

Short Communication

Identification of growth-dependent transcripts in the haloalkaliphilic archaeon *Natrialba magadii*

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The transition from balanced growth to the stationary phase induces changes in the morphology and physiology of most bacteria with the aim of preparing the cells to withstand unfavorable conditions (Nystrom, 2004; Siegele and Kolter, 1992). In *Archaea*, the third domain of life, many species are extremophiles, meaning that they have adapted their physiology to optimally live in environments with conditions that are lethal for most life forms. The effect of the growth stage in archaeal physiology has been investigated to a limited extent (Lange et al., 2007) and may reveal conserved as well as novel strategies of adaptation. The accurate regulation of gene expression is essential in all living cells for the adaptation to different environmental conditions (Soppa, 2006). In the haloarchaea (optimum growth at >2 M NaCl) growth phase-dependent gene expression has been investigated through genome-wide analysis of transcription and/or translation in the neutrophilic species *Haloferax volcanii* and *Halobacterium salinarum* (Facciotti et al., 2010; Lange et al., 2007). These studies showed that a significant fraction of genes were subjected to differential transcriptional

and/or translational control; however, neither the fractions nor the identity of the regulated genes were conserved between these archaea.

RNA arbitrarily primed polymerase chain reaction (RAP-PCR) uses random oligonucleotide primers to create a unique cDNA fingerprint for a given microorganism in a particular condition, thus, providing a useful tool for detecting genes with differential expression. This technique has been successfully applied to a variety of prokaryotes (Bidle and Bartlett, 2001; Li et al., 2006) including for the detection of changing salinity-mediated differential gene expression in *H. volcanii* (Bidle, 2003).

Analysis of gene expression at the transcriptional level helps to explore the physiological significance of gene products. In this study we have used RAP-PCR to identify genes differentially regulated in response to the transition from the exponential to the stationary phase in the dual extremophilic haloarchaeon *Natrialba magadii* ATCC 43099 (optimum growth in 3.5 M NaCl and pH 10). The screening was also aimed at those genes whose expression responded to molecules secreted/accumulated at high cell density. With this aim, RNA samples corresponding to cells in the exponential (OD₆₀₀ 0.5) and stationary growth phases (OD₆₀₀ 2.7) incubated in the absence or presence of stationary phase-conditioned medium (CM) were compared. CM was prepared as previously described (Paggi et al., 2010). The RAP-PCR assay was per-

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Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')
1. 60% G+C random	ATSSSSSSAT
2. 70% G+C random	ASSSSSSAT
3. L6350 F	GGAGAACGACCAAGAAGGAG
4. L6350 R	ATGCAAGCGTGTGTTGGCA
5. L1500 F	CACCCTGGCGAATCGAATGG
6. L1500 R	AAGCTGACTGCTCGCGACTC
7. 7S F	CCAACGTGGAAGCCTCGTC
8. 7S R	GGTGGTCCGCTGCTCACTTC

formed according to Bidle (2003) and was optimized by using 10 different combinations of random primers containing 50–80% G+C content and performing each RAP-PCR reaction in duplicate to assess reproducibility. RAP-PCR reactions with 60–70% G+C content primers (Table 1) gave the highest number of differential bands and the results were reproducible in duplicate reactions, thus, this condition was used in this study. Several cDNAs were detected to be differentially regulated during the transition from exponential to stationary

