INFECTION AND IMMUNITY, June 2003, p. 3657–3662 Vol. 71, No. 6
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.6.3657–3662.2003

Physical Map and Genome Sequencing Survey of *Mycoplasma haemofelis* (*Haemobartonella felis*)

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Received 11 October 2002/Returned for modification 7 January 2003/Accepted 11 March 2003

***Mycoplasma haemofelis* is an uncultivable red-cell pathogen of cats. Isolated *M. haemofelis* DNA was used to create a bacterial artificial chromosome library and physical map. Random sequencing of this material revealed 75 genes that had not been previously reported for *M. haemofelis* or any other hemotrophic mycoplasma.**

Mycoplasma haemofelis is a member of a newly defined group of mycoplasmas that parasitize the red blood cells of animals and humans. *M. haemofelis*, which was formerly known as *Haemobartonella felis* strain OH (18), was chosen for use in these molecular studies because it is the highly pathogenic causative agent of the syndrome historically reported as feline infectious anemia (8, 11). This syndrome is often associated with feline retroviral infections and may serve as a model for hemotrophic mycoplasmal infection in immunosuppressed human patients (13; M. I. Duarte, M. S. Oliveira, M. A. Shikanai-Yasuda, O. N. Mariano, C. F. Takakura, C. Pagliari, and C. E. Corbett, Letter, J. Infect. Dis. **165:**976–977, 1992). This paper describes the genome size of *M. haemofelis*, the creation of the first genome encyclopedia and physical map of an uncultured mycoplasma, and the collection of *M. haemofelis* genome survey sequences (GSSs) for the purpose of gene discovery.

To acquire large quantities of *M. haemofelis* DNA for the following experiments, a feline leukemia virus- and feline immunodeficiency virus-seronegative cat was splenectomized and infected with *M. haemofelis*. When more than 60% of the red cells contained organisms, blood was aseptically drawn from the jugular vein into a syringe containing 1 ml of anticoagulant-citrate-dextrose solution per 5 ml of blood. The organisms were released from the red cells and embedded in agarose plugs, and the DNA was extracted by using previously described protocols (1, 19). Related organisms *Mycoplasma genitalium* (ATCC 49895) and *Mycoplasma haemosuis* (15), as well as a commercial DNA marker, were used as size controls in subsequent experiments.

Gamma radiation was used to linearize the bacterial DNA prior to pulsed-field gel electrophoresis (PFGE) to determine the full genome length of *M. haemofelis* (20, 31). A 1.0% 0.5× Tris-borate-EDTA gel was run for 24 h at 14°C and 6 V/cm, with 60- to 120-s switch times and a field angle of 120°. This PFGE was repeated four times with *M. haemofelis* plugs from separate blood collections. To confirm the identify of the bands seen, the Southern blotted DNA was probed by using a 393-bp digoxigenin-labeled fragment of the *M. haemofelis* 16S rRNA gene which spanned hypervariable regions 1 to 3 (1, 17). Membranes were prehybridized in 10 to 20 ml of PerfectHyb Plus (Sigma-Aldrich Corp., St. Louis, Mo.) at 68°C for at least 5 min. Labeled probe was boiled for 10 min and added to the prehybridization solution, and the probe was allowed to hybridize overnight at 68°C. Stringency washes and signal detection with CDP-star were performed according to the manufacturer's instructions (Boehringer Mannheim Biochemicals [Roche Molecular Biochemicals], Indianapolis, Ind.).

The average size of the linearized *M. haemofelis* chromosome was 1,199 kb (standard deviation, 13.5 kb). This falls well within the published range (580 to 1,400 kb) for the *Mycoplasma pneumoniae* subgroup of mycoplasmas (14, 20). When membranes were probed with a labeled fragment of the 16S rRNA gene of *M. haemofelis*, a strong signal was seen with the *M. haemofelis* DNA and weaker signals were observed for *M. haemosuis* and *M. genitalium*. This weak heterologous binding of our probe to the other mycoplasmas was expected due to the sequence similarities between the hypervariable regions of the 16S rRNA genes.

