UNIVERSITY OF CALIFORNIA SAN DIEGO

Comparative Metagenomic Analyses of Transporters Within the Archaeal Superphylum, Asgard

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in

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By

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2020

The Thesis of Steven Russum is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2020

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ABSTRACT OF THE THESIS

Comparative Metagenomic Analyses of Transporters Within the Archaeal Superphylum, Asgard

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Upon discovery of the first archaeal species in the 1970s, life has been subdivided into three domains: Eukarya, Archaea, and Bacteria. However, the organization of the three-domain tree of life has been challenged following the discovery of archaeal lineages such as the TACK Superphylum and, more recently, the Asgard Superphylum. The Asgard superphylum has emerged

as the closest archaeal ancestor to eukaryotes and may allow us to improve our understanding of the evolution of life from relatively simple prokaryotes to complex eukaryotes. We characterize the transportomes and their substrates within four metagenomes of Asgard archaea (i.e., Loki-, Odin-, Thor-, and Heimdall-archaeota). Using the Transporter Classification Database (TCDB) as reference, candidate transporters were identified based on sequence similarity, alignment coverage, overlap of hydropathy profiles, TMS topologies and shared Pfam domain content. Identified transport systems are compared within the Asgard superphylum, and to other eukaryotic, archaeal and bacterial transport systems. We found that Asgard organisms rely mostly on the transport of substrates driven by an electrochemical potential. They seem to contain a diverse range of systems required for establishing the membrane potential and proton motive force for subsequent ATP production. To varying degrees, the results indicate that Asgard organisms depend mostly on the intake of organic molecules such as lipids, amino acids, and proteins. The transporters identified clearly resemble prokaryotic transporters more than eukaryotic specific transporters. Taken together, the results support that the suggestion that the Asgard superphylum is largely mixotrophic and anaerobic.

Introduction

In this study, we analyzed the transport systems present within the uncultivated archaeal Superphylum Asgard for two fundamental reasons. Firstly, it is recognized that most of the life on Earth remains uncultivated (i.e., not cultured or isolated in a laboratory setting)[1]. Uncultivated microorganisms contain an abundance of novel mechanisms that can potentially be applied to improve current problems in areas such as bioremediation, biotechnology and public health[1,2]. Secondly, the Asgard superphylum has emerged as an interesting group to research for those seeking to elucidate the progression of life from relatively simple prokaryotes to more complex eukaryotes. Phylogenomic analyses, the intersection of studies of genomes and evolution, of the Asgard group has indicated that it may be the closest known archaeal relative to eukaryotes[3,4].

Microorganisms represent the largest and most diverse group of living organisms. Microbes occupy almost every niche on Earth and have contributed significantly to the evolution of complex eukaryotic life[5]. However, due to their vast prevalence and rapid rates of discovery, most microbes remain uncultivated[1,6]. Thus, experimental research on live cells has been limited to a tremendously small fraction of microbial life[6]. Furthermore, these yet to-be cultured microorganisms are essential to modern biological research, as they contain an abundance of novel mechanisms[1]. Research uncovering the novel molecular mechanisms of unexplored microorganisms is expected to provide essential insights that can potentially be applied to solutions in biotechnology and drug discovery[1,2].

The genome sequences of uncultivated organisms offer unique information regarding their genetic and metabolic properties and serve as a basis for functional genomic research. Metagenomics, the study of genomic data derived from environmental samples, provides researchers with the tools to explore the genomic information of uncultivated microbiomes[7,8].

Metagenomic research of uncultured organisms has revealed unexpected physiological and metabolic capabilities[8,9].

We aim to reveal physiological traits of uncultivated organisms through the characterization of transport systems. Transport proteins are vital to the physiology and survival of a cell, constituting about 10% of the average cell's proteome[10]. They drive crucial cellular functions such as the uptake of nutrients and the export of metabolic end products, drugs, and toxins[11,12]. Comparative genomics, a powerful method utilized by bioinformaticians to observe relationships between genomes and proteomes of different species, has contributed to the characterization of consequential transporters, such as those associated with drug resistance in pathogens and oncogenic cells[13,14]. Additionally, a firm understanding of the structures and functions of transporters is crucial, seeing that defects in transporters can lead to severe diseases such as Cystic Fibrosis[14]. The characterization of transporters involved in these conditions is the first step in the development of targeted treatments. New findings regarding transporter mechanisms may aid the continuing development of vital drugs and treatments.

Research conducted in the Saier lab has identified transport proteins within various species across all domains of life, ranging from bacteria to humans[15]. The characterization of transport proteins has provided a wealth of information regarding pathogenicity in *E. coli*[16] and *P. aeruginosa*, an antibiotic resistant pathogen that is the predominant cause of mortality in Cystic Fibrosis patients[17]. The analyses conducted on these pathogenic organisms have provided a source of information available for future clinical studies. Similar studies will be conducted here on four model metagenomes from a superphylum that may help elucidate the origin of the first eukaryotic cell.

The Asgard Superphylum, which is phylogenetically related to the TACK Archaea superphylum, is a group of uncultivated Archaea of particular interest due to studies revealing their phylogenetic relationship to eukaryotes[3,4]. Prior to the discovery of Asgard, the TACK superphylum was proposed to be the closest known archaeal relative to eukaryotes[18]. Additionally, the number of eukaryotic signature proteins in Asgard archaea surpasses the number found in previously discovered archaeal groups and supports the hypothesis of an archaeal ancestor to eukaryotes[3,4,18–20]. The Asgard superphylum contains four phyla named after the Nordic gods: Lokiarchaeota, Thorarchaeota, Odinarchaeota, and Heimdallarchaeota[3,4,21]. The sampling locations of the extant Asgard reveal that they inhabit various environments, and are most prominent in methane hydrate seafloor sediments[22].

Phylogenomic analyses of the Asgard archaea have challenged the notion that life is separated into three distinct evolutionary lineages (i.e. domains): the Eukarya, Bacteria, and Archaea[23]. These studies led to the proposal that an archaeal lineage is the most probable candidate to act as the phagocytosing host cell involved in the primary endosymbiotic events. This proposed host cell would have acquired the proteobacteria, the precursor to the mitochondrion, resulting in the first eukaryote, in the process termed eukaryogenesis[20,24]. However, this notion has been opposed, due to research noting that although the Asgard archaea possess the cytoskeletal framework for phagocytosis, they are missing essential contributions from bacterial and eukaryotic innovation necessary for phagocytosis as it occurs in eukaryotes[25].

Comparative analyses revealed the presence of eukaryotic signature protein (ESP) homologs in Asgard that, in eukaryotes, are involved in cytoskeleton remodeling, vesicle trafficking, and the endomembrane system. The functions of the ESP homologs, such as actin, GTPases, and longin domains, are not fully known, but their presence suggests that Asgard

organisms contain primitive endolysosomal-like capacities and cytoskeletons[3,4,26]. Intriguingly, Thorarchaeota encodes additional ESPs involved in intracellular trafficking pathways including Endoplasmic Reticulum-to-Golgi transport, secretion, and autophagy[4,26]. These eukaryotic-like proteins, in Thor, include some components of the TRAPP complex, which is a highly conserved multi-subunit complex conserved from yeast to humans[27], and Sec23/Sec24 protein families, which are essential domains in vesicular transport[28].

Although there are gaps in what is known about the physiological capabilities of the Asgard group, the state of current research has concluded that Asgard organisms are anaerobic and mixtotrophic. Thus, the Asgard have been shown to use a mix of energy and carbon sources from different trophic modes such heterotrophy and autotrophy[22,29]. The Wood-Ljungdahl carbon fixation pathway (WLP) [or acetyl-coenzyme A (CoA) pathway], hypothesized to be the oldest carbon fixation pathway on Earth, represents a means for the fixation of acetyl-CoA from two carbon dioxide molecules concomitant with the generation of energy[30]. All Asgard phyla contain key enzymes of the WLP, and therefore may be capable of acetogenesis[21,29,31]. Furthermore, these organisms may use the end products of WLP as electron acceptors and therefore may be capable of heterotrophic growth[21]. Studies have revealed the presence of essential proteins involved in the import and degradation of organic molecules such as carbohydrates and proteins[21,22,31–33]. It should be noted that the Asgard metagenomes contain the essential components of glycolysis except for the key initial enzyme, hexokinase[22]. Thorarchaeota may play a role in reducing intermediate sulfur compounds generated by the oxidation of sulfides in the sulfate-methane transition zones of its sediment sampling location[21]. In contrast to the anaerobic Lokiarchaeota and Thorarchaeota, most recent research has shown that some Heimdallarchaeota metagenomes contain oxygen dependent metabolic pathways[33].

Although Asgard's relationship to eukaryotes is still disputed[34], the findings reported above have been interpreted to support a two-domain tree of life, wherein the eukaryotes would be recognized as a monophyletic group stemming from the domain Archaea[3,4,20]. We plan to contribute to the established research on the Asgard Superphylum by determining the relationships the transporters share with eukaryotes, archaea, and bacteria. Although most recent research has annotated the full metagenomes of Asgard for the purpose of characterizing their physiology[35], there has been little research comparing the presence of proteins responsible for membrane transport between the Asgard phyla. Bulzu et al. have detailed an in-depth characterization of the metabolic capabilities and the transporters in the Heimdallarchaeota metagenome that will be analyzed in this study, Heimdall_LC_3[33]. Therefore, to contribute to the knowledge of the overall physiology of Asgard, we have sought to identify the types of transporters and their probable substrates encoded within the metagenomes of these four groups of Asgard organisms. We hope to gain further insight into their physiology and their capabilities to interact with their environment.

Methods

Identification of Transport Systems

The protein sequences of the four Asgard metagenomes (Loki[3], Thor[4], Heimdall[4], and Odin[4]) were retrieved from NCBI. One proteome from each phylum was chosen for analyses based on the total length of their sequenced metagenomes, the level of completeness, the number of citations associated with the metagenome and their relevance to the evolution of eukaryotic complexity. All chosen metagenomes were the largest of their respective phyla and had been cited in literature regarding Asgard's close phylogenomic relationship to eukaryotes. For Thorarchaeota, we analyzed the second largest Thor metagenome because it was instrumental in the grouping of Thor into the Asgard superphylum. Furthermore, Thor (previously described as a part of

bathyarchaeota[36]) had been rigorously characterized[21,36,37] and we sought to contribute to the metagenome of the lesser annotated Thor metagenome. The proteomes of each of the four Asgard phyla were screened against all of the proteins contained in the Transport Classification Database (www.tcdb.org) for transport protein homologues in July 2018 using GBLAST[38]. GBLAST runs BLAST[39] to compare protein sequences from the metagenomes to proteins within TCDB. GBLAST reports E-values, protein length, sequence identity, alignment coverage, hydropathy profiles estimated with the Web-based Hydropathy, Amphipathicity and Topology (WHAT) program[40], Transmembrane segments (TMS) inferred with HMMTOP[41], and the substrate(s) associated with the matching TC system.

Candidate homologues were identified if alignments showed E-values $< 10^{-5}$ with alignment coverages ≥ 60% when proteins were of comparable size. Lower coverages were also allowed when one of the proteins was > 60% larger than the other to identify potential fusions. Since two proteins may display a significant E-value due to aligned hydrophilic regions, it was necessary to examine the alignments to prevent false positives in assignments where there is no similarity in the transmembrane domain. Confidence in homology inferences was strengthened when hydropathy alignments showed overlapping TMSs and conserved Pfam[42] domains (domains are conserved parts of a protein that can function and form a tertiary structure independently). Putative orthologous proteins were identified across all four metagenomes when high-confidence and high-coverage alignments (allowing for potential fusions) with the homologous protein in TCDB were found.

