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ORIGINAL PAPER

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A membrane-bound nitrate reductase encoded by the *narGHJI* operon is responsible for anaerobic respiration in *Halomonas maura*

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Abstract The halophilic bacterium *Halomonas maura* is capable of anaerobic respiration on nitrates. By insertional mutagenesis with the minitransposon Tn-*5* we obtained the mutant Tc62, which was incapable of anaerobic respiration on nitrates. An analysis of the regions adjacent to the transposon allowed us to characterize the membrane-bound anaerobic-respiratory nitrate reductase *narGHJI* gene cluster in *H. maura*. We identified consensus sequences for fumarate and nitrate reductase regulator (FNR)-like protein-binding sites in the promoter regions of the *nar* genes and consensus sequences corresponding to the NarL binding sites upstream of the *nar* genes. RT-PCR analysis showed that the *narGHJI* operon was expressed in response to anaerobic conditions when nitrate was available as electron acceptor. This membrane-bound nitrate reductase is the only enzyme responsible for anaerobic respiration on nitrate in *H. maura*. In this article we discuss the possible relationship between this enzyme and a dissimilatory nitrate-reduction-to-ammonia process (DNRA) in *H. maura* and its role in the colonization of the rhizosphere.

Keywords *Halomonas* · Halophilic bacteria · *Nar* genes · Nitrate reductase

Introduction

Halomonas maura is a moderately halophilic bacterium capable of growing in salt concentrations of 1–20% w/v and is the most commonly found exopolysaccharide-producing bacterium in saline soils (Martínez-Cánovas et al. 2004). Its respiratory metabolism is chemo-organotrophic and it can respire anaerobically by using nitrate as alternative terminal electron acceptor. Among *Halomonas* species only two of the thirty so far described, *H. maura* (Bouchotroch et al. 2001) and *H. elongata* (Vreeland et al. 1980) have this capacity.

Denitrification is a dissimilatory process by which bacteria when faced with limited oxygen supplies can use oxidized nitrogen compounds as alternative electron acceptors to generate energy by phosphorylation reactions linked to electron transport. Denitrifying microorganisms are frequently found in soil, sediments and aquatic environments (Philippot 2002; Zumft 1997).

Bacteria can express two types of dissimilatory nitrate reductase, which differ both in their location and structural and catalytic properties: one is membrane-bound (NAR) and the other periplasmic-bound (NAP).

Two processes are involved in dissimilatory nitrate reduction: denitrification and ammonium production. Dissimilatory nitrate reductases catalyze the first step of the catabolic, anaerobic respiration pathway. After this step two different pathways can be followed: either nitrite is reduced to nitric oxide, which can then be further reduced to nitrous oxide and dinitrogen by additional enzymes (DNR), or nitrite is reduced directly to ammonia by haem-bearing nitrite reductases, a process known as dissimilatory nitrate reduction to ammonia (DNRA). Although the subsequent steps involve different enzymes and intermediates, the first steps in the processes of DNRA and denitrification are identical and can be carried out by the same enzyme.

The membrane-bound nitrate-reductase complex is composed of three subunits: (a) a catalytic α subunit, encoded by *narG*, containing a molybdopterin cofactor; (b) a soluble β subunit, encoded by *narH*, containing four [4Fe–4S] centres; and (c) the γ subunit, encoded by *narI*, containing two *b*-type haems.

The *narGHJI* genes are arranged in an operon within which the *narJ* gene encodes a chaperone-like compo-

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nent required for the maturation of the $\alpha\beta$ complex (Blasco et al. 1998). The *narGHJI* operon is often linked to at least one *narK* gene, which encodes proteins belonging to the "major facilitator" superfamily of transmembrane transporters (Marger and Saier 1993). Although the mechanism is still not fully understood it has been shown that *narK* is involved in the transport of nitrogen oxyanions (DeMoss and Hsu 1991; Moir and Wood 2001).

