Taxonomic Position of *Campylobacter cryaerophila* sp. nov.

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A total of 90 aerotolerant *Campylobacter* strains, which were isolated mainly from animal abortions, were examined by using a wide range of biochemical and physiological tests. The resulting data, together with data for reference *Campylobacter* strains that were similarly examined, were compared by using several numerical taxonomic analysis methods. Our analyses demonstrated that there is a strong similarity between the aerotolerant strains and strains of *Campylobacter fetus*, *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter sputorum*. For practical purposes this study revealed four possible taxa; one taxon consisted of *C. fetus* strains, one taxon consisted of the *C. sputorum* strains, and a third taxon consisted of the thermophilic strains, having *C. coli* and *C. jejuni* strains as separate entities. The fourth group consisted of all of the aerotolerant strains and was considered a new species of *Campylobacter*. All of the aerotolerant strains exhibited a tolerance to lower temperatures and higher oxygen concentrations than previously observed with strains of *Campylobacter*; the name *Campylobacter cryaerophila* sp. nov. is suggested for these strains. In this paper we present identification characteristics of *C. cryaerophila* strains and a description of the new species.

Since the original observation of "*Vibrio fetus*" in specimens from ovine abortions (40), a variety of microaerophilic vibrios having the same general description have been reported (2, 12, 14, 16, 26, 28, 34, 48, 57, 60). These organisms have been included in the existing genus *Campylobacter* (54). Of the catalase-positive strains, *Campylobacter fetus* strains are usually associated with problems of infertility and abortion in domestic animals. However, we have described the recovery of other spiral, *Campylobacter*-like organisms from abortions in several animal species (8, 9, 45). These isolates were recovered by using methods and media that are not usually used for *Campylobacter* isolation. The isolates were tolerant to atmospheric oxygen on subculture and, although assigned to the genus *Campylobacter*, did not appear to belong to any of the established groups that have been described previously (44).

In the present study, 90 strains recovered from different sources were examined by using physiological and biochemical tests. A numerical taxonomic comparison of these strains and reference *Campylobacter* strains was made to establish their relationship within the genus *Campylobacter* and to formulate a scheme for their differentiation.

MATERIALS AND METHODS

Strains. In this study we used 133 strains, including type and reference strains of some related species (Table 1); 17 of these strains were examined in duplicate to allow us to estimate test error. Most strains had been held for various periods at -70°C or in liquid nitrogen before use, but recently isolated strains were also included. The other reference strains included a "*Vibrio percolans*" strain, which was representative of aerobic *Campylobacter*-like organisms isolated from semen (51). The aerotolerant *Campylobacter* strains are referred to below as undesignated *Campylobacter* strains.

Culture methods. Preliminary studies established the necessity for using two different culture media and incubation temperatures for maintaining the strains in semisolid media. Strains were subcultured at 1-week intervals during the

study. Strains isolated by using method B (Table 1) and reference strains 91, 92, 132, and 133 were grown at 30°C in a medium (4 ml) containing Trypticase soy broth (BBL Microbiology Systems), 0.075% purified agar, and 1% hemin solution; the hemin solution was prepared as described by Holdeman and Moore (22). The remaining isolates and reference and type strains were grown at 37°C in a medium containing 2.8% brucella broth (Difco Laboratories), 0.5% yeast extract, 0.2% sodium succinate, 0.2% monosodium glutamate, 0.1% MgCl₂ · 6H₂O, 0.005% creatine, 0.005% FeSO₄ · 7H₂O, 0.05% cornstarch, 0.025% sodium thioglycolate, and 0.16% agar (B. D. Firehammer, Montana State University, Bozeman, personal communication).

Test media were inoculated from 48- and 72-h growing cultures. Growth in the maintenance media was homogeneously suspended, and dilutions (1:100) made in 0.25× Ringer solution (Oxoid Ltd.) were used as inocula.

Test procedures. For growth on solid media, we used blood agar base no. 2 (Oxoid) modified by adding 1% (vol/vol) hemin as the base medium. Dried plates were inoculated with a multipoint inoculator (Denley Instruments) and, unless otherwise stated, were incubated under reduced oxygen tension (5% O₂, 7% CO₂, 88% N₂). Broth and semisolid test media were inoculated with 0.05 ml of inoculum, and the test preparations were incubated in air unless otherwise stated.

Each test was carried out by using a single batch of media, and all strains were tested in duplicate. Strains showing variable results were retested before final results were recorded.

Colony and cell morphology. Cultures grown on 7% blood agar were examined after 48, 72, and 96 h; colony size and shape were recorded. Smears were Gram stained when colonies become visible, usually after 48 h. Motility and morphology were determined by using dark-field microscopy. Cultures growing in semisolid medium were also examined by using dark-field microscopy.

Pigmentation. Cultures grown on blood agar and on base medium were examined for pigment production by smearing colonies onto white filter paper. Pigment production on King media A and B (29) was also tested.

Hemolysis. Hemolysis ability was tested at both 37 and 30°C by culturing organisms for 4 days on blood agar base

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TABLE 1. Strains examined in this study

Laboratory no.	Strain	Taxon	Isolation method(s) ^a	Origin	Source ^b
1	A102/Pt		B	Peritoneal fluid; aborted bobine fetus	VRLB
2	A171/Eye		B	Aqueous humor; aborted bovine fetus	VRLB
3	A175/K		B	Kidney; aborted bovine fetus	VRLB
4	A175/P		B	Placenta; bovine abortion	VRLB
5	A169/B ^T		B	Brain; aborted bovine fetus	VRLB
6	A169/P		B	Placenta; bovine abortion	VRLB
7	A174/K		B	Kidney; aborted bovine fetus	VRLB
8	A174/P		B	Placenta; bovine abortion	VRLB
9	A179/T		B	Thoracic fluid; aborted bovine fetus	VRLB
10	B2656/U		B	Aborted bovine fetus	VRLB
11	A128/Pc		B	Pericardial fluid; aborted bovine fetus	VRLB
12	A182/FB		B	Blood; aborted bovine fetus	VRLB
13	B816/T		B	Thoracic fluid; aborted bovine fetus	VRLB
14	N1033/P		B	Bovine placenta	VRLB
15	A191/Pt		B	Peritoneal fluid; aborted bovine fetus	VRLB
16	N1049/K		B	Bovine fetal kidney	VRLB
17	N1059/P		B	Bovine placenta	VRLB
18	N1031/P		B	Bovine placenta	VRLB
19	N1058/P		B	Bovine placenta	VRLB
20	B2188/Am		B	Amniotic fluid; aborted bovine fetus	VRLB
21	B2211/Sc		B	Stomach contents; aborted bovine fetus	VRLB
22	B207/T		B	Thoracic fluid; aborted bovine fetus	VRLB
23	N1054/Am		B	Bovine amniotic fluid	VRLB
24	B2016/K		B	Kidney; aborted bovine fetus	VRLB
25	B955/P		B	Placenta; bovine abortion	VRLB
26	B3091/Pt		A and B	Peritoneal fluid; aborted bovine fetus	VRLB
27	B698/B		A and B	Brain; aborted bovine fetus	VRLB
28	B2767/P		A and B	Placenta; bovine abortion	VRLB
29	B109(A)/Eye		B	Aqueous humor; aborted bovine fetus	VRLB
30	B3493/F		B	Bovine feces	VRLB
31	B3494/F		B	Bovine feces	VRLB
32	B3495/F		B	Bovine feces	VRLB
33	B3592/F		B	Bovine feces	VRLB
34	B/2		B	Bovine feces	VRLB
35	B/53		B	Bovine feces	VRLB
36	LC78/35/4		B	Milk; bovine mastitis	CVLW
37	LC78/49		B	Kidney; aborted bovine fetus	CVLW
38	S613(a)/Eye		B	Aqueous humor; aborted porcine fetus	VRLB
39	S613(b)/Eye		B	Aqueous humor; aborted porcine fetus	VRLB
40	S836/Eye		B	Aqueous humor; aborted porcine fetus	VRLB
41	B1904/K		B	Kidney; aborted porcine fetus	VRLB
42	S368/K		B	Kidney; aborted porcine fetus	VRLB
43	B2075/Sc		B	Stomach contents; aborted porcine fetus	VRLB
44	B1963/Sc		B	Stomach contents; aborted porcine fetus	VRLB
45	B2601/P		B	Placenta; porcine abortion	VRLB
46	B2385/Sc		B	Stomach contents; aborted porcine fetus	VRLB
47	S1010/Eye		B	Aqueous humor; aborted porcine fetus	VRLB
48	S314/T		B	Thoracic fluid; aborted porcine fetus	VRLB
49	S568/Eye		B	Aqueous humor; aborted porcine fetus	VRLB
50	B789/L		B	Liver; aborted porcine fetus	VRLB
51	B1019		B	Aborted porcine fetus	VRLB
52	B1049/P		B	Placenta; porcine abortion	VRLB
53	S314/P		B	Placenta; porcine abortion	VRLB
54	B2582/Sc		B	Stomach contents; aborted porcine fetus	VRLB
55	B99/Sc		B	Stomach contents; aborted porcine fetus	VRLB
56	B288/N		B	Umbilical swab; aborted porcine fetus	VRLB
57	B237/Sc		B	Stomach contents; aborted porcine fetus	VRLB
58	B453/P		B	Placenta; porcine abortion	VRLB
59	B396/P		B	Placenta; porcine abortion	VRLB
60	B1018		B	Aborted porcine fetus	VRLB
61	B2331/P		B	Placenta; porcine abortion	VRLB
62	B2268/Sc		B	Stomach contents; aborted porcine fetus	VRLB
63	B3077/T		A and B	Thoracic fluid; aborted porcine fetus	VRLB
64	B243/P		A and B	Placenta; porcine abortion	VRLB
65	B244(b)/B		A and B	Brain; aborted porcine fetus	VRLB
66	B464/Eye		A and B	Aqueous humor; aborted porcine fetus	VRLB
67	B465/K		A and B	Kidney; aborted porcine fetus	VRLB
68	B1508/B		A and B	Brain; aborted porcine fetus	VRLB