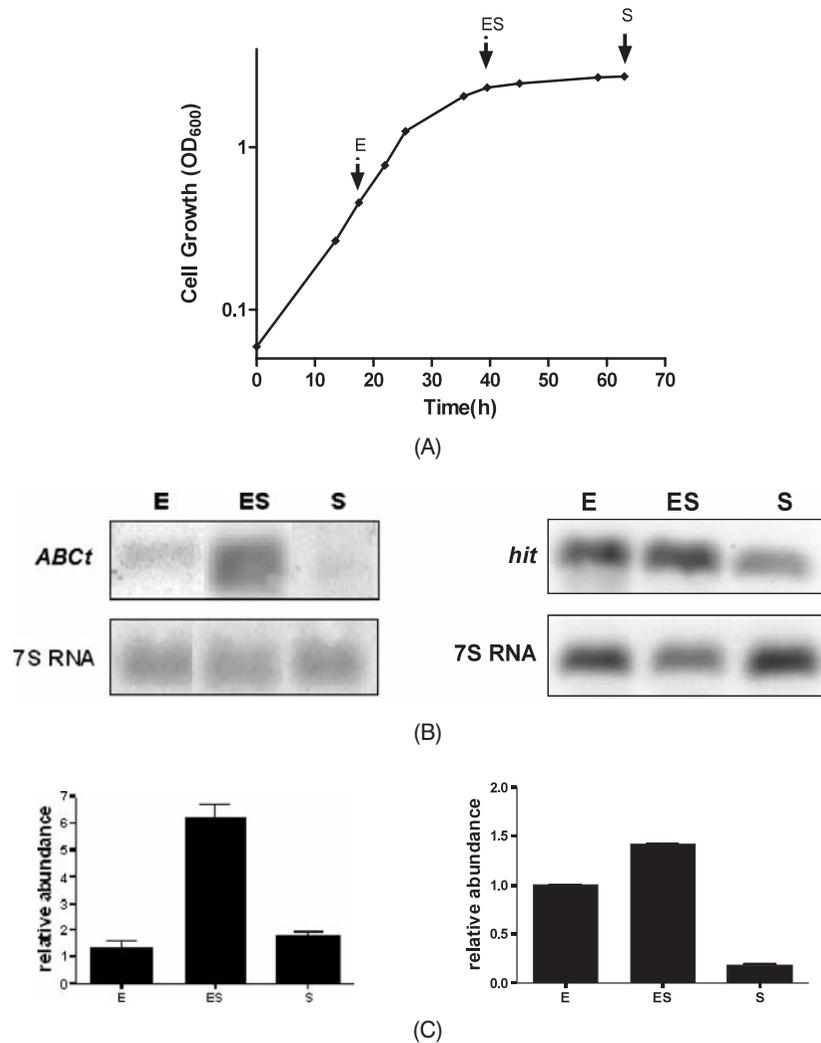


Fig. 1. Expression of HIT and ABCt transcripts during growth in *N. magadii*.

(A) *N. magadii* cells were grown in Tindall medium (Tindall et al., 1984). The arrows indicate the time points at which samples were withdrawn for RNA extraction and RT-PCR analysis: E, exponential (OD₆₀₀ 0.5); ES, early stationary (OD₆₀₀ 1.3–2.3) and S, stationary growth phases (OD₆₀₀ 2.7–2.9). (B) Detection of ABCt and HIT transcripts by RT-PCR. The products were fractionated on agarose gels 1.5% (w/v), stained with SYBR Gold and quantified using Image Quant software. (C) The amount of ABCt and HIT mRNAs was normalized to that of the constitutively expressed 7S RNA. RT-PCR reactions were performed in triplicate. Data are representative of at least two independent experiments.

