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Metagenome-assembled genomes of three Hepatoplasmataceae provide insights into isopod-mollicute symbiosis

--Manuscript Draft--

Metagenome-assembled genomes of three *Hepatoplasmataceae* provide insights into isopod-mollicute symbiosis

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Repositories

Candidatus Tyloplasma littoralis Fukuoka2020: AP027078.1

Candidatus Hepatoplasma vulgare Av-JP: AP027131.1

Candidatus Hepatoplasma scaber Ps-JP: AP027133.1

Candidatus Hepatoplasma crinochetorum Tokyo2021: AP027132.1

Abstract

The digestive organs of terrestrial isopods harbor bacteria of the recently proposed mollicute family *Hepatoplasmataceae*. The only complete genome available for *Hepatoplasmataceae* is that of "*Candidatus* Hepatoplasma crinochetorum". The scarcity of genome sequences has hampered our understanding of the symbiotic relationship between isopods and mollicutes. Here, we present four complete metagenome-assembled genomes (MAGs) of uncultured *Hepatoplasmataceae* members identified from shotgun sequencing data of isopods. We propose three novel species "*Candidatus* Tyloplasma littoralis" identified from the semiterrestrial isopod *Tylos granuliferus*, "*Candidatus* Hepatoplasma vulgare" identified from the common pill bug *Armadillidium vulgare*, and "*Candidatus* Hepatoplasma scaber" identified from the common rough woodlouse *Porcellio scaber*. Phylogenetic analysis of 16S ribosomal RNA sequences showed that "*Candidatus* Tyloplasma littoralis" and other semiterrestrial isopod-associated mollicutes form a sister clade to terrestrial Hepatoplasma members, justifying their assignment to a novel genus. Phylogenomic analysis of 151 mollicutes confirmed that *Hepatoplasmataceae* is a sister clade of *Metamycoplasmataceae* in the order *Mycoplasmoidales*. Our analysis also revealed that that *Hepatoplasmataceae* lack major metabolic pathways but has a likely intact type IIA CRISPR-Cas9 machinery, indicating that these mollicutes have an ectosymbiotic lifestyle with high nutritional dependence on their host. We did not find any evidence that Hepatoplasmataceae encode digestive enzymes that could provide nutritional benefits to the host, which suggests that they may act as defensive symbionts.

Impact statement

Terrestrial isopods, commonly known as pill bugs and woodlice, are a unique group of crustaceans that successfully colonized land. Their digestive organs are home to symbiotic microbes that may support the host's survival. One of the most characteristic microbes associated with terrestrial isopods are hepatoplasmas, a lineage of mycoplasma-like bacteria (Class *Mollicutes*) that reside on the surface of the host's midgut gland. It has been suggested that *Hepatoplasma* mollicutes promote the host's survival, but their exact roles remain unknown. Our aim was to better understand their physiological roles by analyzing the metagenome-assembled genomes of novel *Hepatoplasma* lineages. Our analyses found little evidence that they provide nutritional benefits to the host. This suggests that the symbiotic relationship between isopods and hepatoplasmas is not defined by the exchange of essential nutrients, as is often the case in insect-bacterial symbiosis. Rather, it is more likely that hepatoplasmas are defensive symbionts that limit the growth of pathogenic microbes by occupying the host digestive organs, rather than providing essential nutrients to the host.

Data Summary

The whole genome shotgun sequencing data of isopods are available in DDBJ/ENA/NCBI under the following accession numbers: *Tylos granuliferus*: DRR394944, DRR394945; *Armadillidium vulgare*: DRR394921, DRR394929; *Porcellio scaber*: DRR394922, DRR394930. The complete MAGs of the uncultured mollicutes analyzed in this study are available in DDBJ/ENA/NCBI under the following accession numbers: *Candidatus* Tyloplasma littoralis Fukuoka2020: AP027078.1; *Candidatus* Hepatoplasma vulgare Av-JP: AP027131.1; *Candidatus* Hepatoplasma scaber Ps-JP: AP027133.1; *Candidatus* Hepatoplasma crinochetorum Tokyo2021: AP027132.1. The phylogenetic trees and associated multiple sequence alignments as well as the bioinformatic codes used in this study are available on FigShare (https://figshare.com/s/bb880cf2c37f31455632).

Introduction

Terrestrial isopods, commonly called woodlice or pill bugs, are a group of crustaceans that have adapted to life on land. They play an important ecological role as decomposers, feeding on dead plant material. Their digestive organs are home to symbiotic microorganisms that are thought to enhance the host's fitness (1–6); *Candidatus* Hepatoplasma (7) (Mollicutes: *Hepatoplasmataceae* (8)) are one of the most well-characterized isopod symbionts, which reside on the brush borders of the host's hepatopancreas (2).

There is some evidence that hepatoplasmas are mutualistic symbionts of isopods, as they are found in a variety of terrestrial and semiterrestrial isopods and have the signature of host-symbiont co-evolution (1). Additionally, the presence of hepatoplasmas is correlated with a higher survival rate under a low-quality diet (1); this has led to speculation that hepatoplasmas are nutritional symbionts that provide essential nutrients to the host. However, the exact physiological advantage of harboring these mollicutes remains unclear.

Little genome data is available for hepatoplasmas, which has hampered our understanding of the symbiotic relationship between isopods and mollicutes. The only complete genome available for *Hepatoplasmataceae* is that of "*Candidatus* Hepatoplasma crinochetorum" (7), although several draft metagenome-assembled genomes of hepatoplasmas have been reported (9, 10, 8).

We hypothesized that additional hepatoplasma genomes would help to understand the genetic basis of the physiological benefits they provide. Here, we present complete metagenome-assembled genomes (MAGs) of four *Hepatoplasmataceae* representatives, three of which are novel species. Genomic analysis supports the view

that hepatoplasmas are ectosymbionts with high nutritional dependence on the host. Hepatoplasmas lack biosynthetic pathways or digestive enzymes that could provide nutritional benefits to the host. These results suggest that hepatoplasmas are not nutritional symbionts. If the isopod-mollicute symbiosis is a mutually beneficial one, hepatoplasmas are more likely to be defensive symbionts, whose presence by competing with limiting the growth of other pathogenic microorganisms and ultimately benefit the host.

