Differentiation of Catalase-Positive Campylobacters With Special Reference to Morphology

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Examination of three groups of catalase-positive campylobacters by phase-contrast microscopy showed significant differences in cell size between the groups. These differences were most striking when the spiral forms of the organisms were observed. *Campylobacter jejuni* consistently produced small, tightly coiled spirals with a mean wavelength and amplitude of 1.12 and 0.48 μm , respectively. *Campylobacter fetus* subsp. *fetus* produced intermediate-sized spirals (mean wavelength and amplitude of 1.80 and 0.55 μm , respectively); *C. fetus* subsp. *venerealis* produced the largest spirals (mean wavelength and amplitude of 2.43 and 0.73 μm , respectively). Organisms belonging to the different groups could be distinguished by a trained observer on the basis of size alone, without actual measurement. Two additional features which distinguish *C. jejuni* from *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* are the tendencies of *C. jejuni* to swarm on moist agar plates and to undergo rapid coccal transformation in normal atmospheric air. These findings should help in clarifying the subdivision of catalase-positive campylobacters and in making a rapid presumptive species or subspecies identification.

The genus *Campylobacter* consists of small, microaerophilic, oxidase-positive, gram-negative bacteria with a characteristic curved, S-shaped, or spiral morphology (13, 18). The key differential characteristics of catalase-positive campylobacters (13, 18) are listed in Table 1. In this report, the nomenclature of Véron and Chatelain (18) will be used unless otherwise specified, and furthermore, strains corresponding to the *Campylobacter jejuni*-*Campylobacter coli* group of Véron and Chatelain will be referred to simply as *C. jejuni* (4). It should be noted that the strong hydrogen sulfide-producing, catalase-positive strains listed by Smibert (13) as "*C. fecalis*" (names in quotation marks are not on the Approved Lists of Bacterial Names [12]) were not available for this study.

King (6, 7) was the first to use temperature tolerance to differentiate campylobacters. She distinguished "*Vibrio fetus*" (corresponding to Véron and Chatelain's *Campylobacter fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*) from "related vibrios" (Véron and Chatelain's *C. jejuni* and *C. coli*) on the grounds that the latter had a higher-than-normal optimal growth temperature. "*V. fetus*" grew at 25 and 37°C but not at 42°C, whereas "related vibrios" grew at 37 and 42°C but not at 25°C. Smibert (14), however, has shown that a few aberrant strains of *C. fetus* subsp. *fetus* (Smibert's "*C. fetus* subsp. *intestinalis*") are also able to grow at 42°C.

Nalidixic acid susceptibility (18) differentiates

C. jejuni, which is usually inhibited by 40 μg of this agent per ml, from *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, which are resistant to 40 μg of nalidixic acid per ml. However, Vanhoof et al. (17) have found about 5% of their *C. jejuni* strains to be resistant to nalidixic acid, and Butzler and Skirrow (1) have found that such resistant strains of *C. jejuni* are commonly found in seagulls. Furthermore, Neill et al. (10, 11) have recently described a group of aero-tolerant, catalase-positive campylobacters that conforms to a distinct but previously unrecognized group. These "group 2" strains resemble *C. jejuni* in being susceptible to nalidixic acid but differ from the latter in being unable to grow at 42°C. Thus, nalidixic acid susceptibility should not solely be relied upon as a differential test.

The ability of strains to grow in the presence of 1% glycine (3, 18) may be used to differentiate *C. fetus* subsp. *fetus* (glycine tolerant) from *C. fetus* subsp. *venerealis*, which is unable to grow in the presence of 1% glycine. Chang and Ogg (2) have transduced glycine tolerance from *C. fetus* subsp. *fetus* (Smibert's "*C. fetus* subsp. *intestinalis*") to strains of *C. fetus* subsp. *venerealis* and suggest, therefore, that glycine tolerance may also not be a reliable differential test.

