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# Cholesterol-Reducing Bacterium from Human Feces

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An anaerobic, gram-positive diplobacillus that reduces cholesterol to coprostanol was isolated from human feces and rat cecal contents. The isolates closely resemble a cholesterol-reducing organism isolated by Eyssen et al. (H. Eyssen et al., Eur. J. Biochem. 36:412-421, 1973) from a rat's cecum. These organisms would not form colonies and were isolated and cultivated in an anaerobic medium containing homogenized pork brains (naturally high in cholesterol). These organisms require free or esterified cholesterol for growth. They were isolated by serially diluting feces or cecal contents and inoculating brain medium. Colony-forming organisms, which did not reduce cholesterol, were eliminated by addition of inhibitory agents to the brain medium cultures. This serial dilution procedure was performed until a pure culture of a cholesterol-reducing organism was obtained.

Colon cancer is one of the most common human tumors in the United States (2, 11, 20). This is an environmental disease, most likely related to diet (2, 3, 7, 11, 19). Populations from developed countries, which consume diets high in meat and low in roughage, are at a much higher risk for colon cancer than are more rural populations (9, 14). Investigations conducted by Hill and Aires (9) and later by Reddy and Wynder (14) suggested a correlation between a high degree of bacterial degradation of bile acids and neutral steroids and a high incidence of colon cancer. Our interest in bacterial action on cholesterol is due to the suggestion by several investigators that cholesterol or bile acids might be made into carcinogens by intestinal bacteria (9, 14, 17). However, none of the postulated carcinogens have yet been found in the intestinal tract or have been produced by bacteria either in vivo or in vitro.

A primary reaction of intestinal bacteria on neutral steroids is the conversion of cholesterol to coprostanol. This reaction results in the reduction of the 5,6-double bond of cholesterol to form the saturated derivative coprostanol. Early attempts to obtain a pure culture of the intestinal organism(s) responsible for the formation of coprostanol (6, 16) were unsuccessful; however, in 1973 Crowther et al. (5) reported that 30 of the 90 strains they tested of common isolates of intestinal bacteria were capable of reducing cholesterol to coprostanol. In that same year, Eyssen et al. (8) also reported the isolation from rat cecal contents of an anaerobic, gram-positive diplobacillus that reduced $\Delta^5$-3$\beta$-hydroxy steroids (i.e., cholesterol, campesterol, $\beta$-sitosterol, and stigmasterol) to the corresponding saturated derivatives. This organism (ATCC 21408) has been tentatively placed in the genus *Eubacterium*, and it has an absolute growth requirement for $\Delta^5$-3$\beta$-hydroxy steroids (1 to 2 mg/ml of medium). The *Eubacterium* of Eyssen et al. (8) does not form colonies or grow on conventional media, and the isolation of this organism required the use of consecutive serial dilutions and a filtration step to eliminate other organisms. Eyssen et al. (8) found that the normal intestinal bacteria, which formed colonies on anaerobic agar plates, could not form coprostanol from cholesterol.

In the present work, we confirm the conclusions of Eyssen et al. (8) and report what we believe to be the first isolation of a cholesterol-reducing bacterium from human feces.

## MATERIALS AND METHODS

**Bacterial strains.** The *Eubacterium* isolated by Eyssen et al. (8) was obtained from the American Type Culture Collection (ATCC 21408). Stock cultures of the *Eubacterium* were transferred every 2 weeks in standard pork brain (SPB) medium. Other strains of anaerobic bacteria were from the culture collection of the Anaerobe Laboratory of Virginia Polytechnic Institute and State University (VPI) and are listed in Table 1. The majority of these organisms were isolated by W. E. C. Moore and L. V. Holdeman in their study of the human fecal flora of 20 Japanese-Hawaiians (12). These organisms were identified and cultured by methods described in the *Anaerobe Laboratory Manual* (13).

