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CHARACTERIZATION OF A NADH:FLAVIN OXIDOREDUCTASE INDUCED BY CHOLIC ACID IN A 7α-DEHYDROXYLATING INTESTINAL *EUBACTERIUM* SPECIES

ROBERT H. LIPSKY and PHILLIP B. HYLEMON

Department of Microbiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298 (U.S.A.)

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Summary

A NADH:flavin oxidoreductase was partially purified (seven-fold) from an intestinal *Eubacterium* species V.P.I. 12708 using Bio-Gel A 0.5-M and DEAE-cellulose column chromatography. Enzyme activity was measured spectrophotometrically at 340 nm under anaerobic conditions. A molecular weight of 260 000 was estimated by gel filtration chromatography. The partially purified enzyme preparation exhibited single displacement kinetics with respect to the substrates NADH and FAD. The pH optimum under these conditions was 6.8. NADH:flavin oxidoreductase showed an absolute specificity for NADH as electron donor. However, methylene blue, 2,6-dichlorophenolindophenol, $K_3Fe(CN)_6$, menadione, riboflavin, FMN and molecular oxygen served as alternate electron acceptors with varying degrees of efficiency. Acriflavin, rotenone, *o*-phenanthroline, *p*-chloromercuribenzoate, dicoumarol and 2,4-dinitrophenol inhibited enzyme activity. Surprisingly, 0.1 mM cholic acid, but not 0.1 mM deoxycholic acid, rapidly induced NADH:flavin oxidoreductase activity in growing cultures.

Introduction

There is little information regarding the origin of reducing equivalents necessary for reductive steroid biotransformations by intestinal anaerobes. Feighner et al. [1] have recently shown that NAD(P)H:flavin oxidoreductase can provide reduced flavins which are required for the 21-dehydroxylation of deoxycorticosterone in *Eubacterium lentum*. Moreover, Glass et al. [2] reported that the reduction of 4-androsten-3,17-dione to 5β-androstan-3,17-dione by cell extracts

of *Clostridium paraputrificium* required a reduced pyridine nucleotide and flavin, indicating the possible participation of NAD(P)H:flavin oxidoreductase for the reduction of the C_4-C_5 double bond.

Extensive enzymatic characterization studies of NADH and NADPH specific flavin oxidoreductases have been carried out using enzymes purified from the bioluminescent bacterium, *Beneckea harveyi* [3—6]. Each pyridine nucleotide specific enzyme activity showed a marked difference in their ability to utilize alternate electron acceptors [4]. The NADH:flavin oxidoreductase showed an almost absolute specificity for FMN. In contrast, the NADPH:flavin oxidoreductase from the same organism exhibited appreciable activity with alternate electron acceptors. NAD(P)H:flavin oxidoreductases appear to participate in supplying unbound flavin, which is subsequently reoxidized by luciferase in *B. harveyi* [6].

In the present communication, we report initial characterization of NADH: flavin oxidoreductase from an intestinal *Eubacterium* species and provide evidence for induction of this enzyme by cholic acid but not deoxycholic acid.

Materials and Methods

Bacterial strains and cultural conditions. Eubacterium sp. V.P.I. 12709 was originally isolated by Dr. R. Hamman (Institut für Med. Microbiologie und Immunologie der Universität Bonn, F.R.G.) from a fecal sample taken from a colon cancer patient. The bacterium was characterized by Drs. L.V. Holdeman and W.E.C. Moore at the Virginia Polytechnic Institute (V.P.I.) and State University, Anaerobe Laboratory, and identified as a member of the genus *Eubacterium*, but could not be assigned to a species at that time. Stock cultures were maintained in chopped meat medium under anaerobic conditions as described by Holdeman and Moore [7]. *Eubacterium* sp. V.P.I. 12708 was routinely cultured in complex medium having the following composition: 20 g/l brain heart infusion (Difco); 10 g/l yeast extract (Difco); 2 mg/l hemin; 43 mg/l sodium cholate and standard salts solution as described by V.P.I. Anaerobe Laboratory Manual [7]. Following sterilization of the medium at 121°C (1.06 $kg \cdot cm^{-2}$ for 20 min), sterilized solutions of L-cysteine and Na_2CO_3 were added to give final concentrations of 1.0 and 4.0 g/l, respectively. The pH was adjusted to 7.0 with 6 N HCl. A 0.3% inoculum of frozen (—70°C) cell suspension in 33% glycerol was used to initiate growth under 100% nitrogen atmosphere, Growth at 37°C was determined by measuring culture turbidity with a Klett-Summerson colorimeter equipped with a number 66 (red) filter.

