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Campylobacter butzleri sp. nov. Isolated from Humans and Animals with Diarrheal Illness

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Seventy-eight aerotolerant *Campylobacter* isolates were characterized phenotypically and by DNA hybridization (hydroxyapatite method at 50 and 65°C). Two DNA relatedness groups were found. (i) Sixty-four strains belonged to aerotolerant *Campylobacter* DNA hybridization group 2. These organisms were isolated from humans, primarily with diarrheal illness, and animals on several continents. Strains were aerotolerant at 30 and 36°C and catalase negative or weakly catalase positive, grew in media containing glycine and on MacConkey agar, were susceptible to nalidixic acid, and were resistant to cephalothin. The name *Campylobacter butzleri* sp. nov. is proposed for this group. (ii) DNA hybridization group 1 consisted of the type strain of *Campylobacter cryaerophila* and 13 additional strains isolated from 10 animals outside the United States and from three humans within the United States. This group was genetically diverse; five strains were closely related to the type strain of *C. cryaerophila* (DNA hybridization group 1A), and eight strains were more closely related to one another (DNA hybridization group 1B). Strains in DNA hybridization group 1B were phenotypically diverse, with two of eight strains resembling *C. cryaerophila*. The seven strains from DNA hybridization groups 1A and 1B which resembled *C. cryaerophila* and the *C. cryaerophila* type strain were aerotolerant only at 30°C and catalase positive, did not grow in glycine or on MacConkey agar, were generally susceptible to nalidixic acid, and were resistant to cephalothin. The remaining six strains of DNA hybridization group 1B phenotypically resembled *C. butzleri*; however, they were generally catalase positive and susceptible to nalidixic acid and cephalothin. DNA hybridization group 1B is not designated as a separate species at this time since it cannot, with certainty, be separated genetically from *C. cryaerophila* or phenotypically from *C. butzleri*.

Isolation of aerotolerant spiral or vibriolike organisms from aborted bovine fetuses was first described by Ellis et al. in 1977 (5). Further reports by other investigators (6, 7, 13, 17, 18, 20) associated these aerotolerant strains with mastitis as well as with bovine and porcine abortions. Neill and colleagues (19) classified these strains in the genus *Campylobacter* based on their morphologies and DNA base compositions. They further described these organisms on the basis of a large battery of biochemical and morphologic characteristics and designated these phenotypically heterogeneous organisms as a single new species, *C. cryaerophila* (17).

The initial objective of this study was to characterize 22 isolates of human origin received at the Centers for Disease Control and presumptively classified as *C. cryaerophila* on the basis of oxygen tolerance. When possible new genospecies were identified, additional isolates of human and nonhuman origin resembling *C. cryaerophila* were studied. In all, 78 aerotolerant *Campylobacter* isolates of human and animal origin were examined by DNA hybridization as well as extensive phenotypic testing. Two distinct hybridization groups, which are described here, were found among strains of human and animal origin: aerotolerant *Campylobacter* DNA hybridization group 2 is proposed as *C. butzleri* sp. nov., and aerotolerant *Campylobacter* DNA hybridization group 1 (which is *C. cryaerophila*) is genetically and phenotypically heterogeneous.

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MATERIALS AND METHODS

Strains. All strains thought to be aerotolerant *Campylobacter* species were characterized by gas-liquid chromatographic analysis of cellular fatty acid composition, as described by Lambert et al. (10); several strains were shown to have a profile incompatible with that of any known *Campylobacter* species and were thus excluded. Strains of aerotolerant *Campylobacter* used in DNA relatedness studies and their sources are listed in Table 1. Strains of aerotolerant *Campylobacter* were received from 1975 to 1989 at the Centers for Disease Control and were frozen as soon as possible at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol. Table 2 contains a list of the type and other reference strains of the *Campylobacter*, *Helicobacter*, and *Wolinella* species used in this study.

DNA relatedness. (i) Growth of organisms and extraction of DNA. In order to obtain large quantities of cells, each strain was cultured on 20 to 50 plates (150 by 15mm) containing heart infusion agar (HIA; Difco) with 5% sheep blood (SB) and was incubated for 24 to 72 h at 30°C in an anaerobic incubator containing an atmosphere of approximately 5% oxygen, 7.5% carbon dioxide, 7.5% hydrogen, and 80% nitrogen. Although strains which were later determined to belong to *C. butzleri* produced sufficient cells by using 20 to 25 plates of HIA-SB under these conditions, it was necessary to culture some strains, which were later determined to belong to *C. cryaerophila*, on 40 to 50 plates of chocolate agar with a Mueller-Hinton agar base. Growth of these strains on Chocolate II agar (BBL Microbiology Systems,

TABLE 1. Designations and sources of the aerotolerant *Campylobacter* strains used in this study

