High quality draft genome sequences of *Mycoplasma agassizii* strains PS6\(^\text{T}\) and 723 isolated from *Gopherus* tortoises with upper respiratory tract disease

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**Abstract**

*Mycoplasma agassizii* is one of the known causative agents of upper respiratory tract disease (URTD) in Mojave desert tortoises (*Gopherus agassizii*) and in gopher tortoises (*Gopherus polyphemus*). We sequenced the genomes of *M. agassizii* strains PS6\(^\text{T}\) (ATCC 700616) and 723 (ATCC 700617) isolated from the upper respiratory tract of a Mojave desert tortoise and a gopher tortoise, respectively, both with signs of URTD. The PS6\(^\text{T}\) genome assembly was organized in eight scaffolds, had a total length of 1,274,972 bp, a G + C content of 28.43%, and contained 979 protein-coding genes, 13 pseudogenes and 35 RNA genes. The 723 genome assembly was organized in 40 scaffolds, had a total length of 1,211,209 bp, a G + C content of 28.34%, and contained 955 protein-coding genes, seven pseudogenes, and 35 RNA genes. Both genomes exhibit a very similar organization and very similar numbers of genes in each functional category. Pairs of orthologous genes encode proteins that are 93.57% identical on average. Homology searches identified a putative cytadhesin. These genomes will enable studies that will help understand the molecular bases of pathogenicity of this and other *Mycoplasma* species.

**Keywords:** *Mycoplasma agassizii*, Desert tortoise, Gopher tortoise, *Gopherus*, Upper respiratory tract disease (URTD), PS6\(^\text{T}\), ATCC 700616, 723, ATCC 700617

**Introduction**

The genus *Mycoplasma*, within the bacterial class *Mollicutes* (*Tenericutes*), contains over one hundred species, many of which are pathogenic to vertebrates [1]. An upper respiratory tract disease has been implicated in population declines in Mojave Desert tortoises (*Gopherus agassizii*) found in the desert southwest of the United States and gopher tortoises (*Gopherus polyphemus*) inhabiting forests of the U.S. southeast [2–4]. Pathogens associated with this disease include two *Mycoplasma*, *Mycoplasma agassizii* and *Mycoplasma testudineum* [5–7]. Due to conservation concerns regarding URTD, this disease and its associated pathogens have become a topic of research interest, though our understanding of the biology and progression of URTD is lacking [8, 9]. In particular, disease in tortoises is found with varying levels of morbidity, and one hypothesis for this finding is that there is genetic variation of *M. agassizii* associated with varying levels of virulence [8]. To understand better the amount of genomic differentiation occurring between *M. agassizii* collected from different tortoise host species, and to identify markers associated with virulence, we sequenced the *M. agassizii* genome from two strains, PS6\(^\text{T}\) and 723. This sequencing is part of a larger project to ultimately genetically detect variation in strains and their virulence from field-cultured samples.

**Organism information**

**Classification and features**

*Mycoplasma agassizii* has been isolated from multiple tortoise species, and was found to be pathogenic in Mojave Desert tortoises and gopher tortoises in North America, causing URTD [5, 6, 10]. In infected North American tortoises, *M. agassizii* is most often found in the nasal passages and choana, but can also be isolated...
from the trachea and lungs [10]. This microbe forms a close extracellular association with the nasal epithelium of its host, and severe infections can result in lesions [11]. Infected hosts experience clinical signs of disease including nasal exudate, possibly leading to lethargic behavior and loss of appetite [5, 11].

*M. agassizii* is coccoid to pleomorphic in shape, lacks a cell wall, and has a three-layer membrane (Table 1, Fig. 1). These microbes range in size under 1 μm [10, 11] and grow in culture at an optimal temperature of 30 °C, with an extremely slow growth rate [10, 12]. Mortality of *M. agassizii* occurs at temperatures above 37 °C [12], and it retains viability after prolonged periods of cold temperatures [6, 10], indicating that body temperatures experienced by its ectothermic hosts likely affect the microbe’s success over the seasons. In an experiment to detect co-infection patterns of *M. agassizii* with its close relative *M. testudineum*, there was some indication that the two species form a facilitative relationship in a host-context-dependent manner [13]. Preliminary microbiome data suggest that the presence of *M. agassizii* is associated with a shift in the microbial community composition in Mojave and Sonoran Desert tortoises (*Gopherus morafkai*) (CLW, FCS and CRT, unpublished data).

