Supplemental tables and figures

A regulatory RNA is involved in RNA duplex formation and biofilm regulation in *Sulfolobus acidocaldarius*

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Table S1. Oligonucleotides used in this study

Primer no.	Sequence 5´to 3	description
KO-R-OLfor	GGTGGTTCACATTATTTCTATAGGTCTATTTTTATTTG	Forward primer for upstream region Δ*RrrR* with *Apa*I restriction site
KO-R-for	GATGTCGACTATGAATCACTTAATAGTAGTCACTTTAAC	Reverse primer for upstream region Δ*RrrR*, overlapping region
KO-R-rev	GATGGATCCCTATTTATTTCCTTAAAGAAATCAAGTAAAC	Forward primer for downstream region Δ*RrrR*, overlapping region
KO-R-OLrev	CACAAATAAAAATAGACCTATAGAAATAATGTGAACCACC	Reverse primer for downstream region Δ*RrrR* with *Bam*HI
558	CTATTTTTACGTTCTAAACTAGTTTAAAAAGGGAAAAAAGAAAAAATAACTTTGAGCAGTTCTAG	Δ*saci0301* forward for *pyrEF* exchange
559	AACACACACCAGTTATATTCTGATAATGTATAATATAACTTGGTGATTAAAGACCGGCTATTTTTTCAC	Δ*saci0301* reverse for *pyrEF* exchange
548	CACTGTCCACAGCTCAATTC	*saci0301* forward qPCR primer
549	CTTCCAGGCAATGGCCAGAC	*saci0301* reverse qPCR primer
560	AAGGTGATGAAAGGATAAGGG	*saci0567* forward qPCR primer
561	TTAGTAAGTGGTATAACTGTAGCTG	*saci0567* reverse qPCR primer
562	CTGGTGAACAAACCGCTCAA	*saci1357* forward qPCR primer
563	ACCGAGCCCACCTATATAGTAA	*saci1357* reverse qPCR primer
564	AGTCTACAGCAAGGTCCAAAG	*Saci0667* forward qPCR primer
565	ACTACTCCACTCGCAAGAAAG	*Saci0667* reverse qPCR primer
566	CCTCCTGATAGCTTCTCTCCTA	*Saci0688* forward qPCR primer
567	TGCAGTTAGGAAAGCAGTTAGA	*Saci0688* reverse qPCR primer
568	CGACTTCATCGTGCTCATCTAT	*Saci0906* forward qPCR primer
569	TGCATTGATGAGGCACTAGAC	*Saci0906* reverse qPCR primer
550	GAAGGAGCAACAGCAGTCTG	RrrR(+) forward qPCR primer
555	AAATAAAAATGGTGGTTCAC	RrrR(+) reverse qPCR primer
556	TAAAAATAGACCTATATGGC	RrrR(-) forward qPCR primer
599	GTCCACCTGATTGTTGACC	RrrR(-) reverse qPCR primer
552	GTACCATGGATAAAAATAGACCTATATGGC	Forward primer for cloning RrrR(+) into pSVA1431 with *Nco*I restriction site
553	GTACGGCCGATAGAGAATTCTATCCACGAC	Reverse primer for cloning RrrR(+) into pSVA1431 with *Eag*I restriction site
606	TTCTTCTTCTTTGCCATATAGGTCTATTTTTATTCGGCCGGACAGACTTTATCACGCCCG	Forward primer for cloning RrrR(-) into pSVA1431 with *Eag*I restriction site (inverse PCR)
601	GGAGCAACAGCAGTCTGGTCAACAATCAGGTGGACCATGGTTAACTTAATCACG	Reverse primer for cloning RrrR(-) into pSVA1431 with *Nco*I restriction site (inverse PCR)
614	GTACCGCGGAAAAAAATTATCTTGAGTTAAAAATAAAAATGGTGGTTCACATTATTTCTTTTATGTACTCATTTCCAAATAGCTT	Forward primer for cloning RrrR(-) promoter sequence into pSVA1431(LacS reporter system) with *Sac*II restriction site

