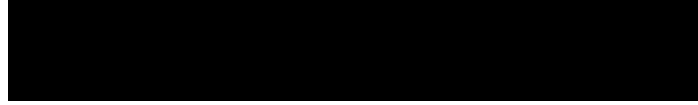




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Prokaryotic silencing (psi)RNAs in *Pyrococcus furiosus*

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ABSTRACT

In many prokaryotes, noncoding RNAs that arise from the clustered regularly interspaced short palindromic repeat (CRISPR) loci are now thought to mediate defense against viruses and other molecular invaders by an RNAi-like pathway. CRISPR loci contain multiple short regions of similarity to invader sequences separated by short repeat sequences, and are associated with resistance to infection by corresponding viruses. It is hypothesized that RNAs derived from these regions, termed prokaryotic silencing (psi)RNAs, guide Slicer-like complexes of partner proteins to destroy invader nucleic acids. Here we have investigated CRISPR-derived RNAs in the archaeon *Pyrococcus furiosus*. Northern analysis revealed multiple RNA species consistent with a proposed biogenesis pathway that includes full-length CRISPR locus transcripts and intermediates generated by endonucleolytic cleavages within the repeat sequences. However, our results identify the principal products of the CRISPR loci as small psiRNAs comprised primarily of invader-targeting sequence with perhaps only 5–10 nucleotides of CRISPR repeat sequence. These RNAs are the most abundant CRISPR RNA species in *P. furiosus* and are likely the guides for the effector complexes of the proposed prokaryotic RNAi (pRNAi) system. We analyzed cell-free extracts fractionated under non-denaturing conditions and found that the various CRISPR RNA species are components of distinct RNA–protein complexes, including at least two complexes that contain mature-length psiRNAs. Finally, RNAs are produced from all seven CRISPR loci present in the *P. furiosus* genome, and interestingly, the most recently acquired psiRNAs encoded proximal to the leader sequence of a CRISPR locus appear to be the most abundant.

Keywords: CRISPR; prokaryotic RNAi; genome defense; viral defense; prokaryotic silencing (psi)RNAs

INTRODUCTION

Small, noncoding (nc)RNAs are found in all domains of life and function in a wide array of essential cellular processes. In eukaryotes, small ncRNAs, including siRNAs and microRNAs, have been shown to function in post-transcriptional gene silencing by targeting exogenous or endogenous RNAs, respectively, in a process called RNA interference, or RNAi (Hannon 2002). Another class of small RNAs referred to as piwi-associated RNAs (piRNAs) or repeat associated small interfering RNAs (rasiRNAs) regulates spreading of selfish genetic elements such as transposons or repeat elements in organisms including mammals, plants, and flies (Kim 2006; Nishida and Siomi 2006; Aravin et al. 2007; Hartig et al. 2007; Lin 2007).

An RNAi-like system that functions in genome defense has recently been proposed to exist in prokaryotes (Makarova et al. 2006; Deveau et al. 2008; Sorek et al. 2008; Tyson and Banfield 2008). The hallmark of the proposed prokaryotic RNAi (or pRNAi) system is the CRISPR locus, a cluster of short direct repeats that separate short variable sequences (i.e., clustered regularly interspaced short palindromic repeat). A number of the variable sequences (also sometimes called “spacers”) found in CRISPR loci display complementarity (or identity) to known prokaryotic viruses, plasmids, and transposons (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005; Lillestol et al. 2006; Makarova et al. 2006). The other signature component of the hypothesized pRNAi system is a set of protein-coding genes referred to as CRISPR-associated or *cas* genes that are found in CRISPR-containing genomes (Jansen et al. 2002; Makarova et al. 2002, 2006; Haft et al. 2005). The *cas* genes are predicted to encode nucleases, helicases, RNA-binding proteins, and a polymerase (Jansen et al. 2002; Makarova et al. 2002, 2006; Haft et al. 2005). These bioinformatically predicted properties of the CRISPR and *cas* gene products led to the hypothesis that they comprise an RNAi-like system of genome defense in prokaryotes, in which RNAs

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the locus number (1, 2, and 4–8 in *P. furiosus*), and the second two digits, separated from the first by a decimal point, indicate the position of the psiRNA within that locus (relative to the leader). For example, the first psiRNA in CRISPR locus 1 is Pf psiRNA 1.01, the first psiRNA in CRISPR locus 2 is psiRNA 2.01, and the last psiRNA in CRISPR locus 1 is psiRNA 1.51 (see Fig. 1). Figure 1 shows the number of times each individual psiRNA was cloned. The psiRNA clones ranged between 17 and 50 nt in length (see Supplemental Table 1 for psiRNA clone sequences). The clones included variable amounts of the psiRNA sequence (12–40 nt) and of repeat sequence at the 5' (0–8 nt) and/or 3' (0–22 nt) end. In addition to the psiRNA clones, we isolated a few CRISPR-derived clones that lacked psiRNA sequence and consisted of a portion of leader sequence upstream of a repeat (see “Leader” section of Supplemental Table 1), indicating that the 3' end of the CRISPR leader is also transcribed.

