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VapC6, a ribonucleolytic toxin regulates thermophilicity in the crenarchaeote Sulfolobus solfataricus

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ABSTRACT

The phylum Crenarchaeota includes hyperthermophilic micro-organisms subjected to dynamic thermal conditions. Previous transcriptomic studies of Sulfolobus solfataricus identified vapBC6 as a heat-shock (HS)-inducible member of the Vap toxin–antitoxin gene family. In this study, the inactivation of the vapBC6 operon by targeted gene disruption produced two recessive phenotypes related to fitness, HS sensitivity and a heat-dependent reduction in the rate of growth. In-frame vapBC6 deletion mutants were analyzed to examine the respective roles of each protein. Since vapB6 transcript abundance was elevated in the vapC6 deletion, the VapC6 toxin appears to regulate abundance of its cognate antitoxin. In contrast, vapC6 transcript abundance was reduced in the vapB6 deletion. A putative intergenic terminator may underlie these observations by coordinating vapBC6 expression. As predicted by structural modeling, recombinant VapC6 produced using chaperone cosynthesis exhibited heat-dependent ribonucleolytic activity toward S. solfataricus total RNA. This activity could be blocked by addition of preheated recombinant VapB6. In vivo transcript targets were identified by assessing the relative expression of genes that naturally respond to thermal stress in VapBC6-deficient cells. Preferential increases were observed for dppB-1 and tetR, and preferential decreases were observed for rpoD and eif2 gamma. Specific VapC6 ribonucleolytic action could also be demonstrated in vitro toward RNAs whose expression increased in the VapBC6-deficient strain during heat shock. These findings provide a biochemical mechanism and identify cellular targets underlying VapBC6-mediated control over microbial growth and survival at temperature extremes.

Keywords: antitoxin; RNA degradation; heat shock; archaea; transcription

INTRODUCTION

Despite the intrinsically high temperatures found in hydrothermal niches, thermotolerance and the heat-shock response are probably essential for endogenous microbial survival and proliferation. Frequent shifts in geological activity foster steep thermal gradients, necessitating the capacity to respond rapidly to this type of challenge, particularly by planktonic organisms. Diverse taxa belonging to the order Sulfolobales within the archaeal phylum Crenarchaeota are common inhabitants of acidic geothermal pools. The heat-shock response of the thermoacidophile, Sulfolobus shibatae, was found to involve a generalized reduction in protein synthesis with continued production of a eukaryotic-like molecular chaperone, termed the thermosome (Trent et al. 1991). Additional components of the heat-shock (HS) response in this organism include adaptive heat resistance by pre-treatment (Trent et al. 1990) and heat-induced shifts in the proteome (Cerchia et al. 2000). In a related species, Sulfolobus solfataricus, the HS response transcriptome associated with a nonlethal temperature shift involved approximately one-third of the genome (Tachdjian and Kelly 2006). However, transcription factors that control thermotolerance are missing in crenarchaeotal genomes such as those characterized in the other major archaeal phylum, the Euryarchaeota (Thompson et al. 1999; Vierke et al. 2003; Liu et al. 2007). Eukaryotic factors (Vuister et al. 1994; Yamamoto et al. 2008; Westerheide et al. 2009) and bacterial sigma factors (Rodriguez et al. 2008; Wagner et al. 2009) and bacterial sigma factors (Rodriguez et al. 2008; Wagner et al. 2009) also are not evident in crenarchaeotal genomes, and an underlying mechanism governing the heat-shock response in these organisms has not been identified.

Type II Toxin–antitoxin (TA) loci are found in nearly all prokaryotic genomes and are thought to promote survival during environmental stress (Gerdes et al. 2005; Makarova

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et al. 2009). Type II TA systems consist of an intracellular protein toxin coexpressed with a cognate protein antitoxin. These two components are usually contiguous and encoded on the genome or plasmids. Antitoxins have been shown to bind and inactivate cognate toxins, while the antitoxin can be susceptible to protease cleavage due to its natively unfolded C-terminal domain (Gerdes et al. 2005; Pandey and Gerdes 2005). When not bound to the antitoxin, some toxins act as ribonucleases and cleave mRNA, thus modulating protein synthesis (Jensen and Gerdes 1995). Consequently, imbalanced concentrations of stable toxin and unstable antitoxin may control protein expression in the cell (Jensen and Gerdes 1995).

Currently, there are eight major TA families encoded on plasmids and chromosomes (Gerdes 2000; Pandey and Gerdes 2005), although this number continues to increase as more genomes are examined (Makarova et al. 2009). In archaea, there are three toxin–antitoxin families that have been identified: RelBE, Phd/Doc, and VapBC (Gerdes et al. 2009). Type II TA systems consist of an intracellular protein toxin coexpressed with a cognate protein antitoxin. These two components are usually contiguous and encoded on the genome or plasmids. Antitoxins have been shown to bind and inactivate cognate toxins, while the antitoxin can be susceptible to protease cleavage due to its natively unfolded C-terminal domain (Gerdes et al. 2005; Pandey and Gerdes 2005). When not bound to the antitoxin, some toxins act as ribonucleases and cleave mRNA, thus modulating protein synthesis (Jensen and Gerdes 1995). Consequently, imbalanced concentrations of stable toxin and unstable antitoxin may control protein expression in the cell (Jensen and Gerdes 1995).