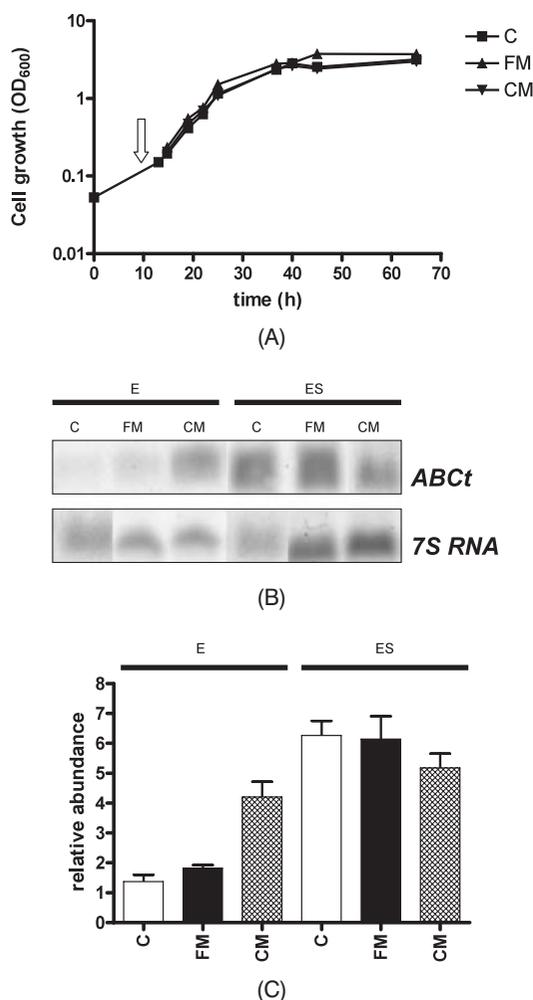


Fig. 2. Expression of ABCt transcript in *N. magadii* cultures in the presence of CM.

A. Cells growing in Tindall medium (OD₆₀₀ 0.3) were suspended (white arrow) in stationary phase conditioned medium (CM) or fresh sterile medium (FM) or allowed to grow without additions (C). The arrows indicate the time points at which samples were withdrawn for RNA extraction and RT-PCR analysis: E, exponential (OD₆₀₀ 0.5); ES, early stationary (OD₆₀₀ 2.3) growth phases. B. Detection of ABCt specific transcripts by RT-PCR. The products were fractionated on agarose gels (1.5%, w/v), stained with SYBR Gold and quantified using Image Quant software. C. The expression of ABCt mRNA was normalized to that of the constitutively expressed 7S RNA. RT-PCR reactions were performed in triplicate. Data are representative of at least two independent experiments.

phase and/or in cells incubated with CM in *N. magadii*. Those with sizes ranging from 300 to 500 bp were re-amplified, cloned and sequenced by using standard methods. Within this group, two partial cDNA fragments were identified based on a similarity search using Blast against the *N. magadii* ATCC 43099 genome (Gene-

bank CP001932, CP001933, CP001934, CP001935) and subjected to further analysis. cDNA 1500 (~450 bp) was an ortholog of the histidine triad protein (HIT) superfamily (Hint branch) (Nmag_2944, E=7e-46) and cDNA 6350 (300 bp) was an ortholog of extracellular solute-binding protein family 5 of the ABC type peptide transporters (ABCt) (Nmag_4050, E=2e-58). Based on the RAP-PCR assay, HIT mRNA was more abundant in the exponential compared to the stationary phase while the ABCt mRNA was preferentially expressed as the culture aged and in exponentially growing cells incubated with CM (not shown). The differential regulation of HIT and ABCt transcripts during growth in *N. magadii* was demonstrated by RT-PCR in samples representative of the mid-exponential (E), early stationary (ES) and stationary (S) growth phases (Fig. 1A). To make the PCR reaction quantitative, the number of amplification cycles was optimized for each gene. The amount of the ABCt mRNA was maximal at the ES phase (6-fold relative to E and S) (Fig. 1B). Based on the RAP-PCR prediction we further investigated the nature of the ABCt gene regulation by analyzing the ABCt mRNA level in cells incubated with and without CM. As expected, the ABCt mRNA was up-regulated (~4-fold) as the culture entered the stationary phase (Fig. 2C) and addition of CM to a low-density culture (OD₆₀₀ 0.3) incremented the level of ABCt mRNA by 3-fold compared to control cultures without additions (C) or incubated with fresh medium (FM) (Fig. 2C). ABC-type transporters are widely distributed in all domains of life and are involved in various processes such as substrate uptake/export and osmoregulation (Albers et al., 2004). Interestingly, the transcription profile of ABCt was similar to that of the extracellular subtilase Nep produced by this archaeon (Paggi et al., 2010). Both *abct* (this work) and *nep* (Paggi et al., 2010) genes are up-regulated at the end of growth and in response to the presence of stationary phase spent medium suggesting that molecules (metabolites or regulatory molecules) secreted to or removed from the stationary phase medium positively affect their expression. Inspection of *N. magadii* genome sequences indicated that *nep* and *abct* genes were not linked at the genomic level; however, their gene products seem to be functionally related. From a physiological view point, high levels of extracellular protease and high-affinity ABC-type oligopeptide transporters at elevated cell densities may allow *N. magadii* to sustain growth on protein substrates derived from dead cells in the nutrient-

