Induction of Ethanol Tolerance on the Production of 17-Ketosteroids by Mutant of *Mycobacterium* sp.

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Tolerance of *Mycobacterium* sp. against organic solvents has been induced for the cholesterol side chain degradation by adding chemicals associated with synthesis of fatty acids or alcohols. Biotin of 300 μg/l and 0.5% aqueous ethanol solution were optima for the enhancement of ethanol tolerance of the microorganism. The induction of ethanol tolerance by biotin was found to be due to increase of degree of unsaturation of the fatty acids in membranous phospholipid of the cell, especially due to increase of oleic acid content. However when 0.5% of ethanol was added for the ethanol tolerance induction, there was an ambiguous correlation between ethanol tolerance and degree of unsaturation of the fatty acids, in spite of the fact that the induction increased the content of unsaturated fatty acids. Addition of 0.5% of ethanol induced several ethanol shock proteins having molecular weight similar to that of heat shock proteins.

Key words: *Mycobacterium* sp., cholesterol, AD, ethanol tolerance, unsaturated fatty acid, ethanol shock protein

Sterols, abundant and cheap, can be degraded to produce pharmaceutical steroids. A major problem in the sterol side chain degradation is the extremely low solubility of substrates and products in aqueous reaction medium. Solubilities of steroids in water mostly range from 0.1 to 0.01 mM and for sterols, it is even lower than 1 μM (12). Therefore organic solvents are inevitably used for sterol side chain degradation. Increase of the substrate solubility in aqueous medium has been achieved by the addition of water miscible or water immiscible solvents. However the reaction rates were significantly slowed down due to the presence of the organic solvents. These phenomena were mainly caused by conformational changes imposed on the intracellular enzyme by the solvents as well as dissolved oxygen content in the reaction medium (9, 17). Several steroid bioconversions have been also carried out in the presence of water-immiscible organic solvents (5, 14). However, the sterol side-chain degradation, being a multienzyme process, has proven to be much more sensitive to the hydrophobic solvent toxicity (10).

Saccharomyces cerevisiae was found to be tolerant to ethanol after it had been anaerobically preexposed to ethanol solution (24), and *Pseudomonas putida* cells adapted to supersaturated concentrations of toluene showed an increased tolerance for ethanol (13). The organic solvent toxicity could be reduced by using the organic solvent-tolerant micoorganisms. Cholesterol side chain degradation to produce 6β-hydroxycholest-4-ene-3-one, cholest-4-ene-3,6-dione has been reported using mutant cells of *Pseudomonas* sp. (2). However there is little information on sterol side chain degradation by *Mycobacterium* sp. cells having induced organic solvent tolerance. Cellular stress tolerance is known to be associated with induction of the synthesis of highly conserved set of proteins, called heat shock proteins. Different stress, including heat, ethanol, UV, heavy metals, sodium arsenite shock also promote the synthesis of different subsets of these shock proteins (21, 23).

Mycobacterium sp. NRRL B-3683 ioslated from soil lacking 9α-hydroxylase produced androstadiene-3,17-dione (ADD) (18). After further u.v. irradiation on NRRL B-3683, a new mutant NRRL B-3805 was isolated which lacked the ability to 1-dehydrogenate the steroid and was found to accumulate androstenedione (AD) in significant amounts (18).

In this paper, the organic solvent tolerance of *Mycobacterium* sp. NRRL B-3805 has been induced by adding chemicals associated with synthesis of fatty acids or alcohols. Relationship between organic solvent tolerance and

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phosphoryl fatty acid composition as well as shock protein synthesis was exploited.

Materials and Methods

Strain and media

A bacterial strain, *Mycobacterium* sp. NRRL B-3805 was obtained from the KCTC. The composition of stock culture medium was yeast extract (1 g/l), peptone (5 g/l), glycerol (20 g/l) and agar (20 g/l). The bacteria were grown aerobically in a liquid medium having composition equal to that of stock culture medium at 30℃ to an early-exponential phase.