Preparation of cell extracts. All manipulations in preparing the crude cell extract were performed aerobically. Early stationary phase cells (180—200 Klett Units) were harvested by centrifugation at 13 700 $\times g$ for 20 min at 20°C. Cells were suspended in 10 mM sodium phosphate buffer (pH 6.8)/0.1 mM EDTA/0.1 mM dithiothreitol/5% glycerol (Buffer A). The suspended cells were broken by two passages through a French Pressure cell (1054—1266 $kg \cdot cm^{-2}$) at 4°C. DNAase was added and the broken cells incubated at 23°C for 15 min. 1% protamine sulfate (pH 7.0) was added and the broken cells centrifuged at 13 700 $\times g$ for 15 min at 4°C. The supernatant fluid was removed and centri-

fuged at 105 000 × *g* at 4°C. The supernatant fluid (crude cell extract) was collected for NAD(P)H:flavin oxidoreductase assays. Extract was stored aerobically at 4°C. Very low (<0.020 U/mg protein) NAD(P)H:flavin oxidoreductase activity was found associated with the pellet fraction.

Enzyme assays. The standard reaction mixture (total volume 3.0 ml) for NADH:flavin oxidoreductase contained 10 mM sodium phosphate buffer (pH 6.8)/0.16 mM NAD(P)H/0.16 mM FAD/0.1 mM tetrasodium EDTA and enzyme preparation. All stock solutions and reaction mixtures were maintained anaerobically by sparging with argon gas that had been passed over a hot (350°C) column containing copper filings. Assays were performed anaerobically in septum-capped cuvettes (3.5 ml capacity). Specific activities were always determined with protein concentrations that were directly proportional to initial reaction velocities.

Enzymes were assayed spectrophotometrically at 37°C, and the initial rates of oxidation of reduced pyridine nucleotides were determined at a wavelength of 340 nm. Initial rates were converted to μmol of NAD(P)H oxidized by assuming a molar extinction coefficient of $6.22 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$. Specific activities are expressed as micromoles of NAD(P)H oxidized per min per mg protein. Protein estimations were performed according to the methods of Kalb and Bernlohr [8] and Lowry et al. [9].

Column chromatography procedures. The crude cell extract preparation was chromatographed on a Bio-Gel 0.5-M column (2.6 × 85 cm) which was equilibrated with Buffer A. The column was calibrated with blue dextran 2000, NAD-dependent 7α-hydroxysteroid dehydrogenase (M_r 320 000) from *Bacteroides thetaiotaomicron* NCTC 10852, catalase (M_r 240 000), aldolase (M_r 158 000) and bovine serum albumin (M_r 68 000) as molecular weight standards. The 7α-hydroxysteroid dehydrogenase was in the form of a crude cell extract prepared from *B. thetaiotaomicron* and assayed according to Sherrod and Hylemon [10]. The enzyme preparation was eluted with equilibration buffer and collected in 5-ml fractions at a flow rate of 12 ml/h. Fractions were assayed for NADH and NADPH-dependent flavin oxidoreductase activity. Those fractions having the greatest NADH-dependent specific enzyme activity were pooled and used for diethylaminoethyl (DEAE)-cellulose column chromatography.

Pooled fractions obtained from the Bio-Gel column were applied to a DEAE-cellulose column (2.6 × 30 cm) previously equilibrated with Buffer A and protein was eluted with a gradient formed by mixing 500 ml of Buffer A and 500 ml Buffer A which was 0.4 M in NaCl (5-ml fractions; flow rate 10 ml/h). Fractions were assayed for NADH and NADPH-dependent flavin oxidoreductase activities. Fractions having the highest NADH-dependent specific enzyme activity were pooled for use in subsequent enzyme characterization studies.

