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Original Research Article

Insights into the evolutionary origin of *Mycoplasma species*

Karzan R. Sidiq [1,*],Dana Khdr Sabir [2], Rebin M. Salih [1],Tre N. Nasir [2]

[1]Charmo Centre for Research, Training and Consultancy, Charmo University, Sulaimani, Kurdistan Region, Iraq
[2]Department of Medical Laboratory Sciences, Charmo University, Sulaimani, Kurdistan Region, Iraq
***Corresponding author:** Karzan R. Sidiq, Charmo Centre for Research, Training and Consultancy, Charmo University, Sulaimani, Kurdistan Region, Iraq.E-mail: karzan.sidiq@charmouniversity.org*

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ABSTRACT

Mycoplasma species are cell wall deficient intracellular microorganisms that are related to respiratory and urogenital infections in human being. In this study, we are providing insights into the *Mycoplasma* evolutionary origin based on the taxonomical characterization of such microorganism and also searching for homology sequences of the bacterial cell wall biosynthetic proteins in *Mycoplasma* species using bioinformatics approach. Our results indicated that the ancestor of *Mycoplasma* is eubacteria. In addition, the presence of homologous proteins to the teichoic acids biosynthetic proteins in *Mycoplasma* strongly suggests that *Mycoplasma* was originally a Gram-positive bacterium and had lost the cell wall as a part of the adaptation mechanism to be intracellular parasite.

***Keywords*:** Mycoplasma, evolution, eubacteria, cell wall biosynthesis, sequence homology

INTRODUCTION

The members of the genus *Mycoplasma* are the smallest self-replicating intracellular microorganisms that belong to a distinct class called *Mollicutes*. So far, more than 200 species of *Mycoplasma* have been identified that are responsible for a number of clinically important infections such as respiratory tract and genitourinary tract infections [1,2]. This group of microorganism are also characterized by having a small genome size (0.58 to 2.20 Mb) and importantly lacking of cell wall [2,3]. This feature makes

Mycoplasmas have polymorphic shape and insensitive to β-lactam group of antibiotics [3]. This is due to the important role of bacterial cell wall, which is known to play roles in defining bacterial cell shape and protecting the cell from bursting in hypotonic environments [4,5].

Structurally, bacterial cell wall is mainly made of peptidoglycan, a mesh-like polymer of two amino-sugar repeats. Based on cell wall structure and composition, bacteria are categorized into Gram-positive and Gram-negative bacteria. In Gram positive bacteria, the cell wall contains a thick layer of peptidoglycan and anionic polymers called teichoic acids, which cannot be found in Gram negative bacteria [6,7]. Whereas, the cell wall of Gram-negative bacteria is composed of only a thin layer of peptidoglycan and an outer membrane, containing endotoxic lipopolysaccharide [8,9].

The biosynthesis of bacterial cell wall components is a complicated process and it is carried out in a series of enzymatic reactions through different complex pathways. The peptidoglycan biosynthesis starts with the synthesis of cytoplasmic cell wall precursor (lipid II) by Alr, Ddl. MurA. MurB, MurC, MurD, MurE, MurF, MraY, MnaA and MurG enzymes and assembly of peptidoglycan polymer by penicillin binding proteins (PBPs) (Figure 1). In Gram-positive bacteria, the synthesis of wall teichoic acid requires TagA, TagB, TagD, TagG, TagH and TagO enzymes, whereas the lipoteichoic acid biosynthesis needs LtaS, YvgJ, YqgS, YfnI and YfhO enzymes (Figure 2). Moreover, lipid A is the most conserved moiety of lipopolysaccharides in Gram-negative bacteria. The synthesis of Lipid A is carried out by LpxA, LpxB, LpxC, LpxD, LpxH, LpxK, KdtA, LpxL, LpxM and WaaL enzymes (Figure 3). Thus, a considerable number of genes in bacterial genome contribute in cell wall biosynthesis.

In the evolutionary point of view, adaptation of *Mycoplasma* species to live without cell well is intriguing. It is initially thought that *Mycoplasma* had been evolved either from primitive ancestral cells through gaining of foreign genetic materials or they are degenerated from complex bacterial ancestors through losing genes [10,11]. The latter is more reliable, depending on the common evolutionary prospect “use it or lose it”, in which microorganisms lose the gene(s) that are not essential for unnecessary metabolic activity [12].

Despite reports from studies about conversion of eubacteria to L-form and vice versa, and the suitability of animal cell as a favourable niche for *Mycoplasma* growth [13,14], the evolutionary origin of

Mycoplasma remains unclear. Here, we are going to give insights into the evolutionary emergence of *Mycoplasma* depending on the protein sequence analysis of more than 30,000 *Mycoplasma* strains in contemporary public databases. Our focus are re-examining the taxonomical characterization of *Mycoplasma* based on 16s rRNA sequence and also analyzing the sequences of the protein homologs that known to be involved in bacterial cell wall biosynthesis.