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TABLE 1—Continued

Laboratory no.	Strain	Taxon	Isolation method(s) ^a	Origin	Source ^b
69	B2346/Sc		A and B	Stomach contents; aborted porcine fetus	VRLB
70	B986/T		B	Thoracic fluid; aborted porcine fetus	VRLB
71	B1066/P		B	Placenta; porcine abortion	VRLB
72	B1171/P		B	Placenta; porcine abortion	VRLB
73	B463/T		B	Thoracic fluid; aborted porcine fetus	VRLB
74	C/79/Q		B	Aborted porcine fetus	UM
75	LC78/35/1		B	Stillborn piglet	CVLW
76	LC78/35/2		B	Stillborn piglet	CVLW
77	LC78/35/3		B	Stillborn piglet	CVLW
78	B3286/F		B	Porcine feces	VRLB
79	B3497/F		B	Porcine feces	VRLB
80	B3540/F		B	Porcine feces	VRLB
81	B750/P		B	Placenta; ovine abortion	VRLB
82	B858/P		B	Placenta; ovine abortion	VRLB
83	B938		B	Placenta; ovine abortion	VRLB
84	B1056		B	Aborted ovine fetus	VRLB
85	B391/K		B	Kidney; aborted ovine fetus	VRLB
86	B3525/FB		B	Blood; aborted ovine fetus	VRLB
87	B3426/T		B	Thoracic fluid; aborted ovine fetus	VRLB
88	B3387/P		B	Placenta; ovine abortion	VRLB
89	B837/S		B	Spleen; aborted equine fetus	VRLB
90	LC78/35/5		B	Kidney; dog	VRLB
91	8193	" <i>V. percolans</i> "			NCIB
92	17802 ^T	<i>V. parahaemolyticus</i>			ATCC
93	1380	<i>C. fetus</i> subsp. <i>jejuni</i> or <i>V. coli</i>		Porcine fetus	TIGU
94	10354	<i>C. fetus</i> biotype A		Bovine vaginal mucus	NCTC
95	6945	<i>C. fetus</i> subsp. <i>venerealis</i>		Bovine prepuce	CIP
96	5419	<i>C. fetus</i> subsp. <i>venerealis</i>		Bovine vagina	CIP
97	18011	<i>C. fetus</i> group A, biotype 1		Bovine cervical mucus	MSU
98	5443	<i>C. fetus</i> subsp. <i>venerealis</i> biotype <i>intermedius</i>		Human blood	CIP
99	5399	<i>C. fetus</i> subsp. <i>venerealis</i> biotype <i>intermedius</i>		Human blood	CIP
100	13831	<i>C. fetus</i> subsp. <i>venerealis</i> biotype <i>intermedius</i>		Bovine fetus	MSU
101	12351	<i>Vibrio fetus</i> subsp. <i>intestinalis</i> group A, biotype 2		Ovine fetus	MSU
102	688	<i>C. fetus</i> subsp. <i>fetus</i>		Bovine prepuce	CIP
103	A168	<i>C. fetus</i> subsp. <i>fetus</i>		Human blood	CIP
104	5396 ^T	<i>C. fetus</i> subsp. <i>fetus</i>		Ovine fetus	CIP
105	6944	<i>C. fetus</i> subsp. <i>fetus</i>		Bovine prepuce	CIP
106	13136	<i>Vibrio fetus</i> subsp. <i>intestinalis</i> group C		Ovine fetus	MSU
107	10348	<i>C. fetus</i> biotype B		Bovine vaginal mucus	NCTC
108	10355	<i>C. sputorum</i> subsp. <i>bubulus</i>		Bovine semen	NCTC
109	53103 ^T	<i>C. sputorum</i> subsp. <i>bubulus</i>		Bovine sperm	CIP
110	7080 ^T	<i>C. coli</i>		Porcine feces	CIP
111	7054	<i>C. coli</i>		Porcine feces	CIP
112	99/888	<i>C. coli</i> or <i>C. jejuni</i>		Human feces	CHE
113	99/965	<i>C. coli</i> or <i>C. jejuni</i>		Human feces	CHE
114	702 ^T	<i>C. jejuni</i>		Bovine feces	CIP
115	BP/0065	<i>C. coli</i> or <i>C. jejuni</i>		Human feces	BPHB
116	BP/1663	<i>C. coli</i> or <i>C. jejuni</i>		Human feces	BPHB
117	A29/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
118	A70/P	<i>C. fetus</i>	A	Placenta; bovine abortion	VRLB
119	A178/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
120	N1022/F	<i>C. fetus</i>	A	Bovine feces	VRLB
121	N1023/F	<i>C. fetus</i>	A	Bovine feces	VRLB
122	N1014/F	<i>C. fetus</i>	A	Bovine feces	VRLB
123	A36/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
124	A158/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
125	A128/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
126	A192/P	<i>C. fetus</i>	A	Placenta; bovine abortion	VRLB

