A Small RNA Is Linking CRISPR–Cas and Zinc Transport

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The function and mode of action of small regulatory RNAs is currently still understudied in archaea. In the halophilic archaeon Haloferax volcanii, a plethora of sRNAs have been identified; however, in-depth functional analysis is missing for most of them. We selected a small RNA (s479) from Haloferax volcanii for detailed characterization. The sRNA gene is encoded between a CRISPR RNA locus and the Cas protein gene cluster, and the s479 deletion strain is viable and was characterized in detail. Transcriptome studies of wild-type Haloferax cells and the deletion mutant revealed upregulation of six genes in the deletion strain, showing that this sRNA has a clearly defined function. Three of the six upregulated genes encode potential zinc transporter proteins (ZnuA1, ZnuB1, and ZnuC1) suggesting the involvement of s479 in the regulation of zinc transport. Upregulation of these genes in the deletion strain was confirmed by northern blot and proteome analyses. Furthermore, electrophoretic mobility shift assays demonstrate a direct interaction of s479 with the target znuC1 mRNA. Proteome comparison of wild-type and deletion strains further expanded the regulon of s479 deeply rooting this sRNA within the metabolism of H. volcanii especially the regulation of transporter abundance. Interestingly, s479 is not only encoded next to CRISPR–Cas genes, but the mature s479 contains a crRNA-like 5′ handle, and experiments with Cas protein deletion strains indicate maturation by Cas6 and interaction with Cas proteins. Together, this might suggest that the CRISPR–Cas system is involved in s479 function.

Keywords: small RNA, archaea, CRISPR-Cas, zinc transport, haloarchaeae

INTRODUCTION

Small RNAs have been well established as key regulators of gene expression in both pro- and eukaryotic species (Storz et al., 2011; Wagner and Romby, 2015; Buddeweg et al., 2018a), but still, understanding of small RNAs (sRNAs) in the archaeal domain lags behind (Gomes-Filho et al., 2018). RNomics and the more recent high-throughput approaches have been applied to several archaeal species to uncover the wealth of small transcripts found in this domain of life [reviewed in Marchfelder et al. (2012); Kliemt and Soppa (2017), Buddeweg et al. (2018a), and Gelsinger and DiRuggiero (2018a)]. With numbers in the hundred (Archaeoglobus fulgidus, Methanosarcina mazei, Sulfolobus solfataricus, Thermococcus kodakarense, Pyrococcus abyssi, and Haloferax mediterranei) or even thousand (Haloferax volcanii and Methanolobus psychrophilus), sRNAs are well established as widespread and abundant players within the transcriptome of archaea.
for the CRISPR–Cas system. Differential transcriptome and proteome analysis revealed changes in several zinc-related mRNAs and proteins, respectively. Analyses of the deletion strain in conjunction with electrophoretic mobility shift data and impaired growth under elevated zinc concentrations confirm a role of s479 within the zinc regulon. Differential proteome analysis reveals a role for s479 in the adjustment of a network of ABC transporters. Furthermore, s479 seems to depend on Cas proteins for maturation and stability.

RESULTS

Characterization of s479

The s479 RNA was identified in an early RNomics study, sequencing cDNA clones after RNA size selection (Straub et al., 2009). It is an intergenic sRNA located on the genomic plasmid pHV4 downstream of the CRISPR locus P1 and upstream of the type I-B cas gene cassette (Figure 1). A genome-wide high-throughput study analyzing transcriptional start sites of *H. volcanii* (Babski et al., 2016) revealed an enrichment of transcript starts at position 207,660.

Expression of s479 was confirmed by northern blot analysis revealing long RNAs of about 220 and 160 nucleotides and a very strong cluster of signals of approximately 51 nucleotides (Figure 2), showing that the primary s479 transcript is processed yielding an RNA of about 51 nucleotides. The northern data confirm the dRNA-seq results presented in Figure 1, which show that RNAs starting at the mature s479 5′ end (position 49 in Figure 3) with about 50 nucleotides length have the most reads.

This is also supported by a serendipitous finding: an RNA-seq study of small RNAs to identify CRISPR RNAs revealed reads mapping to the s479 locus; a 57 and a 60 nt as well as a 73 nt form (Figure 3; Supplementary Figure 1; Maier et al., 2013). These correspond in size to the cluster of bands visible in the northern blot analysis (Figures 2, 4).

To elucidate the importance of s479 in the context of *H. volcanii* metabolism in more detail, we first compared the growth of the s479 deletion strain (Δs479) obtained in a previous study (Jaschinski et al., 2014) with the H66 wild-type strain (Figure 5). Growth curves show a diauxic growth: doubling times of the wild-type and deletion strains in phase 1 (0.5–10.5 h) are not very different with the doubling times of 4.7 h for the wild-type strain and 4.5 h for the deletion strain (see also Supplementary Figure 5C for doubling time details). However, in phase 2 (14.5–24.5 h), the doubling time of the deletion strain is longer with 24 h compared with 19 h of the wild-type strain. In addition, the deletion strain reaches a lower OD in stationary phase, showing that the sRNA has an important function in the cell.

