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# Characterization of *Acholeplasma laidlawii ftsZ* Gene and Its Gene Product

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**The *ftsZ* gene was found among representatives of all bacterial groups. FtsZ protein is an essential component of cell division ring. Contraction of this cytoskeleton-like ring is believed to be the universal way of bacterial division. *Acholeplasma laidlawii* possesses all features of the minimal mycoplasma cell and some traits of cell-wall bacteria and seems to be a promising object for study of basic principles of the bacterial division process. We cloned an *A. laidlawii* chromosomal fragment containing *ftsZ* gene and two flanking *orf* which also were identified. *A. laidlawii* FtsZ protein has been determined with polyclonal antibodies raised in rabbit. It was demonstrated that *ftsZ* gene of *A. laidlawii* could be expressed in *E. coli* cells. We also revealed that *A. laidlawii* FtsZ had a low similarity to proteins of *Mycoplasma genitalium* and *M. pneumoniae*. The comparison of FtsZ structures may be used for investigation of bacterial phylogenetic relations.** 

***Key Words: ftsZ* gene; *Mycoplasma; Acholeplasma;* gene expression.**

*Acholeplasma laidlawii* belongs to mycoplasmas (class *Mollicutes*), which are the smallest and cell-wall free prokaryotic organisms. Mycoplasmas used to be considered a *Clostridial* branch of Gram positive eubacteria and supposed to lose a large part of genomic material and metabolic pathways during their evolution (1). About 15 genes and corresponding proteins are responsible for division process in *E. coli*, but only 3 genes (*ftsH,Y,Z*) of cell division group were found in the completely sequenced genomes of *Mycoplasma genitalium* and *M. pneumoniae* (2, 3). Functions of *ftsH* and *ftsY* gene products in bacterial proliferation are uncertain, whereas FtsZ plays a pivotal role in cell division (4).

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Polymerization of FtsZ into cytokinetic ring at predivision site is considered the earliest event of the prokaryotic cell division process. The FtsZ protein possesses GTPase activity (5), and *in vitro* it forms filaments similar to those of tubulin (6). FtsZ is widely conservative and was found in all bacterial groups. The FtsZ proteins have conservative N-terminal part, GTP-binding motive and variable, hydrophilic C-terminal region (7). Overproduction of FtsZ in *E. coli* cells results in transgression of cell division. High level expression of *Bacillus subtilis* or *M. pulmonis ftsZ* genes in *E. coli* inhibited its cell division, leading to filamentation (8, 9). In bacterial cells, FtsZ forms a scaffold of contractile ring and is located at the leading edge of the invaginating septum during the division process (10). The other cell-wall bacterial proteins responsible for the site division formation and constriction of cytokinetic ring in mycoplasmas are unknown. This observation suggest that FtsZ plays a basic role in the mycoplasma division process, and its characterization is necessary to understand fundamental principles of the eubacterial cell proliferation. *A. laidlawii,* one of the most common mycoplasmas in nature, possesses almost all features of *Mollicutes* and special traits of cell-wall bacteria. Acholeplasmas are able to synthesize sterols in contrast to other *Mollicutes* (11, 12). *A. laidlawii* uses the universal genetic code, whereas mycoplasmas are used to change UGA codon to Trp and TA(A/G) to stop codon (13). The possibility of the *A. laidlawii* gene expression from their own promoters in *E. coli* cells was confirmed experimentally (14).

The *A. laidlawii ftsZ* gene was revealed previously (9). The goal of this work was cloning of the *A. laidlawii ftsZ* complete chromosomal copy, characterization of its gene product, and study of the possibility of its expression in *E. coli*. Besides, a comparative analysis of translated FtsZ sequences of *A. laidlawii* and other microorganisms was carried out. Antibodies to conservative epitops of *A. laidlawii* FtsZ obtained in this work might be used for further studies of morphological aspects of the cell division process in *A. laidlawii*

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and other mycoplasmas by immunobloting and immunoelectron microscopy.