Our sampling is not apparently at saturation; however, we cloned psiRNAs from the beginning, middle, and end of CRISPR loci (Fig. 1), indicating that RNAs are produced from across the length of the loci. Interestingly, however, the likelihood of cloning was significantly higher for psiRNAs encoded within the first part of a CRISPR locus, suggesting a greater abundance in the organism of psiRNAs from these regions. Two-thirds of the psiRNAs that we cloned were from the first third of their CRISPR locus, and 45% were one of the first four psiRNAs in a given locus (Fig. 1). With the exception of locus 2, this trend was observed within each individual CRISPR locus.

Comparison of the percentage of psiRNAs cloned from a given locus (Table 1, percent of clones) to the percentage of the total psiRNAs encoded by that locus (Table 1, percent of psiRNAs) revealed that most of the loci are represented proportionately within the clones. However, locus 6 seems to be significantly underrepresented in the cDNA library. Locus 6 encodes ~22% of the psiRNAs in *P. furiosus*; however, only ~12% of the cloned RNAs were derived from this locus. This suggests that the psiRNAs encoded within locus 6 are less abundant in *P. furiosus* than those encoded by the six other CRISPR loci.

TABLE 1. Distribution of cloned psiRNAs

CRISPR locus	Number of psiRNAs	Number of clones	Percent of psiRNA (%)	Percent of clones (%)
1	51	40	25	30
2	20	12	10	9
4	22	20	11	15
5	30	20	15	15
6	45	16	22	12
7	21	17	10	13
8	11	7	5	5
Total	200	132		

The results of the RNA cloning suggest the presence of novel, small psiRNAs in *P. furiosus*. However, the cloned RNAs were not of a uniform size or composition. To determine whether discrete psiRNA species are present in *P. furiosus*, we undertook additional analysis.

Northern analysis of RNAs derived from the CRISPR loci in *P. furiosus*

In order to further investigate the RNAs that arise from the CRISPR loci in *P. furiosus*, we undertook Northern analysis with probes against both repeat and psiRNA sequences. Probes were designed for detection of both sense (transcription from the leader sequence) and antisense RNAs. Figure 2A shows results obtained with a probe that recognizes the repeat sequence (sense orientation) that is common to *P. furiosus* CRISPR loci 1, 5, and 6. This probe detected a prominent band at ~65 nt, a less prominent band at ~130 nt, and an unresolved set of bands of greater than 150 nt near the top of the gel. For this and all other probes tested, no significant differences in the patterns were observed from total RNA samples prepared with and without DNase treatment, indicating that the bands represent RNAs. Consistent with the observations and CRISPR RNA processing pathway proposed by others (Tang et al. 2002, 2005; Lillestol et al. 2006; Makarova et al. 2006; Sorek et al. 2008), the set of bands above 150 nt likely represents a mixture of primary transcripts from the three loci as well as larger intermediates generated by cleavages within repeat regions. The most prominent band detected with the repeat probe in *P. furiosus* (~65 nt) corresponds well to the primary product of the CRISPR loci reported previously in other organisms (Tang et al. 2002, 2005; Lillestol et al. 2006); however, in this work this RNA is identified as the “1X intermediate” (Fig. 2). This band corresponds in length to a psiRNA (~35–40 nt) and repeat (~30 nt) and likely represents psiRNAs with flanking repeat sequences generated by cleavages within the adjacent repeats (see Fig. 2C). The detection of this RNA by the repeat probe suggests that cleavage may be asymmetric within the repeat sequence, leaving a substantial contiguous region of the 30-nt repeat on one side (e.g., the 3' end as modeled in Figure 2C) for efficient detection by Northern probes. A less abundant band of ~130 nt corresponds in length to two psiRNAs with flanking repeat sequences and likely represents the immediately upstream 2X intermediates (Fig. 2C; Tang et al. 2002, 2005; Lillestol et al. 2006).

