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Comparative Analysis of the Genomes of Intestinal Spirochetes of Human and Animal Origin

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The aim of the present work was to compare the genomes of 21 strains of intestinal spirochetes, which were isolated from patients suffering intestinal disorders, with those of *Treponema hyodysenteriae* (strain P18), the known etiological agent of swine dysentery (bloody scours), and of a nonpathogenic strain (M1) of *Treponema innocens*. The percent guanine-plus-cytosine value of the 23 DNAs was found to be 25.5 to 30.1, as determined by a double-labeling procedure based on nick-translation by DNA polymerase I. The genome size of two spirochetal strains, of human and porcine origin, was found to be similar (4×10^6 base pairs) and close to that of the reference bacterium *Escherichia coli* (4.2×10^6 base pairs). Restriction analysis showed the presence of two modified bases in spirochetal DNA. Methyladenine was present in the GATC sequence of DNA from 15 spirochetes of human origin, and methylcytosine was present in several sequences occurring in all strains. The DNA of *T. hyodysenteriae* displayed a 30 to 100% homology with respect to that of 21 spirochetes from humans, thus suggesting the occurrence of a genetic heterogeneity in the latter group. These data indicate that the intestinal spirochetes analyzed in the present work are related; hence there is a possibility of domestic animals being reservoirs of microorganisms pathogenic for humans. A classification of intestinal treponemes into subgroups has been proposed on the basis of restriction analysis and hybridization experiments.

Swine dysentery (bloody scours), a mucohemorrhagic enteritis affecting pigs of all countries, was described as a disease entity in 1921 (37). Vibriolike species, including *Vibrio coli*, were suggested to be responsible for the disease, although transient nonhemorrhagic diarrhea was produced by these organisms (12, 18, 24). It was only in 1971, however, that a spirochete, *Treponema hyodysenteriae*, was recognized as the true etiological agent on the basis of its ability to reproduce epithelium invasion and hemorrhagic dysentery in experimental animals (15, 23, 33–35). This microorganism was described as a motile, spiral-shaped, gram-negative bacterium, which proved to be a facultative anaerobe and to possess hemolytic properties in vitro.

There have been numerous observations on the occurrence of spirochetes in the gut of domestic and wild animals (6, 7, 21). These organisms were also found in the intestinal tract of both healthy subjects and diarrhea cases (10, 14, 17, 20). More recently, spirochetes closely resembling *T. hyodysenteriae* were isolated from the intestine of patients affected by hemorrhagic diarrhea and other intestinal disorders (9, 22, 31, 32, 36). An immunological relatedness of spirochetes isolated from humans and pigs has been reported (13, 31). Some homology between human intestinal spirochetes and *T. hyodysenteriae* has been related (1). The presence of plasmid in spirochetes has been observed (30).

The aim of the present work was to analyze the DNA of 21 strains of intestinal spirochetes of human origin and 2 strains from pigs. The guanine-plus-cytosine (G+C) content and the hybridization levels of these DNA were determined to assess the genetic kinship of these organisms. In addition, the restriction patterns of the genomes of spirochetes from humans and of *T. hyodysenteriae* were analyzed to gather information on DNA methylation. Finally, the genome size of two treponemal strains of human and porcine origin has been measured.

MATERIALS AND METHODS

Multiplication of spirochetes. The 21 strains of human spirochetes (HRM-1 to -21) that were analyzed in the present work were isolated (31, 32) from feces of patients affected by intestinal disorders (Table 1). They were propagated under strict anaerobic conditions in Trypticase-soy broth supplemented with 10% bovine fetal serum. As reference strains, the following spirochetes were used: *Borrelia burgdorferi* (from H. W. Wilkinson, Atlanta, Ga.), *T. hyodysenteriae* P18A (from R. J. Lysons, New Compton, England), and *Treponema innocens* M1, *Treponema pallidum* (Nichols), and *Treponema phagedenis* (from S. Kellog, Atlanta, Ga.). Strains of human intestinal spirochetes were characterized as treponemes by the conventional biochemical tests (nutritional requirements, fermentation ability, and enzymatic analysis) (31) (Table 2) as well as by morphological studies (9). Electron micrographs of human intestinal spirochetes, measuring 6 by 0.2 and 5.7 by 0.25 μm and bearing five subterminal flagella, are displayed in Fig. 1. The main morphological features of these organisms are summarized in Table 3.

Purification of treponemal DNA. Lyophilized spirochetes suspended in 1 mM EDTA–10 mM Tris hydrochloride buffer (pH 7.6) were lysed by incubation with 0.5% sodium dodecyl sulfate for 30 min at 37°C. Lysates were sequentially incubated (30 min at 37°C) with pancreatic RNase (50 μg/ml) and pronase (100 μg/ml) before repeated extractions with water-saturated redistilled phenol. From the aqueous layer, after phenol removal by ether, treponemal DNA was precipitated twice with 3 volumes of ethanol (15 min at −70°C). The DNA sediment obtained by centrifugation was dissolved in 0.1 mM EDTA–10 mM Tris hydrochloride (pH 7.6), and its concentration was spectrophotometrically measured.

