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SHORT COMMUNICATION

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Resistance to androstanes as an approach for androstandienedione yield enhancement in industrial mycobacteria

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Abstract The resistance to androstandienedione (ADD) of industrial mycobacteria was demonstrated as a valuable approach to increasing ADD yield in sterol fermentations. Colonies growing at 1 mg/ml ADD in culture medium after nitrosoguanidine mutagenesis showed a differential behavior in respect to parentals in cholesterol biotransformation. In the presence of exogenous ADD, a substantial depletion of ADD production was observed in parental strains B3683 and Ex4, whereas it was unaffected, and even increased, in resistant colonies. An apparent reduction from ADD to androstandione and testosterone was also noticed. Furthermore, the ADD resistance phenotype may be related to the increase in steroid 1,2 dehydrogenase activity.

Keywords Biotransformation · Steroids
Mycobacteria · Androstanes

Introduction

Selective microbial degradation of the sterol side chain while maintaining the steroidal nucleus intact has enabled the utilization of natural sterols (e.g., cholesterol or β-sitosterol) as raw materials for the production of commercial steroids. The sterol (e.g., cholesterol) breakdown pathway in bacteria is shown in Fig. 1.

Mutants of mycobacteria capable of transforming sterols into steroid precursors such as androstandione (AD) or androstandienedione (ADD) have been used for partial chemical synthesis of various steroids of biomedical interest [1]. These include blocking mutants for the synthesis of the enzyme 9α-hydroxylase. Strategies varying from specific mutant isolation [12] and fermentation medium design as well as modification and solubilization of the substrates have been assayed to improve yields [11].

Although the toxicity of these compounds has been bypassed by the use of adherent resins, which sequester the product [1], the isolation of mutants resistant to ADD or AD has been proposed [10]. Although this strategy has long been used in genetic research, it seems to represent a valuable alternative for the production process. This paper describes the microbiological and biochemical behavior of various mutants resistant to ADD as well as final yields reached in fermentation broth.

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Materials and methods

Microorganisms

Throughout this study, the morphological mutant (Ex4) and the collection strain NRRL-B3683 of *Mycobacterium* sp. were used as parentals [13].

Media and culture conditions

The nutrient broth (NB) medium used contained nutrient broth (oxoid), 0.8%; yeast extract, 0.1%; and glycerol, 0.5% [16]. All cultures were inoculated from single colonies.

For the susceptibility test, ADD was added at different concentrations to NB, which was then inoculated and incubated at 30°C, 200 rpm. After 24 h incubation, optical density at 540 nm was used to measure growth. Fermentations were carried out in NB plus cholesterol (1 mg/ml).

Mutagenesis and selection of mutants

Mutagenesis was carried out as previously described [15]. Briefly, after growing in NB + 1% Tween 80, for desegregating mycobacterial cells, cultures were mutated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (1 mg/ml) in 0.2 M citrate buffer, pH 5.6, 30°C,