The creation of the bacterial artificial chromosome (BAC) library was done by using the *Escherichia coli* host strain DH10β and the vector pBeloBAC11 (28). Full-length *M. haemofelis* genomic DNA was partially cut with *Hin*dIII (24), size-selected by using PFGE, and isolated from the gel by using standard techniques (26). A molar ratio of vector to insert of 5:1 was used in the ligation experiments. An aliquot of the ligation mix was added to freshly prepared DH10β electrocompetent cells, and the cells were transformed in a 0.2-cm cuvette at 200 Ω, 25 μF, and 12.5 V/cm.

A probe made from full-length *M. haemofelis* genomic DNA was used to screen colony hybridization blots for clones containing *M. haemofelis* DNA and eliminate clones possibly containing *E. coli* or cat genomic DNA. One hundred and fifty colonies that exhibited strong hybridization signals were then used for mapping experiments. The average size of these clones was 42.6 kb, and there was a 99.7% probability that the entire genome was represented (6).

The BAC clones containing *M. haemofelis* DNA were cut

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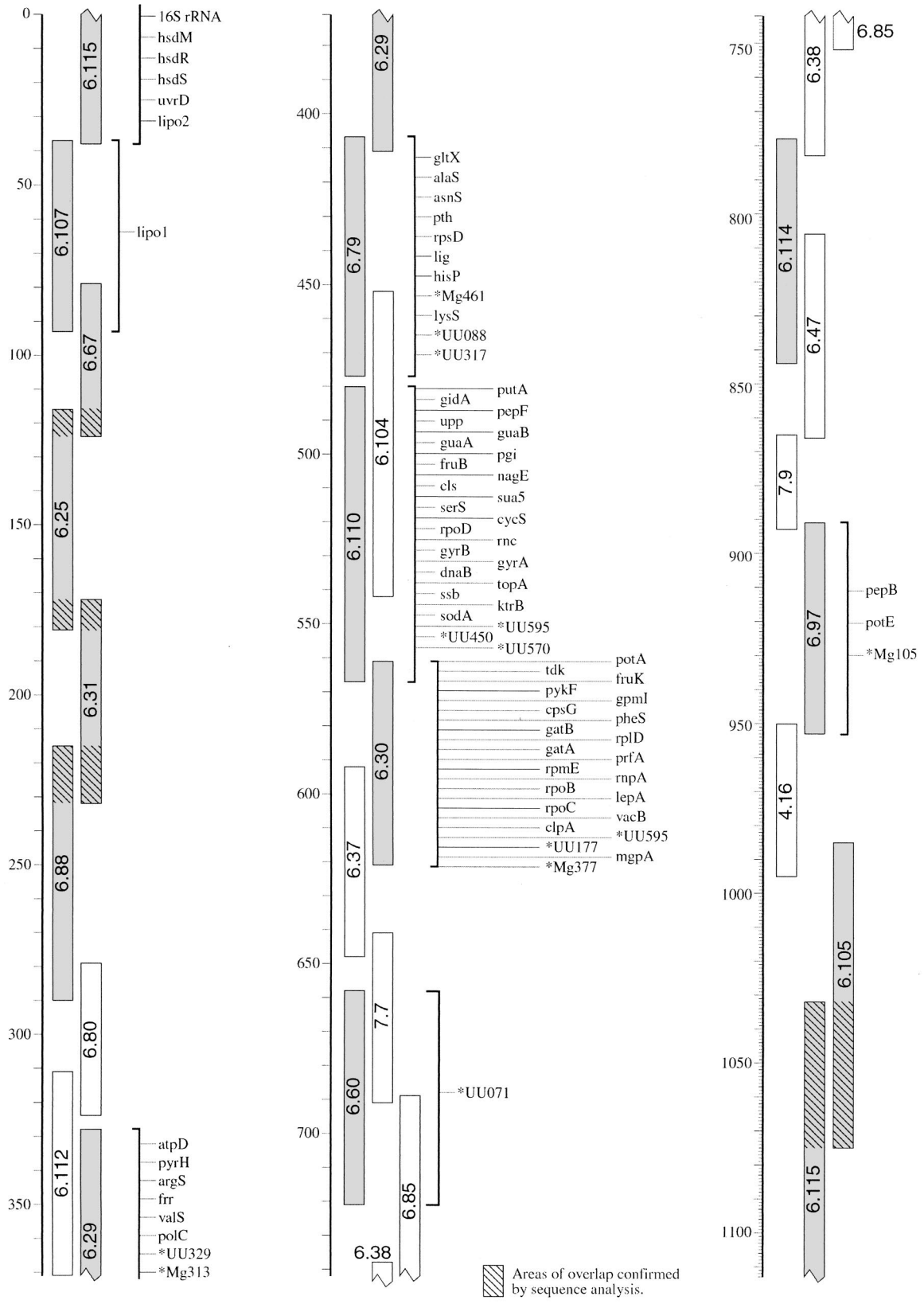

