J Ind Microbiol Biotechnol (2008) 35:1235–1239
DOI 10.1007/s10295-008-0419-5

ORIGINAL PAPER

Enhanced biotransformation of sitosterol to androstenedione by *Mycobacterium* sp. using cell wall permeabilizing antibiotics

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Received: 24 March 2008 / Accepted: 27 July 2008 / Published online: 21 August 2008
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Abstract Mycobacterial cell wall is rigid and offers a high resistance to the transport of sitosterol into cytosol. The effect of ethambutol, penicillin, polymixin and bacitracin on biotransformation of sitosterol to androstenedione by modification of cell wall permeability was examined. Drug sensitivity assay results established that bacitracin increased the permeability of the cell wall to hydrophobic compounds. Growth inhibitory study of bacitracin and rifamycin, individually as well as in combination showed that these two antibiotics act synergistically to reduce cell growth. A comparison of transmission electron micrograph results of the bacitracin-treated cells with untreated cells, revealed deformities caused in the cell wall structure by bacitracin treatment. These deformities increased the cell wall permeability and transport of sitosterol inside the cell, and thus enhanced androstenedione (AD) production. A maximum of 1.37, 1.44, 1.65 and 1.76 g AD per gram dry cell weight of mycobacterial cells was produced in the presence of ethambutol, penicillin, polymixin and bacitracin, respectively. Below the minimum inhibitory concentration, bacitracin can be used as potent enhancer of permeability of hydrophobic substances across the mycobacterial cell wall.

Keywords Androstenedione · Antibiotics · Biotransformation · Cell wall permeability · Sitosterol

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Introduction

Androstenedione (AD), an immediate precursor of testosterone, is used as the starting material for preparation of various kinds of pharmaceutically important steroids [6]. It is prepared by selective side chain degradation of β-sitosterol, mediated by a cascade of 11 enzymes [12]. As the enzymes involved in sitosterol to AD biotransformation are intracellular, the substrate needs to be transported inside the cell for biotransformation to take place. However, the low solubility (1 µM) of β-sitosterol in aqueous system limits the sitosterol available for biotransformation. The cell wall structure of *Mycobacterium* sp., normally used as biocatalyst, also impedes the transport of sitosterol into the cytosol. Mycobacterial cell wall consists of peptidoglycan, arabinogalactan and mycolic acids, which form the mycobacterial cell wall skeleton. Although mycobacterial cell wall is extremely rich in lipids, it exhibits a high level of resistance to hydrophobic compounds such as sterols. Mycobacterial cell wall forms an asymmetric bilayer, containing a moderately fluid outer leaflet and an inner leaflet of extremely low fluidity. The highly ordered structure of inner leaflet of extremely low fluidity mainly accounts for the low permeability of the mycobacterial cell wall [4]. The rate of penetration of hydrophobic compounds across the highly ordered bilayer is about one-hundredth the rate through the normal glycerophospholipid bilayer [8, 13]. Barry [1] studied the changes in permeability of mycobacterial cell wall by altering the biosynthesis of cell wall components, which effectively resulted in disruption of structural and physical architecture of mycobacterial cell wall skeleton. Various agents, such as glycine, lecithin, polycations, have been effectively used to improve the cell wall permeability of sitosterol across the mycobacterial cell wall [3, 5, 11, 15]. These results indicate that in the native

state, cell wall skeleton as well as the components of outer most cell wall layer is a permeability barrier for sterols.

In this paper, we present a comparative study of the effect of four different antibiotics, ethambutol, penicillin, bacitracin and polymixin, on sitosterol penetration through mycobacterial cell wall. A drug sensitivity assay with a hydrophobic antibiotic rifamycin was carried out. In addition, transmission electron micrographs of the antibiotic treated cells were compared with untreated cells and the results discussed.

