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# Interplay between transcriptional regulators and VapBC toxin–antitoxin loci during thermal stress response in extremely thermoacidophilic archaea

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## Abstract

Thermoacidophilic archaea lack sigma factors and the large inventory of heat shock proteins (HSPs) widespread in bacterial genomes, suggesting other strategies for handling thermal stress are involved. Heat shock transcriptomes for the thermoacidophilic archaeon *Saccharolobus* (*f. Sulfolobus*) *solfataricus* 98/2 revealed genes that were highly responsive to thermal stress, including transcriptional regulators $YtrA_{Ss}$ (Ssol_2420) and $FadR_{Ss}$ (Ssol_0314), as well as type II toxin–antitoxin (TA) loci VapBC6 (Ssol_2337, Ssol_2338) and VapBC22 (Ssol_0819, Ssol_0818). The role, if any, of type II TA loci during stress response in microorganisms, such as *Escherichia coli*, is controversial. But, when genes encoding $YtrA_{Ss}$, $FadR_{Ss}$, VapC22, VapB6, and VapC6 were systematically mutated in *Sa. solfataricus* 98/2, significant up-regulation of the other genes within this set was observed, implicating an interconnected regulatory network during thermal stress response. VapBC6 and VapBC22 have close homologues in other Sulfolobales, as well as in other archaea (e.g. *Pyrococcus furiosus* and *Archaeoglobus fulgidus*), and their corresponding genes were also heat shock responsive. The interplay between VapBC TA loci and heat shock regulators in *Sa solfataricus* 98/2 not only indicates a cellular mechanism for heat shock response that differs from bacteria but one that could have common features within the thermophilic archaea.

## INTRODUCTION

Thermal stress response in bacteria involves the orchestration of specific heat shock proteins (HSPs), universal stress proteins (USPs), chaperones (e.g. DnaK, DnaJ, GroEL, GroES and GrpE), and proteases (e.g. Lon, ClpXP) to minimize cellular disruption arising from protein misfolding and to coordinate protein turnover (Richter et al., 2010). This same response in thermophilic archaea involves a different and more limited set of proteins. Thermophilic archaea lack versions of bacterial HSPs, HSP7, HSP90, and HSP100, and rely on prefoldins, small heat shock proteins (HSP20), and the thermosome (HSP60-like) to function as molecular chaperones (Lemmens et al., 2018), and on the proteasome to process misfolded proteins (Bauer et al., 1997; Madding et al., 2007). Small heat shock proteins (sHSPs) are molecular chaperones that assemble into multi-subunit complexes to avert protein aggregation (Baes et al., 2020; Li et al., 2012; Wang et al., 2010). Additionally, the thermosome chaperonin structure is conserved across archaea, typically with α and β subunits making up either 8- or 9-fold rotational symmetry in two stacked rings (Kagawa et al., 1995; Trent et al., 1990, 1991). The archaeal prefoldin is a hexameric chaperone that binds denatured proteins and ushers them to the thermosome. Moreover, prefoldins are composed of two α subunits that control the

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shape of the prefoldin and four β subunits that are responsible for protein binding (Lemmens et al., 2018). On the other hand, protein degradation in archaea is mainly executed by the proteasome that is composed of stacked α and β subunit rings that create a cylindrical structure with an internal cavity containing the active site for protein degradation. A proteasome-activating nucleotidase (PAN) associates with the entrance of the proteasome, requiring ATP to unfold proteins marked for degradation (Bar-Nun & Glickman, 2012; Maupin-Furlow, 2012). The coordination of these molecular chaperones and the proteasome serves to either refold or degrade heat damaged cytosolic proteins (Baes et al., 2020; Bar-Nun & Glickman, 2012; Li et al., 2012; Maupin-Furlow, 2012; Trent et al., 1991; Wang et al., 2010).

There are other protections against heat shock damage in thermophilic archaea. Ether-linked and tetraether lipids with potential for forming multiple cyclopentyl rings add an additional layer of heat stability to the archaeal membrane; this is not observed in bacteria or eukaryotes (Boyd et al., 2011; Choquet et al., 1994; Elferink et al., 1994; Feyhl-Buska et al., 2016; Komatsu & Chong, 1998; Uda et al., 2001; van de Vossenberg et al., 1998). Furthermore, only thermoacidophiles belonging to the order Sulfolobales, such as *Saccharolobus solfataricus* (f. *Sulfolobus solfataricus*) and *Sulfolobus acidocaldarius*, have a cyclopentyl head group (calditol) that is ether-linked to their lipid membranes, thereby imparting acid resistance to these microbes that can be important during thermal stress (Zeng et al., 2018).

Transcriptional regulators that are linked to the heat shock response have been identified in thermophilic archaea. A heat shock responsive regulator in *Pyrococcus furiosus*, Phr (PF1790), acts as a repressor that directly binds to a conserved sequence located across the transcriptional start site of genes for AAA+ ATPase, a sHSP, and in its own promoter region (Keese et al., 2010; Vierke et al., 2003). This body of evidence suggests that Phr sits on the promoter blocking transcription during unstressed growth conditions, then dissociates from the promoter during heat shock, thereby allowing for increased transcriptional expression of the associated heat shock genes (Keese et al., 2010, Vierke et al., 2003). A homolog of Phr, named HSR1 (AF1298), in *Archaeoglobus fulgidus* is similarly responsive to thermal stress (Rohlin et al., 2005).

Bacterial heat shock systems are typically governed by sigma factors, such as RpoH ($\sigma^{32}$) in *Escherchia coli* (Grossman et al., 1984) and the alternative sigma factor B ($\sigma^{B}$) in *Staphylococcus aureus* (Kullik & Giachino, 1997). However, a similar transcriptional regulatory mechanism has not been identified in archaea, although thermophilic archaea, like many prokaryotes, have numerous type II toxin–antitoxin (TA) systems (Fraikin et al., 2020). Type II TA systems that belong to the VapBC family are composed of a VapC stable ribonuclease (toxin) and a VapB antitoxin protein that, when bound to the toxin, silences ribonucleolytic activity. The VapB antitoxin is susceptible to proteolysis, which prevents the neutralization of the VapC toxin ribonuclease activity. VapB antitoxins also contain DNA-binding domains that autoregulate their own promoter and can regulate other distal gene targets (Wang et al., 2011). The role of TA loci in stress response is controversial. It has been suggested that they play no specific role in *E. coli* (LeRoux et al., 2020). Previously, however, type II TA systems have been implicated as a nontraditional regulator of the heat shock response in *Sa. solfataricus*. Certain TA loci (e.g. VapBC6 and VapBC22) responded to thermal stress in *Sa. solfataricus* and deletion of the gene encoding the VapB6 antitoxin rendered the archaeon labile to thermal stress (Cooper Charlotte et al., 2009; Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Tachdjian & Kelly, 2006).

Previous work with *Sa. solfataricus* P2 reported thermal responses of transcriptional regulators and type II TA systems (Cooper Charlotte et al., 2009; Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Tachdjian & Kelly, 2006). Here, an interplay between transcriptional regulators and type II TA systems to manage heat shock response was examined in *Sa. solfataricus* 98/2. This examination was accomplished by utilizing available molecular genetic tools to compare the heat shock response of the *Sa. solfataricus* 98/2 parent strain (PBL2025) to the responses of genetically modified strains of this archaeon in which genes encoding toxins, antitoxins and transcriptional regulators were systematically mutated.

## EXPERIMENTAL PROCEDURES

### Generation of *Sa. solfataricus* PBL2025 mutants

Gene disruptions were achieved by insertion of a *lacS* gene into the target gene sequence in the genome, as described previously (Sowers et al., 2006). Gene disruption of $fadR_{Ss}$ (Ssol_0314) created strain PBL2084, disruption of *vapC22* (Ssol_0818) created strain PBL2067 and disruption of $ytrA_{Ss}$ (Ssol_2420) created strain PBL2026. In-frame gene deletions were also made, as described previously (Maezato et al., 2011). Deletion of *vapB6* (Ssol_2337) created strain PBL2078 and deletion of *vapC6* (Ssol_2338) created strain PBL2080.