Materials and Methods

Isopod genome survey sequencing

Tylos granuliferus animals, originating from Fukuoka Prefecture, Japan, were purchased in October 2020. *Armadillidium vulgare* and *Porcellio scaber* animals were caught at the Shinagawa Campus, Tokyo University of Marine Science and Technology, Japan, in 2021. For all three isopod species, the animals were starved in a humidified chamber for several days before DNA extraction. Genomic DNA was extracted from a single animal per species by phenol-chloroform-isoamyl alcohol extraction and MagAttract HMW DNA Kit (Qiagen). Nanopore sequencing libraries were prepared using the Ligation Sequencing Kit (SQK-LSK109) according to the manufacturer's instructions and were sequenced on R9.4.1 flow cells. The ONT .fast5 files were base-called using Guppy v. 5.0.13 in super accuracy mode. Illumina paired-end sequencing was performed by Eurofins Genomics (Tokyo, Japan) on a HiSeq 4000 instrument.

Genome assembly of "*Candidatus* Tyloplasma littoralis Fukuoka2020"

The *T. granuliferus* ONT reads were filtered using SeqKit (11) at lengths of 5, 10, and 20 kb, and the three sets of length-filtered reads were *de novo* assembled by metaFlye v. 2.8.3 (12). The three assemblies all contained a circular, mollicute-like contig with a

length of approximately 600 kb. The contig from the 20-kb assembly was used as a bait to map back the ONT reads by Minimap2 v.2.19 (13), and the mapped reads were reassembled by Flye v. 2.9 in normal mode. For downstream analyses, we selected the assembly generated from the 10 kb-filtered reads, as we assumed that this read length would the best read coverage and repeat resolution after discovering out that the genome contained a large repetitive region spanning over 5 kb. The resulting assembly was polished using POLCA v.4.0.9 (14).

Genome assembly of "*Candidatus* Hepatoplasma vulgare. Av-JP"

Length-filtered *A. vulgare* ONT reads were *de novo* assembled using metaFlye v. 2.9. A circular contig was identified using Bandage and was used as a bait to map back the ONT reads by Minimap2. The mapped reads were then reassembled using Flye v. 2.9 and polished with Medaka v. 1.6.0 and POLCA v.4.0.9. Alignment of the reads revealed that the ribosomal RNA (rDNA) and transfer RNA (tRNA) cluster sequences of this assembly belonged to *Candidatus* Hepatoplasma crinochetorum Tokyo2021 (AP027132.1), which had higher sequencing coverage. As a result, we manually patched the corresponding regions using the Illumina assembly generated by SPAdes v. 3.15.5, producing the finished assembly.

Genome assembly of "*Candidatus* Hepatoplasma scaber Ps-JP"

P. scaber Illumina reads were *de novo* assembled by SPAdes v. 3.15.5. The assembly contained a circular genome sequence, which was adopted as a MAG without any polishing. The Illumina reads were mapped against the assembly using Minimap2, and the alignment was visualized using IGV to assess the integrity of the sequence.

Genome assembly of "*Candidatus* Hepatoplasma crinochetorum Tokyo2021"

A circular contig was identified from the metaFlye assembly of *A. vulgare* ONT reads described above. The ONT reads reads were mapped back by Minimap2 and reassembled using Flye v. 2.9, followed by polishing with Medaka v. 1.6.0 and POLCA v.4.0.9.

Genome annotation

The polished genome sequences were rotated to start at 100 bp upstream of the DnaA gene and annotated using DFAST v. 1.2.18 (15). BUSCO v. 5.4.3 (16) was used to assess the completeness of the assembly.

Phylogenetic analysis

A total of 25 mollicute 16S rDNA sequences (1, 17) were downloaded from NCBI (accessed October 12, 2022) and aligned with MAFFT v. 7.505 (18). The alignment was used for phylogenetic analysis with IQ-TREE v.2.2.0.3 (19), and the resulting tree was visualized with FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

We downloaded the predicted amino acid sequences of 151 mollicute genomes from NCBI RefSeq (last accessed October 2022). Single-copy orthologs were identified by OrthoFinder v. 2.5.4 (20), and aligned with MAFFT v. 7.505. The multiple sequence alignments were trimmed using trimAl v. 1.4.1 (21) and used for maximum likelihood phylogenetic analysis with IQ-TREE 2.2.0.3. The phylogenetic tree was visualized with FigTree.

Reconstruction of metabolic pathways and exploration for digestive enzymes

KEGG Pathway maps were constructed on the BLASTKOALA server (https://www.kegg.jp/blastkoala/) (22). The results were visualized on the KEGG Mapper (https://www.kegg.jp/kegg/mapper/) (23). Carbohydrate-degrading enzymes were searched on the dbCAN2 meta server (https://bcb.unl.edu/dbCAN2/) (24).

Results

Metanenome assembled genome sequences of novel Hepatoplasma relatives

We generated 11.0 to 39.6 Gb of ONT reads and 21.3 to 23.8 Gb of 2×150-bp Illumina paired-end reads (Table 1). From these shotgun sequence data, we recovered four MAGs representing isopod-associated mollicutes. *Candidatus* Tyloplasma littoralis Fukuoka2020 (AP027078.1) was likely the dominant bacterial symbiont of the *T. granuliferus* animal analyzed, as it was the only genome that was successfully assembled from the ONT reads. The *A. vulgare* assembly contained a *Paracoccus*-like genome and a *Rickettsia*-like genome, in addition to *Candidatus* Hepatoplasma crinochetorum Tokyo2021 (AP027132.1) and *Candidatus* Hepatoplasma vulgare Av-JP (AP027131.1). *P. scaber* reads contained a low proportion of bacterial reads; *Candidatus* Hepatoplasma scaber Ps-JP (AP027133.1) was the only complete bacterial genome recovered from the *P. scaber* reads, as a circular contig in the Illumina-base assembly.

