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Putative Nucleotide-Based Second Messengers in the Archaeal Model Organisms *Haloferax volcanii* and *Sulfolobus acidocaldarius*

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Research on nucleotide-based second messengers began in 1956 with the discovery of cyclic adenosine monophosphate (3′,5′-cAMP) by Earl Wilbur Sutherland and his co-workers. Since then, a broad variety of different signaling molecules composed of nucleotides has been discovered. These molecules fulfill crucial tasks in the context of intracellular signal transduction. The vast majority of the currently available knowledge about nucleotide-based second messengers originates from model organisms belonging either to the domain of eukaryotes or to the domain of bacteria, while the archaeal domain is significantly underrepresented in the field of nucleotide-based second messenger research. For several well-stablished eukaryotic and/or bacterial nucleotide-based second messengers, it is currently not clear whether these signaling molecules are present in archaea. In order to shed some light on this issue, this study analyzed cell extracts of two major archaeal model organisms, the euryarchaeon *Haloferax volcanii* and the crenarchaeon *Sulfolobus acidocaldarius*, using a modern mass spectrometry method to detect a broad variety of currently known nucleotide-based second messengers. The nucleotides 3′,5′-cAMP, cyclic guanosine monophosphate (3′,5′-cGMP), 5′-phosphoadenylyl-3′,5′-adenosine (5′-pApA), diadenosine tetraphosphate (Ap_4A) as well as the 2′,3′-cyclic isomers of all four RNA building blocks (2′,3′-cNMPs) were present in both species. In addition, *H. volcanii* cell extracts also contain cyclic cytosine monophosphate (3′,5′-cCMP), cyclic uridine monophosphate (3′,5′-cUMP) and cyclic diadenosine monophosphate (3′,5′-c-di-AMP). The widely distributed bacterial second messengers cyclic diguanosine monophosphate (3′,5′-c-di-GMP) and guanosine (penta-)/tetraphosphate [(p)ppGpp] could not be detected. In summary, this study gives a comprehensive overview on the presence of a large set of currently established or putative nucleotide-based second messengers in an eury- and a crenarchaeal model organism.

Keywords: archaea, *Haloferax volcanii*, *Sulfolobus acidocaldarius*, cyclic nucleotides, second messengers, signaling molecules

INTRODUCTION

During cellular signal transduction, most external/environmental stimuli do not directly interact with their respective cellular target, but rather cause the intracellular production/release of specific small molecules, which transmit the initial signal and eventually trigger a specific cellular response. In this concept, the initial stimulus is referred to as "first messenger," while the intracellularly transducing small molecules are called "second messengers" (Newton et al., 2016). Various second messengers have been identified and they can be grouped into four different categories according to their chemical properties: ions, gases and free radicals, lipid-based and nucleotide-based second messengers (Newton et al., 2016). Calcium (Ca^{2+}) is a well-established ionic second messenger, which plays a crucial role in a plethora of eukaryotic signal transduction processes such as the excitability of neural cells, exocytosis, motility, apoptosis, and transcription (Clapham, 2007). A well-characterized example of a gaseous second messenger is nitric oxide (NO). Molecules of this gas are involved in various prokaryotic and eukaryotic signal transduction pathways, such as the regulation of the mammalian nervous system and bacterial quorum sensing and biofilm formation (Zhou and Zhu, 2009; Nisbett and Boon, 2016). Examples of lipid-based second messengers are diacylglycerol or ceramide, which are involved in various eukaryotic signal cascades (Liscovitch and Cantley, 1994; Hilgemann et al., 2018). The most diverse category of second messengers consists of nucleotide-based signaling molecules. These second messengers can either be based on a single (mono-nucleotide-based), two (di-nucleotide-based), or several (oligo-nucleotide-based) nucleotide molecules. **Table 1** shows an overview on the majority of currently established or putative nucleotide-based second messengers, including their presence and some examples of their functions in eukaryotes and bacteria.

The information summarized in **Table 1** originates from bacteria and eukaryotes. For archaea, only very limited information about the occurrence and the physiological functions of nucleotide-based second messengers is currently available. Until now, only the presence of 3′,5′-cAMP, 3′,5′-c-di-AMP and cyclic oligo adenylate (cOA) has been reported in archaea. 3′,5′-cAMP was identified in the euryarchaea *Haloferax volcanii* and *Methanothermobacter thermoautotrophicus* and in the crenarchaea *Saccharolobus solfataricus* (previously known as *Sulfolobus solfataricus*) (Leichtling et al., 1986). Additionally, in the euryarchaeon *Halobacterium salinarum* the levels of 3′,5′-cAMP were shown to fluctuate during the cell cycle (Baumann et al., 2007). Analogous to its reported function in bacteria, cOA was shown to be involved in type III CRISPR system mediated immunity in the crenarchaeon *Sa. solfataricus* (Rouillon et al., 2018). In *H. volcanii*, 3′,5′-c-di-AMP was shown to be essential and has been implicated in osmoregulation (Braun et al., 2019). Noteworthy, analysis of the presence of the alarmone (p)ppGpp using radioisotope-labeling approaches in a few archaeal species suggested the absence of this signaling nucleotide (Beauclerk et al., 1985; Cimmino et al., 1993; Scoarughi et al., 1995; Cellini et al., 2004). For all other nucleotide-based second messengers there is, to the best of our knowledge, currently no data available whether or not they are produced in archaea. To study the presence of nucleotide-based second messengers in archaea, cell extracts of the euryarchaeal model organism *H. volcanii* and the crenarchaeal model organism *S. acidocaldarius* were analyzed using liquid chromatography tandem mass spectrometry (LC−MS/MS) for the presence of representatives of mono-, di-, and oligo-nucleotide-based second messengers. These measurements unveiled that *H. volcanii* cells contain, besides the already known 3′,5′-cAMP and 3′,5′-c-di-AMP, detectable levels of 3′,5′-cGMP, 3′,5′-cCMP, 3′,5′-cUMP, 2′,3′-cAMP, 2′,3′-cGMP, 2′,3′-cCMP, 2′,3′-cUMP, 5′-pApA and Ap_4A. Compared to that, *S. acidocaldarius* cells contained a reduced variety of nucleotides. Besides all four 2′,3′-cNMPs, only 3′,5′-cAMP, 3′,5′-cGMP, Ap_4A and very minor amounts of 5′-pApA could be detected. The well-established bacterial second messenger 3′,5′-c-di-GMP, the alarmone (p)ppGpp as well as all three physiologically appearing isomers of cGAMP (2′,3′-cGAMP, 3′,3′-cGAMP, and 3′,2′-cGAMP) could not be detected, suggesting their absence in *H. volcanii* and *S. acidocaldarius* when grown under standard laboratory conditions. The same applies for 3′,5′-cTMP, 3′,5′-cIMP, 3′,5′-cXMP, 5′-pGpG and cOA (n = 4; c-tetra-AMP), which were all not present in the examined *H. volcanii* and *S. acidocaldarius* cell extracts.

Taken together, the results of this study show that the nucleotide-based second messenger pools of *H. volcanii* and *S. acidocaldarius* contain several signaling molecules, whose presence in archaea has not been shown so far. Assuming that many other euryarchaeal and crenarchaeal species make use of similar nucleotide-based second messenger pools, these results offer important leads to further investigate the role and importance of these nucleotides for these organisms.

MATERIALS AND METHODS

Unless stated otherwise all chemicals were purchased from Carl Roth.

Strains and Growth Conditions

H. volcanii strain H26 was grown in selective CA medium (Allers et al., 2004) (0.5 g/L Bacto™ Casamino acids; pH 7.2 adjusted with KOH) modified with an expanded trace element solution (referred to as CAB) (Duggin et al., 2015). Cells were grown at 45°C in liquid medium while rotating (volumes up to 5 ml) or shaking (volumes > 5 ml).