The strains of *M. agassizii* that we have sequenced were isolated from two host species. Strain PS6<sup>T</sup> was isolated from the upper respiratory tract of a Mojave Desert tortoise in the Las Vegas Valley, Nevada, USA [10], while strain 723 was obtained from an ill gopher tortoise in Sanibel Island, Florida, USA [6]. Strains were cultured in SP4 broth, and have been used in experiments to demonstrate their pathogenic effects on their tortoise hosts [5, 6].

### Table 1 Classification and general features of *Mycoplasma agassizii*, strains PS6<sup>T</sup> and 723

| MIGS ID | Property          | Term                         | Evidence code<sup>a</sup> |
|---------|-------------------|------------------------------|---------------------------|
|         | Classification    | Domain Bacteria              | TAS [38]                  |
|         | Phylum            | Firmicutes                  | TAS [39]                  |
|         | Class             | Mollicutes                  | TAS [40]                  |
|         | Order             | Mycoplasmatales             | TAS [41, 42]              |
|         | Family            | Mycoplasmataceae            | TAS [42]                  |
|         | Genus             | Mycoplasma                  | TAS [10]                  |
|         | Species           | Mycoplasma agassizii        | TAS [10]                  |
|         | Strains PS6<sup>T</sup> and 723 |                         | TAS [5, 6, 10, 20]        |
|         | Gram stain        | Negative                    | NAS                       |
|         | Cell shape        | Coccoid to pleomorphic      | TAS [10]                  |
|         | Motility          | Non-motile                  | TAS [10]                  |
|         | Sporulation       | Nonspore-forming            | NAS                       |
|         | Temperature range | Not reported                |                           |
|         | Optimum temperature | 30 °C                      | TAS [10]                  |
|         | pH range; Optimum | Not reported                |                           |
|         | Carbon source     | Glucose                     | TAS [10]                  |
|         | Habitat           | Tortoise respiratory tract  | TAS [10]                  |
|         | Salinity          | Not reported                |                           |
|         | Oxygen requirement | Aerobic                    | TAS [10]                  |
|         | Biotic relationship | Symbiont               | TAS [11]                  |
|         | Pathogenicity     | Pathogenic                  | TAS [5, 6]                |
|         | Geographic location | North America            | TAS [6, 10]               |
|         | Sample collection | 1991 (PS6<sup>T</sup>), 1992 (723) | TAS [43]                  |
|         | Latitude          | Approx.: 36 N (PS6<sup>T</sup>), 26.4 N (723) | TAS [6, 10]               |
|         | Longitude         | Approx.: 115 W (PS6<sup>T</sup>), 82.1 W (723) | TAS [6, 10]               |
|         | Altitude          | Approx.: 800 m (PS6<sup>T</sup>) , 0 m (723) | TAS [6, 10]               |

<sup>a</sup>Evidence codes

*Evidence codes for the Gene Ontology project [44]

IDA Inferred from Direct Assay, TAS Traceable Author Statement (i.e., a direct report exists in the literature), NAS Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [44].
To determine the placement of \textit{M. agassizii} in the mycoplasmal phylogeny, all 16S rRNA gene sequences from the type strains of \textit{Mycoplasma} species were obtained from the SILVA database [14] and aligned using MUSCLE 3.8.31 [15], and a phylogenetic tree was constructed using the maximum likelihood method implemented in MEGA7 [16] (Fig. 2). Consistent with prior results [17, 18], \textit{M. testudineum} is a sister group of \textit{M. agassizii} in the resultant tree, and the \textit{M. agassizii}/\textit{M. testudineum} clade is a sister group of \textit{Mycoplasma pulmonis}, the agent of murine respiratory mycoplasmosis. All three species fall within the hominis group of \textit{Mycoplasma} (see ref. [19] for group definitions). The 16S rRNA gene sequence from \textit{M. agassizii}, strain PS6\textsuperscript{T}, is 99.8, 93.2 and 89.2% identical to those of \textit{M. agassizii} strain 723, \textit{M. testudineum} strain BH29\textsuperscript{T}, and \textit{M. pulmonis} strain PG34\textsuperscript{T}, respectively.

