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Full Length Article

L-form transformation phenomenon in *Mycobacterium tuberculosis* associated with drug tolerance to ethambutol

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ABSTRACT

Objective/background: Cell wall-deficient bacterial forms (L-forms) may occur along with resistance to factors that trigger their appearance. It is of interest to study the relationship between the L-form transformation of Mycobacterium tuberculosis and the exhibition of drug tolerance to ethambutol (EMB), an inhibitor of cell wall synthesis. Methods: L-form variant was produced from a sensitive EMB strain of M. tuberculosis through a cryogenic stress treatment protocol and was subsequently cultivated in Middlebrook 7H9 semisolid medium, containing EMB in a minimal inhibitory concentration of 2 mg/L. Susceptibility to EMB of the parental strain and its L-form variant was evaluated phenotypically and using polymerase chain reaction-restriction fragment length polymorphism assay targeting a mutation in the *embB306* gene fragment. *Results:* In contrast to the sensitivity to EMB of the parental strain, its L-form variant showed phenotypic resistance to high concentrations of EMB (16 mg/L), but the mutation in *embB306* was not found. Electron microscopy observation of the L-form variant showed a heterogenic population of bacteria, with different degrees of cell wall deficiency, as well as cells of protoplastic type without cell walls. Of special interest were the observed capsule-like structures around the L-form cells and the biofilm-like matrix produced by the L-form population. *Conclusion:* We suggest that the expression of phenotypic resistance to EMB in *M. tuberculosis* can be associated with alterations or loss of cell walls in L-form bacteria, respectively, which results in a lack of a specific target for EMB action. In addition, production of capsule-like structures and biofilm matrix by L-forms could contribute to their resistance and survival in the presence of antibacterial agents.

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Introduction

Drug tolerance or phenotypic resistance of bacteria to antibiotics without occurrence of alterations in their genome is not well understood [1,2]. This phenomenon can be observed under some circumstances and can be realized by different mechanisms linked to the physiological state of bacteria [3]. Wallis et al. [1] have reported isolates of *Mycobacterium tuberculosis*, which became drug tolerant after prolonged persistence in vivo, but the bacilli remained genetically drug susceptible. Noninherited resistance is usually related to specific processes, such as growth in biofilms, a stationary growth phase, or persistence [4]. In a previous study, we have recognized the phenomenon of antimicrobial tolerance occurring in cell wall deficient forms (L-forms) of *M. tuberculosis* during experimental infection in rats [5]. Because L-forms may occur along with resistance to factors that trigger their appearance [6], it was of interest to study the relationship between the formation of *M. tuberculosis* L-forms in vitro and the appearance of drug tolerance to ethambutol (EMB), which acts through the inhibition of cell wall synthesis. EMB targets the mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalactan and lipoarabinomannan biosynthesis [7].

Materials and methods

A clinical strain of *M. tuberculosis*, isolated from the sputum of a newly diagnosed tuberculosis patient (without history of previous tuberculosis treatment) at the Sofia State Hospital for Tuberculosis Treatment in Bulgaria, was used for the production of L-form variants. The strain was grown on Löwenstein–Jensen medium (LJ) medium at 37 °C for 28 days. It was determined as sensitive by the standard absolute concentration method of Canetti et al. [8]. Cryogenic stress treatment protocol was used for the induction of L-form variants, as described in our previous study [9]. In brief, 1 mL of sterile saline was inoculated with 0.2-g biomass, harvested from fresh, well-developed LJ culture of *M. tuberculosis*, and the suspension was frozen at −20 °C for 72 h. Subsequently, the cell suspension was thawed and centrifuged at 2862 g for 20 min. The supernatant was removed and the sediment was resuspended in 500-μL saline and this volume was then plated on Middlebrook 7H9 semisolid medium (Difco, Sofia, Bulgaria), containing EMB in a minimal inhibitory concentration of 2 mg/L. The semisolid medium was prepared from Middlebrook 7H9 broth which was solidified with 0.8% (w/v) Bacto Agar (Difco, Sofia, Bulgaria).

