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New Markers for *Eubacterium lentum*

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Of 37 strains of *Eubacterium lentum* and phenotypically similar organisms, 26 (70%) synthesized a corticoid 21-dehydroxylase and/or a 3α-hydroxysteroid dehydrogenase. It appeared that the corticoid 3α-hydroxysteroid dehydrogenase was identical to the bile acid 3α-hydroxysteroid dehydrogenase. Steroid-metabolizing enzymes were found both in *E. lentum* and in phenotypically similar organisms. *E. lentum* is characterized by nitrate reduction and enhanced growth in the presence of arginine. Many phenotypically similar organisms possess either one or the other of the two markers. In contrast, using the steroid-metabolizing enzymes as markers, a "steroid-active" and a "steroid-inactive" group were established with minimal overlapping of metabolic characteristics. Synthesis of the steroid enzymes was positively correlated with production of gas from H_2O_2 and formation of H_2S. A simple method for the detection of corticoid 21-dehydroxylase and 3α-hydroxysteroid dehydrogenase, one or both of which were present in 92% of the steroid-active group, is described.

Eubacterium lentum is a gram-positive, nonsporeforming, nonmotile, obligate anaerobe characterized by its few positive biochemical reactions (4). In 1976 Sperry and Wilkins (12) noted that strains of *E. lentum* derive their energy from arginine rather than from carbohydrates. The latter authors proposed to adopt the growth-stimulating effect of arginine and the production of cytochromes as characteristics of *E. lentum*.

Working on the bacterial metabolism of bile acids, Macdonald et al. (6) demonstrated that 25/32 strains of organisms characterized as *E. lentum* or phenotypically similar organisms possess one or both of two hitherto little recognized enzymes: bile acid 3α-hydroxysteroid dehydrogenase (BA-3α-HSDHase) and bile acid 12α-hydroxysteroid dehydrogenase (BA-12α-HSDHase). These enzymes also occur in other species, such as *Clostridium perfringens* (8) and *Pseudomonas testosteroni* (7, 11).

Available evidence indicates that 21-dehydroxylase (21-DOHase, Fig. 1) is not produced in mammalian tissues (13). Nor was it synthesized by some 30 bacterial strains of fecal origin (1, 2), but it is manufactured by about 0.01% of the organisms in normal fecal flora (1). By means of specialized technique, a 21-dehydroxylating organism, strain 116, was recovered in pure culture (3). Strain 116 is phenotypically similar to *E. lentum*. The neotype strain (ATCC 25559) of *E. lentum* (9) produces not only a 21-DOHase, but also a corticoid (C)-3α-HSDHase. In the present communication, we report on the distribution of these two enzymes in 37 stock strains of *E. lentum* and phenotypically similar organisms.

Definitions. In this paper the term *E. lentum* is used for organisms that are phenotypically indistinguishable from the neotype strain of *E. lentum*, reduce nitrate, and are stimulated by arginine. "Phenotypically similar organisms" lack one or both of the two latter characteristics. "Steroid-active" organisms synthesize at least one of these enzymes: 3α-HSDHase, 12α-HSDHase, or 21-DOHase. In "steroid-inactive" strains, none of the three enzymes is detectable.

MATERIALS AND METHODS

Media. Brain heart infusion broth with cysteine was prepared as follows: dehydrated brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.), 37 g; cysteine hydrochloride, 0.5 g; $NaHCO_3$, 1 g; 4 ml of 0.025% aqueous resazurin (J. T. Baker Chemical Co., Phillipsburg, N.J.); and distilled water to 1 liter. The dissolved medium was distributed in 200-ml amounts in 500-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. To create anaerobic conditions, 0.1 ml of a 24-h-old culture of *Escherichia coli* (3) was added to the medium (BHIC-Ec) and cocultured with

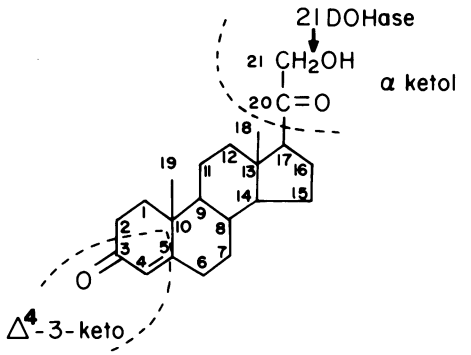

FIG. 1. *DOC, a C21-steroid.*

the organism being tested. Prereduced broth (PR; 45 ml of BHIC in 60-ml vials) was purchased from Scott Laboratories, Inc., Fiskeville, R.I. Tyrpticase soy broth (50 ml/vial) was obtained from BBL Microbiology Systems.

pH and Eh. pH and Eh of the incubated cultures 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; invariably these fell within 10 mV of the theoretical values (pH 4, 218; pH 7, 41; 25°C).

