

## Formation of Ursodeoxycholic Acid from Chenodeoxycholic Acid by a 7 $\beta$ -Hydroxysteroid Dehydrogenase-Elaborating *Eubacterium aerofaciens* Strain Cocultured with 7 $\alpha$ -Hydroxysteroid Dehydrogenase-Elaborating Organisms

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A gram-positive, anaerobic, chain-forming, rod-shaped anaerobe (isolate G20-7) was isolated from normal human feces. This organism was identified by cellular morphology as well as fermentative and biochemical data as *Eubacterium aerofaciens*. When isolate G20-7 was grown in the presence of *Bacteroides fragilis* or *Escherichia coli* (or another 7 $\alpha$ -hydroxysteroid dehydrogenase producer) and chenodeoxycholic acid, ursodeoxycholic acid was produced. Time course curves revealed that 3 $\alpha$ -hydroxy-7-keto-5 $\beta$ -cholanoic acid produced by *B. fragilis* or *E. coli* or introduced into the medium as a pure substance was reduced by G20-7 specifically to ursodeoxycholic acid. The addition of glycine- and taurine-conjugated primary bile acids (chenodeoxycholic and cholic acids) and other bile acids to binary cultures of *B. fragilis* and G20-7 revealed that (i) both conjugates were hydrolyzed to give free bile acids, (ii) ursocholic acid (3 $\alpha$ ,7 $\beta$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholanoic acid) was produced when conjugated (or free) cholic acid was the substrate, and (iii) the epimerization reaction was at least partially reversible. Corroborating these observations, an NADP-dependent 7 $\beta$ -hydroxysteroid dehydrogenase (reacting specifically with 7 $\beta$ -OH groups) was demonstrated in cell-free preparations of isolate G20-7; production of the enzyme was optimal at between 12 and 18 h of growth. This enzyme, when measured in the oxidative direction, was active with ursodeoxycholic acid, ursocholic acid, and the taurine conjugate of ursodeoxycholic acid (but not with chenodeoxycholic, deoxycholic, or cholic acids) and displayed an optimal pH range of 9.8 to 10.2.

The 7 $\alpha$ -hydroxyl group is very actively metabolized by intestinal bacteria, as is evidenced by the following: (i) oxidation to the 7-ketone is catalyzed by a variety of 7 $\alpha$ -hydroxysteroid dehydrogenase-containing organisms, including *Escherichia coli* (5, 18), *Bacteroides fragilis* (11, 20), and certain "afermentative" clostridia (21); (ii) reduction to the alkane is catalyzed by a variety of 7-dehydroxylase-containing anaerobes, including *Clostridium leptum* (24), a *Eubacterium* species (26, 27), *Clostridium bifermentans* (4), and other anaerobic isolates from feces and sewage (19); and (iii) a third competing reaction, 7 $\alpha$ -OH epimerization, can be performed by a single organism, such as *Clostridium absonum* (15), which contains both 7 $\alpha$ - and 7 $\beta$ -hydroxysteroid dehydrogenases or similarly by certain lecithinase-lipase-negative clostridial isolates (2), or can be performed by two cocultured organisms elaborating 7 $\alpha$ - and 7 $\beta$ -hydroxysteroid dehydrogenases, respectively. A

recent paper of Hirano and Masuda (7) exemplified just such a system. Their 7 $\beta$ -hydroxysteroid dehydrogenase-containing isolate was described as a gram-positive, elongated, spheroid anaerobe, but its species could not be readily identified (7). The rationale behind the present study was to isolate, from human feces, an organism actively participating in 7 $\alpha$ -hydroxy epimerization and to determine its species. We will then compare the properties of this organism and the associated transformation reaction with similar data on other microbes performing the same reaction. In this communication we describe another fecal isolate, identified as *Eubacterium aerofaciens*, elaborating a 7 $\beta$ -hydroxysteroid dehydrogenase and the partial characterization of this enzyme.