Phenotypic drug susceptibility testing of the parental strain and its L-form variant was performed using the absolute concentration method of Canetti et al. [8], using different drug concentrations of EMB (1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, and 16 μg/mL) on LJ medium. Interpretation of the results was done after 42 days of incubation at 37 °C. The minimal inhibitory concentration of EMB for the parental strain was 2 mg/L, while for the L-form variant it was 16 mg/L.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay targeting the mutation in the *embB306* gene fragment was used. The PCR protocol was designed by Mokrousov et al. [10]. A primer pair EmbF (5′ATTCGGCTTCCTGCTCTGG3′) and EmbR (5′GAACCAGCGGAAATAGTTGG3′) was used to amplify a shorter *embB* fragment-spanning codon 306 under the following PCR conditions: initial denaturation at 95 °C for 2 min, followed by 40

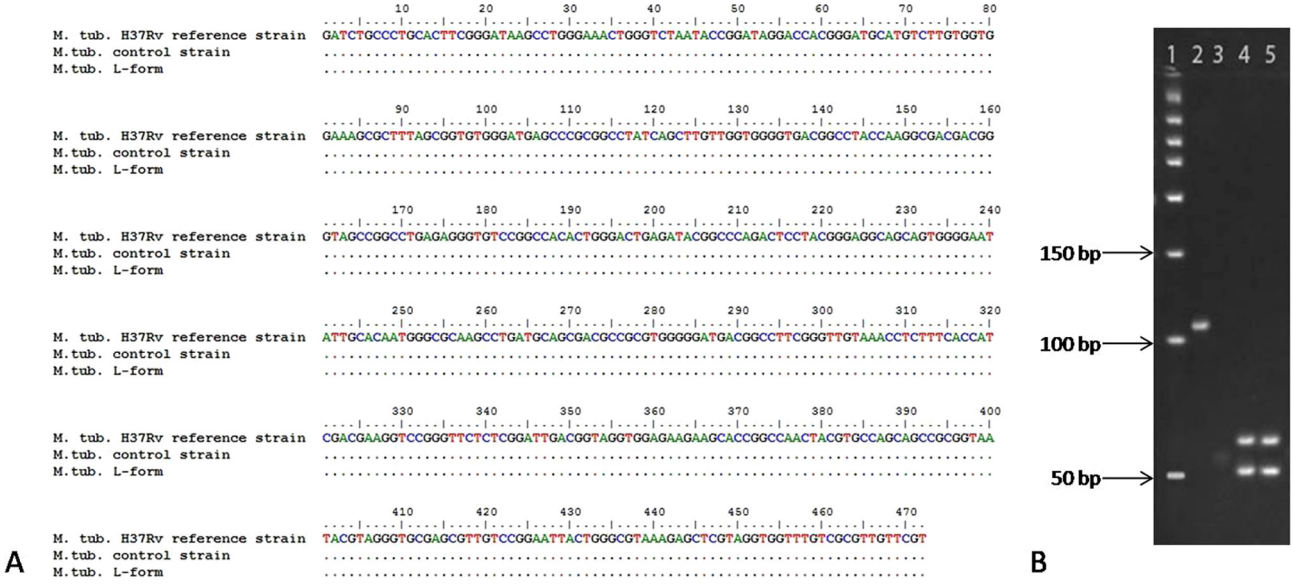

Fig. 1 – Genetic analysis of parental and L-form variant of *Mycobacterium tuberculosis*. (A) *16S ribosomal RNA* partial gene sequencing. Identical nucleotides are indicated by dots; (B) polymerase chain reaction-restriction fragment length polymorphism analysis of the amplified 118-bp *embB306* fragment with *HaeIII*. Lanes: 1, DNA ladder 50 bp; 2, *M. tuberculosis* control strain with *embB* codon 306 mutated in the third base (ATG → ATH); 3, water; 4, *M. tuberculosis* wild-type strain; 5, *M. tuberculosis* L-form variant. Note. M. tub = *Mycobacterium tuberculosis*.

