Polish Journal of Microbiology
2005, Vol. 54, No 1, 5–11

The Effect of Ethambutol on Mycobacterial Cell Wall Permeability to Hydrophobic Compounds

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Received 8 November 2004, received in revised form 21 December 2004,
accepted 22 December 2004

Abstract

Ethambutol (EMB), the first line drug in the treatment of tuberculosis, is an inhibitor of the biosynthesis of the cell wall compound – arabinogalactan. It was found that EMB at sub-inhibitory concentration increases the permeability of the *M. vaccae* cell wall, which was monitored by cell sensitization to erythromycin and rifampicin. The high permeability of the cell wall to hydrophobic compounds allows enhanced intracellular bioconversion of β-sitosterol to 4-androsten-3,17-dione (AD) and 1,4-androstadien-3,17-dione (ADD).

K e y w o r d s: ethambutol, mycobacterial cell wall, permeability

Introduction

Microbiological degradation of sterols yields androstane derivatives, 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) that are the starting material for the production of almost all kinds of medically important steroids (Sedlaczek, 1988). Selective side chain cleavage of β-sitosterol is performed by saprophytic, fast-growing members of the genus *Mycobacterium*. The complex of intracellular enzymes catalyses this process. Most of them form the classical route of β-oxidation of fatty acids, although the system includes additional enzymes specific for β-sitosterol (Szentirmai, 1990). β-sitosterol is a highly hydrophobic substance of very low solubility in water (Hesselink, 1988). The architecture and composition of the mycobacterial cell wall could be a factor facilitating the solubility of sterols and their uptake by the cell.

Recent studies have contributed to the better knowledge of the composition and structure of the mycobacterial cell wall. Mycobacterial cell wall is composed of peptidoglycan (PG) linked to the arabinogalactan (AG) that is esterified with mycolic acids (Barry, 2001; Daffe and Draper, 1998; Brennan and Nikaido, 1995; Liu *et al*., 1995;1996; Nikaido, 1994). These three structures: peptidoglycan, arabinogalactan and mycolic acids form mycobacterial cell wall skeleton (CWS). Peptidoglycan (syn. murein), one of the most common types found in bacteria, serves as a scaffolding for arabinogalactan, that is anchored by phosphodiester bonds. Arabinogalactan structure consists of D-arabinose and D-galactose units in the furanose form. Branching in some residues of the arabinan region produces a pentaarabinose motif. Four of the residues of this motif serve as the point of attachment for mycolic acids (Crick *et al.*, 2001; Daffe and Draper, 1998; Daffe *et al.*, 1990; Dover *et al.*, 2004; McNeil, 1999).

Mycolic acids are high molecular weight α-alkyl, β-hydroxy fatty acids containing 70 to 90 carbon atoms and form the inner leaflet of the unique asymmetric bilayer. They are the most abundant molecules in

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the mycobacterial cell wall; the types produced by different mycobacterial species vary widely in structure. As shown in X-ray diffraction studies, the hydrocarbon chains of the cell wall skeleton mycolic acids occur as a quasicrystalline layer perpendicular to the peptidoglycan (Nikaido *et al.*, 1993). It is believed that mycolic acids, the main component of the mycobacterial cell wall, form the primary hydrophobic barrier; their highly ordered structure of very low fluidity is of crucial importance to drug and hydrophobic compounds impenetrability.

The outer leaflet of the bilayer is composed of the easily extractable free lipids (species- and type-specific glycolipids, phenolic glycolipids, trehalose dimycolate, phtiocerol dimycocerosate and others) (Brennan, 2003; Chatterjee, 1997). These lipids intercalated into the hydrocarbon chains of the mycolic acids, thus that free lipids with longer fatty acids complementing the shorter α-chains, while the free lipids with shorter fatty acids complementing the meromycolic chain (Brennan, 2003; Brennan and Draper, 1994; Colston, 1996; Minnikin, 1982)

Disrupting structure and physical architecture of mycobacterial cell wall layer (PG, AG, mycolic acids, free lipids) by interfering with the biosynthesis their component, results in changes of permeability of the cell wall (Barry, 2001).

In the present study, we used ethambutol (EMB) – an inhibitor of arabinogalactan biosynthesis. We show for the first time that *M. vaccae* treated with EMB, more efficiently performed the intracellular degradation of β-sitosterol side chain. The presence of the inhibitor enhanced susceptibility to antimicrobial agents as well. It is very likely that the observed effect is due to more general changes in the composition of arabinogalactan.