Thus, whereas it is clear that most workers in this field recognize differences among campylobacters, it is equally clear that tests for differentiating these organisms are at present inadequate and need to be extended. We have exam-

TABLE 1. Key differential characteristics of catalase-positive campylobacters^a

Bacterium	Growth at:		Susceptibility to 40 µg of nalidixic acid per ml	Growth in the presence of 1% glycine
	25°C	42°C		
<i>C. jejuni</i> - <i>C. coli</i> group ^b	—	+	S	+
<i>C. fetus</i> subsp. <i>fetus</i> ^c	+	—	R	+
<i>C. fetus</i> subsp. <i>venerealis</i> ^d	+	—	R	—

^a Nomenclature of Véron and Chatelain (18) is used. S, susceptible; R, resistant.

^b *C. fetus* subsp. *jejuni* (Smibert; 13); related vibrios (King; 6).

^c "*C. fetus* subsp. *intestinalis*" (Smibert; 13).

^d *C. fetus* subsp. *fetus* (Smibert; 13).

ined cultures of different groups of catalase-positive campylobacters by phase-contrast microscopy to determine whether they can be differentiated reliably on morphological grounds. We have, in addition, evaluated two further criteria for differentiating *C. jejuni* from *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. These are the tendency of *C. jejuni* to swarm and its recognized ability to degenerate readily into coccoid "granules" (3).

MATERIALS AND METHODS

Bacterial strains. The 32 *Campylobacter* strains used in this project were as follows: 15 human fecal isolates of *C. jejuni* cultured in our laboratory from patients with gastroenteritis; 8 human blood culture isolates of *C. fetus* subsp. *fetus* obtained from N. Chahardjian, S. McDonald, M. Tischler, J. Richter, J. L. Whitby, and W. J. Martin; 2 strains of *C. fetus* subsp. *venerealis* and 1 strain of *C. fetus* subsp. *fetus* obtained from A. J. Winter; 1 strain of *C. fetus* subsp. *venerealis* obtained from J. F. Prescott; and 5 reference *Campylobacter* strains obtained from the American Type Culture Collection (ATCC). The five ATCC strains were: (i) ATCC 15296, J. F. Reback ("*Vibrio fetus*"); (ii) ATCC 19438 = NCTC 10354 = CIP 6829 (*Campylobacter fetus* subsp. *venerealis*); (iii) ATCC 25936, J. H. Bryner = NADL 1083-2255 ("*Vibrio fetus*"); (iv) ATCC 27374 = CIP 5396 = NCTC 10842 (type strain of *Campylobacter fetus* subsp. *fetus* (Smith and Taylor), Véron and Chatelain, 1973); and (v) ATCC 29428, N. R. Kreig = VPI H840 (*Campylobacter fetus* subsp. *jejuni*).

Media. The media used were: (i) blood agar (BA; Columbia blood agar base [GIBCO] containing 7% defibrinated horse blood); (ii) thioglycolate medium (TM; fluid thioglycolate medium [BBL Microbiology Systems]); and (iii) triple sugar iron (TSI) agar (Difco), dispensed in tubes to form a slant and butt.

Cultural conditions. All BA plates were incubated in an atmosphere in which the oxygen tension was reduced to approximately 7%. Such an atmosphere was obtained by evacuating two-thirds of the air from an anaerobic jar (without catalyst) and replacing the evacuated air with carbon dioxide.

Characterization of strains. Strains were characterized as follows. (i) The catalase reaction was determined by adding drops of 3% hydrogen peroxide solution to 48-h-old TSI and 48-h-old TM cultures and

examining cultures for the formation of gas bubbles. (ii) The effect of temperature on growth was determined by culturing all strains in parallel on BA plates and in TM at 25, 37, and 42°C and by examining cultures for growth after 5 days. (iii) The ability of strains to grow in the presence of 1% glycine was determined by inoculating each strain in parallel into TM and TM-containing 1% glycine and examining cultures for the presence or absence of growth after 5 days of incubation at 37°C. (iv) Susceptibility to nalidixic acid was determined by inoculating each strain onto a BA plate containing 40 µg nalidixic acid per ml (18) as well as onto a control BA plate lacking this antibiotic. The plates were incubated at 37°C under reduced oxygen tension and examined for growth after 48 to 72 h. The presence of any growth on the nalidixic acid plate indicated resistance, whereas the complete absence of growth on the nalidixic acid plate, but growth on the control plate, indicated susceptibility.

