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Molecular evolution of the actin-like MreB protein gene family in wall-less bacteria

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ABSTRACT

The *mreB* gene family encodes actin-like proteins that determine cell shape by directing cell wall synthesis and often exists in one to three copies in the genomes of non-spherical bacteria. Intriguingly, while most wall-less bacteria do not have this gene, five to seven *mreB* homologs are found in *Spiroplasma* and *Haloplasma*, which are both characterized by cell contractility. To investigate the molecular evolution of this gene family in wall-less bacteria, we sampled the available genome sequences from these two genera and other related lineages for comparative analysis. The gene phylogenies indicated that the *mreB* homologs in *Haloplasma* are more closely related to those in Firmicutes, whereas those in *Spiroplasma* form a separate clade. This finding suggests that the gene family expansions in these two lineages are the results of independent ancient duplications. Moreover, the *Spiroplasma mreB* homologs can be classified into five clades, of which the genomic positions are largely conserved. The inference of gene gains and losses suggests that there has been an overall trend to retain only one homolog from each of the five *mreB* clades in the evolutionary history of *Spiroplasma*.

1. Introduction

For a long time, the existence of actin or actin-like proteins in prokaryotic cells had remained questionable. It was only since the beginning of this century that the presence of diverse bacterial actin homologs and their crucial roles in cell shape determination have been widely appreciated [1–6]. In both Gram-positive and Gram-negative bacteria such as *Bacillus subtilis*, *Escherichia coli*, and *Caulobacter crescentus*, the mutation or depletion of actin-like MreB proteins resulted in disrupted cell shapes [1,7,8]. Experimental evidence demonstrated that MreB homologs form helical filaments beneath the cell envelope that encircle the cell along its length and determine cell shapes through the control of cell wall synthesis [1,3,8–10]. This MreB-dependent mechanism of cell-shape determination is phylogenetically widespread in bacteria [1,9].

In *Spiroplasma* and *Haloplasma*, two bacterial genera without a cell wall, high copy numbers of *mreB* homologs have been identified. In all the other bacterial taxa characterized to date, at most three *mreB* homologs are present [1,9]. However, five to seven *mreB* homologs were found in the draft genome sequences of *S. citri* [11], *S. melliferum* [12,13], and *H. contractile* [14]. In addition to being wall-less, these two genera are both characterized by their contractile helical cells [15] or protrusions [16], which are suggestive of the involvement of helical cytoskeletal elements. Based on cryo-electron tomography, it has been proposed that MreB possibly forms a helical ribbon along the length of *Spiroplasma* cells [17].

To investigate the molecular evolution of the *mreB* gene family in wall-less bacteria, we took advantage of the recent availability of multiple complete genome sequences spanning most of the phylogenetic diversity within the genus *Spiroplasma* [18–21] to perform phylogenomic analysis. These *Spiroplasma* genomes, together with those of other Mollicutes (*Mycoplasma*, *Mesoplasma*, and *Acholeplasma*), *Haloplasma*, and two representatives of Firmicutes (Table 1), were examined for their gene repertoire of *mreB* homologs. A species phylogeny based on shared single-copy genes was reconstructed for Mollicutes, *Haloplasma*, and Firmicutes, which are closely related taxonomic groups based on 16S rRNA [16]. This

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Table 1
Genome sequences analyzed in this study.