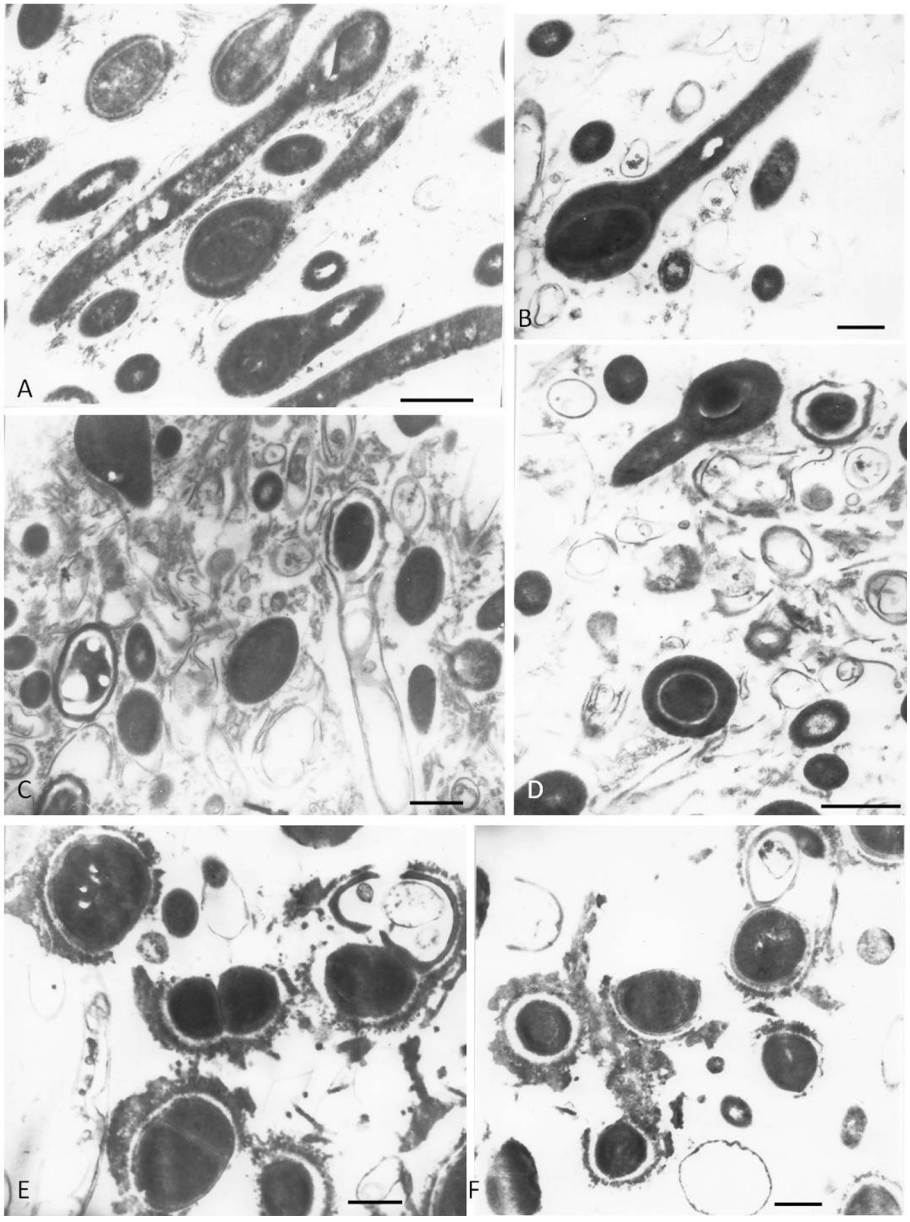

Fig. 2 – Transmission electron microscopy of *Mycobacterium tuberculosis* L-forms cultivated in Middlebrook 7H9 semisolid medium containing ethambutol in a concentration of 2 mg/L. Polymorphism of abnormally dividing cells with different size, shape, and substructure. (A, B, D) Elongated cells with elongated cells with electron dense swelling at the poles; (C, E, F) coccoid cells of protoplast type without cell walls and others with defective cell walls, numerous electron dense granules, vesicles, and membranous reminds of detached cell walls, as well as capsule like substance around the cells. Bar = 0.5 μm.