615	GTACCGCGGAAAAAAATTATCTTGAGTTGGGGG TGGGGGTGGTGGTTCACATTATTTCTTTTATGTA CTCATTTCCAAATAGCTT	Forward primer for cloning RrrR(-) mutated promoter sequence into pSVA1431(LacS reporter system) with *Sac*II restriction site
616	GTACCGCGGAATTTTTATGTGAGTATGGCTTAAT AACTTGTTATTTCACAAATAAAAATAATGTACTCA TTTCCAAATAGCTT	Forward primer for cloning RrrR(+) promoter sequence into pSVA1431(LacS reporter system) with *Sac*II restriction site
617	GTACCGCGGAATTTTTATGTGAGTATGGCGGGG GGGCTTGTTATTTCACAAATAAAAATAATGTACT CATTTCCAAATAGCTT	Forward primer for cloning RrrR(+) mutated promoter sequence into pSVA1431(LacS reporter system) with *Sac*II restriction site
L7PRHAnew	AGCCAACGGCCGTTAGGCGTAGTCTGGAACAT CGTAAGGGTAGTGCCTTAATGGCTTTAC	Reverse primer for cloning RrrR(+) and RrrR(-) promoter sequences into pSVA1431(LacS reporter system) with *Eag*I restriction site

Table S2. Strains and plasmids used in this study

Strain/plasmid	Genotype	Source/reference
Strain		
E. coli		
DH5α	*Escherichia coli* K-12 cloning strain, F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Gibco
ER1821	F- glnV44 e14-(McrA-) rfbD1 relA1 endA1 spoT1	New England Biolabs
S. acidocaldarius		
MW001	Deletion of *pyrEF (91-412 bp)* in *S. acidocaldarius*	Wagner et al. (2012)
ACE100	Deletion of non-coding RNA RrrR in MW001	this study
ACE101	Deletion of *saci0301::pyrEF* in MW001	this study
ACE102	Deletion of *saci0301::pyrEF* in ACE100	this study
ACE103	MW001 carrying pACE113	this study
ACE104	MW001 carrying pACE114	this study
ACE105	MW001 carrying pACE115	this study
ACE106	MW001 carrying pACE116	this study
ACE107	ACE100 carrying pACE106	this study
ACE108	ACE100 carrying pACE107	this study
ACE109	MW001 carrying pACE106	this study
ACE110	MW001 carrying pACE107	this study
Plasmid		
pSVA406	Gene targeting plasmid, pGEM-T Easy backbone, *pyrEF*cassette of *S. solfataricus*	Wagner et al. (2012)
pSVA1431	pRN1-based shuttle vector with maltose inducible promoter and *lacS* reporter gene of *S. solfataricus*	Wagner et al. (2012)
pVT135	In-frame deletion of *RrrR* cloned into pSVA406 with *Apa*I, *Pst*I	this study
pACE106	RrrR sense strand cloned into pSVA1431 with *Nco*I, *Eag*I	this study
pACE107	RrrR antisense strand cloned into pSVA1431 with *Nco*I, *Eag*I	this study
pACE113	RrrR sense strand promoter sequence cloned into pSVA1431 with *Sac*II, *Eag*I	this study
pACE114	RrrR antisense strand promoter sequence cloned into pSVA1431 with *Sac*II, *Eag*I	this study
pACE115	RrrR sense strand mutated promoter sequence cloned into pSVA1431 with *Sac*II, *Eag*I	this study
pACE116	RrrR antisense strand mutated promoter sequence cloned into pSVA1431 with *Sac*II, *Eag*I	this study

Table S3. Differentially expressed ncRNAs. Expression of identified ncRNAs in biofilm versus planktonic (B vs P) cells.

ncRNA ID	Genome coordinates		Length (nt)	RPKM		B vs P (Log2)
	start	end		Planktonic	Biofilm	
ncRNA_529	1,638,356	1,638,576	220	7	311	5.39
ncRNA_332	1,042,083	1,042,172	89	23	187	2.94
ncRNA_636	1,765,999	1,766,204	205	29	167	2.44
ncRNA_322	995,929	996,022	93	14	53	1.83
ncRNA_454	1,510,205	1,510,377	172	10	30	1.50
ncRNA_742	1,900,049	1,900,241	192	10	30	1.50
ncRNA_530	1,639,129	1,639,360	231	13	37	1.42
ncRNA_792	2,010,283	2,010,091	192	13	37	1.42
ncRNA_468	1,533,841	1,534,021	180	47	131	1.39
ncRNA_668	1,800,041	1,800,283	242	23	61	1.32
ncRNA_496	1,581,340	1,581,520	180	20	53	1.32
ncRNA_664	1,797,848	1,798,017	169	10	26	1.29
ncRNA_127	417,429	417,671	242	35	85	1.19
ncRNA_239	805,730	805,902	172	428	1029	1.18
ncRNA_634	1,764,515	1,764,604	89	19	45	1.16
ncRNA_593	1,648,780	1,649,002	222	7	16	1.10
ncRNA_317	972,593	972,683	90	11	25	1.10
ncRNA_700	1,825,621	1,825,765	144	53	119	1.08
ncRNA_533	1,643,581	1,643,819	238	17	38	1.07
ncRNA_592	1,716,356	1,716,445	89	10	22	1.05
ncRNA_61	248,056	248,275	219	30	64	1.01
ncRNA_795	2,019,138	2,019,339	201	15	32	1.01
ncRNA_319	986,262	986,440	178	29	15	-1.04
ncRNA_83	304,215	304,314	99	683	335	-1.12
ncRNA_804	2,032,119	2,032,246	127	66	30	-1.23
ncRNA_826	2,107,679	2,107,904	225	26	11	-1.33
ncRNA_91	331,519	331,631	112	104	44	-1.33
ncRNA_760	1,949,554	1,949,876	322	1127	441	-1.44
ncRNA_475	1,547,152	1,547,302	150	47	17	-1.56