Phylum	Class	Order	Family	Taxon	Accession numbers
Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmataceae	*Acholeplasma laidlawii* PG-8A	NC_010163
		Entomoplasmatales	Entomoplasmataceae	*Mesoplasma florum* L1^T	NC_006055
			Spiroplasmatacea	*Spiroplasma apis* B31^T	CP006682
				Spiroplasma chrysopicola DF-1^T	CP005077
				Spiroplasma citri GII3-3X	AM285301–AM285339
				Spiroplasma culicicola AES-1^T	CP006681
				Spiroplasma diminutum CUAS-1^T	CP005076
				Spiroplasma melliferum IPMB4A	AMGI01000001–AMGI01000024
				Spiroplasma syrphidicola EA-1^T	CP005078
				Spiroplasma taiwanense CT-1^T	CP005074
		Mycoplasmatales	Mycoplasmataceae	*Mycoplasma capricolum* ssp. *capricolum* ATCC 27343^T	NC_007633
				Mycoplasma leachii PG50^T	NC_014751
				Mycoplasma mobile ATCC 43663^T	NC_006908
				Mycoplasma mycoides ssp. *mycoides* SC PG1^T	NC_005364
				Mycoplasma penetrans HF-2	NC_004432
				Mycoplasma putrefaciens KS1^T	NC_015946
Unclassified	Unclassified	Haloplasmatales	Haloplasmataceae	*Haloplasma contractile* SSD-17B^T	AFNU01000001–AFNU01000109
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus subtilis* ssp. *subtilis* 168	AL009126
	Clostridia	Clostridiales	Clostridiaceae	*Clostridium botulinum* A str. ATCC 3502	AM412317
Proteobacteria	Gamma-proteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia coli* str. K-12 substr. MG1655	U00096

species phylogeny provides a framework for our inference of the gains and losses of *mreB* homologs.

2. Materials and methods

2.1. Taxon sampling for phylogenetic analyses

Genome sequences of one *Haloplasma* species (the only species in this monotypic genus [16]) and eight *Spiroplasma* species were obtained from GenBank (Table 1). Because the two *S. melliferum* strains, IPMB4A [13] and KC3 [12], are 99.9% identical at the nucleotide sequence level in single-copy protein-coding genes [13], only the IPMB4A genome was used as a representative. Other Mollicutes taxa that cover the diversity of this group were also included: four *Mycoplasma* species and *Mesoplasma florum* in the Mycoides-Entomoplasmataceae clade [22], two *Mycoplasma* species belonging to two clades outside of the Spiroplasma-Entomoplasmataceae-Mycoides clade [18], and *Acholeplasma laidlawii* (representing the Acholeplasmatales-Anaeroplasma-Phytoplasma clade of Mollicutes). One representative from each of the two major Firmicutes classes was selected: *B. subtilis* (Bacilli) and *Clostridium botulinum* (Clostridia).

2.2. Identification of mreB homologs

Homologous gene clusters among the genome analyzed were inferred by OrthoMCL [23] with the *e*-value cutoff set to 1×10^{-15}. Genes that were assigned the KEGG [24,25] Orthology (KO) number K03569 (*mreB*) and other genes that were clustered in the same orthologous groups were regarded as *mreB* homologs (Supplemental Table S1). Multiple homologs of *mreB* in *Spiroplasma* were numbered according to their order in the chromosome (starting from *dnaA*). For visualization of the chromosomal locations of these *mreB* homologs, the circular genome maps of the six complete *Spiroplasma* genome sequences were drawn using Circos [26].

2.3. Molecular phylogeny of the mreB homologs

Sequences of *mreB* homologs were parsed from the genomes. A maximum likelihood phylogeny was inferred from amino acid sequences with the *ftsA* gene from *S. melliferum*, *S. apis*, and *B. subtilis* as the outgroup. The sequences were aligned using MUSCLE v3.8 [27] with the default settings. A gene tree was inferred using PhyML v3.0 [28] with the WAG + I + G model and six substitution rate categories. Bootstrap supports were estimated from 1000 samples of alignment generated by the SEQBOOT program of PHYLIP v3.69 [29]. Another maximum likelihood phylogeny was inferred for *mreB* nucleotide sequences of all eight *Spiroplasma* species and *M. florum*. The amino acid sequences from these nine species were aligned using MUSCLE, converted into nucleotide sequence alignment with PAL2NAL [30], and analyzed using PhyML with the GTR + I + G model and six substitution rate categories. Bootstrap supports were estimated as described above.

2.4. Species phylogeny

Two species phylogenies were reconstructed, one of which included *E. coli* K12 (Table 1) as the outgroup for rooting. Protein-coding genes were clustered using OrthoMCL as described above. Amino acid sequences of shared single-copy genes were extracted, aligned with MUSCLE, concatenated, and used for PhyML phylogenetic analyses with the WAG + I + G model. Bootstrap supports were estimated from 1000 samples of alignment generated by the SEQBOOT program of PHYLIP.