The regulation of anaerobic metabolism in denitrification has been studied in such microorganisms as *Paracoccus denitrificans* (Van Spanning et al. 1999), *Rhodobacter sphaeroides* (Tosques et al. 1996), *Pseudomonas stutzeri* (Vollack et al. 1999), and *Pseudomonas aeruginosa* (Arai et al. 1997). Denitrification genes are usually expressed as a response to anaerobiosis in the presence of nitrate and nitrite (Zumft 1997). The transcriptional regulator protein FNR is the anaerobic activator of *narGHJI* in *Escherichia coli* and *B. subtilis*. Many bacteria have been found to harbour an FNR-like protein (Spiro 1994; Unden and Schirawski 1997). An additional regulatory system is made up of two component regulatory systems: NarX, which has kinase-histidine activity and acts as a sensor for nitrate before activating the second protein and NarL, which recognises a sequence in the regulatory region for the genes that regulate and bind to it, thus activating these genes. The DNA binding sequences are known as NarL heptamers (TACYNMT, where Y = C or T; M = A or C), and although these sequences are preserved certain variations sometimes occur (Berks et al. 1995b; Dong et al. 1992; Tyson et al. 1994).

There is another enzyme that catalyses the first step of denitrification, known as periplasmic nitrate reductase, which is a heterodimer encoded by the genes *napA* and *napB*. These genes have been identified in *Thiosphaera pantotropha* (Berks et al. 1995c), *R. sphaeroides* (Liu et al. 1999), and *E. coli* (Siddiqui et al. 1993).

We describe here the cloning and characterization of the *narGHJI* gene cluster in *H. maura* strain S-31^T and the expression of this operon under various conditions.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids are listed in Table 1. *H. maura* strain S-31^T was routinely cultured at 32°C in MM complex medium containing a balanced mixture of sea salts (without $MgSO_4$) to a final concentration of 2% w/v (Rodriguez-Valera et al. 1981), plus 2 g/l proteose peptone and 0.4 g/l yeast extract. For conjugation experiments it was grown in SWYE minimal medium (Nieto et al. 1989) containing the same mixture of sea salts plus $MgSO_4$. *E. coli* strains were grown at 37°C in Luria-Bertani broth (LB) (Sambrook and Russel 2001). Antibiotics (Sigma-Aldrich, Tanfkirchen, Germany) were added to the different media at the following concentrations: ampicillin 50 μg/ml, kanamycin 50 μg/ml, tetracycline 15 μg/ml, and rifampicin 50 μg/ml. The media were adjusted to pH 7.2 with 1 M NaOH. Solid media contained an additional 20 g/l Bacto-agar (Difco, USA).

Determination of nitrate and nitrite reduction and respiration in nitrate

Nitrate was reduced to nitrite in MD medium with 7.5% salts (pH 7.2) at 32°C using Skerman's method (Skerman 1967). Respiration in nitrate was carried out in MD or MM medium with 7.5% salts supplemented with KNO_3 at 32°C, with a paraffin stopper and phenol red as indicator.

Transposon mutagenesis and isolation of the Tc62 mutant

Transposon mutagenesis was carried out via conjugation by biparental mating (Llamas et al. 2000). The donor strain was *E. coli* S17-1 *λpir*, harbouring the suicide vector pUT mini-Tn*5* Km2 (De Lorenzo et al. 1990). The recipient strain was *H. maura* S-31R2, a spontaneous, rifampicin-resistant mutant of *H. maura* S-31^T. The transconjugants were isolated on SWYE medium (Nieto et al. 1989) containing 2% w/v salts (Rodríguez-Valera et al. 1981), rifampicin (50 μg/ml), and kanamycin (50 μg/ml).

Southern-blot analysis

Genomic DNA of *H. maura* S-31^T was completely digested with *Bgl*II, *Eco*RI, *Hind*III, *Nco*I, *Sal*I, or *Sma*I. DNA fragments were separated on agarose gel and transferred onto a nylon filter by standard techniques (Sambrook and Russel 2001). Fragments containing mini-Tn*5* were located with a digoxygenin-labelled mini-Tn*5* probe using a digoxygenin DNA labelling and detection kit from Boehringer, Mannheim, Germany and following the manufacturer's instructions.