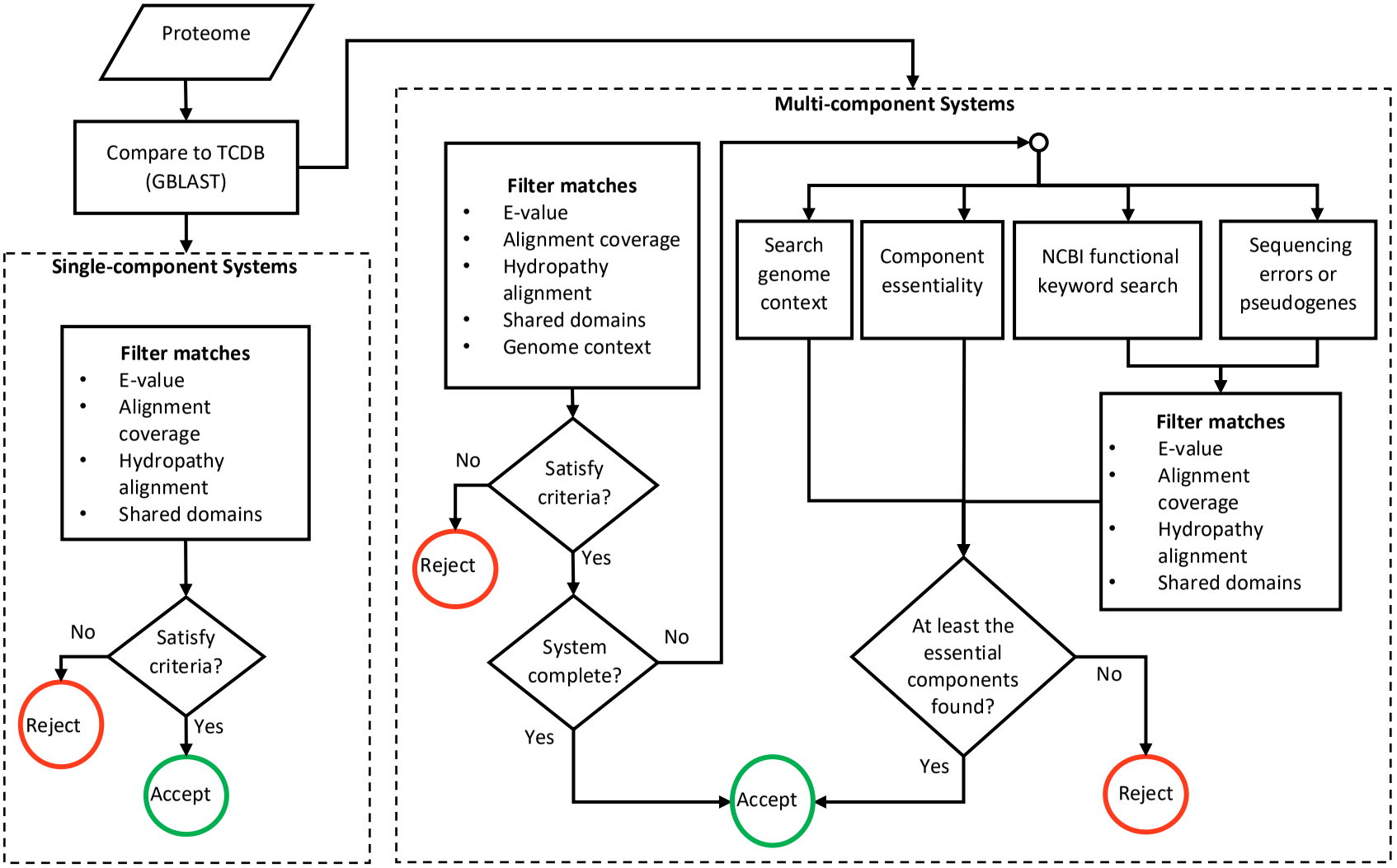
Proteins with no BlastP hits or showing poor scoring alignments (E-values $\geq 10^{-5}$) when compared to TCDB were also examined to identify distant members of established TC families and novel families of transporters. The main goal in this case was to increase the scope of

transporter sequence diversity represented in TCDB. Distant members of a given family were identified when candidate transporters in the metagenomes (having at least 4 TMSs) shared the same Pfam domains or clan as established members of the family in TCDB. If no Pfam domains were found in candidate transporters, we attempted to project the characteristic domain(s) of the target TC family onto the candidate transporter using a method previously published by the Saier group[43,44].

Identification of Multicomponent sequences.

In addition to the strategies listed above, multicomponent transport systems were identified by further taking into consideration the genomic context of the components (multicomponent systems are often found in operons), comparison against transporters with the expected annotation from other databases (e.g., UniProt, NCBI), the essentiality of the components (missing components are tolerated when they are not essential for transport function), and the possibility that different transport systems may share components (e.g., ATPases, some receptors, and other accessory proteins). When no matches in TCDB were found for a specific component, we queried UniProt/NCBI for all proteins with the annotation of the missing component. These proteins were then collected and BLASTed against the corresponding meta-proteome. If at this point, an essential component could not be found in the genome, we searched for the footprint of the gene at the DNA level with BlastX, since it may have not been included in the meta-proteome because the sequence is not yet complete, or there are sequencing errors, or because the component turned into a pseudogene. A flowchart presenting the strategy used to identify homology between protein sequences from the Asgard metagenomes to annotated systems in TCDB is illustrated in Figure 1.

Figure 1. Flowchart illustrating the strategy for characterizing single-component and multi-component transport systems. Candidate transporters homologous to TCDB systems were initially selected using a maximum BlastP E-value threshold of 10^{-5} and an alignment coverage above 60%. Candidate transporters were then subjected to the multiple criteria shown in this diagram. Shared domains were identified with Pfam. Hydropathy curves were analyzed with our in-house program QUOD.


Proteome
Compare to TCDB (GBLAST)
Single-component Systems
Filter matches
E-value
Alignment coverage
Hydropathy alignment
Shared domains
Satisfy criteria?
No
Yes
Reject
Accept
Multi-component Systems
Filter matches
E-value
Alignment coverage
Hydropathy alignment
Shared domains
Genome context
Satisfy criteria?
No
Yes
Reject
System complete?
No
Yes
Accept
Search genome context
Component essentiality
NCBI functional keyword search
Sequencing errors or pseudogenes
Filter matches
E-value
Alignment coverage
Hydropathy alignment
Shared domains
At least the essential components found?
No
Yes
Reject
Accept

Results

Metagenomes of Asgard

Asgard genomes are represented by their metagenomes, which consist of segments of overlapping DNA (i.e., contigs or scaffolds). The metagenomes of Lokiarchaeota (Loki) and Heimdallarchaeota (Heimdall) contained over 5 million base pairs while Thorarchaeota (Thor) and Odinarchaeota (Odin) contained roughly 3 million and 1.5 million base pairs, respectively[3,4]. Further information on the metagenomes of the four selected Asgard metagenomes that we examine here is provided in Table 1.

Table 1. Overview of metagenomes of the four selected Asgard phyla. Relevant information provided in NCBI of the four Asgard metagenomes. "Total proteins" indicates predicted proteins based on the metagenome assemblies as of 2019. "Total number of transport proteins" indicates the number of proteins that were predicted in this study to be homologous to a TCDB transporter. "Contig count" refers to the number of overlapping DNA sequences that constitute the metagenomic assembly.

Metagenome	Candidatus Heimdallarchaeota archaeon LC_3 (Heimdall)	Lokiarchaeum sp. GC14_75 (Loki)	Candidatus Thorarchaeota archaeon AB_25 (Thor)	Candidatus Odinarchaeota archaeon LCB_4 (Odin)
Assembly Accession	GCA_001940645	GCA_000986845	GCA_001940705	GCA_001940665
Total proteins	5514	5384	2914	1584
total number of transport proteins	419	336	296	166
transport proteins per total proteins	7.6%	6.2%	10.2%	10.5%
metagenome size (Mbp)	5.68	5.14	2.86	1.46
Contig Count	157	504	264	9
sampling location	Loki's castle hydrothermal vent sediment	Loki's castle hydrothermal vent sediment	Marine sediment Aarhus Bay, Baltic Sea	Lower Culex Basin hot spring

Overview of Transport types in Four Asgard Metagenomes

The Transporter Classification (TC) system classifies transport systems using a five-tier system based on their structures and functions. The first tier is divided into 5 well-defined categories (TC classes 1-5) and 2 less well-defined categories (TC classes 8-9). The five well defined classes are designated: (1) channels/pores, (2) electrochemical potential-driven transporters known as secondary carriers, (3) primary active transporters, (4) group translocators, and (5) transmembrane electron carriers. The 2 less well-defined classes are (8) auxiliary transport proteins and (9) putative or incompletely characterized transport systems. Following the TCDB convention, transporters are further ranked in increasingly specific groups: subclass, family, subfamily, and lastly, the actual transport system[12].

As outlined in Methods, the four Asgard metaproteomes were screened using GBLAST and TCDB to identify the transporters present. Of importance to note is that since we are analyzing metagenomes, which are partial genomes, we can only identify what homologs are present. Moving forward, note that transport proteins that are regarded as missing from a particular metagenome does not reflect proteins missing from the actual organism because the genomic data is not complete. Examination of transport proteins revealed that Heimdall contains the most identified transport proteins (419) and Odin contained the least (166) (Table 1). The remaining Asgard metagenomes, Thor and Loki, contain 296 and 336 total recognized transport proteins, respectively. Loki contains the smallest percentage of transport proteins (6.2%) compared to the other Asgard metagenomes while Odin, despite containing the smallest metagenome size, encodes the largest percentage of transport proteins (10.5%) (Table 1).

TC subclass 1.A consists of α-type channels. These are ubiquitously found throughout all domains of life. They usually catalyze the transport of substrates through a pore or channel within

a membrane by an energy-independent process. The four Asgard transportomes were found to include 2.7-6.1% α-type channels. Heimdall and Odin contains approximately two times the percentage of α-type channels as compared to Loki and Thor (Table 2).

TC subclass 1.C consists of pore-forming toxins. These systems are synthesized in one cell, secreted, and form transmembrane pores in another which leads to lysis of the cell. Loki was the only Asgard metagenome that contained proteins of this subclass, which were two homologs from the bacterial hemolysin A (B-Hemolysin A) family (TC# 1.C.109). Loki B-Hemolysin A homologs presumably form pores in membranes of cells and consequently cause cell lysis[45].

TC subclass 1.E consists of holins. Holins perform a variety of functions in bacteria, but mainly serve to promote cell death[46,47]. They are encoded by bacteria or bacteriophages. In both cases, they induce cell death by forming pores in the cytosolic membrane of the bacteria that produce them, and thereby, release endolysins that hydrolyze the cell wall to cause cell death[46]. Holins make up approximately 1% of the transportomes of Heimdall and Thor but were not found in the metagenomes of Loki and Odin (Table 2). These putative holin homologs, of unknown function, all belong to the Putative 3-4 TMS Transglycosylase-associated Holin (T-A Hol) family (TC# 1.E.43).

TC subclass 2.A consists of porters. Porters (e.g., antiporters, symporters, and uniporters) are secondary carriers that permit transport in a carrier-mediated process that is usually driven by an electrochemical potential. Porters make up the largest known transporter type in all Asgard metagenomes, constituting 27.8-36.7% of the total transport systems, where they perform a diverse variety of functions (Table 2).

TC subclass 3.A consists of primary active transporters that derive their energy for transport from the hydrolysis of diphosphate bonds. Based on the total number of proteins involved

in transport, this group of transporters is the most abundant transport type for all Asgard metagenomes. However, most primary active transporters are multicomponent systems. Thus, primary active transport systems make up the second largest known transport type based on the total number of transport systems present. Loki encodes the smallest percentage of these systems (17.1%) while the other Asgard metagenomes contain approximately 22% (Table 2).

TC subclass 3.D includes the oxidoreduction-driven transporters. These systems derive energy for transport of a solute (e.g., an ion) from the transfer of electrons from a reduced substrate to an oxidized substrate. The abundance of oxidoreduction-driven transport systems ranges from 1.2-4.1% (Table 2). Odin contains (percentagewise) approximately 2 times more of such transporters than Heimdall and Thor, and about 4 times more than Loki

TC class 4 consists of group translocators. Group translocators either loosely or tightly couple substrate modification with the transport process. TC subclass 4.C represents the Acyl CoA ligase-coupled transporters and is the best represented group translocator subclass in Loki and Thor. Loki contains the largest fraction of such transport systems, 9.7%, while these proteins only comprise 1% of Odin's transportome. Heimdall and Thor's transportome includes Acyl CoA ligase-coupled transporters at 2.2% and 4.8%, respectively (Table 2). TC subclass 4.D includes the polysaccharide synthase/exporters and comprises the largest percentage of group translocators in Heimdall and Odin. These transporters make up 1.1-3.3% of the total transport proteins in all Asgard metagenomes. TC subclass 4.F consists of the choline/ethanolamine phosphotransferase 1. This subclass's representation in the Asgard metagenomes is like that of 4.D, comprising 1-2.3% of the total transport systems.

TC class 5 represents the transmembrane electron carriers. This class influences the membrane potential by transporting either one or two electrons across the membrane in either

direction. This class is divided into two subclasses depending on the number of electrons transported across the membrane; TC subclass 5.A transports two electrons as a pair, while 5.B transports single electrons across the membrane. While Odin contains no recognized transmembrane electron carriers of any kind, two-electron transmembrane carriers represent 0.5-2.9% of the remaining Asgard metagenomes. Heimdall contained over 3 times the percentage of two-electron transmembrane carriers compared to Loki and Thor. Loki is the sole metagenome encoding a single homolog from subclass 5.B (Table 2).

