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Isolation and Characterization of a Virus-Like-Particle from a Novel Strain of Sulfolobus Acidocaldarius

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Abstract

Archaea have evolved to survive in the harshest niches this planet has to offer and alongside the archaea, viruses have coevolved retaining their ability to infect these extremophilic populations. Over the last 30 years, archaeal viruses were shown to be morphologically and genetically very diverse. With each new isolation of an archaeal virus, scientists gain further insight into this enigmatic world and into the mechanisms of evolution that have allowed the emergence of such diversity. In this study, we have cultivated a novel, Virus-Like-Particle(VLP)-producing strain of *Sulfolobus acidocaldarius* from an nvironmental sample collected in Beppu, Japan, and attempted to characterize the produced VLPs. Transmission electron microscopy studies of the VLPs showed a unique, vesicle-like morphology with a filamentous tail extending from one point of the main body. Although we were unable to isolate nucleic acid from the VLP, we succeeded in initiating VLP production in a previously uninfected strain of *S. acidocaldarius* after exposure to a cell free concentrate of VLPs. Further, a protein extraction from a cell free VLP fraction showed high concentrations of proteins that were not visible in the crude cellular extract. The evidence gathered so far supports the hypothesis that this isolated VLP could be the first S-layer encapsulated archaeal virus. Alternatively, since its infectivity could not unambiguously been proven, it might represent an S-layer coated vesicle released from *S. acidocaldarius* strains.

Zusammenfassung

Archaeen haben sich an die extremsten Habitate, die unser Planet zu bieten hat, nicht nur angepasst, sondern haben Wege gefunden dort zu gedeihen und diese Nischen für sich zu erobern. Hand in Hand mit den Archaea haben sich zahlreiche Viren in diesen Habitaten entwickelt und die Fähigkeit beibehalten, diese extremen Wirte zu infizieren. Über die letzten 30 Jahre wurden Einblicke in die morphologische und genetische Vielfalt dieser Vieren erhalten, und mit jeder Isolierung eines weiteren archaeellen Virus wird unser Verständnis der Entwicklung und Vielfalt dieser faszinierend geheimnisvollen Welt etwas erweitert. Im Verlauf dieser Studie wurde ein neuer *Sulfolobus acidocaldarius* Stamm aus Beppu, Japan, isoliert, der Virus-ähnliche Partikel (VAP) produziert. Dieser wurde in Reinkultur gebracht und die produzierten VAPs wurden elektronenmikroskopisch charakterisiert. Es war nicht möglich, ein VAP-spezifisches Genom zu isolieren, jedoch konnte gezeigt werden, dass ein zuvor uninfizierter Stamm von *S. acidocaldarius* nach der Inkubation mit einem Zell-freien VAP Konzentrat VAPs produzierte. Des Weiteren enthielt das Zell-freie VAP-Konzentrat Proteine, die im Zellextrakt des Wirts nicht sichtbar waren. Zusammenfassend deuten die gesammelten Resultate darauf hin, dass es sich bei diesem Virus-ähnlichen Partikel um den ersten von einem S-Layer umschlossenen, archaeellen Virus handeln könnte.

1 Introduction

1.1 Archaea and their place in the tree of life

Until the 1970s, when DNA was proposed as the primary semantophoretic molecules and used as a marker for the inquiry into evolutionary history and phylogenetic studies [1] , the field of phylogeny was limited to metazoans and metaphyta which only covered 20% of the evolutionary time span [2]. At the time, the newly discovered possibility of sequencing nucleic acids opened the door to the next era of genetics, medicine, and microbiology. This gave scientists a glimpse at the historical document that is a cell's genome, which would forever change our perception of evolution [3]. Over the last two centuries, the ancient dichotomist view of life, in which an organism was either plant or animal, has been restructured and since developed into the phylogenetic system widely accepted today. The dichotomist notion was first challenged by Haeckl in 1866 with the addition of Protista as the third kingdom of life (Figure 1 Left) [4]. It was expanded again to include Bacteria as a fourth kingdom in 1938 and once more in 1959 with the addition of Fungi as the fifth and final kingdom [5]. In 1990, Carl Woese and Otto Kandler proposed a fundamental change in the classification of organisms. They introduced a phylogeny based on 16S rRNA sequences and asserted that the existing, morphology based system was not an option. Rather, the five kingdom system as a whole had to be abolished and replaced with a sequence based three domain system (Figure 1 Right) .This was the birth of modern phylogenetics which established Eukarya, Bacteria, and Archaea as the three domains of life. [2]

The first archaea that were isolated and characterized thrived in extreme environments of high salinity, high temperature, low pH, and/or strict anoxia. This led scientists to the believe that archaea had found a way to outcompete other organisms and thus thrive in these niche habitats that were previously thought to be uninhabitable. However, this ecological perspective was overturned when archaea were discovered in the world's oceans [6]. With the aid of advances in metagenomics, scientists have shown that archaea do not only compete successfully in all ecosystems [7], but are also omnipresent across the globe. According to recent studies, archaea are expected to make up approximately 20% of the biomass in the oceans and are expected to have major roles in global nutrient cycles. Nevertheless, it is their existence in the most unhospitable habitats on the planet that continues to fascinate us.

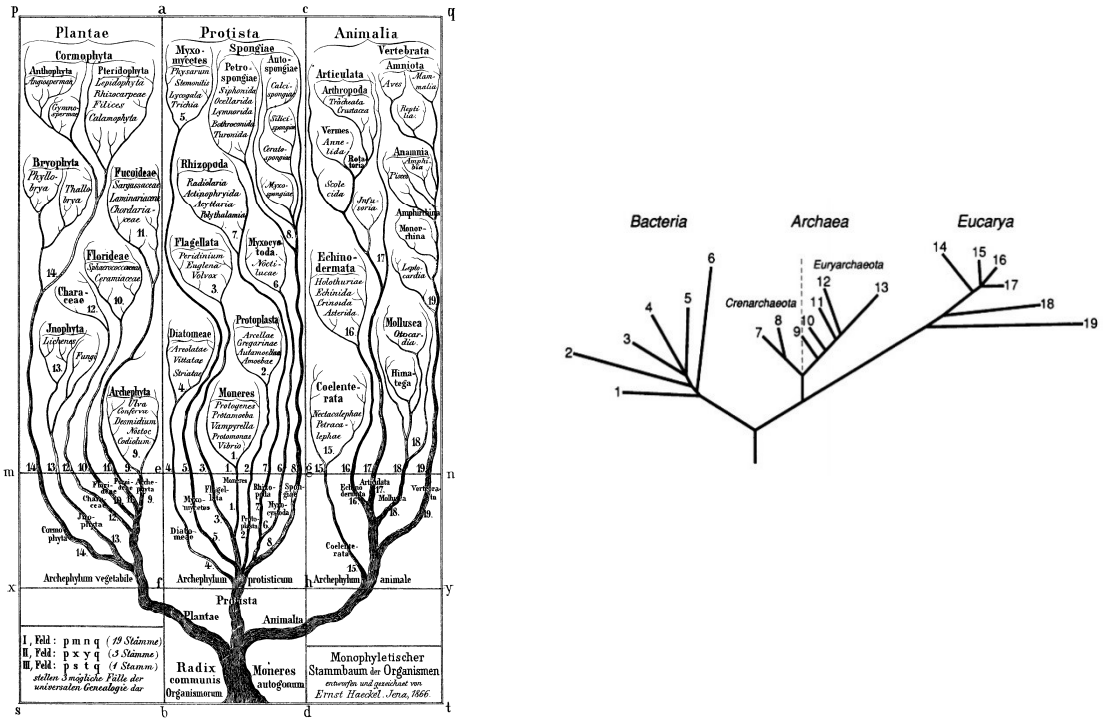


Figure 1 - Left: Haeckel's phylogenetic tree of 1866 [4]; Right Proposed Rooted Tree for the three Domains of Life [2]

Archaea have found a way of not only surviving, but thriving in habitats that are intolerable for eukaryotic, and sometimes even bacterial, life. For example, hyperthermophilic archaea thrive in volcanic environments and deep-sea hydrothermal vents. Hyperthermophiles grow at temperatures above 60°C with the iron reducing archaeon *G. barossii (Strain 121)*maintaining a doubling time of 24 hours in an autoclave at 121°C [8] and the record holder Methanopyrus kandleri doubling every 48hours at 122°C[9]. Many hyperthermophiles are found in highly acidic hot springs producing sulfuric acid as a metabolomic product of their sulfur oxidation pathway. At the other end of the spectrum, psychrophilic archaea proliferate at 0-10°C, are capable of utilizing a functional metabolism down to -20°C, and have been shown to survive prolonged exposures to -45°C [10]. Additionally, halophilic archaea can endure high osmotic stress with extreme halophiles favoring life in hypersaline environments with salt concentrations near the saturation limit. Archaea have evolved a wide range of specific features customized to cope with each of these individual habitats [11]. Archaea living in these environments, are a showcase of life's relentless ability to adapt and survive. Among others, these adaptations include thermostable proteins, novel packaging and modifications of nucleic acids and a high resistance against acidics and saline stress. Not only have Archaea evolved to strive in these environments, but a vast number of viruses have developed strategies of successfully infecting populations of archaea under these conditions. Archaea do not only hold the key to vital questions in the evolution of life but their repertoire of unique adaptations have immense biotechnological potential.

1.2 What are viruses?

Viruses are considered either to be alive or not-alive depending on the source of information and the opinions of the author. It is simply a question of how life, or rather, the concept of "aliveness"[12], [13] is defined. Raoult and Forterre have previously categorized life into two fundamental categories, the capsid encoding and ribosome encoding organisms [14]. This definition suggests that viruses are alive. On the other hand, there are those advocating that due to their inability to replicate autonomously, viruses cannot be considered alive and should be excluded from the tree of life. [15]

If viruses cannot be conclusively defined as dead or alive, then what are they? It is generally agreed that viruses are obligate intracellular parasites that infect all known forms of cellular life. Viruses are essentially genetic material that is encased in an assembly of proteins that require a cellular host to proliferate. In their hosts, the viruses highjack the host's transcription, translation and replication machinery to produce proteins and replicate their own genetic material. Due to the recognition specificity of the host's extracellular proteins, most viruses have a very narrow host range. Viruses normally possess a relatively small genome that encodes for at least one capsid protein, but can also include genes encoding an array of proteins for nucleic acid replication, virus-host interaction, DNA processing, and even non-coding fragments of DNA as remnants from previous hosts. With the exception of the Mimivirus, viruses do not have nucleic acids that code for ribosomes or ATP synthesis and therefore cannot carry out protein synthesis or energy production and depend on their host to provide these services. [16]

Even though the majority of discovered human and animal viruses are mostly known for their pathogenicity , it is becoming increasingly evident that viruses play a vital role on an ecological level [17]. Viruses have been shown to influence compositions of bacterial communities, bacterial abundance, and mediate gene transfer within populations. By influencing bacterial populations in this way, viruses contribute to the control of global nutrient cycles. In marine environments, bacteria have two fundamental roles: producing food for micro-grazers by generating bacterial biomass, and remineralizing nutrients through bacterial respiration. Viral lysis of cells in oligotrophic environments regulates the supply of organic matter[18]. As bacterial abundance increases, the number of produced virions also increases. This leads to an increase in the chance of infection and therefore an increase in the virus-caused bacterial mortality [19]. This pattern can be seen in phytoplankton, bacteria, and viruses during the annual phytoplankton bloom and subsequent collapse [19], [20].

Although much is known about interactions of pathogenic bacteria and viruses, the interactions with our archaeal, bacterial and fungal symbionts, which make up 80% of the cells that can be found in the human body, are only just being unraveled [21], [22]. Our symbionts help us develop and prime our immune system, outcompete pathogens, and digest our food. A dysbalanced

in their communities can cause obesity, type 2 diabetes, inflammatory bowel syndrome and a variety of other problems [23]–[30]. Although we are starting to understand the complexity of microbiota-host interactions, there is little to no information on the effects of virus interactions with microbiota [31] that are bound to be vital for the stability of the ecosystem within and on the human body. The omnipresence of viruses as an intrinsic part of the ecosystem will continue to be elucidated as high-throughput research methods, such as metagenomics and transcriptomics, are improved.

1.3 Archaeal Viruses – A Wondrous World of Diversity

The first archaeal virus was discovered as early as 1974 [32], even before archaea were recognized as a separate domain (Figure 2). At the time of its discovery, the virus infecting *Halobacterium salinarium* was categorized as a bacteriophage. As such, it was mistakenly considered unremarkable because its head and tail morphology was shared by 96% of the viruses in its classification [33]. Its significance was only realized after the dichotic tree of life was replaced by the current triadic system in 1990 [2]. Over the next two decades viruses infecting halophilic archaea continued to be discovered in globally dispersed hypersaline habitats [34]–[39]. Nowadays the study of archaeal head-tail viruses has been vital in understanding the complex function of the viral genome and virus-host interactions [40]–[43]. The discovery in the field of archaeal virology, that changed the perspective on archaeal viruses, was that of the filamentous and spindle shaped viruses (Figure 2) from sulfuric hot spring by Wolfram Zillig and colleagues [37], [44].

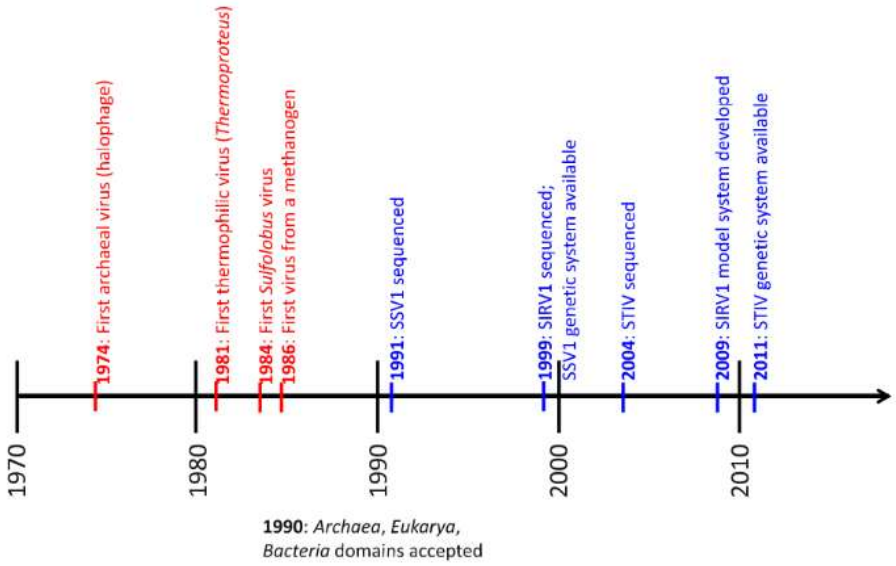


Figure 2 - Timeline of important events in the endeavor into archaeal viruses*[45]*

Not only were these viruses morphologically unique, but also their doubled-stranded DNA (dsDNA) genomes which contained novel genes often without known homologues [16]. The pioneering work of Zillig incited multiple groups worldwide to isolate new archaeal viruses, one of the most influential being the virologist David Prangishvili. The joined efforts of scientists from around the world, have led to the discovery of 117 new viruses to date, many of which showed unique morphologies and demonstrated novel mechanisms underlying virus-host interactions (Figure 3, Table 1).

Table 1 – Viruses infecting Archaea (Compiled from [16], [45], [46], Blue: halophilic, Orange: hyperthermophilic host)

Morphology	Family	Virus	Host	Genome	
	Myoviridae	HF1	*Halorubrum*	Linear	75.9 kb
		HF2	*Halorubrum*	Linear	77.7 kb
		HGTV1	*Halorubrum*	Circular	143.9 kb
		HRTV4	*Halorubrum*	Linear	35.7 kb
		HRTV5	*Halorubrum*	Linear	76.1 kb
		HRTV7	*Halorubrum*	Linear	69.0 kb
		HRTV8	*Halorubrum*	Linear	74.5 kb
		HSTV2	*Haloarcula*	Linear	68.2 kb
		ϕCh1	*Natrialba*	Linear	58.5 kb
		ΦH	*Halobacterium*	Linear	59.0 kb
 Head-tail	Siphoviridae	BJ1	*Halorubrum*	Linear	42.3 kb
		HRPV3	*Halorubrum*	Linear	8.8 kb
		HRPV6	*Halorubrum*	Circular	8.6 kb
		HCTV1	*Haloarcula*	Linear	103.3 kb
		HCTV2	*Haloarcula*	Linear	54.3 kb
		HCTV5	*Haloarcula*	Linear	102.1 kb
		Hh1	*Halobacterium*	ND	32.7 kb
		Hh2	*Halobacterium*	ND	29.4 kb
		HHTV1	*Haloarcula*	Linear	49.1 kb
		HHTV2	*Haloarcula*	Linear	52.6 kb
		HVTV1	*Haloarcula*	Linear	101.7 kb
		ΦN	*Halobacterium*	Linear	56.0 kb
	Podoviridae	HRPV2	*Halorubrum*	Circular	10.7 kb
		HSTV1	*Haloarcula*	Linear	32.2 kb
	Siphoviridae	ΨM2	*Methanobacterium*	Linear	26.11 kb
		ΨM100	*Methanothermobacter*	Linear	28.8 kb
	Fuselloviridae	ASV1	*Acidianus*	Circular	24.2 kb
		PAV1	*Pyrococcus*	Circular	18.0 kb
		SMF1	*Sulfolobus*	Circular	14.8 kb
		SSV1	*Sulfolobus*	Circular	15.5 kb
		SSV2	*Sulfolobus*	Circular	14.8 kb
		SSV4	*Sulfolobus*	Circular	15.1 kb
		SSV5	*Sulfolobus*	Circular	15.3 kb
		SSV6	*Sulfolobus*	Circular	15.6 kb
 Spindle-shaped		SSV7	*Sulfolobus*	Circular	17.6 kb
		SSVK1	*Sulfolobus*	Circular	17.4 kb
		SSVRH	*Sulfolobus*	Circular	16.4 kb
		TPV1	*Thermococcus*	Circular	21.1 kb
	Not Identified	ATSV	*Acidianus*	Circular	71 kb
		APSV1	*Aeropyrum*	Circular	38.1 kb
		STSV1	*Sulfolobus*	Circular	75.3 kb
		STSV2	*Sulfolobus*	Circular	17.6 kb
	Bicaudaviridae	ATV	*Acidianus*	Circular	62.7 kb
	Monocaudavirus	SMV1	*Sulfolobus*	Circular	48.8 kb
	Salterprovirus	HIS1	*Haloarcula*	Linear	14.5 kb