Gene cloning and sequencing

Plasmid pB62 was obtained as follows: chromosomal DNA of *H. maura* S-31^T was completely digested with *Bgl*II, ligated into p-SCRIPT, digested with *BamH*I, then dephosphorylated and transformed into *E. coli* XL1-Blue. Transformants were then selected and purified on LB medium plates supplemented with ampicillin at 100 μg/ml and kanamycin at 50 μg/ml (Table 1 and Fig. 1). Plasmid DNA (pB62) was subjected to DNA sequencing with a universal primer and a synthetic oligonucleotide primer (5′-GCCGCACTTGTGTATAAGAGTC-3′) based on the sequence determined for the

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. coli S17-1 *λpir*	Tp^r Sm^r *rec*A, *thi*, *pro*, *hds*R$^-$M$^+$ RP4:2-c : Mu : Km Tn7, *λ*pir	(Miller and Mekalanos 1988)
P. aeruginosa PAO1 CECT4122	Denitrifying strain with *nap* genes	(Philippot and Hojberg 1999)
H. maura CECT5298^T	Type strain	(Bouchotroch et al. 2001)
H. maura S-31R2	(Rif^r) Spontaneous rifampicin-resistant mutant of S-31^T	This article
H. maura Tc62	Transconjugant mini-Tn*5* (Rif^r Km^r). Phenotype anaerobic respiration on NO_3- negative	This article
H. desiderata DSM 9502	Denitrifying strain with *nap* genes	(Berendes et al. 1996; Dobson and Franzmann 1996)
H. campisalis ATCC 700597	Denitrifying strain with *nap* genes	(Mormile et al. 1999)
pUTmini-Tn*5*Km2	Ap^r Km^r, *ori* R6K, *oriT* RP4	(De Lorenzo et al. 1990)
pPCR-Script Amp SK(+)	Ap^r	Stratagene
pGEM-T ©	High-copy-number cloning vector, Ap^r, *bla lacZ*	Promega
PB62	pPCR-script with a *Bgl*II fragment carrying mini-Tn*5* insertion	This article
pPv62	pGEM-T with a inverse PCR product amplified from a *SmaI* fragment	This article
pSm62	pGEM-T with an inverse PCR product carrying the mini-Tn*5* insertion amplified from a *Pvu*II fragment	This article

O-end of the mini-Tn*5* transposon (De Lorenzo et al. 1990). This allowed us to determine the nucleotide sequences of the chromosomal regions adjacent to the transposon in these plasmids. The complete chromosomal nucleotide sequence was obtained using synthetic oligonucleotides.

Contiguous sequences were obtained by inverse PCR (Hartl and Ochman 1996). The chromosomal DNA of *H. maura* S-31^T was completely digested by restriction endonucleases and no restriction sites were found within the first 500 base-pair region of the *nar* gene sequence. The appropriate restriction enzymes were selected empirically by Southern blotting and hybridization procedures using the first 200 base pairs as probe. We selected the *Sma*I and *Pvu*II restriction endonucleases. The probe was labelled with digoxygenin-11dUTP by PCR (kit from Boehringer, Germany). The following oligonucleotides (Pharmacia, Kalamazoo, MI, USA) were used as primers:

5′-TCTACAGCGCCAACCGTCTCAAGCACCC-3′ (forward), 5′-CCAGGTGACCAGGCCGTTCTTGAC GTAG-3′ (reverse) for the *Sma*I fragment and 5′-GGA TGTGCATCTCCGGCTGTCCCTACAA-3′ (forward), and 5′-GTGTTCTCGAACTGGCCGTAGATGTCGG-3′ (reverse), for the *Pvu*II fragment. The labelled DNA was hybridized for 16 h at 42°C to a Southern blot of digested genomic DNA on a nylon membrane.

The DNA fragments produced by the restriction enzymes were diluted and ligated with T4 DNA ligase (Promega, Mannheim, Germany) for 16 h at 16°C under