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TABLE 1—Continued

Laboratory no.	Strain	Taxon	Isolation method(s) ^a	Origin	Source ^b
127	A87/V	<i>C. fetus</i>	A	Vaginal mucus; bovine abortion	VRLB
128	N1022/V	<i>C. fetus</i>	A	Vaginal mucus; bovine	VRLB
129	N1020/F	<i>C. fetus</i>	A	Feces; bovine	VRLB
130	A163/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
131	A70/F	<i>C. fetus</i>	A	Feces; aborting cow	VRLB
132	NCIB 9072	<i>A. polymorphum</i>		Pond water	NCIB
133	NCIB 9070	<i>A. itersonii</i>		Freshwater	NCIB
134	A102/Pt		B	Peritoneal fluid; aborted bovine fetus	VRLB
135	A171/Eye		B	Aqueous humor; aborted bovine fetus	VRLB
136	A175/K		B	Kidney; aborted bovine fetus	VRLB
137	S613(a)/E		B	Aqueous humor; aborted bovine fetus	VRLB
138	S613(b)/E		B	Aqueous humor; aborted bovine fetus	VRLB
139	B750/P		B	Placenta; ovine abortion	VRLB
140	10354	<i>C. fetus</i> biotype A		Bovine vaginal mucus	NCTC
141	5396 ^T	<i>C. fetus</i> subsp. <i>fetus</i>		Bovine prepuce	CIP
142	53103 ^T	<i>C. sputorum</i> supsb. <i>bubulus</i>		Bovine sperm	CIP
143	7080	<i>C. coli</i>		Porcine feces	CIP
144	A29/F	<i>C. fetus</i>	A	Bovine feces	VRLB
145	A70/P	<i>C. fetus</i>	A	Placenta; bovine abortion	VRLB
146	702 ^T	<i>C. jejuni</i>		Bovine feces	CIP
147	B858/P		B	Placenta; ovine abortion	VRLB
148	S836/E		B	Aqueous humor; aborted bovine fetus	VRLB
149	A70/F	<i>C. fetus</i>	A	Bovine feces	VRLB
150	A128/Pc		B	Pericardial fluid; aborted bovine fetus	VRLB

^a See reference 46.^b ATCC, American Type Culture Collection, Rockville, Md.; BPHB, Belvoir Park Hospital, Belfast, Northern Ireland; CHE, City Hospital, Edinburgh, Scotland; CIP, Collection d'Institut Pasteur, Paris, France; CVLW, Central Veterinary Laboratories, Weybridge, England; MSU, Montana State University, Bozeman; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England; TIGU, Tierärztliches Institut, Göttingen University, Göttingen, Federal Republic of Germany; VRLB, Veterinary Research Laboratories, Belfast, Northern Ireland; UM, University of Montreal, Montreal, Canada.

no. 2 containing 7% sterile blood (human, bovine, equine, ovine, caprine, and porcine).

Growth on different media. Strains were tested for growth on MacConkey agar (Oxoid), deoxycholate-citrate agar (Oxoid), brucella agar (Oxoid), cysteine heart agar (Difco), brain heart infusion agar (Oxoid), blood agar base no. 2 (Oxoid), and EMJH medium (Difco) containing 1.2% agar. Plates were examined for up to 4 days.

Growth at different temperatures. Growth on the base medium and on 7% horse blood agar at 5, 10, 15, 20, 22, 25, 30, 33, 35, 37, 40, 42, and 43 ± 0.5°C was recorded for up to 7 days.

pH. Growth on the base medium at initial pH values which ranged from 5.0 to 11.0 in 0.5 pH unit intervals was recorded. Inoculated plates were incubated for up to 4 days.

Growth in different atmospheres. All strains were examined for up to 7 days for growth on the base medium and on horse blood agar in air, in air containing 10% CO₂, under reduced oxygen tension (5% O₂, 7% CO₂, 88% N₂), and under anaerobic conditions (12% H₂, 8% CO₂, 80% N₂, in the presence of a palladium catalyst).

Catalase test. Catalase activity was determined (15) by using 48-h cultures grown in semisolid maintenance medium.

Oxidase test. Oxidase activity was determined (31) by using 48-h cultures grown on base medium.

Indole production. Cultures grown in Trypticase soy semisolid medium were examined for indole production by using method 2 of Cowan (7).

Methyl red and Voges-Proskauer tests. The methyl red test was performed by the method of Cowan (7), and the Voges-Proskauer test was performed by method 1 of Cowan (7).

Nitrate and nitrite reduction. Cultures incubated for 7 days

in Trypticase soy semisolid medium containing 0.1% (wt/vol) KNO₃ were examined for nitrate and nitrite reduction by using method 1 of Cowan (7). Cultures grown for 7 days in Trypticase soy semisolid medium containing 0.1% (wt/vol) NaNO₂ were also examined for nitrite reduction (7).

Decarboxylase activity. Cultures grown in Moller medium (42) and Falkow medium (10) modified to contain 0.075% purified agar and supplemented with either 1% (wt/vol) lysine, 1% (wt/vol) ornithine, or 1% (wt/vol) arginine were examined for decarboxylase activity after 7 days by using methods 1 and 2 of Cowan (7).

β-Galactosidase production. The method of Cowan (7) was used to examine β-galactosidase activity. Agar (0.075%, wt/vol) was added to *o*-nitrophenyl-β-D-galactopyranoside broth.

Urease production. Method 1 of Cowan (7) was used to examine strains for urease activity. Slants of Christensen medium (6) were inoculated with a heavy inoculum, incubated under reduced oxygen tension, and examined after 4 days.

Deoxyribonuclease production. Deoxyribonuclease test agar (Oxoid) was examined after 4 days for enzyme activity, which appeared as a clear zone around the area of bacterial growth after the medium was flooded with 1 N HCl.

Phosphatase production. Medium containing blood agar base no. 2 as the base medium and method 1 of Cowan (7) were used to determine phosphatase activity after 4 days.

Hydrogen sulfide production. Strains were examined for H₂S production in triple sugar iron medium by using method 1 of Cowan (7) and in standard and sensitive media by using the method of Véron and Chatelain (62).

Tributylin hydrolysis. Inoculated plates of tributyrin agar

(Oxoid) were examined for zones of clearing for up to 7 days. The growth was then removed, and the underlying agar was examined for clearing.

Casein hydrolysis. The medium of Cowan (7), modified by using blood agar base no. 2, was used to determine casein hydrolysis; hydrolysis was determined as described above for tributyrin hydrolysis.

Starch hydrolysis. The medium of Cowan (7), modified by using blood agar base no. 2, was used to determine starch hydrolysis. Plates were incubated for 4 days, and hydrolysis was determined by method 1 of Cowan (7).

Esculin hydrolysis. The method and medium of Cowan (7) were used to determine esculin hydrolysis; hydrolysis was recorded after 4 days.

Hippurate hydrolysis. The medium and method 2 of Cowan (7) were used to determine hippurate hydrolysis for up to 7 days.

Gelatin hydrolysis. Blood agar base no. 2 and method 3 of Cowan (7) were used to detect gelatin hydrolysis after 4 days.

Serum liquefaction. The medium and method of Cowan (7) were used to determine serum liquefaction; the results were recorded after 7 days.

Decomposition of tyrosine. The method and medium of Cowan (7) were used to test for decomposition of tyrosine; the plates were examined after 14 days of incubation.

Citrate utilization. Koser and Simmons citrate media containing 0.075% agar and methods 1 and 2 of Cowan (7) were used to determine citrate utilization. The media were incubated in air for 7 days.

Malonate utilization. Method 1 of Cowan (7), modified so that the medium contained 0.075% agar, was used to determine malonate utilization. Cultures were incubated in air for 7 days.

Gluconate oxidation. The medium of Cowan (7), modified so that it contained 0.075% agar, was used to determine gluconate oxidation. Cultures were incubated in air for 7 days; Labstix (Ames Co. and Miles Laboratories) and method 2 of Cowan (7) were used.

Oxidative or fermentative utilization of glucose. The method and medium of Hugh and Leifson (23) were used to determine oxidative or fermentative utilization of glucose.

Phenylalanine deamination. Malonate-phenylalanine medium containing 0.075% agar and method 1 of Cowan (7) were used to study phenylalanine deamination.

Reaction in milk. Litmus milk (7) and Crossley milk (Oxoid) were incubated under reduced oxygen tension and read after 7 days.