### MATERIALS AND METHODS

**Abbreviations.** The following abbreviations are used: HSDH, hydroxysteroid dehydrogenase; CDC,

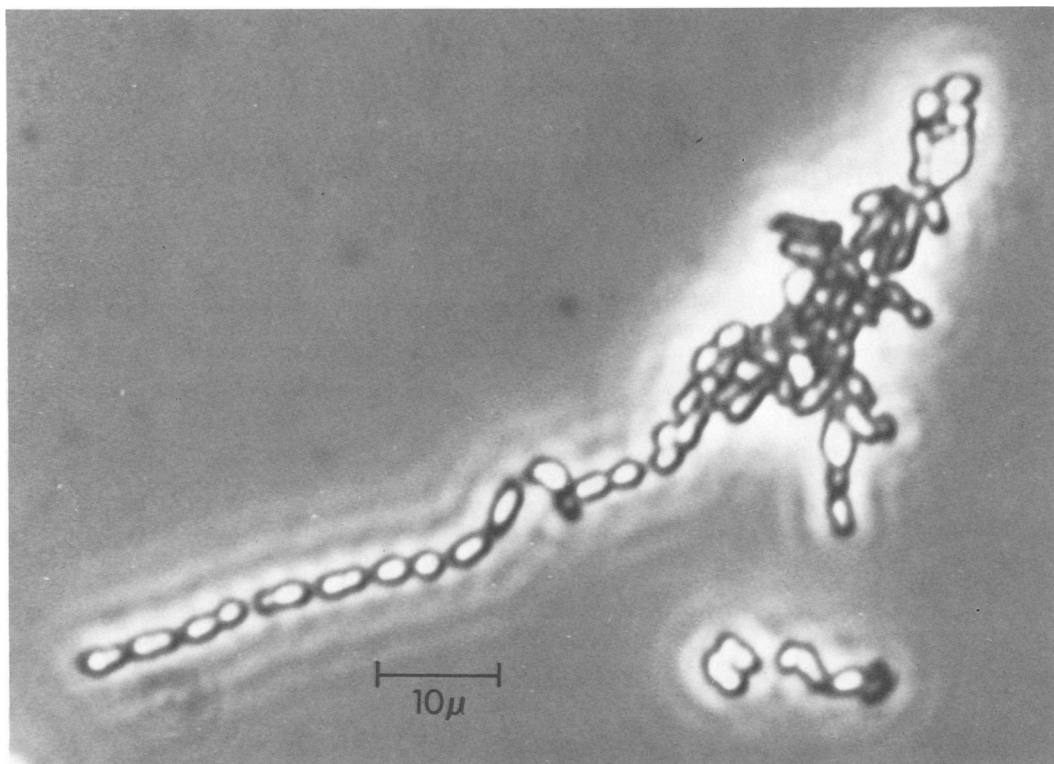


FIG. 1. Phase-contrast micrograph of *E. aerofaciens* G20-7.

chenodeoxycholic acid (3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholanoic acid); UDC, ursodeoxycholic acid (3 $\alpha$ ,7 $\beta$ -dihydroxy-5 $\beta$ -cholanoic acid); UC, ursocholic acid (3 $\alpha$ ,7 $\beta$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholanoic acid); 7K-LC, 3 $\alpha$ -hydroxy-7-keto-5 $\beta$ -cholanoic acid; 7K-DC, 3 $\alpha$ ,12 $\alpha$ -dihydroxy-7-keto-5 $\beta$ -cholanoic acid; BHI, brain heart infusion; TLC, thin-layer chromatography.

**Bacterial strains.** *B. fragilis* 18 (20), *E. coli* 23 (19), *Clostridium perfringens* 4 (16) were originally isolated from clinical material and retained in our laboratory in lyophilized vials. Wild-type strains of *Alcaligenes faecalis*, *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, and *Clostridium sporogenes* were obtained from the culture collection of the Department of Microbiology, Victoria General Hospital, Halifax, Nova Scotia.

**Materials.** BHI broth, agar, and cooked meat broth were products of Difco Laboratories, Detroit, Mich. Ether, methanol, chloroform, acetic acid, and *p*-hydroxybenzaldehyde (Komarowsky reagent) were from Canadian Laboratories, Montreal, Quebec, Canada. Cholic acid, mono- and dibasic sodium phosphate, glycine, and sodium hydroxide were products of J. T. Baker Chemical Co., Phillipsburg, N.J.; CDC, UDC, and NADP were from Sigma Chemical Co., St. Louis, Mo.; 7K-LC and 7K-DC were from Steraloids, Wiltshire, N.H.; and UC was kindly donated by R. A. DiPietro and A. C. Hofmann of the Division of Gastroenterology, School of Medicine, University of Califor-

nia, San Diego. Labeled primary bile acids, [24-<sup>14</sup>C]cholic acid and [24-<sup>14</sup>C]CDC, were products of New England Nuclear Corp., Lachine, Quebec. 24-<sup>14</sup>C-labeled 7K-LC, UDC, and UC were made from [24-<sup>14</sup>C]CDC and [24-<sup>14</sup>C]cholic acid (15).

**Isolation of 7 $\beta$ -OH-dehydrogenating bacterium from human feces.** Fecal suspensions of approximately 0.5 g/10 ml of BHI broth containing  $2 \times 10^{-4}$  M CDC were prepared with feces obtained from four healthy human volunteers. Six serial dilutions, each by a factor of 10, were made in each case, and the organisms were incubated at 37°C for 18 h. A 5-ml portion of each dilution was then transferred to centrifuge tubes equipped with glass stoppers and acidified to pH 3 to 4 with 0.3 ml of 1 M HCl. Each sample was extracted with an equal volume of ether, and the ether was evaporated to dryness. The samples were reconstituted in 50  $\mu$ l of methanol-water (4:1, vol/vol), and 25  $\mu$ l of each sample was spotted along with pure samples of UDC, CDC, and 7K-LC on a Bakerflex TLC plate (20 by 20 cm) (J. T. Baker Chemical Co.) and chromatographed in chloroform-methanol-acetic acid (40:2:1, vol/vol). The plates were sprayed with Komarowsky reagent (13), gently heated with a heat gun, and inspected under visible light. We then selected the fecal suspension which actively produced a spot corresponding to UDC at the highest fecal dilution. A series of six duplicate aliquots of 0.1 ml of the third serial dilution of the most active sample were then streaked onto blood agar plates in such a fashion that isolated colonies would form on incubation. Plates were anaer-