TC subclass 8.A consists of auxiliary transport proteins. These proteins do not participate directly in transport, but in some way influence or facilitate transport and do not belong to another TC multicomponent system. This subclass makes up 1.2-2.6% of each Asgard transportome (Table 2).

TC subclass 9.A contains the transporters of unknown biochemical mechanism. These transporters' functions are known, but no described mode of transport or energy coupling mechanism has been reported. Each of the Asgard transportomes is made up of 0.4-1.6% from TC subclass 9.A. TC subclass 9.B is comprised of putative uncharacterized transport proteins. This subclass is the second-best represented group of transport system (23.5-29.4%) of the Asgard transportomes. An overview of the presence of transport systems in the four Asgard metagenomes can be found in Table 2. This table was generated based on the ChEBI ontology[48].

Table 2. Overview of the Asgard transport protein numbers based on TC subclass. Values in parentheses represent the total number of transport proteins per subclass if the number of systems is different from the number of systems per subclass.

TC subclass and description	Number of transport systems/subclass				Percent (%) of transport systems			
	Heimdall	Loki	Thor	Odin	Heimdall	Loki	Thor	Odin
1.A, Alpha-type channel	16	8	5	6	5.9	3.1	2.7	6.1
1.C, Pore-forming toxins	0	2	0	0	0	0.8	0	0
1.E, Holins	3	0	1	0	1.1	0	0.5	0
2.A, Porters (uniporters, symporters, antiporters)	76 (80)	85	62	36 (39)	27.8	32.9	33.2	36.7
3.A, P-P-bond-hydrolysis-driven transporters	62 (170)	44 (100)	40 (110)	22 (63)	22.7	17.1	21.4	22.4
3.D, Oxidoreduction-driven transporters	5 (32)	3 (25)	4 (41)	4 (28)	1.8	1.2	2.1	4.1
4.C, Acyl-CoA ligase-coupled transporters	6	25	9	1	2.2	9.7	4.8	1.0
4.D, Polysaccharide synthase exporters	9	3	2	2	3.3	1.2	1.1	2.0
4.F, Choline/Ethanolamine Phosphotransferase	4	6	2	1	1.5	2.3	1.1	1.0
5.A, Transmembrane two-electron transfer carriers	8 (15)	2	1	0	2.9	0.8	0.5	0
5.B, Transmembrane one-electron transfer carriers	0	1	0	0	0	0.4	0	0
8.A, Auxiliary transport proteins	7	3	3 (4)	2	2.6	1.2	1.6	2.0
9.A, Recognized transporters of unknown biochemical mechanism	3	1	3 (4)	1	1.1	0.4	1.6	1.0
9.B, Putative transport proteins	74	75	55	23	27.1	29.1	29.4	23.5
Total	273 (419)	258 (336)	187 (296)	98 (166)	100	100	100	100

α-type Channel Proteins (TC Subclass 1.A)

The ubiquitous[49] Voltage-gated Ion Channel (VIC) superfamily (TC# 1.A.1) was missing from all Asgard genomes except for Heimdall. Heimdall has one protein homolog of a VIC transport system (TC# 1.A.1.13.8), predicted to be capable of transporting potassium at low membrane potentials[50].

The four Asgard metagenomes have multiple homologs within different families involved in regulating calcium ion levels across lipid bilayers. Each of the four Asgard metagenomes contain 1-2 proteins homologous to transporters within the Calcium Load-activation Calcium Channel (CLAC) Family (TC# 1.A.106). These Asgard proteins display high levels of sequence similarity (E-value < 10^{-15}) and exhibit over 90% shared coverage to proteins from the TC subfamily 1.A.106.2, which is exclusively made up of archaeal proteins. Each Asgard metagenome, except Thor, contains a homolog of the Presenilin ER Ca^{2+} Leak Channel (Presenilin) Family (TC# 1.A.54). Odin and Heimdall both contain one protein homologous to characterized eukaryotic presenilins, while Loki and Heimdall have homologs of presenilins that have been biochemically shown to act both as proteolytic enzymes and cation channels in archaea[51]. In these four Asgard metagenomes, these channels may function in controlling the influx of calcium ions into the cell[52].

Each Asgard metagenome examined here, except for that of Odin, contains 1-2 homologs of the Small Conductance Mechanosensitive Ion Channel (MscS) Family (TC# 1.A.23). These proteins relieve osmotic pressure in hypotonic solutions through the release of anions in response to cellular expansion[53,54]. Notably, no member of the Large Mechanosensitive Ion Channel (MscL) family was present in any of the four metagenomes.

All four Asgard metagenomes contain homologs of the Cation Channel-forming Heat Shock Protein-70 (Hsp70) family (TC# 1.A.33). Hsp70 has been identified across all domains of life, and in eukaryotes, they are capable of forming membrane channels[55,56]. However, no evidence of this characteristic has been shown in prokaryotes. All Asgard metagenomes have 1-2 homologs of the chaperone protein DnaK (TC# 1.A.33.1.4). The significantly high sequence similarities of these Asgard homologs (E-value < 10^{-90}) are clearly indicative of the orthologous nature of these proteins.

We provide support for previous research[4] that has identified homologs of the eukaryotic specific Magnesium Transporter1 (MagT1) family (TC# 1.A.76) in all Asgard metagenomes. MagT1 and its homologs, determined by strong sequence similarities (E-value < 10^{-15}) and coverage of above 70% to proteins in NCBI, were all of eukaryotic organisms. MagT1 homologs in these four Asgard metagenomes are inferred by homology to probably be Mg^{2+}-specific, voltage dependent, ion channels. In addition to MagT1 transporters, ubiquitous magnesium transporters[57] are present in Heimdall and Odin. Heimdall encodes a single homolog from the Mg^{2+} Transport-E (MgtE) family (TC# 1.A.26) and contains a homolog of a putative system from the Cylcin M Mg^{2+} Exporter (CNNM) family (TC# 1.A.112). Odin also encodes a homolog of a Magnesium transport protein (TC# 1.A.35.3.2) of the CorA Metal Ion Transporter (MIT) family (TC# 1.A.35).

The remaining channels are only present in half or less of the Asgard metagenomes. Only Heimdall contains a homolog of the Calcium Transporter A (CaTA) family (TC# 1.A.14). Odin contains a single putative fluoride ion transport homolog (TC# 1.A.43.1.4) of the Camphor Resistance or Fluoride Channel (Fluc) family (TC# 1.A.43).

α-type Channels Substrates & Conclusion

In conclusion, Heimdall and Odin contain roughly twice the percentage of alpha-type channels, approximately 6%, as compared to Thor and Loki, which contain around 3%. Most of these channels seem to be unknown, nonspecific for ions, or specific for cations such as calcium and magnesium (Figure 2). Some archaeal homologs of the calcium channels in the four Asgard metagenomes are uncharacterized and are therefore grouped into the unknown substrate category in Figure 2. However, eukaryotic channels that have been shown to transport calcium are homologous to these Asgard proteins. While these eukaryotic channels have been reported to function in calcium ion level regulation between endomembranes[52], we can infer that the function of these transport systems in Asgard may be to transport calcium across the plasma membrane. Taken together, the calcium channels in Asgard, which are homologous to characterized and putative calcium channels, may play a role in calcium signaling or the regulation of calcium influx into the cell.

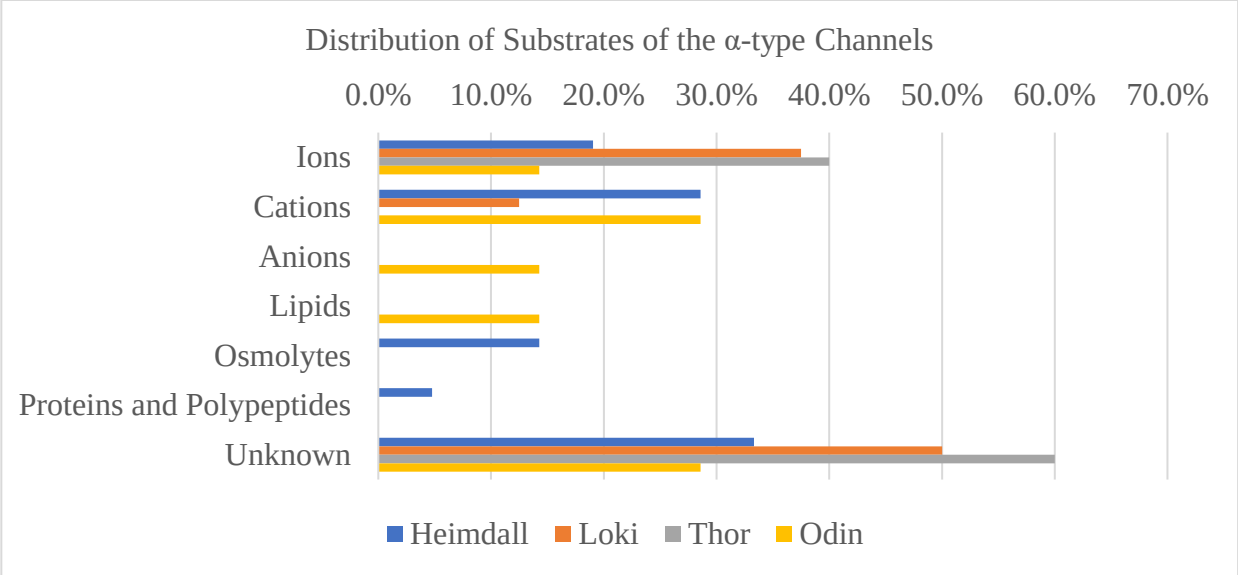


Figure 2. Overview of the predicted substrates transported by α-type channels within the four Asgard metagenomes. The number of each substrate type transported is normalized by dividing the number of transport systems that have been shown to catalyze the transport of a substrate type by the total amount of substrate types transported by the α-type channels for each Asgard metagenome.

Secondary Carriers (TC Subclass 2.A)

The largest group of porters present in nature, the Major Facilitator Superfamily (MFS; TC# 2.A.1), is the best represented group of secondary carriers in Asgard. The MFS is a large and diverse group of uniporters, symporters, and antiporters[58]. MFS transport systems in the Asgard metagenomes make up the smallest percentage of the transportome in Odin (7.1%) and the largest percentage in Loki (14.3%). In Heimdall and Thor, they represent 11.4% and 13.3% of the transportome, respectively. Of the MFS porters, the most common type in all Asgard phyla is the Drug:H^+ Antiporter-1 (DHA1) Family (TC# 2.A.1.2). Although there is extensive evidence regarding DHA1 family's capability to catalyze drug efflux in all domains of life[58,59], it cannot be assumed that drug efflux is the primary physiological function of these transporters. Homologs from the Drug:H^+ Antiporter-2 (DHA2) Family (TC# 2.A.1.3) are present in Thor and Heimdall, including suggested riboflavin and siderophore exporters[60,61]. Indeed, numerous homologs from

the Drug:H^+ Antiporter-3 (DHA3) Family (TC# 2.A.1.21) are present in all Asgard metagenomes except Odin. These DHA3 homologs have been shown to confer macrolide, a type of microbial antibiotic, resistance in *Streptococcus pneumoniae*[62,63]. In conclusion, we observe that a hydrogen gradient must be essential in all Asgard metagenomes to sustain the antiport of toxins and promote a drug resistant phenotype.

Interesting homologs of other MFS homologs include: homologs of a pyruvate/H^+ symporter (TC# 2.A.1.11.3) are present in Odin, Heimdall, and Loki. This transporter is known to take up pyruvate in *E. coli*[64]. Homologs of the Putative Aromatic Compound/Drug Exporter (ACDE) family are found in all Asgard metagenomes, except that of Thor. A homolog of the putative ACDE family (TC# 2.A.1.32.1) is found in Loki, while Odin and Heimdall encode a putative corynebactin (siderophore) exporter[65] (TC# 2.A.1.32.2). Thor encodes a member of the Enterobactin Exporter (EntS) family (TC# 2.A.1.38), whose homolog is known to export the siderophore, enterobactin, in *Salmonella*[66]. Families capable of siderophore export (ACDE, EntS, and DHA2) were not found in Loki.

