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Isoprenoid Quinone Content and Cellular Fatty Acid Composition of *Campylobacter* Species

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A total of 36 strains of *Campylobacter* species were examined for isoprenoid quinones and cellular fatty acids. The isoprenoid quinone content was determined by reverse-phase high-pressure liquid chromatography, and the fatty acids were determined by capillary gas-liquid chromatography. All *Campylobacter* species contained menaquinone-6 (2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone) and a methyl-substituted menaquinone-6 (2,[5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-napthoquinone) as the major isoprenoid quinones. The latter menaquinone has not been reported in other bacteria and may prove to be a useful chemical marker of *Campylobacter* species. *Campylobacter jejuni* and most *Campylobacter coli* were distinguished from other campylobacteria by the presence of a C_{19} cyclopropane acid, and *Campylobacter sputorum* subsp. *mucosalis* differed from other species by the presence of lauric acid.

Isoprenoid quinones are widely distributed in nature and have been shown to be useful for identifying and classifying some bacteria (5, 12). They are divided into two major structural groups: napthoquinones, which include menaquinones and demethylmenaquinone, and benzoquinones, which include ubiquinones. Menaquinones (vitamin K_2) and ubiquinones (coenzyme Q) occur most frequently in bacteria and are located in the bacterial plasma membrane where they function in electron transport (5). Menaquinones have a 1,4-napthoquinone ring nucleus, whereas ubiquinones have a 1,4-benzoquinone ring. Both menaquinones and ubiquinones have polyprenyl side chains which vary in the number of isoprene units as well as in the degree of unsaturation. This structural variation in the polyprenyl side chain is the basis for bacterial classification (5, 12).

Recently, Carlone and Anet (4) have investigated the respiratory quinones of one strain each of *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus* and reported that both strains contained menaquinone-6 (MK-6) and a methyl-substituted menaquinone-6 (*MK-6) as major components. The latter menaquinone has not been previously reported in bacteria and may be unique for *Campylobacter* species. Our study was conducted to determine the isoprenoid quinone content of additional strains and species of *Campylobacter* and to evaluate their potential usefulness for identification and classification. Cultures were also examined for their content of cellular fatty acids to further evaluate the value of these compounds in the taxonomy of *Campylobacter* species (3, 6, 10, 11, 14).

MATERIALS AND METHODS

Cultures. A total of 36 strains were studied. The origin and strain designations are listed in Table 1. The nomenclature used is that of Owen (17). The identity of all strains was confirmed by conventional cultural and biochemical tests (7, 9, 10). Cultures were grown on heart infusion agar plates with 5% defibrinated rabbit blood (Nolan Biological Laboratories, Atlanta, Ga.). Plates were incubated at 37°C in an atmosphere of approximately 5% oxygen–8% carbon dioxide–87% nitrogen for 48 h, except for the three subspecies of *Campylobacter sputorum*, which were incubated anaerobically. Three of the anaerobic strains (D1179, D1180, D1181) were incubated for 72 h.

Determination of isoprenoid quinones. For isoprenoid quinone determinations, cultures were grown for 48 to 72 h on heart infusion agar plates with 5% defribinated rabbit blood. Cells were removed by scraping and saponified by the modified procedure of Abe et al. (1) as described previously (12). The isoprenoid quinones were removed from the saponified cells by extracting four times with 5 ml of a 1:1 mixture of diethyl ether-hexane. The combined diethyl ether-hexane layer was evaporated to approximately 1.0 ml under nitrogen, and the extracted isoprenoid quinones were analyzed by thin-layer chromatography (TLC) (5), reverse-phase TLC (RPTLC) (12), and reverse-phase high-pressure liquid chromatography (RPHPLC) (16). Identification was established by these techniques by comparison with menaquinone standards supplied by Hoffmann-La Roche Co., Basel, Switzerland. Identification was confirmed by collecting fractions from RPHPLC followed by analysis with both electron impact and chemical ionization mass spectrometry (MS) (12, 13).