Cholesterol side chain degradation and subtrate or product analysis

Minimal medium (NH_4NO_3 1 g/l, K_2HPO_4 0.25 g/l, KH_2PO_4 0.4 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, $FeSO_4 \cdot 7H_2O$ 0.05 g/l and glycerol 20 g/l) was used for cholesterol side chain degradation reaction. A cell suspension containing 0.15 g (dry weight) cell was added to the minimal medium to be incubated for 5 days at 30℃, pH 7.2. Cholesterol concentration was 1 g/l. The reaction were terminated by the addition of ethyl acetate. The cholesterol and AD were extracted by vortexing; the ethyl acetate phase was separated, dried; and the residues resuspended in LC eluent (n-heptane : ethanol=95 : 5) were analyzed by HPLC (Waters, 600) on a Linchrosorb Si 60 column (Merck) at 214 nm. As a result, LC chromatogram was obtained (Fig. 1). Internal standard of cholesterol and AD were purchased from Sigma Chemical Co.

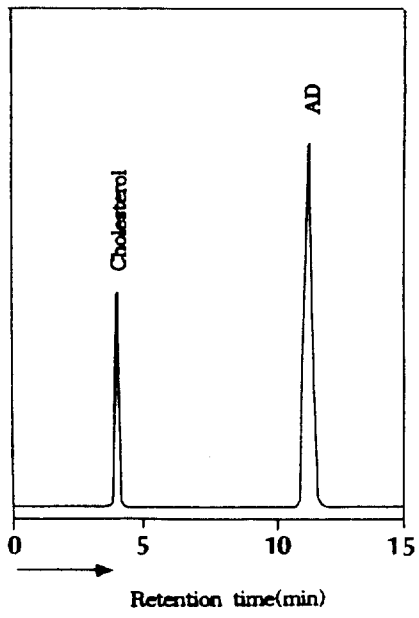

Fig. 1. LC chromatogram of cholesterol and AD.

Induction of ethanol tolerance

For induction of ethanol tolerance, oleic acid (0.1 g/l), linoleic acid (0.1 g/l), linloenic aicd (0.1 g/l), Tween 80 (0.1 g/l), $CaCl_2$ (0.0075 g/l), $CoCl_2$ (0.0075 g/l), or biotin (300 μg/l) was added to the reaction medium containing cells grown for 48 hours. The reaction medium was incubated for another 5 hours.

0.5, 1, 2, 4% of ethanol, methanol, 0.05, 0.1, 0.2, 0.4% of propanol, butanol or 6.25×10^{-3}, 1.25×10^{-2}, 0.25×10^{-1}, 0.5×10^{-1}% of pentanol was added to the culture incubated for 50 hours. The culture was allowed to grow for additional 3 hours in the presence of alcohols to induce the ethanol tolerance.

Fatty acid analysis

Phospholipid of ethanol tolerant strain was extracted according to the procedure reported by Beavan *et al.* (4). The fatty acids of phospholipid was methylated following Allen and Good's method (1). A composition of methylated fatty acids was analysed by GC on a Shimadzu HiCap-CBP 20 under N_2 flow of 40 ml/min and column, injection port, and detector temperature of 180, 220, 230℃ respectively.

Isolation of total protein

To investigate the difference of total protein pattern between ethanol-tolerant strain and native strain, 0, 0.5, 1, 2, 4% ethanol was added to the culture at early log phase. Cell extract was prepared according to the method proposed by Chart (7) with some modifications. Protein samples (20 μg) were analysed by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with Coomassie brilliant blue solution for 1~2 hours and then destained with a destaining solution (10% acetic acid sol.). The stained gels were scanned with a densitometer (LKB 2202) at 630 nm.

