Taxonomic Study of Helically Coiled, Sporeforming Anaerobes Isolated from the Intestines of Humans and Other Animals: *Clostridium cocleatum* sp. nov. and *Clostridium spiroforme* sp. nov.

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Thirty-three anaerobic, gram-positive, helically coiled microorganisms, of which 26 were freshly isolated from the intestinal contents of humans, mice, rats, and chickens, were the subjects of a taxonomic study. These and 48 freshly isolated *Clostridium ramosum*-like, noncoiled organisms were found to belong to the genus *Clostridium* because of their sporeforming ability. They were divided into five groups on the basis of numerous biochemical properties, the guanine-plus-cytosine (G+C) contents of their deoxyribonucleic acids, and their deoxyribonucleic acid homologies. Strains of group I had straight cells and a G+C content of about 28 mol% and were identified as members of *C. ramosum*. Cells of the strains in the other groups were coiled. The strains of group II had a G+C content of about 28 mol% and were regarded as belonging to a new species, *Clostridium cocleatum*, of which strain I50 (= ATCC 29902 = NCTC 11210) is designated the type strain. Those of group III had a G+C content of about 27 mol% and were placed in *Clostridium spiroforme* sp. nov., of which strain VPI C28-23-1A (= ATCC 29900 = NCTC 11211) is designated the type strain. The single strain of group IV, VPI C22-44-2 (*Eubacterium* AQ of Moore and Holdeman), had a G+C content of 35.8 mol% and was regarded as a new but unnamed organism in the genus *Clostridium*. Those of group V, including the original strains of this sort reported by Fitzgerald et al., had a G+C content of about 46 mol% and were also considered to be a new but unnamed organism in the genus *Clostridium*.

An anaerobic, gram-positive, helically coiled microorganism from the cecum of rats was first reported by Bladen et al. (1) in 1964. Fitzgerald et al. (4) studied similar strains in detail and identified them as members of *Eubacterium*. A number of similar organisms have been isolated from the feces and intestines of humans and other animals (6, 8, 18, 19, 26, 34) and from a case of human abdominal abscess (30). Most of these strains have been considered to be non-sporeformers (*Eubacterium*) (1, 4, 6, 26, 30, 34). Recently, however, Koopman et al. (18, 19) observed in vivo spore formation with the strains from mice. Hazenberg et al. (8) identified their strains from mice as members of *Clostridium ramosum*.

In the course of studies on the intestinal flora of humans and other animals, a number of strains that appear to be closely related to the above-mentioned coiled organisms were isolated. Some of these isolates were found to have spores; this was an indication that they were clostridia. They were also found to share a number of features in common with *C. ramosum*. A

study was undertaken, therefore, to determine the systematic positions of the previously reported coiled organisms and of the fresh isolates, in particular with respect to *C. ramosum* (9, 10, 32).

MATERIALS AND METHODS

Bacterial strains. The 84 strains studied and their sources are listed in Table 1. Of these, 33 were coiled organisms isolated from the feces of healthy men or from the cecal contents of mice, rats, or chickens (including the original strains of Fitzgerald et al. [4]); 48 were fresh isolates which had straight cells and resembled *C. ramosum*; and the remaining 3 were *C. ramosum* strains ATCC 25582 (neotype strain), VPI 8546, and VPI 4496A, used as references. VPI 4496A was formerly identified as a strain of *Eubacterium filamentosum*, a name which has been shown to be synonymous with *C. ramosum* (10).

Isolation of strains and maintenance of cultures. Fecal and cecal samples diluted in an anaerobic buffer solution were plated out on modified Eggerth-Gagnon (EG) agar (25), glucose-blood-liver (BL) agar (25), and medium 10 agar (3). Medium 10 agar was supplemented with 1% (wt/vol) fresh mouse feces and