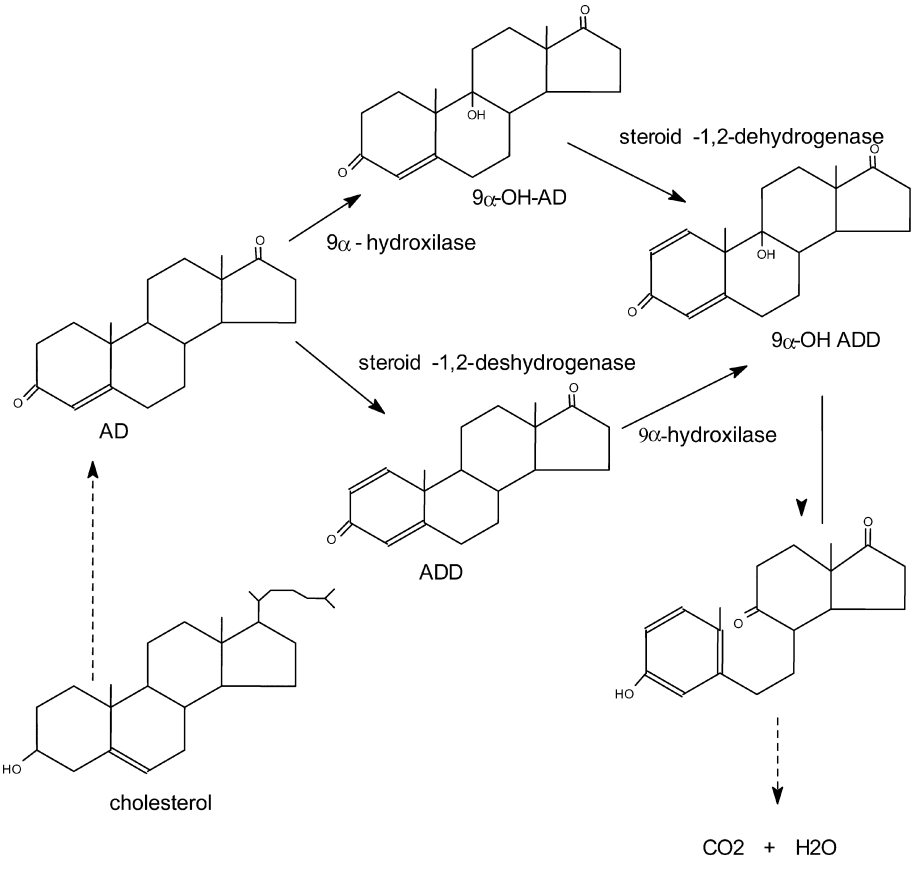

Fig. 1 Generalized pathway of steroid ring degradation by bacteria [16]

for 30 min, at survivals up to 10%. Colonies that grew well on NB plates containing ADD (1 mg/ml) were picked and subcultured.

Fermentation experiments and chemical analysis of products

Protocols for biotransformation were carried out as previously described [13]. Cells were pre-grown in NB medium for 48 h, 200 rpm; one-tenth (v/v) from a common culture was then added to Erlenmeyer flasks containing 50 ml fermentation medium. After a 5-day incubation at 30°C, samples were autoclaved and extracted with ethyl acetate. For further chemical analysis of steroids an RP-8 LiChoCART 125 mm ×4 mm column was employed with a mixture of 65:35 methanol:water and a flow-rate of 1.5 ml/min. Detection was at 254 nm wavelength and 17-a-methyl-testosterone was used as an internal standard.

Enzyme activity determinations

To determine steroid-1,2-dehydrogenase activity, crude extracts were prepared by re-suspending cells in 0.1 M Tris-HCl buffer, pH 8.2 at 4°C. After sonication, samples were centrifuged and dialyzed on a PD-column. The reaction was carried out at room temperature and contained 100 µl crude extract, 840 µl 0.1 M Tris-HCl pH 8.2 buffer, AD (1.04×10^{-3} M) as substrate; phenazine methosulphate (PMS), 6.53×10^{-4} M was used as an electron acceptor. The reaction was stopped with trichloroacetic acid after 1 h. Proteins were precipitated for 30 min. After centrifugation, the pellet was extracted with methanol and the supernatants were pooled for steroid quantification by HPLC using a reverse phase column (RP-8 LIChroCART; 125 mm ×4 mm) and a methanol:water mixture (60:40) as mobile phase at a flow-rate of 1.4 ml/min. Detection was at 242 nm.

Results

ADD sensitivity test and characterization of resistant mutants

Growth inhibition was observed after incubation of mycobacterium cells in various concentrations of ADD. Inactivation of parental strains became evident even at the lower doses. A value of 1.0 mg/ml ADD was selected as the most suitable for further selection of mutants, corresponding to a survival rate of 3.6%. The frequency of resistant colonies growing on NB + ADD (1 mg/ml) vs NB was evaluated as 1.62×10^{-4}.

Two colonies showing a good appearance were picked for additional studies. Growth response in liquid NB plus 1 mg/ml ADD was different in the presumed resistant mutants with respect to controls B3683 and Ex4 (Fig. 2). The selected colonies, M2 and M10, exhibited better growth. ADD was less toxic to Ex4 than to B3683.

This behavior suggests that resistance to ADD might lead to increasing amounts of the product in sterol fermentation. Such mutants can overcome ADD toxicity, which is imposed in this kind of fermentation. Thus, a biochemical control must be bypassed, possibly by de-repression of a particular enzyme. Similar effects have been described for other systems in different bacteria [4].

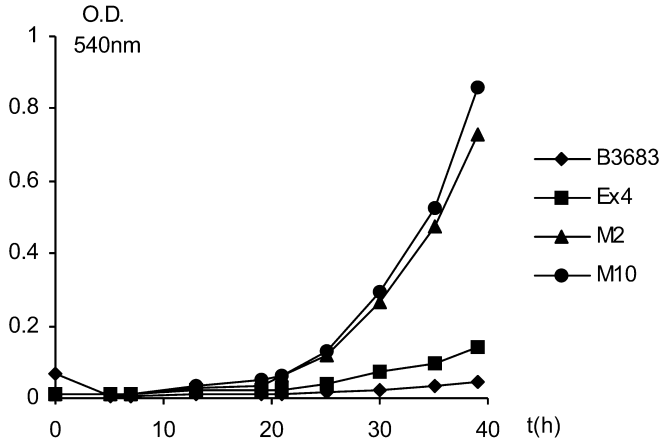

Fig. 2 Growth response of resistant mutants (*M2*, *M10*) and parent strains (*B3683* and *Ex4*) of mycobacteria in the presence of androstandienedione (ADD). Cells were inoculated in nutrient broth (NB) medium plus 1 mg/ml ADD. Growth was followed by measuring optical density at 540 nm. Each value represents the average of three independent determinations

In order to support this assumption, an additional experiment was carried out. Parentals and mutants (M2 and M10) were pre-grown in liquid NB and tested for bioconversion of 50 mg cholesterol in the presence of exogenous ADD at the inhibitory concentration, 1 mg/ml (Table 1).

