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HMG-CoA reductase is regulated by salinity at the level of transcription in *Haloferax volcanii*

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Abstract The moderately halophilic archaeon *Haloferax volcanii* was surveyed for protein profile changes correlated with growth at high and low salinity. A single polypeptide with an approximate mass of 46 kDa was conspicuously more abundant during growth at high salinity. This protein was identified as HMG-CoA reductase (HMGR), encoded by the *hmgR* gene. HMGR is a key enzyme in the mevalonate pathway of isoprenoid biosynthesis, the sole route in haloarchaea for lipid and carotenoid production. Enzymatic assays confirmed that HMGR activity is more abundant in cells grown at high salinity. Low salt cultures of *H. volcanii* contained lower amounts of *hmgR* transcript compared to cells grown in high salt suggesting that the observed regulation occurs at the level of transcription. Paradoxically, both lipid and carotenoid content decreased in *H. volcanii* grown at high salinity despite the increased levels of HMGR specific activity. To our knowledge, this is the first report demonstrating that the expression of HMGR is regulated in response to non-optimal salinity in a halophilic archaeon.

Keywords Haloarchaea · HMG-CoA reductase · Salt regulation · Lipids · Carotenoids

Introduction

Haloferax volcanii is an obligate halophile belonging to the domain Archaea. In contrast to extreme haloarchaea, which cannot grow below salinities of ~ 2.5 M NaCl and grow optimally above 4 M NaCl, *H. volcanii* is a moderate halophile that grows over a wide range of salinities, ranging from ~ 1.5 to 4 M NaCl, with optimal growth between 2 and 2.5 M NaCl (Mullakhanbhai and Larsen 1975). Due to its ability to cope with widely varying salinities, *H. volcanii* has been used as a model to examine both salinity-mediated gene regulation and protein expression (Daniels et al. 1984; Ferrer et al. 1996; Mojica et al. 1997; Bidle 2003). *H. volcanii* is also capable of growing over a relatively wide temperature range (~ 25–50°C) (Mullakhanbhai and Larsen 1975), and is a model for thermally responsive gene regulation as well (Daniels et al. 1984; Kuo et al. 1997; Thompson and Daniels 1998; Thompson et al. 1999). While these studies suggest that genome-wide regulation occurs in response to changing salinity, no specific molecular mechanisms for hypersaline adaptation have been elucidated to date.

The cell membrane is an essential factor in cell survival, particularly for extremophilic microorganisms, which by nature are subjected to vast and rapid environmental changes, be it in temperature, pressure, or salinity. These changes force adjustments in lipid composition and membrane permeability, via transport proteins, to facilitate survival (van de Vossenberg et al. 1998; Konings et al. 2002). Thus, the cell membrane of haloarchaea is a major factor in offering protection to the organism from the desiccating effects of high salt. Haloarchaea maintain osmotic homeostasis by utilizing a membrane bound H^+/Na^+ antiporter for the expulsion of Na^+ ions from the interior of the cell. Of equal importance is an inwardly oriented K^+ transporter that helps to maintain osmotic equilibrium with the environment. Haloarchaeal maintenance of osmotic homeostasis as well as cell membrane composition, structure, and function have all been extensively reviewed (Oren 2000; Kates and Moldoveanu 1991; Kamekura 1993; Kates and Kushwaha 1995; Kates 1996). However, despite the wealth of information on the haloarchaeal cell membrane, a clear picture detailing

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the how its adaptation to high salt is regulated at the molecular level is currently lacking.