Strain	Origin[a]	Source	Previously published no./designation
C. cryaerophila			
D2792	ATCC[b]	Bovine; brain	*C. cryaerophila* type 43158
D2077[c]	Ireland[d]	Ovine; placenta	02797:B750/P: phenon A2[e]
D2078[c]	Ireland	Porcine; aqueous humor	02772:S613/E(b): phenon A3[e]
D2079[c]	Ireland	Bovine; brain	02766:A169/B: phenon A2[e]
D2080[c]	Ireland	Ovine; placenta	02798:B858/P: phenon A2[e]
D2081[c]	Ireland	Porcine; kidney	02774:B1904/K: phenon A2[e]
D2883	Ireland	Porcine; aqueous humor	02773:S/836/EYE phenon A2[e]
D2884	Ireland	Ovine; blood	02930:B3525/FB phenon A2[e]
D0460	Wisconsin	Human; blood	
D2610	Illinois	Human; blood	
D2639	Iowa	Human; stool	
D2887	Ireland	Porcine; placenta	02770:B/2331/P phenon A3[e]
D2888	Ireland	Bovine; amniotic fluid	02743:N/1054/AM phenon A3[e]
D2891	Ireland	Porcine; stillborn piglet	04957:LC/78/35/1 phenon B2[e]
C. butzleri			
D0232	Georgia	Human; mitral valve	
D1106	Montana	Human; stool	
D1751	Texas	Human; stool	
D1780	Massachusetts	Human; abdominal contents	
D2076 w[f]	California	Human; rectal swab	
D2076 c[f]	California	Human; rectal swab	
D2197	Idaho	Human; stool	
D2280	Tennessee	Human; stool	
D2322	Yerkes Primate Research Center	Primate; rectal swab	RNm1[j]
D2446	Texas	Human; stool	
D2451	Iowa	Human; stool	
D2563	Massachusetts	Human; blood	
D2568	Florida	Human; blood	
D2576	California	Human; peritoneal fluid	
D2630	Washington	Human; stool	
D2638	Arizona	Human; stool	
D2685	California	Human; peritoneal fluid	
D2686	California	Human; stool	
D2703	South Dakota	Ostrich; yolk sac	
D2709 t[f]	Colorado	Human; stool	
D2709 c[f]	Colorado	Human; stool	
D2720	Indiana	Human; stool	
D2725	Massachusetts	Human; stool	
D2775	Thailand	Human; stool	CP22[g]
D2776	Thailand	Human; stool	CP182[g]
D2778	Thailand	Human; stool	P245[g]
D2779	Thailand	Human; stool	P305[g]
D2780	Thailand	Human; stool	P315[g]
D2782	Thailand	Human; stool	CP66[g]
D2783	Thailand	Human; stool	CP18[g]
D2784	Thailand	Human; stool	CP23[g]
D2785	Thailand	Human; stool	CP43[g]
D2786	Thailand	Human; stool	CP71[g]
D2787	Thailand	Human; stool	P42[g]
D2788	Thailand	Human; stool	P304[g]
D2789	Thailand	Human; stool	P218[g]
D2790	Thailand	Human; stool	P232[g]
D2791	Thailand	Human; stool	P337[g]
D2810	Ohio	Human; stool	
D2815	Australia	Human; stool	02811[h]
D2828	Canada	Human; stool	
D2829	Canada	Human; stool	
D2873	Kansas	Human; stool	
D2885	Ireland	Porcine; aqueous humor	04955:S/568/E phenon A3[e]
D2892	Ireland	Porcine; stillborn piglet	02834:LC/78/35/3 phenon B2[e]
D2893	Ireland	Porcine; thoracic fluid	02937:B/986/T phenon B2[e]
D2894	Ireland	Bovine; stomach contents	02741:B/2211/SC phenon B2[e]
D2895	California	Human; stool	
D2896	Massachusetts	Human; stool	

Continued on following page

TABLE 1—*Continued*

Strain	Origin[a]	Source	Previously published no./designation
D2899	Yerkes Primate Research Center	Primate; rectal swab	MF403[j]
D2900	Yerkes Primate Research Center	Primate; rectal swab	OPE342[j]
D2901	Colorado; VHMC[i]	Human; stool	
D2905	Yerkes Primate Research Center	Primate; feces	RKg[j]
D2906	Yerkes Primate Research Center	Primate; rectal swab	RZt[j]
D2907	Massachusetts	Human; stool	
D2912	California	Human; stool	
D2914	Iowa	Human; stool	
D2916	Yerkes Primate Research Center	Primate; stool	PVj[j]
D2917	Yerkes Primate Research Center	Primate; rectal swab	RWk[j]
D2918	Yerkes Primate Research Center	Primate; rectal swab	RDb-1[j]
D2919	Yerkes Primate Research Center	Primate; rectal swab	ROo[j]
D2920	California	Human; stool	
D2921	California	Human; stool	
D3615	Maryland	Primate; stool	

[a] Submitted by United States state public health laboratory unless noted otherwise.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] Strains were obtained from a third party, as provided by S. Neill.
[d] All strains received from S. Neill were from aborted fetuses unless stated otherwise.
[e] As described by Neill et al. (17).
[f] Both strains were isolated from the same patient but differed phenotypically.
[g] As described by Taylor et al. (23a).
[h] As described by Tee et al. (24).
[i] VHMC, Veterans Hospital and Medical Center.
[j] Richardson et al. (22a).

Cockeysville, Md.) prepared from a GC agar base was better than that on HIA-SB, but it was inferior to that obtained on chocolate agar with a Mueller-Hinton agar base.

Cells from most strains were harvested and lysed, and the resulting DNA was purified by the method described by Brenner et al. (4), except that lysis occurred within 5 min after the addition of sodium dodecyl sulfate and incubation at 37°C was not necessary. Strains later determined to belong to DNA hybridization group 1 were harvested from chocolate agar in lysing solution prepared without pronase and were lysed in 0.125% rather than 0.5% sodium dodecyl sulfate.

