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Transcriptome Analysis of Infection of the Archaeon *Sulfolobus solfataricus* with *Sulfolobus* Turreted Icosahedral Virus▽†

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Microarray analysis of infection by *Sulfolobus t*urreted *i*cosahedral *v*irus (STIV) revealed insights into the timing and extent of virus transcription, as well as differential regulation of host genes. Using a microarray containing genes from both the host and the virus, the infection cycle of STIV was studied. Following infection of *Sulfolobus solfataricus* strain 2-2-12 with STIV, transcription of virus genes was first detected at 8 h postinfection (p.i.), with a peak at 24 h p.i. Lysis of cells was first detected at 32 h p.i. There was little temporal control of the transcription of virus genes, although the three open reading frames on the noncoding strand were transcribed later in the infection process. During the infection, 177 host genes were determined to be differentially expressed, with 124 genes up-regulated and 53 genes down-regulated. The up-regulated genes were dominated by genes associated with DNA replication and repair and those of unknown function, while the down-regulated genes, mostly detected at 32 h p.i., were associated with energy production and metabolism. Examination of infected cells by transmission electron microscopy revealed alterations in cell ultrastructure consistent with the microarray analysis. The observed patterns of transcription suggest that up-regulated genes are likely used by the virus to reprogram the cell for virus replication, while the down-regulated genes reflect the imminent lysis of the cells.

*Sulfolobus t*urreted *i*cosahedral *v*irus (STIV) was originally isolated from a high-temperature (82°C) acidic (pH 2.2) hot spring in Yellowstone National Park. STIV is known to infect members of the archaeal hyperthermophiles belonging to *Sulfolobus* spp., but the full extent of its host range is not known. Characterization of both the virion structure (17) and its components, along with sequence analysis of the viral genome (23), indicates that the virus will be the type member of a new family of archaeal viruses. Further interest in STIV stemmed from the discovery of its structural similarity to bacteriophage PRD1, adenoviruses and *Paramecium bursaria Chlorella* virus 1, and bacterial and eukaryal viruses, suggesting a shared evolutionary history between viruses from all three domains of life (8, 23).

Sequence analysis of the circular double-stranded DNA genome of STIV raised several important questions about the replication cycle of the virus. Analysis of the ~17-kb genome of STIV suggested there were 37 open reading frames (ORFs) (36 in the original annotation and an additional gene product revealed by proteomic analysis of the purified virion) (23). A total of nine virus-encoded proteins were identified in purified virions (17), including the major capsid protein, for which the crystal structure has been determined (8). The remaining 36 ORFs showed little or no similarity to sequences in the database, with most of the similar sequences belonging to other viruses infecting *Sulfolobus* spp. The lack of sequence similarity between any of the STIV genes and known genes led to structural studies to investigate function. The putative functions of three more proteins have been elucidated by these structural studies. STIV proteins B116 and F93 are both believed to be DNA binding proteins (10, 11), while A197 appears to be a glycosyltransferase (12). The remaining 25 ORFs identified in STIV have no known function, nor is it clear that they are transcribed and code for actual proteins. Although the genetic and structural studies have revealed a great deal about the virion itself, little is known about the replication cycle of STIV.

The dependence of STIV on the replication and transcription machinery of its host suggests that there must be other host proteins that are important in the production of virus progeny. Using a proteomics approach to study purified STIV virions, 11 proteins were identified, including 2 from the host (Sso7d and SSO0881) and 1 previously unannotated virus protein (A78) (17). The identified virus-encoded proteins were the major capsid protein B345 and other structural proteins, C557, B109, B130, A55, B164, A223, and C381. One small ORF (A51) in the initial annotation is thought to be the end of a larger ORF (C557). None of the ORFs in STIV appear to code for either a DNA or an RNA polymerase; thus, it is likely that the virus uses the host machinery for replication and transcription. This is not uncommon in viruses, especially archaeal vi-

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ruses, few of which have been identified as coding for a polymerase.

One way to approach questions about the unknown ORFs in the STIV genome and to begin to understand how the virus interacts with the host is to measure the global transcriptome during the infection process. Microarray analysis to determine the transcriptional responses of the host and the virus has been applied to better characterize host-virus interactions. For example, this approach has been successfully used to study PRD1 infections of *Escherichia coli* (19) and P-SSP7 infection of *Prochlorococcus* (13). Microarrays have also been used to determine the response of SSV1, a temperate virus infecting *Sulfolobus* spp., following induction with UV light (4). In all of these studies, the use of microarrays enabled the detection of differentially regulated host genes and, in some studies, viral genes, providing insights into the viruses' replication cycles.

Using custom microarrays containing both the STIV and *Sulfolobus solfataricus* genomes, a time course of STIV infection was investigated to determine how both host and virus transcriptions change. In addition, we followed changes in host cell growth, cell ultrastructural changes, and virus genome production. Our goal was to identify which virus and host genes were differentially transcribed as a consequence of STIV infection and to determine if there is any temporal pattern in transcription.

MATERIALS AND METHODS

Isolation of a *Sulfolobus* strain highly susceptible to STIV. *S. solfataricus* P2 (DSM 1617), obtained from the ATCC, was grown in liquid medium 182 (http://www.dsmz.de/microorganisms/html/media/medium000182.html) at pH 3.5. The cells were plated on the same medium solidified with Phytogel (Sigma) and subjected to five rounds of single-colony isolation (30). Liquid cultures of the single-colony isolates were tested for susceptibility to STIV by inoculating the culture with concentrated STIV. The susceptibilities of the single-colony strains to STIV were determined by following cell growth using the optical density at 650 nm (OD_{650}) and collecting samples for immunoblot detection of the major capsid protein of STIV and for quantitative PCR (qPCR) using primers to the major capsid protein. One isolate was characterized by production of high levels of the STIV major capsid protein and viral genomes coinciding with a decrease in OD and cell numbers. This isolate, designated *S. solfataricus* strain 2-2-12, was used for microarray analysis.