Experimental

Materials and Methods

Microorganism. The *Mycobacterium vaccae* NRRL B 3805 was used in the present study. This strain, identified as non-pathogenic (Seidel and Horhold, 1992), is able to perform the selective cleavage of β-sitosterol side chain yielding 4-androsten-3,17-dione (AD) with trace amount of 1,4-androstadien-3,17-dione (ADD).

Culture media. Medium NB containing (g/l): nutrient broth (Difco) 8.0 and glucose 10.0 supplemented with 0.2% (v/v) Tween 80 was used in all experiments for both cell growth and β-sitosterol transformation with or without inhibitors. After autoclaving, the medium pH was 6.0–6.2. The bacteria were maintained on slants, on medium NB solidified with 2% (w/v) agar (Difco).

Sterols and steroids. A preparation of β-sitosterol containing 16% (w/w) campesterol was obtained from Triple Crown (Sweden). For all biotransformation experiments it was prepared as described previously (Sedlaczek *et al.*, 1994). Cholesterol from Serva, and androst-1-ene-3,11,17-trione from Sigma were used as internal standards to GC analysis for quantitative determination of β-sitosterol and AD, respectively. AD and ADD were used as standards (Koch-Light).

Other chemicals. Ethambutol (EMB) – an inhibitor of arabinogalactan biosynthesis (Wolucka *et al.*, 1994) was used. The inhibitor was dissolved in distilled water, sterilized by filtration and stored at 4°C for no longer than 2 days. In the experiments, the inhibitor was added to the culture medium at the time of inoculation at various concentrations ranging from 3 to 50 μg ml^{-1}.

Growth and β-sitosterol transformation. Medium NB (20 ml in 100 ml flasks) was inoculated with *Mycobacterium vaccae* NRRL B 3805 washed off from 48 h cultures on agar slants, and incubated for 24 h at 32°C with shaking at 180 rev/min. From this culture 10 ml were transferred to 90 ml new NB medium in 1-litre flasks. At the time of inoculation, β-sitosterol (0.2 g/l) and the inhibitor was added to the medium, that was then incubated in conditions as described above. To determine the cell dry mass, at the start of the experiments and at 12 h intervals, samples (2×5 ml) were withdrawn from the culture, filtered through Synpor filters (pore diameter 0.2 μm) of known weight, and the sediment was dried to constant weight. The progress in β-sitosterol side chain degradation was determined in another 2-ml culture samples to which cholesterol and androst-1-ene-3,11,17-trione as internal standards were added (each at 100 μg in 50 μl chloroform), and extracted 3 times with equal volume of chloroform. The extracts were dried under vacuum; the residue was dissolved in 0.5 ml acetone and steroids were analysed by chromatography as described previously (Rumijowska *et al.*, 1997). The selective side chain degradation of β-sitosterol proceeds intracellularly, catalysed by an enzyme complex, a part of which is involved in fatty acid β-oxidation (Szentirmai, 1990). The substrate must permeate the cell wall to be transformed to androstene derivatives AD(D). The rate of AD(D) formation and accumulation is thus a measure of sterol penetration rate for normal cells, as well as those whose cell wall permeability has been altered by means of deliberate procedures.

Drug sensitivity assay. Twofold dilution series were prepared in 4.5 ml NB medium in glass tubes, from sterile stock solutions of 0.25 mg ml^{-1} erythromycin and 0.20 mg ml^{-1} rifampicin. The final drug concentrations ranged from 25 to 0.045 μg ml^{-1} and from 20 to 0.04 μg ml^{-1}, respectively. The tested inhibitor was added to each tube at previously determined concentration that did not affect growth, the medium was inoculated with a 24-h liquid culture and incubated as described. Bacterial growth was monitored spectrophotometrically (Specol 20, Carl Zeiss, Jena) at 560 nm. To test the effect of the drug alone and in combination with the EMB, samples (0.5 ml) were removed at the start, after 24 h and 48 h, diluted adequately, and OD_{560} was measured. The initial OD_{560} was 0.1–0.15. Moreover, cultures containing ethambutol alone, and those without any supplement were analysed. From the relationship between OD_{560} and bacterial mass content in the samples, MIC_{50} was determined for the drug and its combination with EMB.