Morphological examination. All strains were cultured on BA plates and incubated under reduced oxygen tension at 37°C for 2 days, except for one strain of *C. fetus* subsp. *venerealis*, which required 3 days of incubation for visible growth. Suspensions of each culture were made in normal saline, and a drop of the suspension was examined by phase-contrast microscopy under oil immersion. High-power fields were carefully chosen to include one or more spiral forms of the organism and photographed with exactly the same magnification for each strain, the final magnification on the negative being ×400. Photomicrography was performed with a photomicroscope with automatic exposure control; Kodak photomicrography daylight color film 2483 was used. Subsequently, direct prints were made from the 35-mm slides at ×18 magnification, giving an overall magnification of ×7,200 for each organism. The dimensions of between 2 to 4 wavelengths of the spiral forms of each strain were measured, and the average wavelength (λ) and average amplitude (α) of the wave forms were recorded. In this study, α represented the full vertical distance between the trough and the crest of the wave form.

It should be noted that photomicrography was performed on live organisms. Although this was more difficult than photographing dead organisms, it was considered preferable in view of the possible effects of shrinkage and distortion resulting from the drying and fixing of organisms.

After the placement of the strains into the three species, or into subspecies, by the classification of

Véron and Chatelain (Table 1), the ranges of λ and α of the strains in each group were determined. The mean λ and α of each of the groups were calculated, and differences in λ and α among the three groups were tested for significance with Duncan's multiple range test (8).

Swarming. To demonstrate swarming, it was necessary to use moist agar plates. Freshly made BA plates, dried in air at room temperature just long enough (about 0.5 h) to allow surface water to evaporate, were found to be suitable. Strains to be tested were spotted on each quadrant of a plate. All plates were incubated at 37°C under reduced oxygen tension and examined after 36 to 48 h.

Coccal transformation. All strains were subcultured onto duplicate sets of BA plates which were incubated at 37°C in jars containing a reduced oxygen environment. After incubation for 48 h, both sets were removed from the incubator. Jars containing one set of BA plates were opened, and the plates were left on the bench in room air for 6 days, whereas jars containing the duplicate set of BA plates were left unopened for a further 2 days so that the plates remained in a reduced oxygen atmosphere on the bench at room temperature.

Suspensions of colonies from the first set of BA plates were examined by phase-contrast microscopy for coccal forms after the plates had been on the bench in room air for 1, 2, 4, and then 6 days.

Suspensions of colonies from the duplicate set of BA plates were similarly examined after the plates had remained on the bench in unopened jars (i.e., in reduced oxygen tension) for 2 days.

RESULTS

All strains tested were strictly microaerophilic, oxidase- and catalase-positive, gram-negative bacteria which had the characteristic morphology and motility of campylobacters. None of the strains produced hydrogen sulfide in TSI agar after 5 days of incubation. On the basis of the differential tests used in this study, it was possible to classify all strains by the scheme of Véron and Chatelain (Table 1). A total of 16 strains corresponded to *C. jejuni*, 12 corresponded to *C. fetus* subsp. *fetus*, and 4 corresponded to *C. fetus* subsp. *venerealis*. The mean wavelength and amplitude of each strain as well as the mean wavelength and amplitude of each of the three groups of strains are shown in Table 2. The differences in wavelength between the groups were highly significant ($P < 0.001$). It should be noted that there was no overlap at all between the ranges of wavelengths of each of the three groups (Table 2). The differences in

TABLE 2. Wavelength (λ) and amplitude (α) of 16 strains of *C. jejuni*, 12 strains of *C. fetus* subsp. *fetus*, and 4 strains of *C. fetus* subsp. *venerealis*^a