In the Vap family, VapB is the unstable antitoxin and VapC is the stable toxin. The VapC toxin is identified by the presence of a PIN (PilT N-terminal) domain, which is indicative of RNase activity (Anantharaman and Aravind 2003). The crystal structure of an archaeal PIN domain toxin, PAE2754, shows homology with RNase H, but exhibits DNA not RNA nuclease activity (Arcus et al. 2004; Bunker et al. 2008). Bacterial VapC’s have been studied in some detail. VapC-1 of nontypeable Haemophilus influenzae has ribonuclease activity that could be inhibited by the VapB-1 antitoxin (Daines et al. 2007). FitB of Neisseria gonorrhoeae modulates virulence through transcellular trafficking and structurally resembles PAE2754, although insolubility of the recombinantly produced protein precluded assessment of its activity (Hopper et al. 2000; Mattison et al. 2006). NtrR of Sinorhizobium meliloti controls metabolism under symbiosis and stress (Bodogai et al. 2006). Finally, while VapC5 from several species of Mycobacteria have undergone structural (Miallau et al. 2009) and in vivo characterization, a biologic function has yet to be identified (Robson et al. 2009). In S. solfataricus, the strong and rapid heat-shock induction of certain vapBC loci (Tachdjian and Kelly 2006), combined with preliminary information on an uncomplemented vapBC mutant (Cooper et al. 2009), provided the impetus to investigate the function of the toxin and antitoxin belonging to a single family member, vapBC6, and a role in thermotolerance.
strain PBL2025 (Fig. 2A). Lactose utilizing recombinants arose as a result of a simultaneous triple recombination event. These were shown to encode a \( \text{lacS} \)-disrupted copy of \( \text{vapB6} \) by PCR (Fig. 2B) and by DNA sequence analysis. The insertion was placed 51 nt 3’ of the \( \text{vapB6} \) start codon, resulting in strain PBL2066 (Fig. 1A).

Thermotolerance was measured as the percentage of culturable cells surviving exposure to lethal heat shock (Fig. 2C). After exposure, cells were transferred to solid medium and incubated at 80°C until colonies formed. A normally nonlethal temperature shift from 80°C to 95°C resulted in preferential killing of the \( \text{vapB6} \) disruption strain PBL2066. Killing was evident after 1 h at 95°C and became more pronounced with continued exposure. Thermotolerance of the mutant was reduced 100-fold relative to the wild type after 5 h of heat shock. Under the conditions used, the wild-type strain exhibited a 50% reduction in thermotolerance after 2 h of exposure, but then stabilized at this level for the duration of the exposure period. No significant cell lysis was apparent for either strain during the entire exposure period, indicating that cells retained their integrity despite a loss of viability.

Since \( \text{vapBC6} \) is expressed during growth at normal temperatures (Fig. 1B), an effect resulting from mutation of this locus could also have an impact at other temperatures. To assess the role of VapBC6 on biological fitness, the rate of growth at temperatures ranging from 70°C to 90°C was evaluated for the \( \text{vapB6} \) disruption mutant and wild-type strain (Fig. 2D). Generation times were determined across the temperature growth range of the organism and the results are depicted in an Arrhenius plot, where growth rate is plotted as a function of the reciprocal of absolute temperature. Cells were cultivated in a glucose minimal medium to maximize anabolic requirements, and thereby enhance detection of differences in fitness. Reduced rates of growth were observed for the \( \text{vapB6} \) disruption mutant at all temperatures above 70°C relative to the wild-type control strain.

Complementation analysis was conducted to confirm that reduced thermotolerance and growth rate of the \( \text{vapB6} \) disruption mutant arose from \( \text{vapBC6} \) deficiency. Two genetic markers were required to conduct this test; \( \text{lacS} \) was used for \( \text{vapB6} \) disruption, and a new genetic marker, \( \text{Sso malA} \) (Rolfsmeier et al. 1998), was used as the genetic marker gene to select for a \( \text{vapBC6} \) recombinant shuttle plasmid (Table 1). Simultaneous use of these genetic markers required construction of a strain (PBL2069) that was deficient in utilization of both lactose and maltose as sole carbon and energy sources (Table 2). Although \( \text{Sso} \) encodes six annotated \( \alpha \)-glucosidases (She et al. 2001; Kim et al. 2004), deletion of only three was sufficient to create an absolute catabolic defect for maltose utilization. \( \alpha \)-glucosidase-specific activities for several strains were measured to assess the relative importance of each of these genes. Simultaneous inactivation of \( \text{malA} \) (SS03051), \( \text{xylS} \) (SSO3022), and SSO0990, resulting in strain PBL2069, reduced \( \alpha \)-glucosidase activity over 100-fold (Table 2) and blocked growth on maltose as the sole carbon and energy source. A \( \text{vapB6} \) disruption mutant derivative of PBL2069 was then constructed by linear DNA recombination using the PCR-amplified \( \text{vapB6} \) mutation as the recombination template, creating strain PBL2081. Plasmid pBN1080 encoding the wild-type \( \text{vapBC6} \) operon, and \( \text{malA} \) as a selectable marker (Fig. 3A), was then