Archaea possess unique ether-linked polar membrane lipids, a unique trait not shared by Bacteria or Eukarya. These polar lipids are composed of isoprenoid side chains linked to a glycerol phosphate backbone unlike fatty acid side chains in polar lipids found in the other two domains of life. Archaea and many eukaryotes are thought to synthesize isoprenoids solely via the mevalonic acid or mevalonate (MVA) pathway while some bacteria and photosynthetic eukaryotes utilize a non-MVA pathway, i.e., 1-deoxyxylulose-5-phosphate, or DXP pathway (Lange et al. 2000). To date, there has been no confirmation of the existence of a non-MVA pathway in Archaea. Thus, the only known mechanism for the biosynthesis of haloarchaeal polar lipids is via the MVA pathway (Tachibana et al. 1996). The reductive deacylation of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) catalyzed by HMG-CoA reductase (HMGR) is a signature of the MVA pathway that culminates in the production of isopentenyl diphosphate (IPP). IPP, and its isomer dimethylallyl diphosphate (DMAPP), are thought to be the sole biosynthetic precursors for all Archaeal isoprenoids and isoprenoid derivatives. Aside from polar lipids, IPP is also thought to be the precursor for all archaeal carotenoid pigments (Lange et al. 2000).

We have taken a dual genetic and biochemical approach towards identifying specific genes involved in hypersaline adaptation in *H. volcanii*. Using the molecular technique of RNA arbitrarily primed PCR (RAP-PCR) we were able to identify specific genes involved in adaptation to changing salinity in *H. volcanii* (Bidle 2003). Here, we present results from a complementary approach to identify proteins in *H. volcanii* that whose abundance changes in response to varying salinity. This approach has led to the unexpected discovery that HMGR expression is regulated by salinity in *H. volcanii*.

Materials and methods

Strains and growth conditions

Haloferax volcanii strain WFD11 (DSM 5716) was used in these studies (Charlebois et al. 1987). All cultures for these experiments were grown aerobically with vigorous shaking at 42°C in a medium containing, per liter, 125 g (2.1 M, "low-salt") or 206 g (3.5 M, "high-salt") NaCl, 45 g $MgCl_26H_2O$, 10 g $MgSO_47H_2O$, 10 g KCl, 1.34 ml 10% $CaCl_22H_2O$, 3 g yeast extract, and 5 g tryptone (Robb et al. 1995). For growth on solid medium, agar was added at a concentration of 15 g l^{-1}.

Protein analysis and identification

Haloferax volcanii cultures were grown to mid-exponential phase in low or high salt medium. Cells were harvested in a microcentrifuge, cell pellets were resuspended in gel loading buffer (525 mM Tris–HCl, pH 6.8, 5 mM EDTA, pH 8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol, 15% (v/v) glycerol) and heated at 100°C for 5 min prior to loading. Equal amounts of protein, as determined by modified Lowry protein assays (Pierce, Rockford, IL, USA), were resolved on a pre-cast 10% acrylamide gel (Bio-Rad, Hercules, CA, USA).

Individual protein bands were obtained for N-terminal sequencing analysis by separation of crude extracts on an 8% acrylamide gel. The gel was transferred onto a PVDF membrane, stained with Coomassie blue, and specific bands were excised from the blot with a sterile scalpel. Protein identification by mass spectrometry (LC–MS/MS) was performed using nanoscale LC–/MS/MS analysis methods (Link et al. 1999; Washburn et al. 2001). All MS/MS spectra were analyzed using the SEQUEST program.

RT-PCR analysis

Mid-exponential phase cultures of *H. volcanii* were harvested and RNA was extracted as previously described (Bidle 2003). For RT-PCR analysis, 10 µg of RNA was subjected to DNAseI digestion at 37°C for 30 min. The reaction was stopped with 0.5 M EDTA, pH 8, extracted with phenol:chloroform, ethanol precipitated, and quantified. A 20 µl first stand cDNA reaction was initiated by the addition of the reverse primer MevR (5′CAGGTTCTTGCGTGTGTTGAG TTC) and MMLV–RT (Ambion) to 1 µg of RNA obtained from either low or high salt cultures. First strand reactions were incubated at 37°C for one hour. Following first strand cDNA synthesis, both forward (MevF–5′CCGCTACGACACCAAGGC) and reverse primers targeting a 283 bp fragment of the *hmgR* gene were added into a standard PCR reaction (92°C 1 min, 50°C 1 min, 72°C 1 min for 30 cycles) using 10 µl of cDNA:mRNA template. 16S rDNA primers were used to perform a second RT-PCR analysis as an internal control performed in conjunction with the *hmgR* analysis. For this analysis, ten fold less starting material (0.1 µg of RNA) was used to create first strand cDNA.