The four MAGs ranged in size from 606 kb to 662 kb and had GC contents of 22.6 to 24.4 % (Figure 1, Table 2). Small genome sizes and low GC contents are characteristic to mollicute genomes. 530 to 597 protein-coding genes were predicted. BUSCO completeness for the mycoplasmatales_odb10 dataset ranged from 87.4% to 90.8% in genome mode. These BUSCO scores are low and are likely due to real gene

loss or extensive sequence divergence, rather than assembly incompleteness, as the complete genome of "*Candidatus* Hepatoplasma crinochetorum" (NZ_CP006932.1) had similar BUSCO values (Table 2). Variant calling based on Illumina read alignment detected an average of 20.8 structural variants per MAG, suggesting that the assembled MAGs represent clonal populations.

A maximum likelihood phylogenetic tree based on 16S ribosomal RNA sequences placed Fukuoka2020 into a monophyletic clade consisting of semiterrestrial isopod-associated mollicutes (Figure 2). This clade was a sister clade of the terrestrial isopod-associated mollicutes, including "*Candidatus* Hepatoplasma crinochetorum" and its closest relatives. Based on the phylogenetic divergence between semiterrestrial isopod-associated mollicutes and their terrestrial relatives, we propose the novel species name "*Candidatus* Tyloplasma littoralis" in the novel genus "*Candidatus* Tyloplasma", for the MAG Fukuoka2020. We also propose novel species names "*Candidatus* Hepatoplasma vulgare" for MAG Av-JP and "*Candidatus* Hepatoplasma scaber" for MAG Ps-JP. The novelty of these genomospecies was justified based on the low average nucleotide identities when compared to other members of the family *Hepatoplasmataceae*. "*Candidatus* Hepatoplasma scaber" was the closest relative of "*Candidatus* Hepatoplasma crinochetorum", with an extensive genome collinearity (Figure 1B).

Hepatoplasmatacaeae is a sister clade of _Metamycoplasmataceae_

To further investigate the phylogenetic position of *Hepatoplasmataceae*, we built a maximum-likelihood phylogenomic tree of mollicutes based on single-copy protein-coding genes. Ortholog analysis using OrthoFinder2 identified 64 single-copy protein-coding genes that were universally conserved among 151 mollicutes. Multiple sequence

alignments containing a total of 15,307 sites were used for maximum likelihood phylogenetic analysis by IQ-TREE2. The resulting tree recovered *Hepatoplasmataceae* as a sister clade of *Metamycoplasmataceae* (formerly known as the Bovis group) (25) (Figure 3).

Hepatoplasma group retains an intact type IIA CRISPR-Cas9 system

We identified three phage defense mechanisms on the *Candidatus* Tyloplasma littoralis genome: type I and II restriction modification systems and a likely intact type IIA CRISPR-Cas9 system (Table 2). *Candidatus* Hepatoplasma spp. appeared to lack the restriction modification systems. The initial report on the "*Candidatus* Hepatoplasma crinochetorum" genome suggested that the CRISPR/Cas machinery is no longer functional due to the loss of the helper protein Csn2 (7). However, using HHpred, we found Csn2 homologs in the vicinities of the CRISPR arrays. This indicates that *Hepatoplasmataceae* is equipped with a complete set of Type IIA CRISPR/Cas9 machinery.

Nutritional dependence on the host

Hepatoplasmas, like other mollicutes, lack many of the biosynthetic pathways necessary for the production of amino acids, nucleic acids, and carbohydrates. Instead, these pathways are likely substituted by various transport proteins, such as ABC transporters (26, 27) and the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) (28, 29). A summary of hepatoplasma metabolic pathways is shown in Figure 4.

Hepatoplasmas are only able to catabolize carbohydrates through glycolysis. The PTS catalyzes the uptake and concomitant phosphorylation of carbohydrates in bacteria (28, 29). *Candidatus* Tyloplasma littoralis Fukuoka2020 encode PTS systems for five sugars: glucose, fructose, trehalose, N-Acetyl-D-glucosamine (GlcNAc), and N-

239 Acetyl-muramic acid (MurNAc), while *Candidatus* Hepatoplasma spp. lacked the PTS
240 for GlcNAc (Table 3). A mannose isomerase was present in all hepatoplasma genomes,
241 suggesting the ability to utilize mannose, but we could not identify a mannose
242 transporter protein. The sugars are converted to beta-D-fructose 6-phosphate and enter
243 the glycolysis pathway (Figure 4). The ability to metabolize GlcNAc and MurNAc, the
244 building blocks of bacterial cell walls, means that *Hepatoplasmataceae* can utilize
245 debris from other cell wall-containing bacteria.

246 *Hepatoplasmataceae* lack most of amino acid synthesis pathways and therefore
247 must import them from the environment through yet unknown transporters. The
248 conversion of glycine to serine by serine hydroxymethyltransferase (EC 2.1.2.1) is
249 coupled with the conversion of 5,10-methylenetetrahydrofolate to tetrahydrofolate,
250 which is part of the one-carbon pool. Aspartate is converted to asparagine by the
251 aspartate-ammonia ligase (EC 6.3.1.1). The ammonia moiety could be derived from the
252 purine nucleotide cycle in the nucleotide savage pathway and the arginine deaminase
253 (ADI) pathway (discussed below).

254 The ADI pathway, composed of arginine deiminase (EC 3.5.3.6), carbamate
255 kinase (2.7.2.2), and arginine/ornithine antiporter, produces ATP through the conversion
256 of arginine to citrulline to ornithine (30) (Table 3). This pathway has been suggested to
257 act as a pH buffer in order to counteract acidification resulting from glycolysis (8, 10).
258 A complete set of ADI pathway components was present in *Candidatus* Hepatoplasma
259 spp., whereas *Candidatus* Tyloplasma littoralis lacked them altogether. The presence of
260 the ADI pathway has been reported in other *Hepatoplasma* draft MAGs (8, 10). This
261 suggests that the absence of the ADI pathway in *Candidatus* Tyloplasma littoralis is due
262 to a secondary loss.

Mycoplasmas are able to synthesize glycerophospholipids, the main components of the cell membrane (31). Most of the enzymes required for the biosynthesis of glycerophospholipids were successfully identified, but the phosphatidylglycerophosphatase (EC:3.1.3.27) was absent from the hepatoplasma genomes. The lack of this gene in Mycoplasma genomes has been noted in the comparative genomic analyses of swine respiratory tract mycoplasmas (32). As the authors of (32) noted, this enzymatic reaction should be present and is likely to be replaced by other gene(s).

