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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

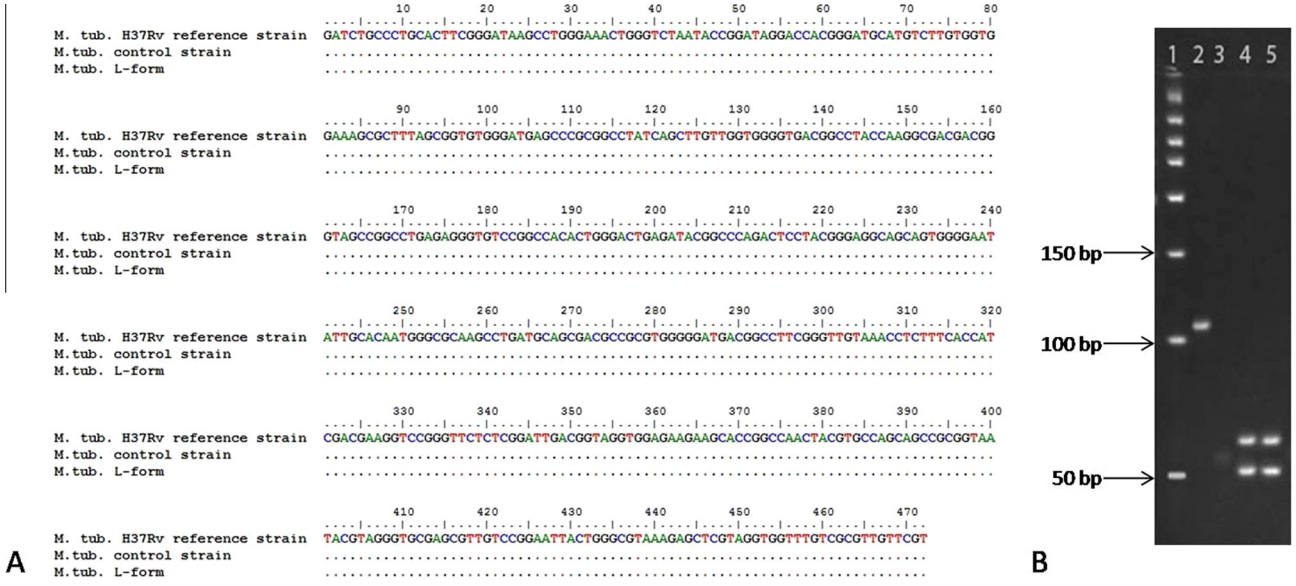

Fig. 1 – Genetic analysis of parental and L-form variant of *Mycobacterium tuberculosis*. (A) *16S ribosomal RNA* partial gene sequencing. Identical nucleotides are indicated by dots; (B) polymerase chain reaction-restriction fragment length polymorphism analysis of the amplified 118-bp *embB306* fragment with *Hae*III. 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*.

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