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TABLE 1. Sources of human intestinal spirochetes

Spirochete (isolate designation)	Patient		
	Sex	Age	Clinical features
HRM-1	F	25	Nausea, bowel sounds, hypogastric cramping, anorexia, and weight loss
HRM-2	M	65	Abdominal pain, vomiting, diarrhea; acute gastroenteritis
HRM-3	M	40	Constipation and diarrhea
HRM-4	M	56	Diverticulosis of duodenum and colon; cirrhosis; diabetes mellitus
HRM-5	F	60	Abdominal pain, vomiting, diarrhea; acute gastroenteritis
HRM-6	M	69	Diarrhea, rectal bleeding; carcinoma of rectum
HRM-7	M	57	Carcinoma of liver
HRM-8	M	28	Duodenitis
HRM-9	F	22	Constipation and diarrhea
HRM-10	M	50	Fever, vomiting, diarrhea; duodenal ulcer
HRM-11	M	5	Intestinal parasites in epileptic child
HRM-12	M	73	Chronic hepatitis
HRM-13	F	46	NA[a]
HRM-14	F	49	NA
HRM-15	M	61	NA
HRM-16	M	22	NA
HRM-17	M	40	Acquired immunodeficiency syndrome
HRM-18	M	35	Acquired immunodeficiency syndrome
HRM-19	M	41	Acquired immunodeficiency syndrome
HRM-20	M	71	Abdominal pain; hepatitis
HRM-21	M	24	Diarrhea

[a] NA, Data not available.

Determination of base composition of DNA. The G+C-content (% G+C) of spirochetal DNA was determined by a recently developed double-labeling procedure which required microquantities of ultrapure DNA (4), but controls were made with the usual procedure of buoyant density measurement in CsCl gradients. A DNA sample of about 1 μg of DNA was added to 10 μl of a reaction mixture as follows: 7.5 mM $MgCl_2$; 0.75 mM dithiothreitol; 30 mM Tris hydrochloride buffer (pH 7.4); 5.25 μM each dGTP, dTTP, and dATP (Boehringer, Mannheim, Federal Republic of Germany); 5.25 μM [5-^{3}H]dCTP (28.5 Ci/mmol; New England Nuclear Corp., Dreieich, Federal Republic of Germany); 0.004 μM [α-^{32}P]dATP (3,200 Ci/mmol, New England Nuclear Corp.); and 0.5 U of DNA polymerase I (type 104485 [Boehringer], an endonuclease-containing preparation). After 25 min of incubation at 20°C, the nick-translation reaction was stopped by 100-fold dilution with 10 mM EDTA–10 mM Tris hydrochloride (pH 7.4). DNA was precipitated with 10% trichloroacetic acid and 1% bovine serum albumin at 0°C for 30 min. Samples were filtered through glass fiber filters (MN85; Macherey-Nagel, Düren, Federal Republic of Germany), which were repeatedly washed with trichloroacetic acid, air dried, and counted for both ^{3}H and ^{32}P in a scintillation spectrometer. After subtraction of background values (no DNA samples) and correction for ^{32}P contamination of the ^{3}H channel, base composition was obtained from the relation $1/\%G+C = 0.01\ K(^{32}P/^3H) + 0.01$ by graphic interpolation on a linear plot of 1/%G+C against the ^{32}P/^{3}H ratio, 0.01 K being the slope and 0.01 the ordinate intercept. The K value was determined for each experiment by using two or more reference DNAs of known composition. This procedure has been described in detail elsewhere (4).

Restriction analysis of DNA. The following restriction endonucleases were used: *Hpa*II, *Eco*RI, *Hin*dIII, *Hha*I, *Alu*I, *Hae*III, *Dpn*I, *Sau*96I, *Taq*I, and *Sau*3A1 (Boehringer

TABLE 2. Biochemical characterization of intestinal spirochetes

Strain	Hemolysis[a]	Indole production[b]	Enzymatic activity[c]					
			α-Galactosidase	β-Galactosidase	α-Glucosidase	β-Glucosidase	C-4 esterase	C-8 esterase
Swine								
M1[d]	wβ	−	1	5	0	0	4	5
P18A[e]	β	+	0	5	0.5	2	3	3
Human								
HRM-4, -5	wβ	+	0.5	5	0	0	4	3
HRM-15	wβ	−	0.5	5	0	5	3	3
HRM-1, -2, -3, -6	wβ	−	1	5	0	0	3	2
HRM-7, -8, -9, -10	wβ	−	1	5	0	0	4	2
HRM-11, -12, -13, -14, -16	wβ	−	0.5	5	0	0	4	3
HRM-17, -18, -19, -20, -21	wβ	−	0.5	5	0	0	3	2

[a] wβ, Weakly beta-hemolytic; β, beta-hemolytic.
[b] −, Negative reaction; +, positive reaction.
[c] Values from 0 to 5 were assigned to the colors developed; 0 was a negative reation and 5 was a reaction of maximum intensity.
[d] Nonpathogenic.
[e] Pathogenic.