## MATERIALS AND METHODS

*Bacterial strains and plasmids.* The *E. coli* strains used as general cloning hosts were DH5$\alpha$ and JM109. Bacteria were grown in LB media supplemented with the appropriate antibiotics (ampicillin at 100 $\mu$g/ml and chloramphenicol at 25 $\mu$g/ml). *A. laidlawii* PG8, *M. capricolum* ATCC 27343, *M. fermentans* ATCC 19989, *M. pneumoniae* FH, and *M. gallisepticum* S6 (Cell Culture Collection of the Institute of Cytology of the Russian Academy of Sciences) were cultivated in a modified PPLO broth (15). Vectors exploited were pUC18, pBluescriptKS+/−, pBeloBAC11, and also expression vectors pGEX3X and pQE42.

*Amplification.* DNA from *A. laidlawii* cells was extracted by the phenol method. Arbitrary primers approximately corresponded to two highly conserved regions (130–150 and 630–650 bp) of the *B. subtilis ftsZ* gene (8). The final primers structure was as follows: 5′-AAT-AC(A/T)-GAT-GC(A/T)-CAA-GC-3′ and 5′-TCC-G(G/C)A-C(A/G)T-C(A/G/T)G-C(A/G)A-AGT-C-3′. Amplification was carried out with *Taq* polymerase (Fermentas, Litva) in a PROGENE termocycler (Techne, GB). *A. laidlawii* DNA was used as a template, and PCR was performed at 35 cycles, each of 1 min 93°C, 1 min 42°C, 1 min 72°C. The amplified fragment of expected length 520 bp was incubated with Klenov DNA polymerase and cloned into SmaI site of pUC18 vector. The resulting plasmid was called pNA17.

*Cloning of ftsZ gene.* To find fragments containing *ftsZ* gene, *A. laidlawii* DNA was hydrolyzed with different restriction enzymes and analyzed by Southern hybridization. The insert of pNA17 (amplification product) was labeled with an ECL kit (Amersham) and used as a probe. HindIII fragment of 2.4 kb was isolated from agarose gel, purified with Qiagene kit and ligated with pBeloBAC11. The *E. coli* DH5$\alpha$ strain was used for transformation. Positive transformants were identified by colony hybridization. One of the recombinant plasmids was selected and called pBB1.

*DNA sequencing.* The XhoI-HindIII fragment, 1.7 kb long, from insert of pBB1 was subcloned into pBluescript KS+/−, and the resulting plasmid was called pBLK25. Double-stranded DNA of pBLK25 and pBB1 was sequenced by the dideoxynucleotide method with T7 Sequenase II from Amersham. Additional primers were synthesized and used to sequence any remained gaps and the complementary strands.

*Expression and purification of FtsZ fusion polypeptides.* The insert of pNA17 was subcloned into expression vector pGEX3X using BamHI and EcoRI sites to get recombinant pGEX-17. To obtain a fusion protein for generating antiserum, JM109(pGEX-17) was grown to log phase and was induced by addition of 10 mM IPTG for 3 h (16). The expressed protein (45 kDa) which contained glutathion S-transferase (GST) peptide (26.5 kDa) and FtsZ peptide was insoluble at low ionic strength and was isolated in a form of inclusion bodies. *E. coli* cells were disintegrated by ultra-sonication at 22 kGz, and inclusion bodies were collected at 10000 rpm. The pellet was dissolved in guanidine-HCL, 6 M, and the solution was clarified at 20000 rpm (see Fig. 3A). Supernatant was dialyzed 20 h at +4°C against $H_2O$. During dialysis the protein coagulated. It was collected by centrifugation, solubilized with 2% SDS in a sample buffer and additionally purified by preparative electrophoresis in a tube with polyacrylamide gel (17). This sample of the protein was used to raise antiserum.

Another fusion protein was obtained as a result of recloning of the 1.7 kb XhoI-HindIII fragment from pBLK25 into the expression vector pQE42. In *E. coli* JM109 cells transformed with the plasmid obtained (pQF55), a fusion protein of 55 kDa was expressed. It also accumulates in the cells in a form of inclusion bodies that were solubilized in a sample buffer with detergent (see Fig. 2A). The protein consists of DHFR (26.0 kDa) and FtsZ peptide. This 55 kDa fusion protein was used for affinity purification of polyclonal antibodies.