**FIGURE 2.** Construction and characterization of a \( \text{vapB6}::\text{lacS} \) disruption. (A) Multiplex linear recombination for \( \text{vapB6} \) disruption. Two overlap extension PCR products containing \( \text{lacS} \) and either the 5’ or 3’ end of \( \text{vapB6} \) were cotransformed into PBL2025. Recombinants arose by three crossover events (X’s) between the two incoming fragments and the corresponding chromosomal allele. Primers used to verify identities of \( \text{vapB6} \) alleles are indicated. (B) Mutant genotype. PCR amplification of the wild-type (lane 1) and \( \text{lacS} \) disrupted alleles of \( \text{vapB6} \). (C) Thermotolerance of the \( \text{vapB6} \) mutant. Wild-type (●) and \( \text{vapB6}::\text{lacS} \) disruption mutant (○) were cultivated in rich medium and shifted from 80°C to 95°C for the times indicated. Culturable cells were enumerated on solid medium with incubation at 80°C. (D) Arrhenius plot. The growth rate constant (\( k \)), which is related inversely to generation time, is portrayed on the y-axis. The temperature in °K is indicated on the x-axis, and the temperature in °C is indicated across the top of the figure. Strains were: wild-type (●) and \( \text{vapB6}::\text{lacS} \) disruption mutant (○). Values for the average of two independent experiments are shown.
alternative mechanisms such as VapC6. VapB6 was responsible for HS sensitivity, and the phenotype suggested that a deficiency of normal growth temperatures could also influence survival during heat shock. Complementation of vapB6 disruption mutant (PBL2080) lacked detectable vapC6 mRNA and the ΔvapB6 mutant (PBL2078) lacked detectable vapB6 mRNA. Thermal conditions producing rates of killing of the wild type similar to those used to test complementation (Fig. 3B) indicated that both vapB6 and vapC6 in-frame deletions mutants were thermally sensitive after at least 1 h of heat shock relative to the wild type (data not shown). To clarify the basis for this result, levels of vapB6 and vapC6 transcript abundance were determined by qRT–PCR in each of the respective deletion mutants, as well as both the vapB::lacS disruption and wild-type strains, during normal growth and in response to heat shock (Fig. 4B). Abundance of vapBC6 mRNA was normalized to the transcript abundance of tbp as an internal control for each RNA sample tested, variation was <10% in all cases. In general, both vapB6 and vapC6 mRNA levels were heat-shock inducible in all strains examined; however, their abundance under steady-state growth conditions was differentially affected by the various mutations. At 80°C, the abundance of vapB6 mRNA increased eightfold in the vapC6 in-frame deletion strain relative to the wild type. In contrast, the abundance of vapC6 mRNA decreased over 10-fold in both the vapB6 disruption mutant and the in-frame

**Coupling of vapB6 and vapC6 expression**

Though the vapB6 disruption mutant phenotype suggested that a deficiency of VapB6 was responsible for HS sensitivity, alternative mechanisms such as VapC6 toxin deficiency or VapB6 and C6 deficiency remained plausible. To discriminate between these possibilities, in-frame deletion mutants lacking either vapB6 or vapC6 were constructed and evaluated. In-frame deletions were created by markerless exchange using internally truncated reading frames (Fig. 1A; Schelert 2006). RT–PCR using reverse primers complementary to sequences within the deleted region confirmed loss of each respective transcript (Fig. 4A). Total RNA derived from cells growing at normal growth temperatures (80°C) was used as the template in these reactions. The ΔvapC6 mutant (PBL2080) lacked detectable vapC6 mRNA and the ΔvapB6 mutant (PBL2078) lacked detectable vapB6 mRNA. Thermal conditions producing rates of killing of the wild type

### TABLE 1. Archaeal strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL2009</td>
<td>malA::lacS</td>
<td>PBL2002 (Hoang et al. 2004)</td>
</tr>
<tr>
<td>PBL2025</td>
<td>Δ::SSO3004–SSO3050</td>
<td>PBL2000 (Schelert et al. 2004)</td>
</tr>
<tr>
<td>PBL2027</td>
<td>Δ::SSO3017–SSO3052</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2069</td>
<td>Δ vapC6 (nt 691–1854) in PBL2027</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2066</td>
<td>vapB6::lacS</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2078</td>
<td>Δ vapB6 (nt 39–267)</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2080</td>
<td>Δ vapC6 (nt 36–291)</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2081</td>
<td>vapB6::lacS in PBL2069</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2082</td>
<td>PBL2081 with pBN1096</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2089</td>
<td>PBL2081 with pBN1080</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>bla</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pBN1074</td>
<td>pJlacS, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Berkner et al. 2007)</td>
</tr>
<tr>
<td>pBN1080</td>
<td>pJmalA, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>pBN1074 (this work)</td>
</tr>
<tr>
<td>pBN1096</td>
<td>vapBC6 in pBN1080</td>
<td>pBN1080 (this work)</td>
</tr>
</tbody>
</table>

### TABLE 2. S. solfataricus alpha-glucosidases

<table>
<thead>
<tr>
<th>Strain</th>
<th>Orf (Mutation)</th>
<th>Sp. act. (μmol of p-nitrophenol/min/mg)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>none</td>
<td>47.8 27.4</td>
</tr>
<tr>
<td>PBL2025</td>
<td>SSO3022 (Δ xy/S)</td>
<td>39.9 29.1</td>
</tr>
<tr>
<td>PBL2009</td>
<td>SSO3051 (malA::lacS)</td>
<td>1.87 1.57</td>
</tr>
<tr>
<td>PBL2027</td>
<td>SSO3051 (Δ malA)</td>
<td>0.18 0.02</td>
</tr>
<tr>
<td>PBL2069</td>
<td>SSO3022 (Δ xy/S)</td>
<td>&lt;0.01 &lt;0.01</td>
</tr>
<tr>
<td>PBL2069</td>
<td>SSO3051 (Δ malA)</td>
<td>SSO3022 (Δ xy/S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSO0990 (Δ SSO0990)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All assays were conducted in replicate and varied by <0.05.
vapB6 deletion mutant during growth at 80°C. This latter observation is consistent with the heat-shock sensitivity of both mutant strains. No transcript was evident for vapB6 in the vapB6 in-frame deletion strain or for vapC6 in the vapC6 in-frame deletion strain. Examination of the vapBC6 locus identified a single putative intergenic terminator located between vapB6 and vapC6 (Fig. 1A). This stem–loop would sequester the C-terminal 33 nt of the vapB6 coding region and 23 nt of the flanking vapB6–vapC6 intergenic region. Analysis using MFOLD (ver. 2.3) (Zuker 2003) indicates that this stem–loop has a ΔG of -2.45 kcal/mol and Tm 86.3°C at standard conditions (80°C, 1 M NaCl, 0 Mg) and, therefore, could play a role in the expression of vapC6 in a manner that would depend on vapB6 translation. These data indicate that expression of these genes is coupled, and suggest that RNA degradation, perhaps mediated by VapC6, could be involved in modulating transcript abundance in response to heat shock.