Time course of infection and sample collection. Three independent pairs of *S. solfataricus* strain 2-2-12 cultures were infected with STIV and sampled over time. For each time course, a 25-ml culture of *S. solfataricus* strain 2-2-12 was started from the glycerol stock in medium 182 at pH 3.5 and 80°C. These cultures were grown for ~48 h, reaching an OD_{650} of ~0.3 (approximately late-log culture). The cultures were subsequently diluted to 500 ml in medium 182 at pH 2.5 and grown to an OD_{650} of ~0.3 before being further diluted to 2 liters and split into two separate cultures. The 1-liter cultures were grown until the OD_{650} was ~0.29, and then one culture was inoculated with concentrated STIV at a multiplicity of infection (MOI) of ~1.5 to 2, while the other culture remained uninfected as a control.

Sample aliquots were collected at time zero (at the point of culture infection with STIV) and every 8 h for 56 h postinfection (p.i.) from both the infected and control cultures. At each sampling time point, cell growth was measured by OD_{650} and STIV genome production was measured by qPCR, and total RNA and total protein were extracted. For RNA and protein analyses, 50-ml samples were collected and centrifuged at 1,610 × *g* for 3 min, and the cell pellet was washed with yeast extract-free medium 182 at pH 2.5. The samples for RNA analysis were resuspended in 0.5 ml of RNALater (Qiagen) and stored at −20°C until the time course was completed. The protein samples were resuspended in 0.5 ml of sterile PBS, flash-frozen in liquid nitrogen, and stored at −80°C until further analysis was performed. STIV was detected by qPCR using primers designed to amplify a 280-bp section of the major capsid protein; 7.5 pmol of each primer was added to 12.5 μl of master mixture from a QuantiTect Sybr green PCR kit (Qiagen) with 2 μl of sample, and the volume was adjusted to 25 μl with double-distilled H_2O. The reaction was carried out using a RotorGene 2000 real-time cycler (Corbett Research) with the following protocol: 95°C for 15 m, then 30 cycles of 95°C for 1 min, 50°C for 30 s for annealing, and 72°C for 1 m, with a final melt analysis ramping from 50 to 99°C in 0.5°C steps. The standard curve was generated using a known amount of plasmid containing the target sequence.

Transmission electron microscopy (TEM) analysis. During an STIV infection time course, cells from 1-ml samples were collected by low-speed centrifugation and fixed for several days in a small volume of 3% glutaraldehyde before being centrifuged again and resuspended in a small volume of cool 2% Noble agar. Once the agar had solidified, the cell pellet was teased out of the microcentrifuge tube, cut into smaller pieces, and fixed overnight with 3% glutaraldehyde in 0.05 M potassium sodium phosphate buffer, pH 7.2. Agar pieces were washed three times with potassium sodium phosphate buffer for 10 min each time, followed by postfixation in 2% osmium tetroxide at room temperature for 4 h. After dehydration in an ethanol series and the addition of the transitional solvent propylene oxide, the cell pieces were gradually infiltrated with Spurr's resin (27) and baked overnight at 70°C. Thin sections, 60 to 90 nm, were cut with a Diatome diamond knife on a Reichert OM-U2 ultramicrotome. Sections were floated onto 300 mesh copper grids and stained with uranyl acetate and Reynold's lead citrate (22). All sections were viewed with a LEO 912AB transmission electron microscope.

RNA extraction and cDNA synthesis. After each time course was completed, total RNA was extracted using the total RNA protocol for the *mir*Vana kit (Ambion). RNA was quantified using a Nanodrop spectrophotometer, and quality measures were obtained using a Bioanalyzer 2100 (Agilent). For cDNA synthesis, 2 to 5 μg of RNA was mixed with 5 μg of random hexamers and incubated at 70°C for 10 min. Following 2 min of incubation on ice, deoxynucleoside triphosphates (50 mM dCTP, dATP, and dGTP; 10 mM dTTP [Promega]; and 40 mM of aminoallyl dUTP (Sigma)), 0.01 M dithiothreitol, and 400 units of Superscript II (Invitrogen) in the supplied buffer were added. The mixture was incubated at 42°C for 2 h and then stored at −20°C until it was processed for hybridization.

Microarray hybridizations and analysis. The microarrays were custom-designed oligonucleotide arrays containing 3,042 *S. solfataricus* P2 probes (26) and 52 probes to STIV (23), as well as seven other crenarchaeal viruses, three plasmids, and *Arabidopsis thaliana* oligonucleotides as negative controls (Ocimum Biosolutions). Each probe was represented twice on every slide.

Samples for hybridization were thawed on ice and incubated for 15 min at 70°C following the addition of EDTA and NaOH to hydrolyze the RNA. After the heating step, HCl was added to neutralize the solution. The enzyme and free deoxynucleoside triphosphates were removed using the MinElute Enzymatic Cleanup kit (Qiagen), except that 80% ethanol was substituted for the wash buffer. The cDNA was eluted off the column into 20 μl of 0.1 M Na_2CO_3 at pH 8.7, added to a dried aliquot of either Cy3 or Cy5 dye (GE Healthcare), and incubated in the dark for 1.5 h. Excess dye was removed using the MinElute Enzymatic Cleanup kit, following manufacturer's protocols. The labeled cDNA was quantitated using a Nanodrop spectrophotometer to determine the amount of cDNA and the percentage of dye incorporation. Samples with at least 800 pmol of cDNA and an incorporation ratio between 20 and 70 (pmol cDNA/pmol Cy3 or Cy5) were utilized for hybridizations.

Two-color hybridizations were carried out with samples hybridized in pairs. For each slide, one infected sample and one control sample from the same time course and the same time point were hybridized. This hybridization protocol enabled us to control for variations in physiology over time and only detect changes due to virus infection. Each pair of samples was hybridized twice, in order to incorporate dye-swapping protocols. For each pair of samples, the cDNAs were combined and added to a hybridization solution containing 51% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1% sodium dodecyl sulfate (SDS). Ten micrograms of herring sperm single-stranded DNA and 10 μg of tRNA were added to the hybridization solution, and the entire mixture was incubated at 95°C for 2 min before being placed on ice for at least 1 min. The hybridization mixture was applied to a washed and dried prehybridized slide (40 min in 1% bovine serum albumin in 5× SSC and 0.1% SDS). The slides were sealed in hybridization chambers (Array-It) with water and incubated in the dark for 20 h at 42°C. Following hybridization, the slides were washed twice in SSC, 0.1% SDS for 5 min at 42°C; in 0.1× SSC, 0.1% SDS for 20 min at 42°C; and then five separate washes in 0.1× SSC at room temperature before being dried. The slides were scanned immediately using an Axon 4000B scanner (Molecular Devices).