obically incubated in a GasPak system (BBL Microbiology Systems, Cockeysville, Md.) for 18 h, and six replicate plates were generated from each of the original plates. All plates were stored at 4°C after the growth period. The area of isolated colonies (approximately 60 to 200 per plate) was identified on each of the original duplicates, and the corresponding area on one set of six replicate plates (each derived from a different original plate) was scraped off with a sterile wire and inoculated into freshly boiled 10-ml tubes of BHI broth containing  $2 \times 10^{-4}$  M CDC. The tubes were incubated for 18 h, and UDC formation was detected as described above. Formation of UDC was assessed simply on the basis of the relative intensity as perceived visually in the UDC area of the plate versus the CDC area, and one of the duplicates and its remaining series of replicates were selected on this basis. We then divided the test plate into four sections, tested each quarter, selected the active quarter, and again divided this quarter into four and tested each section until activity was narrowed to one of seven colonies in the subsection. One colony which was active in converting CDC to UDC was restreaked and grown overnight in a GasPak. As many as three different colony types on blood agar plates were evident when this colony was restreaked, and none of them alone converted CDC to UDC. However, a small colony type (which was anaerobic) produced UDC when grown with a larger colony type (which was subsequently shown to be *E. coli*) in the presence of CDC. We restreaked this anaerobic isolate (subsequently called G20-7) until it was free of contamination and lyophilized a number of samples of the pure organism.

**Identification of isolate G20-7 as *Eubacterium aerofaciens*.** Fermentation and biochemical tests on isolate G20-7 were performed according to the procedure of Holdeman et al. (10).

**Time course studies with CDC and binary cultures of *E. aerofaciens* G20-7 and 7 $\alpha$ -HSDH-containing organisms.** Pure 10-ml cultures of *E. aerofaciens* G20-7 and *B. fragilis* 18 (20) or *E. coli* 23 (19) were grown overnight at 37°C (*E. aerofaciens* G20-7 was grown in a GasPak system). The *E. aerofaciens* G20-7 and one of the 7 $\alpha$ -HSDH-containing organisms were inoculated into a 100-ml culture of BHI broth in a graduated cylinder (10% inoculum each) containing  $2 \times 10^{-4}$  M CDC and about 0.10  $\mu$ Ci of [24-<sup>14</sup>C]CDC. Samples were removed periodically as described previously (15), extracted, and subjected to TLC studies.

*E. aerofaciens* G20-7 was similarly cocultured with wild-type strains of the 10 organisms listed above (those other than *B. fragilis* and *E. coli*); 10-ml cultures were used. The organisms were grown at 37°C for 24 or 48 h in the presence of  $2 \times 10^{-4}$  M CDC (unlabeled), and the spent medium was extracted and subjected to TLC.

**Time course studies with 7K-LC and *E. aerofaciens* G20-7 in pure culture.** These studies were performed as described above except that the medium was BHI broth containing 0.1% thioglycolate and 1.0% glucose, and labeled 7K-LC was used instead of labeled CDC.

**Time course incubation study with 7K-LC and *E. aerofaciens* in sterile sodium phosphate buffer.** An overnight (ca. 18 h) 10-ml culture of isolate G20-7 in BHI broth in a GasPak system (ca.  $10^9$  organisms per ml) was grown, and the culture was centrifuged at

TABLE 1. Fermentative and bacteriological data on 7 $\beta$ -HSDH-elaborating organisms<sup>a</sup>

Characteristic	Isolate G20-7 <sup>b</sup> ( <i>E. aerofaciens</i> )	Isolate a-16 <sup>c</sup> (species unidentified)	<i>C. absonum</i> 6905 <sup>d</sup>
Amygdalin	—	—	—
Arabinose	—	W	—
Cellobiose	A	A	A
Erythritol	—	—	—
Esculin (pH)	A	W	W
Esculin (hydrolysis)	+	+	+
Fructose	A	A	A
Glucose	A	A	A
Glycogen	—	—	—
Inositol	—	—	—
Lactose	A	A	A
Maltose	A	A	A
Mannitol	W	—	—
Mannose	A	W	A
Melezitose	—	—	—
Melibiose	—	A	—
Raffinose	—	A	—
Rhamnose	A	W	—
Ribose	W	A	W
Salicin	A	—	A
Sorbitol	—	—	—
Starch (pH)	—	—	—
Starch (hydrolysis)	—	—	+
Sucrose	A	A	A
Trehalose	A	—	W
Xylose	—	A	—
Gelatin (liquefaction)	—	—	+
Milk	Curd	Curd	Curd
Meat	—	—	—
Indol production	—	—	—
Nitrate	—	—	+
Catalase	—	—	—
Lecithinase	—	—	+
Lipase	—	—	—
Hemolysis	—	—	+( $\beta$ )
Mobility	—	—	—

<sup>a</sup> Abbreviations: —, negative; +, positive; W, weak reaction; A, acid produced; curd, curds formed.

<sup>b</sup> Current study.

<sup>c</sup> Reference 7.

<sup>d</sup> Reference 15.

1,000  $\times$  g in a clinical centrifuge. The cell pellet was suspended in 5.0 ml of sterile, anaerobic 0.1 M sodium phosphate buffer at pH 6.0, 7.0, or 8.0 and transferred under sterile conditions into 100 ml of the same buffer containing  $4 \times 10^{-4}$  M 7K-LC and about 0.10  $\mu$ Ci of [24-<sup>14</sup>C]7K-LC. Samples were removed periodically, extracted, and subjected to TLC as described previously (15, 25).