TABLE 1. *Strains used in this study*

Group	Strain ^a	Source and date of isolation	Obtained from:
I	<i>Clostridium ramosum</i> ATCC 25582 ^b		ATCC
	<i>C. ramosum</i> VPI 8546 ^b		E. P. Cato ^c
	<i>C. ramosum</i> VPI 4496A ^b		W. E. C. Moore ^c
	A2-E3 ^b ; A2-F3; A2-F4; A2-R3; A3-E1; A3-R1; A4-F5; A4-C2; A4-R2; A5-E3; A5-C1; A5-R2; A8-F4; A9-C3; CKR-3; CKR-7; CKR-8; CKR-10; MMR-1; MMR-2; MMR-11; HCR-1	Human feces, 1974-1977	Our collection
	R16-12 ^b ; R16-31; R18-4; R33-13; R36-24; R58-6; R59-4; R60-7; R60-16	Cecum, rat, 1975-1976	Our collection
	MO101-3 ^b ; MO101-10; MO102-5; MO103-4; MO105-8; MO105-11; MO109-2; MO109-9; MO111-5	Cecum, mouse, 1974-1977	Our collection
	CH1-5; CH1-6; CH2-8; CH3-2; CH5-6; CH7-4; CH7-7; CH9-4	Cecum, chicken, 1977	Our collection
II	E3-1 ^b	Cecum, rat, 1976	Y. Morishita ^d
	VPI 8723 ^{b, e}	Cecum, mouse	E. P. Cato
	147 ^b ; I49 ^b ; I50 ^b	Cecum, SPF ^f mouse, 1975	Our collection
	R-S-1 ^b ; R-S-2; R18-9	Cecum, rat, 1975-1977	Our collection
	MO-S-1 ^b ; MO-S-2; MO-S-3 ^b ; MO-S-4; MO-S-5; MO-S-6; MO-S-7; MO-S-8; MO-S-9	Cecum, mouse, 1976	Our collection
	CH2-3; CH2-9; CH6-5; CH7-12	Cecum, chicken, 1977	Our collection
III	VPI C28-23-1A ^b ; VPI 8872 ^b	Human feces	E. P. Cato
	18H-39 ^b ; H17-60 ^b ; H19-24	Human feces, 1976	Our collection
	CH46-27; CH48-103; CH49-55	Cecum, chicken, 1977	Our collection
IV	<i>Eubacterium</i> -AQ ^g VPI C22-44-2 ^b	Human feces, 1974	E. P. Cato
V	F#6 ^b ; F#10 ^b	Cecum, rat, 1964	R. J. Fitzgerald ^h
	CH-C-41 ^b	Cecum, chicken, 1977	Our collection

^a ATCC, American Type Culture Collection, Rockville, Md.; VPI, Virginia Polytechnic Institute and State University, Blacksburg, Va.

^b Strains for which G+C contents were determined in this study.

^c Virginia Polytechnic Institute and State University, Anaerobe Laboratory, Blacksburg, Va.

^d National Institute of Health, Tokyo, Japan.

^e Originally isolated by F. Wensinck, Laboratory of Medical Microbiology, Medical Faculty, Rotterdam, The Netherlands.

^f Specific pathogen free.

^g Reference 26.

^h Veterans Administration Hospital, Miami, Fla.

was prepared "plate-in-bottle" fashion (24). Decimal dilutions of most samples were also plated on the three media after they had been either heated at 70°C for 10 min or treated for 2 h with ethyl alcohol added to give a final concentration of 80% (vol/vol) (7) or for 1 h with chloroform added to give a final concentration of 0.02% (vol/vol). EG and glucose-blood-liver agar plates were incubated at 37°C in an anaerobic steel-wool jar (29) filled with an atmosphere of 90% N₂ and 10% CO₂. After incubation for 3 days, all colonial types were picked onto EG agar or medium 10 agar. Gram-

positive, curved or coiled organisms and *C. ramosum*-like organisms were selected. Strains were maintained on prereduced EG liver agar slants (with H₂CO₃-CO₂ buffer) (25) stored at 4°C. New transfers were usually made at 3-month intervals.

Cultural and biochemical tests. The methods for cultural and biochemical characterization of the strains used in this study were described previously (16). β -Glucuronidase activity was tested by the method of Kent et al. (17) with a partial modification. Lecithinase activity was detected in peptone-yeast

extract-Fildes solution (PYF) broth (16) with 0.2% (wt/vol) glucose and 5% (vol/vol) fresh egg yolk added. Lipase activity was tested in PYF deep agar (0.5% [wt/vol] agar) with 1% (vol/vol) glycerol tri-*n*-butyrate added (12). A positive reaction was detected by a zone of clearing along the stab.

Spore formation test. Organisms were grown on the surfaces of chopped-meat (CM) agar slants (10) and medium 10 agar at 30°C and stained at intervals of 5 to 7 days during a 4-week period by the Gram stain (Hucker) (13) or the spore stain (Wirtz) (13). In addition, prereduced PYF starch broth in rubber-stoppered tubes (16) was inoculated with growth from CM agar slants or medium 10 agar (which had been incubated for 7 days at 30°C), heated to 70 or 80°C for 10 min by immersing the tubes in a water bath, cooled immediately, and incubated at 37°C for up to 2 weeks. Spore production was indicated by the development of turbidity in the broth and the subsequent development of growth on EG agar or medium 10 agar after inoculation with the broth culture.

Fermentation products. Strains were grown for 3 days in peptone-yeast extract-Fildes solution-glucose (PYFG) broth (16). Alcohols, volatile acids, and nonvolatile acids were analyzed, for the most part, by the method of Holdeman and Moore (10). Formic acid could not be detected by the method used.

Media and growth conditions for cells in the preparation of DNA. For the preparation of deoxyribonucleic acid (DNA), most organisms were grown at 37°C in EG broth with 4% (vol/vol) Fildes solution (16) and 0.5% (wt/vol) glucose added. Three strains, F#6, F#10, and CH-C-41, were grown in prereduced EG broth. Cells were harvested by centrifugation in the late logarithmic phase and were washed twice with distilled water and once with 0.15 M NaCl-0.1 M ethylenediaminetetraacetic acid (pH 8.0). For preparation of tritium-labeled DNA, the organisms were grown in prereduced EG broth supplemented with about 5 μ Ci of [*methyl*-³H]thymidine per ml (The Radiochemical Centre Ltd., Amersham, England).

