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Isolation and Characterization of New Strains of Cholesterol-Reducing Bacteria from Baboons

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We isolated and characterized nine new strains of cholesterol-reducing bacteria from feces and intestinal contents of baboons. Cholesterol-brain agar was used for the primary isolation, and subsequent biochemical tests were done in a lecithin-cholesterol broth containing plasmenylethanolamine and various substrates. All strains had similar colony and cell morphology, hydrolyzed the β-glucosides esculin and amygdalin, metabolized pyruvate, and produced acetate and acetoin. Unlike previously reported strains, the nine new strains did not require cholesterol and an alkenyl ether lipid (e.g., plasmalogen) for growth; however, only two strains reduced cholesterol in the absence of the plasmalogen. These two strains also produced succinate as an end product. Carbohydrate fermentation was variable; some strains produced weak acid (pH 5.5 to 6.0) from only a few carbohydrates, whereas other strains produced strong acid reactions (pH ≤ 5.5) from a wide variety of carbohydrates.

Cholesterol is converted to coprostanol in the intestine by microbial reduction of the 5,6 double bond. Bacteria which reduce cholesterol to coprostanol have been isolated from the rat cecum (3) and from human (7) and baboon (5) feces and have been tentatively assigned to the genus *Eubacterium*. These organisms have a unique requirement for alkenyl ether lipids (e.g., plasmalogens) (5), require cholesterol or other steroids for growth, and do not form colonies on conventional agar media (3, 5, 7). We recently described an agar medium, cholesterol-brain agar (CBA), which facilitates isolation of these organisms (1). This report describes the isolation and characterization of nine new strains of cholesterol-reducing bacteria which have some characteristics which are significantly different from those shown by the two organisms that we previously characterized (6).

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MATERIALS AND METHODS

Bacteria. *Eubacterium* strains 403 and ATCC 21408, which we previously characterized (6), were included in this study for comparison and to include additional tests on these two isolates. New cholesterol-reducing bacteria were isolated as described below.

Culture conditions. The isolation procedures and biochemical testing were performed in a modified stainless steel Blickman chamber (S. Blickman Co., Weehawken, N.J.) under anaerobic conditions (2). The gas mixture contained 10% hydrogen, 5% carbon dioxide, and 85% prepurified nitrogen (Union Carbide Corp., Houston, Tex.). The chamber humidity was maintained at 40 to 50% relative humidity by a mechanical condenser. The cultures were incubated at 35°C in the chamber. All media were prepared aerobically and passed into the chamber at least 24 h before use.

Media. Standard brain medium was similar to that previously described (5, 8) and contained the following components per liter: lyophilized bovine brain, 30 g; Casamino Acids (Difco Laboratories, Detroit, Mich.), 10 g; yeast extract (Difco), 10 g; K_2HPO_4, 5 g; sodium thioglycolate, 0.5 g; and resazurin, 1 mg. The dry ingredients were suspended with water, and the mixture was homogenized in a blender (Waring model 31BL91, New Hartford, Conn.). The pH was adjusted to 7.2, and 4.5 ml of medium was dispensed into screw-capped tubes (16 by 100 mm) and autoclaved.

CBA was prepared as previously described (1), except that the brain was reduced to 2% and it was not papain treated. The dry ingredients were suspended with water, and the mixture was homogenized in a Waring blender for 5 min.

The base medium used in the biochemical characterization was similar to that previously described (6) and contained the following components per liter: Casitone (Difco), 10 g; yeast extract, 10 g; lecithin (type IIS; Sigma Chemical Co., St. Louis, Mo.), 1 g; sodium thioglycolate, 0.5 g; plasmenylethanolamine (PLE; Supelco, Inc., Bellefonte, Pa.), 0.2 g; cholesterol, 2 g; K_2HPO_4, 40 mg; KH_2PO_4, 40 mg; and resazurin, 1 mg (pH 7.2). This medium was prepared in the same manner as described previously for lecithin-cholesterol medium (5). Brain heart infusion agar (Difco) was supplemented with 0.5% sodium pyruvate (Sigma), 1% yeast extract, and resazurin (1 mg/liter).

Isolation procedure. Feces or intestinal contents were obtained from baboons which were fed a baboon chow diet (Special Monkey Chow 25, Ralston-Purina,

St. Louis, Mo.) supplemented with 20% fat (wt/wt) and 0.4% cholesterol (wt/wt). Feces from two baboons were collected and immediately placed in a GasPak (BBL Microbiology Systems, Cockeysville, Md.) anaerobe jar. Transport time to the anaerobe laboratory was 20 to 30 min. Sections of the upper colon and ileum were removed from seven animals and transported in a GasPak jar to the laboratory. All subsequent procedures were performed in the anaerobic chamber. The contents of the colon and ileum sections were removed inside the anaerobic chamber.