**Media.** The SPB medium was similar to that described by Snog-Kjaer et al. (16) and contained,

Table 1. *Bacterial strains which were tested for their ability to reduce cholesterol to coprostanol*

| Organism | VPI Strain Number |
|---|---|
| Strains used with Crowther et al.'s medium and methods: | |
| *Bacteroides thetaiotaomicron* | 5951, 3512, 3051, 2302, 0940-1, 8601, 6387, 5444-A, 3936, 0061-1 |
| The 16 most frequently occurring bacterial species of the fecal flora of 20 Japanese-Hawaiians (11): | |
| *Bacteroides vulgatus* | C9-28, C7-2, C6-7, C1-13 |
| *Fusobacterium prausnitzii* | C23-32, C22-30, C18-22, C17-4 |
| *Bacteroides adolescentis* | C23-4, C22-37, C17-29, C14-20 |
| *Eubacterium aerofaciens* | C22-5, C18-13, C14-9, C13-8A |
| *Peptostreptococcus productus - II* | C23-1, C21-38, C19-4, C18-23 |
| *Bacteroides thetaiotaomicron* | C22-15, C14-5, C11-15, C9-11 |
| *Eubacterium eligens* | C23-19, C22-19, C19-20, C18-6 |
| *Peptostreptococcus productus - I* | C22-23, C21-31, C19-2, C18-37 |
| *Eubacterium biforme* | C17-5, C15-18, C12-6, C10-14 |
| *Eubacterium aerofaciens - III* | C19-24, C17-39, C15-8A, C14-10 |
| *Eubacterium rectale - I* | C18-17, C13-45, C12-18, C10-16 |
| *Bacteroides distasonis* | C21-1, C19-17, C18-7, C14-2 |
| *Eubacterium rectale - II* | C21-15, C13-4, C11-50, C10-17 |
| *Bacteroides fragilis ss. a* | C12-24, C8-19, C1-23, C2-19 |
| *Eubacterium rectale - IV* | C23-32, C17-1, C15-7, C13-3 |
| *Bifidobacterium longum* | C23-5, C14-6, C10-21, C9-8 |

per liter: homogenized pork brain, 130 g; Trypticase (BBL), 18 g; yeast extract (Difco), 0.5 g; sodium chloride, 2.2 g; dipotassium phosphate, 1.3 g; agar (Difco), 0.5 g; L-cystine (Sigma), 0.4 g; cholesterol (Sigma), 0.5 g; sodium thioglycolate (Sigma), 0.3 g; and methylene blue, 2 mg. All medium ingredients were combined and thoroughly blended in a Nova I Waring blender (Waring Products Div., New Hartford, Conn.) for 1 min. The pH was adjusted to 7.2 with 8 N sodium hydroxide, and the medium was boiled for 3 to 5 min. Within 10 min, the medium was placed inside an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) for 45 to 60 min and dispensed into test tubes (18 by 142 mm), 9 ml/tube, and no. 1 black rubber stoppers were inserted. The tubes were autoclaved (121°C, 15 min) in a press. The composition of the medium (4) used by Crowther et al. (5) contained, per liter: acetone-extracted pork brains, 20 g; dipotassium phosphate, 5 g; cysteine-hydrochloride (Sigma), 0.5 g; Tween 80 (Baker), 2.5 ml; cholesterol (Sigma), 2.5 g; and inorganic salt solution, 10 ml. This medium was made as described for SPB medium. Acetone-extracted pork brains were prepared by extracting 500 g of homogenized pork brains with 1 liter of acetone for 24 h on each of 10 consecutive days with constant stirring. Cholesterol could not be detected after this procedure. The extracted brains were air dried before being used.

**Medium for cholesterol esters.** The medium for cholesterol esters contained, per liter: acetone-extracted pork brain, 28.8 g; Trypticase (BBL), 20 g; NaCl, 2.5 g; $K_2HPO_4$, 1.5 g; yeast extract (Difco), 1.0 g; sodium thioglycolate, 0.75 g; L-cystine, 0.4 g; $Na_2SO_3$, 0.2 g; methylene blue, 1 mg; and NaOH to pH 7.2. The medium was boiled, taken into the glove box, dispensed at 10 ml/tube, stoppered, and autoclaved (121°C, 15 min) in a press. Either 0.5 ml of cholesterol (20 mg/ml) in ether or 0.5 ml of cholesterol palmitate (28 mg/ml) in ether was added per 10 ml of media under $O_2$-free $N_2$, and the $N_2$ was allowed to evaporate the ether. This medium was inoculated with either 0.5 ml of *Eubacterium* (ATCC 21408) or our human isolate. Cultures were incubated for 5 to 7 days at 37°C.

Conventional anaerobic broth media were prepared as described in the VPI *Anaerobe Laboratory Manual* (13). We used the following media for agar plates: brain heart infusion agar with and without 5% (vol/vol) sheep blood, chopped-meat agar, egg yolk agar, rumen fluid-glucose-cellobiose-agar, peptone-yeast extract-glucose agar, and Trypticase soy agar. The plate cultures were incubated (1) in an anaerobic chamber.