**Viable count estimation.** Samples of *E. aerofaciens* G20-7 broth cultures at 24 and 72 h of growth in flasks in GasPak systems were diluted serially into ice-cold BHI broth, and 0.1-ml aliquots were spread over blood agar plates which were incubated anaerobically (GasPak system). Colonies were counted after 48 h of incubation.

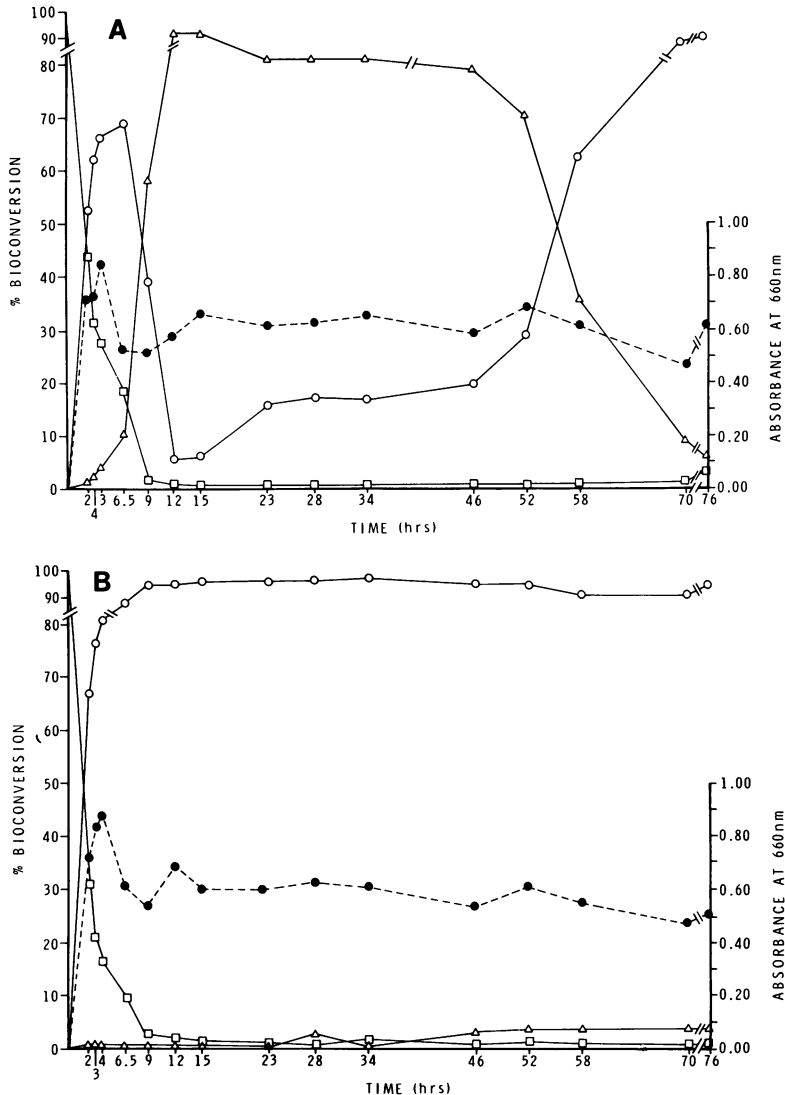


FIG. 2. Time course curves for cultures of *B. fragilis* and *E. aerofaciens* G20-7 (A), *B. fragilis* alone (B), *E. coli* and *E. aerofaciens* G20-7 (C), and *E. coli* alone (D). The substrate was  $2 \times 10^{-4}$  M [24- $^{14}$ C]CDC. Symbols: □, CDC; ○, 7K-LC; △, UDC; ●, absorbance at 660 nm. Cultures and estimations were in singlet.

**Extraction, TLC, and counting procedure.** These procedures were performed as described previously (15).

**Mass spectroscopy identification of UDC synthesis from 7K-LC by *E. aerofaciens*.** Identification of UDC synthesis was performed as described for *C. absonum* (15) except that *E. aerofaciens* was incubated with  $4 \times 10^{-4}$  M 7K-LC for 24 h in a GasPak system, and then the product (putatively UDC) was separated by TLC (as described above), methylated (25), and subjected to mass spectroscopy.

**Production of cell-free preparations of *E. aerofaciens* G20-7.** Cultures (100 ml) of *E. aerofaciens* G20-7 were grown in BHI broth-thioglycolate-glucose as described above except that cultures were grown in GasPak

systems and  $4 \times 10^{-4}$  M bile acid was added or deleted. The cells were harvested at 18 h, and cell-free preparations were made as described previously for *C. absonum* (17).