3. Results and discussion

3.1. Numbers of mreB homologs in Spiroplasma and relatives

A total of 57 *mreB* homologs in the selected taxa were identified by a BLAST-based approach (Supplemental Table S1). In *Acholeplasma* and *Mycoplasma*, no *mreB* homolog was found, which is consistent with the original genome annotations. In *M. florum*, a gene (Mfl003) with the product description "actin-like protein" was identified as an *mreB* homolog. It is notable that all *Spiroplasma* genomes characterized to date have at least five *mreB* homologs. Seven copies were found in *S. culicicola* and *H. contractile*, which is the highest number reported among the bacteria genomes examined [1,9]. In comparison, the Firmicutes representatives, *B. subtilis* and *C. botulinum*, have only three and two *mreB* homologs, respectively.

3.2. Relationships among mreB homologs

To understand the relationships among the *mreB* homologs of Mollicutes, *Haloplasma*, and Firmicutes, we first reconstructed a gene tree based on the amino acid sequences (Fig. 1a). This phylogenetic tree was rooted using the single-copy gene *ftsA*, the bacterial actin homolog most closely related to *mreB* [31]. To avoid overrepresentation of the *Spiroplasma* sequences (44 out of 57), only those from the four more distantly related species [18,19] were included. The *mreB* homologs from Mollicutes, *Haloplasma*, and Firmicutes form three separate clades, among which the *Haloplasma* clade and the Firmicutes clade are more closely related. This result indicates that despite the similar motility observed in *Spiroplasma* and *Haloplasma*, their multiple *mreB* homologs may have originated from independent gene family expansions. The observation that the *Mesoplasma mreB* is nested within a subclade of *Spiroplasma mreB* is consistent with the species phylogeny [18,19,22].

We then utilized all the *mreB* homologs from *Spiroplasma* and *Mesoplasma* to build a phylogeny, which was rooted at the point inferred in the first tree. Nucleotide sequences were used to provide better resolution. This nucleotide sequence phylogeny (Fig. 1b) is largely congruent with that based on amino acid sequences. Five clades of *Spiroplasma mreB* homologs can be identified in both trees, which mainly differ in the placement of Clade V.

3.3. Genomic organization of mreB homologs in Spiroplasma

In our previous study [18], we found that the chromosomal locations of *mreB* homologs are highly conserved in *S. chrysopicola* and *S. syrphidicola*, and even in the highly rearranged virus-invaded genomes of *S. citri* [11] and *S. melliferum* [13]. The examination of four additional species indicated that this positional conservation exists in the more diverse Apis clade as well (Supplemental Fig. S1). In terms of the relative positions from *dnaA* (the first gene downstream of the chromosome replication origin), the last four (five in *S. culicicola* and *S. diminutum*) homologs are located in a segment with positive GC-skew just upstream of *dnaA*. This segment is prominent in the second half of these chromosomes, where most other parts have negative GC-skew. The first (second in *S. culicicola*) homolog is located about one third of the chromosome after *dnaA*. This homolog is invariably within 8 kb downstream of *ppa* (coding for an inorganic pyrophosphatase). This relationship is disrupted only in *S. melliferum* and *S. citri*, where viral and other sequences are found between *ppa* and the *mreB* homolog.

By comparing the phylogeny of the *mreB* homologs (Fig. 1) and their genomic positions, we found that the order of members from the five *mreB* clades is also relatively conserved. Using the *S. taiwanense* genome as a reference, where a single member from each clade is found, the five clades can be named after their genomic position using Roman numerals, which are distinguished from the Arabic numbers in gene names that indicate the order of homologs in respective genomes (Fig. 1b).