MATERIALS AND METHODS

Phylogenetic tree analysis

The taxonomical relationship of *Mycoplasma* with a number of Gram-positive and Gram-negative genera were constructed using ClustalX 2.1 [15] and MEGA 7.0.26 [16] as described before [17]. Initially, accession numbers and 16S rRNA nucleotide sequence of the genes from the studied genera were retrieved from National Center for Biotechnology Information (NCBI). Phylogenetic tree were build using the neighbor-joining method, bootstrapped with 1,000 replicate runs, using the MEGA 6 tree-building program.

Protein sequences

The amino acid sequences and the accession numbers of the proteins known to be involve in the cell wall biosynthesis in a Gram-positive (*Bacillus subtilis*) and a Gram-negative (*Escherichia coli*) bacteria were collected from NCBI, EcoliWiki and SubtiWiki databases (Tables 1 and 2). Searching for homology of the protein sequences was performed using protein BLAST tool (BLASTP) against *Mycoplasmas* (taxid: 31969) in NCBI. Then, the sequence cover similarity and the percentage of similarity of *Mycoplasma* proteins to the cell wall characterized proteins in *B. subtilis* and *E. coli* were recorded.

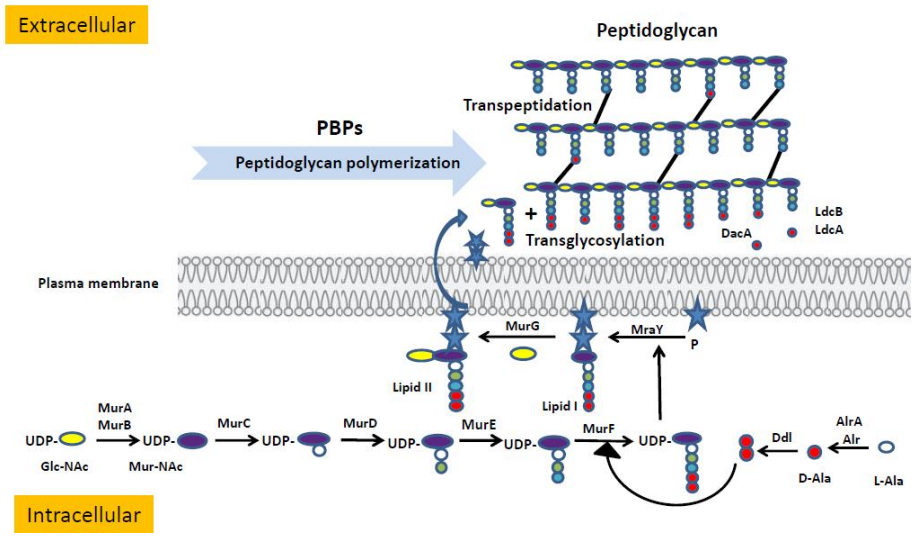

Figure 1. Peptidoglycan biosynthesis in bacteria**.** It includes two processes; a cytoplasmic biosynthetic pathway for peptidoglycan precursor (lipid II) by AlrA, Ddl. MurA. MurB, MurC, MurD, MurE, MurF, MraY, MnaA and MurG enzymes and peptidoglycan polymerization by pencilling-binding proteins (PBPs). The Details of the above enzymes are shown in (Tables 1 and 2).

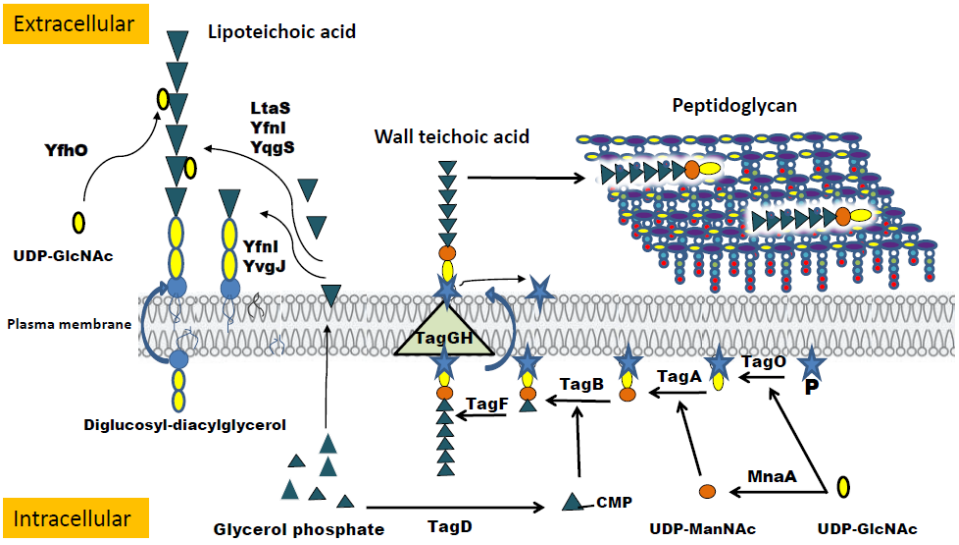

Figure 2. Wall teichoic acid and lipoteichoic acid biosynthesis pathways in Gram-positive bacteria. The enzymes of wall teichoic acid pathway are TagA, TagB, TagD, TagG, TagH and TagO, whereas, the enzymes that synthesize lipoteichoic acid are LtaS, YvgJ, YqgS, YfnI and YfhO. The Details of the above enzymes are shown in (Table 1).

