Ethambutol-induced alterations in *Mycobacterium bovis* BCG imaged by atomic force microscopy

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Abstract

Progress in understanding the structure–function relationships of the mycobacterial cell wall has been hampered by its complex architecture as well as by the lack of sensitive, high-resolution probing techniques. For the first time, we used atomic force microscopy (AFM) to image the surface topography of hydrated *Mycobacterium bovis* bacillus Calmette Guérin cells and to investigate the influence of the antimycobacterial drug ethambutol on the cell wall architecture. While untreated cells showed a very smooth and homogeneous surface morphology, incubation of cells in the presence of ethambutol caused dramatic changes of the fine surface structure. At 4 μg mL^{-1}, the drug created concentric striations at the cell surface and disrupted a ~8 nm thick cell wall layer, attributed to the outer electron-opaque layer usually seen by electron microscopy, while at 10 μg mL^{-1} an underlying ~12 nm thick layer reflecting the thick electron-transparent layer was also altered. These noninvasive ultrastructural investigations provide novel information on the macromolecular architecture of the mycobacterial envelope as well as into the destructuring effects of ethambutol.

Introduction

Mycobacterium tuberculosis is a Gram-positive pathogen which infects over one-third of the population in the world, causing annually 10 million new cases and 3 million deaths (Raviglione, 2003). The mycobacterial cell wall is highly complex and essential for growth and survival in the infected host. It also protects mycobacteria by restricting permeability to most hydrophilic antimicrobial compounds. Our present day understanding of its structure reveals an insoluble matrix as a crosslinked peptidoglycan linked to arabinogalactan, esterified at the distal ends to the mycolic acids. The other dominant feature is the lipoarabinomannan, somewhat embedded into the framework of the mycolylarabinogalactan, and anchored into the cell membrane via its phosphatidylinositol portion (Brennan & Nikaido, 1995). Surrounding the wall, a capsule may also be present (Daffe & Draper, 1998). Although the major components of the cell envelope have been identified, their 3-dimensional spatial arrangement remains largely unknown.

In the absence of an effective vaccine, treatment with antimycobacterial drugs is the key weapon to limit the expansion of the tuberculosis pandemic. Some of the most effective drugs are known to inhibit the biosynthesis of cell wall components (Kremer & Besra, 2002; Schroeder *et al.*, 2002). Among these, ethambutol inhibits the synthesis of the polysaccharidic portion of the envelope, i.e. arabinomannan and arabinogalactan (Wolucka *et al.*, 1994). Yet, due to the very complex architecture of the mycobacterial cell wall components, the molecular mechanisms by which antibiotics affect the cell wall structure and properties remain poorly understood.

In the past decades, various characterization methods have been developed to probe the structural properties of microbial cell walls. While valuable information on the dynamics of cell wall assembly can be obtained using fluorescence microscopy (Daniel & Errington, 2003), our current view of the cell wall ultrastructure essentially relies on electron microscopy techniques (Beveridge & Graham, 1991). Particularly, transmission electron microscopy (TEM) combined with freeze substitution has allowed to visualize the mycobacterial cell wall in conditions close to their native hydrated state (Paul & Beveridge, 1992). This elegant cryotechnique combines ultrarapid freezing with mild chemical fixation, thereby avoiding shrinkage of hydrated structures that would occur otherwise. These studies

and others (for a review, see Brennan & Nikaido, 1995) have revealed that mycobacterial cell walls are complex, multilayered structures composed of an inner layer of moderate electron density which contains peptidoglycan and is ~4 nm thick, a ~10 nm thick electron-transparent layer (ETL) which appears to be the hydrophobic domain of the cell wall and is dominated by mycolic acids covalently bound to arabinogalactan, and an outer electron-opaque layer (OL) of variable thickness, containing polysaccharides, glycoproteins and glycolipids. Clearly, it is interesting to attempt complementing these thin section TEM analyses with three-dimensional views of the cell surface obtained in aqueous solution.