The following MFS families occur in 50% or less of the Asgard metagenomes. Heimdall contains a homolog of the Organophosphate:P_i Antiporter (OPA) family (TC# 2.A.1.4). This porter (TC# 2.A.1.4.10) takes up both 2-phosphonoacetate and 2-phosphonopropionate[67]. A homolog of the putative thiazole transporter (TC# 2.A.1.6.12) was found in the metagenome of Loki. Heimdall encodes a homolog of the 2-component, NarK1 and NarK2, transport system of the Nitrate/Nitrite Porter (NNP) family (TC# 2.A.1.8). NarK1, the nitrate:proton symporter, and NarK2, the Nitrate:Nitrite antiporter, are likely fused in *P. denitificans*, but are capable of functioning independently[68]. The proximity of these two proteins in the Heimdall metagenome suggests that these proteins may be capable of functioning together, thereby closely coupling transport of nitrate

and nitrite. Thor encodes homologs of a probable glucarate and/or D-galactarate:H^+ symporter[69] (TC# 2.A.1.14.14) and a cis,cis-muconate porter[70] (TC# 2.A.1.15.4). Odin encodes a homolog of the proteobacterial Intraphagosomal Amino Acid Transport family (TC# 2.A.1.53.9). Heimdall contains an uncharacterized porter from the Microcin C51 Immunity Protein family (TC# 2.A.1.61). Loki and Heimdall encode a homolog of the putative 4-hydroxybenzoate uptake transporter (TC# 2.A.1.66.2) that may also transport S-adenosyl methionine. Loki and Heimdall contain homologs of the Glucose Transporter family (TC# 2.A.1.68). Loki encodes a homolog of a niacin uptake porter[71] (TC# 2.A.1.82.4). In addition, 11 uncharacterized homologs from 11 different, poorly characterized, TC families were present in the Asgard metagenomes.

The Glycoside-Pentoside-Hexuronide (GPH):Cation Symporter family (TC# 2.A.2) is a large and ubiquitous family encoded only in the Loki and Thor metagenomes. GPH:cation symporters catalyze the uptake of a range of sugars, mostly glycosides and oligosaccharides, in symport with one or more monovalent cations (H^+ or Na^+)[72]. In Loki, homologs probably capable of transporting the oligosaccharides, cellobiose and melibiose, were identified. Overall, the GPH family may mediate the uptake of small sugars in Loki and Thor.

The transport of amino acids and polyamines is mediated by the Amino Acid-Polyamine-Organocation (APC) Superfamily (TC# 2.A.3). The APC family is represented in all domains of life, and its members transport a variety of substrates by solute:cation symport or solute:solute antiport. The Asgard metagenomes, except for Loki, contain prokaryotic homologs of proteins from this family. Odin is the only Asgard metagenome that encodes a system likely to be capable of importing the polyamine, putrescine (TC# 2.A.3.1.13). Furthermore, the rest of the homologs from the APC superfamily present in Heimdall, Thor, and Odin may take up amino acids and amino acid derivatives[73].

The following homologs are members of the APC superfamily and are present in only one of the analyzed Asgard metagenomes. Thor encodes a sodium-dependent, tyrosine transporter (TC# 2.A.22.5.2) homolog and a putative amino acid uptake porter (TC# 2.A.120.1.11) of the Putative Amino Acid Permease family (TC# 2.A.120).

The Cation Diffusion Facilitator (CDF) Superfamily is represented by 2 different constituent families across Asgard. The Homologs of the CDF family (TC# 2.A.4), present in all four metagenomes of Asgard, likely maintain intracellular homeostasis of zinc, iron and manganese via the export of such ions when present at toxic levels[74,75]. Furthermore, the CDF homologs present in Asgard showed no eukaryotic-specific features, such as histidine rich cytoplasmic loops[76]. All of the Asgard metagenomes, except for that of Odin, contain putative Ca^{2+}:Na^{+} antiport homologs from the Ca^{2+}:Cation Antiporter family (TC# 2.A.19)[77]. The calcium ions that might enter the cell via α-type channels, may be extruded from the cells of Heimdall, Loki, and Thor by Ca^{2+}:cation antiporters.

Multiple proteins in Loki were homologous to a single Zinc uptake protein[78] of the Zinc-Iron Permease (ZIP) family (TC# 2.A.5). The Heimdall, Odin, and Thor metagenomes lacked homologs of the ZIP family.

The Resistance-Nodulation-Cell Division (RND) superfamily (TC# 2.A.6) is present in all Asgard metagenomes due to the presence of HMG-CoA reductase (TC# 2.A.6.6.11) orthologs. These orthologs are homologous to the C-terminal, hydrophilic domain of the essential membrane protein of the Eukaryotic Sterol Transporter (EST) family (TC# 2.A.6.6). Recognizing that these homologs lack TMSs, their function in Asgard may not be involved in transport, but rather as a cytosolic reductase (as annotated in NCBI). Heimdall encodes multidrug resistance proteins of the

RND constituent family, the Hydrophobe/Amphiphile Efflux-2 (HAE2) family (TC# 2.A.6.5), which are commonly found in gram positive bacteria[79].

The Drug/Metabolite Transporter (DMT) Superfamily (TC# 2.A.7) is the second largest family of secondary carriers represented in these Asgard metagenomes. Throughout nature, this superfamily claims a range of transport proteins that can function as nutrient uptake porters, drug/metabolite efflux pumps, and solute:solute exchangers[80,81]. Numerous homologs of the 10 TMS Drug/Metabolite Exporter (DME) family (TC# 2.A.7.3) are present in all Asgard metagenomes. These homologs are mostly uncharacterized or putative DME proteins. A single member of the 4 TMS Small Drug Resistance family (TC# 2.A.7.1), which is an exclusively prokaryotic family, is present in Odin. A choline uptake transporter (TC# 2.A.7.18.1) and a putative choline transporter (TC# 2.A.7.18.4) are present in Heimdall and Thor, respectively. Furthermore, four uncharacterized DMT families were represented in Odin, Thor, and Loki.

Two different families of the Cation:Proton Antiporter (CPA) superfamily, CPA1 (TC# 2.A.36) and CPA2 (TC# 2.A.37), are represented in Thor and Heimdall. These homologs resemble Na^+:H^+ antiporters from prokaryotes.

Three different family constituents of the Ion Transporter (IT) superfamily are present in all Asgard metagenomes, which includes primary active transporters and secondary carriers that exclusively transport organic and inorganic ionic substrates[82]. Asgard contains only secondary carriers of the IT superfamily. All Asgard metagenomes contain 1-5 putative ion transporters similar to one found in magnetosome membranes of *Magnetospirillum* (TC# 2.A.45.2.2) of the Arsenite-Antimonite Efflux (ArsB) family, suggesting their paralagous nature. Loki encodes a homolog of a glycolate permease (TC# 2.A.14.1.2), and Thor contains a putative Na^+-coupled

dicarboxylate transporter homolog (TC# 2.A.47.1.1; this last assignment was inferred from homology to 2.A.47.1.13).

Two different constituent families of the Bile/Arsenite/Riboflavin Transporter (BART) Superfamily are present in all 4 metagenomes of Asgard. Homologs from the Arsenical Resistance-3 (ACR-3) family (TC# 2.A.59) are encoded by these Asgard metagenomes, except for Thor. These ACR-3 homologs may confer resistance to the toxic metalloid, arsenite[83]. An uncharacterized homolog (TC# 2.A.69.4.3) from the Auxin Efflux Carrier (AEC) family is encoded in the Thor and Heimdall metagenomes.

Three different constituent families of the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily (TC# 2.A.66) are represented in the metagenomes of Loki and Odin. They both contain uncharacterized homologs of the Polysaccharide Transport (PST) family (TC# 2.A.66.2). Odin is the only Asgard metagenome to contain homologs of the Putative Exopolysaccharide Exporter (EPS-E) family (TC# 2.A.66.6) and the Uncharacterized MOP-12 (U-MOP12) family (TC# 2.A.66.12).

The L-Lysine Exporter (LysE) superfamily has six different constituent families represented within the Asgard metagenomes. The proteins within the LysE Superfamily share a common ancestral origin which suggests that they share common structural and functional attributes[84]. Loki and Thor contain putative amino acid efflux transporters of the Resistance to Homoserine/Threonine (RhtB) family (TC# 2.A.76). The Ca^{2+}:H^{+} Antiporter-2 (CaCA2) family (TC# 2.A.106) is conserved across eukaryotes and prokaryotes and catalyzes the transport of calcium ions[85]. Uncharacterized homologs from the CaCA2 family are present in all Asgard metagenomes except that of Loki. Loki is the only metagenome to contain a putative manganese exporter of the Mn^{2+} exporter (MntP) family (TC# 2.A.107). Homologs of ferrous iron uptake

porters of the Iron/Lead Transporter (ILT) family (TC# 2.A.108) are present in all Asgard metagenomes, except Odin. A Putative Nickel/Cobalt efflux system (TC# 2.A.113.1.4) and an uncharacterized protein (TC# 2.A.113.1.9) of the Nickel/Cobalt Transport (NicO) family (TC# 2.A.113) are present in Loki and Heimdall, respectively.

Three different families from the Transporter-Opsin-G Protein-coupled Receptor (TOG) Superfamily are represented across these Asgard metagenomes. Loki encodes a high Affinity Ni^{2+}-specific transporter[86] of the Ni^{2+}-Co^{2+} Transporter (NiCoT) family. All Asgard metagenomes encode 1-3 paralogs of a putative permease of the 4-Toluene Sulfonate Uptake Permease (TSUP) family (TC# 2.A.102). An additional TSUP homolog (TC# 2.A.102.4.6) was identified in Thor and Loki. Although the TSUP family has not been rigorously characterized, bioinformatic approaches have shown that the TSUP family is ubiquitous in nature and often mediates the uptake of sulfur-containing compounds[87]. Heimdall contains a putative transporter homolog of the Lipid-linked Sugar Translocase (LST) family (TC# 2.A.129).

Secondary Carriers Substrates

Asgard porters from TC subclass 2.A transport a diverse range of substrates, as shown in Figure 3. The most common substrates transported by this subclass are cations, drugs, and anions. Odin has the only metagenome that encodes more anion porters than cation porters. Anions transported by Asgard include inorganic anions such as phosphate, arsenite, and chloride, and organic anions such as pyruvate and lactate. The cations transported by the porter subclass are mostly inorganic cations such as sodium, hydronium ions, and various heavy metals. Heimdall and Thor have the only metagenomes to encode an organic cation porter, which mediates the transport of the substrate, choline. Polyamines are exclusively transported in Asgard by secondary carriers.

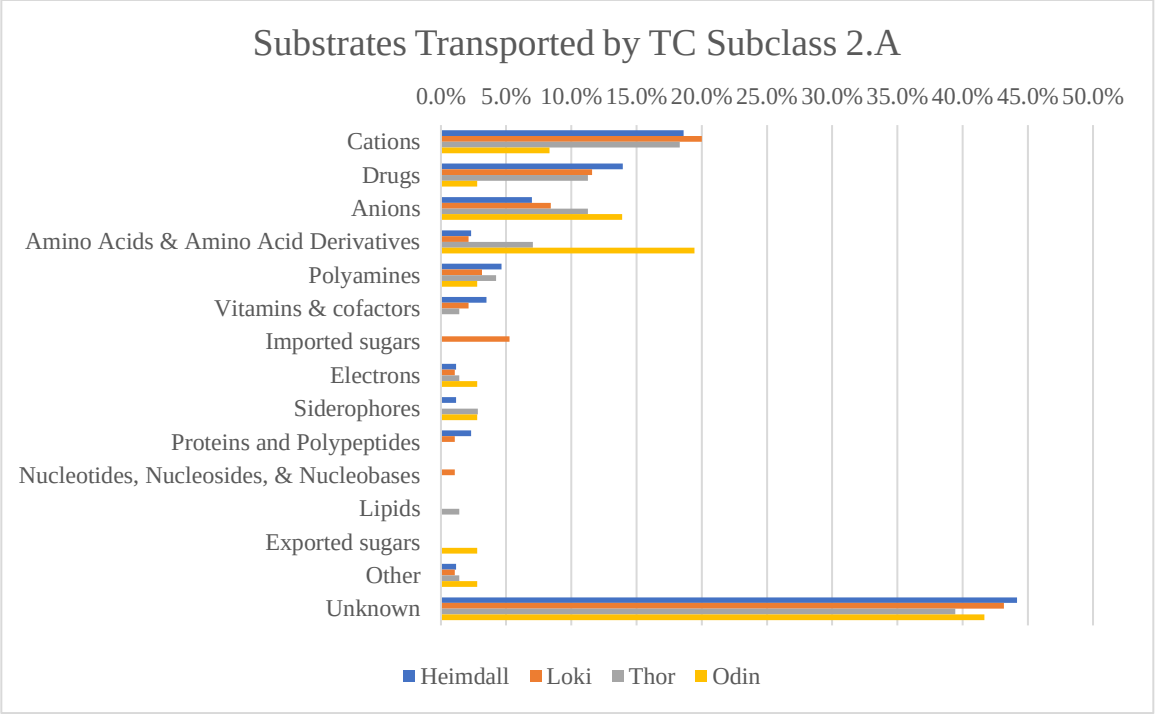


Figure 3. Overview of the predicted substrates transported by porters from the four Asgard metagenomes. The number of each substrate type transported is normalized by dividing the number of transport systems that have been shown to catalyze the transport of a substrate type by the total amount of substrate types transported by the secondary carriers for each Asgard metagenome.

