Aug 3rd, 9:00 AM - 12:00 PM

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Phylogenetic Studies of Newly Isolated Freshwater Magnetospirilla Using *cbb* and *mam* Genes

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Abstract

The phylogeny and general relatedness of prokaryotes is determined by comparisons of the sequences of rRNA genes, most commonly the 16S rRNA gene. Comparisons between other gene sequences have been used for this purpose and some have supported conclusions from 16S rRNA genes while others have not. In this study, 13 new magnetospirilla were phylogenetically characterized using the sequences of the 16S rRNA gene as well as the genes for forms I and II ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (*cbbL* and *cbbM*, respectively) and for two magnetosome membrane proteins unique to magnetotactic bacteria, *mamJ* and *mamK*. Polymerase chain reaction (PCR) with degenerate primers designed for the specific genes under study was used to amplify a large portion of the genes. PCR products were cloned and sequenced and used for the construction of phylogenetic trees. Based on 16S rRNA gene sequences, the magnetospirilla phylogenetically span, more as a continuum rather than as clearly delineated groups, over two genera based on the current accepted sequence divergence between organisms for genera (>5%). While almost all strains appear to fit into the genus *Magnetospirillum*, strain LM-1 appears to represent a new genus. Phylogeny of these strains based on *cbbM* sequences was reasonably consistent with that from 16S rRNA genes. The *cbbL* gene was not a good choice for this study as most strains did not possess this gene. Relatedness and phylogeny of the strains based on *mamJ* and *mamK* sequences was more complex. Although our data set is not complete, some specific strains shown to be closely related by 16S rRNA gene sequence, also appeared to be closely related based on one or both of the *mam* gene sequences (e.g., strains UT-1, LM-2 and *M. gryphiswaldense* strain MSR-1). Other strains did not show this type of relationship. Because of these somewhat inconsistent results, those from *mam* gene sequences might reflect evolution of the magnetosome gene island (MAI) in magnetospirilla rather than relatedness between strains.

Introduction

Magnetotactic bacteria (MTB) are a unique group of motile, gram-negative, mainly aquatic prokaryotes that passively align along geomagnetic field lines while they swim (Bazylinski & Frankel 2004). This passive alignment and active motility along magnetic field lines is called magnetotaxis and is due to the presence of intracellular structures called magnetosomes. Magnetosomes are membrane-bounded crystals of the magnetic minerals magnetite (Fe_3O_4) and greigite (Fe_3S_4). MTB are a morphologically, phylogenetically and physiologically diverse group that are phylogenetically affiliated with the *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* classes of the *Proteobacteria* phylum and the *Nitrospirae* phylum. One of the most studied groups of MTB are the magnetospirilla, most cultured strains of which are species of the genus *Magnetospirillum* in the *Alphaproteobacteria*.

Known magnetotactic *Magnetospirillum* species are facultatively anaerobic microaerophiles that biomineralize a chain of cubo-octahedral crystals of magnetite. Some strains do not produce magnetosomes although all have the same helical cell morphology and possess a single flagellum at each end of the cell (Figure 1). Several species are well-characterized. All grow chemoorganoheterotrophically using organic acids as sources of electrons and carbon and some have been shown to grow chemolithoautotrophically using reduced sulfur compounds as an electron source and the Calvin-Benson-Bassham cycle for autotrophy (Geelhoed et al. 2009, 2010). Several others have not yet been shown to grow autotrophically but show a strong potential for this metabolic feature as they possess a form II ribulose-1,5-bisphosphate carboxylase/oxygenase gene (*cbbM*) (Bazylinski et al. 2004).

The genes presumably responsible for the biomineralization of magnetite were originally discovered in *Magnetospirillum* species and are referred to the *mam* and/or *mms* genes (Bazylinski & Schübbe 2007). Many of these genes encode for proteins in the cytoplasmic membrane-derived magnetosome membrane (MM), some of which are present no where else in the cell. Most, if not all, these genes are located in the genome as clusters within a magnetosome gene island (MAI) in *Magnetospirillum* species and other MTB that is thought to be distributed between non-magnetotactic species via horizontal gene transfer (HGT) (Jogler et al. 2009). The roles of most of these genes are unknown. The *mamJ* and *mamK* genes are located within the *mamAB* gene cluster in *Magnetospirillum* species and are cotranscribed and their respective proteins have been clearly shown to be responsible for magnetosome chain formation (Schübbe et al. 2006). Magnetosomes are attached by the acidic MM protein MamJ to a series of cytoskeletal filaments that traverse the cell along its long axis that are composed of MamK (Komeili et al. 2006; Scheffel et al. 2006). All cultured characterized magnetotactic *Magnetospirillum* species synthesize these proteins. Phylogeny of MTB based on *mam* gene sequences may provide may help to discern how the MAI is evolving as it is transferred via HGT to other bacteria.