TABLE 2. Reference and type strains of *Campylobacter*, *Helicobacter*, and *Wolinella*

Name	ATCC[a] no.	Centers for Disease Control no.
C. cinaedi	35683	D418[b]
CLO1B		D419[b]
C. coli	33559	D145
C. concisus	33237	D2082
C. fennelliae	35684	D420[b]
C. fetus subsp. *fetus*	27374	D373
C. hyointestinalis	35217	D1932
C. jejuni	33560	D133
C. lari (formerly *C. laridis*)	11352	D373
C. mucosalis	43265	D1179
C. nitrofigilis	33309	D2711
"*C. upsaliensis*"	11541[c]	D1914[d]
C. sputorum subsp. *bubulus*	33562	D1248
C. sputorum subsp. "*fecalis*"	11415[c]	D1169
H. mustelae (*C. mustelae*)	43772	D2793[e]
H. pylori (*C. pylori*)	43504	D2371
W. curva	35224	D2712
W. recta	33238	D2083
W. succinogenes	29543	D2713

[a] All strains obtained from the American Type Culture Collection (Rockville, Md.) unless indicated otherwise.
[b] Strains provided by C. Fennell, Seattle, Wash.
[c] Strain designations are those of the National Collection of Type Cultures, Central Public Health Laboratory (London, England). Strains were obtained from the National Collection of Type Cultures unless indicated otherwise.
[d] Strain provided by J. Ursing, as given to P. Edmonds, Atlanta, Ga.
[e] Strain provided by P. Edmonds, Atlanta, Ga.

(ii) DNA studies. DNAs from strains A169/B (D2079), ATCC 43158 (D2792), D2610 (ATCC 49615), and D2686 (ATCC 49616) were labeled with $^{32}PO_4$ using commercial nick-translation reagents (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Promega, Inc., Madison, Wis.) and tested for reassociation with unlabeled DNA from the same strain (homologous reaction) as well as other clinical isolates and type and reference strains (heterologous reactions) as described by Brenner et al. (4). Because the guanine plus cytosine (G+C) content of *C. cryaerophila* has previously been reported (17, 19) to be 31 ± 1 mol%, temperatures of 50 and 65°C were chosen for optimal and stringent reassociation temperatures, respectively. Results represent the relatedness under optimal conditions, unless indicated otherwise. The G+C content of 11 aerotolerant *Campylobacter* strains was determined spectrophotometrically by thermal denaturation (4, 14).

Phenotypic tests. (i) Standard tests. All strains were subcultured from frozen stock onto HIA containing 5% rabbit blood (BBL), incubated at 30°C under the microaerobic conditions described above, and further subcultured one time prior to the performance of phenotypic tests. Unless indicated otherwise, all tests were performed by using an inoculum prepared by suspending 48-h growth into heart infusion broth to the density of a no. 1 McFarland standard. All aerotolerant *Campylobacter* strains, the type strain of *C. nitrofigilis*, three strains each of *C. fetus* subsp. *fetus* and "*C. upsaliensis*," and one strain of *C. jejuni* subsp. *doylei* were included in phenotypic testing. *C. nitrofigilis*, a nitrogen-fixing organism associated with plants (15), was included in phenotypic testing, although it has not previously been

described as being aerotolerant, because investigation of the type strain in our laboratory demonstrated that this strain grows in the presence of atmospheric oxygen at 25°C (unpublished data), and might therefore be misidentified as an aerotolerant *Campylobacter* species.

All isolates were characterized by using the phenotypic tests described by Barrett et al. (2), with three exceptions: (i) the rapid H_2S test was not performed, (ii) trimethylamine *N*-oxide results were considered invalid when we observed that some strains grew anaerobically in the trimethylamine *N*-oxide base medium in the absence of trimethylamine *N*-oxide (unpublished data), and (iii) the test for growth on MacConkey agar was done by inoculating plates, rather than slants, containing MacConkey agar with a streak from a swab saturated with the standardized suspension. Up to four strains were inoculated per plate. A series of 50 organisms was tested in parallel by using both slants and plates; growth was more apparent on plates, and no discrepancies were noted. Strains that did not demonstrate adequate growth in nitrate broth or in basal brucella broth were retested in the alternative media suggested by Barrett et al. (2) for nitrate reduction, hydrogen sulfide production, and tolerance to glycine and NaCl.

(ii) Additional tests. In addition to the standard battery of tests, 13 tests were chosen for use in characterizing these *Campylobacter* strains. These tests were selected because they are in common use by investigators working with *Campylobacter* species and were thought to be of potential use in distinguishing among the DNA hybridization groups or between aerotolerant *Campylobacter* and other *Campylobacter* species. Additional temperature tolerance tests, at 4, 15, and 30°C, were included to determine more precisely the growth ranges of these organisms. The ability of the organisms to grow on brucella agars prepared from dehydrated media from several manufacturers (GIBCO Laboratories, Grand Island, N.Y.; Difco; and BBL) was tested because growth on brucella agar has been shown to be inconsistent (17). Hydrolysis of indoxyl acetate (Sigma Chemical Co., St. Louis, Mo.) was performed by using disks prepared as described by Mills and Gherna (16). DNase production was tested by using the toluidine blue DNA agar described by Lior and Patel (12) in parallel with methyl green DNase test agar. Bacteria were also tested for their ability to grow on Campy-BAP (BBL) and Campy-CVA (BBL) selective media, which are commonly used for primary *Campylobacter* isolation in clinical laboratories.

Additional tests were included to determine the ability of strains to grow in the presence of 0.04 and 0.1% 2,3,5-triphenyltetrazolium chloride (TTC), cadmium chloride, sodium succinate, 0.1% selenite, and esculin. TTC was sterilized by filtration before suspension in yeast nutrient agar (blood agar base no. 2 [CM 271; Oxoid, Columbia, Md.] containing 1 g of yeast extract per liter) essentially as described by Benjamin et al. (3). A positive result was defined as brick red growth on this medium. Disks containing 2.5 and 20 μg of cadmium chloride were prepared locally as described by Kazmi et al. (8). Zone sizes were measured following the observation of satisfactory growth (usually 24 to 48 h). A resistant organism was not inhibited by the presence of cadmium chloride (zone size, 6 mm), whereas some degree of inhibition was seen for susceptible strains (zone size, >6 mm). Sodium succinate (0.5%)-containing medium was prepared by adding 3.35 g of yeast nitrogen base agar (Difco) to a 5% solution (filter sterilized) of sodium succinate (Sigma) in a final volume of 50 ml and diluting the resulting suspension in 450 ml of 1.5% agar (Bacto-Agar; Difco) before dispensing it into plates (100 by 15 mm). Strains were examined for growth or lack of growth on this medium (17). Control plates containing yeast nitrogen base agar were inoculated to ensure that growth was due to the presence of the succinate. Type strains of *C. jejuni* and *C. coli* were included with each series of tests as negative and positive controls, respectively.