A 0.5-g portion of feces or intestinal contents was serially 10-fold diluted in standard brain medium to 10^{10}. A drop of the 10^2 and 10^3 dilutions was transferred with a sterile Pasteur pipette to CBA plates which were streaked for isolation. The CBA plates and the 10 serial dilution tubes were incubated at 35°C. The plates were observed after 3 to 5 days of incubation. Colonies showing the unusual morphology previously described (1) were inoculated into standard brain medium and incubated until the medium clotted (5), which occurs as the cholesterol-reducing bacteria approach 10^8 to 10^9 cells per milliliter of medium (unpublished data). The clotted cultures were streaked to CBA to check purity and analyzed for coprostanol by gas-liquid chromatography (GLC) (5). If suspect colonies were not present on the initial plates, we observed the serial dilution tubes for clotting. Since we know from experience that large numbers of cholesterol-reducing bacteria are present when the medium clots, we streaked a drop of medium from freshly clotted tubes of the dilution set on CBA. In some cases, several subcultures were made in standard brain medium to enhance the numbers of cholesterol-reducing bacteria before streaking to CBA.

Cholesterol and plasmalogen requirements. All isolates were streaked on supplemented brain heart infusion agar to test their ability to grow without cholesterol and plasmalogen. Isolates which grew were repeatedly subcultured on brain heart infusion agar to ensure that cholesterol and plasmalogen in the inoculum were not responsible for the growth.

All isolates were inoculated into lecithin-cholesterol medium (5) to test for cholesterol reduction in the absence of plasmalogens. Each isolate was also inoculated into lecithin-cholesterol medium containing 0.5 mg of PLE as a positive control. Cultures which reduced cholesterol in the lecithin-cholesterol medium without PLE were repeatedly subcultured to confirm the results.

Biochemical characterization. All new isolates plus *Eubacterium* strains 403 and ATCC 21408 were tested for reduction of nitrate, production of indole, hydrolysis of starch, gelatin, and esculin, and fermentation of amygdalin, arabinose, cellobiose, erythritol, fructose, glucose, glycogen, inositol, lactate, lactose, maltose, mannitol, mannose, melezitose, melibiose, pyruvate, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, threonine, trehalose, and xylose. We added sterile solutions of substrate to sterile base medium to yield a final volume of 2 ml; the concentrations of substrate were those recommended by Holdeman et al. (4). The test media were inoculated with 0.05 ml of cultures of the isolates grown in base medium. All cultures were incubated at 35°C for 7 days and observed for clotting as an indicator of growth (5). Fermentation of carbohydrates was determined by a change in pH compared with uninoculated controls and cultures in base medium without carbohydrate (4). Fermentation of lactate, pyruvate, and threonine, reduction of nitrate, production of indole, and hydrolysis of starch, gelatin, and esculin were tested by the method of Holdeman et al. (4).

We assayed for the arginine dihydrolase pathway in separate cultures of all 11 isolates grown for 7 days in 2 ml of base medium containing 1% L-arginine. We analyzed the cultures for arginine loss and products of the arginine dihydrolase pathway by thin-layer chromatography as previously described (6).

The short-chain organic acids produced in the base medium and media containing glucose, amygdalin, lactate, pyruvate, and threonine were determined by GLC as previously described (2). The extraction and methylation procedures described by Holdeman et al. were used (4).

Production of CO_2 and H_2 by the bacteria in standard brain medium with and without 0.5% sodium pyruvate was analyzed by GLC on a Gow-Mac series 550 gas chromatograph (Gow-Mac Instrument Co., Bridgewater, N.J.) equipped with a thermal conductivity detector. The media were contained in Vac Vials (The VirTis Co., Inc., Gardiner, N.Y.) which we modified by drilling a small hole in the plastic cap to allow sampling of the headspace through the rubber stopper. CO_2 was detected by injecting a 0.5-ml sample of the headspace gas onto a glass column (2 mm [inside diameter] by 1.83 m) packed with 80/100 mesh Chromosorb 102 (Johns-Manville, Denver, Colo.) at 65°C with helium as carrier. H_2 was detected by injecting a 0.5-ml sample of the headspace gas onto a glass column packed with 60/80 mesh molecular sieve 5A (Supelco, Inc.) at 65°C with N_2 as carrier. An analyzed gas mixture (Scott Environmental Technology, Inc., Plumsteadville, Pa.) containing 1% CO_2 and 1% H_2 was used as a standard. The CO_2 and H_2 concentrations in the cultures were compared with the concentration in the headspace over uninoculated media.

