Relationships Among Catalase-Positive *Campylobacter* Determined by Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization

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Deoxyribonucleic acid-deoxyribonucleic acid hybridization among 66 strains of catalase-positive *Campylobacter* and *Campylobacter*-like organisms delineated seven genetically distinct groups. Our results support the species designations of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*, although our data contraindicate the division of the latter species into *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Our deoxyribonucleic acid relatedness values also suggest that the "*Campylobacter fecalis*" and nalidixic acid-resistant thermophilic *Campylobacter* strains, as well as *Campylobacter*-like organisms isolated from mice, may deserve species status. *Campylobacter*-like isolates from bovine and ovine abortions possess morphological and metabolic characteristics that are incompatible with the genus description, and these isolates need further study to clarify their taxonomic status.

The genus *Campylobacter* was created by Sebald and Véron (19) to accommodate microaerophilic nonfermentative organisms which have low guanine-plus-cytosine (G+C) contents (29 to 36 mol%) and were previously classified as *Vibrio* species. At present, this genus contains the following four officially recognized species: *Campylobacter fetus*, *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter sputorum* (20). In addition, *C. fetus* and *C. sputorum* are each divided into two subspecies (*C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. sputorum* subsp. *sputorum*, and *C. sputorum* subsp. *bululus*). A third subspecies of *C. sputorum* (*C. sputorum* subsp. *mucosalis*) has been described, but it is not yet included on the Approved Lists of Bacterial Names (11). In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (23), Smibert presents a different taxonomic grouping of these organisms. This classification is used extensively, especially in the United States, and lists the following three species in the genus *Campylobacter*: *C. fetus*, *C. sputorum*, and "*Campylobacter fecalis*," with *C. fetus* and *C. sputorum* each divided into three subspecies (23). In the classification system of Smibert (24), the separate species *C. jejuni* and *C. coli*, which are recognized by Véron and Chatelain (26), are combined into one subspecies of *C. fetus* (*C. fetus* subsp. *jejuni*). In addition, *C. fetus* subsp. *fetus* of Véron and Chatelain is equivalent to *C. fetus* subsp. *intestinalis* of Smibert, and *C. fetus* subsp. *venerealis*

of Véron and Chatelain is equivalent to *C. fetus* subsp. *fetus* of Smibert. This conflict in classification schemes and the difficulties encountered in biochemically and serologically differentiating among strains have resulted in taxonomic and epidemiological confusion. We undertook this study in an attempt to resolve some of the confusion concerning the genetic relationships among the catalase-positive campylobacters.

MATERIALS AND METHODS

Bacterial strains. In this study we used 66 strains (Table 1) of *Campylobacter* species and *Campylobacter*-like organisms previously characterized by us with a standard battery of tests (see Table 4).

DNA isolation. To isolate deoxyribonucleic acids (DNAs), 10-ml cultures in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 μ M FeSO₄ (5) were inoculated from growth on Columbia agar (Oxoid Ltd., London, England) supplemented with 5% sheep blood that had been incubated overnight at 35°C in an atmosphere containing 5% O₂, 10% CO₂, and 85% N₂. The brucella broth cultures were incubated with shaking for 18 h at 35°C, and then each one was added to 400 ml of fresh medium in a 500-ml flask. The flasks were incubated for 24 h at 35°C on a rotary shaker. The cells were harvested by centrifugation and then suspended in 50 ml of saline-ethylenediaminetetraacetate (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, pH 8.0). A 1-ml amount of 25% (wt/vol) sodium dodecyl sulfate and 2.5 mg of pronase (grade B; nuclease-free; Calbiochem, La Jolla, Calif.) were added, and the mixture was incubated overnight at 35°C. The subsequent isolation-purification technique was essentially that described by Marmur (14).