Sodium selenite-containing medium was prepared by adding 0.1% sodium selenite to sterile brucella agar base (Albimi formulation; Pfizer Inc., New York, N.Y.). The *C. coli* type strain was included with each set of tests as a positive control. A control plate prepared from the brucella agar base was included to ascertain that all strains were capable of growing on the base medium (17). All but three strains of DNA hybridization group 1 were capable of growth on the base medium. These media were inoculated with the organisms as described above for MacConkey agar plates. All plated media were incubated under microaerobic conditions at 36°C as described above and were examined at 1, 2, and 5 days. Plates inoculated with slower, poorly growing strains were held for 7 days. The ability of the organisms to grow in the presence of esculin was determined by inoculating them into esculin broth (prepared locally) with 1 drop of the McFarland suspension. A strain of *Aeromonas hydrophila* known to reduce esculin was included as a positive control with each experiment. Inoculated esculin broths were incubated for 5 days and were examined at 2 and 5 days for the production of a black pigment (positive result). When phenotypic results within a DNA hybridization group were discrepant, the tests were repeated at least once.

RESULTS

In DNA hybridization studies, the *C. cryaerophila* type strain, A169/B (D2079), was approximately 30% related under optimal conditions to 23 representative strains of human and animal origin from the United States that were tested (data not shown). We then chose one of the human strains (D2686) to be used for hybridization against the human and animal isolates from the United States. This strain (D2686) was related at the species level (72 to 100% related under optimal conditions, with divergence between related sequences generally being between 1.0 and 2.5%) to all but three strains of human origin and to all isolates of animal origin from the United States (Table 3). This group of isolates was then classified as a genospecies and designated DNA hybridization group 2.

The *C. cryaerophila* type strain, A169/B (D2079), was 68 to 100% related to 12 strains, primarily isolates of animal origin from outside the United States (data not shown). However, although this type strain (A169/B [D2079]) was originally used for hybridization studies, it demonstrated biochemical test results that were not consistent with those of the type strain 43158 (D2792) obtained from the American Type Culture Collection (ATCC; Rockville, Md.). In addition, the two cultures of the type strain demonstrated different restriction endonuclease profiles (data not shown). Therefore, all further hybridization experiments were conducted by using the type strain ATCC 43158. Strains A169/B (D2079) and ATCC 43158 (D2792) were classified as *C. cryaerophila* by DNA relatedness, although their level of relatedness was much lower than expected for two cultures presumed to be the same strain (Table 3). Hybridization experiments with ATCC 43158 (D2792) demonstrated high levels of relatedness to 13 strains (64 to 79%), with divergence between related sequences of 4.0 to 6.5%. Ten strains

TABLE 3. Relatedness of reference strains ATCC 43158 (D2792), ATCC 49616 (D2686), and ATCC 49615 (D2610) to other aerotolerant *Campylobacter* strains[a]

Strain	DNA hybridization group 1A (*C. cryaerophila*; label = ATCC 43158 [D2792])			DNA hybridization group 1B (label = ATCC 49615 [D2610])			DNA hybridization group 2 (*C. butzleri*; label = ATCC 49616 [D2686])		
	50°C RBR[b]	*D*[c]	65°C RBR	50°C RBR	*D*	65°C RBR	50°C RBR	*D*	65°C RBR
D2792 (43158[d])	100	0.0	100	61	8.0	62	50		21
D2079 (A169/B)	77	4.0	64	70	6.0	62	42		26
D2080	74	5.5	73	73	6.5	59			
D2077	73	4.0	62	72	7.0	59			
D2078	70	5.0	58						
D2081	64	5.5	63						
D2884	64	5.5	66	72	4.0	79	50		
D2883	64	6.5	55	74	5.0	75	49		
D2610	79	5.5	70	100	0.0	100	56		38
D0460	74	6.0	66	89	1.5	93	44		28
D2887	70	6.0	68	74	3.0	82	47		
D2891	70	6.0	69	79	4.0	66	56		
D2639	69	6.0	64	73	3.0	78			15
D2888	67	6.5	66	83	3.0	88	54		
D2686	42	15.0	19	41	10.5	21	100	0.0	100
D2778							100		
D2779							100		
D2780							100		
D2896							100		
D2810							98		
D2873							98		
D2895							98		
D2914							98		
D2829							97		
D2451	40	12.5	20				95	4.0	83
D2576							95	2.5	83
D2782							95		
D2828							95		
D2907							94		
D2900							93		
D2920							93		
D2921							93		
D2901							92		
D2906							92		
D2916							92		
D2775							91		
D2893							91		
D2899							91		
D2919							91		
D2685							90	2.5	84
D2783	54	10.0	35				90		
D2815				43	12.5	33	90		
D2912							90		
D2563							89	2.5	89
D2703							89	2.0	84
D2725				38	12.5		89	2.5	84
D2894							89		
D1106							88	2.5	85
D2776							88		
D2917							88		
D2918							88		
D2076w							87	2.0	83
D2720				37	12.5	19	87	3.0	84
D1751				49	10.5	30	86	1.5	86
D2076c							86	2.0	86
D2788							86		
D2784							85		
D2787							85		
D2785							84		
D2892							84		
D2446							83	1.0	79
D2709c							83	1.0	80
D2709t							83	1.5	80
D2885				42	12.5	34	83		