Influence of s479 on the *H. volcanii* Transcriptome

As deletion of s479 led to a modest growth phenotype, we wanted to identify genome-wide changes in the *H. volcanii* gene expression profile resulting from loss of sRNA expression. We performed RNA-seq analysis of the sRNA deletion strain Δs479 and the wild-type strain H66 grown to exponential phase. To
pinpoint transcripts affected by the absence of s479, we applied a stringent differential transcriptome analysis.

Bioinformatic analysis identified one transcript as downregulated, the s479 RNA, and five transcripts as upregulated with a log2 fold change ≥ 2. As shown in Table 1, two of the upregulated genes are derived from a single genomic region on the main chromosome comprising among others the operon znuA1C1B1 encoding a putative zinc ABC transporter (Figure 6).

Abundance of the two transcripts, znuC1 and znuB1, increases more than four-fold in response to s479 deletion. znuA1, encoding the periplasmic substrate-binding protein of the said putative ABC transporter, is also present within the set of differentially expressed genes but fell just below the threshold of log2FC ≥ 2 with a score of 1.8 (Supplementary Table 1).

As the abundance of all three genes of the znu operon is altered upon s479 deletion, we further concentrated our analysis on the znu operon comprising znuA1 (HVO_2397), znuB1 (HVO_2399), and znuC1 (HVO_2398). TSS analyses (Babski et al., 2016) show that expression is governed by a single TSS four nucleotides upstream of the znuA1 start codon, resulting in a multicistronic mRNA of approximately 3,000 nt (Figure 6).

Such a short 5′ UTR is typical for H. volcanii which has a high percentage of leaderless mRNAs and 5′ UTRs shorter than six nucleotides (Babski et al., 2016).

The transcript differences seen for the znu operon were validated using northern blot analysis probing for part of the znuC1 coding sequence (Figure 7). The znu transcript is more abundant in the strain without s479. In addition, results of the northern blot analysis confirm transcription of the znu operon as a single polycistronic transcript of about 3,000 nucleotides (Figure 7).

Influence of s479 on Protein Abundance
Taking the analysis of the 479 target sphere a step further, we compared proteomes of the wild type and the s479 deletion strain. Since we saw differential expression of transporter...
FIGURE 3 | RNA-seq analysis of small RNAs reveals sequences matching the s479 locus. The s479 precursor RNA is 213 nucleotides long, shown in light orange. RNomics analysis of size-selected RNA samples (Maier et al., 2013) reveals shorter versions of s479: 57, 60, and 73 nt in length, shown below in dark orange. These data confirm the results of the northern blot (Figure 2). The sequence of the shorter sRNAs starts at nucleotide 49 of the precursor. The first 8 nucleotides are identical to crRNA 5' handle sequence (shown in bold: ATTGAAGC) (Supplementary Figure 2).

FIGURE 4 | The processed s479 species are lost upon deletion of genes for the Cas6 or Cas7 protein. Total RNA was separated on a polyacrylamide gel. After transfer, a probe against s479 was used for hybridization (upper panel). A size marker is given at the left. Lane w: RNA from H. volcanii H119 wild-type strain, lane Δ6: RNA from H. volcanii cas6 deletion strain Δcas6, lane Δ7: RNA from H. volcanii cas7 deletion strain Δcas7. In the lower panel, hybridization with a probe against 5S rRNA is shown.

For 12 proteins, a significant differential abundance with log2 fold change ≥2 was measured. Eight of them accumulated and four were depleted in the deletion strain. A summary of the proteomic changes detected with a log2 fold change ≥0.8 is given in Supplementary Table 2. Comparison with the transcriptome data shows that the increase in transcript level seen for the znu operon is paralleled by an increase in protein level (znuC1, HVO_2398) or the exclusive detection of the gene product in the deletion strain (znuA1, HVO_2397). Analysis of the KEGG pathway assignment of the uniquely or differentially present proteins reveals an enrichment of transporter proteins exclusively present in the deletion strain (5 of 22) (Table 2 and Supplementary Table 2). However, the majority of proteins present in the deletion strain only are hypothetical proteins (9 of 22).

Since deletion of the s479 gene alters the expression of zinc transporters, we compared the growth of the wild-type and s479 strains in low zinc and high zinc concentrations. Under low zinc concentrations, wild type and s479 show the same growth behavior (data not shown). However, upon addition of high zinc concentrations, Δs479 shows defects in growth (Supplementary Figure 5).