Bioconversion from cholesterol to ADD seemed to be strongly affected in strains B3683 and Ex4 after the addition of extra ADD. On average, final amounts of ADD were lowered from around 16 mg to 5 mg, suggesting a greater reduction to AD and testosterone of exogenous ADD. In contrast, ADD formation in mutants M2 and M10 was apparently unaffected, amounts being similar with or without extra ADD. Therefore, the resistance to ADD in these mutants may be related to regulatory control of a key enzyme or enzymes of the steroid breakdown pathway.

Reduction from ADD to AD and testosterone was also remarkable in mutant M2, in the presence of extra ADD. Nevertheless, reduction was depressed in mutant M10, leading finally to higher ADD yields.

Table 1 Cholesterol (50 mg) bioconversion in the presence of exogenous androstandienedione (ADD). All cultures were inoculated at an optical density at 540 nm of 1.0. Values (in milligrams) represent the mean of three independent determinations. (*AD*) Androstandione, *TES* testosterone

Strain	−ADD[a]			+ADD[a]		
	ADD	AD	TES	ADD	AD	TES
B3683	14.60	2.97	0	5.28	20.97	11.96
Ex4	17.47	2.51	0	5.20	22.10	9.00
M2	16.11	2.24	0	14.57	27.37	2.96
M10	18.81	1.92	0	38.99	9.87	1.79

[a]Biotransformation without (−) or with (+) 1 mg/ml ADD in 50 ml nutrient broth (NB)

Table 2 Specific activity of steroid 1,2 dehydrogenase

Strain	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]
B3683	60.7
Ex4	62.6
M2	78.8
M10	88.6

The finding that AD arises from ADD reduction in mycobacteria [17] and the behavior of 17-oxido reductase activity (data not shown) also supports these results.

On the other hand, the steroid 1,2-dehydrogenase activity of parental strains and mutants (Table 2) must be associated with a higher ADD yield [8]. Both M2 and M10 exhibited a higher specific activity than parental strains, which is partially in agreement with control biotransformation experiments.

The resistance to ADD may be due to an alteration in the regulation of the enzymes involved in steroid metabolism. Reduction to AD and testosterone could affect the expression of the trait, so that the best ADD accumulating mutant must lack this reduction activity.

Discussion

Low ADD yields in sterol fermentations may be due to the well-known toxicity of this molecule to microbial cells [10]. This effect seems to be related to a block at the level of the respiratory chain in *Nocardia corallina* [9], as well as of any of the first enzymes in the sterol degradation pathway in *Pseudomonas testosteroni* [18].

Hesselink et al. [6,7] reported that cyclodextrins improve AD and ADD yields in *Mycobacterium* sp. NRRL B-3683 by inhibiting side oxidation of sterol ring structures, or by sequestering ADD in fermentation broth. Although some authors have suggested that ADD or AD toxicity might cause cell death and lysis, the actual nature of this toxicity has not been elucidated, nor how the microbial cell might bypass the effect [14].

One of the most important and well-studied enzymes in microbial sterol metabolism is Δ-1,2 steroid dehydrogenase, which introduces a 1,2 double bond in the A-ring of the steroidal molecule [5]. The enzyme has been described in *Cylindrocarpo radicicola*, *P. testosteroni*, *Mycobacterium* sp. and *Nocardia* sp. [2] and used extensively for producing the 1-dehydro derivatives of cortisone and cortisol, i.e., prednisone and prednisolone.

Direct ADD reduction to AD, testosterone and dehydrotestosterone has been demonstrated in *Mycobacterium* sp. NRRL B-3683 [14]. These authors also reported 1-dehydrotestosterone yield enhancements in ADD-adapted mycobacterial cells.

The frequency of colonies resistant to ADD (1.62×10^{-4}) was in agreement with those figures reported previously for some other induced mutations in bacteria [4]. Furthermore, they were stable as the resistant phenotype remained after subculturing.

Mutants resistant to ADD were demonstrated to harbor a distinctive behavior in respect to parentals in the presence of exogenous ADD (Table 1), although it is not possible to distinguish exogenous ADD from the ADD produced by cholesterol conversion.

According to our results, the mutation must enable a mycobacterial cell to not only overcome ADD toxicity, but also to diminish ADD reduction to AD and testosterone, thereby raising final ADD yield. The higher 1,2 steroid dehydrogenase activity of both mutants (Table 2) also contributes to better yields. The screening of new industrial strains having a higher 1,2-steroid dehydrogenase activity has been one of the most promising trends in steroid biotransformation protocols [3].

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