Growth in potassium cyanide agar. KCN broth (50) containing 0.075% agar and the method of Cowan (7) were used to determine growth in potassium cyanide agar. Cultures were incubated in air for 7 days.

Tolerance tests. Plates of basal medium containing the following compounds were examined after 7 days: acriflavine (0.002, 0.001, and 0.0005%, wt/vol); azaguanine (0.01, 0.025, 0.05, 0.2, 0.12, 0.15, 0.2, and 0.25%, wt/vol); basic fuchsin (0.002, 0.001, and 0.0005%, wt/vol); bile salts (0.1, 0.5, 1.0, and 2.0%, wt/vol; Oxoid); bile salt no. 3 (0.1, 0.5, 1.0, and 2.0%, wt/vol; Oxoid); bile (1%, wt/vol; Oxoid); brilliant green (0.004, 0.002, 0.001, 0.0005, and 0.00025%, wt/vol; Difco); cetrinide (0.1, 0.01, and 0.001%, wt/vol); copper sulfate (0.01, 0.001, and 0.0001%, wt/vol); crystal violet (0.01, 0.001, and 0.0001%, wt/vol); glycine (0.1, 0.5, 1.0, and 1.5%, wt/vol); glucose (2, 4, 6, 8, and 10%, wt/vol); lysozyme (0.05, 0.005, and 0.0025%, wt/vol); methyl violet (0.01, 0.002, and 0.001%, wt/vol); malachite green (0.002,

0.001, and 0.005%, wt/vol); potassium tellurite (0.01 and 0.001%), pyronine (0.001, 0.00005, and 0.00001%, wt/vol); safranin (0.05, 0.01, and 0.001%, wt/vol); sodium azide (0.01 and 0.001%, wt/vol); sodium chloride (1, 1.5, 2.0, 2.5, 3.0, 3.5, and 5%, wt/vol); sodium lauryl sulfate (0.01, 0.1, and 1.0%, wt/vol); sodium selenite (0.01, 0.05, 0.1, and 0.5%, wt/vol); thallous acetate (0.01 and 0.001%, wt/vol); thionine (0.002, 0.001, and 0.0005%, wt/vol); 2,3,5-triphenyl tetrazolium chloride (0.01, 0.025, 0.05, and 0.1%, wt/vol); and vibriostatic compound 0/129 (2,4-diamino-6,7-diisopropylpteridine; 1%, wt/vol).

The following antibiotics were used (the ranges of concentration were prepared in doubling dilutions [e.g., 1/2, 1/4, etc.]): ampicillin (0.5 to 32 µg/ml); carbenicillin (2.0 to 256 µg/ml); chloramphenicol (1 to 16 µg/ml); dimetridazole (0.125 to 128 µg/ml); kanamycin (1, 2, and 4 µg/ml); metronidazole (0.125 to 128 µg/ml); nalidixic acid (5 to 40 µg/ml); penicillin G (0.75 and 1.5 IU/ml); polymyxin B (1 to 128 µg/ml), and streptomycin (12.5, 25, and 50 µg/ml).

Tolerance tests were performed as described by Véron and Chatelain (62) for the following compounds: glucose (8%, wt/vol), glycine (1%, wt/vol), NaCl (3.5%, wt/vol), ox bile (1%, wt/vol), sodium selenite (0.1%, wt/vol), brilliant green (0.001 and 0.003%, wt/vol), 2,3,5-triphenyl tetrazolium chloride (1 mg/ml), and nalidixic acid (40 µg/ml).

Acid from carbohydrates. Broth sugar medium (7) containing 0.075% agar and 0.002% (wt/vol) bromocresol purple as an indicator was used to study acid production from carbohydrates. Filter-sterilized carbohydrates were added aseptically to final concentrations of 1.0% (wt/vol). Starch, esculin, and glycogen were added before autoclaving. Duplicate samples were inoculated; one was layered with liquid paraffin, and both were incubated in air for up to 14 days. Acid production was scored as positive; no growth or no reaction was scored as negative. The following carbohydrates were used: esculin, L-arabinose, cellobiose, dextrin, erythritol, D-fructose, D-galactose, galactitol, D-glucitol, D-glucose, glycogen, *meso*-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-ribose, ribitol, starch, sucrose, trehalose, and D-xylose.

Nutritional requirements. Carbon utilization tests were performed by using yeast nitrogen base agar (Difco). The carbon sources were sterilized as 5% solutions at 100°C for 1 h on 3 consecutive days or by filtration and were added to the base medium at final concentrations of 0.01 to 0.1% (wt/vol or vol/vol). The media were dispensed into sectioned plates (Sterilin). Dry plates were inoculated by using a multipoint inoculator and were incubated under reduced oxygen tension. Growth was assessed for up to 14 days. Yeast nitrogen base medium containing no carbon source was used as a control. Horse blood agar was replica-plated at the beginning and end of each inoculation to check strain growth and to check for contaminants. The following compounds were used as sole sources of carbon: D-arabinose, L-arabinose, cellobiose dextrin, erythritol, D-fructose, D-galactose, D-glucose, D-glucitol, glycogen, glycerol, lactose, maltose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, ribitol, salicin, starch, sucrose, trehalose, D-xylose, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine hydrochloride, L-glutamic acid, L-glutamine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, acetic acid, propionic acid, butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, palmitic acid, stearic acid, palmitoleic acid, lignoceric acid, oleic acid, methanol,

ethanol, 1-propanol, propan-2-ol, 1-butanol, D-lactic acid, oxalic acid, DL-malic acid, tartaric acid, DL- β -hydroxybutyric acid, adenine, cytosine, guanine, uracil, thymine, xanthine, hypoxanthine, 5-methylcytosine hydrochloride, sodium citrate, sodium pyruvate, sodium succinate, sodium hippurate, creatine, acetamide, Tween 40, Tween 60, Tween 80, Tween 85, progesterone, oestrogene, stilbestrol, and albumin.

Coding of data. Tests were carried out in duplicate. Most characters were coded as 1 (present), 0 (absent), or 2 (inconsistent), but some were multistate characters (59). When tests recorded the tolerance levels for a substance or the upper and lower limits of growth, the uncoded results were used directly in the analysis. Missing or inapplicable data were coded as -1 (no comparison).

The results were scrutinized before coding, and those tests which gave identical results for all strains examined (e.g., gram-negative or identical results for the *Campylobacter* strains and the undesignated strains) were omitted from the analysis, as the object was to obtain useful differential tests for these organisms.

Analyses. Cluster and ordination techniques were used. To obtain clusters, single-linkage, complete-linkage, and unweighted-average-linkage methods were used, along with the simple matching (S_{SM}) coefficient, in which both positive and negative matches between character measurements are included (58), and the coefficient of Jaccard (S_J coefficient), in which only positive matches are considered. Cophenetic correlation coefficients were calculated to assess the goodness of fit of the resulting dendrograms with the calculated similarities. Additional evidence of groupings among the organisms was obtained from an ordination of the similarity coefficients by using the principal coordinate technique (19).

These analyses were carried out by using the DUST computer package (63) on the Queen's University of Belfast computer.

RESULTS

Most strains gave negative results in the majority of the tests used. The results of the classical biochemical tests, such as sugar reaction and nutritional requirement tests, only accentuated the difference between *Vibrio parahaemolyticus* and the other strains and were therefore omitted from the computer analyses. Thus, tests of a physiological and inhibitory nature predominated.

Single-linkage analysis. The simplest clustering was obtained by using single-linkage analysis. Using the S_{SM} coefficient resulted in four phenon at a similarity level of 80%; these phenon contained all but 4 of the 150 strains examined. One phenon (44 strains) contained all but three of the *Campylobacter* strains. Of these three strains, strains 126 and 127 were ungrouped, and strain 93 was incorporated into the largest phenon (98 strains), which contained 98% of the undesignated strains. "*V. percolans*" and strain 36 formed a minor phenon linkage at a similarity level of 75%. Both *Aquaspirillum* strains were members of a separate phenon, which was linked to the phenon described above at a similarity level of 70%. *V. parahaemolyticus* was related to all strains at a similarity level of 60%.

