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Differential expression of genes influenced by changing salinity using RNA arbitrarily primed PCR in the archaeal halophile Haloferax volcanii

Abstract

Extreme halophiles belonging to the domain Archaea require a minimum of ~10% NaCl for growth. Many of these obligate halophiles will continue to grow even as NaCl concentrations approach saturation. The haloarchaeon Haloferax volcanii is a model organism in which to study the effects of changes in medium salinity on gene expression, as this organism grows over a wide range of NaCl concentrations, between 12% and 23%, with little effect on growth rate. An RNA arbitrarily primed PCR (RAP-PCR) approach has been applied to identify those genes that are differentially expressed in response to changing salinity. Differences in gene expression can be detected using this methodology, as each sample generates its own unique RNA fingerprint for each growth condition examined. RNA was prepared from H. volcanii cultures grown with two different NaCl concentrations in the medium, RAP-PCR was performed, and seven differentially expressed transcripts were identified. These fragments were cloned, sequenced, and subjected to transcript analysis to confirm their regulation. One of the sequences identified in this study displays homology to the eukaryotic Ser/Thr protein kinase Ire1p, a sensor of protein unfolding in yeast and mammalian cells. Evidence for serine phosphorylation in H. volcanii is also presented.

Keywords

Gene expression · Haloferax volcanii · Halophile · RAP-PCR · Sensor kinase

Introduction

Haloferax volcanii is an extreme halophile belonging to the domain Archaea. H. volcanii can be used as a model microorganism for examining the adaptations necessary for dealing with changes in salinity due to its ability to grow over a wide range of NaCl concentrations, from ~12% to 23%, while maintaining a relatively constant growth rate (Mullakhanbhai and Larsen 1975). The study of haloarchaeal adaptations to hypersaline environments has mainly been limited to examination of protein modifications and function [reviewed in Dennis and Shimmin (1997) and Madern et al. (2000)] and the physiological response of the cell to its high-salt environment (Christian and Waltho 1962; Lanyi 1974, 1984; Stoeckenius and Bogomolni 1982; Meury and Kohiyama 1989). There is little known about the molecular and genetic adaptations used by the extreme halophiles for existence in hypersaline environments. Prior studies have identified differentially expressed proteins (Daniels et al. 1984; Mojica et al. 1997) and transcriptionally active regions within the genome (Ferrer et al. 1996) that are responsive to changes in medium salinity in H. volcanii. In these studies H. volcanii was exposed to several different salinities, ranging from the lower to upper limits for the organism, and examined for a global response to osmotic shifts. It was found that different sets of proteins are expressed dependent on medium salinity (Daniels et al. 1984; Mojica et al. 1997), and that differential expression of transcripts can be identified in situations of both high- and low-salt induction (Ferrer et al. 1996). While little molecular information was revealed in these studies (i.e., no DNA sequence information was obtained from the activated transcripts), they clearly indicated that there are specific genes, thus far unidentified, involved in hypersaline adaptation in the extreme halophiles, which are awaiting detailed investigation.

In this report, a putative sensor kinase and six other genes regulated in response to a shift in salinity have
been identified using the technique of RNA, arbitrarily primed PCR (RAP-PCR). This powerful method is a useful tool for examining differential gene expression in prokaryotes, since accurate identification of differentially expressed transcripts is accomplished using random 10-mer oligonucleotides to create first and second strand cDNA from differing RNA populations. A unique fingerprint is created for each sample and primer used, thereby allowing easy comparison between the strains or conditions examined. Recently, this technique has been used in the deep-sea bacterium *Photobacterium profundum* SS9 to identify genes under the control of the global regulator ToxR (Bidle and Bartlett 2001). This method has also been successfully applied to several other bacterial systems to examine stress response (Wong and McClelland 1994; Benson et al. 2000), in situ growth conditions (Fleming et al. 1998), and response to (Wong and McClelland 1994; Benson et al. 2000), in situ global regulator ToxR (Bidle and Bartlett 2001). This method relies first on the creation of random cDNA molecules from a RNA population, followed by denaturing acrylamide gel (20 × 42 cm) and electrophoresed at 1,700 V for ~2 h until the xylene cyanol band reached the bottom of the gel. The gels were subsequently dried, marked asymmetrically with a phosphorescent pen for orientation, and exposed to film (Kodak) for 1–3 days at −80°C. Following identification of differentially expressed fragments, bands of interest were isolated from the gel as described in Bidle and Bartlett (2001). Eluted bands were subjected to secondary PCR using the original primers used in RAP-PCR amplification to facilitate cloning into pCR2.1 (Invitrogen) according to the manufacturer’s instructions. Clones verified for the correct sized insert were then sequenced to determine putative identity.

**Materials and methods**

**Bacterial strains and culture conditions**

*H. volcanii* strain WFD11 (DSM 5716) was generously provided by Dr. H.J. Schreier. Cultures were grown aerobically with vigorous shaking at 42°C in a medium containing 12% (optimal) or 20% (high) NaCl (2.1 or 3.5 M), 45 g MgCl₂·6H₂O, 10 g KCl, 1.34 ml 10% CaCl₂·2 H₂O, 3 g yeast extract, and 5 g tryptone. Inclusive of magnesium, calcium, and potassium, which remained constant regardless of NaCl concentrations used, these culture conditions represent a total medium salinity of either 18.5% or 26.5%. However, for the purpose of these studies, only the effects of changing NaCl concentrations were examined. *Escherichia coli* strains used for plasmid maintenance were grown in liquid or solid LB medium at 37°C. When necessary, antibiotics and chromogenic substrates were used in the following concentrations: kanamycin (50 µg/ml); X-gal (40 µg/ml).