Densitometry analyses both of RT-PCR reactions and protein gel results were performed using the Kodak Digital Science 1D Image Analysis Software package.

Lipid extraction and analysis

Haloferax volcanii lipids were extracted using the Bligh-Dyer method (Bligh and Dyer 1959) as adapted from http://www.sfu.ca/bisc/bisc-429/lipidextract.html. Briefly, 500 mg of wet packed cells were resuspended in 800 µl of nanopure H_2O. HPLC grade chloroform (1.0 ml) and HPLC grade methanol (2.0 ml) were added and samples were homogenized with a Pasteur pipette.

After 10 min at room temperature, chloroform and water (1.0 ml each) were added and samples homogenized again. Centrifugation for 30 min at $2{,}500 \times g$ produced two layers, the lower of which was transferred into clean glass vials. The solvent was evaporated under a stream of nitrogen and samples stored at −20°C after sealing with a Teflon lid. The lipid sample was resuspended in Bligh-Dyer solvent to a concentration equivalent to 3×10^{10} cells ml^{-1} based on direct cell counts of the original cultures. TLC was performed as described by Sprott et al. (2003) on 10 µl samples of each extract.

Lipids were identified by comparison of the R_f and relative abundance of lipids observed in this study (Table 2) with those reported by Sprott et al. (2003). The presence of the phosphate group for ArGlyMeP and ArGlyP was confirmed with molybdenum blue staining (Table 2) (Kundu et al. 1977). Lipids were quantified using ImageQuaNT software after primuline staining using phosphatidylcholine, myristylphosphatidylcholine and phosphatidylethanolamine (Sigma-Aldrich, St Louis, MO, USA) as standards. Phospholipids were stained with molybdenum blue. Each strain was analyzed in duplicate from independent cultures harvested at the same stage of growth.

HMGR activity measurements

The HMGR activity was assayed as previously reported (Bischoff and Rodwell 1996) with the exception that cells were lysed by two stage sonication using a model 450 sonifier equipped with a microtip probe (Branson Ultrasonics, Danbury, CT, USA). The first stage used a power setting of 2, 50% duty cycle for 2 min followed by cooling on ice for 10 min. The second stage used a power setting of 4, 50% duty cycle for 1 min followed by cooling on ice. Extracts were clarified by centrifugation at $10{,}000 \times g$ for 5 min at room temperature.

Membrane and soluble fractions were prepared from clarified extracts by centrifugation at $100{,}000 \times g$ for 60 min at 15°C in a TLA 55 rotor using an Optima Max tabletop ultracentrifuge (Beckman-Coulter, Fullerton, CA, USA). The supernatant was transferred to a fresh tube and the membrane pellet was rinsed with 0.5 ml lysis/assay buffer and then resuspended in 0.5 ml of lysis/assay buffer by sonication.

Extracts of *H. volcanii* were also produced using a French pressure cell (SLM Aminco, Rochester, NY, USA). Cell pellets were resuspended in the same buffer reported by Bischoff and Rodwell (1996) and lysed by two passages through the pressure cell at 16,000 psi. Membrane and soluble fractions were prepared as above.

Absorption spectroscopy

Absorption spectra of Bligh-Dyer extracts and soluble protein extracts were recorded from 250–750 nm in a DU7400 spectrophotometer (Beckman-Coulter, Fullerton, CA, USA). Soluble extracts were converted to Bligh-Dyer extracts by adding appropriate amounts of methanol and chloroform to the sample. Precipitated salt and protein were removed by centrifugation at $16{,}000 \times g$ for 5 min sat room temperature. Spectra were recorded from samples at a concentration of 3×10^{10} cells ml^{-1} of Bligh-Dyer solvent and are averages of duplicate samples.