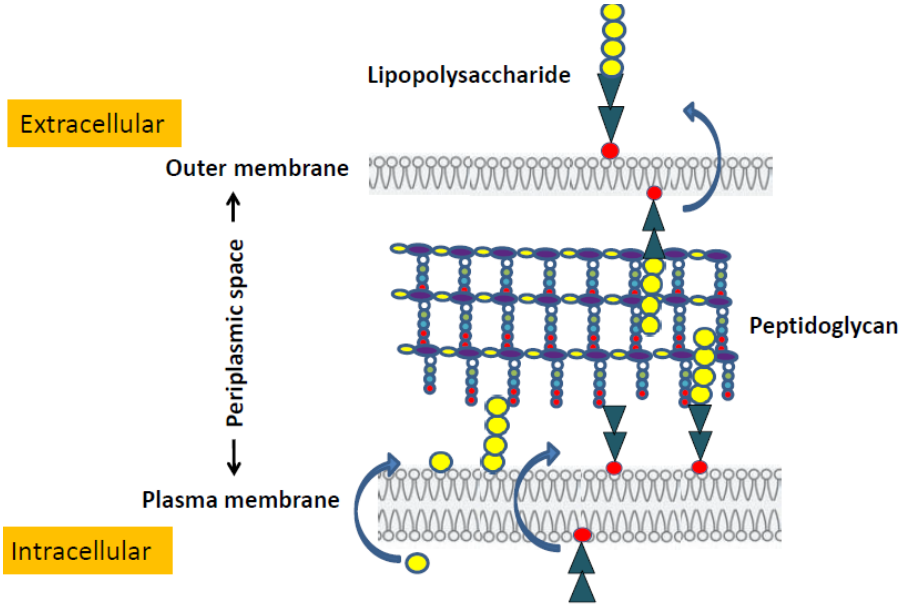

Figure 3. Biosynthetic pathway of lipopolysaccharide in Gram-negative bacteria. The enzymes involved in the pathway are LpxA, LpxB, LpxC, LpxD, LpxH, LpxK, KdtA, LpxL, LpxM and WaaL. The Details of the above enzymes are in (Table 2).

Table 1. The accession numbers and sequences of the proteins, known to be involve in the cell wall synthesis in a Gram-positive (*B. subtilis*)

Pathways	Protein name	Accession No.	Enzymatic activity
Biosynthesis of peptidoglycan precursor (Lipid II) in cytoplasm [18, 19]	AlrA	WP_120322737.1	Alanine racemase
	Ddl	WP_003234325.1	D-alanine-D-alanine ligase
	MurA	WP_048654789.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
	MurC	WP_038429473.1	UDP-N-acetylmuramoyl-L-alanine synthetase
	MurD	WP_064671357.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
	MurE	WP_021479338.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimelate synthetase
	MurF	WP_101501920.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine synthetase
	MraY	WP_038428918.1	Phospho-N-acetylmuramoyl-pentapeptide undecaprenyl phosphate transferase
	MnaA	WP_063694956.1	UDP-N-acetylglucosamine 2-epimerase
	MurG	WP_128473890.1	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
Extra-cytoplasmic polymerization and maturation of peptidoglycan by Penicillin binding proteins [20-23]	PBP2a	WP_014480276.1	Transpeptidase
	PBP2b	WP_080479550.1	
	PBP3	WP_032728924.1	
	PBP4	WP_072175137.1	Glycosyltransferase and Transpeptidase
	PBP1	WP_088467250.1	
	LdcB	WP_046340243.1	LD-carboxypeptidase
	DacA	WP_128992133.1	DD-carboxypeptidase

Wall Teichoic acid biosynthesis [24,25]	TagA	WP_032726979.1	UDP-N-acetyl-D-mannosamine transferase
	TagB	WP_041519545.1	Putative CDP-glycerol:glycerol phosphate glycerophosphotransferase
	TagD	WP_003227921.1	Glycerol-3-phosphate cytidylyltransferase
	TagG	WP_003227928.1	ABC transporter for teichoic acid translocation (permease)
	TagH	WP_017697343.1	ABC transporter for teichoic acid translocation (ATP-binding protein)
	TagO	WP_014481064.1	UDP-GlcNAc-1-phosphate transferase, teichoic acid linkage unit synthesis
Lipoteichoic acid biosynthesis [26,27]	LtaS	WP_003244191.1	Major polyglycerolphosphate lipoteichoic acid synthase
	YvgJ	WP_116315876.1	Lipoteichoic acid synthesis primase
	YqgS	WP_129093023.1	Polyglycerolphosphate lipoteichoic acid synthase
	YfnI	WP_133954001.1	Polyglycerolphosphate lipoteichoic acid synthase
	YfhO	WP_009969309.1	Lipoteichoic acid glycosylation

Table 2: The accession numbers and sequences of the proteins, known to be involve in the cell wall synthesis in a Gram-negative (*E. coli*)