Results

The effect of ethambutol on growth and cell susceptibility to antimicrobial agents. To reveal the effect of an ethambutol on the cell wall permeability for the β-sitosterol, it was necessary to precede the experiments to estimate the most effective inhibitor concentration. These concentrations should be high enough to cause the disorganization of the cell wall, but be below the point requiring for complete cell growth inhibition. In preliminary experiments, EMB was used at concentration ranging from 3 μg ml^{-1} to 50 μg ml^{-1}.

The effect of the increasing concentrations of EMB on the bacterial growth was shown on Fig. 1. No measurable effect on cell growth was observed with low concentrations of EMB. The cell biomass formation was at the same level as in the control cells. At higher concentrations (10–50 μg ml^{-1}) the cell biomass content was about 30–50% lower compared to the control.

The drug sensitivity test has been frequently used for the evaluation of the permeability barrier under conditions in which the barrier is expected to be weakened (Rastogi *et al.*, 1990; Yuan *et al.*, 1998; Mdluli *et al.*, 1998). A comparison of synergistic activity of drugs with ethambutol was illustrated in Fig. 2. The

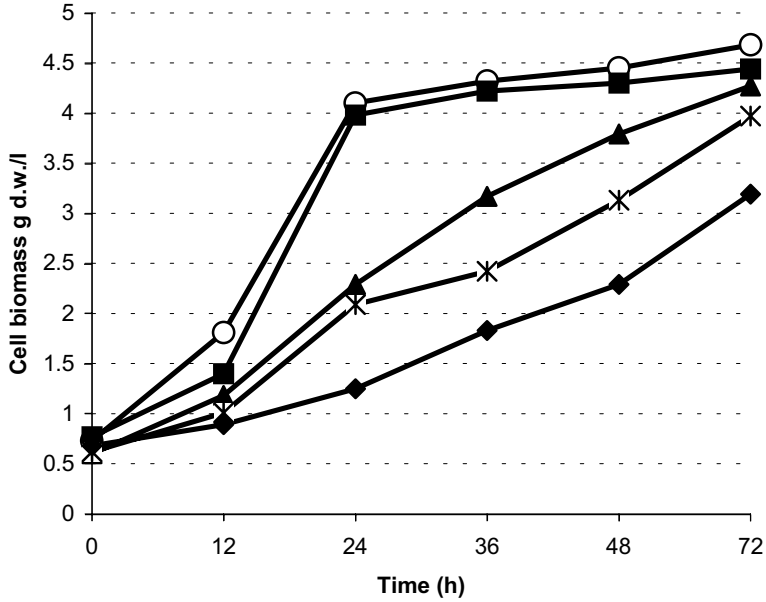

Fig. 1. Growth of *Mycobacterium vaccae* B 3805 in the absence (○) and presence of ethambutol at 3 μg ml^{-1} (■), 10 μg ml^{-1} (▲), 20 μg ml^{-1} (✳) and 50 μg ml^{-1} (◆).