Table S4. Biofilm volume quantitation. Biofilm volumetric determinations were performed in three biological replicates and 9 images at different microscopy fields were recorded for each replicate. Biofilm volumes are expressed as $\mu m^3 * 10^6$.

Strain	Biofilm volume ($\mu m^3 * 10^6$)	Biofilm thickness (µm)	DAPI (%)	ConA (%)	IB4 (%)
MW001	4.14 ± 0.57	71.9 ± 9.9	57.7	23.1	19.2
ΔRrrR	2.51 ± 0.22	43.6 ± 3.8	44.0	35.5	20.5
ΔRrrR + p_RrrR (+)	4.05 ± 0.52	70.3 ± 9.0	54.7	28.6	16.7
ΔRrrR + p_RrrR (-)	2.48 ± 0.29	43.0 ± 5.0	41.8	39.8	18.4
MW001 + p_RrrR (+)	5.03 ± 0.41	87.3 ± 7.1	26.0	58.4	15.6
MW001 + p_RrrR (-)	3.40 ± 0.36	59.0 ± 6.2	46.0	33.9	20.1

Table S5. *In silico* prediction of the potential target mRNAs of ncRNA RrrR. The bioinformatics tool CopraRNA was used to identify RrrR-mRNA interactions. The ten highest-ranking hits are shown. Differential gene expression ratios (fold change (FC) or non-significant (n.s.)) are indicated for each potential target gene between biofilm-associated (B) cells and planktonic (P) cells.

Rank	CopraRNA p-value	Locus	Energy (kcal/mol)	Position mRNA	Position ncRNA	Annotation	FC (B/P)
1	4.03e-05	saci0567	-18.43	247-272	16-43	Hypothetical protein	2.19
2	6.78e-04	saci1357	-16.92	350-382	19-45	Nucleotide-binding protein, UspA family	n.s
3	7.02e-04	saci0765	-13.45	217-229	27-39	Predicted nucleotidyltransferase	n.s
4	8.64e-04	saci0667	-16.75	219-259	24-52	HerA helicase	n.s
5	9.03e-04	saci0449	-13.51	350-377	26-53	Transcriptional regulator	2.86
6	1.23e-03	saci0688	-18.82	266-291	26-48	Ribosomal protein S12	n.s
7	1.84e-03	saci0906	-17.51	244-267	17-41	ATPase involved in DNA replication HolB, large subunit	n.s
8	1.90e-03	saci2353	-14.67	217-246	20-48	Transcriptional regulator	n.s
9	1.23e-03	saci0301	-16.70	24-54	16-39	Predicted membrane protein	21.03
10	1.953e-03	saci1430	-15.06	358-370	26-38	Valyl-tRNA synthetase	n.s

Figure S1

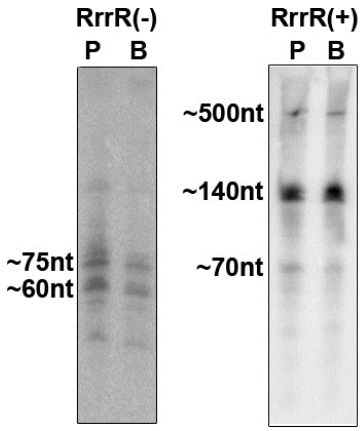

Figure S1. Northern blot analysis of RrrR transcripts. Small RNA molecules of *S. acidocaldarius* planktonic (P) or biofilm (B) cells were separated on denaturing polyacrylamide gels and hybridized with radioactively labeled probes complementary to the RrrR(-) or RrrR(+) duplex region.