Direct cell counting

Paraformaldehyde fixed cell suspensions were diluted with 2.5 M NaCl and loaded into a hemacytometer (Hausser Scientific, Horsham, PA, USA). Cells were manually counted at 400× magnification under phase contrast on a BX61 microscope equipped with a 40× UPlanFl Ph2 objective (Olympus, Melville, NY, USA).

Results

HmgR abundance increases with increased salinity in *H. volcanii*

As part of ongoing efforts to examine differential gene expression in *H. volcanii* in response to changing salinity, crude protein extracts from cultures grown in optimal low salt (12% or 2.1 M) or high salt (20% or 3.5 M) medium were compared by one-dimensional SDS-PAGE analysis. This analysis clearly revealed that a 46 kDa protein accumulated to significantly higher levels (∼ six fold) in extracts prepared from high salt cultures (Fig. 1). This protein was gel-purified and subjected to N-terminal sequencing and LC–MS/MS analysis for identification. The protein was identified as HMGR, an essential enzyme involved in the synthesis of mevalonic acid, an obligate intermediate of the IPP biosynthetic pathway leading to archaeal membrane lipids and carotenoids.

HmgR mRNA abundance is increased by high salinity in *H. volcanii*

Total RNA was isolated from *H. volcanii* grown under high or low salt conditions and *hmgR* mRNA abundance was identified using an RT-PCR approach. The observed 283 bp internal fragment of the *hmgR* transcript was found to increase in abundance with increasing salinity by a factor of 2.2-fold (Fig. 2). Indeed, replicate experiments demonstrated that *hmgR* transcript abundance consistently increases in response to high salinity by an average factor of 2.3 ± 0.2-fold. This result indicates that the salinity-mediated regulation of HMGR protein abundance occurs at the level of transcription. Northern analyses performed in conjunction with RT-PCR yielded similar results (data not

shown). The specific mechanism of transcriptional regulation, i.e., increased transcription by relief of repression, enhancement of transcription initiation or stabilization of *hmgR* mRNA, remains to be determined.

HMGR activity is regulated by salinity in *H. volcanii*

HMGR activity was assayed to determine whether the changes in HmgR protein abundance significantly affected the level of activity observed. HMGR activity was found to be completely soluble in *H. volcanii* (> 96% of activity in 100,000 × *g* supernatant, Table 1) in agreement with prior observations (Bischoff and Rodwell 1996). *H. volcanii* HMGR activity was found to increase three fold when extracts from high salt grown cells were compared to those from low salt grown cells. As all extracts were assayed at the same salt concentration, this change in specific activity suggests that there is more active HmgR protein in cells grown at high salinity. This corroborates the observed increase in both *hmgR* transcript abundance and HmgR protein levels in high salt cultures and provides further support to the notion that *H. volcanii* regulates the amount of HMGR activity in response to salinity.

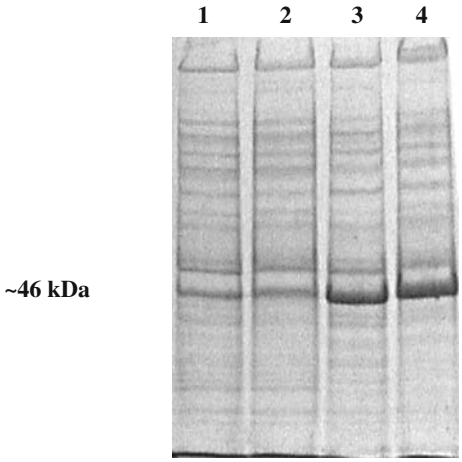

Fig. 1 SDS-PAGE analysis of wild-type *Haloferax volcanii*. *Lanes* 1 and 2, duplicate cultures of wild-type grown in low salt medium; *lanes* 3 and 4, duplicate cultures of wild-type grown in high salt medium