TABLE 1. Designations and sources of the *Campylobacter* strains tested

Strain	Source ^a	History
<i>C. fetus</i> subsp. <i>fetus</i>		
C-16 ^T (= ATCC 27374 ^T)	ATCC	Sheep
C-15 (= ATCC 15296)	ATCC	Blood
C-2 (= Pickett Ra63)	Pickett	Unknown
C-3 (= Sutter 4273)	Sutter	Human blood
C-4 (= Sutter 4275)	Sutter	Human blood
C-5 (= Sutter 4276)	Sutter	Human blood
C-41 (= VPI Barr)	VPI	Unknown
C-42 (= VPI 18151-β-76)	VPI	Unknown
C-47 (= CCM 5682)	CCM	Intestinalis A (Morgan); serotype I (Mitscherlich)
C-48 (= CCM 5683)	CCM	Intestinalis B (Morgan); serotype II (Mitscherlich)
<i>C. fetus</i> subsp. <i>venerealis</i>		
C-14 ^T (= ATCC 19438 ^T)	ATCC	Heifer
C-44 (= VPI 82214)	VPI	Unknown
C-46 (= CCM 5681)	CCM	venerealis A (Morgan); serotype I (Mitscherlich)
C-49 (= CCM 5992)	CCM	venerealis A (biotype intermedius); serotype III (Marsh, Firehammer)
C-51 (= CCM 5994)	CCM	Marsh
<i>C. jejuni</i>		
C-61 (= ATCC 29428)	ATCC	Human stool
C-127 ^T (= NCTC 11351 ^T = CIP 702 ^T)	NCTC	
C-8 (= Cohen 199)	Cohen	Unknown
C-17	Original isolate	Human stool
C-18 (= Holmes A/40/74)	Holmes	Human rectal swab
C-20 (= Holmes A/36/75)	Holmes	Human gall bladder
C-25 (= Holmes A/106/77)	Holmes	Human stool
C-26 (= Holmes A/118/77)	Holmes	Human stool
C-30 (= Holmes A/52/78)	Holmes	Human stool
C-55 (= Reference Lab 2)	Reference Lab	Human stool
C-56 (= Greenwood 3311)	Greenwood	Human stool
C-73	Original isolate	Chicken intestine
C-82 (= Greenwood 8554)	Greenwood	Human stool
C-84 (= Blaser A1625)	Blaser	Human stool
C-85 (= Blaser A1616)	Blaser	Human stool
C-92 (= Luechtefeld 80-170)	Leuchtefeld	Human stool
C-117 (= Bryner 958)	Bryner	Sheep fetus
C-134 (= NCTC 11392)	NCTC	<i>C. jejuni</i> biotype 2
<i>C. coli</i>		
C-38 (= Butzler P2)	Butzler	Pig feces
C-128 ^T (= NCTC 11366 ^T = CIP 7080 ^T)	NCTC	Pig feces
C-39 (= VPI 12141)	VPI	Unknown
C-59 (= Greenwood 4641)	Greenwood	Human stool
C-63	Original isolate	Chicken intestine
C-64	Original isolate	Chicken intestine
C-69	Original isolate	Chicken intestine
C-71	Original isolate	Chicken intestine
C-72	Original isolate	Chicken intestine
C-83 (= Blaser A1618)	Blaser	Human stool
C-95 (= Luechtefeld 79-451)	Leuchtefeld	Colobus
C-98 (= Luechtefeld 80-23)	Leuchtefeld	Saki
C-100 (= Luechtefeld 80-215)	Leuchtefeld	Hog
C-105 (= Luechtefeld 79-489)	Leuchtefeld	Turkey
C-106 (= Luechtefeld 79-458)	Leuchtefeld	Duck
C-107 (= Luechtefeld 80-21)	Leuchtefeld	Black lemur
C-108 (= Luechtefeld 80-22)	Leuchtefeld	Chimp
C-113 (= Plorde 17140)	Plorde	Human blood
C-114 (= Ferguson N-156-79)	Ferguson	Human stool
C-115 (= Ferguson N-220-79)	Ferguson	Human stool
C-116 (= Bryner 1380)	Bryner	Hog fetus
" <i>C. fecalis</i> "		
C-32 (= Firehammer 14227A)	Firehammer	Sheep feces

TABLE 1—Continued

Strain	Source ^a	History
C-33 (= Firehammer 14223A)	Firehammer	Sheep feces
C-34 (= Firehammer 14146)	Firehammer	Sheep feces
C-35 (= Firehammer 14220A)	Firehammer	Sheep feces
C-36 (= Firehammer 11759)	Firehammer	Sheep feces
Nalidixic acid-resistant thermophilic <i>Campylobacter</i> ^b		
C-129 (= Weaver E7879)	Weaver	Skirrow, seagull
C-130 (= Weaver E7886)	Weaver	Skirrow, fowl pond
C-133 (= NCTC 11352)	NCTC	Skirrow, avian
<i>Campylobacter</i> -like		
C-58	Higgins and Degre	Pig fetus
C-80	Original isolate	Mouse feces
C-131	Original isolate	Mouse feces
C-132	Original isolate	Mouse feces

^a ATCC, American Type Culture Collection, Rockville, Md.; Pickett, J. Pickett, Department of Microbiology, University of California, Los Angeles; Sutter, V. Sutter, Wadsworth Hospital Center, Veterans Administration, Los Angeles, Calif.; VPI, Virginia Polytechnic Institute and State University, Blacksburg; CCM, Czechoslovak Collection of Microorganisms, Veterinary Research Institute, Brno, Czechoslovakia; NCTC, National Collection of Type Cultures, London, England; Cohen, H. Cohen, Clinical Laboratories, Kaiser Permanente Hospital, Los Angeles, Calif.; Holmes, B. Holmes, Computer Identification Laboratory, National Collection of Type Cultures, London, England; Reference Lab, Reference Laboratory, Los Angeles, Calif.; Blaser, M. Blaser, Department of Medicine, Veterans Administration Medical Center, Denver, Colo.; Leuchtefeld, N. Leuchtefeld, Veterinary Research Laboratory, Montana State University, Bozeman; Bryner, J. Bryner, National Animal Disease Center, Ames, Iowa; Butzler, J. Butzler, Department of Microbiology, St. Pierre Hospital, Brussels, Belgium; Plorde, J. Plorde, Veterans Administration Medical Center, Seattle, Wash.; Ferguson, D. Ferguson, Department of Microbiology, East Tennessee State University, Johnson City; Firehammer, B. Firehammer, Department of Veterinary Science, Montana State University, Bozeman; Weaver, R. Weaver, Bacteriology Division, Centers for Disease Control, Atlanta, Ga.; Higgins and Degre, R. Higgins and R. Degre, Department of Pathology and Microbiology, University of Montreal, Montreal, Quebec, Canada.