Pathways	Protein name	Accession No.	Enzymatic activity
Biosynthesis of peptidoglycan precursor (Lipid II) in cytoplasm [28,29]	Alr	WP_001147329.	Alanine racemase
	Ddl	WP_074512685.1	D-alanine-D-alanine ligase A
	MurA	WP_104730778.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
	MurB	WP_119186487.1	UDP-N-acetylenolpyruvoylglucosamine reductase
	MurC	WP_134240128.1	UDP-N-acetylmuramate-L-alanine ligase
	MurD	WP_112031069.1	UDP-N-acetylmuramoylalanine--D-glutamate ligase
	MurE	WP_027868356.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase
	MurF	WP_000626686.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine synthetase
	MraY	WP_101348531.1	Phospho-N-acetylmuramoyl-pentapeptide-transferase
	MurG	WP_000016570.1	N-acetylglucosaminyl transferase
Extra-cytoplasmic polymerization and maturation of peptidoglycan by Penicillin binding proteins [20,30-32]	Pbp1A	WP_089625239.1	Glycosyltransferase and Transpeptidase
	Pbp1B	WP_096123974.1	Glycosyltransferase and Transpeptidase
	Pbp1C	WP_108118785.1	Transglycosylase
	PbP2	WP_100679055.1	Transpeptidase
	FtsL	WP_000642202.1	Transpeptidase
	Pbp7	WP_077039566.1	D-alanyl-D-alanine endopeptidase
	LdcA	WP_097735320.1	LD-carboxypeptidase
	DacA	WP_044869612.1	DD-carboxypeptidase

Lipopolysaccharide Biosynthesis [33,34]	LpxA	WP_032280282.1	UDP-N-acetylglucosamine acyltransferase
	LpxB	WP_111724754.1	Lipid-A-disaccharide synthase
	LpxC	WP_104778318.1	UDP-3-O-acyl-N-acetylglucosamine deacetylase
	LpxD	WP_097471763.1	UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase
	LpxH	WP_121855960.1	UDP-2,3-diacylglucosamine diphosphatase
	LpxK	WP_000570549.1	Tetraacyldisaccharide 4'-kinase
	KdtA	WP_000891571.1	3-deoxy-D-manno-octulosonic acid transferase
	LpxL	WP_042091265.1	Lauroyl/palmitoleoyl acyltransferase
	LpxM	WP_096938928.1	Lauroyl-Kdo(2)-lipid IV(A) myristoyltransferase
	WaaL	WP_064262091.1	O-antigen ligase family protein

RESULTS

Phylogenetic analysis

The nucleotide sequence of the 16S rRNA genes was used to construct the taxonomical relationship of *Mycoplasma* with a number of Gram-positive and Gram- negative genera. As shown in Figure 4, *Mycoplasma* appears to be a neighbours and have the same internal nodes with a small pleomorphic wall–deficient bacteria *Ureaplasma spp*. In addition, Spiroplasma spp, which are also small bacterium without cell walls, are neighbours with other wall-less bacteria belong to *Acholeplasma spp.* All the above four mentioned genera of the cell wall less bacteria form a monophyletic group and share a common ancestor with Gram-positive bacteria group. This is a clear indication of the closer evolutionary relationship of the *Mycoplasma sp. t*o Gram-positive bacteria and also suggesting that *Mycoplasma sp.* and other cell wall less bacteria are emerged from Gram-positive bacteria.

Homologs of peptidoglycan biosynthetic proteins in Mycoplasma spp.

Homology sequence searching was carried out for proteins that participate in peptidoglycan biosynthesis pathways in both Gram-positive (*B. subtilis*) and Gram-negative *(E. coli*) bacteria. This was done by BLASTP searching for the proteins that involved in the biosynthesis of peptidoglycan precursor (lipid II) and penicillin binding proteins (Tables 1 and 2). The result showed that the homologs of all the proteins involve in biosynthesis of peptidoglycan precursor (lipid II) and penicillin binding proteins in *B. subtilis* are present in *Mycoplasma species.* The AlrA, Ddl. MurA. MurC, MurD, MurE, MurF, MraY, MnaA and MurG proteins that required for the biosynthesis of cytoplasmic

precursor (lipid II) of peptidoglycan showed high sequence coverage (> 90 %) and intermediate (> 50 %) sequence similarity to *Mycoplasma* proteins (Figure 5A). Our results also showed slightly lower percentage of sequence coverage and similarity for the presence of homologies of *B. subtilis* penicillin binding proteins in *Mycoplasma* (Figure 5 B). As above results, the proteins that are involved in biosynthesis of peptidoglycan precursor (lipid II) and the penicillin binding proteins (Tables 1 and 2) of *E. coli* have homologies in *Mycoplasma* with high percentage of sequence coverage (> 90 %) and intermediate sequence similarity (50 %) (Figures 6A and B). Only LcdA of *E. coli* seems to have no homolog in *Mycoplasma species*.

Homologs of Teichoic acids and lipopolysaccharides biosynthetic proteins in Mycoplasma spp.