cycles of 94 °C for 1 min, 60 °C for 40 s, and 72 °C for 20 s, with a final elongation step at 72 °C for 2 min. The amplified 118-bp fragment was subjected to cleavage by *Hae*III (Fermentas) restriction endonuclease, and the digests obtained were separated in 3% agarose gels. The *Hae*III site can be altered only if a mutation occurs in the *embB306* third base (G). In such a mutant allele (ATG 224 ATH) the PCR fragment will remain uncut by *Hae*III (118 bp; Fig. 1B, Lane 2), whereas a two-band profile (50 bp and 68 bp) will be observed in *embB306* wild-type or differently mutated strains (Fig. 1B, Lanes 4 and 5).

16S ribosomal RNA (*rRNA*). Gene segment sequencing was performed as follows: chromosomal DNA was isolated as described by Van Embden et al. [11]. PCR was used to amplify the segment of the *16S rRNA* gene with primers: g2R-F 5′GA GAATTCGTGCTTAACACATGCAAGTCG3′ and rM582R-R 5′ATG GATCCGTGAGATTTCACGAACAACGC3′. PCR protocol has been described by Devulder et al. [12]. The amplification of the *16S rRNA* gene fragment was done using a 50-μL reaction mixture containing 10× PCR buffer (Tris.Cl, KCl, $(NH_4)_2SO4$, 15 mM $MgCl_2$, pH 8,7; Qiagen, SGP Bio Dynamics Ltd., Sofia 1202, Bulgaria) at a final concentration of 1×; 1.25 U