Initial analysis was performed using GenePix Pro 6.0 (Molecular Devices). Features were aligned with the data file, and bad features were flagged. The criteria for flagging were a signal-to-noise ratio of <3 in both of the channels or

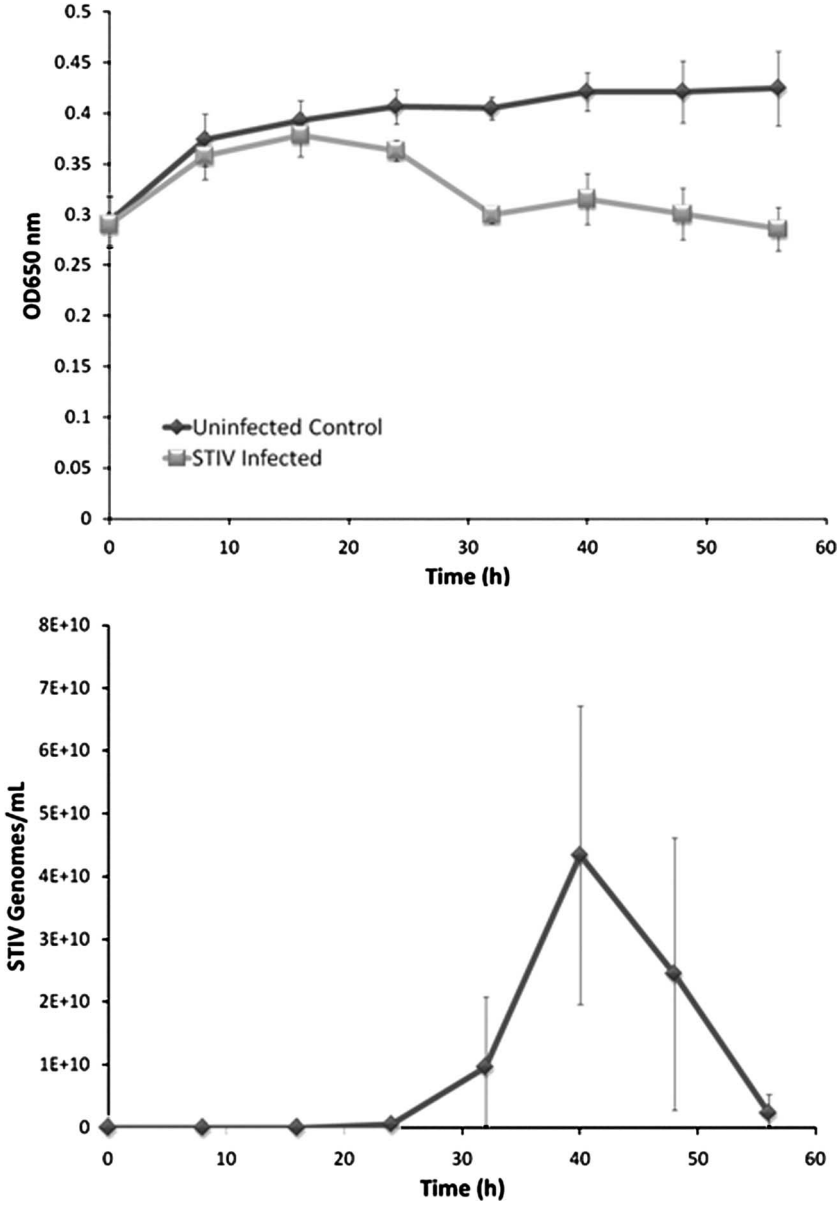

FIG. 1. Growth of cells and detection of STIV across a time course. (Top) The average OD_{650}s for triplicate cultures are presented with standard deviations. The diamonds represent the uninfected control cultures, while the squares represent the STIV-infected cultures. (Bottom) Averages and standard deviations for STIV genomes detected with qPCR.

a regression ratio of <0.2 times or >1.8 times the ratio of the medians. These criteria were designed to remove features that were similar in intensity to the background or were not uniform.

The results of the GenePix Pro analysis were then imported into Genespring GX (Agilent) for normalization and further analysis. The slides were normalized using the Lowess normalization using only probes for *S. solfataricus* P2 and STIV. This was done due to nonrandom cross-hybridization between *S. solfataricus* and STIV and some of the other genomes on the array. Following normalization and removal of data that had been flagged in GenePix Pro, the data in Genespring were filtered based on the expression level to remove any genes for which the raw data were <75, based on an average background value of 50. The final list of genes (2,916) was exported from Genespring into Excel as ratios (see Table S1 in the supplemental material). The ratio was calculated by dividing the expression value for the infected sample by the corresponding value from the control sample hybridized on the same slide.

For each probe, the Genespring value represented the average ratio of the two features on each slide. Because the dye-swapped slides were technical replicates and not biological replicates, the values for each probe were averaged between the two slides, and subsequently, the $\log_2$ was calculated. For each time point, three $\log_2$ expression ratios were used to determine differentially expressed genes using the SAM (Stanford Tools) Excel plug-in (29). Two different analyses were performed. The first was a time course analysis, looking for genes with expression ratios that changed significantly over time. The second analysis was a one-factor analysis performed at each time point to determine which genes had expression ratios that were significantly different from zero. The two gene lists were combined for analysis of the results.