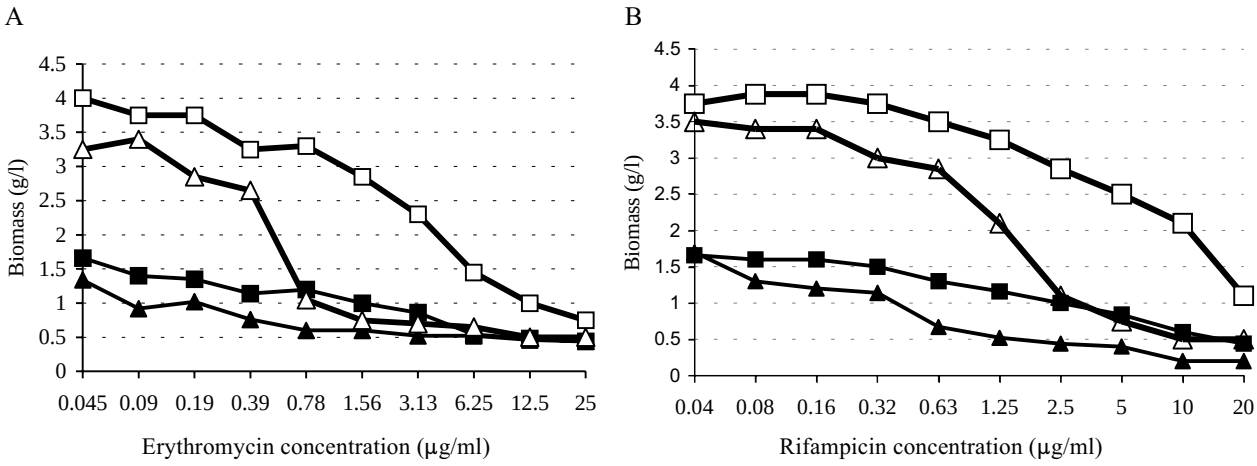

Fig. 2. Inhibition of *Mycobacterium vaccae* B 3805 growth by erythromycin (A) and rifampicin (B) with and without ethambutol at 3 μg ml^{-1}.
OD_{560} measurements of adequately diluted cultures were converted into cell dry biomass by the use of a standard graph depicting the relationship between the OD_{560} value and cell biomass content in the culture. Growth at 24 h with (▲) and without (■) ethambutol. Growth at 48 h with (△) and without (□) ethambutol.

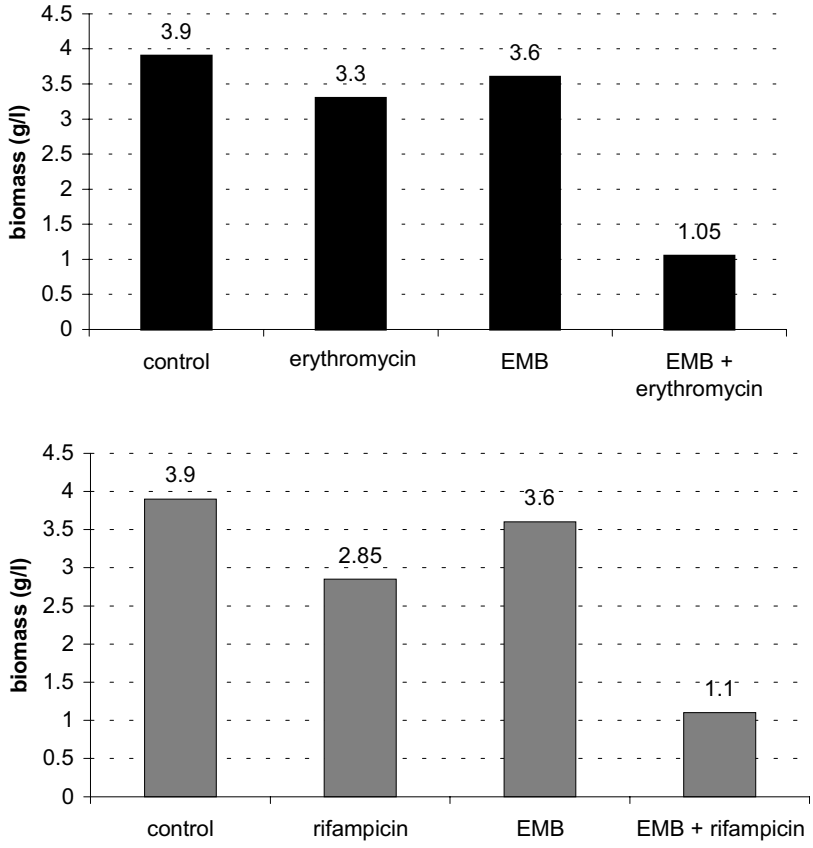

Fig. 3. Synergistic effect for drug combination using erythromycin 0.78 μg ml^{-1} and EMB 3 μg ml^{-1} (A) and rifampicin 2.5 μg ml^{-1} and EMB 3 μg ml^{-1} (B) after 48 hours.

susceptibilities to various concentrations of rifampicin (from 20 to 0.04 μg ml^{-1}) and erythromycin (from 25 to 0.045 μg ml^{-1}) were determined on NB liquid medium.

Ethambutol increased the action of antibiotic in all tested combinations within 24 h, but the effect was stronger in 48 h cultures. At lower concentrations, up to 0.19–0.39 μg ml^{-1} erythromycin (Fig. 2A), and 0.16–0.32 μg ml^{-1} rifampicin (Fig. 2B), ethambutol produced a slight effect on growth inhibition. Profound enhancement of the antibiotic action by ethambutol was observed at 0.78 μg ml^{-1} erythromycin and 2.5 μg ml^{-1} rifampicin. These concentrations could be regarded as MIC_{50} of the antibiotics in combination with ethambutol, as growth reached approximately half that the control one.

