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Unique biological properties of *Mycobacterium tuberculosis* L-form variants: impact for survival under stress

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Summary. Bacteria can, under certain conditions, enter into a cell-less state known as L-form conversion. This phenomenon is universal, but also recognized with difficultly by microbiologists. The current study addresses several aspects concerning the ability of tubercle bacilli to use L-form conversion as a unique adaptive strategy to survive and reproduce under unfavorable conditions. Nutrient starvation of *M. tuberculosis* in vitro followed by passages in Middlebrook 7H9 semisolid medium was used for stress induction and the selective isolation of mycobacterial L-form variants. Light and electron microscopy images evidence the peculiar characteristics of mycobacterial L-forms. For example, mycobacterial L-forms were observed to lose their acid-fastness and change their morphology. In addition, wide morphological variability, the presence of large and elementary bodies, coccoids and small granular forms, as well as the appearance of unusual modes of irregular cell division were observed. Unlike classical tubercle bacilli, L-form variants grew and developed typical "fried-egg" colonies faster. L-forms were verified as *M. tuberculosis* by spoligotyping. The results provide insights into the nature of L-form phenomena in *M. tuberculosis* and link them to the mechanisms allowing mycobacterial survival under stress. [**Int Microbiol** 2012; 15(2):61-68]

Keywords: *Mycobacterium tuberculosis* · L-form conversion · bacterial survival · starvation stress

Introduction

The persistence of *Mycobacterium tuberculosis* in hosts is thought to underlie latent tuberculosis, but whether and how tuberculosis (TB) bacilli are able to survive under unfavorable conditions in vivo and to remain unrecognized by the immune system for extended periods of time remains unclear [4,14,17,27]. *Mycobacterium tuberculosis* has adapted its genetic structure and regulatory systems specifically to respond to environmental stresses. Studies of the expression of selected genes in *M. tuberculosis* in the Wayne model of dormancy have shown that, under hypoxic conditions, different tuberculosis genotypes are able to transcribe genes involved in DNA replication and cellular division [2].

Atypical, non-acid fast, cell-wall deficient forms of *M. tuberculosis* have been found in patient specimens [3,10,12,15,20]. Essentially, the ability of these bacteria to exist as populations of self-replicating forms despite defective or entirely missing cell walls (L-forms) is considered an adaptive strategy allowing the bacteria to survive and reproduce under unfavorable circumstances [1,5].

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Mycobacteria are unique among prokaryotes due to their cell wall structure, which contains tightly packed mycolic acids. These provide tuberculosis bacilli with efficient protection and a remarkable capacity to resist various exogenous stresses. The envelope of tuberculosis bacilli is a major determinant of exceptional impermeability that limits nutrient uptake and is responsible for the long doubling time and slow growth of mycobacteria. The mycobacterial cell wall appears to be a dynamic structure that can be remodeled during the growth or persistence of the bacterium in different environments [13]. However, under conditions that are unfavorable to mycobacteria, e.g., when they are exposed to host defense mechanisms, they may produce the above-noted cell-wall-deficient forms (L-forms) [16,17]. The important characteristics of bacterial L-forms are pleomorphism and a loss of rigidity, due to the lack of a murein layer. Cell-wall deficient mycobacteria are characterized by uncoordinated propagation, the appearance of highly pleomorphic forms, and a significantly changed bacillary physiology. Their spontaneous growth is assumed by some authors to be a natural phase of their life style [1,3,16,18].

Many questions concerning the nature of mycobacterial L-forms remain unanswered and deserve re-examination using modern molecular biology techniques [28]. The present study began as an attempt to establish an experimental model for studying the formation of *M. tuberculosis* L-form variants and their survival under stress starvation. Further aims included observation of the unusual morphological and growth characteristics of L-form mycobacteria, as well as the assessment of methods for their DNA-based identification.