RESULTS

Isolation of a *Sulfolobus* isolate that is highly susceptible to STIV. Initial STIV infection studies with the ATCC culture collection isolate of *S. solfataricus* P2 indicated that only ap-

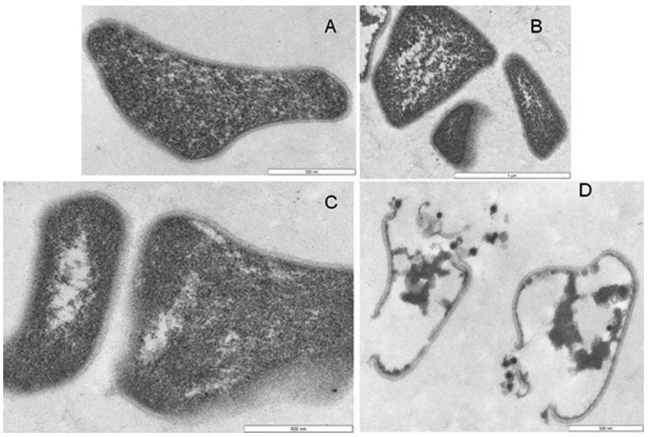

FIG. 2. TEM images from 8 (A) and 48 (B) h p.i. for uninfected controls showing intact cells. The cells infected with STIV at 8 h p.i. appear similar to the uninfected 8-h cells (C), but at 48 h p.i., the cells have been lysed and intact STIV particles have been released (D).

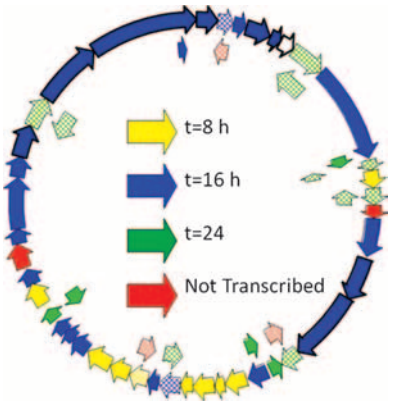

FIG. 3. Functional and transcriptional characteristics of the STIV genome. The sections of the genome are colored based on when transcripts were first determined to be differentially regulated, although all transcript levels peaked at 24 h p.i. The cross-hatched arrows represent intergenic regions, and the thickly outlined ORFs are associated with the virion. A78, not represented on the microarray, is shown by the white arrow.

proximately 10% of the cells became infected, even when inoculated at a wide range of MOIs (data not shown). This suggested that the collection isolate of *S. solfataricus* P2 was a mixed culture of cells with regard to susceptibility to STIV infection. By performing several rounds of single-colony isolations, we were able to isolate *S. solfataricus* strain 2-2-12, which was found to be highly susceptible to STIV infection at an MOI of >1.5. In contrast to the original *S. solfataricus* P2 culture, *S. solfataricus* strain 2-2-12 infected with STIV showed a reduction of cell growth (death) ~30 h p.i. Cell counts and live/dead staining (Invitrogen) suggested that the death of almost 100% of the cells was a result of STIV infection, as the pattern was not observed in uninfected controls (data not shown). Limited sequencing of *S. solfataricus* strain 2-2-12 did not reveal any differences compared to the genome of the original culture of *S. solfataricus* P2. This susceptible strain was used for the microarray study to ensure that measured signals were a result of infected cells responding to STIV without confounding signals from uninfected cells.

Effects of STIV infection on viral genome production and cell growth (lysis). Three time course experiments were carried out using *S. solfataricus* strain 2-2-12. The cells were infected in late log phase, and the infected cells continued growing at a rate similar to that of the uninfected control cells for the first 16 h (Fig. 1, top). The generation time for the control culture was calculated to be 38 h over the first 16 h, while the infected cultures' generation time was 41 h. At 32 h p.i., a decrease in OD was detected in the infected cultures, while the control cultures continued to grow. Throughout the remainder of the experiment, the OD of the infected cultures continued to decrease slightly, while the uninfected control reached stationary phase. This observation corresponded to the TEM analysis, which first detected lysed/broken cells at 32 h p.i. but saw an increase in dead cells at 40 h p.i. Based on counts of dead and broken cells at 40 h p.i., it is estimated that ~91% of the cells were infected with STIV.

STIV genomes were quantified by qPCR and used as a proxy for virus production. The original STIV inoculum was not detected at the initial sampling point (zero hour p.i.). STIV genomes were first detected in one of the cultures at 16 h p.i. (Fig. 1, bottom); however, the levels were low until 32 h p.i. Although the absolute number of genomes varied between the three experiments (within the same order of magnitude), the peak of genome detection for all cultures was at 40 h p.i., followed by a decrease in numbers to 56 h p.i. The maximum number of genomes estimated by qPCR ranged from 2.8×10^{10} to 7.1×10^{10} genomes/ml of culture for the three independent replicates. qPCR did not detect virus genomes in the uninfected controls. Samples collected from one time course were prepared for thin sectioning and imaging using TEM (Fig. 2). The images clearly showed major ultrastructural changes consistent with STIV-directed lysis of the host cell and the release of particles.

Microarray analysis. Although samples were collected through 56 h p.i., only the samples from time zero to 32 h p.i. were used for microarray analysis. RNA was extracted for all samples, but both Bioanalyzer results and the inability to synthesize good-quality cDNA from 40, 48, and 56 h p.i. suggest that the RNA from the later time points was highly degraded. This is likely a result of cell lysis/death caused by STIV infection, resulting in severely degraded RNA. As cDNA could be successfully labeled and hybridized for time points up to 32 h, the microarray analysis was restricted to the initial 32 h p.i.