Preparation of DNA. DNA was prepared by the Marmur method (21) modified to include phenol extraction steps in place of the chloroform-isoamyl alcohol deproteinization treatment (31). At least two steps of each of phenol, ribonuclease, and isopropyl alcohol treatments and one step of Pronase (Kaken Chemical Co. Ltd., Tokyo; 1 mg/ml) treatment were included. Ribonucleic acid in the crude DNA solution was digested at 37°C for 30 min by addition of 40 μ g of ribonuclease A (Sigma Chemical Co.) per ml, which was preheated at 80°C for 10 min to destroy deoxyribonuclease. DNA finally precipitated by isopropyl alcohol on a glass rod was washed successively in ethyl alcohol solutions of increasing concentration (70, 80, 90, and 99% [vol/vol]), each for 10 to 30 min with occasional gentle stirring by hand, and dissolved in 0.2 $\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) after drying in air. Turbidity of the DNA solution, if any, was reduced by repeated centrifugation at 15,000 rpm for 30 min. The DNA purity was assessed from the 230 nm/260 nm extinction ratio (21). DNA thus prepared was found to contain less than 10% RNA determined by the orcinol reaction (23) and less than 5% protein detected by the method of Lowry et al. (20). The amount of DNA was deter-

mined by the method of Burton (2), using calf thymus DNA (Worthington Biochemicals Corp., Freehold, N.J.) as a standard.

DNA base composition. The guanine-plus-cytosine (G+C) content of the DNA was determined by the methods of Marmur (21) and Marmur and Doty (22), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo). The DNA from calf thymus was included in each set of analyses as a standard; the G+C content of the calf thymus was regarded as 42 mol%.

Preparation of DNA filter (disk DNA). The DNA filter was prepared by the method of Gillespie and Spiegelman (5) with partial modification. An appropriate amount of DNA in 0.2 $\times$ SSC, about 50 μ g per filter, was denatured by heating to 100°C for 10 min and quickly cooling. The DNA solution was adjusted to a final concentration of 50 μ g/ml of 2 $\times$ SSC with distilled water and 10 $\times$ SSC. One milliliter of the adjusted solution was passed at low speed (about 1 ml/min) through a nitrocellulose membrane filter (24 mm in diameter, 0.45- μ m pore size; Toyo Roshi Ltd., Tokyo) that had been presoaked in 2 $\times$ SSC. The DNA filter was washed with 2 ml of 2 $\times$ SSC at low speed and with 10 ml of 2 $\times$ SSC at moderate speed (about 3 ml/min) and dried for 2 h at room temperature and for 2 h in a vacuum oven at 80°C.

DNA-DNA hybridization. DNA-DNA hybridization was carried out as described by Gillespie and Spiegelman (5) with partial modification. [³H]DNA was sheared by ultrasonic disruptor (model UR-200P, Tomy Seiko Co., Ltd., Tokyo) at 140 W, 20 kHz, for about 3 s in 5 ml of 1 $\times$ SSC and dissociated at 100°C for 10 min in 0.5 $\times$ SSC. The DNA concentration was finally adjusted to a specific activity of about 3,000 cpm/ml in 2 $\times$ SSC containing 0.25% (wt/vol) sodium dodecyl sulfate. One milliliter of this DNA solution (input DNA) was incubated with the disk DNA in a capped, small glass vial at 55°C for 20 h with occasional gentle shaking by hand. At the end of incubation, the filter was washed by filtration with 2 ml of 2 $\times$ SSC and then with 5 ml of 5 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 9.0) at moderate speed. The washed filter was dried under an infrared lamp and placed in 10 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene. The amount of input DNA bound to the disk DNA was estimated by counting the radioactivity of the membrane filter, using a liquid scintillation counter (Packard model 3330). The DNA homology was represented as the homology index, i.e., the percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

Dissociation of hybrid DNA. The thermal dissociation assay was carried out by the method of Nakamura et al. (28). A DNA-DNA hybrid was formed on a membrane filter as described above, except that 1 ml of annealing mixture with 6,000 instead of 3,000 cpm was used. The filter on which the hybrid duplex was formed was punched into small disks (10 mm in diameter) which were transfixed with an insect pin and placed in a tube (13 by 100 mm) containing 1.2 ml of 0.5 $\times$ SSC. The tube was then placed in a water bath. The small disks were transferred at 10-min intervals through a series of tubes with a 5°C degree

increase at each interval. The water bath reached each new temperature in 2 to 4 min. Each tube was emptied into a scintillation vial and washed with 0.5 ml of 0.5× SSC. Ten milliliters of a preparation of Triton X-100 counting solution (three parts of the toluene counting solution and two parts of Triton X-100) was added to each vial. The radioactivity of the vial was counted after the vial had been left in the liquid scintillation counter for more than 24 h.