^b See reference 21.

The only modifications were the addition of several more purification steps. After completion of the Mar-mur technique, each isolated DNA was subjected to an additional ribonuclease step, which was followed by repeated (three or four) chloroform-isoamyl alcohol deproteinizations before final precipitation of the DNA in isopropyl alcohol. The purified DNA was stored in dilute standard saline citrate (0.1× SSC; 1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) over chloroform at 4°C.

Preparation of radioactive DNA. Radioactive DNAs were produced by adding 2.0 mCi of [2,8-³H]adenine (34 Ci/mmol; ICN, Irvine, Calif.) to flasks containing 400 ml of brucella broth. These flasks were then inoculated and incubated as described above. The cells were harvested, and the DNAs were extracted and purified by the techniques described above for unlabeled DNA. All DNA concentrations were determined by using a model 710 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at 260 nm and a standard curve.

DNA-DNA hybridizations. Numbered microfilters (diameter, 6 mm) were punched from filter membranes (type HAWP; Millipore Corp., Bedford, Mass.) and soaked in 6× SSC overnight. Solutions of DNA in 0.1× SSC were denatured for 10 min in a boiling water bath, chilled quickly on ice, and made up to 6× SSC. Microfilters were individually clamped between a fritted glass filter holder (Millipore) and a 10-mm-thick piece of Plexiglass with a 5-mm-diameter hole which was positioned over the microfilter. After the filters

were washed with 6× SSC, solutions containing 10 µg of denatured DNA were slowly passed through them (2). The filters were washed with additional 6× SSC and dried at room temperature overnight in a vacuum desiccator. Control filters were treated in the same manner, except that loading was with 10 µg of DNA extracted from "*Yersinia intermedia*." The desiccator containing the filters was then placed in an oven at 80°C for 2 h.

Solutions (0.5 ml) of radioactive reference DNA were sonically treated for 1 min in test tubes (diameter, 13 mm) by using the microtip of an Ultratip Labsonic System (Lab-Line Instruments, Inc., Melrose Park, Ill.) at 40% intensity (4). Sonically treated DNA was denatured by heating as described above.

DNA-loaded filters and blank control filters were placed individually in test tubes (10 by 75 mm) containing 0.2 ml of 50% formamide (Eastman Kodak, Rochester, N.Y.) in 2× SSC (pH 7.0) (4, 10, 15). With a digital microliter pipette (Pipetman; Cole Scientific, Calabasas, Calif. 1 µg of the appropriate reference DNA was added to each tube, and the tubes were incubated without shaking at a stringent temperature for this system (34°C) for 20 h (10, 15). Hybridizations were routinely done in duplicate. After incubation, the filters were transferred to 50 ml of 2× SSC-50% formamide in a 100-ml beaker and rinsed with magnetic stirring for 30 min at room temperature. This was followed by repeated (six to eight) manual rinses in small volumes of 1× SSC. The filters were dried in an oven at 110°C for 3 h and counted by using a model

3385 liquid scintillation system (Packard Instrument Co., Inc., Downers Grove, Ill.) and a toluene-based scintillation fluid (Beckman Instruments, Inc., Fullerton, Calif.). Relative binding ratios were calculated for heterologous strands by using a value of 100% for the mean binding of homologous strands (measured in counts per minute).

Phenotypic tests. The rapid hippurate hydrolysis method was performed as initially described by Hwang and Ederer (9) for use in differentiating *Streptococcus agalactiae* (group B) from *Streptococcus pyogenes* (group A). A 1% aqueous solution of sodium hippurate (Eastman Kodak) was prepared and dispensed in 0.4-ml amounts. Tubes of hippurate that were not used on the day of preparation were corked and frozen at -20°C until they were used. For the test, cells grown for 24 to 48 h microaerophilically on brucella agar (Difco) supplemented with 5% sheep blood (BSB agar) were emulsified in the substrate. The inoculated tubes of hippurate substrate were incubated for 2 h in an air incubator at 35°C . The end product of hippurate hydrolysis, glycine, was detected with a ninhydrin solution (1.75 g of ninhydrin in 50 ml of a 1:1 mixture of acetone and butanol). After incubation, 0.2 ml of the ninhydrin solution was layered on top of each tube. Without shaking, the tubes were returned to the incubator for 10 min and then removed for reading. A deep purple color in the ninhydrin layer was scored as a positive test for hydrolysis of sodium hippurate. Negative tubes showed no color reaction or occasionally a faint tinge of lavender.