Fatty acid analysis. Growth from one agar plate was removed by scraping, and it was then placed in a screw-capped tube (20 by 150 mm) fitted with a Teflon-lined cap. Cells were saponified by adding 4 ml of 15% NaOH in 50% aqueous methanol and heating at 100°C for 30 min. The sample was cooled to ambient temperature, 5 ml of 25% hydrochloric acid-methanol reagent was added, and the mixture was heated at 85°C for 15 min. The resulting fatty acid methyl esters (FAME) were extracted twice with diethyl ether-hexane (1:1), evaporated to 0.3 ml with nitrogen, and stored at −20°C (13). To ensure that all bound hydroxy acids were liberated, the aqueous layer (remaining after saponification, methylation, and FAME extraction) was mixed with 2.5 ml of concentrated HCl and heated at 85°C for 16 to 18 h. The sample was cooled to room temperature and extracted two times with diethyl ether-hexane. The organic phase, which contained methyl esters of acid-labile fatty acids, was concentrated to 0.3 ml and combined with the FAME from the base hydrolysis as described above. The combined FAME fraction was washed with basic buffer as

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described previously (13) and then analyzed by gas-liquid chromatography.

The FAME samples were analyzed on a fused-silica capillary column (50 m by 0.2 mm [inside diameter]) with cross-linked methyl silicone (OV-1) as the stationary phase (Hewlett-Packard, Avondale, Pa.). The column was installed in a 5790 gas chromatograph (Hewlett-Packard) equipped with a flame-ionization detector. For analysis of FAME, the

TABLE 1. *Campylobacter* strains studied

Species and strain	Other strain designation[a]	Source	Supplied by
C. jejuni			
D133	NCTC 11351^T; CIP 702	Bovine feces, Belgium	—
D109	KB 16	Human, Japan	R. Sakazaki[b]
D114	KB 3	Human, Japan	R. Sakazaki
D128	KB 295	Human, Japan	R. Sakazaki
D1368	NCTC 11168	Feces, England	M. B. Skirrow[c]
C. coli			
D145	NCTC 11366^T; CIP 7080	Pig feces, Belgium	M. Veron[d]
D115	19494	Bovine feces, United States	R. Walker[e]
D118	C 4196	Human gall bladder, United States	R. E. Weaver[f]
D122	KB 14	Chicken, Japan	R. Sakazaki
D111	KB 28	Pig, Japan	R. Sakazaki
C. laridis			
D375	NCTC 11352^T	Seagull	
D67	82-2363	Human feces, United States	CDC[f]
D70	1788	Human feces, United States	CDC
D71	583	Human feces, United States	CDC
D110	25438/76	Seagull, England	M. B. Skirrow
D120	177/79	Seagull, England	M. B. Skirrow
C. fetus subsp. *fetus*			
D373	NCTC 10842^T; CIP 5396	Brain, sheep fetus	
D1162	18986	Unknown	R. Walker
D1175		Unknown	
D1176	CF 83-80	Monkey, Japan	T. Itoh[g]
C. fetus subsp. *venerealis*			
D374	NCTC 10354^T; CIP 6829	Vaginal mucous, heifer, England	
D1133	1285	Unknown	J. Bryner[h]
D1134	662	Unknown	J. Bryner
D1177	Gifu 8741	Unknown	E. Yabuuchi[i]
C. sputorum subsp. *bubulus*			
D1248	NCTC 11367^T; CIP 53103	Bull sperm, Belgium	
D1166	7508	Unknown	R. Walker
D1172	11770	Ram sperm	R. Walker
C. sputorum subsp. *sputorum*			
D1163	VPI 11767	Unknown	L. Holdeman[j]
D1180	VPI S17	Unknown	L. Holdeman
C. sputorum subsp. *mucosalis*			
D1179	NCTC 11000^T	Pig, Scotland	
D1181	NCTC 11001	Pig, Scotland	
"*Campylobacter fecalis*"			
D1135	11362	Unknown	B. Firehammer[k]
D1136	11411	Unknown	B. Firehammer
D1137	NCTC 11416; 14223	Sheep feces	B. Firehammer
D1138	14479	Unknown	B. Firehammer
D1169	NCTC 11415; 14227	Sheep feces, United States	B. Firehammer