Materials and methods

Bacterial strains. A clinical strain of *Mycobacterium tuberculosis* (S-14) isolated from sputumspecimens of newly diagnosed tuberculosis patients at Sofia State Hospital for Tuberculosis Treatment in Bulgaria and *M. tuberculosis* H_{37}Rv strain purchased from NBIMCC in Bulgaria were used. Both strains were grown on Löwenstein-Jensen medium (LJ) medium at 37 °C for 42 days before the experiments.

Experimental design. Nutrient starvation of *M. tuberculosis* strains for stress induction of L-form formation was performed as follows. Two ml of sterile saline was inoculated with 0.2 g of mycobacterial biomass harvested from *M. tuberculosis* strains grown on LJ medium. After incubation at 37 °C for 30 days without shaking, the cell suspension was centrifuged at 4000 rpm for 20 min. The supernatant was removed and the sediment was re-suspended in 500 µl saline followed by plating on semisolid medium in Petri dishes (94 × 15 mm). The semisolid medium (SsM) was prepared from Middlebrook 7H9 broth (Difco), which was solidified with 0.8 % (w/v) Bacto Agar (Difco). The Petri dishes were wrapped with Parafilm and incubated at 37 °C for one week.

Five serial passages in SsM at weekly intervals were then performed by the following technique: The surface of the Petri dish with SsM was flooded with 1500 µl of Middlebrook 7H9 broth and gently scraped with a spreader. Five hundred µl of the washing liquid was aspirated and then dispensed on the surface of new SsM, These plates were incubated at 37 ºC for one week, during which they were examined daily for macroscopic or microscopic evidence of growth. Ziehl-Neelsen (ZN)-stained preparations from cultures were observed weekly at each passage. In control experiments for the sterile performance of the technical procedures, plates were inoculated with sterile saline and subsequent transfers were done by the schema described above. Microscopic and macroscopic growth was not observed in control plates during all passages until the end of experiments. All experiments were repeated five times.

Electron microscopy. L-form variants of *M. tuberculosis* strains were observed at the fourth and fifth passages in SsM after starvation as described above. Bacterial 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, followed by dehydration in serial ascending ethanol concentrations. For scanning electron microscopy, specimens were placed on membrane filters with a pore size of 0.22 µm (Millichrom, Isopore), covered with 15–20 Å gold, and then visualized and photographed in a scanning electron microscope Phillips SCM 515. For transmission electron microscopy (TEM), after dehydration in an ethanol and propylene-oxide series, cell pellets were embedded in epoxy resin Epon-Araldite (Serva, Heidelberg, Germany). Resin blocks polymerized at 56 °C for 48 h. Ultrathin cell sections were cut with crystal glass knives on a Reichert-Jung Ultracut Microtome and stained with 5 % (w/v) uranyl acetate in 70 % (v/v) methanol, and 0.4 % (w/v) lead citrate. The samples were observed with a Zeiss 10C electron microscope at 60 kV.

DNA-based identification. Single colonies of mycobacterial L-form variants were picked for genetic testing at the fifth passage. Several precautions were taken to avoid contamination during the extraction procedure and in the PCR. The extraction, PCR, and post-PCR analyses were conducted in separate laminar boxes and rooms. Sterile aerosol protection filter tips were used to avoid cross-contamination. Two extraction blanks were always included in the same procedure and an additional PCR blank, containing no DNA template, was included in each PCR**.** Chromosomal DNA was isolated as described by Van Embden et al. [26].

Spoligotyping. The *M. tuberculosis* complex specific spoligotyping (spacer oligonucleotide typing) method was performed as described by Kamerbeek et al. [9]. Spoligotyping is used to analyze variations in mycobacteria-specific direct repeats (DRs), especially in determining the absence or presence of 43 different spacers.