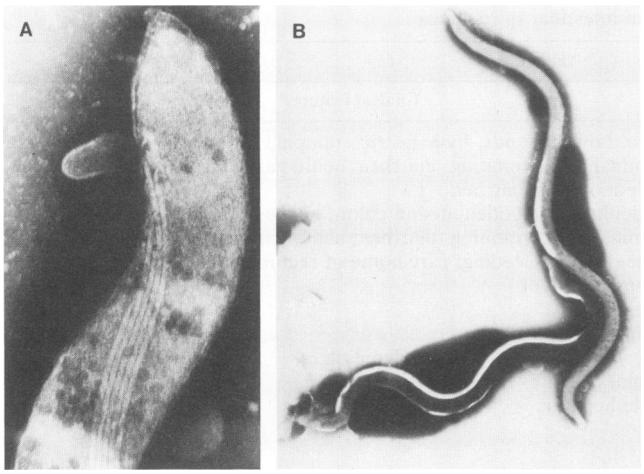

FIG. 1. Electron micrographs of human intestinal treponemes. (A) End of a cell with five flagella. Magnification, ×44,100. (B) Magnification, ×8,750. Dimensions of the treponemes: (A) 6.1 by 0.2 μm; (B) 5.7 × 0.25 μm.

Mannheim), *Fnu*4HI (New England Biolabs, Beverly, Mass.), and *Mbo*I (Pharmacia, Uppsala, Sweden). DNA samples were incubated with 5 U of a given endonuclease for 60 min at 37°C, and hydrolysis was halted by 10 min of heating at 65°C. A staining solution (5 μl of bromophenol blue [Sigma Chemical Co., St. Louis, Mo.] per 30 μl of DNA solution) was added, and the mixture was submitted to horizontal electrophoresis in 0.5% purified agarose gel in TBE buffer (0.089 M Tris-H_3BO_3 buffer, pH 8.5, 10 mM EDTA) containing 1 μg of ethidium bromide per ml. After electrophoresis at 250 V for 2 h, gels were photographed under UV light at 260 nm, using a red filter. The molecular weight of the DNA fragments was determined from that of simultaneously run standards of known size. The use of isoschizomers to disclose the presence of unusual and modified bases in bacterial DNA has been recently stressed (16).

DNA hybridization in liquid medium. The two DNA species to be annealed were submitted to 30 s of sonication at 60 W (B12 sonifier; Branson Sonic Power Co., Danbury, Conn.) in ice. DNA segments had an average size of 10^3 base pairs, as determined by electrophoresis on agarose gels with *Hin*dIII-cleaved phage λ DNA as reference. One DNA species (0.02 μg in 10 μl of 0.1 mM EDTA plus 10 mM Tris hydrochloride, pH 7.4) was labeled by nick-translation (4), using a precursor mixture of dGTP, dTTP, and dCTP (5.25 μM each) plus 0.166 μM [α-^{32}P]dATP (3,200 Ci/mmol, 3.1×10^{-3} μmol/ml). The reaction was started by the addition of polymerase I (15 U/μg of DNA) in 1 mM dithiothreitol–10 mM $MgCl_2$–50 mM Tris hydrochloride buffer (pH 7.5). The reaction was stopped by 10 min of incubation at 100°C. Specific activity of the probes was, on the average, 5×10^7 dpm/μg of DNA. Unlabeled DNA (1.5 μg/62 μl, a 75-fold excess with respect to the labeled DNA species) was added, and the mixture was denatured for 10 min at 100°C. The final concentration of NaCl was increased to 0.3 M, and the mixture was incubated at the annealing temperature $t^0 = T_m - 25°C = 60°C$, where the midpoint melting transition (T_m) was the average of the T_m values of annealing DNAs. The T_m value of a given DNA was obtained from the corresponding G+C value by the relation $T_m = 69.3 + 0.41(G+C) + 18.5 \log M$, where M is the ratio of the NaCl concentrations of the unknown (0.3 M in this case) and of reference DNA (that of *Escherichia coli*, the T_m of which was determined in 0.195 M NaCl) (11). The length of the annealing reaction was made equal to twice the $C_0t_{1/2}$ value (19). The latter represents the product of the single-stranded DNA concentration (in moles of nucleotides per liter) by the time (in seconds) required to render double-stranded half of the input DNA. DNA mixtures hybridized under stringent conditions were incubated with S1 nuclease (5 U/μg of DNA) for 30 min at 37°C (8). Nuclease buffer contained 0.045 M NaCl, 0.033 M sodium acetate (pH 4.5), and 3×10^{-4} M $ZnSO_4$. Hydrolysis was halted by addition of 1 ml of ice-cold 10 mM EDTA–10 mM Tris hydrochloride buffer (pH 7.4) followed by 2 ml of 10% trichloroacetic acid containing 2% bovine serum albumin. After 30 min in ice, mixtures were filtered on glass fiber filters and counted in a scintillation spectrometer (5). For each experiment, several controls were made. Homologous DNA reassociation, which varied between 60 and 90% of the input, was taken as 100% hybridization. A blank sample was prepared with an unrelated DNA possessing a %G+C value close to that of the unknown sample (in our case, *Clostridium perfringens* DNA, 26.5 mol% G+C). In addition, the kinetics of single- and double-stranded DNA hydrolysis by S1 nuclease, under our experimental conditions, were monitored for each annealing DNA. The optimum concentration affording 89% digestion of single-stranded DNA and 9.8% cleavage of the double-stranded species was found to be 5 enzyme units of S1 endonuclease per μg of DNA (I. Antoine, M. Coene, and C. Cocito, J. Med. Microbiol., in press).

