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Isolation and Characterization of Human Fecal Bacteria Capable of 21-Dehydroxylating Corticoids

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It has been known for a decade that human intestinal flora include organisms capable of 21-dehydroxylating corticoids. Yet the identity of the organisms synthesizing 21-dehydroxylase has remained unknown. Using diluted human feces, we determined the prevalence of colonies of 21-dehydroxylating organisms on a variety of media. Isolation from the medium of colonies with the highest prevalence yielded an obligate anaerobe capable of 21-dehydroxylating deoxycorticosterone and tetrahydrodeoxycorticosterone. This transformation could be carried out in a prereduced medium by the microbial culture alone or in an aerobic medium reduced by growth of *Escherichia coli*. The culture shares many characteristics with *Eubacterium lentum*, the neotype strain of which elaborated both 21-dehydroxylase and 3α-hydroxysteroid dehydrogenase.

21-Dehydroxylated metabolites of many corticoids are found in human urine (5, 14, 15, 18). It appears that 21-dehydroxylation of corticoids is restricted to steroids undergoing enterohepatic circulation (8, 10, 16). Dehydroxylation of steroids and bile acids has not been demonstrated in mammalian tissue.

In 1971, Eriksson and Gustafsson (6) demonstrated 21-dehydroxylation of 3β,21-dihydroxy-5α-pregnan-20-one (systematic names according to IUPAC/IUB, Steroids **13**:277-310, 1969) with a slurry of human intestinal contents, and, in 1975, Bokkenheuser et al. (2) reported that cultures of mixed fecal flora convert 11-deoxycorticosterone (DOC; 21-hydroxy-4-pregnene-3,20-dione) to 3α-hydroxy-5β-pregnan-20-one (pregnanolone). This conversion involves a reduction of DOC to tetrahydrodeoxycorticosterone (THDOC; 3α,21-dihydroxy-5β-pregnan-20-one) followed by the removal of the 21-OH group (3) (Fig. 1).

Reduction of Δ^4-3-ketone is effectively accomplished by *Clostridium paraputrificum* (3, 17), a member of the normal human intestinal flora. Many nonsporulating intestinal bacteria, a few nonintestinal bacteria, two yeasts, and three molds also reduce DOC to THDOC, but in much lower yields (3, 17, 21).

Organisms synthesizing 21-dehydroxylase have not been isolated heretofore. Recently, however, we provided strong evidence that 21-dehydroxylating organisms in mixed fecal flora form colonies on solid media (P. Dehazya, J. Winter, W. G. Kelly, and V. D. Bokkenheuser, Mt. Sinai J. Med. N.Y., in press). In the present communication, we report the isolation of an organism with 21-dehydroxylase activity and the demonstration of this property in a similar, if not related, bacterial species.

MATERIALS AND METHODS

Media. Conventional bacteriological media were employed as described by Bokkenheuser et al. (2, 3). The following additional media were used: 5% sheep blood agar plates from Scott Laboratories Inc., Fiskeville, R.I., and Mueller-Hinton agar from Difco Laboratories, Detroit, Mich. Bacterial transformation of steroids was examined in BHIC, a medium of the following composition per liter of water: 37 g of dehydrated brain heart infusion broth (Baltimore Biological Laboratories, Cockeysville, Md.); 0.5 g of cysteine hydrochloride; 1 g of $NaHCO_3$; and 4 ml of 0.025% aqueous resazurin (J. T. Baker Chemical Co., Phillipsburg, N.J.). The medium was distributed in 200-ml amounts in 500-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. Prereduced broth (PR; 45 ml of BHIC in 60-ml bottles) was purchased from Scott Laboratories.

pH and Eh. pH and Eh both were measured on a Beckman Zeromatic II instrument (Beckman Instruments Inc., Fullerton, Calif.). Eh measurements were done with a platinum electrode. The readings were checked regularly with quinhydrone-saturated buffers at pH 4 and 7 and invariably fell within 10 mV of the theoretical values.