Morphology	Family	Virus	Host	Genome	
Filamentous	Lipothrixviridae	AFV1	*Acidianus*	Linear	21.5 kb
		AFV2	*Acidianus*	Linear	31.8 kb
		AFV3	*Acidianus*	Linear	40.5 kb
		AFV6	*Acidianus*	Linear	39.6 kb
		AFV7	*Acidianus*	Linear	36.9 kb
		AFV8	*Acidianus*	Linear	38.2 kb
		AFV9	*Acidianus*	Linear	41.2 kb
		SIFV	*Sulfolobus*	Linear	40.9 kb
		TTV1	*Thermoproteus*	Linear	15.9 kb
		TTV2	*Thermoproteus*	Linear	15.9 kb
		TTV3	*Thermoproteus*	Linear	15.9 kb
Rod-shaped	Rudiviridae	ARV1	*Acidianus*	Linear	24.7 kb
		SIRV1	*Sulfolobus*	Linear	32.3 kb
		SIRV2	*Sulfolobus*	Linear	35.5 kb
		SRV	*Stygiolobus*	Linear	28.1 kb
		SMR1	*Sulfolobus*	Linear	27.4 kb
Pleomorphic 	Pleolipoviridae	HRPV1	*Halorubrum*	Circular	7.1 kb
		HRPV2	*Halorubrum*	Circular	10.7 kb
		HRPV3	*Halorubrum*	Circular	8.8 kb
		HRPV6	*Halorubrum*	Circular	8.6 kb
		HIS2	*Haloarcula*	Linear	16.1 kb
	Not Identified	HHPV1	*Haloarcula*	Circular	8.1 kb
		HGPV1	*Halorubrum*	Circular	9.7 kb
Tailless	Sphaerolipoviridae	HHIV2	*Haloarcula*	Linear	30.6 kb
		PH1	*Haloarcula*	Linear	28.1 kb
		SH1	*Haloarcula*	Linear	30.09 kb
		SNJ1	*Natrimena*	Circular	16.3 kb
Spherical 	Globulaviridae	PSV	*Pyrobaculum*	Linear	28.3kb
		TTSV1	*Thermoproteus*	Linear	28.3kb
Droplet-shaped 	Guttaviridae	APOV1	*Aeropyrum*	Circular	13.8 kb
		SNDV	*Sulfolobus*	Circular	20.0 kb
Icosahedral 	Turriviridae	STIV1	*Sulfolobus*	Linear	17.7 kb
		STIV2	*Sulfolobus*	Circular	17.6 kb
Unclassified Spherical	Not Identified	HAV1	*Not Identified*	Linear	22.7 kb
		HAV2	*Not Identified*	Linear	17.7 kb
Bottle-shaped 	Ampullaviridae	ABV	*Acidianus*	Linear	23.9 kb
Coil-shaped	Spiraviridae	ACV	*Aeropyrum*	Circular	24.9 kb
Bacilliform	Clavaviridae	APBV1	*Aeropyrum*	Circular	5.2 kb

1.4 The Enigmatic Morphologies and Abilities of Viruses

To date, more than 6,300 viruses have been identified that infect prokaryotes; Of those, only 117 have the ability to infect archaea [33]. The 117 archaeal viruses are classified into 29 species which represent 16 viral families and infect 16 genera of archaea [47]. The 6300+ bacterial viruses, however, cluster into 10, morphologically defined viral families and infect over 200 genera of bacteria [33]. All isolated archaeal viruses infect archaea from the phyla *Euryarchaeota* (77 viruses) and *Crenarchaeota* (40 viruses). All viruses replicating in *Crenarchaeota* show unique morphologies that have not been observed in any other domain. Some of these viral morphotypes include spindle shaped, bottle shaped, droplet shaped with beard-like fibers, spherical, and ones that grow two tails once they have left the host (Figure 6). In contrast, almost the entirety of viruses infecting *Euryarchaeota* have a classical bacterial head and tail morphology. The absence of the head-tail morphology in viruses replicating in *Crenarchaeota* might be due to the fact that all *Crenarchaeotal* viruses isolated to date, were isolated from hyperthermal acidic environments. In these harsh environments, such morphologies might not be stable. Alternatively, the lytic life-cycle typical of a head-tail phage would not be the best strategy of survival in hot springs because the nucleic acids in the viral particles can not be stabilized over longer time periods. This would coincide with the observation that the viral concentration in hot springs is much lower than that of other analyzed environments ([48], [49]). Instead of lysing the cell, many viruses of extremely thermophilic archaea tend to replicate in the host and then egress the cell without inducing lysis. Many archaeal viruses have been observed to live inside of their hosts in a carrier state and are regularly produced and excreted from the cell [50]. The Spindle Shaped Virus 2 (SSV2), for example, has been shown to bud off its host *Sulfolobus solfataricus P2* in a manner similar to many eukaryotic viruses (Figure 4)[51]. The *Sulfolobus islandicus* Rod-Shaped-Virus 2 (SIRV2) on the other hand was shown to have a unique manner of egressing from its host. The virus codes for a protein that assembles to form a Virus-Associated-Pyramid (VAP)(Figure 5). The VAPs assemble on the cytoplasmic side of the membrane and then pierce through the membrane and S-layer creating pores for the infecting virus to egress through [52]. The other known lytic hyperthermal viruses are the Acidianus Two Tailed Virus ATV, which completes its replication cycle once it has lysed the cell, the *Pyrobaculum filamentous virus 1* PFV1 and the *Thermoproteus tenax viruses* TTV1 and TTV4 [44], [53], [54].

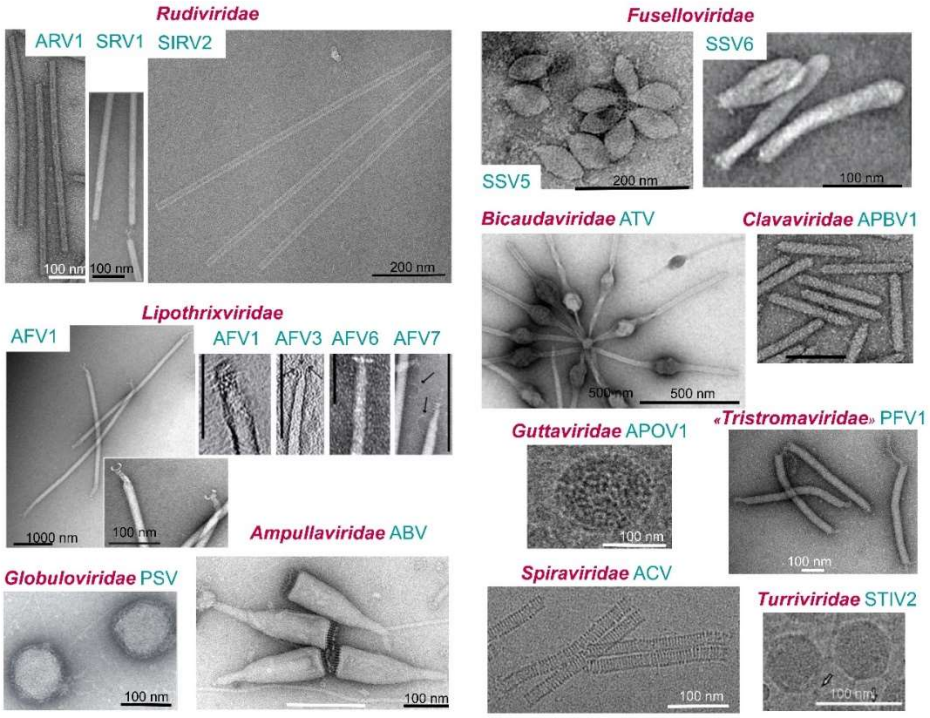


Figure 3 -Transmission electron micrographs (TEMs) showing representatives of the most prominent viral families of hyperthermophilic arachaea [50]

Interestingly, it was later shown that the high morphological differences are accompanied by vastly heterogenous genetic sequences. The most prominent example of this is shown by four viruses isolated from a hot acidic spring near Pozzuoli, Italy. The *acidianus bottle-shaped virus* (ABV), *Acidianus filamentous virus 2* (AFV2), *Acidianus rod-shaped virus* (ARV1) and the *Acidianus two-tailed virus* (ATV) are all able to infect the same host but show no homologies or signs of genetic exchange [55]–[58].

The field of archaeal virology is still young, and most viruses known today come from a very limited number of hosts that are primarily from extreme environments. As discussed above, viruses play a vital ecological role in the recycling of biologic materials and population control. As we know, archaea are present not only in extreme environments, but also inhabit a much broader range of habitats including soils, marine environments and within and on the human body. Therefore, it is of great interest and importance to deepen our understanding of not only archaea, but also their associated viruses, in the effort to understand microbial communities and their effect on the planet.

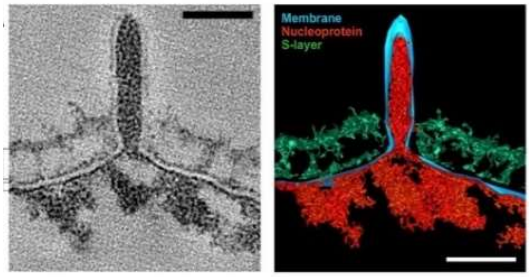


Figure 4 - Thin section images of SSV1 *egress from its host* S. solfataricus [51]

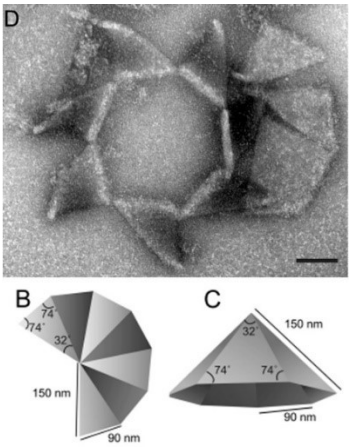


Figure 5 - SIRV2 egress from its host with the aid of Virus-Associated-Pyramids (VAP) [52]

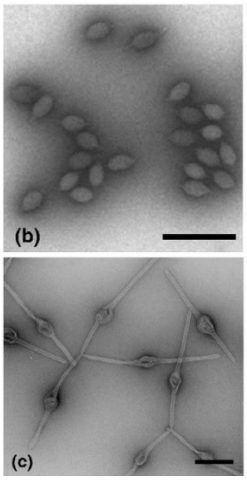


Figure 6 - TEM images showing the morphological change of ATV [54]

1.5 Constitutive and Inducible Defense Systems of Achaea

All organisms, regardless from which domain, are faced with the challenge of handling the threat of viral infections, which can either kill the organism or drastically reduce its fitness. No matter how fit the organism is in other aspects of life, if it cannot defend itself against viral pathogens, it will not be successful. This reality explains the copious viral defense mechanisms found throughout nature. Traditionally, it was believed that only animals possessed both an innate and an adaptive immune system, as it was assumed that the adaptive immune system required a complex multi-cellular organization. This notion changed when the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) -Cas System was proposed as an adaptive immune system of microorganisms in 2005 [59]–[61] and later verified in 2007 [62]. The system stores fragments of foreign DNA in a CRISPR locus in the host genome. Once a CRISPR locus is transcribed into CRISPR RNA (crRNA), the crRNA guides CRISPR associated (Cas) proteins to the corresponding foreign DNA upon reinfection. The foreign genetic material containing a sequence matching the crRNA is subsequently cut and then degraded, protecting the host cell from further infection and potential lysis. After its discovery, the CRISPR system was found to be present in 45% of bacteria, and almost 85% of archaea [63]. These numbers show that CRISPR is important, but not essential, for microbial life. Strains lacking the CRISPR system may perhaps have

developed alternate defense machineries. Only a miniscule amount of the identified CRISPR sequences have been successfully matched to isolated viruses. If the amount of known CRISPR spacers is taken as a measure of viral diversity in their corresponding environments, the discovery of many more unique viruses can be expected.

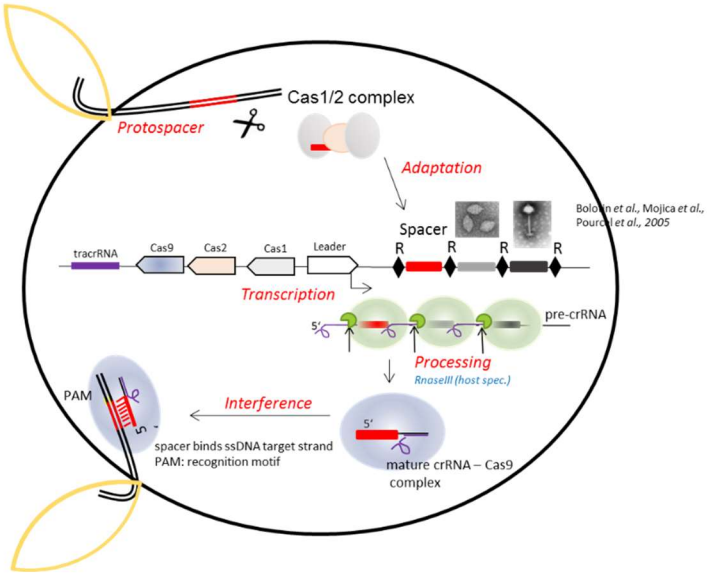


Figure 7 - Schematic depiction of the CRISPR-Cas system found in Sulfolobus solfataricus. *©Isabelle Zink*

1.6 Vesicles – A highly conserved aspect of microbial life

The production of membrane vesicles is a trait that is conserved across all three domains [64]–[67]. The ubiquitous and highly conserved nature of vesicles indicates that they are vital for microbial life. Therefore, elucidating their specific functions is necessary in order to fully understand microbial physiology. Some of the functions attributed to the release of membrane vesicles include quorum-sensing [68]; the release of virulence factors, antigens and toxins [69]; creating decoys for immune systems and viruses; and the horizontal transfer of genetic material [70]. (Figure 8) One of the particularly interesting vesicle associated toxins excreted by archaea are the Sulfolobicines, a compound produced by several species of *Sulfolobus* [69]. Sulfolobicines consist of two proteins associated with membrane vesicles, SulA and SulB, that are produced by *S. islandicus*, *S. tokodaii* and *S. acidocaldarius* and inhibit the growth of other *Sulfolobus* species [69], [71].

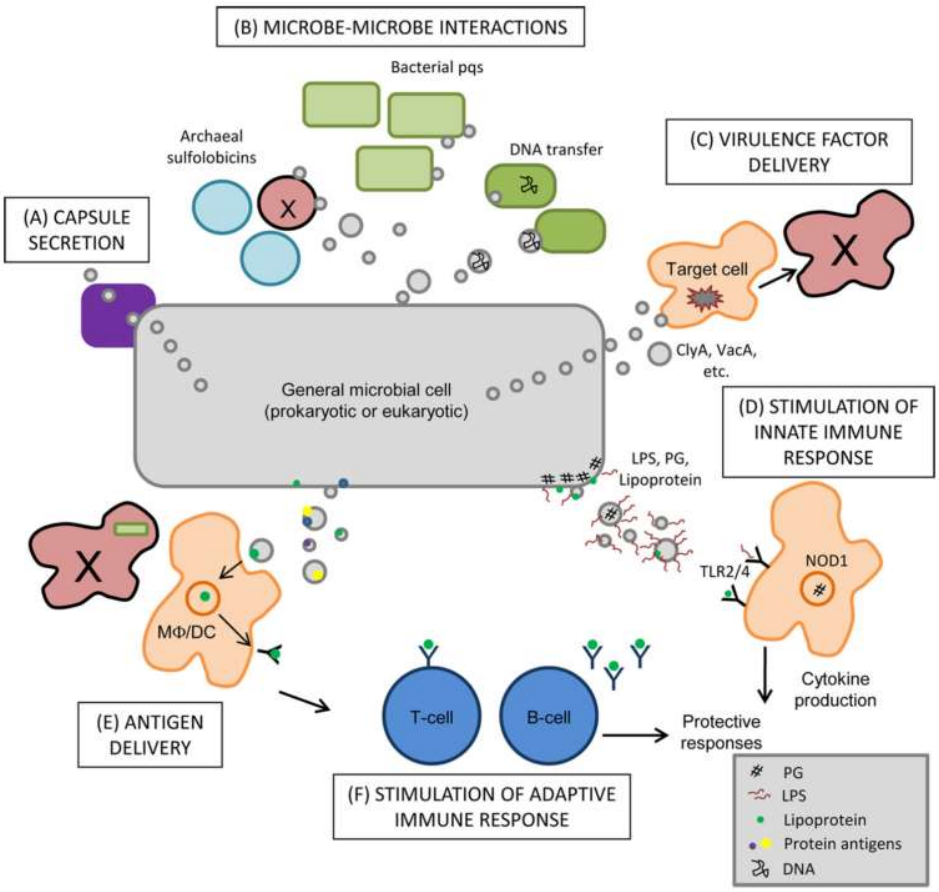
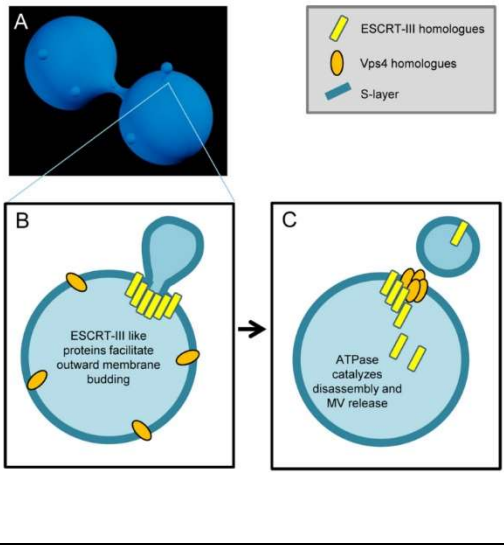
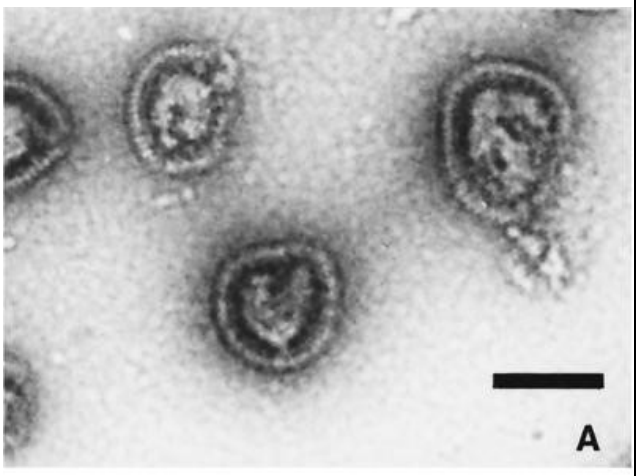


Figure 8 - Schematic depiction of vesicle functions described in archaea, bacteria and eukaryotes [72].

