Full Length Research Paper

Genetic and morphologic variations during L-form conversion in *Mycobacterium tuberculosis*

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The loss of cell wall in bacteria may result in uncoordinated propagation and occurrence of highly pleomorphic forms with new biologic characteristics, known as L-forms. The ability of *Mycobacterium tuberculosis* to survive and reproduce as L-forms is assumed to be an adaptive reaction towards unfavourable factors. In this respect, stress induced L-form conversion could provide an interesting opportunity to study morphologic and genetic changes in mycobacteria. Stressful starvation followed by passages in semisolid medium was used for induction and selective isolation of *M. tuberculosis* L-forms from three strains (S-18, S-43 and CRBIP7.10). Colonial growth and morphologic ultrastructure, as well as genetic changes in *16S rRNA* gene and mycobacteria specific direct repeats (DRs) were evaluated during L-form conversion. Electron microscopy showed exhibition of specific ultrastructure of cell wall deficient bacteria, which were consistent with the formation of typical "fried eggs" shaped L-form colonies. Based on sequence analysis of *16S rRNA* gene fragment, we found variable sites in L-form of strain S-18. Spoligotyping analysis demonstrated appearance of genetic variations in DRs locus of all L-forms. In conclusion, we suggested that appearance of genetic changes in *M. tuberculosis* could be driven and accelerated by L-form transformation. Both morphologic and genetic variations found in L-forms of *M. tuberculosis*, can be regarded as a manifestation of adaptive plasticity and capability of mycobacteria to survive under unfavorable environmental factors.

Key words: *Mycobacterium tuberculosis*, L-form conversion, genetic plasticity, survival.

INTRODUCTION

Mycobacterium tuberculosis has adapted its genetic structure and regulatory systems specifically to infect humans, as well as to respond to environmental stresses and thus to survive under harsh conditions for a prolonged period (Cole et al., 1998). The ability of *M. tuberculosis* to survive and reproduce as L-forms is assumed as one of the adaptive reactions of mycobacteria towards unfavourable factors- absence of nutrients, pH of the medium, damaging physical and chemical factors, inhibitors of cell wall biosynthesis such as antibiotics and host immune factors such as lysozyme, compliment, antibodies, etc. (Beran et al., 2006; Mattman, 2001; Markova, 2012).

Bacterial L-form conversion, that is, existence without rigid walls, is a unique phenomenon well described by many authors (Mattman, 2001; Domingue, 1982; Allan et al., 2009; Prozorovski et al., 1981). However, studies concerning gene analysis of bacterial L-forms have largely been restricted because of difficulties in their isolation and cultivation.

The mechanisms of mycobacterial responses to particular types of stress have been studied and discussed in detail by many authors (Gupta and Chatterji, 2005; Ramage et al., 2009; Li and He, 2012) but L-form conversion of mycobacteria adopted as stress response is a little

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studied phenomenon. In this respect, L-form conversion of *M. tuberculosis* provides an interesting opportunity to study the driving forces of biological diversity. Drawing parallels between phenotypic and genetic processes can provide new insights into genetic heterogeneity and the unusual nature of *M. tuberculosis* L-form variants.

The aim of the present study was to investigate genetic changes in *M. tuberculosis* during L-form conversion based on the sequence analysis of *16S rRNA* gene fragment. Spoligotyping analysis was also used to demonstrate appearance of genetic variations in *M. tuberculosis* L-forms.

MATERIALS AND METHODS

Bacterial strains

Two clinical strains (S-18 and S-43) of *Mycobacterium tuberculosis* isolated from sputum samples of newly diagnosed tuberculosis patients at Sofia State Hospital for Tuberculosis Treatment in Bulgaria and *Mycobacterium tuberculosis* CRBIP7.10 (Beijing clade, isolated from a human lymph node) purchased from Institute Pasteur, Paris were used.

Production of *M. tuberculosis* L-forms

For the experiments, bacterial biomass was harvested from cultures of three *M. tuberculosis* strains which were grown on Löwenstein-Jensen medium (LJ) at 37°C for 42 days. 2 ml of sterile saline (solution of sodium chloride; 0.9% w/v) was inoculated with bacterial biomass in amount of 0.2 g and was incubated at 37°C for 30 days (starvation phase). After incubation, the cell suspension was centrifugated at 4000 rpm for 20 min, the supernatant was removed and the sediment was re-suspended in 500 µl saline and the entire volume was plated on Middlebrook 7H9 semisolid medium. The semisolid medium was prepared from Middlebrook 7H9 broth (Difco) which was solidified with 0.8% (w/v) Bacto Agar (Difco). Middlebrook 7H9 ADC enrichment was not added. The Petri dishes were enveloped with parafilm and incubated at 37°C for one week followed by five serial passages of starved mycobacteria in the same medium at weekly intervals. The passages were performed by the following technique: The surface of Petri dish was flooded with 1500 µl of Middlebrook 7H9 broth and was gently scraped with spreader. Then, 500 µl of the washing liquid was aspirated, transferred and dispensed on the surface of new medium which was incubated at 37°C for a week.