S. acidocaldarius strain MW001 was grown in basal Brock medium (pH 3.5) (Brock et al., 1972) supplemented with 0.1% (w/v) NZ-amine (Sigma) and 0.2% (w/v) dextrin. Cells were grown at 75°C in liquid medium while shaking.

Since both strains are auxotroph for uracil (H26, Δ*pyrE2* and MW001, Δ*pyrEF*), growth media were supplemented with uracil (Sigma) at a defined concentration (50 µg/ml for H26; 10 µg/ml

TABLE 1 | Overview on occurrence and exemplary function(s) in eukaryotes and bacteria of all signaling nucleotides addressed in this study.

Nucleotide(s)	Presence in eukaryotes	Exemplary functions in eukaryotes	Presence in bacteria	Exemplary functions in bacteria
Mono-nucleotide-based signaling molecules				
3′,5′-cAMP	In a multitude of uni- and multicellular species (Shemarova, 2009; Gancedo, 2013; Blanco et al., 2020)	• Regulation of carbohydrate metabolism (Sutherland and Wosilait, 1956; Berthet et al., 1957; Rall and Sutherland, 1958) • Synaptic transmission (Marx, 1972; Maiellaro et al., 2016) • Seed germination (Uematsu and Fukui, 2008) • Chemotaxis (Escalante et al., 1997)	In species of various phyla (Botsford and Harman, 1992)	• Catabolite repression (Crasnier, 1996) • Infection and host colonialization (McDonough and Rodriguez, 2011) • Biofilm formation (Liu et al., 2020)
3′,5′-cGMP	Mainly found in ciliated eukaryotes (Johnson and Leroux, 2010)	• Phototransduction (Stryer, 1986) • Smooth muscle relaxation (Rybalkin et al., 2003) • Osmoregulation (Kuwayama et al., 1996)	Identified in f.i.: • Cyanobacteria (Cadoret et al., 2005) • α-proteobacteria (Marden et al., 2011) • γ-proteobacteria (An et al., 2013)	• UV-stress adaption (Cadoret et al., 2005) • Cyst formation (Marden et al., 2011) • Biofilm and virulence (An et al., 2013)
3′,5′-cCMP 3′,5′-cUMP	Using modern mass spectrometry detected in various mammalian cell lines and different organs (Burhenne et al., 2011; Bähre et al., 2015). Additionally also found in zebrafish (Dittmar et al., 2015) and cUMP in a plant (Hartwig et al., 2014)	• In general functions are currently mostly unknown (Seifert, 2017) • Both can activate some cAMP/cGMP effectors *in vitro* (Wolter et al., 2011; Zong et al., 2012) • cCMP potentially involved in processes such as tissue development and cell proliferation, immune responses modulation and platelet aggregation (Bloch et al., 1974; Anderson, 1982; Desch et al., 2010)	• Specific cytidylate and uridylate cyclases just recently discovered in species of various phyla (Tal et al., 2021) • Synthesis stimulated by phage infection; activate downstream defense mechanisms (Tal et al., 2021) • Bacterial toxins [e.g., ExoY from *P. aeruginosa* (Beckert et al., 2014), CyaA from *Bordetella pertussis* (Göttle et al., 2010), edema factor from *Bacillus anthracis* (Göttle et al., 2010)] have been demonstrated to be capable of forming cCMP and cUMP	
3′,5′-cIMP	Identified in isolated porcine coronary arteries (Chen et al., 2014)	• In general functions are currently mostly unknown (Leung et al., 2015) • Involved in hypoxia−induced constriction of porcine coronary arteries (Chen et al., 2014; Nan et al., 2020)	• Only very few data available • Detected in *Corynebacterium murisepticum* (Newton et al., 1998); specificity of the used method is however questioned (Seifert, 2015)	
3′,5′-cTMP 3′,5′-cXMP	Not yet detected in any biological samples using modern and sensitive mass spectrometry techniques (Beste and Seifert, 2013) cXMP can be formed by purified guanylate cyclase and activate certain cAMP effectors *in vitro* (Wolter et al., 2011; Beste et al., 2012)			
2′,3′-cNMPs	Identified in several mammalian cell lines (Ren et al., 2009; Pabst et al., 2010; Bähre and Kaever, 2014), different organs (Jia et al., 2014) and plant tissue (Van Damme et al., 2014)	• Originate from mRNA degradation by transphosphorylation (Thompson et al., 1994) or RNA cyclase activity (Shigematsu et al., 2018) • Actual utilization as second messengers currently unknown; reporting of tissue damage as possible function (Jackson, 2011, 2017; Van Damme et al., 2014)	Identified in f.i.: • *Pseudomonas fluorescens* (Bordeleau et al., 2014) • *E. coli* (Fontaine et al., 2018)	• In *E. coli* originating from RNase I-dependent RNA degradation (Fontaine et al., 2018) or RNA cyclase activity (Genschik et al., 1997) • Levels of 2′,3′-cNMPs influenced the biofilm formation of *E. coli* (Fontaine et al., 2018)
(p)ppGpp	• For a long time believed to be absent in eukaryotes, with the notable exception of chloroplasts (Tozawa and Nomura, 2011) • Recently found in *Drosophila melanogaster* and human cell lines (Ito et al., 2020) • Altered levels caused metabolic changes and cell death in *D. melanogaster* (Ito et al., 2020)		In species of various phyla (Atkinson et al., 2011)	• Stress signaling related alarmones (Cashel and Gallant, 1969; Lazzarini et al., 1971) • Synthesis triggered by diverse metabolic or physical stresses (Cashel and Gallant, 1969; Gallant et al., 1977; Flärdh et al., 1994; Spira et al., 1995; Vinella et al., 2005; Battesti and Bouveret, 2006; Hood et al., 2016; Tarusawa et al., 2016) • Globally regulates gene transcription in the context of the stringent response (Magnusson et al., 2005)
Di-nucleotide-based signaling molecules				
3′,5′-c-di-GMP	• Synthesizing and degrading enzymes bioinformatically predicted in lower eukaryotes (Römling et al., 2013) • Synthesis confirmed in social amoebae of the class of Dictyostelia with a function for stalk cell differentiation (Chen and Schaap, 2012) • Recognized by human innate immune system (Burdette et al., 2011)		In species of various phyla (Römling et al., 2013)	• Involvement in various physiological functions observed (Römling et al., 2005) • Major functions: Transition from motile to sessile lifestyle (Wolfe and Visick, 2008; Valentini and Filloux, 2016), virulence (Valentini and Filloux, 2019)

(Continued)

TABLE 1 | (Continued)