**Assay for 7 $\beta$ -HSDH in cell-free preparations.** A 100- $\mu$ l portion of a freshly prepared cell-free preparation of *E. aerofaciens* G20-7 was assayed in a cuvette containing 1.0 mM NADP, 0.3 M glycine-NaOH (pH 10.1), and  $10^{-3}$  M bile acid in a total volume of 1.0 ml. The formation of NADPH was followed at 340 nm and 25°C with a Beckman DB-GT grating spectrophotometer and a 10-in. (ca. 25.4 cm) recorder. Initial velocities were expressed in terms of units of 7 $\beta$ -HSDH per 100 ml of culture or units of 7 $\beta$ -HSDH per milligram of protein, where one unit is defined as the amount of

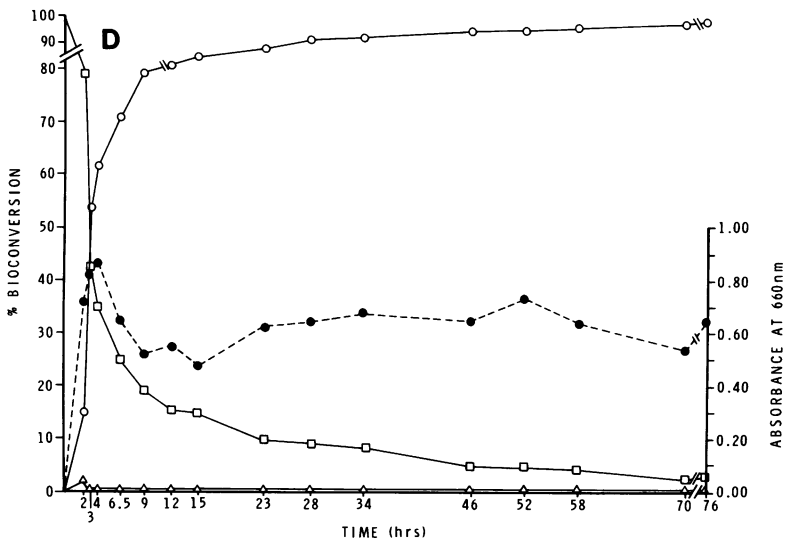
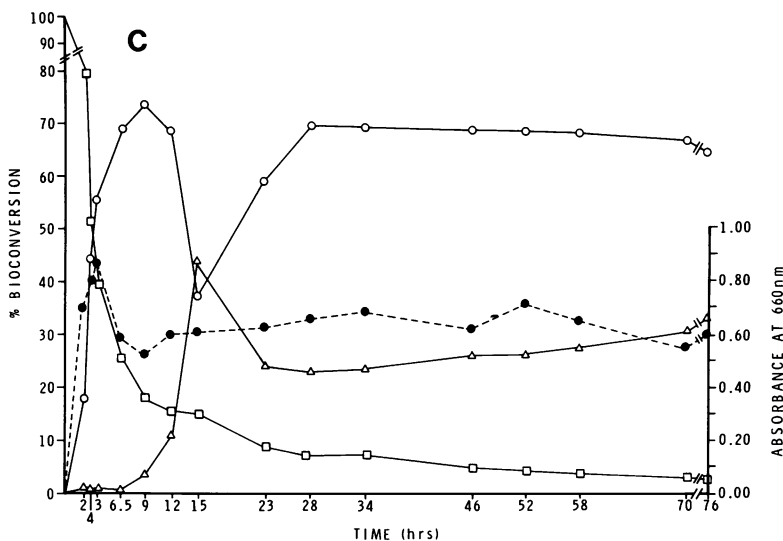


FIG. 2—Continued

enzyme sufficient to reduce 1 μmol of NADP to NADPH per min at 25°C.

**Protein estimation.** Total protein concentrations were estimated according to the method of Bradford (1), using the commercially prepared reagent and globulin standard (Bio-Rad Laboratories, Richmond, Calif.).

**RESULTS**

Mass spectroscopy data showed that the mass spectrum of the methyl ester of putative UDC made from 7K-LC and *E. aerofaciens* G20-7 and that of commercially available UDC were identical. Additionally, these two sources of UDC

cochromatographed in three different solvent systems (13).

Of the four human stool samples studied in the initial phase of this work, only one demonstrated a high number of 7β-hydroxyl group-epimerizing organisms (approximately 10<sup>4</sup> organisms per g of wet stool). We demonstrated approximately 10<sup>2</sup> such organisms per g of wet stool in a second person and were unable to demonstrate any 7α-OH epimerization in two others. The isolated G20-7, a gram-positive, anaerobic, plump rod occurring in chains (Fig. 1), was shown by fermentation and biochemical characteristics (Table 1) to be *E. aerofaciens* as described by

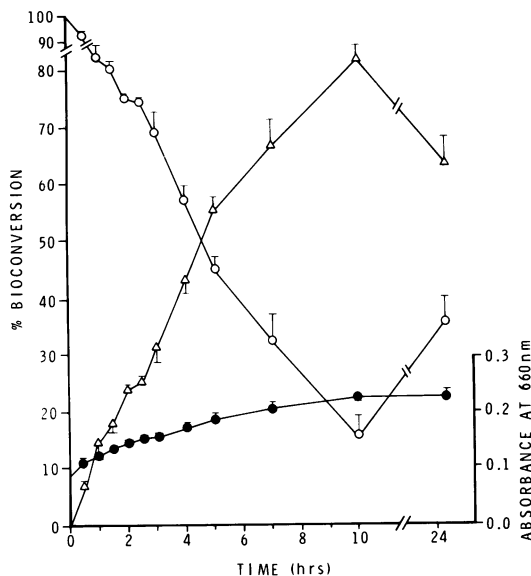


FIG. 3. Time course curve for pure cultures of *E. aerofaciens* G20-7 incubated in the presence of  $4 \times 10^{-4}$  M 7K-LC. Symbols:  $\circ$ , 7K-LC;  $\Delta$ , UDC;  $\bullet$ , absorbance at 660 nm. Estimations were performed on two identical cultures; standard deviations are shown.