Measurement of genome size. The size of labeled spirochetal DNA was measured at 60°C by renaturation kinetics analysis, and the $C_0t_{1/2}$ value, which is the product of denatured DNA initial concentration (moles of nucleotides per liter) by the time (seconds) affording 50% reannealing, was determined by graphic interpolation from the kinetics plots (25). The technique used was similar to that outlined in the preceding section, except that samples were periodically withdrawn from the reaction mixture for incubation with S1 nuclease. The enzyme action was stopped by addition of ice-cold trichloroacetic acid, filtration, and radioactivity

TABLE 3. Morphological features of human and animal spirochetes

Microorganism	Cell width (μm)	Cell length (μm)	Cell waves (type)	Cell ends (type)	Flagella (no.)
Human intestinal spirochetes	0.25	7.11	Loose, irregular	Pointed or blunt	4–6
T. hyodysenteriae	0.33	7.86	Loose, irregular	Blunt	7–9
T. innocens	0.23	6.23	Loose, irregular	Blunt	4–7
T. pallidum	0.13	8.80	Tight, regular	Pointed	3
T. phagedenis	0.26	13.15	Regular	Blunt	5–7
B. burgdorferi	0.22	12.00	Regular	Pointed	6–8

TABLE 4. Base composition of DNAs from intestinal treponemes

Strain	% G+C (mol/100 mol)[a]
P18	27.9 ± 3.0
M1	25.5 ± 2.1
HRM-1	30.1 ± 6.5
HRM-2	29.3 ± 1.1
HRM-3	27.7 ± 1.9
HRM-4	26.5 ± 1.6
HRM-5	25.9 ± 0.6
HRM-6	29.8 ± 4.3
HRM-7	29.1 ± 2.5
HRM-8	28.3 ± 0.8
HRM-9	28.0 ± 2.4
HRM-10	27.8 ± 2.5
HRM-11	25.8 ± 2.1
HRM-12	28.1 ± 1.8
HRM-13	25.6 ± 2.7
HRM-14	26.1 ± 1.7
HRM-15	26.6 ± 2.0
HRM-16	27.4 ± 2.8
HRM-17	26.8 ± 3.2
HRM-18	25.8 ± 3.2
HRM-19	29.8 ± 3.1
HRM-20	28.8 ± 1.2
HRM-21	26.6 ± 2.4

[a] Average of three independent determinations ± standard deviation.

measurement of residual labeled DNA. For this purpose, air-dried micropore filters (BA85; Schleicher and Schüll, Dassel, Federal Republic of Germany) in Omnifluor (New England Nuclear Corp; 4 g/liter of toluene) were counted in a scintillation spectrometer. The $C_0t_{1/2}$ value was calculated by linear regression from six measurements with a correlation coefficient higher than 0.96 (Antoine et al., in press).

RESULTS

Base composition of treponemal DNA. The base composition of microbial DNA, which is widely used in bacterial classification, is usually calculated from either the thermal denaturation midpoint (T_m) or the buoyant density. These measurements, however, can be altered by the presence of modified and unusual bases (the apparent G+C values of virus 2C DNA are 80% by CsCl gradient centrifugation, 15% from its T_m value, and 38% by hydrolysate analysis, a discrepancy related to thymine replacement by hydroxymethyluracil [4]). A recently developed micromethod, based on DNA labeling by nick-translation with two radioactive precursors, was found to be unaffected by the presence of modified bases (4). This procedure has been applied to analysis of DNA from intestinal spirochetes: the presence of modified bases in this DNA (see the following section of this work) has prompted the use of the double-labeling technique in this instance.