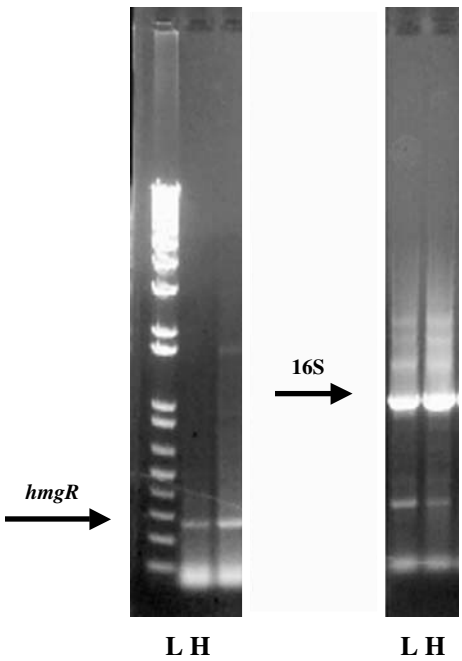

Fig. 2 RT-PCR analysis of *hmgR* expression in *H. volcanii* strains (*L*), low salt and (*H*), high salt. An *arrow* indicates the position of the ~ 283 bp partial fragment of the *hmgR* transcript. An *arrow* also indicates the position of the 937 bp partial fragment of the 16 S transcript used as an internal control

Haloferax volcanii HMGR activity detected in this study, 0.69 U (mg protein)$^{-1}$ (Table 1), was considerably higher than that previously reported, 0.014 U (mg protein)$^{-1}$, using the same assay conditions (Bischoff and Rodwell 1996). This may be due to the use of sonication in this study, rather than a French pressure cell, to produce the cell free extracts used in the assay. To address this, *H. volcanii* extracts were also prepared with a French pressure cell in this study. These extracts contained a soluble HMGR activity that was measured at 0.063 ± 0.025 U (mg protein)$^{-1}$ in low salt grown cells, a value much closer to the activity observed in the original studies of HMGR activity in *H. volcanii*. The activity in high salt grown cells was 0.31 ± 0.12 U (mg protein)$^{-1}$ in French pressure cell extracts, a five fold increase over the low salt grown cells. Thus, while the total level of HMGR activity varies in extracts prepared by sonication or French pressure cell, the increase in activity observed in high salt cultures is consistently observed.

Lipid content and composition of *H. volcanii* is strongly affected by salinity

To determine the effect of increased HMGR activity on the end products of isoprenoid biosynthesis, the bulk lipid composition of *H. volcanii* grown in low or high

Table 1 HMGR activity in *H. volcanii* grown at different salinities

Growth salinity/extract fraction	Specific activity[a]
2.1 M NaCl	
100,000 × *g* supernatant	0.69 ± 0.05
100,000 × *g* pellet	0.023 ± 0.003[b]
3.5 M NaCl	
100,000 × *g* supernatant	2.01 ± 0.07
100,000 × *g* pellet	Not Detected

[a]One unit of specific activity is the HMG-CoA dependent oxidation of 1 µmol NADPH min^{-1} (mg protein)$^{-1}$. Values are the means ± SD of three determinations

[b]Upon repeating ultracentrifugation, this activity was found to be soluble and was likely non-specifically trapped (data not shown)

salt culture conditions was compared by TLC analysis of Bligh-Dyer extracts (Fig. 3 ; Table 2). This procedure has been successfully utilized for lipid analysis of both *Natronobacterium magadii* and *Halobacterium salinarum* (Qin et al. 1998, 2000). Three abundant lipids were routinely detected in all samples: archaetidyl glycerol methylphosphate, archaetidyl glycerol phosphate, and the sulfated glycolipid S-DGD-1. Surprisingly, our results indicate that the lipid content of *H. volcanii* cells grown under high salt conditions is ~ 60% lower than cells cultured in low salt. The relative abundance of the three major lipids also varies in response to salinity. At high salt, archaetidyl glycerol phosphate abundance decreases ~ 10% coupled to a ~ 10% increase of sulfated glycolipid. This change is expected to increase the negative charge in the membrane due to a greater abundance of ionized head groups associated with the sulfated glycolipid. An increase in negative charges in the membrane at non-optimal salinities in haloarchaea has been hypothesized to lend increased stability to the cell wall structure via increased interactions with positively charged Na^+ and Mg^{2+} ions (Grant 2004).

