Aerobic and Anaerobic Biotransformation of Bile Acids by *Escherichia coli* (I)

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The effects of *Escherichia coli* on the transformation of bile acids were examined in aerobic and anaerobic culturing systems for 4 days. Bile acids were converted to methyl ester dimethylethylsilyl ether derivatives and determined by capillary gas liquid chromatography. *E. coli* oxidized cholic acid (CA) and chenodeoxycholic acid (CDCA) to 3 α ,12 α -dihydroxy-7-oxo-5 β -cholanoic acid (3 α ,12 α 7=O) and 3 α -hydroxy-7-oxo-5 β -cholanoic acid (3 α 7=O), and reduced these oxo-bile acids to CA and CDCA, respectively. These oxidation and reduction reactions occurred in both aerobic and anaerobic cultures, finally attaining certain equilibrium values. The values, or oxidation/reduction ratios, were 9 or more in the aerobic culture, and about 1 in the anaerobic culture. *E. coli* also oxidized taurocholic acid in the aerobic culture, but not in the anaerobic culture.

Key words: *Escherichia coli*; cholic acid; chenodeoxycholic acid; taurocholic acid; *in vitro* biotransformation

INTRODUCTION

Escherichia coli possesses 7 α -hydroxysteroid dehydrogenase (7 α -HSDH) (2, 12). 7 α -HSDH is a non-inducible intracellular enzyme of substrate specificity for bile acids (both free and conjugated) having a 7 α -hydroxyl group (7, 12) and requiring NAD⁺ as a cofactor for the activity (12, 15). Therefore, 7 α -hydroxyl bile acids are oxidized to the corresponding 7-oxo-bile acids when incubated with *E. coli* *in vitro* (1, 9, 13). However, no detectable transformation of endogenous bile acids was observed in gnotobiotic rats inoculated with *E. coli* (4, 10, 17).

We speculated that this discrepancy between *in vitro* and *in vivo* experiments was due to the presence or absence of oxygen, and so we examined the activity of *E. coli* on bile acid transformation in both aerobic and anaerobic culturing systems.

MATERIALS AND METHODS

Chemicals. Cholic acid (CA), chenodeoxycholic acid (CDCA), and taurocholic acid (TCA) were purchased from Sigma Chemicals Co., Missouri, USA. 3 α ,12 α -Dihydroxy-7-oxo-5 β -cholanoic acid (3 α ,12 α 7=O)

and 3 α -hydroxy-7-oxo-5 β -cholanoic acid (3 α 7=O) were prepared by the oxidation of CA and CDCA with *N*-bromosuccinimide (5), respectively. 7 β ,12 β -Dihydroxy-5 β -cholanoic acid ($\beta\beta$), an internal standard for analysis by capillary gas liquid chromatography (GLC), was synthesized as previously described (3). These bile acids were estimated to be more than 96% pure by capillary GLC. Piperidino hydroxy propyl dextran gel (PHP GEL) was purchased from Shimadzu, Kyoto, Japan, and dimethylethylsilylimidazole from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. The other reagents and solvents of analytical grade were obtained from Wako Pure Chemical Industries, Osaka, Japan. If not otherwise stated, the solvents were distilled once before use.

Bacteriological procedures. Two strains of *E. coli* were used. *E. coli* K-12 was obtained from the American Type Culture Collection, Virginia, USA, and *E. coli* HB101 was obtained from Takara Shuzo Co. Ltd., Shiga, Japan. Brain-heart infusion (BHI; Becton Dickinson, Maryland, USA) was used in all tests.

E. coli was precultured aerobically in the BHI medium at 37°C for 1 day. Aliquots (3 $\times$ 10⁷ /10 μ l) of *E. coli* were added to 2 ml of BHI medium containing 1 mmol/l of bile acids and cultured at 37°C for 4 days. Aerobic culturing was performed by standing in a thermostat and anaerobic culturing was performed using the gas-pack method (BBL™ Gas Pak Pouch™ System; Becton Dickinson, Maryland, USA).

