Phylogenomics of “Candidatus Hepatoplasma crinochetorum,” a Lineage of Mollicutes Associated with Noninsect Arthropods

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Abstract

Bacterial gut communities of arthropods are highly diverse and tightly related to host feeding habits. However, our understanding of the origin and role of the symbionts is often hindered by the lack of genetic information. “Candidatus Hepatoplasma crinochetorum” is a Mollicutes symbiont found in the midgut glands of terrestrial isopods. The only available nucleotide sequence for this symbiont is a partial 16S rRNA gene sequence. Here, we present the 657,101 bp assembled genome of Candidatus Hepatoplasma crinochetorum isolated from the terrestrial isopod Armadillidium vulgare. While previous 16S rRNA gene-based analyses have provided inconclusive results regarding the phylogenetic position of Candidatus Hepatoplasma crinochetorum within Mollicutes, we performed a phylogenomic analysis of 127 Mollicutes orthologous genes which confidently branches the species as a sister group to the Hominis group of Mycoplasma. Several genome properties of Candidatus Hepatoplasma crinochetorum are also highlighted compared with other Mollicutes genomes, including adjacent tryptophan tRNA genes, which further our understanding of the evolutionary dynamics of these genes in Mollicutes, and the presence of a probably inactivated CRISPR/Cas system, which constitutes a testimony of past interactions between Candidatus Hepatoplasma crinochetorum and mobile genetic elements, despite their current lack in this streamlined genome. Overall, the availability of the complete genome sequence of Candidatus Hepatoplasma crinochetorum paves the way for further investigation of its ecology and evolution.

Key words: Hepatoplasma, Mollicutes, genome sequence, symbiont.
using the 16S rRNA gene. All phylogenetic analyses concur in placing Hepatoplasma within Mollicutes (Wang et al. 2004; Kostanjsek et al. 2007; Fraune and Zimmer 2008; Nechitaylo et al. 2009). The membership of Hepatoplasma to Mollicutes is also supported by the total lack of a cell wall (Wang et al. 2004), which is a physiological specificity of Mollicutes (Razin 2006). However, there are still uncertainties on the evolutionary relationships of Hepatoplasma relative to the four currently defined groups of Mollicutes: the AAA group (Acholeplasma/Asteroplasma/Anaeroplasma), which also includes plant parasites Candidatus Phytoplasma spp.; the Entomoplasmatales group containing insect-associated Spiroplasma/Mesoplasma species and mammal-associated Mycoplasma mycoides subgroup species; and two major groups of Mycoplasma, Hominis and Pneumoniae, which infect various vertebrates including humans (Tully et al. 1993; Razin 2006; Oshima and Nishida 2007). Depending on studies, Hepatoplasma has been inferred to be sister to the Pneumoniae group (Wang et al. 2004; Kostanjsek et al. 2007), to the Entomoplasmatales group (Fraune and Zimmer 2008) or to a monophyletic group composed of the Pneumoniae and Hominis groups (Nechitaylo et al. 2009), and generally with little statistical support. To elucidate the phylogenetic relationships of Hepatoplasma and provide new molecular tools for the study of the evolution and ecology of these symbionts, we determined the genome sequence of Hepatoplasma symbionts from the terrestrial isopod Armadillidium vulgare and performed a phylogenomic analysis providing conclusive evidence for Hepatoplasma constituting a sister lineage to the Hominis group of Mollicutes.

**Phylogenomic Analyses**

The genome sequence of Hepatoplasma strain Av consists of a circular DNA molecule of 657,101 bp with an average G+C content of 22.5% and it harbors a total of 582 CDS, 433 of which have a predicted function (table 1). The genome is highly reduced with a coding density of 94% and only four identified pseudogenes, in line with most other Mollicutes genomes. We conducted a phylogenomic analysis using a set of 127 orthologous gene families concurrently detected in Hepatoplasma, 45 available representative Mollicutes genomes, and the three outgroups Bacillus subtilis, Streptococcus pneumoniae, and Clostridium perfringens (see Materials and Methods). Two maximum-likelihood analyses were performed, using the standard LG-I amino acid substitution model (Le and Gascuel 2008) and the Mollicutes-optimized MOLLI60 substitution model (Lemaitre et al. 2011), respectively.