Nucleotide(s)	Presence in eukaryotes	Exemplary functions in eukaryotes	Presence in bacteria	Exemplary functions in bacteria
3′,5′-c-di-AMP	• Currently believed to be absent from eukaryotic cells (He et al., 2020) • Recognized by human innate immune system (Barker et al., 2013)		In species of various phyla (Corrigan and Gründling, 2013)	• Involvement in several physiological functions observed (Fahmi et al., 2017) • Major function: Regulation of cellular osmotic homeostasis (Stülke and Krüger, 2020)
2′,3′-cGAMP	Currently believed to only be present in metazoa (Kranzusch, 2019)	• Endogenous activator of the innate immune system leading to type-I interferon production (Wu et al., 2013; Zhang et al., 2013)	Isomer containing the atypical 2-′5′ phosphodiester linkage currently believed to be exclusively found in metazoa (Davies et al., 2012; Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013; Li et al., 2019)	
3′,3′-cGAMP	Produced by some lower metazoa like the anemone *Nematostella vectensis* (Kranzusch et al., 2015)	• Same function as 2′,3′-cGAMP as endogenous activator of the innate immune system (Kranzusch et al., 2015)	Synthesizing enzymes identified in various phyla (Whiteley et al., 2019)	• Biofilm formation and motility (Li et al., 2019) • Protection against viral infection (Severin et al., 2018; Cohen et al., 2019)
3′,2′-cGAMP	Very recently identified in *D. melanogaster*; synthesizing enzymes also conserved in metazoa (Holleufer et al., 2021; Slavik et al., 2021)	• Very similar function as 2′,3′-cGAMP as an endogenous activator of innate immune response (Holleufer et al., 2021; Slavik et al., 2021)	Currently no data available on the existence in bacteria; Presence of the atypical 2-′5′ phosphodiester linkage suggest its absence in bacteria (see 2′,3′-cGAMP above)	
5′-pGpG 5′-pApA	• Actual function as second messenger currently unclear • In bacteria, both are the degradation product of 3′,5′-c-di-GMP and 3′,5′-c-di-AMP, respectively (Rao et al., 2010; Stelitano et al., 2013) • Both have the capability to bind to some targets which are regularly binding the respective, unhydrolyzed c-di-NMP (Smith et al., 2012; Stelitano et al., 2013; Bowman et al., 2016; Kuipers et al., 2016) • As a nanoRNA, both have the potential to affect gene transcription on the level of transcription initiation (Goldman et al., 2011; Nickels and Dove, 2011)			
Ap_4A	In a multitude of uni- and multicellular species (Plesner and Ottesen, 1980; Flodgaard and Klenow, 1982; Garrison and Barnes, 1984; McLennan and Prescott, 1984)	• Potential stress signaling related alarmone (Brevet et al., 1985; Baltzinger et al., 1986; Coste et al., 1987; Garrison et al., 1989) • In metazoa: regulatory effects on the cardiovascular and immune system and neuronal signal transduction (Vahlensieck et al., 1999; Miras-Portugal et al., 2003; Lee et al., 2004)	Synthesizing and hydrolyzing enzymes present in various phyla (Ferguson et al., 2020)	• Potential stress signaling related alarmone (Lee et al., 1983; Pálfi et al., 1991; Kimura et al., 2017) • Cellular development (Nishimura et al., 1997; Kimura et al., 2017) • Biofilm formation (Monds et al., 2010)
Oligo-nucleotide-based signaling molecules				
cOA	According to current knowledge absent in eukaryotes		In species utilizing a type III CRISPR system (Kazlauskiene et al., 2017; Niewoehner et al., 2017)	• Produced upon presence of invader RNA (Kazlauskiene et al., 2017; Niewoehner et al., 2017) • Activate effectors leading to invader RNA/DNA degradation (Kazlauskiene et al., 2017; Niewoehner et al., 2017; Koonin and Makarova, 2018)

3′,5′-cAMP, 3′,5′-cyclic adenosine monophosphate; 3′,5′-cGMP, 3′,5′-cyclic guanosine monophosphate; 3′,5′-cCMP, 3′,5′-cyclic cytidine monophosphate; 3′,5′-cUMP, 3′,5′-cyclic uridine monophosphate; 3′,5′-cIMP, 3′,5′-cyclic inosine monophosphate; 3′,5′-cTMP, 3′,5′-cyclic thymidine monophosphate; 3′,5′-cXMP, 3′,5′-cyclic xanthosine monophosphate; 2′,3′-cNMPs, 2′,3′-cyclic isomers of nucleotides with N here: adenosine, guanosine, cytidine or uridine; (p)ppGpp, guanosine (penta-)/tetraphosphate; 3′,5′-c-di-GMP, 3′,5′-cyclic diguanosine monophosphate; 3′,5′-c-di-AMP, 3′,5′-cyclic diadenosine monophosphate; 2′,3′-cGAMP, 2′,3′-cyclic guanosine monophosphate-adenosine monophosphate (cyclic [G(2′,5′)pA(3′,5′)p]); 3′,3′-cGAMP, 3′,3′-cyclic guanosine monophosphate-adenosine monophosphate (cyclic [G(3′,5′)pA(3′,5′)p]); 3′,2′-cGAMP, 3′,′-cyclic guanosine monophosphate-adenosine monophosphate (cyclic [G(3′,5′)pA(2′,5′)p]); 5′-pGpG, 5′-phosphoguanylyl-3′,5′-guanosine; 5′-pApA, 5′-phosphoadenylyl-3′,5′-adenosine; Ap_4A, diadenosine tetraphosphate; cOA, cyclic oligoadenylate (with n = 3–6); CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; f.i., for instance.

for MW001). Further details on H26 and MW001 are listed in **Supplementary Table 1**.

Nucleotide Extraction From *Haloferax volcanii* and *Sulfolobus acidocaldarius* Cells

The extraction of nucleotides from total cell pellets of *H. volcanii* and *S. acidocaldarius* cells was performed as described previously (Spangler et al., 2010; Braun et al., 2019). Briefly, H26 was grown in 340 ml CAB + uracil, MW001 in 250 ml supplemented Brock + uracil. Samples were taken during exponential growth and at the beginning of the stationary phase. For nucleotide extraction from exponentially growing cultures 25 ml were harvested, for stationary grown cultures 15 ml were harvested. For each nucleotide sample, an additional 2 ml aliquot of each culture was harvested for the determination of the total protein content [bicinchoninic acid (BCA) Protein Assay Macro Kit (Serva)]. Cell pellets were snap—frozen in liquid nitrogen. Unless mentioned otherwise the experiments were performed as three biological replicates with three technical replicates each. The cell pellets were resuspended in 300 μl extraction solution [acetonitrile/methanol/water (ultrapure): 2:2:1 (v/v/v)]. Resuspended pellets were incubated on ice for 15 min followed by a heating step at 95°C for 10 min. After cooling on ice, the solution was centrifuged at 21,100 × *g* for 10 min at 4°C.

The resulting supernatant was transferred to a fresh vial. The extraction was repeated two times (three extraction steps in total) with 200 µl fresh extraction solution, omitting the heating step. The supernatants were combined and stored overnight at -20°C to precipitate proteins. To remove precipitates, the samples were centrifuged again (10 min, 4°C; 21,100 × *g*) and the supernatant was transferred to a fresh vial. Final extracts were desiccated using a vacuum concentrator (Eppendorf) at 45°C.

Nucleotide Extraction From *Haloferax volcanii* Cell Lysate via Solid Phase Extraction

Nucleotide extracts from total cell pellets of *H. volcanii* could not be analyzed for their (p)ppGpp content due to certain specific fragmentation patterns of unknown molecular species overlapping with the internal (p)ppGpp standard. Therefore, extraction of (p)ppGpp was performed using a solid phase extraction approach as described previously (Ihara et al., 2015). For solid phase extraction from exponentially growing cultures 15 ml were harvested, for stationary grown cultures 9 ml were harvested. For each sample, an additional 2 ml aliquot of each culture was harvested for the determination of the total protein content (BCA assay). Growth of H26 and time points of sample acquisition were as described above. Cell pellets were snap—frozen in liquid nitrogen and subsequently resuspended in 2 ml ultrapure water on ice. Resuspended pellets were lysed by the addition of formic acid (to a final concentration of 1 M) and incubated for 1 h on ice. The cell lysate was mixed with 2 ml ammonium acetate (pH 4.5) and centrifuged for 5 min at 4°C and 3,000 × *g* to remove cell debris. The lysate was further purified on an OASIS Wax cartridge 1 cc (Waters) using centrifugation steps of 1 min at 4,300 × *g* at 4°C. The cartridge was equilibrated with 1 ml methanol followed by 1 ml ammonium acetate (pH 4.5), the lysate was loaded in four consecutive loading steps of 1 ml, the cartridge was washed with 1 ml ammonium acetate (pH 4.5) followed by 1 ml methanol and the sample was eluted with 1 ml elution solvent [water (ultrapure)/methanol/ammonium hydroxide (25% (w/v)): 7:2:1 (v/v/v)]. The elution fractions were desiccated using a vacuum concentrator (Eppendorf) at 45°C.

Quantification of Nucleotides From Cell Extracts by Liquid Chromatography With Tandem Mass Spectrometry

Desiccated nucleotide extracts were resuspended in 200 µl water, centrifuged and diluted 1:2 with the respective standard solution (containing stable isotope labeled nucleotides as well as 100 mg/ml Tenofovir as internal standards) and analyzed by a LC—MS/MS method.