Source and preparation of microorganisms. Fecal samples from healthy adults on the usual Western diet were obtained in stool cups under ordinary atmospheric conditions; processing began within 30 min after defecation (2). Serial 10-fold dilutions of the specimens were prepared in supplemented peptone broth (Becton, Dickinson & Co., Rutherford, N.J.). The neotype strain of *Eubacterium lentum* (VPI 0255)

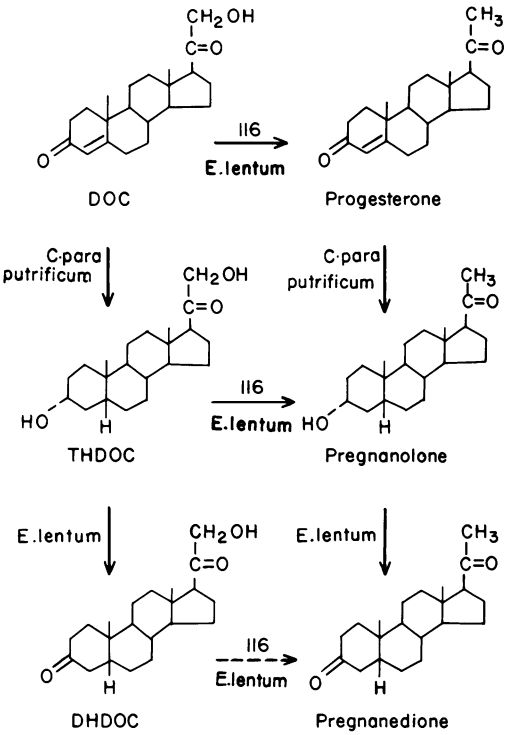

FIG. 1. *Transformation of C_{21} steroids by cultures of C. paraputrificum and E. lentum and culture 116.*

was supplied by L. V. Holdeman and W. E. C. Moore, Virginia Polytechnic Institute and State University, Blacksburg. The strain was grown in PR and maintained as a lyophilized culture.

Steroids. [1,2-^{3}H]DOC was purchased from New England Nuclear Corp., Boston, Mass. The steroid was at least 97% pure by isotopic dilution analysis. [1,2-^{3}H]THDOC was prepared by biosynthesis in our laboratories as previously described (3). Unlabeled steroids were purchased from Steraloids, Inc., Wilton, N.H. Before incubation, carrier and radioactive steroid were mixed in methanol and added to the sterilized medium to give a concentration of 16 μg of steroid per ml, 0.5% methanol (vol/vol), and 2×10^6 cpm per culture, regardless of the amount of carrier added to the medium (2).

Incubation. In conversion experiments, BHIC and PR were supplemented with substrate as described above, seeded with bacterial flora, and incubated at 37°C for 7 days (2). Anaerobic surface cultures were incubated in GasPak jars (Becton, Dickinson & Co.) at 37°C for 2 days.

Thin-layer chromatography. Five milliliters of culture was extracted with 5 ml of methylene chloride for 1 min; the organic solvent was dried over sodium sulfate and evaporated under nitrogen at 45°C, and the dry residue was dissolved in 100 μl of acetone and subjected to thin-layer chromatography on Bakerflex silica gel with fluoresence indicator (no. 1B2F, J. T. Baker Chemical Co.), as previously described (2).

Extraction of steroids, partition chromatography, and identification. Steroid extraction, chromatography, and identification were carried out as previously described (2, 3). Briefly, the steroids were extracted with organic solvents, concentrated, and subjected to partition chromatography on Celite with a heptane-ethylene dichloride gradient as the mobile phase. The metabolites were identified by color reactions, R_f, and infrared spectroscopy.

RESULTS

Our earlier experiments provided strong evidence that the occurrence of 21-dehydroxylating organisms in the fecal flora of our donors is about 10^7/g (2; Dehazya et al., in press). Since there are approximately 10^{11} viable organisms per g of feces, the ratio of 21-dehydroxylating organisms to the total number of viable bacteria is about 1:10,000 (a prevalence of 10^{-4}). Therefore, selective enrichment is required.

Development of selective media. Portions (0.1 ml) of fecal suspension diluted to a concentration of 10^3 to 10^7 were spread on a variety of solid media and incubated anaerobically for 48 h. The colonies were counted, and the growth was harvested in 5 ml of sterile saline and inoculated into 200 ml of BHIC containing 16 μg of THDOC per ml. After incubation of the cultures, the steroids were extracted and analyzed. 21-Dehydroxylation required the presence of 85 to 300 colonies on 5% sheep blood agar plates (average, 200) and 15 to 59 colonies on Mueller-Hinton agar (average, 42). We interpret this to mean that 1 colony out of 42 on Mueller-Hinton agar consists of 21-dehydroxylating organisms. Occasionally, conversion was obtained with growth from Mueller-Hinton agar containing colistin (50 μg/ml), but not with growth from the same medium supplemented with erythromycin (25 μg/ml), kanamycin (75 μg/ml), penicillin G (50 U/ml), or vancomycin (7.5 μg/ml).