RESULTS

The characteristics of the 84 strains studied are given in Tables 2 through 4. The strains were divided into five groups (I, II, III, IV, and V) on the basis of cellular morphology, carbohydrate fermentation, and G+C content. The groups were considered to differ from each other at the species level. This differentiation of groups was substantiated by the results of DNA homology assays; the homology indexes among strains of the five groups were less than 65%. Except for five fresh isolates of groups II and III, all of the strains studied were found to have spores and be heat resistant. Consequently, they were all regarded as sporeformers belonging to the genus *Clostridium*.

Characters common to all strains. All of the strains studied shared certain features in common. They were all obligately anaerobic, nonmotile, sporeforming, gram-positive rods with rounded ends; they occurred singly, in pairs, and in short or long chains. They had distinctive coiling characteristics, except for the strains of group I, which had a straight form. Cultures on solid or in liquid media, at 48 h or less, revealed long, tightly or loosely coiled forms with various proportions of semicircular or circular rods and partially coiled elements (Fig. 1C). Old cultures had an increasing number of gram-negative cells. Cells on glucose-blood-liver agar containing a relatively large amount of carbohydrates were larger or stouter than those on EG agar. All of the strains had cells with Y-shaped branching. The branching was not easily observed, but it was often clearly seen with gram-negative, partially autolyzed cells in old (3 weeks) cultures. Terminal to subterminal spores were produced in medium 10 agar and/or CM agar slants incu-

bated at 30°C for 2 weeks. Spore formation was not demonstrated easily, particularly with fresh isolates. Among the media used, the decreasing order of detection of spores was: medium 10 agar > CM agar slant ≥ EG agar plate. These strains survived heating at 70°C for 10 min, but their resistance to 80°C for 10 min was variable not only between strains but also within strains after incubation on medium 10 agar and/or CM agar slants at 30°C for 7 days.

The temperature for optimal growth was 37°C. No growth usually occurred at 15°C. Growth in PYG broth (10) was slightly enhanced by the addition of 10% rumen fluid or both 0.0005% hemin and 0.00005% menadione but was not affected by the addition of 0.1% Tween 80. Surface colonies on EG agar (2 days) and on medium 10 agar (3 days) were whitish to brownish gray, convex to slightly pulvinate, circular, smooth, shiny, and semiopaque to opaque; they had an entire or slightly erose margin. The colonies were 0.7 to 1.5 mm in diameter, except those of group I strains, which were larger (3.0 to 4.0 mm).

Group I. Group I consisted of 51 strains, including *C. ramosum* strains ATCC 25582, VPI 8546, and VPI 4496A, with straight form (Fig. 1A).

Cells on EG agar (2 days old) measured 0.4 to 0.6 by 0.8 to 13.0 µm. Long filamentous forms were often observed, particularly in media containing 1% (vol/vol) detergent Triton X-100. Coiled forms were not detected. The fresh isolates frequently stained gram negative, especially at initial isolation. No, or only slight, growth occurred in PYF broth. PYFG cultures had moderate to abundant growth, usually with a smooth sediment.

All the strains of this group readily formed round, terminal spores (0.6 µm in diameter) in cultures on EG agar plates as well as on medium 10 agar and CM agar slants.

The G+C contents of the DNAs from ATCC 25582, VPI 8546, VPI 4496A, A2-E3, R16-12, and MO101-3 were 28.8, 27.9, 28.4, 28.2, 28.6, and 28.5 mol%, respectively. These values were consistent with those reported for the neotype and other strains of *C. ramosum* (9, 10, 32).

TABLE 2. Positive characteristics of the helically coiled strains studied and of a related organism, *Clostridium ramosum*

Acetic acid production from PYFG
Acid from glucose
Y-shaped branching of cells
Growth enhancement by: Hemin (0.0005%) and menadione (0.00005%)
Rumen fluid (10%)
Neutral red reduction
Round, terminal to subterminal spores

TABLE 3. *Negative characteristics of the helically coiled strains studied and of a related organism, Clostridium ramosum*^a

Acid from:
Arabinose
Erythritol
Inositol
Melezitose
Sorbitol
Sorbosose
Xylose
Ammonium production
Gas from PYF
Gelatin liquefaction
Growth enhancement by Tween 80 (0.05%)
Hemolysis
Lactate utilization to propionic acid
Litmus milk digestion
Motility
Nitrate reduction
Production of:
Catalase
Glutamic acid decarboxylase
Hydrogen-sulfide (SIM)
Indole
Lecithinase (EYA)
Products from PYFG:
Butyric acid
Isobutyric acid
Propionic acid
Succinic acid
Valeric acid
Isovaleric acid
Threonine conversion

^a SIM, Hydrogen sulfide-indole-motility medium; EYA, McClung-Toabe egg yolk agar (modified) (10).