3.4. Duplications and losses of mreB homologs in Spiroplasma and relatives

Although the deep phylogenetic relationships between Mollicutes and Firmicutes remain largely uncertain [32,33], our genome-wide phylogenies point out that Mollicutes form a clade distinct from *Haloplasma* (Fig. 2 and Supplemental Fig. S2). By integrating this species tree and the *mreB* gene tree (Fig. 1b), we reconstructed the most probable history of gene duplications and losses (Fig. 2). In total, we inferred two duplications and seven losses in *Spiroplasma* lineages. We noted that the Citri-Chrysopicola clade has not retained a Clade I *mreB* homolog. However, at approximately the same genomic position as in the Apis clade, the Citri-Chrysopicola clade has gained a Clade IV homolog, which is presumably a duplicate of the original Clade IV homolog located upstream of *dnaA*. This is intriguing because Clade IV and Clade I are strongly supported as a monophyletic group (Fig. 1). The replacement by a duplicate of the closest homolog suggests that there is functional divergence between different *mreB* clades. Despite the overall tendency to lose homologs, a minimum of five *mreB* homologs are maintained at relatively conserved positions of the genome. However, the functional significance of this conservation in genomic locations is unclear.

In the common ancestor of the Mycoides-Entomoplasmataceae clade, all *mreB* genes, except for one Clade III homolog, had been lost (Fig. 2). This coincided with the loss of helical cell morphology and the fibril protein (*fib*), the constituent of a *Spiroplasma*-specific cytoskeletal filament [15]. Two features of the remaining *mreB* homolog in *M. florum* are worth noting: (1) the sequence divergence is extremely high (Fig. 1), and (2) the position had changed to the downstream of *dnaN* (near the replication origin). Although the function of this homolog is unknown, the release from selective constraint appears to be a plausible explanation for these observations.

3.5. Implications

Although MreB was first demonstrated as a cell-shape determining protein in bacteria [1], the major role of MreB seems not to be maintenance of a fixed shape in *Haloplasma*, which has a pleomorphic cell body with one or two protrusions that change drastically between straight and helical forms [16]. More recent works have shown that MreB filaments control cell shapes mainly through guiding cell wall synthesis and do not have any structural role by themselves [3,5,8–10]. Therefore, MreB may play different roles in walled and wall-less bacteria.

Based on the similar motility and the presence of multiple *mreB* homologs in both *Spiroplasma* and *Haloplasma*, we argue that the major role of MreB proteins in these bacteria is related to motility rather than shape determination. The characteristic helical shape of *Spiroplasma* cells is likely attributed to their unique cytoskeletal element, the fibril protein, which is the building block for two helical cytoskeletal ribbons attached to the cell membrane from underneath [15,17]. The fibril ribbons are elastic, relatively stable, and were first isolated only one year after the discovery of spiroplasmas [34]. In contrast, MreB filaments, which possibly form a third cytoskeletal ribbon between the two fibril ones [17], have never been isolated from living spiroplasmas. It indicates the instability of the MreB filaments, which is consistent with the dynamic nature of actin homologs [7,31]. The dynamics of the MreB filaments may contribute to spiroplasmas' ability to lengthen or shorten the pitch of their helical cells [15]. Similarly, it may enable *Haloplasma* to alternate between a straight form and a contracted helical form with a period of only a few seconds [16]. Given the distant relationship between *Spiroplasma* and *Haloplasma* (Fig. 2) and the independent *mreB* gene family expansions (Fig. 1a), their similarity in motility may have resulted from functional convergence. A possible mechanism for the alteration between contracted and relaxed states may be the regulation of MreB polymer structure by small molecules. For example, it has been shown that in the presence of ATP/GTP or ADP/GDP, *Thermotoga maritima* MreB forms helical or linear protofilaments, respectively [35,36].

Phylogenetic analyses indicate that the multiple *mreB* homologs in *Spiroplasma* and *Haloplasma* are the products of independent ancient duplications (Fig. 2). In *Spiroplasma*, the different homologs may have assumed different functions and at least five homologs are maintained throughout their evolutionary history. Since cryo-electron tomography revealed only one putative MreB ribbon in