The isolated subcultures at the fifth passage of cultivation in Middlebrook 7H9 semisolid medium were completely transformed L-forms, which gave rise to typical L-form colonies as examined by electron microscopy. The colonies were observed by light microscope (Carl Zeiss Axiostar Plus).

Electron microscopy

Observations of *M. tuberculosis* L-forms isolated at the fifth passage of cultivation in Middlebrook 7H9 semisolid medium, were performed by electron microscopy. Bacterial L-form cultures were fixed with 4% (v/v) glutaraldehyde in 0.1 M cacodylate buffer with 4.5% w/v sucrose, pH 7.2 and post-fixed in 1% (w/v) osmium tetroxide in the same buffer at room temperature for 2 h and dehydrated in serial ascending ethanol concentrations. For scanning electron microscopy (SEM), specimens were placed on membrane filters with pore size diameter of 0.22 µm (Millichrom, Isopore), covered with 15 - 20 Å gold, visualized and photographed with scanning

electron microscope Phillips SCM 515. For transmission electron microscopy (TEM), after dehydration in ethanol and propylene-oxide series, cell pellets were embedded in epoxy resin Epon-Araldite (Serva, Heidelberg, Germany). Resin blocks were polymerised at 56°C for 48 h. Ultrathin cell sections were made with crystal glass knives on a Reichert-Jung Ultracut Microtome and were stained with 5% (w/v) uranyl acetate in 70% (v/v) methanol and 0.4% (w/v) lead citrate. Observations were made with Zeiss 10C electron microscope at 60 kV.

Sequence analysis of *16S rRNA*

Colonies of mycobacterial L-forms isolated at the fifth passage of cultivation in Middlebrook 7H9 semisolid medium, were picked up for genetic testing. Chromosomal DNA was isolated as described by Van Embden et al. (1993). PCR was used to amplify segment of *16S rRNA* gene with primers: g2R-F 5'GAGAATTCGTGCTTAACACATGCAAGTCG3' and rM582R-R 5'ATGGATCCGTGAGATTTCACGAACAACGC3'. PCR protocol has been described by Devulder et al. (2005). The amplification of 16Sr RNA gene fragment was done with a 50µl reaction mixture containing 10x PCR buffer (Tris.Cl; KCl; $(NH_4)_2SO_4$; 15mM $MgCl_2$, pH 8,7, Qiagen) at final concentration of 1x; 1,25 U HotStarTaq DNA polymerase (Qiagen); 0,2 mM of each dNTPs (dNTP Mix, Qiagen); 1 mM $MgCl_2$ (Qiagen), 0,2 µM of each primer; 5 µl template DNA and PCR-grated water up to 50 µl. The thermal profile involved initial denaturation for 10 min at 90°C, 40 cycles of denaturation for 25 s at 94°C, annealing for 30 s at 60°C and extension for 45 s at 72°C followed by 1 cycle at 72°C for 10 min. The PCR product (5 µL) was examined and the amount estimated visually on a 3% agarose gel. All PCR products were purified and subjected for sequencing by Macrogen Company. Sequence analysis was performed by BioEdit Software.

Spoligotyping

M. tuberculosis complex specific spoligotyping method was performed as described by Kamerbeek et al. (1997). Spoligotyping (spacer oligonucleotide typing technique) was used to analyze variation in mycobacteria specific direct repeats (DRs), especially in determining the absence or presence of 43 different spacers.