Glycerol utilization has been linked to the virulence of *Mycoplasma* (31, 33). An aquaporin protein gene was identified in all *Hepatoplasmataceae* genomes, which could functions as a glycerol importer (34) (Table 3). Additionally, *Candidatus* Hepatoplasma genomes encoded a gene cluster associated with glycerol utilization and therefore are likely able to utilize glycerol as carbon source, while *Candidatus* Tyloplasma littoralis lacks components of this pathway.

As with other mollicutes, hepatoplasmas lack *de novo* synthesis pathway of nucleotides and therefore rely on import and the salvage pathway. Purine nucleobases are converted to nucleosides by purine nucleoside phosphorylase (EC 2.4.2.1), and then to nucleotide by deoxyadenosine kinase (EC 2.7.1.76). Hepatoplasmas and *Mycolasma pneumoniae* encode deoxyadenosine/deoxycytidine kinase (EC:2.7.1.76 2.7.1.74), but not deoxyguanosine kinase (DGK; EC 2.7.1.113), even though DGK activity has been detected in *M. pneumoniae* (35). Hypoxanthine is converted to inositol monophosphate (IMP) by hypoxanthine phosphoribosyltransferase (EC:2.4.2.8). IMP is converted to adenosine monophosphate in the purine nucleotide cycle. However, xanthine dehydrogenase/oxidase (EC:1.17.1.4 1.17.3.2) and guanine deaminase (EC:3.5.4.3)

were missing from hepatoplasma genomes, suggesting that the interconversion between purine bases is not possible (36, 37). In addition, nucleoside-diphosphate kinase (EC:2.7.4.6) was missing from hepatoplasma and other mollicute genomes, which is likely to be compensated for by monophosphate kinases (38).

Our findings suggest that hepatoplasmas do not contribute to host nutrition by synthesizing cofactors. They lacked nicotinamide phosphoribosyltransferase (EC:2.4.2.12) and nicotinamide adenine dinucleotide kinase kinase (EC:2.7.1.23), both of which are present in *M. pneunmoniae*. Flavin mononucleotide (FMN) and derivatives can potentially be imported by specific transporters, which seem to be uniquely expanded in *Candidatus* Tyloplasma littoralis (BDU67363.1, BDU67639.1, BDU67349.1, BDU67732.1, and BDU67420.1). Riboflavin is converted to FMN and then to flavin adenine dinucleotide. Thiamine kinase (EC 2.7.1.89), thiamine-monophosphate kinase (EC 2.7.4.16), and thiamine pyrophosphokinase (EC 2.7.6.2) were not detected in the hepatoplasma genomes. The one-carbon pool seems to be functioning in hepatoplasmas.

ABC transporters represent the largest group of active membrane transport proteins in bacteria (33, 39). *Candidatus* Tyloplasma littoralis Fukuoka2020 encoded at least five ABC transporter systems. The substrate molecules could not be determined based on homology search due to ambiguous search outcome.

Overall, *Hepatoplasmataceae* are highly dependent on the host for nutrition and are unlikely to code for biosynthetic pathways providing essential nutrients to the host. A few differences in metabolic pathways exist among the four *Hepatoplasmataceae* species; while *Candidatus* Tyloplasma littoralis Fukuoka2020 seems to be more versatile in terms of its ability to utilize various sugars, *Candidatus*

311 Hepatoplasma spp. seem to exploit the arginine deaminase pathway as a means of
312 generating ATP.
313 **Hepatoplasmataceae do not encode enzymes suggestive of nutritional benefits to**
314 **the host**
315 We searched for genes encoding polysaccharide-degrading enzymes in hepatoplasma
316 genomes using dbCAN2. The only carbohydrate-degrading enzyme was alpha,alpha-
317 phosphotrehalase in *Candidatus* Hepatoplasma vulgare (BDV02505.1), *Candidatus*
318 Hepatoplasma scaber (BDV03525.1) and *Candidatus* Hepatoplasma crinochetorum
319 (WP_025208682.1 and BDV02972.1). In combination with the discovery that
320 hepatoplasmas lack enzymes required for the synthesis of essential nutrients, it is
321 unlikely that hepatoplasmas provide nutritional benefits to their isopod hosts.
322 **Discussion**
323 The MAGs presented in this study provide important reference materials to analyze the
324 evolution and lifestyles of terrestrial isopod-associated mollicutes. *Hepatoplasmataceae*
325 is characterized by small genome sizes with a highly streamlined genome architecture.
326 The traditional view of the isopod-mollicute symbiosis is that it is a nutritional
327 symbiosis, with the mollicute symbionts providing nutritional benefits to the host (1, 3,
328 17). However, this study did not find evidence that hepatoplasmas encode biosynthetic
329 pathways or digestive enzymes related to polysaccharide degradation; rather,
330 hepatoplasmas lack major biosynthetic pathways and therefore are nutritionally highly
331 dependent on the host, and the only carbohydrate-degrading enzymes present were
332 alpha,alpha-phosphotrehalase in *Candidatus* Hepatoplasma species. Regardless, it is
333 still possible that hepatoplasmas code for yet unknown enzymes that could not be
334 identified by the homology search algorithms used in this study.

Some bacterial symbionts benefit the host by conferring resistance against invading pathogens, collectively called defensive symbionts (40, 2, 41). Defensive symbionts often encode toxin genes to kill competing microorganisms (42), but hepatoplasmas do not seem to encode toxins. This suggests that they may use other means to provide defense to the host, such as providing physical barriers that block the access of competitors to the host's tissues, absorbing free nutrients to deny them to competitors, and enhancing the host's immune response through immune priming. For example, *Spiroplasma* endosymbiont of *Drosophila* confer protection from parasitoid wasps by competing with the parasitoid for lipids (34). Given the lack of evidence for nutritional benefits provided by hepatoplasmas, we speculate that they may act as defensive symbionts of terrestrial isopods (34, 41, 43).

Overall, the availability of new data on isopod-associated mollicutes has provided valuable insights into the evolution of *Hepatoplasmataceae*. However, it is important to note that the analyses in this study are based on a limited number of genome sequences and lack experimental validation. Further sequencing and characterization of additional hepatoplasma lineages would greatly improve our understanding of the evolution and significance of the isopod-mollicute symbiosis.

