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Identification and transcriptional analysis of nitrate assimilation genes in the halophilic archaeon *Haloferax mediterranei*

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Abstract

Sequencing a 6,720-bp segment of the extreme halophilic archaeon *Haloferax mediterranei* genome has revealed the genomic organization of the putative structural genes for nitrate assimilation. We report a gene operon containing *nasABC* and *nasD* gene. *nasA* encodes an assimilatory nitrate reductase, *nasB* codes for a membrane protein with similarity to the NarK transporter, *nasC* encodes a protein with similarity to MobA; and *nasD* codes for an assimilatory ferredoxin-dependent nitrite reductase. Reverse transcription-PCR and primer extension experiments have demonstrated the existence of one polycistronic messenger *nasABC* and one monocistronic *nasD* initiated from a different promoter. The gene order and the grouping in two adjacent transcriptional units constitutes a novel organization of *nas* genes. The promoter regions harbor direct palindromes reminiscent of target sites for binding of a hypotetical regulatory protein(s). Transcription of the *nasABC* and *nasD* regions was found to be repressed by the presence of ammonium as nitrogen source.
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Keywords: Archaea; Nitrate assimilatory pathway; Operon; Transcriptional regulation; *Haloferax mediterranei*

1. Introduction

In the biosphere, nitrate assimilation is the major pathway converting inorganic nitrogen to organic forms. It is a property common to many heterotrophic (Stolz and Basu, 2002) and photosynthetic (Qingfeng et al., 2000) organisms. Assimilation of nitrate involves three pathway-specific steps: uptake, reduction to nitrite, and further reduction to ammonium. External nitrite can also be taken up and reduced directly to ammonium. The resulting ammonium is then metabolized through central pathways (Lin and Stewart, 1998). Nitrate assimilation requires the reducing activity of two enzymes, nitrate reductase and nitrite reductase. Three forms of nitrate reductase have been described: respiratory (Nar), periplasmic (Nap) and assimilatory (Nas) (Moreno-Vivian et al., 1999). Nar systems are involved in nitrate respiration under anaerobic conditions and Nar is distributed more widely in nitrate-respiring microorganisms (Richardson et al., 2001). In general, Nar is a heterotrimeric enzyme. Nap has a role in redox balancing, found mainly in the denitrifying microorganisms. Nap is a soluble heterodimeric enzyme. Nas carries out the assimilation of nitrate as a nitrogen source, and is a cytoplasmic molybdoenzyme classified in two groups: one group depends upon ferredoxin or flavodoxin as an electron donor, while the other depends upon NAD(P)H (Stolz and Basu, 2002). Nas also has a putative N-terminal [4Fe-4S] cluster. The molybdopterin cofactor in bacteria consists of a unique pterin complexed with Mo, forming Mo-MPT, and modified by the addi-

Abbreviations: *bp*, base pair(s); *cDNA*, DNA complementary to RNA; *DNase*, deoxyribonuclease; *dNTP*, deoxyribonucleoside triphosphate; *kb*, kilobase; *kDa*, kilodalton(s); *NAD(P)H*, (phospho)nicotinamide-adenine dinucleotide; *ORF*, open reading frame; *RNase*, ribonuclease; *S-D*, Shine-Dalgarno.

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tion of a guanosine to form Mo-MGD. In the present study we have sequenced the *mob*A gene, its product is implied in Mo-MGD synthesis, for this reason, *Hfx. mediterranei* Nas could be included in the bacterial group, they form a prokaryotic group. Cyanobacteria and plants synthesize ferredoxin-dependent nitrite reductase, whereas other bacteria and fungi synthesize enzymes that are dependent upon NADH and/or NADPH (Lin and Stewart, 1998). Both types of assimilatory nitrite reductase contain iron–sulfur clusters and sirohaem. NAD(P)H-dependent Nirs contain also non covalent FAD (Lin and Stewart, 1998).

The structural genes code for the Nas and Nir have not yet been identified in Archaea. Generally, bacterial assimilatory genes expression is controlled by general and pathway-specific regulatory systems, both at transcriptional levels. General control responds to the availability of the preferred nitrogen source (ammonium), whereas pathway-specific control responds to the availability of nitrate and/or nitrite (Lin and Stewart, 1998).