Using positive matches only (S_J coefficient) gave five phenon (similarity level, 80%), which contained 145 (96.7%) of the strains examined. The undesignated strains were all members of a single phenon, whereas a second phenon contained 41 of the 44 catalase-positive *Campylobacter* strains. At a similarity level of 75% all of the catalase-posi-

tive *Campylobacter* strains and the undesignated strains were members of a single large cluster that was separated from the other phenon. *Campylobacter sputorum* (three strains) were members of a single phenon, as were the two *Aquaspirillum* strains. "*V. percolans*" and strain 36 also formed a single phenon.

The cophenetic correlation coefficients of these analyses were 0.82 and 0.85 with the S_{SM} and S_J coefficients, respectively.

Complete-linkage and unweighted-average-linkage analyses. More compact clusters were obtained by using complete-linkage and unweighted-average-linkage analyses. Very similar arrangements of strains resulted from the use of both of these methods, and therefore only the results of the latter method are presented and discussed below. The simplified dendrogram in Fig. 1 represents the results obtained when average-linkage analysis and the S_{SM} coefficient were used. Where possible, the phenon were named after the authentic strains found within them. Encouragingly, the phenon composition was not affected by the exclusion of negative matches (S_J coefficient). In general, the phenon formed at a similarity level of 80% by using the S_{SM} coefficient were equivalent to those formed at a similarity level of 75% by using the S_J coefficient. In these analyses (S_{SM} and S_J coefficients), the cophenetic correlation coefficients were 0.88 and 0.87, respectively.

The test error, which was calculated from the results of tests performed on duplicate strains, was less than 4% (59).

Three distinct areas (similarity level, 70%) are recognizable in Fig. 1. Areas A and B contained all of the undesignated strains linked at a similarity level of 80%. These areas were linked to area C (the *Campylobacter* strains) and to phenon D (*Aquaspirillum*) at a similarity level of 60%.

The phenon in areas A and B contained 84 undesignated strains. These phenon did not reflect the origin or the clinical condition of the hosts from which the strains were isolated. The strain composition of each phenon was affected only to a minimal degree by using the S_J coefficient. The phenon in area A contained only undesignated strains that were isolated in Northern Ireland.

Phenon B1 contained "*V. percolans*" and an undesignated strain from outside Northern Ireland.

Phenon B2 consisted of seven Northern Ireland strains and four strains from other laboratories. Closely associated with these strains was another strain isolated outside Northern Ireland; this strain remained ungrouped at the 80% similarity level.

Phenon C1 (similarity level, 80%) contained 32 *Campylobacter fetus* strains, including strains from culture collections and laboratory isolates. However, subphenon within this phenon did not correspond to the existing *C. fetus* subspecies. The compositions of the phenon and subphenon were not altered when the S_J coefficient was used, but the strain positions within the phenon were altered slightly.

Phenon C2 consisted of nine strains and had two distinct subphenon. These subphenon contained strains of *Campylobacter coli* (subphenon C2i) and *Campylobacter jejuni* (subphenon C2ii) and appeared in analyses when S_{SM} and S_J coefficients were used. In the analysis shown, one strain (strain 93) received as *C. coli* was found among the undesignated strains. When only positive matches were used (S_J coefficient), this strain clustered close to phenon C2 at a similarity level of 80%.

Phenon C3 contained the three *C. sputorum* reference cultures.

Phenon D contained the two *Aquaspirillum* strains.

FIG. 1. Simplified dendrogram derived by unweighted-average-linkage analysis, using the S_{SM} coefficient.

TABLE 2. Centrotype strains determined by using average-linkage analysis

Centrotype strain	Phenon ^a	Taxon
A169/B ^T	A2	Unassigned
S568/E	A3	Unassigned
B3495/F	A4	Unassigned
LC78351	B2	Unassigned
6944	C1	<i>C. fetus</i>
702 ^T	C2	<i>C. jejuni</i>
53103 ^T	C3	<i>C. sputorum</i> subsp. <i>bubulus</i>

^a See Fig. 1.

The relevant centrotype strains (i.e., the strains closest to the centroids) were computed and are shown in Table 2.

Principal coordinate analysis. Principal coordinate analysis was initially carried out by using the test results for only the *Campylobacter* strains. Figure 2 shows the computation from these data on the first two principal component axes and displays 33.0% of the total spatial variation. The three distinct groups, which contained all but two of the strains, were named after the authentic strains found within them. This distinct two-dimensional representation of *Campylobacter* species was achieved regardless of whether negative matches were included or excluded. The principal coordinate analysis for all of the strains tested is shown graphically in Fig. 3 by using two of the first three principal coordinate axes. This plot included 27.5% of the total variation displayed in the analysis and retained the three distinct species groups displayed previously (Fig. 2). This representation was affected very little by the inclusion of negatively matched results.

Characterization of strains. Each of the analyses separated

V. parahaemolyticus from the remainder of the strains. These strains shared many phenotypic features. Strains in areas A, B, and C (Fig. 1) failed to catabolize any of the carbohydrates tested. This was a characteristic of *Aquaspirillum polymorphum*, although *Aquaspirillum itersonii* produced acid oxidatively from one carbohydrate. Similarly, most carbon sources at the concentrations used appeared to be of little value for discriminating among the majority of the strains tested. The aquaspirilla differed from the other strains by their oxidative activity in Hugh-Leifson medium, their hydrolysis of tributyrin, esculin, and starch (one strain), and their ability to grow in the presence of potassium cyanide.

Comparison of the strains from areas A, B, and C. All of the strains grew on base medium containing rabbit, cow, horse, sheep, pig, or human blood. Incomplete hemolysis (alpha-hemolysis) was observed with some strains on some of these media, but lack of consistent hemolysis invalidated the use of this character as a differentiating character. The colony appearance on blood agar was variable and was obviously influenced by the surface moisture on the agar medium. In general, the colony appearance of area A and B strains after 24 to 48 h under reduced oxygen tension was similar to that of area C strains. After continued incubation, the colonies of area C strains usually became larger, coarser, and more opaque.

For the purpose of objective differentiation among strains from areas A, B, and C, no distinctive pigmentation was observed on blood agar or King media A and B. Colonies on the former medium ranged from off-white to pale yellow.

All strains were gram-negative, motile rods that exhibited varying degrees of cell curvature and after prolonged culture could become elongated, helical forms of different lengths.

For area A, B, and C strains the following tests were

FIG. 2. Ordination plot from the principal coordinate analysis of named *Campylobacter* strains only.

FIG. 3. Ordination plot from the principal coordinate analysis of all of the strains examined.

positive: oxidase activity (100% of the strains); nitrate reduction (98.6%); nitrite reduction (95.2%); growth in air containing 10% CO₂ (90.0%) or under anaerobic conditions (85.9%); growth in the presence of 1% NaCl, 4.0% glucose, 0.1% glycine, or vibriostatic agent 0/129 (100%).

The following tests were negative for area A, B, and C strains: methyl red (100% of the strains); Voges-Proskauer (100%); production of indole, ornithine, lysine, and arginine decarboxylases, urease, ribonuclease, and deoxyribonuclease (97.9%); utilization of citrate, malonate, and gluconate (100%); liquefaction of serum and gelatin (100%); hydrolysis of casein (100%), starch (100%), hippurate (100%), tributyrin (99%), and esculin (100%); growth in the presence of KCN (98.6%) and on deoxycholate agar (100%); and catabolism of a range of carbohydrates (100%). The strains decolorized litmus milk (92.5% of the strains), were inert or produced no reaction in Crossley milk, and produced an alkaline reaction in Hugh-Leifson medium.