Description of Tyloplasma gen. nov.

Tyloplasma (Ty.lo.plas'ma. Gr comp. *Tylos*, referring to the host isopod *Tylos*; Gr. neut. n. *plasma*, something formed or molded; N.L. neut. n. *tyloplasma*, intended to mean associated with the host isopod *Tylos*). The type species is *Candidatus* Tyloplasma littoralis gen. nov. sp. nov. The members of this genus can be distinguished from other species in the family *Hepatoplasmataceae* and order *Mycoplasmoidales* by their

358 phylogenetic positions based on 16S ribosomal RNA sequences and their host range

359 specific of semiterrestrial isopods, such as *Tylos* and *Ligia*.

360

Description of "*Candidatus* Tyloplasma litoralis"

Tyloplasma litoralis (li.tto.ra'lis. L. neut. adj. *littoralis* referring to the littoral habitat of the host). This taxon is distinguished from other members of *Hepatoplasma* based on low average nucleotide identities and distinct phylogenetic positions. A likely ectosymbiont of the semiterrestrial isopod *Tylos granuliferus*. The circular genome is 615,622 bp in size with 24.4% GC, coding for 530 protein-coding genes, six rRNA genes and 25 tRNA genes; lacks major metabolic pathways including biosynthesis of amino acids, nucleic acids, lipids, and cofactors; arginine deiminase pathway absent; predicted to utilize glucose, fructose, trehalose, N-Acetyl-D-glucosamine, and N-Acetyl-muramic acid; so far uncultivated. This taxon is represented by the MAG AP027078.1.

Description of "*Candidatus* Hepatoplasma vulgare"

Hepatoplasma vulgare (vul.ga're. L. adjective *vulgare*, common; referring to the host isopod *Armadillidium vulgare*). This taxon is distinguished from other members of *Hepatoplasma* based on low average nucleotide identities. The circular genome is 662,079 bp in size with 22.7% GC, coding for 597 protein-coding genes, three rRNA genes and 26 tRNA genes; lacks major metabolic pathways including biosynthesis of amino acids, nucleic acids, and cofactors; arginine deiminase pathway present; predicted to utilize glucose, fructose, trehalose, N-Acetyl-muramic acid, and glycerol; so far uncultivated. This taxon is represented by the MAG AP027131.1.

Description of "*Candidatus* Hepatoplasma scaber"

Hepatoplasma scaber (sca'ber. L. adjective *scaber*, rough, scabrous; intended to show association with the host *Porcellio scaber*). This taxon is distinguished from other members of *Hepatoplasma* based on low average nucleotide identities. The circular

genome is 606,194 bp in size with 24.7% GC, coding for 543 protein-coding genes, three rRNA genes and 28 tRNA genes; lacks major metabolic pathways including biosynthesis of amino acids, nucleic acids, and cofactors; arginine deiminase pathway present; predicted to utilize glucose, fructose, trehalose, and N-Acetyl-muramic acid, and glycerol; so far uncultivated. This taxon is represented by the MAG AP027133.1

Author Statements

Author contributions

Conceptualization: S.K. Data curation: S.K. Formal analysis: S.K. Funding acquisition: S.K., I.H. Investigation: S.K., R.N. Methodology: S.K. Project administration: I.H., H.K. Resources: I.H., H.K. Supervision: I.H., H.K. Visualization: S.K. Writing-original draft: S.K. Writing-review & editing: I.H., H.K.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Consent for publication

The authors consent for publication.

Ethical approval

For the handling of the terrestrial isopods, we complied with the relevant institutional guidelines in Tokyo University of Marine Science and Technology.