Catalase, oxidase, H_2S , and nitrate reduction tests were performed with 48-h-old growth from BSB agar plates incubated microaerophilically at 35°C . The oxidase test was performed by rubbing a loopful of cell paste onto a moistened strip of filter paper previously saturated with a solution of 1% tetramethyl-*p*-phenylenediamine dihydrochloride in 0.2% ascorbic acid. Development of a purple color within 10 s was read as positive. For detection of catalase activity, a loopful of cell paste was placed on a cover slip, and 2 to 3 drops of 3% H_2O_2 were added. The appearance of bubbles was recorded as a positive result. Evolution of H_2S was detected by heavily inoculating cystine medium approximately 5 mm below the surface with a loopful of growth. The cystine medium was incubated aerobically for 5 days at 35°C . Blackening of the medium was recorded as positive for H_2S production. The medium used contained brucella broth (Difco) prepared according to the directions of the manufacturer, 0.025% sodium pyruvate, 0.025% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% L-cystine, 0.02% ferric ammonium citrate, and 0.2% agar. Nitrate reduction was tested in indole-nitrate medium (Difco) inoculated with a large loopful of growth. After 48 and 72 h of incubation of the inoculated medium, nitrite formation was detected with a reagent composed of 500 mg of α -naphthylamine, 500 mg of sulfanilic acid, 1 ml of glacial acetic acid, and 99 ml of distilled water. Two strains (strains C-20 and C-51) were negative for nitrite production with this reagent but positive when they were retested with a 1:1 mixture of reagents A and B; reagent A was composed of 8 g of sulfanilic acid in 1,000 ml of 5 N acetic acid, and reagent B was composed of 6 ml of *N,N*-dimethyl-1-naphthylamine in 1,000 ml of 5 N acetic acid.

Growth at 42°C , growth in 1.0% glycine, and growth in 3.5% NaCl were tested at least twice by using fluid thioglycolate medium (Difco) and brucella broth (Difco) containing 0.16% agar as the basal media. Both media were inoculated in the same manner. Growth from a 48-h-old BSB agar plate was suspended in sterile distilled water. The optical density of the suspension was adjusted to approximately that of a MacFarland no. 5 standard. To test for growth at 42°C , two tubes of each kind of medium were inoculated with 0.1 ml of the suspension. One tube of each set was incubated at 42°C , and the other was incubated at 35°C . To test for growth in the presence of 3.5% NaCl or 1.0% glycine, two tubes of each basal medium, one with the appropriate supplement and one without, were inoculated as described above. The tubes were incubated at 35°C . All tests were terminated after 5 days of incubation.

The ability of an organism to grow aerobically was tested on BSB agar plates inoculated with a loopful of a 48-h-old broth culture. The plates were incubated in an air incubator at 35°C .

To detect deoxyribonuclease activity, we used the method of Smith et al. (25), except that the plates were incubated microaerophilically at 35°C for 5 days.

Susceptibility to 30- μg disks of nalidixic acid and cephalothin was tested by placing the disks onto BSB agar inoculated by using a cotton swab with 24-h-old brucella broth cultures. The plates were read after 48 h of incubation at 35°C in a microaerophilic environment.

RESULTS

DNA-DNA hybridization studies. The *Campylobacter* strains tested are listed in Table 2, and the levels of homology between *Campylobacter* strains and *Campylobacter*-like strains are given in Table 3. The values for binding of labeled DNAs to the control filters averaged 1 to 2% of the values for the homologous systems.

The DNA-DNA hybridization data in Table 2 demonstrate a high degree of relatedness between *C. fetus* subsp. *fetus* DNA and *C. fetus* subsp. *venerealis* DNA and a very low degree of relatedness between the DNAs of these organisms and the DNAs of *C. jejuni* and *C. coli*. *C. jejuni* and *C. coli* (thermophilic nalidixic acid-susceptible strains) represent two distinct DNA homology groups that are characterized by intra-group relatedness values of more than 70% and an intergroup relatedness value of less than 50%.

The relatedness values (Table 3) derived from DNA-DNA hybridizations between strains of recognized *Campylobacter* species and strains of *Campylobacter*-like organisms, as well as hybridizations among strains of *Campylobacter*-like organisms, indicate a low level of DNA relationship between the presently recognized catalase-positive species and all of the *Campylobacter*-like organisms tested. The *Campylobacter*-like strains, including "*C. fecalis*" and the nalidixic acid-resistant thermophilic *Campylo-*

bacter strains, were resolved into four DNA homology groups. All strains designated "*C. fecalis*" demonstrated high intragroup DNA relatedness values (73% or higher), as did the nalidixic acid-resistant thermophilic *Campylobacter* strains (90% or higher) and the mouse strains C-80, C-131, and C-132 (93% or higher). DNA from the pig isolate of Higgins and Degre (strain C-58) demonstrated only low relatedness (5% or less) to the DNAs of all of the strains tested.

Phenotypic tests. The following tests gave positive results for all strains: catalase, oxidase, and nitrate. No strain except strain C-58 grew aerobically. Table 4 shows the results of all of the other phenotypic tests performed.