Uniformly and consistently, all of the area A and B strains grew in atmospheric oxygen on solid media. In contrast, the area C strains consistently failed to grow in this atmosphere.

Relatively few characters could be used successfully to distinguish the area A, B, and C strains from one another. These characters are shown in Table 3.

Comparison of phena. Although most of the phenotypic characters examined were common to the majority of strains, many were not uniformly present in all of the strains in any one phenon. Few distinctive characters were recognized as separating the phena produced at any particular level. The phena differed more in the degree to which strains could tolerate a particular substance than in distinct differences in the substances tolerated. The characters that were

TABLE 3. Characters which have the most value for distinguishing among area A, B, and C strains

Area	No. of strains	Growth in air	Growth at 15°C ^a	Growth in the presence of NaCl (2%) ^a	Growth in the presence of carbenicillin (64 µg/ml) ^a
A	88	+ (100) ^b	+ (100)	+ (86.2)	+ (93.1)
B	15	+ (100)	+ (100)	+ (100)	+ (87.6)
C	44	— (0)	— (0)	— (0)	— (2.3)

^a Growth under reduced oxygen tension (85% N₂, 10% CO₂, 5% O₂).

^b +, More than 85% of the strains were positive; —, less than 15% of the strains were positive. The values in parentheses are the percentages of positive strains.

TABLE 4. Characters of discriminating value for distinguishing among phena at a similarity level of 80%^a

Phenon	Designation	Growth in air	Growth in 5% O ₂ at:			Production of:		Tolerance ^b to:											
			15°C	25°C	42°C	Cat	H ₂ S	H ₂ S*	NaCl (2.0%)	Glu (8.0%)	Sel (1.0%)	TTC (0.1%)	BG (.00025%)	NA (40 ug/ml)	Carb (32 ug/ml)	BF (.002%)	PT (.001%)	Az (.25%)	
A1 (2 strains)		+(100) ⁵	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	+(100)	+(100)	-(0)	+(100)	-(0)	+(100)	-(0)	+(100)	-(0)	+(100)	
A2 (37 strains)		+(100)	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	d(72.4)	+(97.1)	-(0)	+(88.8)	-(0)	+(88.8)	-(0)	+(97.1)	d(67.5)	-(2.9)	+(100)
A3 (40 strains)		+(100)	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	+(97.6)	+(97.6)	-(2.4)	+(100)	-(4.8)	+(100)	-(4.8)	+(100)	d(66.7)	+(100)	
A4 (5 strains)		+(100)	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	+(100)	+(100)	-(0)	+(100)	-(0)	+(100)	-(0)	d(50)	d(80)	+(100)	
B1 (2 strains)		+(100)	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	d(50)	-(0)	+(100)	
B2 (11 strains)		+(100)	+(100)	+(100)	-(0)	+(100)	-(0)	-(0)	+(100)	+(100)	-(0)	+(100)	-(0)	+(100)	-(0)	+(100)	+(90.1)	+(90)	+(100)
C1 (32 strains)	<u>C. fetus</u>	-(0)	-(0)	+(100)	-(0)	+(100)	-(0)	-(0)	-(0)	+(100)	d(78.6)	-(0)	+(100)	-(0)	+(100)	-(0)	+(96.8)	-(0)	d(29)
C2i (6 strains)	<u>C. jejuni</u>	-(0)	-(0)	+(100)	+(100)	+(100)	+(100)	+(100)	-(0)	-(0)	d(28.6)	-(0)	-(0)	-(0)	-(0)	+(85.7)	-(0)	-(0)	+(85.7)
C2ii (3 strains)	<u>C. coli</u>	-(0)	-(0)	+(100)	+(100)	+(100)	+(100)	+(100)	-(0)	-(0)	+(100)	+(100)	-(0)	-(0)	-(0)	+(100)	-(0)	+(100)	+(100)
C3 (3 strains)	<u>C. sputorum</u>	-(0)	-(0)	+(100)	-(0)	-(100)	+(100)	+(100)	-(0)	+(100)	+(100)	-(0)	d(33)	d(33)	d(33)	-(0)	d(33)	d(66)	-(0)
Strain																			
A87/V		+	+	-	-	+	+	-	+	+	+	-	+	+	+	+	-	+	+
A192/P		+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+
B1171/P		+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
B2016/K		+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
LC7849		+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
1380		+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	NT	+	+

^a Cat, Catalase; Glu, glucose; Sel, sodium selenite; TTC, triphenyl tetrazolium chloride; BG, brilliant green; NA, naldixic acid; Carb, Carbenicillin; BF, basic fuchsin; PT, potassium tellurite; Az, azoguanine; H₂S*, hydrogen sulfide in "sensitive medium."

^b Growth in reduced oxygen on base medium containing the inhibitory substance at the concentration shown.

^c +, 85 to 100% of the strains were positive; -, 0 to 15% of the strains were positive.

^d 15 to 85% of the strains were positive; NT, not tested. The values are percentages of positive strains.

useful for assigning strains to the phenon at a similarity level of 80% are shown in Table 4.

DISCUSSION

The assignment of the strains studied to the same general arrangement by using several analytic methods strengthened the taxonomic validity of the groupings produced. The cophenetic correlation coefficients produced for the single-linkage and average-linkage analyses indicated a satisfactory correlation between the original similarity values and those of the respective phenon from each analysis.

From the dendrogram (Fig. 1), it could be suggested that generic status should be attributed to each of the areas shown, as the current genus *Campylobacter* was clearly recognizable as area C. However, consideration must also be given to the distinct lack of biochemical activities common to all of the strains in the three areas and to the generic union that can be inferred (4) from the deoxyribonucleic acid base ratios of representative strains shown in Table 5. Therefore, it is more realistic and practical to suggest that the undesigned strains be considered a new and distinct group, which is more distantly related to any of the existing *Campylobacter* species than these species are to each other. In the simplest of the analyses (single-linkage analysis), the undesigned strains clustered significantly more closely to the named *Campylobacter* strains than to the *Aquaspirillum* strains (S_{SM} coefficient), and when only positive matches were used (S_J coefficient), the catalase-positive *Campylobacter* and undesigned strains formed a single phenon (similarity level, 75%). The new group includes some recently isolated strains; some of these strains had been held in storage, and others had had prolonged laboratory passage. The strains also originated from a variety of sources, and this new group therefore complies with the concept of a species (18).

A. polymorphum and *A. itersonii* were included in this study because they are among the smallest spirilla (comparable in size to *Campylobacter* strains) and not because they are representative spirilla, which include bacteria that are morphologically, physiologically, and biochemically quite varied (24, 32). The distinction of the *Aquaspirillum* strains from the named and undesigned *Campylobacter* strains is clearly illustrated in the cluster diagram (Fig. 3).

The *Campylobacter*-like organism "*V. percolans*" (47) was recovered in area B (Fig. 1), yet it possessed the following conspicuous characters that are absent in *Campylobacter* strains: hydrolysis of tributyrin and casein and tolerance to KCN. These characteristics were obviously outweighed by the large number of phenotypic characters shared with *Campylobacter*. The difference between "*V. percolans*" and *Campylobacter* strains, which has been illustrated previously (43), was again more realistically demonstrated in the cluster diagram (Fig. 3).