Results

Production of mycobacterial L-form variants. The ability of *M. tuberculosis* to produce L-form variants was demonstrated in vitro using both clinical (S14) and referent (H_{37}Rv)

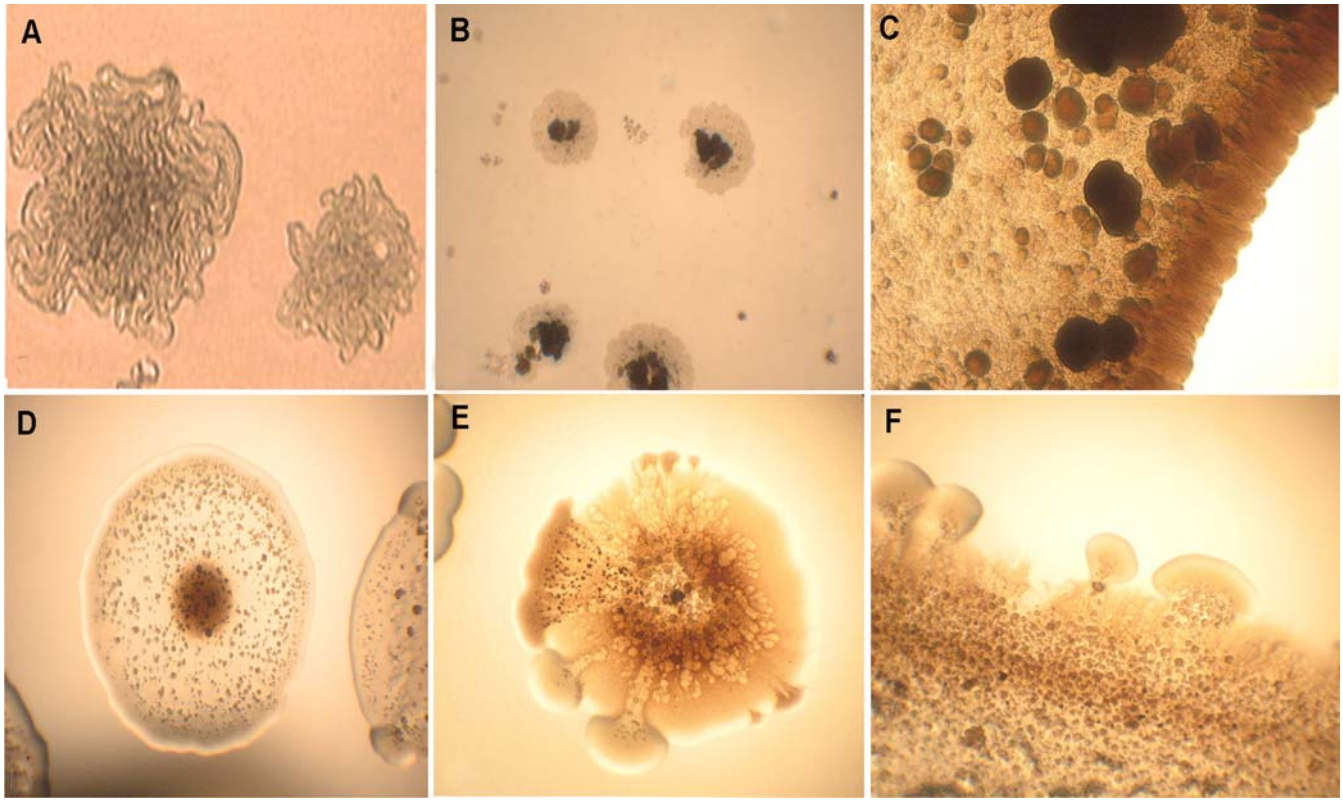

Fig. 1. Light microscopy of *Mycobacterium tuberculosis* colonies. (**A**) Control rough microcolony (S14 strain). (**B–F)** L-form colonies and growth of *M. tuberculosis*. (**B, C)** S14 strain; (**D, E, F)** H_{37}Rv strain. (**B, D, E**) Typical L-form colonies with a "fried-egg" appearance; (**C, F)** confluent L-type growth with granular appearance of the edges of the colonies. Magnifications: (**A, D, E**) 800×; (**B, C, F**) 200×.