**Source of human fecal samples.** Fecal samples were obtained from staff volunteers working at the VPI Anaerobe Laboratory. All volunteers were in good physical health and were not on unusual diets. The samples were collected in 12- by 12-inch (ca. 30.5- by 30.5-cm), interlocking-seal, polyethylene bags (KCL Corp., Shelbyville, Ind.). All samples were processed within 10 to 15 min of defecation. Before a fecal sample was used, it was mixed by vigorously kneading the bag.

**Serial dilutions of feces.** Approximately 1 g of mixed human feces was transferred to 9 ml of prereduced dilution salt solution (13) under $O_2$-free $N_2$. The exact weight of sample was calculated from the increased weight of the dilution tube. The first dilution tube was vigorously shaken for about 1 min until a homogeneous mixture was obtained. Serial 10-fold dilutions were then made. From each

of the dilution tubes a 1-ml sample was inoculated into 9 ml of SPB medium and incubated at 37°C. In the isolation of a cholesterol-reducing organism from human feces, the highest dilution that formed coprostanol was again serially diluted and inoculated again into SPB medium. The serial dilutions were continued in this manner until a total of seven consecutive serial dilutions were made for this isolation.

The set of 11 fecal dilutions (Table 2) was performed by using a blender inside the anaerobic glove box. Approximately 1 g of feces in 9 ml of dilution salt solution was added to 90 ml of dilution solution in the blender. This solution was blended for three 1-min periods. The solution was then diluted in a series of eight 10-fold dilutions. Each dilution (1 ml) was inoculated into 9 ml of SPB medium and incubated at 37°C for 20 days.

**Phenethyl alcohol treatment.** Phenethyl alcohol (10 ml) was filter sterilized with a 0.45-$\mu$m membrane filter (Millipore Corp.). The tube containing the alcohol was flushed with $O_2$-free $N_2$, and the alcohol was kept under this atmosphere. The phenethyl alcohol was added to mixed cultures in SPB medium to achieve concentrations from 0.1 to 1.0% (vol/vol) in 0.1% increments.

**Extraction of steroids.** Feces were extracted by placing approximately 1 g of fecal sample in a test tube (25 by 150 mm) and adding 20 ml of petroleum ether. This mixture was vigorously shaken for 1 min, the solid matter was allowed to settle, and the petroleum ether was removed. The petroleum ether was centrifuged at 935 × *g* for 10 min. The supernatant was assayed by gas-liquid chromatography. Steroids were extracted from SPB medium by adding 1 ml of 1 N sodium hydroxide and 2 ml of petroleum ether to 1 ml of medium in a test tube (15 by 125 mm) and then following the above procedure. The presence of cholesterol and coprostanol in the petroleum ether extracts from SPB cultures was confirmed by gas-liquid chromatography-mass spectrometry.

**Assay of cholesterol esters.** One milliliter of culture was added to 2 ml of petroleum ether in a test tube that was capped and inverted at least 20 times. The tube was centrifuged at 1,000 × *g* for 1 min, and a sample of the petroleum ether extract was assayed by gas-liquid chromatography. Another 1 ml of culture was added to 5 ml of 1 N KOH in 90% ethanol and heated at 90°C for 90 min. The tube was allowed to cool, then 1.5 ml of distilled water and 2 ml of petroleum ether was added, and the sample was extracted and assayed by the above procedure.

**Gas-liquid chromatography for neutral steroids.** Extracted neutral steroids (in petroleum ether) were separated and quantitated with a Hewlett-Packard model 5830-A computer-integrated (18850A GC terminal) gas chromatograph with a hydrogen flame-ionization detector (Hewlett-Packard Co., Avondale, Pa.). $N_2$ was used as carrier gas at a flow rate of 30 cm$^3$/min. A coiled glass column (274.3 cm by 2-mm ID) was packed with 3% QF-1 on GasChrom Q, 100 to 120 mesh (Applied Science Laboratories Inc., State College, Pa.). The column was kept at a constant temperature of 225°C. The injector and detector were at 270°C. The relative retention times were determined with known reference standards (Sigma Chemical Co.). When quantitation of the steroids was required, 5-$\alpha$-cholestane was used as an internal standard.