Continued on following page

TABLE 3—*Continued*

Strain	DNA hybridization group 1A (*C. cryaerophila*; label = ATCC 43158 [D2792])			DNA hybridization group 1B (label = ATCC 49615 [D2610])			DNA hybridization group 2 (*C. butzleri*; label = ATCC 49616 [D2686])		
	50°C RBR[b]	*D*[c]	65°C RBR	50°C RBR	*D*	65°C RBR	50°C RBR	*D*	65°C RBR
D2905							83		
D3615							83	1.5	84
D1780							82	2.0	75
D2789							82		
D2322							81	2.5	80
D2280							80	2.0	83
D0232							79	2.5	82
D2786							79		
D2197							78	2.5	83
D2568							78	1.5	79
D2630							78	2.0	79
D2638							77	2.5	84
D2790							73		
D2791							72		

[a] Boxes indicate hybridization groups.
[b] RBR (relative binding ratio) = (percent heterologous DNA bound to hydroxyapatite/percent homologous DNA bound to hydroxyapatite) × 100. A control without unlabeled DNA was included to determine any self-reaction of the labeled DNA, and this value was subtracted prior to calculation of relatedness. Blank signifies reaction not done.
[c] *D*, percent divergence; percentage of unpaired bases in related DNA sequences to the nearest 0.5%, calculated based on the assumption that each decrease of 1°C in the thermal stability is caused by approximately 1% unpaired bases (4). Blank signifies reaction not done.
[d] ATCC strain.

(including A169/B [D2079]) were strains of animal origin from Ireland and were from the study that defined *C. cryaerophila* (17). An additional three strains (D2610, D460, D2639) isolated from humans in the United States were also included in this hybridization group.

Since the group of 13 strains showing approximately species-level relatedness to the type strain of *C. cryaerophila* (ATCC 43158 [D2792]) appeared to be heterogeneous, we repeated the relatedness studies by using DNA freshly prepared from ATCC 43158 (D2792); essentially the same results were obtained. Next, we labeled D2610, an isolate with a divergence of 5.5% to *C. cryaerophila* ATCC 43158. D2610 was 72 to 89% related to seven other strains under optimal conditions, with divergence of 1.5 to 5.0% between related sequences (Table 3). *C. cryaerophila* isolates still demonstrated genetic heterogeneity, with five isolates (DNA hybridization group 1A) appearing to be more closely related to ATCC 43158 (D2792) and eight isolates appearing to be more closely related to D2610 (DNA hybridization group 1B). However, based on the phenotypic data presented below, we designated phenotypic groups which did not precisely correspond with these genetic groups.

ATCC 43158 was 40 to 54% related to selected representative human strains of DNA hybridization group 2, with divergence between related sequences of >10%. Reciprocal reactions demonstrated essentially the same results (Table 3). Thus, the majority of the U.S. strains were clearly not *C. cryaerophila*. The type strain of *C. cryaerophila* and the reference strains for DNA hybridization groups 1B and 2 were all less than 20% related to reference strains of all other currently recognized *Campylobacter* species, *Helicobacter* species (previously included in the genus *Campylobacter*), and *Wolinella* species (data not shown).

The G+C content was 29 to 32 mol% for the five *C. cryaerophila* isolates and 30 ± 1 mol% for six DNA hybridization group 2 isolates.

Following establishment of the two hybridization groups, extensive testing was done in an attempt to demonstrate phenotypic differences between the clinically important strains contained in DNA hybridization group 2 and results previously reported for *C. cryaerophila* and to determine whether, in fact, there was a valid reason to divide DNA hybridization group 1. Strains clearly identified by hybridization as *C. cryaerophila* (DNA hybridization group 1A) were aerotolerant at 30°C, but not at 36°C, did not grow in the presence of glycine or NaCl (at either 1.5 or 3.5%) or on MacConkey agar, demonstrated large zones (18 mm or greater) in the presence of nalidixic acid (30-μg disk), and were resistant to cephalothin (30-μg disk). Strains grew poorly in the presence of nitrate-containing medium, with trace amounts of nitrate reduced to nitrite. All but one strain of DNA hybridization group 1A (*C. cryaerophila*) were susceptible to cadmium chloride at low concentrations; however, this strain (A169/B [D2079]) was resistant to both 2.5 and 20 μg of cadmium chloride (data not shown).

In contrast, DNA hybridization group 2 isolates were aerotolerant at both 30 and 36°C, grew in glycine- and nitrate-containing media (reducing nitrate to nitrite) and on MacConkey agar, and generally grew in both 1.5 and 3.5% NaCl. DNA hybridization group 2 strains were resistant to cephalothin; 14% of DNA hybridization group 2 strains were resistant to nalidixic acid, with all strains producing a zone size of 18 mm or less (mean, 11 mm). Most DNA hybridization group 2 strains were resistant to both 2.5 and 20 μg of cadmium chloride. Therefore, DNA hybridization group 2 is proposed as a new species, *C. butzleri*, since it can be phenotypically and genetically separated from *C. cryaerophila*.