Vesicles in archaea are secreted by using pathways with homologues in both eukaryotes and prokaryotes. The similarities between these homologous mechanisms might represent a point of evolutionary divergence to the complex systems found today. The ESCRT system (endosomal sorting complex required for transport) and Vps4 (vacuolar sorting protein) for example, are systems that have homologues in many archaea, fungi and mammals [64], [73]. They have been suspected to be involved in vesicle formation in Archaea. Interestingly, there are some vesicle producing archaea that lack the ESCRT system [74], which suggests that the vesicle production might be more diversified than previously thought.



ESCRT system [72]



Sulfolobicines [71]

In the field of viral ecology, a primary method of quantifying viruses in environmental samples is Fluorescence In Situ Hybridization (FISH). The dimensions, morphologies, and molecular composition of membrane vesicles are very similar to those of some virions [75], causing them to sometimes be mistaken for viral particles when screening environmental samples [76]. This could lead to an overestimation of virus populations.

1.7 *Sulfolobus* – The first hyperthermophilic Archaeon

The genus *Sulfolobus* belongs to the Crenarchaeota phylum and the species within this genus are considered hyperthermophilic and acidophilic. The first member of the *Sulfolobus* genus, *Sulfolobus acidocaldarius*, was isolated from a hot spring in the Yellowstone National Park by T. Brock in 1972 [77]. Since then, *Sulfolobus* species have been discovered around the globe in volcanic hot springs with temperatures between 70-80°C and at pH 2-3. They have an irregular coccoid shape (Figure 28) and can use sulfur for their heterotrophic or autotrophic metabolism. *Sulfolobus* species are known to live either chemoheterotrophically by utilizing sulfur to oxidize simple reduced carbon compounds or lithoautotrophically by oxidizing sulfur producing H_2SO_4 or heterotrophically on sugars and peptides, with the main metabolic pathways being the TCA cycle, modified glycolytic pathway and the pentose phosphate pathway. *Sulfolobus* as well as other hyperthermophiles have many adaptations that enable life at extremely temperate and acidic conditions. Reverse gyrase for example is a protein unique to *hyperthermophiles*, that positively supercoils the chromosomal DNA and histone like helix-stabilizing nucleoid proteins (HSNP) increase the thermal stability of DNA [78], [79]

Apart from their numerous transposable-elements, all species of *Sulfolobus* have an stable genome in regard to point mutations. Still, one species stands out among the rest. In a study comparing several strains of *Sulfolobus acidocaldarius* isolated from various locations around the globe, Dominic Mao has found there to be almost no genetic divergence between the different strains (Table 1). This is particularly impressive as the sampling locations were, on average, 8000 km apart [80]. This highly conserved genome can be attributed to a collection of genes such as a UV damage excision repair system, an aromatic ring dioxygenase and a restriction modification system.[81], [82] Although the genome of *S. acidocaldarius* codes for 4 CRISPR systems, a virus has not been isolated that could infect and successfully replicate within the organism.

Table 1 – Genomic divergence of different strains of Sulfolobus Acidocaldarius *compared to one another* [80].

Strain pair	*Separation (km)*	*Polymorphisms (total number)*	*Insertion/deletions (% of polymorphisms)*	*Nucleotid divergenc*
98-3 vs N8	8006	9	44.4	$3.68 \times 10^{-}$
N8 vs Ron12/I	8546	30	46.7	$1.30 \times 10^{-}$
Ron12/I vs 98-3	8075	38	44.7	$1.66 \times 10^{-}$
Average	8209	25.7	45.3	$1.11 \times 10^{-}$

1.8 Goals of the Study

Archaea are hosts to some of the most morphologically diverse viruses discovered to date. These viruses are not only of interest from an ecological and evolutionary aspect, but can be used to establish genetic systems to alter their hosts genomes and can provide novel proteins with technological applications. The goal of this study was to isolate, enrich and characterize an archaeal virus-host system from environmental samples collected in Ischia, Italy and Beppu, Japan. For this interdisciplinary project, approaches and skills from the fields of ecology, microbiology, molecular biology, genetics and diverse imaging technologies had to be learnt, combined and applied. To characterize the Virus-Like-Particle (VLP), it was important to isolate and identify the VLP producing host using diverse cultivation techniques to produce VLPs in a controlled manner and to find new potential hosts. Once the production of VLPs in pure culture was confirmed, the VLPs were described morphologically using state of the art transmission and scanning electron microscopy. Purified VLPs were then used to infect novel, uninfected, closely related hosts to demonstrate virulent behavior. It was further attempted to extract and sequence the nucleic acids contained within the VLP and to analyse its proteome. For this purpose, sufficient amounts of VLP need to be isolated from culture supernatants.

2 Materials

2.1 2Microorganisms

EDTA 0.5M (pH 8)
Organism
Beppu-7 *Enrichment*
Sulfolobus acidocaldarius DSM639
E. coli Top 10 Cells
Sulfolobus solfataricus M18
Sulfolobus solfataricus P1
Sulfolobus tokodaii

2.2 Oligonucleotides

16S & 18S Primers

Specificity	Name	Product size (bp)	Sequence	Annealing temp °C	Reference
16S Archaea	Arch109F Arch1493R	1384	ACKGCTCAGTAACACGT GYYACCTTGTTACGACTT	55 55	[83] [84]
16S Bacteria	Bac27F Bac1492R	1465	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	55 55	[85] [85]
18S Eukarya	Euk1A Euk564R	563	AACCTGGTTGATCCTGCCAGT GGCACCAGACTTGCCCTC	56 56	[86] [86]

M13 Primers

Specificity	Name	Product size (bp)	Sequence	Annealing temp °C	Reference/Company
M13 F	M13 F M13 R	-	GTTTTCCCAGTCACGAC CAGGAAACAGCTATGAC	55 55	Promega

Sulfolobicin Primers - *S. acidocaldarius DSM639*

Specificity	Name	Product size (bp)	Sequence	Annealing temp °C	Reference
DSM639 Saci_1272-1273	Saci_1272_FW Saci_1273_RW		TACTTCCTCGCCCTCCTTGA TTCCGTATCCTTACGTAAAATGGT	65 65	[69] [69]

CRISPR Primers - *S. acidocaldarius DSM639*

Specificity	Name	Product size (bp)	Sequence	Annealing temp °C	Reference
	DSM_CR1_FW	203	TACTTCCTCGCCCTCCTTGA	57	This study

DSM639 CRISPR 1	DSM_CR1_RW_2FW		TTCCGTATCCTTACGTAAAATGGT	57	
DSM639 CRISPR 2	DSM_CR1_RW_2FW DSM_CR2_RW	203	TTCCGTATCCTTACGTAAAATGGT GAGCAGAAAGTAGTAGAGGACAGAG	57 57	This study
DSM639 CRISPR 3	DSM_CR3_FW DSM_CR3_RW	248	CGTTGCAGGCAGATATGAG CCCAACTTAGTCACAACCCCT	57 57	This study
DSM639 CRISPR 4	DSM_CR4_FW DSM_CR4_RW	265	AGTACGCAAACGATTTGAGCTT CTTAAGGTCGCATTAAAGTTCCC	57 57	This study

2.3 Buffers and Solutions

2.3.1 General Buffers

EDTA 0.5M (pH 8)

Chemical Compound	Amount (M, ml, g %)
Tris base	242 g
EDTA 0.5M(pH8)	

Tris Acetate buffer (TAEbuffer)

Chemical Compound	Amount (M, ml, g %)
Tris base	242 g
EDTA 0.5M(pH8)	100 ml
Acetic acid	57.1 ml
Add to 1L ddH2O	

Sodium Acetate 3M

Chemical Compound	Amount (M, ml, g %)
Sodium acetate (3H2O)	20.421 g
Adjust pH with glacial acetic acid	
Add to 50 mL ddH2O	

TE-buffer

Chemical Compound	Amount (M, ml, g %)
Tris/HCl (pH 7.5)	10 mM
EDTA	1 mM

2.3.2 TEM Grids & Staining

Formvar

Chemical Compound	Amount (M, ml, g %)
Formvar	0.75 %
Dissolve in 100ml chloroform	

0.1% Poly-L-Lysine

Chemical Compound	Amount (M, ml, g %)
Poly-L-lysine	0.1%
Add to 50ml ddH_2O	

0.1% Ammonium Molybdate

Chemical Compound	Amount (M, ml, g %)
Ammonium molybdate	0.1%
Add to 50ml ddH_2O	

2.3.3 DNA Extractions

TEN-buffer

Chemical Compound	Amount (M, ml, g %)
Tris/HCl (pH 8)	20 mM
EDTA	1 mM
NaCl	100 mM

TENST-buffer

Chemical Compound	Amount (M, ml, g %)
TEN-buffer	s.a.
Natrium-N-lauroylsarcosin	1.6 %
Triton X-100	0.12 %

3M Sodium-Acetate

Chemical Compound	Amount (M, ml, g %)
Na-Acetate (3M)	40.8 g
Add to 100ml ddH_2O	

2.4 Media

2.4.1 Sulfolobus

2.4.1.1 Brock

Brock Medium 50ml

Chemical Compound	Amount (M, ml, g %)
Brock 100	0.5 ml
Brock 200	0.25 ml
Brock 1000	50 µl
Sucrose 20%	0.5 ml
Yeast 10%	0.25 ml
Tryptone 10%	0.25 ml
H2SO4 50%	17.5 µl

Brock 100 x (1L) – sterile-filtered

Chemical Compound	Amount (M, ml, g %)
$(NH_4)_2SO_4$	130 g
$MgSO_4$ x 7 H_2O	25 g
$FeCl_3$ x 6 H_2O	2 g
50% H_2SO_4	3 ml

Brock 200 x (1L) – autoclaved

Chemical Compound	Amount (M, ml, g %)
KH_2PO_4	56 g
Tracelelemets	10 mg/ml Stock-solution
$MnCl_2$	36 ml
$ZnSO_4$	4.4 ml
$CuCl_2$	1 ml
$VOSO_4$	0.6 ml
$CoSO_4$	0.2 ml
MoO_4	0.6 ml
Na_2B4O_7	90 µl
Autoclave then add 50% H_2S0_4	5 ml

Brock 1000 x (200ml) – autoclaved

Chemical Compound	Amount (M, ml, g %)
$CaCl_2$ x 2H_2O	14g

2.4.1.2 Pasteur Medium

Pasteur for 50ml of Culure

Chemical Compound	Amount (M, ml, g %)
Parisian 10X	5 ml
Metal solution	100 µl
Sucrose 20%	1 ml
Yeast 10%	0.5 ml
Tryptone 10%	0,5 ml

Metal Solution (1L)

$Ca(NO_3)_2 \times 4H_2O$	70.8 g
$MgCl_2 \times 6H_2O$	203.3 g

Pasteur 10X (1L)

Chemical Compound	Amount (M, ml, g %)
$(NH_4)_2SO_4$	30 g
Glycin	7 g
K2HPO4 x 3H2O	5 g
KCl	1 g

Tracelelemets	1% Stock-solution
$Na_2B_4O_7 \times 10H_2O$	2240 µl
$MnCl_2 \times 4H_2O$	900 µl
$ZnSO_4 \times 7H_2O$	110 ml
$CuCl_2 \times 2H_20$	25µl
$Na_2MoO_4 \times 2H_2O$	15 µl
$VOSO_4 \times 5H_2O$	15 µl
$CoCO_4 \times 7H_2O$	5 µl
$NiSO_4 \times 5H_2O$	5µl
Autoclave then add 50% H_2S0_4	8 ml

2.4.2 E. Coli

SOB Medium

Chemical Compound	Amount (M, ml, g %)
Bacto trypton	2 %
Yeast extract	0.5 %
Glucose	20 mM
Mg2+	20 mM
NaCl	10 mM
KCl	2.5 mM

2.5 Other Chemicals, enzymes, size markers and vectors

Name	Amount (M, ml, g %)
Bacto Trypton	2 %
Yeast extract	0.5 %
Glucose	20 mM
Mg2+	20 mM
NaCl	10 mM
KCl	2.5 mM
Taq-Polymerase	
Phusion Polymerase	
Long Range PCR Master Mix (biotechrabbit)	

3 Methods

3.1 Sampling thermoacidophiles

Liquid and sediment samples were collected in Ischia, Italy and Beppu Japan. The samples from Ischia were collected in 2013 as part of a practical course supervised by Prof. Dr. Christa Schleper of the Archaea Biology and Ecogenomics Division at the University of Vienna. The samples in Beppu were collected by Ricardo Alves and Christa Schleper in cooperation with Yoshizumi Ishino and Sonoko Ishino from the University of Fukuoka.

Samples were collected at terrestrial hot springs, sulfur fields and fumaroles with temperatures above 70°C and a pH below 4. The pH was tested with pH paper, because pH electrodes are often inaccurate at high temperatures. Liquid samples were collected using a 50ml Falcon tube attached to a metal pole while solid samples were taken by scraping soil into a 15ml Falcon using a sterilized spatula. After the samples were allowed to cool, the liquid samples were titrated to a pH of 5.5 using $CaCo_3$. Samples collected in this manner were kept at room temperature for up to two weeks before enrichment or up to four weeks at 4°C.

3.2 Enrichment

The original samples were enriched for *Sulfolobus* in Brock Medium (1.4.1.1). The quality of the enrichments was routinely checked using light microscopy and 16S rDNA analysis using both archaeal and bacteria specific primers. The preliminary enrichments were sent to the Institute Pasteur in Paris, where they were grown in the “Pasteur” medium (1.4.1.2), further enriched through dilution during passaging and screened for viruses. The enrichments containing Virus Like Particles (VLPs) were then sent back to Vienna, where they were plated on Gelrite Plates and single colonies were used to inoculate fresh medium. These single colony cultures were then again screened using archaeal specific 16S rDNA primers.

3.3 Light-Microscopy

The enriched cultures were inspected visually using a light-microscope. The slides were prepared by placing 5µl of culture into the middle of a microscope-slide and placing a coverslip on it. To get an overview of the culture, it was fist inspected using 40x magnification. For a more detailed look at the cells, a drop of microscopy oil was placed on the slide, optimizing the light refraction for a 100x magnification. 8

3.4 VLP Preparation

3.4.1 Concentrating VLPs by Ultracentrifugation

50ml of culture were centrifuged at low speed (1500xg) for 30 minutes to pellet the cells. The supernatant was then again centrifuged at 2000xg for 30 minutes, to pellet any remaining cells. The resultant supernatant was then filtered through a 0.45 µm filter. The filtrate was split into 6 ultracentrifugation tubes and spun at 64000xg and 4°C for 4 hours. The supernatant was decanted and the pellet in the first tube resuspended in 200µl TA Buffer. The resuspended VPLs were then transferred into the next ultracentrifugation tube and used to resuspended the second pellet. This was repeated for all remaining tubes, until all pellets were resuspended in the original 200µl of TA Buffer. The Sample was then stored at 4°C.

3.4.2 Concentrating VLPs by Polyethylenglycol-Precipitation

500ml of culture were centrifuged at low speed (1500xg) for 30 minutes to pellet the cells. The supernatant was then again centrifuged at 2000xg for 30 minutes, to pellet any remaining cells. PEG600 and NaCl were added to concentrations of 10.5g/100ml and 5.8g/100ml respectively. The VLP fraction was then allowed to precipitate overnight at 4°C and was subsequently centrifuged at 22000xg and 4°C for 60 minutes. The supernatant was discarded and the remaining pellet was resuspended in a minimal amount of TA buffer. The VLP-concentrate was either be used for DNA extraction or for a CsCl density gradient centrifugation.

3.4.3 Purifying VLPs by Cesium Chloride density gradient centrifugation

The sample was diluted to a total volume of 10ml and $CsCl_2$ was added to a final concentration of 0.45g/ml. The sample was centrifuged in a SW41ti rotor at 256,000 G and 4°C for 4h hours. The visible bands were collected using a needle and a syringe followed by a dialysis of the individual bands against a TA Buffer for 2hours using a MWCO 10kDa protein filter. The band was placed into filter, which was then submerged in TA buffer. The diffusion was allowed to take place for two hours, after which the TA buffer was replaced and diffusion allowed to continue for another 2 hours. This was repeated 4 times.

3.4.4 Concentrating VLPs suing a MWCO Filter

Cells were removed from 500ml of culture through centrifugation at 1500xg and 4°C for 20 minutes and subsequent filtration of the supernatant using a 0.45µm filter. Following the titration of the cell free supernatant to a pH of 7.5 using 10M KOH, it was then concentrated using 25ml MWCO filters with a 30kDa cut-off. Four filters were filled with 25 ml of supernatant each and centrifuged at 1500rpm and 4°C for 15 minutes. The flow through was removed and a further 25 ml of supernatant added onto each filter. This was repeated until all 500ml of culture were filtered. The centrifugation period had to be adjusted, depending on the accumulation of particles and their change on the flow rate. In the last run, the filters were centrifuged until 5ml of concentrate remained in each filter. The samples were pooled into a single filter and centrifuged until 1ml of sample remained. 20ml TA-Buffer were added to the concentrate to wash the sample. A last centrifugation step was performed, to concentrate the sample to 500µl. The final concentrate was transferred into a 1.5ml Eppendorf tube and stored at 4°C.