The purpose of this study was to determine phylogenetic relatedness of a large number of newly isolated freshwater magnetospirilla using sequences from the 16S rRNA gene and to compare these results to those from sequences of the *cbbL*, *cbbM*, *mamJ* and *mamK* genes.

Methods

Isolation of new strains of magnetospirilla. Magnetically-purified MTB from various water samples were inoculated into a semi-solid O_2-gradient enrichment medium similar to that described by Bazylinski et al. (2004) except that the medium was designed for non-marine heterotrophs. Differences were that the basal medium contained (per liter): 5 ml modified Wolfe's mineral elixir (Bazylinski et al. 2000); 0.2 ml 1% aqueous resazurin; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.3 g NH_4Cl; and 0.68 g Na acetate as the electron and carbon source. 30 µM FeSO4 replaced the ferric quinate as the major iron source, 0.5 mM Na_2S and 0.4 g neutralized cysteine were used together as the reducing agent and 0.2 g of agar was used to render the medium semi-solid. The final pH of the medium was adjusted to 7.0.

Most strains were isolated by streaking material from the enrichment medium onto plates of ACA medium (Schultheiss & Schüler 2003) where single colonies were obtained. Certain strains (UT-2, UT-4, LM4 and CB1) did not produce colonies on this medium and were isolated by dilution to extinction in the enrichment medium.

Phylogeny of magnetospirilla strains. A large portion of the 16S rRNA, *cbbL*, *cbbM*, *mamJ*, and *mamK* genes were amplified using PCR with degenerate primers designed specifically for these genes (Table 1). PCR products were cloned into pGEM-T Easy Vector (Promega Corporation, Madison WI) and sequenced (Functional Biosciences, Inc., Madison WI). Alignment of 16S rRNA genes was performed using CLUSTAL W multiple alignment accessory application in the BioEdit sequence alignment editor. Phylogenetic trees were constructed using MEGA version 4 applying the neighbor-joining method. Bootstrap values were calculated with 1000 replicates.

Gene	Primer Designation	Primer Sequence	Approximate Size of amplified DNA fragment
16S rRNA gene	16F	5′- AGAGTTTGATCMTGGCTCAG-3′	1600
	1492R	5′- TACGGHTACCTTGTTACGACTT -3′	1600
cbbL (gene for "green"-like form I RubisCO)	RublgF	5′- GAYTTCACCAARGAYGAYGA-3′	800-900
	RublgR	5′- TCRAACTTGATYTCYTTCCA-3′	800-900
cbbL (gene for "red"-like form I RubisCO)	RubIrF	5′-GCVACCTGGACSGTSGTVTGG -3′	800-900
	RubIrR	5′-TCGCCYTCSAGCTTGCCSAC -3′	800-900
cbbM (gene for form II RubisCO)	RulIF1	5′-GGHAACAACCARGGYATGGGYGA-3′	800-900
	RulIR3	5′-CGHAGIGCGTCCATGCCRCC-3′	800-900
mamJ	MamJF	5′-TCGGTGACGGTCCATCCCWCGCC-3′	1000-1200
	MamJR	5′-AGAAGGTCTTCSACGGGAACKTC-3′	1000-1200
mamK	MamKF	5′-ATGAGTGAAGGTGAAGGCCA-3′	800-900
	MamKR	5′-TGMAGCACGGTGGCCTGATA-3′	800-900

Table 1. Degenerate PCR primers used for the amplification of 16S rRNA, *cbbL*, *cbbM*, *mamJ*, and *mamK* genes.