[a] NCTC, National Collection of Type Cultures, London, England; T, type strain; CIP, Collection of the Institute Pasteur, Paris, France.
[b] National Institute of Health, Tokyo, Japan.
[c] Worcester Royal Infirmary, Worcester, England.
[d] Institute Pasteur, Paris, France.
[e] University of Tennessee, Veterinary Teaching Hospital, Knoxville, Tenn.
[f] Centers for Disease Control, Atlanta, Ga.
[g] Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.
[h] National Animal Disease Laboratory, U.S. Department of Agriculture, Ames, Iowa.
[i] Gifu University School of Medicine, Gifu, Japan.
[j] Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
[k] Montana Veterinary Research Laboratory, Montana State College, Bozeman, Mont.

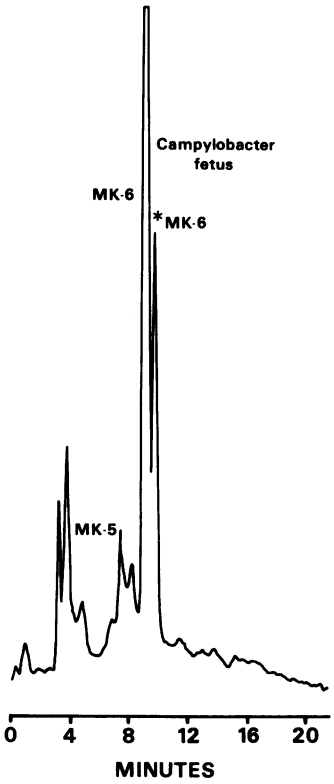

FIG. 1. High-pressure liquid chromatogram of menaquinones from *C. fetus* analyzed on a μBondapak C_{18} reverse-phase column (300 by 3.9 mm). The detector was set at 248 nm. The retention time (in minutes) of menaquinone (MK) standards were as follows: MK-3, 5.0; MK-4, 6.1; MK-5, 7.4; MK-6, 9.4; MK-7, 10.2; MK-8, 11.2; MK-9, 13.0; MK-10, 14.4

column was temperature programmed from 180 to 260°C at 8°C/min and was maintained at 260°C for 8 min before recycling to the initial temperature of 180°C. The injector temperature was 250°C, and the detector temperature was 280°C. Hydrogen was used as carrier gas, with a flow rate of 0.7 ml/min; the sample size was 1 μl, with a split ratio of approximately 50:1. The FAME were identified by comparing retention times to known standards (Supelco, Inc., Bellefonte, Pa.; Applied Science Div., Milton Roy Co., State College, Pa.). Their identity was confirmed after trifluoroacetylation, hydrogenation, and MS (15). A model 3390 Hewlett-Packard reporting integrator was used for quantitation of peak areas.

RESULTS AND DISCUSSION

Carlone and Anet have reported that MK-6 (2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone) and *MK-6 (2,[5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-napthoquinone) were the major isoprenoid quinones in *C. jejuni* and *C. fetus* subsp. *fetus* (4). The identities of these two compounds were established by a combination of procedures including RPTLC, RPHPLC, UV spectrometry, MS, and nuclear magnetic resonance. Data from MS and nuclear magnetic resonance firmly established that *MK-6 contained a methyl group at the C-5 or C-8 on the benzenoid ring (4). In the present study, we did not use nuclear magnetic resonance, but all other chemical and analytical data (from RPTLC, RPHPLC, and MS) are consistent with those reported by Carlone and Anet (4).