The phenon (similarity level of 80% with the S_{SM} coefficient; similarity level of 75% with the S_J coefficient) identified in this study have valid taxonomic significance, as similarity levels of 75 and 80% have been previously suggested as good criteria for phenetic species (36, 59). Both phenetic and cluster presentations (Fig. 1 through 3) contained the reference and type strains appropriately grouped and largely supported the species differentiation within the genus *Campylobacter* described by Véron and Chatelain (62). However, the strains included as authentic members of *C. fetus* subspecies were distinguished as such only in the principal coordinate analysis and were not separated into discrete subphenon in the single-linked analysis. At the level

TABLE 5. Deoxyribonucleic acid base compositions of strains

Taxon	Phenon ^a	Strain	G+C content (mol%)	
			Range ^b	Mean ^c
<i>C. fetus</i>	C1		33.0–35.5	
<i>C. coli</i> or <i>C. jejuni</i>	C2		29.0–34.0	
<i>C. sputorum</i>	C3		29.0–31.0	
" <i>V. percolans</i> "	B1		58.9–65.0	
<i>Aquaspirillum</i>	D		50.0–65.0	
<i>V. parahaemolyticus</i>			42.0–45.0	
Unassigned	A2	A175/P		29.6 (0.5) ^d
		B1019		30.1 (1.2)
		A175/K		30.8 (0.8)
		A169/B		31.5 (0.2)
		A171/E		32.0 (1.3)
Unassigned	A3	B1904/E		33.9 (0.3)
		B1049/P		29.5 (0.2)
		A102/Pt		30.3 (0.7)
		S568/E		31.8 (1.1)
Unassigned	B2	S613A/E		29.9 (0.2)
		B2211/Sc		29.6 (0.6)

^a See Fig. 1.

^b The base ratios of the organisms are generally accepted to be within the ranges shown (5, 32, 38, 46, 51, 55, 62).

^c See reference 43.

^d The values in parentheses are standard errors.

at which *C. fetus* and *C. sputorum* are recognizable (Fig. 1), *C. jejuni* and *C. coli* strains appeared together in one phenon, yet they were separable components of this phenon. Studies of the deoxyribonucleic acids of *Campylobacter* strains have shown that *C. jejuni* and *C. coli* are more similar to each other than to *C. fetus* when guanine-plus-cytosine (G+C) contents and genome sizes are used but are distinct and separate species when deoxyribonucleic acid hybridization techniques are used (35, 46).

Although for practical purposes the undesigned *Campylobacter* strains (areas A and B) were considered members of one group (Fig. 3), they were heterogeneous. Area A contained only strains that were isolated in Northern Ireland, whereas area B contained cultures from geographical areas outside Northern Ireland. At the similarity level (70%) which revealed the previously described *Campylobacter* species, subgroups of strains were evident in areas A and B. None of these subgroups could be correlated with a particular host or with the origin or clinical state of the host, and none included a predominance of recently isolated strains over stored strains or laboratory-passaged cultures. The true extent of the heterogeneity of the undesigned aerotolerant strains and their precise relationship to the other *Campylobacter* species can only be determined by deoxyribonucleic acid hybridization studies. The mean G+C contents of the 11 strains examined was 31 ± 1 mol%, a value more similar to the G+C content of *C. sputorum* than to that of *C. fetus*, *C. jejuni*, or *C. coli*.

The centrotypes strains produced for phenon C1, C2, and C3 (Table 2) are reference cultures held in collections; strain 702 is a type strain. Although centrotypes strain 6944 is not the type strain of *C. fetus*, its closest neighbor in the analysis was strain 5396, which is the type strain of *C. fetus*. Strain A169/B is regarded as the type strain of the aerotolerant undesigned strains.

In taxonomic studies it is desirable to use uniform cultural conditions and a single base medium. Unfortunately, consistent and healthy growth of all of the strains examined could not be sustained at one temperature, and it was also

necessary to maintain the strains in two different media before testing. These different cultural procedures appeared to have little effect on the groups produced in the analyses, as three undesigned strains maintained at 37°C and examined by using tests carried out at 37°C clustered with the remaining undesigned strains, which were maintained and tested at 30°C.

Subjective characters, such as pigmentation, were of little value for distinguishing among the phenotypes or clusters. However, morphology was sometimes distinctive. The colonies of *C. coli* and *C. jejuni* strains were usually larger than the colonies of either *C. fetus* or the undesigned strains, and the former organisms swarmed readily on moist agar. The colony sizes of *C. fetus* and the undesigned strains after 24 to 48 h of incubation were similar, but after continued incubation *C. fetus* colonies became larger, coarser, and more opaque and usually could be distinguished from colonies of the undesigned strains. Microscopically, the cells of *C. coli* and *C. jejuni* were characteristically smaller and more tightly curved than the cells of the other strains; the cells of *C. coli* and *C. jejuni* had a tendency to become coccoid, especially in older cultures. The cells of *C. fetus* and the undesigned strains were not so visibly distinct, and the observed similarities in cell sizes make the use of microscopic appearance for identification fraught with danger. Cell morphology may in fact vary with changes in cultural conditions.

Although at least four possible species could be clearly recognized, the distinguishing characters of these taxa were not so obvious. Strains differed more in the degree to which a compound was tolerated than in the compounds tolerated. This study illustrates the following problem often encountered in bacterial taxonomy: separation of bacteria may not be by sharp divisions, but by slight and subtle differences in characters, so that the strains resemble a spectrum. Interestingly, of the characters examined, those of possible discriminatory value (Tables 3 and 4) were quite similar to the characters previously recognized by other workers as valuable for *Campylobacter* species differentiation (1, 2, 52, 53, 55, 62). In this study we found that all of the *Campylobacter* strains tested were unable to hydrolyze hippurate; however, the test method used was different from that recently recommended (21). Use of this latter method confirmed that the undesigned strains are not able to hydrolyze hippurate (unpublished data).

Table 3 illustrates the limited characters by which the aerotolerant strains can be recognized. By definition, *Campylobacter* strains are microaerophilic; they respire yet are usually inhibited by atmospheric oxygen. The optimum oxygen concentration for *Campylobacter* growth has been reported to be 6% (27, 49), but the aerotolerance of strains can be artificially enhanced on supplemented media (3, 17, 30). Therefore, the aerotolerant undesigned strains could represent one end of a spectrum of natural oxygen tolerance among *Campylobacter* strains; *C. sputorum* represents the other end. This oxygen spectrum concept is supported by the degree of inhibition exhibited to nitroimidazole drugs. *C. sputorum* strains were most susceptible, and the undesigned strains were most resistant. The majority of the area A, B, and C strains (Fig. 1) were resistant to metronidazole at concentrations up to 4 µg/ml; however, at a metronidazole concentration of 64 µg/ml all of the strains of the previously described *Campylobacter* species were inhibited, whereas the undesigned strains were less susceptible (area A strains, 57.5% inhibited; area B strains, 78.9% inhibited). It is of interest that a range of susceptibility to nitroimidazole

drugs was observed with the undesigned strains; the fact that strains isolated from pigs exhibited greater resistance may have been due to the greater contact of pigs with nitroimidazoles.

Differences in temperature tolerance have proved to be useful for *Campylobacter* differentiation (12, 52–54, 62). In this study all of the area A, B, and C strains (Fig. 1) grew at 37°C, with 50% of the undesigned area A strains at growing temperatures up to 40°C. However, the lower limits of growth were more useful for differentiation. Area A and B strains consistently grew at much lower temperatures than area C strains; 43.2% of the area A strains and 21% of the area B strains grew at temperatures as low as 5°C. All area A and area B strains grew at 15°C, whereas none of area C strains grew at this temperature. This provided a unique differential character. This psychrophilic nature of the undesigned strains could place them at one end of a temperature spectrum for *Campylobacter*; at the opposite end of this spectrum are the thermophilic strains.

Differences in sodium chloride tolerance have also been used to group *Campylobacter* strains (13, 20, 25, 37). Our results indicate that the majority of the undesigned strains are inhibited by 3.0% NaCl and that previously described catalase-positive *Campylobacter* strains are inhibited by 2% NaCl. Sensitivity of the undesigned strains to levels of salt above 2% was not consistent for all strains in either of area A or area B. Only a minority (17%) of the undesigned strains grew at salt concentrations of 3.5% or above; these strains are perhaps similar to the aerobic vibrios described by other workers (25, 41). Tolerance to 3.5% NaCl has been cited as a characteristic which distinguishes between pathogenic and saprophytic *Campylobacter* strains (25, 41); when this criterion was used, the majority of the undesigned strains examined could be described as potentially pathogenic.