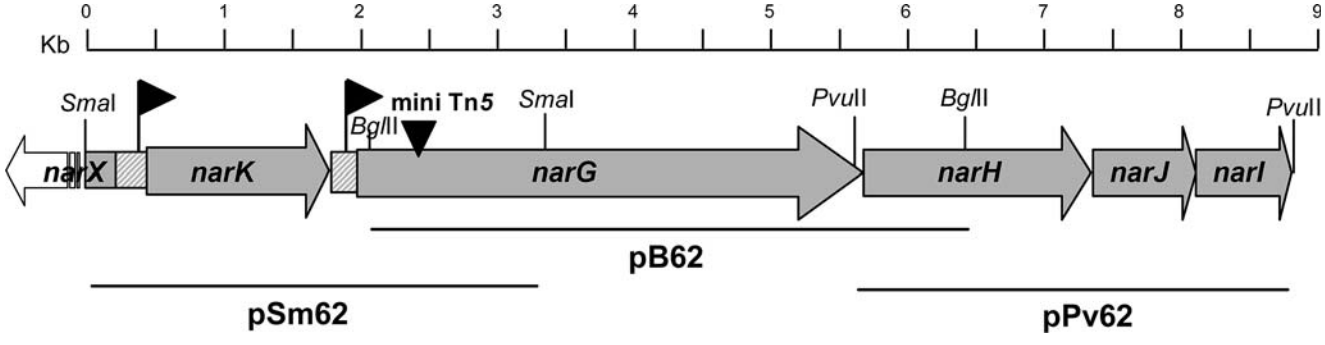

Fig. 1 Physical map of the *nar* genes encoding the membrane-bound nitrate reductase in *H. maura* S-31^T and scheme of the cloning strategy used for DNA sequencing. pB62 was obtained by fusing the pPCR-Script with the *Bgl*II restriction fragment; the transposon (mini-Tn*5* Km2) insertion site is indicated. pSm62 and pPv62 recombinant plasmids were obtained by fusing the pPCR-Script plasmid with the two inverse PCR fragments

conditions favouring the formation of monomeric circles. The resulting intramolecular ligation products formed the substrates for DNA amplification by PCR using oligonucleotide primers homologous to the ends of the known DNA sequence, but facing in opposite directions. The chromosomal DNA of *H. maura* strain S-31^T was amplified by PCR to a final volume of 50 µl containing 30 pM of each primer, 200 µM dNTP, and 1–2.5 U of Taq DNA polymerase PfuTurbo (Stratagene, La Jolla, CA, USA) or Taq Triple Master (Eppendorf, Hamburg, Germany). Amplification was performed for 30 cycles: at 98°C for 30 s and 72°C for 30 s, and then at 72°C for 3.5 min and 72°C for 10 min.

The amplified fragment was purified with a microcon PCR kit (Millipore, Madrid, Spain) and ligated into the pGEM-T plasmid to obtain the recombinant plasmids pSm62 and pPv62, before being transformed into *E. coli* XL1-Blue (Fig. 1).

DNA sequences were determined by the dideoxynucleotide-chain-termination method of Sanger et al. (1977) with both universal and specific oligonucleotide primers and double-stranded plasmid templates.

RNA isolation, primer extension analysis, and RT-PCR

An RNAII Nucleospin kit (Macherey-Nagel, Duren, Germany) was used to extract total RNA from *H. maura* S-31^T samples taken at set times directly from both aerobic and anaerobic cultures containing 10 ml of MM 2% w/v medium or 10 ml of the MM 2% w/v medium supplemented with 50 mM KNO_3.

For the analysis of the primer extension we used 50 µg of RNA to map the 5′ end of the *narG* transcript. Reverse transcription was initiated from the γ-^{32}P-end-labelled primer, 5′-ATGGTCATTGGCGAAGGG-3′, complementary to the 5′ end of the *narG* coding region. The sequencing reaction was performed with the same primer. The primer extension products and sequencing reactions were analysed on a 6.5% v/v denaturing polyacrylamide gel.

We undertook transcriptional analyses of the *narGHJI* genes under different denitrifying conditions by RT-PCR using an Enhanced Avian HS RT-PCR kit (Sigma, Deisenhofen, Germany), following the protocol provided by the manufacturer and using the following specific primers: 5′-TCGTGCAGCTGGAAGATCTA-3′ (forward) and 5′-ATCAGCGACAGGTAACGGCT-3′ (reverse), located at the beginning of the *narG* gene. Total RNA from *H. maura* S-31^T was extracted under denitrifying conditions using an RNAII Nucleospin kit (Macherey-Nagel) followed by rigorous treatment with Turbo DNA-free RNase (Ambion, TX, USA). To create these denitrifying conditions the cells were induced by nitrate (0.1% w/v, 0.5% w/v and 1% w/v for 1 h) under O_2 limitation. The shaker was then stopped and the tubes sealed with sterile paraffin. Samples for RNA extraction were drawn from cell suspensions at an optical density of about 0.8 at 600 nm. All standard precautions to prevent RNase contamination were taken.

