JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2065–2070
0021-9193/96/$04.00+0

Vol. 178, No. 7

Lipid Profile of *Helicobacter* spp.: Presence of Cholesteryl Glucoside as a Characteristic Feature

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Received 25 September 1995/Accepted 17 January 1996

The lipid and fatty acid profiles of eight *Helicobacter* spp. (*H. nemestrinae*, *H. acinonyx*, *H. canis*, *Helicobacter* sp. strain CLO-3, "*H. rappini*" [*Flexispira rappini*], *H. pametensis*, *Helicobacter* sp. strain Bird-B, and *Helicobacter* sp. strain Bird-C) and the fatty acid profiles of five additional species (*H. pylori*, *H. felis*, *H. muridarum*, *H. mustelae*, and *H. fennelliae*) were analyzed and compared. A heterologous fatty acid profile was observed among the *Helicobacter* spp., and on that basis the species could be divided into two groups. Group A had 19-carbon cyclopropane fatty acid (19:0cyc) and tetradecanoic acid (14:0) as the major fatty acids, and group B characteristically lacked the 19:0cyc and had hexadecanoic acid (16:0) and octadecenoic (18:1) acids as the major fatty acids. The species of group A are primarily gastric colonizers, and those of group B are primarily intestinal colonizers. Seven of the eight species studied showed the unusual and characteristic presence of cholesteryl glucosides (CGs), and most of these seven showed a very large amount (9.7 to 27.4% of the weight of total extractable lipid). The types of CGs and their distribution in different species were as follows: cholesteryl-6-*O*-acyl-α-D-glucopyranoside (cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside in *H. nemestrinae* and mainly cholesteryl-6-*O*-dodecanoyl-α-D-glucopyranoside in "*H. rappini*"), cholesteryl-α-D-glucopyranoside (*H. nemestrinae*, *H. acinonyx*, *H. canis*, *Helicobacter* sp. strain CLO-3, and "*H. rappini*"), and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (*H. nemestrinae*, *H. acinonyx*, *H. canis*, and *Helicobacter* sp. strain CLO-3). Besides this, we could also detect cholesteryl acyl glucoside in *H. acinonyx*, cholesteryl glucoside in *Helicobacter* sp. strains Bird-B and -C, and cholesteryl phosphatidyl glucoside in "*H. rappini*" and *Helicobacter* sp. strain Bird-C. A selective accumulation of free cholesterol was observed in the neutral lipid fractions. On the basis of the detection of CGs in 11 of the 13 species studied so far, the presence of CGs appears to be a characteristic feature of the genus *Helicobacter*. In view of this and also because of a simple and rapid detection method described herein, the CGs can be used as a valuable chemotaxonomic marker.

The genus *Helicobacter*, identified as a separate genus in 1989 (13) and later found to be a distinct group within rRNA superfamily VI (43), has continued to expand, and so far 19 species have been identified. *H. pylori*, the most important member of the genus, is responsible for chronic gastritis and is associated with peptic ulcer disease (19, 27). A causal role has also been proposed for gastric cancer (35) and primary B-cell mucosa-associated lymphoid tissue lymphoma of the stomach (22).

Helicobacters occupy specific niches in the mammalian and avian gastrointestinal tracts and have been isolated from variable sites: *H. pylori* (30), *H. felis* (28), *H. mustelae* (10), *H. nemestrinae* (4) and *H. acinonyx* (6) from the stomach; *H. canis* (41), *H. cinaedi*, *H. fennelliae* (7), *H. pametensis*, *Helicobacter* sp. strains Bird-B and -C (5), and *H. pullorum* (40) from the intestinal tract; "*H. rappini*" (17) and *H. muridarum* (37) from the intestinal tract and the stomach; *H. hepaticus* (9) and *H. bilis* (11) from the liver and the gastrointestinal tract; and *Helicobacter* sp. strain mainz from joint effusions (21).