### Cultivation of *Sa. solfataricus* strains

*Sa. solfataricus* PBL2025 and the related mutant strains were grown at 80°C on DSMZ 182 medium,

pH 4.0; cells were enumerated using epifluorescence microscopy with acridine orange stain, by methods previously reported (Chhabra et al., 2002). Thermal stress experiments were carried out with a modified 3-L glass bioreactor (Applikon, Schiedam, The Netherlands), as described previously (Tachdjian & Kelly, 2006). Briefly, using heated re-circulating water and a heating mantel, the culture at mid-exponential phase was shifted from 80 to 90°C (which took ∼7 min) and maintained at 90°C ± 1°C for the duration of the experiment. Samples were taken 10 min before starting the temperature shift and then at 10 and 30 min after reaching 90°C. Samples (400 mL) were harvested for each time point and chilled in a dry ice and ethanol bath before harvesting at 4°C for 10 min. Biological replicates of all transcriptional response experiments were done.

## Cultivation of *M. prunae*

*M. prunae* (DSM 10039) was sub-cultured from freezer stocks and acclimated to DSMZ88 media supplemented with 1 g/L yeast extract (Bacto BD Biosciences) (growth temperature—70°C, shaking rate—100 rpm). DSMZ88 media (2 L) with 1 g/L yeast extract was prepared and sterilized in a 3 L Applikon bioreactor, temperature in the bioreactor was maintained at 70°C by a circulating water bath set at 75.6°C. Air was sparged into the media at a rate of 100 mL/min and agitation rate was set at 150 rpm. The bioreactor was inoculated from a culture growing in early stationary phase, inoculum volume was adjusted to target an initial cell density of $1 \times 10^7$ cells/mL. Cultures were grown to cell density of $\sim 1 \times 10^8$ cells/ml (3–4 doublings) and harvested by chilling quickly in a dry ice, 95% ethanol mix followed by centrifugation at $6000 \times g$ for 15 min. The pellets were washed in 25 mM TE buffer, pH 8.0 and stored in −80°C for RNA extraction. This sample was assigned as a baseline sample indicating normal growth culture. Temperature in the circulating water bath was then set to 90.4°C and simultaneously temperature in Applikon controller was set to 85°C. This was done to expedite the process of raising temperature in the bioreactor. Temperature in the bioreactor increased to 85°C after 10 min and controller was turned off to avoid overheating. After 10 min of heat shock, cultures were harvested using the method described above, this sample was stored as the first heat shock sample (HS10). A second heat shock culture was harvested 30 min after heat shock and stored in −80°C freezer as the second heat shock sample (HS30).

## Transcriptomic analysis

Whole-genome oligonucleotide microarrays for *Sa. solfataricus* P2 (DSMZ, Germany) and *M. sedula* were developed based on the reported genome sequences (Counts et al., 2021) and fabricated, as reported previously (Tachdjian & Kelly, 2006). The *M. sedula* array (Auernik & Kelly, 2008) was used for *M. prunae* since their genomes are nearly identical (Counts et al., 2021). Probes were designed for any missing Vap genes in *Sa. solfataricus* from the previously developed microarray (Tachdjian & Kelly, 2006) in OligoArray 2.0 (Rouillard et al., 2003) and custom synthesized (Integrated DNA Technologies, Coralville, IA). Slides with the updated microarray were printed with four replicates per probe spotted onto each array to fortify statistical analysis. From each sample in the heat shock time course experiments described above, RNA was extracted using the Qiagen RNAqueous kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Equal amounts of RNA from the biological repeats were pooled and cDNA was synthesized; samples were then hybridized in a three-slide loop design, by methods described previously (Chhabra et al., 2003), with minor adjustments for long oligonucleotide platforms (Tachdjian & Kelly, 2006). Microarray slides were scanned with the Axon 400B (Molecular Devices, Sunnyvale, CA) microarray scanner and raw data were analysed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). Data from each experiment were analysed with JMP Genomics version 4.0 (SAS, Cary, NC), using a mixed linear analysis of variance model as described previously (Tachdjian & Kelly, 2006). A ± 2.0-fold change (FC) or higher (where a $\log_2$ value of ±1 equals a 2-FC) with significance values at or above the Bonferroni correction, which was 4.8 (equivalent to a *p*-value of $4.0 \times 10^{-6}$), defined differential expression for these data set. Microarray data are available online through the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO).

## Generation of the thermophilic archaea protein database

Genomes for the species investigated in this study are available in the NCBI database and were used to identify protein homology to support comparison of transcriptomics between species. In the case of *P. furiosus*, *Sa. solfataricus 98/2*, and *M. prunae*, the probes for microarray slides were designed based on the genome of a different strain (or for *M. prunae*, a different species) than the one used in transcriptomic experiments. Therefore, the genome of the experimental strain or species and the genome of the strain or species used for probe design were evaluated to ensure the appropriate transcript was present to bind to each microarray probe. Accession numbers for the genomes can be found in Table S1. Protein homology for whole genomes was evaluated using the GET_HOMOLOGUES suite

(Contreras-Moreira et al., 2017; Contreras-Moreira & Vinuesa, 2013) with default settings for the orthoMCL algorithm (Li et al., 2003) BLASTP and allowing for clusters containing only a single protein sequence. The resulting clusters of protein sequences were used to compare transcriptomic response of genes of interest between species.

## Genome comparison between *Sa. solfataricus* strains P2 and 98/2

*Sa. solfataricus* P2 was the focus of previous heat shock studies (Cooper Charlotte et al., 2009; Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Tachdjian & Kelly, 2006). However, molecular genetic tools are not available for the P2 strain. As such, *Sa. solfataricus* 98/2 was used here to take advantage of genetic tools developed for this strain (Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Sowers et al., 2006). Note also that phylogenetic classification of the Sulfolobales was reexamined, leading to some adjustments (Counts et al., 2021; Lewis et al., 2021) in assigning species to genera; however, the 98/2 and P2 strains were found to be closely related. To validate the use of the *Sa. solfataricus* P2 oligonucleotide microarray for the 98/2 strain (GenBank ID: CP001800.1), the two genomes were compared using Jspecies (Richter & Rossello-Mora, 2009). *Sa. solfataricus* 98/2 and P2 were found to be much more similar to each other (scores above 98/100 on Jspecies) than to other Sulfolabales, including *S. acidocaldarious*, *Sulfurisphaera* (f. *Sulfolobus*) *tokadii*, or *Sulfolobus islandicus* (scores ranging from 66.5 to 89.6 out of 100). When genomes from strains P2 and 98/2 were aligned using MAUVE (Darling et al., 2004), it was apparent that the organization of the two genomes is conserved, although the origin of replication in 98/2 is located downstream of that in P2 (Figure S1). The genome of 98/2 is smaller (~2.6 Mb compared to ~3 Mb for P2), with 98/2 lacking many of carbohydrate-degrading enzymes in P2. BLASTn analysis showed that approximately 2000 ORFs are 100% identical between strains P2 and 98/2 (Deng et al., 2007), several hundred ORFs have 95% or higher identity, and 300–400 P2 ORFs have 70% or less identity with 98/2. There are several regions of the P2 genome that are not found in 98/2, but only a few small regions in 98/2 that are not found in P2. Thus, nearly all of the genes annotated in the 98/2 genome have homologues in P2. Pertinent to this study, the 98/2 genome encodes all type II VapBC TA pairs identified in P2 (Figure S1), except for VapBC2, VapBC3, VapBC5, and VapBC18, none of which were heat shock responsive in P2 (Tachdjian & Kelly, 2006). Furthermore, comparative genomics analysis supports using the *Sa. solfataricus* P2 strain ORF nomenclature for *Sa. solfataricus* 98/2 (PBL2025 strain); nonetheless, the corresponding ORFs in the P2 and 98/2 strains are listed herein.

# RESULTS AND DISCUSSION

## Homologue clusters of known HSPs in extremely thermophilic archaea

Given their already high growth temperatures, questions concerning how extremely thermophilic archaea respond to heat shock have been of great interest (Cooper Charlotte et al., 2009; Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Phipps et al., 1991; Rohlin et al., 2005; Shockley et al., 2003; Tachdjian & Kelly, 2006). As sequence genomes are available for the Euryarchaeote thermophiles *P. furiosus* (Laksanalamai et al., 2001; Robb et al., 2001) and *A. fulgidus* (Klenk et al., 1997), and Crenarchaeote thermophiles *M. sedula* (Auernik et al., 2008), *M. prunae* (Counts et al., 2021), *Sa. solfataricus* strains P2 (She et al., 2001) and 98/2 (McCarthy et al., 2015), and *S. acidocaldarius* (Roy et al., 2018), GET_HOMOLOGUES was used to generate a thermophilic archaea protein database. Table 1 shows the genes identities of the homologues identified in this generated database of known heat shock responsive proteins across a subset of thermophilic archaeal species. These archaea were selected because corresponding heat shock transcriptional data have been reported previously (*Sa. solfataricus* P2 (Tachdjian & Kelly, 2006); *P.* furiosus (Shockley et al., 2003); *A. fulgidus* (Rohlin et al., 2005); *S. acidocaldadius* (Baes et al., 2020)), or were determined here (*M. prunae*, *Sa. solfataricus* 98/2).