In silico Analysis of the sRNA–Target Interaction

As little is known about sRNA–target interactions in the archaeal domain and the few examples described so far reveal a diverse set of interaction modes, the potential interaction of s479 with the znuC1 coding region was further analyzed using bioinformatics. The sequence of the s479 (Figure 8B) was utilized to predict interactions with the znu operon transcript by the IntaRNA suite (Mann et al., 2017). We were able to predict two potential interaction sites, both within the znuC1 open reading frame, using standard settings (Figure 8A). Site 1 is located 120 nt downstream of the first nucleotide of the znuC1 coding sequence and site 2 is located 408 nt downstream of it with predicted energy gain of $E = -7.82$ for site 1 and $E = -10.27$ for site 2, respectively. The predicted interacting sequence of s479 is situated at the 3’ end.

As proteome analysis revealed additional potential targets of s479, the IntaRNA analysis was extended to the transcripts of these proteins as well. Since translational regulation commonly involves sequences at or in close proximity to the first codon, we incorporated the sequences from transcriptional start site to 50 nucleotides downstream1. Analysis was confined to proteins exclusively present or absent in the deletion strain, as these were the most affected. Using standard parameters, interaction

1 For targets without a predicted TSS (Babski et al., 2016) or targets located in operons, we included 100 nt upstream of the first coding nucleotide.
FIGURE 5 | Growth experiment comparing the wild-type strain and Δs479 strain. Both strains were cultivated in triplicate with Hv-Ca medium (supplemented with uracil) in microtiter plates and OD$_{650 \text{nm}}$ was monitored using a heated plate-reader instrument.

TABLE 1 | Six genes are differentially expressed in the deletion strain Δs479.

| Gene            | log$_2$ | p adj | Product                                      |
|-----------------|---------|-------|----------------------------------------------|
| Downregulated   |         |       |                                              |
| s479            | −4.30   | 2.7E-31 | s479                                         |
| HVO_2402; gcvP2 | 3.27    | 1.7E-19 | Glycine dehydrogenase                        |
| HVO_2396; grx4  | 2.16    | 6.5E-07 | Glutaredoxin-like protein                    |
| HVO_2398; znuC1 | 2.11    | 1.6E-07 | Putative zinc ABC transporter ATP-binding protein |
| HVO_2400        | 2.08    | 1.7E-08 | Hypothetical protein                         |
| HVO_2399; znuB1 | 1.98    | 3.2E-07 | Putative zinc ABC transporter permease        |

The log$_2$ fold change (column log$_2$) deletion vs. wild type is given alongside the HVO–gene number (column gene), p value (column p adj), and gene product name (column product).

with s479 was predicted for eight transcripts. We then modified the search parameters to also include seed sequences of only five nucleotides and relaxed specificity and this resulted in 21 predicted interactions. All of those interactions involve a similar region within s479 around position 44 to 54 at the 3′ end of the mature s479 (Figure 9). The WebLogo (Crooks, 2004) created for the s479 nucleotides involved in contacting all these transcripts highlights a nine-nucleotide consensus motif (GCCGAAGCG) corresponding to the interaction surface predicted for the s479–znuC1 contact.

**Verification of the s479–znuC1 Target Interaction**

Assessment of the transcriptome by RNA-seq as well as northern blot analysis confirms that abundance of the znuC1 transcript is altered upon deletion of the s479 (Table 1 and Figure 7). Moreover, target site prediction suggests two interaction surfaces on the znuC1 mRNA (Figure 8). To validate whether the znuC1 transcript is a direct interaction partner of s479 and whether the predicted interaction site is correct, we performed an electrophoretic mobility shift assay (Figure 10).

Labeled s479 RNA was incubated with increasing amounts of an unlabeled znuC1 mRNA fragment, comprising the predicted interaction site 2 (Figure 8D) and flanking nucleotides (28 nt upstream, 40 nt downstream), and gel shift analysis shows that s479 binds to the znuC1 RNA (Figure 10). Competition experiments with unlabeled s479 were also performed (Supplementary Figure 3A) revealing that the unlabeled s479 competes effectively with the radioactively labeled one for binding to the znuC1 RNA. However, sRNA s479 does not bind to a znuC1 mutant RNA, which has the s479 interaction site (Figure 8D) deleted (Supplementary Figure 3B).

**s479 Is Bound by Cascade**

The s479 precursor RNA contains almost a complete CRISPR repeat sequence (Supplementary Figure 2). Processing at the Cas6 cleavage site of this repeat sequence would yield the mature s479 RNAs containing a 5′ sequence identical to the characteristic eight nucleotide long 5′ handle of the mature *H. volcanii* crRNAs of locus P1 (5′ ATTGAAGC 3′) (Maier et al., 2013). This observation led us to investigate whether the Cas6 protein is involved in s479 biogenesis. Northern blot analysis shows that the short RNAs with about 50 nucleotides length are lost upon cas6 deletion (Figure 4), and only an intermediate RNA of about 100 nucleotides is detected, suggesting that Cas6 indeed generates the mature sRNA.