strains. L-form transformation was induced by nutrient starvation stress and L-form variants were selected through transfers of stressed mycobacterial cultures in SsM at weekly intervals. L-form transformation was examined in ZN-stained smears every week at each passage. The first sign of the appearance of L-forms within mycobacterial population was the loss of acid-fastness. After starvation at the first transfer, the mycobacterial population consisted partly of acid-fast bacilli and partly of non-acid-fast coccobacilli. A progressive decrease in ZN positively stained mycobacteria was observed until the third passage, after which they were almost entirely absent. In contrast to classical tubercle bacilli, which typically appeared as characteristic aggregations (cording) of acid-fast rods in ZN-stained smears, mycobacterial L-forms showed marked polymorphism, including non-acid-fast rod, elongated, and coccoid forms of different size.

Growth characteristics of mycobacterial L-forms. At the fourth and fifth passages after starvation, the tested *M. tuberculosis* strains gave rise to typical L-form colonies with a "fried-egg" appearance (Fig. 1B, D, E). L-type growth consisted of many individual colonies of varied size and compactness, as well as abundant confluent growth with a granular appearance of the edges of the colonies (Fig. 1C, F). It should be pointed out that fully developed L-type colonies appeared between 36 and 48 h after plating, in contrast to control *M. tuberculosis* rough microcolonies, which formed on the same medium after a long incubation period of approximately 10 days (Fig. 1A).

Ultrastructure of mycobacterial L-form variants. A very clear and detailed resolution of L-form ultrastructure was achieved by electron microscopy. Samples for electron microscopic examinations were prepared from L-form growths at the fifth passage after starvation, when the mycobacterial populations consisted of non-acid-fast cells. Comparisons of the shape and surface morphology of control *M. tuberculosis* bacilli (Fig. 2A) with that of L-form variants through SEM examinations revealed polymorphic bacteria of variable shape and size within the L-form population (Fig. 2B–M). Short coccobacilli and long rods (Fig. 2B, C, I),