Results and Discussion

Effect of ethanol presence on cholesterol side chain degradation

Fig. 2 shows yield of AD produced from the cholesterol side chain degradation as a function of ethanol concentration. The amount of AD produced in a reaction medium containing 5% of ethanol was three times larger than in a solution free of organic solvents. However further addition of ethanol reduced gradually the AD production. Therefore it can be said there exists optimum concentration of organic solvents for the side chain degradation. Aono *et al.* (2) used mixture of p-xylene and diphenylmethane for the cholesterol side chain degradation by *Pseudomonas* sp. They found the microorganism

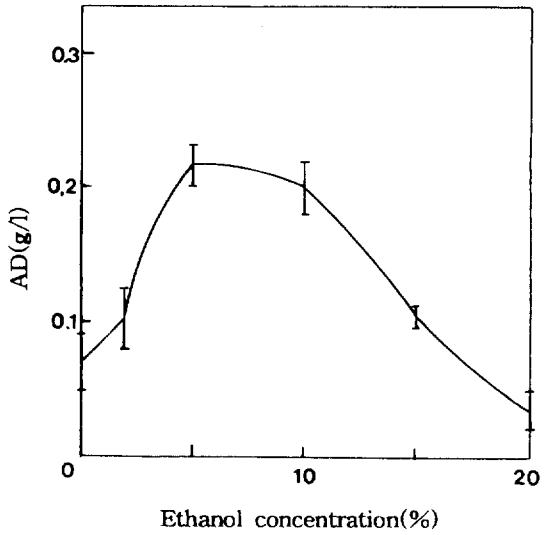

Fig. 2. Production of AD as a function of ethanol concentration in the medium of cholesterol side chain degradation.

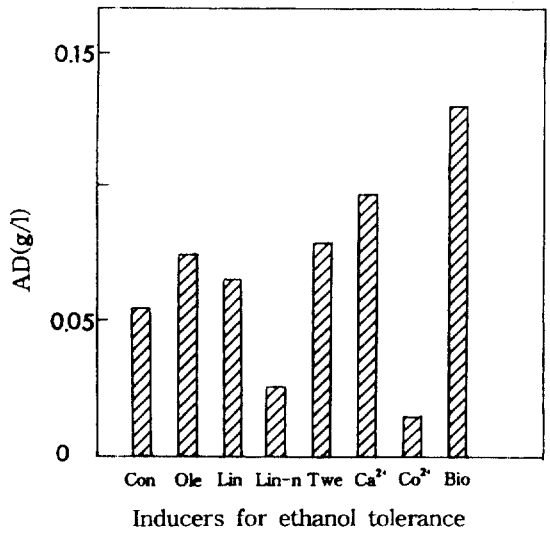

Fig. 3. Induction of ethanol tolerance by chemicals associated with synthesis of fatty acids. Con: control, Ole: oleic acid, Lin: linoleic acid, Lin-n: linolenic acid, Twe: Tween 80, Ca^{2+}: $CaCl_2$, Co^{2+}: $CoCl_2$, Bio: biotin.

transformed almost completely the substrate into the product in less than 8 hours in the presence of the organic solvent, while absence of the organic solvent slowed down the transformation reaction so that only 25% of the substrate was consumed during the same period of time.

Induction of ethanol tolerance by chemicals associated with synthesis of fatty acids

Mycobacterium sp. was liquid cultured in the presence of chemicals associated with synthesis of fatty acids, such as oleic acid, linoleic acid, linolenic acid, Tween 80, Ca^{2+}, Co^{2+} and biotin, to induce tolerance against ethanol. Results of the cholesterol side chain degradation using the cells thus cultivated are demonstrated in Fig. 3. The cells without any chemical pretreatment produced 0.054 g/l of AD, while the cells pretreated to induce ethanol tolerance by oleic acid, Tween 80, Ca^{2+} and biotin yielded respectively 1.4, 1.5, 1.8 and 2.4 times larger amount of AD than the native control strain to proving biotin to be the most effective chemical for the tolerance induction. D'Amore and Stewart (8) found externally added fatty acids during alcohol fermentation process were absorbed in membranous phospholipid of the cell affecting the composition of fatty acids in cell membrane, which had close relationship with organic solvent tolerance. Nabais *et al.* (20) ascribed the positive effect of Ca^{2+} on the solvent tolerance to its deposition on the surface of yeast cells to protect cell membrane from direct contact of organic solvents. Biotin (8) and Co^{2+} (11) are known to promote synthesis of phospholipid of the cell, and Tween 80 (3) abounds in oleic acid to accelerate synthesis of unsaturated fatty acids in the cell membrane. Mishra *et al.* (19) showed fatty acids with multiple double bonds were more effective than those having single double bond for induction of ethanol tolerance of *Saccharomyces cerevisiae.* However, in the present study, order of efficacy of fatty acids for the ethanol tolerance induction was found to be oleic acid (18 : 1)>linoleic acid (18 : 2)>linolenic acid (18 : 3).