We also analyzed whether s479 is present in a cas7 deletion strain. An earlier investigation showed that Cas7 is the central subunit of the *Halofex* Cas protein complex Cascade which binds the crRNAs. In a cas7 deletion strain, the Cascade complex cannot form anymore; thus, crRNAs are not bound to Cascade and, therefore, are not stable anymore (Brendel et al., 2014).
Genomic localization of the znu operon. Depicted is the localization of the znu operon alongside the read coverage at TSS (Babski et al., 2016) confirming the joint transcription of all three znu genes as an approximately 3,000 nt transcript. The location of the PCR probe used for znuC1 detection in northern blot hybridization is given as orange bar. Reads obtained from RNA treated with terminal exonuclease (+TEX) are shown in green, and reads from untreated RNA (−TEX) are shown in orange. Comparison of reads from both fractions allowed us to determine the TSS (indicated by an arrow). Genome coordinates and annotated genes of the main chromosome plus strand are shown in black at the bottom.

Transcript levels of the znu operon in wild-type and s479 deletion strains. We compared the transcript levels of znu in total RNA of wild-type H66 (lanes w) and Δs479 (lanes Δ) cells at exponential and stationary phases (lanes e and s, respectively) using northern blot analysis. The probe used for hybridization in the upper panel is located in the central part of the znuC1 coding sequence (Figure 5). Signals at about 3,000 nucleotides correspond in length to the complete znu operon mRNA, and signals at approximately 2,000 correspond in length to a bicistronic mRNA encompassing either znuA1 and znuC1 or znuC1 and znuB1. In the lower panel, the blot was hybridized with a probe against the 16S rRNA. A size marker is given in kilobytes at the left.

We used RNA from such a cas7 deletion strain to monitor the s479 interplay with the Cascade complex. Indeed, in a Δcas7 strain, s479 is not detectable anymore, suggesting that s479 is bound and protected by Cascade as observed for crRNAs. Such an interaction with Cascade would be the first example for a non-crRNA bound by a type I-B Cascade used for internal gene expression regulation.

To determine whether the znu operon is upregulated in Δcas6 and Δcas7 strains, we probed a northern membrane containing RNA from these deletion strains with the znuC1 probe (Figure 6 and Supplementary Figure 4). Higher concentrations of znu mRNA are detected in the Δcas7 strain (Supplementary Figure 4), as expected when s479 is missing. Interestingly, in the Δcas6 strain, the znu RNA is not upregulated although the mature s479 is not present (Figure 4). Thus, the longer s479 intermediate with about 100 nucleotides length present in the Δcas6 strain seems to be also active in regulating the znu RNA.

**DISCUSSION**

Despite decades of research, archaeal sRNA networks are still enigmatic. It is well established that archaeal sRNAs play crucial roles in gene regulatory networks related to metabolism and, therefore, are essential players in stress responses, but pinpointing their interaction partners remained challenging (Buddeweg et al., 2018a). An inventory of small transcripts has been made for more than seven archaeal species, but sRNA–target pairs have been identified for only four of them (Buddeweg et al., 2018b; Gelsinger and DiRuggiero, 2018b; Orell et al., 2018). We report here data for the second sRNA–target RNA pair of *H. volcanii*.

**Deletion of the s479 Gene Has an Impact on Growth and the Transcriptome**

*Haloferax volcanii* encodes two operons for putative zinc ABC transporters (znuA1-C1 and znuA2-C2 genes); without s479, a higher abundance of transcripts for one of the two operons is observed (znuA1-C1), which is confirmed by northern blot analysis. Moreover, proteome analysis comparing s479 deletion and wild type also identifies znu1 gene products as differentially regulated. In addition, our data support a direct interaction of s479 with the transcript of the zinc transporter gene znuC1. IntaRNA prediction reveals that the 3′ end of the s479 interacts with the coding region of znuC1. The energy gain predicted for the interaction implies a stable sRNA–target pairing (Kliemt et al., 2019). The few examples of archaeal sRNA–target pairs described to date suggest a nonuniversal mode of action and a wide variability in the site of target contact (Buddeweg et al., 2018b; Gomes-Filho et al., 2018). The binding site for s479 within the coding region of the targeted mRNA is a feature shared...
TABLE 2 | Transporter components exclusively present or accumulated in the deletion strain.