Various phenotypic markers, such as oxidase, catalase, urease, nitrate reduction, alkaline phosphatase, sensitivity to nalidixic acid and cephalothin, etc., have been used to differentiate helicobacters from the species of closely related genera or to differentiate between the *Helicobacter* spp. Fatty acids and lipids have been used to characterize and differentiate between different bacterial species (24, 26, 34). On analyzing the lipids of *H. pylori*, we observed three types of cholesteryl glucosides (CGs) (18). Further study also showed CGs in four of five *Helicobacter* species (16). In the present study we analyzed the lipids and fatty acids of eight *Helicobacter* species; the whole-cell fatty acid profiles of two other species are also described for the first time. Besides this, we have also described a rapid and simple technique for the direct detection of CGs in whole cells.

MATERIALS AND METHODS

Strains and culture conditions. Strains from eight *Helicobacter* species were investigated for their lipid and the fatty acid profiles. *H. nemestrinae* (ATCC 49396) isolated from a macaque monkey; *H. acinonyx* (ATCC 51101) isolated from a cheetah; *H. canis* (CCUG32756) isolated from a dog; *Helicobacter* sp. strain CLO-3 (CCUG14564) and "*H. rappini*" (*Flexispira rappini*; CCUG23435) isolated from humans; and *H. pametensis* (CCUG29255), *Helicobacter* sp. strain Bird-B (CCUG29256), and *Helicobacter* sp. strain Bird-C (CCUG29261) isolated from birds were studied. Besides these, the fatty acid profiles of *H. pylori* (ATCC 43505), *H. felis* (ATCC 49179), *H. mustelae* (HM 180), *H. muridarum* (ATCC 49282), and *H. fennelliae* (NCTC11613) were also analyzed and compared. *H. felis*, *H. mustelae*, *H. muridarum*, and *H. fennelliae* were received from Adrian

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The bacteria were grown on brain heart infusion agar supplemented with 5% horse blood at 37°C under microaerophilic conditions (AnaeroPack Campylo; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). For *Helicobacter* sp. strain CLO-3 and *H. fennelliae*, a GasPak Anaerobic System Envelope (BBL, Cockeysville, Md.) was used without a catalyst. To obtain a large mass of whole cells, the bacteria were cultured and processed as described previously (16). All bacteria were grown to the late logarithmic phase, when almost all the cells were either curved or spiral shaped and the coccoid form was minimal.

Extraction, purification, and identification of lipids. Lipid analyses were carried out according to the methods of Kates (23). Briefly, lipids were extracted from the washed whole cells obtained by centrifugation by stirring three times in chloroform-methanol (2:1 [vol/vol]), and each time the cells were separated by filtration. The filtrates were accumulated together, purified by the method of Folch et al. (8), and used as total extractable lipid (TEL).

For thin-layer chromatography (TLC) Silica Gel 60 G (E. Merck AG, Darmstadt, Germany) plates were used and developed with chloroform-methanol-water (70:30:5 [vol/vol/vol]) for one-dimensional TLC and with chloroform-methanol-water (65:25:4 [vol/vol/vol]) and chloroform–methanol–7 N ammonia (60:35:4.5 [vol/vol/vol]) in the first and the second directions, respectively, for two-dimensional TLC. For the neutral lipids the plates were developed with petroleum ether-diethyl ether-acetic acid (41:18:2 [vol/vol/vol]). Individual lipids were identified as described previously (16).

Lipids were fractionated into neutral lipid, glycolipid, and phospholipid fractions by column chromatography using Iatrobeads 6RS-8060 (Iatron Lab. Inc., Tokyo, Japan) and successive elutions with 5 column volumes of the following solvents: chloroform, chloroform-acetone (4:1 and 1:1 [vol/vol]), acetone-methanol (3:1 [vol/vol]), and methanol. The glycolipids, present in the chloroform-acetone (4:1 and 1:1 [vol/vol]) and acetone-methanol (3:1 [vol/vol]) fractions, were purified by one-dimensional TLC. The phosphorus values of the individual phospholipids were estimated by the methods of Kates (23). Briefly, the lipids were chromatographed two dimensionally as described above and the individual phospholipid spots were visualized with iodine vapor. Areas corresponding to the individual spots were scraped off, digested with perchloric acid on a Kjeldahl digestion rack, and then treated with molybdic acid and amidol reagents. The color changes were quantified.