RESULTS

The completely transformed L-form variants derived from three strains of *M. tuberculosis* (S-18, S-43 and CRBIP7.10) were obtained at the fifth passage of cultivation in Middlebrook 7H9 semisolid medium. They gave rise to colonies with typical L-forms "fried eggs" shape (Figure 1b and c), which are quite different from colonies of classical tubercle bacilli (Figure1a). Electron microscopy showed typical L-form cell population morphology and ultrastructure (Figure 1e and f). Polymorphic cells of different sizes and shapes (large spherical bodies, oval or round coccoid cells, short coccobacilli and rods) were observed with SEM (Figure 1e). As shown in Figure 1f, TEM-micrograph of L-form subculture clearly demonstrates L-bodies of varied size, shape and electron density which were bound only by a single unit membrane. Cytoplasmic elements (ribosomal material) in L-bodies were diffuse scattered or condensed at the periphery
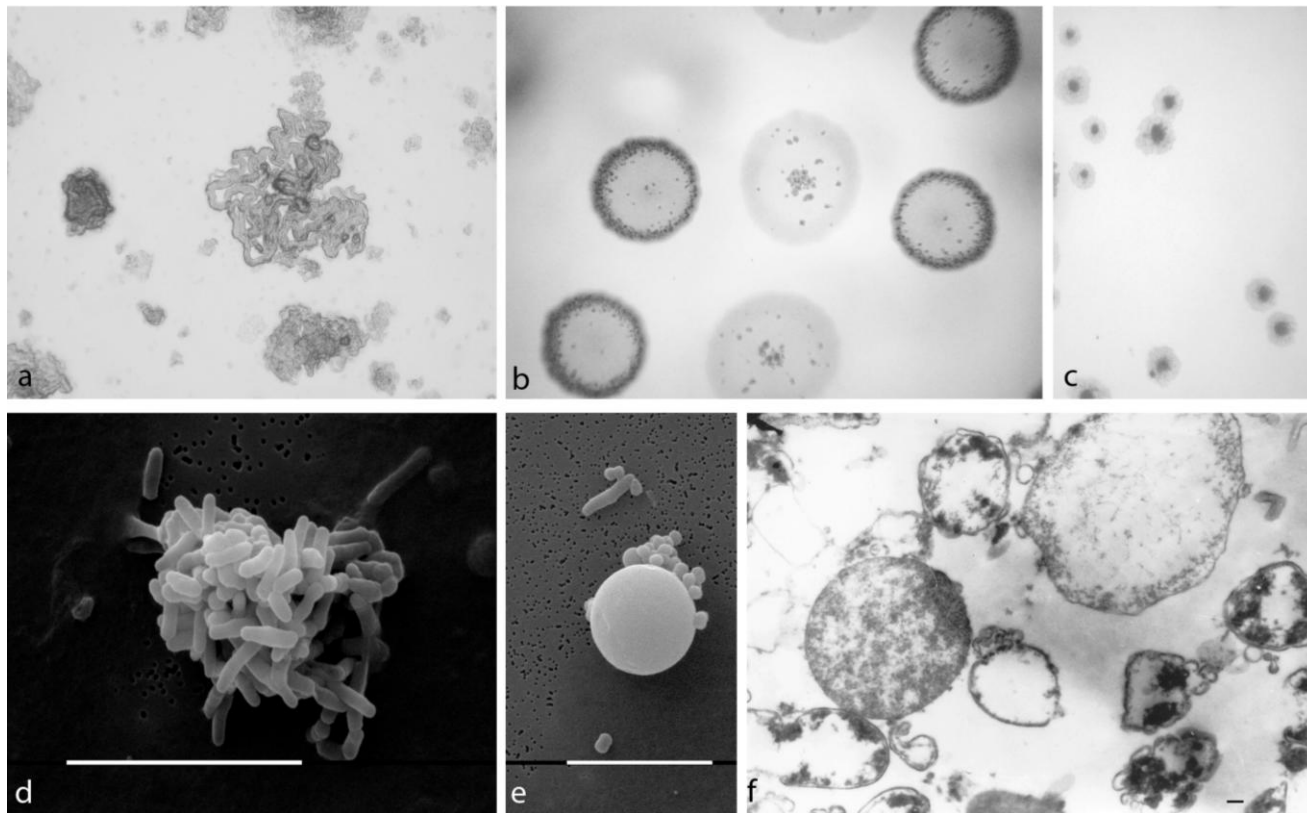
Figure 1. Colonial and ultrastuctural morphology of *M. tuberculosis* L-forms: a. control colonies of *M. tuberculosis,* strain CRBIP7.10; Magnification: 200x; b. L-form colonies of *M. tuberculosis* CRBIP7.10 strain; Magnification: 200x; c. typical "fried eggs" shaped L-form colonies of *M. tuberculosis* S-18 strain; Magnification: 100x; d. SEM of control cells from *M. tuberculosis* S-18 strain, Bar = 10 µm; e. SEM of large spherical body, small ovoid cells, short coccobacilli and rod from *M. tuberculosis* S-18 L-form subculture, Bar = 10 µm; f. TEM- micrograph of *M. tuberculosis* S-18 L-form subculture - L-bodies of varied size, shape and electron density which were bound only by a single unit membrane, bar = 0.2 µm.

of cells along the membrane. In L-bodies which were more loosely filled with cytoplasmic elements, nucleoid strands were well visible. Empty vesicular forms were seen as well.