3.5 DNA Extraction

3.5.1 Sulfolobus

Cells from 10ml of culture were centrifuged at 1´500xg and 4°C for 20 minutes. The resulting pellet was resuspended in 500µl of TEN-buffer and transferred into a 2.5ml Eppendorf tube (Eppi). The cells were then lysed by the addition of 500µl of TENST-buffer and 10s of vortexing, followed by a 30-minute incubation on ice. The proteins and cell debris were isolated from the lysate by adding 500µl of Phenol/Isoamylalcohol/Chloroform (25:24:1) (PIC) titrated to a pH of 7.8, subsequent 10 second vortexing and centrifugation at 15000xg and 4°C for 15 minutes. The aqueous (upper) phase of the post centrifugation gradient was transferred into a new 2.5ml Eppi. This step was repeated to ensure a protein free supernatant. 500µl of chloroform were added to the aqueous phase, which was transferred after the second PIC cleaning step. The resulting solution was vortexed for 10 seconds and centrifuged at 15000xg and 4°C for 15 minutes. As before the aqueous phase was transferred to a new Eppi and the chloroform step was repeated a second time. Having removed the phenol with the help of chloroform, 700µl of 100% isopropanol and 70µl of 3M sodium-acetate are added to the aqueous phase to precipitate the DNA. The solution was then left at 4°C overnight to ensure sufficient time for the precipitation. The next morning, the precipitate was centrifuged at 15000xg and 4°C for 20 minutes and the resulting supernatant was decanted, leaving behind a faint white pellet on the side of the Eppi. Following the precipitation, the pelleted DNA was washed by carefully adding 500µl of 80% (ice cold) ethanol and a last round of centrifugation at 15000xg and 4°C for 10 minutes. As before, the

supernatant is decanted and the remaining ethanol is removed by drying the pellet in a Speed-Vac. The dried pellet is finally resuspended in 30-100µl nuclease-free H_2O and stored at -20°C

3.5.2 VLP fraction

Prior to the lysis of the VLP fraction, the extracellular and free nucleotides in the sample had to be digested. To achieve this, the sample was vortexed incubated at 37°C for 30 minutes after the addition of MgCl2 1M to a final concentration of 10mM, 120U DNAse and 100µg/ml RNAse. After the incubation EDTA 0.5M was added to a final concentration of 20mM to inactivate the enzymes. Next 100µg/ml of proteinase-K and SDS 10 to a final concentration of 0.5% were added to the sample and incubated for 30 minutes at 55°C. The lysate was then transferred to a 2ml Eppi and an equal volume of PCI was added. The sample was carefully inverted 5 times and centrifuged at full-speed for 5 minutes. The aqueous phase was transferred to a new tube and the protein precipitation was repeated. The sample was then washed with chloroform in the same manner to remove the phenol. To precipitate the DNA, 0.3M sodium acetate and twice its volume of 99.9% ethanol were added to the aqueous phase and mixed by inverting the tube several times. The nucleotides were precipitate over night at -20°C and subsequently centrifuged at full-speed and 4°C for 20 minutes. The supernatant was decanted and the remaining pellet washed with 70% Ethanol and again centrifuged at full-speed and 4°C for 50 minutes. The supernatant was discarded, the pellet dried in a Speed -Vac, resuspended in 30µl TE-Buffer and stored at -20°C.

3.6 RNA Extraction of Host and VLP fraction

As described in 4.5.1 but instead of using Phenol Chloroform with a pH of 7.8 a more acidic Phenol/Isoamylalcohol/Chloroform (25:24:1) titrated to a pH of 4.6 was used.

3.7 Glycerol Stocks

Glycerol stocks were produced after every 2nd inoculum, by harvesting cells in the early exponential growth phase of the cultures at OD 0.2-0.3. This provided backups in case a culture did not grow properly or the host no longer produced the virus. To produce the glycerol stocks, the cells of 10ml of culture were pelleted at 2000xg for 30 minutes and the supernatant was decanted. The cells were then resuspended in 1ml of 25% glycerol in the corresponding medium. The 1ml of 10x concentrated cells was split into 5 aliquots and stored at -80°C.

3.8 PCR amplification

3.8.1 Go-Taq

Elongations times were calculated according to the size of the fragment that was being amplified and with an elongation speed of 1kb per Minute

Master Mix:

Reagents	Reaction (µl)
ddH2O	13,6
Buffer	5
FW primer	1,25
RW primer	1,25
dNTPs	0,5
BSA	0,25
Taq-Pol	0,15
MgCl2	2
Template	1

95°C	5min	
95°C	30sec	29x
Primer Specific Annealing Temp.	20sec	
72°C	Fragment specific elongation time	
72°C	4min	
4°C	For ever	

3.8.2 Phusion

Elongations times were calculated according to the size of the fragment that was being amplified and with an elongation speed of 2kb per Minute

Master Mix:

Reagents	Reaction (µl)
ddH2O	13,6
GC Buffer	5
FW Primer	0,5
RW Primer	0,5
dNTPs	0,5
DMSO	0,6
BSA	0,2
Phusion-Pol	0,2
Template	1

95°C	5min	
95°C	30sec	29x
Primer Specific Annealing Temp.	20sec	
72°C	Fragment specific elongation time	
72°C	4min	
4°C	For ever	

3.8.3 Colony PCR for *E. coli*

Master Mix:

Reagents	Reaction (µl)
ddH2O	34,6
Buffer	10
FW Primer	1
RW Primer	1
dNTPs	1
Taq-Pol	0,4
Template	2

95°C	5min	
95°C	30sec	29x
Primer Specific Annealing Temp.	20sec	
72°C	Fragment specific elongation time	
72°C	4min	
4°C	For ever	

3.9 Preparation of Gellan Gum Plates

Gelrite provides an alternative growth matrix to agar and as such retains its stability at higher temperatures and at lower pH levels than agar would. 500ml of double distilled MiliQ water was added into a 1L Schott bottle and microwaved until the water started to boil. 6.4g of gellan gum were then slowly added to the MilliQ water while it was being stirred and heated to 100°C on a magnetic-heating plate. While the gellan gum was dissolving, 500ml of culture-specific growth-medium were prepared as described in 2.4 and heated in the microwave. As soon as all of the gellan gum was dissolved, and while the gellan gum was still stirring, the Brock Medium was poured into the Schott bottle containing the gellan gum. The resulting growth-medium was then allowed to cool to 70°C and poured into petri-dishes while working next to a flame. The plates were dried at 37°C

3.10 Plaque Test

Spot-on-lawn (halo) assay for screening enrichment cultures and isolates for viruses—This protocol is based on Schleper et al. (1992) and modified by Stedman et al. (2003). Gellan gum plates are preincubated ca. 10 min at 80˚C to dry, then 10 mL of Pasteur medium with ca. 0.2 % (w/v) gellan gum is boiled until the gellan gum is dissolved. This “softlayer” is allowed to cool slightly (to ca. 80˚C). Approximately 3 mL of softlayer are added to ca. 0.2 mL of exponentially growing host cells and spread on a plate by swirling. After the gellan gum solidifies, 1–2 µL of culture or supernatant to be screened is spotted on the plate. Plates are incubated as above for 2–3 days, after which the plates were examined for plaques.

3.11 Electron Microscopy

3.11.1 Grid preparation for TEM

3.11.1.1 Covering EM Grid with Formvar

A 50ml beaker was filled with 0.75% Formvar solution and a microscope slide is submerged into the Formvar for 5 seconds, shortly lifted out of the formvar, re-submerged for 5 seconds and then allowed to dry for 30 seconds. The corners of the glass slide were scratched with a scalpel and the slide was subsequently slowly immersed, small edge first, into a 100ml beaker filled with MiliQ, until the formvar film on the glass slide started to peel from the glass slide and floated on the surface of the water. The TEM-Grids were now place onto the formvar film with the glossy side facing up. Once the area of the formvar film was covered with grids, a piece of parafilm, slightly larger than the film of formvar, was slowly placed onto the formvar film. The formvar film adhered to the parafilm, was removed from the water and allowed to dry, formvar facing up. Once dried, one grid at a time was carefully removed from the parafilm, keeping the formvar coat intact and placed into a Grid-box and stored for later use.

3.11.1.2 Sample Application and Staining

The formvar coated grids were prepared with a sample using the D.O.G. (Drop On Grid) procedure. The grids were secured in reverse action tweezers, 15µl of 0.1% Poly-L-lysine were placed onto the formvar coated side of the grid and incubated for two minutes. The excess solution was removed from the grid by blotting it on filter-paper. The grid was then washed by placing 15µl of MiliQ onto the grid, allowing it to sit for a few seconds and then blotting it dry. This washing step was repeated 3 times after which the grid was allowed to dry. Next, 15µl of sample were placed onto the grid and allowed to incubate for 2 minutes before the excess liquid was blotted away. Th grid was again allowed to dry. Finally, 15µl 1% ammonium molybdate were placed onto the grid and allowed to stain the sample for exactly 30 seconds before it was blotted away using filter-paper. The grid was again allowed to dry and placed back into the grid-box.

3.11.2 Sample Preparation for REM

The REM-Sample table was covered with a two-sided adhesive, onto which a round glass cover slip was placed. 15µl of a sample were pipetted onto the coverslip and allowed to dry. Once dry, the sample was sputter-coated with gold using a *Leica EM ACE600* for 80 seconds.

3.12 Clone Library

3.12.1 Ligation:

The Ligation solution was prepared as follows:

Fragment size (kbp) x 50 = ng of Sample for Ligation in 10ul

Ligation Mix:

Reagents	Reaction (µl)
Sample	x
pGemTeasy Vector	1
2x Rapid Ligation Buffer	5
T4 DNA Ligase	1
ddH2O	10-x

The solution was incubated overnight at 4°C.

3.12.2 Transformation of Vectors into *E. Coli*:

20µl of competent *TOP10 E. coli* cells and the ligation mix were combined into a 1,5ml Eppi and incubated on ice for 30 minutes. The reactants were then treated with a 42°C heat shock for 1minute, followed by 2minutes recovery on ice. 250µl of prewarmed LB-medium was added to transformed cells and incubated in a heat-block at 37°C for 1 hour at 500rpm.

3.12.3 Plating of Transformants

LB/Amp plates were prepared and left dried in a 37°C fan oven for 15 minutes. 20µl X-Gal and 100µl IPTG were added to each plate, distributed homogeneously and left to dry in the same oven for 15 minutes. 50µl of the transformed cells were plated onto one and 100µl onto the second plate and incubated at 37°C for 12 hours. A selection of blue colonies was picked from each of the plates and used for colony PCR. The cloned fragments were then amplified via colony-PCR using the M13F/M13R primers.

4 Results:

4.1 Enrichment and Virus Screening

Environmental samples from Ischia, Italy and Beppu, Japan (Table S1) were used as inoculum for cultures grown at 75°C and in the Brock Medium, designed to enrich for archaea of the order *Sulfolobales*. These initial enrichments were grown in the Department of "Ecogenomics and Systems Biology" at the University of Vienna. Cryostocks of the enrichments were taken to the "Viruses of Hyperthermophilic Archaea" group at the Institute Pasteur, Paris where they were screened for viruses.

Samples were prepared for TEM assisted screening for Virus-Like-Particles (VLPs) from 40ml of culture. The cells in the culture were pelleted with low speed centrifugation (4000rpm) for 15 minutes. The supernatant was removed without disturbing the pellet. The remaining cells and particles in the supernatant were pelleted by ultracentrifugation and resuspended in 200µl of TA-Buffer. The sample was then applied to a grid and negatively stained with 1% uranly-acetate for 30 seconds. VLPs were observed by Transmission Electron Microscopy (TEM) in sample enrichments from the environmental samples TAM1 and Beppu-7.

The TAM1 sample preparation contained filamentous VLPs at 1.2-1.5 µm in length and 20µm in diameter that had a spherical structure attached to one end (Figure 9). In the Beppu-7 preparation, vesicle-like VLPs with an average diameter of 100µm and a tail-like structure penetrating the cell envelope were observed (
Figure *10*).

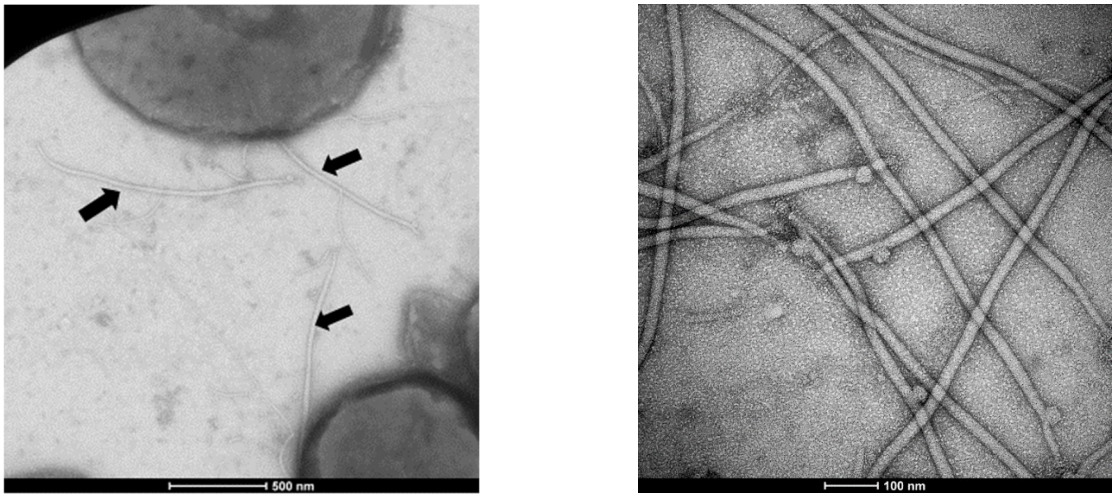


Figure 9 - TEM images of TAM1 VLP preparations negatively stained with 0.1% uranyl-acetate. (Images ©Elena Ransen)

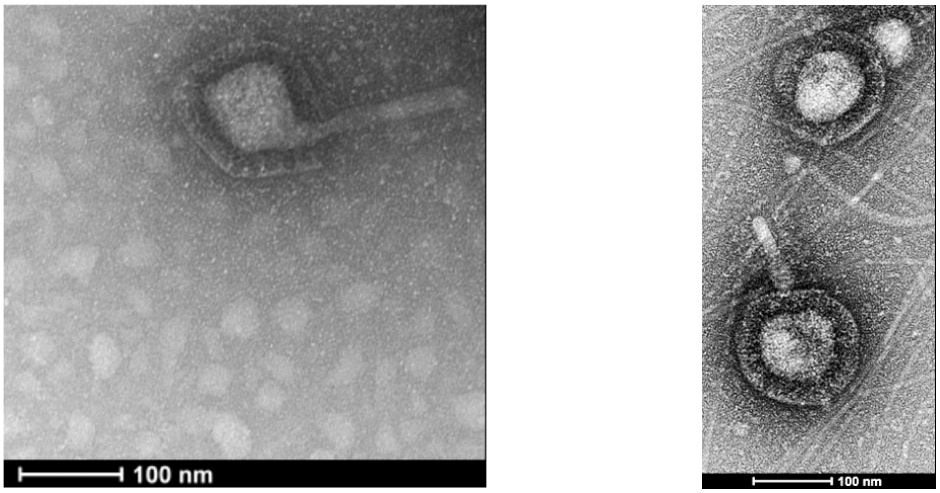


Figure 10 - TEM images of Beppu-7 VLP preparations negatively stained with 2% Uranyl-Acetate. (Images ©Elena Ransen)

4.2 Strain identification

An early enrichment of the Beppu-7 sample was split into four cultures. Each of the cultures was passaged with different innoculums (1%, 2%, 5% and 10%), in the effort to dilute any bacteria from the culture while retaining VLP production. The purity of the culture was regularly checked with bacterial and archaeal 16S rDNA primers. After six passages, sample Beppu-7.*1* did not show products for bacterial 16S rDNA (Figure 11).

16S rDNA of Beppu-7.1 was amplified using Arch109 and Arch1493 primers and Taq-polymerase. The resulting amplicons were used to create a clone library. The individual fragments were amplified from blue/white-screened colonies via colony-PCR using the M13FW and M13RW primers and Taq-polymerase (Figure 12). The resultant amplicons were cleaned to remove excess reactants using the Monarch PCR Cleanup Kit and both strands were sequenced.

