

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

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The oxidation/reduction reactions of bile acids by *Escherichia coli* (*E. coli*) K-12 were examined in both Davis and brain-heart infusion (BHI) media under aerobic and anaerobic conditions. The pH in the Davis medium changed by almost the same amount, around pH 6.5–7.0 in both aerobic and anaerobic cultures, but the pH in the BHI medium was different in both cultures, that is, about pH 9.0 in the aerobic culture but only about 6.5 in the anaerobic culture. The growth curve of *E. coli* in the Davis medium showed a similar pattern in both conditions. Cholic acid (CA) was oxidized to 3 $\alpha$ ,12 $\alpha$ -dihydroxy-7-oxo-5 $\beta$ -cholanoic acid (3 $\alpha$ ,12 $\alpha$ ,7=O) in both cultures, but the reaction in the anaerobic culture was somewhat slower than that in the aerobic culture. On the other hand, reduction of 3 $\alpha$ ,12 $\alpha$ ,7=O to CA did not occur in the aerobic culture, but about 10% reduction was observed in the anaerobic culture after 4 days. These data suggest that the oxidation/reduction reaction of *E. coli* was oxidative in aerobic culture but reductive in anaerobic culture and these characteristics were not due to the changes in the pH of the medium. The reactions of CA and glycocholic acid to crude 7 $\alpha$ -HSDH prepared from *E. coli* were examined and it was found that both free and conjugated CA as a substrate for the 7 $\alpha$ -HSDH showed similar  $K_m$  values.

**Key words:** *Escherichia coli*; cholic acid; glycocholic acid; 3 $\alpha$ ,12 $\alpha$ -dihydroxy-7-oxo-5 $\beta$ -cholanoic acid; in vitro biotransformation

*Escherichia coli* (*E. coli*) possesses 7 $\alpha$ -hydroxysteroid dehydrogenase (7 $\alpha$ -HSDH) and oxidizes 7 $\alpha$ -hydroxyl bile acids to the corresponding 7-oxo-bile acids, or reduces 7-oxo-bile acids to the corresponding 7 $\alpha$ -hydroxyl bile acids (Aries and Hill, 1970; Haslewood and Haslewood, 1976; Macdonald et al., 1973). In a previous paper (Ogura et al., 2003), we reported that *E. coli* in aerobic culture transformed a large proportion of cholic acid (CA) to 3 $\alpha$ ,12 $\alpha$ -dihydroxy-7-oxo-5 $\beta$ -cholanoic acid (3 $\alpha$ ,12 $\alpha$ ,7=O) but hardly transformed 7-oxo-

bile acid to CA, whereas in anaerobic culture about 50% of CA was oxidized and about 50% of 7-oxo-bile acid was reduced. A similar result was also obtained with chenodeoxycholic acid. Therefore, we speculated that the oxidation/reduction reaction of *E. coli* attained different equilibrium values depending on the presence or absence of oxygen. In other words, the reaction equilibrium shifted to the oxidative side in aerobic culture but was almost equally balanced in anaerobic culture.

In previous experiments with brain-heart

Abbreviations: BHI, brain-heart infusion; CA, cholic acid; GCA, glycocholic acid; GLC, gas-liquid chromatography; 7 $\alpha$ -HSDH, 7 $\alpha$ -hydroxysteroid dehydrogenase; Me-DMES, methyl ester dimethylethylsilyl ether; PHP GEL, piperidino-hydroxypropyl dextran gel; 3 $\alpha$ ,12 $\alpha$ ,7=O, 3 $\alpha$ ,12 $\alpha$ -dihydroxy-7-oxo-5 $\beta$ -cholanoic acid;  $\beta\beta$ , 7 $\beta$ ,12 $\beta$ -dihydroxy-5 $\beta$ -cholanoic acid

infusion (BHI) medium, the pH of the cultured medium increased to about 9 in aerobic culture but decreased to around 6.5 in anaerobic culture (Ogura et al., 2003). Since it has been reported that the reaction of 7 $\alpha$ -HSDH is oxidative in alkaline conditions but reductive in acidic conditions (Groh et al., 1993; Macdonald et al., 1983), we suspected our previous results were dependent on the difference of pH of the medium rather than on the difference between aerobic and anaerobic conditions.

Meanwhile, we found that when *E. coli* was cultured in the Davis medium (Davis and Mingoli, 1950) containing 0.2% glucose, the pH of the medium did not differ significantly between aerobic and anaerobic conditions. Therefore, we examined the effect of *E. coli* on bile acids in both aerobic and anaerobic cultures in the Davis medium and compared it with that in the BHI medium.