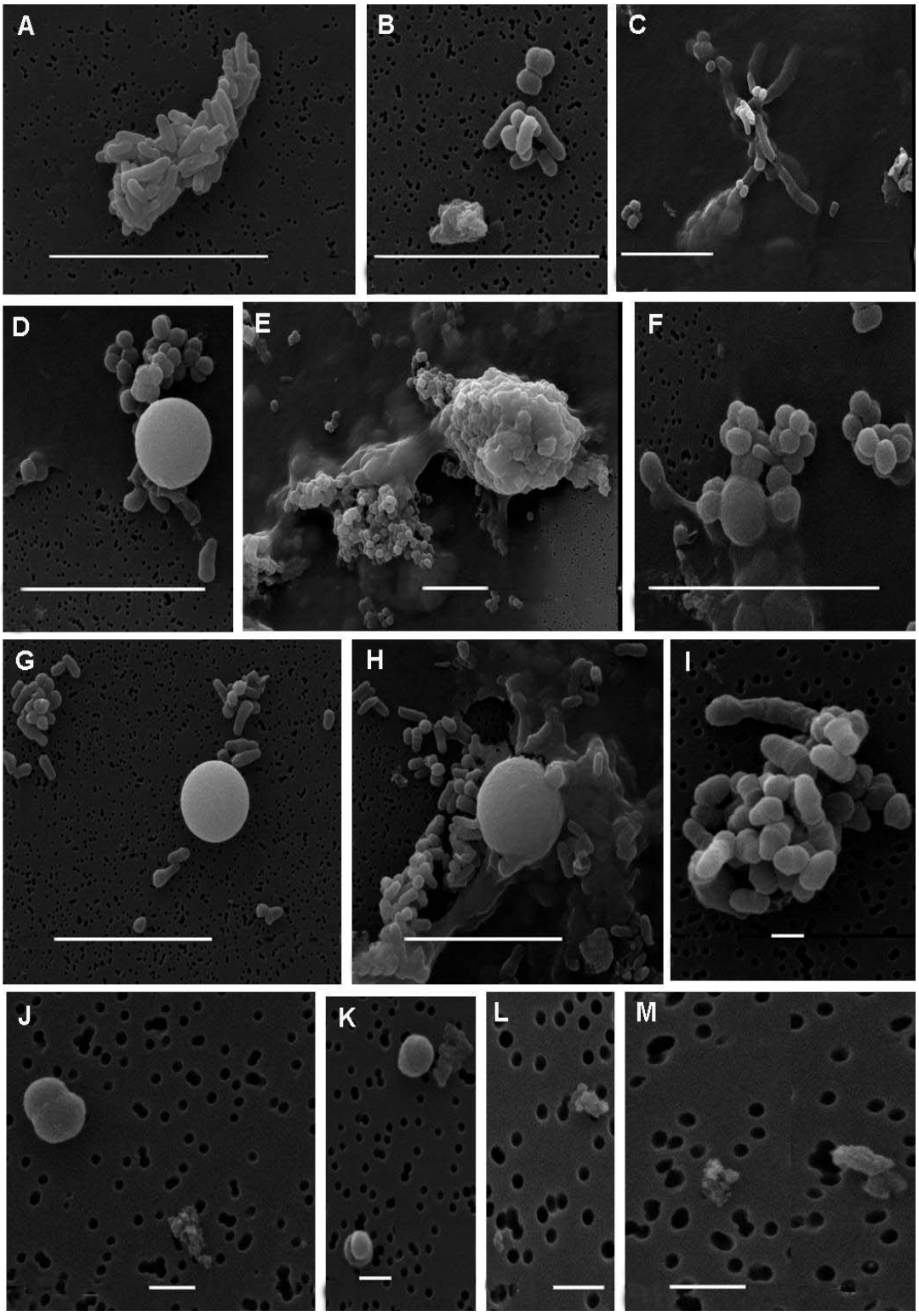

Fig. 2. Scanning electron microscopy: (**A**) control *Mycobacterium tuberculosis* microcolony; (**B–M**) L-form cultures of *M. tuberculosis* strains S14 and H_{37}Rv, obtained after starvation and five passages in SsM. (**B, C, F, I, K**) Small rod-shaped and coccoid cells of different sizes and elongated forms. (**D, G, H, J**) Typical spherical large-body cells, some of which are reproducing by budding (**H**) or binary fission (**J**). (**E, H**) Giant L-form "mother" cells, massively releasing numerous coccoid elementary bodies, and membranous remains. (**L, M**) Small granular bodies on membrane filters with 0.22 μm pores. Bars: (**A**–**H**) 10 μm; (**I**–**M**) 1 μm.

small oval coccoid cells, and large spherical bodies (Fig. 2D, F, G, H, J) were observed. Some of the bacilli were more elongated (Fig. 2C), while others appeared as beaded rods with bulges at the ends or center of the cells (Fig. 2F, J). Some of the spherical large bodies, typical for L-forms, propagated by budding (Fig. 2H). Other, coccoid cells of varied size were seen to divide by binary fission (Fig. 2I, J, K) and were arranged singly, in pairs, or in irregular clusters (Fig. 2F, I). Different arrangements of coccoids suggested either the capability of L-forms to divide in different planes or the possibility that they had arisen en masse from huge L-form bodies. A particularly noteworthy observation was that of a giant L-body, which exhibited rough surface and external bumps (Fig. 2E, H) and was in the process of releasing numerous, previously generated granules through profusion. These granular elements subsequently developed into bigger coccoid or large L-bodies. Figure 2L,M shows the very small granular elements, displayed on membrane filters with a pore size of 0.22 μm, evidencing their ability to pass through bacterial filters, i.e., filterable L-form cells.