**Inhibition of bacterial cholesterol reduction.** The final dilution ($10^{-10}$) of a fecal specimen from a low-cholesterol converter was streaked on a rumen fluid-glucose-cellobiose-agar roll tube for isolation of organisms that might antagonize cholesterol reduction. The original mixture contained three morphologically different colony types. These different colony types were picked and grown in chopped-meat broth under pure $CO_2$ either as pure cultures or as a mixed inoculum.

Tubes of SPB medium (9 ml) were inoculated under $O_2$-free $N_2$ with 0.5 ml of a 7- to 10-day-old SPB culture of either *Eubacterium* (ATCC 21408) or our human fecal isolate. We then tested for inhibition by adding 0.5 ml of the original mixture to

Table 2. *Comparison of 11 different serial dilutions of feces*

| Subject[a] Number | % Coprostanol In Fecal Sample | % Coprostanol Formed in Fecal Dilution Cultures | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ |
| 1 | 4 | 8 | 14 | 33 | 46 | 37 | 6 | 6 | 0 | 0 |
| 2 | 6 | 2 | 5 | 8 | 12 | 8 | 8 | 6 | 7 | 10 |
| 3 | 10 | 4 | 6 | 8 | 19 | 0 | 0 | 0 | 0 | 0 |
| 4 | 15 | 2 | 6 | 13 | 8 | 12 | 26 | 10 | 40 | 0 |
| 5 | 43 | 14 | 3 | 4 | 3 | 0 | 0 | 0 | 0 | 0 |
| 6 | 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 62 | 100 | 100 | 100 | 100 | 100 | 81 | 81 | 63 | 0 |
| 8 | 79 | 44 | 70 | 100 | 82 | 100 | 80 | 79 | 0 | 0 |
| 9 | 86 | 100 | 100 | 100 | 100 | 100 | 100 | 47 | 65 | 0 |
| 10 | 100 | 100 | 100 | 78 | 100 | 80 | 100 | 55 | 75 | 0 |
| 11 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 |

[a]Sample size ranged from 0.90 to 1.16 of wet feces.

some tubes, separate isolates to others, and all possible combinations of the isolates to other tubes. The tubes were incubated for 10 days at 37°C, and then we determined the relative proportions of cholesterol and coprostanol.

## RESULTS

**Isolation of organisms from the rat cecum.** Initial attempts to repeat the isolation as described by Eyssen et al. (8) of a cholesterol-reducing organism from the cecal contents of a rat were not successful. Coprostanol was not found on incubation of SPB medium with cecal contents from a rat fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.). Only small amounts of cholesterol (0.054 mg/g, wet weight) and coprostanol (0.021 mg/g, wet weight) were found in the cecal contents of rats fed this diet, and only about 28% of the cholesterol was converted to coprostanol in such animals. We attempted to increase the concentration of cholesterol and, we hoped, the concentration of coprostanol by feeding rats a high-cholesterol diet (2% Cholesterol Feed, Nutritional Biochemicals, Cleveland, Ohio). In rats fed the high-cholesterol diet there was an increase in the concentration of cholesterol in the feces (5 mg/g, wet weight), but the concentration of coprostanol was still low (0.2 mg/g, wet weight) even after 24 days on this diet.

Rosenheim and Webster (15) reported that rats fed a diet of raw brain excreted very large amounts of coprostanol. Since the mammalian brain contains 12% (dry weight) cholesterol, it was not necessary to supplement the diet with additional cholesterol. After 1 day on the brain diet, a rat excreted 1 mg of cholesterol and 4 mg of coprostanol per g (wet weight) of feces. This high level of coprostanol formation continued through day 6, when the animal was sacrificed. The cecal contents were used in an attempt to isolate the organism(s) responsible for the formation of coprostanol.

Cecal contents (0.57 g) were serially diluted. Tubes containing 9 ml of the SPB medium were inoculated with 1 ml of each dilution. After incubation, the amount of coprostanol in each tube was determined, and the highest dilution containing coprostanol was used as the inoculum for the next dilution series. Data for the first three consecutive serial dilutions are given in Table 3.