Fatty acid, sugar, and sterol analysis. Fatty acids of the whole cells were analyzed by the method of Hotta et al. (20). Briefly, the whole cells were treated with 15% NaOH in 50% methanol at 95°C for 20 h and then the mixture was acidified to pH 2.0 with 6 N HCl. The fatty acids were extracted with hexane, evaporated to dryness, and subjected to methylation with diazomethane for 30 min.

For analyses of the sugars and fatty acids of the TELs, acid methanolysis was done at 95°C for 1 h with 5% hydrogen chloride in methanol (Wako Chemicals, Osaka, Japan). On cooling, the fatty acid methyl esters were extracted with hexane and the lower methanol fractions containing the methylated sugars were trimethylsilylated as described previously (18).

The trimethylsilyl methyl glycosides and the fatty acid methyl esters were analyzed on a G-3000 gas chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with a flame ionization detector and a Hitachi 883 Data Processor using a silicone OV-1 101 column (25 m; 0.53-mm inside diameter) with temperature programming (180 to 230°C).

The peaks were identified by comparing their retention times and patterns (for sugar) with those of the standards. The fatty acids were confirmed by mass spectrometry.

The sterol components were identified by comparing the R_f values of the sterols liberated by acid methanolysis (hexane extracts) with those of authentic samples as described previously (18).

Spectroscopy. Combined gas chromatography-mass spectrometry of the fatty acid methyl esters was carried out on a GCMS9020-DF gas chromatograph-mass spectrometer (Shimadzu Corp., Kyoto, Japan) equipped with a silicone OV-1 glass column (3 mm [internal diameter] by 2 m; 180 to 230°C). The electron mass spectra were recorded at an electron energy of 70 eV.

Fast atom bombardment-mass spectrometry of the glycolipids was performed with a GCMS9020-DF gas chromatograph-mass spectrometer equipped with a tungsten filament and operated at an acceleration potential of 5 kV (fragments accelerated at 3 kV) and a data processing system (SCAP-1123; Shimadzu Corp.). Nitrobenzyl alcohol or glycerol was used as the matrix.

Estimation of cholesterol and cholesterol ester levels. Cholesterol and cholesterol ester levels in neutral lipids were estimated by the enzymatic method of Allain et al. (1) using Boehringer Mannheim GmbH (Mannheim, Germany) kits.

Detection of CGs in whole cells. CGs in whole cells were directly detected by a method described previously by Matsuyama et al. (31) for the identification of lipids of *Serratia marcescens*. The cells were applied to a spot on a TLC plate, air dried, and developed up to one-third of the plate with chloroform-methanol (2:1 [vol/vol]). The plate was dried, redeveloped with chloroform-methanol-water (70:30:5 [vol/vol/vol]) in the same direction, sprayed with orcinol-sulfuric acid reagent (0.2% orcinol in 2 N sulfuric acid) (42), and heated at 120°C until the appearance of purple-red color for the CGs was observed.

RESULTS

Fatty acid profile. Fatty acids of the whole cells and the TELs of the *Helicobacter* spp. are shown in Table 1. On the basis of the fatty acids, the species could be differentiated into two broad groups (groups A and B). Group A was characterized by the presence of 19-carbon cyclopropane fatty acid (19:0cyc) and tetradecanoic acid (14:0) as the major fatty acids; of these species, *H. pylori*, *H. felis*, *H. nemestrinae*, and *H. acinonyx*, having 3-hydroxy octadecanoic acid (3OH 18:0), were assigned to subgroup A1, and *H. mustelae*, having no 3OH 18:0 and having a sizeable proportion of hexadecanoic acid (16:0), was included in subgroup A2. Group B did not show 19:0cyc but had the characteristic presence of 16:0 and octadecenoic (18:1) acids as the major fatty acids and variable amounts of 14:0. This group could be further differentiated into three subgroups, B1, B2, and B3: B1 had 16:0 and 18:1 as the major fatty acids and included *H. canis*, *H. muridarum*, *Helicobacter* sp. strains Bird-B and -C, and *H. pametensis*; B2 had, in addition, a sizeable amount of dodecanoic acid (12:0) and only one hydroxy acid (3-hydroxy tetradecanoic acid) and included *H. muridarum* and "*H. rappini*"; and B3 had, in addition, 16-carbon aldehyde (16:0 Ald) and 16-carbon dimethylacetyl (16:0 Dma) and included *H. fennelliae* and *Helicobacter* sp. strain CLO-3. The hydroxy fatty acids, present in the whole cells, were not observed among the fatty acids of the TELs, and no other major difference between the fatty acid profiles of the whole cells and the TELs was observed.