| Gene       | Product                                                                 | On/log₂ | KEGG pathway/COG assignment |
|------------|-------------------------------------------------------------------------|---------|-----------------------------|
| HVO_B0198  | ABC-type transport system periplasmic substrate-binding protein (probable substrate iron-III) | On      | hvo02010—ABC transporters  |
| HVO_B0047  | ABC-type transport system periplasmic substrate-binding protein (probable substrate iron-III) | On      | hvo02010—ABC transporters  |
| HVO_2375   | Putative phosphate ABC transporter periplasmic substrate-binding protein | On      | hvo02010—ABC transporters  |
| HVO_2397   | Putative zinc ABC transporter periplasmic substrate-binding protein     | On      | hvo02010—ABC transporters  |
| HVO_1705   | Putative iron-III ABC transporter periplasmic substrate-binding protein | On      | hvo02010—ABC transporters  |
| HVO_2398   | Putative zinc ABC transporter ATP-binding protein                       | 2.5     | hvo02010—ABC transporters  |
| HVO_2324   | Pantothenate permease                                                   | 2.1     | COG0591 code ER N₃⁻/proline symporter |

Differential proteome analysis comparing the s479 deletion strain and wild-type strain was used determining the log₂ fold change deletion vs. wild type (column on/log₂). If detected only in the deletion strain, the protein is listed as “on.” Additionally, HVO-gene numbers (column gene) encoding the proteins are given alongside the gene product (column product) and KEGG/COG assignments (column KEGG pathway/COG assignment) informing on the metabolic pathway(s). Proteins encoded by the znu operon are given in bold.

with the only other sRNA-target pair described for H. volcanii (sRNA s132) (Kliemt et al., 2019) and with examples from other species [M. mazei s154, Sulfolobus acidocaldarius RrrR(+)] (Prasse et al., 2017; Orell et al., 2018). EMSA analysis revealed s479 binding to the znuC1 RNA (Figure 10), confirming a direct interaction of both RNAs. This is the first H. volcanii sRNA–target pair for which the interaction has been confirmed by a gel shift experiment. We hereby also confirm that s479 is exerting direct control of the znuC1 transcript. This results in a negative effect on znuC1 abundance in the cellular context which can be released by s479 deletion as seen in northern blot analysis. Destabilizing the target by potentially binding within the coding sequence is also suggested for S. acidocaldarius RrrR(+) (Orell et al., 2018), but archaeal RNases have not been studied in great depth yet, and therefore, no candidate RNase is evident for direct degradation of dsRNA (no RNase III activity has been described in archaea) or for ssRNA cleavage upon dsRNA formation (Clouet-d’Orval et al., 2018).

The s479 deletion strain has a slight growth disadvantage compared to the wild type during late exponential growth. The growth rate of the deletion strain is retarded by 22% compared with wild type during late exponential phase resulting in a lower end point of growth as well, showing that the sRNA is required for normal growth. The fact that s479 only regulates one of the two znu operons might be the reason that deletion of the s479 gene has only a slight impact on growth. The second znu operon could be regulated by another sRNA or other factors and concerted regulation of both znu operons might require an as yet unknown master regulator.

Deletion of the s479 Gene Has a Severe Impact on the Proteome

The differential proteome analysis revealed a much larger regulon for s479 on the protein level than seen on the transcript level suggesting that the primary effect of s479 is at the translational level. s479 is severely affecting the presence of more than 50 proteins. In contrast to the transcriptional level, where s479 acts as negative regulator, proteome analysis revealed proteins less abundant in the deletion strain, too. Such a duality in the direction of regulation achieved by a single sRNA has also been described for the other H. volcanii sRNA s132 (Kliemt et al., 2019). Also, like s132, s479 is implicated in both accumulation and depletion of certain proteins. Whether this effect is direct or indirect via intermediate gene products regulated by s479 must be analyzed in future experiments. However, it already demonstrates that sRNA-based regulation in H. volcanii is a complex and multimodal process in case of both sRNAs as they are addressing a multitude of targets.

Involvement of s479 in a Transporter Regulation Network

The common theme reflected in the functions assigned to the proteins influenced by s479 reveals a network of transporters and transport-related proteins. Among the proteins exclusively present in the s479 deletion strain are six components of ABC transporters including proteins encoded by the znu operon, which is regulated by s479 on the transcriptional level. The transported substances besides zinc are phosphate and iron. All of them are influx transporters and suggest a role of s479 in regulating the cellular network of metal ion and phosphate transporters. Taking into consideration proteins, which are differentially expressed with log₂ fold change 2, this is even more pronounced; 13 components of ABC transporters accumulated upon loss of s479 (Supplementary Table 2) including zinc, iron, phosphate, and peptide substrates. Therefore, a potential role for s479 might be in regulating metallostasis. A cross talk between the regulation of metal ion concentrations can be seen in bacteria, for instance for the regulatory networks of transcription factors Zur regulating zinc response and Fur regulating iron homeostasis in Caulobacter crescentus (Mazzon et al., 2014) or even within the transport itself, e.g., Yersinia pestis possesses a second Zn²⁺ transporter that engages components of the yersiniabactin (Ybt) siderophore-dependent transport system for iron (Bobrov et al., 2017). Further work is needed, to unravel, whether those translational effects are mediated directly or through secondary effects of other members of the regulon, e.g., the translation...
initiation factor aIF-5A. However, the direct effect of s479 is plausible for at least a subset of targets; as for 31 of the proteins on/off regulated in the s479 deletion strain, a site for physical interaction between the mRNA and the sRNA regulator could be predicted. Interestingly, the majority of interaction sites all map to the same part of the s479 sequence already predicted for the s479::znuC1 interaction (Figures 8, 9), which was confirmed as direct contact by gel shift analysis. However, as it is not yet resolved entirely how translation initiation ensues on the mostly leaderless transcripts of H. volcanii (Babski et al., 2016), future work is needed to unravel how translational control by small RNAs is exerted in this archaeal species. Interestingly, despite the
The s479 RNA is encoded between a CRISPR locus and the cas gene cluster. In addition, the s479 primary transcript contains a sequence highly similar to the CRISPR RNA repeat sequences (Supplementary Figure 2). CRISPR RNAs are processed by the endonuclease Cas6 at these repeat sequences to yield the functional crRNAs (Figure 11; Supplementary Figure 2; Maier et al., 2019). Northern experiments confirmed that cells without Cas6 cannot generate mature s479.