Haloferax mediterranei is an extreme halophilic archaeon able to grow in a minimal medium with inorganic salts, glucose as a carbon source and ammonium, nitrate or nitrite as a sole nitrogen source (Martinez-Espinosa et al., 2001a). *Hfx. mediterranei* is also known as a denitrifier, using nitrate as electron acceptor in anaerobic respiratory pathway (Manicelli and Hochstein, 1986). The product of this metabolic pathway is NO (Manicelli and Hochstein, 1986).

Here we report the identification, organization, characterization and regulation of the nitrate assimilation genes from *Hfx. mediterranei*, a member of the Domain Archaea. We demonstrate that the nitrate assimilatory genes are transcribed as two mRNAs, an unprecedented organization from nitrate assimilatory genes. Moreover, we report that control of the assimilatory pathway is at the transcriptional level.

2. Materials and methods

2.1. Strains, media, and growth conditions

The strains used were: *Haloferax mediterranei* (ATCC 33500), *Haloferax volcanii* (DSM 3757), and *E. coli* strain KW251 as the host for the λ EMBL3 vector (Promega).

Hfx. mediterranei cultures were grown aerobically at 37 °C on a rotary shaker platform in either ammonium medium or minimal medium with nitrate or nitrite as the sole nitrogen source. Media contained 25% (w/v) mixtures of salts (Rodríguez-Valera et al., 1980), as well as 0.005 g l^{-1} $FeCl_3$, 0.5 g l^{-1} KH_2PO_4, 5 g l^{-1} glucose, and either 1 g l^{-1} KNO_3, 0.17 g l^{-1} KNO_2 or 5 mM NH_4Cl. The pH was adjusted to 7.3 using KOH. Growth conditions for *Hfx. volcanii* were described previously (Dyall-Smith et al., 2004).

2.2. DNA preparations and manipulations, sequencing strategy, and amino acid sequence comparisons

A probe was prepared from *Hfx. volcanii* genomic DNA by PCR. Genomic DNA was extracted from a stationary phase culture as described by Dyall-Smith (Dyall-Smith et al., 2004). Three oligonucleotide primers were designed using the partial sequence of the *nas* gene from *Hfx. volcanii* obtained from the genome sequence project (http://zdna2.umbi.umd.edu/~haloweb/hvo.html). The oligonucleotide primers were made for amplification of genomic DNA, one sense primer, NASfor, 5′-TCCCCGCGGCGAC-GTGGGGA-3′ and two antisense primers, NASrev1 5′-TCCCGGACGCCGTCGGGAAC-3′ and NASrev2 5′-GCGTCGTCCGGGCTGAGCGT-3′. PCR was performed with *Taq* DNA polymerase (Ecogen) under the following conditions: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 64 °C for 1 min and 72 °C for 2 min, and a final extension of 72 °C for 10 min. Two products of expected sizes 377 and 641 bp were detected by electrophoresis and labeled with digoxigenin-dUTP using the Non-Radiactive DNA labeling and Detection kit (Roche Molecular Biochemicals). The labeled product of 641 bp was used as a probe in λ plaque hybridization experiments (Ausubel et al., 1989).

Hfx. mediterranei DNA was partially digested with *Sau*3A (Fermentas), and size-fractioned by agarose gel electrophoresis. Restriction fragments from 15 to 20 kb were extracted and ligated into the lambda vector using the Genomic Cloning Technical Manual kit (Promega). Ligated DNA was packaged and phages were plated on *E. coli* KW251. For library screening, λ plaques were blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech) and hybridized with a probe prepared as above. Positive λ plaques were isolated after double screening and subsequent DNA extraction was carried out by Lambda Maxi Kit (Qiagen). Nucleotide sequencing was performed by the dideoxy chain termination method using 3100 DNA sequencer (Applied Biosystems). Specific oligonucleotides were designed and used as primers in a primer walking strategy.

Analysis of the DNA and amino acid sequences was performed using the GCG package (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, WI, USA). Searches in the SWISS-PROT and TrEMBL databases for sequence similarities were carried out using the programs BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988). Multiple alignments were constructed using the CLUSTAL W program (Chenna et al., 2003). The trasmembrane regions prediction were performed using the TMHMM algorithm.

2.3. Reverse transcriptase primer extension

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche). Transcription start points were determined by primer extension. A 20-mer oligonucleotide (5′-ACAGGTCGTCGGGACTTGTT-3′), labeled at the 5′

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