Lipid profile. Variable lipid patterns were observed for the species studied. Most of the lipids could be clearly recognized by their chromatographic migration pattern and staining with different reagents; however, a few could not be identified. Two-dimensional thin-layer chromatograms of TELs of four representative *Helicobacter* species are shown in Fig. 1. In *Helicobacter* sp. strain Bird-C (Fig. 1d), an unknown phosphoaminolipid was observed and diphosphatidylglycerol (DPG) was not observed. In addition, some unknown phospholipids were seen in this species (U-2, U-3, P-2, P-3, and P-4) and in "*H. rappini*" (P-1, P-2, and P-3 in Fig. 1c). *Helicobacter* sp. strain Bird-C and *Helicobacter* sp. strain CLO-3 (Fig. 1b) showed negligible amounts of phosphatidylserine. Among the phospholipids, phosphatidylethanolamine (Table 2) was the major one. High percentages of phosphatidylserine were observed in *H. nemestrinae* and *H. canis*. DPG was absent in the species of avian origin, for which phosphatidylglycerol levels were found to be higher than those in species containing DPG.

Steryl glycosides, demonstrating the same R_f values as the cholesteryl acyl glucoside (CAG), cholesteryl glucoside (CGL), and cholesteryl phosphatidyl glucoside (CPG) of *H. pylori* (18), were detected in seven of the eight species studied, and they were named G-1, G-2, and G-3, respectively. When the R_f values of the sterols, liberated by acid methanolysis, were compared with those of the authentic samples, the sterol component was identified as cholesterol, and on the basis of the gas chromatographic pattern, glucose was found to be the only sugar component (data not shown). The steryl glucosides were therefore identified as CGs.

The fatty acid compositions of the CGs are shown in Table 3. G-1 of *H. nemestrinae* had 14:0 as the sole fatty acid, whereas the G-1 of "*H. rappini*" had 12:0 as the predominant one. G-3s of different species showed fatty acid profiles similar to those of the corresponding TELs.

For the determination of molecular weights, fast atom bombardment-mass spectrometric analyses of G-1s, G-2s, and deacylated G-3s of different species were carried out. The G-2s of all the species had a quasimolecular mass ($[M + Na]^+$) of

TABLE 1. Fatty acid compositions of whole cells and TELs of *Helicobacter* spp.

Species	Group	TEL or WC[a]	Fatty acid composition (% of total fatty acids)[b]												
			12:0	14:0	16:0	16:1	18:0	18:1	18:2	19:0cyc	16:0 Ald	16:0 Dma	3OH 14:0	3OH 16:0	3OH 18:0
H. pylori	A1	TEL	—[c]	49	3	—	2	10	2	33	—	—	—	—	—
		WC	—	38	2	—	6	7	—	35	—	—	1	3	6
H. felis	A1	TEL	—	61	6	—	8	2	8	11	—	—	—	—	—
		WC	1	53	6	—	8	9	4	13	—	—	—	2	3
H. nemestrinae	A1	TEL	—	40	10	—	2	16	1	30	—	—	—	—	—
		WC	—	36	8	—	5	13	—	27	—	—	1	3	5
H. acinonyx	A1	TEL	—	35	13	—	25	4	5	17	—	—	—	—	—
		WC	—	41	5	—	14	2	1	29	—	—	—	3	5
H. mustelae	A2	TEL	—	22	40	1	3	2	4	22	—	—	—	—	—
		WC	—	20	33	1	—	2	6	26	—	—	3	5	—
H. canis	B1	TEL	—	9	40	1	1	48	1	—	—	—	—	—	—
		WC	—	23	29	2	1	43	—	—	—	—	1	1	—
Helicobacter sp. strain Bird-C	B1	TEL	—	25	31	—	3	37	2	—	—	—	—	—	—
		WC	—	28	31	2	2	32	1	—	—	—	1	3	—
Helicobacter sp. strain Bird-B	B1	TEL	—	5	37	1	13	41	3	—	—	—	—	—	—
		WC	—	23	36	1	7	28	1	—	—	—	1	3	—
H. pametensis	B1	TEL	—	11	33	—	8	42	6	—	—	—	—	—	—
		WC	2	15	31	—	11	25	7	—	—	—	4	3	—
H. muridarum	B2	TEL	6	17	34	—	3	37	2	—	—	—	—	—	—
		WC	11	23	37	—	3	22	—	—	—	—	3	—	—
"*H. rappini*"	B2	TEL	12	11	37	2	4	30	4	—	—	—	—	—	—
		WC	27	8	31	2	2	19	2	—	—	—	7	—	—
Helicobacter sp. strain CLO-3	B3	TEL	—	11	31	—	6	40	—	—	2	8	—	—	—
		WC	—	15	33	—	10	27	1	—	1	4	2	4	—
H. fennelliae	B3	TEL	—	8	24	—	3	44	4	—	2	12	—	—	—
		WC	—	12	27	—	3	39	2	—	2	5	2	4	—