We also used BLASTP searching tool of NCBI to discover the protein sequences in *Mycoplasma species* may be homologs to the proteins, taking part in teicnoic acids and lipopolysaccharide biosynthetic pathways in Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria respectively (Figures 5C and 6C). It was found that *Mycoplasma* possesses homologs of teichoic acids biosynthetic proteins at high percent of sequence coverage (> 90 % and intermediate (> 50 %) sequence similarity (Figure 5C). However, with exception of LpxA and LpxD proteins all other lipopolysaccharide biosynthetic proteins of *E. coli* do not have protein homologs in *Mycoplasma* (Figure 6C). The LpxA and LpxD proteins *E. coli* also showed almost lower than (50 %) sequence coverage and sequence similarity to their homologs in *Mycoplasma species*.

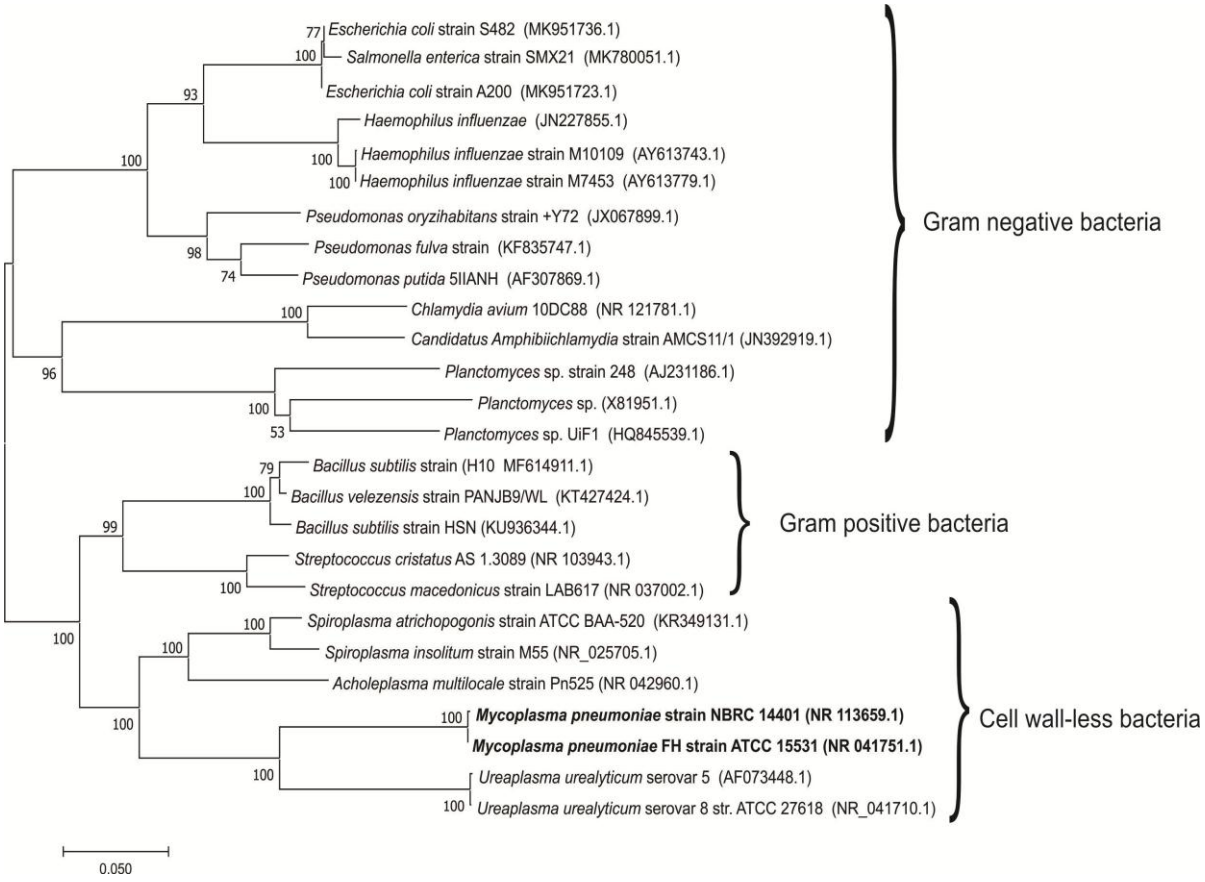

Figure 4. Unrooted phylogenetic tree based on the nucleotide sequence of the 16S ribosomal RNA gene. The two strains of *Mycoplasma spp*., used two represent the genus of *Mycoplasma* are shown in bold. The GenBank accession numbers are shown in parentheses after species names. ClustalX program was used to aligned the nucleotide sequence and was created using the neighbor-joining method, bootstrapped with 1,000 replicate runs, by the MEGA 7 tree-building program. Bootstrap values above 50 (1000 replicates) is shown as a number at nodes.