**RNA preparations**

Mid-exponential phase cultures of *H. volcanii* were harvested and RNA was extracted using RNAzol B (Tel-Test; Friendswood, TX, USA), a guanidinium thiocyanate/phenol-based reagent, according to the manufacturer’s instructions. RNA was quantified using a UV spectrophotometer and its quality was assessed on an agarose gel prior to the experiments.

**RNA arbitrarily primed PCR (RAP-PCR)**

RAP-PCR was performed essentially as in Fleming et al. (1998) and Bidle and Bartlett (2001). Arbitrary 10-mer primer kits with G+C contents of 70% (GEN4–70) and 80% (GEN4–80) were obtained from Genosys Biotechnologies (The Woodlands, TX, USA). First strand cDNA synthesis was performed using 200 ng RNA heat denatured for 10 min at 65°C in 20 µl reactions containing the following: 200 µM each dNTP, 5 mM dithiothreitol, 50 U MMLV-RT (Ambion), 1× RT reaction buffer, and 0.4 µM of an arbitrary 10-mer primer. The first strand reaction was performed in an MJ Research mini-cycler (PTC-150) as follows: touchdown from 50°C to 30°C in 45-s increments, 37°C for 1 h. Second strand synthesis was performed in 30 µl reactions containing the following: 3 µl of first strand reaction, 0.3 U Taq polymerase (BRL), 20 µM each dNTP, 6% dimethyl sulfoxide, 2 µM each primer (primer 1 is from first strand synthesis and primer 2 is a second arbitrary primer), 2.5 µCi 32P-dCTP, 1× PCR buffer, and 1.5 mM MgCl₂. Cycles for RAP-PCR were: 94°C for 30 s, 40°C for 2 min, and 72°C for 1 min for 40 cycles, with a 10 min 72°C extension on the final cycle. All products were stored at −20°C for up to 1 day prior to electrophoresis. RAP-PCR samples were heated at 92°C for 2 min after the addition of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were then loaded onto a 5% denaturing acrylamide gel (20 × 42 cm) and electrophoresed at 1,700 V for ~2 h until the xylene cyanol band reached the bottom of the gel. DNA sequences were determined by thermal cycle dideoxy sequencing with an ABI 373A automated sequencer using fluorescently labeled terminators (Perkin-Elmer) using T7, -M13 reverse, or custom primers. Some sequencing reactions contained 10% DMSO to prevent secondary structure formation within the high-G+C-rich templates. Initial global similarity searches were performed with the BLAST network service (Altschul et al. 1990). Multiple alignments of amino acids were performed using Clustal W (Thompson et al. 1994) in conjunction with the Boxshade program available at http://www.ch.embnet.org/software/BOX_form.html.

**DNA sequencing**

DNA sequencing was performed using the RAP-PCR products. Sequencing reactions were performed at the request of either clone B2 or B5 which was randomly primed with the original primers used in RAP-PCR amplification to facilitate cloning into pCR2.1 (Invitrogen) according to the manufacturer’s instructions. Clones verified for the correct sized insert were then sequenced to determine putative identity.

**Confirming the regulation of RAP-PCR fragments by changing salinity**

To verify differential expression of the fragments identified in RAP-PCR, a quantitative PCR approach was used (Benson et al. 2000; Bidle and Bartlett 2001). This method relies first on the creation of random cDNA molecules from a RNA population, followed by gene-specific PCR. Briefly, first strand cDNA was created using RNA from *H. volcanii* strains following the same RT conditions stated above with the exception that 2 µM of random hexamers were used instead of a single arbitrary primer. Quantitative PCR was then performed using custom made 18-mer primer pairs specific for each sequence being tested and 2.5 µCi of 32P-dCTP. Cycles of PCR were as follows: 92°C, 1 min; 50°C, 1 min; 72°C, 1 min for 25 cycles. Synthesis of a PCR product of the correct size and expression pattern was considered verified for differential expression of the original RAP-PCR result. All quantitative RAP-PCR experiments were performed in duplicate to ensure reproducibility.

**Isolation of the locus-bearing genes encoded by clones B2 and B5**

Genomic DNA from *H. volcanii* was isolated as described in Ng et al. (1995) and digested with either *BamHI, KpnI, NotI, SphiI, or XhoI* restriction enzymes (Gibco-BRL). Restriction digests were electrophoresed through a 0.8% agarose gel in duplicate and subsequently transferred to a nylon membrane (Osmonics) for Southern analysis. Each membrane was probed with the PCR product from either clone B2 or B5 which was randomly primed using [α-32P]dCTP and the RadPrime kit (Gibco-BRL). Hybridizations were performed at 62°C in QuikHyb hybridization solution (Stratagene) overnight. Each membrane was washed under stringent conditions (Ausubel et al. 1995) and exposed to autoradiographic film (Kodak) overnight at −80°C with an intensifying screen.