Data in Table 4 yield average values of 27.4% G+C for 21 spirochetes of human origin and 27.9% for *T. hyodysenteriae*. It can be concluded, therefore, that the DNAs of intestinal spirochetes from human and porcine guts have similar G+C values.

Restriction pattern of treponemal DNA. Restriction endonucleases may furnish useful information on the composition and organization of bacterial and viral genomes (see references 4 and 29, e.g.). The identification of modified bases and of species-specific DNA markers by restriction enzymes has also been related (8).

The possible occurrence of methylcytosine in spirochetal DNA was checked by use of nine restriction enzymes. The DNAs of human spirochetes (HRM-2 to -21), *T. hyodysenteriae* P18, and *T. innocens* M1 were resistant to at least one of these enzymes (Table 5), suggesting the presence of methylated cytosine in one or more sequences of the genomes analyzed. Note that every single case of resistance to a given endonuclease was controlled on mixtures of treponeme and phage λ DNAs: a cleavage of the latter species excluded the possible presence of nuclease inhibitors in treponemal DNA preparations (Fig. 2).

Another modified base, methyladenine, was examined by use of three endonucleases: *Dpn*I, cleaving the sequence GATC only when adenine is methylated; *Sau*3A1, able to cut GATC irrespective of A methylation; and *Mbo*I, to which DNA with a methylated adenine in GATC is resistant. Adenine is methylated in the GATC sequences of the DNA from 15 intestinal spirochetes of human origin plus *T. innocens* M1, but not in the pathogenic strain *T. hyodysenteriae* P18 or four other human intestinal treponemes (HRM-3, -9, -17, and -20) (Table 5). Cleavage of some treponemal DNAs by *Dpn*I and resistance to *Mbo*I provided evidence for the presence of methyladenine in the GATC sequences (Fig. 3).

Homology of the genomes of intestinal spirochetes from humans and pigs. Data in Table 4 indicate a similarity in base composition for the DNAs of intestinal spirochetes of human and porcine origins. A possible genetic kinship between these two groups of microorganisms was challenged by heterologous hybridization of the genomes of *T. hyodysenteriae* with a series of human spirochetes. For this purpose, a procedure of DNA-DNA hybridization in liquid phase was used, whereby a labeled probe of nick-translated *T. hyodysenteriae* DNA was annealed with different spirochetal DNAs. As a negative control (0% homology), *C. perfringens* DNA was chosen since its 26.5% G+C value is very close to that of *T. hyodysenteriae* (27.9% G+C; Table 4). The highest homology level (100%) was that of *T. hyodysenteriae* self-annealing at 60°C; this corresponded to 65% (60 to 70%) real hybridization value equal to 2.5 × $C_0t_{1/2}$ (see below). The annealing of *T. hyodysenteriae* with the genomes of several reference spirochetes of nonintestinal origin was also considered.

The 21 strains from human gut essentially fell into two groups of high (>70%) and low (<50%) homology with respect to *T. hyodysenteriae* (Table 6). The first group included strains HRM-2 to -9 and -15 to -19 (i.e., 13 of the 21 isolates that were analyzed), whereas HRM-10 to -14 and HRM-1, -20, and -21 belonged to the second group. Note that *T. innocens* and *T. hyodysenteriae* shared a very high homology level, while the latter was genetically unrelated to nonintestinal spirochetes.

Sizing of the treponemal genome. Genome size is an additional element of taxonomic value. Such a parameter was evaluated by renaturation kinetics in liquid phase, as outlined in Materials and Methods. The human strain HRM-16 and *T. hyodysenteriae*, two microorganisms sharing 71% homology (Table 6) were chosen for this study. Annealing was carried out at 60°C (the optimum value established on the basis of data in Table 4), and experimental values were analyzed by linear regression.

From the renaturation kinetics curve in Fig. 4, $C_0t_{1/2}$ values of 1.042 for *T. hyodysenteriae* and of 1.096 for HRM-16 were computed. The genome of the reference organism, *E. coli*, yielded, under conditions of DNA renaturation at 70°C, a $C_0t_{1/2}$ value of 1.123, corresponding to a

