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Growth phase-dependent gene regulation in vivo in *Sulfolobus solfataricus*

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Received 23 February 2011; revised 27 April 2011; accepted 13 May 2011.

Final version published online 6 June 2011.

DOI:10.1111/j.1574-6968.2011.02313.x

Editor: Marco Moracci

**Keywords**

rRNA gene; archaeal transcription; archaeal promoter.

**Introduction**

Regulation of rRNA transcription is critical for cellular life and has been investigated extensively in Bacteria and Eukarya, where it is tightly regulated by multiple and overlapping mechanisms including growth phase-dependent regulation (Nomura, 1999; Schneider *et al*., 2003). However, little is known about rRNA transcriptional regulation in Archaea. rRNA genes in Archaea are frequently linked, containing the 23S rRNA gene downstream of the 16S rRNA gene (http://archaea.ucsc.edu). *Sulfolobus solfataricus* and *Sulfolobus shibatae* contain single 16S/23S rRNA gene operons that have been previously studied *in vivo* and *in vitro* (Reiter *et al*., 1990; Qureshi *et al*., 1997).

The basal transcriptional apparatus of Archaea is similar to that of Eukaryotes (reviewed in Bartlett, 2005). However, most putative transcriptional regulators are homologues of bacterial transcription factors and appear to act similarly, by either preventing or facilitating the assembly of the transcriptional preinitiation complex (Bell, 2005; Peng *et al*., 2011). How the regulators function *in vivo* is unclear partly due to the lack of efficient genetic systems for many Archaea. The majority of transcriptional regulation analyses in Archaea, particularly thermoacidophilic Archaea, have been performed *in vitro*. This is changing with the development of genetic tools for *S. solfataricus* (Wagner *et al*., 2009), *Sulfolobus islandicus* (Peng *et al*., 2011), and *Sulfolobus acidocaldarius* (Berkner *et al*., 2010). Regulation of rRNA transcription remains particularly cryptic, as most current approaches specifically exclude stable RNAs, including rRNA (e.g. Wurtzel *et al*., 2010). We used an SSV1-based reporter gene system in the model archaeon *S. solfataricus* (Jonuscheit *et al*., 2003) to determine whether the *S. solfataricus* core 16S/23S rRNA gene promoter (−41 to +1) is functional and regulated *in vivo* in response to the growth phase. The core TF55α and the

**Abstract**

Ribosomal genes are strongly regulated dependent on growth phase in all organisms, but this regulation is poorly understood in Archaea. Moreover, very little is known about growth phase-dependent gene regulation in Archaea. SSV1-based lacS reporter gene constructs containing the *Sulfolobus* 16S/23S rRNA gene core promoter, the TF55α core promoter, or the native lacS promoter were tested in *Sulfolobus solfataricus* cells lacking the lacS gene. The 42-bp 16S/23S rRNA gene and 39-bp TF55α core promoters are sufficient for gene expression in *S. solfataricus*. However, only gene expression driven by the 16S/23S rRNA gene core promoter is dependent on the culture growth phase. This is the smallest known regulated promoter in *Sulfolobus*. To our knowledge, this is the first study to show growth phase-dependent rRNA gene regulation in Archaea.
wild-type lacS promoters from *S. solfataricus* were used as controls.

**Materials and methods**

**Plasmid and recombinant viral vector construction**

Viral vector pKMSW72 containing the wild-type lacS gene in SSV1 was constructed in two steps (primers and plasmids listed in Table 1). First, the lacS gene plus 200 bp of upstream DNA was amplified from *S. solfataricus* P2 (DSM1617) DNA via PCR using Pfu DNA polymerase and primers BG840 and BG841, thereby introducing BamHI sites. The BamHI-cut PCR product was ligated into similarly cut pUC28, yielding plasmid pKMSW70. Plasmid pKMSW70 was cut with PstI, dephosphorylated, and ligated to PstI-cut SSV1 to create pKMSW72 (Fig. 1).