RESULTS

Nine new strains of cholesterol-reducing bacteria were obtained from nine baboons: two strains from feces, four strains from the upper colon, and three strains from the ileum. All of the strains were gram-positive diplobacilli similar to *Eubacterium* strain ATCC 21408 (3) and produced coprostanol in standard brain medium with subsequent clotting of the medium. The nine new strains of cholesterol-reducing bacteria and *Eubacterium* strains 403 and ATCC 21408 segregated into three groups based on their growth requirements and succinate production (Table 1).

All of the new strains formed small colonies (~0.5-mm diameter) when subcultured on supplemented brain heart infusion agar. The colonies were observed microscopically (×100) and appeared as circular, convex colonies with a smooth, glistening surface and an entire edge. Although all of the strains formed colonies with protruding fibers on CBA (1), we did not observe any fibers protruding from the colonies

TABLE 1. Characteristics of cholesterol-reducing bacteria

Test	Group		
	1 (2)[a]	2 (7)	3 (2)
Growth on BHIA[b]	−	+	+
Cholesterol reduced without PLE[c]	−	−	+
Succinate produced	−	−	+

[a] Number in parentheses is the number of isolates per group.
[b] BHIA, Supplemented brain heart infusion agar.
[c] Coprostanol produced in lecithin-cholesterol medium without PLE.

formed on supplemented brain heart infusion agar. The bacteria could be repeatedly subcultured on supplemented brain heart infusion agar, and isolated colonies produced coprostanol when inoculated into standard brain medium.

All strains reduced cholesterol to coprostanol in lecithin-cholesterol medium containing PLE. Only two strains, 103 and 104, reduced cholesterol in the absence of PLE (Table 1).

The strains were characterized by analysis of the organic acids produced during growth as recommended by Holdeman et al. (4). All strains produced a small amount of acetate in the base medium, and some strains produced acetate concentrations greater than 1 meq/100 ml in the presence of a fermentable substrate (e.g., glucose). Strains 103 and 104 also produced a small amount of succinate in the base medium and succinate concentrations greater than 1 meq/100 ml were observed in the presence of a fermentable substrate (Table 1).

Carbohydrate fermentation was variable. Strains 403 and ATCC 21408 did not produce acid from any of the carbohydrates tested, and none of the strains produced acid from erythritol, glycogen, inositol, mannitol, melizitose, rhamnose, sorbitol, or trehalose. The carbohydrate reactions which were differential are listed in Table 2. In general, the group 3 organisms fermented a larger variety of carbohydrates and produced a lower terminal pH (~5.2 to 5.3).

All isolates produced a β-glucosidase which was detected by the hydrolysis of esculin and amygdalin. Esculin hydrolysis was indicated by the conventional test (4), and we previously identified one of the products, esculetin, by GLC-mass spectrometry (6). Since several of the amygdalin cultures had an acid pH, we analyzed all of the amygdalin-containing cultures for carboxylic acids by GLC. We observed a peak response which eluted between isobutyric and butyric acids; we identified the peak as benzaldehyde by mass spectral analysis. Presumably, amygdalin was hydrolyzed to glucose, cyanide, and benzaldehyde, and the acid produced by several of the strains was the result of subsequent glucose fermentation.

Pyruvate was metabolized by all strains. Loss of pyruvate from the medium was detected by GLC analysis. Acetate production by all strains and succinate production by strains 103 and 104 were increased in the presence of pyruvate. Acetoin (3-hydroxy-2-butanone) was also detected by GLC and gave a peak response preceding acetic acid. We identified the peak as acetoin by mass spectral analysis and subsequently all pyruvate-containing cultures gave a positive Voges-Proskauer reaction. Smaller amounts of acetoin were detected in cultures growing in the base medium and in carbohydrate-containing media. CO_2 production was also enhanced in the presence of pyruvate. CO_2 concentrations of 1 to 11% of the headspace gas were observed in cultures of the 11 strains