MIC_{50} of the antibiotics alone was much higher, amounting to 12.5 μg ml^{-1} erythromycin, depending on growth intensity, and about 10–20 μg ml^{-1} rifampicin. The sensitisation factor (Vaara, 1992), *i.e.*, the approximate ratio between MIC_{50} for control bacteria and that exposed to ethambutol ranged from 4 to 16 in various combinations.

The ethambutol-induced increase in sensitivity to antibiotics was retested in separate experiments (Fig. 3), with chosen concentration of antibiotics. This confirmed the synergistic effect of growth inhibition.

β-sitosterol transformation by ethambutol treated *M. vaccae* cells. *M. vaccae* B 3805 used in this study is a mutant able to accumulate AD and ADD, intermediates of β-sitosterol degradation process. The formation of AD starts in the 12–24 h period, reaching the highest level at that time. From four tested concentrations (3; 10; 20; 50 μg ml^{-1}) 10 μg ml^{-1} of EMB appears to be the most effective inhibitory concentration, since the amount of product accumulation increased in compare to the control culture during the period of transformation.

The most appropriate quantity presentation for the transformation effectiveness is the ratio of AD produced per g dry biomass in successive 12-h intervals of the process (Fig. 4).

The lowest and highest concentration of ethambutol did not significantly change the accumulation of β-sitosterol degradation intermediates in compare to control cultures. However, the concentration of ethambutol 10 μg ml^{-1} resulted in accumulation of 36 mg AD gd.w.$^{-1}$ in compare to the 17,9 mg AD gd.w.$^{-1}$ accumulated in the control culture in the 12–24 hour period. This indicate that cells exposed to 10 μg ml^{-1} of EMB were almost 2-fold more active.

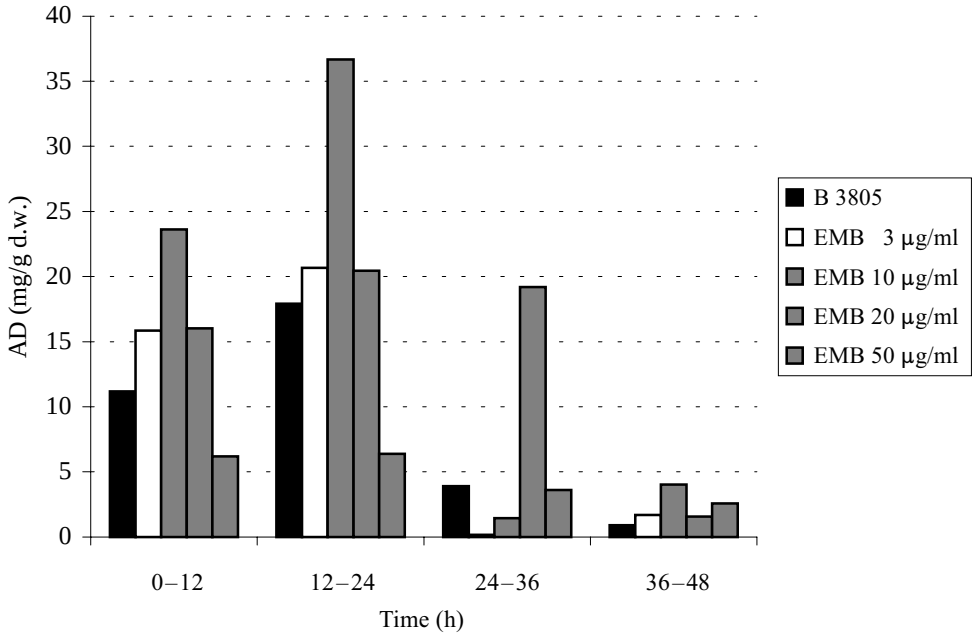

Fig. 4. The activity of *Mycobacterium vaccae* B 3805 cells in β-sitosterol transformation at successive 12-h intervals. The quantity of AD(D) formed during each 12-h period was divided by the cell biomass reached at the end of the period.

The drug susceptibility test showing increased permeability of the cell wall for hydrophobic compounds together with the results obtained in AD(D) accumulation analysis strongly suggest, that the arabinogalactan layer is an important barrier in efficient transformation of steroids by mycobacteria.