TABLE 5. Susceptibility of treponemal DNA to restriction endonucleases

Strain	Endonuclease cleavage[a]											
	*Sau*3AI ($\downarrow$ GATC)[b]	*Mbo*I ($\downarrow$ GATC)	*Dpn*I ($\downarrow$ GA$\downarrow$TC)	*Hae*III (GG$\downarrow$CC)	*Hpa*II (C$\downarrow$CGG)	*Taq*I (T$\downarrow$CGA)	*Alu*I (AG$\downarrow$CT)	*Hha*I (GCG$\downarrow$C)	*Sau*96I (G$\downarrow$GNCC)	*Fnu*4HI (GC$\downarrow$NGC)	*Eco*RI (G$\downarrow$AATTC)	*Hin*dIII (A$\downarrow$ACCT)
P18A	+	+	−	−	+	+	+	−	+	+	−	+
M1	+	−	+	+	+	+	+	−	−	+	−	+
HRM-2	+	−	+	−	+	+	+	−	+	+	−	+
HRM-3	+	+	−	−		+	+	−	+			+
HRM-4	+	−	+	±	+	+	+	−	+	+	−	+
HRM-5	+	−	+	−	+	+	+	±	−	+	−	+
HRM-7	+	−	+	±	+	+	+	−	−	+	−	+
HRM-8	+	−	+	−	+	+	+	±	−	+	−	+
HRM-9	+	+	−	+	+	+	+	−	+	+	−	+
HRM-10	+	−	+	+	+	+	+	±	−	+	−	+
HRM-11	+	−	+	+	+	+	+	−	−	+	−	+
HRM-12	+	−	+	+	+	+	+	−	−	+	−	+
HRM-13	+	−	+	+	+	+	+	−	−	+	−	+
HRM-14	+	−	+	+	+	+	+	−	−	+	−	+
HRM-15			+	+	+	+	+	−	−	+	−	+
HRM-16			+	+	+	+	+	−	−	+	−	+
HRM-17	+	+	−	+	+	+	+	−	−	−	−	+
HRM-18			+	±	+	+	+	−	−	+	−	+
HRM-19			+	−	+	+	+	−	−	+	−	+
HRM-20	+	+	−	±	−	+	+	−	+	+	−	+
HRM-21			+	+	+	+	+	−	−	+	−	+

[a] +, Cleaved; −, not cleaved; ±, partially cleaved.
[b] $\downarrow$, Cleavage site; ° cleavage not influenced by methylation; +, cleavage inhibited by methylation; m, methylation is a prerequisite for cleavage.

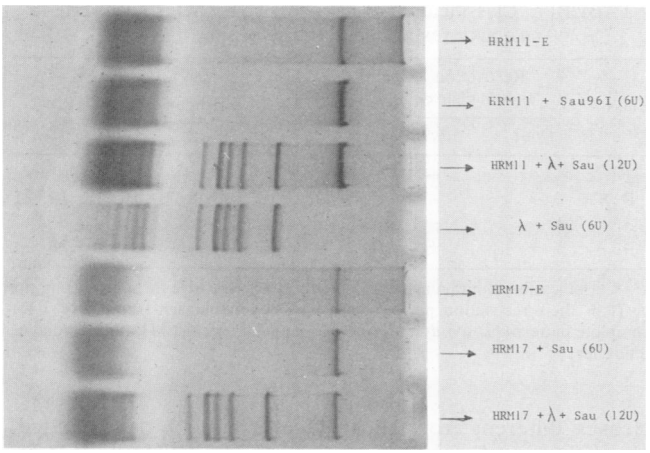

FIG. 2. Analysis of DNA from strains HRM-11 and HRM-17 of human intestinal treponemes. HRM-11 DNA (lanes 1, 2, 3), HRM-17 DNA (lanes 5, 6, 7), and λ DNA (lanes 3, 4, 7), either untreated (lanes 1 and 5) or treated with *Sau*96I (lanes 2, 3, 4, 6, 7).

genome size of 4.2×10^6 base pairs. By comparison, the spirochetal genome had a size of 3.9×10^6 base pairs in the case of *T. hyodysenteriae* and 4.1×10^6 for HRM-16. These values are very close and also similar to those of most bacteria.

DISCUSSION

The taxonomic value of DNA base composition has been proven by numerous studies. It is widely accepted that organisms differing in %G+C values by ≥5% do not belong to the same species, and those bearing a 10% difference in G+C are from different genera. Data in Table 4 clearly indicate that the genomes of all strains of intestinal treponemes analyzed in the present work have %G+C values lying between 25.5 and 30.1. Consequently, these results argue for their inclusion within the same species. Note that the %G+C value of spirochetal DNA has been established with a recently developed double-labeling procedure, which proved to be unaffected by the presence of unusual components. Modified bases were indeed shown to occur in treponemal DNA (Table 5). When the %G+C values in the DNAs of intestinal spirochetes (Table 4) are compared with the literature data concerning spirochete species from tissues (*T. pallidum*, 53% G+C, *Treponema refringens*, 42% G+C; and *T. phagedenis*, 39% G+C) (26, 28), any taxonomic kinship between the two groups of organisms is to be excluded.

Methylation of bacterial DNA, a postduplicational process occurring in the proximity of the replication fork, is directed by template-specific methyltransferases promoting the transfer of methyl groups from *S*-adenosylmethionine to DNA.