Parallel TEM observations revealed the typical ultrastructure of the L-form population, composed of cells of variable shapes and sizes, completely lacking a bacterial cell wall and bounded only by a single unit membrane (Fig. 3B–E).

Figure 3B shows two large L-bodies undergoing division by binary fission. Budding was another mode of L-form replication (Fig. 3B–D). Cytoplasmic condensation at the periphery of the large bodies, ending in the formation of protrusions and buds, was often observed (Fig. 3B–D). Small budding elementary bodies were seen at the periphery and along the membrane of a large L-body (Fig. 3D). These forms appeared to be in the process of detachment from the

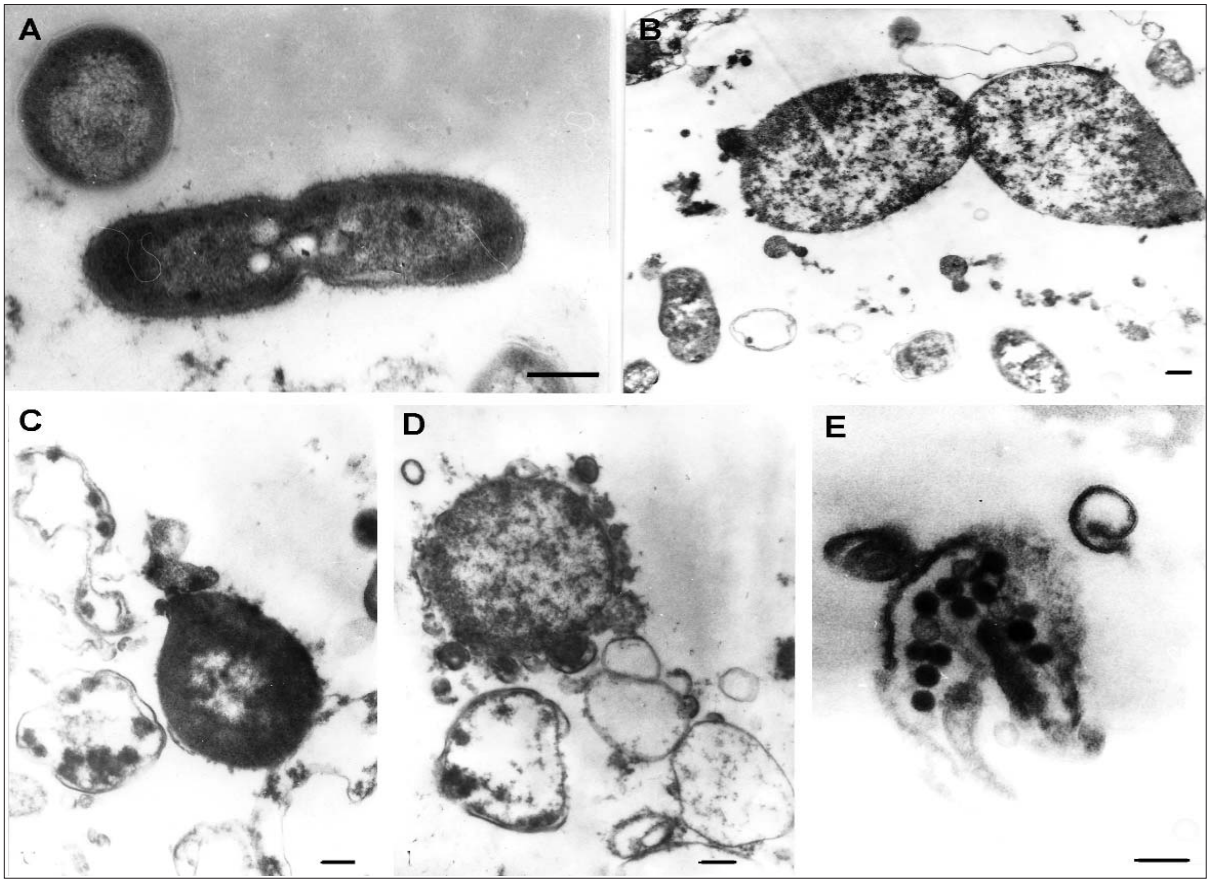

Fig. 3. Transmission electron microscopy of ultrathin sections of *Mycobacterium tuberculosis* (S14 and H37Rv strains): (**A**) Control tubercle bacilli; (**B–E**) L-form cells from mycobacterial cultures obtained after starvation and five passages in SsM. All L-cells are of protoplast type and covered only by a "unit" cytoplasmic membrane. (**B**) Two large L-bodies are dividing by binary fission, one of them also by budding. They are surrounded by small elementary bodies. (**C, D**) Large and small L-bodies, and