To confirm whether the different genes contained in the *narGHJI* operon were transcribed together we carried out transcriptional analysis by RT-PCR following the same procedure as that described earlier. The primers used to amplify the intergenic region sequences were: 5′-CTCTGCATGCTGGTGATG-3′ and CCATGGT CATTGGCGAAG-3′ (*nark–narG*); 5′-GCTTCAACTA CTACGGCACC-3′ and 5′-CCGTTCCACTTCTTCTG GTTC-3′ (*narG–narH*); 5′-CGCAAGATGACCACC AC-3′ and 5′-CTGCAGGTCCATGAGGTC-3′ (*narH–narJ*); 5′-GACGCGATCTGGGAAGAG-3′ and 5′-GA TATGGAAGAGGTTGCTGG-3′ (*narJ–narI*).

Search for periplasmic-bound (NAP) nitrate-reductase genes

To check whether *H. maura* contains an NAP enzyme we used the degenerate primers V16 and V17, as described by Flanagan et al. (1999), to amplify a 1 kb fragment of the *napA* gene, which forms a partial fragment of the active subunit of NAP. Three denitrifying microorganisms were used as controls (Table 1). Amplification was performed for 30 cycles: at 98°C for 1 min and 50°C for 1 min, then at 72°C for 2 min and finally at 72°C for 10 min.

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been assigned Accession No AY641547 by the EMBL data library.

Results and discussion

Isolation and characterisation of strain Tc62

Strain Tc62 of *H. maura* is an anaerobic, nitrate-respiratory-deficient mutant which we obtained by insertional mutagenesis using a mini-Tn*5* Km2 transposon (De Lorenzo et al. 1990). The mutant had completely lost its capacity for anaerobic respiration using nitrate as alternative electron acceptor.

Identification of the gene affected by transposon insertion

To identify the gene affected by transposon insertion into *H. maura* S-31^T we cloned the chromosomal regions flanking mini-Tn*5*. The nucleotide sequence of recombinant plasmids, described in the Materials and methods section, showed five complete ORF's (Fig. 1) which shared high homology with nitrate reductase *nar* genes, the characteristics of which are set out in Table 2.

Table 2 ORF characteristics

ORF	Nucleotides (bp)	Amino acids	Molecular weight (kDa)	% G+C	Location	Transmembrane helices	Protein
ORF1	1,302	433	46.06	72.22	Transmembrane	12	NarK
ORF2	3,777	1,258	141.41	67.14	Cytoplasm	–	NarG
ORF3	1,608	535	60.84	65.98	Cytoplasm	–	NarH
ORF4	771	256	28.69	71.08	Cytoplasm	–	NarJ
ORF5	669	223	24.59	64.71	Transmembrane	6	NarI

The transposon was inserted into ORF2, the derived amino-acid sequence of which revealed a high degree of homology with the corresponding catalytic α subunit of the anaerobic, membrane-bound nitrate-reductase proteins (NarG) from various other microorganisms (Oshima et al. 1996; Philippot et al. 2001; Stover et al. 2000).

Nitrate-reductase tests on *H. maura* S-31^T and its mutant Tc62 confirmed that anaerobic respiration on nitrates was suppressed by the insertion of the mini-Tn*5* transposon into the *narG* gene.