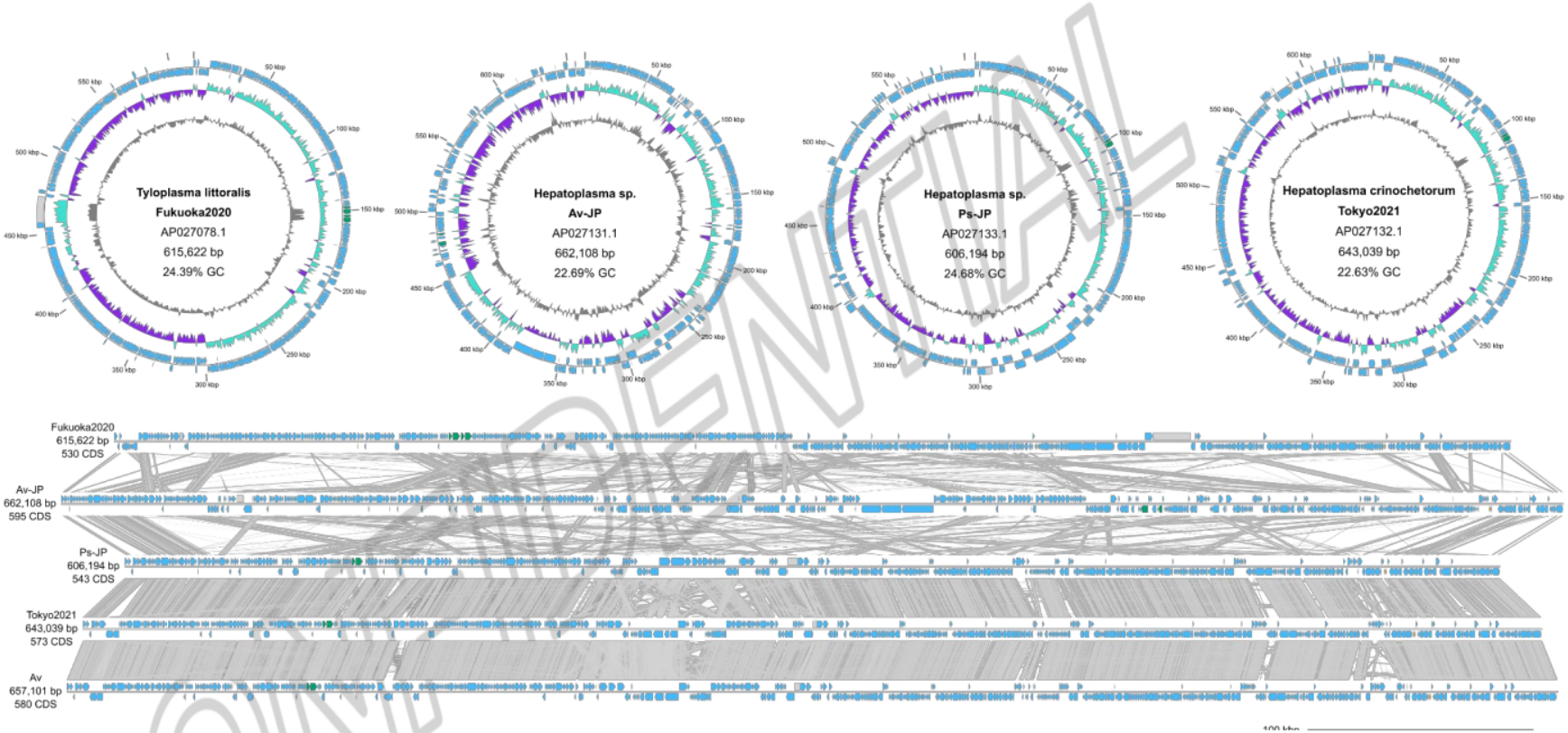
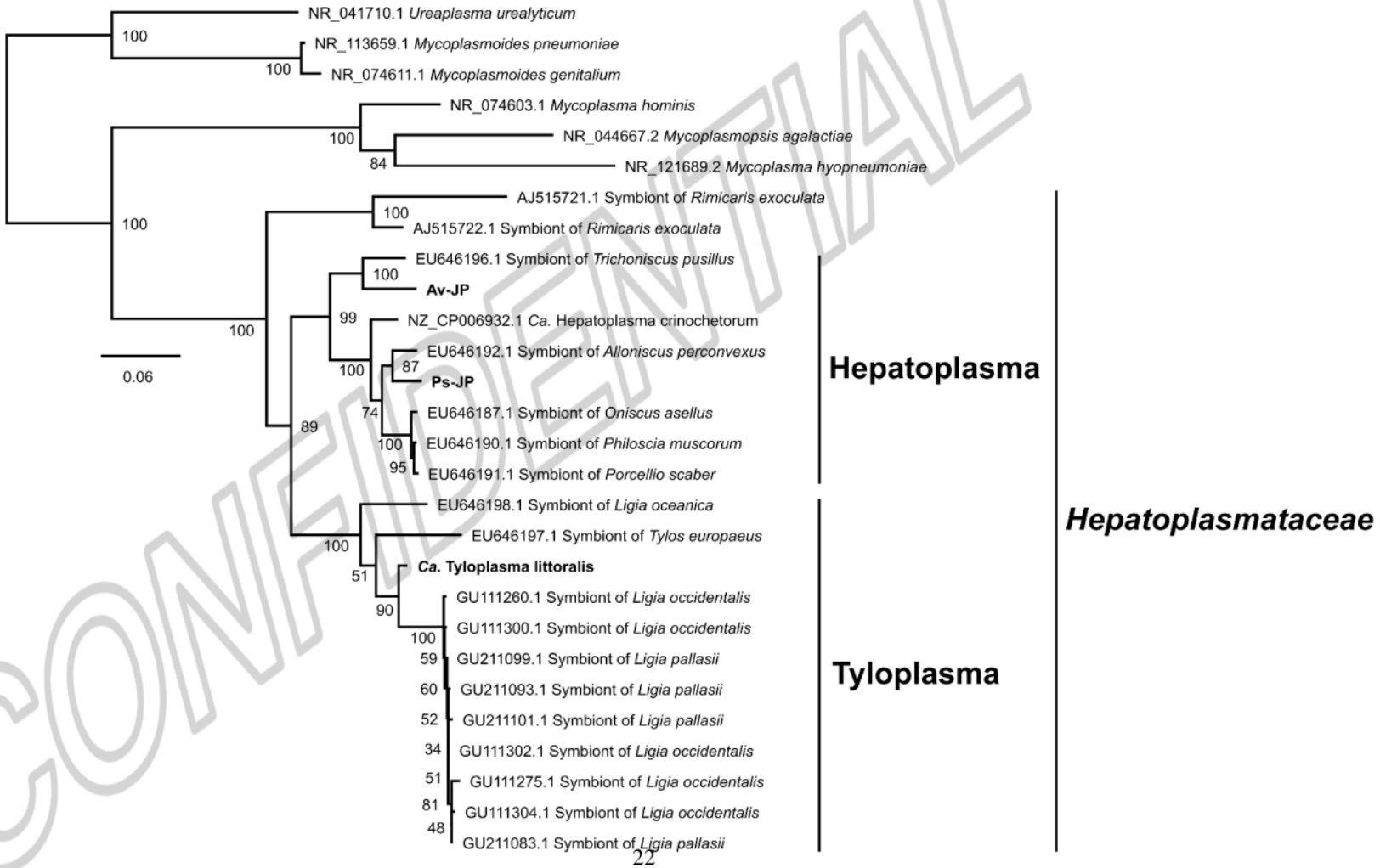


Figure 1. Genome diagrams of *Hepatoplasmataceae* members

(A) Circular genome diagrams of *Hepatoplasmataceae* members. Arrowheads indicate the transcriptional orientation. Outer track: protein-coding genes (blue), ribosomal RNA genes (green), transfer RNA genes (orange), repeat regions (gray). Middle track: GC skew of 100 bp sliding windows with 10-bp increments (positive: emerald, negative: purple). Inner track: Deviation of GC contents from the average, 100 bp sliding windows with 10-bp increments.

(B) Linear diagrams of *Hepatoplasmataceae* genomes. The reference genome for "*Candidatus* Hepatoplasma crinochetorum" is shown at the bottom as isolate "Av" for comparison. TBLASTX hits (e-value: 1e-3, bitscore:50) are shown in gray.



403

Figure 2. Phylogenetic analysis of 16S ribosomal RNA gene sequences

A total of 1462 sites (model: GTR+F+R3) were used in the maximum-likelihood phylogenetic analysis using IQ-TREE2 v. 2.2.0.3. Values beside nodes indicate the ultrafast bootstrap support (1,000 trials).