*Polyclonal antibodies, raising and purification.* After several injections of the purified fusion GST-FtsZ protein into rabbit, polyclonal antiserum was obtained by the method of Ivanov and Fel (18). IgG fraction was separated from the antiserum by precipitation with saturated solution of $(NH_4)_2SO_4$, pH 7.1. Antibodies reacting with GST were removed from IgG fraction by incubation with GST-glutathione-agarose. The next step of the antiserum purification was carried out with DHFR-FtsZ fusion protein by the method of Smith and Fisher (19). After SDS-PAGE electrophoreses the inclusion bodies protein fraction was transferred to nitrocellulose membrane Hybond C. The membrane was stained with Ponceau S (ICN, USA), its part caring major 55 kDa fusion protein was cut off and used for the next step of PAbs purification. The fragment of membrane was blocked with 1 × PBS containing BSA (100 mg/ml), 0.5% Tween 20 during 1 h, then incubated overnight with IgG fraction and washed in PBS-Tw (1 × PBS, 0.5% Tween 20) 3 times, 10 min each. Antibodies were eluted from the membrane with 0.2 M glycine, pH 2.8, and 1 mM EGTA solution. These purified monospecific antibodies (PAbs) were used in Western blot experiments.

*Immunoblotting.* Proteins were separated by 12% SDS-PAGE electrophoresis (20) and transferred to nitrocellulose Hybond C. The membrane was blocked with 1 × PBS containing BSA (100 mg/ml), 0.5% Tween 20 for 1 h, incubated with PAbs for 2 h and washed in PBS-Tw (1 × PBS, 0.5% Tween 20) 3 times, 10 min each. Proteins were detected with ECL-Western kit (Amersham).

## RESULTS AND DISCUSSION

For the *A. laidlawii ftsZ* gene identification, a PCR approach was used. Degenerated primers were synthesized after *ftsZ* sequence comparison of 7 eubacteria (*B. subtilis, E. coli, Micrococcus luteus, Streptomyces coelicolor, Str. griseus, Ryizobium meliloti, Staphylococcus aureus*). In accordance with low GC% in mycoplasmal genomes, A or T nucleotides were used in the third positions of the codons. Four pairs of different primers were checked, which corresponded to two highly conserved regions of *ftsZ*. One pair of the primers yielded an expected PCR product, 520 bp long. The amplified fragment was cloned and pNA17 was obtained. Comparison of nucleotide sequence of this fragment revealed a significant similarity with other bacterial *ftsZ* genes (21). The fragment was used as a hybridization probe for search of *ftsZ* in other mycoplasma genomes. Negative result was obtained in these experiments. It has turned out to agree with our consequence results of comparison of the *A. laidlawii* amplified fragment with the corresponding *ftsZ* fragments of other mycoplasmas: as low similarity level as about 30% with *M. genitalium* and *M. pneumoniae* was detected.

*Cloning and sequencing of the ftsZ gene from A. laidlawii.* Southern hybridization using the 520 bp *ftsZ* fragment as a probe revealed the 2.4 kb HindIII *A. laidlawii* DNA fragment carrying *ftsZ*. The fragment of 2.4 kb was cloned into a low-copy-number vector pBeloBAC11, and recombined plasmid was called pBB1. Nucleotide sequence of the insert was obtained and