Secondary Carriers Conclusions

27.8% of Heimdall's transport systems are secondary carriers while these systems represent 33.1-36.7% in the other Asgard metagenomes. This contrast indicates that Heimdall contains an inferior metabolic repertoire, at least in terms of secondary carriers, compared to the other Asgard metagenomes. Unlike the other Asgard metagenomes, amino acids and amino acid derivatives are mostly transported by porters in Odin. The primary role of MFS transporters in Asgard appears to be multi drug resistance. As for the putative siderophore exporters within these Asgard metagenomes, we cannot confidently claim that their function is to sequester iron for eventual uptake by separate siderophore uptake systems for the following reasons. Firstly, siderophores chelate ferric iron, not ferrous iron which is most likely to be present in the anaerobic

environment from which these Asgard metagenomes were sequenced[88]. Furthermore, the putative siderophore exporters are also homologous to multidrug resistance proteins, which may be their physiological function in Asgard. Lastly, no siderophore uptake systems were found in any of the four Asgard metagenomes. Odin imports polyamines through the putrescine importer (TC# 2.A.3.1.13) while all other Asgard metagenomes contain the DHA1 antiporter (TC# 2.A.1.2.8) that exports the endogenous polyamine, spermidine[89]. In bacteria, polyamines are crucial for growth and are involved in functions such as the synthesis of siderophores, acid resistance, and free radical scavenging[90].

Homologs of secondary carriers throughout the Asgard superphylum may promote a wide variety of physiological capabilities (Table 3). In Loki and Thor, GPH homologs may catalyze the uptake of glycosides and oligosaccharides[72]. The import of amino acids may be performed by the large number of APC homologs in all Asgard metagenomes, except for that of Loki[73]. The sequestration and export of heavy metal ions, such as zinc and manganese, at toxic levels, are likely mediated by CDF homologs in all Asgard metagenomes[74,75]. Additional CDF homologs that may function as Ca^{2+}:cation antiporters are present in Heimdall, Loki, and Thor. Furthermore, putative Ca^{2+}:H^{+} antiporters[85] were identified in all Asgard metagenomes except for Loki. The calcium antiporters that are encoded in all Asgard metagenomes may extrude calcium ions that have entered the cell through calcium specific channels mentioned in the α-type channels subclass section. ArsB and ACR-3 homologs that are encoded within these Asgard metagenomes are likely the major detoxification systems for arsenic via the extrusion of arsenite[83,91]. EPS-E homologs in Odin may play roles in biofilm formation via the export of exopolysaccharides[92]. RhtB homologs, which were only found in Loki and Thor, may function in the regulation of intracellular leucine levels via amino acid efflux[93]. Ferrous iron uptake is likely mediated by porters of the ILT family

in all Asgard metagenomes except Odin, whereas Odin ferrous iron uptake is mediated by FeoB transporters of the TC family 9.A.8.

Table 3. Overview of Secondary Carriers encoded within Asgard. The TC family in the first column is present within a particular Asgard metagenome if an X is marked.

TC Family	Heimdall	Loki	Thor	Odin	Function
Arsenical Resistance-3 (ACR-3) family; Arsenite-Antimonite Efflux (ArsB) family	X	X	X	X	Arsenic detoxification/resistance[83,91]
Cation Diffusion Facilitator (CDF) Superfamily	X	X	X	X	Export, or sequestration, of heavy metal ions, mostly zinc and manganese, at toxic levels[74,75]
Drug/Metabolite Transporter (DMT) Superfamily	X	X	X	X	Mostly uncharacterized, some import amino acids, choline, and riboflavin
Chloride Carrier/Channel (ClC) Family	X		X	X	Chloride efflux via Cl^-:H^+ antiport[94]
Amino Acid-Polyamine-Organocation (APC) Superfamily	X		X	X*	Imports amino acids[73] *polyamine uptake in Odin
Iron/Lead Transporter (ILT) family	X	X	X		Ferrous iron uptake
Glycoside-Pentoside-Hexuronide (GPH):Cation Symporter		X	X		Catalyzes uptake of small sugars[72]
Resistance to Homoserine/Threonine (RhtB) family		X	X		Regulation of intracellular leucine levels[93]
Putative Exopolysaccharide Exporter (EPS-E) family				X	Biofilm production via exopolysaccharide efflux[92]

Primary Pyrophosphate Hydrolysis-driven Active Transporters (TC Subclass 3.A)

The ABC Superfamily (TC Superfamily 3.A.1)

The best represented superfamily in Asgard is the ATP Binding Cassette (ABC) Superfamily (TC# 3.A.1). The ABC superfamily is represented in all domains of life and contains families of proteins responsible for ATP driven translocation of molecules across the cell membrane, for either uptake or export[95]. Of the Asgard metagenomes, Heimdall and Thor contain the most ABC transport systems (~18%) and Loki contains the least (10.1%). 15.3 % of transport systems in Odin are of the ABC type. Odin's metagenome contains more ABC uptake systems than efflux systems. According to TCDB, ABC transporters are divided into three types (ABC1, ABC2, ABC3) based on the distinct evolutionary paths of their integral membrane proteins[96].

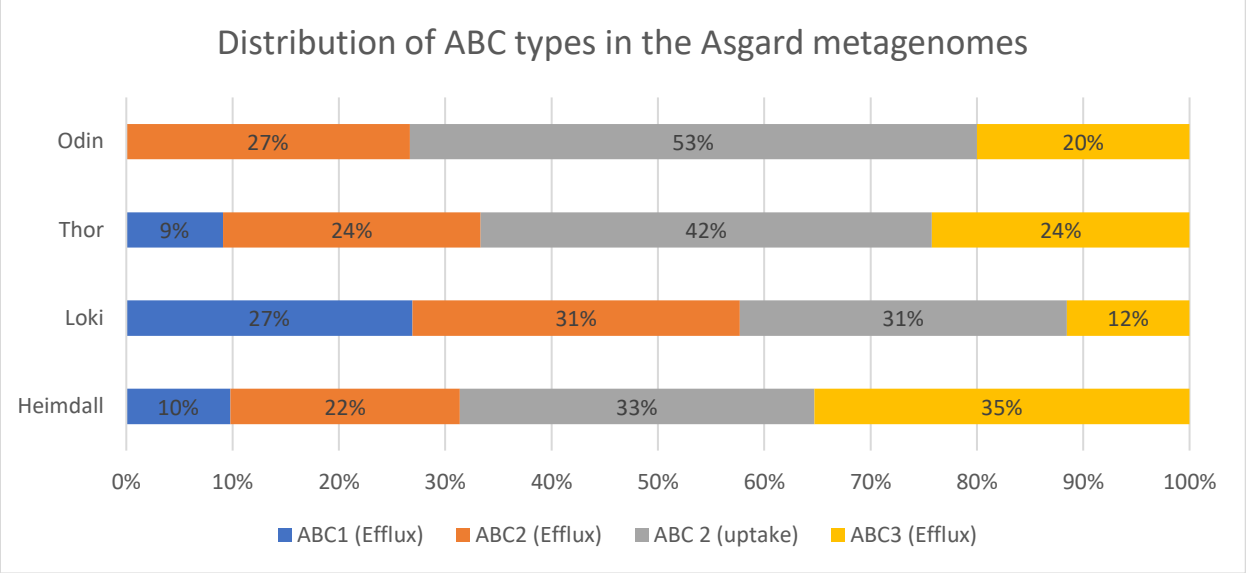


Figure 4. Distribution of ABC types encoded within the four Asgard metagenomes. Only efflux systems from ABC1 and ABC3 were identified in the Asgard metagenomes. ABC2 systems were split into two groups, efflux and uptake.

Type 1 ABC transporters (ABC1) are solely ABC efflux systems. ABC1 systems were not found in Odin and were the least represented ABC type in all Asgard metagenomes except Loki

(Figure 4). ABC1 exporters In these Asgard metagenomes are all multidrug exporter homologs, except for one lipid exporter homolog in Heimdall that has been shown to be essential for acid, salt, and thermal tolerance[97]. Type 3 ABC exporters (ABC3) are the second largest group of ABC transporters encoded within these metagenomes, except for Loki where it is the least populous type of ABC system. Aside from peptide exporters of the peptide-7 Exporter (Pep7E) family (TC# 3.A.1.134) present in Heimdall, the rest of the ABC3 transporters present in Asgard are uncharacterized exporters. In *Staphylococcus aureus*, the Pep7E homolog forms a five-component system with GraXSR sensing cationic antimicrobial peptides (CAMP), signaling to downstream enzymes to confer resistance to such peptides. However, it should be noted that the GraXSR system and systems that confer resistance to antimicrobial peptides such as MprF (TC# 4.H.1.1.1) were not found in the Heimdall metagenome, and therefore CAMP resistance is questionable unless performed by another system in Heimdall, characterized or uncharacterized. Homologs of the Macrolide Exporter family (TC# 3.A.1.122; ABC3) are present in all Asgard metagenomes. However, none of the TC homologs of the Macrolide Exporter family that are homologous to Asgard proteins have been characterized. Heimdall contained ABC3 transport systems showing sufficient sequence divergence from other ABC3 proteins in Asgard and NCBI that it was added to TCDB as a new uncharacterized TC family (TC# 3.A.1.157). Heimdall was the sole metagenome to encode two ABC3 systems homologous to proteins of the Eukaryotic ABC3 (TC# 3.A.1.207) family.

Type 2 ABC (ABC2) transporters are the best represented ABC type in these Asgard metagenomes. Uncharacterized and drug/antimicrobial peptide exporters make up the majority of the ABC2 efflux systems that are abundant across Asgard metagenomes. Proteins of the Drug Exporter-1 family (TC# 3.A.1.105) are present in all Asgard metagenomes and are the best

represented group of ABC2 Efflux homologs in Thor and Odin. Examples of these drug exporter homologs are chromoycin and pyoluteorin exporters in Odin and a putative lantibiotic exporter in Heimdall. Other ABC2 efflux systems present in Asgard are sodium export homologs in Heimdall and Thor and a putative heme exporter in Loki. ABC uptake transporters in Asgard are exclusive to ABC2 and make up the majority of the ABC2 transport systems present. Homologs of the Peptide/Opine/Nickel Uptake Transporter (PepT) family (TC# 3.A.1.5) are present in all four metagenomes. These PepT homologs in Asgard are peptide uptake systems, with one exception, a probable cellobiose transporter (TC# 3.A.1.5.14) in Thor. Homologs from the Sulfate/Tungstate Uptake Transport (SulT) family (TC# 3.A.1.6) are encoded in all Asgard metagenomes, except for Thor. SulT homologs present in Asgard presumably mediate the uptake of tungstate and vanadate, cofactors for enzymes generally involved in the transformation of carbon-, nitrogen-, and sulfur-containing compunds[98,99].

ABC Substrates

Of the ABC exporters in these Asgard metagenomes, the most common recognized function is drug and antimicrobial peptide export. At least 50% of the substrates imported by ABC uptake systems in these Asgard metagenomes are heavy metal cofactors, amino acids and amino acid derivatives, and peptides, as shown in Figure 5. Metal cofactors imported by ABC2 uptake systems are Cobalt, Zinc, Nickel, and Manganese. Loki and Odin's range of substrate types taken in by uptake systems is less diverse than those of Heimdall and Thor.