STIV transcription. There were 52 probes to STIV, covering 36 of the 37 ORFs, as well as intergenic regions in both directions. A total of 47 of these 52 probe regions were determined to be differentially expressed (see Table S2 in the supplemental material). As expected, STIV probes were not detected in the uninfected controls. Intergenic regions were included in both directions to determine if there were missed ORFs or if transcription occurred across these regions. A probe to the A78 gene, corresponding to a protein detected in purified STIV particles, was not in the original genome annotation and was not included on the array. Of the 52 STIV probes, two putative ORFs, A61 and B124, and three intergenic regions on the noncoding strand (between B109 and C67, between C381 and D66, and between C57 and A106) were either not detected or

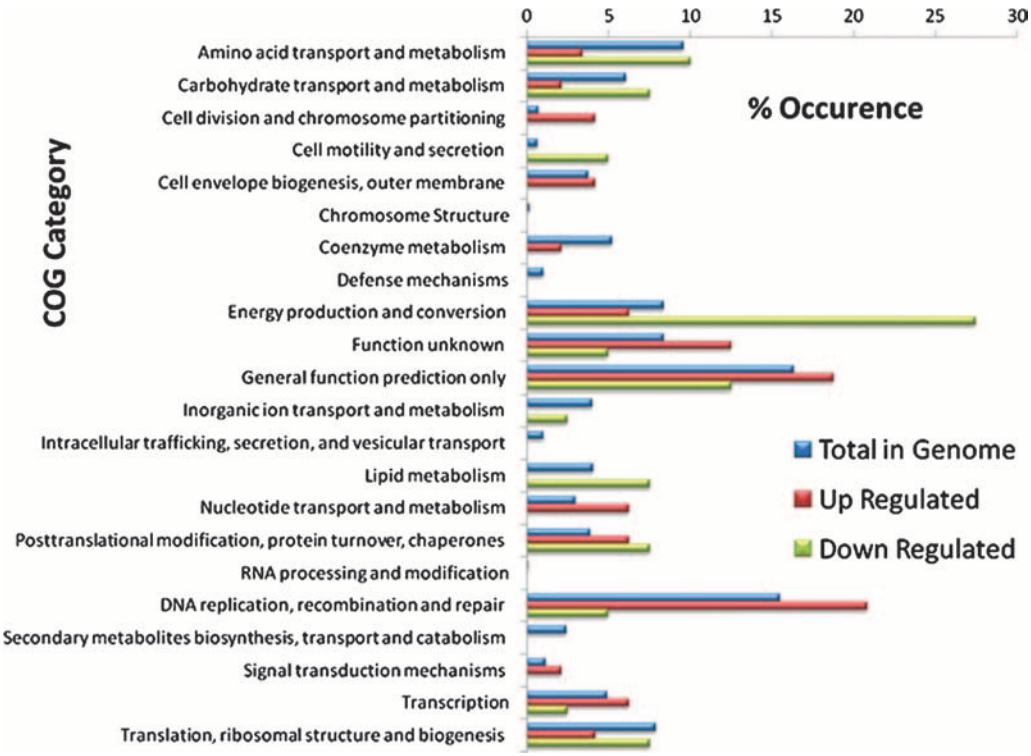

FIG. 4. Distribution of genes assigned to COG categories in the whole genome of *S. solfataricus* P2 (blue), in the up-regulated genes (red), and in the down-regulated genes (green). The percent occurrence represents the distribution of each COG category based on 2,105 assigned genes in the whole genome, 59 up-regulated, genes and 40 down-regulated genes. The unassigned genes are not included in this analysis.

transcribed at low levels and not considered statistically different from the uninfected controls (Fig. 3).

Transcripts from STIV were first detected at 8 h p.i., with nine virus genes and no host genes being differentially expressed. These early genes corresponded to the individual STIV genes C92 and C121 and two STIV gene clusters, (i) B116, A53, C118, and A66 and (ii) the region from C57 to A106, as well as A106 and A137, suggesting that these two clusters may be transcribed as operons. It further suggests that these four transcripts may represent early STIV gene transcripts (Fig. 3). By 16 h, most of the viral genes were significantly expressed (an average of 12-fold up-regulated; $n = 31$ genes); however, there were some distinct exceptions. These included the three putative ORFs on the noncoding strand, F93, F75, and D66, which all appear to be transcribed later in infection (beginning at 24 h p.i.). On the coding strand, genes C63 and B66a were not significantly expressed until 24 h p.i. Several of the intergenic regions in both directions were also not significantly expressed until 24 h p.i. These regions may represent late gene transcription regions.

Of the nine virus-encoded proteins identified in purified STIV particles, eight are represented by nine probes on the array. All were first detected 16 h p.i. and appear to be coordinately expressed. The structural genes occur in three separate areas of the genome, but all of the genes were detected by 16 h p.i. and had maximum expression at 24 h p.i. (Fig. 3). It is likely that the genes from B345 to A55, and presumably gene A78, are transcribed together, as all of the genes showed similar expression levels. Based on the position of gene A51, it is most likely that it is not an individual gene product but a fusion with the end of gene C557. In this set of genes, the role of C67, the product of which was not identified as part of the virion, remains unknown. The region encoding B164, a possible ATPase involved in DNA packing (17), is separated from this cluster of structural genes by an intergenic region. Based on the low expression levels for this noncoding region, it is likely that the gene is transcribed independently of the other structural genes, perhaps along with the gene for A109, a hypothetical protein. The other two structural genes appear to be transcribed together, perhaps with the A197 gene, which encodes a putative glycosyltransferase (12), at slightly lower levels than the other structural genes.

***S. solfataricus* response to STIV infection.** By combining the results of analysis across the time course with the results from the within-time-point analyses, a total of 177 host genes were determined to be differentially expressed during STIV infection (see Table S3 in the supplemental material). Of these genes, 124 were up-regulated, with the majority detected at 24 and 32 h p.i., while 53 genes were down-regulated, most at 32 h p.i. The change in expression tended to be higher for up-regulated genes than for down-regulated genes. The majority of down-regulated genes decreased by 2.5-fold or less, whereas 41 genes were up-regulated 4-fold or greater.