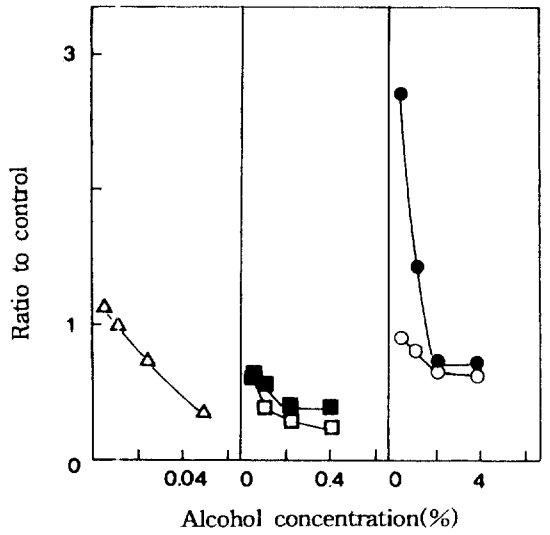

Fig. 4. Induction of ethanol tolerance by alcohols. ○: methanol, ●: ethanol, □: propanol, ■: butanol, △: pentanol.

Induction of ethanol tolerance by alcohols

Alcohols having different chain length were employed for the induction of ethanol tolerance. Fig. 4 gives the side chain degradation results of cells pretreated with

the alcohols. Ethanol at 0.5% concentration was found to be the most efficient agent for the induction of solvent tolerance, shown by the 2.8 times larger quantity of AD produced in the cultivated cells compared to the native control cells. Further increase in ethanol concentration diminished the effectiveness of the solvent tolerance induction. Alcohols other than ethanol were found to be far less effective than ethanol.

Composition of membranous phospholipid in the cell

Biotin and Ca^{2+}, which were proven to be efficient inducers for the ethanol tolerance (Fig. 3), affected the composition of fatty acids in membranous phospholipid of the cell, as collected in Table 1. Biotin and Ca^{2+} enhanced the degree of unsaturation of the fatty acids from 0.3 (control) to 0.49 and 0.48 respectively. Effectiveness of inducers for the ethanol tolerance has a strong correlation with the content of oleic acid, so that increase of the oleic acid content should be one of the most important factors for the ethanol tolerance induction. Phospholipid of the cell became enriched in unsaturated fatty acids as a result of pretreatment with alcohols. The degree of unsaturation of the fatty acids increased as the carbon number of the alcohols rose from 1 to 3, and then decreased with further increase in the alcohol carbon number (Table 2). The proportion of oleic acid to total unsaturated fatty acids increased considerably. However the oleic acid content didn't show any definite correlation with effectiveness of the ethanol tolerance induction by alcohols. Cells pretreated with biotin and 0.5% ethanol yielded respectively 2.4 and 2.8 times larger amount of AD than the native control cells (Fig. 3, 4). However Table 1 and 2 show that increase of oleic acid content was unexpectedly low, in taking into account of the efficacy of the ethanol tolerance induction of the two chemicals. Composition change of fatty acids in phospholipid of the cell owing to the ethanol tolerance induction by alcohols should be ascribed to one of adaptation mechanisms of biomembranes for alcohols. It can be said that the composition change was not a main reason for the induction of ethanol tolerance by alcohols. Ingram (15) pretreated *Escherichia coli* with various alcohols. He observed alcohols having carbon number of 1~4 increased oleic acid content and decreased palmitic acid content to bring about enhanced degree of unsaturation of the fatty acids. However antagonistic results were obtained in the case of pretreatment with long chain alcohols (chain length 5~10). Short chain alcohols could be inserted not only into the exterior of the lipid bilayer but also into the hydrophobic interior. These small alcohols could fill many of the gaps within the hydrophobic layer caused by unsaturated fatty acids and fatty acid-protein interactions. Insertion of small alcohols into gaps within the hydrophobic interior would restrict the motion of fatty acid chains. On the contrary long chain alcohols were too large sterically to fill gaps in the hydrophobic membrane interior, introducing additional gaps in phospholipid packing, thus allowing less restricted movement of the fatty acid chain, and thereby increasing membrane fluidity. The increase in saturated fatty acids would act to compensate for the presence of the long chain alcohols, while unsaturated fatty acid