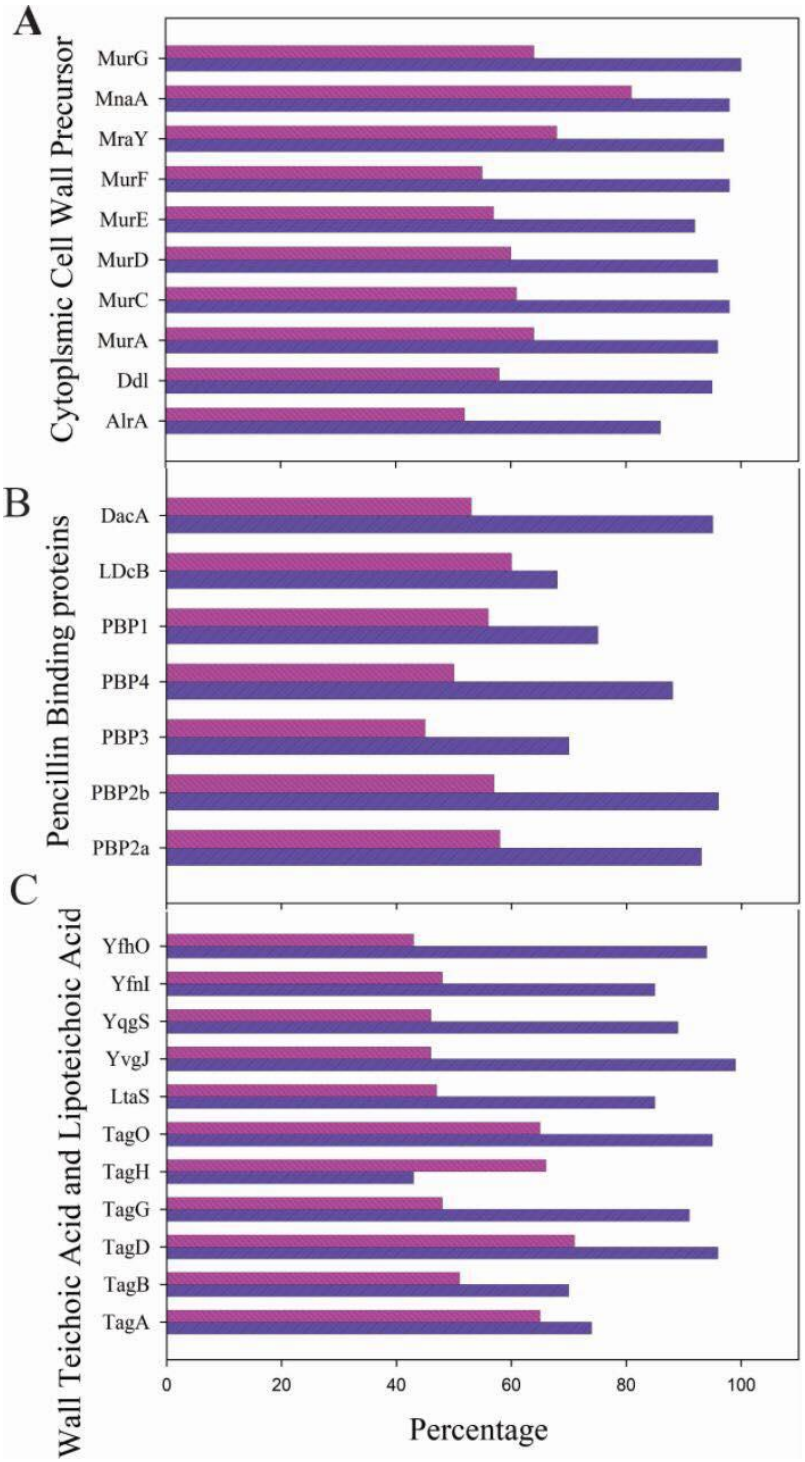

Figure 5. Percentage of the sequence coverage and the sequence similarity of cell wall biosynthetic proteins in *B. subtilis* and *Mycoplasma spp.* **A)** The proteins that take part in cell wall precursor (lipid II) biosynthetic pathway in cytoplasm. **B)** The proteins that play role in extracytoplsmaic peptidoglycan polymerization. **C)** The proteins that catalyse the pathways of wall- and lipo- teichoic acid biosynthesis. The data was generated through blasting the sequence of *B. subtilis* cell wall biosynthetic proteins against more than 30000 *Mycoplasma* strains using NCBI protein blast tool. Purple and violet lines are representing the percentage of the sequence coverage and similarly.