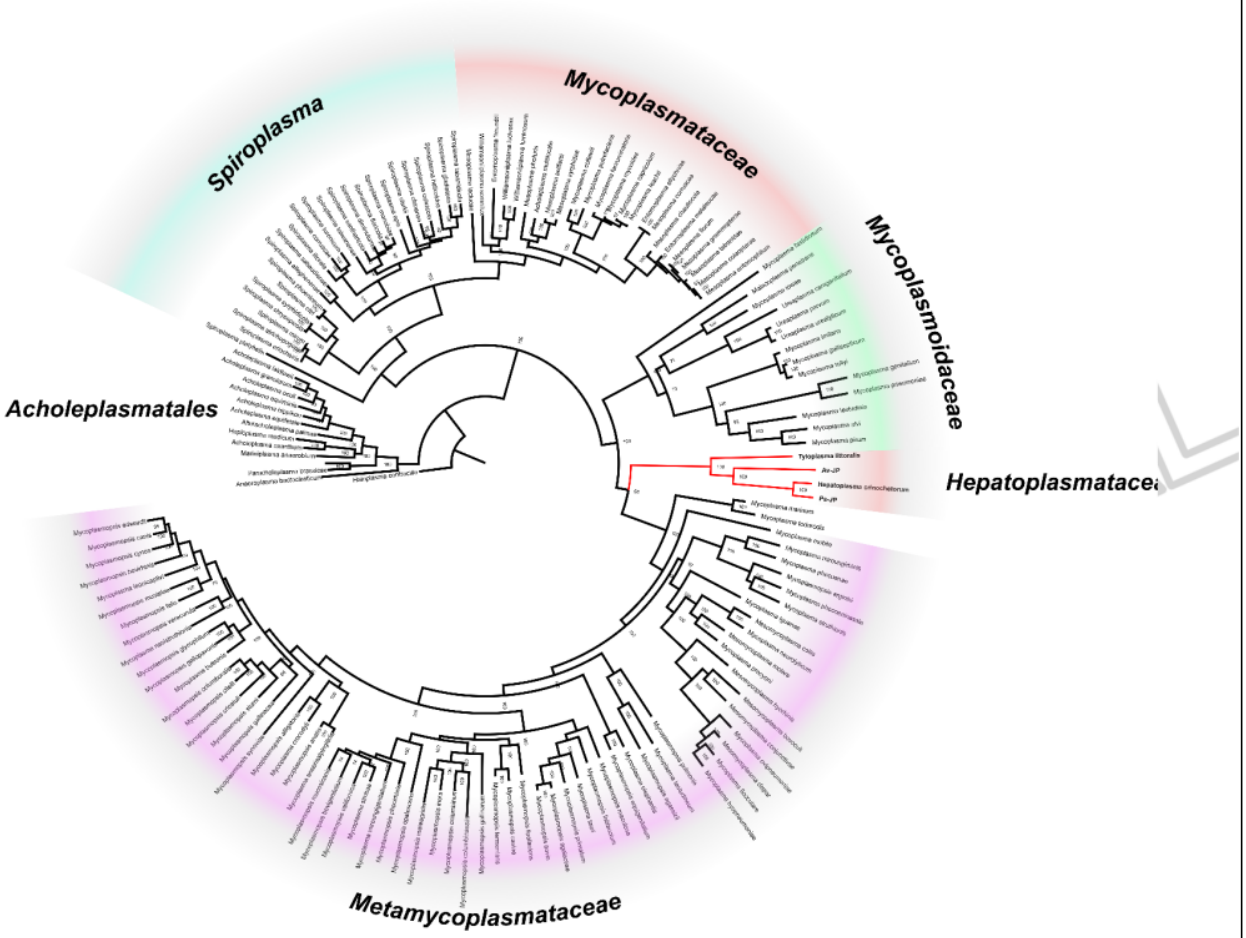


Figure 3. Phylogenomic analysis of Mollicutes

A total of 64 genes (15,307 sites; model: LG+F+I+I+R10) were used in the maximum-likelihood phylogenetic analysis using IQ-TREE2 v. 2.2.0.3. Values beside nodes indicate the ultrafast bootstrap support (1,000 trials).t