Based on phenotypic testing, two strains contained in DNA hybridization group 1B appeared to resemble the type strain of *C. cryaerophila* and six strains more closely resembled *C. butzleri*. Strains D2883 and D2884 resembled *C. cryaerophila* in that they did not grow on MacConkey agar or in the presence of glycine. However, six strains belonging to DNA hybridization group 1B more closely resembled *C. butzleri*, in that they were aerotolerant at both 30 and 36°C

and grew in glycine- and nitrate-containing media and on MacConkey agar. In contrast to *C. butzleri* strains, these DNA hybridization group 1B strains were generally susceptible to both nalidixic acid and cephalothin (with the exception of strain D2639) and did not grow in the presence of 1.5% (except D2610 and D2891) or 3.5% (except D2887 and D2891) NaCl. Also, DNA hybridization group 1B strains were generally resistant to 2.5 μg (except D460), but were susceptible to 20 μg (except D2888 and D2891), of cadmium chloride. Results available from other phenotypic tests were essentially the same for both *C. butzleri* and DNA hybridization group 1B strains. We did note that *C. cryaerophila* and DNA hybridization group 1B strains grew as small, watery, or yellowish colonies on HIA-SB and HIA-rabbit blood in 48 to 72 h, and growth was greatly enhanced on chocolate agar produced with a Mueller-Hinton agar base. *C. butzleri* strains produced larger, white, opaque colonies in 24 h on both HIA-SB and HIA-rabbit blood, and growth was not enhanced on chocolate agar. Tests with several strains of DNA hybridization group 1B indicated that the enhancement of growth on chocolate agar was not due to the presence of either X or V factor (unpublished data). The results shown in Table 4 indicate the phenotypic test results with DNA hybridization group 1 subdivided as indicated to demonstrate the phenotypic heterogeneity among these isolates.

DISCUSSION

Our results indicate that the strains of aerotolerant *Campylobacter* do not make up a homogeneous group. Most of the human strains tested were not *C. cryaerophila*. These observations confirm the need to define new species by DNA relatedness. The majority (49 of 52) of the isolates of human origin, including those from outside the United States, belonged to *C. butzleri*, as did the majority (15 of 26) of strains of animal origin tested. However, the percentage of strains in each DNA hybridization group in this study may not be entirely representative of potential isolates; since *C. cryaerophila* strains (hybridization groups 1A and 1B) grew poorly on blood agar, they would most likely be missed in specimens which contain contaminating flora. In addition, the *C. butzleri* isolates from Thailand and those from the Yerkes Primate Research Center were found as part of special studies; the Thai isolates were isolated by filtration (23a), and the strains of nonhuman primate origin from the Yerkes Primate Research Center were isolated on Campy-CVA agar incubated at 35°C (22a).

Of the *C. butzleri* strains isolated from humans in the United States, 25 isolates were from stools of patients with diarrheal illness, as were the 2 isolates from Canada, 1 isolate from Australia, and the 15 isolates from Thailand (23a). Three isolates were from the abdominal contents or peritoneal fluids of patients with acute appendicitis, and three additional isolates were obtained from blood cultures.

Many of these strains would not have been isolated by customary, primary isolation methods for *Campylobacter* species because they did not grow at 42°C or on Campy-BAP medium. Therefore, more *C. butzleri* and *C. cryaerophila* strains may be isolated if alternative isolation methods are used. Possible alternative methods include the use of Campy-CVA agar incubated at 35°C or filtration methods such as those described by Wells et al. (28).

The three human isolates which were genetically not *C. butzleri* would be phenotypically designated *C. butzleri*, although they were genetically more closely related to *C. cryaerophila*. Two of these strains (D460, D2610) were isolated from blood cultures of patients who had aspirated fluid contaminated with fecal material. The third strain (D2639) was isolated from the stool of a patient with acute gastroenteritis. Three additional strains were from animal abortions (17). At this time, it appears that approximately 10% of isolates identified phenotypically as *C. butzleri* will genetically be *C. cryaerophila*. It will require a genetic technique such as DNA hybridization, multilocus enzyme electrophoresis, or analysis of ribosomal DNA patterns to distinguish precisely which hybridization group organisms phenotypically resembling *C. butzleri* belong to.

The low levels of relatedness to other members of the genus *Campylobacter*, with the exception of *C. cryaerophila*, that are present suggest that it would be technically appropriate to classify both *C. cryaerophila* and *C. butzleri* in another genus. This is in accord with results obtained by other investigators using 16S rRNA sequencing (25). However, we did not choose to do this because we were not able to identify a test which would allow clinical laboratories to distinguish members of *C. cryaerophila* and *C. butzleri* from other members of the genus *Campylobacter*. We feel that these organisms will be identified clinically as *Campylobacter* species, as they produce cytochrome oxidase and demonstrate the typical morphologic characteristics (although appearing somewhat larger and with fewer spirals) and motility associated with other campylobacters. Aerotolerant *Campylobacter* strains are distinguished from strains of other *Campylobacter* species because they grow in the presence of atmospheric levels of oxygen. However, variations in oxygen tolerance are seen among other species of *Campylobacter* and may be present (2) or induced in species which would not normally be considered aerotolerant (11).

Characteristics other than aerotolerance that are important in distinguishing aerotolerant *Campylobacter* species from other *Campylobacter* species include hydrolysis of indoxyl acetate; growth at 15, 25, and 36°C, but not 42°C; and the inability to hydrolyze hippurate. Cadmium chloride susceptibility may also be useful in discriminating isolates of aerotolerant *Campylobacter* species from other *Campylobacter* species. A wide range of susceptibilities to cadmium chloride was demonstrated among isolates of aerotolerant *Campylobacter*. Strains of *C. butzleri* were generally resistant to both concentrations of cadmium chloride tested, strains of DNA hybridization group 1A (*C. cryaerophila*) were generally susceptible to cadmium chloride, and strains of DNA hybridization group 1B were resistant to 2.5 μg of cadmium chloride but were susceptible to 20 μg. Resistance to cadmium chloride is reported to be uncommon among strains of *Campylobacter* (1, 8), but it is a common characteristic of other enteric organisms such as *Salmonella*, *Shigella*, *Pseudomonas*, and *Aeromonas* species.