TABLE 6. DNA-DNA hybridization with the genomes of human intestinal spirochetes and reference strains

Source of unlabeled DNA	% Hybridization of labeled *T. hyodysenteriae* DNA[a]
Human spirochetes	
HRM-1	46.2 ± 13.4
HRM-2	90.5 ± 7.5
HRM-3	83.7 ± 16.3
HRM-4	104.8 ± 9.7
HRM-5	82.3 ± 20.8
HRM-6	103.7 ± 3.6
HRM-7	100.6 ± 6.3
HRM-8	91.8 ± 14.4
HRM-9	82.7 ± 16.7
HRM-10	47.0 ± 10.6
HRM-11	34.8 ± 8.9
HRM-12	31.5 ± 3.4
HRM-13	43.6 ± 19.3
HRM-14	55.6 ± 15.8
HRM-15	90.2 ± 13.5
HRM-16	70.7 ± 11.7
HRM-17	98.5 ± 9.7
HRM-18	96.7 ± 15.3
HRM-19	73.5 ± 22.6
HRM-20	41.3 ± 7.8
HRM-21	44.6 ± 21.0
Reference bacteria	
T. hyodysenteriae P18A	100.0
T. innocens M1	93.3 ± 19.9
T. pallidum	17.0 ± 9.3
T. phagedenis	7.9 ± 6.5
B. burgdorferi	6.4 ± 3.9
C. perfringens	0.0

[a] Average of four independent determinations ± standard deviation.

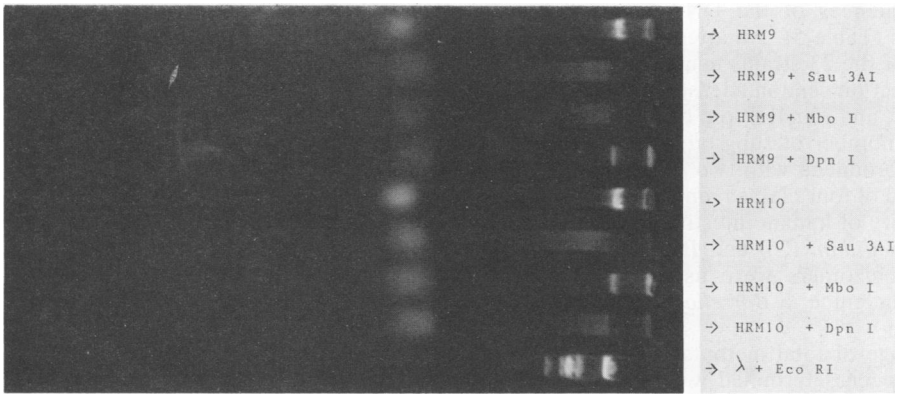

FIG. 3. Restriction analysis of the DNA from two strains of human intestinal spirochetes: identification of methylated adenine by isoschizomers. The genomes of strains HRM-9 and HRM-10 of intestinal spirochetes from human gut were submitted to three isoschizomers identifying methylated adenine in GATC sequences: *Dpn*I, which cleaves only when methyladenine is present; *Mbo*I, which is blocked by methyladenine presence, and *Sau*3A1, which is active in both cases. The DNA was then subjected to agarose gel electrophoresis. Phage λ DNA was treated with *Eco*RI as a reference.

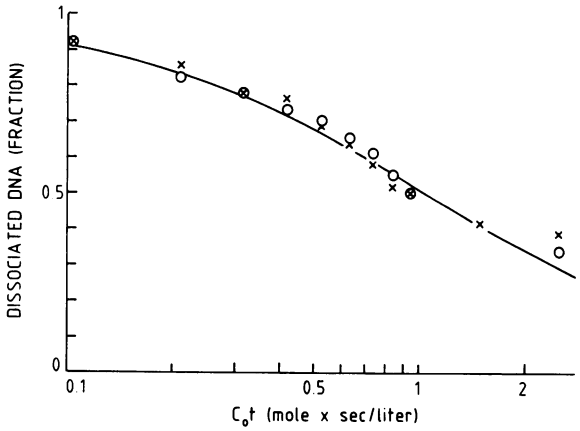

FIG. 4. Reassociation kinetics of spirochetal DNA. ^{32}P-labeled, heat-denatured spirochetal DNA, which was prepared by nick-translation, was reannealed in 0.3 M NaCl at 60°C in the presence of an excess of unlabeled DNA. At intervals, samples withdrawn from the reaction mixture were incubated with the single-strand-specific S1 nuclease, and trichloroacetic acid-resistant DNA was determined. The single-stranded DNA fraction was given by the ratio of S1-sensitive radioactivity to the initial acid-insoluble radioactivity. The curve corresponds to the ideal second-order reaction expressed by the equation $C/C_0 = 1/1 + KC_0t$, where C and C_0 are, respectively, the single-stranded fraction and the total DNA, and K is the second-order rate constant equal to the reciprocal $C_0t_{1/2}$ value (1.042 in this case). Symbols: ×, *T. hyodysenteriae* P18A; ○, HRM-16.