vesicular forms of different sizes and electron densities. Some of the L-forms are propagating by budding and the detachment of small forms from large L-cells. (**E**) L-form "mother" cell filled with numerous electron-dense granules. Bars = 0.2 μm.

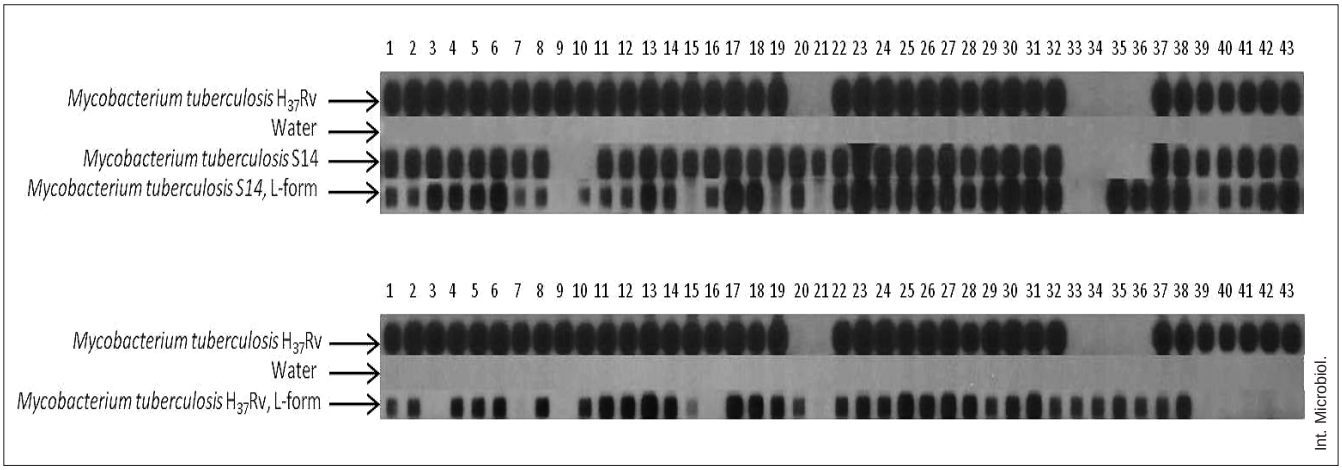

Fig. 4. Spoligotyping of parental *Mycobacterium tuberculosis* $H_{37}Rv$ and S14 strains, and their L-form variants obtained after starvation and five passages in SsM.

large body. Many small electron-dense bodies and granules, usually filled with ribosomes, were present outside large bodies (Fig. 3B) or inside vesicular forms (Fig. 3C, D). Some of the latter were either apparently empty or they contained electron-dense granules (Fig. 3C, D). Of considerable interest was a large body, or "mother cell," filled with numerous electron-dense granules (Fig. 3E). Obviously, this process of fragmentation of the cytoplasmic mass into numerous granular and elementary bodies represents a mode of L-form replication. It should be noted that nucleoid and ribosomal areas within the L-bodies were of variable electron density and intracellular location (Fig. 3C, D). Ribosomes were compact or diffusely scattered, usually in the periphery of the cells (Fig. 3C, D).