**Structure and activity of VapC6**

VapC homologs from bacteria have been reported to have ribonuclease activity (Daines et al. 2007; Miallau et al. 2009), while VapC (PAE0151) from the archaeon Pyrobaculum aerophilum has DNA exonuclease activity (Arcus et al. 2004). To clarify the situation for the Sso VapC6, the amino acid sequence for SSO1493 was analyzed with the 3D-JIGSAW prediction tool (Bates and Sternberg 1999; Bates et al. 2001) using PDB values for the PIN-domain protein and putative toxin from Pyrobaculum aerophilum PAE0151 (PDB:2FE1) (Arcus et al. 2004). The model showed >90% secondary structure homology with the template, and the confidence level of structure predictions (or “accuracy”) was >95%, indicative of a high-quality match.

**FIGURE 3.** Complementation of vapB6::lacS disruption using malA-based selection. (A) Complementation plasmid pBN1096. Plasmid pBN1096 was derived from plasmid pBN1080 by insertion of vapBC6 into the NotI site. Plasmid pBN1080 was derived from pBN1074 (Berkner et al. 2007) by replacement of lacS with malA at the SacII site. (B) Percent survival of the vapB6 disruption mutant and complemented strain during heat shock. Cultures were shifted to 95°C and samples were taken thereafter every 0.5 h. Strains were: wild-type (PBL2025, ●); vapB6::lacS disruption (PBL2081, ○), vapB6::lacS complemented mutant (PBL2082, ▽).

**FIGURE 4.** Expression and coupling of vapB6 and vapC6. (A) Detection of vapBC6 transcript. RT–PCR was used to detect transcripts present in wild-type (PBL2025), the vapC6 deletion strain (PBL2080), and the vapB6 deletion strain (PBL2078). (Lanes 1,2) vapB6 transcript with reverse transcriptase (RTase) added or omitted, respectively; (lanes 3,4) vapC6 transcript with RTase added or omitted, respectively; (lane 5) 7S RNA. Location of vapBC6 deleted regions is shown in Figure 1A. (B) Abundance of vapB6 and vapC6 mRNA. qRT–PCR primers used to assess transcript abundance were complementary to regions that remained present in all strains tested. RNA was extracted from cells growing exponentially at the temperatures indicated: (open bars) 80°C; (closed bars) 95°C. Strain genotypes for vapB6 and vapC6, respectively, are indicated across the top of the figure. Strain and relevant vapBC6 genotypes were: + +, wild-type; d +, vapB6::lacS disruption; – +, vapB6 deletion strain; + –, vapC6 deletion. The tbp mRNA was used as an internal control in each RNA sample to normalize vapBC6 values; tbp mRNA varied in abundance by <15% in these experiments.
VapC6 is missing about 30 residues at the beginning of the template when compared with PAE0151, resulting in a more open conformation at the active site. When comparing this predicted structure to a known ribonuclease, T4 RNAse H (PDB: 1TFR), using an in silico superimposition tool LOCK 2 (Shapiro and Brutlag 2004), all of the predicted catalytic residues in VapC6 matched known T4 RNAse catalytic residues (Bhagwat et al. 1997), and the overall RNAse H active site configuration was conserved in the toxin (data not shown).

To test the prediction that the S. solfataricus VapC6 was a ribonuclease, efforts to produce this and its cognate antitoxin, VapB6, as recombinant proteins in E. coli were undertaken. VapC6 or hexahistidine N- or C-terminal-tagged derivatives expressed in E. coli were determined by tandem mass spectrometry are indicated (arrowheads). 

FIGURE 5. In vivo solubilization of VapC6_His and VapB6_His. SDS-PAGE analysis of E. coli crude extracts (lanes 2,4,6) or Ni-NTA column eluants (lanes 1,3,5). Extracts were prepared from strains containing plasmids expressing vapC6_His (lanes 1,2), both vapC6_His and dnaK (lanes 3,4), and vapB6_His (lanes 5,6). Protein identities that were determined by tandem mass spectrometry are indicated (arrowheads).

Nickel-affinity column-purified VapC63_his was then tested for activity toward homologous total RNA extracted from S. solfataricus. RNase activity was monitored by the extent of 16 and 23S rRNA degradation on agarose gels following incubation under various conditions (Fig. 6A). To exclude any contribution in these assays from contaminating RNase activity that might copurify from E. coli extracts, mock extracts were also assayed that had been prepared from an otherwise identical strain lacking VapC63_his (Fig. 6A, lane 7). At 80°C, S. solfataricus total RNA was rapidly degraded by VapC63_his in a manner that was proportional to the quantity of protein added. Residual activity was still apparent following incubation at 65°C, while no activity was evident at 37°C. In contrast, VapC63_his did not exhibit any exo- or endonucleolytic activity toward DNA at 80°C. DNAase activity was tested using double-stranded circular DNA, double-stranded linear DNA, as well as a single-stranded linear DNA (data not shown, see Materials and Methods). To evaluate the potential interaction between VapC63_his and the antitoxin VapB6, the effect of addition of affinity-purified VapB63_his was tested on ribonuclease activity (Fig. 6B). VapB63_his and VapC63_his were preincubated separately at 95°C for 10 min, then combined and incubated at 90°C for an additional 10 min, followed by RNA addition and incubation for 30 min at 80°C. VapB63_his addition blocked the action of VapC63_his and prevented RNA degradation with increasing potency as its relative amount increased. Addition of similar amounts of a VapB63_his mock affinity-purified extract had a negligible effect on VapC63_His ribonuclease activity even at the highest ratio tested (20:1). Modulation of VapC63_His RNase activity could arise from other proteins present in the heat fractionated affinity-purified recombinant E. coli extracts. However, since VapC63_His RNase activity was both strongly heat dependent and specifically inhibited by addition of the cognate VapB63_his antitoxin, a mechanism whereby copurifying the E. coli protein would confer these properties seems unlikely. In summary, these data suggest that recombinant VapC6 and VapB6 form a heat-activated inactive complex that has lost ribonucleolytic activity, thereby providing a mechanism to regulate the activity of the VapC6 toxin in vivo.