Chemicals. NADH and NADPH were obtained from P-L Biochemicals. Sodium cholate, sodium deoxycholate, and *p*-chloromercuribenzoate were purchased from Calbiochem. The following chemicals were purchased from Sigma Chemical Co.: 2,4-dinitrophenol, rotenone, acriflavin, *o*-phenanthroline, FAD, FMN and 2,2′-dipyridine. Methyl viologen and benzyl viologen were purchased from K. and K. Laboratories. Chromatography supplies were:

Bio-Gel A 0.5-M, Biorad and DEAE-cellulose (DE-52), Whatman Biochemicals, Ltd.

Results

Column chromatography of NADH:flavin oxidoreductase

Both NADH and NADPH-dependent flavin oxidoreductase activities were detected in crude cell extracts prepared from *Eubacterium* sp. V.P.I. 12708. When crude cell extracts were subjected to Bio-Gel A 0.5-M column chromatography (Fig. 1), two peaks of enzyme activity were observed. The large peak corresponded to NADH-dependent flavin oxidoreductase (M_r 260 000) and the small peak to the NADPH-dependent flavin oxidoreductase (M_r 250 000). Pooled Bio-Gel column fractions (42—50) were then subjected to DEAE-cellulose chromatography (Fig. 2). No NADPH-dependent flavin oxidoreductase activity was detected from pooled DEAE-cellulose fractions. The pooled DEAE-cellulose fractions (130—150) were subjected to 6-aminohexanoyl-FMN Sepharose-4B affinity chromatography, prepared according to Hastings et al. [11]. A FMN elution gradient (0—10 mM) was used in an attempt to release bound enzyme from the FMN-substituted affinity column. However, this procedure was unsuccessful in recovering any active enzyme. A NaCl gradient (0—0.6 M) was also attempted with no effect in releasing any enzyme activity. No further attempts were made to purify the enzyme using affinity chromatography.

The undialyzed pooled DEAE-cellulose preparation was utilized in all subsequent characterization studies. This activity was purified seven-fold over crude cell extract with a specific activity of 2.15 μmol NADH oxidized per min per mg protein. The NADH-dependent flavin oxidoreductase had a pH optimum of 6.8 in 50 mM potassium phosphate buffer.

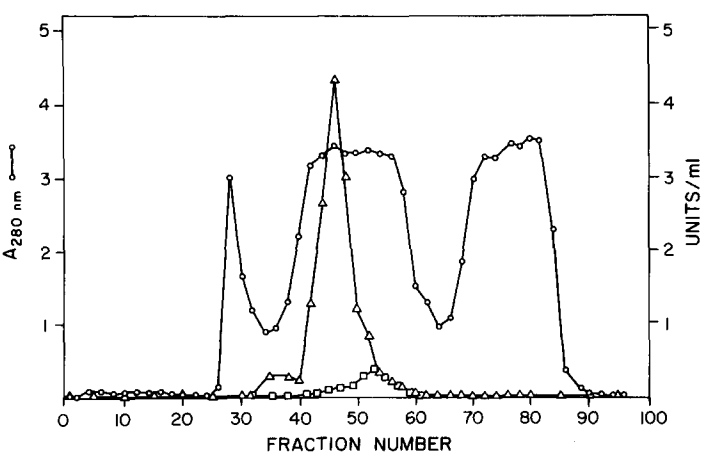

Fig. 1. Elution profile of NADH and NADPH-dependent flavin oxidoreductase from Bio-Gel A 0.5-M column. Crude cell extract was applied to the column (12 ml, 48 mg protein ml^{-1}). Fractions (5-ml) were collected and assayed for absorption at 280 nm (○), NADH (△), and NADPH (□) dependent flavin oxidoreductase activities using standard assay conditions.