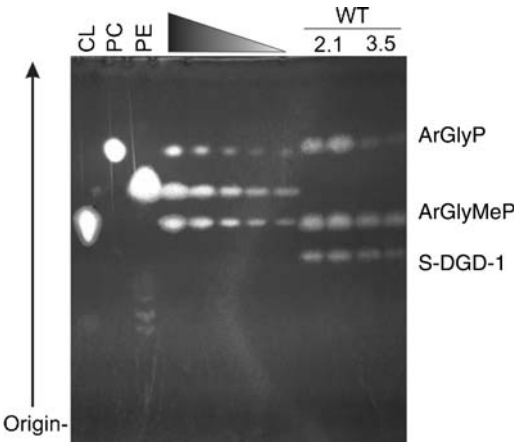

Fig. 3 Analysis of lipids in Bligh-Dyer extracts from *H. volcanii* wild type cultures grown in low or high salt conditions. Lipids were separated by TLC and visualized with primuline staining. Duplicate samples equivalent to 1×10^{10} cells for each strain grown at either 3.5 or 2.1 M NaCl were loaded. Lipid standards for quantification were cardiolipin (*CL*), phosphatidylcholine (*PC*) and phosphatidylethanolamine (*PE*). *ArGlyP*-archaetidyl gycerol methylphosphate, *ArGlyMeP*-archaetidyl glycerol phosphate, *S-DGD- 1*-sulfated glycolipid. The *triangle* represents decreasing amounts of a mixed standard containing from 2.5 µg of each lipid to 0.25 µg of each lipid

Table 2 Analysis of Bligh-Dyer extracts of *H. volcanii* via TLC

Lipid	R_f[a]	PO_4[b]	Lipid content (%)	
			2.1 M	3.5 M
Archaetidyl glycerol phosphate	0.75	+	0.69[c] (37)	0.18 (24)
Archaetidyl glycerol methylphosphate	0.53	+	0.83 (44)	0.35 (47)
Sulfated glycolipid (S-DGD-1)	0.43	–	0.36 (19)	0.21 (29)
Total			1.88 (100)	0.74 (100)

[a]Relative mobility compared to the solvent front. Solvent was described by Sprott (2003)
[b]Result of molybdenum blue staining for phosphate content
[c]Micrograms of lipid per 1×10^8 cells

Total carotenoid content of *H. volcanii* decreases with increased salinity

Changes in carotenoids, the other major end product of isoprenoid metabolism, were also observed when high and low salt grown *H. volcanii* were compared. Absorption spectra of Bligh-Dyer extracts from whole cells indicated that, on a per cell basis, cells grown at high salt contained less carotenoid when compared to cells grown at low salt conditions (Fig. 4). Each peak in the carotenoid region of the absorption spectrum (350–600 nm) decreased by a factor of 1.6- to 1.7-fold in high salt grown cells. This decrease indicates that all carotenoids were affected equally, rather than one particular carotenoid changing in abundance. This observation, together with the changes observed in lipid content per cell, is in stark contrast to the increased HMGR activity seen in high salt growth conditions. While increased HMGR activity might be expected to increase flux through the MVA pathway thereby increasing lipid and carotenoid content per cell, our results indicate this is clearly not the case.