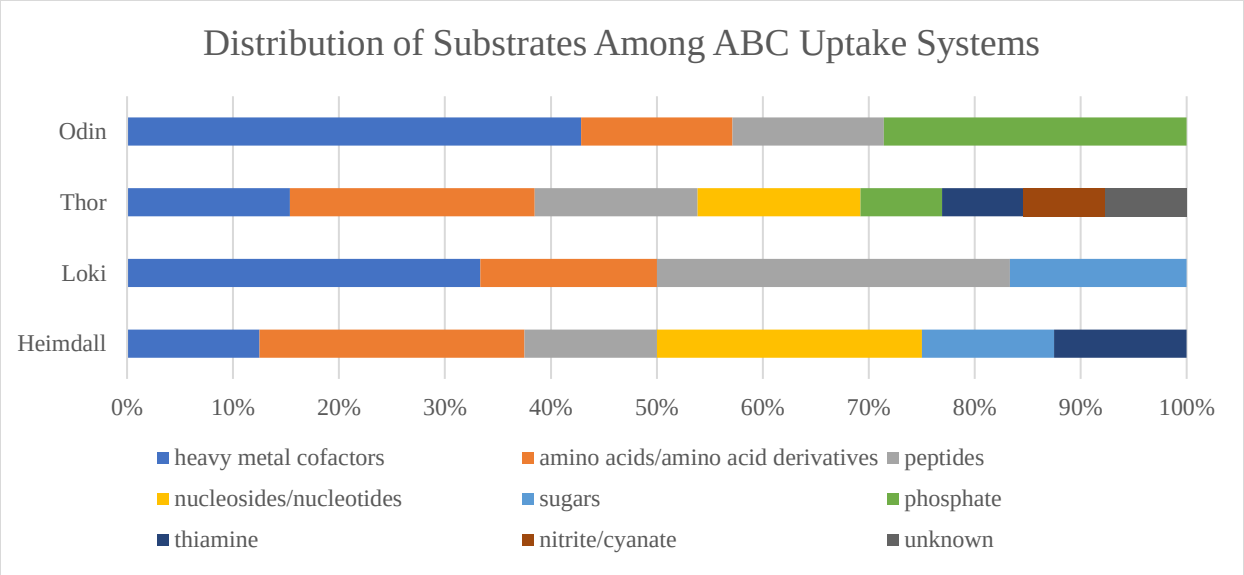


Figure 5. Imported substrates by homologs of recognized ABC systems encoded within the four Asgard metagenomes.

Additional Major families of Pyrophosphate Hydrolysis-driven Active Transporters

The H^+- or Na^+- translocating F-type, V-type, and A-type ATPase (F-ATPase) superfamily (TC# 3.A.2) is represented in all Asgard metagenomes. These ATPases are all proton pump complexes that can synthesize ATP when the rotor complex is driven by a proton motive force (*pmf*). Conversely, these pumps can hydrolyze ATP to export protons across the membrane to establish a *pmf*[100]. Thor, Heimdall, and Odin contain orthologous sets of the integral membrane protein system of the Archaeal-type (A-type) ATP synthase (TC# 3.A.2.3.2). These A-type complexes can use Na^+ or H^+ to synthesize ATP[101]. The eukaryotic-like V-type ATP synthase present in Loki is uncharacterized, but likely, due to homology, transports protons and possibly other ions such as sodium and lithium[102].

The P-type ATPase (P-ATPase; TC# 3.A.3) superfamily is represented by various homologs throughout the Asgard metagenomes, and its members mediate uptake and efflux of cations driven by ATP hydrolysis[103]. All Asgard metagenomes contain orthologs of a putative Ca^{2+} ATPase (TC# 3.A.3.2.21). A variety of members from the Copper Cation P-ATPase family (TC#

3.A.3.5) are present in all Asgard metagenomes. These homologs confer copper resistance and maintain low levels of intracellular copper[104]. All Asgard metagenomes contain the essential components of the General Secretory Pathway (Sec) family (TC# 3.A.5). Homologs of the H^+ or Na^+-translocating Pyrophosphatase (M^+-PPase) family (TC# 3.A.10) are present in all Asgard metagenomes. Heimdall, Thor, and Odin contain homologs of a H^+-translocating PPase while Loki contains a Na^+-translocating PPase. Therefore, these homologs may contribute to *pmf* generation via proton, or in the case of Loki, sodium extrusion coupled to pyrophosphate hydrolysis[105,106]. Orthologous proteins of the Guided Entry of Tail Anchored Protein (GET) family (TC# 3.A.19) are present in all Asgard metagenomes.

Primary Pyrophosphate Hydrolysis-driven Active Transporters Conclusions

For pyrophosphate hydrolysis-driven transporters, larger percentages are found in Heimdall, Thor, and Odin (21.4%-22.7%) than in Loki (16.7%). The ABC uptake systems in Loki and Odin import a more restricted range of substrates than in Heimdall and Thor (Figure 4). This may indicate a poorer metabolic repertoire in Loki than the other Asgard metagenomes. Loki may rely more on the uptake and export of nutrients and toxins by secondary carriers than by primary phosphate hydrolysis-driven transporters, suggesting greater reliance on *pmf*-generating electron flow than on substrate-level phosphorylation. The Asgard metagenomes primarily encode ABC uptake systems for heavy metal cofactors, amino acids, and peptides. ABC1 efflux systems are present in all Asgard metagenomes except Odin. Aside from a lipid exporter that may confer salt, acid, and thermal resistance to Heimdall[97], ABC1 transport systems are all multidrug exporters. ABC3 efflux systems, which are present in all Asgard metagenomes, are mostly uncharacterized. Loki and Heimdall are the only metagenomes to encode ABC sugar uptake systems.

The Na^+ and H^+-translocating Archaeal-type ATP synthase is the only proton pump complex found in Odin, Thor, and Heimdall. The presence of only A-type ATPases and the establishment of two ion gradients (i.e., proton and sodium) is a characteristic that has only been observed in methanogens[101,107]. Loki contains a different A-type ATPase homolog and an additional V-type ATPase that likely transports protons and possibly other ions such as sodium and lithium[102]. A-type ATPases are regarded as the evolutionary origin of eukaryotic V-type ATPases[108]. This V-type ATPase is unique to eukaryotes and provides support for Loki's proposed ancestral relationship to eukaryotes.

The PPases that are present in all Asgard metagenomes likely contribute to pmf generation via proton, or in the case of Loki, sodium, extrusion coupled to pyrophosphate hydrolysis. The presence of GET systems (TC# 3.A.19.1.3) in all Asgard metagenomes further supports a novel membrane protein-targeting pathway in archaea[109]. Homologs within the P-type ATPase Superfamily may confer copper tolerance and contribute to copper homeostasis in each of the Asgard phyla[104].

General secretory (Sec) complexes with similar components were found encoded within all Asgard metagenomes. Sec protein complexes are found universally in the 3 domains of life and are responsible for protein secretion and integration into membranes[110]. The archaeal Sec complexes consist of a large transmembrane SecYEG complex and the ribosome-associating signal recognition particle (SRP) complex. The SRP complex binds to nascent polypeptides that are destined for secretion or membrane insertion as they emerge from translating ribosomes[111]. The newly formed SRP and the ribosome nascent chain complex are then targeted to the membrane embedded receptor, FtsY. The nascent polypeptide is transferred to the SecYEG protein translocon complex that either secretes the polypeptide or inserts it into the membrane[112].

Oxidoreduction Driven Transporters (TC Subclass 3.D)

In accordance with previous research, we identified two transport systems of the H^+ or Na^+-translocating NADH Dehydrogenase (NDH) Family (TC# 3.D.1) in Odin. Although the following transport systems are members of the NDH family, they appear not to be NADH dehydrogenases. The first system is a [Ni^{2+}-4Fe-4S] quinone-independent ferredoxin:H^+ oxidoreductase (TC# 3.D.1.4.2) that translocates protons to drive *pmf* generation[113]. Odin is the only metagenome with a transport complex capable of reducing ferredoxin, an essential step in methanogenesis. The second system in Odin is the formate-dependent [NiFe] hydrogenase (TC# 3.D.1.9.2) that may couple H_2 production with oxidation of formate to carbon dioxide[114]. The H_2:Heterodisulfide Oxidoreductase (HHO) family (TC# 3.D.7) is represented in Heimdall. HHO systems are used in cytochrome containing methanogenic archaea to generate a *pmf* using redox-driven proton extrusion concomitant with the reduction of a heterodisulfide[115–117]. More distantly related HHO orthologs that seem to lack the integral membrane-bound protein are present in Thor, Loki, and Heimdall. Interestingly, tungsten, which is imported via an ABC uptake system (TC# 3.A.1.6.2), is an important cofactor for the reduction of CO_2 to CH_4[98]. The Na^+ or H^+ Pumping Formyl Methanofuran Dehydrogenase (FMF-DH) family (TC# 3.D.8) is represented in all Asgard metagenomes, except for Heimdall. FMF-DH couples Na^+ transport with the initiation of methanogenesis by the archaeal cofactor, methanofuran[118]. Thor, Odin, and Heimdall contain orthologous sets of proteins from the H^+-translocating $F_{420}H_2$ Dehydrogenase ($F_{420}H_2$DH) family (TC# 3.D.9). The $F_{420}H_2$DH proteins form a redox-driven proton pump complex that, in methanogenic archaea, couples the reduction of methylated compounds to proton extrusion[119]. Seitz et al suggested that the $F_{420}H_2$DH in Thor likely performs non-methanogenic functions due to the lack of other genes required for methanogenesis[21]. However, it should be pointed out that

their suggestion is uncertain because no fully sequenced genomic data are available. Orthologous sets of the Prokaryotic Succinate Dehydrogenase (SDH) family (TC# 3.D.10) are present in all Asgard metagenomes except Odin. These QFR systems (TC# 3.D.10.1.4) of the SDH family are mostly from organisms that live in anoxic environments where anaerobic respiration would be necessary for autotrophic growth[31]. The QFR system utilizes fumarate as a final electron acceptor and supports electron transfer across the membrane which is neutralized by proton export by other systems. Although this reaction is electroneutral and does not directly drive ATP production, the produced menaquinones can be used by formate dehydrogenases and hydrogenases to establish a *pmf*[120,121].

Oxidoreduction Driven Transporters Conclusion

Odin has the most diverse means of generating a *pmf* for subsequent ATP generation or electrochemical driven transport. In comparison to the total number of transport systems, Odin has approximately twice the percentage of oxidoreduction-driven transport systems as the other three Asgard metagenomes. Due to the presence of enzymes that could confer upon Odin the ability to ferment organic substrates to formate[35], formate-dependent NDH-like systems may allow Odin to use the products of fermentation to enhance *pmf* generation. We identified transport systems in Odin that are essential for methanogenesis in methanogens without cytochromes[122]. In Odin, the FMF-DH and $F_{420}H_2$DH systems that form H_2 dependent redox-driven proton pumps may convert methylated compounds to CO_2 for subsequent reduction to produce methane[119]. Additionally, CO_2 produced from formate oxidation may be used for methane production. However, the membrane associated methyl-H4MPT–coenzyme M methyltransferase (MtrA-H), which is an essential membrane component for the reduction of carbon dioxide to methane[122,123], was not found in any Asgard metagenome. Odin is also the only Asgard metagenome to lack the heterodisulfide

transport system, which is present in all methanogens[107,119,122]. The evidence suggests that Odin contains the initial membrane components required for the utilization of methylated compounds and formate for methanogenesis but may lack the components necessary for the reduction of carbon dioxide to methane. Therefore, the present oxidoreduction driven transporters suggest that Odin's ability to generate a *pmf* is diverse and relies on various electron acceptors such as formate, hydroxyphenazine, and ferredoxin. However, the potential for methanogenesis cannot be ruled out because the methyltransferase function may be carried out by an uncharacterized transport system. In addition, while Odin contains some of the essential methanogenic transport complexes, there is no evidence for the presence of the various cytosolic enzymes required for methanogenesis[35]. However, further investigation of more metagenomic data for Odin is required to support this suggestion.

Loki and Thor both contain oxidoreduction-driven transport systems like that of methanogens without cytochromes[122]. Consistent with previous findings[35], non-membrane associated HHO systems were found in both Thor and Loki, which is characteristic of methanogens that lack cytochromes. Both Loki and Thor contain FMF-DH proton pumps that are required for the utilization of methylated compounds for methanogenesis[118]. However, the MtrA-H and ferredoxin hydrogenase, which are essential to methanogenesis, were not found in either Asgard metagenome. Like Odin, the oxidoreduction driven transport systems in Loki and Thor likely do not play a role in methanogenesis but contribute to the production of a *pmf* using electron acceptors such as fumarate and organic heterodisulfides.