Figure S2

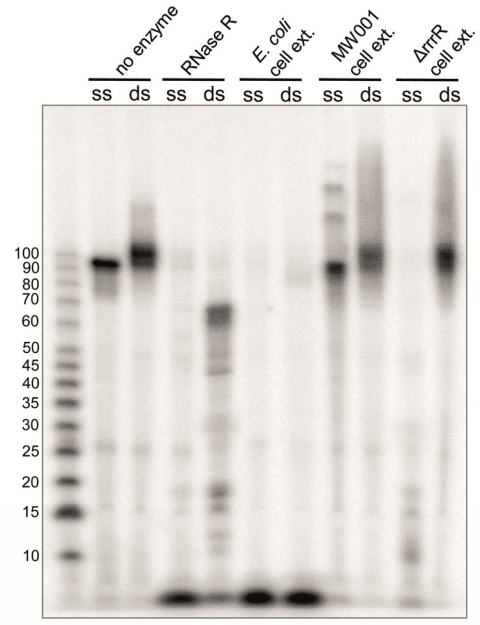

Figure S2 RNaseR exonuclease. (A) Radiolabeled single strand (ss) RrrR(+) and radiolabeled double-stranded (ds) RrrR were subjected to RNaseR digestion assays. 20U of RNaseR (Epicentre) or 5 µg of total protein cell extract were added to the assays. Digestion products were visualized on a 10 % denaturating polyacrylamide gel. The sizes (in nucleotides) of the radiolabeled RNA ladder bands are indicated.

Figure S3

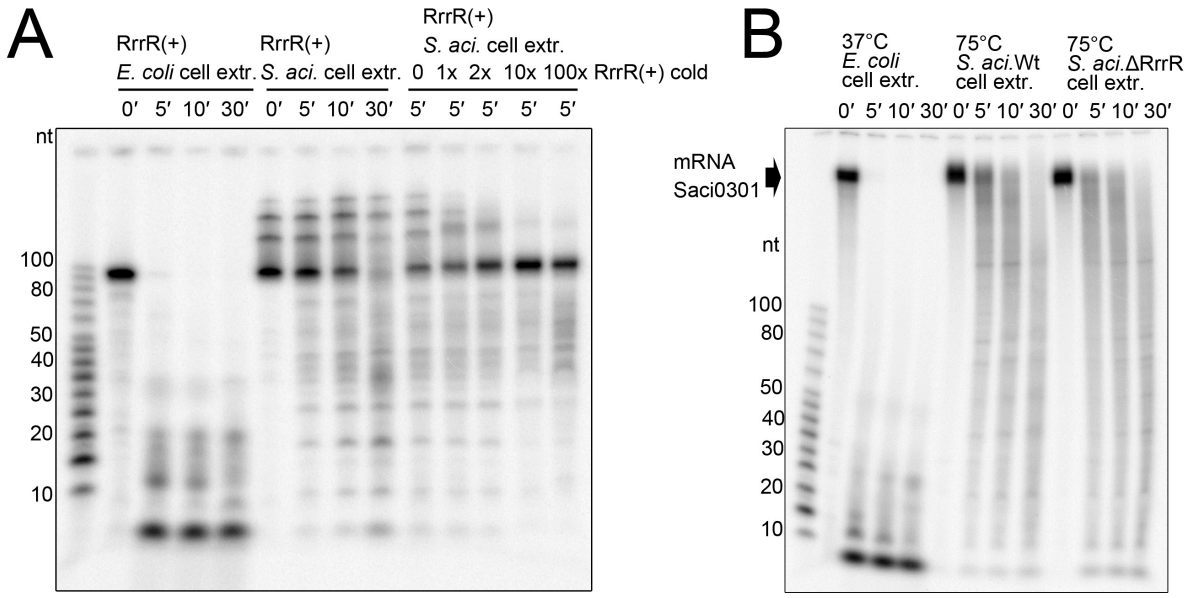

Figure S3. RNA stability assays. (A) Transcripts of radioactively labeled ssRrrR(+) were incubated with different cell extracts at 37°C (*E. coli*) or 75°C (*S. acidocaldarius*) for the indicated time. The addition of excess unlabeled RrrR(+) transcripts (molar ratio 1:1, 2:1, 10:1, 100:1) prevents ssRrrR degradation and upshift formation by RNA binding proteins (e.g. LSm proteins, see Figure S7). (B) Transcripts of radioactively labeled Saci0301 mRNA were incubated with different cell extracts at 37°C (*E. coli*) or 75°C (*S. acidocaldarius*) for the indicated time.