FIG. 1. *M. haemofelis* genomic encyclopedia and map. White bars indicate BAC clones that were not subcloned and gray bars indicate BAC clones used for subcloning and GSS generation. The genes discovered within each BAC clone are listed in random order by the abbreviations listed in Table 2. The scale is in kilobases.

with restriction endonucleases *Not*I, *Nru*I (New England BioLabs, Beverly, Mass.), and *Sal*I (Promega, Madison, Wis.) in either single or double digestions according to the manufacturers' instructions. The enzymes were chosen such that the pBeloBAC11 backbone would be excised and would serve as an internal control for complete digestion. These digestions were run on a 1.0% gel in 0.5× Tris-borate-EDTA buffer at 14°C for 15 h with switch times of 5 to 15 s at 6 V/cm and at an angle of 120°. The DNA was then Southern blotted onto a positively charged nylon membrane by using standard techniques (26).

To define a starting point for the restriction map, the *M. haemofelis* 16S rRNA gene probe was used to screen dot blots of BAC clones and identify a contig of six clones containing this gene. The probe should hybridize just before the *Nru*I site at nucleotide 506 within the 16S rRNA gene (1, 25). Based on the restriction patterns of the clones and the placement of the 16S rRNA gene, it was determined that these six clones represented a single contig and that only one copy of this gene was present in *M. haemofelis*. This *Nru*I site was designated as point zero for the purposes of this mapping project. Probes were then made from restriction fragments flanking the 16S rRNA gene and used to identify adjacent BAC clones. The order of the various restriction fragments from each BAC clone was determined by examining the restriction enzyme fingerprints and by hybridizing single and double restriction digests with probes made from the fragments in question. After repeated cycles of hybridizations of the dot blots, pulsed-field gel blots, and new probe selections, a complete genomic circle was closed. A total of 24 BAC clones were identified which represented the entire genome of *M. haemofelis* with minimal overlap (the genomic encyclopedia). The restriction map of *M. haemofelis* contains 28 *Nru*I sites, 21 *Sal*I sites, and 9 *Not*I sites.

A selection of BAC clones containing known regions on the *M. haemofelis* physical map were subcloned into the vector pGEM-3Zf(+) (Promega) and partially sequenced. A total of 624 GSSs were successfully generated. An average length of 450 bases per GSS was obtained, and this yielded 283,351 bp of genomic sequence. After elimination of duplicate sequences and combination of contigs, 430 unique GSSs were deposited in GenBank. The average guanine-plus-cytosine (G+C) percentage was 38.5% and showed little variation among the subclones. This G+C percentage is less than that found in *M. pneumoniae* (41%) (12) and greater than that seen in *M. genitalium*, *Mycoplasma pulmonis*, and *Ureaplasma urealyticum* (26 to 32%) (3, 9, 10). It also confirms that there is no contamination by *E. coli* DNA, which has a G+C percentage of 48 to 52% (2), or mammalian DNA, which has a G+C percentage of 52 to 54% (16).

Nine different contigs were identified that spanned overlapping areas on the genomic encyclopedia map. These contigs confirmed the overlap between clones 6.115 and 6.105, 6.67 and 6.25, 6.25 and 6.31, and 6.31 and 6.88 (Fig. 1). In addition, no contigs were detected between clones that did not overlap in the physical map. The 16S rRNA gene was found in two subclones of BAC 6.115 (as expected), and the other known *M. haemofelis* gene (*rnpA*) was found on the opposite side of the physical map in BAC clone 6.30. Finally, no GSSs were detected which represent feline or *E. coli* DNA present in the nonredundant database.