Cyclic di-nucleotides were analyzed as described previously (Rao et al., 2010; Bähre and Kaever, 2017). Chromatographic separation was performed by reversed phase chromatography on a C18-column (Nucleodur Pyramid C18 3 µ 50 × 3 mm; Macherey-Nagel; Germany), using water containing 10 mM ammonium acetate and 0.1% acetic acid as eluent A and pure methanol as eluent B, using the following gradient: 0–4 min 0% B, 4–7.3 min 0–10% B, 7.3–8.3 min 10% B, 8.3–11 min 10–30% B, 11–13 min 0% B. The flow rate was 600 µl/min. Mass spectrometric analysis was performed on a tandem mass spectrometer (API4000; MA, United States) performing selected reaction monitoring (SRM). The mass spectrometer was equipped with an electrospray ionization source (ESI) and ionization was performed in positive mode for all analytes.

Cyclic nucleotides, were analyzed as described previously (Bähre and Kaever, 2014). Chromatographic separation was performed by reversed phase chromatography on a C18-column, using methanol-water [3:97 (v/v)] containing 50 mM ammonium acetate and 0.1% (v/v) acetic acid as eluent A and methanol-water [97:3 (v/v)] containing 50 mM ammonium acetate and 0.1% (v/v) acetic acid as eluent B, using the following gradient: 0–5 min 0–50% B and 5–8 min 0% B. The flow rate was 500 µl/min. Cyclic nucleotides were analyzed on a QTRAP 5500 (Sciex, MA, United States). Ionization was achieved with an ESI in positive mode. In SRM mode 3′,5′-cNMPs and the 2′,3′-cNMPs show the same mass transitions due to their high structural similarity. However, these analytes were clearly identified by their different retention times.

Ap_4A, ppGpp, and pppGpp were analyzed as described previously (Schäfer et al., 2020). Chromatographic separation was performed on a Hypercarb column (30 × 4.6 mm, 5 µm particle size; Thermo Fisher, Scientific MA, United States) using 10 mM ammonium acetate (pH 10) as eluent A and acetonitril as eluent B, using an 8 min gradient from 4 to 60% B. The flow rate was 600 µl/min. All analytes were detected by LC-MS/MS on a QTRAP 5500 (Sciex MA, United States). Ionization of analytes was achieved with an ESI in positive ion mode and SRM was used for analyte detection.

For all used LC-MS/MS methods, the control of the LC and the mass spectrometers as well as data sampling was performed using Analyst software (version 1.7 Sciex, MA, United States). For quantification, calibration curves were created by plotting peak area ratios of the analyte, and the internal standard vs. the nominal concentration of the 10 calibrators. The calibration curve was calculated using quadratic regression and 1/× weighing.

The measured concentration of each nucleotide was normalized for each cell extract sample to the total protein concentration of the respective sample.

RESULTS AND DISCUSSION

Haloferax volcanii and *Sulfolobus acidocaldarius* Cells Grown Under Standard Conditions Contain Several Mono-Nucleotide-Based (Putative) Second Messengers

We set out to identify nucleotide-based (putative) signaling molecules in the euryarchaeal and crenarchaeal model organisms *H. volcanii* and *S. acidocaldarius*. In a first step, we screened for mono-nucleotide-based (putative) second messengers. The presence of 3′,5′-cAMP in *H. volcanii* cells has been described 35 years ago (Leichtling et al., 1986). Except for 3′,5′-cAMP,

the presence of other mono-nucleotide-based (putative) second messengers, like other 3′,5′-cyclic nucleotides (3′,5′-cNMPs), 2′,3′-cNMPs or (p)ppGpp, has not been detected in any archaeal species so far. No reports are available on the presence of 3′,5′-cAMP in *S. acidocaldarius,* but the closely related species *Sa. solfataricus* has been shown to produce 3′,5′-cAMP (Leichtling et al., 1986), implying that this nucleotide is most likely also present in *S. acidocaldarius*.

H. volcanii strain H26 and *S. acidocaldarius* strain MW001 were grown under standard laboratory conditions. These cultures were used to obtain cell material from the exponential and stationary growth phases for nucleotide extraction (**Supplementary Figure 1**). Extraction of nucleotides from the cell extracts followed by LC-MS/MS not only confirmed the presence of 3′,5′-cAMP in these two species (**Figures 1A,B**) but also revealed that 3′,5′-cGMP, 3′,5′-cCMP and 3′,5′-cUMP are present in *H. volcanii* as well (**Figure 1A**). These four 3′,5′-cNMPs showed increased levels in exponentially growing *H. volcanii* cells compared to stationary cells (**Figure 1A**). Similar observations were made for 3′,5′-cAMP in exponentially growing *S. acidocaldarius* cells, which contained higher amounts of this cyclic nucleotide compared to stationary growth (**Figure 1B**). 3′,5′-cCMP and 3′,5′-cUMP were not detected in any *S. acidocaldarius* sample (**Figure 1B**), suggesting that these nucleotides, at least under the tested growth conditions, are not synthesized by this crenarchaeon. 3′,5′-cGMP was the only other 3′,5′-cNMP detected in *S. acidocaldarius* (**Figure 1B**). However, the levels of 3′,5′-cGMP were low in both tested conditions. Five of nine replicates from the stationary culture (originating from three biological replicates with three technical replicates each) contained sufficient amounts of 3′,5′-cGMP for a quantitative analysis. In all other samples from *S. acidocaldarius* (four replicates from stationary cells and all nine replicates from exponentially growing cells), 3′,5′-cGMP could be detected as well, but at levels which did not allow for a valid quantification. Therefore no reliable 3′,5′-cGMP level could be calculated for this crenarchaeon.

With (some) 3′,5′-cNMPs being present in *H. volcanii* and *S. acidocaldarius* the question arises by which enzymes they are produced. Synthesis of 3′,5′-cAMP *in vivo* is achieved by adenylate cyclases (ACs) of which six different classes (I–VI) are currently known (Khannpnavar et al., 2020). Very few predicted archaeal ACs fall into class III (Bassler et al., 2018) [cluster of orthologous groups (COG) 2114], while the vast majority belongs to class IV (COG1437), which is characterized by a CYTH (CyaB, thiamine triphosphatase) domain. *H. volcanii* as well as *S. acidocaldarius* each contain a single gene encoding for a putative class IV AC (*HVO*_1648 and *Saci*_0718). The *S. acidocaldarius* gene product of *Saci*_0718 has recently, however, been demonstrated not to function as a cyclase but as a phosphohydrolase of the triphosphate tunnel metalloenzyme (TTM) family (Vogt et al., 2021). An in this context performed systematic sequence similarity network analysis of the CYTH superfamily unveiled that actual class IV ACs only account for a small subgroup, which is entirely of bacterial origin (Vogt et al., 2021). This is in accordance with the observation that a *H. volcanii* deletion mutant lacking *HVO*_1648 has unchanged 3′,5′-cAMP levels (preliminary data). Together, these observations imply that most archaea are likely to synthesize 3′,5′-cAMP with a yet unknown new class of ACs, which is structurally different from the currently established ones.

Enzymes synthesizing 3′,5′-cGMP, called guanylate cyclases, have been characterized from bacteria (Marden et al., 2011; An et al., 2013) and eukaryotes (Kang et al., 2019). Protein BLAST searches using the sequences of the guanylate cyclase domains of such enzymes (Marden et al., 2011; An et al., 2013; Kang et al., 2019) against the translated protein databases of species of the genera *Haloferax* and *Sulfolobus* yielded no results with significant similarity. This finding is supported by the fact that the COG2114 (adenylate and guanylate cyclase catalytic domain) neither contains a homolog for *H. volcanii* nor for *S. acidocaldarius*. All these observations might imply that 3′,5′-cGMP in *H. volcanii* and S. *acidocaldarius* is not generated by a classic guanylate cyclase.