Strain	λ	α	Strain	λ	α
<i>C. jejuni</i>			<i>C. fetus</i> subsp. <i>fetus</i>		
MK 15	1.31	0.49	PC 16	2.13	0.68
MK 118	1.31	0.50	PC 3	1.99	0.56
MK 125	1.26	0.49	ATCC 27374	1.99	0.58
MK 6	1.25	0.63	PC 17	1.86	0.51
MK 1	1.18	0.44	ATCC 15296	1.82	0.50
MK13	1.17	0.61	PC 14	1.81	0.57
MK 5	1.15	0.46	PC 18	1.81	0.49
MK 111	1.11	0.49	PC 7	1.75	0.49
MK 121	1.11	0.49	PC 5	1.74	0.53
MK 86	1.07	0.38	ATCC 25936	1.71	0.50
MK 10	1.06	0.47	PC 4	1.69	0.61
MK 23	1.04	0.46	PC 15	1.36	0.56
ATCC 29428	1.04	0.40	Minimum	1.36	0.49
MK 27	1.03	0.54	Maximum	2.13	0.68
MK 22	1.01	0.46	Mean	1.80	0.55
MK 128	0.89	0.35	SD	0.19	0.06
Minimum	0.89	0.35	<i>C. fetus</i> subsp. <i>venerealis</i>		
Maximum	1.31	0.63	ATCC 19438	2.56	0.69
Mean	1.12	0.48	PC 2	2.54	0.69
SD	0.12	0.07	PC 19	2.39	0.79
			PC 1	2.25	0.74
			Minimum	2.25	0.69
			Maximum	2.56	0.79
			Mean	2.43	0.73
			SD	0.14	0.05

^a Values are given in micrometers. SD, Standard deviation.

amplitude between *C. fetus* subsp. *venerealis* and the other two groups were also significant ($P < 0.01$).

It should be further noted that we were able to distinguish strains from the different groups by simply observing them under a phase-contrast microscope without actually measuring the size (Fig. 1a, 1b, 2a, 2b, 3a, and 3b). When individual strains were examined after various periods of incubation at different temperatures, the size, as judged visually, always corresponded to the size range of the group to which the strain

belonged. Whereas strains of *C. jejuni* and *C. fetus* subsp. *fetus* usually produced regular spirals, strains of *C. fetus* subsp. *venerealis* often produced large, irregular spirals with varying wavelength and amplitude. Flattening and phase variation in the spirals of *C. fetus* subsp. *venerealis* were often noted (Fig. 3a and 3b).

Under the experimental conditions we used, swarming (Fig. 4) was found to be a feature only of *C. jejuni* and not of the other two groups.

All BA cultures of *C. jejuni* that were left on the bench in room air underwent rapid coccal

FIG. 1. Phase-contrast photomicrographs of two strains of *C. jejuni*: MK 125 (a) and MK 15 (b). Magnification, $\times 2,520$.

FIG. 2. Phase contrast photomicrographs of two strains of *C. fetus* subsp. *fetus*: PC 17 (a) and PC 18 (b). Magnification, $\times 2,520$.

transformation in 24 h, and this was virtually complete (Fig. 5) by 48 h. In contrast, all BA cultures of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* left in room air showed predominantly normal morphology (less than 5% coccal forms) at 48 h. Most of the latter cultures retained predominantly normal morphology after 4 days, and some did so even after 6 to 8 days.

In contrast to the rapid coccal transformation seen in BA cultures of *C. jejuni* left in room air, duplicate BA cultures left on the bench in unopened jars (i.e., in a reduced-oxygen atmosphere) failed to undergo coccal transformation in 48 h and showed not only normal morphology but also a high degree of motility. Additional

differential characteristics of catalase-positive campylobacters incorporating our findings are shown in Table 3.

DISCUSSION

Morphological differences between groups of *Campylobacter* were first observed by Florent (3) and later confirmed by King (7), who noted that the undulations in related vibrios tended to be closer together than those in "*Vibrio fetus*." We have extended these observations by actually measuring the wavelength and amplitude of representative strains and confirmed that *C. jejuni*, *C. fetus* subsp. *fetus*, and *C. fetus* subsp. *venerealis* may be distinguished from each other on morphological grounds by direct microscopy.

FIG. 3. Phase-contrast photomicrographs of two strains of *C. fetus* subsp. *venerealis*: ATCC 19438 (a) and PC 19 (b). Magnification, $\times 2,520$.