The differentially expressed genes could be divided into categories based on presumed function or lack of function. Clusters of orthogolous group (COG) numbers and categories could be assigned to 99 of the 177 genes (Fig. 4). Of the remaining 78 genes, 4 corresponded to untranslated RNAs, 4

TABLE 1. Host genes and their products in which transcription was up-regulated fourfold or more during infection with STIV

Gene locus	Gene product[a]	COG category[b]
NT02SS1974	Conserved hypothetical protein	
NT02SS3548	Hypothetical protein	
SSO0034	SOJ protein (*soj*)	Cell division and chromosome partitioning
SSO0035	Conserved hypothetical protein	
SSO0111	Hypothetical protein (predicted metal-dependent protease)	General function prediction only
SSO0237	Thiamine monophosphate kinase, putative (*thiL*)	Coenzyme metabolism
SSO0257	Cell division control 6/orc1 protein homolog(*cdc6-1*)	DNA replication, recombination, and repair
SSO0633	Amidophosphoribosyltransferase (glutaminephosphoribosylpyrophosphate amidotransferase) (ATase) (GPAT) (*purF-2*)	Nucleotide transport and metabolism
SSO0816	Hypothetical protein	
SSO0881	Conserved hypothetical protein, possibly associated with secretion	Cell motility and secretion
SSO0909	AAA family ATPase, p60 katanin	Posttranslational modification, protein turnover, chaperones
SSO0910	Conserved hypothetical protein, possibly associated with secretion	Cell motility and secretion
SSO0911	Conserved hypothetical protein	General function prediction only
SSO0946	Transcription initiation factor IIB (TFIIB) homolog (TFB-2)	Transcription
SSO0963	Reverse gyrase (*topR-2*)	DNA replication, recombination, and repair
SSO1027	Hypothetical protein	
SSO10704	Conserved hypothetical protein	
SSO1124	Not annotated	
SSO1229	Hypothetical protein	
SSO1294	Hypothetical protein (possible ATPase)	Cell division and chromosome partitioning
SSO1580	Molybdopterin oxidoreductase, molybdopterin binding subunit	Energy production and conversion
SSO1634	Conserved hypothetical protein	
SSO2048	Hypothetical protein	
SSO2184	Cell division control 6/orc1 protein homolog(*cdc6-3*)	DNA replication, recombination, and repair
SSO2277	Conserved hypothetical protein	General function prediction only
SSO2369	Hypothetical protein	
SSO2370	Hypothetical protein	
SSO2538	Glucan phosphorylase, putative (*glgP* or *malP*)	Carbohydrate transport and metabolism
SSO2596	Not annotated	
SSO2750	Hypothetical protein (possible helicase)	DNA replication, recombination, and repair
SSO2751	Hypothetical protein	
SSO3207	Ser/Thr protein kinase, putative	Signal transduction mechanisms
SSO3242	Hypothetical protein	
SSO5826	Conserved hypothetical protein	Function unknown
SSO6687	Hypothetical protein	
SSO6778	Hypothetical protein	
SSO6806	Not annotated	
SSO6830	Not annotated	
SSO7086	Not annotated	
SSO9221	DNA-directed RNA polymerase, subunit M (*rpoM-2*)	Transcription
SSO9222	Not annotated	

[a] Gene product determined from annotation on the *S. solfataricus* complete genome sequencing project website (http://www-archbac.u-psud.fr/projects/sulfolobus/).
[b] COG categories verified by BLASTP analysis using the NCBI database.

had predicted functions without a corresponding COG, and 50 were conserved hypothetical proteins or hypothetical proteins with no known function. There was also a group of 20 genes that were identified as putative ORFs in the *S. solfataricus* P2 genome but that had no annotation. The majority of these are small ORFs that may or may not be translated into proteins.

The four genes with functions but no corresponding COG include two down-regulated transposase genes and two up-regulated genes encoding 7-kDa DNA binding proteins (Sso7d). These DNA binding proteins have been shown to function in DNA bending and compaction, as well as negative supercoiling (18). Interestingly, Sso7d was identified in purified STIV particles, suggesting a role in binding the viral genomic DNA. *S. solfataricus* has three genes for 7-kDa DNA binding proteins annotated in its genome, all of which are identified as Sso7d. Because of the sequence similarity, only two of the three Sso7d genes (SSO9180 and SSO9535) were included on the array, whereas the third gene (SSO10610) would likely cross-hybridize with probes for the other two genes.

Of the 41 genes up-regulated fourfold or more, many are associated with DNA replication or transcription functions (Table 1). Of particular interest is the detection of two of the three *cdc6*-like genes, *cdc6-1* (SSO0257) and *cdc6-3* (SSO2184), which are associated with the three origins of replication in *S. solfataricus* (15). These two genes are closely associated with replication origins, while the other gene, *cdc6-2* (SSO0771), is offset from the third origin of replication, and its product may be functionally distinct from those of the other two *cdc6* genes (16). During the time course, *cdc6-2* (SSO0771) was expressed at the same level in both infected and control cultures. Other DNA replication and transcription genes that are up-regulated encode a reverse gyrase (SSO0963), a transcription initiation factor IIB homolog (SSO0946), and the M subunit of a DNA-directed RNA polymerase (SSO9221).

The distribution of genes that could be assigned COGs suggests an overall pattern in the response of *S. solfataricus* strain 2-2-12 to STIV infection (Fig. 4). Compared to the distribution of COGs in the entire genome, some categories are overrepresented by differentially regulated genes, while others are underrepresented or showed few differences. Genes for nucleotide transport and metabolism, DNA replication and repair, transcription, cell envelope biogenesis and outer membrane, cell division, and chromosome partitioning and genes assigned to the general-function or function-unknown categories tend to be up-regulated. Down-regulated genes tended to be associated with energy production and conversion, lipid metabolism, amino acid transport and metabolism, and carbohydrate transport and metabolism. Surprisingly, genes encoding proteins involved with posttranslational modification were overrepresented compared to the entire genome, but similar numbers of genes were up- and down-regulated. Genes associated with translation, although underrepresented in up-regulated genes, were evenly represented in the down-regulated genes compared to the whole genome, resulting in this category being underrepresented in the differentially expressed genes.

DISCUSSION

Analysis of both the viral and host transcriptomes during STIV infection in *S. solfataricus* strain 2-2-12 has revealed new insights into archaeal host-virus interactions. A total of 224 genes were differentially expressed as a consequence of STIV infection; 47 were up-regulated viral genes, while 177 were differentially expressed host genes. This represents approximately 95% of the suspected viral genes and 6% of the *S. solfataricus* strain 2-2-12 genes directly affected by STIV infection.