The atomic force microscope (AFM; Binnig *et al.*, 1986) has recently proven to be a powerful addition to the range of cell surface analysis techniques (for a review, see Dufrêne, 2004). An exciting feature of AFM is the possibility to directly visualize the effect of external agents such as enzymes and antibiotics on the microbial cell surface. In the first such study, the action of penicillin on the morphology of *Bacillus subtilis* cells was investigated using AFM in air (Kasas *et al.*, 1994). In other work, changes in the morphology of *Escherichia coli* induced by cefodizime were demonstrated, again in the dried state (Bragga & Ricci, 1998). Although these reports represent an important preliminary step towards the application of AFM in drug–microorganism interaction studies, their relevance is limited by the drying procedure applied during sample preparation. Here, we provide the first high-resolution AFM images of hydrated *Mycobacterium bovis* bacillus Calmette Guérin (BCG) surfaces before and after exposure to ethambutol. We found that the drug dramatically alters the fine surface architecture of the cells, by (i) removing outer cell wall layers to an extent that depends on the drug concentration and by (ii) revealing concentric striations on top of the remaining layers.

Materials and methods

Bacterial cultures

Mycobacterium bovis BCG was grown in Sauton medium as described elsewhere (Menozzi *et al.*, 1996). Briefly, mycobacteria were cultured at 37 °C for about 3 weeks ($OD_{600\,nm}$~0.6) in static conditions using 75-cm^2 Roux flasks that contain 50 mL of Sauton medium supplemented with Triton WR1339. For some experiments, cells were resuspended for 24 or 72 h in Sauton medium containing ethambutol at concentrations of 4 or 10 $\mu g\,mL^{-1}$, corresponding to *c.* 1 or 2.5 times the minimal inhibitory concentration (MIC), respectively. All cells were harvested by centrifugation, washed three times with deionized water, and resuspended to a concentration of ~10^8 cells mL^{-1}.

AFM measurements

AFM images were recorded in contact mode using an optical lever microscope equipped with a liquid cell (Nanoscope IV Multimode AFM; Veeco Metrology Group, Santa Barbara, CA). To image mycobacteria in their native state by AFM, the bacteria were immobilized by mechanical trapping onto Isopore polycarbonate membranes (Millipore), with pore size similar to the cell size (Kasas & Ikai, 1995; Dufrêne *et al.*, 1999). This approach is well suited to image single cells under aqueous conditions, and it does not involve chemical treatment or drying which would cause rearrangement or denaturation of the surface molecules (Dufrêne, 2004). After filtering a concentrated cell suspension, the filter was gently rinsed with deionized water, carefully cut (1 × 1 cm), attached to a steel sample puck (Veeco Metrology Group LLC) using a small piece of adhesive tape and the mounted sample was transferred into the AFM liquid cell. Height and deflection images were recorded at room temperature (20 °C) with a minimal applied force (< 500 pN), using oxide-sharpened microfabricated Si_3N_4 cantilevers (Microlevers; Veeco Metrology LLC) with spring constant of 0.01 N m^{-1} (manufacturer specified). While height images provide quantitative information on sample surface topography, deflection images often exhibit higher contrast of the morphological details.

Results and discussion

Surface structure of native *M. bovis* BCG cells

To image mycobacteria in their native state by AFM, these were mechanically immobilized onto polycarbonate membranes, thus allowing cell imaging in buffer without pretreatment such as air drying or chemical fixation. Representative images of native *M. bovis* BCG are shown in Fig. 1. Owing to the large curvature of cells, the image obtained in the height mode (Fig. 1a) has fairly poor resolution, while the image obtained in the deflection mode (Fig. 1b) is much more sensitive to the surface relief. Depending on the region investigated, cells were either aggregated (Fig. 1b) or isolated (Fig. 1c). We note that despite the unfavorable rod-like morphology of the cells, their immobilization was very successful. In fact, as opposed to what is usually observed with spherical cells (some bacteria, fungal spores, yeasts), most mycobacteria were found not to be trapped into the pores of the polymer membrane, but to be firmly attached on its surface, a behavior that may reflect strong hydrophobic interactions between the cells and the polycarbonate membrane.