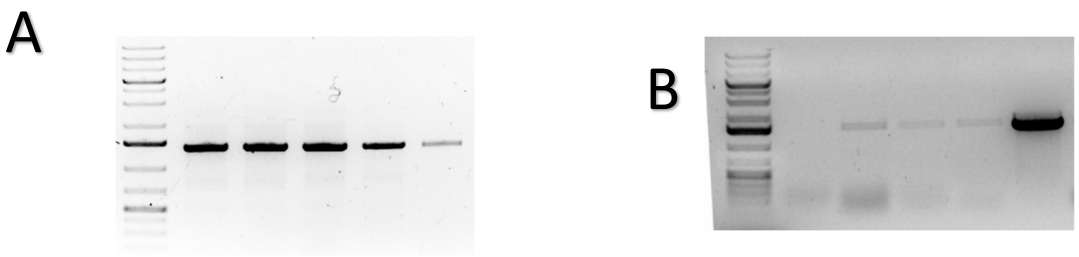


Figure 11 - 1% agarose gel run for 30 minutes at 120V, showing 16S rDNA amplicons of - A: Archaeal 16S rDNA Primers, B: Bacterial 16S rDNA Primers - Lane 1-4: Beppu-7 *enrichments, 5: Positive control, 6: Negative Control - Ladders: 1kb Plus*

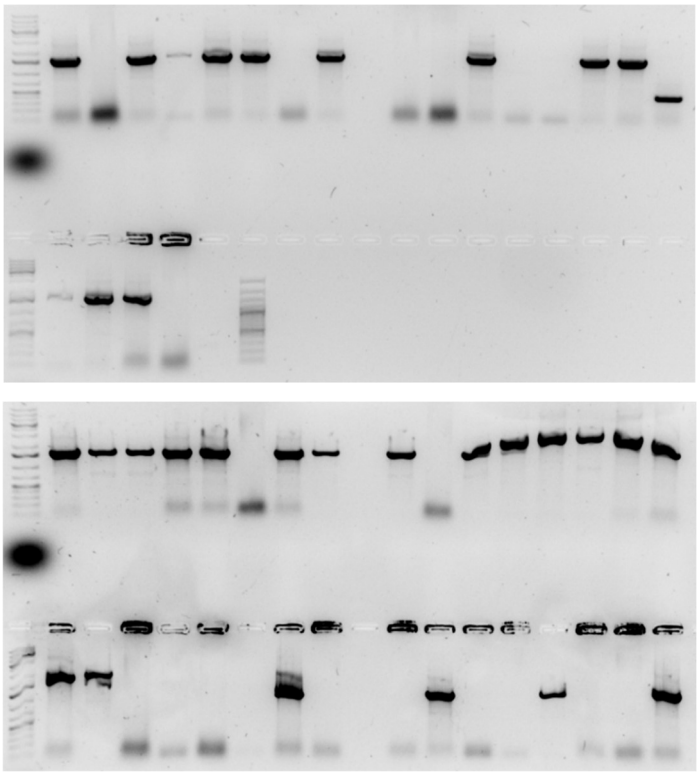


Figure 12 - 1% agarose gel run for 30 minutes at 120V showing M13 amplicons from 52 transformed E. coli *colonies picked after a blue/white staining. - Ladders: 1kb Plus*

According to their chromatograms, the sequences were of poor quality, but when blasted against the NCBI database they uniformly showed a high degree of identity to the 16S rDNA sequence of *Sulfolobus acidocaldarius*. Using the 16S rDNA gene of *Sulfolobus acidocaldarius DSM639* (DSM639) as a reference, all amplicon sequences were aligned using *Bioedit* [87].

The alignment showed that the sequences contained several single-nucleotide polymorphism (SNPs) and point mutations. Comparing these positions to the corresponding chromatogram revealed that some of these positions were of low sequencing quality. A factor that decreased the quality of the amplicons was the use of the mistake prone Taq-polymerase instead of a Phusion-polymerase with proofreading capacity.

To verify the BLAST results, the Beppu-7 enrichment was plated on Gelrite Plates and incubated at 80°C for 72 hours. 15 colonies were picked from the plates and used both to inoculate an individual culture and for culture PCR using Arch109 and Arch1493 primers and Phusion-polymerase. The Phusion-polymerase did not proficiently amplify the desired fragment, nor did the activity improve after the addition of DMSO or BSA. The *biotechrabbit Long-Range-Polymerase* (LR-Polymerase) amplified the target more efficiently (Figure 13).

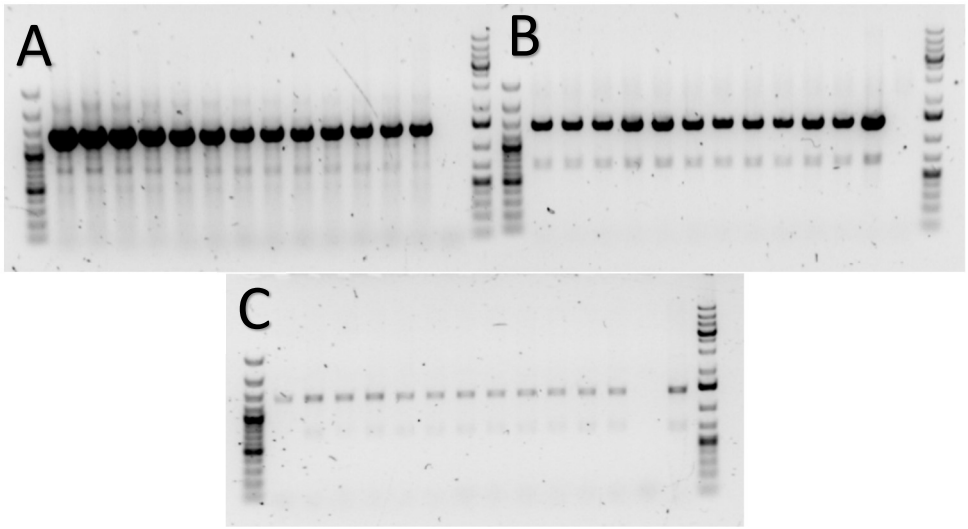


Figure 13 - 1% Agarose gel showing 16S rDNA amplicons of purified Beppu-*7 cultures using different DNA polymerases. A= Taq- Polymerase, B= Long-Range Master, C=Phusion-Polymerase*
Ladders: Left: 100bp Plus, Right: 1kb Plus

The amplicons produced with the LR-polymerase were sent for sequencing and the resulting reads were subsequently processed as described above. Although the sequences still contained SNPs, the overall quality had improved. Forward and reverse reads for each of the cultures were combined to a consensus sequence referenced against the 16S rDNA gene of DSM639. Once aligned, the sequences appeared to be identical to the sequence of DSM639. The 16S rDNA sequences of all *S. acidocaldarius* strains in the *NCBI* database were aligned against DSM639, revealing that all 16S rDNA sequences were identical.

4.3 Infection of new Host

The high degree of similarity between DSM639 and Beppu-7 might mean, that the DSM639 could be an alternate host for the VLP, therefore an infection of a culture of DSM639 was attempted. A culture of DSM639 was grown in Pasteur- Medium and exposed to filtered supernatant of the Beppu-7 culture. The cells of a 500ml Beppu-7 were pelleted by centrifugation at 4500 rpm and 4°C. The remaining cells were filtered from the supernatant using a 0.45µm filter, producing a cell free filtrate. Cell free supernatant of a DSM639 culture was produced in the same manner. Nine cultures were prepared and inoculated with an OD of 0.02 as shown in Table 2 and their growth monitored. At OD 0.2, 20ml of cell-free Beppu-7 supernatant was added to the sample DSM639+B0.2. The exposure of DSM639 to the VLPs had no apparent effect on the growth rate of the cultures (*Figure 14*).

Table 2 – Infection of potential hosts

Culture	1° Innoculum	1° Culture (ml)	Addition of filtrate	Volume (ml)	OD at addition
Control	NONE	30	Medium	20	0.0
Control+D	NONE	30	DSM639 filtrate	20	0.0
Control+B	NONE	30	Beppu-7 filtrate	20	0.0
Beppu7	Beppu-7	30	Medium	20	0.02
Beppu7+D	Beppu-7	30	DSM639 filtrate	20	0.02
DSM639	S. acidocaldarius DSM 639	30	Medium	20	0.02
DSM639+D	S. acidocaldarius DSM 639	30	DSM639 filtrate	20	0.02
DSM639+B0.02	S. acidocaldarius DSM 639	30	Beppu-7 filtrate	20	0.02
DSM639+B0.2	S. acidocaldarius DSM 639	30	Beppu-7 filtrate	20	0.2

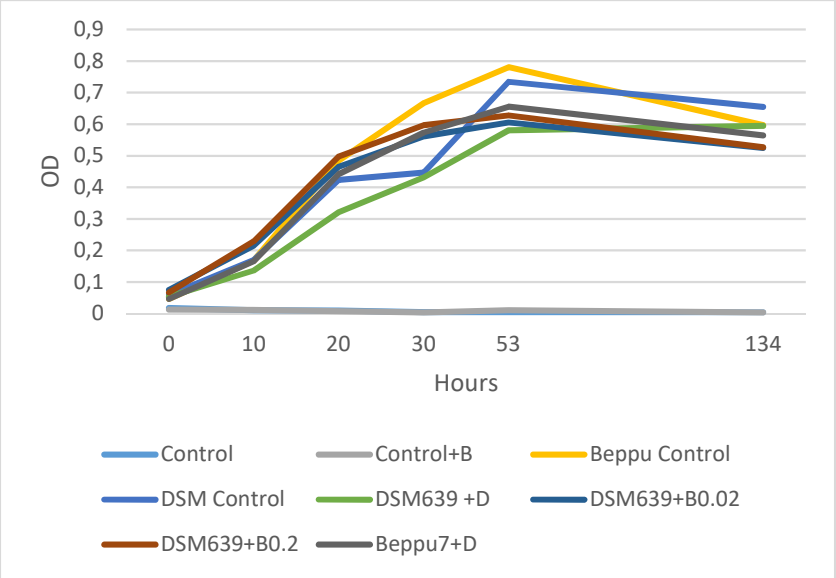


Figure 14 - *Growth curve of attempted infection of DSM639 with VLP, +D: 20ml of cell free* DSM639 *supernatant were added to the culture, +B: 20ml of cell free* Beppu-7 *supernatant were added to the culture*

The cultures exposed to the VLPs were passaged three times using a 1% inoculum, to ensure that any VLP in a later culture was also produced by that culture. VLP concentrates were produced from the 4th passage of *DSM Control,* DSM639*+B0.02,* DSM639*+B0.2* and Beppu-7 *Control* by ultracentrifugation as explained previously. VLPs were observed in all four of the cultures.

4.4 Restriction Digest

A DNA restriction digest of genomic DNA was performed to compare the newly isolated Beppu-7 strain to the closely related *S. acidocaldarius* DSM 639.

DNA from all cultures (Beppu-7, DSM639 and DSM639+V) in both early and late exponential phase was extracted and 1µg aliquots digested with the EcoRI, BamHI, HindI and XohI restriction enzyme. The digested extract was run in a 1% agarose gel for 16 hours at 60V (Figure 15). Interestingly, all patterns were identical. This is unusual, because it would mean, that the strain of *S. acidocaldarius* cultivated from the environmental samples from Beppu, Japan was identical to the strain DSM639 isolated from the Yellowstone National Park, America (Figure 15).

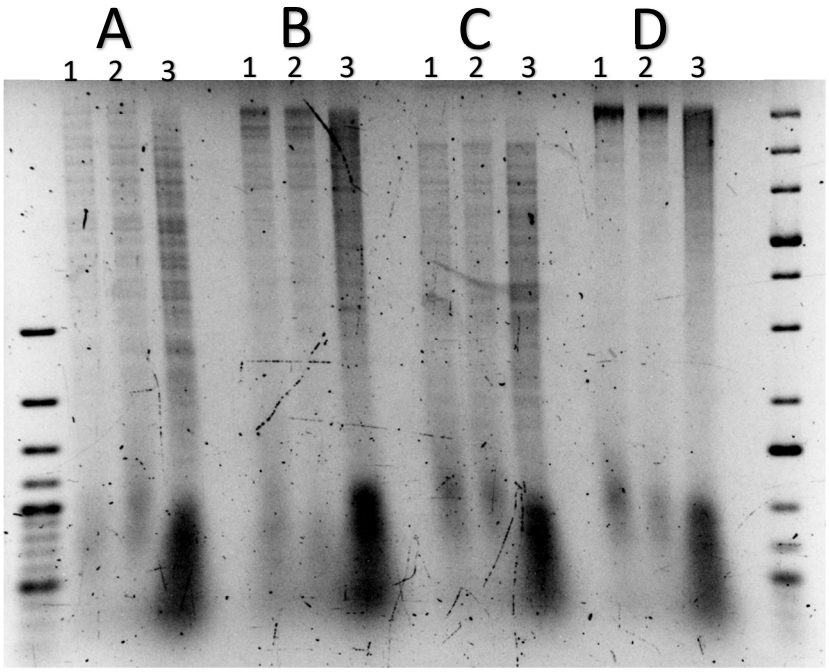


Figure 15 - 1% Agarose gel run for 16hours at 60volts, showing restriction digests of 1µg of Late Stationary phase: 1: Beppu-7, 2: DSM639, 3: DSM639+VLP - With restriction enzymes: A: EcoRI, B: BamHI, C: HindI, D:XhoI - Ladders: Left: 100bp Plus, Right: 1kb Plus

4.5 UV induction

In order to induce virus production, 30ml of the samples Beppu-7, DSM639 and DSM639*+VLP* with an OD of 0.2 were exposed to 7500 µJ/cm^2 of UV radiation for 5 seconds using a UVP CL-1000 Ultra-Violet Crosslinker. The cultures were allowed to recover for 6hours at 75°C, after which DNA was extracted from 10ml of culture and cell free VLP concentrate was prepared by ultracentrifugation at 64000 G for 4 hours using the remaining 40ml of culture.

A change in abundance of viral DNA can be monitored by changes in relative intensity of the bands in a restriction digest [88], therefore 1µg of DNA of each sample was digested with EcoRI and run on a 1% Agarose Gel for 16 hours at 60V(Figure 16). The effect of UV radiation was also analyzed by TEM. Supernatant of each sample was concentrated by ultracentrifugation as described earlier and prepared for TEM. There seemed to be no apparent change in band intensity, vesicle or VLP production in the screened samples.

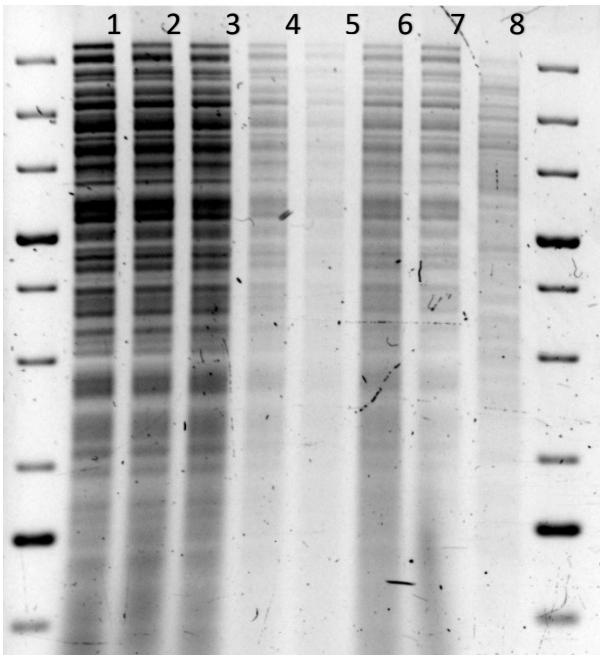


Figure 16 - 1% Agarose Gel showing 1µg of EcoRI digested DNA of whole culture DNA extract run for 16hours at 60V. Lane 1: *Beppu-7*, 2: UV treated *Beppu-7*, 3: *DSM639*+VLP, 4: UV treated *DSM639*+VLP, 5: *DSM639* 6: UV treated *DSM639*, 7: *DSM639* grown in an isolated shaker, 8: *S. solfataricus - Ladders: 1kb Plus*

4.6 VLP DNA extraction

40ml of Beppu-7 culture with an OD of 0.5 were pelleted and plasmid DNA was extracted using the OMEGA E.Z.N.A Plasmid DNA Mini Kit II. 1ng of extracted DNA was digested with either one of the restriction enzymes EcoRI and AseI. Both digests and the untreated DNA appeared only as a smear and did not give a clear pattern (Figure 17).

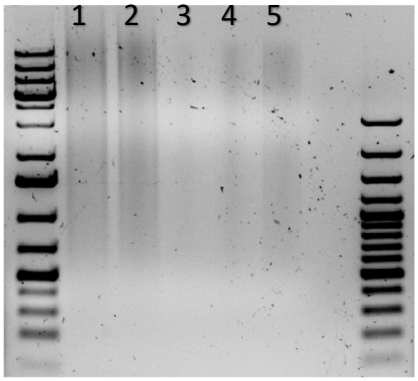


Figure 17 - 1% Agarose gel run at 60V for 4 hours, 1:1ng EcoRI, 2:1ng AseI, 3: 150ng pure, 4: 300ng pure, 5: 600ng pure - Ladders: Left: 1kb Plus, Right: 100bp Plus

DNA from 50ml of cell free Beppu-7 (OD 0.6) supernatant which was concentrated to 200µl via ultracentrifugation was extracted with the *Sulfolobus* specific Phenol-Chloroform protocol (3.51.). 1µg of the extracted DNA was loaded onto a gel but the lane merely showed a smear. The sample was checked for the presence of host DNA by PCR using archaeal 16S rDNA primers. The PCR product was run on a 1% Agarose gel for 30 minutes at 120V and showed a band corresponding to the size of amplified 16S rDNA DNA.

A virus specific DNA extraction protocol (3.5.2) was used to extract DNA from 50ml cell-free Beppu-7 (OD 0.6) supernatant which was concentrated to 500µl using a 30kDa MWCO. The extracted DNA was resuspended in 50µl of ddH2O, but the concentration of nucleotides was too low to be measured with a Nanodrop. The entire extract was loaded onto a 1% Agarose gel and run for 1hour at 100V, but no band was visible.

In a further attempt 500ml of culture were centrifuged at 4500rpm to pellet the cells, the supernatant was carefully decanted and filtered through a 0,45µm filter. The resultant filtrate was concentrated using 30kDa MWCO filters. DNA was extracted from the concentrate using the same virus specific protocol. The extract was run on a 1% agarose gel and although a band was visible on the gel, it was the same size as the host chromosomal DNA and the extract was positive for archaeal 16S rDNA.

4.7 RNA Extraction

With the DNA extractions from VLP concentrates being unsuccessful and the whole DNA extracts showing a difference in RNA bands (Figure 18), RNA was extracted from a sample of Beppu-7, DSM639+Virus and DSM639. RNA was extracted using the standard PCI extraction with a pH 7.6 PCI solution. 500ng of the extracted RNA of each sample was analyzed using gel-electrophoresis running at 50V for 6 hours. The extracts of Beppu-7 and DSM639+Virus showed faint bands approximately the size of the host genome (Figure 19). An aliquot of 500ng of the extracts were treated with DNAse for 1hour and then run on a 1% agarose gel for 6hours at 50V (Figure 20). It can be clearly seen, that the bands are on the same height as the host chromosome and disappeared after the treatment with DNAseI.