Table 1. Concentration of fatty acids in phospholipid of *Mycobacterium* sp. treated with various chemicals associated with synthesis of fatty acids for the induction of ethanol tolerance.

Fatty acids	Concentration (%) of fatty acids			
	Control	Biotin	Ca^{2+}	Co^{2+}
Myristic acid	2	3	2	2
Palmitic acid	29	29	31	31
Palmitoleic acid	6	11	11	6
Stearic acid	17	10	11	16
Oleic acid	24	38	37	24
Arachidic acid	8	5	4	8
Behenic acid	11	4	4	10
Degree of unsaturation	0.30	0.49	0.48	0.30

Table 2. Concentration of fatty acids in phospholipid of *Mycobacterium* sp. treated with various alcohols for the induction of ethanol tolerance.

Fatty acids	Concentration (%) of fatty acids					
	Control	Methanol	Ethanol	Propanol	Butanol	Pentanol
Myristic acid	2	2	1	3	2	3
Palmitic acid	29	28	28	30	31	30
Palmitoleic acid	6	9	10	11	9	7
Stearic acid	17	14	15	10	15	15
Oleic acid	24	30	34	38	28	27
Arachidic acid	8	7	7	4	8	7
Behenic acid	11	8	6	4	7	9
Degree of unsaturation	0.30	0.39	0.44	0.49	0.37	0.34

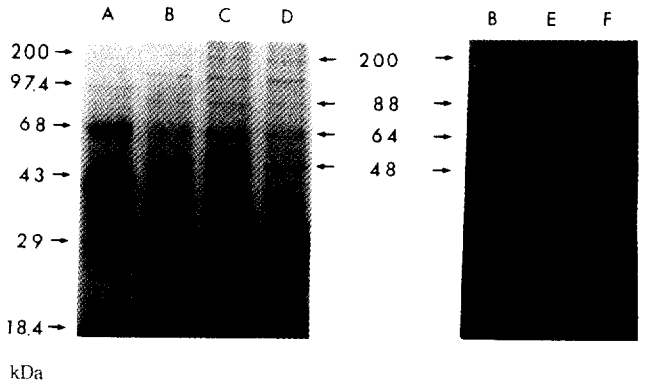

Fig. 5. SDS-polyacrylamide gel electrophoresis of total proteins produced by *Mycobacterium* sp. before and after ethanol stress. Lanes: A, size marker; B, control; C, 0.5%; D, 1%; E, 2%; F, 4% ethanol stress.