Discussion

The mycobacterial cell wall is an effective permeability barrier for both lipophilic and hydrophilic substances, including those of great importance in biotechnology. The fundamental impermeability of the mycobacterial cell is determined by the native structure of cell wall skeleton (CWS), which is composed of mycolic acids, peptidoglycan and arabinogalactan (Daffe and Draper, 1998; Nikaido *et al*., 1993). Mycolic acids play a crucial role in the permeation compounds through the mycobacterial cell wall envelope. Using mutants lacked 50% of cell-wall-bound corynemycolates (Puech *et al.*, 2000) or mutant strain of *M. tuberculosis* H37Rv with an inactivated *hma* gene (Dubnau *et al.*, 2000) autors shown significant changes in the permeability of inner layer to hydrophobic compounds.

The permeability of the mycobacterial cell wall can be changed by means of partial disintegration of its components. Rastogi *et al.* (1990) using m-fluorophenylalanine and D,L-norleucine as a specific inhibitor outer layer were able to demonstrate increased drug entry into the cells of *Mycobacterium avium*, resulting in enhanced susceptibility to hydrophobic antibiotics. The results obtained in our previous study with m-fluorophenylalanine and D,L-norleucine suggest that *Mycobacterium vaccae* complex lipids forming the outer leaflet of the cell wall bilayer may contribute to the permeability barrier (Rumijowska-Galewicz *et al.*, 2000).

We have also shown that glycine disturbed the peptidoglycan structure and caused partial disintegration of mycolic acids as well (Lisowska *et al*., 1996; Sedlaczek *et al*., 1999). The enhanced uptake of β-sitosterol and hydrophobic antibiotic (rifampicin and erythromycin) in the presence of polycations (protamine, polymyxin B nonapeptide and polyethyleneimine) was accompanied by disorganization of non-covalently bound lipids (Korycka-Machała *et al.*, 2001).

According to our best knowledge the effect of ethambutol on penetration of β-sitosterol through mycobacterial cell wall has not been described yet.

Ethambutol is one of the drugs recommended for the treatment of disease caused by *Mycobacterium tuberculosis* as well as opportunistic infections of AIDS patients caused by *Mycobacterium avium* complex (Häusler *et al.*, 2001). Ethambutol is known to rapidly inhibit biosynthesis of the arabinan component of the mycobacterial cell wall core polymer, arabinogalactan (Takayama and Kilburn, 1989; Mikusova *et al.*, 1995; 2000). A possible target for the action of ethambutol is one or several of the arabinosyl transferases

involved in the formation of the diverse motifs of arabinan (Belanger *et al.*, 1996; Deng *et al.*, 1995; Han *et al.*, 2003; Kordulakova *et al.*, 2003; Pathak *et al.*, 2004; Ramaswamy and Musser, 1998).

Synergistic activities of antituberculous drugs with ethambutol are well documented (Kaur and Khuller, 2001; Schiavano *et al.*, 2001). Rastogi *et al.* (1990; 1998) showed that ethambutol and other inhibitors (m-fluorophenylalanine, cerulenin and *trans*-cinnamic acid) caused significant enhancement of *M. avium* and *M. tuberculosis* drug susceptibility.

We have previously reported that one of the limiting factors inhibiting the steroid biotransformation process by mycobacteria is permeability of the cell wall. In this paper we have shown that arabinogalactan layer is one of the important barrier for steroids substrates. The inhibition of the arabinogalactan biosynthesis results in double accumulation of the AD(D) intermediates. The earlier study show that inhibition of the biosynthesis of peptidoglycan layer increases AD(D) accumulation twice (Sedlaczek *et al.*, 1999). In addition, changes in organization of free lipids increases β-sitosterol degradation and product formation 3-fold (Rumijowska-Galewicz *et al.*, 2000; Korycka-Machała *et al.*, 2001). It is likely that lower amount of arabinogalactan influences more general composition of the CWS resulting in loss of integrity of arabinogalactan and mycolic acids. Our results indicate that the biosynthesis of arabinogalactan process is a reliable target for inhibitors that would help to intensify the AD(D) accumulation in biotechnological processes.

Acknowledgement. We thank Prof. Dr Leon Sedlaczek for critical comments on the manuscript and discussion. This work was supported by grant 3 PO4C 069 23 of the Committee for Scientific Research.

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