In addition, *E. coli* dehydrogenates taurine- or glycine-conjugated CA to the corresponding taurine- or glycine-conjugated 3 $\alpha$ ,12 $\alpha$ ,7=O without deconjugation, but the conversion rates for conjugated CA were lower than for free CA (Ogura et al., 2003). In the present experiments, we analyzed the effect of concentrations of conjugated and free CA on crude 7 $\alpha$ -HSDH prepared from *E. coli* to clarify the difference mentioned above.

## Materials and Methods

### Chemicals

CA was purchased from Sigma Chemical, St. Louis, MO. 3 $\alpha$ ,12 $\alpha$ -Dihydroxy-7-oxo-5 $\beta$ -cholanoic acid (3 $\alpha$ ,12 $\alpha$ ,7=O) was prepared by oxidation of CA with N-bromosuccinimide (Fieser and Rajagopalan, 1949). 7 $\beta$ ,12 $\beta$ -Dihydroxy-5 $\beta$ -cholanoic acid ( $\beta\beta$ ), an internal standard for analysis by capillary gas-liquid chromatography (GLC), was synthesized as described previously (Arimoto et al., 1982). Piperidinoxypropyl dextran gel (PHP GEL) was purchased from

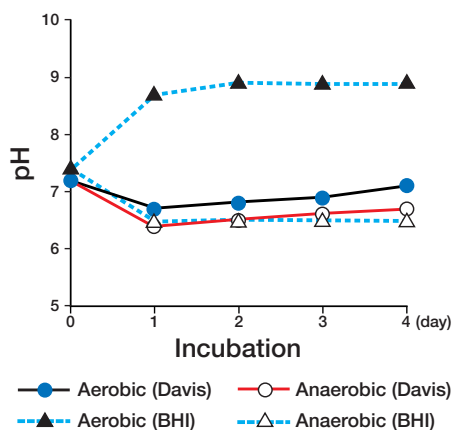
Shimadzu, Kyoto, Japan, and dimethylethylsilylimidazole from Tokyo Kasei Kogyo, Tokyo, Japan. The other reagents and solvents of analytical grade were obtained from Wako Pure Chemical Industries, Osaka, Japan. If not otherwise stated, the solvents were distilled once before use.

### Bacteriological procedures

*E. coli* K-12 was obtained from the American Type Culture Collection (Manassas, VA). The BHI medium (Becton Dickinson, Sparks, MD) and the Davis medium (pH 7.2) were used for the culture experiments. The Davis medium contains 0.7% K<sub>2</sub>HPO<sub>4</sub>, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 0.047% sodium citrate, 0.005% MgSO<sub>4</sub>, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0.2% glucose. The pH was adjusted to 7.2. *E. coli* was precultured aerobically in the BHI medium at 37°C for 1 day. Aliquots of the precultured *E. coli* ( $3 \times 10^7$  in 10  $\mu$ L medium) was added each to 2 mL of the fresh BHI medium, or to the fresh Davis medium, containing 1 mM of bile acids and cultured at 37°C for 4 days. The aerobic culture was performed in the open air with an incubator (PCI-100; Iuchi, Osaka, Japan) adjusted at 37°C, and the anaerobic culture was performed with the use of a gas-pack (BBL Gas Pak Pouch System; Becton Dickinson). Viable cells of *E. coli* in both media were counted by colony formation on an agar plate after seeding the diluted culture medium.

### Analytical methods

Bile acids were analyzed according to the method reported previously (Ogura et al., 2003). A portion of the medium cultured, 100  $\mu$ L, was heated with 8 volumes of ethanol at 80°C for 10 min and then filtered through a glass microfiber filter (GF/C; Whatman, Maidstone, United Kingdom). The filtrate was evaporated to dryness under a stream of nitrogen and the residue was acidified with diluted hydrochloric acid. The bile acids were extracted with ethylether, converted to methyl ester dimethylethylsilyl ether (Me-DMES) derivatives



**Fig. 1.** Changes in pH of the Davis medium and the brain-heart infusion (BHI) medium during aerobic and anaerobic cultures of *Escherichia coli* K-12.

and quantified by gas-liquid chromatography with a Hicap CBP-1 capillary column (25 m  $\times$  0.25 mm i.d.; Shimadzu). Conversions were expressed as % of bile acids converted in the culture medium.

### Enzyme activity of cell-free $7\alpha$ -HSDH

*E. coli* K-12 was cultured aerobically for one day in the BHI medium. Crude  $7\alpha$ -HSDH was prepared according to the method of Macdonald et al. (1973). The enzyme activity was assayed by the method of Macdonald et al. (1974, 1975) with a slight modification. Briefly, the reaction was performed at 37°C for 10 min in 0.18 M glycine-NaOH buffer (pH 9.5) containing 7 mM NAD<sup>+</sup>, 0 to 4 mM bile acid and 45  $\mu$ L of the crude enzyme preparation in a final volume of 1 mL.

