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Research paper

Global transcriptome analysis of *Halolamina* sp. to decipher the salt tolerance in extremely halophilic archaea

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ABSTRACT

Extremely halophilic archaea survive in the hypersaline environments such as salt lakes or salt mines. Therefore, these microorganisms are good sources to investigate the molecular mechanisms underlying the tolerance to high salt concentrations. In this study, a global transcriptome analysis was conducted in an extremely halophilic archaeon, *Halolamina* sp. YKT1, isolated from a salt mine in Turkey. A comparative RNA-seq analysis was performed using YKT1 isolate grown either at 2.7 M NaCl or 5.5 M NaCl concentrations. A total of 2149 genes were predicted to be up-regulated and 1638 genes were down-regulated in the presence of 5.5 M NaCl. The salt tolerance of *Halolamina* sp. YKT1 involves the up-regulation of genes related with membrane transporters, CRISPR-Cas systems, osmoprotectant solutes, oxidative stress proteins, and iron metabolism. On the other hand, the genes encoding the proteins involved in DNA replication, transcription, translation, mismatch and nucleotide excision repair were down-regulated. The RNA-seq data were verified for seven up-regulated genes as well as six down-regulated genes via qRT-PCR analysis. This comprehensive transcriptome analysis showed that the halophilic archaeon canalizes its energy towards keeping the intracellular osmotic balance minimizing the production of nucleic acids and peptides.

1. Introduction

Archaea are the prokaryotic microorganisms generally living in extreme environments such as high salinity, pH, and temperature (Swan et al., 2010). The haloarchaea (order *Halobacteriales*) require at least 10% salt for growth, and survive up to 32% NaCl in the environments such as salt mines, solar salterns, salt lakes, brine pools, and deep-sea brine. Generally, they produce carotenoid pigments conferring protection against ultraviolet light, and this causes red color formation in the brines when the cell density increases (McGenity et al., 2000; Elevi et al., 2004; Cha et al., 2014).

Halophilic microorganisms are good sources to be used in diverse biotechnological applications. For instance, *Halomonas smyrnensis* AAD6T was reported for high levels of levan production capacity, which is a long exopolisaccharide (EPS) of β(2-6)-linked fructose residues and has a potential to be used in food, chemical and pharmaceutical industries as a bioactive polymer (Diken et al., 2015). In addition, receptor binding activity of secondary metabolites from a halophilic marine *Streptomyces* was shown to be acting against quorum-sensing signals of the uropathogen *Proteus mirabilis* (Younis et al., 2016). Moreover, haloarchaeal enzymes such as cellulase and chitinase from *Haloarcula* sp. LLSG7 and *Haloferax mediterranei*, respectively, were successfully used in bioethanol production and bioconversion of chitin to bioplastic, respectively (Li and Yu, 2013; Hou et al., 2014). The ability of an organism to survive at highly saline environments is defined as the halotolerance. Under the conditions involving excess ion concentrations, the halotolerant cell withstands the detrimental effects of ionic imbalance producing resistance proteins with increased quantity of acidic and basic amino acids, high amount of enzymes, gas vesicles, and compatible solutes (Mahapatra and Mishra, 2014; Leuko et al., 2015). Compatible solutes are highly soluble and small organic molecules either synthesized or accumulated from outside. Mostly they are neutral or zwitterionic such as amino acids, sugars, polyols, betaines, trehalose and ectoines. When accumulated inside the cell they stabilize turgor pressure, protect cell volume and electrolyte concentration thereby helping maintenance of cell integrity at high salt concentrations (Roberts, 2005). Transcriptome analysis, the global evaluation of transcript profiles, is a widely utilized technique for the investigation of the halotolerance. The studies have been conducted on plants such as

Abbreviations: ABC, ATP-binding cassette; Cas, CRISPR-associated protein; COGs, Cluster of Orthologous Groups; CRISPR, Clustered regularly interspaced short palindromic repeats; DEG, Differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NR, Non-redundant; NT, Nucleotide database; qRT-PCR, Quantitative real time polymerase chain reaction; RNA-seq, RNA sequencing.

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Brassica napus (Yong et al., 2014; Long et al., 2015), *Zoysia japonica* (Xie et al., 2015), *Artemisia sphaerocephala* (Zhang et al., 2016a), or *Caragana korshinskii* (Li et al., 2016) as well as microorganisms such as *Deinococcus radiodurans* (Im et al., 2013), *Staphylococcus* sp. (Choi et al., 2014), *Enterococcus faecalis* (Solheim 2014), *Mesorhizobium alhagi* (Liu et al., 2014) or *Tetragenococcus halophilus* (Liu et al., 2015). Recently, an RNA seq analysis was performed on *Wallemia ichthyophaga*, an extremely halophilic fungus, grown at 10% NaCl and 30% NaCl, corresponding to 1.71 and 5.13 M NaCl, respectively to reveal osmoadaptation mechanisms (especially for ion transporters) of halotolerant eukaryotes to extreme salinity (Zajc et al., 2013). However, to our knowledge, there have been no studies regarding RNA-seq analysis of salt tolerance mechanisms in an extreme halophilic archaeon when grown at 2.7 M and 5.5 M NaCl which is the highest salt concentration ever reported.

Deciphering the salt tolerance of extremely halophilic archaea at high salt concentrations which are lethal to most living organisms will contribute to a better understanding of the molecular level of resistance and defense against salt toxicity. Here, we conducted a comprehensive RNA-seq analysis in *Halolamina* sp. YKT1, an extreme halophilic archaeon isolated from a salt mine in Turkey, to reveal the molecular mechanisms related with the halotolerance of the microorganism. *Halolamina* sp. YKT1 was grown in the presence of two limiting salinities, in a medium containing either 2.7 M or 5.5 M NaCl, and the resulting differential gene expression was evaluated. In addition, metabolic pathways playing role in the salt tolerance of the microorganism were determined. Thus, the resultant RNA-seq data will support future genetic engineering studies to improve salt tolerance of many organisms such as salt-adapted crops or to be used in biotechnological applications such as development of halotolerant enzymes.