Northern analysis with a probe against one of the variable psiRNA sequences revealed novel CRISPR RNA species. Using a probe against psiRNA 4.02 (sense orientation), we detected a band at ~60 nt and a very faint signal near the top of the gel, but the most prominent band is ~46 nt (Fig. 2B) and corresponds in size to that of the psiRNA (35 nt in the case of psiRNA 4.02) and ~10 nt of repeat sequence. A significant secondary band was detected at ~39 nt (Fig. 2B).

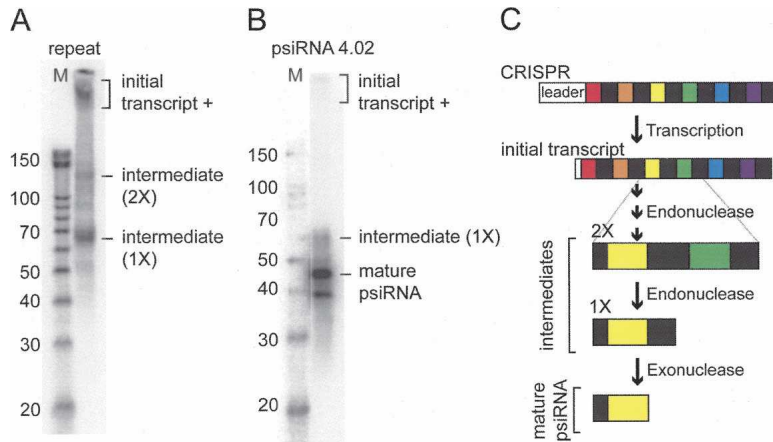


FIGURE 2. Northern analysis of RNAs containing CRISPR repeat and psiRNA 4.02 sequences. (A, B) Northern blots were performed with 10 μ g total RNA using a degenerate oligonucleotide probe designed to detect the repeat sequences for CRISPR loci 1, 5, and 6 (A) or a probe for psiRNA 4.02 sequence (B). Radiolabeled RNA marker sizes are indicated (M). The positions of the initial transcript and large intermediates, 2X intermediate, 1X intermediate, and primary mature psiRNA described in the text and Figure 4C are indicated. (C) Proposed psiRNA biogenesis pathway. The CRISPR locus is transcribed from a start site within the leader sequence to produce an initial transcript that includes a portion of the leader and the alternating psiRNA and repeat sequences. The initial transcript is cleaved within the repeats to produce intermediates. The endonucleolytic cleavage site may be asymmetrically located within the repeat. The 2X intermediate and 1X intermediates are illustrated. Our results indicate that the 1X intermediate is further processed by an exonuclease to remove most of the repeat sequence, resulting in a primary mature psiRNA species that contains 5–10 nt of the repeat sequence.

Importantly, similar results were observed in Northern analysis of RNAs from other CRISPR loci. Results for all RNAs analyzed, both sense and antisense, are compiled in Figure 3. First, like the repeat 1, 5, 6 probe, a probe for the repeat sequence common to loci 2, 4, and 7 (sense orientation) detected prominent diffuse bands of \sim 65 and \sim 130 nt (theoretical 1X and 2X processing intermediates) (see Fig. 2C). In addition, we probed for psiRNAs from the first part of each CRISPR locus (1.01, 2.01, 4.02, 5.02, 6.01, 7.01, and 8.01) as well as for psiRNA sequences from the middle and end of locus 7 (7.11 and 7.21). Strikingly, probes for each of the psiRNA sequences (sense orientation) detected a single predominant RNA species (indicated with dots in Fig. 3). Most of these predominant RNAs were \sim 43 to \sim 46 nt. The observed size of the major RNA species was generally 5–10 nt longer than the encoded psiRNA sequence. The psiRNA with the longest observed primary product (psiRNA 1.01) has an unusually long psiRNA sequence. These findings, together with the observation that these RNAs are recognized by psiRNA but not repeat sequence probes, suggest that the primary mature psiRNA species in *P. furiosus* consists of a psiRNA with \sim 5–10 nt of repeat.

In addition to the primary psiRNA species, each of the psiRNA probes detected other RNAs. These often included an RNA close to the size of the \sim 65-nt 1X intermediates that were detected by repeat probes, and in some cases (e.g., psiRNAs 101 and 402), an RNA was detected that was the size

of the theoretical 2X intermediate. Many of the psiRNA probes detected other faint bands. However, in many cases the most prevalent secondary species was a slightly smaller RNA of \sim 38 to \sim 45 nt.

We did not detect antisense RNAs with most of the CRISPR probes (Fig. 3B). Prominent bands were detected with probes from psiRNAs 2.01 and 7.11; however, the absence of corresponding bands with the repeat probes suggests that these are not CRISPR locus-derived RNAs.