All species surveyed in Table 1 have a representative of each of the chaperones sHSP, the prefoldin, and the larger chaperonine structure, the thermosome, which all serve to refold misfolded proteins. The sHSPs are ubiquitous across all domains of life (Haslbeck et al., 2005), so it is not surprising that at least one sHSP gene is encoded in the genomes of all species examined in Table 1. These archaea have two genes encoding sHSPs, except for *P. furiosus*, which has a single sHSP. The lone sHSP in *P. furiosus* is not only heat shock responsive, but recombinantly expressed *P. furiosus* sHSP increased the thermal resistance of the mesophilic *E. coli* 6-fold (Laksanalamai et al., 2001). Genomes of thermophilic archaea typically also contain a larger chaperonin, referred to as the thermosome (or rosettasome) (Kagawa et al., 1995; Trent et al., 1990, 1991). The main difference among thermosomes is the variable occurrence of 1–3 subunit types (α, β, and γ). For example, among the Sulfolobales in Table 1, a γ subunit is present in *S. acidocaldarius* and *Sa. solfataricus* but absent in *Metallosphaera* species.

**TABLE 1** Homologues of known heat shock proteins across thermophilic archaeal species.

| | Gene ID | | | | | | |
|---|---|---|---|---|---|---|---|
| **Protein** | **Mpru** | **Msed** | **Sso 98/2** | **Sso P2** | **Saci** | **Pfu** | **Af** |
| sHSP | DFR88_03380 | Msed_0640 | Ssol_0231 | SSO2427 | Saci_0922 | PF1883 | AF1296 |
| | DFR88_11850 | Msed_1294 | Ssol_0413 | SSO2603 | Saci_1665 | | AF1971 |
| HtpX | DFR88_01450 | Msed_0242 | Ssol_0965 | SSO1859 | Saci_0415 | PF1135 | AF0235 |
| | DFR88_05405 | Msed_1012 | Ssol_2626 | SSO3231 | Saci_0871 | | |
| | | | Ssol_0508 | SSO2694 | Saci_1676 | | |
| | | | | | | PF1597 | |
| Thermosome subunit α | DFR88_06875 | Msed_1710 | Ssol_1851 | SSO0862 | Saci_1401 | PF1974 | AF2238 |
| Thermosome subunit β | DFR88_09690 | Msed_2264 | Ssol_1258 | SSO0282 | Saci_0666 | | AF1451 |
| Thermosome subunit γ | | | Ssol_0790 | SSO3000 | Saci_1203 | | |
| Proteasome subunit α | DFR88_06295 | Msed_0074 | Ssol_1795 | SSO0738 | Saci_0613 | PF1571 | AF0490 |
| Proteasome subunit β | DFR88_06855 | Msed_2268 | Ssol_1254 | SSO0278 | Saci_0662 | | |
| Proteasome subunit β | DFR88_07900 | Msed_2052 | Ssol_1825 | SSO0766 | Saci_0909 | PF1404 | AF0481 |
| Proteasome subunit β | | | | | | PF0159 | |
| PAN | DFR88_06820 | Msed_2275 | Ssol_1248 | SSO0271 | Saci_0656 | PF0115 | AF1976 |
| Prefoldin subunit α | DFR88_10125 | Msed_1630 | Ssol_1321 | SSO0349 | Saci_1463 | | |
| Prefoldin subunit α | | | | | | PF0375 | AF2063 |
| Prefoldin subunit β | DFR88_06255 | Msed_0082 | Ssol_1787 | SSO0730 | Saci_0605 | PF0382 | AF1150 |

*Note*: Colour indicates protein homologue cluster in the thermophilic archaea protein database. PF1974 could not be classified as a specific thermosome subunit type.
Abbreviations: Af, *Archaeoglobus fulgidus*; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; PAN, proteasome-activating nucleotidase; Pfu, *Pyrococcus furiosus*; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.

The archaea listed in Table 1 all contain at least an α and a β thermosome subunit, except for *P. furiosus*, which has a single thermosome subunit that cannot be classified as α, β, or γ based on amino acid sequence homology alone. All species in Table 1 also have identifiable α and β prefoldin subunits, a chaperone that works in conjunction with the thermosome to refold proteins (Lemmens et al., 2018). Interestingly, the β subunit is homologous across the Euryarchaeota and Crenarchaeota, while the homology of the α subunit seems to be phylum-specific.

Degradation of archaeal proteins is performed by the proteosome and the HtpX. HtpX, a membrane-bound metalloprotease with a cytosolic active site, is conserved across bacteria and archaea (Shimohata et al., 2002). The *A. fulgidus* genome has one predicted HtpX gene, while *Sa. solfataricus* strains have three. The variability across species may be due to redundancy or perhaps other cellular proteases compensating in the case of fewer HtpX proteases. However, the proteosome is the primary protein degrader. The archaea in Table 1 each contain one gene encoding a proteasome α subunit and one PAN, but all except *A. fulgidus* have two genes encoding proteasome β subunits. The reason for the two β subunits is not certain, although the *P. furiosus* proteasome varies in the relative amount of the two β subunits during thermal stress (Madding et al., 2007).

## Thermal stress response of known HSPs and proteases in extremely thermophilic archaea

Table 2 compares reported transcriptional response information for HSPs and proteases in *Sa. solfataricus* P2, *P. furiosus*, *S. acidocaldarius*, and *A. fulgidus* to the transcriptional response of *M. prunae*, and *Sa. solfataricus* 98/2 reported here. Heat shock transcriptomes for *M. prunae* and *Sa. solfataricus* 98/2 were determined here for temperature shifts of 70°C–85°C and 80–90°C, respectively. Note that DNA microarrays were used to determine transcriptomes, except for *S. acidocaldarius* (Baes et al., 2020), where qPCR was used for selected genes and may explain the large differences in magnitude for fold changes. The sHSP's of *P. furiosus*, *S. acidocaldarius*, and *A. fulgidus* have the largest response to thermal stress, with at least one sHSP that is >7-fold up-regulated. Similarly, subunits of the HSP60-type thermosome were up-regulated, the magnitude of which depended on the specific thermosome subunit and species. Decreasing γ subunit expression with increasing temperature has been observed; for example, an increase in the thermosome's γ subunit expression occurs at suboptimal temperatures in *Saccharolobus shibatae* (Kagawa et al., 2003). Also, in *Sa. solfataricus* P2, the γ subunit response was undetectable at 80°C, which may relate

**TABLE 2** Heat shock response of known heat shock genes across thermophilic archaeal species.

| | Heat shock response | | | | | |
|---|---|---|---|---|---|---|
| **Protein** | **Mpru** | **Sso 98/2** | **Sso P2** | **Saci** | **Pfu** | **Af** |
| sHSP | 4.0 | 2.8 | 3.5 | 47.3 | 7.3 | 2.8 |
| | 1.6 | 2.4 | 3.9 | 7.0 | | 7.8 |
| HtpX | 1.5 | N.C. | 1.7↓ | 5.2 | 3.7 | 2.1 |
| | N.C. | N.C. | N.C. | | | |
| | 1.5 | 2.3↓ | N.C. | | | |
| | | | | | 1.8↓ | |
| Thermosome subunit α | N.C. | 1.9 | N.C. | 72.9 | 4.1 | 3.8 |
| Thermosome subunit β | 8.2 | 1.5 | N.C. | 29.7 | | 3.4 |
| Thermosome subunit γ | | 2.2↓ | 5.2↓ | N.C. | | |
| Proteasome subunit α | 2.1↓ | 1.7↓ | N.C. | | 2.9↓ | 2.4↓ |
| Proteasome subunit β | 2.2↓ | N.C. | N.C. | | | |
| Proteasome subunit β | 2.4↓ | 1.7↓ | N.C. | | 2.0 | 1.6 |
| Proteasome subunit β | | | | | 1.6 | |
| PAN | 1.5↓ | 2.0↓ | 7.5↓ | | N.C. | 1.8 |
| Prefoldin subunit α | 2.0↓ | 2.2↓ | N.C. | 4.0 | | |
| Prefoldin subunit α | | | | | | 2.1↓ |
| Prefoldin subunit β | 2.0↓ | 2.6↓ | N.C. | 6.4 | 1.9↓ | 1.7↓ |