Materials and methodology

Microorganisms and culture conditions

Mycobacterium sp. DSM 2966 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany and maintained on nutrient agar slants. A mutant strain obtained from *Mycobacterium* sp. DSM 2966 was used in the present study. For development of a mutant strain, the parent strain was initially acclimatized to 20 g/l sitosterol concentration by growing them on increasing concentration (2–20 g/l) of sitosterol in a step wise manner. The strain acclimatized to 20 g/l sitosterol concentration, was subjected to NTG mutagenesis. The strain tolerant to 1 g/l AD concentration and exhibiting highest sitosterol biotransformation ability was used in experiments. The basal culture medium for cell growth was prepared using reconstituted nutrient broth to which 0.2 % (v/v) tween 80 was added. Cultures for all experiments were grown at a temperature of 30 °C and a constant agitation speed of 200 rpm in an orbital shaking incubator (Orbitek, India). The nutrient broth (Himedia, India) used, contained 5 g/l peptic digest of animal tissue, 5 g/l sodium chloride, 1.5 g/l beef extract and 1.5 g/l yeast extract.

Sitosterol biotransformation study in the presence of different cell wall permeabilizing antibiotics

Biotransformation experiments were carried out in 250-ml flasks containing 20 ml of the basal culture medium supplemented with 2 g/l of β-sitosterol (Acros, USA). For each of the four different antibiotics studied, namely, bacitracin, ethambutol, penicillin and polymixin, flasks were prepared containing these antibiotics individually in concentrations of 2, 4, 6, 8 and 10 mg/l. The flasks were inoculated with mycobacterial cells (2 ml culture containing approximately 1.12 g/l dry cell weight) and incubated at 30 °C with constant shaking at 200 rpm for 72 h. Cells required for biotransformation experiments were obtained from 48-h cultures of the mutant *Mycobacterium* sp. DSM 2966. These cultures were prepared in 250-ml flasks containing 50 ml of culture medium that were inoculated from 48 h agar slants. Experiments were carried out in duplicate.

Extraction and analysis

For extraction of the metabolites produced by biotransformation of sitosterol, 5 ml of the culture broth was extracted twice for 2 h on each occasion, with equal volume of chloroform. The organic phase was pooled together and evaporated to dryness. The solid residue thus obtained was dissolved in 5 ml of ethanol. This was filtered through a 0.45-μm nylon syringe filter and transferred to sampling vial.

The extracts were spotted in 2 μl aliquots along with internal AD standards, on Kieselgel 60 F_{254} fluorescent thin layer chromatography (TLC) plates as 5-mm bands using a Linomat V sample applicator (Camag, Switzerland). Linear ascending development, with 1:1 v/v composition of chloroform and diethyl ether as mobile phase, was performed in a 20 cm × 10 cm twin trough glass chamber (Camag, Switzerland). Developing chamber was previously saturated with mobile phase for 30 min at room temperature (25 ± 2 °C). The plates were developed upto a distance of 80 mm. After development, the TLC plates were dried using a hair dryer. AD was observed as black spots on a yellow green fluorescent background. Densitometric scanning of these TLC plates was performed at 251 nm with a Camag TLC Scanner III in absorbance mode operated by WinCats software (Version 1.2.0). The concentration of AD in the samples was determined from the internal AD standard calibration curve.

Drug sensitivity assays

Drug sensitivity assays were carried out using bacitracin and rifamycin. In one set of experiments, mutant *Mycobacterium* sp. DSM 2966 was grown in basal medium containing 2, 4, 6, 8 and 10 mg/l bacitracin while the other set was performed in basal medium containing 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mg/l rifamycin. Cells grown in basal medium served as the control in each set. The synergistic effect of bacitracin and rifamycin was examined by growing the mutant *Mycobacterium* sp. DSM 2966 in basal medium containing 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mg/l rifamycin and 4 mg/l of bacitracin. All experiments were performed taking 25 ml of the required medium in a 250-ml flask. Flasks were harvested after 72 h and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the cell pellet was washed twice in 0.5 M phosphate buffer (pH 7.2), centrifuged at 10,000 rpm for 10 min. The cell pellet thus obtained was suspended in 2 ml of 0.5 M phosphate buffer and transferred into a pre-weighed aluminum foil cup. The dry cell weight was measured after drying at 90 °C for 12 h. Experiments were performed in duplicate.