**Genome sequencing information**

**Genome project history**

Two strains of \textit{M. agassizii} were selected for sequencing, strains PS6\textsuperscript{T} and 723, both isolated from tortoises with signs of URTD [5, 6, 10, 20]. Sequencing was conducted in October 2016. The Whole Genome Shotgun projects were deposited at DDBJ/ENA/GenBank under the accession numbers NQMN00000000 (strain PS6\textsuperscript{T}) and NQNY00000000 (strain 723). The versions described in this paper are the first versions. A summary of the information of both projects in compliance with MIGS version 2.0 [21] is shown in Table 2.

**Growth conditions and genomic DNA preparation**

Freeze-dried \textit{M. agassizii} strains were obtained from the ATCC in March 2011 (strain PS6\textsuperscript{T}) and May 2016 (strain 723). Strain PS6\textsuperscript{T} was cultured on SP4 media and re-pelleted in-lab prior to DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit protocol for Gram-negative bacteria and eluted with water. Extracted DNA was quantified on a Qiagen QIAxpert system and with Picogreen analysis.

**Genome sequencing and assembly**

Genome sequencing was conducted using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, USA) with the Illumina NextSeq500 platform (150 bp, paired-end) and 2 ng of starting genomic DNA at the Nevada Genomics Center (University of Nevada, Reno). Sequencing was performed in multiplex with multiple samples, using dual index sequences from the Illumina Nextera XT Index Kit, v2 (PS6 indices: index 1 N702, index 2 S510; 723 indices: index 1 N702, index 2 S511). A total of 349,251 and 332,967 read pairs were obtained for strains PS6\textsuperscript{T} and 723, respectively. Using Trimmomatic, version 0.36 [22], reads were trimmed to remove Nextera adapter sequences and low quality nucleotides from either end (average Phred score Q ≤ 5, four bp sliding window), and sequences trimmed to < 35 bp were removed. After trimming, 330,351 (PS6\textsuperscript{T}) and 305,002 (723) read pairs, and 16,438 (PS6\textsuperscript{T}) and 25,017 (723) single-reads (the pairs of which were removed) remained. De novo genome assembly was performed using SPAdes 3.10.1 [23], using as inputs the trimmed paired reads, and the trimmed single reads (assembly k-mer sizes 21, 33, 55, and 77, with read error-correction enabled and ‘–careful’ mode mismatch correction). After removing scaffolds of less than 500 bp, the final assemblies consisted of 8 (PS6\textsuperscript{T}) and 40 (723) scaffolds with a total length of 1,274,972 bp (PS6\textsuperscript{T}) and 1,211,209 bp (723), an average length of 159,372 bp (PS6\textsuperscript{T}) and 30,280 bp (723), and an N50 of 654,010 bp (PS6\textsuperscript{T}) and 56,701 bp (723). The coverage was 38.51× for the PS6\textsuperscript{T} assembly and 37.73× for the 723 assembly.

**Genome annotation**

Gene prediction was carried out using the NCBI Prokaryotic Genome Annotation Pipeline 4.2 [24]. For each predicted protein, (i) families were identified using the Pfam 31.0 [25] batch search tool (“gathering threshold” option), (ii) Clusters of Orthologous Groups categories...
Insights from the genome sequence

The small genome size and low G+C content of both *M. agassizii* genomes described here are consistent with those of other *Mycoplasma* genomes sequenced [18, 32, 33]. However, the *M. agassizii* genomes are significantly larger than the genome of *M. testudineum*, strain BH29 
(960,895 bp, 788 protein-coding genes; ref. [18]). The difference in the genome size of both sister species might account for the fact that *M. agassizii* is associated with URTD, whereas the link between *M. testudineum* and URTD is less clear [13]; i.e., genes present in *M.
but not in *M. testudineum* might be responsible for pathogenicity.