High-resolution images of the cell surface could be recorded without significant modification of the surface morphology (Fig. 1d). The surface was rather homogeneous and smooth, the root mean square (rms) roughness being

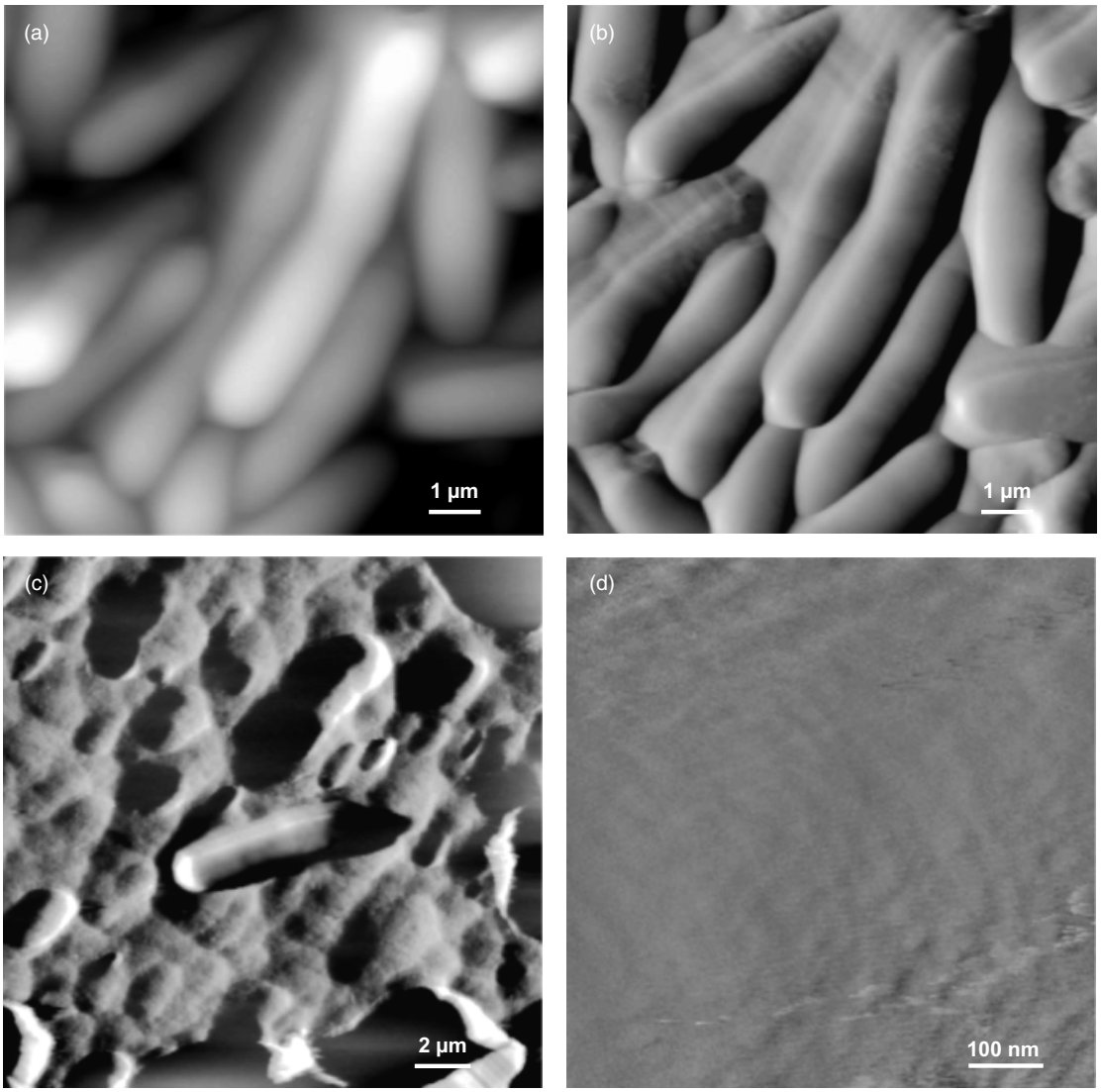

Fig. 1. AFM images of *Mycobacterium bovis* BCG cells recorded in water. Height (a; *z*-range: 2.5 μm) and deflection (b, c) images showing aggregated cells (a, b) and a single cell (c) immobilized onto a porous polymer membrane. (d) High-resolution deflection image of the cell surface.

0.34 ± 0.02 nm (on 400 nm × 400 nm height images), which is very close to the value obtained for *Saccharomyces cerevisiae* cells using similar imaging conditions. We suggest it represents essentially the electron-dense outer layer (OL), rather than a loosely bound capsule, for the following reasons. First, mycobacterial capsular material is known to be loosely bound and easily displaced during the conventional processing of samples for microscopy or upon treatment with glass beads (Daffe & Etienne, 1999). As our sample preparation procedure involves washing by successive cycles of resuspension/centrifugation and filtering through a porous polymer membrane, it should cause detachment of the loosely bound capsular material. Second, the surface was remarkably smooth and not affected by repeated scanning. This is not consistent with the presence of loosely bound macromolecules, which should result in imaging artifacts in the form of streaks oriented in the scanning direction (van der Aa *et al.*, 2001). Third, approach force curves (data not shown) did not show significant deformation in the contact region, indicating the surface was rather stiff, while retraction force curves never showed any elongation forces expected for soft, loosely bound macromolecules (van der Aa *et al.*, 2001).

Nanoscale surface alterations induced by ethambutol

The surface morphology of mycobacteria was then investigated following incubation for 24 h with 4 μg mL^{-1} ethambutol, a concentration close to the MIC (Fig. 2). As can be