Vector pMAD107, containing the core 16S/23S rRNA gene promoter–lacS fusion, was constructed in three steps. First, the lacS promoter in pKMSW70 was deleted using long-inverse PCR (Clore & Stedman, 2007) using primers pKMSW70MasterF and pKMSW70MasterR. The PCR product was phosphorylated and ligated to produce pMT95. This plasmid was cut with PstI and PacI, dephosphorylated, and ligated to annealed oligonucleotides p16S/23SrRNAF and p16S/23SrRNAR. For annealing, oligonucleotides were incubated at 94°C for 10 min followed by slow cooling to room temperature. The resulting plasmid, pMT95, was digested with PstI, dephosphorylated, and ligated into SSV1 cut with PstI to yield pMAD107. In the same manner, primers pTF55xF and pTF55xR were annealed then ligated to pMT95 to produce the TFS55x promoter–lacS construct pMAD109. This plasmid was inserted into PstI-cut SSV1 to create pMAD110. All constructions were confirmed by restriction endonuclease digestion and sequencing of the promoter and part of the lacS gene (data not shown). XL-10 Gold supercompetent *Escherichia coli* cells (Stratagene) were utilized for all steps in vector construction.

**Transformation of recombinant virus vectors**

The pMAD107, pMAD110, and pKMSW72 plasmids, purified from *E. coli* by alkaline lysis (Feliciello & Chinali 1993), were electroporated into *S. solfataricus* PH1 as described previously (Albers & Driessen, 2008). Successful transformation was confirmed by PCR using SSV1-specific primers UnivSSV#7F and UnivSSV#8R (Snyder et al., 2004) or B49F and B49R. For UnivSSV#7F and UnivSSV#8R, PCR conditions were as follows: 95°C 1 min, then 35 cycles, 95, 60, and 72°C for 30 s each, then 4 min at 72°C. For B49F and B49R, 95°C 1 min, then 35 cycles, 95, 60, and 72°C for 30 s each, then 4 min at 72°C.

<table>
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<th>Primer name</th>
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<tr>
<td>pMAD110</td>
<td>pMAD109+SSV1</td>
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**Culture conditions**

*Sulfolobus solfataricus* strains were grown aerobically at 76 °C on plates or in liquid media, both as in Jonuscheit *et al.* (2003). *Sulfolobus solfataricus* strains PH1 and P1 were from Wolfram Zillig’s collection. Single *Sulfolobus* colonies containing recombinant viral vectors were isolated by blue-white screening on rich media as described (Schleper *et al.*, 1994). Virus infection was confirmed by PCR. Before all experiments, all strains containing viral vectors were grown to the stationary phase in minimal media containing 0.2% lactose and shifted to room temperature for 2 h to synchronize growth (Hjort & Bernander, 2001). Each culture was then diluted to OD$_{600}$nm = 0.05 in yeast sucrose media, divided into three flasks, and incubated at 76 °C with moderate shaking.

**Cell-free extracts**

Cell-free extracts were prepared from 8.0 mL of OD$_{600}$nm = 0.05 cultures 1 h after dilution for lag, 2.0 mL of OD$_{600}$nm = 0.2 cultures for mid-exponential, and 0.3 mL of OD$_{600}$nm = 1.2 cultures for stationary phase. Cultures were centrifuged for 10 min at 3000 g and cells were washed once in 1 sample volume of 10 mM Tris pH 8. Cells were resuspended in 400 μL 10 mM Tris pH 8 and lysed by two freeze/thaw cycles of −80 and 50 °C for 5 min each, and then diluted 1:10 in 10 mM Tris pH 8. Protein concentrations of cell-free extracts were determined by micro Bradford assay (Bio-Rad) compared with bovine serum albumin.

**β-Galactosidase assay**

β-Galactosidase activities were determined by colorimetric endpoint enzyme assay (Jonuscheit *et al.*, 2003). Briefly, 20 μL of each crude cell extract was added to 480 μL preheated 5 mM pNPG in 0.1 M sodium acetate pH 5. After 15 min at 95 °C (optimal temperature for lacS; Kaper *et al.*, 2002), 1.0 mL of ice-cold 0.5M NaHCO$_3$ was added and OD$_{405}$nm was measured spectrophotometrically. The amount of enzyme catalyzing the hydrolysis of 1 μmol of pNPG in 15 min at 95 °C is 1 U. The extinction coefficient of pNPG is 15.8 mM$^{-1}$ cm$^{-1}$ in sodium acetate pH 5 (Kaper *et al.*, 2002). Extracts from *S. solfataricus* PH1 (lacS$^-$) and
S. solfataricus P1 (lacS wild type) served as negative and positive controls, respectively.