Transcriptomic effects of VapBC6 deficiency

To identify the in vivo targets of VapBC6, the impact of VapBC6 deficiency was examined on the heat-shock transcriptome. A qRT–PCR analysis was conducted surveying 13 different mRNAs identified previously as the strongest responders to heat-shock stimulus in this organism (Tachdjian and Kelly 2006). Levels of the target mRNAs were evaluated in the wild-type and vapB6 disruption strains during growth under normal conditions at 80°C, and 5 min after culture temperatures had increased to...
either 90°C or 95°C. The level of the *tbp* mRNA was evaluated as an internal control in all reactions. Several transcripts were more abundant in the mutant relative to the wild type (Fig. 7A). The most apparent was the Oligo/dipeptide transport permease (*dppB-1*, SSO1274), followed by TetR a transcriptional regulator (SSO2506). Transcripts whose abundance was less in the mutant relative to the wild type (Fig. 7B) included RNA polymerase subunit RpoD (SSO0271), translation initiation factor subunit eIF-γ (SSO0412), Acetyl-CoA c-acetyltransferase (acetoacetyl-CoA thiolase; *AcaB1*, SSO0534), and transcription factor GntR (SSO1589). All transcript abundance values for both strains are indicated (Supplemental data; Supplemental Table 3). The variations between replicate determinations are provided for all determinations along with internal values for *tbp*. While the mechanism underlying the relative reduction in abundance of some transcripts in the *vapB6* disruption mutant is as yet unclear, VapC6 may act in a direct manner to modulate the abundance of those transcripts whose levels increased preferentially in cells lacking the ribonuclease. This hypothesis was tested by measuring the action of VapC6 at 80°C toward several RNAs. Runoff RNAs were prepared as described (Hoang et al. 2004) for four genes; SSO1274 (*dppB-1*), SSO2506 (*tetR*), SSO1494 (*vapB6*), and SSO091 (*7SRNA*). While three of these (*dppB-1*, *tetR*, *vapB6*) underwent an increase in abundance in the *vapC6* disruption mutant upon heat shock (Fig. 7), the *7SRNA* did not vary appreciably under these conditions or, as demonstrated previously, in response to heat shock in wild-type cells (Tachdjian and Kelly 2006). Treatment of purified runoff RNAs with recombinant VapC6 at 80°C resulted in preferential degradation of the RNAs.
**DISCUSSION**

The *S. solfataricus* VapBC6 toxin–antitoxin (TA) is the first example of a cellular component required for heat-shock (HS) survival in the archaeal phylum, Crenarchaeota. The toxin, VapC6, appears to play an essential ribonucleolytic role in this process. The *vapBC6* genes are also the first example of a gene pair that are required for normal rates of growth in this organism. This finding, together with the apparent absence of heat-shock transcription factors found in Crenarchaeota, suggest that TA pairs like VapBC6 contribute strongly to heat-shock gene regulation in this phylum. Since VapC6 was found to be a ribonuclease, RNA depletion mediated by an increase in ribonuclease abundance is a requirement for cell survival. This mechanism could explain the basis for prior reports of a strong repression in protein synthesis during the heat-shock response of the related species, *S. shibatae* (Trent et al. 1990).

VapC6 rapidly degraded total *S. solfataricus* RNA in vitro; therefore, this capacity in vivo could provide a rapid response mechanism for modulating protein synthesis. An initial survey of 13 genes selected because of their response to heat shock (HS) (Tachdjian and Kelly 2006) revealed strong changes in the abundance of several transcripts during HS of the *vapB6: lacS* disruption mutant relative to the wild type. Those undergoing large preferential increases included the oligopeptide permase, *dppB-1*, as well as the transcription factor, *tetR*, suggesting that these transcripts are direct targets of the VapC6 ribonuclease. Genes whose abundance preferentially decreased during HS in the *vapB6: lacS* disruption mutant included the RNA polymerase subunit, *rpoD*, and the translation initiation factor, *eIF-2 gamma*. Since depletion of the corresponding two proteins would block both transcription and translation, their modulation could underly the HS sensitive phenotype of the *vapB6: lacS* disruption mutant. While the mechanism for this effect remains to be elucidated, it demonstrates that the VapB6 proteins are required to maintain normal levels of essential cellular components.