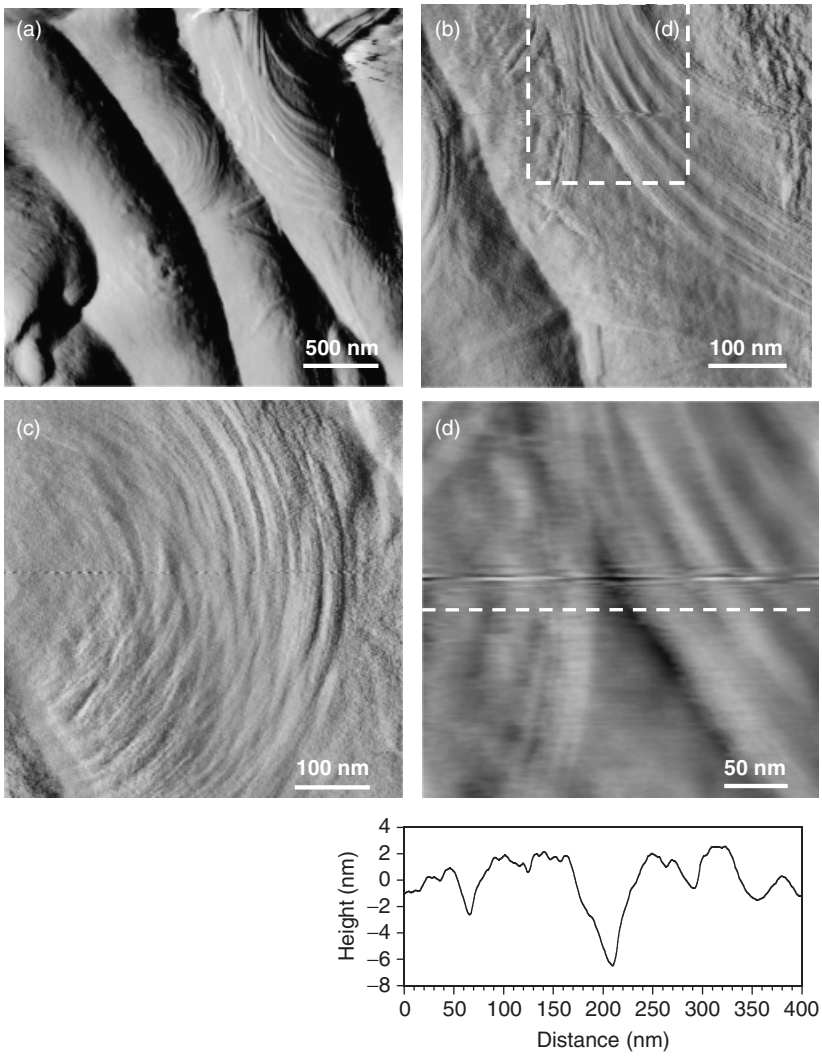

Fig. 2. AFM images of *Mycobacterium bovis* BCG cells recorded in water following incubation for 24 h with 4 μg mL^{-1} ethambutol. (a–c) Deflection images recorded at increasing resolutions and (d) high-resolution height enlargement (*z*-range: 50 nm) corresponding to the box shown in Fig. 2b. A vertical cross-section taken along the dashed line is shown beneath the height image.

seen in Fig. 2a, the ultrastructural integrity of the cell was maintained and the cell surface was clean and well-resolved, indicating that the treatment was gentle and did not cause visible cell breakage. However, significant alterations of the fine surface structure were seen on closer examination: most cells showed a nanostructured external layer possessing concentric rings or striations (Fig. 2b and c). This layer covered only part of the cell surface and was 8 ± 1 nm thick, as revealed by cross-sections taken in height images (Fig. 2d). To assess whether the observed changes depended on the drug concentration, we also imaged cells following incubation with 10 μg mL^{-1} ethambutol, corresponding to 2.5 times the MIC (Fig. 3). Clearly, cell surface alterations were more pronounced, i.e. striations were more evident, and the surface showed two superimposed layers of 8 ± 1 and 12 ± 1 nm, respectively.

These AFM observations are consistent with electron microscopy data, which revealed a multilayered organization for the mycobacterial cell wall (Brennan & Nikaido, 1995). In fact, the ~8 nm thick striated layer observed at both 4 and 10 μg mL^{-1} ethambutol may reflect the outer layer (OL), while the ~12 nm thick layer observed only at 10 μg mL^{-1} would correspond to the underlying electron-transparent layer (ETL) of mycolic acids esterified to arabinogalactan. This is consistent with the mode of action of ethambutol, which primarily inhibits arabinan polymerization, thus its incorporation in the cell wall. Hence, ethambutol used at the MIC would impair the formation of the OL. Above the MIC, ethambutol seems to cause detachment of the mycolic acids, which could no longer be esterified to arabinogalactan. This global destructuration of the wall could be amplified by the inhibition of lipoarabinomannan biosynthesis that occurs at such concentration of the drug. We also note that our AFM images showing two layers are in agreement with the known observation that mycobacterial cell wall can be cleaved by the process of freeze-fracture and