Discussion

The integrity of cell membranes is essential for cellular fitness and survival particularly for microorganisms inhabiting an extreme environment. Haloarchaea inhabit niches composed of extremely high, and often varying, salinity. During the course of our investigations

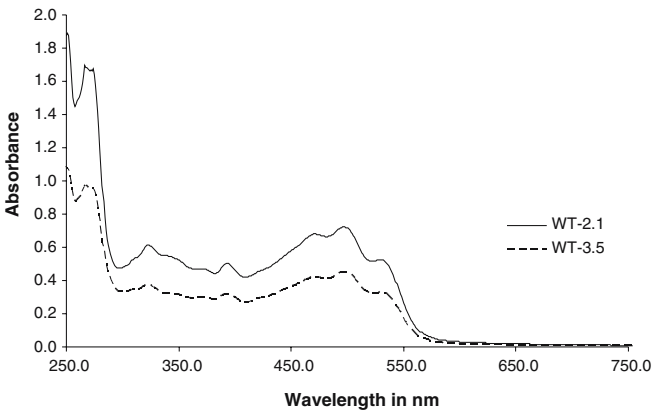

Fig. 4 Absorption spectra of Bligh-Dyer extracts from whole cells. Spectra are normalized to a cell concentration of 3×10^{10} cells ml^{-1}. *Solid lines*,wild-type cultured in low salt; *broken lines*, wild-type cultured in high salt

into hypersaline adaptive strategies employed by *H. volcanii*, we have discovered that the enzyme HMGR is regulated by salinity. HMGR is a key step in the MVA pathway of isoprenoid biosynthesis leading to the synthesis of unique archaeal lipids and carotenoids. To our knowledge, this is the first report demonstrating that *H. volcanii* regulates the expression of HMGR in response to non-optimal salinity, although it has been observed in a halophilic yeast (Petrovic et al. 1999). These observations suggest that salt may regulate the rate of membrane and carotenoid biosynthesis at the level of MVA pathway flux in those halophilic organisms that utilize the MVA pathway for isoprenoid synthesis. While the specific pathway of salt sensing and response is unknown, we have isolated a *H. volcanii* strain, HvM7, which displays a pleiomorphic phenotype which includes altered membrane properties, deficiencies in carotenoid localization, irregular morphology, and non-existent HMGR activity. HvM7 will be a useful tool for dissecting salinity regulation in the haloarchaea as the mutation is not associated with the *hmgR* gene (K. Bidle, T. Hanson, K. Howell, and J. Nannen, unpublished data).

Wild-type *H. volcanii* induces increased production of *hmgR* transcript, HmgR protein, and displays higher levels of HMGR specific activity while growing under high salt conditions. At first glance this enhanced activity would be expected to produce increased lipid and carotenoid content in the membrane. Paradoxically, we have shown on a per cell basis that both lipid and carotenoid content actually decrease under non-optimal high salt growth in *H. volcanii*. Previous reports of salinity-mediated changes in lipid and/or carotenoid content in haloarchaea have described similar observations. In a study by Kushwaha et al., two haloarchaeal strains, *Hbt. salinarum* (formerly *Hbt. cutirubrum*) and *H. mediterranei* (formerly strain R-4) were shown to display both decreased lipid and carotenoid content as salinity deviated from optimal (Kushwaha et al. 1982). In the case of *Hbt. salinarum*, as salinity decreased from its optimal of 30% NaCl, total lipid content decreased and the cells became virtually unpigmented at 15% NaCl. Compatible results were obtained upon examination of *H. mediterranei* which also displayed decreased lipid profiles as salinity increased away from its optimal of $\sim$ 15% NaCl. In addition, *H. mediterranei* displayed stronger pigmentation at its optimal salinity and this pigmentation decreased as salinity deviated from optimal. Taken together, these results in conjunction with our own observations suggest the possibility that increased membrane turnover rates may occur in haloarchaea when subjected to non-optimal salinity. Increased membrane turnover would require an increased flux through the MVA pathway of isoprenoid biosynthesis to replace lost or damaged lipids and/or carotenoids. Our results demonstrating increased HMGR activity at high salinity would corroborate this hypothesis.

Here we have presented the first molecular evidence demonstrating that *H. volcanii* cell membrane biosynthesis is regulated in response to changes in salinity. Ongoing studies in *H. volcanii* are focused on dissecting the molecular mechanisms regulating *hmgR* expression, investigating the rates of membrane turnover at differing salinity, and further examining how the interplay of lipid and carotenoid composition helps *H. volcanii* survive and grow in hypersaline environments.

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