limited hypersaline and hyperalkaline environments in which this extremophile thrives. The *N. magadii* genome encodes several extracellular proteases and peptide transporters most likely as an adaptation to survive in such hostile habitats. Up-regulation of ABCt towards the stationary phase was not surprising as this type of transporter was recently shown to be induced at the end of growth in the neutrophilic haloarchaea *Halobacterium salinarum* and *Haloferax mediterranei* using global transcript and protein analysis (Facciotti et al., 2010; Lange et al., 2007). This observation indicates that scavenging peptide substrates by means of this type of transporter is a conserved strategy in haloarchaea. In addition to their role during starvation, ABC-type transporters are induced and/or activated upon hyperosmotic shock in methanogenic archaea probably as a means to concentrate compatible solutes (Albers et al., 2004). We performed preliminary experiments with *N. magadii* cells transferred from high (3.5 M NaCl) to low salinity (1.75 M NaCl) and observed a tendency of the ABCt mRNA to accumulate at a higher level in cells subjected to hyposmotic shock (not shown), suggesting that the ABC-type peptide transporters may be needed for survival under various stress conditions in this extremophile.

On the other hand, by RT-PCR we confirmed that HIT transcripts show elevated expression in growing cells, being slightly higher at ES (1.4-fold), and decrease significantly (5-fold) during the S phase (Fig. 1C). HIT proteins are nucleotide hydrolases and transferases which contain the conserved motif His-X-His-X-His-XX, X being a hydrophobic amino acid (Brenner et al., 1999). Within this superfamily, the Hint branch is widespread in all domains of life suggesting that these proteins play a central role in cell physiology; however, their function has not been clearly addressed. While in eukaryotic cells Hint proteins have been involved in tumor suppression and regulation of apoptosis (Weiske and Huber, 2006), the prokaryotic counterparts have not been characterized and their physiological role remains elusive. In this study we show that Hint nucleotide hydrolases are expressed at the mRNA level during the exponential phase in this haloalkaliphilic archaeon, suggesting that these enzymes may have a function in active cells in this microbe. To the best of our knowledge, the expression of this protein family has not been previously explored in the haloarchaea (at the mRNA, protein and/or activity levels).

Moreover, hit homologs have not been reported as

differentially expressed genes in *Haloferax volcanii* or *Halobacterium salinarum* in which genome-wide transcription/translation analysis was performed (Facciotti et al., 2010; Lange et al., 2007). However, HIT homologs have been detected at the protein level in the hyperthermophilic archaeon *Pyrococcus furiosus* and shown to decrease in response to a temperature downshift (Trauger et al., 2008). In *E. coli* the expression of a Hint homolog (*hin T*) was required for growth at high salinity (Chou et al., 2005). Recently, it was demonstrated that *E. coli* HiT protein 1 (ecHin T) is a catalytic regulator of D-alanine dehydrogenase (DadA) activity in vivo and it is required for growth on this amino acid as a sole carbon source (Bardaweel et al., 2011). This finding links for the first time the catalytic activity and structure of a Hint protein with a bacterial physiological function. Interestingly, during this study, we detected a basal level of HIT mRNA in cells incubated in 3.5 M NaCl and 1.75 M NaCl (not shown) suggesting that the *hit* gene product may be needed in this obligate extreme halophile under optimal and suboptimal salinity conditions. Further studies at the transcript, protein and/or activity levels will help to get insight into the role of these enzymes in archaeal (and prokaryote) physiology.

The effect of the growth stage was examined for the first time in haloalkaliphilic archaea. This exploratory strategy showed up-regulation of a gene involved in peptide scavenging as well as down regulation of an uncharacterized gene product at the onset of the stationary phase in *N. magadii*.

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