Moore et al. (22). This species has been described as a low-population organism present in the feces of healthy human donors (12). Its fermentation characteristics and cellular morphology are distinct from that of the isolate of Hirano and Masuda (7) (compare row 1 with row 2 in Table 1), and it is also very different from *C. absonum* (6, 23), another bacterium which participates in  $7\alpha$ -OH epimerization (compare row 1 with row 3 in Table 1). Fermentation in peptone-yeast extract-glucose broth revealed the presence of acetic, formic, and lactic acids with a trace of succinic acid. This fatty acid pattern and the cellular morphology distinguish *E. aerofaciens* from *E. contortum*, a closely related organism (10, 22).

In our initial approach to the isolation of a  $7\alpha$ -OH-epimerizing organism, we conceived of a bacterium which was self-sufficient in converting CDC to UDC, such as *C. absonum* (15) or certain lecithinase-negative, lipase-negative clostridia (2). It was evident, however, that when isolate G20-7 was free of various contaminating organisms, including *E. coli*, it was, by itself, incapable of epimerizing the  $7\alpha$ -OH group of CDC. Binary cultures with known organisms clearly demonstrated  $7\alpha$ -OH epimerization of CDC with *E. coli*, *B. fragilis*, and *C. sporogenes*, all of which contain  $7\alpha$ -HSDH. However, this did not occur with *A. faecalis*, *S. aureus*, *S. typhimurium*, *P. mirabilis*, *E. aerogenes*, *S.*

*faecalis*, *P. aeruginosa*, or *C. perfringens* strains, all of which are devoid of  $7\alpha$ -HSDH. In Fig. 2, time course curves of binary cultures of isolate G20-7 with *B. fragilis* and *E. coli* (Fig. 2A and C, respectively) and control cultures of *B. fragilis* 28 and *E. coli* 23 alone (Fig. 2B and D, respectively) are shown. Viable counts of the two organisms in each binary culture at 24 h were  $1.1 \times 10^{10}$  and  $1 \times 10^8$  organisms per ml for *B. fragilis* 18 and *E. aerofaciens* G20-7, respectively, and  $5.0 \times 10^9$  and  $1 \times 10^7$  organisms per ml for *E. coli* 23 and *E. aerofaciens* G20-7, respectively.

However, when *E. aerofaciens* G20-7 was grown alone in BHI broth-thioglycolate-glucose containing  $4 \times 10^{-4}$  M labeled 7K-LC, the 7K-LC clearly was converted to UDC (Fig. 3). No conversion to CDC was observed. Additionally, in both the binary culture systems with CDC (Fig. 2) and the pure culture of *E. aerofaciens* with 7K-LC (Fig. 3), some oxidation back to 7K-LC occurred in the latter stages of the time course, particularly when *E. aerofaciens* was cultured with *E. coli* or alone (Fig. 2C and 3). No reaction occurred when either *E. coli* or *B. fragilis* was incubated with UDC in the absence of *E. aerofaciens*.

In spite of the ability of isolate G20-7 to transform 7K-LC to UDC, viable counts of this organism during growth at  $37^\circ\text{C}$  in graduated cylinders on the bench revealed that it grew very poorly; in fact, the viable counts at 24 h were fewer than those at the time of inoculation, which were approximately  $2 \times 10^7$  organisms per ml. Thus far the only successful way of culturing this organism appears to be in a Gas-Pak system, in which we consistently obtain about  $10^9$  organisms per ml. When an identical 10% inoculum ( $10^9$  organisms per ml) of isolate G20-7 was suspended in freshly boiled, sterile 0.1 M phosphate at pH 6.0, 7.0, or 8.0, no measurable transformation of 7K-LC occurred over a 6-h test period. The viable counts for the three buffer systems at 6 h revealed the presence of about  $1.5 \times 10^4$ ,  $3.1 \times 10^7$ , and  $2.1 \times 10^7$  organisms per ml, respectively (with about  $1.0 \times 10^8$  organisms at time zero).

When binary BHI broth cultures of *E. aerofaciens* and *B. fragilis* were tested by using both conjugated and unconjugated primary bile acids, it could be seen that both taurine- and glycine-conjugated bile acids were quantitatively deconjugated and that epimerization at the  $7\alpha$ -OH group occurred both with cholic acid and CDC. In fact, the yields of UC from cholic acid were somewhat greater than the yields of UDC from CDC (Fig. 4). Additionally, a binary culture of *B. fragilis* and *E. aerofaciens* contained at 24 h a fairly constant CDC/UDC/7K-LC (or cholic acid/UC/7K-DC) ratio, regardless of what sub-