Heimdall contains some membrane components involved in methanogenesis, similar to those of the order Methanosarcinales. This type of methanogen forms an electrochemical gradient using cytochromes, as opposed to other methanogens that do not contain cytochromes, and

involves redox driven ion translocation across the membrane that is catalyzed by an anaerobic respiratory chain[119]. Heimdall contains the $F_{420}H_2DH$ redox driven proton pump that couple's proton extrusion to the conversion of methylated compounds to carbon dioxide for potential reduction to produce methane. Heimdall also contains the quinol:fumarate reductase (QFR) system of the SDH family which may function as a quinone reductase that does not contribute to *pmf* generation[124]. Heimdall has the only Asgard metagenome that contains the key membrane components of methanogens with cytochromes, such as a transport system of the Proton-translocating Cytochrome Oxidase (COX) Superfamily (TC# 3.D.4) and the membrane-bound heterodisulfide reductase of the HHO family. The membrane constituents of the cytochrome containing methanogens of the Methanosarcinales are present except for the MtrA-H system and the ferredoxin oxidoreductase. In addition, the homologous COX transport systems in Heimdall are probably oxygen-dependent proton pumps and therefore may not play a role in methanogenesis[125]. This supports previous research identifying Heimdall as a possible facultative anaerobe[33,35,126]. Due to the findings mentioned, it is doubtful that Heimdall is capable of methanogenesis, but it may use oxygen, fumarate, and other organic heterodisulfides as final electron acceptors.

No oxidoreduction-driven transport system was found to be ubiquitous to all Asgard metagenomes, but there are possible orthologous transport systems encoded within three of the four metagenomes. Therefore, it is difficult to determine the origin of these genes and the overall metabolic capabilities of the Asgard superphylum. All Asgard metagenomes studied here seem to depend on generating a proton motive force using protons rather than sodium ions and depend on a variety of transport systems unique to methanogens. However, we were unable to confidently establish the capability of methanogenesis due to incomplete metabolic and transport pathways.

The proton motive force generated is likely used to drive ATP synthesis and proton driven transport by secondary carriers. Additionally, the lack of uniformity amongst the observed metabolic capabilities inferred for the Asgard makes it difficult to determine the metabolic capabilities of the last Asgard archaeal common ancestor.

Group Translocators (TC Class 4)

The presence of homologs of the Fatty Acid Group Translocation (FAT) family (TC# 4.C.1) are variable across Asgard metagenomes, with Odin containing just 1 homolog and Loki containing 25 homologs. Research has shown that the FAT family's role in linking the translocation of fatty acids with acylation by CoA to form thioesters can be distinct as well as overlapping[127–129]. Asgard metagenomes encode multiple homologs of putative FAT systems and some well characterized FAT systems that reportedly couple translocation to esterification. The majority of FAT homologs in Thor, Loki, and Heimdall (~59%) match the same TC system, a long chain fatty acyl CoA synthase (TC# 4.C.1.1.4), suggesting that they may be orthologous. These systems transport, concomitant with esterification, fatty acids for subsequent degradation or incorporation into phospholipids[130].

Three different families from the Polysaccharide Synthase/Exporter (TC# 4.D) subclass are represented in these Asgard metagenomes. Two of these families (TC# 4.D.1, 4.D.3) belong to the Glycosyl Transferase/Transporter Superfamily. Homologs from the Putative Vectorial Glycosyl Polymerization (VGP) family (TC# 4.D.1) are only present in Odin and Heimdall. These proteins may couple the export of glycosyl groups when added to growing polysaccharide chains[131,132]. A suggested cellulose synthase and a putative integral membrane glycosyl transferase homolog of the Glycan Glucosyl Transferase family (TC# 4.D.3) were only found in Heimdall.

All Asgard metagenomes, except for that of Odin, contain homologs from the Glycosyl Transferase 2 family (TC# 4.D.2). Multiple proteins from Thor and Loki, and one protein from Heimdall are homologous to the same putative glycosyl transferase (TC# 4.D.2.1.6). This TC protein may function as a glycosyl transferase and a membrane transporter (See entry 4.D.2.1.6 in TCDB).

A range of 1-6 homologs of the Choline/Ethanolamine Phosphotransferase 1 (CEPT1) family (TC# 4.F.1) are present in all Asgard metagenomes. All of these metagenomes encode a homolog of the CDP-alcohol phosphatidyltransferase (TC# 4.F.1.3.1), thus suggesting their orthologous nature. This system is uncharacterized, but is inferred by homology to play a role in phospholipid synthesis through the displacement of CMP from a CDP-alcohol by a second alcohol[133].

Group Translocators Conclusions

Group translocators are more common in Loki than in the other Asgard metagenomes, mostly due to the presence of FAT transporters. These paralogs may have been selected for via duplication events, likely due to natural pressures. This may indicate the vital physiological role that FAT transport proteins may have in Loki. These FAT transporters, which are present in all Asgard, catalyze the esterification of exogenous long-chain fatty acids, concomitant with uptake, yielding cytoplasmic metabolically active CoA thioesters[127–129]. As observed in *E. coli*, these active CoA thioesters may be degraded or incorporated into the phospholipid membrane in Loki, Thor, and Heimdall[128,130]. Interestingly, ester-linked phospholipids are unique to eukaryotes and bacteria while ether-linked phospholipids are characteristic of archaea[134]. This raises the question as to whether esterified phospholipids are produced in Asgard, and if they make up the

phospholipid bilayer, like that of bacteria and eukaryotes, or if they are degraded as a source of carbon.

All Asgard metagenomes contain polysaccharide synthase exporters that add glycosyl groups to growing polysaccharide chains destined to the exterior of the cell[131]. However, only Odin and Heimdall contain glycosyl transferase homologs that may be essential for biofilm formation and intracellular adhesion[135]. The prokaryotic orthologs of CDP-alcohol phosphotransferases are ubiquitous among these Asgard metagenomes. These homologs may catalyze phospholipid biosynthesis in a process coupled to phospholipid insertion into the membrane[133,136].

Transmembrane Electron Carriers (TC Class 5)

Homologs from the Disulfide Bond Oxidoreductase D (DsbD) family (TC# 5.A.1), a constituent of the LysE superfamily, are present in all Asgard metagenomes except for Odin. Loki, Heimdall, and Thor contain 1-3 homologs of the same TC entry, CcdA (TC# 5.A.1.2.10). The CcdA protein shuffles electrons into the periplasm from the cytoplasm via 2 cysteines, cycling between oxidized and reduced states[137]. Heimdall encodes homologs of the Prokaryotic Molydopterin-containing Oxidoreductase (PMO) family (TC# 5.A.3), which are three multi-component DmsABCE systems (TC# 5.A.3.3.3) and one NarGHI system (TC# 5.A.3.1.1). Loki is the only metagenome containing a bacterial-type phenol hydroxylase homolog of the $gp91^{phox}$ Phagocyte NADPH Oxidase-associated Cytochrome b_{558} (PHOX) family (TC# 5.B.1).

Transmembrane Electron Carriers Conclusions

Compared to the other Asgard metagenomes, Heimdall contains the greatest percentage of transmembrane electron carriers (2.9%). Aside from Odin that contains no transmembrane electron carriers, these transport systems make up approximately 1% of the other Asgard transportomes. Transmembrane electron carriers can shuttle electrons from the cytoplasm to substrates in the

external milieu of these Asgard organisms, or vice versa, thus affecting membrane potentials. These homologs likely introduce reducing equivalents from the cytoplasm to the periplasm to carry out essential reducing pathways[138]. Reduced substrates in the periplasm of Heimdall, Thor, and Loki may promote correction of non-native sulfide bonds, defense against oxidative damage, and cytochrome c biogenesis[138,139]. Additional PMO systems that were found only in Heimdall may generate a *pmf* across its cell membrane. This suggests that Heimdall is capable of proton translocation and reduction of dimethyl sulfoxide/trimethylamine N-oxide and nitrate via 2 different systems, DmsC[140] and NarA[115], respectively. Loki's sole one-electron transmembrane carrier homolog may be essential for the utilization of phenol as a carbon source[141].

Auxiliary transporters (TC Subclass 8.A)

TC subclass 8.A contains proteins that facilitate transport, but do not participate directly in the transport process. They function in conjunction with one or several established TC systems. In all Asgard metagenomes, the Voltage-gated K^+ Channel β-subunit (Kvβ) family (TC# 8.A.5) is well represented with numerous homologs. Five other families from TC subclass 8.A were present in 50% or less of the Asgard metagenomes. One such family is the Stomatin/Podacin/Band7/Nephrosis.2/SPFH (Stomatin) family (TC# 8.A.21) that is represented by two homologs in Heimdall and Thor.

Auxiliary Transporters Conclusions

Multiple homologs of auxiliary transport proteins of the Kvβ family are ubiquitous among the Asgard. These homologs may function in deactivation of voltage-gated K^+ channels[142]. However, as mentioned earlier, voltage-gated K^+ channels are missing in all Asgard metagenomes aside from Heimdall. Therefore, these proteins may be ubiquitous oxidoreductases that function

as bacterial stress response proteins. The stomatin homologs in Heimdall and Thor are membrane bound proteases that may function to open ion channels[143].

Poorly Characterized Transporters (TC Subclass 9.A)

Proteins in this subclass function in transport across a membrane via an unknown mechanism. Homologs from the Ferrous Iron Uptake (FeoB) family (TC# 9.A.8) are present in all Asgard metagenomes. Thor contains two uncharacterized homologs of the Mitochondrial Cholesterol/Porphyrin Uptake Translocator Protein family.

Poorly Characterized Transporters Conclusions

FeoB homologs represent the majority of proteins from this subclass. These homologs import ferrous iron, which is predominantly present in highly acidic, reducing, and anaerobic environments and cannot be taken up complexed to siderophores. The uptake of ferrous iron is possibly mediated in a process powered or regulated by the action of GTP binding proteins[144]. The FeoB proteins present in Asgard are of the prokaryotic type and likely associate with GTPases encoded nearby in the metagenome, as in the case of Thor (TC# 9.A.1.8.17)[3]. Paralogs of the SαR family are putative integral membrane steroid 5α-reductases that may be essential to the physiology of these Asgard organisms.

Putative Transport Proteins (TC Subclass 9.B)

Proteins in this subclass are either awaiting classification upon elucidation of function or will be deleted from TCDB if their suggested transport function is disproven. This subclass is the third largest group of proteins in the Asgard metagenomes aside from that of Odin where it is the fourth largest subclass. Homologs from the Integral Membrane CAAX Protease (CAAX protease) family (TC# 9.B.1) and the Integral Membrane CAAX Protease-2 (CAAX Protease-2) family (TC# 9.B.2), belonging to the CAAX Superfamily, are present in all Asgard metagenomes. CAAX

proteases have been shown to cleave and degrade transmembrane α-helices anchored in the cell membrane[145,146]. Putative heme exporters[147,148] of the Heme Handling Protein family (TC# 9.B.14) are well represented in Asgard metagenomes, although missing in Thor. The Integral Membrane Glycosyltransferase 39 (GT39) family (TC# 9.B.142) is present in all Asgard metagenomes. The GT39 family may be distantly related to other glycosyl transferases (TC# 4.D.1, 4.D.2). Orthologs from the eukaryotic, GT39 system (TC# 9.B.142.3.3) are present in all Asgard metagenomes.

The following families are represented in Asgard metagenomes, unless noted missing in one of the four phyla: The DedA family (TC# 9.B.27) is missing only in Heimdall; members of the Acid Resistance Membrane Protein family (TC# 9.B.36), the Acyltransferase-3/Putative Acetyl-CoA Transporter family (TC# 9.B.97), the Lipoprotein Signal Peptidase/Phosphatase/Lead Resistance Fusion Protein family (TC# 9.B.105), the Putative Integral Membrane Steroid 5α-reductase (SαR) family (TC# 9.B.115), the Putative Undecaprenyl-phosphate N-Acetylglucosaminyl Transferase family (TC# 9.B.146), the M50 peptidase family (TC# 9.B.149) (TC system 9.B.149.1.10 is a possible ortholog), the Prenyl Transferase family (TC# 9.B.241), and three other uncharacterized families (TC# 9.B.288; 9.B.289; 9.B.303).

Major Superfamilies

In TCDB, Superfamilies are sequence divergent families that are derived from a common ancestral system and often share similar functions, determined by several shared characteristics such as protein domains, motifs, etc. The distribution of the major superfamilies within the Asgard metagenomes are shown in Figure 6. Most Asgard transport systems do not belong to a superfamily, and they are therefore assigned to the unclassified group in Figure 6. Most of the subfamilies within the unclassified group are only present in just one of the four metagenomes. Furthermore, most of the conserved transporters, those present in all Asgard metagenomes, belong

to the unclassified group. Of these unclassified transport systems, numerous homologs of the FAT family and SαR family are present in all Asgard metagenomes, indicating the potential orthologous nature of these homologs. The large number of FAT homologs in Loki, and the large number of SαR homologs in all Asgard metagenomes, except that of Odin, indicates that many of these are paralogous, suggesting that they are important to the survival of these organisms.