TABLE 1. Database match categories of GSSs from *M. haemofelis*

Database match	No. of GSSs (%)
Any	165 (26.4)
Mycoplasma or *Ureaplasma* spp.	116 (18.6)
Bacillus-Clostridium group bacteria	24 (3.8)
Other organisms	25 (4.0)
None	459 (73.6)
Total	624 (100)

The GSSs were compared to known sequences in the nonredundant database by using the BLASTn, BLASTx, and tBLASTx programs (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) with the mycoplasmal translation code. Among the GSSs obtained, 165 (26.4%) had a significant BLAST score ($[E]$ of $\leq 10^{-5}$) (27) with at least one entry in GenBank (Table 1). The majority of the most significant BLAST scores were for genes and proteins in the *Mycoplasma* and *Ureaplasma* genera. The remaining GSSs with significant BLAST scores were split evenly between members of the *Bacillus-Clostridium* group and other miscellaneous bacteria. Four hundred fifty-nine (73.6%) of the *M. haemofelis* GSSs failed to yield a significant database match ($[E]$ of $\geq 10^{-5}$). These results indicate that the *M. haemofelis* genome likely encodes a large number of unique proteins. Unlike most other mycoplasmas, which tend to colonize mucosal surfaces, the host-adapted survival of *M. haemofelis* is achieved through surface parasitism of the red blood cell, and it is therefore not surprising that this bacterium contains many unique genes.

The 165 GSSs with significant BLAST scores were further scrutinized to eliminate GSSs of the same gene. After deleting redundant GSSs, 77 out of 165 remained as nonredundant GSSs, representing 75 distinct genes in *M. haemofelis* that had not been previously reported. Two GSSs represent the 16S rRNA and RNase P RNA genes of *M. haemofelis*, both of which had been previously described (GenBank accession numbers U95297 and AF407210). Sequences were further grouped according to the cluster of orthologous groups (COG) and the presumptive gene function (29). The largest number of genes found were related to translation, ribosomal structure, and biogenesis (25%). Other groups with significant numbers of genes included those related to DNA replication (14%), carbohydrate metabolism (9%), transcription (6%), and nucleotide transport (6%) (Table 2).

Several putative virulence factors were detected in this analysis which merit further study. These include membrane lipoproteins, possibly related to antigenic variation systems (5), and other well-described virulence factors such as VacB and MgpA-like proteins (4, 7).

The superoxide dismutase (SOD) gene appears to be unique to *M. haemofelis* among the mycoplasmas. There are studies describing SOD activity in some mycoplasmas (21), but there are currently no available reports of a mycoplasma SOD gene sequence available in GenBank or from the fully sequenced mycoplasmas (3, 9, 10, 12). The best BLAST scores found when comparing the putative *M. haemofelis* SOD gene to the nonredundant database were with bacteria from the *Bacillus-Clostridium* group. This suggests that the SOD gene was

TABLE 2. Putative genes identified in the GSSs of *M. haemofelis,* the BAC clone in which the gene is located, the affiliated COG, the functional category, and the GenBank accession number(s)[a]