Structure of the *Halomonas maura narGHJI* operon

The amino-acid sequence obtained for these five ORF's revealed a high degree of homology with the Nar proteins of various other microorganisms, including *E. coli* (Oshima et al. 1996) and *P. denitrificans* (Berks et al. 1995b) , and thus we also refer to them as *narK* (ORF1), *narG* (ORF2), *narH* (ORF3), *narJ* (ORF4), and *narI* (ORF5) (Table 2). Transcriptional analysis carried out by RT-PCR using primers based on their intergenic region sequences (see Materials and methods section) confirmed that *narGHJI* genes are transcribed as a single transcriptional unit (data not shown) just as they are in other microorganisms. In *H. maura* we found a single gene similar to *narK* upstream of *narGHJI* (Fig. 1 and Table 2); this pattern differs from that found in most other microorganisms studied to date, such as *Corynebacterium diphteriae*, *Brucella melitensis, Pseudomonas fluorescens*, *P. aeruginosa* (Stover et al. 2000), and *Thermus thermophilus* (Ramirez et al. 2000), all of which have two copies (Philippot and Hojberg 1999). As predicted by the SOSUI system, this nitrate or nitrite transporter, located in the membrane, is made up of 12 transmembrane helices, which is characteristic of this type of transporter.

The ORF2 encodes the active α subunit of an anaerobic nitrate reductase, NarG, bound to the membrane. According to SOSUI analysis this protein is soluble, contains no membrane helix and contains preserved amino-acid residues in the N-terminus region.

The ORF3 is very similar to the NarH protein, which corresponds to the β subunit of the NAR enzyme (Berks et al. 1995a). This protein contains four motifs in its amino-acid sequence, which bind to the [Fe–S] complexes via which they transport the electrons from the γ subunit located in the membrane to the α subunit in the cytoplasm. In the NarH protein belonging to *H. maura* we have located the four characteristic motifs, the first two of which possess all four cysteines with normal spacing between each other; in the fourth motif, however, they are separated by 11 amino acids, whilst in the third motif the second cysteine is replaced by a tryptophan residue, indicating that it may bind to [3Fe–4S].

The ORF4 is similar to the other NarJ proteins that make up the δ subunit of the NAR enzyme. This soluble protein is a chaperone that helps in the assembly of the NarGHI complex and no characteristic motif has been described in it.

The ORF5 is similar to the NarI protein, which constitutes the γ subunit of the NAR enzyme. This subunit is integrated into the membrane and is that which accepts the electrons released by quinones. In the *H. maura* NarI protein we have found structural characteristics similar to the ones proposed from sequence comparisons of *E. coli* NarI and NarV and *T. pantotropha* NarI (Berks et al. 1995b).

Regulation of nitrate reductase

Primer extension assays allowed us to identify precisely the +1 site of the corresponding -10 and -35 regions (Fig. 2a). The *narGHJI* system functions under anaerobic conditions and is regulated by the FNR protein, which binds to the inverse consensus sequence (TTGAT-N_{4-6}-ATCAA), known either as box or anaerobox FNR, and is located in the promoter region (Spiro 1994). In the promoter region upstream of *H. maura narGHJI* genes we found an inverse repeated sequence at -97.5 bp from the +1 site of *narG* with the sequence cg**G**cgccggg**ATCA**c (Fig. 2b), which, because it only preserves one of the parts of the symmetry axis, may be thought of as a partial box FNR, just as it is in various species of *Pseudomonas* (Härtig et al. 1999; Philippot 2002; Philippot et al. 2001).

Upstream of *narKGHJI* genes there is normally an operon known as *narXL*, which encodes the nitrate/nitrite regulatory system of the two components, as has been described in *E. coli* (Dong and DeMos 1992; Walker and DeMoss 1994) *P. stutzeri* (Härtig et al. 1999), *P. aeruginosa* and *P. fluorescens* (Philippot 2002). *P. denitrificans* does not possess this operon, however, and nitrate-reductase expression is regulated by another nitrate-dependent system (Moir and Wood 2001). In *H. maura* we found a region of 270 bp upstream from the