Unexpectedly, deletion of *vapC6* resulted in the elevated abundance of the *vapB6* transcript during growth at 80°C. This finding could mean that VapC6 normally degrades the mRNA of its cognate antitoxin, VapB6, suggesting that there is another level of regulatory control operating to coordinate the relative abundance of the toxin and antitoxin proteins. The HS-sensitive phenotype of the *vapB6* disruption reflects reduced *vapC6* mRNA abundance due to premature transcription termination mediated by the *lacS* terminator (transcriptional polarity). Despite this effect, levels of *vapC6* mRNA retained HS inducibility, albeit at reduced levels. This regulatory pattern most likely reflects readthrough of the *lacS* terminator from transcripts initiating at the *vapBC6* promoter and not an internal initiation site, since RNAseq data of *S. solfataricus* fail to detect any 5’ primary transcript reads from within this region (Wurtz et al. 2010). In addition, *vapB6* deficiency (by in-frame deletion) resulted in a reduction of *vapC6* mRNA. This effect could be mediated through premature transcription termination at a putative intergenic terminator identified at the 3’ end of *vapB6*. Translation of *vapB6* would normally compete with formation of this structure, preventing premature transcription termination and reduction in *vapC6* expression. Inhibition of *vapB6* translation resulting from either *vapB6* mutation, might compromise stem–loop formation. This type of coupling mechanism could coordinate expression of both Vap products in the wild-type strain. Alternatively, VapB6 deficiency may allow VapC6 to degrade itself. As the VapC6 protein is likely to be longer lived than *vapC6* mRNA, an autodegradatory mechanism is plausible. Such a strategy resembles that used by other TA families like *ccd*, which regulate plasmid replication where the toxin is long lived and antitoxin is short lived; if cells lose the TA genes, they die because the long-lived toxin inactivates plasmid replication (Madl et al. 2006). Since recombinant VapB6 was found to be a potent inhibitor in vitro of VapC6 ribonucleolytic activity, coordinate increases in both gene products may not elevate ribonuclease activity without a corresponding reduction in antitoxin abundance. While this could arise by elevated rates of antitoxin proteolysis, in vivo measurements of toxin and antitoxin abundance are necessary to resolve this process.

VapBC6 is only one of 22 copies of this two-gene family (and one solitary toxin) in *Sso* (Tachdjian and Kelly 2006; Jorgensen et al. 2009). While several other *vap* loci are heat-shock inducible, including, for example, *vapBC8* and *vapBC22* (Tachdjian and Kelly 2006), most others are not. The finding that a single *vapBC* locus was required for thermotolerance leaves open the possibility that other heat-shock-inducible *vap* loci are also involved in the heat-shock response. In addition, it also suggests that other *vap* loci may play distinct and important physiologic roles. The occurrence in *S. solfataricus* of multiple *vapBC* loci that are closely related in sequence composition raises the possibility that interactions occur between noncognate pairs. Such

**FIGURE 8.** Treatment of runoff RNAs at 80°C using VapC6. RNA standards (lanes 1,10); *dppB-1* RNA (lanes 2,3); *tetR* RNA (lanes 4,5); *vapB6* RNA (lanes 6,7); *7SRNA* (lanes 8,9). RNAs treated with VapC6 (lanes 3,5,7,9). Amounts of RNA and VapC6 added to reactions were identical to those described in the legend to Figure 6.
noncognate interactions or “cross talk” could increase the functional role of these proteins by expanding their capacity to respond to diverse stimuli and control diverse genes. However, while vapC6 inactivation by in-frame deletion had a strong impact on long-term heat-shock genes. However, while vapC6 inactivation by in-frame deletion had a strong impact on long-term heat-shock survival, the absence of vapB6 may be more important for the initial HS response. This may indicate that VapB6 plays a specific regulatory role toward VapC6, but has little control over other VapC proteins. Experiments are underway to determine the affinity of VapB6 toward other recombinant VapCs.

Initial efforts to prepare recombinant soluble VapC6 were not successful because of protein insolubility. As described previously for other proteins (Blum et al. 1992b), molecular chaperones can be used to overcome this constraint. DnaK is a highly characterized molecular chaperone and a heat-shock protein that plays a critical role in protein folding impacting thermotolerance, stress, and other physiologic traits. Simultaneous overproduction of DnaK in E. coli greatly improved the solubility of VapC6. This effect may have general applicability for promoting recovery of soluble forms of other S. solfataricus VapC proteins. The results presented here also introduce a genetic advance for S. solfataricus. Complementation of the vapB6::lacS disruption mutant depended on the development of a new genetic marker encoded by malA and a host strain that had lost the catabolic ability to grow on maltose. The S. solfataricus genome is thought to encode several α-glucosidases (Kim et al. 2004). Deletion of three of these genes was sufficient to disable maltose catabolism and create a strong selection for restoration of this trait by malA introduction. The malA marker expands the list of genes that can be used to conduct studies with S. solfataricus (Martusewitsch et al. 2000; Worthington et al. 2003b; Sowers et al. 2006; Berkner et al. 2007).

This study reinforces the importance of TA loci such as the Vaps for post-transcriptional regulation in microorganisms. Given the apparent lack of several bacterial post-transcriptional regulation mechanisms in the archaea, the prospect that TA loci play an even more critical role in stress response cannot be ruled out and is currently under investigation.

MATERIALS AND METHODS

Archaeal cultivation and strains

All S. solfataricus cell lines were derivatives of the wild-type strain 98/2 (Rolfsmeier and Blum 1995) and are listed with plasmids in Table 1 (primers are listed in the Supplemental Materials). S. solfataricus strains were cultivated in a basal salts medium (Allen 1959) with added carbon sources and adjusted to a pH of 3.0 at 80°C in screw-top flasks, with aeration in orbital baths (Rolfsmeier et al. 1998; Worthington et al. 2003a). Sugars were prepared as 10% (w/v) stocks, filter sterilized, and added at a final concentration of 0.2% (w/v). Rich medium contained tryptone 0.2% (w/v). Growth in liquid medium was followed spectrophotometrically at a wavelength of 540 nanometers. Solid medium contained 0.6% (w/v) gelrite (Kelco) and 8.0 mM magnesium chloride added to rich liquid medium.

The vapB6::lacS disruption strain, PBL2066, was created by simultaneous transformation of multiple overlap extension PCR products, and recombinants were enriched in a lactose minimal medium, as described previously (Sowers et al. 2006). The lacS gene including promoter and terminator was inserted 51-bp downstream from the vapB6 start codon. Primers Vap6B-LF and Vap6B-LR-lacS were used to amplify 527-nt upstream of the lacS insertion, including an overlap portion that complements the 5´ end of lacS. Primers Vap6B-RF-lacS and Vap6B-RR amplified 557-bp downstream from the lacS insertion site, including a portion that complements the 3´ end of lacS. A total of 1630 bp of lacS, including flanking regions was amplified using primers Vap6B-lacS-F and Vap6B-lacS-R. OLE PCR was used to join the upstream PCR product with lacS, producing a 2157-bp amplicon to join the downstream PCR product with lacS, producing a 2189-bp amplicon. The two chimeric PCR products were cotransformed into cells, and recombinants arising from three crossover events were recovered. Purified isolates were recovered as described (Schele et al. 2006). The genotype of strain PBL2066 was confirmed by colony PCR using primers Vap6B-LF and Vap6B-RR to amplify the wild-type (1073 bp) or mutant alleles (2706 bp).