Northern analysis of CRISPR RNA distribution in fractionated *P. furiosus* extract

The CRISPR RNAs are hypothesized to function in complex with proteins in various aspects of RNA-guided genome defense in prokaryotes (Makarova et al. 2006). To assess whether the CRISPR-derived RNA species that we identified may be components of complexes, we analyzed the distribution of the RNAs across fractions from anion exchange chromatography of *P. furiosus* S100 cell extract performed under anaerobic conditions. Fractions were evaluated by Northern analysis using probes against the repeat sequence common to loci 1, 5, and 6, and psiRNA 4.02 and 7.01 sequences (Fig. 4). For reference, the profile of RNAs detected in unfractionated extract is shown in the first lane.

The distribution of the various RNAs across the fractions suggests the presence of several distinct CRISPR RNA-containing complexes. The novel primary and secondary psiRNAs (\sim 45 and \sim 39 nt) from both loci co-fractionated in a distinct set of fractions denoted as peak A (Fig. 4, fractions 10–14). Other larger CRISPR RNA species were not observed in peak A. The primary mature psiRNA is also present in peak B (Fig. 4, fractions 20–23) along with a fraction of the 1X intermediate RNA and some of the variable-size psiRNA species. However, the highest concentration of the 1X intermediate is found in a distinct set of fractions that lack small psiRNAs, termed peak C (Fig. 4, fractions 23–26). The mixture of RNAs that likely includes full CRISPR locus transcripts and larger intermediates and the 2X intermediate are found primarily in peak D (Fig. 4, fractions 31–34). These results suggest the presence of multiple complexes each containing distinct subsets of CRISPR-derived RNAs in *P. furiosus*.

To verify the presence of CRISPR RNA–protein complexes in the fractionated *P. furiosus* extract, we examined the complexes on native gels. Using the probe for psiRNA 7.01, we compared the mobility of the RNAs, both in the

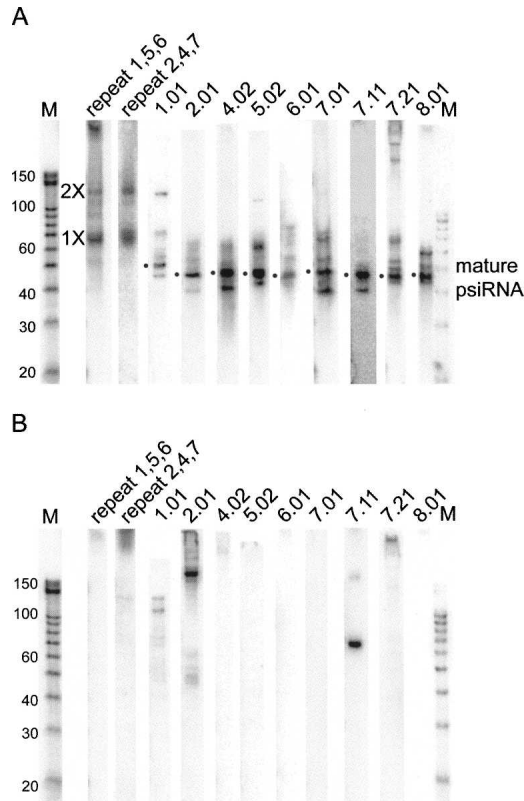


FIGURE 3. Northern analysis of RNAs from the seven *P. furiosus* CRISPR loci. (A, B) Northern analysis was performed with 10 μ g of total RNA using probes to detect sense (A; transcribed from the leader sequence) or antisense (B) psiRNA or repeat sequence-containing RNAs as indicated. Lanes are approximately aligned on the basis of adjacent marker lanes (not shown except for repeat 1, 5, 6 and psiRNA 8.01 lanes). Dots located to the left of lanes indicate the primary mature psiRNA species. 1X and 2X intermediates detected by repeat probes are indicated.

presence of the cofractionating proteins in peaks A, B, and C and following protein extraction, by non-denaturing PAGE and Northern analysis. In each case, a significant shift in the mobility of the RNAs was observed in the presence of the proteins. Peak D was not examined on native gels. Together our results indicate that the novel primary mature psiRNA is a component of at least two chromatographically distinct RNA–protein complexes (peaks A and B), the 1X intermediate is found primarily in a third complex (peak C), and a fourth complex includes larger psiRNA precursors. These complexes are likely candidates for the mediators of psiRNA production, invader destruction, and CRISPR element integration in the proposed prokaryotic RNAi pathway.