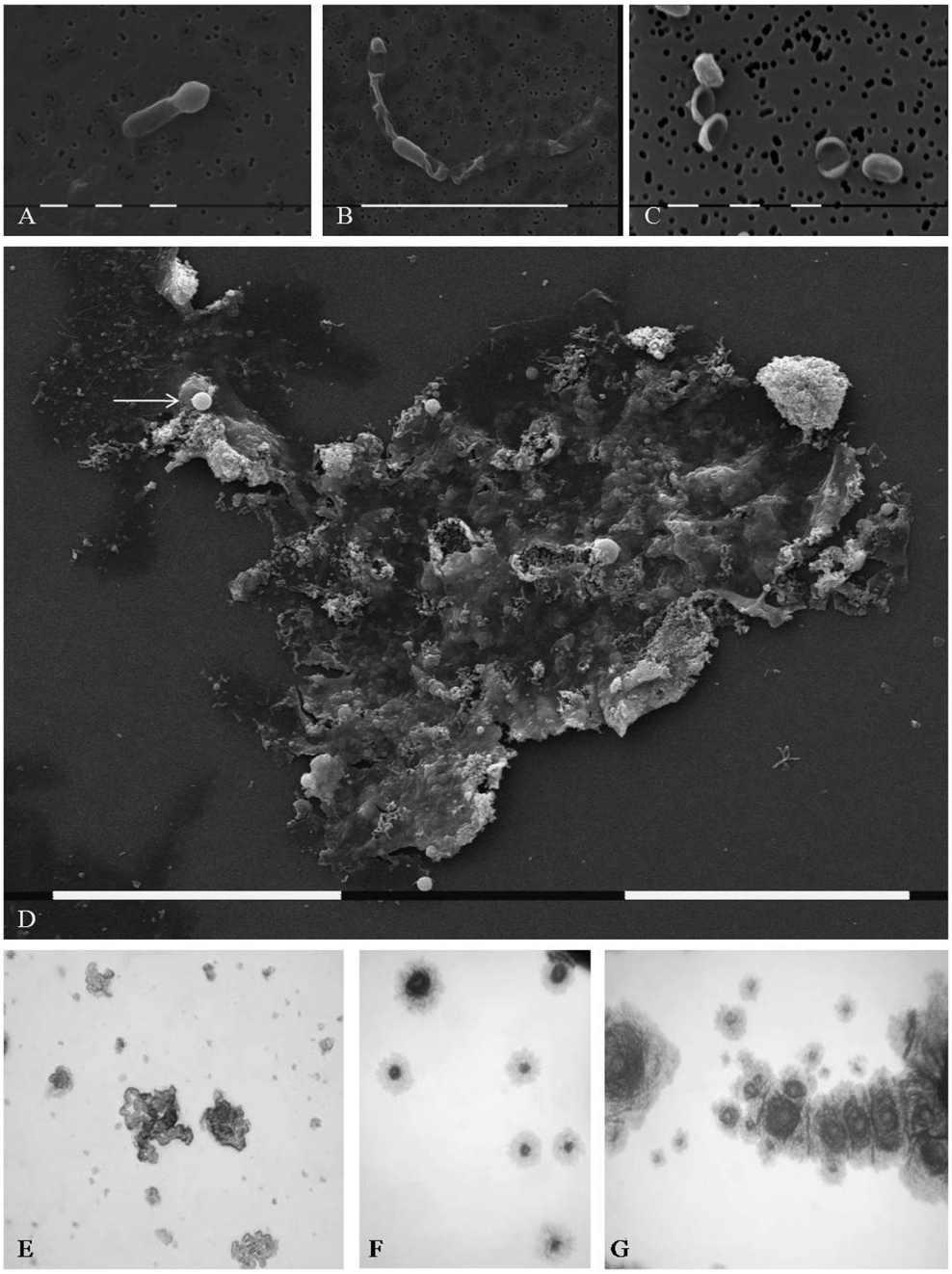

Fig. 3 – Scanning electron microscopy and colonial morphology of *Mycobacterium tuberculosis* L-forms cultivated in Middlebrook 7H9 semisolid medium containing ethambutol in a concentration of 2 mg/L. (A–C) Polymorphic cells of different shape and size; (D) large spherical L-body (arrow) and microcolonies of coccoids located within biofilm-like material. Bar = 1 μm and 10 μm; (E) control colonies of *M. tuberculosis*; (F, G) typical "fried egg" shaped colonies formed by L-forms. Magnification: ×200.

HotStarTaq DNA polymerase (Qiagen, SGP Bio Dynamics Ltd., Sofia 1202, Bulgaria); 0.2 mM of each deoxynucleotide (Deoxynucleotide Mix, Qiagen, SGP Bio Dynamics Ltd., Sofia 1202, Bulgaria); 1 mM $MgCl_2$ (Qiagen, SGP Bio Dynamics Ltd., Sofia 1202, Bulgaria), 0.2 μM of each primer; 5-μL template DNA, and PCR grade water up to 50 μL. The thermal profile involved initial denaturation for 15 min at 95 °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 one 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, Amsterdam-Zuidoost, Netherlands. Sequence analysis was performed using BioEdit Software.

Observation of *M. tuberculosis* L-form variant was performed using 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 postfixed 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, specimens were placed on membrane filters with a pore size diameter of 0.22 μm (Millichrom, Isopore), covered with 15–20 Å gold, and visualized and photographed using a 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 polymerized at 56 °C for 48 h. Ultrathin cell sections were made using 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 using a Zeiss 10C electron microscope at 60 kV.