Isolation of 21-dehydroxylating organisms. Mueller-Hinton agar was employed as the selective medium of choice; 0.1 ml of the 10^{-7} fecal suspension was spread on this medium and incubated anaerobically for 2 days. Sixty-five consecutive colonies were removed from the plates with Pasteur pipettes, transferred under a stream of N_2 to PR, and incubated at 37°C for 48 h. After incubation, portions of four to seven cultures were mixed and tested for 21-dehydroxylase activity in PR. Individual cultures in positive mixtures were tested; two of these cultures possessed 21-dehydroxylase activity.

Identification of the 21-dehydroxylating organisms. L. V. Holdeman and W. E. C. Moore kindly characterized the two isolates and found them to be identical. Accordingly, only

one of the isolates, culture 116, was studied further. This isolate was an obligatory anaerobic, somewhat pleomorphic, gram-positive, nonmotile, nonsporulating rod, 0.6 to 1.4 μm by 0.3 to 0.6 μm, that failed to metabolize carbohydrates and proteins. The growth of culture 116 was stimulated by the presence of 1% arginine in the medium (22), but, whether or not this amino acid was added, the organism was oxidase negative, hippurate negative, nitrate negative, H_2S negative, and catalase negative (glucose agar slant growth flooded with 3% H_2O_2). The growth was inhibited by chloramphenicol (12 μg/ml), clindamycin (1.6 μg/ml), erythromycin (3.0 μg/ml), penicillin G (2 U/ml), and tetracycline (6.0 μg/ml) in the broth-disk susceptibility test (23).

Steroid metabolism by the neotype strain of *E. lentum*. Culture 116 shares many characteristics with *E. lentum* (12). However, in media supplemented with arginine, the latter is usually oxidase positive, catalase positive, nitrate positive, and H_2S positive. Nevertheless, we decided to examine the neotype strain of *E. lentum* for 21-dehydroxylase activity. Seeded together with *Escherichia coli* in BHIC, the neotype strain of *E. lentum* 21-dehydroxylated THDOC (16 μg/ml) to pregnanolone and small amounts of pregnanedione and 3β-pregnanolone. On the other hand, *E. lentum* grown in PR metabolized THDOC to dihydrodeoxycorticosterone. However, if PR was seeded with both *E. coli* and *E. lentum*, THDOC was metabolized to a mixture of dihydrodeoxycorticosterone and pregnanolone (Table 1). Differences in metabolic pattern could not be attributed to dissimilarities between BHIC and PR, because a mixture of *E. coli* and *E. lentum* incubated aerobically in PR converted THDOC quantitatively to pregnanolone. The 21-dehydroxylation by *E. lentum* correlated with a low Eh, whereas 3α-dehydrogenation was associated with a higher Eh (Table 1).

Culture 116, in contrast to the neotype strain of *E. lentum*, transformed THDOC to pregnanolone both in BHIC (seeded with *E. coli*) and in PR. Control experiments showed that *E. coli* did not metabolize THDOC.

Air tolerance of culture 116. In an earlier work, it was speculated that the 21-dehydroxylating organism is an obligate anaerobe, quite resistant to atmospheric oxygen (3). To test this hypothesis, culture 116 was streaked on three 5% sheep blood agar plates. The control was incubated anaerobically for 48 h; the remaining two plates were kept aerobically at 5 and 20°C, respectively, for 24 h, and then incubated anaerobically at 37°C for 48 h. All three plates yielded good growth which converted DOC to progesterone both in PR and in BHIC.

DISCUSSION

Metabolic pathways of DOC and THDOC. It is now possible to present diagrammatically the bacterially mediated metabolic pathways of DOC (Fig. 1). DOC is rapidly reduced to THDOC mainly by *C. paraputrificum* (3, 17, 21) and, to a much lesser degree, by other isolates of human intestinal bacteria (3). At an Eh of −250 to −300 mV, culture 116 and *E. lentum* both 21-dehydroxylated THDOC to pregnanolone. At an Eh of −120 to −180 mV, *E. lentum* oxidized the 3-OH group of THDOC to dihydrodeoxycorticosterone, a transformation that cannot be performed by culture 116. Dihydrodeoxycorticosterone, in turn, may be reduced by *C. paraputrificum* to THDOC. The 3α-hydroxysteroid dehydrogenase of *E. lentum* also oxidizes pregnanolone to pregnanedione, which may be reduced to pregnanolone and 3β-pregnanolone. This pathway of pregnanolone metabolism adequately explains the epimerization of the compound observed in many cultures.