However, a 100-nucleotide RNA is still present and seems to be sufficient for regulating the znu mRNA. In addition, cells without Cas7 and thereby without Cascade contain neither the mature s479 nor the 100 nucleotide RNA (Figure 4), suggesting that s479 is bound and protected by Cascade. The znu RNA is clearly upregulated in a Δcas7 strain similar to the upregulation in the Δs479 strain. The fact that the mature s479 contains a typical crRNA 5′ handle and crRNAs are bound to Cascade via the conserved 5′ handle makes it even more likely that s479 is bound by Cascade. Such a dependence and interaction of s479 with Cas6 and Cascade, respectively, would be the first example for a non-crRNA bound by a type I-B Cascade used for internal gene expression regulation, pointing to a connection between the evolutionary origin of this sRNA as a drift from the CRISPR-Cas machinery, evolved to control gene expression at the mRNA level. A similar mode of gene regulation by Cas proteins has so far only been shown in detail for type II systems (Dugar et al., 2018; Ratner et al., 2019). In Campylobacter jejuni, for instance, Cas9 is guided by a crRNA to mRNA targets, inducing RNA cleavage by Cas9 thereby regulating the expression of these genes (Dugar et al., 2018).

Further work will show whether s479 guides Cascade to the znuC1 mRNA and triggers degradation of the mRNA (Figure 11).

CONCLUSION

s479 supports a role for sRNAs as substantial regulatory players within the metabolic networks of *H. volcanii* especially in regulating metabolite availability as s479 seems to harmonize the abundance of several influx transporters of the ABC-type regulating the zinc, iron, peptide, and phosphate flux of the cell. We demonstrate the direct interaction of s479 to its target znuC1 mRNA. Furthermore, our data show that s479 is linked to the CRISPR-Cas system and might act together with Cascade to regulate zinc transport proteins.

MATERIALS AND METHODS

Strains and Growth Conditions

Strains and oligonucleotides used in this study are listed in Supplementary Tables 3, 4. A detailed description of the media used can be found in the Supplementary Data. Cloning procedures were performed using *E. coli* strain DH5α and standard culture (aerobically, 37°C, 2YT media) as well as molecular biological techniques (Sambrook, 2006). *H. volcanii* strains were grown aerobically at 45°C in either YPC, Hv-Ca, or Hv-MM with appropriate supplements (Allers et al., 2004; Duggin et al., 2015; de Silva et al., 2020). For in-depth comparison of growth, transcriptome, and proteome, H66 (ΔpyrE2, ΔleuB) was used as wild type, since in the s479 deletion strain, the s479 gene is replaced by a tryptophan marker in the genome of the parent strain H119 (ΔpyrE2, ΔtrpA, ΔleuB). Thus, Δs479 and H66 require both the addition of uracil and leucine to media for growth (Allers et al., 2004; Jaschinski et al., 2014).

Growth Experiments

Growth experiments were carried out in microtiter plates using a heated plate reader (Epoch 2 NS Microplate Spectrophotometer, BioTek Instruments). Strains H66 (wild type) and Δs479 were precultured in Hv-Ca medium supplemented with uracil to OD650 nm = 0.4–0.7 and then diluted to OD650 nm = 0.05 and transferred to microtiter plates in triplicates. These were then cultured (aerobically, orbital shaking, 45°C) while OD650 nm was measured every 30 min. Outer wells were filled with salt water as evaporation barriers (Jaschinski et al., 2014). For stress conditions, adjusted media preparations were used (see section “Strains and Growth Conditions”). Doubling time \[d\] (h) and growth rate \[\mu\] (h⁻¹) were calculated as growth rate \[\mu = (\ln(x_t) - \ln(x_0)) / (t - t_0)\] and doubling time \[d = \ln(2) / \mu\]. Calculations were carried out separately for all replicates.
before calculating mean value and standard deviation. Phases of exponential growth were identified using fitted trendlines and corresponding R² values (Supplementary Figures 5B,C).