[a] WC, whole cells.
[b] The number before the colon indicates the number of carbon atoms, and the number after the colon indicates the number of double bonds. cyc, cyclopropane; Ald, aldehyde; Dma, dimethylacetyl.
[c] —, not detected or less than 1%.

m/z 571, which is identical to that of the CGL of *H. pylori*. The $[M + Na]^+$ of the G-1 of *H. nemestrinae* was observed to be identical to that of the CAG of *H. pylori* (*m/z* 782). However, the $[M + Na]^+$ of the "*H. rappini*" CAG was found to be *m/z* 754, reflecting the presence of 12:0 as the major component. On ^1H nuclear magnetic resonance spectroscopic analysis of G-1, G-2, and deacylated G-3 and comparison of the results with those for the CAG, CGL, and deacylated CPG of *H. pylori*, respectively, the newly identified lipids were found to be identical to the corresponding ones of *H. pylori* (18) except for the fatty acids of "*H. rappini*." The anomeric configurations in all the CGs were found to be α.

From the observations presented above, it was apparent that the G-1s, G-2s, and G-3s, respectively, of the strains studied and their species distributions were as follows: cholesteryl-6-*O*-acyl-α-D-glucopyranoside (cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside in *H. nemestrinae* and mainly cholesteryl-6-*O*-dodecanoyl-α-D-glucopyranoside in "*H. rappini*"), cholesteryl-α-D-glucopyranoside (*H. nemestrinae*, *H. acinonyx*, *H. canis*, *Helicobacter* sp. strain CLO-3, and "*H. rappini*"), and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (*H. nemestrinae*, *H. acinonyx*, *H. canis*, and *Helicobacter* sp. strain CLO-3). The G-1 of *H. acinonyx*, the G-3 of "*H. rappini*," and the steryl glucosides of *Helicobacter* sp. strains Bird-B and -C could not be analyzed because only very small amounts were present. However, because of identical R_f values they were considered to be same as the corresponding CGs (CGL in *Helicobacter* sp. strains Bird-B and -C, CAG in *H. acinonyx*, and CPG in "*H. rappini*" and *Helicobacter* sp. strain Bird-C).

To estimate the amounts of CGs present in different species, the percent distributions (weight/weight) of the major lipids were determined (Table 4). A very large accumulation of CGs