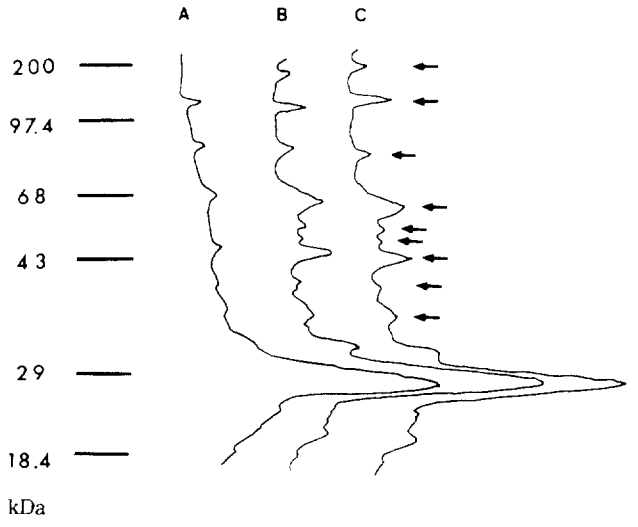

Fig. 6. Densitometric tracing of SDS-PAGE of total proteins produced by *Mycobacterium* sp. before and after ethanol stress. The arrows indicate induced shock proteins. A, control; B, 0.5%; C, 1% ethanol stress.

content increased in case of short chain alcohols for the same reason.

Protein pattern of native and ethanol tolerant strains

Relationship between induction of ethanol tolerance and synthesis of shock proteins was examined by extracting total proteins of cells pretreated with ethanol through SDS-PAGE. Synthesis of proteins having molecular weight less than 30 kDa was inhibited and presence of several bands corresponding to proteins of higher molecular weight was observed by the naked eye (Fig. 5) as a result of pretreatment with ethanol above 0.5%. Analysis by densitometer confirmed enlarged peak area of the corresponding bands (Fig. 6). This can be attributed to the fact that the ethanol shock inhibited synthesis of some proteins while accelerated synthesis of the shock proteins in the cell. Molecular weights of shock proteins were estimated to be 200, 88, 64 and 48 kDa compared with standard proteins (Fig. 7). Proteins of 88 and 64 kDa were synthesized as heat shock proteins of *Vibrio cholerae* and *Bacillus subtilis* respectively (6, 22). 48 kDa protein was also reported to be a heat shock protein (16, 22). Proteins from native and ethanol tolerant strain differed from each other especially the 200 kDa band. However they could correspond to some constituents of the cell other than proteins. Heat shock proteins of molecular weight around 200 kDa have not been reported yet. The tolerance induction by 0.5% and 1% ethanol brought about enhanced yield of AD but pretreatment with 2% and 4% ethanol reduced the AD yield in spite of the fact that the shock proteins were synthesized in all cases. Kim *et al.* (16) examined the survival rate of *Campylobacter jejuni* after ethanol treatment. The survival rate decreased rapidly as the ethanol concentration increased from 0~1% to 3%. Nevertheless shock proteins having molecular weight of 90, 66, 60, 45 and 24 kDa were synthesized after treatment even with 5% ethanol.

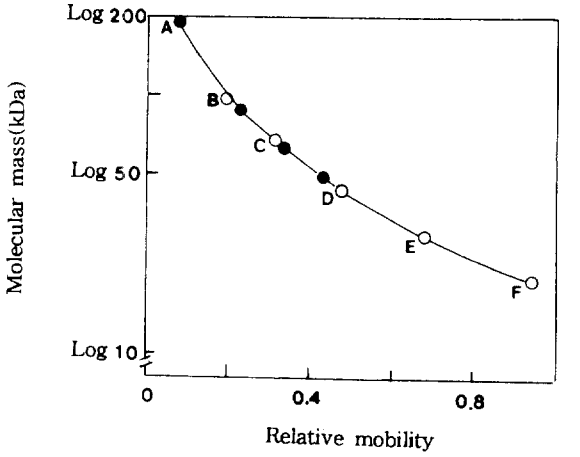

Fig. 7. Determination of molecular weight of ethanol shock proteins. ○: marker proteins, ●: ethanol shock proteins. A, 200; B, 97.4; C, 68; D, 43; E, 29; F, 18.4 kDa.

Acknowledgement

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