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Analytical methods. A portion of the medium cultured, 100 μ l, was treated with 8 volumes of ethanol by warming for 10 min and then filtered. The filtrate was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in alkaline solution (20). After hydrolysis, the reaction mixture was acidified with diluted hydrochloric acid and bile acids were extracted with ethylether. The extracted bile acids were converted into methyl ester dimethylethylsilyl ether (Me-DMES) derivatives as described previously (18, 19). Briefly, bile acids were methylated with diazomethane, and then left in 70 μ l of dimethylethylsilylimidazole in an air-tight vessel at room temperature overnight.

In the case of TCA, bile acids in the medium were

separated to free, glycine-conjugate and taurine-conjugate fractions using a PHP GEL column (18). Glycine- and taurine-conjugate fractions were hydrolyzed in alkali and analyzed by GLC as described above.

3 α -Hydroxy-7-oxo-5 β -cholanoic acid methyl ester was converted to its methoxime derivative as described by Horning et al. (8), and then to a DMES derivative to separately determine this compound and CDCA by GLC.

Capillary gas liquid chromatography. An aliquot of the bile acid derivatives dissolved in *n*-hexane was injected into a gas chromatograph (Model GC-14A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, a solventless injector, and a comput-

Table 1. Relative retention times of bile acid derivatives.

Derivatives	Retention time (min)	Relative retention time
7 β ,12 β -Dihydroxy-5 β -cholanoic acid Me-DMES	12.57 ^{a)}	1.00
Chenodeoxycholic acid Me-DMES		1.16
3 α -Hydroxy-7-oxo-5 β -cholanoic acid Me-DMES		1.16
3 α -Hydroxy-7-oxo-5 β -cholanoic acid Me-MO-DMES		1.05
Cholic acid Me-DMES		1.47
3 α ,12 α -Dihydroxy-7-oxo-5 β -cholanoic acid Me-DMES		1.57
Unknown		1.76

^{a)} This value varies slightly depending on individual experiments.

Me-DMES: Methyl ester dimethylethylsilyl ether.

Me-MO-DMES: Methyl ester methoxime dimethylethylsilyl ether.

Fig. 1. Cholic acid (CA) and 3 α ,12 α -dihydroxy-7-oxo-5 β -cholanoic acid (3 α ,12 α ,7=O) conversions by *Escherichia coli* K-12.

Fig. 2. Chenodeoxycholic acid (CDCA) and 3α-hydroxy-7-oxo-5β-cholanoic acid (3α 7=O) conversions by *Escherichia coli* K-12.

erized data system (Model C-R4A, Shimadzu, Kyoto, Japan). A Hicap CBP-1 capillary column (25 m × 0.25 mm i.d., Shimadzu, Kyoto, Japan) was used. The column temperature was maintained at 285°C and helium was used as the carrier gas.

Relative retention times of bile acid derivatives obtained during the GLC analysis are given in Table 1.

RESULTS

The time course of changes in the 7α-dehydrogenation of CA and 7α-hydrogenation of 3α 12α 7=O by *E. coli* K-12 are shown in Fig. 1. In the aerobic culture, CA was rapidly oxidized to 3α 12α 7=O, about 80% in 1 day and almost to a maximum in 2 days. Small amounts of unidentified metabolites were detected after 2 days. On the other hand, the reverse reaction, reduction of 3α 12α 7=O to CA, was minor in this system. In the anaerobic culture, the oxidation of CA progressed, but more slowly than in the aerobic culture, and attained a constant value (about 50%) after 2 days. The reduction of 3α 12α 7=O also progressed in a similar manner as the oxidation of CA and attained a constant value after 2 days. The activity of another strain of *E. coli*, HB101, on CA conversion in the aerobic culture was examined, and it was found the activity was similar to that of K-12 (data are not given).

Figure 2 shows the time course of changes in the 7α-dehydrogenation of CDCA and the reverse reaction by *E. coli* K-12. The changes are very similar to those in

Fig. 3. Taurocholic acid (TCA) conversion by *Escherichia coli* K-12.