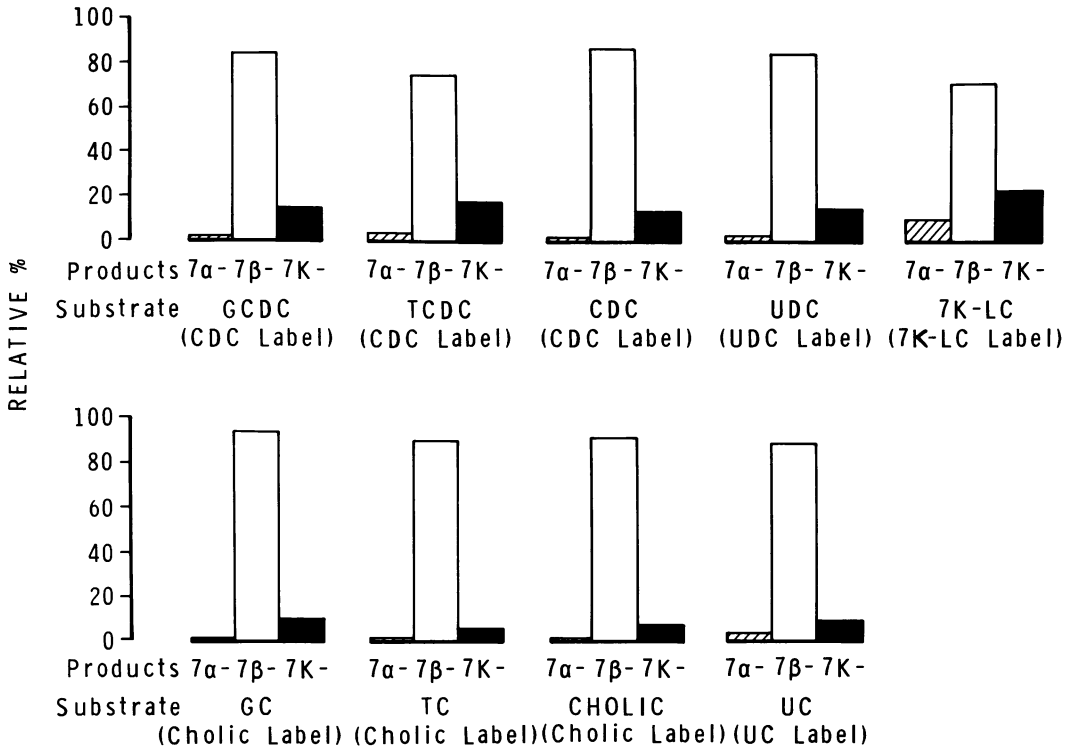


FIG. 4. Relative distribution of primary bile acid (7 $\alpha$ ), urso bile acid (7 $\beta$ ), and 7-keto bile acid (7K) after 24 h of incubation of *B. fragilis* and *E. aerofaciens* G20-7 with various bile acid substrates as indicated. GCDC and TCDC, Glycine and taurine conjugates of CDC; GC and TC, glycine and taurine conjugates of cholic acid.

strate was used initially (Fig. 4). *E. aerofaciens* also deconjugated bile acids (e.g., taurine conjugates of CDC or UDC), whereas *E. coli* and *B. fragilis* under the same conditions were inactive, as has been shown previously (14).

To demonstrate the presence of 7 $\beta$ -HSDH in *E. aerofaciens* G20-7, we made cell-free preparations of 100-ml cultures grown in GasPak systems and assayed samples with UDC for HSDH activity (oxidative direction) at pH 10.1. No HSDH activity was evident against 3 $\alpha$ -, 7 $\alpha$ -, or 12 $\alpha$ -OH groups of various bile acids (cholic acid, CDC, and deoxycholic acid). No NAD-dependent activity was present. We demonstrated the presence of an NADP-dependent 7 $\beta$ -HSDH with a pH optimum of 9.8 to 10.3 for the oxidative direction (Fig. 5). Time course studies revealed an optimal growth period for 7 $\beta$ -HSDH production of about 14 to 18 h (Fig. 6). Additions of  $4 \times 10^{-4}$  M 7K-LC and CDC suppressed both the growth of the organisms (Fig. 6B) and the amount of 7 $\beta$ -HSDH. In fact, poorer growth and enzyme yields resulted from the addition of any bile acid, including 7K-LC, CDC, UDC, deoxycholic acid, or 7K-DC, with the notable exception of cholic acid, which had no effect. The addition of 1.0% glucose to the medium mildly enhanced both growth and enzyme level (by 35

to 40%). No specific induction effect analogous to that of *C. absonum* (17) was evident.

When the enzyme was tested with 7 $\beta$ -OH group-containing substrates, both UDC and UC were substrates and so was the taurine-conjugated form of UDC, but UC and the taurine-conjugated form of UDC were relatively poor substrates, each reacting at approximately one-fifth of the velocity measured with UDC under saturating conditions.