DISCUSSION

The genetic relationships delineated by this study confirm and extend the present taxonomic ordering of the catalase-positive species of genus *Campylobacter*. It is generally accepted that relatedness values of 70% or more imply species relationship, whereas values less than 50% denote no more than genus relationship. Our DNA-DNA hybridization data affirm the validity of species status for *C. fetus*, *C. coli*, and *C. jejuni*, and deny any genetic basis for the division of *C. fetus* into subspecies. It has been argued that *C. fetus* should be subdivided because the biochemical and ecological characteristics of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* are so different (26). Support for this argument appears to depend primarily on the characteristic of growth in the presence of 1% glycine; that is, the organism which is said to be restricted to the genital tract of cattle and to be the etiological agent of enzootic sterility and abortion in these animals is designated *C. fetus* subsp. *venerealis* and is unable to grow in the presence of 1% glycine. However, the organism designated *C. fetus* subsp. *fetus* is said to be more versatile with regard to both its ecology and its pathogenicity, as well as in its ability to grow in the presence of 1% glycine. It is accepted that the ecological niche and pathogenicity of *C. fetus* subsp. *venerealis* is unvaried (warranting it subspecies status) because allegedly no organism which fits the species description of *C. fetus* but is unable to grow in the presence of 1% glycine has ever been isolated except from the genital tracts of cattle (18, 24, 26). It is our experience that the glycine tolerance of a single strain of *C. fetus* can differ in the same basal medium or in different basal media. Furthermore, there are at least two published reports of *C. fetus* glycine-intolerant organisms isolated from animals other than cattle. In their study of chemically defined media for microaerophilic vibrios, Fletcher and Plastring (3) used 10 gly-

TABLE 2. DNA relatedness values among *Campylobacter* strains

Strain	% relatedness to strain:		
	C-16 ^T	C-61	C-38
<i>C. fetus</i> subsp. <i>fetus</i>			
C-16 ^T	100	6	4
C-15	84	5	
C-2	94		
C-3	90		
C-4	93		
C-5	95		
C-41	99	5	
C-42	97	5	
C-47	91		
C-48	90		
<i>C. fetus</i> subsp. <i>venerealis</i>			
C-14 ^T	92	6	4
C-44	108		
C-46	94	3	4
C-49	90		
C-51	98	6	5
<i>C. jejuni</i>			
C-61	8	100	37
C-127 ^T		101	26
C-8		72	
C-17		97	33
C-18		78	22
C-20	8	95	35
C-25		79	
C-26		94	36
C-30	7	81	28
C-55		86	
C-56		104	37
C-73	10	75	27
C-82		88	32
C-84		86	34
C-85		93	38
C-92		85	38
C-117		99	31
C-134		102	25
<i>C. coli</i>			
C-38	8	39	100
C-128 ^T		39	79
C-39			99
C-59	4	37	83
C-63		34	85
C-64	12	35	86
C-69			76
C-71	8	32	77
C-72	13		85
C-83		30	79
C-95		36	80
C-98		36	86
C-100		37	83
C-105		49	99
C-106		35	76
C-107		34	104
C-108		44	80
C-113		29	75
C-114		47	84
C-115		39	71
C-116		46	72

TABLE 3. DNA relatedness values among *Campylobacter*-like organisms and *Campylobacter* species

Strain	% Relatedness to strain:					
	C-16 ^T	C-61	C-38	C-32	C-131	C-133
" <i>C. fecalis</i> "						
C-32	9	7	4	100	4	7
C-33				73		
C-34				84		
C-35				78		
C-36				73		
Nalidixic acid-resistant thermophilic <i>Campylobacter</i> ^a						
C-129	9	19	12		5	90
C-130	9	19	14		5	101
C-133	10	14	9		4	100
<i>Campylobacter</i> -like						
C-58	4	3	4	4	3	5
C-80	4	3	4	3	93	
C-131	6	6	4		100	
C-132	8	5	3		94	

^a See reference 21.

cine-intolerant strains. Of these, eight were bovine isolates, 1 was an ovine isolate, and 1 was from a human. In a recent study of the fatty acid compositions of campylobacters Leaper and Owen (12) used a number of reference strains. One of these, strain CIP 5443, is listed as *C. fetus* subsp. *venerealis*, and the source given is human blood. In this same paper, seven *C. fetus* subsp. *fetus* reference strains were used. By definition, all of these strains should be glycine tolerant, yet one is recorded as being glycine intolerant. Unfortunately, the source of this strain is not identified, but it serves as yet another illustration of the variability in the glycine tolerance test. In our opinion, there is no justification for recognizing subspecies within *C. fetus*.

Unfortunately, in the work published in the last 10 years, very few attempts were made to distinguish among the thermophilic campylobacters. This was due in part to the taxonomic trend to classify all such organisms as *C. jejuni* and in part to the lack of convenient differential tests. Before our genetic work, we discovered the usefulness of the rapid hippurate hydrolysis test in differentiating among campylobacters (6). Our results have since been confirmed and expanded by a number of workers (12, 13, 22). Skirrow and Benjamin (22) used hippurate hydrolysis in conjunction with H₂S production to distinguish not only *C. coli* from *C. jejuni* but two biotypes of *C. jejuni* as well. Our genetic data confirm the accuracy of the biotyping scheme, although not with 100% infallibility. Of the 22 hippurate-negative, nalidixic acid-susceptible, thermophilic campylobacters tested, 21 were in the relatedness group which included the type strain of *C.*