Group II. Group II included 21 strains with distinctive coiling characteristics. In contrast with the uniformity of group I strains (*C. ramosum*), they had a variety of carbohydrate fermentation patterns from strain to strain (Table 4).

Cells on EG agar (2 days old) measured 0.3 to 0.4 by 1.0 to 5.0 μm . They showed a preponderance of semicircular to circular forms with a few relatively short helical filaments (Fig. 1B) and sometimes had partially swollen, stout forms (0.8 μm in width) in old cultures. Slight growth occurred in PYF broth. PYFG cultures had moderate to abundant growth, with a smooth to flocculent sediment in 7 days. Round, terminal to subterminal spores (0.7 μm in diameter) were formed by all of the strains of this group except for strains MO-S-2, CH2-3, and CH2-9.

The average G+C content of the DNAs from eight strains (E3-1, VPI 8723, I47, I49, I50, R-S-1, MO-S-1, and MO-S-3) was 28.0 mol%.

Group III. Group III consisted of eight coiled strains.

Cells on EG agar (2 days old) measured 0.3 to

0.5 by 2.0 to 10.0 μm . They were relatively large and long, with various degrees of coiling (Fig. 1C). Cells of strain VPI C28-23-1A were sometimes nearly straight after having been heated to 80°C for 10 min without alteration of any other property; subsequent subcultures did not show coiled cells. Slight growth occurred in PYF broth. PYFG cultures had moderate to abundant growth, with a smooth to flocculent sediment in 7 days. Round, terminal to subterminal spores (about 0.7 μm in diameter) were formed by six strains. Two strains, 18H-39 and H17-60 (fresh isolates from humans), neither formed spores nor survived heating at 70°C for 10 min.

The average G+C content of the DNAs from four strains (VPI C28-23-1A, VPI 8872, 18H-39, and H17-60) was 27.2 mol%.

Group IV. Group IV consisted of a single strain, VPI C22-44-2 (*Eubacterium-AQ*) (26), with coiling characteristics.

Cells on EG agar (2 days old) measured 0.3 to 0.4 by 1.5 to 5.0 μm . A small number of nearly straight cells in chains or in filaments was usually observed among masses of the tightly coiled forms. The diameter of the coiled form was about 1.0 μm . Slight growth occurred in PYF broth. PYFG cultures had moderate growth, with a smooth to flocculent sediment, sometimes with no turbidity. This strain was the least fermentative of the strains in the five groups. Round, terminal to subterminal spores (0.7 μm in diameter) were seen in old cultures (2 weeks old) on medium 10 agar.

The G+C content of the DNA from VPI C22-44-2 was 35.8 mol%.

Group V. Group V was comprised of three strains (F#6, F#10, and CH-C-41) with coiling characteristics. The former two are the original strains of this sort described by Fitzgerald et al. (4).

No growth usually occurred on EG or glucose-blood-liver agar plates incubated in the steel-wool jar. Moderate growth was obtained on medium 10 agar and on prereduced EG agar plates. Poor growth occurred in prereduced PYF broth, but prereduced PYFG cultures had moderate growth, with a smooth to stringy sediment, mostly without turbidity. Cells on medium 10 agar (3 days old) measured 0.3 to 0.4 by 1.5 to 3.0 μm . Long filaments with tight coils predominated. Round, terminal or subterminal spores (about 0.7 μm in diameter) were formed by the three strains in old cultures on medium 10 agar incubated at 30°C for 2 weeks (Fig. 1D).

The G+C contents of the DNAs were 46.8, 46.7, and 45.9 mol% for F#6, F#10, and CH-C-41, respectively.

DNA homologies. Table 5 shows the percent homologies among seven [³H]DNAs and 21 un-

TABLE 4. Characteristics of the helically coiled strains studied and of a related organism, *Clostridium ramosum*, in which there are group differences^a

Characteristics	Group I (<i>C. ramosum</i>) (51 strains)		Group II (<i>C. cocleatum</i>)		Group III (<i>C. spiroforme</i>)		Group IV (<i>Clostridium</i> sp.) (VPI C22-44-2)	Group V (<i>Clostridium</i> sp.) (F#6, F#10, and CH-C-41)
	21 strains	Type strain (I50)	8 strains	Type strain (VPI C28-23-1A)				
Acetylmethylcarbinol	+	+	+	+				
Acid from:								
Amygdalin	+	-	+-	-			w	-
Cellobiose	+	+	+-	+			-	w
Dextrin	-	-	-	-			-	+
Dulcitol	-w	-	-	-			-	+
Esculin	+w	w	-w	w			-	-
Fructose	+	+	+	+			- or w	-
Galactose	+	+	+	+			-	+
Glycogen	-	-	-	-			-	+
Inulin	-	+	+w	+			-	-
Lactose	+	+	+	+			-	+
Maltose	+	+	+	+			-	+
Mannitol	+-	-	-	-			-	-
Mannose	+	+	+	+			-	+
Melibiose	+	+	+-	+			-	-
α -Methylglucoside	+	-	-	-			-	+
α -Methylmannoside	+	-	-	-			-	+
Raffinose	+	+	+-	+			-	+
Rhamnose	+-	-	-	-			+	-
Ribose	-	-	-	-			+	+
Salicin	+w	w	+-	w			-	+
Starch	-	w	-	w			-	+
Sucrose	+	+	+	+			-	-
Trehalose	+	-	-	-			-	-
Cellular morphology	Straight	Coiled	Coiled	Coiled			Coiled	Coiled
Esculin hydrolysis	+	+	+-	+			-	+
Final pH in PYFG	4.4-4.9	5.0-5.3	4.8-5.1	4.9-5.1			4.8-5.1	5.6-6.0 ^b
Gas from PYFG	-	-	-	-			++ ^c	++
G+C content of DNA (mol%) ^d	28.3(5)	27.9	27.2(4)	27.4			35.8(1)	46.5(3)
β -Glucuronidase	-	w	-	-			-	-
Growth at:								
15°C	-w	-	-	-			-	-
25°C	w	w	-w	w			-	-
45°C	w	-	-w	-			-	-
Growth in 20% bile	w	-	-w	-			w	w