*Note*: Fold changes in transcription are derived from DNA microarray from Sso P2 (Tachdjian & Kelly, 2006), Pfu (Shockley et al., 2003), and Afu (Rohlin et al., 2005). Mpru and Sso 98/2 data are from this work (highlighted in purple) and ≥2-fold changes have met the Bonferroni correction of 4.8. Fold changes for Saci are from qPCR data (Baes et al., 2020). Data are from a 60 min heat shock of Pfu and a 30 min heat shock for all other thermophiles.
Abbreviations: Af, *Archaeoglobus fulgidus*; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; N.C., no change; PAN, proteasome-activating nucleotidase; Pfu, *Pyrococcus furiosus*; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.

to the fact that the recombinant γ subunit was heat labile at 75°C (Chaston et al., 2016). Proteomics analysis of the *M. sedula* thermosome showed that it increased 6-fold upon heat shock, with one thermosome isoform increasing 73-fold (Han et al., 1997). It is interesting that prefoldin subunit expression was down-regulated 2- to 3-fold upon heat shock in every archaeal species, except for the *S. acidocaldarius* β subunit, which was up-regulated ≥4-fold. HtpX protease genes were either unchanged or up-regulated across all species, except for one HtpX gene (PF1597) in *P. furiosus* and one in *Sa. solfataricus* P2 (SSO2694), which are both down-regulated by 2-fold. The difference in thermal response of the two HtpX proteases in *P. furiosus* may indicate specialization, where HtpX 1 (PF1135) is important for thermal stress response and HtpX 2 (PF1597) plays a different role. The genes encoding proteasome subunits were largely unresponsive to heat shock, and in fact were slightly down-regulated in most cases; this was also the case for PAN.

## Heat shock responsive of transcriptional regulators of thermophilic archaea

Certain transcriptional regulators were highly responsive to heat shock in the representative thermophilic archaea and have homologues across archaeal species, as summarized in Table 3 and Table 4. The HTH family putative transcriptional regulator in *Sa. solfataricus* 98/2 (Ssol_0975) has homologues only in the Sulfolobales (*Sa. solfataricus* P2, *M. sedula*, *M. prunae*, and *S. acidocaldarius*). This response of this HTH regulator varied across these species; it was significantly down-regulated in *Sa. solfataricus* 98/2 (down 20-fold), less so in *M. prunae* (down 3-fold), and unchanged in *S. acidocaldarius*. The *S. acidocaldarius* homologue, Saci_1012 (Sulf12a), has been shown to function in chromatin-organization by binding DNA nonspecifically (Lemmens et al., 2022). Although this HTH regulator appears to have a major function in *Sa. solfataricus* heat shock response, this role is not common across the Sulfolobales.

Similarly, although homologues of a phosphate uptake PhoU-type regulator are in all thermophilic archaea examined, the heat shock response varied across thermophilic species. For instance, this PhoU-type regulator was highly up-regulated in *Sa. solfataricus* 98/2 (>8-fold) but unresponsive in *M. prunae*. Furthermore, the two PhoU-type homologues in *A. fulgidus* differed in their heat shock response with one being modestly up-regulated (∼2-fold) and the other unresponsive. As with Ssol_0975 and its homologues, the importance of this PhoU-type regulator to heat shock may be specific to the thermophilic archaeal species.

Another regulator of note is the Lrp/AsnC family transcriptional regulator with homologues only in *Metallosphaera* species and the *Sa. solfataricus* strains. This

**TABLE 3** Homologues of heat shock responsive transcriptional regulators across thermophilic archaeal species.

| Protein family | Function | Gene ID | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Mpru | Msed | Sso 98/2 | Sso P2 | Saci | Pfu | Af |
| GntR | Repressor | DFR88_00895 | Msed_1126 | Ssol_2213 | SSO1255 | | | |
| TetR-AcrR | Repressor | | | (*ytrA*$_{Ss}$) Ssol_2420 | SSO1589 | Saci_1851 | | |
| | | | | (*fadR*$_{Ss}$) Ssol_0314 | SSO2506 | Saci_1107 | | |
| Heli-turn-helix | Chromatin Organization | DFR88_05660 | Msed_0192 | Ssol_0975 | SSO3242 | Saci_1012 | | |
| PhoU | Phosphate Uptake Regulator | DFR88_08335 | Msed_1968 | Ssol_1682 | SSO0618 | Saci_1600 | PF0141 | AF1355 AF1797 |
| Lrp/AsnC | Repressor/Activator | DFR88_00500 | Msed_1202 | Ssol_2860 | SSO2131 | | | |
| ArsR | Repressor | | | | | | (*phr*[a]) PF1790 | (*hsr1*[b]) AF1298 |

*Note*: Colour indicates protein homologue cluster in the thermophilic archaea protein database.
Abbreviations: Af, *Archaeoglobus fulgidus*; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; Pfu, *Pyrococcus furiosus*; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.
[a]Vierke et al. (2003).
[b]Rohlin et al. (2005).

**TABLE 4** Heat shock response of transcriptional regulators across thermophilic archaeal species.

| Protein family | Function | Heat shock response | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Mpru | Sso 98/2 | Sso P2 | Saci | Pfu[a] | | Af |
| GntR | Repressor | 2.3 | 2.1 | 3.0 | | | | |
| TetR-AcrR | Repressor | | (*ytrA*$_{Ss}$) 10.9 | 8.3 | 28.3 | | | |
| | | | (*fadR*$_{Ss}$) 10.9 | 12.7 | 3.6 | | | |
| Heli-turn-helix | Unknown regulator | 3.0↓ | 20.0↓ | 8.1↓ | N.C. | | | |
| PhoU | Phosphate uptake regulator | N.C. | 8.5 | 6.0 | | 2.3↓ | N.C. | 1.5 N.C. |
| Lrp/AsnC | Repressor/Activator | 4.7 | 4.9 | N.C. | | | | |
| ArsR | Repressor | | | | | 1.8 | 2.5 | 4.7 |

*Note*: Fold changes in transcription are derived from DNA microarray data from Sso P2 (Tachdjian & Kelly, 2006), Pfu (Shockley et al., 2003), and Afu (Rohlin et al., 2005). Mpru and Sso 98/2 data are from this work (highlighted in purple) and ≥2-fold changes have met the Bonferroni correction of 4.8. Fold changes for Saci are from qPCR data (Baes et al., 2020). Data are from a 60 min heat shock of Pfu and a 30 min heat shock for all other thermophiles.
Abbreviations: Af, *Archaeoglobus fulgidus*; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; N.C., no change; Pfu, *Pyrococcus furiosus*; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.
[a]Pfu fold changes in the left column are from 30 min heat shock cultures grown on maltose and yeast extract and values in the right column are from 30 min heat shock cultures grown on tryptone and elemental sulfur.

regulator responded ~5-fold in both *M. prunae* (DFR88_00500) and *Sa. solfataricus* 98/2 (Ssol_2860), suggesting a similar role in both species. The homologue in *Sa. solfataricus* P2 (Sso2131) has been named Ss-LrpB, able to bind its own promoter (Peeters et al., 2004), and has been characterized as an activator of a pyruvate ferredoxin oxidoreductase operon and two permease genes (Peeters et al., 2009). However, no heat shock response was measured in *Sa. solfataricus* P2 ss-LrpB, which may indicate that it does not have the same thermal stress response function as other homologues.

Additionally, a homologue of Phr (PF1790) in *P. furiosus* (Keese et al., 2010; Vierke et al., 2003) was identified in the only other Euryarchaeota examined here, *A. fulgidus*. HSR1 (AF1298). The Phr homologue in *A. fulgidus*, was also responsive to thermal stress in previous studies (Rohlin et al., 2005). Interestingly, no homologue was found in the Crenarchaeota, suggesting that this heat shock regulator may be phylum specific.