DISCUSSION

Novel CRISPR RNAs

The CRISPR loci found in many prokaryotes encode alternating repeat and spacer or psiRNA sequences and

have been shown to give rise to a series of RNAs that decrease in increments from the full-length locus transcript to a single psiRNA and repeat (i.e., 1X intermediate) (see Fig. 2C; Tang et al. 2002, 2005; Lillestol et al. 2006). Current evidence indicates that processing occurs by endonucleolytic cleavages within the repeat sequences (Tang et al. 2002, 2005). The RNA products of the CRISPR loci are hypothesized to guide silencing of viruses and other genome invaders. In this work, we have identified a novel class of smaller discrete CRISPR-derived RNAs that we have termed psiRNAs, which appear to be the ultimate gene products of the CRISPR loci (Fig. 2C).

The mature psiRNA species that we have identified is the most abundant CRISPR-derived RNA detected in *P. furiosus*. The primary mature psiRNAs are \sim 5–10 nt longer than the corresponding psiRNA sequence (i.e., \sim 45 nt long). These RNAs are shorter than the smallest

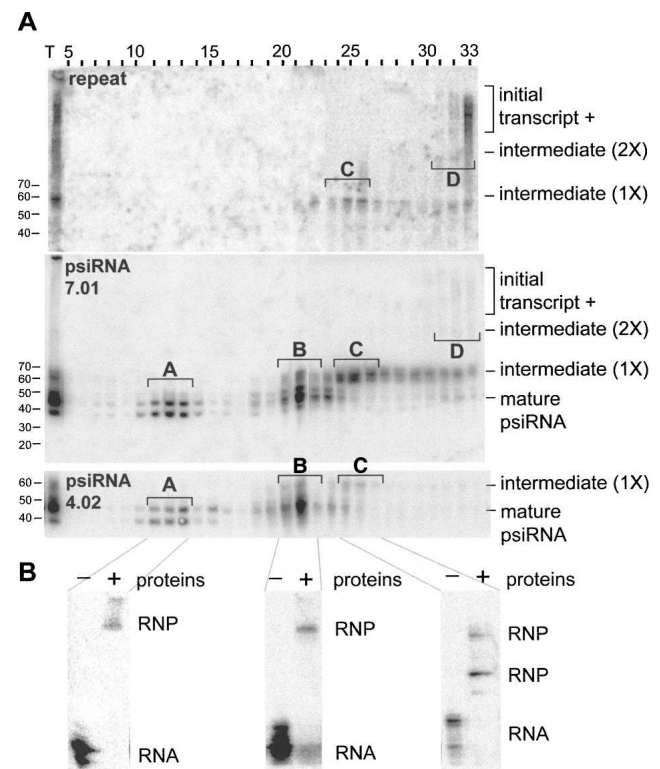


FIGURE 4. CRISPR RNA–protein complexes in fractionated *P. furiosus* cell extract. (A) *P. furiosus* S100 cell extract was separated by DEAE anion exchange chromatography and CRISPR RNAs present in the fractions were examined by Northern analysis using probes against repeat 1, 5, 6, and psiRNAs 7.01 and 4.02 as indicated. Unfractionated extract (T) was coanalyzed for reference. Positions of 1X intermediate, 2X intermediate, and primary mature psiRNA, peaks A–D (see text), and markers are indicated. (B) Fractions corresponding to peaks A (left), B (center), and C (right) were analyzed by non-denaturing gel electrophoresis and Northern blotting using a probe against psiRNA 7.01. For comparison, proteins were extracted from a portion of each sample and analysis of the RNAs with (+) and without (–) proteins is shown. The positions of the RNAs (–proteins) and potential RNPs (+ proteins) on the native gel are indicated.

discrete CRISPR RNA products previously reported (i.e., the ~60- to 65-nt 1X intermediate species) (Tang et al. 2002, 2005; Lillestol et al. 2006) and are presumably generated by exonucleolytic processing of the 1X intermediate. Our Northern and sequence analysis indicates that these RNAs are composed primarily of psiRNA sequence and do not contain substantial repeat sequence. A secondary psiRNA of ~39 nt was also consistently observed among psiRNA profiles.