FIG. 1. Photomicrographs of Gram-stained cells of helically coiled microorganisms and *Clostridium ramosum*. (A) Cells of *C. ramosum* VPI 8546 (group I) from a 48-h-old EG agar plate; (B) cells of E3-1 (group II, *C. cocleatum*) from a 2-week-old culture on medium 10 agar; (C) cells of VPI 8872 (group III, *C. spiroforme*) from a 48-h-old EG agar plate; (D) cells of F#10 (group V, *Clostridium* sp.) from a 2-week-old culture on medium 10 agar. Spores or prespore-like swellings are indicated by arrows. Bar represents 10 μ m.

mology index, 63%) had a lower thermal elution midpoint (about 65°C) than the hybrid duplexes formed between group I strains ATCC 25582 and A2-E3 and between group II strains I50 and VPI 8723. Thus, it seems that the homology regions of hybrid DNA duplexes among three groups of strains may include more unpaired base sequences than those of the strains of a group. These findings confirmed the appropriateness of differentiating groups I, II, and III from each other even though they have similar G+C contents (ca. 28%).

DISCUSSION

The first report of an anaerobic, gram-positive, helically coiled microorganism from the cecum of rats was by Bladen et al. in 1964 (1). Since then, a number of similar organisms have been isolated from the intestines of humans and other animals (4, 6, 8, 18, 19, 26, 34) and from a human abscess (30). Recently, Sugihara et al. (P. T. Sugihara, V. L. Sutter, K. S. Bricknell, and S. M. Finegold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C146, p. 59) isolated 18

strains of this organism from 18 of 135 fecal specimens from adult humans. The mean number of cells per gram (dry weight) of feces was 2×10^9 . In this study, 3 strains from 3 of 22 fecal specimens from adult humans, 3 strains from 2 of 10 cecal specimens from rats, 12 strains from 7 of 15 cecal specimens from mice, and 8 strains from 7 of 21 cecal specimens from chickens were isolated. They were found in amounts of 10^6 to 10^9 cells per gram (wet weight) of feces. Consequently, this particular kind of organism is considered to be distributed in a wide range of animals including humans.

Most authors (1, 4, 6, 26, 34) have regarded the coiled organisms as nonsporeformers (*Eubacterium*) because of the difficulty of demonstrating their spore formation. The original strain of this sort was, however, isolated by Fitzgerald et al. (4) from specimens heated to 80°C for 5 min. Recently, Koopman et al. (19) and Hazenberg et al. (8) observed spores, particularly in vivo, with their strains. In this study, a number of strains were also isolated from the specimens treated with heat, ethyl alcohol, or chloroform. Furthermore, all of the strains of coiled organisms were found to have spores and

TABLE 5. DNA homologies among four groups of coiled strains and *Clostridium ramosum*^a

Unlabeled DNA from:	Labeled DNA from:						
	Group I (<i>C. ramosum</i> ATCC 25582)	Group II			Group II (C28-23-1A)	Group IV (C22-44-2)	Group V (F#10)
		E3-1	I50	VPI 8723			
Group I							
<i>C. ramosum</i> ATCC 25582	100	35	50	38	53	4	2
<i>C. ramosum</i> VPI 8546	97	41	47	40	51	5	2
<i>C. ramosum</i> VPI 4496A	106	38	45	44	49	1	3
A2-E3	92	42	42	36	47	2	2
R16-12	91	40	39	39	50	— ^b	—
MO101-3	83	35	38	42	45	—	—
Group II							
E3-1	38	100	77	91	47	2	3
R-S-1	41	76	86	84	51	—	—
VPI 8723	52	94	96	100	56	2	2
I47	39	82	95	78	52	2	3
I50	43	84	100	89	48	3	—
CH2-3	40	87	91	75	49	—	—
Group III							
VPI C28-23-1A	53	56	63	58	100	2	3
VPI 8872	43	60	61	60	87	5	4
18H-39	32	53	52	54	88	—	1
H17-60	39	47	48	59	85	—	—
CH46-27	35	46	50	55	74	—	—
Group IV							
VPI C22-44-2	1	3	1	2	2	100	5
Group V							
F#6	3	5	2	1	1	3	96
F#10	5	2	3	2	3	4	100
CH-C-41	2	2	2	1	3	1	83