Strains of *C. cryaerophila* might be confused with *C. fetus* subsp. *venerealis* because they did not grow in media (either brucella or brain heart infusion agar) containing glycine but did grow at 25°C; however, *C. fetus* subsp. *venerealis* strains grow on MacConkey agar (2) and do not hydrolyze indoxyl acetate (16, 22). *C. butzleri* strains were catalase negative or weakly catalase positive, which could result in confusion with strains of "*C. upsaliensis*." However, unlike *C. butzleri* strains, "*C. upsaliensis*" strains do not grow under aerobic and anaerobic conditions, at 25°C, or on MacConkey agar and are susceptible to both nalidixic acid and cephalothin (2). In fact, several isolates included in this study were tentatively identified by state or other reference laboratories as *C. fetus* or "*C. upsaliensis*." The most noticeable sources

TABLE 4. Phenotypic characteristics of aerotolerant *Campylobacter* species[a]

Characteristic	% Strains demonstrating positive reaction			
	DNA hybridization group 2 (*C. butzleri*)	DNA hybridization group 1 (*C. cryaerophila*)		*C. nitrofigilis*
		C. butzleri phenotype	*C. cryaerophila* phenotype	
No. of strains	64	6	8	1
Growth under:				
Aerobic conditions	100	100	100[b]	100[c]
Anaerobic conditions	97	83	60	100[d]
Growth at:				
5°C	0	0	0	0
15°C	100	100	100	100
25°C	100	100	100	100
30°C	100	100	100	100
36°C	100	100	100	100[d]
42°C	**33**[e]	**0**	0	0
Growth on or in the presence of:				
Glycine (1.0%)	100	100	0	0
MacConkey agar	100	100	0	0
NaCl (3.5%)	61	33	0	100
NaCl (1.5%)	84	33	0	100
Glucose O/F	0	0	0	0
Glucose (8%)	**100**	**83**	25	100
Succinate	100	100	25	100
Campy-CVA	100	100	100	0
Campy-BAP	14	17	0	0
Production or presence of:				
Oxidase	100	100	100	100
Catalase	**33 negative/66 weak**	**66 positive**/33 negative	75 positive/25 weak	100 positive
Motile	92	100	100	100
Flagella	81	83	100	100
H_2S-triple sugar iron	0	0	0	0
H_2S-lead acetate	16	17	13	100
Resistance to:				
Nalidixic acid	14	17	0	0
Cephalothin	98	33	100	0
Cadmium chloride (2.5 μg)	**100**	**83**	13	100
Cadmium chloride (20 μg)	**72**	**33**	13	100
Reduction or hydrolysis of:				
Nitrate	100	83[d]	90[d]	100
Nitrite	0	0	0	0
Hippurate	0	0	0	0
Indoxyl acetate	100	100	100	0
Urea	0	0	0	0
DNA (TB)[f]	28	40	63	100
DNA (MG)[g]	**25**	**0**	13	100
TTC (0.04%)[h]	**31**	**0**	0	0

[a] Reactions in bold type may be used to distinguish between *C. butzleri* organisms and *C. cryaerophila* organisms with a *C. butzleri* phenotype.
[b] Growth at 30°C, but not 36°C.
[c] Growth at 25°C, but not 36°C.
[d] Trace or light growth.
[e] A total of 26 of 26 strains tested demonstrated 2+ to 3+ growth at 40°C, in contrast to trace growth at 42°C.
[f] DNase medium containing toluidine blue (TB); production of DNase was detected in a mean of 2 days.
[g] DNase medium containing methyl green (MG); production of DNase was detected in a mean of 3 days.
[h] No strains were found which grew in the presence of 0.1% TTC, selenite, or esculin.

of confusion were the failure of the submitting laboratories either to test for aerotolerance or to test for aerotolerance by use of standardized conditions. We did not find any of the "*C. upsaliensis*" strains tested in our study to be aerotolerant, nor were any of the 12 "*C. upsaliensis*" strains tested by Patton et al. (21) aerotolerant. Several *C. fetus* isolates were received that were thought to be aerotolerant by the submitting laboratory. However, on repeat testing with a standardized suspension of organisms, these isolates were not aerotolerant. Also included in this study was the isolate classified as *C. cryaerophila* by Tee and colleagues (24) based on DNA-DNA dot blot testing; it was identified in this study by hydroxyapatite DNA hybridization to be *C. butzleri*. Although we did not examine the strain (E11117-88)

described by Taylor and colleagues (23) that did not hybridize with their "*C. upsaliensis*" or *C. jejuni* probe, it would be phenotypically consistent with *C. butzleri*.

Reference strains D2610 and D2686 have been deposited in the American Type Culture Collection as representatives of DNA hybridization groups 1B and 2, respectively (ATCC 49615 [CDC D2610] and ATCC 49616 [CDC D2686], respectively).

Emended description of *C. cryaerophila* (Neill, Campbell, O'Brien, Weatherup and Ellis, 1985). Cells of *C. cryaerophila* are aerotolerant at 30°C; some strains are also capable of light growth under anaerobic conditions. Growth occurs at 15, 25, and 36°C, but not at 5 or 42°C. No growth on MacConkey agar. Poor growth present on brucella agar medium in the absence of blood. No growth in medium (brain heart infusion or brucella agar) containing 1% glycine or NaCl (at concentrations of 1.5 or 3.5%). Variable reduction and growth in medium containing nitrate. Variable growth in 8% glucose (25% positive) and sodium succinate (25% positive). Indoxyl acetate hydrolyzed. Hydrogen sulfide is not produced in triple sugar iron medium; trace amounts of hydrogen sulfide occasionally (13%) present on lead acetate paper suspended over cysteine-containing media. Resistant to a 30-μg disk of cephalothin and susceptible to a 30-μg disk of nalidixic acid. Generally susceptible to both 2.5 and 20 μg of cadmium chloride. The type strain is Veterinary Research Laboratories isolate A169/B^T (02766 [NCTC 11885 = ATCC 43158]) as described by Neill et al. (17).