Both trees yielded essentially the same topology with few minor branching inconsistencies (supplementary fig. S1, Supplementary Material online). The main inconsistency occurred in the Hominis group, in which the MOLLI60 analysis branched M. pulmonis strain with the M. mobile strain, while the LG-I analysis branched it at the root of an internal group (including the M. synoviae strain, among others). Both topologies were statistically very robust, with bootstrap procedures values slightly higher for the MOLLI60 topology in general (all but one nodes with 100% confidence) than for the LG-I topology (most nodes with 90–100% confidence), suggesting that the former better reflects the true species relationships. Interestingly, the conflicting branching of M. pulmonis was previously highlighted in a whole-genome-based Mollicutes phylogeny, in which M. pulmonis branched with M. synoviae instead of M. mobile, with only 61% bootstrap support (Oshima and Nishida 2007). These results emphasize the relevance of using optimized amino acid substitution models to conduct protein sequence comparisons, especially for highly compositionally biased bacterial genomes such as Mollicutes.

Although previous phylogenetic analyses of Hepatoplasma exclusively based on 16S rRNA have produced conflicting results (Wang et al. 2004; Kostanjsek et al. 2007; Fraune and Zimmer 2008; Nechitaylo et al. 2009), our phylogenomic approach provides unprecedented support for the branching of Hepatoplasma as sister lineage of the Hominis group with 100% confidence under both the LG-I and MOLLI60 models (fig. 1; supplementary fig. S1, Supplementary Material online). From an evolutionary perspective, the nesting of Hepatoplasma within a clade grouping the Hominis and Pneumoniae groups (all known strains of which are vertebrate-associated parasites), could at first sight suggest that the ancestor of Hepatoplasma was associated to vertebrates and that the presence of this bacterium in arthropods results from a vertebrate-to-invertebrate transfer. However, horizontal transfers of Mollicutes from nonvertebrate to vertebrate hosts have seemingly been common place during Mollicutes evolution. For example, the mammal-infecting subgroup M. mycoides originated from insect-associated species (Spiroplasma and Mesoplasma species) and various Acholeplasma and all Anaeroplasma strains infecting mammals are phylogenetically related to plant-associated Phytoplasma species (Razin 2006). This observation suggests that the alternative hypothesis of an invertebrate-associated Hepatoplasma ancestor transferred multiple times to vertebrate hosts remains highly probable. It will be interesting to address this question more comprehensively in the future by

**Table 1**

| General Properties of the “Candidatus Hepatoplasma cuniculorum” strain Av Genome |
|------------------------------------------|
| Total length (bp) | 657,101 |
| GC content (%) | 22.5 |
| CDS number | 582 |
| Coding density (%) | 94 |
| Pseudogenes | 4 |
| tRNA number | 27 |
| rRNA number | 3 |
Fig. 1.—Phylogeny of the 46 Mollicutes strains from which proteomes are available. The tree is based on a set of 127 orthologous proteins and results from a maximum likelihood analysis computed by RAxML using a gamma model of rate heterogeneity, a proportion of invariable sites, and the Mollicutes-optimized substitution matrix MOLLI60. Bootstrap supports were computed using 1,000 iterations and the three Firmicutes Bacillus subtilis, Streptococcus pneumoniae, and Clostridium perfringens were used as outgroups.
adding more invertebrate-associated Mollicutes genomes to the phylogeny.

Evolution of Tryptophan tRNAs

The *Hepatoplasma* genome contains a single 5S-23S-16S operon, as well as 27 tRNAs (table 1). Interestingly, both Tryptophan tRNAs (UCA and CCA anticodons) are adjacent, a feature previously detected in *M. capricolum* and *S. citri* (Yamao et al. 1988; Citti et al. 1992). Investigation of tRNA-Trp gene positions in Mollicutes genomes reveals that this feature is restricted to the Entomoplasmatales group in addition to *Hepatoplasma* (fig. 2). Moreover, gene synteny is conserved between the two groups over ~7 kb, while there is no or very few conservation with the 12 other representative genomes analyzed (fig. 2). According to the aforementioned Mollicutes phylogeny, the occurrence of adjacent tRNA–Trp in both *Hepatoplasma* and Entomoplasmatales suggests that this region has been horizontally transferred between the two groups, or that this gene order is ancestral to all groups but AAA, and subsequent chromosomal rearrangements occurred independently around tRNA–Trp genes in Hominis and Pneumoniae groups.