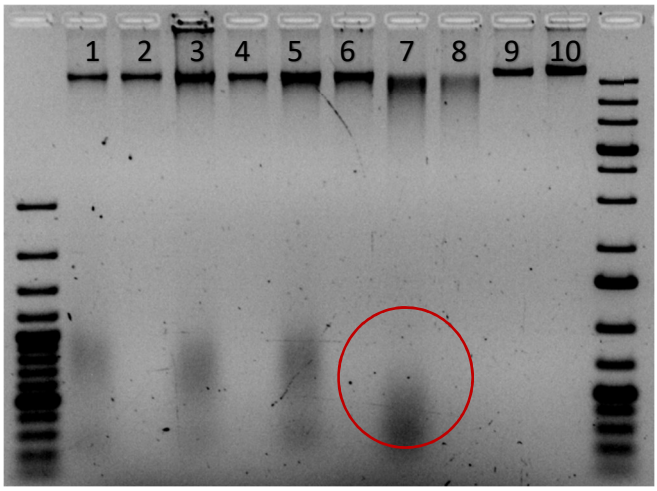


Figure 18 - 1% Agarose Gel run at 60V for 6 hours. - 1µg of each sample was loaded onto the gel - Lane 1: Beppu-7 *DNA extract, 2: RNAse treated* Beppu-7 *DNA extract, 3:* DSM639*+VLP 0.02 DNA extract, 4: RNAse treated* DSM639*+VLP 0.02 RNA extract, 5:* DSM639*+VLP 0.02 DNA extract, 6: RNAse treated* DSM639*+VLP 0.02 RNA extract, 7:* DSM639 *RNA extract 8: RNAse treated* DSM639 *RNA extract, 9: RNAse treated* S. solfataricus *DNA extract, 10: RNAse treated* S. tokodaii *DNA extract - Ladders: Left: 100bp Plus, Right: 1kb Plus*

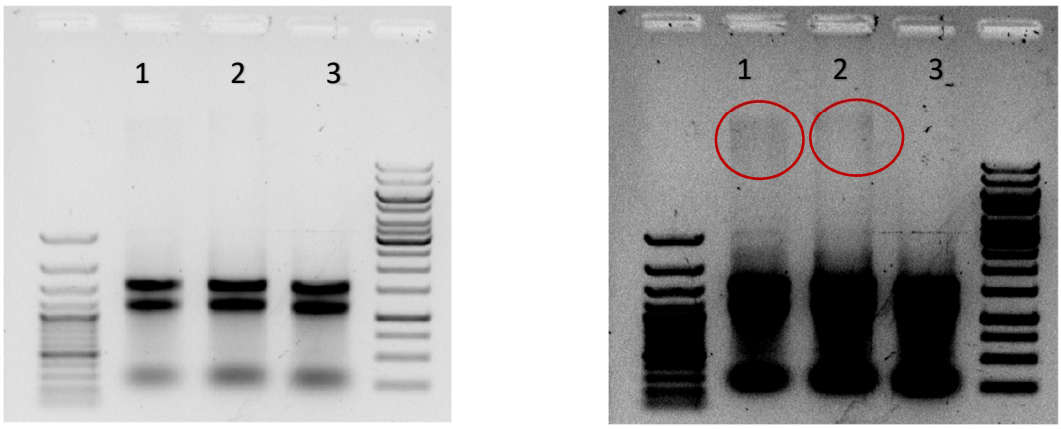


Figure 19 - 1% Agarose gel run at 60V for 16 hours - 1µg of each sample was loaded onto the gel. - Lane 1: Beppu-7 *RNA extract, 2: DNAse treated* Beppu-7 *RNA extract, 3:* DSM639*+VLP RNA extract, 4: DNAse treated* DSM639*+VLP RNA extract, 5:* DSM639 *RNA extract 6: DNAse treated* DSM639 *RNA extract, 7:* Beppu-7 *DNA extract, 8: DNAse treated* Beppu-7 *DNA extract, 9:* DSM639*+VLP DNA extract, 10: DNAse treated* DSM639*+VLP DNA extract. - Ladders: Left: 100bp Plus, Right: 1kb Plus*

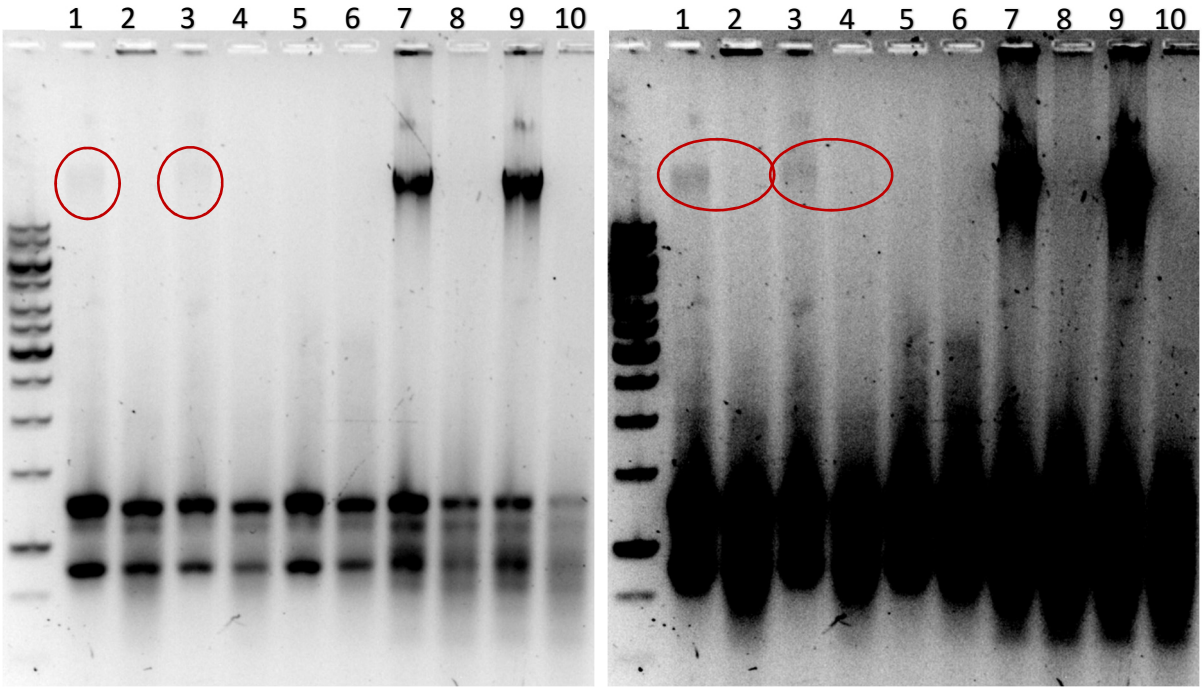


Figure 20 - 1% Agarose gel run at 60V for 16 hours - 1µg of each sample was loaded onto the gel. - Lane 1: Beppu-7 *RNA extract, 2: DNAse treated* Beppu-7 *RNA extract, 3:* DSM639*+VLP RNA extract, 4: DNAse treated* DSM639*+VLP RNA extract, 5:* DSM639 *RNA extract 6: DNAse treated* DSM639 *RNA extract, 7:* Beppu-7 *DNA extract, 8: DNAse treated* Beppu-7 *DNA extract, 9:* DSM639*+VLP DNA extract, 10: DNAse treated* DSM639*+VLP DNA extract.* - Ladder: 1kb Plus

4.8 Analysis of CRISPR

CRISPR, as the cells adaptive immune system can be seen as a registry of viruses and other extrachromosomal nucleotides the cell has come into contact with, and should therefore vary, even between closely related strains. Primers for all four CRISPR systems of *S. acidocaldarius* DSM639 were designed to bind to a region spanning from roughly 100bp into each CRISPR leader to the second spacer in each system. Because of the adaptiveness of the CRISPR system, the primers should not bind to the genome of Beppu-7 and if the infection took place, the amplicon of the de novo infected culture should be slightly larger than that of the uninfected strain. Figure 21 shows that all amplicons were identical in size and that the primers bound to a sequence in the Beppu-7 genome. The bands were excised, the amplicons removed for the gel with the Monarch GEL Clean-Up Kit and were sent for sequencing.

The sequences from all samples were aligned against the sequences of the four CRISPR systems found in *S. acidocaldarius* DSM639. The alignment revealed that the first two spacers in all four of the CRISPR systems were identical in all samples (Figure 22).

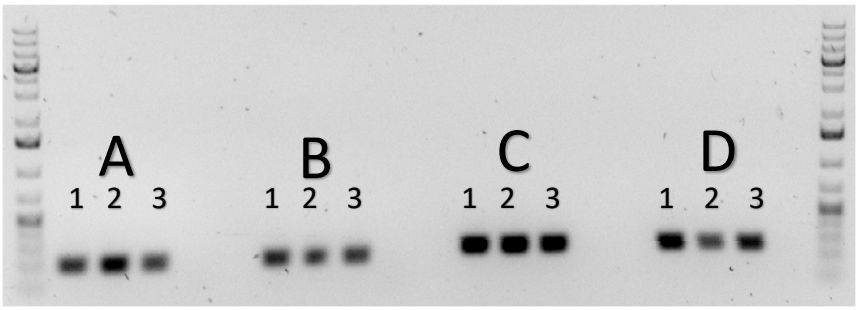


Figure 21 - 1% Agarose gel run for 30 minutes at 120V showing: 1: Beppu-7, *2:* DSM639, *3:* DSM639+VLP
A: CRISPR System 1, B CRISPR System 2, C: CRISPR System 3, D: CRISPR System 4 - *Ladder: 1kb Plus*

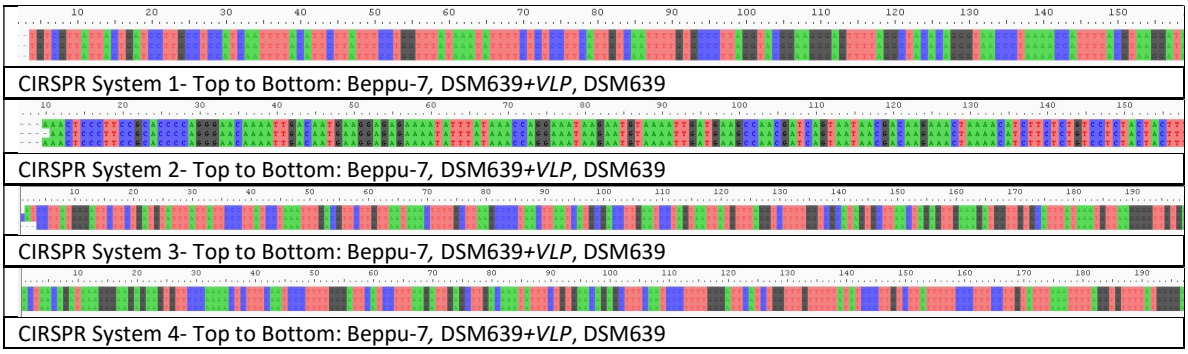


Figure 22 - Aligned CRISPR sequences; Color code: Red: Thymine, Gray: Guanine, Green: Adenine, Blue: Cytosine; Each CRISPR System shows 3 sequences: 2nd: DSM639, 1st: Beppu-7, 3rd : DSM639+VLP

4.9 Analysis of Sulfolobicines

Sulfolobicines have been defined as a cross-species growth inhibitor produced by different archaea and Beppu-7 and DSM639+*VLP* both showed to cause growth retardation of DSM639 on gellan gum plates but DSM639 did not inhibit its own growth. Therefore, the Saci1271 and Soci1272 genes corresponding to the two Sulfolobicine proteins were amplified using the Saci_1272_FW and Saci_1273_RW primers designed by Albert et al [69](Figure 23).

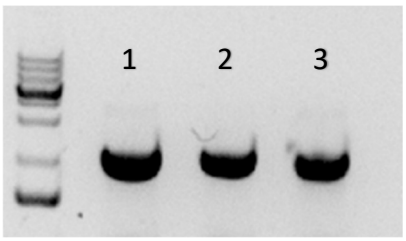


Figure 23 - 1% Agarose gel run for 30 minutes at 120V showing Sulfolobicine-amplicons of: 1: Beppu-7, 2: DSM639, 3: DSM639+VLP - Ladder: 1kb Plus

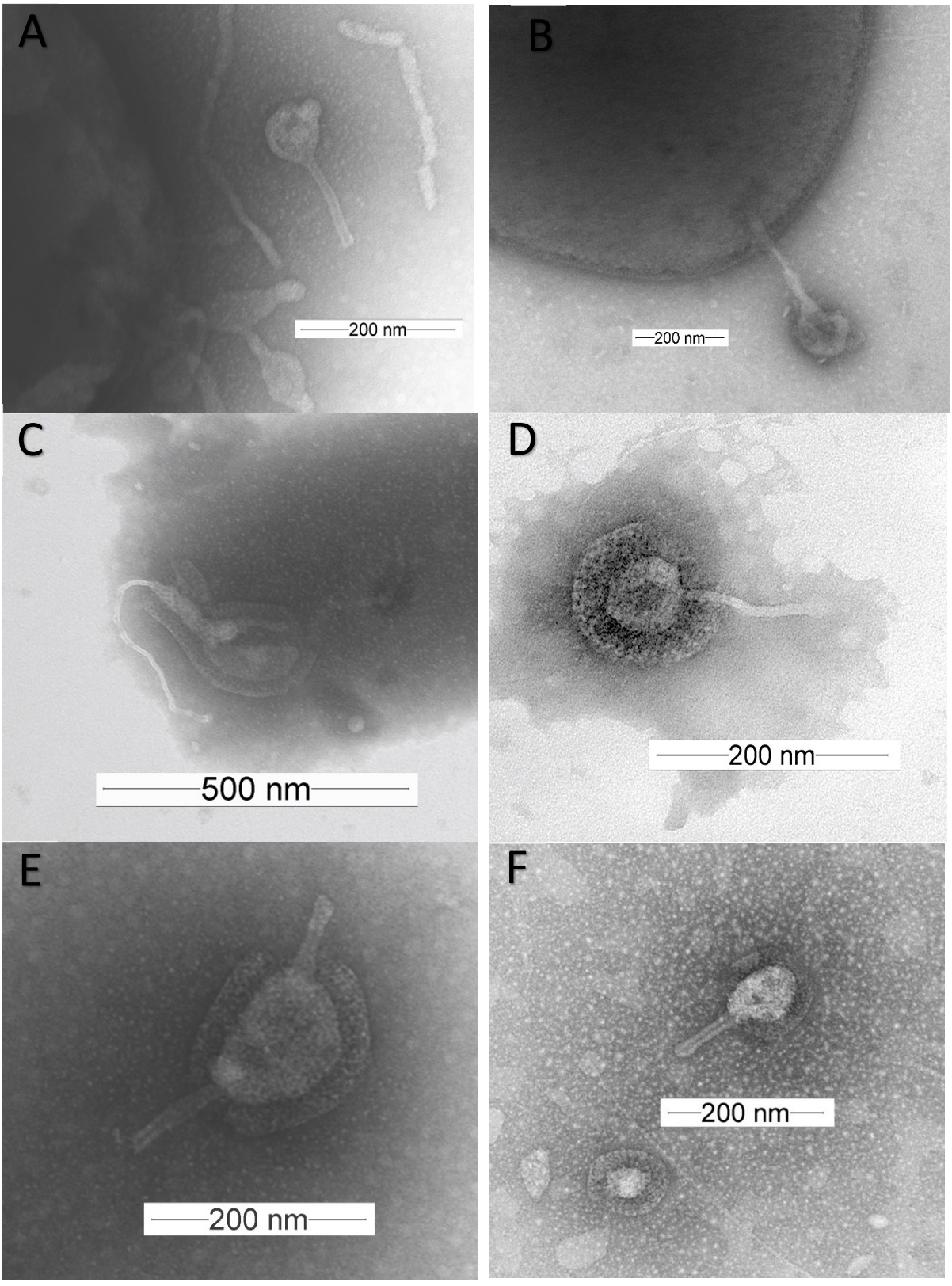
The amplicons were sent for sequencing and aligned to the sulfolobicine genes of DSM639, and although the amplicons were sequence using both the forward and reverse primers, the amplified region with ~2kbp was too long to sequence with just one primer pair. To close the gap in the middle of the sequence, a forward primer would have to be designed for the center of the gene. The portions of the genes that were successfully sequenced, show a complete match to the DSM639 genes (Figure S.2).

4.10 Concentration of VLP by PEG precipitation and $CsCl_2$ Density Gradient

In order to produce a concentrated sample of VLPs, 500ml of Beppu-7 culture was harvested at the beginning of the stationary growth phase (OD 0.8). The cells were removed from the culture by two rounds of centrifugation at 1500xg and 2000xg for 20minutes and 30 minutes respectively. The resultant supernatant was filtered using a 0.45µm filter, after which the pH of the culture was raised to 6.5 with 1M NaOH. 52.5g of PEG600 and 29g of $NaCl_2$ were added to the supernatant and left to stir over night at 4°C. The next day, the supernatant was centrifuged at 23000xg for 30 minutes and the resulting pellet was resuspended in 5ml of TA-buffer. For the density gradient, 4.5g of $CsCl_2$ were dissolved in 5ml of MillQ and added to the 5ml VLP concentrate. The resultant solution was centrifuged at 265000xg for 24hours. This should have resulted in a density gradient showing a clearly visible light gray bad and containing the concentrated VLPs, but this was not the case in several attempts. Instead, the ultracentrifugation vials always showed a thick brown pellet, which could not be fully resuspended.