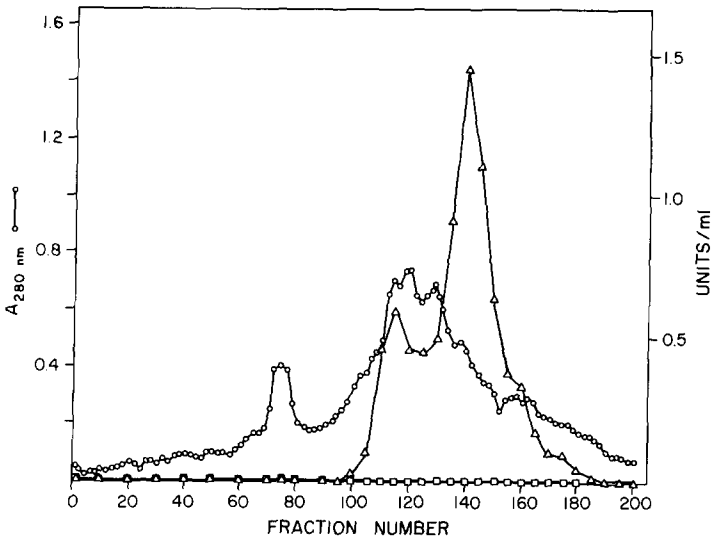

Fig. 2. Elution profiles of NADH-dependent flavin oxidoreductase from DEAE-cellulose column chromatography. Fractions (5-ml) were collected and assayed for absorbance at 280 nm (○), NADH (△), and NADPH (□) dependent flavin oxidoreductase activities using standard assay procedures.

Kinetic properties of NADH:flavin oxidoreductase

Figs. 3 and 5 show saturation curves for NADH and FAD, respectively, at different concentrations of their complement substrate. A Lineweaver-Burk plot of the data in Fig. 3 yielded non-parallel lines which intersected behind the ordinate (Fig. 4).

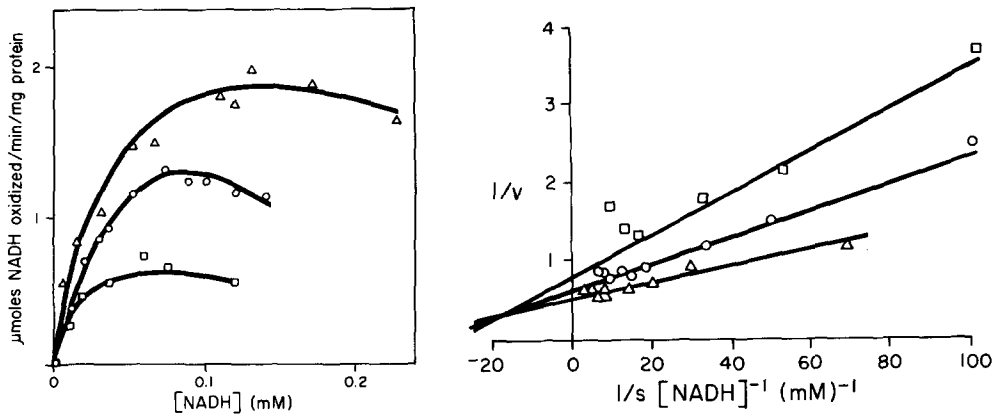

Fig. 3. Initial velocities of NADH oxidation as a function of NADH concentration. FAD concentrations were: 0.13 mM (△), 0.07 mM (○), and 0.04 mM (□).

Fig. 4. Double reciprocal plot of the data from Fig. 3. FAD concentrations are, from top line downward, 0.04, 0.07, and 0.13 mM, respectively.

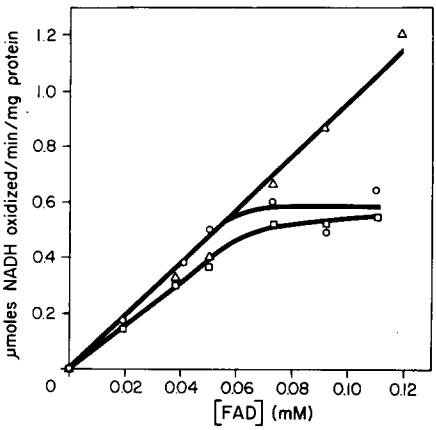

Fig. 5. Initial velocities of NADH:flavin oxidoreductase as a function of FAD concentration at constant NADH concentration. NADH concentrations were: 0.12 mM (△), 0.08 mM (○), and 0.04 mM (□).