Although Phr-type regulators appear to be restricted to members of the phylum Euryarchaeota, a GntR superfamily heat shock regulator (designated here as YtrA$_{Ss}$; Ssol_2420, SSO1589; in *Sa. solfataricus*) may have a similar function in the phylum Crenarchaeota. Two GntR family transcriptional repressors in *Sa. solfataricus* 98/2 (Ssol_2213, Ssol_2420) have homologues in *Metallosphaera species* or *S. acidocaldarius*, respectively. GntR family regulators have a HTH N-terminal

DNA-binding domain and a signal receptor domain at the C-terminus; others in the GntR family also function as transcriptional regulators (Haydon & Guest, 1991). While Ssol_2213 and the *M. prunae* homologue (DFR88_00895) had only a modest response to thermal stress, both were up-regulated ~2-fold, all homologues of Ssol_2420 ($ytrA_{Ss}$) responded with >8-fold transcriptional up-regulation (Table 4). $YtrA_{Ss}$ has a GntR superfamily homologue (Saci_1851; $YtrA_{Sa}$) identifiable in the genome of *S. acidocaldarius* that is responsive to thermal stress (Baes et al., 2020). However, $YtrA_{Ss}$ is not ubiquitous in the Sulfolobales, as it is notably absent in some species (e.g. *M. prunae*) (Table 3). Additionally, the regulon of $YtrA_{Sa}$ of *S. acidocaldarius* under normal growth conditions has been defined to including its own operon and one encoding a putative membrane protein (Lemmens et al., 2019). However, the regulon of $YtrA_{Sa}$ in *S. acidocaldarius* or $YtrA_{Ss}$ in *Sa. solfataricus* have not been defined during periods of thermal stress. Additionally, using the14 bp primary motif determined for *S. acidocaldarius* $YtrA_{Sa}$ (Lemmens et al., 2019) and the FIMO tool of the MEME Suite (Grant et al., 2011), a putative $YtrA_{Ss}$ binding site is found in not only its own promoter region but also within the *vapB6* gene, indicating a possible direct regulation of this TA operon.

In addition to $YtrA_{Ss}$, another heat shock responsive regulator (designated here as $FadR_{Ss}$) can be identified in *Sa. solfataricus* (Ssol_0314, SSO2506) that belongs to the TetR family of transcriptional regulators (Tachdjian & Kelly, 2006). TetR-AcrR family regulators are transcriptional repressors that are structurally similar to the GntR family regulators, consisting of an N-terminal helix-turn-helix (HTH) DNA-binding domain and a C-terminal ligand binding domain (Deng et al., 2013). Like $YtrA_{Ss}$, $FadR_{Ss}$ has close homologues present only in *Sa. solfataricus* strains (SSO2506, Ssol_0314) and *S. acidocaldarius* (Saci_1107) (Table 3) and was upregulated in response to heat shock in these archaea (Table 4). The *S. acidocaldarius* $FadR_{Ss}$ homologue (Saci_1107; $FadR_{Sa}$) has been shown to regulate fatty acid metabolism during normal growth and acyl-CoA binding $FadR_{Sa}$ causes dissociation from its DNA binding sites (Wang et al., 2019). Interestingly, both $ytrA_{Ss}$ (Ssol_2420) and $fadR_{Ss}$ (Ssol_0314) in *Sa.solfataricus* 98/2 had similarly large (~11-fold) responses to thermal stress.

## Heat shock response of toxins–antitoxins in thermophilic archaea

Type II TA systems are the most common type in archaea, and some have been implicated in the heat shock response of *Sa. solfataricus* P2 (Cooper Charlotte et al., 2009; Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011; Tachdjian & Kelly, 2006). Tables 5 and 6 summarize the TA systems in which the toxin and/or the antitoxin was highly up-regulated (≥5-fold) in response to heat shock in the selected thermophilic archaea. Although no highly heat shock responsive TA system was identified with homologues across all species investigated, the *Sa. solfataricus* 98/2 VapC22 (Ssol_0818) Toxin has homologues in all species examined here. Although TA systems are prevalent in prokaryotes they are thought to be rarely conserved (LeRoux & Laub, 2022). However, VapC22 homologues are not constrained to just the species in Table 5 as BLASTp results identified homologues throughout the Crenarchaeota and Euryarchaeota Phyla Supplemental File S1. Though *vapC22* was not highly responsive to thermal stress in both *Sa. solfataricus* strains, with fold changes less than 3-fold across species listed in Table 6, *vapC22* was among the most highly transcribed genes in the transcriptome (top 5%); thus, larger fold changes are possible since the response was at the upper end of the dynamic range for the oligonucleotide microarray used. Note that the VapC22 homologue in *M. prunae*, VapC8 (DFR88_00130), extensively degrades rRNA (Mukherjee et al., 2017). The gene encoding another rRNA degrader in *M. prunae*, VapC7 (DFR88_00435) (Mukherjee et al., 2017), responded similarly to heat shock as its *Sa. solfataricus* homologue VapC13 (Ssol_2713, SSO1914); *M. prunae* VapC7 has homologues across the Sulfolobales, but not in the Euryarchaeota *P. furiosus* or *A. fulgidus*.

There were also major differences in the thermal stress response of TA loci in the representative extreme thermophiles in Table 6. For instance, the two most heat shock responsive TA genes in *Sa. solfataricus* 98/2, *vapC8* (Ssol_2442; 10-fold) and the PilT N-terminus (PIN) domain containing putative VapC Toxin gene Ssol_2721 (16-fold), have no homologues in the other species (Tables 5 and 6). *Sa. solfataricus* 98/2 *vapC6* (Ssol_2338), highly responsive to elevated temperatures (9-fold), has homologues in *S. acidocaldarius* and *P. furiosus*, which are also up-regulated. The two most highly responsive TA genes in *M. prunae*, *vapB2* (DFR88_04525) and *vapC10* (DFR88_11390), have homologues in all surveyed Sulfolobales. *M. prunae's vapB2* has a similar thermal stress response to its *Sa. solfataricus* 98/2 homologue *vapB11* (Ssol_2492), 10- and 8-fold, respectively. Additionally, *M. prunae's vapC10* (9-fold) was responsive to thermal stress as were its homologues in the *Sa. solfataricus* species.

## Impact of VapBC and transcriptional regulator mutants on the *Sa. solfataricus* 98/2 heat shock transcriptomes

Given the heat shock response of genes encoding VapC22, VapB6, VapC6, and the transcriptional regulators $YtrA_{Ss}$ and $FadR_{Ss}$, mutants were created from the

**TABLE 5** Homologues of heat shock responsive toxin–antitoxins across thermophilic archaeal species.

| Toxin/antitoxin name | | Gene ID | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sso** | **Mpru/ Msed** | **Mpru** | **Msed** | **Sso 98/2** | **Sso P2** | **Saci** | **Pfu** | **Af** |
| VapB6 | | | | Ssol_2337 | SSO1494 | Saci_1947 | PF1353 | AF0608 |
| | | | | | | Saci_1980 | | |
| VapC6 | | | | Ssol_2338 | SSO1493 | Saci_1981 | PF1352 | |
| VapB8 | | | | Ssol_2441 | SSO8620 | Saci_1970 | | |
| VapC8 | | | | Ssol_2442 | SSO1657 | | | |
| VapB11 | VapB2 | DFR88_04525 | Msed_0412 | Ssol_2492 | SSO_RS08625[a] | Saci_1791 | | |
| VapC11 | VapC2 | DFR88_04530 | Msed_0411 | Ssol_2491 | SSO1786 | Saci_1790 | | |
| VapB22 | | | | Ssol_0819 | SSO11914 | Saci_1955 | PF0573 | AF2359 |
| VapC22 | VapC8 | DFR88_00130 | Msed_1245 | Ssol_0818 | SSO3078 | Saci_1954 | PF0574 | AF2360 |
| | | | | | | Saci_2030 | | |
| PIN putative VapC toxin | | | | Ssol_2721 | SSO1921 | Saci_1953 | | |
| HTH putative antitoxin | | | | Ssol_2443 | SSO1658 | | | |
| | VapB1 | DFR88_04920 | Msed_0338 | | | Saci_1813 | | |
| | VapC1 | DFR88_04915 | Msed_0339 | | | Saci_1812 | | |
| VapB13 | VapB7 | DFR88_00430 | Msed_1215 | Ssol_2714 | SSO_RS09275[a] | Saci_1882 | | |
| | | | | Ssol_2776 | SSO_RS09595[a] | | | |
| VapC13 | VapC7 | DFR88_00435 | Msed_1214 | Ssol_2713 | SSO1914 | | | |
| | VapB10 | DFR88_11390 | Msed_1386 | | | Saci_2003 | | |
| PIN Putative VapC Toxin | VapC10 | DFR88_11395 | Msed_1385 | Ssol_2482 | SSO1701 | Saci_0323 | | |