As the number of consecutive serial dilutions increased, the number of cell types discernible with the light microscope and the number of colony types that occurred on a variety of anaerobic plates decreased. None of the colony types picked from these agar plates formed coprostanol in pure culture in SPB medium. After the third serial dilution, the $10^{-7}$ dilution tube contained: a gram-negative rod, a gram-positive rod, and a gram-positive diplobacillus. Two colony types were found on anaerobic brain heart infusion agar plates that corresponded to the gram-negative and gram-positive rods. Neither of these two organisms could form coprostanol.

The gram-negative rod was eliminated from the culture by addition of phenethyl alcohol in 0.1% increments from 0.1 to 1.0%. Microscopic examination of the 0.5% culture and its subsequent subcultures revealed that the gram-negative rod had been eliminated. After four more consecutive serial dilutions, the gram-positive bacillus was eliminated (Table 3). A small (0.4 by 1.0 $\mu$m), gram-positive diplobacillus was observed with the light microscope in the cholesterol-reducing cultures. We could not obtain

Table 3. *Results of 7 consecutive serial dilutions of rat cecal contents*

| Dilution Series | Highest Dilution To Form Coprostanol | % Coprostanol In Highest Dilution | Incubation Period (days) |
|---|---|---|---|
| First | $10^{-8}$ | 70 | 7 |
| Second | $10^{-9}$ | 61 | 37 |
| Third[a] | $10^{-7}$ | 27 | 9 |
| Fourth | $10^{-9}$ | 68 | 7 |
| Fifth | $10^{-7}$ | 70 | 8 |
| Sixth | $10^{-8}$ | 70 | 14 |
| Seventh | $10^{-6}$ | 80 | 23 |

[a]The $10^{-7}$ tube of the third dilution was used to inoculate tube of SPB media containing phenethyl alcohol. The one containing 0.5% (vol/vol) phenethyl alcohol was used to inoculate the fourth dilution series.

colonies on any of a variety of aerobic and anaerobic agar media, and the organism did not grow in conventional broth media either aerobically or anaerobically. Thus, the cholesterol-reducing rat isolate appeared to be very similar to that described by Eyssen et al. (8), which was also isolated from rat cecal contents.

**Isolation of organisms from human feces.** Initial attempts to isolate such organisms from human feces were based on the use of a fecal inoculum from a person whose feces contained more coprostanol than cholesterol. A fecal sample (0.75 g) was diluted serially in 1:10 dilutions, and then samples of these dilutions were inoculated into SPB medium. Again, the highest dilution in which coprostanol was found was serially diluted. A total of seven consecutive serial dilutions were performed during this isolation (Table 4).

After the seven consecutive serial dilutions, only three morphological types of bacteria could be found: a gram-negative rod, a gram-positive coccus, and a small gram-positive diplobacillus. Only the gram-negative rod and gram-positive coccus formed colonies on anaerobic brain heart infusion agar plates; however, in pure cultures these two organisms did not form coprostanol.

In an attempt to eliminate these organisms, subcultures were made in tubes of SPB medium which contained different antibiotics. The following concentrations of antibiotics were tested: chloramphenicol, 12 μg/ml; clindamycin, 1.6 μg/ml; erythromycin, 3 μg/ml; penicillin, 2 U/ml; and tetracycline, 6 μg/ml. After 5 days of incubation, coprostanol was detected in those cultures containing clindamycin, erythromycin, and penicillin G. The erythromycin-treated culture contained only a gram-negative bacillus and gram-positive diplobacillus. The gram-positive coccus had been killed by the erythromycin.

The mixture was subcultured into tubes of SPB medium supplemented with phenethyl alcohol as previously described. After 7 days of incubation, coprostanol was detected in the tubes containing less than 0.8% phenethyl alcohol. This eliminated the gram-negative rod, and a gram-positive diplobacillus appeared to be the only remaining organism. The cholesterol-reducing diplobacillus did not form colonies on any of a variety of aerobic and anaerobic agar media, and the organism did not grow in conventional broth media aerobically or anaerobically in prereduced anaerobic broth media.

This organism is very similar to the organism isolated by Eyssen et al. (8); both organisms have slightly pointed cell ends and a characteristic diploid cell shape, and the cell dimensions are essentially the same (0.4 by 1.0 μm).

Eyssen et al. (8) reported that their cholesterol-reducing isolate did not reduce the double bond of cholesterol when the cholesterol was present as an ester. Since we found moderately high levels of esterified coprostanol in the feces of rural South African natives (J. F. Sperry, unpublished data), we wondered whether our human cholesterol-reducing isolate could reduce the double bond of esterified cholesterol. We found that Eyssen's organism converted 16% of the cholesteryl palmitate to coprostanol palmitate, and our human cholesterol-reducing isolate converted 23% of the cholesteryl palmitate to coprostanol palmitate. There were essentially no unesterified neutral steroids in these cultures.