Moreover, the 21-dehydroxylase activity of culture 116 and *E. lentum* also explains the presence in urine of 21-dehydroxylated compounds of C_{21} steroids undergoing enterohepatic circulation. For example, urinary 3α,20α-dihydroxy-5β-pregnane, a 21-dehydroxylated deriv-

TABLE 1. *Demonstration of THDOC-metabolizing enzymes in bacterial strains*[a]

Bacterial strain	Enzyme activity[b]		
	BHIC + *E. coli*[c]	PR[c]	PR + *E. coli*[c]
Culture 116	21-DOH	21-DOH	21-DOH
E. lentum (neotype)	21-DOH	3-DH	21-DOH + 3-DH
E. coli	None	None	None
Eh after incub. (mV)	−280 ± 50	−160 ± 40	−220 ± 20

[a] EH of media before inoculation: −130 ± 20 mV.
[b] 21-DOH, 21-Dehydroxylase activity; 3-DH, 3α-hydroxysteroid dehydrogenase activity.
[c] Medium.

ative of DOC (4, 13), may be formed in the following way: DOC is reduced to THDOC in the liver, conjugated, excreted in the bile, transported into the intestine, probably deconjugated, and dehydroxylated by culture 116, *E. lentum,* or allied bacteria to pregnanolone. Although the fate of pregnanolone is unclear, it is probably absorbed, reduced in the liver to 3α,-20α-dihydroxy-5β-pregnane, conjugated, and returned to the blood for renal excretion.

Occurrence of 21-dehydroxylating organisms in human intestinal flora. Since 21-dehydroxylated metabolites of biliary steroids are normal compounds in human urine (4, 9, 13, 16, 19), and in view of the apparent inability of mammalian tissue to carry out 21-dehydroxylation, it must be expected that 21-dehydroxylating organisms form a part of the normal intestinal flora in human adults. The prevalence of these organisms in our donors was of the order of 10^{-4} (2; Dehazya et al., in press). This agrees with Moore and Holdeman's findings of an *E. lentum* prevalence of less than $10^{-3.3}$ in fecal flora of a Japanese population (20). But it is at variance with Finegold's observation of a prevalence of $10^{-1.5}$ in groups of Japanese, whether on an Eastern or a Western diet (7).

E. lentum and culture 116 are not necessarily the only 21-dehydroxylase-producing organisms. Other intestinal bacteria in yet lower numbers may also synthesize this enzyme. The age at which colonization with 21-dehydroxylating bacteria takes place is unknown.

Effect of antibiotic treatment on bacterial metabolism of steroids. Culture 116 and the neotype strain of *E. lentum* are highly susceptible to the same broad range of antibiotics. If this sensitivity pattern is typical for steroid-metabolizing bacteria, it is likely that appropriate antibiotics reaching the intestinal localization of the bacteria could interfere with the formation of certain metabolites. Whether or not such an intervention of a normal metabolic pathway is of consequence to the host is a matter of conjecture. It is noteworthy, however, that the suppression of intestinal 7-dehydroxylating bacteria by neomycin prevents gallstone formation in cholestanol-fed rabbits (1, 11).

Role of *E. coli* in the transformation of steroids. There was no evidence, either in this work or in a previous investigation (3), that *E. coli* is capable of metabolizing THDOC. We believe that the role of *E. coli* in the transformation of steroids in vitro is to provide a suitable redox environment not only for the multiplication of the bacteria, but also for the function of the steroid-metabolizing enzymes. It is noteworthy, however, that while *E. coli* promotes the 21-dehydroxylase activity of both culture 116 and *E. lentum,* it seems to suppress the 3α-hydroxysteroid dehydrogenase activity (Table 1). The latter was observed in PR at a comparatively high Eh. One may speculate that the low Eh created by *E. coli* is incompatible with the oxydation activity of the dehydrogenase. Why the 21-dehydroxylase is so poorly expressed in a culture that dehydrogenates is not clear.

Taxonomic markers for *E. lentum.* Sperry and Wilkins (22), noting that the speciation of *E. lentum* is based mainly on negative features, offered two positive criteria for identification: growth stimulation by arginine and production of cytochromes. Our investigation has revealed that the neotype strain of *E. lentum* elaborates at least two enzymes: a 21-dehydroxylase detectable at a low Eh and a 3α-hydroxysteroid dehydrogenase demonstrable at a somewhat higher Eh. It remains to be determined whether these markers are characteristic for *E. lentum* or, more likely, whether the strains presently classified as *E. lentum* form a heterologous group from which some strains may be singled out by their ability to transform steroids.

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