In spite of the fact that the two *M. agassizii* strains sequenced here were obtained from geographically distant locations (the Mojave Desert and Sanibel Island) and from different tortoise species (*G. agassizii* and *G. polyphemus*; refs. [5, 6, 10, 20]), the two genomes are very similar, exhibiting very similar sizes, numbers of genes (Table 3), functional composition (Table 4), and a high degree of synteny (Fig. 3a). A best-reciprocal-hit approach (based on BLASTP searches, *E*-value ≤ 10\(^{-10}\)) identified 828 pairs of putative orthologs within both genomes. The sequences of proteins encoded by pairs of orthologous genes were aligned using ProbCons version 1.12 [34], and were 93.57% identical on average (median: 96.84%). In contrast, comparison of the genomes of *M. agassizii*

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**Table 2** Project information

| MIGS ID | Property | Term |
|---------|----------|------|
| MIGS-31 | Finishing quality | High quality drafts |
| MIGS-28 | Libraries used | Illumina Nextera XT |
| MIGS-29 | Sequencing platforms | Illumina NextSeq500 |
| MIGS-31.2 | Fold coverage | 38.51 (strain PS6\(^6\)); 37.73 (strain 723) |
| MIGS-30 | Assemblers | SPAdes 3.10.1 |
| MIGS-32 | Gene calling method | NCBI Prokaryotic Genome Annotation Pipeline 4.2 |
|         | Locus Tag | CJF60 (strain PS6\(^6\)); CJJ23 (strain 723) |
|         | GenBank ID | NQMN000000000 (strain PS6\(^6\)); NQNY000000000 (strain 723) |
|         | GenBank Date of Release | August 28, 2017 (strain PS6\(^6\)); August 29, 2017 (strain 723) |
|         | GOLD ID | – |
|         | BIOPROJECT | PRJNA397947 (strain PS6\(^6\)); PRJNA398096 (strain 723) |
| MIGS-13 | Source Material Identifier | ATCC 700616 (strain PS6\(^6\)); ATCC 700617 (strain 723) |
|         | Project relevance | Animal parasite |

**Table 3** Genome statistics

| Attribute | Strain PS6\(^6\) | % of Total | Strain 723 | % of Total |
|-----------|-----------------|------------|------------|------------|
| Genome size (bp) | 1,274,972 | 100.00 | 1,211,209 | 100.00 |
| DNA coding (bp) | 1,124,547\(^a\) | 88.20\(^c\) | 1,072,218\(^a\) | 88.52\(^c\) |
| DNA G + C (bp) | 362,520 | 28.43\(^c\) | 343,241 | 28.34\(^c\) |
| DNA scaffolds | 8 | 100.00 | 40 | 100.00 |
| Total genes | 1,027 | 100.00 | 997 | 100.00 |
| Protein coding genes | 979 | 95.33\(^d\) | 955 | 95.79\(^d\) |
| RNA genes | 35 | 3.41\(^d\) | 35 | 3.51\(^d\) |
| Pseudo genes | 13 | 1.27\(^d\) | 7 | 0.70\(^d\) |
| Genes in internal clusters | – | – | – | – |
| Genes with function prediction | 467\(^b\) | 47.70\(^e\) | 301\(^b\) | 31.52\(^e\) |
| Genes assigned to COGs | 581 | 59.35\(^e\) | 577 | 60.42\(^e\) |
| Genes with Pfam domains | 608 | 62.10\(^e\) | 607 | 63.56\(^e\) |
| Genes with signal peptides | 160 | 16.34\(^e\) | 150 | 15.71\(^e\) |
| Genes with transmembrane helices | 294 | 30.03\(^e\) | 288 | 30.16\(^e\) |
| CRISPR repeats | 0 | – | 0 | – |

\(^a\)Protein-coding sequences, not including stop codons  
\(^b\)Proteins not annotated as "hypothetical protein" by PGAP  
\(^c\)Relative to genome size  
\(^d\)Relative to total number of genes  
\(^e\)Relative to protein-coding genes
strain PS6T and *M. testudineum* strain BH29T [18] revealed much less synteny (Fig. 3b) and protein identity (average: 54.78%, median: 54.71%).