Gene description	BAC clone	COG	Function	GenBank accession no.
Lipoprotein (lipo1)	6.107	None	None	BH792935–BH792936
DNA helicase II (uvrD)	6.115	COG0210	L	BH792933
Type I restriction-modification system DNA methylase (hsdM)	6.115	COG0286	L	BH792934
Type I restriction enzyme R protein (hsdR)	6.115	COG0610	L	BH792929–BH792930
Type I restriction enzyme S protein, truncated homolog (hsdS)	6.115	COG0732	L	BH792931–BH792932
16S rRNA gene (16S rRNA)	6.115	None	None	BH792926
Lipoprotein (lipo2)	6.115	None	None	BH792927–BH792928
ATP synthase B chain precursor (atpD)	6.29	COG0055	C	BH792937
UMP kinase (pyrH)	6.29	COG0528	F	BH792939
Arginyl-tRNA synthetase (argS)	6.29	COG0018	J	BH792946
Ribosome releasing factor (frr)	6.29	COG0233	J	BH792938
Valyl-tRNA synthase (valS)	6.29	COG0525	J	BH792944
DNA polymerase III alpha chain (polC)	6.29	COG2176	L	BH792940–BH792943
Hypothetical protein similar to UU329 (∗UU329)	6.29	None	None	BH792945
MG313 homolog (∗MG313)	6.29	None	None	BH792947
Glutamate-tRNA ligase (gltX)	6.79	COG0008	J	BH792991
Alanyl-tRNA synthetase (alaS)	6.79	COG0013	J	BH792979–BH792981
Aspartyl/asparaginyl-tRNA synthetase (asnS)	6.79	COG0017	J	BH792983–BH792984
Peptidyl-tRNA hydrolase (pth)	6.79	COG0193	J	BH792990
Ribosomal protein S4 (rpsD)	6.79	COG0522	J	BH792988
DNA ligase (lig)	6.79	COG0272	L	BH792977–BH792978
Histidine transport ATP-binding protein (hisP)	6.79	COG1122	P	BH792989
Hypothetical protein homolog of MG461 (∗MG461)	6.79	COG1078	R	BH792985–BH792986
Lysyl-tRNA synthetase (lysS)	6.79	COG1190	J	BH792987
Uncharacterized conserved protein, YbaB family (∗UU088)	6.79	COG0718	S	BH792987
Conserved hypothetical protein similar to UU317 (∗UU317)	6.79	None	None	BH792982
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (putA)	6.110	COG1012	C	BH792968
Glucose-inhibited division protein A (gidA)	6.110	COG0445	D	BH792948, BH792957–BH792958
Zinc metalloproteinase oligoendopeptidase F (pepF)	6.110	COG1164	E	BH793355
Uracil phosphoribosyltransferase (upp)	6.110	COG0035	F	BH792948
Inosine-5′-monophosphate (IMP) dehydrogenase (guaB)	6.110	COG0516	F	BH792967
Guanosine 3′,5′-monophosphate (GMP) synthase (guaA)	6.110	COG0519	F	BH792967
Glucose-6-phosphate isomerase (pgi)	6.110	COG0166	G	BH792976
ptsH 3′-region protein 1 (fruB)	6.110	COG1925	G	BH792963
Glucose permease IIa (nagE)	6.110	COG2190	G	BH792955–BH79295
Possible cardiolipin synthase (phospholipase D family) (cls)	6.110	COG1502	I	BH792954
Hypothetical protein similar to UU005m (sua5)	6.110	COG0009	J	BH792969
Seryl-tRNA synthetase (serS)	6.110	COG0172	J	BH792971
Cysteine-tRNA ligase (cysS)	6.110	COG0215	J	BH792975
RNA polymerase sigma 43 factor (rpoD)	6.110	COG0568	K	BH792959–BH792960
Ribonuclease III (rnc)	6.110	COG0571	K	BH792974
DNA topoisomerase, DNA gyrase subunit B (gyrB)	6.110	COG0187	L	BH792966
DNA gyrase subunit A (gyrA)	6.110	COG0188	L	BH792949–BH792951
Replicative DNA helicase (dnaB)	6.110	COG0305	L	BH792961
DNA topoisomerase I (topA)	6.110	COG0550	L	BH792952–BH792953
Single-stranded DNA binding protein (ssb)	6.110	COG0629	L	BH792965
Potassium uptake protein (ktrB)	6.110	COG0168	P	BH792973
Superoxide dismutase (sodA)	6.110	COG0605	P	BH792962
GTP-binding protein (∗UU595)	6.110	COG0012	R	BH792972
Conserved hypothetical protein similar to UU450 (∗UU450)	6.110	COG0392	R	BH792970
Protein similar to single-stranded RNA (DNA) processing enzymes (∗UU570)	6.110	COG0595	R	BH792964
Spermidine/putrescine transport ATP-binding protein (potA)	6.30	COG3842	E	BH793015
Thymidine kinase (tdk)	6.30	COG1435	F	BH793013
Carbohydrate kinase, pfkB family (fruK)	6.30	COG1105	G	BH793016
Pyruvate kinase (pykF)	6.30	COG0469	G	BH793012
Phosphoglycerate mutase (gpmI)	6.30	COG0696	G	BH793001–BH793002
Phosphomannomutase (cpsG)	6.30	COG1109	G	BH793014
Phenylalanine-tRNA synthetase alpha chain (pheS)	6.30	COG0016	J	BH793003–BH793004
Glutamyl-tRNA amidotransferase subunit B (gatB)	6.30	COG0064	J	BH793006–BH793007
Ribosomal protein L4 (rplD)	6.30	COG0088	J	BH793017
Glutamyl-tRNA amidotransferase subunit A (gatA)	6.30	COG0154	J	BH793008
Translation releasing factor RF-1 (prfA)	6.30	COG0216	J	BH793000
50S ribosomal protein L31 (rpmE)	6.30	COG0254	J	BH793016
Ribonuclease P RNA gene (rnpA)	6.30	COG0594	J	BH793011
RNA polymerase beta subunit (rpoB)	6.30	COG0085	K	BH792992–BH792995