coli. One hippurate-negative organism, strain C-92, was 85% related to our reference strain of *C. jejuni* and only 38% related to *C. coli*. This organism, a human isolate, was one of several sent to us by Nancy Leuchtefeld and was one of the organisms used in a recent study by Leuchtefeld and Wang (13). These authors listed strain C-92 (Leuchtefeld 80-170) as a Penner serotype 30, 34w strain (the w denotes a weak reaction). Coincidentally, we included in our study six additional strains discussed by Leuchtefeld and Wang. These strains (strains C-95 [= Leuchtefeld 79-451], C-98 [= Leuchtefeld 80-23], C-100 [= Leuchtefeld 80-215], C-106 [= Leuchtefeld 79-458], C-107 [= Leuchtefeld 80-21], and C-108 [= Leuchtefeld 80-22]) were all hippurate negative and in the *C. coli* relatedness group. Three of these strains are Penner serotype 34 strains, one is a Penner serotype 28 strain, and two are listed as not typable. In view of the fact that an organism genetically classified as *C. jejuni* (strain C-92) reacted with the same antisera as organisms genetically classified as *C. coli*, it seems plausible that these two species share some common antigenic components detectable by the Penner technique.

All 17 hippurate-positive organisms included in our study were grouped by their DNA relatedness values with the type strain of *C. jejuni*. Two of these strains showed the additional characteristic of H₂S production in our cystine medium. One of these is a Skirrow-Benjamin (22) *C. jejuni* biotype 2 strain (strain C-134), and the other is a strain sent to us by M. Blaser as a fecal isolate from an asymptomatic human (strain C-84). Blaser sent us six human fecal isolates; three of these were strains from asymptomatic individ-

uals, and three were strains from symptomatic patients. We included two of the strains from asymptomatic humans (strains C-83 and C-84) and one strain from a symptomatic individual (strain C-85) in our study. Of the strains from asymptomatic humans, strain C-83 was genotypically and phenotypically classified as *C. coli*, whereas strain C-84 was genotypically and phenotypically classified by us as a *C. jejuni* biotype 2 strain (Blaser A1633). Phenotypic classifications were made on the basis of routine tests in biotype 1 strain by both criteria. The remaining three organisms sent by M. Blaser were not genetically grouped, but the one additional isolate from an asymptomatic individual was phenotypically classified by us as *C. jejuni* biotype 2 strain (Blaser A1633). Phenotypic classifications were made on the basis of routine tests in conjunction with the tests for hippurate hydrolysis and H₂S production in cystine medium. Based on our genetic and phenotypic results with strains from known asymptomatic and symptomatic individuals, distinguishing between the species *C. coli* and *C. jejuni*, as well as the biotypes of the latter, should provide useful epidemiological data. Based on the correlation of hippurate hydrolysis activity with species (21 of 21 genetically studied *C. coli* strains were negative, whereas 17 of 18 *C. jejuni* strains were positive), the majority of the strains in our culture collection which were known to be isolated from cases of human enteritis are hippurate-positive *C. jejuni* strains (37 of 43 strains). Of the six hippurate-negative strains, two were not genetically studied, three were genetically confirmed as *C. coli* strains, and one was genetically confirmed as a *C. jejuni* strain.

Unfortunately, with few exceptions (13, 21), in most epidemiological studies there has been no attempt to differentiate among the thermophilic strains of *Campylobacter*, and thus the relative importance of various animal reservoirs is still largely a matter for speculation. Our work with chickens (unpublished data) has shown that both *C. jejuni* (strain C-73) and *C. coli* (strain C-63) can be isolated from the intestinal tract of the same bird. We hope that with the recognition of *C. coli* and *C. jejuni* as distinct species, future studies will distinguish between these organisms for more precise epidemiological information.

The organisms which we received from B. Firehammer designated "*C. fecalis*" were all isolated from sheep feces, and this organism is said to be a nonpathogenic commensal inhabitant of ovine intestinal tracts (24). These strains are phenotypically and genotypically distinct from all other catalase-positive *Campylobacter* strains, yet they have certain characteristics in common with *C. coli* and *C. jejuni*, including growth at 42°C, no growth at 25°C (24), and

variable susceptibility to nalidixic acid. Thus, if only these criteria are used to classify a *Campylobacter*-like organism, misidentifications can result. The following distinctive characteristics are shared by our "*C. fecalis*" strains: copious quantities of H₂S produced in cystine medium, resulting in blackening of the entire medium after prolonged incubation; heavy growth in fluid thioglycolate containing 3.5% NaCl; and large zones of inhibition around 30-μg cephalothin disks. In these characteristics, the strains of "*C. fecalis*" are identical to the one strain of *C. sputorum* subsp. *bubulus* in our collection. Phenotypically, the characteristics of this strain, which was sent to us from the Czechoslovak Collection of Microorganisms (strain CCM 5888), are similar to those of our "*C. fecalis*" strains, with the exception of catalase activity. "*C. fecalis*" strains display vigorous evolution of O₂ from 3% H₂O₂ when they are grown on a non-blood-containing medium, whereas strain CCM 5888 gives no evidence of catalase activity when it is incubated and tested under identical conditions. Phenotypically, there is no reason to dispute the classification of strain CCM 5888 as *C. sputorum* subsp. *bubulus*, but genetically this strain is 75% related to "*C. fecalis*" strain C-32, as determined by DNA-DNA hybridization. The published DNA base composition values (G+C contents) for "*C. fecalis*" differ from those for *C. sputorum* subsp. *bubulus*, although relative genome sizes have apparently not been determined. The mean G+C content of *C. sputorum* subsp. *bubulus* DNA is 30.5 ± 1.1 mol%, whereas the most recent value determined for "*C. fecalis*" DNA is 36.6 mol% (17). However, this value for "*C. fecalis*" differs considerably from values given in earlier work (32.0, 32.5, and 32.8 mol% for three strains of "*C. fecalis*") (1). We were not able to obtain any other strains designated *C. sputorum* subsp. *bubulus* and, therefore, could not resolve the enigma; we note it here in the hope that other investigators can perform additional relevant DNA-DNA hybridizations.