CA and 3α 12α 7=O, but the oxidation of CDCA in the aerobic culture seemed to proceed more rapidly than that of CA.

E. coli also dehydrogenated TCA. This reaction was observed in the aerobic culture, but not in the anaerobic culture (Fig. 3). The aerobic dehydrogenation of

Fig. 4. Gas-chromatograms of neutral, free, glycine- and taurine-conjugated fractions by PHP GEL column from the cultured medium containing taurocholic acid (TCA) for 4 days.

Fig. 5. Changes in pH of the medium and viable *E. coli* counts during culturing with *Escherichia coli* K-12.

TCA occurred more slowly than CA.

In the case of TCA, the 7-oxo-bile acid formed was found only in the taurine-conjugate fraction (Fig. 4). This suggests that TCA was oxidized without deconjugation. No bile acids, including CA, were detected in the free-fraction, suggesting that *E. coli* has no activity of deconjugation.

Figure 5 shows changes in the pH of the medium with *E. coli* and in viable *E. coli* counts during aerobic and anaerobic culturing. The pH increased to about 9

in the aerobic culture, but decreased slightly to around 6.5 in the anaerobic culture from day 1, and these values remained the same thereafter (Fig. 5A). The viable *E. coli* counts increased about 3×10^9 /ml in the aerobic culture on day 1, but decreased remarkably after the second day. In the anaerobic culture, it increased about 1×10^9 /ml on day 1, and then decreased gradually. As for the viable *E. coli* count, there was more in the anaerobic culture than in the aerobic culture after the second day (Fig. 5B). These changes were reproducible in all the other experiments.

DISCUSSION

E. coli possesses 7 α -HSDH (2, 12) and effectively oxidizes CA and CDCA to the corresponding 7-oxo-bile acids when incubated *in vitro* (9, 13, 14, 16), while *E. coli* exhibits only minor activity on endogenous bile acids in gnotobiotic rats (4, 10).

So far, as we have examined in the present culturing systems, *E. coli* oxidized CA and CDCA to 3 α 12 α -7=O and 3 α 7=O, and reduced these oxo-bile acids to CA and CDCA, respectively. These oxidation and reduction reactions seem to attain different equilibrium values depending on the presence or absence of oxygen. When the equilibrium value was expressed by an oxidation/reduction (O/R) ratio, it was 9 or more under aerobic conditions and around 1 under anaerobic conditions for both CA and CDCA. The equilibrium was attained in about 2 days.

How oxygen affects the equilibrium value is not yet known, but the reaction of this enzyme is reported to be dependent on the pH of the medium. Oxidation is predominant in values above 7 as reduction is in values

below 7 (6, 11). In the present experiments, the pH of the medium increased to about 9 in the aerobic culture but decreased to about 6.5 in the anaerobic culture. These changes are consistent with changes in the equilibrium values (O/R ratios).

It is reported that the enzyme has about one order of magnitude higher affinity for CDCA than for CA (12, 15). In the present experiments, the oxidation of CDCA was faster than that of CA in the aerobic culture, but was almost the same in the anaerobic culture.

TCA was also oxidized to the corresponding taurine-conjugated 7-oxo-bile acid. However, this reaction occurred only in the aerobic cultures, and not in the anaerobic culture (Fig. 3). Aries and Hill (2) and Imamura et al. (9) also noted that TCA was hardly dehydrogenated by *E. coli* under anaerobic conditions.

These data explain the fact that *E. coli* possesses 7 α -HSDH but causes almost no effect on the transformation of bile acids *in vivo*. Bile acids in germ-free rats consist of mainly taurine-conjugated primary bile acids such as CA, CDCA and α - and β -muricholic acids (10). Therefore, taurine-conjugated 7 α -hydroxyl bile acids are rarely oxidized in *E. coli* inoculated gnotobiotic rats (4, 10).

An anaerobic condition is considered to be one of the reasons that the conversion of bile acids was not observed *in vivo*, but there are many factors that affect the biotransformation of bile acids *in vivo*. Further studies are necessary to clarify the exact mechanisms.

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