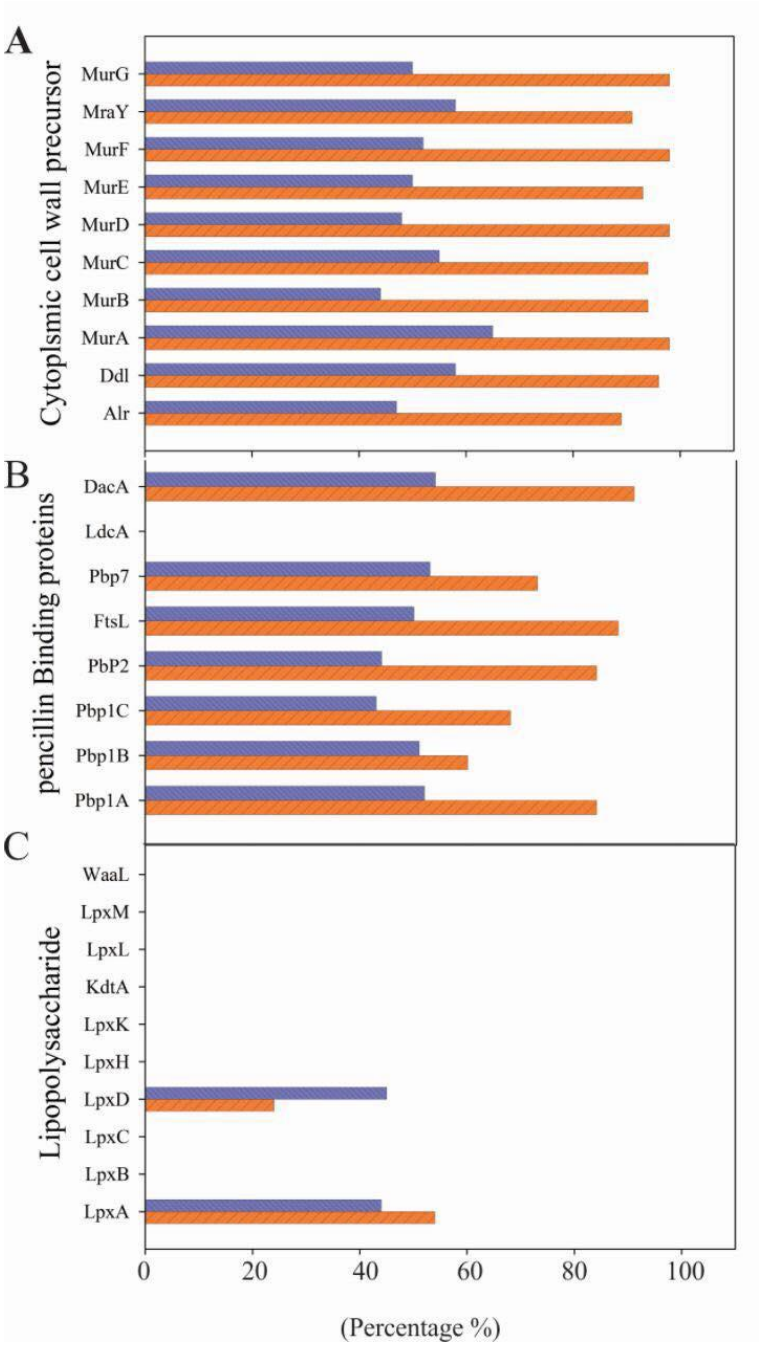

Figure 6. The percentage of sequence coverage and similarity of cell wall biosynthetic proteins in *E. coli* and *Mycoplasma spp.* **A)** The proteins that run the steps of cell wall precursor (lipid II) biosynthetic pathway in cytoplasm. **B)** The proteins that take part in extracytoplsmaic peptidoglycan polymerization. **C)** The proteins that play roles in lipopolysaccharide biosynthetic pathway. The data was generated through blasting the sequence of *E coli* cell wall biosynthetic proteins against more than 30000 *Mycoplasma* strains using NCBI protein blast tool. Blue and orange lines are representing the percentage of the sequence coverage and similarly.

DISCUSSION

It has been known that mutations in the genetic material play vital roles in the evolution and the adaptation of the living organisms. Motoo Kimura is a pioneer in the field of evolution that developed the "Neutral Theory of Molecular Evolution". In which, he developed a mathematical calculation to estimate nucleotide substitution rates. He proposed that the variation among the species is mainly results of the random genetic variations, which are selectively neutral and spread through genetic drift [35]. Such mutations can be silent or neutral. They may not have effect on the fitness of the organism, or can be deleterious and has lethal effect. In some cases mutations may be advantageous and improve the fitness of the organism within an environmental niche [36].

Like bacteria, *Mycoplasmas* are prokaryotes and they are classified as a group of cell wall-deficient microorganism in the kingdom of Monera. The cell biology of Mycoplasma has been investigated well. However, there is no enough data about the evolutionary origin of *Mycoplasmas*. In this study, contemporary online data mining method of several *Mycoplasma* spp. were analyzed to answer this question. In the first step, the 16S rRNA sequence alignment was used to construct a phylogenetic tree and characterize the bacterial genus (Figure 4). The results showed that *Mycoplasma* form a monophyletic group with other Gram-positives, rather than Gram negative. This suggests that Mycoplasma is closely related to the Gram-positive bacteria. Our result is consistent with a study that used traditional technique of oligonucleotide catalogue of 16S rRNA to show the evolutionary relationship between *Mycoplasma* and other bacteria [37].