The 16S rRNA gene sequences of *M. agassizii*, strains PS6T and 723, differed at 3 nucleotide positions (Fig. 4). Surprisingly, our 16S sequence for strain PS6T and that obtained by Brown et al. (also for strain PS6T; ref. [20]) exhibit 8 differences (4 point differences and 4 indels; Fig. 4). These differences may represent mutations accumulated since the isolation of the strain, or sequencing errors.

To initiate pathogenesis, *Mycoplasma* cells usually require adhering to the host mucosa. Adhesion mechanisms are relatively well understood in *Mycoplasma pneumoniae* and its close relatives, but poorly understood in other *Mycoplasma* species [35]. In a prior study, we searched all available *Mycoplasma* genomic data (nr database, including the genome of *M. testudineum* BH29T) for homologs of *M. pneumoniae* cytadhesins P1, P30, P65, P40 and P90 and cytadhesin accessory proteins hmw1, hmw2 and hmw3, finding homologs only in species closely related to *M. pneumoniae* (*Mycoplasma genitalium*, *Mycoplasma gallisepticum*, *Mycoplasma pirum*, *Mycoplasma alvi*, *Mycoplasma imitans*, and *Mycoplasma testudinis*) [18]. Here, we expanded these analyses (BLASTP and TBLASTN searches; $E < 10^{-5}$ and low-complexity regions filtering) to the two *M. agassizii* proteomes/genomes, also with negative results. In addition, none of the predicted *M. agassizii* proteins exhibit any of the Pfam domains present in the *M. pneumoniae* (domains “CytadhesinP1”, “Adhesin_P1”, “Cytadhesin_P30”, “MgpC” and “EAGR_box”). This could be attributed either to (i) *M. pneumoniae* adhesion proteins being specific to this species and its close relatives, or (ii) adhesion proteins evolving very fast, perhaps due to co-evolutionary races, precluding detection of homologs in distantly related species. The first possibility is supported by the fact that *M. pulmonis*, the most closely related known species to the *M. agassizii/
Fig. 3 Comparison of the genomes of *M. agassizii* strains PS6<sup>T</sup> and 723 (a), and *M. agassizii* strain PS6<sup>T</sup> and *M. testudineum* strain BH29<sup>T</sup> (b). The figure was generated using Circoletto 07.09.16 [45], a web interface for Circos [46]. The relative order of scaffolds is unknown. For strain PS6<sup>T</sup>, scaffolds are sorted by size.
Fig. 4 Comparison of the 16S rRNA gene sequences generated by Brown et al. [20] (M. agassizii strain PS6 T; GenBank accession: U09786) and in our study (M. agassizii strains PS6 T and 723). Asterisks represent identical sites