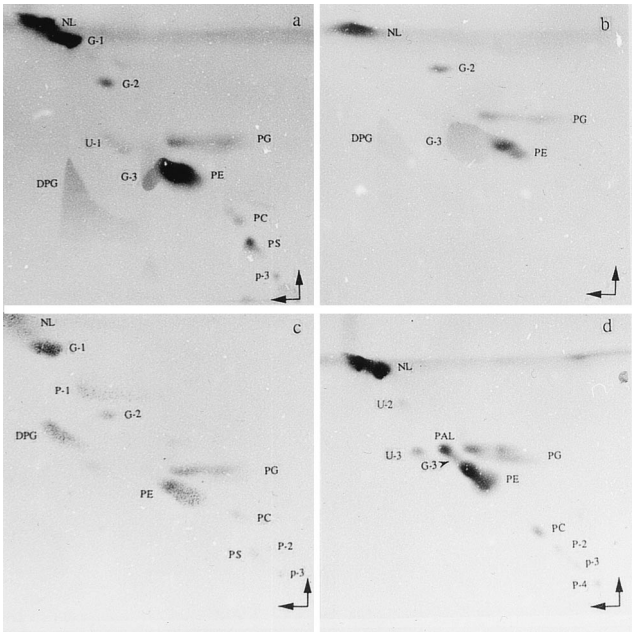

FIG. 1. Two-dimensional thin-layer chromatograms of TELs of *Helicobacter* spp. (a) *H. nemestrinae*; (b) *Helicobacter* sp. strain CLO-3; (c) "*H. rappini*"; (d) *Helicobacter* sp. strain Bird-C. NL, neutral lipid; G-1, CAG; G-2, CG; G-3, CPG; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PAL, phosphoaminolipid; U-1, -2, and -3, unknown lipids 1, 2, and 3 (these lipids are phosphorus negative); P-1, -2, -3, and -4, unknown phospholipids 1, 2, 3, and 4. The plates were stained with 30% sulfuric acid.

TABLE 2. Phospholipid distribution in *Helicobacter* spp.

Species	% of phosphorus in[a]:						
	PE	PG	DPG	PS	PC	CPG	Other phospholipids
H. nemestrinae	55.8	10.8	17.4	10.1	1.2	3.0	1.7
H. acinonyx	44.0	11.7	15.8	1.7	6.6	11.0	9.2
H. canis	45.7	10.1	16.8	8.0	2.0	13.8	3.6
Helicobacter sp. strain CLO-3	54.1	15.5	17.4	1.3	T	10.9	T
"*H. rappini*" (*Flexispira rappini*)	49.2	17.0	17.3	2.1	1.5		12.9
Helicobacter sp. strain Bird-C	57.4	34.3		2.8	3.3		1.8
Helicobacter sp. strain Bird-B	63.1	21.7			8.5		6.7
H. pametensis	53.0	25.6		4.2	5.9		11.3

[a] PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; T, less than 1%.

(9.7 to 27.4% of the weight of TEL) was observed in all the species studied except those of avian origin, in which very little (*Helicobacter* sp. strains Bird-B and -C) or no (*H. pametensis*) CG but a large accumulation of neutral lipid was observed.

On analysis of the neutral lipids, a large accumulation of free cholesterol was observed in all of the species studied (Table 5). The compounds containing cholesterol moieties (free cholesterol, cholesterol esters, and CGs) are the next major lipids after the phospholipids, varying from about 10.0 to 41.4% of the weight of TEL (CGs in Table 4 plus total cholesterol in Table 5) in different *Helicobacter* spp.

Direct detection of CGs in whole cells. We could also directly detect the CGs in the whole cells and compare their R_f values with those of the already identified CGs of *H. pylori* (Fig. 2). Variable combinations of CGs in different *Helicobacter* spp. were shown in one-dimensional TLC of TELs (Fig. 2a) and whole cells (Fig. 2b). The CGs in whole cells of different species showed R_f values identical to those of the CGL, CAG, and CPG of *H. pylori.*

DISCUSSION

The *Helicobacter* spp. have shown a number of phenotypic traits which differentiate them from other related bacteria. Their fatty acid profiles show differences from those of the campylobacters, members of the family *Vibrionaceae*, and related bacteria (25). Lipopolysaccharide of the type species, *H. pylori*, shows obvious differences (i.e., it has 3-OH18:0 as its major fatty acid and also has 18:0) from that of the other related bacteria (12). Also, we found unique CGs in *H. pylori* (18) and four other *Helicobacter* species (16).