Shown in Fig. 1 is an HPLC chromatogram of the isoprenoid quinone fraction from a strain of *C. fetus* subsp. *fetus* analyzed on a reverse-phase C_{18} column with the detector at 248 nm. There are two major peaks in the chromatogram at retention times of 9.4 min (MK-6) and 9.9 min (*MK-6), with two smaller peaks at 7.4 (MK-5) and 8.3 min (unlabeled). Each of these components was tentatively identified as menaquinones by UV absorbance, TLC on silica gel, and RPTLC (4, 5, 12). Fractions corresponding to each of the four peaks were collected from RPHPLC and analyzed by MS (12). Peaks MK-5 and MK-6 were confirmed as menaquinones since the mass spectra of each showed a base peak ion at *m/e* 225 and an intense *m/e* 187 ion indicating the naphthoquinone nucleus (4, 5). The base peak ion for *MK-6 was at *m/e* 239, with an intense ion at *m/e* 201, which is consistent with the presence of an additional methyl group on the naphthoquinone ring (4). Prominent molecular ions (M+) were observed at *m/e* 512 for MK-5, at *m/e* 580 for MK-6, and at *m/e* 594 for *MK-6. The M+ ions were verified by chemical ionization spectra which showed intense $(M+1)^+$ ions at the expected mass values of *m/e* 513, *m/e* 581, and *m/e* 595 for MK-5, MK-6, and *MK-6, respectively. Preliminary data suggest that the unlabeled peak at 8.3 min is a methyl-substituted menaquinone, but additional purified material is required to complete the chemical identification.

A total of 36 strains of *Campylobacter* were examined for isoprenoid quinones, and each was found to contain only menaquinones. The identity and relative amounts of the menaquinones detected in these strains are summarized in Table 2. Thirty-three strains contained MK-6 as the major menaquinone, with smaller amounts of *MK-6 (10 to 30% of the amount found for MK-6), and trace amounts of MK-5 (less than 10% of the amount found for MK-6). Two strains of *C. sputorum* subsp. *mucosalis* and one strain of *C. sputorum* subsp. *sputorum* D1180 differed from the other cultures in that they contained *MK-6 as the major menaquinone with smaller amounts of MK-6. These latter three strains were regrown and retested by gas-liquid chromatography and RPHPLC with essentially identical results. Only small quantitative differences in menaquinone content were observed among strains within a species, and with the exception of the above three strains, no significant differences were observed among species.

The fatty acid composition of the 36 strains was deter-

TABLE 2. Menaquinone (MK-*n*) content of *Campylobacter* species

Species and strains	No. of strains tested	Menaquinone identified[a]		
		MK-5	MK-6	*MK-6
C. jejuni	5	<1	10	2
C. coli	5	<1	10	2
C. laridis	6	<1	10	2
C. fetus				
subsp. *fetus*	4	<1	10	2
subsp. *venerealis*	4	<1	10	2
C. sputorum				
subsp. *bubulus*	3	<1	10	2
subsp. *sputorum* D1163		<1	10	1
subsp. *sputorum* D1180		<1	3	10
subsp. *mucosalis*	2	<1	2	10
C. fecalis	5	<1	10	3

[a] The major peak in the chromatogram was assigned a value of 10, and the areas of other peaks were related to this value (i.e., 2 is a peak with 20% of the area of a peak designated 10; <1 is a peak with an area less than 10% of a peak designated 10).

TABLE 3. Cellular fatty acid composition of *Campylobacter* species

Species	No. of strains	Fatty acid composition (% of total)											
		12:0[a]	14:0	15:0	3-OH 14:0	16:1	16:0	3-OH 16:0	18:2	18:1 Δ9	18:1 Δ11	18:0	19 cyc
C. jejuni	5	–	8	3	6	4	36	–	1	1	21	2	18
C. coli	5	–	5	T	7	3	41	–	1	1	27	2	13[b]
C. laridis	6	–	5	–	6	6	36	–	1	T	45	1	–
C. fetus													
subsp. *fetus*	4	–	8	T	3	17	33	3	2	1	32	1	–
subsp. *venerealis*	4	–	11	1	3	19	38	3	1	1	22	1	–
C. sputorum													
subsp. *bulbulus*	3	–	21	T	5	7	31	5	2	1	27	1	–
subsp. *sputorum*	2	–	19	1	4	7	31	5	5	2	25	1	–
subsp. *mucosalis*	2	10	12	1	8	14	26	4	7	4	11	3	–
C. fecalis	5	–	21	T	5	7	29	5	2	1	29	1	–

[a] Numbers before the colon indicate the number of carbon atoms, and numbers after colon refer to number of double bonds; Δ9, Δ11 indicate double bond at the 9, 10 and 11, 12 carbon atoms, respectively; 2-OH and 3-OH indicate an hydroxyl group at the 2 or 3 carbon atom, respectively. Values are percentage of total fatty acids and are arithmetic means. T, <1%; —, not detected.