*Note*: Colour indicates protein homologue cluster in the thermophilic archaea protein database. Toxin–antitoxin system with a ≥ 5-fold change in either Sso or Mpru were included in the above table. The *vapBC22* toxin-antitoxin system was also included due to the high basal expression of *vapC22* in Sso.
Abbreviations: Af, *Archaeoglobus fulgidus*; HTH, helix-turn-helix; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; Pfu, *Pyrococcus furiosus*; PIN, PilT N-terminus domain; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.
[a]These locus tags were assigned after the initial annotation of the genome.

parent strain PBL2025 of *Sa. solfataricus* 98/2 in which these genes were systematically disrupted or deleted. Figure S2 shows that growth was halted in all strains at the onset of the temperature shift of 80–90°C for *Sa. solfataricus* 98/2; a similar response was noted for 70–85°C for *M. prunae*. Table S2 provides an overview of the genome-wide transcriptional response for all strains. The importance of these genes in *Sa. solfataricus* 98/2 is reflected in the reduction of fitness, as evidenced by the extended doubling times for normal growth compared to the parent. As an indicator of heat shock impact, total counts for genes differentially transcribed were determined (up- and down-regulated) (Tables 7 and S2). Transcriptomes for all strains reflected a significant impact of thermal stress. The parent strain had the lowest number of genes that were 2-fold heat shock-responsive (770 total, 400 ↑/370 ↓). The $fadR_{Ss}$ mutant had the most genes responding (959 total, 520↑/439↓), while the *vapC22* mutant had the next highest number (951 total, 533 ↑/418 ↓); the *vapC22* mutant also had many more genes >8-fold up-regulated or down-regulated (65 vs. 34, respectively) than the parent strain or the other mutants (Tables 7 and S2). Note that while 872 total, 488↑/384↓ genes responded to heat shock in the $ytrA_{Ss}$ mutant, only 95 genes met the Bonferroni criterion; however, fitness was significantly affected. Given the fact that *vapC22* is within the top 5% of genes in the *Sa. solfataricus* transcriptome under normal growth conditions, the impact of its absence is not surprising.

For the most part, the transcriptional responses of sHSPs, HtpX, thermosome, proteasome, and prefoldin genes in the mutants tracked the parent strain (Table 8). Genes encoding many transcriptional regulators and TA loci, however, were significantly affected (Tables 7 and S3). The phosphate uptake PhoU-type regulator (SSO0618, Ssol_1682), already up-regulated 8.5-fold in the parent strain during heat shock, was more than 3-fold further up-regulated in the $ytrA_{Ss}$ and *vapC6* mutants. Similarly, the Lrp/AsnC family regulator (SSO2131, Ssol_2860), up-regulated 5-fold in the parent strain, was further up-regulated ~7-fold in the $ytrA_{Ss}$ mutant, 2-fold in the $fadR_{Ss}$ mutant, and 3-fold in the *vapC22* mutant (Table S3). Conversely, the HTH-type

**TABLE 6** Heat shock response in toxin–antitoxin genes across thermophilic archaeal species.

| Toxin/antitoxin name | | Heat shock response | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sso** | **Mpru/Msed** | **Mpru** | **Sso 98/2** | **Sso P2** | **Saci** | **Pfu[a]** | | **Af** |
| VapB6 | | | 4.9 | 7.0 | | | | N.C. |
| VapC6 | | | 9.3 | 8.1 | | 4.3 | 8.8 | |
| VapB8 | | | 7.1 | 6.6 | | | | |
| VapC8 | | | 10.1 | 3.4 | | | | |
| VapB11 | VapB2 | 9.7 | 7.6 | | | | | |
| VapC11 | VapC2 | 1.7 | 5.4 | 2.4 | | | | |
| VapB22 | | | N.C. | N.C. | | 3.2 | 3.0 | 1.8↓ |
| VapC22 | VapC8 | 2.5 | N.C. | N.C. | | 1.7 | 3.9 | 1.5 |
| PIN putative VapC toxin | | | 15.6 | 5.3 | | | | |
| HTH putative antitoxin | | | 7.1 | 16.5 | | | | |
| | VapB1 | 7.0 | | | | | | |
| | VapC1 | 2.5 | | | | | | |
| VapB13 | VapB7 | 7.1 | 4.6 | | | | | |
| | | | | | | | | |
| VapC13 | VapC7 | 2.6 | 4.0 | 4.6 | | | | |
| | VapB10 | 7.6 | | | | | | |
| PIN Putative VapC Toxin | VapC10 | 9.0 | 4.0 | 14.6 | | | | |

*Note*: Fold changes in transcription are derived from DNA microarray data from Sso P2 (Tachdjian & Kelly, 2006), Pfu (Shockley et al., 2003), and Afu (Rohlin et al., 2005). Mpru and Sso 98/2 data are from this work (highlighted in purple) and ≥2-fold changes have met the Bonferroni correction of 4.8. Fold changes for Saci are from qPCR data (Baes et al., 2020). Data are from a 60 min heat shock of Pfu and a 30 min heat shock for all other thermophiles. Toxin-Antitoxin system with a ≥ 5-fold change in either Sso or Mpru was included in the above table. The *vapBC22* Toxin-Antitoxin system was also included due to the high basal expression of *vapC22* in Sso.

Abbreviations: Af, *Archaeoglobus fulgidus*; HTH, helix-turn-helix; Mpru, *Metallosphaera prunae*; Msed, *Metallosphaera sedula*; N.C., no change; Pfu, *Pyrococcus furiosus*; PIN, PilT N-terminus domain; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2; Saci, *Sulfolobus acidocaldarius*.

[a]Pfu fold changes in the left column are from 30 min heat shock cultures grown on maltose and yeast extract and values in the right column are from 30 min heat shock cultures grown on tryptone and elemental sulfur.

regulator (SSO3242, Ssol_0975) was down-regulated 20-fold due to heat shock in the parent strain, but much less so when *ytrA*$_{Ss}$ or *fadR*$_{Ss}$ were disrupted (Table S3). Of note was the fact that disruption of either *ytrA*$_{Ss}$ or *fadR*$_{Ss}$ affected transcription of the other regulator. Specifically, when *ytrA*$_{Ss}$ was disrupted, the heat shock response of *fadR*$_{Ss}$ is reduced 6-fold compared to in the parent strain. On the other hand, disruption of *fadR*$_{Ss}$ triggered a 5-fold increase in transcription of *ytrA*$_{Ss}$ (to 52.2-fold), suggesting that FadR$_{Ss}$ could be a repressor of YtrA$_{Ss}$ (Tables 7 and S3). In addition, disruption of *vapC22* increased the transcriptional response of both *ytrA*$_{Ss}$ and *fadR*$_{Ss}$ >2-fold more than their heat shock response (both 10.9-fold) in the parent strain (Tables 4, 7, and S3).