4.11 Host and VLP Morphology

Throughout the process of purifying the culture, it was regularly screened for the presence of VLPs. Cultures were prepared for TEM at different stages of the growth curve. Samples prepared from cultures at the beginning of the stationary phase, showed a higher number of VLPs than samples prepared from cultures in the early or mid-log phase. The VLPs showed to be enclosed in vesicles ranging from 70–100nm and had tail-like structures egressing from the vesicles ranging in length from 25-130nm and 15-25nm in width. Many vesicles without egressing structures were observed (Figure 24-F), but no uniform structures were observed that were egressed fully from the vesicles. Figure 24-C shows a filamentous structure associated to a larger, electron dense structure egressing from a ruptured vesicle. A change in electron density can be observed in the regions of the vesicle that no longer contain the electron dense structure. Figure 24-F-K show that there is clearly defined, electron dense structure enclosing the filamentous structure egressing from the vesicles, a morphology common for virus capsid proteins. Figure 24-L shows a VLP without a membrane/s-layer coat. This stripped structure can also be seen attached to a cell in Figure 29-B.


A
200 nm
B
200 nm
C
500 nm
D
200 nm
E
200 nm
F
200 nm

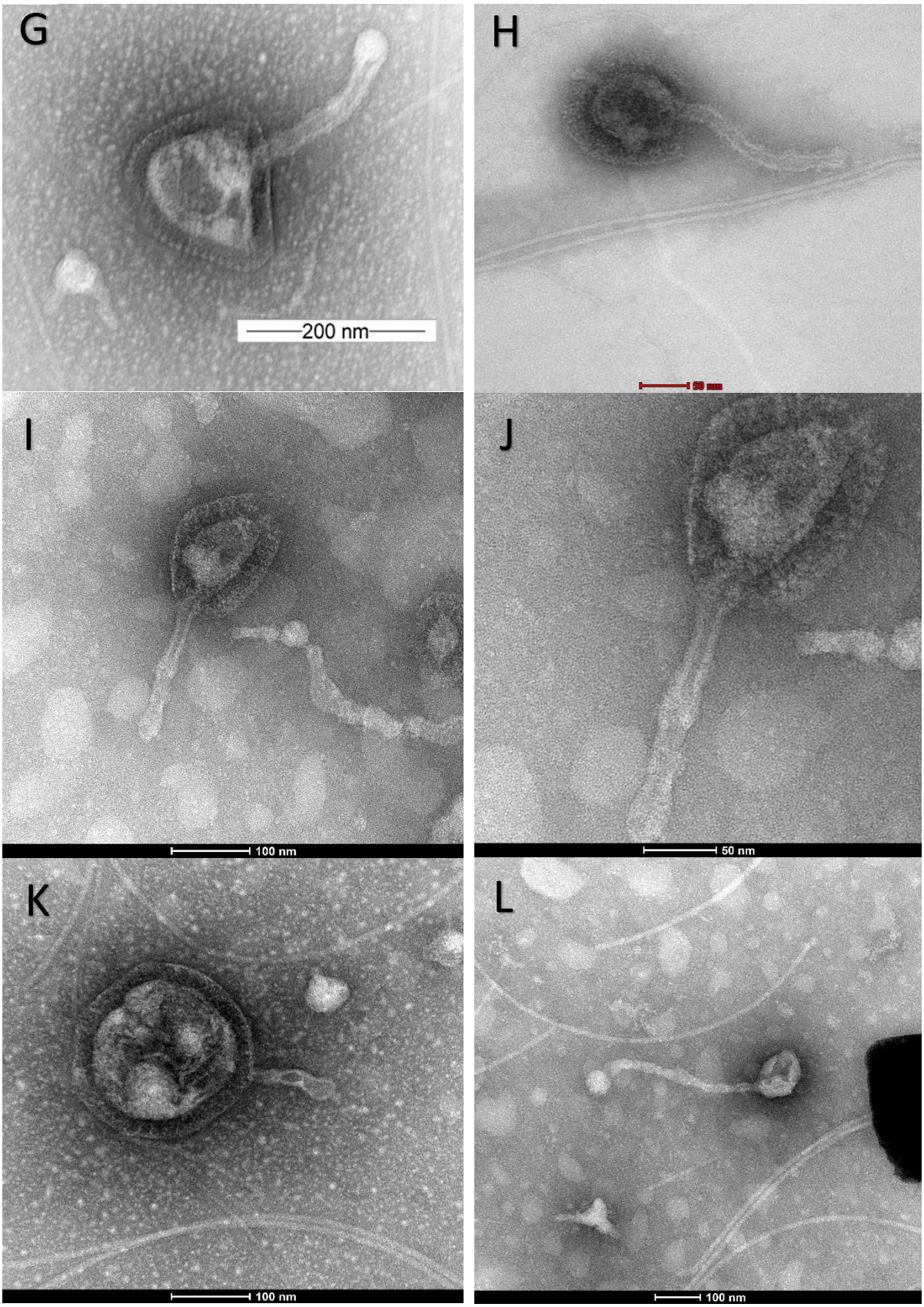


Figure 24 - TEM Images of Beppu-7 *cells and VLPs stained with 1% ammonium molybdate. Scale bar can be found in the individual pictures.*

The VLPs were encapsulated by a structure similar to that of the hosts S-layer covered membrane (Figure 25). In addition to the envelope structure enclosing the vesicle, a lattice pattern similar to that produced by an S-layer could be observed in Figure 24-C,E and H and in Figure 26-B. The hosts bi-layer had a width of 7.5nm and an S-layer with a width of 23nm, totaling to 30.5nm [89][90]. Measurement of the envelope of 30 VLPs and 10 cells showed an average width of 29.4nm and 28.5nm respectively.

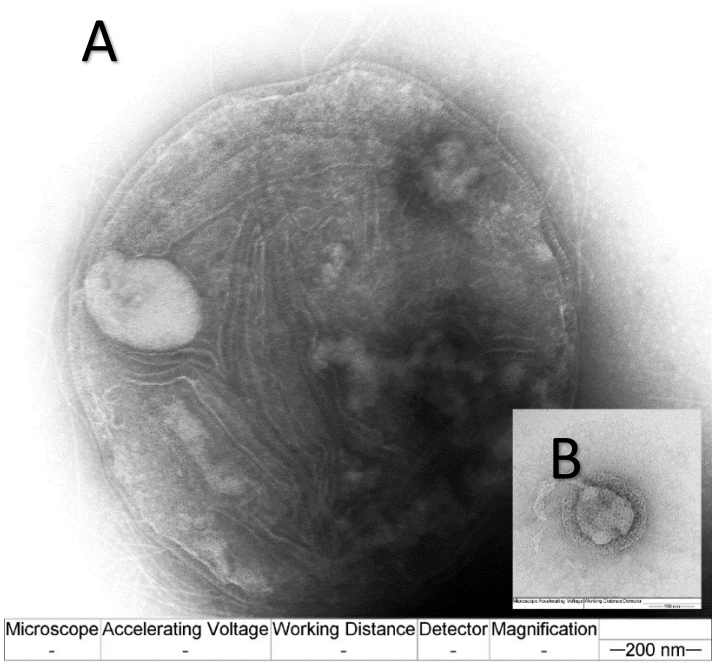


Figure 25 - TEM analysis of a Beppu-7 *concentrate stained with 1% ammonium molybdate. - A: Cell from a culture of Beppu-7*
B: VLP from a 1:200 VLP-concentrate from the same culture

A concentrated VLP fraction with residual cells was prepared as explained above and screened using TEM. Several cells showed an accumulation of spherical structures with an average diameter of 75nm, in close vicinity to the cells membrane. These structures showed the same electron density as the structures enclosed within the VLP associated vesicles with an average diameter of 71nm (Figure 26).

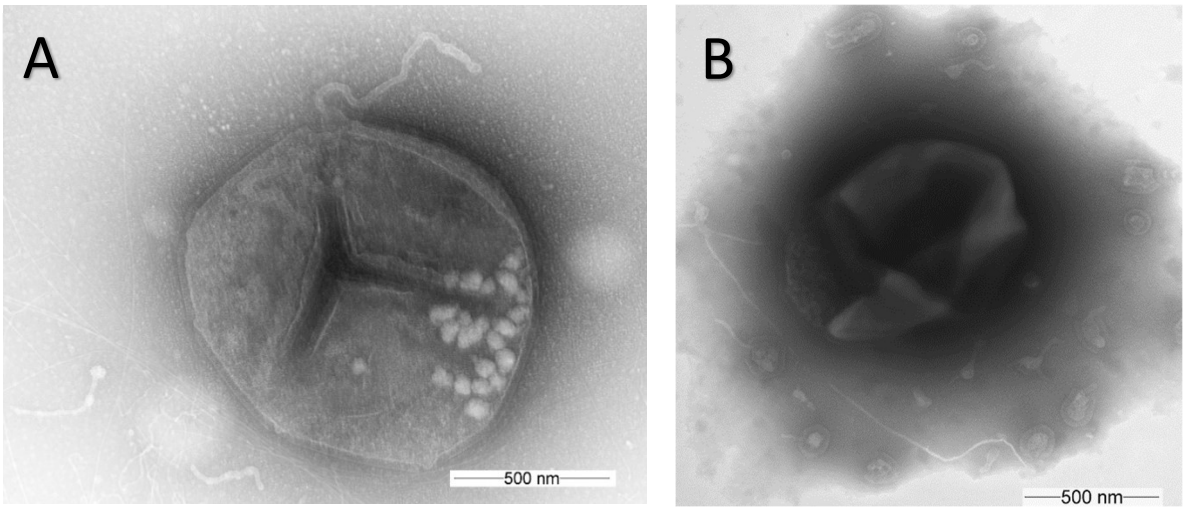


Figure 26 - TEM Images of Beppu-7 *cells and VLPs stained with 1% ammonium molybdate. - Scale bar can be found in the individual pictures. -A: A* Beppu-7 *cell containing spherical structures located in close vicinity to the cell membrane – B: A* Beppu-7 *cell from the same sample showing a accumulation of VLPs surrounding it*

The morphology of the host cells was further studied by scanning electron microscopy (SEM) (Figure 27). The cells appeared to be uniform in size (1.2µm) und morphology, speaking to the purity of the culture. Beppu-7 cells appeared to be indented in a manner to produce a star shaped morphology similar to that of the tropical carambola fruit.

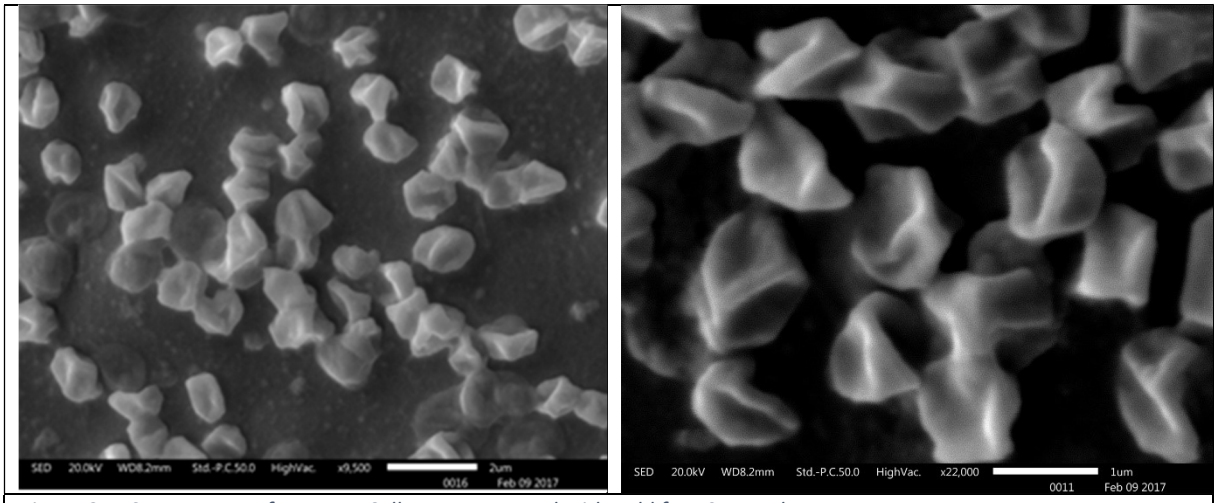


Figure 27 - SEM Images of Beppu-7 *Cells sputter coated with gold for 50seconds*

4.12 Plaque Test:

The plaque test was performed as described in 3.10 using *Sulfolobus acidocaldarius D639*, *S. solfataricus p1* and *S. tokodaii* as lawns and either 100µl of culture, cell free VLP concentrate or super-filtered supernatant were added (Table 3). The super-filtered supernatant was a byproduct of concentrating the VLPS with MWCO30kDa filters and was therefore free of any molecules larger than 30kDa. None of the plates blotted with the 30kDa filtered supernatant showed any plaques, therefore the agent causing the growth retardation had to be larger than 30kDa. The Beppu-7 and DSM639+VLP concentrates caused growth retardation of DSM639 (*Figure 28*) whereas a concentrate of DSM639 supernatant did not cause plaque formation on itself (*Figure 28*).

Table 3 – Plaque Test

		Blot						
		DSM639 CONC (OD=0.6)	DSM639 Cells (OD=0.6)	DSM639+V CONC (OD=0.6)	DSM639+V Superfilt (OD=0.6)	Beppu-7 CONC (OD=0.6)	Beppu-7 Superfilt (OD=0.6)	STK Cells OD=0.2
Lawn	DSM639							
	SSO							
	STK							

CONC= Cell free VLP concentrate, Super filt= Supernatant filtered through a MWCO with a cut-off of 30kDa - DSM639 = Sulfolobus acidocaldarius DSM639, SSO = S. solfataricus P1, STK= S. tokodaii; Green: Plaque was observed, Red: No plaque was observed, Yellow: Plaque Test was not performed.

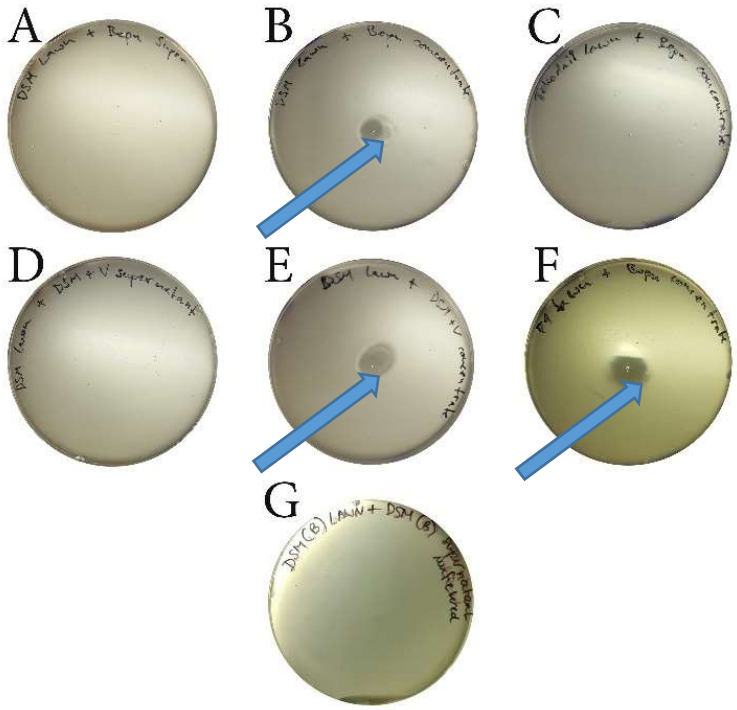


Figure 28 -A: 10µl of 30kDa filtered Beppu-7 supernatant blotted on DSM639 lawn, B: 10µl of concentrated cell-free Beppu-7 supernatant on DSM639 lawn, C: 10µl of concentrated cell-free Beppu-7 supernatant on d10µl of 30kDa filtered DSM639+VLP supernatant blotted on DSM639 lawn, E: 10µl of concentrated cell-free DSM639+VLP supernatant blotted on DSM639 lawn, F: 10µl of concentrated cell-free Beppu-7 supernatant on S. solfataricus lawn, G: Control DSM639 Concentrated Supernatant on DSM639 lawn; Blue arrows show the position of the plaques.

4.13 Protein Analysis

A 200µl cell free concentrate of VLPs was produced from 500ml of culture as described. 20µl of the concentrate, 20µl of pure culture and 20µl of a concentrate containing cells were prepared for SDS-Page analysis using the *Novex NuPAGE SDS-PAGE gel system.* The gel was run for 1hour at 200V, stained with *Coomassie Blue®* for 1hour and destained overnight in MilliQ (Figure 29).

The lane containing the highly concentrated VLPs showed a very strong band at roughly 80kDA, that was not very prominent in the two other samples (Figure 29 – red arrow). All three samples showed a band at 140kDa which could correspond to SlaA subunit of the S-layer samples (Figure 29 – green arrow)[89]. This band shows a high relative abundance in the samples containing cells compared to the very faint band in the cell free VLP concentrate. Furthermore, the cell free concentrate shows a band at about 250kDa that is not present in the other samples (Figure 29 – orange arrow), or at least in comparably very low amounts.

