12, 15, 19, 21 and 23 h), using five tubes for each incubation time. Although growth always occurred, the conditions under which ethylene was detected...
corresponded to the assay in which 500 µl of inoculate and 1% w/v of glucose was added to the nitrogen-free medium adjusted to 7.5% w/v salts. The total quantity of ethylene produced versus incubation time under these conditions, measured as described in Section 2, is shown by the graph in Fig. 2, in which it can be seen that nitrogen fixation reached a peak between 19 and 23 h incubation time, which coincides with the end of the exponential growth phase, and that after this it was inhibited, possibly due to the quantity of nitrogen already fixed. Finally, to determine nitrogenase activity under these conditions we repeated the assay at 21 h incubation time, this being the time of maximum ethylene production in the earlier assay. The result was one of $0.47 \pm 0.12 \text{nmol} \ C_2\text{H}_4 \ (10^7 \text{cfu})^{-1} \text{h}^{-1}$, corresponding to the mean of 15 test tubes, each measured twice chromatographically.

Lastly, to provide further proof that *H. maura* S-31T is a diazotroph we looked for *nif* genes in its genome using PCR reactions with some degenerate oligonucleotides described elsewhere [18] and others designed in our own laboratory. Following the methods described in Section 2 we amplified a 330 bp DNA fragment corresponding to the *nifH* gene in *H. maura* S-31T. A phylogenetic tree based on a comparison of this partial *nifH* nucleotide sequence of *H. maura* and those from other nitrogen-fixing bacteria is shown in Fig. 3. As can be seen in this figure, the partial sequence of the *nifH* gene

![Fig. 2. Ethylene levels produced by the strain S-31T of *Halomonas maura* grown in tubes containing 10 ml of nitrogen-free medium adjusted to 7.5% w/v salts versus incubation time.](image)

**Fig. 2.** Ethylene levels produced by the strain S-31T of *Halomonas maura* grown in tubes containing 10 ml of nitrogen-free medium adjusted to 7.5% w/v salts versus incubation time.

![Fig. 3. Phylogenetic relationship between the *nifH* partial gene sequence of *Halomonas maura* and other partial *nifH* gene sequences of nitrogen-fixing bacteria obtained from the GenBank database (Accession Numbers are indicated next to the bacterial names). Locations of the *nifH* fragments used for the analysis correspond to a sequence fragment of $\approx 250 \text{bp}$ at positions 214–476 (with reference to the *A. vinelandii* *nifH* coding sequence [M20568]). The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 40% are shown (1000 replications). Bar, 5% estimated sequence divergence.](image)
shows considerable homology with the sequences of the same gene in *A. brasilense* and *A. lipoferum* and with *Bacillus megaterium* in spite of the fact that all three bacteria are phylogenetically quite distant from *H. maura* according to their 16S rRNA analysis.

Nitrogenase and the ability to fix N\textsubscript{2} are present in a wide variety of bacteria and some methanogenic archaea [2], and in fact new diazotrophic microorganisms, such as the acid-tolerant *Clostridium acidisoli* [22], continue to be found. Nevertheless, to date no moderately halophilic microorganism capable of fixing nitrogen has been described.

Interestingly there are several features which coincide between our bacterium and other diazotrophic microorganisms that may be significant: all *H. maura* strains possess plasmids ranging from 70 to 600 kb [15] and in some other soil bacteria, megaplasmids are known to carry information essential for plant interaction, such as the *Agrobacterium* virulence (vir) genes and those for nodulation (nod) and host-specific nodulation (hsp) in *Rhizobium* [23]. Nevertheless, we are unsure at the moment whether these plasmids in *H. maura* do play a role in the interaction between the bacterium and plants and or have some other function. In fact, by Southern analysis, we have observed that *nifH* in *H. maura* is located in the chromosome. Apart from this, as we have mentioned above, *H. maura* produces considerable quantities of exopolysaccharide; it has been suggested that *A. brasilense* uses similar extracellular polysaccharides to anchor itself to plant roots [24]. Moreover, it is known that the EPS succinoglycane is essential in the symbiotic process between the legume alfalfa and *S. meliloti* [10].

The inoculation of certain crops with nitrogen-fixing microorganisms produces excellent results in terms of production and profitability. Several species of free-living bacteria and also *Azotobacter chroococcum* have been used as symbionts, and commercial inocula of *Rhizobium* have been available for some eighty years for use on legumes [25]. It has been shown, however, that saline stress impedes nitrogen fixation quite significantly, inhibiting both the synthesis and activity of nitrogenase, and/or reducing bacterial adhesion to plant roots [26]. Thus the existence of a bacterial diazotroph capable of thriving under a wide range of saline concentrations is of considerable potential interest to agriculture and forestry.

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### References