### DISCUSSION

Three categories of organisms which participate in 7 $\alpha$ -OH epimerization are evident. Organisms such as *C. absonum* (15, 17) and certain lipase- and lecithinase-negative clostridia (2) are capable of 7 $\alpha$ -OH epimerization independently of other organisms (i.e., by implication or practical demonstration they contain both 7 $\alpha$ - and 7 $\beta$ -HSDH). Another group consists of organisms such as that isolated by Hirano and Masuda (7) and *E. aerofaciens* G20-7, which contain only a 7 $\beta$ -HSDH and therefore require coculturing with a 7 $\alpha$ -HSDH-containing organism. Logically, the third and last known group consists of organisms such as *B. fragilis* (11, 20) and *E. coli* (5, 18), which contain only 7 $\alpha$ -HSDH and there-

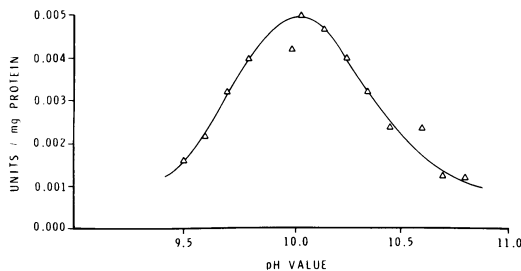


FIG. 5. Effect of pH on  $7\beta$ -HSDH activity with UDC as the substrate and crude cell-free preparations of *E. aerofaciens*.

fore require coculturing with a  $7\beta$ -HSDH-containing organism. Clearly, the organisms described above which can participate in  $7\alpha$ -OH epimerization do so via an oxidative-reductive mechanism in which a 7-keto intermediate is involved. As pointed out by Hirano and Masuda (7), the two-step, two-organism redox mechanism may be the predominate in vivo mechanism of UDC formation and is one example of a metabolic interspecies relationship in the gut flora. The data in Fig. 4 suggest that a binary culture of *B. fragilis* and *E. aerofaciens*, like a pure culture of *C. absonum* (15), can perform  $7\alpha$ -OH epimerization which is at least partially reversible and can deliver a primary bile acid/urso bile acid/7-keto bile acid ratio comparable to that seen at anaerobic equilibrium in *C. absonum* cultures.

It appears that with time and oxygen exposure, cultures of *E. aerofaciens* G20-7, as well as those of *C. absonum*, tend to oxidize UDC back to 7K-LC. The explanation proposed for *C. absonum*, and which may be applicable here, is that oxygen exposure gradually reduces the intracellular NADPH/NADP ratio and thereby shifts the reaction in the oxidative direction. Clearly, cultures of *E. aerofaciens* G20-7 are very oxygen sensitive and can be grown to yield a high viable number of organisms per milliliter

only under strict anaerobic conditions.

It is also evident that during the 7-epimerization there is considerably more 7K-LC formed as a transient intermediate in the binary culture systems (Fig. 2A and C) than in pure cultures of *C. absonum*. This is a predictable result since two organisms instead of one are involved. It is not clear why binary cultures with *B. fragilis* give a higher yield of UDC than those with *E. coli* (compare Fig. 2A with Fig. 2C).

There is little doubt, also, that *E. aerofaciens* G20-7 is a distinctly different species from isolate a-16 described by Hirano and Masuda (7), from the clostridia described by Edenharder and Knaflic (2), and from *C. absonum* (15).

In contrast to the  $7\beta$ -HSDH in *C. absonum* (17), the *E. aerofaciens* G20-7 enzyme is apparently not induced by the addition of bile acids to the medium and is found in a very much lower titer than that in *C. absonum* when the latter is appropriately induced (17). When cocultured with *B. fragilis* 18, relatively low numbers (less than  $10^8$  organisms per ml) of *E. aerofaciens* were capable of catalyzing an efficient reaction. The yields of UDC produced from CDC by *B. fragilis* and *E. aerofaciens* (greater than 95%) were substantially greater than that for *C. absonum* grown in the same medium.

In whole-cell cultures, it appears that  $7\alpha$ -OH epimerization can take place in mixed fecal cultures even though we found by fecal dilution only approximately  $10^2$  organisms per g of wet feces in some samples (Macdonald, unpublished data). Several independent studies (3, 8; R. P. Serva et al., *Gastroenterology*, **78**:1252, 1980) on the conversion of CDC to UDC by mixed fecal cultures also suggest that a low population of organisms is instrumental in  $7\alpha$ -OH epimerization. It is not yet clear why UC is not similarly formed from cholic acid in vivo.

The pH optimum (about 9.8 to 10.2) for  $7\beta$ -HSDH was just slightly higher than that for *C. absonum*  $7\beta$ -HSDH, which is 9.5 to 10. Enzyme purification and further substrate specificity

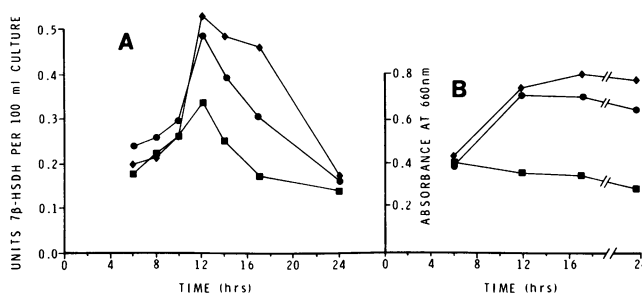


FIG. 6. Time course curves of pure cultures of *E. aerofaciens* grown in GasPak systems (anaerobically) in the presence of CDC (■) or 7K-LC (●) or in the absence of added bile acid (◆).  $7\beta$ -HSDH levels per 100-ml culture (A) and absorbance at 660 nm (turbidity) (B) are shown. The enzyme assay system was as described in the text.



studies will be required to extend comparisons between these two forms of 7 $\beta$ -HSDH.

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