The best known bacterial DNA methylases are those concerned with methylation of adenine in the nitrogen N^6 position (methyladenine) and of cytosine in the 5 position (methylcytosine). In *E. coli*, the product of the *dam* gene brings about formation of 6-methyladenine in the sequence GATC, and the *mec* gene encodes production of 5-methylcytosine in CC^A_TGG. It is known that, in bacteria, most methylatable sites, which are unequally distributed within the chromosome, are actually methylated under normal conditions. They seem to play a major role at the level of regulatory genes and to affect recognition of the corresponding DNA sites by proteins, as suggested by the hypermethylation of both replication origin and operators, the increased mutability and recombination level of *dam* mutants, and the lethality induced by overposition of mutations in *dam*, *rec*, and *pol* genes. Methylation of both adenine and cytosine occurs in many sequences of the DNA from all human intestinal spirochetes (Table 5). An attempt at ordering the restriction patterns of the 23 strains analyzed was made by considering 6 restriction endonucleases (*Dpn*I, *Hae*III, *Hpa*II, *Hna*I, *Sau*96I, and *Fnu*4HI) out of the 12 reported in Table 5; a too large number of subgroups was obtained. A simpler pattern was produced with two restriction enzymes (Table 7). It consisted of four classes, one of which included 13 strains, the majority of human intestinal strains. Restriction analysis of *T. pallidum* (26) and *Borrelia* species has been reported. Isoschizomers were used to disclose the presence of 6-methyladenine in *Borrelia hermsii* (see reference 3 for review).

Since the percentage of total methylated bases in spirochetal DNA has not been determined, we cannot say which fraction of total modified bases is represented by the identified sequences. All spirochetal DNAs tested were proved to be resistant to several restriction enzymes (four on the average, and two at least). The possible occurrence of additional methylated sequences, identifiable by endonucleases different from those tested, cannot be excluded. Note that in *E. coli*, 6-methyladenine (m^6A) in GATC corresponds to 100% of the total m^6A, and 5-methylcytosine (m^5C) in CC^T_AGG corresponds to 50% of the total m^5C.

TABLE 7. Classification of intestinal spirochetes according to selective restriction patterns of their DNA[a]

Class	Restriction endonuclease		Swine strains	Human strains (HRM-)
	*Dpn*I	*Sau*96I		
1	−	+	P18	3, 9, 20
2	+	−	M1	5, 7, 8, 10–16, 18, 19, 21
3	−	−		17
4	+	+		2, 4

[a] Classification of human intestinal spirochetes (HRM-2 through -21) on the basis of their restriction pattern with two endonucleases (see Table 5 for complete pattern). *T. hyodysenteriae* P18 and *T. innocens* M1 are included as reference.

According to the hybridization data in Table 6, intestinal human spirochetes can be divided into two groups, those (HRM-2 through -9 and HRM-15 through -19) with high (>70%) homology with respect to *T. hyodysenteriae* DNA, and those (HRM-1, -10 through -14, -20, and -21) with low (<50%) homology level. Most of the human strains within the first group clearly belong to the same species as *T. hyodysenteriae* and *T. innocens*. Members of the second group are to be assigned to a different subspecies.

On the other hand, a very low homology level (6 to 17%) between intestinal and nonintestinal spirochetes has been observed (Table 6). These data are in good agreement with previous reports indicating a genetic unrelatedness of *T. hyodysenteriae* with respect to *T. pallidum*, *T. phagedenis*, and *T. refringens* (26–28). The only discrepancy between the work of Miao et al. (28) and ours is the low homology level (28%) observed by the former authors when the DNAs from pathogenic and nonpathogenic isolates of *T. hyodysenteriae* were annealed. According to our data, *T. hyodysenteriae* and *T. innocens* are 93% homologous. An antigenic cross-reactivity between *T. pallidum* and several pathogenic members of the family *Spirochaetaceae* has been reported (2).

Sizing of spirochetal DNA by renaturation analysis yielded the surprising finding that *T. pallidum* and *T. phagedenis* had genomes more than threefold larger than that of *E. coli* and most bacteria (26). By contrast, the data in Fig. 4 indicate a similarity in the genome size of intestinal spirochetes and *E. coli*. This represents yet another piece of evidence for the genetic unrelatedness of intestinal and nonintestinal spirochetes: the wide differences in %G+C values and hybridization levels are consistent with such an inference.

The overall conclusion is that many spirochetes isolated from the human gut are closely related, if not identical, to pathogenic treponemes from swine intestines (HRM-3, e.g., is practically indistinguishable from *T. hyodysenteriae*; Tables 4, 5, and 6). This points to domestic animals as sources of spirochetes pathogenic for humans.

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