To investigate these hypotheses, we performed a phylogenetic analysis of the two degV genes, which are located immediately downstream of tRNA–Trp genes in Entomoplasmatales and *Hepatoplasma* genomes. Orthologs of these two genes were retrieved in other Mollicutes genomes using the orthogroup information provided by the OrthoMCL procedure (see Materials and Methods), and Neighbor-Joining and ML trees were computed. Both analyses produced congruent trees (supplementary fig. S2, Supplementary Material online), with a well-supported clade for each degV gene of Entomoplasmatales and a separate clade for the two *Hepatoplasma* degV genes. Although phylogenetic relationships between these clades are poorly resolved, the monophyly of each *Hepatoplasma* and Entomoplasmatales degV clades does not support the hypothesis that *Hepatoplasma* degV genes originate from those of Entomoplasmatales (or vice versa), i.e., shared gene synteny between these two groups probably does not result from horizontal gene transfer.

Yamao et al. (1988) proposed a hypothesis in which a tRNA–Trp with UGA anticodon may appear in bacterial lineages when all (or almost all) TGA termination codons have shifted to TAA, because of the evolution toward AT-biased genomes observed in highly reduced genomes (Moran et al. 2008). In such a case, the tRNA$_{CCA}$ may be duplicated and mutated to tRNA$_{UCA}$ through a C to T mutation in the anticodon, allowing TGG Trp codons present in the genome to mutate to TGA and being still correctly translated. Under this hypothesis, the *Hepatoplasma* tRNA–Trp tandem duplication may represent the ancestral state from which the new anti-codon recognition has appeared.

**CRISPR/Cas System**

The *Hepatoplasma* genome contains no detectable phage, plasmid, transposon, insertion sequence, or group I or II intron gene. The absence of mobile genetic elements is a common feature of streamlined bacterial symbiont genomes (Moran et al. 2008; Moya et al. 2008), although notorious exceptions exist such as *Wolbachia* (Cerveau et al. 2011; Leclercq et al. 2011). Interestingly, we detected a CRISPR/Cas system using the CRISPRFinder interface (Grissa et al. 2007). CRISPR/Cas systems are molecular systems involved in defense of prokaryotes against phage and other mobile genetic element infections (Deveau et al. 2010). CRISPR/Cas systems are composed of a CRISPR locus, and a variable number of CRISPR-associated (cas) genes located in the vicinity of the CRISPR locus (Deveau et al. 2010; Horvath and Barrangou 2010). Several CRISPR/Cas subtypes have been described, each with a specific subset of cas genes (Makarova et al. 2011). In *Hepatoplasma*, the CRISPR locus is located at genome coordinates 320,594–322,939 and consists of 35 spacers separated by 36-bp repeat units. The genome harbors cas1 and cas2 genes, considered as universal markers of CRISPR/Cas systems, and the cas9 gene, which is typical of the type II CRISPR/Cas subtype (Makarova et al. 2011). The type II CRISPR/Cas subtype is usually composed of cas9 (formerly csn1), cas1, cas2, and csn2 (for type IIA) or cas4 (for type IIB) genes, all located in a single transcriptional unit directly upstream of the CRISPR locus. However, in *Hepatoplasma*, neither csn2 nor cas4 is present, cas9 is in reverse orientation compared with the CRISPR locus, and cas1 and cas2 are located 13 kb upstream of the CRISPR locus, in reverse orientation, and separated from cas9 by 7 genes involved in other metabolic functions (fig. 3). Contrary to *Hepatoplasma*, the eight other Mollicutes genomes in which we found CRISPR/Cas systems all show the typical type IIA subtype organization (with some minor rearrangements/pseudogenization in *M. mobile*, *M. ovipneumoniae*, and *M. arthritidis*), indicating that the unusual organization we recorded in *Hepatoplasma* does not reflect a general feature of CRISPR/Cas systems in Mollicutes (fig. 3; supplementary table S1, Supplementary Material online). Previous studies on the *Streptococcus thermophilus* type IIA CRISPR/Cas subtype suggest that csn2 is involved in the adaptation stage of immunity acquisition, that is, the acquisition of invaders’ DNA fragments used to prevent further infections (Barrangou et al. 2007; Garneau et al. 2010). Bacterial strains devoid of this gene, such as *Hepatoplasma*, may thus not update their defense against new infections with mobile genetic elements, which strongly reduces the efficiency of the CRISPR/Cas system. Therefore, the CRISPR/Cas system in *Hepatoplasma* may have become largely inefficient, allowing cas gene rearrangements without counterselection. Altogether, these observations suggest that the CRISPR/Cas system has likely been inactivated in *Hepatoplasma*. 