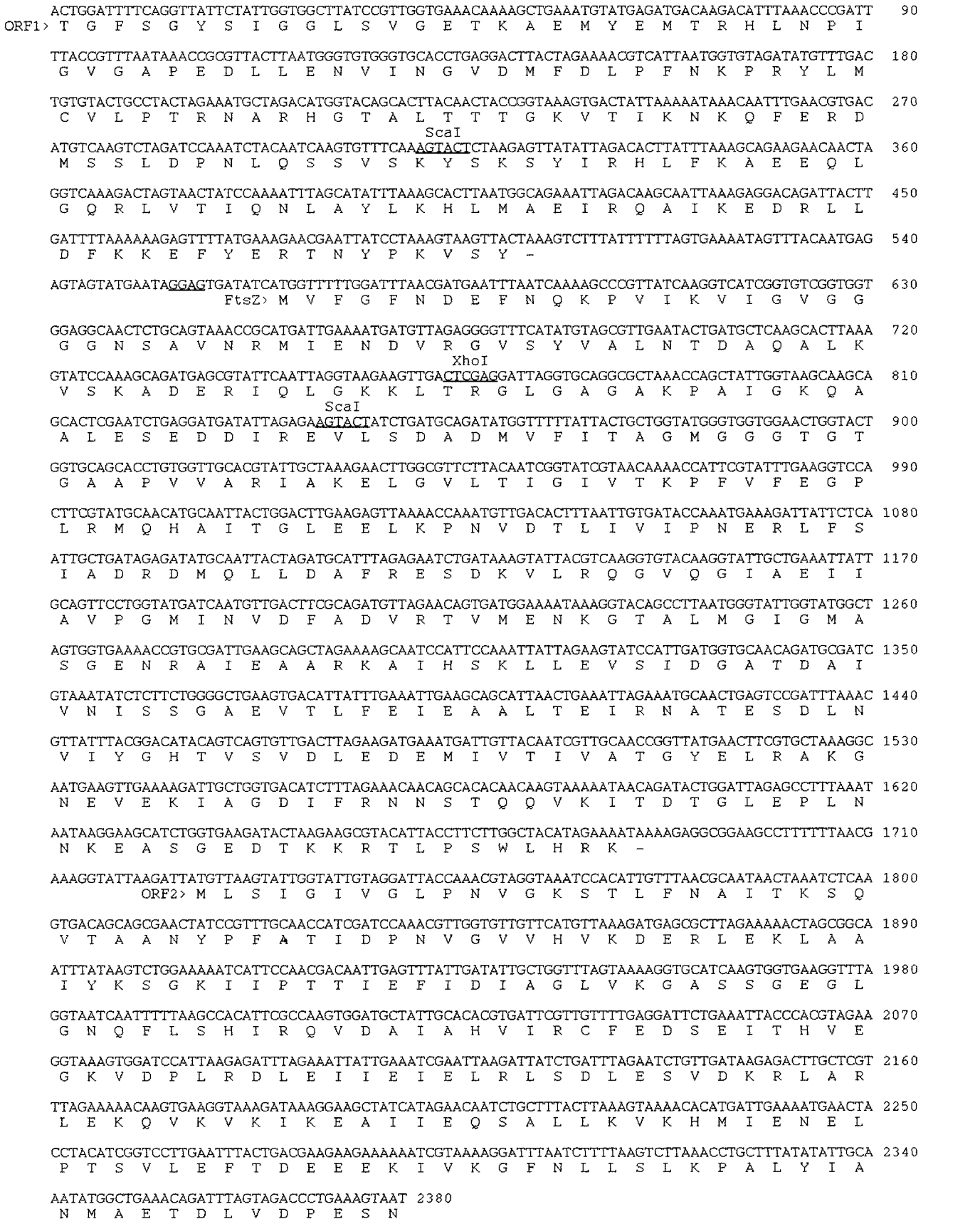


**FIG. 1.** Nucleotide sequence of *A. laidlawii ftsZ* gene and flanking regions. The predicted amino acid sequences are given in single-letter code. Putative RBS is underlined and relevant restriction sites (ScaI and XhoI) are indicated.

submitted to EMBL database (accession number AJ239100). Analysis of the insert sequence revealed a large open reading frame corresponding to the full-size copy of *A. laidlawii ftsZ* flanked with two other ORFs (Fig. 1). Recognizable RBS (AGGAG) presides the frame and situates 7 nucleotides upstream of the start codon. In many microorganisms the general structure *dcw* (division cell wall) cluster, including *ftsZ*, is strongly conservative (22). But among phylogenetically distant organisms there are some differences in the *dcw* cluster organization (23). ORF1 and ORF2 adjoining *ftsZ* in *A. laidlawii* chromosomal DNA were identified based on GenBank/EMBL searches (BLAST). ORF1 encodes an upstream polypeptide of 52% identity with eubacterial tRNA guanine transglycosylase and ORF2-downsteam polypeptide of 57% identity with a hypothetical 40.1 kDa GTP-binding protein of *E. coli*. Genes encoding similar proteins were also found in completely sequenced genomes of *M. genitalium* and *M. pneumoniae*. In the cell-wall bacteria and mycoplasmas studied these three genes found in *A. laidlawii* chromosome close to each other are positioned in different and distantly situated genome sites.