Figure S4

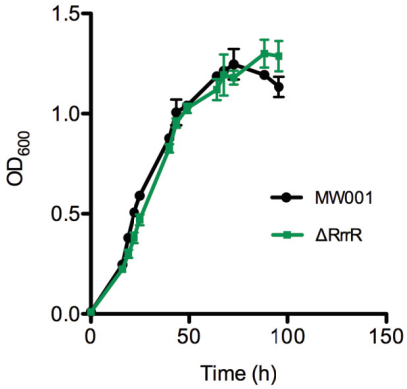

Figure S4. Planktonic growth of the *S. acidocaldarius* RrrR deletion mutant strain (ΔRrrR). A shaking cultured RrrR deletion mutant strain was sampled at the indicated time points to measure cell density at OD_{600}. Growth of the reference strain MW001 (black curve) and the markerless deletion mutant ΔRrrR (green curve) was compared. Each point represents the mean of 3 biological replicates.

Figure S5

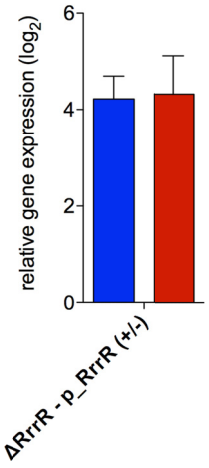

Figure S5. Complementation of the ΔRrrR deletion mutant. Transcript levels of both RrrR(+) (blue bar) and RrrR(-) (red bar) were quantified by qRT-PCR after RNA isolation from the *S. acidocaldarius* RrrR deletion mutant strain (ΔRrrR) overexpressing either RrrR(+) or RrrR(-). Total RNA was isolated from cells grown as biofilms. Relative transcript expression was normalized to the internal control gene *sec*Y. The means and standard deviations of 3 biological replicates are shown.

Figure S6

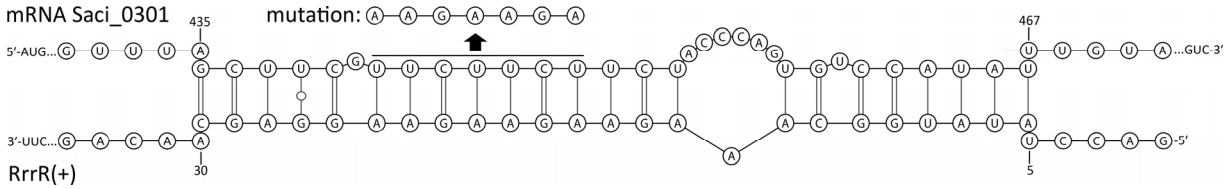

Figure S6. Proposed interactions between RrrR(+) and the mRNA of Saci_0301. Potential mRNA targets for RrrR(+) were predicted *in silico* using the CopraRNA tool (34). The indicated seven nucleotide stretch was exchanged to assess duplex formation in the presence of recombinant LSm1/LSm2 proteins (Fig. 3B).

Figure S7

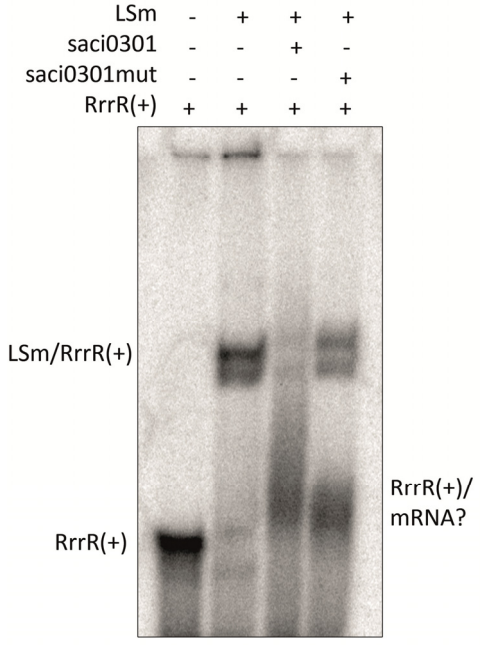

Figure S7. LSm binding assays. EMSAs demonstrate binding of RrrR(+) transcripts to 500 nM of recombinant LSm1-LSm2 complexes. A 2-fold excess of unlabeled saci0301 mRNA or a mRNA variant with a mutation of the predicted interaction region (mut, Fig. S4) was added to the reaction. A lower shift was observed for the native mRNA, suggesting that RrrR(+) – mRNA duplexes are formed. Reaction mixtures were separated by 8 % native PAGE.