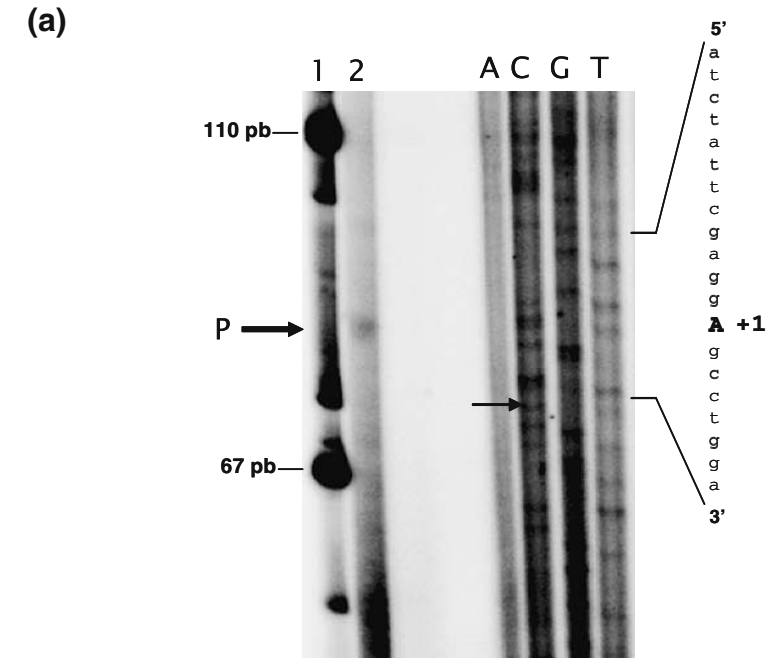

Fig. 2 **a**. Primer extension mapping of the 5′ end of the *narGHJI* mRNA. RNA was extracted from *H. maura* S-31^T grown on MM medium under anaerobic conditions and supplemented with KNO_3. Sequencing ladders constructed with the oligonucleotide (5′-ATGGTCATTGGCGAAGGG-3′, see its location in Fig. 2b) used for the primer extension (*lanes* A, C, G and T) were loaded beside the reaction mixture in *lane* 2. In the DNA sequence of the sense strand, the first nucleotide in the transcript is indicated as +1 and the promoter as P. *Lane* 1 shows the DNA markers (Marker VIII, Roche, Penzberg, Germany) in bases pairs (bp). **b** Structure of the promoter region upstream from the *narG* gene. The transcriptional initiation site for the *narGHJI* transcript (+1) and the putative -10 and −35 regions of the promoter are indicated. A Shine–Dalgarno (*SD*) sequence is also shown. A possible FNR box located at −97.5 bp of the initial codon of the *narG* gene and three possible NarL heptamers (−50, −123 and −144) are indicated in *boxes*

narGHJI operon that is very similar to the end of the N-terminal of the NarX protein, together with three possible NarL heptamers in the promoter zones of both *narK* and *narGHJI* (Fig. 2b), which would seem to confirm the existence of this type of regulation.

Reverse transcription-PCR experiments confirmed that the *narG* denitrifying gene was not expressed under aerobic conditions (Fig. 3, lane 4), whereas it was expressed after incubation under anaerobic conditions using different nitrate concentrations as electron acceptor. Figure 3 (lanes 1–3) shows the expected 464-bp RT-PCR product under all anaerobic conditions but not in aerobiosis. Altogether, these results and the presence of a possible FNR box located upstream from the *narK*

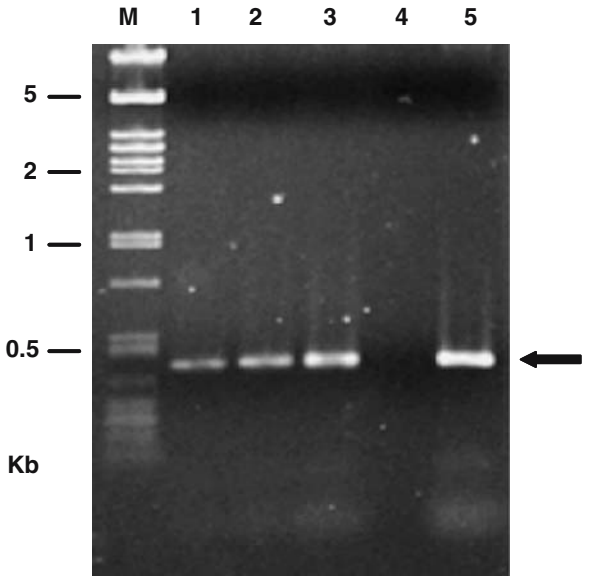

Fig. 3 Expression of *narG* in *H. maura* S-31^T was determined by RT-PCR of 5 μg of total RNA obtained 1 h after the switch from aerobic to anaerobic conditions plus 0.1% w/v nitrate (*lane 1*), 0.5% w/v (*lane 2*) and 1% w/v (*lane 3*). *Lane 4* was a negative control RT-PCR of *H. maura* S-31^T under aerobic conditions plus 0.5% w/v nitrate, and *lane 5* a PCR product using chromosomal DNA as a template. RT-PCR products were electrophoresed on a 1% w/v agarose gel. The *narG* products are indicated by the *arrow* to the right. The sizes (kb) of the DNA markers (*lane M*) are indicated on the left