TABLE 2. Differential carbohydrate reactions

Group[a]	Strain	Fermentation of[b]:													
		Glu	Fru	Mne	Amg	Sal	Lac	Mel	Ara	Cel	Xyl	Mal	Rib	Raf	Suc
1	ATCC 21408	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	403	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	79	a	a	w	a	a	−	−	−	−	−	−	−	−	−
	83	w	w	w	w	a	−	−	−	−	−	−	−	−	−
	92	w	w	w	w	w	−	−	−	−	−	−	−	−	−
	99	w	w	w	w	w	−	−	−	−	w	−	−	−	−
	100	w	w	w	w	w	w	w	−	−	−	−	−	−	−
	2402	w	w	w	w	w	−	−	w	w	−	−	−	−	−
	1274	a	a	a	a	a	a	a	w	w	w	w	w	w	w
3	103	a	a	a	a	a	a	a	w	w	w	w	w	w	−
	104	a	a	a	a	a	a	a	w	w	w	w	w	−	−

[a] See Table 1.
[b] Symbols for fermentation reactions: −, pH > 6.0; w, pH 5.5 to 6.0; a, pH < 5.5. Abbreviations: Glu, glucose; Fru, fructose; Mne, mannose; Amg, amygdalin; Sal, salicin; Lac, lactose; Mel, melibiose; Ara, arabinose; Cel, cellobiose; Xyl, xylose; Mal, maltose; Rib, ribose; Raf, raffinose; and Suc, sucrose.

growing in standard brain medium; these concentrations increased to 9 to 20% in the presence of pyruvate. H_2 was not produced.

All isolates were negative for indole production, nitrate reduction, urease production, and gelatin and starch hydrolysis. No products of the arginine dihydrolase pathway were detected by thin-layer chromatography, and lactate and threonine were not fermented as analyzed by GLC.

DISCUSSION

This study indicates that organisms which reduce cholesterol to coprostanol are a much more diverse group of bacteria than previously described. The cholesterol-reducing bacteria previously reported have limited activity in routine biochemical reactions (3, 5–7), whereas some of our new isolates fermented a variety of carbohydrates, had different requirements for cholesterol and plasmalogen, and produced additional end products of growth.

The diversity we have observed seems to be related to the metabolism of substrates for energy production. In a carbohydrate-free medium, all of the strains produced acetate presumably from one or more of the amino acids. Several of the new strains produced a low terminal pH (<5.5) and increased amounts of acetate when certain carbohydrates were added to the medium. The addition of pyruvate to the base medium also resulted in an increase in acetate with a concurrent loss of pyruvate. The most probable mechanism for ATP production is the dehydrogenation of pyruvate to acetyl-CoA, since acetyl-CoA is the most frequently used precursor of high-energy phosphate in anaerobically growing bacteria (9). ATP and acetate are produced from acetyl-CoA via the phosphotransacetylase and the acetate kinase reactions. Therefore, we propose that all of the strains probably produce energy through the same basic mechanism but vary in their ability to use different substrates leading to pyruvate.

The role of cholesterol in growth of these organisms is not understood. Eyssen et al. (3) suggested that cholesterol is the terminal electron acceptor, and all of the isolates previously reported require cholesterol for growth (3, 5, 7). Since all of our new isolates grew in the absence of cholesterol, an alternate electron acceptor must be used by these strains when cholesterol is not available. Strains 103 and 104 produced succinate which may be the result of a two-step reduction of oxalacetate through malate and fumarate (9). However, this could not be the mechanism for the remaining seven strains. Further studies are needed to determine the role of cholesterol and to identify the actual mechanisms for energy production by these organisms.

The role of alkenyl ether lipids in cholesterol reduction also is not known. We have shown that *Eubacterium* strains 403 and ATCC 21408 require an alkenyl ether lipid (e.g., PLE) for growth (5). Although all of our nine new isolates grew without cholesterol and PLE, only strains 103 and 104 reduced cholesterol in the absence of PLE. This finding suggests that the PLE is involved in cholesterol reduction by the remaining strains.

The taxonomical status of these organisms is unclear. Based on the criteria established by Holdeman et al. (4), most of our new isolates would belong in the genus *Eubacterium* along with the previously described cholesterol-reducing bacteria. Since strains 103 and 104 produced significant amounts of succinate, a characteristic of *Actinomyces*, they do not fit the description for the genus *Eubacterium*. However, since we have never observed production of lactate by these strains or observed any branching or other irregular cell morphology, these strains also would be atypical of the genus *Actinomyces*. Since strains 103 and 104 share so many characteristics with the other isolates we feel that all of these bacteria should at present remain in a single taxon.

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