3′,5′-cCMP and 3′,5′-cUMP were just recently identified to play an important role as signaling molecules in prokaryotic phage-defense systems (Tal et al., 2021). A recently performed phylogenetic analysis of proteins containing pyrimidine cyclase domains revealed that these types of enzymes are also found in few euryarchaeal species (Tal et al., 2021). However, BLAST searches of these identified putative euryarchaeal pyrimidine cyclases in the proteome of *H. volcanii* yielded no hit. The same applies when experimentally characterized bacterial pyrimidine cyclases (Tal et al., 2021) were used as template. This observation might suggest the existence of additional and more distantly to the recently discovered pyrimidine cyclases related types of specific cytidylate/uridylate cyclases. Nevertheless, since guanylate and adenylate cyclases were identified or are assumed to be capable of not only producing their respective intrinsic products but also 3′,5′-cCMP and 3′,5′-cUMP under certain conditions (Beste et al., 2012; Bähre et al., 2014; Seifert, 2017), it is possible that the detected 3′,5′-cCMP and 3′,5′-cUMP originate from a divergent enzymatic activity of these two types of cyclases.

In addition to 3′,5′-cAMP, 3′,5′-cGMP, 3′,5′-cCMP, and 3′,5′-cUMP, the cell extracts from both species were also checked for the presence of 3′,5′-cTMP, 3′,5′-cIMP, and 3′,5′-cXMP, however, none of these cyclic nucleotides could be detected (**Table 2**). Only very few studies show the presence 3′,5′-cIMP in biological systems (Newton et al., 1998; Chen et al., 2014), with some of these reports called into question when it comes to the specificity of the detection method used (Seifert, 2015). Therefore, it is difficult to speculate whether the absence of 3′,5′-cIMP observed in *H. volcanii* and *S. acidocaldarius* indicates a general absence of this nucleotide or an absence under the standard conditions used in this study. The absence of 3′,5′-cTMP and 3′,5′-cXMP in cell extracts from both model organisms is in line with the fact that these cyclic nucleotides have not been unequivocally identified in any living cell yet.

Additionally to 3′,5′-cNMPs, *H. volcanii* and *S. acidocaldarius* cell extracts were also analyzed for the presence of 2′,3′-cNMPs. All four examined 2′,3′-cNMPs, namely 2′,3′-cAMP, 2′,3′-cGMP, 2′,3′-cCMP and 2′,3′-cUMP, were present in both species in samples from at least one of the two tested growth stages (**Figures 2A,B**). For *H. volcanii* cell extracts,

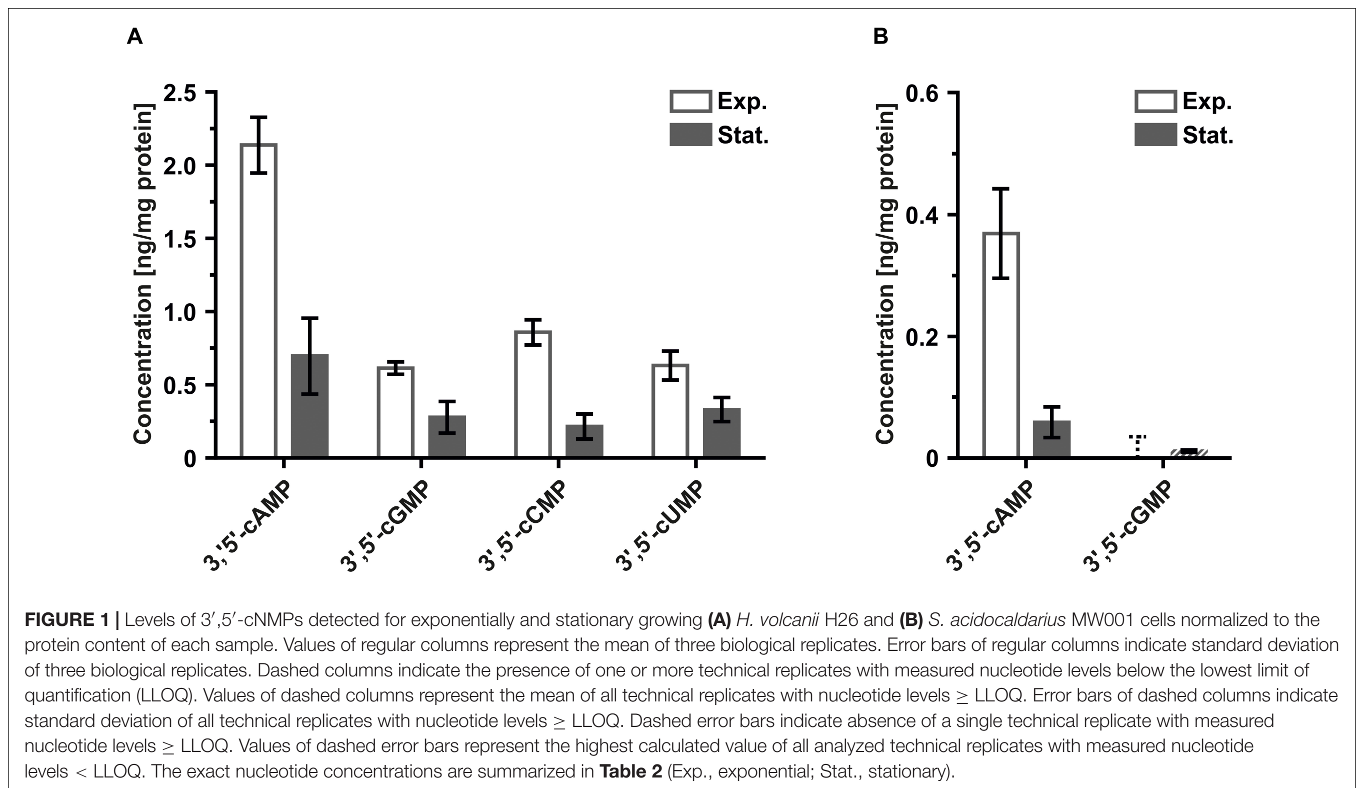

FIGURE 1 | Levels of 3′,5′-cNMPs detected for exponentially and stationary growing **(A)** *H. volcanii* H26 and **(B)** *S. acidocaldarius* MW001 cells normalized to the protein content of each sample. Values of regular columns represent the mean of three biological replicates. Error bars of regular columns indicate standard deviation of three biological replicates. Dashed columns indicate the presence of one or more technical replicates with measured nucleotide levels below the lowest limit of quantification (LLOQ). Values of dashed columns represent the mean of all technical replicates with nucleotide levels ≥ LLOQ. Error bars of dashed columns indicate standard deviation of all technical replicates with nucleotide levels ≥ LLOQ. Dashed error bars indicate absence of a single technical replicate with measured nucleotide levels ≥ LLOQ. Values of dashed error bars represent the highest calculated value of all analyzed technical replicates with measured nucleotide levels < LLOQ. The exact nucleotide concentrations are summarized in **Table 2** (Exp., exponential; Stat., stationary).

TABLE 2 | Summary of measured and detected mono-nucleotide-based (putative) second messengers.