Results

Because L-forms are a heterogenic and constantly fluctuating population of polymorphic bacteria, several mechanisms of resistance to antibacterials can take place. In the present study, we utilized experimentally generated L-forms of *M. tuberculosis* to elucidate the mechanisms of their resistance to EMB. *M. tuberculosis* L-forms were produced in vitro using cryogenic stress treatment, as described in our previous paper [9]. After cryogenic stress treatment, mycobacteria were exposed to EMB by cultivation in EMB, containing Middlebrook 7H9 semisolid medium, in a concentration of 2 mg/L. Susceptibility to EMB of both the parental strain and its L-form culture was evaluated phenotypically by the absolute concentration method of Canetti et al. [8], using different drug concentrations of EMB (1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L, and 16 mg/L) on LJ medium and by PCR-RFLP assay targeting the mutation in the *embB306* gene fragment. It is interesting to point out that in contrast to the parental strain which was sensitive to EMB (2 mg/L), its L-forms showed resistance to high concentrations of EMB (16 mg/L). Notably, the mutation of *embB306* was not found in the L-form variant (Fig. 1B). In addition, both parental strain and L-forms do not differ genetically by amplifying and sequencing the *16S rRNA* gene segment (Fig. 1A). The lack of genetic changes in the L-form variant at the tested specific sites leads us to seek an explanation of phenotypic resistance to EMB through the exhibition of nonspecific defense mechanisms and formation of morphological structures distinctive for L-forms. As demonstrated in our previous papers [8,9], we observed here that under stress, mycobacteria can undergo drastic morphological and functional changes, leading to L-form conversion. TEM and scanning electron microscopy revealed that L-form culture consisted of highly pleomorphic and abnormally dividing cells of different size, shape, and internal ultrastructure (Figs. 2 and 3A–D). Bacteria with different degrees of cell wall deficiency, and cells of protoplastic type without cell walls, numerous electron dense granules, and vesicles were detected using TEM (Fig. 2). Debris of detached walls and amorphous material which extend around the cells as capsule-like substances were observed as well (Fig. 2C,D, E and F). As seen in Fig. 3D, spherical L-bodies, or microcolonies of coccoid L-form cells, were located within abundant biofilm-like matrixes. The polymorphic L-form bacteria gave rise to the formation of typical “fried egg” colonies (Fig. 3F and G).

Discussion

The L-form transformation could be considered as a phase state of bacteria, induced in response to unfavorable conditions, threatening the vital functions of cells. L-forms can survive unfavorable conditions much longer than the classic bacteria [13,14]. The stress environmental factors imply occurrence of alterations in the constitution of the bacterial cell wall, resulting from the deletion and faulty synthesis of wall components. Therefore, the cryogenic treatment used in our study resulted in the appearance of a highly pleomorphic population of cell wall defective cells. We suggest that the loss of cell wall, i.e., the loss of specific targets for EMB in L-forms of *M. tuberculosis*, may underlie the phenotypic drug tolerance to this drug. However, the insensitivity of the L-form variant to antimicrobials can be greatly assisted by additional structures, such as capsule-like substances and biofilm-like formations, enveloping the L-form population entirely. They can act as a barrier, which prevents the penetration of antibiotics.

The observed phenomenon of phenotypic drug tolerance to EMB raises additional questions about the ineffectiveness of antituberculosis therapy in cases when it is not possible to kill cell wall defective L-forms.

Conclusion

Phenotypic drug resistance without genetic mutations, also known as drug tolerance, can be realized by different mechanisms and can be attributed to the peculiar physiological states of bacteria occurring under certain circumstances. The observed phenomenon of antimicrobial tolerance to EMB in L-forms of *M. tuberculosis* suggests a new view on resistance mechanisms as regards the loss of specific drug targets and the production of barrier structures against drug penetration. The interpretation of drug tolerance exhibition, as a result of L-form transformation, is an important explanation which contributes to a better understanding of the reasons for therapeutic failures for mycobacteria, classified as susceptible.

Conflicts of interest

All authors have no conflicts of interest to declare.

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