Continued on following page

TABLE 2—*Continued*

Gene description	BAC clone	COG	Function	GenBank accession no.
GTP-binding protein (lepA)	6.30	COG0481	N	BH792996
DNA-directed RNA polymerase beta′ chain (rpoC)	6.30	COG0086	K	BH792997
Virulence-associated protein, exoribonuclease II (vacB)	6.30	COG0557	K	BH793005
Heat shock ATP-dependent protease (clpA)	6.30	COG0542	O	BH793011
GTP-binding protein (∗UU595)	6.30	COG0012	R	BH793010
Probable inorganic polyphosphate/ATP-NAD kinase (∗UU177)	6.30	COG0061	R	BH792999
M. genitalium adhesin MgPa operon hypothetical protein MG190 (mgpA)	6.30	COG0618	R	BH792998
Hypothetical protein similar to MG377 (∗MG377)	6.30	None	None	BH793009
Probable ABC substrate-binding protein, iron (∗UU071)	6.60	None	None	BH793018–BH793019
Probable cytosol aminopeptidase (leucine aminopeptidase) (pepB)	6.97	COG0260	E	BH793020
Amino acid permease (potE)	6.97	COG0531	E	BH793021–BH793022
Hypothetical protein MG105 homolog (∗MG105)	6.97	COG1624	S	BH793023

[a] Abbreviations: L, DNA replication; C, energy production; F, nucleotide transport; J, translation; P, inorganic ion transport; R, general function prediction only; S, unknown function; D, cell division; E, amino acid metabolism; G, carbohydrate metabolism; I, lipid metabolism; K, transcription; N, cell secretion; O, posttranslational modification. The letters in parentheses indicate the gene abbreviations used on the genomic map in Fig. 1. ∗indicates a protein without a defined gene name that has a homolog in either *U. urealyticum* (UU) or *M. genitalium* (MG).

present in a common ancestor and either that other mycoplasmas may have lost the gene or that a product of a different gene has assumed the role of SOD production.

Of particular interest are two genes found in *M. haemofelis* that are involved in purine biosynthesis and encode proteins similar to IMP (inosine-5′-monophosphate) dehydrogenase and GMP (guanosine 3′,5′-monophosphate) synthase. There are no reports describing the enzymatic activity of these proteins in *Mollicutes* spp., and thus it was assumed that all *Mollicutes* spp. require a source of preformed guanine (23, 30). The mere presence of these genes in *M. haemofelis* does not definitively prove that the enzymes have the predicted activity (22). If *M. haemofelis* could be grown, the culture could be examined for the enzyme activity, but with *M. haemofelis* we are limited to using an expression vector to study this protein in the future.

This genomic encyclopedia and collection of survey sequences provides the first physical map and genomic survey of an uncultured hemotrophic mycoplasma. It is our hope that this information will ultimately lead to the development of more effective treatments and detection of *M. haemofelis* by characterizing its virulence and biosynthetic pathways.

Nucleotide sequence accession numbers. The 430 unique GSSs were deposited in GenBank under the accession numbers BH792926 through BH793355.

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Editor: J. T. Barbieri