[b] One strain of *C. coli* (D118) did not contain 19 cyc acid.

mined after cells were hydrolyzed with both base and acid (13). The data presented in Table 3 are in general agreement with our previous results which showed that the presence of a C_{19} cyclopropane acid (19 cyc) was a distinguishing characteristic for *C. jejuni* (3). Since our original publication, we have examined more than 100 strains of *C. jejuni* and found only 1 strain (hippurate positive) which did not contain 19 cyc (10, 11). The data in Table 3 show that the fatty acid composition of *Campylobacter coli* is essentially identical to that in *C. jejuni*, including 19 cyc. However, we observed that approximately 20% (14 of 66) of *C. coli* strains do not contain 19 cyc. The six strains of nalidixic acid-resistant thermophilic *Campylobacter* species, for which the name *Campylobacter laridis* was recently proposed (2), have an overall fatty acid profile like *C. jejuni* and *C. coli*, except for the absence of 19 cyc and larger amounts of 18:1 (45 versus 21 or 27%). A common characteristic of *C. jejuni*, *C. coli*, and *C. laridis* is the presence of 3-hydroxytetradecanoic acid ($C_{14:0}$) and the absence of 3-hydroxyhexadecanoic acid ($C_{16:0}$); all other *Campylobacter* species contained small amounts of both of these hydroxy acids. The relative amounts of hydroxy acids shown in Table 3 are greater than that reported previously (3) and are due to the fact that cells were hydrolyzed with both base and acid to ensure maximum liberation of bound hydroxy acids (13). The two strains of *C. sputorum* subsp. *mucosalis* differed from all other *Campylobacter* species by the presence of moderate amounts (10%) of lauric acid ($C_{12:0}$). This acid was reported previously in *C. sputorum* subsp. *mucosalis* and an atypical *C. fetus* (6); however, we found that it is absent (or present in only trace amounts) in all *Campylobacter* species except *C. sputorum* subsp. *mucosalis*.

Results from this study provided additional information on the chemical composition of *Campylobacter* species. MK-6 and a *MK-6 are the major quinones, and these were present in all strains and species tested. Since *MK-6 has not been reported in other bacteria (5), it may prove to be a useful chemical marker of *Campylobacter* species. The presence of *MK-6 as the major menaquinone of *C. sputorum* subsp. *mucosalis* suggests that this compound may be a useful marker to distinguish this organism from other *Campylobacter* species. However, this observation must be tentative until additional strains of all subspecies of *C. sputorum* are studied. Tests for menaquinones can be accomplished by simple treatment and extraction of fresh whole cells with subsequent chromatographic analysis. However, at present, RPHPLC is required for analysis since MK-6 and *MK-6 were not resolved with the simpler RPTLC technique in the present or in a previous study (4).

The data from the present study provide strong support to our earlier observation on the value of 19 cyc acid as a distinguishing marker of *C. jejuni* (3, 10). The presence of this acid in *C. jejuni* was confirmed by others, who have also found significant amounts of 19 cyc in *C. coli* (6, 14). We also observed this acid in *C. coli* but found that approximately 20% of *C. coli* strains do not contain 19 cyc. The fatty acid content of these latter strains (19 cyc-negative *C. coli*) are thus essentially identical to *C. laridis*, and their differentiation requires conventional cultural and biochemical testing. The absence of 3-hydroxyhexadecanoic acid and a high ratio of $C_{16:0}$ to $C_{16:1}$ acids (Table 3) (6, 14) in *C. jejuni*, *C. coli* and *C. laridis* may prove valuable in the chemotaxonomy of *Campylobacter* species and may be particularly useful in defining physiologically atypical strains of *C. coli* and *C. laridis* from *C. fetus* subsp. *fetus* (8). The presence of lauric acid ($C_{12:0}$) appears to be a useful marker for distinguishing *C. sputorum* subsp. *mucosalis* from other *Campylobacter* species. However, it is recognized that the various subspecies of *C. sputorum* show some degree of heterogenity in their DNA characteristics (17); it is possible that similar heterogeneity in fatty acid composition may be observed as additional strains of *C. sputorum* are isolated and tested.

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