CRISPR RNA–protein complexes

The common primary and secondary psiRNA species are likely candidates for the guide RNA component of the effector complex in the proposed pRNAi genome defense pathway. Both of these psiRNA species, but not larger intermediate CRISPR RNAs, are found in RNA–protein complexes in anion exchange chromatography peak A (Fig. 4A,B); thus, peak A could contain the effector complex. The primary mature psiRNA is even more abundant in peak B, which contains relatively less of the secondary psiRNA but also contains some 1X intermediate RNA (Fig. 4). Peak B may also contain the effector complex and/or a complex involved in the exonucleolytic processing of the 1X intermediate to psiRNAs. The 1X intermediate RNA is most abundant in peak C, which is adjacent to peak B, and processing may occur across peak B and C fractions. Longer CRISPR RNAs are found in peak D. Our results indicate that the various RNA species are components of distinct RNA–protein complexes in *P. furiosus*. Extensive purification and analysis will determine whether these hypothesized activities and the Cas proteins predicted to function in CRISPR RNA biogenesis and invader silencing (e.g., RNA binding proteins and nucleases) are present in these complexes.

psiRNA expression

The psiRNAs are hypothesized to act in a manner similar to the antibodies of the human immune system, and expression would be expected even in the absence of active infection to patrol for returning invaders. Our results indicate that psiRNAs are actively produced from all seven CRISPR loci in *P. furiosus*. Moreover, expression levels appear to be equivalent between the loci under the growth conditions examined, with the possible exception of one locus that yielded 50% fewer psiRNA clones than expected (see Table 1, locus 6). Our results confirm that CRISPR RNAs are transcribed from the leader sequence in *P. furiosus* and indicate that a portion of the leader sequence is also transcribed.

Interestingly, we found evidence of significantly higher levels of expression of psiRNAs encoded proximal to the leader of a CRISPR locus. Current data indicate that these

are the most recently acquired psiRNA sequences within CRISPR loci (Pourcel et al. 2005; Barrangou et al. 2007). Increased levels of these psiRNAs may be important for targeting current invaders. Distal psiRNAs may be produced at lower levels to provide surveillance for past invaders. It is not clear whether the increased level of leader-proximal psiRNAs results from differences in RNA transcription (e.g., partial transcription of the loci), processing, stability, or other factors. This is an important new aspect of understanding the regulation of psiRNA expression that remains to be explored.

MATERIALS AND METHODS

Small RNA cloning and sequencing

Total RNA was isolated from *P. furiosus* cells using the Trizol reagent (Invitrogen) as indicated by the manufacturer. Approximately 300 µg of total RNA was separated on an 8cm × 8.5cm 15% polyacrylamide 7 M urea gel using DNA size standards (pGEM markers, Promega) for size determination. RNAs between 20 and 50 nt were isolated and passively eluted overnight in 0.5 M ammonium acetate, 0.1% SDS, 0.5 mM EDTA, followed by ethanol precipitation. In order to remove potential 5' triphosphates or cap structures, RNAs were treated with 50 U tobacco acid pyrophosphatase (TAP) (Epicentre) for 2 h at 37°C. The eluted RNAs were cloned using standard microRNA cloning protocols (Lau et al. 2001) using the following primers containing EcoRI restriction sites: 3' adapter, 5'-AppTTTAACCGGAATTC CAGddC-3' (IDT); 5' adapter, 5'-ACGGAATTCCTCACTrArA-3' (IDT); RT/PCR primer, 5'-GACTAGCTGGAATTCGCGGT TAAA-3' (IDT); and PCR primer, 5'-CAGCCAACGGAATTCCT CACTAAA-3'. An additional PCR was performed in order to add a BanI restriction site (GGYRCC) for concatamerization of the PCR products using the following primers: PCR2 5' primer 5'-GA CTAGCTTGGTGGCGAATTCGCGGTAAA-3', PCR2 3' primer 5'-GAGCCAACAGGCACCGAATTCCTCACTAAA-3'. The products were subject to restriction digestion and DNA ligation by standard methods (Lau et al. 2001). cDNAs were cloned into the pCRII TOPO vector (Invitrogen) and transformed into TOP10 cells (Invitrogen) as described by the manufacturer. Plasmid preparation and sequencing was performed in a 96-well plate format using standard M13 forward, reverse, and T7 promoter primers. Sequences were analyzed using BLAST (NCBI).