Transmission electron microscopy of the bacitracin-treated mycobacterial cells

Mycobacterium sp. cells were grown in basal medium containing 6 mg/l bacitracin along with a control culture without bacitracin. After growing the cultures for 48 h, cells were harvested and quickly washed with 0.1 M phosphate buffer (pH 7.4). These were then fixed in 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were further processed according to the procedure of Fraud et al. [2]. Transmission electron micrographs were recorded in TEM Philips, CM-10 model.

Results and discussion

β-Sitosterol biotransformation by antibiotic-treated *Mycobacterium* sp. DSMZ 2966 cells

Results indicated that, within the tested range of antibiotics concentration (2–10 mg/l), all the four antibiotics favor enhanced AD production as compared to the control. It was observed that the concentration for maximal AD production was different for each antibiotic. An average of 99.1 mg/l AD was produced in control culture at the end of 72 h of biotransformation. Compared to this, a maximum of 153.19, 161.31, 184.69 and 196.85 mg/l AD was produced, respectively, when 8 mg/l ethambutol, 8 mg/l penicillin, 6.0 mg/l polymixin and 6.0 mg/l bacitracin was used (Fig. 1). In terms of AD per unit of dry cell weight, these values, respectively, correspond to 0.87, 1.37, 1.44, 1.65 and 1.76 g AD per gram of dry cell weight. Hence, among these antibiotics, bacitracin induces a suitable degree of cell wall permeabilization.

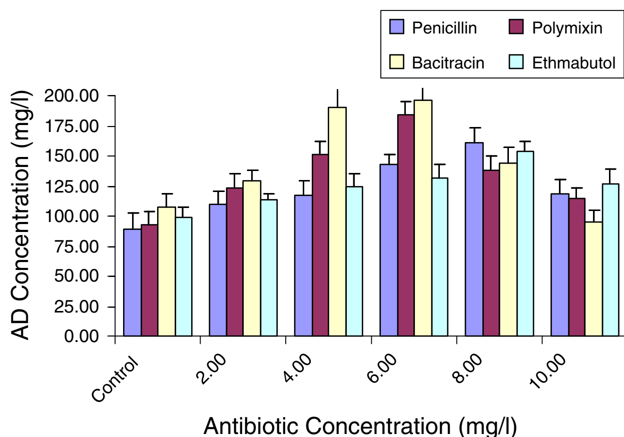

Fig. 1 Biotransformation of β-sitosterol to androstenedione in the presence of varying concentrations of the antibiotics penicillin, polymixin, bacitracin and ethambutol. Control experiment was performed in basal medium without antibiotic addition

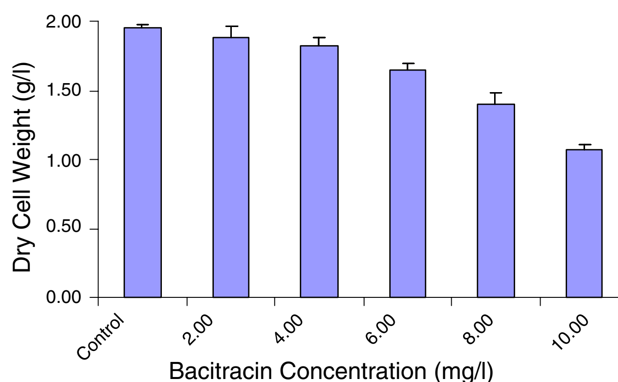

Fig. 2 Effect of varying concentration of bacitracin (2–10 mg/l) on mycobacterial cell growth