## Results and Discussion

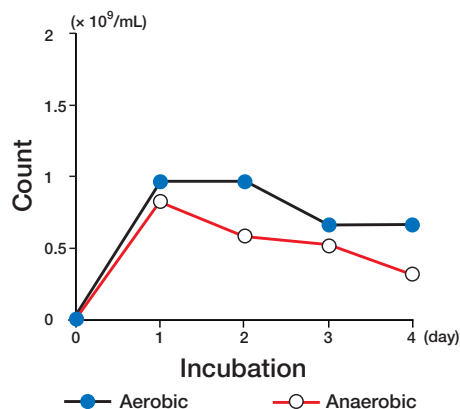
Major conversions of bile acids by intestinal bacteria include deconjugation, oxidation of hydroxy groups at C-3, C-7 and/or C-12 positions, and  $7\alpha/\beta$ -dehydroxylation. *E. coli* possesses  $7\alpha$ -HSDH for the oxidation, but has neither deconjugation nor dehydroxylation activity. In the present study, we examined the effect of *E. coli* on bile acids in

both Davis and BHI media, which showed different pH after cultures in aerobic and anaerobic conditions.

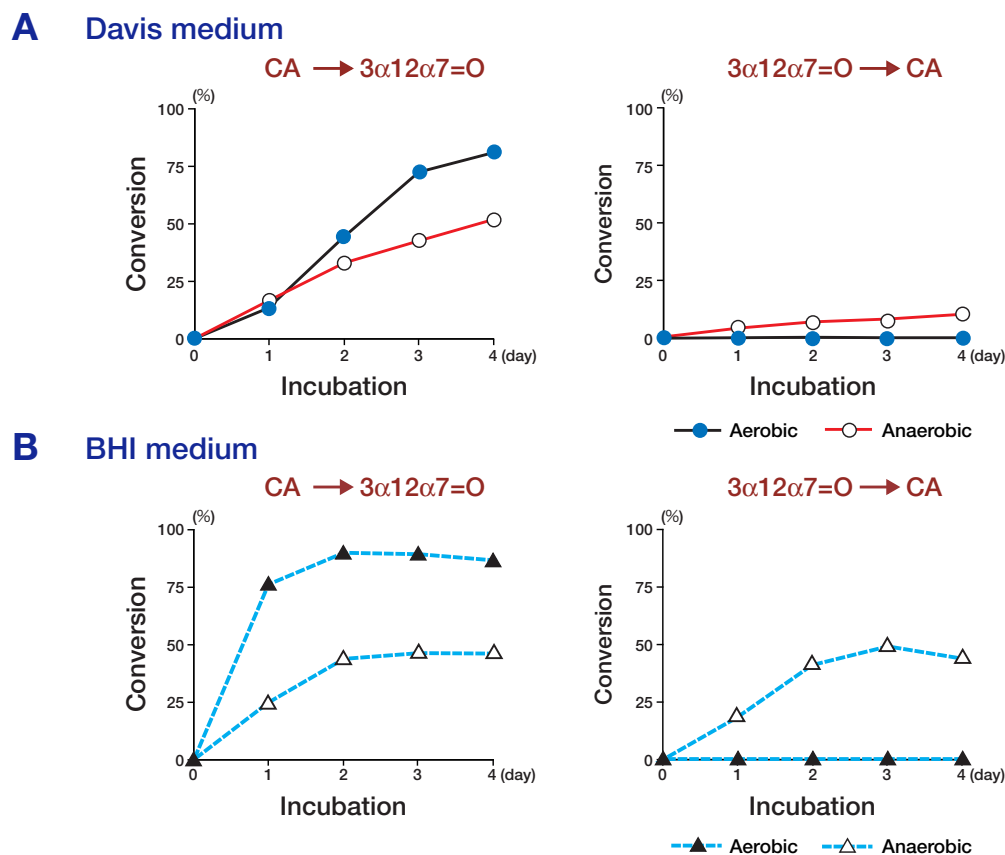
Changes in the pH of the BHI and Davis media cultured with *E. coli* are given in Fig. 1. The pH in the Davis medium changed almost by a similar value under both aerobic and anaerobic conditions; it decreased slightly to pH 6.5 on the first day of incubation and remained almost constant or very slightly increased thereafter, while the pH in the BHI medium markedly increased to about pH 9.0 in the aerobic condition but decreased to about pH 6.5 in the anaerobic condition. The changes in the Davis medium were almost the same to the change in the BHI medium in the anaerobic condition.