Northern Blot Analysis

For the analysis of s479 transcripts (Figure 2), Haloferax strains H119 and Δs479 were cultivated in Hv-MM supplemented with leucin and uracil (for H119, tryptophan was also added); for the detection of znu mRNAs (Figure 7), Haloferax strains H66 and Δs479 were grown in YPC. For the detection of s479 transcripts in Figure 4, the Haloferax strain H119 was cultivated in Hv-MM supplemented with leucin, tryptophan, and uracil; deletion strains Δcas6 and Δcas7 were cultivated in YPC. For the investigation of znu mRNAs in Cas protein deletion strains Δcas6 and Δcas7 (Supplementary Figure 4) H119, Δcas6 and Δcas7 were grown in YPC. TRIzol reagent (Invitrogen, Thermo Fisher Scientific) or NucleoZOL™ (Machery and Nagel) was used to isolate total RNA from H. volcanii cells. Ten micrograms of total RNA was separated using a 1.5% agarose (transcript size > 500 nt) or 8 % denaturing polyacrylamide gel (PAGE) and then transferred to a nylon membrane (Biodyne A, PALL or Hybond-N+, GE Healthcare). After transfer, the membrane of PAGE blots was hybridized with oligonucleotide s479spacert (primer sequences are listed in Supplementary Table 4) to detect the s479 transcript, and the membrane was subsequently hybridized with an oligonucleotide against the 5'S rRNA, both radioactively labeled with [γ-32P]-dCTP via polynucleotide kinase treatment. Membranes of agarose blots were hybridized with a probe against the 16S rRNA. The probe was generated with PCR using primers 16Sseqf and 16Sseqrev and genomic DNA from H. volcanii as template. Using the DECAprime™III kit (Invitrogen), the PCR fragment was radioactively labeled with [α-32P]-dCTP. For oligonucleotide probes and PCR primers, see Supplementary Table 4.

Sample Preparation for Transcriptome Analysis and RNA-Seq Analysis

Three replicates of wild type (H66) and deletion strain (Δs479) were cultured in Hv-Ca medium supplemented with uracil at 45°C and grown to OD650 nm = 0.6–0.7. Total RNA was isolated using NucleoZOL™ (Machery and Nagel), and RNA samples were sent to Vertis Biotechnologie AG ( Martinsried, Germany) for further treatment. Total RNA was treated with T4 Polynucleotide Kinase (NEB) and rRNA depleted using an Agencourt AMPure XP kit (Beckman Coulter Genomics), samples were pooled (equimolar), and the pool size fractionated (200–550 bp) by preparative agarose gel electrophoresis and sequenced on an Illumina NextSeq 500 system using 1 × 75 bp read length. TruSeq barcode sequences which are part of the 5' TruSeq sequencing adapter are included in Supplementary Table 4. Sequencing reads are deposited at the European Nucleotide Archive (ENA) under the study accession number PRJEB41379. For data analysis, reads were mapped to the genome using bowtie2 (version 2.3.4.1) with the "--very-sensitive" option and defaults otherwise (Langmead et al., 2009). Then,
reads per feature were counted using featureCounts (version 1.6.4) and analyzed for differential expression with DeSeq2 (version 1.2.11) (Liao et al., 2014; Love et al., 2014).

**Sample Preparation for MS/MS Analysis**

Three biological replicates (250 ml) of the wild type (H66) and deletion strain (Δs479) were cultivated in Hv-Ca media supplemented with uracil, 45°C, and grown to OD$_{650}$ nm = 0.6–0.74. Cells were harvested and washed in enriched PBS buffer, 100,000 g and treated as separate samples. The cytosolic protein sample was directly used for 1D SDS-PAGE, whereas the pelleted membrane protein fraction was solubilized in 2 ml HTH buffer [(6 M thiourea/2 M urea); 10 min 37°C ultrasonication]. Twenty micrograms of both samples were separated by 1D SDS-PAGE and in-gel digested as previously described (Bonn et al., 2014). Briefly, Coomassie-stained gel lanes were cut resulting in 10 gel pieces per sample before gel pieces were cut into smaller blocks and transferred into low binding tubes. Samples were destained and dried in a vacuum centrifuge before being covered with trypsin solution. Digestion was carried out at 37°C overnight before peptides were eluted in water by ultrasonication. The peptide-containing supernatant was transferred into a fresh tube and desiccated in a vacuum centrifuge, and peptides were resolubilized in 0.1% (v/v) acetic acid for mass spectrometric analysis.