Tolerance to the antibiotics examined was similarly not uniform for all of the strains in each of the areas. *Campylobacter* strains (area C) were markedly more resistant to nalidixic acid and to polymyxin than were area A and B strains, but were generally more susceptible to carbenicillin, ampicillin, the nitroimidazoles, and chloramphenicol. Susceptibility to penicillin, streptomycin, and kanamycin appeared to be similar for all strains. Only carbenicillin susceptibility (64 µg/ml) could be used as a distinguishing character for the undesigned aerotolerant strains.

Inhibition by most of the other substances examined was not held in common by all strains in any of the groups observed. The majority (93.2%) of the strains examined tolerated media with initial pH values of 6.8 to 8.0. However, greater proportions of area A and B strains (35.7 and 36.8%) tolerated pH 9.5 than area C strains (16.7%). No area C strains and few area A and B strains (8.0 and 10.5%, respectively) grew on media with initial pH values above 9.5. None of the area A strains survived on medium with an initial pH of 5.5, whereas area B and C strains did survive (58 and 88%, respectively). None of the strains examined grew on media with an initial pH of 5.0.

It was not totally unexpected to find that the majority of the Northern Ireland strains clustered closely together and separate from the strains isolated in other geographical areas. Most of the isolates examined were from animals, and, as antibiotic susceptibility may be influenced by the antibiotic usage in different localities, the clustering of strains could reflect to some extent the geographical origins.

Unexpectedly, strain 93 (*C. fetus* subsp. *jejuni* or *Vibrio coli*) and strains 126 and 127 (*C. fetus*) were excluded from

the phena and clusters that contained the appropriate reference cultures (Fig. 1 and 3). Under test conditions, strain 93 grew at both 42 and 25°C, failed to produce hydrogen sulfide, and grew aerobically; this strain was probably misidentified originally. Strains 126 and 127, although correctly identified as *C. fetus* (62), had characteristics different from those of the strains in the *C. fetus* cluster. Both of these strains failed to reduce nitrate, a character which is found positive for all of the *C. fetus* strains tested, and both strains grew in atmospheric oxygen. In addition, strain 126 grew at temperatures below 15°C and tolerated 4% NaCl. The recognition of these rogue strains highlights the potential hazard of relying on the small number of discrete phenotypic characters described above and a predominance of inhibitory-type tests. The distinguishing pattern of correlating properties should be large enough to permit variation of a few properties by a strain without excluding the strain from the species (18). This problem has been previously encountered with some *Campylobacter* strains, as identification difficulties have been described by other workers (12, 56, 62).

Table 4 shows those characters that are common to the majority of the strains in each of the phena produced by group average-linkage analysis (Fig. 1). This table shows that the phena differ by relatively few phenotypic characters. However, these characters permitted separation of the named *Campylobacter* strains included in the study into recognized species, even though only a few strains of some of these species were included. No practical significance could be established for the subdivision of the undesigned aerotolerant strains into the phena shown, and separation may only be of purely taxonomic interest, as some phena differed by as little as one or two phenotypic characters.

Topical interest in enteric *Campylobacter* strains has highlighted the taxonomic diversity of the intestinal strains (52, 53), and subsequent characterization studies have identified several new and potentially new species for inclusion within the expanding genus *Campylobacter* (2, 11, 16, 33, 39, 60, 61).

This study showed the phenotypic relationship of novel aerotolerant *Campylobacter* strains to at least some strains of the previously established species. The aerotolerant strains clearly appear to be unrecognized species within the genus, and *Campylobacter cryaerophila* is proposed as a name for this species.

Description of *Campylobacter cryaerophila* sp. nov. *Campylobacter cryaerophila* (cry.ae.ro.philá. Gr. adj. cry cold; Gr. n. *aero* air; L. adj. *phila* fond of; *cryaerophila* fond of cold and air) cells are gram-negative nonsporeforming rods (0.4 µm wide by 1.8 µm long); unusually long cells (>20 µm) have been observed. Cells are usually curved, S shaped, or helical. Darting motility; single polar flagella (amphitricate). Growth occurs at 30°C, and colonies grown under reduced oxygen tension for 48 to 72 h are small (1 mm in diameter), smooth, and convex with entire edges. Colonies may become flattened and irregular and may vary in size. Strains in subculture grow in air, in air containing 10% CO₂, and anaerobically. Some strains grow between 5 and 40°C, but all strains grow at 15°C. The strains are positive for catalase and oxidase activities and reduce nitrate (98.6% of the strains) and nitrite (85.9%). The strains are tolerant to 2% NaCl, 4% glucose, 0.1% glycine, and pteridine vibriostatic compound 0/129. The strains do not grow in medium containing 1.0% glycine and 1 mg of 2,3,5-triphenyl tetrazolium chloride per ml, and sodium selenite is not reduced. No H₂S is produced in triple sugar iron medium or medium containing cystine. The strains produce alkaline reactions in Hugh-

Leifson medium. All strains are negative in methyl red and Voges-Proskauer tests and do not produce indole, ornithine, lysine, and arginine decarboxylases, urease, ribonuclease, or deoxyribonuclease (97.9% of the strains). The strains do not utilize citrate, malonate, or gluconate, do not liquify serum or gelatin, and do not hydrolyze casein, starch, hippurate, tributyrin, or esculin. Growth does not occur in KCN or on deoxycholate agar, and all strains fail to catabolize carbohydrates. The strains are susceptible to nalidixic acid (40 µg/ml) and resistant to carbenicillin (64 µg/ml).

Strains have been isolated from reproductive tracts and aborted fetuses of several species of farm animals, from animal feces, and from milk of mastitis cows.

The mean G+C content is 31 ± 1 mol%.

Strain A169/B (= 02766) is the type strain of *C. cryaerophila* and has been deposited in the National Collection of Type Cultures as strain NCTC 11885. This strain conforms to the description given above for the species with respect to morphology and growth characteristics. No growth occurs below 5°C or above 37°C; the optimum growth temperature is 30°C. Strain A169/B^T (T = type strain) is catalase and oxidase positive and reduces nitrate and nitrite. Methyl red and Voges-Proskauer tests are negative, and indole is not produced; ornithine, lysine, and arginine decarboxylases, urease, ribonuclease, and deoxyribonuclease are not produced. Citrate, malonate, and gluconate are not utilized; serum and gelatin are not liquified, and casein, starch, hippurate, tributyrin, and esculin are not hydrolyzed. No acid is produced from carbohydrates. No reaction is produced in litmus milk or Crossley milk, and an alkaline reaction occurs in Hugh-Leifson medium.

Growth occurs on medium containing 8% glucose and pteridine vibriostatic compound 0/129. No growth occurs on medium containing >2% NaCl, 1.0% glycine, or 1 mg of 2,3,5-triphenyl tetrazolium chloride per ml. Sodium selenite is not reduced, and no H₂S is produced in triple sugar iron medium or medium supplemented with cystine. No growth occurs on McConkey or deoxycholate agar.

Antibiotic susceptibility: resistant to ampicillin (18 µg/ml), carbenicillin (64 µg/ml), chloramphenicol (4 µg/ml), dimetridazole (1 µg/ml), kanamycin (2 µg/ml), metronidazole (8 µg/ml), nalidixic acid (5 µg/ml), penicillin G (1 IU/ml), and polymyxin B (2 µg/ml); susceptible to streptomycin (12 µg/ml).

The G+C content of the type strain is 31.5 mol%.

Strain A169/B^T was isolated from an aborted bovine fetus.

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