The nucleotide composition of *A. laidlawii ftsZ* corresponds to 61% A+T, i.e., somewhat lower as compared with *ftsZ* of all other mycoplasmas studied. The translated FtsZ protein sequence consists of 373 amino acids, and the predicted molecular weight of the polypeptide is 39.9 kDa, with an isoelectric point of 4.49. The protein is rich in Ala (10.1%), Gly (9.9%), Val (9.1%), and much poorer in Trp (0.2%), with the absence of Cys. The evolutionary conservative GTP-binding domain of *A. laidlawii* (GGGTGTG) completely coincides with the glycine-rich domain of cell-wall bacteria and differs by one amino acid from that of mycoplasmas.

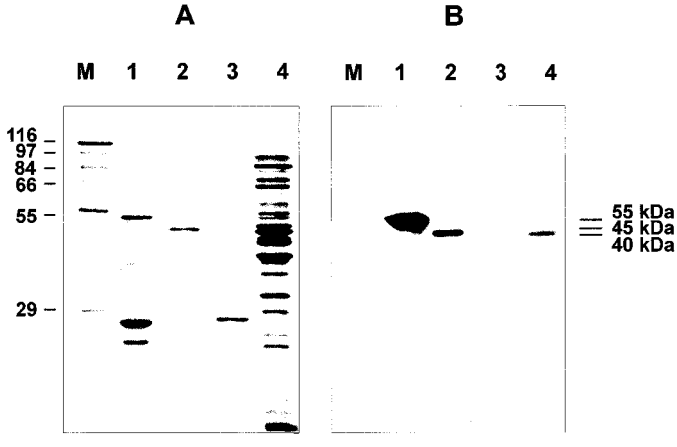


**FIG. 2.** SDS-PAGE electrophoresis (A) and Western analysis (B) of *A. laidlawii* FtsZ protein. (1) Fusion protein (55 kDa), DHFR (26 kDa) and *A. laidlawii* FtsZ peptide. (2) Fusion protein (45 kDa) composed of GST peptide and *A. laidlawii* FtsZ peptide. (3) GST protein (26.5 kDa). (4) *A. laidlawii* cells. M, molecular weight markers.

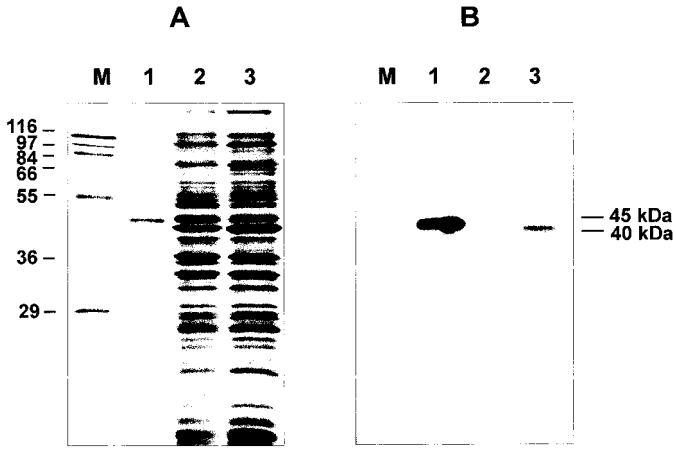


**FIG. 3.** Expression of *A. laidlawii ftsZ* in *E. coli* cells. Electrophoresis (A) and Western analysis (B). (1) Fusion protein (45 kDa) consisting of GST peptide and *A. laidlawii* FtsZ peptide. (2) *E. coli* cells transformed with pBeloBAC11. (3) *E. coli* cells transformed with pBB1. M, molecular weight marker.

*Detection of the A. laidlawii FtsZ protein.* In Western blot experiments with monospecific polyclonal antibodies (Pabs) a single *A. laidlawii* polypeptide, migrating in SDS-PAGE gel as 40 kDa protein, was identified (Fig. 2B, line 4). The molecular weight of this polypeptide corresponds to the calculated one (39.9 kDa) and is similar to the size of some eubacterial FtsZ proteins (7).