DNA-based identification of L-form variants as *Mycobacterum tuberculosis*. The mycobacterial identity of L-form variants was confirmed by spoligotyping results (Fig. 4). Spoligotyping showed the occurrence of certain polymorphisms in L-form variants, i.e., the insertion or deletion of spacer signals, compared to the spoligoprofile of the parental *M. tuberculosis* strains.

Discussion

The view that L-form conversion is a universal feature of bacteria is widely supported [1,3,5]. The uniqueness of bacterial L-forms stems from the fact that, independently of their species origin, they exhibit a common morphological phenotype and similar growth characteristics. In this respect, morphologically modified L-forms of *M. tuberculosis* are difficult to identify and often go unrecognized [16,18]. Various morphological forms of mycobacteria have been observed by many authors, who described them as large "amoeba-like cells" [7], giant non-cellular structures or "budding yeast-like structures" [11], "elementary bodies and filament structures" [19], "endospores" [6,25], and "ovoid cells" [24].

Our experiments aimed to induce L-conversion of *M. tuberculosis* by means of nutrient starvation stress. The L-form variants were then selectively separated through passages in SsM. As seen by ZN staining, L-form variants of *M. tuberculosis* lost their acid fastness, a characteristic that is dependent on both the integrity and cell-wall alterations of tubercle bacilli [23]. The appearance of polymorphic non-acid-fast forms and coccoids in cultures of mycobacteria has been previously reported [8,21]. Using electron microscopy, we were able to characterize the ultrastructure of L-form transformation in detail, through sequential images of the morphogenesis of different L-form units. L-conversion involved the appearance of polymorphic cell-wall-deficient forms, such as giant spherical and small elementary bodies. Unusual modes of cell replication, such as irregular binary fission, budding or the destruction of giant L-structures ("mother" cells) and the release of numerous small granular bodies were noted. Small granular bodies, in turn, grew into larger coccoid cells. Domingue [5] has suggested that small, electron-dense L-bodies are capable of developing along several different routes, depending on the stimulus received, in addition to possessing the potential for unlimited growth and division.

Of special interest was the finding that mycobacterial L-forms developed colonies at 36–48 h, which is much earlier than classical tubercle bacilli. When *M. tuberculosis* is grown on LJ medium or Middlebrook agar, rough colonies usually appear only after 4 weeks of incubation, due to the slow doubling time of mycobacteria. In the present study, however, mycobacterial L-forms exhibited phenomenally faster growth. Pla Armengol found in 1931 that a large inoculum of tubercle bacilli was able to grow rapidly on all routine media, appearing as large L-body spheres [22]. It may be that the unique ability of mycobacterial L-forms to grow faster than classical tubercle bacilli is due to their lack of cell walls, facilitating the permeation of nutrients.

That the L-forms derived from *M. tuberculosis* was confirmed by spoligotyping. This technique provides new insight into the occurrence of a certain genetic polymorphism in L-forms of *M. tuberculosis*.

Mycobacterial L-forms offer interesting and intriguing aspects for experimentation, as well as for drawing parallels between the phenotypic and genetic processes that occur during L-conversion. These data provide the basis for further investigations aimed at determining both the origin of the genetic variations in *M. tuberculosis* in association with its L-form conversion and the mechanisms by which they appear.

In conclusion, the unique property of *M. tuberculosis* to exist as polymorphic, rapidly growing non-acid-fast L-form variants contributes to its extraordinary capacity to adapt to environmental changes. Finally, our results have shed light on several specific aspects of the *M. tuberculosis* L-form phenomenon and link them to the mechanisms of survival under stress conditions.

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Competing interests. None declared.

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