Bacterial strains. Strains of *E. lentum* and phenotypically similar organisms were from the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg, from the Wadsworth Anaerobic Bacteriology Laboratory, Los Angeles, and from the Department of Pathology, St. Luke's Hospital Center, New York. Using the diagnostic criteria recommended by the Virginia Polytechnic Institute Anaerobe Laboratory (4), all strains were recharacterized immediately before, and some strains after, the investigation for 21-DOHase and C-3α-HSDHase.

The cultures were maintained in lyophilized form and, before testing, passaged two to three times in PR at 37°C.

Steroids and solvents. [1,2,-^{3}H]deoxycorticosterone (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]tetrahydrodeoxycorticosterone (THDOC) was prepared by biosynthesis as previously described (2). Solvents were reagent grade except for methanol, which was technical grade.

Conversion experiments. Carrier and radioactive steroids were dissolved in methanol and added to the medium to give a concentration of 20 μg of steroid per ml and 10^6 cpm of ^{3}H per culture. The modified medium was then seeded with a 24-h-old PR culture of the organism to be tested: 20 ml of broth to 0.1 ml of inoculum. The cultures were incubated at 37°C for 7 days. The pH and Eh were measured at the end of the incubation.

Determination of enzymatic transformation. (i) Thin-layer chromatography. A 5-ml sample of the incubated culture was extracted with 5 ml of methylene dichloride for 30 to 60 s. The organic phase was separated, dehydrated over Na_2SO_4, filtered, and evaporated under N_2 at 40 to 50°C. The residue was redissolved in 50 μl of acetone and spotted on Baker-flex silica gel plates with fluorescence indicator (1B2F; J. T. Baker Chemical Co.). A good separation was obtained in a solvent system consisting of isooctane-ethyl acetate-acetic acid (5:25:0.2, vol/vol/vol). R_f values were determined and compared with those of authentic samples (DOC, 0.36 ± 0.02; progesterone, 0.46 ± 0.02; THDOC, 0.28 ± 0.03; dihydrodeoxycorticosterone, 0.40 ± 0.02). The Δ^4-3-oxo structure and the α-ketol group, depicted in Fig. 1, were detected under ultraviolet light (254 nm) and after spraying with blue tetrazolium, respectively. When both reactions were negative, the steroid molecule was located by spraying with H_2SO_4-ethanol (1:1, vol/vol).

(ii) Partition chromatography. The remaining culture was extracted as previously described (1). Briefly, the steroids were chromatographed on a column (diameter, 1.5 cm) packed with 5 g of celite moistened with 2.5 ml of water (10), followed by 15 g of celite mixed with ethylene glycol-water (9:1, vol/vol). The column was first developed with 50 ml of heptane; the gradient, generated in an apparatus consisting of two connected cylinders of equal cross-sectional areas, was then started. The mixing chamber was initially charged with 250 ml of heptane, and the reservoir was charged with an equal weight of ethylene dichloride (150 ml). Radioactivity of the fractions was measured on a Packard scintillation counter. Fractions comprising a peak were combined and purified by rechromatography as previously described (2). The infrared spectra were determined in KBr (5) and compared with spectra of authentic samples. Mass spectra of the metabolites were described by Winter et al. (13).

Electron microscopy. For negative staining, colonies were harvested with a sterile swab and dispersed in distilled water. A drop of cell suspension was then added to a spot-plate well containing 1 drop of 4% aqueous potassium phosphotungstate adjusted to pH 6.8, ca. 20 drops of distilled water, and 1 drop of 1% bovine serum albumin (Cohn fraction V). After mixing gently, the preparations were sprayed onto carbon-coated collodion-filmed grids with a Vaponefrin type all-glass nebulizer (Ted Pella Co., Tustin, Calif). Contrast, particle distribution, and spreading were optimized by varying the final concentrations of potassium phosphotungstate, sample, or bovine serum albumin, as required. Grids were examined immediately in a Philips EM-200 electron microscope operated at 60 kV with double condenser illumination and a 30- to 35-μm copper disk aperture in the objective.