^a Most values (given as percentages) were averages of two to three tests.^b Not tested.

be heat resistant except for the five fresh isolates of groups II and III. The two nonsporeforming strains of group III, however, showed high degrees of DNA homology with the sporeforming strain VPI C28-23-1A of the same group. It therefore is considered appropriate that all of the coiled organisms are sporeformers and should be placed in the genus *Clostridium*. These organisms are gram-positive, obligately anaerobic, nonmotile, sporeforming, rod-shaped cells that grow in long chains with tight coils, particularly in young broth cultures; these features distinguish these organisms from the members of the other genera of the family *Bacillaceae*, viz., *Bacillus*, *Sporolactobacillus*, *Desulfofotomaculum*, and *Sporosarcina*.

All of the strains with cells of straight form were included in a single group (group I) and were identified as members of *C. ramosum* be-

cause their overall characteristics conformed closely to those of the neotype strain of *C. ramosum*—ATCC 25582—and to the current descriptions of *C. ramosum* (9, 10, 32).

The coiled strains studied were divided into four groups (II, III, IV, and V) at the species level mainly on the basis of carbohydrate fermentation, DNA base composition, and DNA homology. They differ from the previously described species of the genus *Clostridium* principally in morphology: their tightly coiled cells, particularly in young broth cultures, are rather distinctive. Unlike most clostridia, they do not produce butyric acid as an end product of glucose fermentation (10). They can also be distinguished from the species which do not produce butyric acid (such as *C. aminovalericum*, *C. histolyticum*, *C. indolis*, *C. limosum*, *C. oroticum*, *C. putrefaciens*, *C. ramosum*, and *C. sphe-*

FIG. 2. Thermal dissociation profiles of DNA hybrids. The DNA hybrid duplex was formed by incubation of appropriate amounts of ^3H -labeled input DNA having a radioactivity of 6,000 cpm with 50 μg of unlabeled DNA in 1 ml of $2\times$ SSC containing 0.25% (wt/vol) sodium dodecyl sulfate at 55°C for 20 h. The hybrid duplex of DNA was dissociated by heating the filter at various temperatures for 10 min in 1.0 ml of $0.5\times$ SSC. The dissociated DNA at each temperature was determined by counting the radioactivity as described in the text. Symbols: ○, hybrid duplex of DNAs from ATCC 25582 (labeled) and A2-E3 (unlabeled); ●, hybrid duplex of DNAs from ATCC 25582 (labeled) and VPI 8723 (unlabeled); △, hybrid duplex of DNAs from I50 (labeled) and VPI 8723 (unlabeled); ▲, hybrid duplex of DNAs from I50 (labeled) and VPI C28-23-1A (unlabeled).

oides), by carbohydrate fermentation, by a number of biochemical properties, and/or by DNA base composition (Table 6) (10, 32). Among these organisms, however, *C. ramosum* shares a number of features in common with the coiled strains of groups II and III such as G+C content of DNA (about 28%), numerous biochemical properties, and a cellular morphology which sometimes even includes coiling characteristics (10, 26). Indeed, Hazenberg et al. (8) identified their coiled strains from mice as members of *C. ramosum*. Their strains may correspond to those of group II or group III rather than groups IV and V in this study in view of the biochemical features and a G+C content of 29.5% observed by the authors. For these reasons, a number of fresh *C. ramosum* isolates from the same intes-

tinal origin as the coiled organism as well as the neotype strain, ATCC 25582, were included in this study for comparison with other clostridia. In contrast with the uniformity of group I strains (*C. ramosum*), however, the coiled strains of groups II and III are quite variable in carbohydrate fermentation, differing from each other in five to eight carbohydrate fermentations. This difference was also pointed out by Sugihara et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C146, p. 59), Pollock and Rintala (30), and E. P. Cato (Virginia Polytechnic Institute, Blacksburg, Va.; personal communication), who did not consider their coiled strains to be members of *C. ramosum*. Furthermore, DNA homology experiments in this study indicate that groups II and III of coiled strains are clearly different from *C. ramosum* and from each other. Thus, the two groups of coiled strains represent new species in the genus *Clostridium*. For the group II strains, we propose the name *Clostridium cocleatum* (L.n. *coclea* a whirlpool; M. L. neut. adj. *cocleatum* in the shape of a whirlpool). The type strain of *C. cocleatum* is I50 (= ATCC 29902 = NCTC 11210). This strain was isolated from the cecum of mice (10^8 to 10^{10} cells per gram [wet weight] of cecal material). For the group III strains, we propose the name *Clostridium spiroforme* (Gr.n. *spira* a coil; L.n. *forma* shape; M. L. neut. *spiroforme* in the shape of a coil). The type strain of *C. spiroforme* is VPI C28-23-1A (= ATCC 29900 = NCTC 11211). This strain was isolated from human feces collected at the Virginia Polytechnic Institute, Blacksburg, Va.