An in-frame vapB6 deletion strain (PBL2078) was constructed using plasmid pPB1093 by markerless exchange as described (Scheleert et al. 2006). Overlap extension PCR (OLE PCR) was used to create a 228-bp in-frame deletion that begins 39 bp downstream from the vapB6 start codon and ends 267 bp downstream from the start codon, leaving a 72-bp gene product. A 487-nt amplicon upstream of the deletion was created using primers XmaVap6Bout-F and Vap6BdelOR-R. A 530-bp amplicon 3’ to the deletion site was made using forward primers Vap6BdelOR-F and SbfVap6Bout-R. An OLE PCR amplicon was cloned at Xmal and SbfI sites in plasmid pPB1035, creating plasmid pPB1093, used subsequently to make strain PBL2078. Presence of the vapB6 in-frame deletion was confirmed by DNA sequence of PCR amplicons using primers XmaVap6Bout-F and SbfVap6Bout-F for the wild-type (1244 bp) and mutant alleles (1016 bp). An in-frame vapC6 deletion strain (PBL2080) was constructed following the strategy used for the vapB6 in-frame deletion strain. A 507-bp OLE PCR product used to create the deletion was generated using primers XmaVap6Cdel-F and VapC6delOR-R. A 427-bp amplicon located 3' of the deletion was produced using primers Vap6CdelOR-F and SbfVap6Cdel-R. An OLE PCR amplicon was cloned at Xmal and SbfI sites in plasmid pPB1035, creating plasmid pPB1093, used subsequently to make strain PBL2078. Presence of the vapB6 in-frame deletion was confirmed by DNA sequence of PCR amplicons using primers XmaVap6Cdel-F and SbfVap6Cdel-R for the wild-type (1189 bp) and mutant alleles (934 bp).

Maltose-based selections using malA

PBL2069 was derived from strain PBL2027 (Scheleert et al. 2006), which carried a spontaneous chromosomal deletion
from SSO3017 through SSO3052 that removed lacS (SSO3019), xyfS (SSO3022), and malA (SSO3051). While a malA-deficient strain (malA::lacS, PBL2009) exhibited normal growth rates on maltose as the sole carbon and energy source, simultaneous loss of SSO3022 reduced the growth rate by only one-third. Therefore, SSO0990 was inactivated, creating strain PBL2069 by in-frame deletion, resulting in loss of positions 691–1854 of the open reading frame. This region was removed by markerless exchange (Schelert et al. 2006) following recombination with an overlap PCR amplicon consisting of two paired segments, generated using primers 0990-BamHI-F and 0990-Del-R and primers 0990-Del-F and 0990-BamHI-R. PBL2069 could not grow on maltose as the sole carbon and energy source.

Strain PBL2081, a vapB6::lacS-disrupted derivative of PBL2069, was created by linear DNA transformation of the vapB6::lacS disruption mutation amplified using genomic DNA from strain PBL2066 and primers Vap6B-LF and Vap6B-RR. PCR and DNA sequencing were used to confirm the vapB6 genotype of strain PBL2081. Complementation of PBL2081 was performed using a derivative of pLacS (Berkner et al. 2007), in which lacS had been replaced with malA, including its promoter and terminator at the SacI site; vacB6C was inserted at the NotI site using primers NotIVap6out-F and NotIVap6out-R to make plasmid pPB1096. To maintain the plasmid in strain PBL2081, cells were grown in 0.2% maltose minimal media prior to heat-shock analysis. α-Glucosidase assays were conducted as described (Rolfsmeyer and Blum 1995) using the fluorescent maltose analog, p-nitrophenylglucoside (10 mM) as substrate in 100 mM sodium acetate (pH 4.5) buffer at 80°C. All samples were assayed in duplicate, and the averages of the sample results are shown.

Heat-shock analysis

Heat-shock experiments designed to determine heat inactivation rates for S. solfataricus used 50-mL cultures of rich medium grown to an optical density at A540 of 0.1 at 80°C, which is equivalent to 1.0 ¥ 10⁸ cells/mL. To minimize gas exchange, flask caps were lined with neoprene seals (0.66-mm thick). Heat shock was initiated by transferring flasks to a second orbital-shaking glycerol bath equilibrated at 95°C; flask contents required 20 min to reach the new temperature. Samples were removed periodically to determine cell density and viability. Viable counts were determined by transferring flasks to a second orbital-shaking glycerol bath equilibrated at 95°C; flask contents required 20 min to reach the new temperature.