Organism	*H. volcanii*		*S. acidocaldarius*	
Molecule	**Levels during exponential growth [ng/mg protein]**	**Levels during stationary growth [ng/mg protein]**	**Levels during exponential growth [ng/mg protein]**	**Levels during stationary growth [ng/mg protein]**
3′,5′-cAMP	2.14 ± 0.19	0.70 ± 0.26	0.37 ± 0.07	0.06 ± 0.03
3′,5′-cGMP	0.61 ± 0.04	0.28 ± 0.11	≥ 0	≤ 0.01 ± 0.002
3′,5′-cCMP	0.86 ± 0.09	0.22 ± 0.08	n.d.	n.d.
3′,5′-cUMP	0.63 ± 0.10	0.33 ± 0.08	n.d.	n.d.
3′,5′-cTMP	n.d.	n.d.	n.d.	n.d.
3′,5′-cIMP	n.d.	n.d.	n.d.	n.d.
3′,5′-cXMP	n.d.	n.d.	n.d.	n.d.
2′,3′-cAMP	50.03 ± 3.70	32.23 ± 5.86	1.65 ± 0.16	1.13 ± 0.31
2′,3′-cGMP	36.33 ± 2.66	21.91 ± 4.80	0.92 ± 0.10	0.85 ± 0.25
2′,3′-cCMP	25.21 ± 1.99	18.72 ± 3.02	0.36 ± 0.18	0.53 ± 0.15
2′,3′-cUMP	0.84 ± 0.19	0.66 ± 0.17	n.d.	≤ 0.04 ± 0.006
ppGpp	n.d.	n.d.	n.d.	n.d.
pppGpp	n.d.	n.d.	n.d.	n.d.

(± , gives standard deviation; n.d., not detectable; ≤, average of all technical replicates ≥ LLOQ; ≥ 0, nucleotide detected but all technical replicates < LLOQ).

the measured concentrations of 2′,3′-cAMP, 2′,3′-cGMP, and 2′,3′-cCMP (**Figure 2A**) were much higher than the ones of the corresponding 3′,5′-isomer (**Figure 1A**). Only 2′,3′-cUMP was present at concentrations similar to 3′,5′-cUMP. Similar to the 3′,5′-cNMPs, the concentrations of 2′,3′-cNMPs in *H. volcanii* generally increased during exponential growth (**Figure 2A**). In *S. acidocaldarius* extracts, 2′,3′-cNMP levels were in the same range for exponential and stationary growth (**Figure 2B**). Similarly, to what was observed for the 2′,3′-cUMP levels of *H. volcanii*, levels of this 2′,3′-cyclic nucleotide in *S. acidocaldarius* were also considerably lower in comparison to the other three 2′,3′-cNMPs. Of all stationary samples, only three technical replicates (out of nine in total) contained quantifiable amounts of 2′,3′-cUMP, whereas samples of exponentially growing *S. acidocaldarius* cells did not contain any 2′,3′-cUMP. Production of the detected 2′,3′-cNMPs in both species has most likely to be linked to the process of RNA-degradation, a major source of 2′,3′-cNMPs in eukaryotes and bacteria

(Thompson et al., 1994; Fontaine et al., 2018), and/or to the activity of homologs of certain RNA cyclases/ligases, which are also known to form 2′,3′-cyclic phosphates at the 3′-ends of RNAs (Shigematsu et al., 2018). As currently no distinct function as second messenger is ascribed to any 2′,3′-cNMP it appears likely that they are not used as such in *H. volcanii* and *S. acidocaldarius* as well. Still, it also cannot be excluded that they may act in some yet to be discovered signaling network.

Next to 3′,5′-cNMPs and 2′,3′-cNMPs, the cell extracts were also analyzed for the presence of the alarmone ppGpp and its precursor pppGpp. Analysis of extracts from *S. acidocaldarius* did not detect any (p)ppGpp at the tested conditions (**Table 2**). Since cell extracts of *H. volcanii* contained substances that interfered with the (p)ppGpp internal standard signal, an alternative solid phase extraction protocol was used (see section "Materials and Methods"). For these extracts the internal (p)ppGpp standard signal was unaffected and the corresponding measurements revealed that neither ppGpp nor pppGpp was present in the *H. volcanii* samples (**Table 2**). These observations are in accordance with former studies examining the occurrence of (p)ppGpp in both species which showed that this alarmone was not produced, even when cells were subjected to stress factors such as starvation (Scoarughi et al., 1995; Cellini et al., 2004). In line with this, a study on the distribution of (p)ppGpp synthetases and hydrolases across the tree of life suggests that *H. volcanii* and *S. acidocaldarius* do not contain any of these enzymes (COG0317 contains no hit for both organisms) and that they are in general only very rarely found in archaea (Atkinson et al., 2011).

Cyclic Diadenosine Monophosphate, 5′-Phosphoadenylyl-3′,5′-Adenosine, and Diadenosine Tetraphosphate Are the Only Di-Nucleotide-Based (Putative) Second Messengers Measured in at Least One of the Two Archaeal Model Organisms

In a second step, we screened for di-nucleotide-based (putative) second messengers. The only di-nucleotide-based second messenger detected in any archaeon so far is 3′,5′-c-di-AMP. It was recently shown to be produced in the euryarchaeon *H. volcanii* by the corresponding di-adenylate cyclase DacZ and osmoregulation had been implicated as a major function of this nucleotide (Braun et al., 2019). Extraction of nucleotides from the cell extracts followed by LC-MS/MS confirmed the presence of 3′,5′-c-di-AMP during both the exponential and the stationary phase at concentrations similar to what was detected previously (Braun et al., 2019; **Figure 3A**). In contrast to *H. volcanii*, *S. acidocaldarius* cell extracts generated in this study did not contain any 3′,5′-c-di-AMP. A protein BLAST search using the sequences of an established bacterial (Rosenberg et al., 2015) and an archaeal (Braun et al., 2019) di-adenylate cyclases against the proteome of *S. acidocaldarius* yielded no significant hits. These observations are consistent with previous bioinformatical analyses showing that proteins containing the 3′,5′-c-di-AMP synthesizing diadenylate cyclase (DAC)-domain (COG1624) are absent in crenarchaeota, while being frequently found in euryarchaeota (Römling, 2008; Witte et al., 2008; He et al., 2020). This suggests that 3′,5′-c-di-AMP is likely to be used as second messenger by many euryarchaea, whereas crenarchaea do not seem to utilize this signaling nucleotide.

With 3′,5′-c-di-AMP being present in *H. volcanii*, it was not surprising that its (intermediate) degradation product 5′-pApA, was also found in this euryarchaeon (**Figure 3A**). The concentration of 5′-pApA was, however, significantly lower than the concentration of 3′,5′-c-di-AMP. Especially samples from exponentially growing cultures contained only low amounts of 5′-pApA. Although all nine replicates of exponential cultures included 5′-pApA, only two of them contained sufficient amounts for a quantitative analysis. These very low levels of 5′-pApA suggests that most of this linear di-nucleotide in *H. volcanii* is further degraded to 5′-AMP, the final degradation product of 3′,5′-c-di-AMP (Commichau et al., 2019). The phosphodiesterases which are degrading 3′,5′-c-di-AMP and/or 5′-pApA in *H. volcanii* are currently unknown, as well as a potential function of 5′-pApA as second messenger. Samples from exponentially growing *S. acidocaldarius* cells did not exhibit any 5′-pApA. Yet, most samples (eight of nine replicates) from stationary *S. acidocaldarius* cultures included minor amounts of 5′-pApA (**Figure 3B**). However, as these amounts were in all samples below the quantification limit no valid average 5′-pApA level could be calculated. As *S. acidocaldarius* does not contain any 3′,5′-c-di-AMP these minor amounts of 5′-pApA do certainly not originate from the degradation of 3′,5′-c-di-AMP. As the genome of *S. acidocaldarius* has an A + T-content of 63% (Chen et al., 2005) it appears possible that the detected molecules of 5′-pApA are intermediate fragments from degraded genomic DNA.