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**Fig. 2.**—Gene synteny of regions harboring tRNA–Trp in *Hepatoplasma* and different representative Mollicutes genomes. The tRNA anticodon (CCA or UCA) is indicated, and surrounding regions for each tRNA–Trp gene are displayed when the copies are not in tandem. Gene lengths are not to scale. Genes with the same color between genomes are homologous, except for tRNAs which are all colored in light blue, and mobile genetic element genes colored in gray. Phylogenetic relationships displayed on the left are retrieved from figure 1. Phylogenetic group names are indicated on the right. "Hyp prot": hypothetical protein.
It is noteworthy that despite the absence of crs2, the cas9, cas1, and cas2 genes do not show any apparent hallmark of pseudogenization and all 35 repeat units of the CRISPR locus are strictly identical, suggesting that the CRISPR/Cas system, if inactive, was inactivated recently. In any event, the occurrence of a CRISPR-Cas system in the Hepatoplasma genome, even though it is probably nonfunctional, reveals that Hepatoplasma has been confronted to mobile genetic elements in its evolutionary past. Therefore, the current lack of mobile genetic elements in this genome does not reflect that it has never had any. Rather, it indicates that remnants of past invasions have been eliminated through the process of reductive evolution that symbiont genomes usually experience (Moran et al. 2008; Moya et al. 2008).

Materials and Methods

Genome Sequencing and Assembly

The Hepatoplasma genome from the terrestrial isopod A. vulgare was assembled from data generated as part of the ongoing A. vulgare genome project (Leclercq S and Cordaux R, unpublished data). Briefly, total genomic DNA was extracted from a single A. vulgare individual. A paired-end library with ~370 bp inserts was prepared and sequenced on an Illumina HiSeq2000. Reads were filtered with FastQC and assembled using the SOAP de novo software version 1.05. The best assembly (obtained with a k-mer size of 49) was screened for Hepatoplasma-derived sequences using the proteomes of Mesoplasma florum L1, M. agalactiae ASM8986v1, M. suis Illinois, Spiroplasma melliferum IPMB4A, S. melliferum KC3, and Ureaplasma urealyticum ATCC27618 downloaded from the NCBI FTP website (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria, last accessed February 14, 2014) as queries for a BlastX search (minimal identity of 37% over 80% of the query protein size), to identify contigs containing Mollicutes-like genes. Twenty-four contigs stood out of the analysis as likely Hepatoplasma contigs because of an atypical but consistently homogeneous coverage of 350× or more (40× on average for A. vulgare contigs). These 24 contigs were subsequently concatenated into 3 contigs with SSPACE version 2.0 (Boetzer et al. 2011), which were then assembled into a circular sequence of 657,101 bp using reads from a mate-pair
library with ~4,100bp inserts sequenced on an Illumina HiSeq2000 and several GapFiller iterations (Boetzer and Pirovano 2012). Assembly errors were experimentally corrected and validated using PCR and Sanger sequencing at the junctions between the 24 initial contigs, and through mapping of the whole paired-end reads set onto the reconstructed sequence. The genome sequence of Hepatoplasma strain Av is available under the GenBank accession number CP006932.

**Genome Annotation**

Annotation was performed using the Prokka annotation pipeline version 1.5.2 (Prokka: Prokaryotic Genome Annotation System—http://victioinformatics.com/, last accessed February 14, 2014). Parameters “genus” and “genetic code” were set to “Mycoplasma” and “4,” respectively, to fit with the properties of Mollicutes genomes. Prokka executed Aragorn 1.2.34, RNAmmer 1.2, Prodigal 2.60, and HMMER3/BlastP to retrieve tRNAs, mRNAs, open reading frames, and gene annotations, respectively. Pseudogenes were detected using the ψ–φ program suite (Lerat and Ochman 2004) with all other Mollicutes genomes as references. Because of the specific Mollicutes genetic code (not implemented in ψ–φ) and high genetic distance between Hepatoplasma and other Mollicutes, many detected pseudogenes were false positives and were manually curated. Genes related to mobile genetic elements were searched in annotated genes using keywords “transposase/Tpase” and “IS” for insertion sequences; “phage/prophage,” “integrase,” and “recombinase” for prophages, transposons, and group I introns; “reverse-transcriptase” and “RNA-directed DNA polymerase” for group II introns.