RESULTS AND DISCUSSION

21-DOHase and C-3-α-HSDHase in strains of *E. lentum* and phenotypically similar organisms. 21-DOHase removes the C21—OH of DOC and THDOC, converting the substrates to progesterone and 3α-pregnanolone, respectively (Fig. 2). C-3α-HSDHase oxidizes the C3—OH of THDOC or 3α-pregnanolone to a keto group and is responsible for the epimerization at carbon 3 (3).

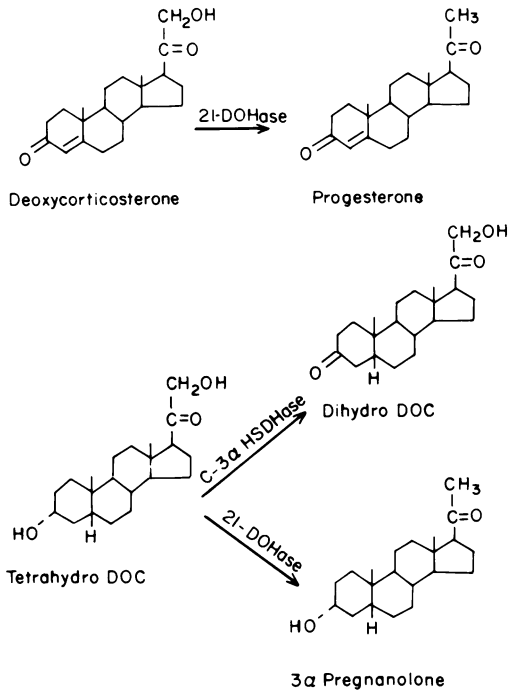

FIG. 2. *Transformation of corticoids by E. lentum and related organisms.*

Quantitative studies with strain 116 showed that the yield of 21-dehydroxylated metabolites was 80 to 90% of the theoretical value in the highly reduced BHIC-Ec (Eh, ± −280 mV), compared to 50 to 60% in the less reduced PR (Eh, ± −150 mV). When the neotype strain of *E. lentum* was employed, 21-dehydroxylation was confined to BHIC-Ec, whereas C-3α-HSDHase was observed in PR. The failure to demonstrate significant amounts of C-3α-HSDHase (<5%) at a low Eh could be expected because the dehydrogenase is an oxidizing enzyme.

Of 37 strains tested, 16 (43%) possessed C-3α-HSDHase and 21 (57%) synthesized 21-DOHase (Table 1). Some strains elaborated both enzymes; others, one or none. From the above observations it follows that, to demonstrate both enzymes, it is necessary to use media of different Eh values.

Bile acid- and corticoid-metabolizing enzymes. Thirty strains were examined both by Macdonald et al. (6) and by us (Table 2). Enzymes metabolizing bile acids and corticosteroids were found predominantly in strains of *E. lentum,* but were also present in phenotypically similar organisms. BA-3α-HSDHase and C-3α-HSDHase are probably one and the same enzyme. They are both dehydrogenases operating at carbon 3; individual strains usually were active on both substrates or neither. However, three discrepancies were observed between our data and those of Macdonald et al. (6): in strains 3999 and 9066, 3α-HSDHase was demonstrated against bile salts but not against THDOC, and in 8662C 3α-HSDHase was only detected against THDOC. These results were completely reproducible in both laboratories. Two explanations are possible: (i) vastly different oxidation rates are measurable for a single organism whether the living bacteria or the cell-free preparation is tested against cholic acid, deoxycholic acid, and THDOC (Winter et al., unpublished data); and (ii) there may be a sensitivity difference between the enzymatic determination (6) and the thin-layer chromatography estimation of metabolites. 12α-HSDHase was the most common steroid enzyme in our strains, but it is not specific for these organisms. It also occurs in *C. perfringens* (8). It is unstable and nicotinamide adenine dinucleotide dependent (7). The 21-DOHase appears to be specific for *E. lentum* and phenotypically similar organisms (1, 2).

TABLE 1. *21-DOHase and C-3α-HSDHase in 37 strains of E. lentum and phenotypically similar organisms*

No. of strains	Enzymes observed at (Eh):	
	−280 ± 50 mV[a]	−150 ± 30 mV[b]
11	21-DOHase	C-3α-HSDHase
5	21-DOHase	21-DOHase
5	21-DOHase	None
5	None	C-3α-HSDHase
11	None	None

[a] Medium: BHIC-Ec.
[b] Medium: PR.