**MS/MS Analysis**

LC-MS/MS analyses were performed on an LTQ Orbitrap Velos Pro (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using an EASY-nLC II liquid chromatography system. Tryptic peptides were subjected to liquid chromatography (LC) separation and electrospray ionization-based mass spectrometry (MS) applying the same injected volumes in order to allow for label-free relative protein quantification. Therefore, peptides were loaded on a self-packed analytical column (OD 360 μm, ID 100 μm, length 20 cm) filled with 3 μm diameter C18 particles (Dr. Maisch, Ammerbuch-Entringen, Germany) and eluted by a binary nonlinear gradient of 5–99% acetonitrile in 0.1% acetic acid over 86 min with a flow rate of 300 nl/min. For MS analysis, a full scan in the Orbitrap with a resolution of 30,000 was followed by collision-induced dissociation (CID) of the 20 most abundant precursor ions. MS2 experiments were acquired in the linear ion trap.

**MS Data Analysis**

Database search was performed with MaxQuant 1.6.17.0 against a *H. volcanii* database (Jevtić et al., 2019) containing 4,106 entries. MaxQuant’s generic contamination list as well as reverse entries was added during the search. The following parameters were applied: digestion mode, trypsin/P with up to two missed cleavages; variable modification, methionine oxidation and N-terminal acetylation, and maximal number of five modifications per peptide; activated LFQ option with minimal ratio count of two and “match-between-runs” feature. The false discovery rates of the peptide spectrum match and the protein level were set to 0.01. A protein was considered to be identified if two or more unique peptides were identified in a biological replicate. Only unique peptides were used for protein quantification.

The comparative proteome analyses based on MaxQuant LFQ values were performed separately for cytosolic and membrane protein samples. Proteins were considered to be quantified if a quantitative value based on at least two unique peptides was available in at least two biological replicates. LFQ values as proxy for protein abundance were used for statistical analysis. Student’s *t* test was performed to analyze changes in protein amounts between wild type and mutant. Proteins with significantly changed amount exhibited a *p* value < 0.01 and an average log$_2$ fold change > [0.8].

**Electrophoretic Mobility Shift Assay**

For electrophoretic mobility shift assay (EMSA), RNAs were obtained from Biomers (Ulm, Germany) (sequences are listed in Supplementary Table 4). The s479 RNA was labeled at the 3’ end using [α-32P]-pCp and T4 RNA ligase (Fermentas, Thermo Fisher Scientific). For the EMSA in Figure 10, 100 cps s479-RNA was mixed with 50, 100, or 200 pmol of the unlabeled znuC1 RNA fragment encompassing interaction site 2 (Figure 8D). For the EMSA in Supplementary Figure 3A, 100-cps labeled s479 RNA was mixed with 50 pmol unlabeled znuC1 RNA encompassing interaction site 2 (Figure 8D); in addition, 0, 50, 200, or 400 pmol of the unlabeled s479 was added. For the EMSA in Supplementary Figure 3B, 100-cps labeled s479 RNA was mixed with 50 pmol unlabeled znuC1 RNA mutant, which has the s479 interaction site deleted (Figure 8D). All reactions were performed in 20 μl reaction volume containing 10 mM Tris–HCl, pH 7.5, 5 mM MgCl$_2$, and 100 mM KCl. After incubation at 37°C for 30 min, 1 μl 50% glycerol containing 0.1% (w/vol) bromphenol blue was added, and the samples were separated on a native 8% (w/vol) polyacrylamide gel at 4°C which was subsequently analyzed by autoradiography.

**In silico Target Site Prediction**

To predict the target sites of s479 in *silico*, we applied IntaRNA (version 3.2.0) (Mann et al., 2017). For the prediction of potential s479 interaction sites, we used the s479 sequence corresponding to pHV4: 207,716–207,770. This corresponds to the start point of the potential spacer sequence of the shortened s479 versions (Figure 3). The spacer is the sequence located downstream of the 5’ handle sequence within crRNAs. In crRNAs, the spacer sequence is the sequence used for target recognition. Therefore, we chose this part of the sequence for analysis and set the spacer length to 55 nt. IntaRNA was used with default settings for the prediction of the s479::znuC1 interaction sites. For the prediction of the interaction sites on the proteome–targets, we first used default settings and then also included predictions for a seed
sequence of five nucleotides as this is the increment of protein contacts seen for spacer sequences within Cascade complexes (Maier et al., 2019).

DATA AVAILABILITY STATEMENT

The transcriptome data were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under study accession number: PRJEB41379. Proteome data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2016) with the dataset identifier PXD022750.

AUTHOR CONTRIBUTIONS

PM, L-KM, SM, CH, and JB did the experiments. BV carried out the bioinformatics analyses. PM, L-KM, AM, SM, DB, and BV performed data curation. AM conceptualized the project. L-KM, PM, and AM wrote the original draft. SM, JB, DB, BV, L-KM, and AM reviewed and edited the draft. DB, BV, and AM provided the resources and funding. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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