As had been reported previously, heat shock of *Sa. solfataricus* P2 impacted the TA transcriptome significantly (Tachdjian & Kelly, 2006), and this is in line with the response of the *Sa. solfataricus* 98/2 strain observed here (see Tables 7 and S3). Many VapB/VapC genes were up-regulated upon heat shock in the parent strain; of note *vapC6*, *vapC8*, and a putative PIN domain/toxin were up-regulated 9-, 10- and 15.6-fold, respectively. As is clear from Tables 7 and S3, the mutations of *vapB*/*C6*, *vapC22*, *ytrA*$_{Ss}$, and *fadR*$_{Ss}$ significantly impacted the transcription of genes encoding toxins and antitoxins. In addition to the 5-fold up-regulation of *ytrA*$_{Ss}$, the disruption of *fadR*$_{Ss}$ led to increased transcription of several *vapBCs* over the parent strain (Tables 7 and S3). The disruption of *ytrA*$_{Ss}$ had the opposite effect; compared to the parent strain, *vapBC8* were further down-regulated 2- to 3-fold, *vapBC11* were reduced 2- to 4-fold, and *vapBC6* were decreased 4- to 9-fold. In general, the deletion of *vapB6* and vapC6 did not have a major impact on other *vapBC*s, although a putative PIN domain protein (SSO1921, Ssol_2721), and presumed VapC, was up-regulated from 15.6-fold in the parent to >25-fold in the *vapB6* and *vapC6* deletion strains. The disruption of *vapC22* had a major effect on both transcriptional regulators and VapBC loci. *ytrA*$_{Ss}$, *fadR*$_{Ss}$, *lrp*/*asnC* (SSO2131, Ssol_2860) and *phoU* (SSO0618, Ssol_1682) were up-regulated ~2- to 3-fold more in the *vapC22* mutant than their heat shock response in the parent strain. The *vapC22* disruption led to an increased transcription of several *vapBC* genes, most

**TABLE 7** Heat shock response of *Saccharolobus solfataricus* 98/2 regulators and toxin–antitoxin mutants.

| Gene product | Gene ID | | Heat shock response | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sso 98/2 | Sso P2 | Parent (PBL2025) | *ytrA*$_{Ss}$ *gntR*::*lacS* (PBL2124) | *fadR*$_{Ss}$ *tetR*::*lacS* (PBL2026) | Δ*vapB6* (PBL2078) | Δ*vapC6* (PBL2080) | *vapC22*::*lacS* (PBL2067) |
| ORFs[a] | | | 400↑/370↓ | 488↑/384↓[b] | 520↑/439↓ | 427↑/403↓ | 451↑/314↓ | 533↑/418↓ |
| $t_d$ (pre-HS) | | | 3.5–4 | 7–9 | 6–9 | 5–6 | 5–6 | 6 |
| Transcriptional regulator genes | | | | | | | | |
| GntR family repressor | Ssol_2213 | SSO1255 | 2.1↑ | 5.0↑ | 3.0↑ | 2.4↑ | 2.5↑ | 4.0↑ |
| | Ssol_2420 | (***ytrA*$_{Ss}$**) SSO1589 | 10.9↑ | N.C | 52.2↑ | 9.0↑ | 7.5↑ | 24.7↑ |
| TetR-AcrR family repressor | Ssol_0314 | (***fadR*$_{Ss}$**) SSO2506 | 10.9↑ | 1.7↑ | 5.3↑ | 7.5↑ | 8.6↑ | 27.2↑ |
| Toxin–antitoxin genes | | | | | | | | |
| VapB6 | Ssol_2337 | SSO1494 | 4.9↑ | N.C. | 25.9↑ | 3.1↑ | 1.7↑ | 13.5↑ |
| VapC6 | Ssol_2338 | SSO1493 | 9.3↑ | N.C. | 22.5↑ | 6.1↑ | 5.5↑ | 11.9↑ |
| VapB22 | Ssol_0819 | SSO11914 | N.C. | 2.8↑ | 2.2↑ | N.C. | 2.2↑ | 3.8↑ |
| VapC22 | Ssol_0818 | SSO3078 | N.C. | 2.7↑ | N.C. | 1.7↑ | 2.1↑ | 3.2↑ |

*Note*: Data are for 30 min heat shock of Sso 98/2 strains.
Abbreviations: N.C., no change; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2.
[a]ORFs differentially transcribed ≥2-fold 30 min post-HS versus pre-HS that met the Bonferroni correction of 4.8.
[b]Only 95 ORFs out of 872 transcribed ≥2-fold 30 min post-HS versus pre-HS met the Bonferroni correction.

notably the 6-fold up-regulation above the parent (to 92-fold) of the putative VapC annotated as a PIN domain protein (SSO1921, Ssol_2721).

## Interplay among transcriptional regulators and VapBC loci

Through these mutant transcriptional comparisons, an interconnected heat shock network composed of both traditional transcriptional regulators and type II TA systems begins to emerge (Figure 1). Both *ytrA*$_{Ss}$ and *fadR*$_{Ss}$ mutants have a large impact on several TA loci, especially *vapBC6*, which was shown previously to have a major effect on *Sa. solfataricus* 98/2 thermal lability (Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011). Directly or indirectly, YtrA$_{Ss}$ appears to function as an activator to the *vapBC6* locus, while FadR$_{Ss}$ functions as a repressor. The opposing roles of YtrA$_{Ss}$ and FadR$_{Ss}$ could be to maintain VapBC6 at an optimum level for effective heat shock response. Furthermore, there appears to be a feedback loop between these two transcriptional regulators in that YtrA$_{Ss}$ activates expression of FadR$_{Ss}$, while FadR$_{Ss}$ represses YtrA$_{Ss}$, another possible way these regulators keep the balance of the *Sa. solfataricus* 98/2 heat shock response.

The role of VapC22 in *Sa. solfataricus* strains heat shock response appears to be important. Its gene is among the top 5% of transcripts in this archaeon under both normal and stressed conditions (Tachdjian & Kelly, 2006); as mentioned, the limited dynamic range of DNA microarray analysis likely underreports the transcription level of *vapC22* under thermal stress. The fact that VapC22 has homologues in other archaea, especially to total RNA-degrading VapC8 in *M. prunae*, is intriguing. Structural predictions indicate significant homology between *Sa. solfataricus* VapC22 and VapC8 in the *Metallosphaera* species (Figure 2). Finally, the VapC22 toxin seems to function as a brake for the whole thermal stress response system by possibly degrading transcripts of YtrA$_{Ss}$, FadR$_{Ss}$, VapB6, and VapC6. In fact, VapC22 may function as a targeted brake of these specific heat shock genes or a coarse brake of total cellular function by generalized RNA degradation. Amino acid and structural homology of *Sa. solfataricus* VapC22 to the known total RNA degrader VapC8 in *M. prunae* points to the latter (Table 5, Figure 2). Additionally, VapC6 and YtrA$_{Ss}$ ease this heat shock response brake by having a repressive effect on *vapC22* expression (Figure 1).

## CONCLUSION

Despite the fact that TA loci were discovered decades ago, their role in microbial physiology and stress

**TABLE 8** Heat shock response of *Saccharolobus solfataricus* 98/2 heat shock proteins and proteases.

| | Gene ID | | Heat shock response | | | | | |
|---|---|---|---|---|---|---|---|---|
| Gene product | Sso 98/2 | Sso P2 | Parent (PBL2025) | *ytrA*$_{Ss}$ *gntR*:: *lacS* (PBL2124) | *fadR*$_{Ss}$ *tetR*:: *lacS* (PBL2026) | Δ*vapB6* (PBL2078) | Δ*vapC6* (PBL2080) | *vapC22*:: *lacS* (PBL2067) |
| Heat shock proteins and proteases | | | | | | | | |
| sHSP | Ssol_0231 | SSO2427 | 2.8 | 2.6 | 2.5 | 1.8 | 1.6 | 2.4 |
| | Ssol_0413 | SSO2603 | 2.4 | N.C. | 2.4 | 1.8 | 2.0 | 3.9 |
| HtpX | Ssol_0965 | SSO1859 | N.C. | 1.8 | N.C. | N.C. | N.C. | 1.7↓ |
| | Ssol_2626 | SSO3231 | N.C. | 2.0 | 1.7↓ | N.C. | N.C. | N.C. |
| | Ssol_0508 | SSO2694 | 2.3↓ | N.C. | N.C. | 2.5↓ | 2.0↓ | N.C. |
| Thermosome subunit α | Ssol_1851 | SSO0862 | 1.9 | 3.6 | N.C. | N.C. | 2.1 | N.C. |
| Thermosome subunit β | Ssol_1258 | SSO0282 | 1.5 | 2.7 | N.C. | N.C. | 1.8 | N.C. |
| Thermosome subunit γ | Ssol_0790 | SSO3000 | 2.2↓ | 2.0↓ | 2.0↓ | 2.0↓ | 2.0↓ | N.C. |
| Proteasome subunit α | Ssol_1795 | SSO0738 | 1.7↓ | N.C. | 2.0↓ | 1.7↓ | N.C. | N.C. |
| Proteasome subunit β | Ssol_1254 | SSO0278 | N.C. | 1.9 | 1.7↓ | N.C. | 1.7↓ | N.C. |
| Proteasome subunit β | Ssol_1825 | SSO0766 | 1.7↓ | N.C. | 2.5↓ | 2.0↓ | N.C. | 1.7↓ |
| PAN | Ssol_1248 | SSO0271 | 2.0↓ | 5.0↓ | 3.3↓ | 3.3↓ | N.C. | 5.0↓ |
| Prefoldin subunit α | Ssol_1321 | SSO0349 | 2.2↓ | 2.5↓ | N.C. | 1.7↓ | 1.7↓ | N.C. |
| Prefoldin subunit β | Ssol_1787 | SSO0730 | 2.6↓ | 2.5↓ | 1.7↓ | 2.0↓ | 2.0↓ | 1.7↓ |