Results

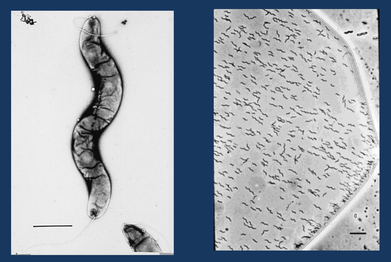

Figure 1. Morphology of the magnetospirilla. Left, transmission electron microscopy image of a negatively-stained cell of (*Magnetospirillum magnetotacticum* strain MS-1). Note the helical cell morphology and the bipolar pattern of flagellation. Bar = 0.5 µm. Right, suspension of magnetospirilla in liquid medium in a magnetic field. Bar = 5 µm.

Results (cont.)

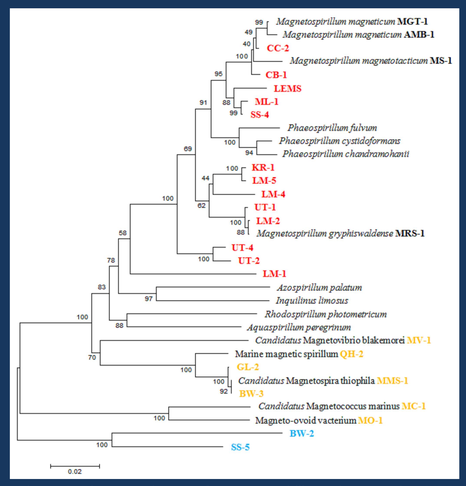

Figure 2. Phyogenetic tree of magnetospirilla and some other MTB based on 16S rRNA gene sequences. Red = new strains of magnetospirilla; black = *Magnetospirillum* species; orange = other MTB; and blue = magnetotactic *Gammaproteobacteria* as outgroup. Bootstrap values at nodes are percentages of 1,000 replicates. Bar represents 2% sequence divergence.

Magnetospirillum Strain	*cbbL* (gene for "green" – like form I RubisCO)	*cbbL* (gene for "red" – like form I RubisCO)	*cbbM* (gene for form II RubisCO)	*mamJ*	*mamK*
CC-2	-	-	+	+	+
LEMS	-	-	+	+	+
ML-1	-	-	-	-	-
SS-4	-	-	+	+	+
KR-1	ND	ND	+	+	+
LM-1	ND	ND	+	-	+
LM-2	ND	ND	+	+	+
LM-4	-	-	+	-	+
LM-5	ND	ND	+	+	+
UT-1	ND	ND	+	+	+
UT-2	-	-	+	-	-
UT-4	+	+	+	-	-
CB-1	-	-	+	+	-
AMB-1	ND	ND	ND	+	+
MSR-1	ND	ND	ND	+	+
SS-5	+	-	+	ND	ND
BW-2	+	-	-	ND	ND

Note: ND = Not Determined

Table 2. Presence of *cbbL*, *cbbM*, *mamJ* and *mamK* genes in strains of magnetospirilla as determined by PCR. A negative result does not mean the gene is necessarily absent, it could mean that the sequence of the gene is different enough that the degenerate primers used are not effective. SS-5 and BW-2 are *Gammaproteobacteria* MTB used as positive PCR control for presence of *cbbL* gene.

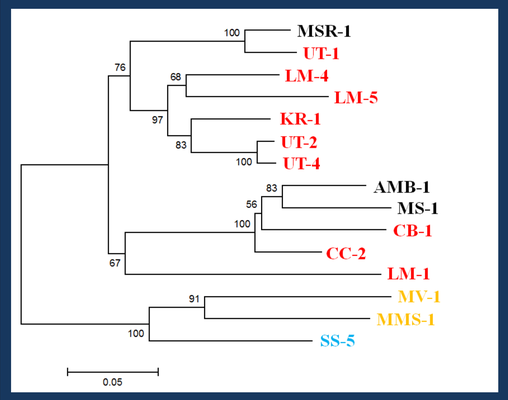

Figure 3. Phylogenetic tree of magnetospirilla and other MTB based on *cbbM* gene sequences. Red = new strains of magnetospirilla; black = known *Magnetospirillum* species; orange = other MTB in *Alphaproteobacteria*; and blue = magnetotactic *Gammaproteobacteria* as outgroup. Bootstrap values at nodes are percentages of 1,000 replicates. Bar represents 5% sequence divergence.

Results (cont.)