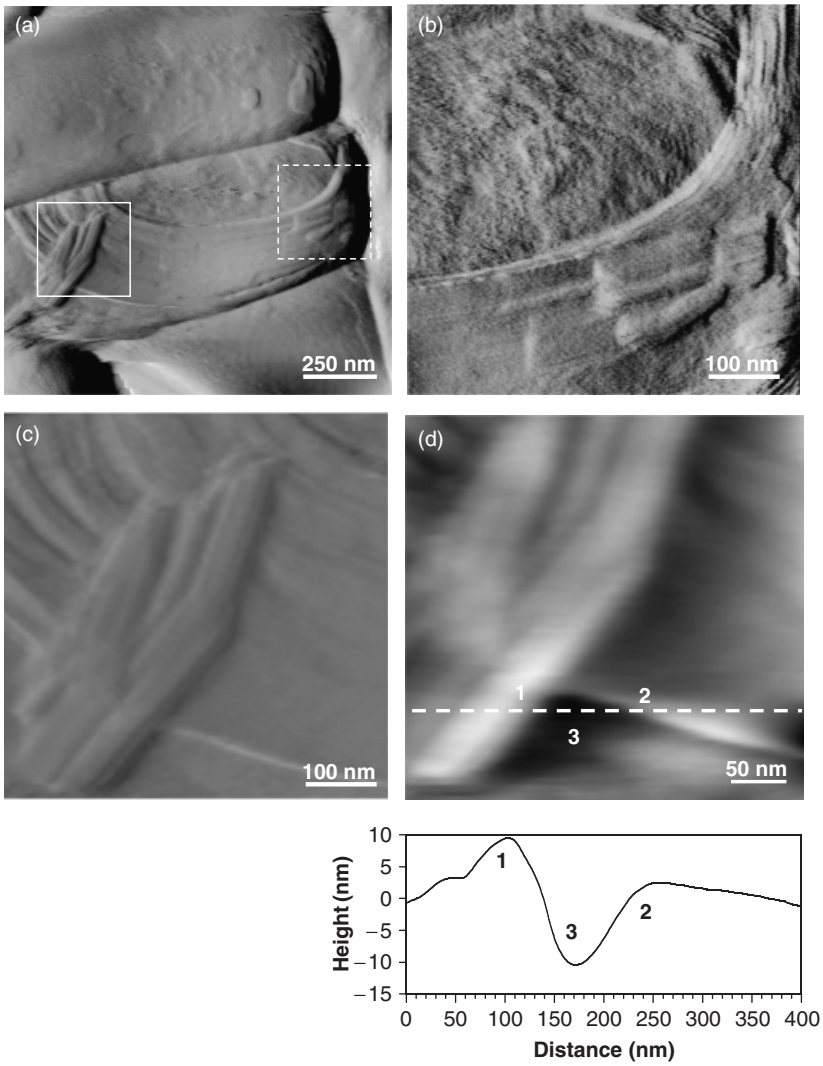

Fig. 3. AFM images of *Mycobacterium bovis* BCG cells recorded in water following incubation for 24 h with 10 μg mL^{-1} ethambutol. (a) Deflection image and (b, c) deflection and (d) height enlargements (*z*-range: 75 nm) corresponding to the dashed (b) and solid (c, d) boxes shown in Fig. 3a. A vertical cross-section taken along the dashed line is shown beneath the height image.

with the notion that the wall is constructed basically as a lipid bilayer (Brennan & Nikaido, 1995). The original smooth surface of the mycobacterial cell and its transformation in a fibrous structure has been previously observed by electron microscopists (Kölbel, 1984). It was proposed that these alterations must be regarded more as artifacts due to drastic conditions of sample preparation that induce rearrangements of hydrophilic–hydrophobic lipids to form rope-like structures. Importantly, the observation of such structures following ethambutol treatment using an *in situ* noninvasive technique suggests they may reflect the global organization of the structural polar lipids of the cell wall. Further work is needed to understand the relevance of these striations and to establish whether they are related to the mode of assembly of arabinogalactan or other cell wall components. Also, using AFM force spectroscopy it would be most interesting to investigate to what extent the cell wall mechanical properties are altered upon incubation with the drug, while varying the concentration and incubation time.

In summary, our results represent the first *in situ*, i.e. under physiological conditions, visualization of nanoscale alterations induced in mycobacterial cell walls upon incubation with ethambutol. The drug causes disruption of cell wall layers, presumably the OL and ETL layers, and induces characteristic concentric striations that may be related to the mode of assembly of arabinogalactan. We feel that the nondestructive high-resolution imaging capability of AFM exploited here is a valuable complementary approach to electron microscopy techniques and has a great potential for exploring antibiotic–cell wall interactions on the nanoscale.

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