**Southern hybridization**

Total DNA (Stedman et al., 1999) was extracted from exponentially growing cultures (OD$_{600}$ nm = 0.2–0.3) of S. solfataricus PH1 infected with pMAD107 (16S/23S rRNA-lacS), pMAD110 (TF55Zp-lacS), or pKMSW72 (lacSp-lacS), digested with PstI, separated by gel electrophoresis, transferred and fixed to nitrocellulose membranes. Sulfolobus solfataricus PH1 chromosomal DNA and pKMSW72 plasmid DNA were included as size markers. The lacS gene was detected by a chemiluminescent probe complementary to the N-terminus of the gene and exposure to X-ray film (Supporting Information, Fig. S2). The vector copy number was determined from multiple exposures by comparing the intensity of the signals from the chromosomal and vector copies of lacS using IMAGEQUANT (Molecular Dynamics).

**Quantitative PCR (qPCR)**

The absolute vector copy number in all cell-free extracts used for growth-phase dependent enzyme assays was determined by qPCR using the QuantiTect SYBR Green PCR kit (Qiagen) on a Strategene iCycler (Table S1). Vector-specific primers B49F and B49R were used at 0.5 μM each. Linearized pKMSW72 quantified spectrophotometrically was used as the standard for qPCR quantification (Fig. S3). Sulfolobus solfataricus PH1 cell-free extract was the no template control. The qPCR settings were as follows: one cycle at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. A melting curve was determined after the last cycle to ensure that the measured fluorescence was due to the specific product. The qPCR was performed in triplicate for all samples.

**Results and discussion**

**The 16S/23S rRNA gene and TF55Z core promoters are sufficient for S. solfataricus lacS gene expression in vivo**

In order to determine whether the core promoters of the 16S/23S rRNA gene (42 bp) and TF55Z genes (39 bp) were sufficient for expression of the lacS reporter gene in vivo, we measured β-galactosidase activity in cell-free extracts of S. solfataricus PH1 (lacS::ISC1217) (Schleper et al., 1994) transformed with viral vectors containing the respective promoter–lacS gene fusions (Fig. 2). A construct containing 200 bp upstream of lacS was used as a positive control. Cell-free extracts from transformants with all three promoters had higher levels of β-galactosidase activity than host back-ground activity, indicating that even the 39/42 bp core promoter sequences were sufficient for lacS expression in vivo (Fig. 2a). The pattern of β-galactosidase activity did not change significantly when normalized for the relative copy number of the lacS reporter gene by Southern hybridization (Fig. 2b).

Previous Sulfolobus in vivo gene expression studies using similar SSV1-based reporter gene constructs have shown that 448 bp for the TF55Z promoter (Jonuscheit et al., 2003) or 241 bp for the araS promoter (Lubelska et al., 2006) are sufficient for expression of the lacS gene. A 55-bp core promoter plus an ‘ara-box’ is sufficient for expression of lacS when in a pRN2-plasmid-based vector, but not when the ‘ara-box’ is removed (Peng et al., 2009).

**The Sulfolobus 42-bp 16S/23S rRNA gene core promoter is sufficient for growth phase-dependent gene regulation in vivo**

To determine whether the core 16S/23S rRNA gene promoter is regulated in vivo in response to the growth phase in S. solfataricus PH1, we measured the β-galactosidase activity in S. solfataricus PH1 containing the 16S/23S rRNA gene core promoter–lacS gene fusion during lag, mid-exponential, and stationary growth phases. Similar constructs with the TF55Z core and wild-type lacS promoters were tested to determine whether regulation is promoter specific. Sulfolobus solfataricus strains PH1 and P1 were included as negative and positive controls for β-galactosidase activity, respectively. The β-galactosidase activity did not change drastically between different phases of the growth cycle in wild-type S. solfataricus P1 or S. solfataricus PH1 containing the TF55Zp–lacS fusion, indicating that the wild-type lacS promoter and the core TF55Z promoter are not regulated with growth phase (Fig. 3). However, β-galactosidase activity produced by S. solfataricus PH1 containing the 16S/23S rRNA gene–lacS fusion increased approximately threefold during exponential growth compared with lag phase (Fig. 3), indicating that the region from −41 to +1 is sufficient for specific regulated transcription in response to entry into exponential growth phase. β-Galactosidase activity due to the core 16S/23S rRNA gene promoter in Sulfolobus was 1.7–3-fold lower in the stationary phase than in exponential growth (Fig. 3). The pattern of β-galactosidase activity did not change significantly when normalized for the absolute copy number of the lacS gene by qPCR, indicating that the increase in activity in exponential growth was due to regulation of the 16S/23S rRNA gene promoter, not gene dosage (Fig. 3b). The 42-bp 16S/23S rRNA gene core promoter is the smallest reported regulated promoter for Sulfolobus.