TABLE 3. Additional differential characteristics of catalase-positive campylobacters^a

Bacterium	Rapid coccal transformation	Swarming on moist agar media	Size	$\bar{\lambda}^b$ (μm)	$\bar{\alpha}^c$ (μm)
<i>C. jejuni</i> - <i>C. coli</i> group	+	+	Small	1.12	0.48
<i>C. fetus</i> subsp. <i>fetus</i>	—	—	Medium	1.80	0.55
<i>C. fetus</i> subsp. <i>venerealis</i>	—	—	Large	2.43	0.73

^a Amplitude in this study represents the full vertical distance between the crest and the trough of the wave form.

^b $\bar{\lambda}$, Mean wavelength (see Table 2).

^c $\bar{\alpha}$, Mean amplitude of spiral forms (see Table 2).

We have also confirmed Florent's earlier observation (3) that strains of *C. jejuni* rapidly degenerate into coccal forms or granules. Our results showed that rapid coccal transformation

occurred in strains of *C. jejuni* that were left in room air but not in duplicate strains maintained in a reduced-oxygen environment. This suggests that exposure to air was an important factor

FIG. 4. Moist BA plate spotted with different *Campylobacter* strains and incubated for 48 h at 37°C under reduced oxygen tension. Swarming occurred only with *C. jejuni* (top and bottom) and not with *C. fetus* subsp. *fetus* (left) or *C. fetus* subsp. *venerealis* (right).

FIG. 5. Coccal granules of *C. jejuni* seen in a 48-h-old BA culture that was left in room air for a further 48 h and subsequently examined by phase-contrast microscopy ($\times 2,300$).

leading to rapid coccal transformation. The absence of rapid coccal transformation in *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* further serves to differentiate these two subspecies from *C. jejuni*, as does their inability to swarm on moist media.

The differentiation of catalase-positive campylobacters requires improvement. Our observations should help in clarifying the subdivision of the genus *Campylobacter* and in classifying those strains which react aberrantly in currently available differential tests. Our findings will also be readily applicable to a routine diagnostic laboratory and will be of particular value in making a rapid presumptive species or subspecies identification not only of pure cultures but also of organisms in clinical specimens, such as stools, examined by phase-contrast microscopy (5).

The results of our studies may also help to resolve one of the controversies surrounding the nomenclature of catalase-positive campylobacters. The term "*Vibrio fetus*" was first proposed by Smith and Taylor in 1918 for organisms (now considered to be campylobacters) that were found to be associated with abortion in cattle (16). In 1959, Florent (3) showed that there were in fact two different groups of campylobacters involved in bovine abortion. One group, which Florent called "*V. fetus intestinalis*," originated in the intestinal tract and caused sporadic bovine abortion. The second group, which he termed "*V. fetus venerealis*," was transmitted venereally and caused enzootic sterility as well as abortion in herds of cattle.

It is impossible to know for certain whether the original strains of Smith and Taylor were of the venereal or the intestinal type, and thus the difficulty arises as to which variety should constitute the type species of the genus *Campylobacter*. In 1973, Véron and Chatelain (18) argued for selecting "*V. fetus intestinalis*" (Florent) as the type species and, in accordance with the rules of the International Code of Nomenclature of Bacteria (9), renamed the latter organism *C. fetus* subsp. *fetus*. Smibert (13), on the other hand, speculated that the original strains of Smith and Taylor were more likely to be "*V. fetus venerealis*" (Florent) and thus designated the latter organism as the type species and renamed it *C. fetus* subsp. *fetus*. Véron and Chatelain's nomenclature was validly published first, although Smibert's nomenclature continues to be widely favored.

Smith's original report (15) does have a clue that might indicate whether he was dealing with the intestinal or the venereal strains. He recorded the average wavelength and amplitude of his strains as being about 2.0 and 0.5 μm ,

respectively. From the results obtained in our study, these dimensions conform most closely to those of Véron and Chatelain's *C. fetus* subsp. *fetus* ("V. *fetus* subsp. *intestinalis*" of Florent) and therefore support the designation of the latter organism as the type species of the genus *Campylobacter*.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of M. M. Wood, who performed the statistical analysis.

This work was supported by the Medical Research Council of Canada (grant no. MA7063).

REPRINT REQUESTS

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