Change in mycobacterial cell wall permeability by partial disintegration of its components has been demonstrated by several researchers [5, 10, 11]. The four antibiotics used in this investigation brought about different degrees of permeabilization of *Mycobacterium* cell wall and consequently increased sitosterol transport into the cell. Within the cell, sitosterol is converted to AD by intracellular enzymatic reactions. Therefore, the amount of AD produced was used as a measure of sterol penetration. Based on the results shown in Fig. 1, it was concluded that within the tested range of antibiotics, bacitracin followed by polymixin, was the most potent sterol permeabilizing agent. The role of polymixin B nonapeptide in increasing the cell wall permeability for sitosterol has been reported by Korycka-Machala et al. [5], where it was postulated that binding of polycations with negative charge of the outermost cell wall layer results in an evident disorganization of the native arrangement of noncovalently bound lipids resulting in enhanced uptake of β-sitosterol and hydrophobic antibiotics [5]. Both polymixin and bacitracin are nonribosomal peptides that act nonspecifically as membrane-inserting cationic hydrophobic peptides [14]. Therefore the enhanced sitosterol

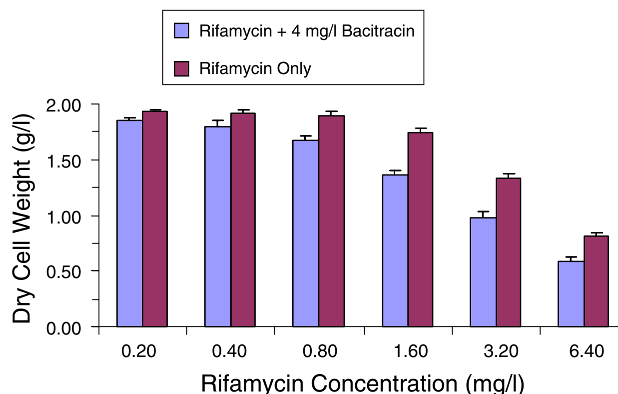

Fig. 3 Effect of varying concentration of rifamycin, individually and in combination with bacitracin (constant at 4 mg/l) on mycobacterial cell growth

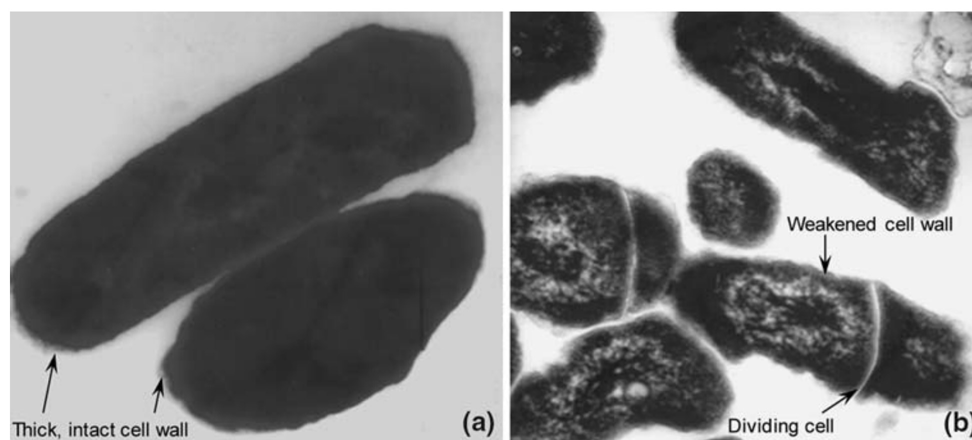

Fig. 4 Transmission electron micrographs of *Mycobacterium* sp. **a** Cells grown in the absence of bacitracin. **b** Cells grown in presence of 6 mg/l bacitracin

biotransformation by bacitracin may be attributed to the mechanism similar to that discussed by Korycka-Machala et al. [5]. In addition to this, bacitracin interferes indirectly with the biosynthesis of bacterial cell wall by inhibiting the dephosphorylation of C55-isoprenyl pyrophosphate (IPP). IPP needs to be dephosphorylated before being reused as a disaccharidepentapeptide carrier. Bacitracin thus leads to deformities in bacterial cell wall synthesis [9]. The cumulative effect on cell membrane and cell wall synthesis is postulated as the probable mechanism for enhanced sitosterol transport rate across the *Mycobacterium* cell wall. This is supported by the observed increase in AD production in presence of bacitracin.