Northern analysis

Approximately 10 µg of *P. furiosus* total RNA was separated on 15% polyacrylamide 7 M urea gels (Criterion, Bio-Rad) alongside [³²P]-5'-end radiolabeled RNA markers (Decade, Ambion). The RNAs were transferred onto nylon membranes (Zeta-Probe, Bio-Rad) using a Trans-Blot SD Semi-Dry Cell (Bio-Rad). Membranes were baked at 80°C for at least an hour before prehybridization in a ProBlot hybridization oven (LabNet) for at least 1 h at 42°C. Prehybridization and hybridization was performed in either Oligo-UltraHyb (Ambion) buffer or hybridization buffer containing 5× SSC, 7% SDS, 20 mM sodium phosphate (pH 7.0), and 1× Denhardt's solution. Deoxyribonucleotide probes (MWG)

TABLE 2. Probes used in Northern analysis

Probe	Sequence
Repeat 1,5,6 (antisense)	CTTTCAATTCTATTTT(AG)GTCTTATT(C)TAAAC
Repeat 1,5,6 (sense)	GTT(AC)CAATAAGAC(TC)AAAATAGAATTGAAAG
Repeat 2,4,7 (antisense)	CTTTCAATTCTTTTGTAGTCTTATTGGAAC
Repeat 2,4,7 (sense)	GTTCCAATAAGACTACAAAAGAATTGAAAG
psiRNA 1.01 (antisense)	GGTCAGATCAGATTGCTTAAGACAAGAAATG
psiRNA 1.01 (sense)	CATTTCTGTCTTAAGCAATCTGATCTGACC
psiRNA 2.01 (antisense)	GTGGAGCAGAGTCAGAAGAAGAAGTGCCG
psiRNA 2.01 (sense)	CGCACTTCTTCTTCTGACTCTGCTCCAC
psiRNA 4.02 (antisense)	TCTGATAGGCTTCAAAGAGTGCGCTTCAAC
psiRNA 4.02 (sense)	GTTGAAGCGCCACTCTTTGAAAGCCTATCAGA
psiRNA 5.02 (antisense)	GGGAATGGTTCACGTAGTACTTGAGGGCGC
psiRNA 5.02 (sense)	GCGCCCTCAAGTACTACGTGAACCATCCC
psiRNA 6.01 (antisense)	CTAAGGACATTTGTACGTCAAATCTTCCAC
psiRNA 6.01 (sense)	GTGAAGAATTTGACGTACAATGTCCTTAG
psiRNA 7.01 (antisense)	GCTCTCAGCCGCAAGGACCGCATAC
psiRNA 7.01 (sense)	GTATGCGGTCCTTGGCGCTGAGAGC
psiRNA 7.11 (antisense)	CCTTATATGGGTGTTGTGAAGCAGGATAGAAC
psiRNA 7.11 (sense)	GTTCTATCCTGCTTCACAACACCCATATAAGG
psiRNA 7.21 (antisense)	GGCTCTACCTAATCATCTCTTGACACAAC
psiRNA 7.21 (sense)	GTTGTGTCAAGAGGATGATTAGGTAGAGCC
psiRNA 8.01 (antisense)	GACTGTGTGGAGCAGCTATTTGCTTCGGC
psiRNA 8.01 (sense)	GCCGAAGCAAATAGCTGCTCCACACACAGTC

(20 pmol) were 5' end-labeled with T4 Polynucleotide Kinase (Ambion) and γ -[³²P]-ATP (specific activity >7000 Ci/mmol, MP Biomedicals) using standard protocols. Labeled probes were added to the prehybridization buffer, followed by hybridization overnight at 42°C. Following hybridization, two washes were performed in 2× SSC, 0.5% SDS for 30 min at 42°C. Resulting blots were exposed to a PhosphorImager screen for 24–72 h and scanned. The probes and sequences that were used are given in Table 2.

Chromatography

Fifteen grams of *P. furiosus* cells were lysed anaerobically in 200 mL 50 mM Tris (pH 8.0) in the presence of 4 mg/L RNase-free DNase (Sigma). The extract was subject to ultracentrifugation at 113,000g for 2 h (Optima L-90K, Beckman-Coulter). The resulting S100 extract was applied to a 60 mL DEAE Sepharose-FF column and eluted using a 0–500 mM NaCl gradient. The resulting fractions were analyzed by isolating RNAs from 250 μ L of each 30 mL fraction using the Trizol LS protocol (Invitrogen). The RNAs were separated on 10% polyacrylamide 7 M urea gels, blotted, and subject to Northern analysis as described above, using the Oligo-UltraHyb hybridization buffer for both pre-hybridization and hybridization. See above for probe sequences.