Alternate electron donors and acceptors

The data in Table I shows the effects of different electron acceptors on NADH:flavin oxidoreductase activity. The enzyme utilized a number of alternate electron acceptors (Table I). Methylene blue (0.25 mM) was a more effective electron acceptor than FAD. Moreover, FMN, riboflavin, menadione, $K_3Fe(CN)_6$, and 2,6-dichlorophenolindophenol also served as electron acceptors. It is noteworthy that molecular oxygen could serve as electron acceptor for this enzyme to a limited extent (Table I). Acriflavin, janus green B, methyl viologen, and benzyl violgen could not serve as electron acceptors. The following compounds could not serve as electron donors for this enzyme: NADPH (0.13 mM); $Na_2S_2O_4$ (0.1 mM); reduced methyl viologen (0.1 mM), benzyl viologen (0.1 mM) and phenosafranin (0.1 mM).

TABLE I

THE RELATIVE ACTIVITY OF PARTIALLY PURIFIED NADH-DEPENDENT FLAVIN OXIDOREDUCTASE FROM *EUBACTERIUM* sp. V.P.I. 12708 WITH DIFFERENT ELECTRON ACCEPTORS

Acceptor concentration ($\times 10^{-4}$ M)	Activity *
Methylene blue (2.5)	108
FAD (1.3)	100
2,6-Dichlorophenolindophenol (0.7)	94
Riboflavin (1.3)	38
FMN (1.3)	26
O_2 (2.0) **	11
$K_3Fe(CN)_6$ (1.0)	+ ***
Menadione (1.0)	+ ***

* All values of activity expressed as the percent activity normalized to the activity found with NADH (0.13 mM) and FAD (0.13 mM). Values were obtained by measuring the decrease in absorption at 340 nm under anaerobic conditions.

** O_2 concentration (Chappell, 1964).

*** +, indicates a decrease in absorption at 340 nm. However, because of the overlapping absorption spectra of these compounds with NADH these results were not quantitated.

TABLE II

EFFECT OF SELECTED COMPOUNDS OF NADH: FLAVIN OXIDOREDUCTASE ACTIVITY FROM *EUBACTERIUM* SP. V.P.I. 12708

Inhibitor	Concentration (M)	Inhibition * (%)
No additions	—	0
Acriflavin **	$1 \cdot 10^{-4}$ g/ml	79
o-Phenanthroline	$5 \cdot 10^{-3}$	54
Rotenone	$1.5 \cdot 10^{-4}$	53
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	25
Dicoumarol	$3 \cdot 10^{-4}$	18
2,4-Dinitrophenol	$5 \cdot 10^{-6}$	9
2,2'-Dipyridine	$5 \cdot 10^{-5}$	0

* Expressed as that activity relative to NADH (0.13 mM) and FAD (0.13 mM).
** Acriflavin is a mixture of 3,6-diamino-10-methylacridinium chloride and of 3,6-diaminoacridine.

Inhibition of NADH:flavin oxidoreductase

The data in Table II show the effects of various inhibitors on enzyme activity of NADH:flavin oxidoreductase. Acriflavin gave the highest percent inhibition of any of the inhibitors tested. All electron transport inhibitors used gave varing degrees (9% to 53%) of inhibition (Table II).

Induction of NADH:flavin oxidoreductase

Parallel cultures of *Eubacterium* sp. V.P.I. 12708 were grown in complex anaerobic medium (see Materials and Methods), but lacking any added bile acids (Fig. 6a and b). After harvesting two culture samples from each growing

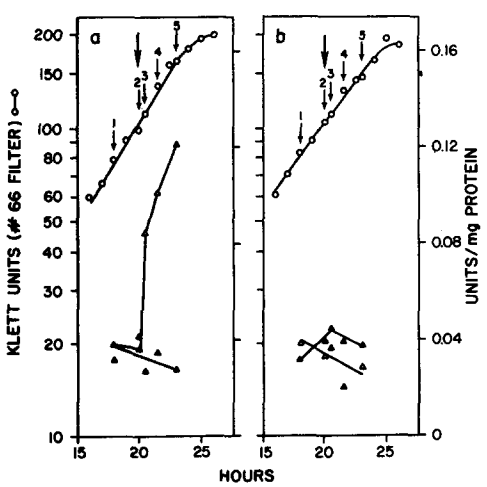

Fig. 6. Change in the specific activities of NADH and NADPH-dependent flavin oxidoreductase in crude cell extracts prepared from culture samples taken during the different growth stages of *Eubacterium* species V.P.I. 12708 following the addition (large arrows) of cholic (0.1 mM) or deoxycholic acids (0.1 mM) (panels a and b, respectively). NADH (△), and NADPH (▲) dependent flavin oxidoreductase activities were assayed using standard conditions.