Diadenosine tetraphosphate (Ap_4A), which was shown in several bacteria to function as an stress induced second messenger (Lee et al., 1983; Pálfi et al., 1991; Kimura et al., 2017; Ferguson et al., 2020), could be detected in cell extracts from both archaeal model organisms (**Figures 3A,B**). For both species, levels of Ap_4A were higher during exponential growth. However, samples from exponentially growing *H. volcanii* cells exhibited a quite broad fluctuation among individual technical replicates and among the biological replicates, with some samples even exhibiting a complete absence of Ap_4A (**Figure 3A**). Intriguingly, Ap_4A levels in exponentially growing *S. acidocaldarius* cells were the highest of all putative nucleotide-based second messengers detected in this study (**Figure 3B**). Noteworthy, exponential samples from *S. acidocaldarius* did not show a similar broad fluctuation for their Ap_4A levels as observed for exponential samples from *H. volcanii*. The 16-fold difference between Ap_4A levels in *S. acidocaldarius* cells during exponential growth and the stationary phase is the largest difference that was observed between the two phases within this study. Whether Ap_4A is actually used in a second messenger context and what possible biological functions this di-nucleotide could have in *H. volcanii* and *S. acidocaldarius* is currently not known. The observed differences between exponential and stationary growth phases as well as the high amounts of Ap_4A specifically produced by

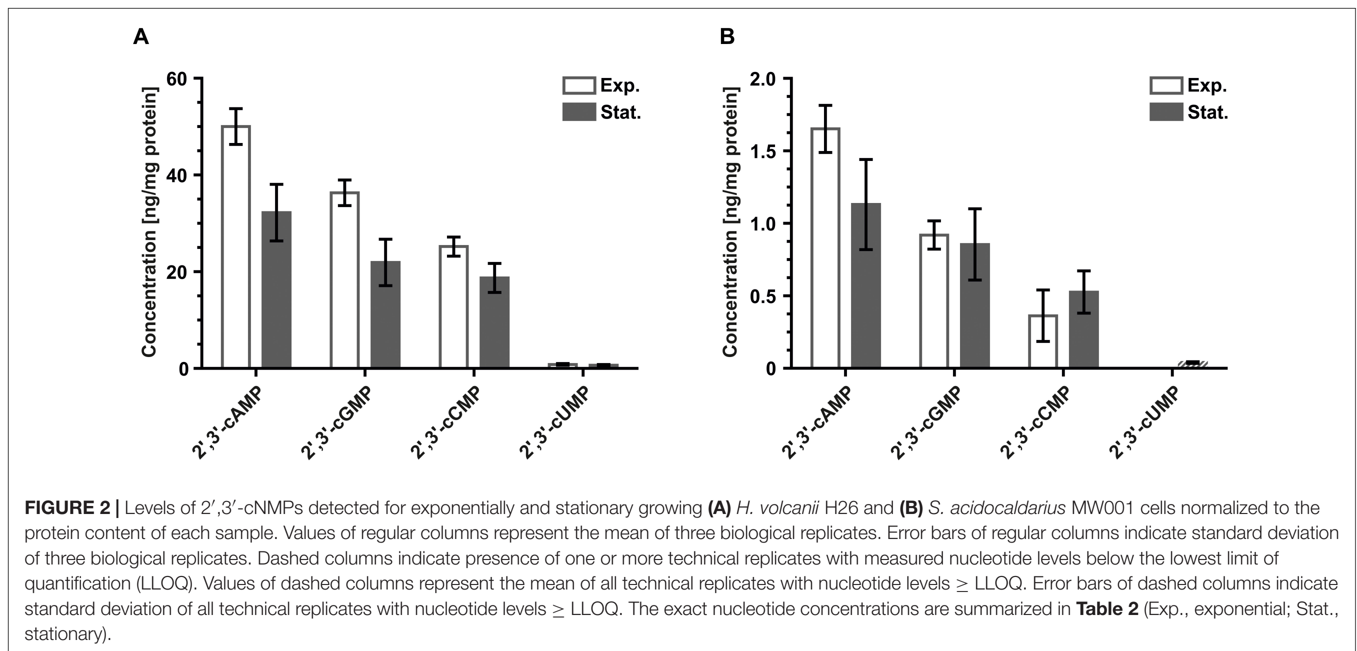

FIGURE 2 | Levels of 2′,3′-cNMPs detected for exponentially and stationary growing **(A)** *H. volcanii* H26 and **(B)** *S. acidocaldarius* MW001 cells normalized to the protein content of each sample. Values of regular columns represent the mean of three biological replicates. Error bars of regular columns indicate standard deviation of three biological replicates. Dashed columns indicate presence of one or more technical replicates with measured nucleotide levels below the lowest limit of quantification (LLOQ). Values of dashed columns represent the mean of all technical replicates with nucleotide levels ≥ LLOQ. Error bars of dashed columns indicate standard deviation of all technical replicates with nucleotide levels ≥ LLOQ. The exact nucleotide concentrations are summarized in **Table 2** (Exp., exponential; Stat., stationary).

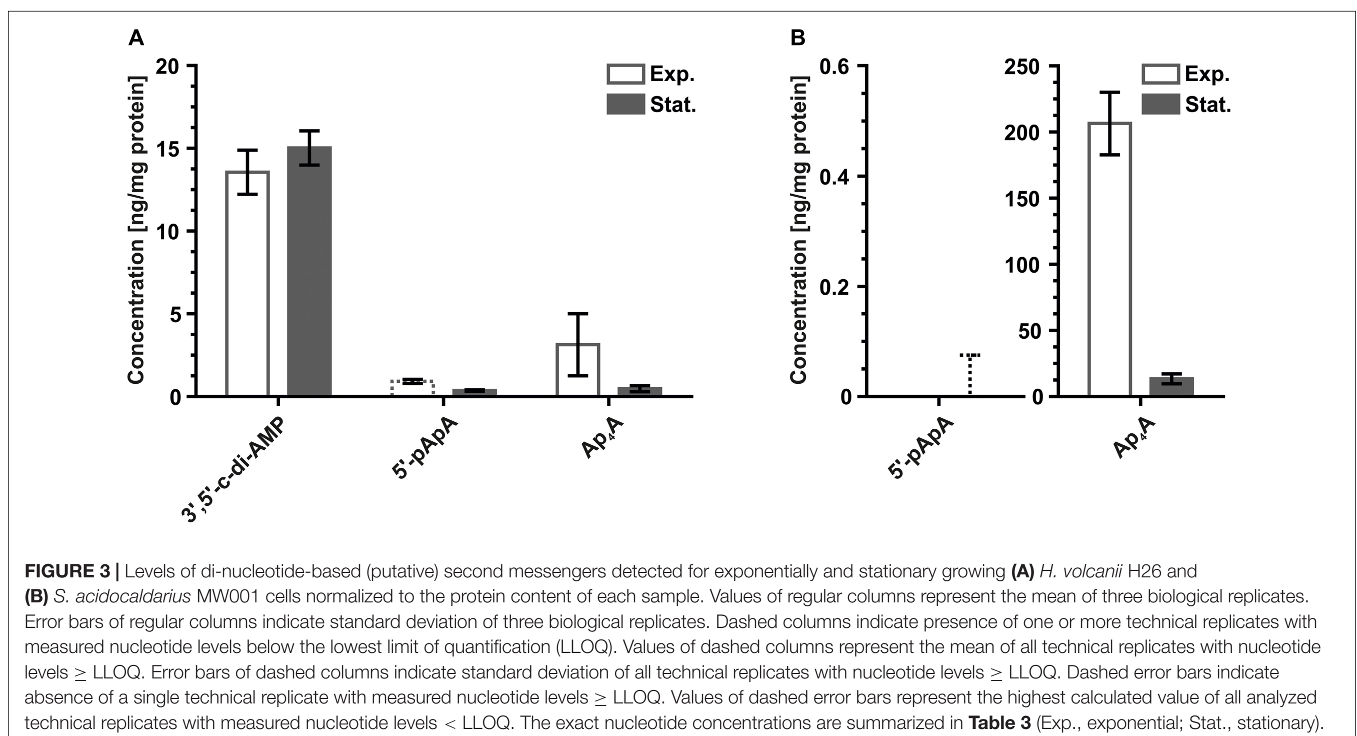

FIGURE 3 | Levels of di-nucleotide-based (putative) second messengers detected for exponentially and stationary growing **(A)** *H. volcanii* H26 and **(B)** *S. acidocaldarius* MW001 cells normalized to the protein content of each sample. Values of regular columns represent the mean of three biological replicates. Error bars of regular columns indicate standard deviation of three biological replicates. Dashed columns indicate presence of one or more technical replicates with measured nucleotide levels below the lowest limit of quantification (LLOQ). Values of dashed columns represent the mean of all technical replicates with nucleotide levels ≥ LLOQ. Error bars of dashed columns indicate standard deviation of all technical replicates with nucleotide levels ≥ LLOQ. Dashed error bars indicate absence of a single technical replicate with measured nucleotide levels ≥ LLOQ. Values of dashed error bars represent the highest calculated value of all analyzed technical replicates with measured nucleotide levels < LLOQ. The exact nucleotide concentrations are summarized in **Table 3** (Exp., exponential; Stat., stationary).

exponentially growing *S. acidocaldarius* cells imply a general physiological relevance of this di-nucleotide. Noteworthy, bioinformatical identification of any Ap_4A synthesizing enzyme in *S. acidocaldarius* and *H. volcanii* is particularly complicated as a broad variety of aminoacyl-tRNA synthetases and also other enzymes like, for example, DNA and RNA ligases, are known to be capable of forming this di-nucleotide (Fraga and Fontes, 2011; Ferguson et al., 2020).