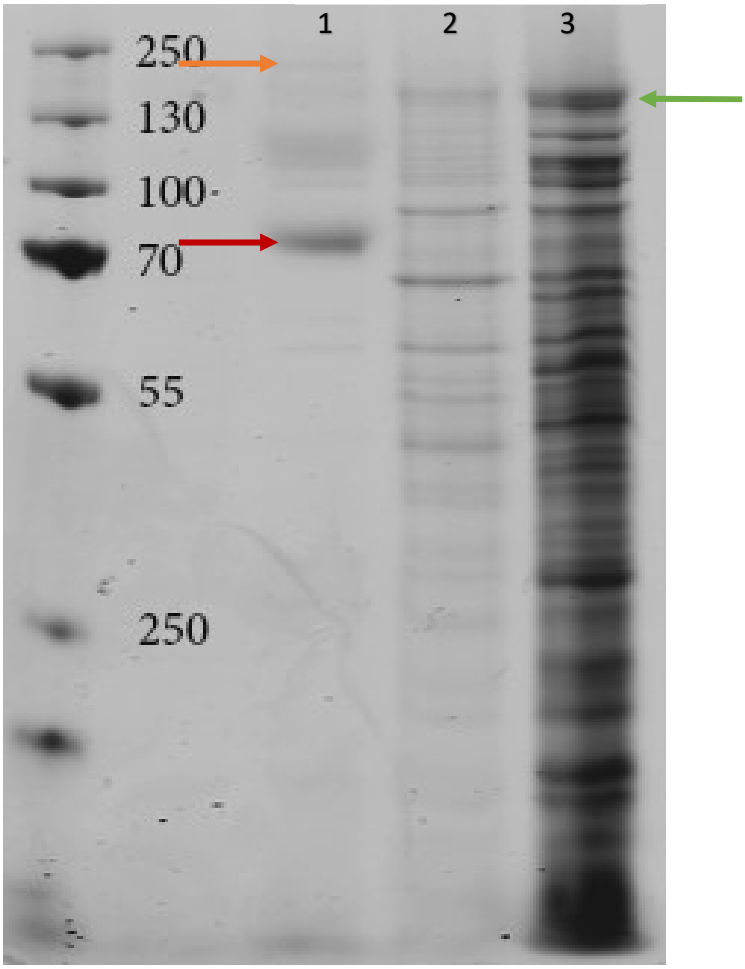


Figure 29 - NuPage *SDS-Gel was run for 1hour at 200V and stained with* Coomassie Blue®. *- Lane 1: Ultracentrifugation of unfiltered supernatant, 2: Cells harvested from a culture with OD 0.5, 3: 10X concentrated* Beppu-7 *culture with OD 0.5*

5 Discussion:

In this study, we attempted to enrich VLP producing strains of *Sulfolobus* from environmental samples and characterize the VLPs found in these samples. The most promising enrichment showing vesicle associated VLPs was Beppu-7 isolated from thermal springs in Beppu, Japan. After establishing a pure culture of the VLP producing Beppu-7, nucleotide analysis of 16S rDNA, CRISPR spacers and Sulfolobicine genes of Beppu-7 showed that the isolated strain was identical to previously described strains of *Sulfolobus acidocaldarius* isolated on different continents. Transmission electron microscopy was used in visualizing and morphologically describing the VLP produced by Beppu-7. The main body of the VLP had an average diameter of 70nm and appeared to be enveloped in a structure that has high similarity to the cellular membrane and S-layer of its host. The envelope of the vesicle-like VLP was pierced at one point, from where a 100-200nm long tail-like structure emerged from the main body of the VLP. This tail-like structure appeared to be used as an attachment anchor to new host cells. It was also shown that previously uninfected strains of *S. acidocaldarius* were producing VLPs after having been exposed to cell free supernatant of a Beppu-7 culture. Although the extraction of nucleotides from the VLPs has not yet been successful, protein extractions from highly concentrated VLP fractions have shown distinct VLP associated bands.

When culturing a new organism, and trying to purify the enrichment, it is imperative to know its position in the phylogenetic tree. Its highly-conserved code give the ribosomal 16S rDNA subunit the property necessary for a genetic marker of evolutionary relation between organisms. Despite its slow rate of mutation, a perfectly matching 16S rDNA sequence normally means that the compared organisms belong to the same species or even strain. In this case, the geographical separation of the two habitats in which DSM639 and Beppu-7 were collected should make this highly improbable. Nonetheless, the 16S rDNA sequence of Beppu-7 matches not only that of DSM639, but those of every isolated *S. acidocaldarius* perfectly. Interestingly, the genomes of 4 strains of *s. acidocaldarius* collected more than 8000km apart seem to be completely identical [80]. This fact speaks for an extremely stable genome that has withstood evolutionary pressure ever since the divergence of the individual strains from a last common ancestor. The stability of the genome and the modifications responsible for it would explain the difficulties in amplifying parts of the genome by PCR.

Even a species of organisms that have such highly conserved genome still must cope with habitat specific complications such as viruses. The adaptive immune system CRISPR found in archaea and bacteria provide the microorganisms with the ability to defend themselves against a changing landscape of viruses. Newly acquired CRISPR spacers have been shown to be integrated at the first position in the existing CRISPR system. It is therefore interesting to see, that even the first spacers in all four CRISPR systems of the newly isolated Beppu-7 and the lab strain DSM639 are identical.

The next step in characterizing this newly cultured archaeon, would be to compare its genome composition to the previously described strains of *S. acidocaldarius* by sequencing its entire genome. This would also aid in understanding the evolutionary impact and of such a conserved genome in organisms living in ecologically diverse environments.

Numerous attempts at DNA extractions form the virus like particles seemed to have failed, as DNA bands were visible on the agarose gels. In order to test the DNA extraction from viral particles, DNA was extracted form 50ml of cell free supernatant of a *S. solfataricus P1* infected with *SSV1*. The extraction was analyzed by gel electrophoresis but failed to show a DNA band, even though DNA was measured to be present at a concentration of 100ng/µl via Nanodrop. A subsequent PCR amplification of a fragment of *SSV1* showed a DNA band when analyzed by gel electrophoresis. This opens the possibility to the fact that DNA was successfully extracted from Beppu-7 VLPs but was not concentrated enough to be shown quantitively by gel electrophoresis. To test this theory, DNA from larger volume (1L) of both *S. solfataricus* and Beppu-7 cultures should be extracted and run on a 0.5% agarose gel.

The standardized procedure for concentrating viruses from copious amounts of culture is to produce a cell free supernatant by low speed centrifugation and subsequent filtration. Polyethylenglycol (PEG) and sodium chloride are then added to the supernatant to aid in the precipitation of the virus particles. The resuspended virus pellet is then further purified and concentrated by a cesium chloride density gradient. This process had failed for the VLP preparation of Beppu-7 numerous times. We can postulate several reasons for the inability to purify the VLPs in this manner. Either the concentrations of salt and PEG for the precipitations were not optimal, and the salt and PEG concentrations must be optimized, or the VLPs were not stable once solubilized in cesium chloride. According to several reports the manufacturer of the used chemicals also seems to play a vital role, since certain viruses are stable and can be precipitated and concentrated in the chemicals produced by one company but are unstable in identical compounds produced by another company. I order to test this theory, all essential chemicals for the precipitation and density gradient would have to be purchased from different producers and the experiment repeated with different combinations and concentrations.

Koch's postulates dictate the process of showing a causative relationship between microbes/viruses and disease. A microbe can only be named as a causative agent for a disease, once all criteria of the postulates have been verified. The potential agent must be isolated from a diseased host and grown in pure culture. The pure agent must then be used to cause the disease in a previously healthy host and again be isolated from the newly diseased host. For viruses the growth in pure culture can be seen as producing a cell free virus fraction, thus removing all possibilities of the transfer of disease from previous host cells. Attempts to verify these postulates for the newly isolated VLPs were

not explicitly successful. Although VLPs were found in newly exposed cultures of DSM639, the VLPs were also observed in an unexposed culture of DSM639. The plaque test on the other hand showed that a concentrate of the cell-free supernatant of both Beppu-7 and DSM639+VLP had a growth retarding effect on DSM639, whereas the concentrate of DSM639 cell-free supernatant did not show this effect. This could imply one of two things, either the growth retardation was induced by VLPs produced by DSM639+V, or the culture was contaminated with Beppu-7 cells during the infection attempt. Due to the high similarity of the Beppu-7 and DSM639 genomes, it is impossible to prove a contamination at this moment.

Transmission electron microscopy images of the VLPsfound in Beppu-7*,* DSM639*+VLP* and DSM639 show VLPs (Figure 25). The fact that the VLPs were also observed in the lab strain DSM639 could mean one of several things. Although great care was taken to prevent cross-contamination when passaging the cultures, this remains a possibility, because the cultures were grown simultaneously in the same oil baths and air shakers. Either viruses or even cells could have been accidentally transferred to the DSM639 culture throughout the experiments. Another explanation might be that these structures were previously observed in cultures of DSM639 but ignored because unrelated hypotheses were being pursued. A definitive answer to this question can only be achieved, by culturing and screening a culture of DSM639 that has not been grown in spatial or temporal vicinity to the Beppu-7 strain.

The exposure of the lab strain DSM639 to the VLPs produced by Beppu-7 did not trigger the integration of a new spacer. This is not unusual, seeing that the lab stain *S. solfataricus* P1, which is readily infected with *SSV1* has yet to show a spacer acquisition against *SSV1*. The lack of spacer uptake is therefore not an indication of a failed infection, but could be a result of the growth conditions of lab strains. CRISPR spacer uptake was shown to work best under low resource conditions when the cell density and thus virus production is relatively low. In a culture with a high number of resources, cells and viruses, the constant activation of the CRISPR system becomes too expensive for the cell and thus the innate immune defenses are mutated [91].

Vesicle production is conserved among all domains of life and has shown to have a diverse array of function, including but not limited to cell-to-cell communication, secretion of antimicrobials, delivery of toxins and virulence factors and acting as decoys for immune cells or phage infections [72]. The association of viruses to vesicles was also observed in a cell free concentrate of *SSV1* produced by *S. solfataricus P1*. Studies of viral secretion in archaea have shown that especially in hyperthermophilic archaea, viruses have developed strategies other than the lytic life cycle known in head-tail phages of bacteria. [50] This difference in egress methodologies is believed to be mostly due to the instability of the viral genome in the harsh environments inhabited by their hosts. *SSV1* which has a spindle shape

when found free in the medium for example, undergoes morphological changes as it egresses from its host. First elongating as it emerges from the host membrane, then morphing to its characteristic lemon shape [51]. Conformations similar to those of *SSV1* mid egress from cellular hosts were seen in association with vesicles when a *SSV1* virus fraction was analyzed by transmission electron microscopy (Figure 32).

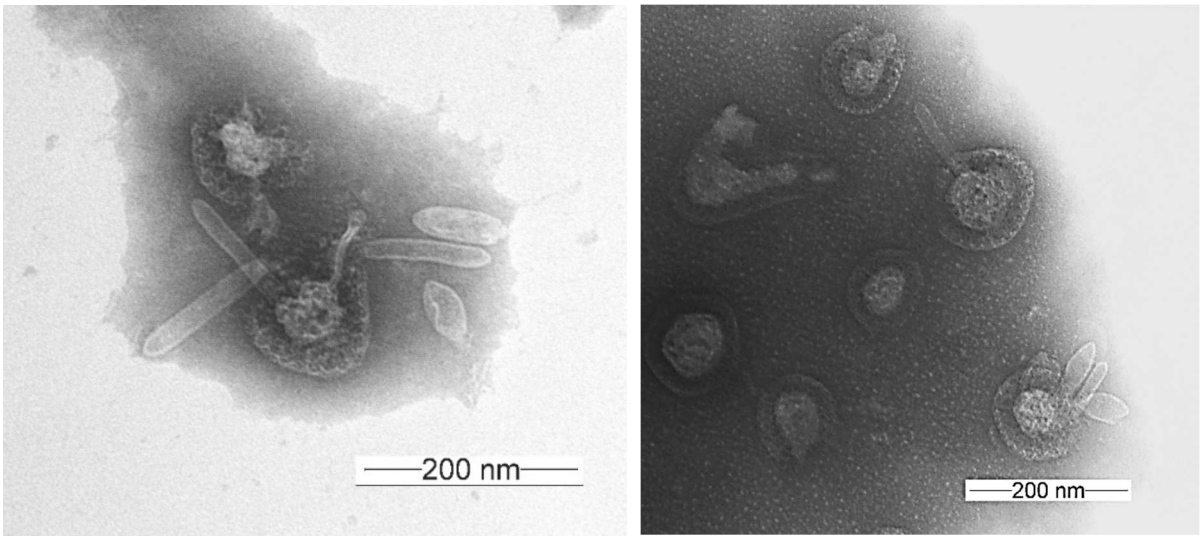


Figure 30
SSV1 interaction with vesicles produced by S. solfataricus P1

We therefore hypothesize that vesicles of acido-thermophiles have an additional function. The observations made throughout this project allow for the speculation that viruses can highjack the vesicle production and ESCRT system of their hosts and thus a fraction of the produced viruses could be exported from the host, packaged inside of vesicles. This would provide the virus with an additional layer of protection from the environment and could increase the time it can spend outside of its host, before DNA degradation begins. The viruses would then egress from the vesicles at a later time, using the identical mechanism used when egressing from the host itself. Figure 34 shows a 3D rendering of what we believe the egress of the VLPs produced by Beppu-7 from the vesicles looks like.

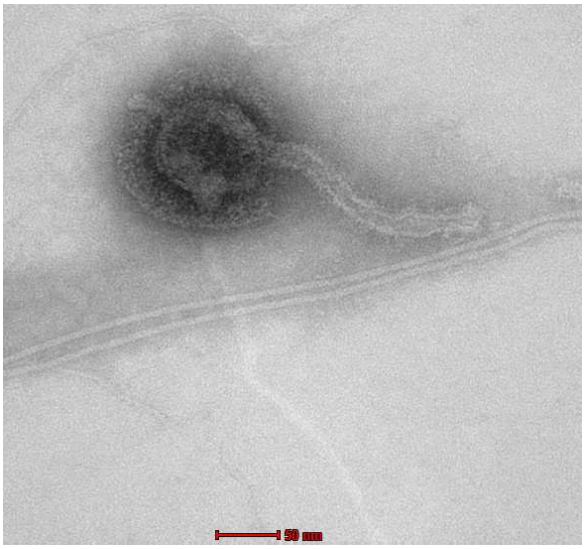


Figure 31
TEM image of a negatively stained VLP found in Beppu-7

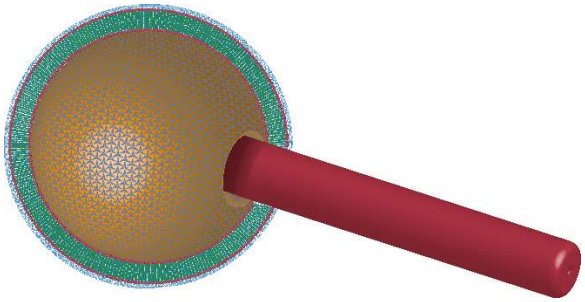


Figure 32
3D Rendering of the predicted structure of the VLP exiting a vesicle.

A protein analysis of a cell free VLP concentrate by SDS-Page has shown a very prominent band at about 74kDa(Figure 31), which is not present in such an intensity in the cellular protein extract, nor does it correspond to prominent vesicle associated proteins. The S-layer protein SlaA has been shown to run slightly above 120kDa and the Sulfolobicines associated proteins run reportedly have a size of roughly 48kDa [69], [71], [89]. Therefore, the band at 70-80kDa could be a virus-associated protein. In the future, a Mass-Spectrometry (MS) analysis and de novo sequencing of the proteins isolated from the VLP enrichment could provide further insight into this elusive virus like particle.

6 Conclusion

In this study, we were able to isolate a novel, VLP producing, *Sulfolobus* strain from the environmental sample Beppu-7 collected at geothermal hot springs in Beppu, Japan, and cultivate it in pure culture. The strain was shown to have high genetic similarities to all previously described strains of *Sulfolobus acidocaldarius* and to sustainably produce VLPs. Protein analysis has shown that the concentrated VLP fractions contain high amounts of proteins that are not in the size range of previously described vesicle-associated proteins, such as the S-layer proteins SlaA, SlaB or Sulfolobicines. De novo sequencing of these proteins will hopefully provide insight into the possible function and origin of these proteins. Further research on the isolated VLP and sequencing of the host genome could reveal this VLP to be the first virus isolated from *Sulfolobus acidocaldarius* and/or a new function of archaeal vesicles.

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8 Supplementary Data:

S1: Environmental Samples Used for Enrichment

Country	Sample	Location	Sample Number	Description	Temperature (°C)	pH
Italy	MCU	Monte Chito Unten	3	ca. 3m above footpath, sulfur smell	96,3	3,9
			4	Steap hill (wett from rain), sample was taken about 7m up the hill and 30cm bellow ground.	95	2,5
			5	vertical cliff, dark-brown --> full of sulfur convections, aprox. 10m above foot path 20cm under ground	100	3,5
	MCO	Monte Cito Oben	1	turned right after first hill, Aluvit, Sulfur, Iron-Oxide(red); 40cm under ground	85	3
			2	cream coloured dirt, turned left after first hill	84,6	2
			3	Dirt was white with lemon yellow crystals, hole on the peak, close to cliff	98,6	3,5
			4	Hole directly on the peak, 50cm under ground, red dirt with white enclosements	83,5	3,8
	SCU	Cava Scura	2	untere Stufe, schlammig	77	7 bis 8
	MRO	Monte Rossi	3		?	5
			4		98,3	6
	SOL	Solfatara	1	Mud-pools, gray, bubbeling, stong H2S smell	39,9	0-1
			2	bubbly, hot water	92,6	0-1
	PIS	Pisciarelli	3a	Pool uphill of Fumerole	93	3
			3b	pH adjusted to 4.5	93	3
			3c	turbid fluid in a otherwise clear pond uphil of fumarole	92	2,9
			3d	pH adjusted to 4.5		
			4	clea liquid (2x50ml)	92	1,5
	TAM	Vale de Tamburo	1	Fumarole at the end of the valley, directly under algea	93	4
			2	slightly deaper	92,4	6
Japan	Bep-2	Tatsumaki = tornado (8)	1	soil (we don't have it), geyser, every 40 min	52	3
	Bep-7	Kamado = oven (4)	1	slurry (red) pond iron(III) oxide (Fe2O3) 510 mg/L	80	3
	7 side	Kamado = oven (4)	1	slurry (red)		2,4
	Bep-8	Kamado = oven (4)	1	red mud	90	
	Bep-10	Yama = mountain (3)	1	transparent, sulfur vapor	99	4,9
	Bep-12	Bozu = skin head (2)	1	mud pond (1) upper, mud (gray)	87	4,5
			2	mud (gray), gray mud pond (2)	87	3,3
			3	mud (gray), gray mud pond (3)	88	3,3
			4	mud (gray), gray mud pond (4) lower	87	3,4
	Bep-13	Myoban = alums	1	slorry mud (gray), gray mud pool, mixture of 2	83	3,6
			2	soil (gary)	90	1,8

	
Figure S1.A Thermal spring in Beppu, Jaban	Figure S1.B Thermal spring in Ischa, Italy

Data S2: Sequence alignment for Sulfolobicine genes