Figure 2 shows changes in viable cell counts during aerobic and anaerobic cultures of *E. coli* K-12 in the Davis medium. Although the viable cell counts in the anaerobic culture were higher than those in the aerobic culture, they changed almost by the same amount in both conditions.

Figure 3 shows the time course of changes in  $7\alpha$ -dehydrogenation of CA and  $7\alpha$ -hydroxylation of  $3\alpha,12\alpha,7\text{-O}$  by *E. coli* in the Davis and BHI media. In the Davis medium, CA was oxidized to  $3\alpha,12\alpha,7\text{-O}$  in both aerobic and anaerobic cultures, but the reaction in anaerobic culture was somewhat slower than that in aerobic culture. On



**Fig. 2.** Changes in viable cell counts during aerobic and anaerobic cultures of *Escherichia coli* K-12 in the Davis medium.



**Fig. 3.** Conversion of cholic acid (CA) and  $3\alpha,12\alpha$ -dihydroxy-7-oxo- $5\beta$ -cholanoic acid ( $3\alpha,12\alpha,7=O$ ) by *Escherichia coli* K-12 in the Davis medium and brain-heart infusion (BHI) medium.

the other hand, the reduction of  $3\alpha,12\alpha,7=O$  to CA did not occur in aerobic culture, but about 10% reduction was observed in anaerobic culture after 4 days. In the BHI medium, the oxidation of CA in aerobic culture was much higher than that in anaerobic culture and the reduction of  $3\alpha,12\alpha,7=O$ , though it was not observed in aerobic culture, occurred obviously in anaerobic culture.

These changes in the Davis medium were less marked than those in the BHI medium, but nevertheless showed the same characteristics for CA oxidation and 7-oxo-bile acid reduction, suggesting that this  $7\alpha$ -HSDH reaction was oxidative in aerobic culture but reductive in anaerobic culture.

Therefore, these data indicate that the oxidation/reduction reaction of  $7\alpha$ -HSDH is not affected by extracellular pH. In addition, it has been

reported that the intercellular pH of *E. coli* is maintained at around 7.6 in a wide range of extracellular pH (Padan et al., 1976; Shimamoto et al., 1994). Therefore, we conclude that the  $7\alpha$ -HSDH activity of *E. coli* is not affected by changes in pH of the culture medium.

$NAD^+$  is a cofactor required for the oxidative activity of  $7\alpha$ -HSDH. The content of  $NAD^+$  in *E. coli* was reported to increase in aerobic culture (Leonardo et al., 1996). This would explain the shift to the oxidative side in the transformation of bile acids by  $7\alpha$ -HSDH.

In our previous paper, we found that conjugated CA was dehydrogenated by *E. coli*, but to a lesser extent than free CA (Ogura et al., 2003). The reactions of CA and glycocholic acid (GCA) by the crude enzyme preparation were then examined in vitro. As shown in Fig. 4, CA and GCA

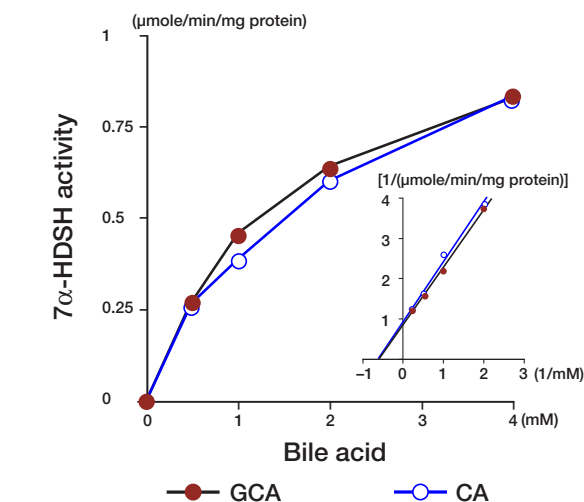
showed almost the same reactions, suggesting that both free and conjugated CA have similar affinities for the enzyme. This result coincides with the data demonstrated by Haslewood and Haslewood (1976). Therefore, the data with the crude enzyme preparation suggest that the lower reaction for conjugated CA is due to a slower penetration rate of conjugated CA into living cells than free CA.

In conclusion, the oxidation/reduction reaction of *E. coli* was oxidative in aerobic culture but rather reductive in anaerobic culture. These results were not due to the changes in the pH of the medium. In addition, the transport step through the cell membrane will be one of the rate limits for the transformation of bile acids in whole cells.

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**Fig. 4.** Dehydrogenation of cholic acid (CA) and glycocholic acid (GCA) in vitro by 7 $\alpha$ -hydroxysteroid dehydrogenase prepared from *Escherichia coli* K-12.

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