culture, 0.1 mM sodium cholate (Fig. 6a) or 0.1 mM sodium deoxycholate were individually added (Fig. 6b). The NADH-dependent flavin oxidoreductase specific activity increased approx. two-fold within 30 min following the addition of 0.1 mM sodium cholate, increasing to greater than three-fold after 3 h (Fig. 6a). In contrast, the parallel culture (Fig. 6b) showed a slight decrease in NADH:flavin oxidoreductase specific activity over the 3 h time course following addition of 0.1 mM sodium deoxycholate. Levels of NADPH:flavin oxidoreductase specific activity for both cultures remained essentially unchanged throughout the time course of these experiments (Fig. 6). The addition of sodium cholate (0.1 mM) to enzyme reaction mixtures did not directly affect NADH:flavin oxidoreductase activity. Moreover, the addition of chloramphenicol (50 μg/ml) to growing cultures 10 min prior to the addition of sodium cholate (0.1 mM) totally blocked the increase in NADH:flavin oxidoreductase specific activity.

Discussion

NAD(P)H:flavin oxidoreductase catalyzes the oxidation of reduced pyridine nucleotides with the concomitant reduction of a flavin. We previously reported that the 21-dehydroxylation of deoxycorticosterone by anaerobically dialyzed cell extracts of *E. lentum* required a reduced flavin for activity. The reduced flavin could be generated by photochemical reduction or by NAD(P)H:flavin oxidoreductase also present in the crude cell extracts. Strictly anaerobic conditions are required for the accumulation of reduced free flavins because these compounds are rapidly auto-oxidized by molecular oxygen [12].

The NADH:flavin oxidoreductase characterized in the present study showed an absolute specificity for NADH. In contrast, Duane and Hastings [6] and Michaliszyn et al. [3] reported that purified NADPH-dependent flavin oxidoreductase from *B. harveyi* and *Photobacterium fischeri* could also utilize NADPH as an alternate electron donor. The NADH-dependent flavin oxidoreductase from *Eubacterium* sp. V.P.I. 12708 used a variety of alternate electron acceptors, including FMN and riboflavin (Table I).

NADH:flavin oxidoreductase obeyed a sequential mechanism for the substrates NADH and FAD (Fig. 4). Similar results were observed when the data in Fig. 5 were plotted as double reciprocal plots. Jablonski and DeLuca [5], using a highly purified preparation of NADH:flavin oxidoreductase isolated from *B. harveyi*, reported similar enzyme kinetics.

A relative molecular weight of 260 000 was estimated for the NADH:flavin oxidoreductase isolated from *Eubacterium* sp. V.P.I. 12708 based on peak elution volume from Bio-Gel A 0.5-M column chromatography. This apparent molecular weight is much higher than that reported for NADH or NADPH-dependent flavin oxidoreductases isolated from other bacteria, which have ranged between 23 000 to 30 000 for the NADH:flavin oxidoreductases and approx. 43 000 for the NADPH:flavin oxidoreductase [3,4,6,13]. Whether the relatively high molecular weight represents aggregation or an association with another enzyme is not known. However, no NADH:flavin oxidoreductase activity was detected in column fractions eluting within the range of M_r

23 000—43 000. Moreover, changes in ionic strength of the elution buffer did not change the molecular weight estimation.

The physiological relationship of NADH:flavin oxidoreductase and 7α-dehydroxylase in this bacterium is not yet clear. However, 7α-dehydroxylation activity in anaerobically dialyzed cell extracts of this organism is highly stimulated by chemically reduced flavins. Furthermore, 7α-dehydroxylase activity and NADH:flavin oxidoreductase activity are both rapidly induced by sodium cholate, but not sodium deoxycholate (Hylemon, P.B., unpublished data). Hence, NADH:flavin oxidoreductase may be supplying reduced free flavins for 7α-dehydroxylase. The purification and characterization of 7α-dehydroxylase from *Eubacterium* sp. V.P.I. 12708 will be necessary to determine the relationship between NADH:flavin oxidoreductase and 7α-dehydroxylase in this anaerobic intestial bacterium.

Acknowledgments

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