In order to trace changes in target *16S rRNA* gene fragment during L-form conversion, PCR samples of the three studied strains (S-18, S-43 and CRBIP7.10) and their L-forms were subjected to gene sequence analysis. All sequences were aligned to referent strain of *M. tuberculosis* $H_{37}Rv$ by using Software and blasted in NCBI data base. As seen in Figure 2, sequencing of *16S rRNA* gene fragment in completely transformed L-forms of S-43 and CRBIP7.10 strains showed 100% sequence homology in comparison with the referent *M. tuberculosis* $H_{37}Rv$ strain, respectively no changes were found during L-form transformation of these strains. Although the *16S rRNA* gene is assumed to be highly conserved, some allelic diversity was uncovered in the L-form subculture of S-18 strain. The changes in this strain resulted in 18 base substitutions and 2 deletions at positions 211-212 and 459-470.

The completely transformed L-forms isolated at the fifth passage of cultivation in Middlebrook 7H9 semisolid me-

dium and their parental *M. tuberculosis* strains (S-18, S-43 and CRBIP7.10) were studied by spoligotyping (Figure 3). It is interesting to note that spoligotyping pat-terns of L-form subcultures showed occurrence of certain poly-morphisms, that is, the deletion or insertion of spacer signals, ascompared to the spoligoprofile of the parental *M. tuberculosis* strains. In L-forms of *M. tuberculosis* S-18 was found deletion of spacer 39, while in L-forms of *M. tuberculosis* S-43 were detected deletions of spacers 3,5,7, 21, 22, 39, 42 and insertions of 33, 34, 35, 36. The most remarkable finding was that L-form of *M. tuberculosis* CRBIP7.10 (Beijing clade) drastically differed from classi-cal parental strain by insertion of 30 additional spacers (spacers 1-8; 17-20;22-34).

DISCUSSION

The loss of cell wall in bacteria has been found to result in uncoordinated propagation and occurrence of highly pleomorphic forms with new biologic characteristics, known as L-forms (Mattman, 2001; Domingue, 1982; Allan et al., 2009; Prozorovski et al., 1981). The ability of *M. tuberculosis* to survive and reproduce as L-forms is assumed to be

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                                       10        20        30        40        50
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     AATACCGGATAGGACCACGGGATGCATGTCTTGTGGTGGAAAGCGCTTTA
M.tub S18 L-form               ...........T.......A.GC.....C.................--...T
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                       60        70        80        90       100
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     GCGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGC
M.tub S18 L-form               .............GA...................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      110       120       130       140       150
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     CTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACT
M.tub S18 L-form               ..................................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      160       170       180       190       200
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     GGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
M.tub S18 L-form               ..................................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      210       220       230       240       250
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     TGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGG
M.tub S18 L-form               ..................................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      210       220       230       240       250
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     TGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGG
M.tub S18 L-form               ..................................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      260       270       280       290       300
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     CCTTCGGGTTGTAAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTC
M.tub S18 L-form               .........................G..GG.......CG.AA.--------
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      310       320       330       340       350
                               ....|....|....|....|....|....|....|....|....|....|
M.tub H37Rv control strain     GGATTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGC
M.tub S18 L-form               ----........CC..C.................................
M.tub S43 L-form               ..................................................
M.tub CRBIP7.10 L-form         ..................................................