Further investigation was carried out by homology sequence searching for proteins that participate in cell wall biosynthesis pathway in both Gram-positive and Gram-negative bacteria. This was done by BLASTP searching for the proteins that involved in the biosynthesis of peptidoglycan precursor (lipid II), penicillin binding proteins, and teichoic acids biosynthetic proteins of *B. subtilis* (Table 1) and lipoteichoic acids biosynthetic proteins of *E. coli* (Table 2). Homologies of all the proteins involve in

biosynthesis of peptidoglycan precursor (lipid II) and penicillin binding proteins in *B. subtilis* were found in *Mycoplasma species*. AlrA, Ddl. MurA. MurC, MurD, MurE, MurF, MraY, MnaA and MurG proteins that required for the biosynthesis of cytoplasmic precursor (lipid II) of peptidoglycan showed high sequence coverage (> 90 %) and intermediate (> 50 %) sequence similarity to *Mycoplasma* proteins (Figure 5A). The same result, with slightly lower percentage of sequence coverage and similarity, was observed for the presence of homologies of penicillin binding proteins in Mycoplasma (Figure 5 B). Similarly, the cell wall biosynthetic proteins of *E. coli*, except LcdA, have homologies in *Mycoplasma species* with high percentage of sequence coverage (> 90 %) and intermediate sequence similarity (50 %) (Figures 6A and B). LdcA of *E. coli* is a LD-carboxypeptidase that functionally similar to LdcB of *B. subtilis*. This could support our phylogenetic analysis that *Mycoplasma* evolved from Gram-positive bacteria because a homologous protein to LdcB is present in *Mycoplasma species* (Figure 5B). The conservation of the Gram-positive and Gram- negative peptidoglycan biosynthetic proteins (Figure 5A and B; Figure 6A and B) by *Mycoplasmas* belong to the fact that the basic mechanisms of peptidoglycan biosynthesis is almost identical in all bacteria (Figure 1 and Tables 1 and 2). Moreover, D-alanine is an essential amino acid for eubacterial peptidoglycan synthesis [19]. The alanine racemase (Alr) and D-alanine-D-alanine ligase (Ddl) are two essential enzymes for peptidoglycan biosynthesis and both are targets of D-cycloserine [38,39]. Both Alr and Ddl showed homologes in *Mycoplasma* Figures 5A and 6A). Thus, the above finding confirms that *Mycoplasma* species had evolved from eubacteria, which possess peptidoglycan in their cell wall.

The different percentage of protein sequence similarity between *B. subtilis* and *Mycoplasma* (Figure 5), and *E. coli* and *Mycoplasma* (Figure 6) arise from high rate of gene mutation in *Mycoplasma* [37]. This rate of mutation is possible because *Mycoplasma* lost the genes that play roles in DNA repair mechanism [40]. It was previously assumed that the cell wall biosynthetic genes are lost in *Mycoplasma* that is why the genome of such microorganism is small [37,41]. However, the high percentage of protein sequence coverage in our data reveal that the cell wall related genes still exist on the *Mycoplasma* genome, but they are mutated and/or truncated (Figures 5 and 6). It is interesting to investigate whether the cell wall related

genes are expressed in *Mycoplasma* and/or metabolically functional? A study previously reported the absence of all the high affinity penicillin binding proteins and DD-carboxypeptidase in isolated membrane of three species of *Mycoplasma* [42]. This indicates that; although our data suggested the presence of the cell wall biosynthetic genes in *Mycoplasma*, the genes are either not expressed or metabolically inactive. Another study was recently able to reverse the L-form of *B. subtilis* to walled cells in a genetically manipulated strain [14]. This process is not feasible in *Mycoplasma* because all the genes required for cell wall biosynthesis are seemed to be mutated and/or truncated (Figures 5 and 6).

In addition, teichoic acids are important secondary polymers in the cell wall of Gram-positive bacteria [6]. The absence of wall teichoic acid and lipoteichoic acid individually resulted in growth and shape defects in *B. subtilis* [27,43]. However, the lack of both types of teichoic acids together is lethal in the same bacterium [44]. Searching for the presence or absence of teichoic acids biosynthetic enzymes in *Mycoplasma* is logic to further investigate the Gram-positive ancestress of *Mycoplasma*. So, homology sequence searching was carried out for the teichoic acids biosynthetic proteins of *B. subtilis* and lipopolysaccharide biosynthetic proteins of *E. coli*. It was found that *Mycoplasma*s possess homologs of teichoic acids biosynthetic proteins at high percent of sequence coverage (> 90 % and intermediate (> 50 %) sequence similarity (Figure 5C). However, the majority of lipopolysaccharide biosynthetic proteins (LpxB, LpxC, LpxH, LpxK, KdtA, LpxL, LpxM and WaaL) of *E. coli* do not have protein counterparts in *Mycoplasma* (Figure 6C). These results interestingly and supportively indicate that *Mycoplasma* was initially a Gram-positive eubacteria, whose cell wall contains teichoic acids.

CONCLUSION

To conclude, the phylogenetic tree based on 16S rRNA alignment and the presence of homologs to peptidoglycan and teichoic acid biosynthetic proteins indicate that *Mycoplasmas* are evolved from Gram-positive bacteria. It also shown that the cell wall related genes are not lost in *Mycoplasma* genome, but they are mutated and/or truncated. Finally, biochemical investigations should be done on the putative cell wall related genes in *Mycoplasma*.

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