**Orthology and Phylogenomic Analyses**

A set of 30 representative Mollicutes proteomes was downloaded from the NCBI FTP website (ftp://ftp.ncbi.nlm.nih.gov/ genomes/Bacteria/, last accessed February 14, 2014) on March 21, 2013, and 15 additional proteomes were retrieved directly from NCBI Genome Projects. Proteomes of Bacillus subtilis 168, Streptococcus pneumoniae 670-EB, and Clostridium perfringens ATCC 13124 were also downloaded as outgroups.

Orthology relationships among all proteomes (Hepatoplasma and the 48 other species) were inferred using the OrthoMCL pipeline (Li et al. 2003), based on all-against-all BlastP hits with an e-value of 10^-3. Among the 5,392 orthology groups returned, 377 contained at least one Hepatoplasma protein.

Phylogenomic analyses were performed on a subset of 127 orthogroups harboring at least 40 orthologs including one in Hepatoplasma and in each of the three outgroups, and not containing any paralog in any taxon. Proteins of each orthogroup were aligned independently using MAFFT v.7.037 (Katoh et al. 2005) with the Mollicutes-optimized MOLLI60 substitution matrix (Lemaitre et al. 2011), available at (http://www.biomedcentral.com/1471-2105/12/457/additional, last accessed February 14, 2014). Resulting alignments were then concatenated in a single alignment of 46,607 residues, in which missing proteins in some orthogroup alignments were replaced by tracks of gaps.

To select the model of protein evolution that best fitted our Mollicutes data set, we performed a PROTEST 3 preanalysis (Abascal et al. 2005). The program returned the LG model (Le and Gascuel 2008) with a gamma model rate heterogeneity, a proportion of invariable sites, and an empirical residue frequency as the best model. RAxML (Stamatakis 2006) was used to compute maximum likelihood trees in two ways. First, we ran the algorithm using the PROTGAMMA/LGF amino acid model, which means that the estimation follows a gamma model rate heterogeneity (four discrete rate categories, and all parameters estimated by RAxML), with the LG substitution matrix, a proportion of invariable sites, and uses empirical residue frequency. The second run was performed under the same gamma model rate heterogeneity and empirical residue frequency, but using the earlier-mentioned MOLLI60 substitution matrix. Branching confidence was estimated with 1,000 bootstraps for each run.

**Tryptophan tRNA Analysis**

Tryptophan tRNA positions were retrieved in each genome by a manual search of GenBank annotation files. tRNA–Trp sequences were extracted and aligned with MAFFT (“linsi” option) to the described tRNA–Trp of M. capricolum (Yamao et al. 1988), to assign the correct anticodon sequence to each tRNA. Genes surrounding each tRNA–Trp were investigated through visual inspection under the GenBank graphical interface. DegV orthologs were extracted from all proteomes using orthology information returned by the OrthoMCL procedure. The 66 resulting sequences were aligned using the “linsi” option of MAFFT and the MOLLI60 substitution matrix. A maximum likelihood tree was computed using RAxML with the same options as for the global phylogenomic analysis (see above), and a Neighbor-Joining tree was computed using Mega 5.1 (Tamura et al. 2011), with the JTT model of substitution, a gamma model of rate heterogeneity, a pairwise deletion comparison, and 500 bootstrap iterations.

**CRISPR/Cas System Detection**

Cas genes were first retrieved from Mollicutes genomes using GenBank annotations. Hepatoplasma, M. canis, M. cynos, M. gallisepticum, M. iowae, and M. ovisneumoniae were found to harbor cas genes. Orthologous genes in other genomes were then recovered from orthogroups returned by OrthoMCL, which added M. synoviae, M. arthriditis, and M. mobile to the list of Mollicutes harboring cas genes. For these nine genomes, a CRISPR region was searched by using the CRISPRFinder webpage (Grissa et al. 2007). A single
CRISPR region was detected in every genome, and their position relative to the cas genes was then investigated through visual inspection under the GenBank graphical interface. The CRISPR/Cas comparison figure was created with EasyFig version 2.1 (Sullivan et al. 2011).

Supplementary Material

Supplementary figures S1 and S2 and table S1 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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