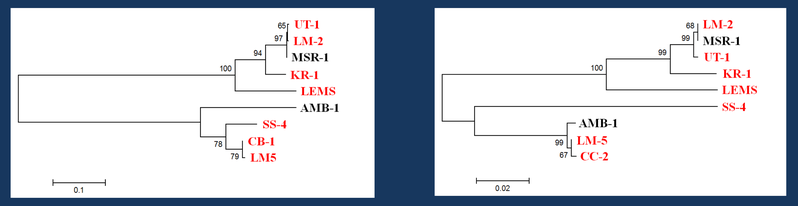

Figure 4. Phylogeny of magnetospirilla based on *mamJ* and *mamK* gene sequences. Left, phylogenetic tree based on *mamJ* gene sequences. Bar represents 10% sequence divergence. Right, phylogenetic tree based on *mamK* gene sequences. Red = new magnetospirilla strains; black = known *Magnetospirillum* strains. Bootstrap values at nodes are percentages of 1,000 replicates. Bar represents 2% sequence divergence.

Discussion

Phylogeny and relatedness of magnetospirilla based on 16S rRNA gene sequences. The current standard method of determining phylogeny of prokaryotes is by the comparison of 16S rRNA gene sequences. This analysis was performed on a number of newly isolated MTB with the typical morphology of the magnetospirilla. The results of the analysis show that most of strains belong in the genus *Magnetospirillum*, either as new strains of existing species (sequence identity >97%) or a new species (<97% but >95% identity). When the data is taken in its entirety, magnetospirilla appear to phylogenetically span, more as a continuum rather than as clearly delineated groups, over two genera based on the current accepted sequence divergence between organisms for genera (>5%). Strain LM-1 appears to represent a new genus. The data also show that species of the *Phaeospirillum* should be reclassified and included in *Magnetospirillum*.

Phylogeny and relatedness of magnetospirilla based on RubisCO gene sequences. Because most of the magnetospirilla possess a *cbbM* gene and not *cbbL* genes, *cbbM* was used for phylogenetic analysis of these organisms based on RubisCO. Phylogeny and relatedness of the magnetospirillum strains based on *cbbM* sequences was reasonably consistent with that from 16S rRNA genes. Strain LM-1 remained the outlier of the magnetospirilla. These results, in general, might indicate that the *cbbM* gene evolved similarly to the 16S rRNA gene and that it was not acquired by HGT.

Phylogeny and relatedness of magnetospirilla based on the magnetosome membrane proteins MamJ and MamK gene sequences. Phylogeny and relatedness of the strains based on *mamJ* and *mamK* sequences was more complex than that based on 16S rRNA and *cbbM* gene sequences. Some specific strains shown to be closely related by 16S rRNA gene sequence (as strains of the same species; e.g., strains UT-1, LM-2 and *M. gryphiswaldense* strain MSR-1) also appeared to be closely related based on one or both of the *mam* gene sequences. Other strains did not show this type of relationship. Because of these somewhat inconsistent results, the results presented here from *mam* gene sequences might reflect evolution of the magnetosome gene island (MAI) in magnetospirilla rather than relatedness between strains. However, our data set is incomplete and more data are needed to make such a conclusion. In addition, the general divergence of the *mamJ* gene within magnetospirilla is much greater than that for *mamK* and might not be a good choice for this type of study. Lastly, another problem is the issue of how much divergence of these genes must take place before our PCR primers are not effective in amplifying these genes. At the moment, we cannot estimate this.

Future Directions for Research

In this study, we have shown some interesting genetic trends. However, before major conclusions can be drawn from the data, some additional PCR and sequencing reactions must be completed. Because it is unlikely that magnetospirilla lack *mamJ* and *mamK* genes, as they have been shown to be necessary or magnetosome chain formation (Komeili et al. 2006; Scheffel et al. 2006), additional primers may need to be constructed to amplify these genes from certain strains. The same may be true for *cbbM*. Lastly, based the fact that most of these new magnetospirilla show great potential for chemolithoautotrophic growth, it would be interesting to compare the substrates they use to support autotrophic growth.

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Acknowledgements

Research in the Bazylinksi Lab is made possible by the National Science Foundation (NSF) Grant Number EAR-0715492 and Research Experience for Undergraduates (REU) Grant Number 0649267.