Recombinant Vap protein synthesis and purification

Recombinant plasmids were constructed using E. coli DH5α, and characterized by restriction analysis and DNA sequencing, then transferred into BL21 (DE3) for expression analysis. VapC6 and vacB6 C-terminal polyhistidine-tagged expression plasmids were constructed by cloning vacB6 or vacB6 at the Ndel and Xhol sites of pET21b (Novagen), using primers SSO1493-F and SSO1493-R or SSO1494-F and SSO1494-R, respectively. Expression of these plasmids was evaluated in E. coli strain BL21(DE3), with or without plasmid pBNI9. Plasmid pBNI9 was a derivative of pBNI3 (Blum et al. 1992a) in which a 2.4-Kb MscI–ScaI fragment encoding ColEI and part of bla was replaced with a 2.3-Kb XmnI–MspI fragment from pACYC184 encoding p15A and tet, thereby conferring compatibility with pET21b. To obtain the Vap proteins, recombinant E. coli strains pregrown in Luria Broth medium with ampicillin (100 μg/mL) and tetracycline (10 μg/mL) were stored at 4°C overnight and used as inocula for large-scale batch cultures. Induction of recombinant proteins used 1-L LB cultures with antibiotics grown to an OD600 of 0.1 and treated with 0.1 mM IPTG, followed by incubation at room temperature overnight as indicated previously (Blum et al. 1992a,b). Cells were recovered by centrifugation at 4000g, resuspended in Buffer A (50 mM Tris-Hcl at pH 7.8, 500 mM NaCl, 10% glycerol, 20 mM imidazole) at 50 OD600 U/mL, sonicated (80W x 30 sec × 10 times), and clarified by centrifugation (34,220g for 20 min). The supernatant was then fractionated by Ni-NTA chromatography. The sample was concentrated sixfold using centrifugal columns (Millipore, 3500 MWCO) and examined by SDS PAGE using 16% resolving gel and 5% stacking gel. Protein identities were determined by tandem mass spectrometry electrospray analysis using material excised from SDS–polyacrylamide gels (Worthington et al. 2003b; Schelert 2006). Gel slices were infused with trypsin to digest the protein, and the resulting peptides were recovered and separated by capillary electrophoresis prior to analysis. To assess the extent of contamination by E. coli ribonuclease, mock samples were prepared from induced cultures of a strain lacking vap expression plasmids, and these were processed exactly as for recombinant protein samples, including heat fractionation and Ni-NTA chromatography followed by centrifugal concentration.

Ribonuclease and deoxyribonuclease assays

For VapC6 assays using total cellular RNA as a substrate, S. solfataricus RNA was prepared as described previously (Bini et al. 2002). Four micrograms of S. solfataricus total RNA or runoff product abundance (Noonan et al. 1990) was determined for all targets, and product qualities were verified by examination of melting curves. Parallel RT–PCR amplifications were used to evaluate RNA levels from experimental genes relative to those of the reference gene, 7S RNA, as described elsewhere (Lesur and Campbell 2004). RNA was treated to remove DNA by addition of 1 U of DNase I (Fermentas) per microgram of total RNA at RT for 15 min and then neutralized with 2 μL of 25 mM EDTA and incubated at 70°C for 10 min. cDNA synthesis used 20 pmol of PCR antise primer, 20 mM dNTPs mix (Invitrogen), and 200 U of M-MuLV RT (Fermentas) for 60 min at 37°C. Synthesized cDNA was subjected to standard PCR and analyzed using 2% (w/v) TBE agarose gels.
RNAs was used as substrate for all ribonuclease assays unless otherwise specified. Ribonuclease A (Sigma) was used as a positive control for the assay in amounts of 0.0246 U. The reactions were carried out in 10 mM MgCl$_2$, 150 mM NaCl, 50 mM Tris-HCl at pH 6.0 at 80°C for 1 h. A total of 250 mM KCl was also added to each reaction to ensure stability of the RNA substrate at high temperature as described (Hethke et al. 1999; Bini et al. 2002). All recombinant and mock samples were dialyzed against 50 mM Tris-HCl (pH 6.0), 100 mM NaCl before assay. Reactions were initiated by addition of RNA substrate to preincubated components and then incubated for 1 h at the temperatures indicated. Protein concentrations of Ni-column-purified and dialyzed samples were VapC6 (0.75 mg/mL), VapB6 (0.61 mg/mL). VapB6 and C6 proteins were incubated first at 95°C for 10 min separately, then various volumetric ratios as indicated were combined and incubated at 80°C for 2 h prior to RNA substrate addition. Reactions were fractionated by gel electrophoresis using 0.8% TBE agarose submersed gels and visualized by Ethidium Bromide staining with a UVP EC3 4210 Imaging System.

DNase activity was assessed using VapC6 and three different DNA substrates, including (1) a circular dsDNA comprised of plasmid pUC19 and a 4.0-kb S. solfataricus insert consisting of a disruption of SSO2651 by SSO6015–6016; (2) linear dsDNA consisting of a PCR amplicon of the pUC19 insert, and (3) ssDNA generated from the PCR amplicon denatured by heating at 95°C followed by transfer to the 80°C reaction mixture. S. solfataricus total RNA was used as a positive control for enzyme activity. Nuclease assays were carried out in 10 mM MgCl$_2$, 150 mM NaCl, 250 mM KCl, 50 mM Tris-HCl (pH 6.0) at 80°C for 1 h. Reactions were initiated by addition of 0.1 mL of VapC6 (0.75 mg/mL) to reactions containing substrates (1 μg of DNA or 2 μg of RNA) and then incubated for 1 h. Reactions were fractionated by gel electrophoresis using 0.8% TBE submersed gels and visualized by Ethidium Bromide staining with a UVP EC3 4210 Imaging System.

Runoff RNAs for VapC6 assays used specific DNA templates prepared as described (Hoang et al. 2004). The selected genes were cloned into plasmid pJT73/18U at HindIII and BamHI sites. Runoff RNA lengths (indicated in parentheses) and the following runoff RNA lengths (indicated in parentheses) and the following are initiated by addition of 1 μL of VapC6 (0.75 mg/mL) to reactions containing substrates (1 μg of DNA or 2 μg of RNA) and then incubated for 1 h. Reactions were fractionated by gel electrophoresis using 0.8% TBE submersed gels and visualized by Ethidium Bromide staining with a UVP EC3 4210 Imaging System.

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**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article (Table 3: Transcriptionic effects of VapBC6 deficiency; Table 4: List of Primers).

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