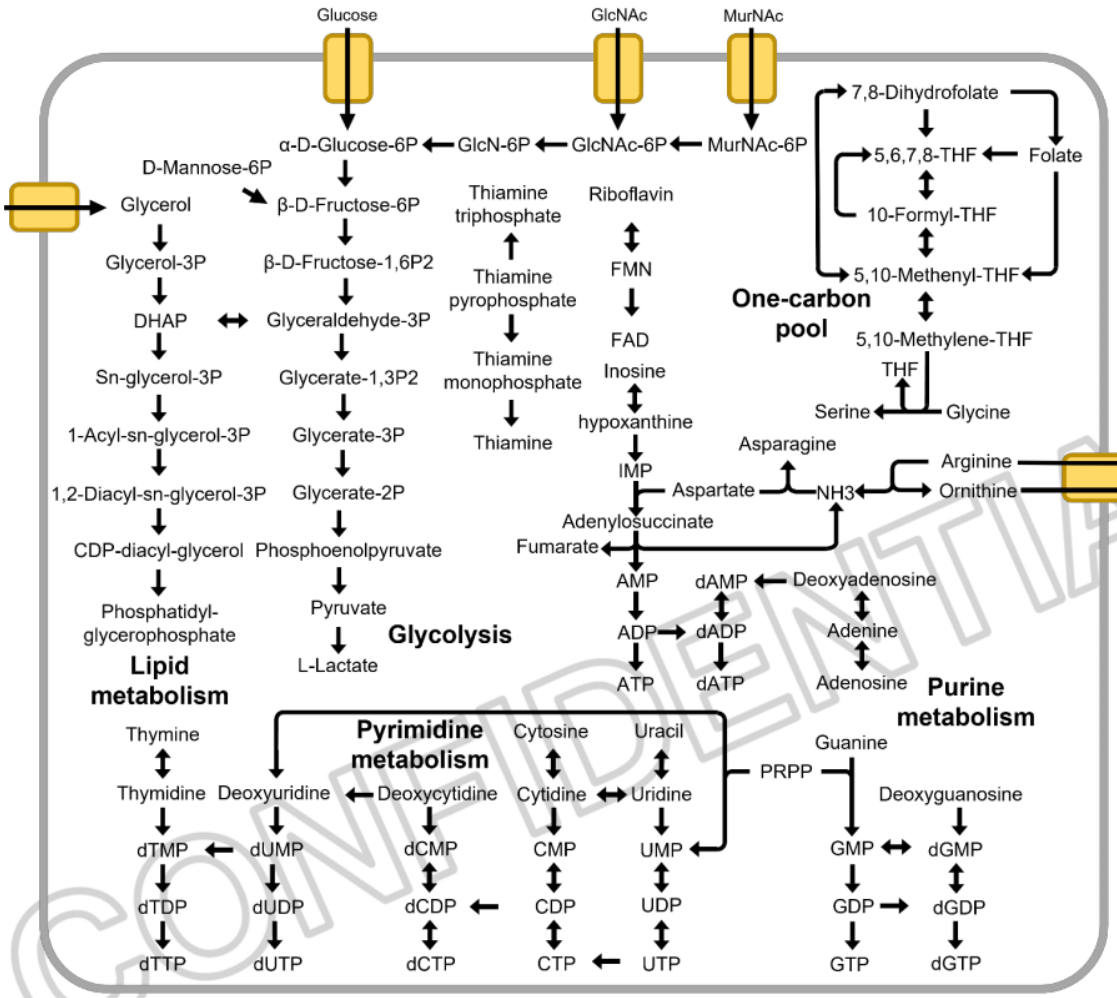


Figure 4. Summary of metabolic pathways in *Hepatoplasmataceae*

Table 1. Genome assembly statistics of *Hepatoplasmataceae* representatives

Species		***Ca.* Tyloplasma littoralis**	***Ca.* Hepatoplasma vulgare.**	***Ca.* Hepatoplasma scaber.**	***Ca.* Hepatoplasma crinochetorum**	
Isolate		Kyushu2020	Av-JP	Ps-JP	Tokyo2021	Av*
Reference		This study	This study	This study	This study	(2, 6)
Host		*Tylos granuliferus*	*Armadillidium vulgare*	*Porcellio scaber*	*Armadillidium vulgare*	*Armadillidium vulgare*
Accession no.		AP027078.1	AP027131.1	AP027133.1	AP027132.1	NZ_CP006932.1
Length (bp)		615,622	662,079	606,194	643,039	657,101
GC content (%)		24.4	22.7	24.7	22.6	22.5
CDSs		530	597	543	573	577
rRNAs		6	3	3	3	3
tRNAs		25	26	28	27	28
CRISPRs		2	1	1	1	1
BUSCO completeness [mycoplasmatales_odb10 (n=174), genome mode]	Complete BUSCOs (C)	158 (90.8%)	158 (90.8%)	156 (89.7%)	152 (87.4%)	152 (87.4%)
	Complete and single-copy BUSCOs (S)	158 (90.8%)	158 (90.8%)	156 (89.7%)	152 (87.4%)	152 (87.4%)
	Complete and duplicated BUSCOs (D)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Fragmented BUSCOs (F)	1 (0.6%)	3 (1.7%)	2 (1.1%)	3 (1.7%)	3 (1.7%)
	Missing BUSCOs (M)	15 (8.6%)	13 (7.5%)	16 (9.2%)	19 (10.9%)	19 (10.9%)

Coverage	Illumina	527.422	38.2049	19.997	761.155	-
	ONT (5kb>)	223.069	14.9362	4.92264	298.474	-
	Illumina SVs	10	42	30	1	-

*Shown for comparison

Table 2. Genome defense-related genes in *Hepatoplasmataceae*

		Ca. Tyloplasma littoralis	*Ca*. Hepatoplasma vulgare	*Ca*. Hepatoplasma scaber	*Ca*. Hepatoplasma crinochetorum
Type I restriction endonuclease	subunit S	BDU67866.1 BDU67868.1 BDU67869.1			
	subunit R	BDU67870.1			
	subunit M	BDU67867.1			
Type II restriction endonuclease	MjaI	BDU67724.1			
Type II CRISPR/Cas9	Cas1	BDU67747.1	BDV02176.1	BDV03524.1	WP_025208688.1
	Cas2	BDU67748.1	BDV02174.1	BDV03523.1	WP_128571630.1
	Cas9	BDU67745.1	BDV02189.1	BDV03530.1	WP_025208688.1
	Csn2	BDU67749.1	BDV02173.1	BDV03522.1	WP_025208679.1

422 **Table 3. Examples of metabolism-related genes in *Hepatoplasmataceae***

Functions	Description	Gene	*Ca.* Tyloplasma littoralis	*Ca.* Hepatoplasma vulgare	*Ca.* Hepatoplasma scaber	*Ca.* Hepatoplasma crinochetorum
Glycerol utilization	Aquaporin		BDU67486.1	BDV02492.1, BDV02493.1	BDV03539.1	WP_025208695.1
	Glycerol uptake facilitator protein	glpF		BDV02358.1	BDV03527.1	WP_025208684.1
	Glycerol kinase	glpK		BDV02359.1	BDV03528.1	WP_025208685.1
	Glycerol-3-phosphate dehydrogenase	glpA		BDV02360.1	BDV03529.1	WP_128571633.1
Arginine deiminase pathway	Arginine deiminase	arcA		BDV02199.1	BDV03492.1	WP_038462236.1
	Ornithine carbamoyltransferase, catabolic	ArcB		BDV02200.1	BDV03493.1	WP_025208634.1
	Carbamate kinase	ArcC		BDV02202.1	BDV03495.1	WP_025208637.1
	Arginine/ornithine antiporter	ArcD		BDV02201.1	BDV03494.1	WP_025208636.1
PTS	Trehalose transporter		BDU67689.1	BDV02506.1	BDV03526.1	WP_025208683.1
	Glucose transporter		BDU67775.1	BDV02499.1	BDV03671.1	WP_025208828.1
	GlcNAc transporter		BDU67558.1	BDV02647.1	BDV03283.1	WP_025208424.1
	MurNAc transporter		BDU67422.1			
	Fructose transporter		BDU67439.1	BDV02545.1	BDV03409.1	WP_025208549.1

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