The nalidixic acid-resistant thermophilic *Campylobacter* strains of Skirrow and Benjamin (21) are also phenotypically and genotypically distinct from all of the catalase-positive campylobacters which we tested, although the G+C content (32.6 mol%) determined for the labeled strain (strain NCTC 11352) is within the range recognized for the genus *Campylobacter* (17). Despite the similarity of the nalidixic acid-resistant thermophilic *Campylobacter* strains to "*C. fecalis*," *C. coli*, and *C. jejuni* in the ability to grow at 42°C, these strains are less than 20% related to all of these organisms and possess characteristic phenotypic features. In our tests, no strain produced H₂S in cystine medium, grew

TABLE 4. Results of phenotypic tests^a

Strain	H ₂ S test	Hippurate hydrolysis	Diam of inhibition zone (mm)		Deoxyribo-nuclease	Growth at 42°C	Growth in 1% glycine	Growth in 3.5% NaCl
			Nalidixic acid	Cephalothin				
<i>C. fetus</i> subsp. <i>fetus</i>								
C-16 ^T	—	—	— ^b	28	—	—	+	—
C-15	—	—	13	30	—	—	+	—
C-2	—	—	—	30	—	—	+	—
C-3	—	—	—	24	—	—	+	—
C-4	—	—	—	23	—	—	+	—
C-5	—	—	—	25	—	—	+	—
C-41	—	—	—	23	—	—	+	—
C-42	—	—	—	30	—	—	+	—
C-47	—	—	—	23	—	—	+	—
C-48	—	—	—	25	—	—	+	—
<i>C. fetus</i> subsp. <i>venerealis</i>								
C-14 ^T	—	—	13	35	—	—	—	—
C-44	—	—	—	52	—	—	—	—
C-46	—	—	—	40	—	+	+ (—) ^c	—
C-49	—	—	12	40	—	—	—	—
C-51	—	—	16	54	—	—	—	—
<i>C. jejuni</i>								
C-61	—	+	33	—	—	+	+	—
C-127 ^T	—	+	28	—	+	— (—)	+	—
C-8	—	+	25	—	+	+	+	—
C-17	—	+	30	—	—	+	+	—
C-18	—	+	32	—	+	+	+	—
C-20	—	+	35	—	+	+	+	—
C-25	—	+	34	—	+	+	+	—
C-26	—	+	32	—	—	+	+	—
C-30	—	+	34	—	+	+	+	—
C-55	—	+	31	—	+	+	+	—
C-56	—	+	32	—	—	+	+	—
C-73	—	+	37	—	+	+	+	—
C-82	—	+	32	—	—	+	+	—
C-84	+	+	38	—	—	+	+	—
C-85	—	+	35	—	—	+	+	—
C-92	—	—	33	—	+	+	+	+ (—)
C-117	—	+	34	—	+	+	+	+ (—)
C-134	+	+	26	—	+	+	—	+ (—)
<i>C. coli</i>								
C-38	—	—	32	—	+	+	+	—
C-128 ^T	—	—	21	—	+	+	+	—
C-39	—	—	33	—	+	+	+	—
C-59	—	—	35	—	—	+	+	—
C-63	—	—	37	—	—	+	+	—
C-64	—	—	38	—	—	+	+	—
C-69	—	—	34	—	+	+	+	—
C-71	—	—	35	—	+	+	+	—
C-72	—	—	34	—	+	+	+	—
C-83	—	—	37	—	—	+	—	—
C-95	—	—	32	—	+	+	+	—
C-98	—	—	40	—	—	+	+	—
C-100	—	—	33	—	+	+	+	—
C-105	—	—	32	—	+	+	+	—
C-106	—	—	36	—	+	+	+ (—)	—
C-107	—	—	35	—	+	+	+ (—)	—
C-108	—	—	32	—	+	+	+	—
C-113	—	—	36	—	—	+ (—)	+	—
C-114	—	—	28	—	+	+	+	—
C-115	—	—	27	—	+	+	+	—
C-116	—	—	30	—	—	+	—	—
" <i>C. fecalis</i> "								
C-32	+	—	—	50	+	+	+	+ (—)