The coiled strains of groups IV and V are readily distinguished from one another and from those of groups I, II, and III by their relatively high G+C contents and by numerous biochemical properties (Table 6). These distinctions were clearly confirmed by the results of the DNA homology tests (Table 5). They also differ from the presently known clostridia in morphology, a number of biochemical characteristics, and/or G+C contents. Table 7 shows the characteristics most useful in distinguishing the two coiled organisms from some clostridia with high G+C contents. Accordingly, these organisms are considered as belonging to new species of *Clostridium*. However, more strains should be studied before the establishment of these new species.

A small number of nearly straight cells was often observed among masses of coiled cells in cultures of some strains. Cells of VPI C28-23-1A once lost their coiling characteristics after heating at 80°C for 10 min without alteration of any other property and never returned to the coiled form thereafter. This might have been a "straight mutant" selected by heat treatment.

TABLE 6. Characteristics most useful in differentiating the four groups of coiled organism from the *Clostridium* species which do not produce butyric acid from glucose fermentation^a

<i>Clostridium</i> species	Gelatin liquefaction	Indole production	Nitrate reduction	Acid from:		
				α -Methyl-glucoside	Lactose	Ribose
Group II (<i>C. cocleatum</i>)	—	—	—	—	+	—
Group III (<i>C. spiroforme</i>)	—	—	—	—	+	—
Group IV (<i>Clostridium</i> sp.)	—	—	—	—	—	+
Group V (<i>Clostridium</i> sp.)	—	—	—	—	+	—
<i>C. ramosum</i>	—	—	—	+	+	—
<i>C. oroticum</i>	—	—	+	—	+	+
<i>C. putrefaciens</i>	—	—	—	—	—	—
<i>C. sphenoides</i>	—	+	v	.	+	w—
<i>C. indolis</i>	—	+	+	.	w	—
<i>C. limosum</i>	+	—	—	—	—	—
<i>C. histolyticum</i>	+	—	—	—	—	—
<i>C. aminovalericum</i>	+	—	—+	—	—	—

^a Data from references 10 and 32. Symbols: +, positive reaction; —, negative reaction; v, variable reaction depending on the strain; w, weak reaction; —+, w—, etc., first reaction more than second.

TABLE 7. Characteristics useful in differentiating coiled organisms of groups IV and V from *Clostridium* species with high G+C contents^a

<i>Clostridium</i> species	G+C content (mol%)	Acid from:			
		Fructose	Galactose	Glycogen	Rhamnose
Group IV (<i>Clostridium</i> sp.)	36	—+	—	—	+
Group V (<i>Clostridium</i> sp.)	46	—	+	+	—
<i>C. barkeri</i>	45	—	—	—	—
<i>C. leptum</i>	51	—+	—	—	—
<i>C. symbiosum</i>	46	+	+	—	—
<i>C. nexile</i>	40–41	+	+	—	—
<i>C. innocuum</i>	43–44	+	+	—	—+
<i>C. indolis</i>	44	+	+	—	—+
<i>C. oroticum</i>	44	+	+	—	+
<i>C. coccoides</i>	44	+	+	—	+
<i>C. sphenoides</i>	41–42	+	+	—+	+
<i>C. clostridiiforme</i>	47–50	+	+	—+	+

^a Data from references 9, 11 (*C. nexile*), 15 (*C. coccoides*), 16 (*C. clostridiiforme* and *C. symbiosum*), 27 (*C. leptum*), and 32. Symbols: +, positive reaction; —, negative reaction; +—, —+, etc., first reaction more frequent than second.

The reproducibility of this phenomenon was not tested. On the other hand, coiling morphology may be observed in some strains of *C. ramosum* (10, 26). Tilby (33) reported that helices predominated among the abnormally shaped cell filaments that occurred in cultures of *Bacillus subtilis* in media containing chlorpromazine or penicillin and that certain mutants of *B. subtilis* resistant to the detergent Triton X-100 grew predominantly in a helical form, especially near the end of exponential growth. Therefore, coiling may not always be regarded as a consistent attribute.

On the basis of DNA base composition, two groups are roughly recognizable within the clostridia (14, 32): the one contains strains with a relatively low G+C content (21 to 30%) and the other contains strains with a high G+C content

(40 to 45%) in the DNA. The G+C contents (27 to 29%) of the DNA of the group II and III strains used in this study fell within the lower range and those (46 to 47%) of the group V strains were in the higher range. The G+C value (35.8%) of the DNA of strain VPI C22-44-2 (group IV) lies between the two ranges, suggesting the existence of a *Clostridium* species whose DNA has a base content intermediate in value between these two ranges.

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