Drug sensitivity assay

To establish the proposition stated in the previous section, that permeabilization of the mycobacterial cell wall by bacitracin increases the transport of hydrophobic compounds, the effect of using hydrophobic antibiotic rifamycin in combination with bacitracin on mycobacterial cell growth was studied. The sensitivity of the *Mycobacterium* sp. in presence of bacitracin and rifamycin, individually and in combination was determined. Cell growth measured after 72 h was used as an indicator of sensitivity in these experiments. An examination of mycobacterial cell growth in basal medium containing 2–10 mg/l bacitracin showed that at 4 mg/l concentration, the growth was reduced by 7%. At concentrations above 4 mg/l, the reduction in growth was more than 15% (Fig. 2). Similar experiments were performed using 0.2–12.8 mg/l rifamycin (Fig. 3). It was observed that with rifamycin, a decrease in growth occurs at concentrations above 1.6 mg/l. This reduction is about −1.9 g dry weight per mg of rifamycin added. However, when examined at the highest concentration of 12.8 mg/l, cell lysis and precipitation occurred and hence data for this point is absent. Considering a 10% reduction in cell growth due to cell wall permeabilization as acceptable for a biotransformation process, 4 mg/l bacitracin concentration was used during the study of the synergistic effect of rifamycin and bacitracin on mycobacterial cell growth.

From Fig. 3, it may be observed that a faster reduction in growth occurs when bacitracin is used in combination with rifamycin. When rifamycin is used individually, a change in slope is observed after 1.6 mg/l rifamycin from −0.08 to −1.9 g dry weight per mg of rifamycin added. The slope changes at a lower concentration of 0.8 mg/l in the presence of 4 mg/l of bacitracin. The degree of growth reduction was observed to be nearly same at −1.9 g dry weight per mg of rifamycin added. Rifamycin is hydrophobic in nature and acts by inhibiting the bacterial RNA polymerase. Therefore, in order to act on RNA polymerase, this hydrophobic antibiotic needs to be transported inside the cell. The reduction in cell growth during synergistic study can be attributed to enhanced transport of rifamycin across the bacterial cell wall due to deformities caused by bacitracin. Hence, a mechanism similar to the transport of rifamycin across the mycobacterial cell wall may be active for sitosterol transport. Drug sensitivity assays have been used as a measure of weakening of the permeability barrier [5, 7, 10, 16]. These results, along with transmission electron micrographs discussed in the following section corroborates that deformities in cell wall may have resulted in enhanced transport of sitosterol across bacterial cell wall.

Transmission electron microscopy of the bacitracin-treated mycobacterial cells

TEM of cells treated with bacitracin was compared to those of untreated cells. The TEM in Fig. 4b, shows a weakened and deformed cell wall of treated cells that are absent in the untreated cells (Fig. 4a). This observation further

establishes the postulate that controlled treatment of bacitracin results in the weakening and partial deformation of mycobacterial cell wall skeleton, resulting in enhanced sitosterol permeability across the cell wall. Presence of dividing cells during the TEM study of bacitracin-treated mycobacterial cells revealed that the used concentration of bacitracin in sub-lethal dose results only in the partial disintegration of bacterial cell wall, and consequently enhanced sitosterol permeability across the cell wall.

Conclusion

It was concluded that below sub-lethal concentration, antibiotics acting on cell wall can be effectively used as mediator of enhanced sitosterol permeability across mycobacterial cell. Bacitracin exhibited the best results among all the tested antibiotics with respect to the enhancement in sitosterol transport across cell wall. Drug sensitivity assay as well as the TEM study has revealed that bacitracin results in deformity and weakening of mycobacterial cell wall. Although the exact mechanism of enhanced transport of sitosterol across the cell wall in presence of bacitracin cannot be given, the available experimental data suggests a common mechanism of transport of hydrophobic compounds through these induced deformities.

Acknowledgments Research fellowship from the Council of Scientific and Industrial Research (CSIR) is deeply acknowledged by A. Malaviya. A partial grant from IIT Delhi and travel grant from Department of Biotechnology (DBT), Government of India, was received for attending BioMicroworld 2007. TEM facility was provided by SAIF, AIIMS, New Delhi.

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