TABLE 4—Continued

Strain	H ₂ S test	Hippurate hydrolysis	Diam of inhibition zone (mm)		Deoxyribonuclease	Growth at 42°C	Growth in 1% glycine	Growth in 3.5% NaCl
			Nalidixic acid	Cephalothin				
C-33	+	—	12	50	+	+	+	+
C-34	+	—	12	50	+	+	+	+
C-35	+	—	12	52	+	+	+	+
C-36	+	—	—	42	+	+	+	+
Nalidixic acid-resistant thermophilic <i>Campylobacter</i>								
C-129	—	—	—	—	—	+	+	—
C-130	—	—	—	—	—	+	+	—
C-133	—	—	—	—	—	+	—	—
<i>Campylobacter</i> -like								
C-58	—	—	30	—	—	—	—	—
C-80 ^d	—	—	20	—	—	—	—	—
C-131 ^d	—	—	15	—	—	—	—	—
C-132 ^d	—	—	18	12	—	—	—	—

^a The following tests gave positive results for all strains: catalase, oxidase, and nitrate. All strains except strain C-58 were negative for aerobic growth. In the nalidixic acid and cephalothin tests 30-μg disks were used.

^b —, No zone of inhibition.

^c Where differences in growth results occurred depending on whether the basal medium was fluid thioglycolate medium or brucella broth containing 0.16% agar, the results obtained with the latter medium are given in parentheses.

^d The basal medium used for all growth tests was brucella broth containing 0.16% agar.

in 3.5% NaCl, hydrolyzed hippurate, or was susceptible to nalidixic acid and cephalothin.

The organisms isolated from laboratory mice form a separate DNA relatedness group and are more fastidious in their growth requirements than any other *Campylobacter* or *Campylobacter*-like organisms tested. These strains do not grow in fluid thioglycolate broth at any temperature. All growth tests were done in brucella broth supplemented with 0.16% agar as the basal medium and the control. These isolates are nonfermentative, microaerophilic organisms which conform to the definition of the genus *Campylobacter* in all respects, including the presence of polar flagella and an average G+C content of 35.4 mol% (buoyant density method). A more complete description of these organisms will be given in a subsequent paper.

The one remaining organism in our study, strain C-58, was set apart from all other strains by DNA-DNA hybridization data. This organism was sent to us by Higgins and Degre and was reported by these workers (7) to be similar to the porcine and bovine abortion isolates of Neill et al. (16). This similarity between isolates was later confirmed by Ellis (8). Neill et al. proposed that this group of organisms should be designated *Campylobacter* on the basis of G+C contents, which ranged from 29 to 34 mol% in 11 strains (16). Despite the similar DNA base composition, these isolates have features not characteristic of known *Campylobacter* strains. Promi-

nent among these is the ability to grow aerobically. Although we do not know whether the strain sent to us by Higgins and Degre was represented among those used in the study of Neill et al., this strain conforms to all of the characteristics described by Neill et al. for the group. It is catalase positive, does not produce H₂S, does not grow at 42°C; does grow aerobically, and is susceptible to nalidixic acid. Phase-contrast microscopy of 48-h-old fluid thioglycolate cultures demonstrates motile vibroid to very long spiral forms. Flagellar straining of microaerophilic 24-h-old BSB agar cultures shows rod-shaped and spiral forms that typically are polarly monotrichous. When this organism is viewed by phase-contrast microscopy, rod-shaped forms predominate in BSB agar cultures, whereas spirals and comma-shaped forms predominate in fluid thioglycolate medium cultures. After 48 h of incubation at 35°C in defined medium, strain C-58 contained granules when it was viewed by phase-contrast microscopy. No other strain tested produced cytoplasmic inclusions in this medium. The medium used was a modification of a medium described by Fletcher and Plastringe (3) and contained the following: 0.2 g of DL-aspartate, 0.05 g of L-cystine, 0.3 g of L-glutamic acid, 0.5 g of sodium acetate, 0.1 g of MgCl₂ · 6H₂O, 0.005 g of NaCl, 0.005 g of K₂HPO₄, 0.005 g of KH₂PO₄, 0.00001 g of niacin, and 100 ml of distilled water. The pH was adjusted to 7.2, and the medium was filter steril-

ized. We believe that despite the appropriateness of the DNA base composition of these organisms for inclusion in the genus *Campylobacter*, other important inconsistencies exist. We feel that further work will be necessary before accurate conclusions regarding the proper classification of these strains can be reached.

Owen and Leaper have recently published a limited DNA-DNA hybridization study which lends support to some of our conclusions (17). Using in vitro-labeled DNA from *C. coli* NCTC 11366, these workers performed DNA-DNA hybridizations between this organism and three strains of *C. jejuni*, one strain of *C. fetus* subsp. *fetus*, and one nalidixic acid-resistant thermophilic *Campylobacter* strain. Owen and Leaper concluded that the *C. coli* and *C. jejuni* type strains belong to separate species and that *C. fetus* is distinct from both, although the relevant hybridizations with *C. jejuni* DNA were not done. The DNA of the nalidixic acid-resistant thermophilic *Campylobacter* strain exhibited considerable heterogeneity in base sequence compared with *C. coli* DNA (relative binding ratio, 13%), a result very comparable to our results.

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