Electron microscopy of *E. lentum* (neotype) and strain 116. Strain 116, the first organism shown to synthesize a 21-DOHase, has not been accurately classified (3). It shares many characteristics with the neotype strain of *E. lentum,* yet it does not reduce nitrate and is H_2S negative. To examine whether there was morphological evidence for assigning the two organisms to different species, electron micrographs were prepared. In a potassium phosphotungstate preparation of strain 116 (Fig. 3), most cells appeared slightly plasmolyzed (turgidity loss), with an average diameter of 0.6 μm. Cell lengths varied from 0.8 to >3 μm. Chain formations with conspicuous mesosomal-like structures at the septa were common. Many cells exhibited a characteristic polar tuft of dense fibrillar material. In chains, this tufting usually was confined to cells at the ends. This suggests that the structure may represent a carry-over from previous cell divisions wherein separation of the sister

TABLE 2. *Enzymes metabolizing bile acids and corticoids in strains of E. lentum and phenotypically similar organisms*

Enzymes metabolizing bile acids[a]	No. of strains	Strains with corticoid-metabolizing enzymes:			
		21-DOHase and C-3α-HSDHase	21-DOHase	C-3α-HSDHase	None
3α-12α-HSDHase	13	25559 (ATCC)[b] 9758 3995 8056 6266C 5483 7725 8123 8114	3999	8330TA 11450 8371B	
12α-HSDHase	9	8662C	8135A 9104A 3162P 6701K$_2$ 6718E 116 (SLH)[b] 8902		9130
3α-HSDHase	2	3197-1			9066
None	6				3060D1 7616B 9262 3219D 9391B 9489

[a] Enzymes described by Macdonald et al. (6).
[b] Except when noted in parentheses, the numbers refer to the Virginia Polytechnic Institute collection.

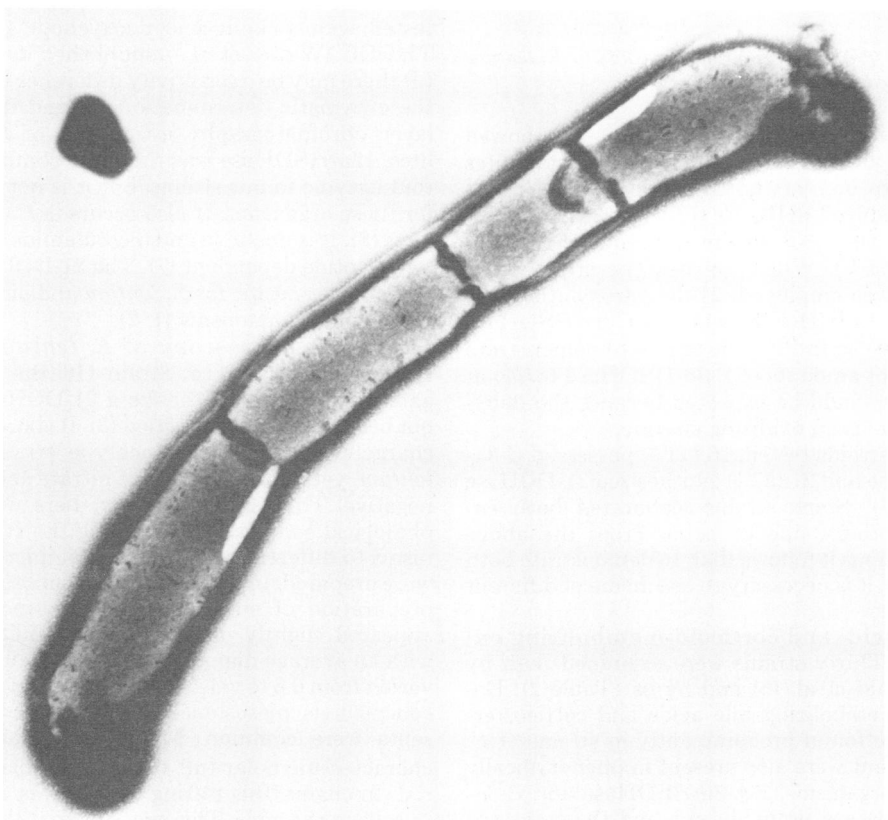

FIG. 3. *Strain 116. Magnification, ×24,000.*

cells was defective, allowing extrusion of remnants of the mesosomal structures associated with the septa. No other surface appendages were noted. The cell surface was generally smooth and finely textured. The morphology of the neotype strain of *E. lentum* (Fig. 4) was similar to that of strain 116.