These findings are consistent with evidence of upregulation of mRNA transcription during exponential growth in E. coli and Saccharomyces cerevisiae (yeast) (Nomura, 1999).
and with microarray data from halophilic archaea showing that ribosomal protein gene transcription is higher during exponential growth than in the stationary phase (Lange et al., 2007). Moreover, rRNA in crude preparations from *Natronococcus occultus* decreases in the stationary phase (Nercessian & Conde, 2006).

The mechanism for core rRNA promoter regulation in *S. solfataricus* is obscure. The decrease in β-galactosidase activity observed during the stationary phase may be due to growth rate-dependent transcriptional regulation or stringent control in response to decreasing nutrient availability and/or charged tRNAs. The latter has been shown to decrease total stable RNA accumulation in *Sulfolobus* (Cellini et al., 2004).

As in *E. coli* and yeast, it is likely that there are multiple mechanisms contributing to regulation of the *Sulfolobus* 16S/23S rRNA gene operon. There is considerable evidence that archaeal transcriptional regulators interact with core promoters, either binding between or overlapping the TATA box and the transcriptional start site (Peng et al., 2011). *In vivo* and *in vitro* analyses have determined several regulatory regions and the start site of the 16S/23S rRNA gene in *S. shibatae* (Hudepohl et al., 1990; Reiter et al., 1990; Hain et al., 1992; Qureshi et al., 1997). The core promoter sequences necessary for transcription initiation *in vitro* are between −38 and −2 bases relative to the transcription start, identical to those used here *in vivo*. This region encompasses the proximal promoter element (PPE) (an AT-rich sequence −11 to −2 conserved in *Sulfolobus* stable RNA promoters), the TATA box, and several bases upstream thereof (Reiter et al., 1990), later identified as a transcription factor B (TFB) recognition element (BRE) (Qureshi & Jackson, 1998). A weak positive regulatory region between −354 and −190 and a negative regulatory region between −93 and −38 were also found (Reiter et al., 1990). Transcription initiates more efficiently *in vitro* from 16S/23S rRNA gene promoters with purified RNA polymerase, TFB, and the TATA-box binding protein than in cell extracts obtained from stationary phase cells, indicating a negative regulatory factor therein (Qureshi et al., 1997).

**Identification of a possible rRNA-specific regulatory motif in the 16S rRNA gene promoter**

In order to define motifs in the *S. solfataricus* 16S/23S rRNA gene core promoter possibly important for regulation, the 42-bp sequence was compared with the core promoters from *S. solfataricus* ribosomal protein genes (http://

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**Fig. 2.** β-Galactosidase activities in cell-free extracts of *Sulfolobus solfataricus*. (a) Enzymatic activities of cell-free extracts from wild-type *S. solfataricus* P1, *S. solfataricus* PH1 (β-galactosidase lacS mutant), and strains of *S. solfataricus* PH1 transformed with promoter–lacS gene fusions. (b) Enzymatic activity in crude extracts normalized for relative lacS copy number determined by Southern hybridization. Activity and copy number were determined for each biological replicate in triplicate. Mean and SD of three biological replicates are shown.
The only clearly conserved motifs are the TATA box and a potential BRE (Fig. 4a) and these are not conserved with the rRNA promoter (Fig. 4b). Moreover, the BRE sequence is noncanonical (Bartlett, 2005) and the distance between the transcription start site and the TATA box is considerably longer in the rRNA promoters (Fig. 4b), indicating that transcription may be differently regulated between rRNA and ribosomal protein genes. There is also no obviously conserved PPE or downstream BRE, unlike the minimal arabinose-regulated promoters analyzed in vivo (Peng et al., 2009) although this region is rich in A/T base pairs and mutations therein reduced activity of the 16S/23S rRNA gene promoter in vitro (Hain et al., 1992). To determine whether there was an rRNA-specific regulatory motif, predicted rRNA promoters from other Sulfolobus species were compared. The rRNA promoter is identical in S. solfataricus, S. shibatae, and seven ‘S. islandicus’ genomes (Reno et al., 2009), but is less conserved in S. acidocaldarius.
and *Sulfolobus tokodaii* (Durovic & Dennis, 1994; Kawarabayasi *et al*., 2001; Fig. 4). Nonetheless, a conserved possible regulatory sequence between −9 and −14, ‘5′-ACAANA-3′’, was identified and remains to be tested.