gene and three possible NarL heptamers indicate that both anaerobiosis and nitrogen oxides are required for any significant activation of the expression of the *nar* denitrification genes, as occurs in *P. fluorescens* (Philippot et al. 2001), *P. aeruginosa* (Hernández et al. 1991) and *P. stutzeri* (Härtig et al. 1999).

The *narGHJI* genes encoding membrane-bound nitrate reductase are essential for nitrate respiration in *Halomonas maura*

In recent years considerable evidence has been found to support the idea of an additional pathway via which many bacteria are able to respire nitrate in the presence of oxygen (Carter et al. 1995; Davies et al. 1989; Lloyd 1993; Patureau et al. 1994). In all cases reported so far the biochemical apparatus for aerobic nitrate respiration is provided by a soluble periplasmic nitrate reductase, NAP (Berks et al. 1995c). This enzyme appears to be more versatile than NAR because it can also be induced under anaerobic conditions when NAR is inactivated by mutation (Bell et al. 1993). Thus, many bacteria can express multiple functionally and biochemically distinct nitrate reductases (Richardson et al. 2001).

In *H. maura* NAR appears to be the only enzyme responsible for anaerobic respiration on nitrate because if an alternative NAP were to exist it would be induced after the inactivation of NAR by mutagenesis and the *narG* mutant would continue to grow under anaerobic conditions via nitrate provided by the medium. Although this would seem to rule out the presence of an NAP enzyme in *H. maura* we decided to check by using degenerate primers, as described by Flanagan et al. (1999). All the Gram-negative strains found by Flanagan et al. (1999) to be capable of aerobic nitrate respiration also turned out to have periplasmic nitrate-reductase genes. Fragments of the *nap* gene were successfully amplified from *P. aeruginosa*, *Halomonas desiderata*, and *Halomonas campisalis*, all capable of aerobic nitrate respiration. In *H. maura* one single enzyme is responsible for respiration on nitrates, the membrane-bound nitrate reductase. We have shown that the mutant Tc62, in which the genes of *narGHIJ* operon are suppressed, is incapable of respiration on nitrates and that there is no alternative Nap enzyme, as there is in other bacteria.

The biochemistry of dissimilatory denitrification has been studied in some depth in *E.coli*. This bacterium is only capable of carrying out the first step in the process, that is, the reduction of NO_3^-–NO_2^- and subsequently ammonia. The denitrification process in *H. maura* occurs in a similar way. When we tested for nitrate reduction neither nitrite nor nitrogen gas was found in the culture medium, which leads us to suspect that nitrite is converted into ammonia via DNRA, as it is in *E. coli*. In DNRA, NO_3^- is used during dissimilatory NO_3^- reduction to NH_4^+ and N_2 is preserved in a form that is available to organisms (Patrick et al. 1996). Although the occurrence of DNRA has been demonstrated in various different environments, such as marine sediments (Tobias et al. 2001), its ecological significance is not yet fully understood (Cornwell et al. 1999).

In conclusion, *H. maura* is a bacterium capable of colonising the rhizosphere and in this it is helped by the anchoring potential of its expolysaccharide (EPS) (Arias et al. 2003) and the biofilm it produces (Llamas et al. 2006). It would also seem reasonable to believe that the possibility of growing in anaerobic conditions on nitrates, thanks to a membrane-bound nitrate reductase, affords it an advantage in its colonisation due to the low-oxygen conditions prevailing around the biofilm. Future studies into the mechanisms operating in the colonisation of the rhizosphere should lead us to a greater understanding of the relationship between this process and the respiration on nitrates observed in *H. maura* and other such nitrate-reducing microorganisms.

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