Abbreviations: HTH, helix-turn-helix; N.C., no change; PAN, proteasome-activating nucleotidase; PIN, PilT N-terminus domain; Sso 98/2, *Saccharolobus solfataricus* strain 98/2; Sso P2, *Saccharolobus solfataricus* strain P2.
*Note*: Fold changes in transcription are from microarray data for Sso 98/2 (before and after 30 min heat shock). ORFs differentially transcribed ≥2-fold 30 min post-HS versus pre-HS that met the Bonferroni correction of 4.8.

response is still controversial (Fraikin et al., 2019). Chromosomally encoded TA systems seem to be associated with mobile genetic elements, are rapidly acquired and lost in microbial genomes, and exhibit little conservation even among closely related species (LeRoux & Laub, 2022). However, as is clear from Table 5, this is not the case for certain TA systems in thermophilic archaea. *Sa. solfataricus* toxins VapC6, important for surviving thermal stress (Maezato, Dana, & Blum, 2011; Maezato, Daugherty, et al., 2011), and VapC22, have homologues in several thermophilic archaea. VapC22 had a mild response to heat shock in *Sa. solfataricus*, but this response might have been much larger had not VapC22 already been transcribed among the top 5% of all genes in the transcriptome; its basal transcription level was at the top of the dynamic range of the DNA microarray used so that larger fold changes would not be detected (Tachdjian & Kelly, 2006). Efforts to clone and express an active form (as a ribonuclease) of the *Sa. solfataricus* VapC22 gene were unsuccessful. However, previous work with VapC8 from *M. prunae*, a VapC22 homologue, revealed that it is a ribonuclease capable of degrading

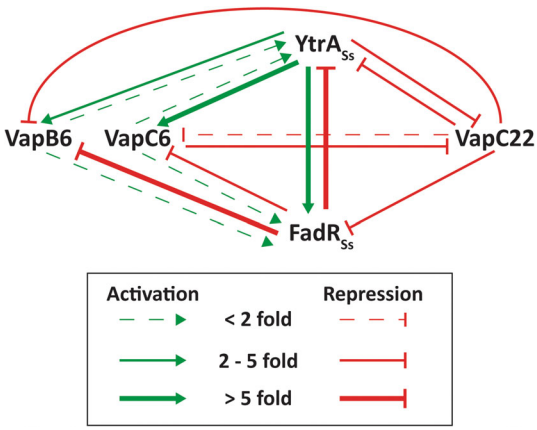


**FIGURE 1** Model of *Saccharolobus solfataricus* transcriptional regulator and toxin–antitoxin heat shock regulatory network. Based on mutant transcriptional responses to thermal stress, the proposed direct or indirect activation or repression of the transcriptional regulators YtrA$_{Ss}$ and FadR$_{Ss}$, the toxins VapC6 and VapC22, and the antitoxin VapB6 by other members of this same group are depicted.

rRNA (Mukherjee et al., 2012; Mukherjee et al., 2017). Furthermore, structural predictions showed that VapC22 and VapC8 are closely related, as are the

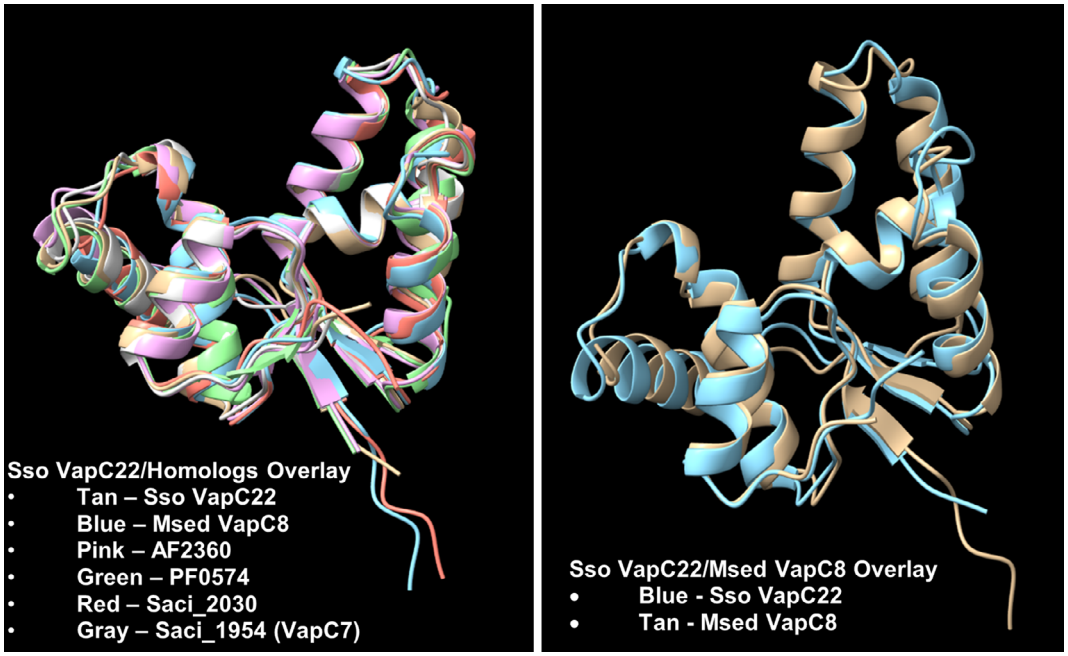


**FIGURE 2** Overlays of *Saccharolobus solfataricus* VapC22 and homologues structural predictions. Structural predictions using the RoseTTAfold option of the Robetta tool. (Left) Overlay of one representative model for each protein of the *Sa. solfataricus* VapC22 and homologues shown. (Right) Comparison of VapC22 and Msed VapC8. VapC8 is a rRNA degrader.

other homologues from *S. acidocaldarius*, *P. furiosus* and *A. fulgidus* (see Figure 2). Whether these homologues, like VapC8 from *M. prunae*, are also ribonucleases with broad specificity, remain to be seen. However, when the *vapC22* in *Sa. solfataricus* was mutated, fitness was affected ($t_d$ went from 4 h in parent to 6 h in the mutant) and the heat shock transcriptome was impacted (more than 100 more ORFs were up-regulated, 20 of which increased 16-fold compared to just 1 ORF in the parent strain; Table S2).

What is clear from this study is that TA loci are not only transcriptionally responsive to stress but also play a significant role in survival. Whether this role is transcriptional (serving as regulators of distal genes) or post-transcriptional (through ribonucleolytic action of toxins) or both remains to be seen. The conservation of VapBC loci across thermophilic archaea (Table [5]) also contrasts with the notion that these systems are randomly inserted into genomes in conjunction with mobile genetic elements. In any case, the results here point to the important role that type II VapBC TAs play in thermophilic archaea under normal and stressed conditions. It also raises the prospect that the interplay between type II TA loci and heat shock regulators in the thermophilic archaea may substitute for the global regulatory systems found in bacteria.

## AUTHOR CONTRIBUTIONS

**Charlotte R. Cooper:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); writing – original draft (equal); writing – review and editing (equal). **April M. Lewis:** Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (lead); methodology (equal); writing – original draft (equal); writing – review and editing (lead). **Jaspreet S. Notey:** Investigation (supporting); methodology (supporting). **Arpan Mukherjee:** Formal analysis (supporting); investigation (supporting); methodology (supporting). **Daniel J. Willard:** Data curation (lead); formal analysis (equal); methodology (supporting); writing – review and editing (supporting). **Paul H. Blum:** Investigation (supporting); writing – review and editing (supporting). **Robert M. Kelly:** Conceptualization (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); project administration (lead); supervision (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead).

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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