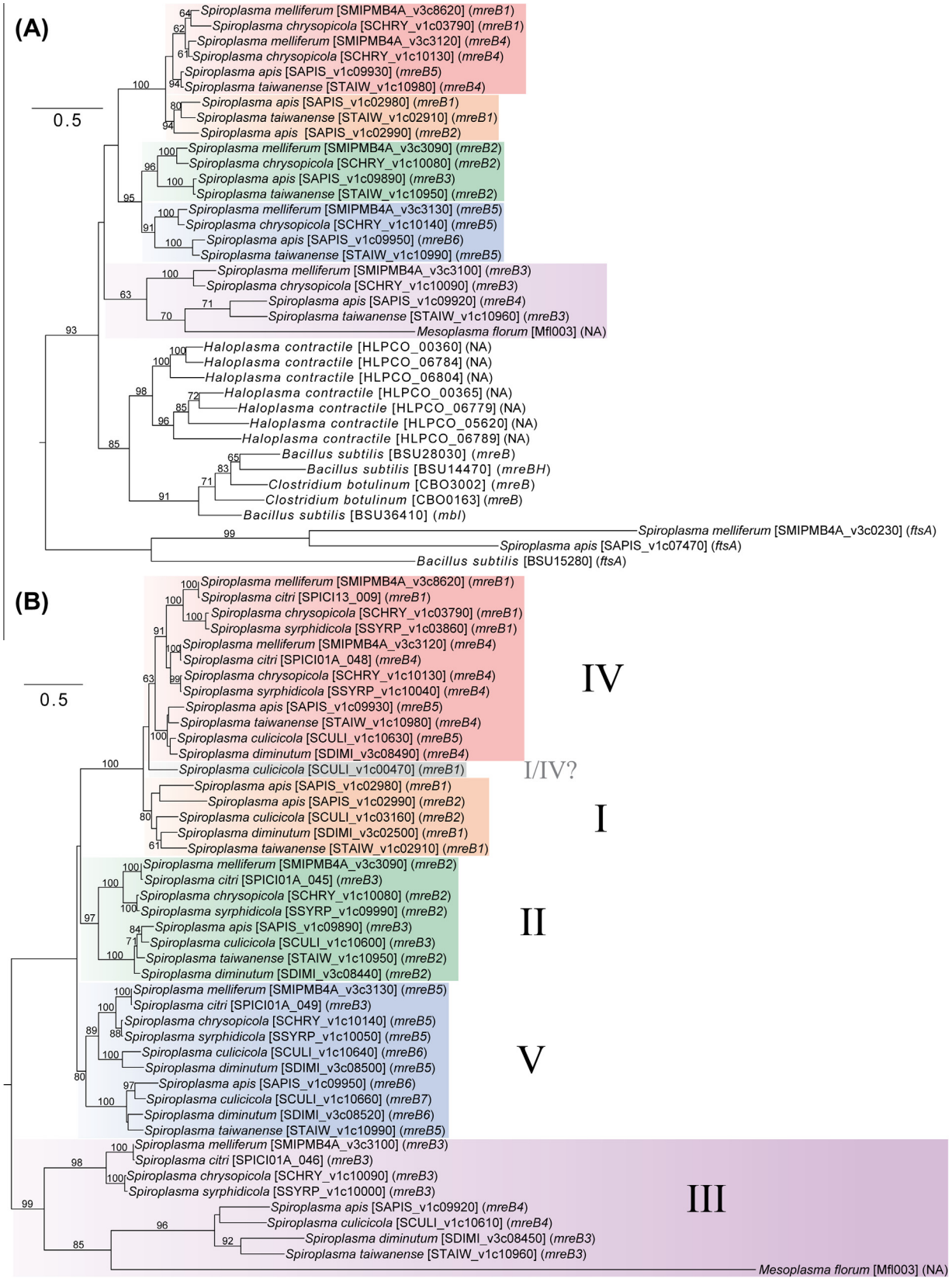

Fig. 1. Maximum likelihood phylogenies of *mreB* homologs. (a) A tree based on amino acid sequences, including homologs from *Mesoplasma*, *Haloplasma*, Firmicutes, and selected *Spiroplasma* species. (b) A tree based on nucleotide sequences of homologs from eight *Spiroplasma* and one *Mesoplasma* species. Bootstrap supports are indicated for values ⩾60%. Locus tags and gene names are indicated in square brackets and parentheses, respectively. The numbers in gene names of the *Spiroplasma* homologs indicate their order (from *dnaA*) within the respective genome. Color shading indicates the five clades of *Spiroplasma* homologs, which are numbered according to their genomic positions with Roman numerals. SCULI_v1c00470 in *S. culicicola* was clustered with Clades I and IV, but could not be placed into either with high confidence.