Description of *C. butzleri* sp. nov. *C. butzleri* (butz'ler.i.) is named in honor of Jean-Paul Butzler, a Belgian clinician and microbiologist, in recognition of his many contributions to the field of *Campylobacter*. Butzler and colleagues were the first to describe a selective medium which allowed hospital-based laboratories to isolate and identify *C. jejuni* and *C. coli* organisms on a routine basis. More recently, they modified the selective medium and used less-selective techniques to isolate other campylobacter pathogens, such as *C. fetus* subsp. *fetus*, and "*C. upsaliensis*." Additionally, Butzler and colleagues have contributed to our current understanding of the disease process and the role of antimicrobial therapy in campylobacteriosis and has provided valuable information about the epidemiology of *C. jejuni* and *C. coli*. Butzler is currently the director of the World Health Organization Collaborating Centre for *C. jejuni*, a member of the International Subcommittees on *Campylobacter* taxonomy and serotyping, and actively involved with the organization of the biennial International Workshops on *Campylobacter* Infections. Cells are gram-negative, nonsporeforming curved rods; some S-shaped forms are observed. Aerotolerant; light growth under anaerobic conditions. Darting motility; single, polar flagella at one or both ends. Catalase negative or weakly positive; oxidase positive. Growth occurs at 15, 25, 30, 36, and 40°C; no growth occurs at 5°C; variable, light growth at 42°C. D-Glucose is not oxidized or fermented. Growth occurs in medium containing 1% glycine; variable growth occurs in the presence of 1.5% (84%) and 3.5% (61%) NaCl. Isolates reduce nitrate, but not nitrite or esculin, hydrolyze indoxyl acetate, but not urea or hippurate, and variably produce DNase (25 to 28%). Growth in media containing 8% glucose and sodium succinate. Hydrogen sulfide not produced in triple sugar iron agar; variable production (16%) on lead acetate strips suspended over media containing cysteine. Growth on MacConkey agar. Variable (31%) red-pigmented growth on medium containing 0.04% TTC; no growth on medium containing sodium selenite. All strains resistant to 2.5 μg of cadmium chloride, and most strains (72%) resistant to 20 μg of cadmium chloride. Good growth on HIA and brucella media with and without blood. Growth not enhanced by the use of chocolate agar. Generally resistant to cephalothin and other cephalosporins, trimethoprim, and trimethoprim-sulfamethoxazole; intermediate susceptibility to nalidixic acid and erythromycin; susceptible to aminoglycosides, quinolones, polymyxin B, colistin, and rifampin (9).

Strains isolated most frequently from stools of human and nonhuman primates with diarrheal illness. Additional strains isolated at necropsy from an ostrich with diarrheal illness and from aborted bovine and porcine fetuses.

The mean G+C content of six strains is 30 ± 1 mol%.

Strain D2686 (ATCC 49616) is the type strain of *C. butzleri*. This strain conforms to the species description given above. Trace growth under anaerobic conditions and light growth at 42°C. Weak production of catalase. Growth in 1.5% NaCl, but not 3.5% NaCl. Hydrogen sulfide not produced in triple sugar iron agar, but a trace amount detected on lead acetate strips suspended over cysteine-containing medium. Negative for DNase, as detected by both toluidine blue- and methyl green-containing media. Growth does not occur in the presence of TTC-containing medium. Resistant to a 30-μg cephalothin disk (no zone of inhibition) and a 20-μg disk of cadmium chloride (no zone of inhibition) and intermediately susceptible to a 30-μg nalidixic acid disk (10-mm zone of inhibition).

Antibiotic susceptibility as determined for the following antimicrobial agents by broth microdilution (26) (unless otherwise noted, antimicrobic concentrations are in micrograms per milliliter): resistant to ampicillin (>32), ampicillin-sulbactam (32.0/16.0), nalidixic acid (>32), erythromycin (8.0), azithromycin (16.0), roxithromycin (16.0), chloramphenicol (>32), cephalothin (>32), cefuroxime (>32), cefoperazone (>32), cefotaxime (32.0), and clindamycin (>8); susceptible to ciprofloxacin (≤0.03), enoxacin (0.05), norfloxacin (0.5), ofloxacin (0.5), tetracycline (0.25), doxycycline (0.5), minocycline (0.25), gentamicin (0.25), amikacin (1.0), and trimethoprim-sulfamethoxazole (1/19 U). No growth in polymyxin B (5 U), colistin (10 U), rifampin (10.0), or metronidazole (4.0); growth in novobiocin (15.0), trimethoprim (5.0, 10.0, and 50.0), vancomycin (10.0), cefazolin (15.0), cycloheximide (50.0), bacitracin (25 U), and amphotericin B (2.0). Growth is not demonstrated on Campy-BAP agar, but does occur on Campy-CVA agar.

The mean G+C content of D2686 is 31 ± 2 mol%. This strain was isolated in the United States from a male patient with diarrheal illness.

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ADDENDUM

Following the submission of the manuscript, we learned of a proposal to place *C. cryaerophila* and *C. nitrofigilis* in a new genus, *Arcobacter* (27). Since *C. butzleri* is approxi-

mately 40% related to *C. cryaerophila*, it would become *Arcobacter butzleri* if this proposal is accepted.

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