*Expression of the A. laidlawii ftsZ gene in E. coli cells.* It is known that overproduction of the *E. coli* FtsZ protein (10) as well as expression of heterologous *ftsZ* genes at the high level cause transgression of the *E. coli* cell division (8, 9, 24). Expression of the *A. laidlawii ftsZ* gene in *E. coli* cells transformed with the low-copy number plasmid (pBB1) was checked. No additional band was found in the Coomassie-stained gel (Fig. 3A, line 3), but it appeared as a result of the Western bloting experiment with the expression of *A. laidlawii ftsZ* in *E. coli* (pBB1) cells (Fig. 3B, line 3). The monospecific PAbs did not cross-react with the *E. coli* FtsZ protein. In this case the translation of *A. laidlawii* FtsZ in *E. coli* cells obviously took place from its own RBS. Our microscopic observations did not reveal any effect of the low level *A. laidlawii ftsZ* expression on *E. coli* cell morphology.

*Variability of FtsZ proteins in the evolution.* Amino acids alignment revealed a high identity level of *A. laidlawii* FtsZ with gram-positive cocci FtsZ (52% with *St. aureus*) and with other cell-wall eubacteria (55% with *B. subtilis* and 47% with *E. coli*). Conservation at N-terminus and the divergence toward the C-terminus were found as a result of the FtsZ protein structure