Unlike that of many other DNA viruses (14), STIV transcription does not appear to demonstrate strong temporal regulation. There are likely four transcripts, representing eight genes and one intergenic region, detected as early as 8 h p.i., suggesting they may be important in initiating early infection processes, but all of these genes continue to be transcribed during the remainder of the infection, peaking at 24 h p.i. with the majority of the virus transcripts. Transcription of the genes on the reverse strand (ORFs F93, F75, and D66) appears to be initiated later in the infection process but also peaks at 24 h p.i. None of these early genes or reverse-strand genes are structural genes. It is likely that the products of these genes play central roles in directing the viral replication cycle toward assembly and virion release. All of the structural genes are significantly transcribed by 16 h p.i., although the two smaller clusters, composed of genes A223, C381, and B164, show lower expression levels than the B345-to-A78 cluster of genes.

The pattern seen for transcription of STIV genes contrasts with the patterns observed for SSV1, a temperate *Sulfolobus* spp. virus that is known to integrate into the host genome (21, 25). In a microarray study of this virus following induction with UV, three distinct sets of genes were transcribed, representing immediate-early, early, and late genes (4). UV exposure induced transcription of a single transcript, which was not produced constitutively in lysogenized cells. Following the production of the induced transcript, three early transcripts were up-regulated relative to cells without UV exposure. The up-regulation of structural genes for SSV1 occurred late in infection, shortly before the release of virus particles. The total transcription process occurred in less than 9 h, unlike the extended transcription of STIV, and did not result in cell lysis.

Temporal transcription in STIV more closely parallels temporal transcription patterns observed for SIRV1 and SIRV2. SIRV1 and SIRV2 also infect *Sulfolbus* spp. but do not integrate into the host chromosomes (20). Infected cells have a depressed growth rate relative to uninfected cells, but lysis has not been documented. The transcription of both SIRV1 and SIRV2 shows little temporal regulation, with most genes transcribed by 30 min p.i. (7). The only significant change in transcription was the addition of two monocistronic transcripts later in the infection cycle, which is 8 h for SIRV1 and 4 h for SIRV2. The lack of strong temporal regulation of transcription in both SIRVs and STIV suggests that nontemperate viruses infecting *Sulfolobus* spp. are less dependent on differential gene expression for controlling different phases of their replication cycles.

During infection, 177 host genes were determined to be differentially expressed, with 124 genes up-regulated and 53 genes down-regulated. The up-regulated genes were dominated by genes of unknown function and those associated with DNA replication and repair. It is likely that many of the products of these genes are integral to the replication of STIV, as their up-regulation was highest at 24 h p.i. The down-regulated genes were associated with energy production and metabolism. These genes were significantly down-regulated at 32 h p.i., before lysis of the cells.

The overall response of *S. solfataricus* to STIV infection shows some similarity to the response of *E. coli* to infection with PRD1 (19). In the case of PRD1 infection, 90% of the genes on the microarray were not differentially expressed, which compares to STIV infection, where 94% of the host probes on the array were not differentially expressed. The response of *Prochlorococcus* sp. strain MED4 to infection by P-SSP7 is different (13), with 75% of the host genes down-regulated in response to infection. These dramatic differences may be a result of different virus strategies to maximize production of virus progeny. P-SSP7 appears to shut down much of the host metabolism and trigger stress responses. Using host-like genes in its own genome and exploiting the stress response in the host, P-SSP7 reprograms the host for virus production. STIV appears to take an approach to virus production similar to that of PRD1 and utilizes the machinery present in the cell, reprogramming only a small subset of host genes and avoiding a major stress response.

The majority of genes differentially expressed by infection with STIV are not typically associated with the known stress response in *S. solfataricus*. This is evident from a comparison of the response to infection by STIV with the induction of SSV1 by UV (3, 4) and exposure of *S. solfataricus* to heat shock (28). When exposed to UV light to induce SSV1 production or heat shock, *S. solfataricus* regulates the transcription of several known stress response genes. In response to UV exposure, transcription of genes encoding the three Sso7d proteins is down-regulated, as is the gene for a proteosome-associated nucleotidase (SSO0271). Of the 55 host genes identified as responding to UV exposure, 21 are shared with STIV infec-

tion; however, genes down-regulated by UV tend to be up-regulated by STIV infection (3). Heat shock of *S. solfataricus* results in a strong stress response with SSO271 down-regulated, but the genes for Sso7d proteins are up-regulated. Also detected in response to heat shock is the down-regulation of exosome-related genes and the up-regulation of genes encoding sHSPs (small heat shock proteins) and most USP (universal stress proteins) genes. The only genes associated with stress response that are differentially regulated in response to STIV infection encode the Sso7d proteins, which are found in the virion and are likely associated with viral DNA packaging.

Of the 177 host genes determined to be significantly differentially regulated, only 53 are down-regulated. All of these genes were found to be differentially regulated at 32 h p.i. or showed a downward trend (when detected as significant in the time course analysis). As the majority of these genes are associated with energy production and metabolism and the down-regulation precedes lysis of the cells, it appears that the decrease in transcription of these genes may be related to imminent cell death. The decrease in the transcription of these genes is also associated with a decrease in the expression levels of the virus genes. After 32 h p.i., following a large decrease in OD, maximum production of STIV genomes and cell lysis occur. It appears that between 24 h p.i., when the largest number of genes is up-regulated, and this final time point, there is a shift from virus replication and assembly to preparation for lysis of the cell.

Most of the 124 up-regulated host genes likely encode proteins used by the virus for replication and assembly, as well as lysis of the host. Unlike many bacteriophages, STIV does not have recognizable DNA polymerase, RNA polymerase, or lysis genes. Most likely, STIV is dependent on host enzymes to catalyze these processes. Comparisons of the up-regulated genes relative to the gene distribution in the entire *S. solfataricus* genome support this conclusion. The major category of up-regulated genes for which functions are known is associated with DNA replication and repair. Many of these genes have been associated with the replication of the host genome and may be co-opted for virus genome replication. Although STIV is known to have a circular double-stranded DNA, it is not clear whether this genome is negatively or positively supercoiled. Up-regulated with STIV infection are both the gene encoding a reverse gyrase, a protein implicated in positive supercoiling of DNA (2), and genes for the Sso7d proteins, which have been shown to induce negative supercoiling in DNA or compaction of positively supercoiled DNA (18). The Sso7d proteins have also been shown to inhibit the activity of the reverse gyrase. The up-regulation of genes for both of these proteins, which appear to counteract each other, may suggest a method of packing the STIV genome. It is possible that the reverse gyrase is involved in positively supercoiling the STIV genome, which is then further packed by the addition of the Sso7d proteins. The Sso7d proteins have been detected in the virion and may assist in relaxation of the STIV genome following infection of a new cell in preparation for replication and transcription.