**Requirement of cholesterol for cell growth.** The cholesterol-reducing *Eubacterium* isolated by Eyssen et al. (8) required the presence of a $\Delta^5$ sterol for growth. Growth of our rat and human cholesterol-reducing isolates in media

Table 4. *Results of consecutive serial dilutions of human feces*

| Dilution Series | Highest Dilution To Form Coprostanol | % Coprostanol In Highest Dilution | Incubation Period (days) |
|---|---|---|---|
| First | $10^{-5}$ | 44 | 14 |
| Second | $10^{-7}$ | 77 | 11 |
| Third | $10^{-7}$ | 70 | 26 |
| Fourth | $10^{-6}$ | 71 | 9 |
| Fifth | $10^{-6}$ | 72 | 10 |
| Sixth | $10^{-7}$ | 54 | 7 |
| Seventh | $10^{-7}$ | 82 | 11 |

made with acetone-extracted pork brain (cholesterol-free) was checked. No growth was observed, even after weeks of incubation. When cholesterol was added back to the medium prepared with acetone-extracted pork brain, both the rat and the human isolates grew and reduced the cholesterol to coprostanol.

**Variation in percent coprostanol formed with different fecal inocula.** A fecal sample that contained more coprostanol than cholesterol was used for isolation of the organism from human feces. However, many people normally excrete feces in which most of the cholesterol has not been reduced to coprostanol (18). We were interested in knowing what influence the original fecal inoculum would have on the in vitro conversion of cholesterol to coprostanol. Therefore, fecal samples from 11 volunteers were homogenized in a blender, diluted, and inoculated into SPB medium. The cultures were incubated for 20 days and then tested for the presence of coprostanol. Data for the serial dilutions of these 11 samples are given in Table 2. Fecal samples with low amounts of coprostanol (subjects 1 to 5) consistently produced a low level of coprostanol in the dilution cultures. Similarly, samples with high amounts of coprostanol (subjects 7 to 11) produced a high percentage of coprostanol in the dilution cultures. It appeared, from the dilution studies, that the relative numbers of cholesterol-reducing organisms are similar in high and low converters.

**Inhibition of cholesterol reduction.** Since the number of cholesterol-reducing organisms appears similar in high and low converters, the lower amount of coprostanol formed by mixed cultures obtained from certain individuals might be explained in two ways. The organism(s) responsible for coprostanol formation could be different and have different degrees of efficiency in reducing cholesterol, or other organisms present in the mixed cultures could inhibit the reduction of cholesterol. Therefore, we attempted to isolate the cholesterol-reducing organism(s) from the feces of an individual who routinely excreted very little coprostanol. The same general isolation scheme was used as previously described. We were able to reduce the number of organisms to three that form colonies on agar plates, but do not form coprostanol alone or in combination, and a gram-positive diplobacillus that will grow only in SPB broth. This mixed culture reduced only about 50% of the cholesterol in SPB broth to coprostanol. These isolated organisms were assayed for inhibition of coprostanol formation when mixed with pure cultures of the *Eubacterium* of Eyssen et al. (8) and our human isolate. These organisms did not significantly inhibit the formation of coprostanol by the human isolate, but the mixture inhibited the reduction of cholesterol by 50% with the *Eubacterium*. The most active inhibitory organism (47%) has been tentatively identified as a strain of *Fusobacterium russii* and a less inhibitory isolate (34%) has been identified as *Bacteroides vulgatus*.

**Anaerobic intestinal bacteria not capable of forming coprostanol.** Investigators (6, 15, 16) have been unsuccessful in attempts to demonstrate coprostanol formation by organisms that will grow or form colonies on cholesterol-free agar media either aerobically or anaerobically. During their isolation of the cholesterol-reducing *Eubacterium*, Eyssen et al. (8) also found that none of the colony-forming organisms could form coprostanol. Crowther et al. (5), however, reported that numerous anaerobic intestinal bacteria, which they isolated on conventional media, formed coprostanol from cholesterol. To resolve these conflicting reports, the following experiments were performed.