### Table 5 Results of a BLASTP search using CJF60_05070 as query against the nr database

| Accession       | Description                               | Total score | Query cover | E-value   | Identity |
|-----------------|-------------------------------------------|-------------|-------------|-----------|----------|
| WP_094254640.1  | hypothetical protein [Mycoplasma testudineum] | 1254        | 98%         | 0.0       | 34%      |
| CAC13384.1      | unknown; predicted coding region [Mycoplasma pulmonis] | 683         | 98%         | 0.0       | 27%      |
| WP_04136975.1   | hypothetical protein [Mycoplasma pulmonis] | 682         | 98%         | 0.0       | 26%      |
| WP_011264623.1  | Gil349 adhesion and gliding protein [Mycoplasma mobile] | 310         | 67%         | 10^-80    | 25%      |
| CCG15197.1      | fNIP repeat-containing protein [Mycoplasma sp. CAG:1193] | 105         | 3%          | 3x10^-4   | 38%      |
| WP_015135277.1  | hypothetical protein [Leptolyngbya sp. CAG:1193] | 58.5        | 5%          | 0.001     | 30%      |
| WP_01876155.1   | hypothetical protein [Lactobacillus nodensis] | 57.8        | 2%          | 5x10^-4   | 40%      |
| CCG15197.1      | fNIP repeat-containing protein [Mycoplasma sp. CAG:1193] | 55.1        | 5%          | 0.001     | 30%      |
| WP_05788036.1   | hypothetical protein [Lactobacillus paucivorans] | 53.5        | 4%          | 0.010     | 30%      |
| WP_06654743.1   | hypothetical protein [Caryophanon tenue] | 53.1        | 5%          | 0.012     | 29%      |
| WP_08178032.1   | hypothetical protein [Porphyromonas uenonis] | 97.8        | 2%          | 0.150     | 37%      |
| BAB92076.1      | truncated adhesin protein [Mycoplasma mobile] | 47.4        | 9%          | 0.770     | 24%      |
**M. testudineum** clade, exhibits adhesion mechanisms different from *M. pneumoniae*, lacking an attachment organelle [36]. In support of the second scenario, our analysis of orthologous sequences revealed poor protein conservation among the sister groups *M. agassizii* and *M. testudineum*.

We repeated our similarity searches using as query a list of known *Mycoplasma* adhesins, which we obtained by searching the text “Mycoplasma adhesion” in the UniProt database [37]. Our prior searches against the *M. testudineum* BH293 proteome/genome failed to detect any significant hits. In the current study, we detected significant similarity between a *Mycoplasma mobile* protein annotated as a “Truncated adhesion protein” (UniProt ID: Q8L3E5_9MOLU) and the proteins CJF60_05070 (strain PS6T, 3308 amino acids) and CJJ23_03020 (strain 723, also 3308 amino acids) of *M. agassizii*. CJF60_05070 and CJJ23_03020 are 92% identical. The C-terminal part of the *M. mobile* protein exhibits homology to three regions of the *M. agassizii* proteins (positions 958–1261, 1296–1597 and 1717–1924 of CJF60_05070; positions 956–1259, 1294–1595 and 1715–1922 of CJJ23_03020). A BLASTP search using CJF60_05070 as query sequence against the nr database identified a total of 17 hits, including three adhesion proteins (Table 5). Of note, the first hit is a *M. testudineum* protein (34%), which was not detected in our prior analyses [18]. Equivalent results were obtained using the CJJ23_03020 protein sequence as query (data not shown). The TMHMM server v. 2.0 [29] predicted both CJF60_05070 and CJJ23_03020 to contain a transmembrane domain at the N-terminal part of the protein (positions 7–29), and most of the protein (positions 30–3308) to be extracellular. Taken together, these observations point to these proteins as potential *M. agassizii* adhesins.

**Conclusions**

We have obtained draft genome sequences for *M. agassizii*, strains PS6T and 723, both isolated from tortoises of the *Gopherus* species. The two assemblies were very similar, in terms of synteny and protein sequences, in spite of the fact that they were obtained from different hosts and geographical locations. We identified a putative cytadhesin in both genomes. The new genomes will facilitate future studies that will help understand the molecular bases of pathogenicity of this and other *Mycoplasma* species.

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**Authors’ contributions**

DAP, CLW, FCS and CRT conceived the work. CLW conducted laboratory work. DAP and RLT conducted bioinformatic analyses. DAP and CLW drafted the manuscript. All authors contributed to interpreting data and improving the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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**Abbreviations**

ATCC: American Type Culture Collection; BLAST: Basic local alignment search tool; COG: Clusters of Orthologous Groups; MIGS: Minimum information on the genome sequence; MRW: Murine respiratory mycoplasmosis; NCBi: National Center for Biotechnology Information; PGAP: Prokaryotic Genome Annotation Pipeline; URTD: Upper respiratory tract disease

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