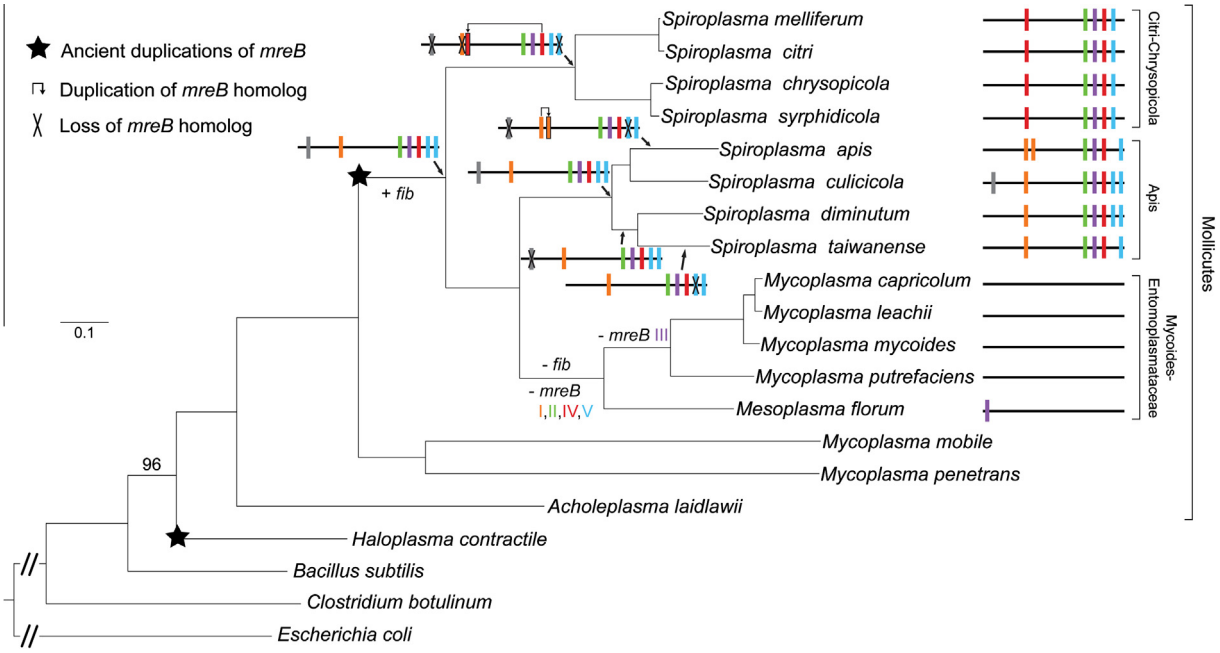

Fig. 2. Maximum likelihood species phylogeny of Mollicutes, *Haloplasma*, and Firmicutes based on 117 shared single-copy protein-coding genes. *Escherichia coli* was used to root the tree. Except where noted, all branches received 100% bootstrap support. Putative gains and losses of *mreB* homologs (color coded for the five clades and one unplaced homolog) and the *Spiroplasma* fibril gene (*fib*) are labeled. The within-genome positions of *mreB* homologs are drawn (not to scale) on an imaginary linearized chromosome (beginning with *dnaA*) for the extant species from two *Spiroplasma* clades and the Mycoides-Entomoplasmataceae clade.

S. melliferum, which is composed of nine filaments [17], it is likely that all different MreB homologs are involved in this ribbon. Such an association among different MreB homologs has been observed in *B. subtilis*, where all three homologs (MreB, Mbl, and MreBH) are almost completely co-localized, indicating they lie in close proximity in a single helical structure [5]. Whether each of the *Spiroplasma* filaments is composed of one or more specific MreB homologs remains to be investigated. Using the six complete *Spiroplasma* genome sequences as a guide, the functional role of these *mreB* homologs shall be testable with genetic manipulations involving deletion and replacement. Additionally, investigations at the RNA and protein levels may provide insights into the expression levels of different homologs and their relative proportions in the MreB ribbon.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.039.

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