Interestingly, the very well established bacterial second messenger 3′,5′-c-di-GMP and its (intermediate) degradation product 5′-pGpG were not detected in the cell extracts of *H. volcanii* and *S. acidocaldarius*. This suggests that this cyclic di-nucleotide is, unlike to various bacteria, not a key regulatory molecule in these two species. Indeed, a protein BLAST search using the GGDEF-domain, the domain responsible for 3′,5′-c-di-GMP formation (Paul et al., 2004), of a di-guanylate cyclase

TABLE 3 | Summary of measured and detected di-nucleotide-based (putative) second messengers.

Organism	*H. volcanii*		*S. acidocaldarius*	
Molecule	**Levels during exponential growth [ng/mg protein]**	**Levels during stationary growth [ng/mg protein]**	**Levels during exponential growth [ng/mg protein]**	**Levels during stationary growth [ng/mg protein]**
3′,5′-c-di-GMP	n.d.	n.d.	n.d.	n.d.
3′,5′-c-di-AMP	13.56 ± 1.34	15.02 ± 1.03	n.d.	n.d.
5′-pGpG	n.d.	n.d.	n.d.	n.d.
5′-pApA	≤ 0.92 ± 0.13	0.37 ± 0.04	n.d.	≥ 0
2′,3′-cGAMP	n.d.	n.d.	n.d.	n.d.
3′,3′-cGAMP	n.d.	n.d.	n.d.	n.d.
3′,2′-cGAMP	n.d.	n.d.	n.d.	n.d.
Ap_4A	3.14 ± 1.88	0.48 ± 0.18	206.51 ± 26.61	13.40 ± 3.77

(± , gives standard deviation; n.d., not detectable; ≤, average of all technical replicates ≥ LLOQ; ≥ 0, nucleotide detected but all technical replicates < LLOQ).

from *E. coli* (Ryjenkov et al., 2005) against the genera *Haloferax* and *Sulfolobus* yielded no hits for the genus *Sulfolobus* and only three hits for the genus *Haloferax*, of which only one contained the complete GGDEF-motive (hypothetical protein; overall query cover: 40%, percent identity: 38.89%). This is in agreement with previous bioinformatical analyses which showed that not only proteins with a GGDEF-domain are almost completely absent in archaea, but also proteins with all other domains associated with 3′,5′-c-di-GMP signaling (e.g., EAL- or PilZ-domain) (Römling et al., 2013). Nevertheless, an analogous BLAST search against the entire domain of archaea yielded more than 300 hits of putative GGDEF-domain containing enzymes with many of them being predicted to belong to species of the recently discovered Asgard- and DPANN-superphyla. This suggests that 3′,5′-c-di-GMP might not entirely be absent in the archaeal domain of life.

In addition to 3′,5′-c-di-GMP and 5′-pGpG, cell extracts were also analyzed for the presence of the eukaryotic di-nucleotide-based second messengers 2′,3′-cGAMP and 3′,2′-cGAMP and their bacterial analog 3′,3′-cGAMP. None of these three isomers could be detected. The absence of 2′,3′-cGAMP in *H. volcanii* and *S. acidocaldarius* fits with the current idea of 2′,3′-cGAMP only being present in metazoa (Kranzusch et al., 2015). The recently discovered isomer 3′,2′-cGAMP was also not detected in any sample. As this isomer also contains an atypical 2-′5′ phosphodiester linkage it appears very likely that it is also only produced by metazoa. The fact that 3′,3′-cGAMP is absent in both species might suggest that both do not use any of the prokaryotic anti-phage defense mechanisms, which have been previously linked to bacterial 3′,3-′cGAMP production (Severin et al., 2018; Cohen et al., 2019). This idea is also supported by a complete lack of proteins in the genera of *Haloferax* and *Sulfolobus* sharing significant similarities with so far characterized bacterial cyclic GMP-AMP synthases (cGASs) (BLAST search using two characterized bacterial cGASs; Davies et al., 2012; Li et al., 2019). A look at the COGs for both, bacterial (ENOG5028K9C) and metazoan (KOG3963), cGASs unveils that each of them is specific for its respective domain and that they do not root in a common COG which would also include any archaeon.

Cyclic Tetra-AMP Could Not Be Detected in *Haloferax volcanii* and *Sulfolobus acidocaldarius* Cell Extracts

Only a few cyclic oligo-nucleotide-based second messengers have been identified. An example of this is c-tetra-AMP ($n = 4$), which is known to occur, alongside with other isomers of cyclic oligo adenylate ($n = 3$–6) (cOA), in some crenarchaeal species, such as *Sa. solfataricus*, a species closely related to *S. acidocaldarius* (Rouillon et al., 2018). There, cOA was shown to be involved in type III CRISPR system mediated immunity (Kazlauskiene et al., 2017; Niewoehner et al., 2017; Rouillon et al., 2018). No c-tetra-AMP could be detected in any of the samples prepared for this study. As *S. acidocaldarius* encodes a functional type III CRISPR system that contains a Cas10 subunit (Zink et al., 2021) detection of c-tetra-AMP in extracts from this species could have been expected. However, as the cells in this study were not challenged with any invading virus it is plausible that the observed lack of c-tetra-AMP originates from the type III CRISPR system of *S. acidocaldarius* being inactive at the tested conditions. *H. volcanii* and other euryarchaea of the order Halobacteriales contain type I, but lack type III CRISPR systems (Maier et al., 2019), which certainly explains the absence of c-tetra-AMP (and thereby most likely the absence of cOA in general) in *H. volcanii*.

CONCLUDING REMARK

The results of this study represent the first screening of cell extracts from an euryarchaeal and a crenarchaeal species for a multitude of currently known (and established) nucleotide-based second messengers using a modern and highly sensitive mass spectrometry method. It gives a comprehensive overview on a broad spectrum of (potential) small signaling molecules which are present in the archaeal model organisms *H. volcanii* and *S. acidocaldarius* under standard growth and experimental conditions. The function of second messengers includes rapid variations within their levels depending on changing environmental conditions. It thus appears reasonable that the here measured nucleotide levels might considerably change when different cultivation methods/circumstances are used. Even

the appearance of a nucleotide species totally absent here (or vice versa) appears then possible. Determining the nucleotide levels in such differentiating growth experiments could help to elucidate the functions of several of the here reported (putative) second messengers in *H. volcanii* and *S. acidocaldarius*, and thereby also in archaea in more general.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon request.

AUTHOR CONTRIBUTIONS

FB designed the experiments and analyzed data under supervision from S-VA. FB performed the growth of *H. volcanii* and the nucleotide extractions. AR performed the growth of *S. acidocaldarius*. HB and RS supervised LC-MS/MS nucleotide measurements. FB wrote the manuscript with input from HB, AR, RS, and S-VA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.779012/full#supplementary-material

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