Selection of markers. Using NO_3 reduction and stimulation by arginine as markers, the 30 strains examined in this study may be subdivided into *E. lentum* and phenotypically similar organisms. As may be seen from Fig. 5, there was a considerable overlapping in the metabolic characteristics of the two groups. Indeed, 3α-HSDHase was the only characteristic that did not appear in both subgroups. However, this could change, when larger groups of organisms are examined.

If one subdivided the 30 strains according to their ability to synthesize steroid enzymes (Fig. 6), a group of 24 strains consisting of steroid-active organisms and a smaller group (6 strains) of steroid-inactive organisms were obtained. There was minimal overlapping of metabolic characteristics. Organisms producing gas from H_2O_2 as well as those forming H_2S belonged to the steroid-active group.

Practical method for demonstration of 21-DOHase and 3α-HSDHase. A simple, rapid technique for the detection of 21-DOHase and C-3α-HSDHase was developed. Two media of different Eh values are required (Fig. 7). First, 50 ml of Trypticase soy broth is supplemented with 1 mg of DOC in 0.25 ml of methanol and seeded with 0.1 ml of a 24-h culture of *Escherichia coli*. Second, 50 ml of PR is supplemented with 1 mg of THDOC in 0.25 ml of methanol. Both media are inoculated with 0.25 ml of a 24-h-old culture of the organism under investigation. After 2 to 3 days of incubation at 37°C, 5-ml samples are extracted with methylene dichloride. The organic phase is collected, and the

FIG. 4. *Neotype strain of E. lentum. Magnification, ×24,000.*

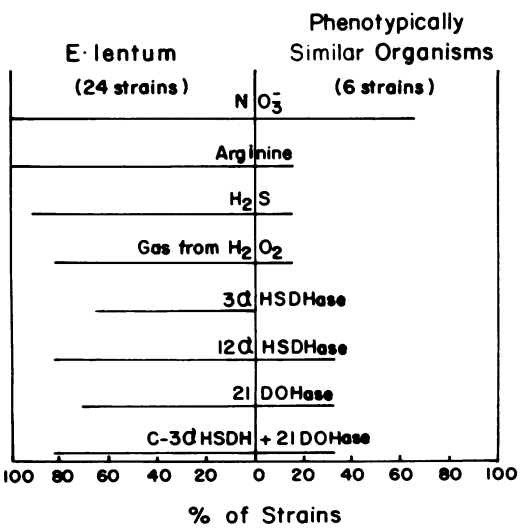

FIG. 5. *Metabolic characteristics of 30 strains classified according to their arginine requirements and ability to reduce nitrate.*

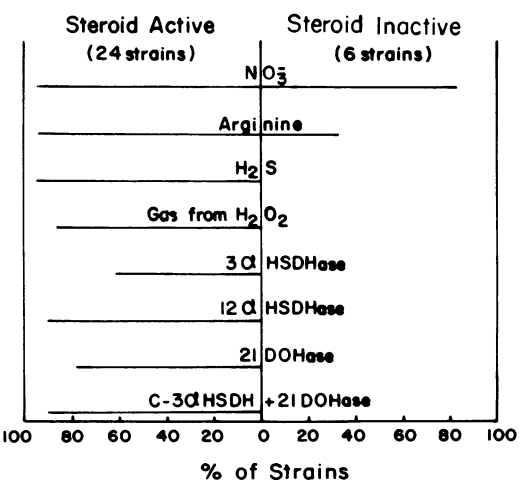

FIG. 6. *Metabolic characteristics of 30 strains classified according to their production of steroid enzymes.*

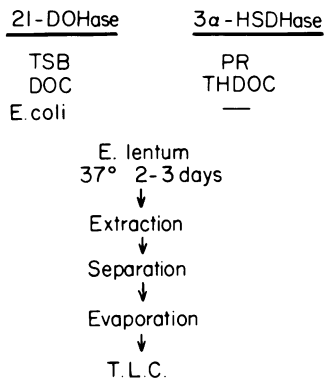

FIG. 7. *Simple method for detection of 21-DOHase and 3α-HSDHase. TSB, Trypticase soy broth; TLC, thin-layer chromatography.*

solvent is evaporated under a stream of N_2. The residue, containing the steroids, is dissolved in 50 μl of acetone and, with appropriate controls, subjected to thin-layer chromatography as described in Materials and Methods.

ACKNOWLEDGMENTS

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