Two host genes of particular interest that are highly up-regulated during STIV infection are *cdc6-1* and *cdc6-3*. The products of these two genes are associated with two of the three origins of replication of the *S. solfataricus* chromosome (15). Sequences in the *S. solfataricus* genome in which these proteins bind to initiate chromosome replication have been identified (24); however, a search of the STIV genome did not reveal similar sequences. Recent analysis of the cell cycle in synchronized cultures of *Sulfolobus acidocaldarius*, a close relative of *S. solfataricus*, suggested that these two genes are up-regulated as the cell enters the growth phase in which the chromosome is replicated (16). The cell cycle of *Sulfolobus* spp. has an extended G_2 phase, in which cdc6 proteins occur at low levels (15). Transcription of *cdc6-1* and *cdc6-3* genes upon STIV infection suggests that these proteins may play a role in virus genome replication. There are four DNA polymerases of the B family in *S. solfataricus* and one that is a homolog of the SP01 bacteriophage polymerase. None of the polymerases are differentially expressed during STIV infection, suggesting that there are likely sufficient polymerase molecules already present in the cell to support STIV genome replication. A microarray study of the *Sulfolobus* cell cycle detected only a small increase in transcription of the DNA polymerase I prior to chromosome replication, supporting this observation (16).

Transcription of the STIV genome appears to be completely dependent on the host machinery. Of the four differentially expressed genes associated with transcription, one is down-regulated (encoding the B subunit of the DNA-directed RNA polymerase), while the other three are up-regulated (genes encoding a TFIIB paralog, the M subunit of the DNA-directed RNA polymerase, and a putative transcriptional regulator). The archaeal DNA-directed RNA polymerase is composed of 10 subunits (1); however, there are 13 genes annotated in the *S. solfataricus* genome, with 3 of them being gene duplicates. Although transcripts for all of the subunit genes were detected on the microarray, 11 genes showed no differential expression during STIV infection. The gene encoding the M2 subunit, characterized as a transcription elongation factor (9), is one of the most highly up-regulated host genes, suggesting its product has an important role in the transcription of STIV. The M2 protein may be used by STIV to recruit transcriptional machinery to the virus genome. The B subunit, whose gene is down-regulated near the end of infection, just before cell lysis, contains the polymerase active site (9).

Perhaps the most interesting genes are the 78 differentially expressed genes that have no known function. The group of 20 genes that have not been annotated in the genome are of particular interest. These genes are generally small and represent ORFs that were initially included in the genome annotation but were removed for unknown reasons. The fact that they are differentially transcribed suggests that they are important in the infection process, but it is not known whether they are likely to be proteins or untranslated RNAs. Either way, these genes, especially SSO9222, the most highly up-regulated host gene, provide tantalizing targets for future investigation.

Of the known viruses infecting *Sulfolobus* spp., STIV is the first that has been demonstrated to be lytic. In these studies, a single round of viral replication was observed, followed by host lysis and release of progeny virus. The doubling time of the hosts was similar to the length of the replication cycle of STIV. The similarity in the growth rate of the host and the length of the virus lytic cycle has been seen in several other host/virus systems (5). It is possible that STIV infection directs a repro-

gramming of the cell for maximal use of the host resources for viral replication.

Previous experiments with STIV infections of the ATCC strain of *S. solfataricus* P2 suggested that the virus did not lyse the cell, due to little, if any, decrease in growth of infected cultures compared to uninfected controls. Because most crenarchaeal viruses behave in this manner, with the exception of the lytic virus *Acidianus* two-tailed virus (6), STIV was thought to be released from infected cells without killing the host. Following further study, evidence suggested that STIV was infecting only a small proportion of the entire culture, leading us to use single-colony isolation to find a cell line highly susceptible to STIV infection.

The single-colony isolate *S. solfataricus* strain 2-2-12 showed a decrease in growth, an increase in membrane-compromised cells, and a decrease in cell numbers following infection with STIV. Further study, including TEM analysis, showed that this virus assembles inside the cell and then lyses the cell for virion release. This is the first documentation of a lytic virus infecting a *Sulfolobus* sp. The inability of the virus to infect most of the cells in an *S. solfataricus* P2 culture suggests that the cultures represent a heterogeneous collection of genotypes, most of which are resistant to STIV infection. The nature of this heterogeneity is unknown; however, the genome of *S. solfataricus* P2 is known to contain high numbers of transposable elements and has been suggested to be unstable (26).

The TEM micrographs of lysing cells clearly show a disruption of the S layer and breaking of the cell membrane, leading to the release of virus particles. Unlike some lytic viruses (19), no ORF in STIV has been identified as encoding a holin or lysin. Although it remains possible that one of the ORFs with unknown function is involved in the lysis of the cell, it is also possible that STIV uses host proteins. One of the proteins identified in the virion is SSO0881 (16), a conserved hypothetical protein from the host. This protein, along with SSO0451, SSO0619, and SSO0910, is annotated as being associated with secretion, based on a conserved domain. The inclusion of SSO0881 in the virus particle may indicate how the virus enters the host cells, while the up-regulation of genes encoding the other three proteins may suggest a possible mechanism for virus release.

Overall, the studies presented here provide a defined list of candidate viral and host genes for further investigation. Clearly, an analysis of differential gene expression is only a starting point, and it will be interesting to correlate the protein expression patterns with the transcription profiles in this same data set. By combining both biochemical and genetic approaches, it should be possible to move toward a better understanding of the replication cycle of these unusual crenarchaeal viruses.

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ERRATUM

Transcriptome Analysis of Infection of the Archaeon *Sulfolobus solfataricus* with *Sulfolobus* Turreted Icosahedral Virus

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