Changes in β-galactosidase activity are not due to gene dosage changes

To eliminate the possibility that differences in β-galactosidase activity were due to gene dosage effects, the relative or absolute copy numbers of the lacS gene in each sample were determined by Southern hybridization or qPCR, respectively. The relative copy number was calculated as the ratio of the signal from the stable vector-borne lacS gene to the disrupted chromosomal lacS gene (Fig. S2). The average relative vector copy number per chromosome is approximately one (Fig. S2). This is consistent with evidence that the number of plaque-forming units (PFU) per cell of SSV1-based shuttle vectors in *Sulfolobus* cultures remains relatively constant at 1.5 PFU per cell (Stedman *et al*., 1999). The relative lacS copy number was sometimes less than one, suggesting that these cultures contained a mixture of infected and noninfected cells (Fig. S2). When normalized for the relative lacS copy number, relative β-galactosidase activities did not change drastically (Fig. 2).

For growth-phase dependent experiments, the absolute copy number of each vector in each culture in all growth phases was determined by qPCR (Fig. S3 and Table S1). Again, this normalization did not drastically change the results (Fig. 3a and b). Hence, gene dosage effects are negligible in cultures grown from single-colony isolates regardless of the different conditions applied (Figs 2 and 3) and are not responsible for changes in the β-galactosidase activity observed at different growth phases.

SSV1-based reporter genes can be used without pyrEF selection

Many viruses affect regulation of the host cell’s genes in order to redirect the host’s machinery to support virus replication. Because little is known about the effects of SSV1 infection on *Sulfolobus*, we cannot rule out that infection with viral vectors caused changes in gene expression. However, growth rates of SSV1-infected cells are very similar to that of uninfected cells (Fig. S1; Frols *et al*., 2007). Additionally, microarray analyses of stably SSV1-infected compared with uninfected *S. solfataricus* strains indicated minimal transcriptional changes (Frols *et al*., 2007). It has been reported that similar vectors containing the lacS reporter gene were not stably maintained in culture and required the addition of pyrEF to stabilize the vector (Jonuscheit *et al*., 2003; Berkner *et al*., 2010). We also experienced loss of the vector from primary transformations (not shown). However, isolation of single colonies infected with the recombinant viral vector and subsequent outgrowth in selective media was sufficient for stable vector maintenance (data not shown). Thus, at least under these conditions, the addition of pyrEF as a selectable marker is not absolutely necessary and makes the vector somewhat smaller and easier to manipulate. We also did not observe recombination of the viral vector in *S. solfataricus* PH1 cells.

Conclusions

To our knowledge, this is the first experimental evidence for promoter-dependent regulation of the 16S/23S rRNA gene operon in *S. solfataricus* in response to changing cellular conditions and the first evidence for rRNA regulation in hyperthermophilic Archaea in response to growth phase. The severely truncated 16S/23S rRNA gene core promoter is the smallest reported regulated *Sulfolobus* promoter and provides an excellent target for future *in vitro* and *in vivo* studies.

Acknowledgements

The authors would like to thank Adam Clore for design of primers B49F and B49R, Michael Bartlett and Justin Courcelle for critical comments, the American Heart Association Pacific-Mountain Affiliate Beginning Grant in Aid Award #046002Z, the National Science Foundation MCB:0702020, and Portland State University for financial support.

References


### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Growth curve of infected and uninfected cells in early and exponential growth.

**Fig. S2.** Representative Southern blot for copy number determination.

**Fig. S3.** Typical qPCR standard curve

**Table S1.** qPCR data.

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