                                      360       370       380
                               ....|....|....|....|....|....|.
M.tub H37Rv control strain     CGCGGTAATACGTAGGGTGCGAGCGTTGTCC
M.tub S18 L-form               ...............................
M.tub S43 L-form               ...............................
M.tub CRBIP7.10 L-form         ...............................
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Figure 2. 16S rRNA partial gene sequencing in completely transformed L-forms of *M.tuberculosis* strains (S-18, S-43 and CRBIP7.10). Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by dots.

an adaptive reaction towards unfavoura-ble factors (Brenan et al., 2006; Mattman, 2001; Markova, 2012). In this respect, stress induced L-form conversion could provide an interesting opportunity to study pheno-typic and genetic changes, driving adaptive plasticity of mycobacteria.
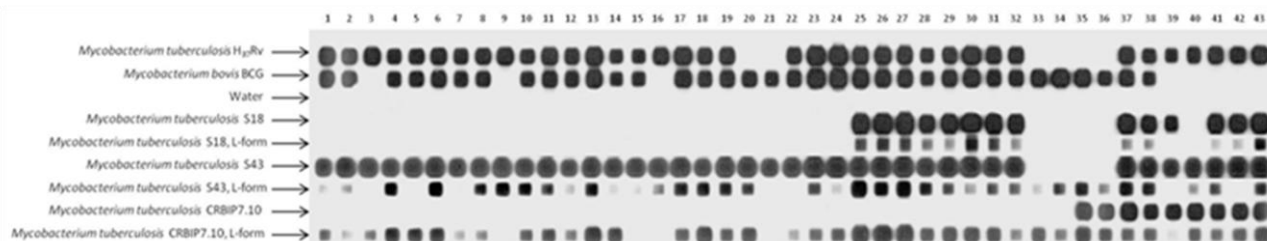
Figure 3. Spoligotyping pattern of control *M. tuberculosis* strains (S-18, S-43 and CRBIP7.10) and their completely transformed L-forms.

The current study addresses the question whether L-form conversion can induce mutations and hence can promote biodiversity. L-forms were induced after nutrient starvation stress and were selected through transfers in semisolid medium. Under constructed experimental conditions, tubercle bacilli converted completely to cell wall deficient forms with typical morphological and growth characteristics (L-forms). The process, by which genetic diversity was generated during L-form transformation, was studied by isolation and analysis of subcultures after five passages in Middlebrook 7H9 semisolid medium. Based on sequence analysis of *16S rRNA* gene we found that variable sites (18 base substitutions and 2 deletions) were identified in L-form of S-18 strain. It is known that mutations are events arising from growth dependent processes. We supposed that the specific for production of L-forms serial subculture transfers had a selective pressure driving morphological transformations and generation of adaptive mutations in the current model system. In fact, some of the mutations arise as a result of spontaneous events during replication, but factors such as uncoordinated and irregular division (characteristic for propagation and biology of L-forms) should not be underestimated, as they could also contribute to the appearance of genetic changes.

Given that 16SrRNA gene sequence analysis of mycobacteria is assumed as the reference standard for accurate identification of species, it should be noted that L-forms of S-43 and CRBIP7.10 strains showed sequences identical to referent *M. tuberculosis* $H_{37}Rv$ strain or 100% sequence homology. According to some authors, the absolute rate of change in the *16S rRNA* gene sequence is not known, but it can be used to mark evolutionary distance and relatedness of organisms (Clarridge, 2004; Harmsen and Karch, 2004; Pace 1997; Thorne et al., 1998).

Spoligotyping analysis additionally demonstrated appearance of genetic variations in L-forms of *M. tuberculosis* strains. We analyzed three pairs of parental and L-form strains by using spoligotyping with standard 43 spacers and we found variations in their spoligoprofiles. The most drastic change was observed in the spoligoprofile of CRBIP7.10 strain (Beijing spoligotype) after L-form transformation including insertion of 30 additional spacers. Van Embden et al. (2000) tried to explain spoligoprofile

changes in pairs of isogenic strains. The authors suggest that genetic rearrangements can appear in direct repeat region of *M. tuberculosis*, namely a single genetic event comprising either the insertion or the deletion of a single, discrete direct variant repeat or a set of contiguous direct variant repeats. The variations have been also explained by insertion or deletion of IS*6110* in the direct repeat region. Norris (2011) admitted that recombination with noncoding DNA or with silent genes, as well as DNA insertion might occur easily in population of cell wall deficient bacteria.

A question of considerable interest is how far mutations and DNA rearrangements are associated with a cycle of morphological transformations and unusual division processes characteristic for L-form populations. It is known that L-form cells divide without peptidoglycan structures, respectively chromosome segregation does not follow normal physical and spatial rules like in normal bacterial cells (Allan et al., 2009). So, in this respect, chromosome segregation and cell division defects are likely to result from irregular propagation of L-forms.

In conclusion, we suggest that mutations can take place in *M. tuberculosis* under conditions of L-form transformation. L-form conversion can be regarded as a stress induced process that in turn is reflected in mutations and genetic diversity in *M. tuberculosis* population enhancing its adaptability to environmental factors.

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