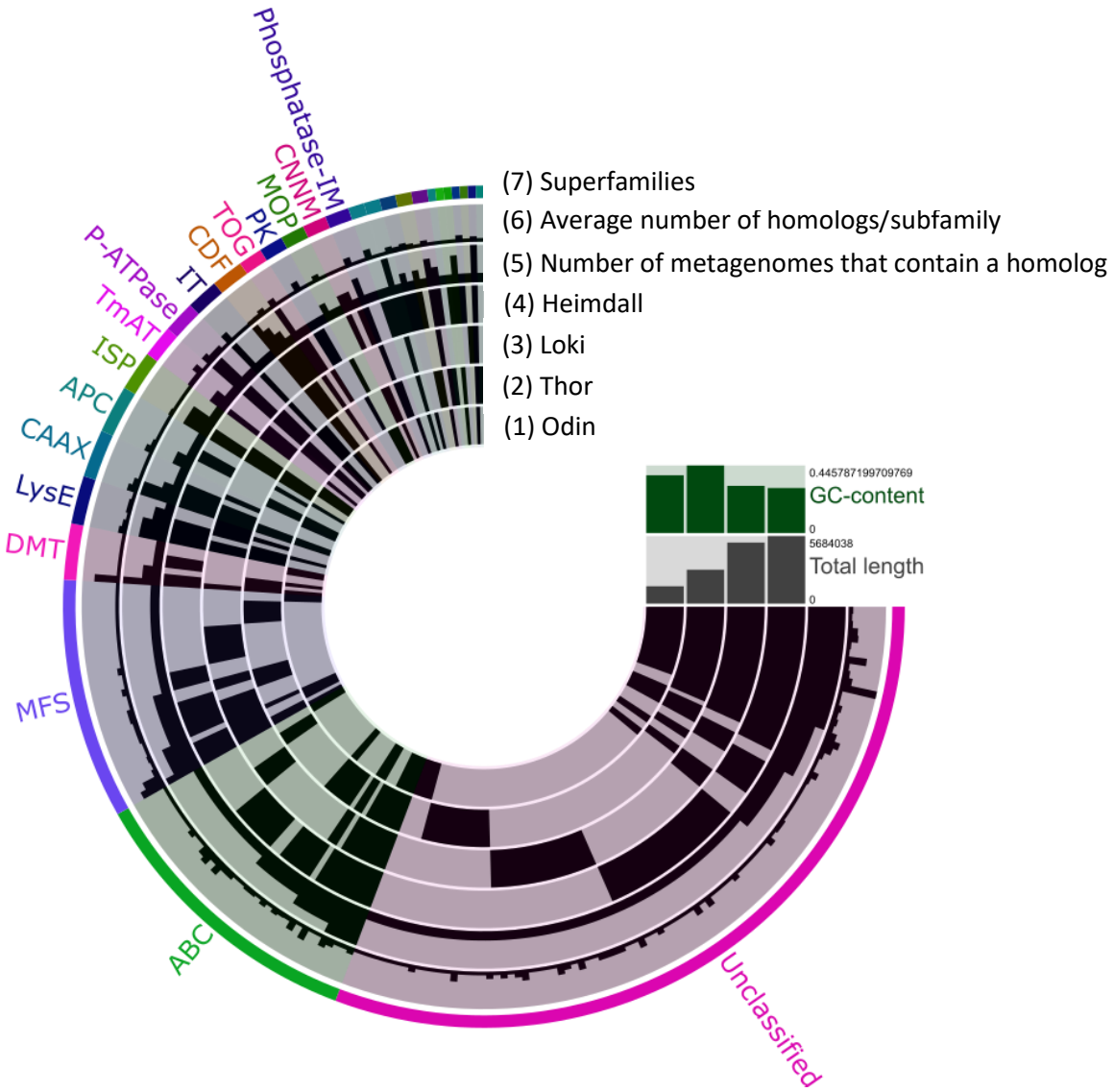


Figure 6. TC Superfamilies within the metapangenome of four Asgard transportomes. Bars in the first four layers from the center indicate the occurrence of a transport subfamily in each of the four metagenomes. The fifth layer shows the number of metagenomes that contains at least one homolog from that particular subfamily within a superfamily. The sixth layer describes the average total number of transport systems within a subfamily. The outermost layer labels the superfamily based on the TC superfamily groupings. Transport systems are organized based on their respective superfamily, and superfamilies are organized based on the number of metagenomes containing a particular type of transport system. This figure was generated using anvi'o[149,150].

The ABC and the MFS are the largest superfamilies represented within the four Asgard metagenomes examined here. Loki is the only metagenome that contains more MFS transport systems than ABC transport systems. Within the ABC superfamily, the transport homologs present

in the largest numbers within all metagenomes are: the peptide/oligosaccharide uptake systems (TC# 3.A.1.5), membrane associated ATPases of the ABC superfamily that are essential for translation and are ubiquitous among eukaryotes and archaea (TC# 3.A.1.31), peptide antibiotic efflux systems (TC# 3.A.1.105), and two different types of uncharacterized exporters. Drug:H^+ antiporters are the most conserved homologs within the MFS, indicated by their ubiquity within the Asgard metagenomes and the average number of homologs present within each Asgard metagenome (average of 7 for DHA1; 5.3 for DHA3). The 10 TMS Drug/Metabolite Exporter (DME) family is the largest family within the DMT superfamily, accounting for about 80% of the transport systems within this superfamily. The largest number of DME systems present within the Asgard metagenomes are most similar to two archaeal uncharacterized transport systems (TC# 2.A.7.3.5; 2.A.7.3.36). Homologs of the LysE superfamily are present in all Asgard metagenomes, but no one subfamily is present in all.

Substrates

Table 4. Predicted substrates transported within four Asgard metagenomes. The number of substrates in "predicted substrates" were determined by the number of systems within an Asgard metagenome that catalyze the transport of the predicted substrate type**.**

Substrate type	Predicted substrates				Predicted substrates/total substrates (%)			
	Heimdall	Loki	Thor	Odin	Heimdall	Loki	Thor	Odin
Cations	38	40	24	15	13.9	15.3	12.3	16.0
Drugs	18	20	17	4	6.6	7.6	8.7	4.3
Anions	11	16	11	8	4.0	6.1	5.6	8.5
Lipids	5	22	8	3	1.8	8.4	4.1	3.2
Amino Acids & Amino Acid Derivatives	6	6	10	8	2.2	2.3	5.1	8.5
Proteins & Polypeptides	12	5	6	5	4.4	1.9	3.1	5.3
Vitamins & cofactors	7	4	4	1	2.6	1.5	2.1	1.1
Polyamines	4	3	3	1	1.5	1.1	1.5	1.1
Ions	4	3	2	1	1.5	1.1	1.0	1.1
Imported sugars	2	6	0	0	0.7	2.3	0.0	0.0
Electrons	4	2	1	1	1.5	0.8	0.5	1.1
Nucleotides, Nucleosides, & Nucleobases	4	1	2	0	1.5	0.4	1.0	0.0
Siderophores	1	0	2	1	0.4	0.0	1.0	1.1
Exported sugars	1	0	0	1	0.4	0.0	0.0	1.1
Other	14	5	6	3	5.1	1.9	3.1	3.2
Unknown	143	129	99	42	52.2	49.2	50.8	44.7
Total	**274**	**262**	**195**	**94**	**100**	**100**	**100**	**100**

To gain a better understanding of Asgard's physiology and its interactions with its surrounding environments, substrates were predicted based on the quality of GBLAST alignments as detailed in Methods (Table 4). Approximately half of the transport systems in the four Asgard metagenomes are of unknown function. The largest group of predicted substrates transported are cations, mostly hydronium ions and inorganic cations. These cations, such as copper and calcium, may function as enzyme cofactors and signaling molecules. Furthermore, hydronium ions likely play a large role in generating electrochemical potentials and driving ATP synthesis. With respect

to drug and antimicrobial agent exporters, Odin contains the smallest percentage while Thor contains the largest percentage (Table 4). For lipid transporters, Loki contains over twice the percentage observed for the other three Asgard metagenomes. The transported lipids include R-camphor, diterpene, and fatty acids. For amino acid and amino acid derivative transporters, Heimdall and Loki transport the smallest percentage (2.2-2.3%) while Thor and Odin's amino acid transport systems make up 5% and 9% of their transportome, respectively. Notably, no characterized sugar transporters were found in Thor and no sugar uptake systems were found in Odin. Loki is the only metagenome lacking putative siderophore transporters.

Using Arturo Medrano's data collection protocol, Katie Jing Kay Lam and Nicholas Alan Wong provided substantial contributions to data collection. Kevin Hendargo designed Figure 6, and Vasu Iddamsetty produced the predicted substrate table in supplemental information.

Discussion

The Asgard superphylum is hypothesized to be the closest known archaeal relative to the first eukaryote[3,4,31]. The Asgard metagenome encodes eukaryotic signature protein (ESP) homologs involved in cytoskeleton remodeling and primitive ER-golgi vesicular trafficking. Based on the publicly available metagenomic data from the four phyla of the Asgard (Loki, Thor, Heimdall, and Odin), the consensus of research has concluded that most metabolism in the Asgard superphylum is mixotrophic and anaerobic. Bulzu et al. have provided an evaluation of some of the transport and metabolic mechanisms of Heimdall[33]. Limited capabilities of sugar, protein, and polypeptide import have been noted for Loki and Thor[35].

All Asgard metagenomes contain a diverse range of transport systems capable of generating a *pmf*. The *pmf* thus generated could drive oxidative phosphorylation via the proton pump complexes present universally in the Asgard. Alternatively, the *pmf* could be utilized to

support electrochemical potential-driven transport by the secondary carriers. The latter is more likely due to the greater percentage of secondary carriers than ATP-driven transporters within these Asgard organisms, especially Loki. To a lesser extent, the sodium membrane potential is likely to be generated in all Asgard metagenomes.

Based on the analysis of the transportome encoded within these four Asgard metagenomes, we conclude that organisms in the Asgard Superphylum are metabolically versatile with multiple means of acquiring energy for growth. Loki and Thor likely take up a diverse range of organic compounds such as lipids, sugars, amino acids, and proteins. However, while Thor's intake of organic molecules is more balanced, Loki contains approximately double the number of lipid transporters. Additionally, both Loki and Thor seem to be capable of anaerobic respiration, and they contain some components necessary for methanogenesis. Odin and Heimdall take up organic molecules, mostly amino acids, amino acid derivatives and proteins. Odin has the most diverse metabolic repertoire of all the Asgard metagenomes observed and contains the most components of transport systems required for methanogenesis. Due to the presence of systems that utilize oxygen and fumarate as electron acceptors, Heimdall may be capable of respiration in both anaerobic and aerobic environments. The primary type of transport systems present across the Asgard Superphylum are the secondary carriers, which indicates Asgard's reliance on sustaining membrane potentials. All Asgard metagenomes contain a large percentage of drug exporters, which may confer drug and toxin resistance to Asgard within their respective environments. Further analyses of the transportome of multiple species within each phylum will be required to conclude that the trends observed in this study are uniquely characteristic of these phyla.

The transportomes of the four Asgard metagenomes contain a limited number of eukaryotic specific proteins. Thus, for transporters, these findings weakly support claims regarding Asgard's

close phylogenomic relationship to eukaryotes. Most transport systems in these Asgard metagenomes were either ubiquitous to all domains or prokaryotic, although a few eukaryotic specific transporters were identified. These homologs include Mg^{2+}-specific ion channels and eukaryotic-like orthologs of glycosyl transferases in all Asgard metagenomes. Furthermore, multiple systems in Heimdall were homologous to uncharacterized ABC transporters unique to eukaryotes. Odin and Heimdall contained proteins homologous to eukaryotic presenilins. In addition, Loki contains a putative eukaryotic-like V-type ATP synthase. Contingent on Asgard's ancestral relationship, these findings suggest that eukaryotic complexity of the transportome may have developed after the primary endosymbiotic event that is claimed to involve the Asgard host cell. However, it is important to note that only metagenomic data were available for this study. Therefore, it is questionable whether the scarcity of eukaryotic transport components has a direct bearing on the question of the origin of the eukaryotic cell. While more work is needed to determine if the unique traits of these four Asgard transportomes are characteristic of each phylum, it is essential that the genomic data for all Asgard organisms are expanded.

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