2. Materials and methods

2.1. Isolation of halophilic archaea

The salt sample was obtained from Yozgat salt mine (39°40′38.6″N 34°13′25.9″E) in Turkey. The halophilic archaea were isolated using slightly modified procedure as stated in Nagaoka et al. (2011) and Enache et al. (2013). One to three grams of samples were inoculated into 10 ml of Sehgal–Gibbons (SG) medium. The cultures were incubated at 37 °C for 2–4 weeks in an orbital shaker with 200 rpm. Single pink colored colonies were obtained after inoculation of the broth cultures onto SG agar plates. The medium consists of the following components (in g/l): NaCl, 250; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; sodium citrate (trisodium salt), 3; casamino acids, 7.5; yeast extract, 1; $FeSO_4 \cdot 7H_2O$, 0.0023; agar, 15 g, pH:7.55 (Sehgal and Gibbons, 1960).

2.2. Identification of the isolates

To determine the salt range that the isolates grow, 1.0 to 5.5 M NaCl concentrations with 0.1 M increments in SG agar medium were tested. As the 5.5 M NaCl corresponds to saturation molarity upper concentrations could not be tested for the salt resistance of the isolates. The microorganisms were incubated at 37 °C for one week, and the growth situations were recorded.

The genomic DNA isolation was performed using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Universal primers, ArchF (5′-TTCCGGTTGATCCTGCCGGA-3′) and AIIR (5′-GGTTACCTTGTTACGACTT-3′), were used for amplification of 16S rDNA (DeLong, 1992; Lane et al., 1985). The applied PCR reaction includes the following components as final concentrations: *Taq* DNA polymerase buffer (1×), $MgCl_2$ (1.5 mM), forward and reverse primers (0.2 μM for each), dNTP mix (200 μM), template DNA (50 ng) and *Taq* DNA polymerase (1 U). The PCR conditions were as follows; an initial denaturation step (5 min at 94 °C), 35 cycles of amplification (45 s at 95 °C, 30 s at 55 °C, 1 min.40 s at 72 °C) and a final extention step (10 min at 72 °C). PCR products were sequenced at BGI Europe. The BLAST (Basic Local Alignment Search Tool) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyze the 16S rDNA sequences.

2.3. RNA isolation

Halolamina sp. YKT1 isolate was grown at 37 °C and 200 rpm for one week in SG medium containing either 2.7 M or 5.5 M NaCl. Two millilitre of samples taken from cultures were used for RNA isolation. The RNA was purified using GeneJET RNA purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The isolated RNAs were treated with a DNA-free™ kit (Ambion) and cleaned up by phenol/chlorophorm/isoamylalcohol using Phase lock gel heavy tubes (5 PRIME). The amounts of RNA were determined in a NanoDrop® ND-2000 Spectrophotometer (Thermo Fisher Scientific). The integrity and quality of RNA samples were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies).

2.4. Library construction and RNA sequencing

A total RNA of 5 μg was used for library construction. The RNA samples had >65 ng/μl concentration and included $OD_{260/280} \geq 1.8$ and $OD_{260/230} \geq 1.8$ quality values. In addition, RIN values higher than ≥6.0 were preferred. Libraries were prepared and sequenced on the platform of Illumina Hiseq 2000 with a paired-end protocol (BGI, China).

2.5. Bioinformatic analysis

The sequences were assembled *de novo*. The contigs were assembled and gene homologies were determined using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The unigene function annotation, Gene Ontology (GO) classification, Cluster of Orthologous Groups (COGs) analysis, metabolic pathway analysis, protein coding region prediction (CDS) and unigene expression difference analysis were performed. The GO classification was determined using the tools Blast2GO (https://www.blast2go.com; Conesa et al., 2005) and WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). The detected unigenes were annotated by aligning to protein databases Non-redundant (NR), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and COGs (e-value < 0.00001) by Blastx, and the Nucleotide database (NT) (e-value 0.00001) by Blastn, retrieving proteins with the highest sequence similarity with the given unigenes along with their protein functional annotations. Resultant bioinformatic data was analyzed by TRAPID program (http://bioinformatics.psb.ugent.be/webtools/trapid). Differentially expressed genes were analyzed using RNAseqViewer (http://bioinfo.au.tsinghua.edu.cn/software/RNAseqViewer).

2.6. qRT-PCR validation

Gene-specific primers were designed using Primer3plus program (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Suppl. Table 1). A RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used for synthesis of complementary DNA (cDNA) using 1 μg RNA and oligodT primers. qRT-PCR reactions were carried out on BioRad CFX96 using HOT FIREPol® EvaGreen® qPCR Supermix (SolisBioDyne). The qRT-PCR reaction mixture included the following components: 5× Reaction Buffer, 4 μl; forward primer (10 μM), 0.4 μl; reverse primer (10 μM), 0.4 μl; cDNA, 1 μl; and dH_2O, 14.2 μl, in a final volume of 20 μl. The qRT-PCR reactions were started with an initial denaturation step (12 min at 95 °C) and proceeded with 40 cycles of amplification (15 s at 95 °C and 20 s at 60 °C). Melting curve analyses were performed to check for specificity of the amplifications. *secA* and *rpoB* were chosen as reference genes in relative quantification of gene expression (Florindo et al., 2012; Pinto et al., 2012). All reactions were performed