In the first experiment, the same methods and media used by Crowther et al. (5) were used (B. Drasar, personal communication). These workers found that strains of *Bacteroides thetaiotaomicron* produced the largest amounts of coprostanol. Unfortunately, all of the original strains used by Crowther et al. (8) were lost (M. J. Hill, personal communication). Therefore, 10 fecal isolates of *B. thetaiotaomicron* (Table 1) were tested for coprostanol formation. Coprostanol was not detected in any of the cultures. This experiment was repeated with the same results.

We next tested four strains of each of the 16 most frequently isolated bacterial species from the feces of 20 Japanese-Hawaiians (12) for their ability to form coprostanol in SPB medium (Table 1). Coprostanol was not formed in any culture.

To further test the ability of colony-forming organisms to form coprostanol, samples from seven of the last dilution tubes showing coprostanol formation in the experiment reported in Table 2 were streaked on rumen fluid-glucose-cellobiose-agar roll tubes (13) and incubated for 4 days. Each type of colony was picked, grown in pure culture in chopped-meat–carbohydrate broth (13) inoculated into SPB medium, and incubated for 10 days. Coprostanol was not formed by any of the 48 colony-forming organisms isolated from these cultures.

## DISCUSSION

The cholesterol-reducing organism isolated from human feces in this study appears to be the first such isolation. The report by Crowther et al. (5) of coprostanol formation by large numbers of anaerobes was not confirmed. We could not repeat their results, and they could not supply us with cultures that performed this reaction. Moreover, earlier investigators found that the double bond in cholesterol was not reduced by common fecal bacteria (6, 8, 15, 16).

The human isolate is very similar to th *Eubacterium* isolated by Eyssen et al. (8) fro.. the cecum of a rat. Both of these cholesterol-reducing organisms are small (0.4 by 1.0 μm), gram-positive, anaerobic diplobacilli that require cholesterol for growth. Both organisms could use cholesteryl palmitate for limited growth, and the double bond of the cholesterol in the ester was reduced. The lower level of the double bond reduction was probably due to the low solubility of this substrate in the medium.

The inability of these organisms to form colonies necessitated laborious and unconventional isolation procedures. Although the SPB medium supported growth when used as a broth medium, it did not support growth as a solid medium. This may be due to the small amount of cholesterol available at the surface of the agar. If the cholesterol is unable to diffuse through the solid media, growth would be limited by the availability of this substrate since these organisms require cholesterol for growth. Eyssen et al. (8) reported that pinpoint, watery colonies developed on brain agar medium after 7 days of incubation. We observed pinpoint droplets of a lipoidal substance on the solid medium; however, these droplets did not contain cells when examined microscopically. Failure to obtain colonies of the organism also means that we cannot be absolutely certain that we have a pure culture. It certainly is possible that two species that look identical could be present, but it does not seem likely.

Fecal samples with initial low coprostanol levels produced low amounts of coprostanol in vitro, and the converse was true for samples with high levels. Wilkins and Hackman (18) have reported that 25% of the individuals in a North American population converted less than 50% of their fecal cholesterol to coprostanol, and the percentage of cholesterol converted in an individual was relatively constant. Since inocula of feces with a low percentage of coprostanol retained their low cholesterol-reducing ability in the dilution cultures, the reasons for differences in coprostanol formation in vivo would seem to be related to the bacterial flora of an individual rather than to differences in the colonic environment.

Cholesterol-reducing organisms from low converters appear to be inhibited by certain members of the fecal flora. Eyssen's *Eubacterium* (ATCC 21408) was significantly inhibited (50%) by the mixed flora isolated from a low converter. One organism, tentatively identified as *Fusobacterium russii*, caused over 40% inhibition of coprostanol formation by *Eubacterium* (ATCC 21408). Our human cholesterol-reducing isolate was only slightly inhibited by *F. russii*, suggesting that the differences in the amount of coprostanol in the feces of individuals may be due to differing susceptibilities of the cholesterol-reducing organisms to certain antagonistic bacteria of the fecal flora.

The cholesterol-reducing organisms appear to be uniquely adapted to a specialized niche in the environment of the colon. Most other intestinal bacteria are not capable of attacking the abundant cholesterol of feces; however, the cholesterol-reducing organisms seem to have evolved a means of using it as a terminal electron acceptor as originally proposed by Eyssen et al. (8). This could be advantageous since it might allow adenosine 5'-triphosphate to be generated via a compound that is not utilized by the rest of the fecal flora.

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