In this report, the whole-cell and TEL fatty acid profiles of eight species have been described for the first time. The fatty acid profiles of the rest of the species studied (*H. pylori*, *H. mustelae*, *H. felis*, *H. nemestrinae*, *H. fennelliae*, and *Helicobacter* sp. strain CLO-3) were almost similar to those of earlier reports (14, 25), except for *H. mustelae* (HM-180), which showed a much lower percentage of 18:1 (2% in this study compared with 17 to 23% in the earlier report [14]). A heterologous pattern of fatty acids was observed among the *Helicobacter* spp., and because of this they could be differentiated into two broad groups. Group A showed a high proportion of 19:0cyc and a low proportion of 18:1, whereas group B showed a high proportion of 18:1 and an absence of 19:0cyc. Since all the bacteria were grown to the same stage of the logarithmic phase and since cyclopropane fatty acids are formed by the methylation of monounsaturated fatty acid (for example, 18:1 is converted to 19:0cyc) (38), group B species are likely to have a defect in the conversion of 18:1 to 19:0cyc.

The species of group A have been primarily isolated from the gastric mucosa, while those of group B have been isolated primarily from the lower gastrointestinal tract. The presence of such a high proportion of 14:0 and 19:0cyc in group A is unique to the helicobacters and can help to differentiate them from closely related bacteria like campylobacters, species of *Wolinella*, and members of the *Vibrionaceae* (14, 24, 25). Because of the increasing numbers of *Helicobacter* spp. being identified and their quite diverse phenotypic characteristics, it may be necessary to subgroup the helicobacters on the basis of some definite characteristics, and the fatty acids appear to be a good candidate.

Besides their chemotaxonomic importance, the fatty acids may have some pathophysiological significance. In vitro studies have shown that 14:0 and 19:0cyc fatty acids show antisecretory effects on parietal cells by blocking H^+/K^+ ATPase activity and also by a detergent action (3). This effect is also seen with *H. pylori* (i.e., with intact organisms, sonicates, methanolic extracts, and extracts from the culture medium) (3). It is therefore likely that the above-mentioned fatty acids have in vivo

TABLE 3. Fatty acid compositions of CGs of *Helicobacter* spp.

Species	CG[a]	Fatty acid composition (% of total fatty acids)[b]										
		12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:2	19:0cyc	16:0 Ald	16:0 Dma
H. nemestrinae	G-1	—[c]	100	—	—	—	—	—	—	—	—	—
	G-3	—	29	—	3	—	13	12	—	44	—	—
H. acinonyx	G-3	—	35	—	2	—	9	9	—	43	—	—
H. canis	G-3	—	21	—	21	3	—	53	—	—	—	—
Helicobacter sp. strain CLO-3	G-3	—	7	—	42	—	2	34	—	—	2	9
"*H. rappini*"	G-1	84	8	—	5	—	—	3	—	—	—	—

[a] G-1, CAG; G-3, CPG.
[b] The number before the colon indicates the number of carbon atoms, and the numbers after the colon indicates the number of double bonds. cyc, cyclopropane; Ald, aldehyde; Dma, dimethylacetyl.
[c] —, not detected or less than 1%.

TABLE 4. Percent distribution of lipids in *Helicobacter* spp.

Species	% Distribution of lipids (wt/wt)					
	Neutral lipids	CGs				Phospholipid
		CGL	CAG	CPG	Total	
H. nemestrinae	11.4	2.5	20.4	4.5	27.4	61.1
H. acinonyx	16.4	3.5	T[a]	6.1	9.7	74.0
H. canis	11.3	0.5		13.1	13.6	87.0
Helicobacter sp. strain CLO-3	8.1	2.1		20.0	22.1	69.8
"*H. rappini*" (*Flexispira rappini*)	4.1	2.0	23.1	T	25.3	69.6
Helicobacter sp. strain Bird-C	22.4	T		0.9	1.1	76.4
Helicobacter sp. strain Bird-B	23.1	0.5			0.5	76.4
H. pametensis	20.0					80.0

[a] T, less than 0.5%.

inhibitory effects on the parietal cells. The functional significance of the fatty acids of group B is not clear. However, *H. muridarum* and "*H. rappini*" of group B2 selectively incorporated 12:0 into the CAG and "*H. rappini*" shows morphological differences from other helicobacters (25). The species included in group B3 showed 16:0 Ald and 16:0 Dma, which are associated with the plasmalogen moiety found in some anaerobic bacteria (25). These species showed better growth with the BBL GasPak Anaerobic System Envelope without a catalyst than with the AnaeroPack Campylo system (Mitsubishi Gas Chemical Company, Inc.) used for the isolation of campylobacters.