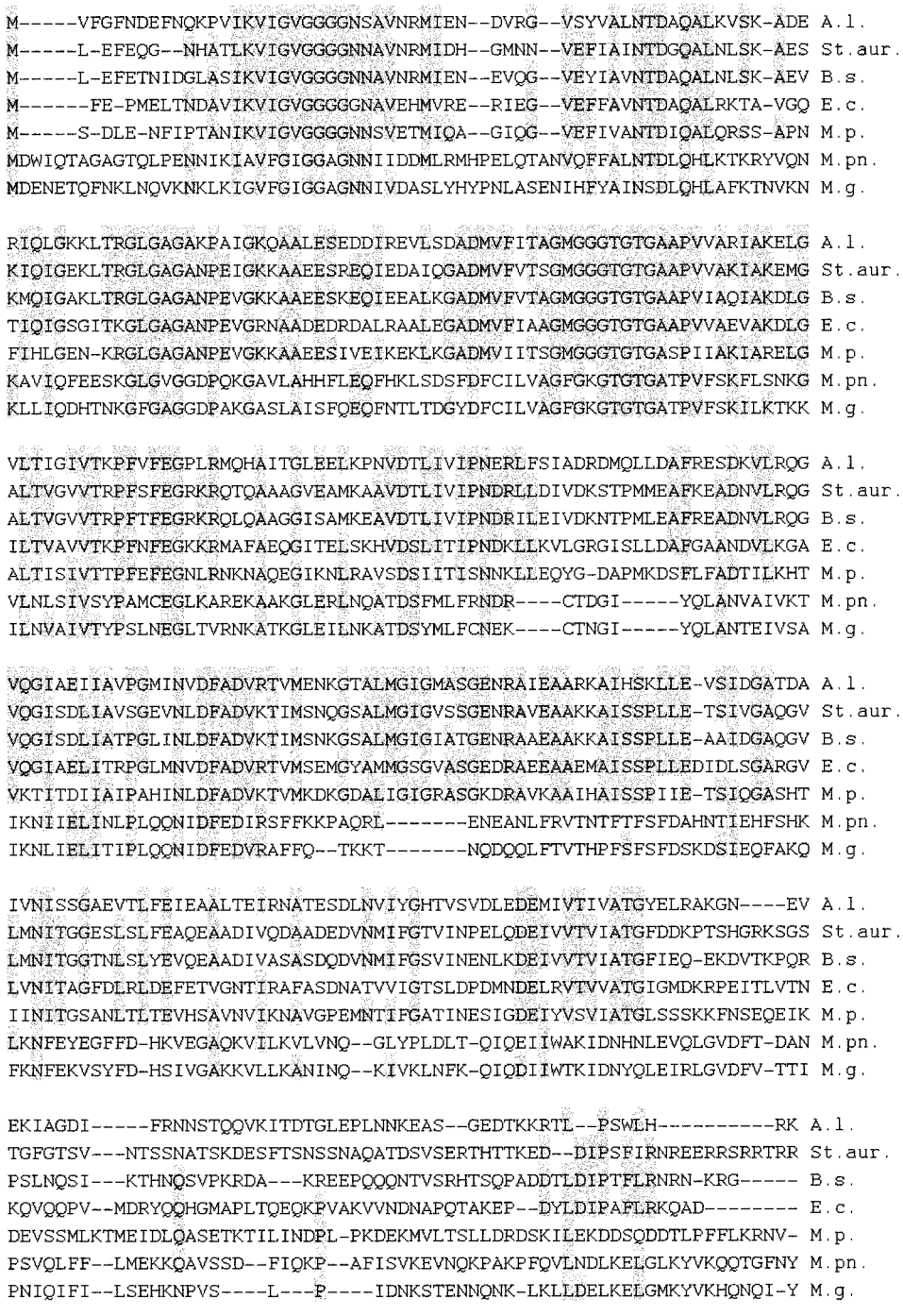


**FIG. 4.** Primary sequence alignment of FtsZ. Deduced amino acid sequences of *Acholeplasma laidlawii* (A.l.), *Staphylococcus aureus* (St. aur.), *Bacillus subtilis* (B.s.), *Escherichia coli* (E.c.), *Mycoplasma pulmonis* (M.p.), *Mycoplasma pneumoniae* (M.pn.), and *Mycoplasma genitalium* (M.g.).

comparison (Fig. 4). The analysis of FtsZ-FtsZ interactions in *E. coli* (25) indicated that the N-terminal conserved domain (between amino acids 100 and 326) is necessary for FtsZ polymerization to form a division ring. *A. laidlawii* FtsZ lacks the conserved part of the extreme carboxyl tail, as it is demonstrated as a result of the amino acid sequence alignment (Fig. 4). This conservative carboxyl end was found in FtsZ protein of cell-wall bacteria and seems to be important for the FtsZ and FtsA interaction in the process of septa formation (26). Removal of 57 amino acids from the C-terminus of *E. coli* FtsZ resulted in elimination of the interaction (25). In the complete sequenced genomes of *M. pneumoniae* and *M. genitalium*, *ftsA* was not found.

These data as well as the lack of the conservative region at FtsZ C-terminus of *A. laidlawii* and other mycoplasmas indicate the existence of another way of septa formation in mycoplasmas, probably caused by the absence of peptidoglycan cell wall. Among mycoplasma FtsZ proteins a comparatively high identity level was revealed between *A. laidlawii* and *M. pulmonis* (39%). The identity level of *A. laidlawii* FtsZ with proteins of *M. pneumoniae* and *M. genitalium* is 25% and 23%, correspondingly. Translated amino acid sequences were used for alignment of FtsZ due to the known difference in the genetic code among mycoplasmas, acholeplasmas, and other microorganisms (Fig. 4).

The differences in identity of FtsZ proteins of *A. laidlawii,* cell-wall eubacteria and *Mycoplasma spp.* are in agreement with the theory of the polyphyletic origin of mycoplasmas. Based on metabolism comparison data, H. Neimark and J. London (27) suggested that sterol-nonrequiring mycoplasmas evolved from gram-positive cocci. In result of comparison of nucleotide sequences the highest identity level was found between *ftsZ* of *A. laidlawii* and *Staphylococcus aureus* (65%). The maximal similarity was revealed between nucleotide sequences of the central region of *ftsZ* (67%).

All the above data taken together allow us to assume that in the structure of FtsZ there are both some conservative and some evolutionary variable regions. Mutations in a variable regions probably do not affect polypeptide conformation and function of the protein. Gene *ftsZ* is widespread, functionally important, and sufficiently extended: this gene was found in all bacterial groups and encodes the inevitable protein. So it fits the basic criteria for the sequences useful for phylogenetic purposes (1).

Comparison of the deduced FtsZ amino acid sequence of *A. laidlawii,* strain PG8 (this work), with the published earlier (9) 100-amino acid-long, corresponding fragment of *A. laidlawii* (strain not indicated) revealed 3 deletions and one amino acid change. The inter-strain differences in *ftsZ* gene structures were also found recently in comparing two strains of *M. fermentans* (28). Studies of bacterium *Wolbachia* spp. (*Rickettsia,* reproductive parasite of *Arthropodes*), based on *ftsZ* sequence, revealed 38 new strains of this bacteria, and a new, more detailed phylogenetic tree of the wolbachia was constructed (29).

The comparative analysis of *ftsZ* sequences can be used as a sensitive method for investigation of short-range prokaryote evolution relationships.

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