Native Northern analysis

Forty microliters of DEAE fractions from peaks A–C were separated on a 4%–20% polyacrylamide gel (Bio-Rad) using SDS-free running buffer (25 mM Tris, 19.2 mM glycine). Deproteinized samples were analyzed in parallel. Gels were run at 50 V for 3–4 h at room temperature. The gel was soaked in 5 M

urea, 45 mM Tris, 45 mM boric acid, and 1 mM EDTA for 15 min, then subject to blotting and Northern analysis as described above, using Ultra-Hyb Oligo hybridization buffer (Ambion) and a probe against psiRNA 7.01.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

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REFERENCES

- Aravin, A.A., Hannon, G.J., and Brennecke, J. 2007. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **318**: 761–764.
- Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**: 1709–1712.
- Bolotin, A., Quinquis, B., Sorokin, A., and Ehrlich, S.D. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**: 2551–2561.
- Deveau, H., Barrangou, R., Garneau, J.E., Labonte, J., Fremaux, C., Boyaval, P., Romero, D.A., Horvath, P., and Moineau, S. 2008. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. *J. Bacteriol.* **190**: 1390–1400.
- Godde, J.S. and Bickerton, A. 2006. The repetitive DNA elements called CRISPRs and their associated genes: Evidence of horizontal transfer among prokaryotes. *J. Mol. Evol.* **62**: 718–729.
- Grissa, I., Vergnaud, G., and Pourcel, C. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics* **8**: 172.
- Haft, D.H., Selengut, J., Mongodin, E.F., and Nelson, K.E. 2005. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput. Biol.* **1**: e60.
- Hannon, G.J. 2002. RNA interference. *Nature* **418**: 244–251.
- Hartig, J.V., Tomari, Y., and Forstemann, K. 2007. piRNAs—The ancient hunters of genome invaders. *Genes & Dev.* **21**: 1707–1713.
- Horvath, P., Romero, D.A., Coute-Monvoisin, A.C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C., and Barrangou, R. 2008. Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. *J. Bacteriol.* **190**: 1401–1412.
- Jansen, R., Embden, J.D., Gastra, W., and Schouls, L.M. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol. Microbiol.* **43**: 1565–1575.

- Kim, V.N. 2006. Small RNAs just got bigger: Piwi-interacting RNAs (piRNAs) in mammalian testes. *Genes & Dev.* **20**: 1993–1997.
- Klein, R.J., Misulovin, Z., and Eddy, S.R. 2002. Noncoding RNA genes identified in AT-rich hyperthermophiles. *Proc. Natl. Acad. Sci.* **99**: 7542–7547.
- Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294**: 858–862.
- Lillestol, R.K., Redder, P., Garrett, R.A., and Brugger, K. 2006. A putative viral defence mechanism in archaeal cells. *Archaea* **2**: 59–72.
- Lin, H. 2007. piRNAs in the germ line. *Science* **316**: 397.
- Makarova, K.S., Aravind, L., Grishin, N.V., Rogozin, I.B., and Koonin, E.V. 2002. A DNA repair system specific for thermophilic Archaea and bacteria predicted by genomic context analysis. *Nucleic Acids Res.* **30**: 482–496.
- Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., and Koonin, E.V. 2006. A putative RNA-interference-based immune system in prokaryotes: Computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biol. Direct* **1**: 7.
- Mojica, F.J., Diez-Villasenor, C., Garcia-Martinez, J., and Soria, E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol.* **60**: 174–182.
- Nishida, K.M. and Siomi, M.C. 2006. Molecular mechanisms of RNA silencing by siRNA, miRNA and piRNA. *Tanpakushitsu Kakusan Koso* **51**: 2450–2455.
- Pourcel, C., Salvignol, G., and Vergnaud, G. 2005. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* **151**: 653–663.
- Sorek, R., Kunin, V., and Hugenholtz, P. 2008. CRISPR—a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat. Rev. Microbiol.* **6**: 181–186.
- Tang, T.H., Bachelierie, J.P., Rozhdestvensky, T., Bortolin, M.L., Huber, H., Drungowski, M., Elge, T., Brosius, J., and Huttenhofer, A. 2002. Identification of 86 candidates for small non-messenger RNAs from the archaeon *Archaeoglobus fulgidus*. *Proc. Natl. Acad. Sci.* **99**: 7536–7541.
- Tang, T.H., Polacek, N., Zywicki, M., Huber, H., Brugger, K., Garrett, R., Bachelierie, J.P., and Huttenhofer, A. 2005. Identification of novel non-coding RNAs as potential antisense regulators in the archaeon *Sulfolobus solfataricus*. *Mol. Microbiol.* **55**: 469–481.
- Tyson, G.W. and Banfield, J.F. 2008. Rapidly evolving CRISPRs implicated in acquired resistance of microorganisms to viruses. *Environ. Microbiol.* **10**: 200–207.