Since all the bacteria were grown to the late logarithmic phase, the absence of DPG in the avian species shows some sort of defect in the conversion of phosphatidylglycerol to DPG. This is why phosphatidylglycerol is found to be present in these species at levels in excess of those observed for other bacteria. Phosphatidylcholine, although not commonly seen in bacteria, was observed in almost all the species. The phosphatidylcholine observed in the *Helicobacter* spp. may have been a contaminant from the serum, as in serum-free cultures of *H. pylori* and *H. mustelae* phosphatidylcholine was not found (15).

The present study shows the presence of CGs in most of the species studied. This study, along with the previous studies, shows that of the 13 *Helicobacter* species studied so far, 11 contain CGs (16, 18). Furthermore, the culture media supplemented with horse serum did not show any CG, even though it has large amounts of cholesterol and cholesterol esters and all the species except *H. cinaedi* and the ones of avian origin showed a very high percentage of CGs (9.7 to 33.1%) (16).

TABLE 5. Percent distribution of cholesterol in *Helicobacter* spp.

Species (NL as % of TEL)[a]	Amt of TC as[b]:		% of TC present as:	
	% of TEL	% of NL	Free cholesterol	Cholesterol ester
H. nemestrinae (11.4)	1.8	15.9	88.8	11.2
H. acinonyx (16.4)	5.5	33.6	70.2	29.8
H. canis (11.3)	1.0	8.4	81.4	18.6
Helicobacter sp. strain CLO-3 (8.1)	1.6	20.1	85.1	14.9
"*H. rappini*" (*Flexispira rappini*) (4.1)	0.6	16.2	92.1	7.9
Helicobacter sp. strain Bird-C (22.4)	12.8	57.3	80.5	19.5
Helicobacter sp. strain Bird-B (23.1)	9.5	41.1	91.0	9.0
H. pametensis (20.0)	11.6	58.2	91.6	9.4

[a] NL, neutral lipid.
[b] TC, total cholesterol.

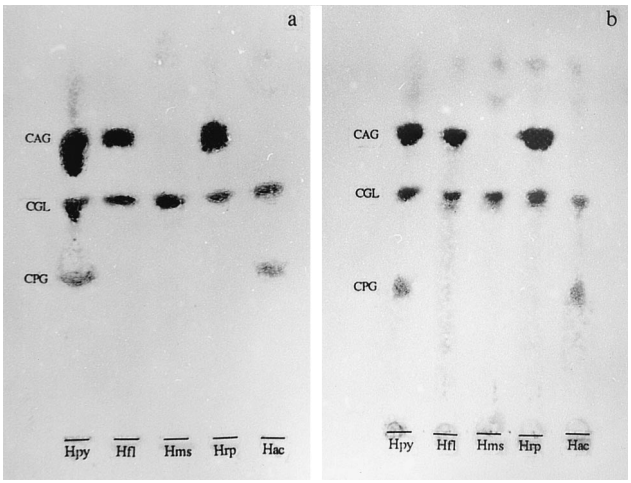

FIG. 2. One-dimensional thin-layer chromatograms showing the CG profiles of TELs (a) and whole cells (b) of *Helicobacter* spp. Hpy, *H. pylori*; Hfl, *H. felis*; Hms, *H. mustelae*; Hrp, "*H. rappini*"; Hac, *H. acinonyx*. The CGs (components containing sugars) were visualized only with orcinol-sulfuric acid stain.

These CGs appear to be synthesized by the selective de novo uptake of cholesterol from the medium, as trace amounts of CGs could be observed in the cultures of *H. pylori* in cholesterol-deficient serum-free media (lacking detectable amounts of cholesterol) (15).

The presence of CGs in most of the *Helicobacter* spp. studied is an unusual characteristic, and that is why it can be considered an important phenotypic marker of the genus. This is particularly important because closely related bacteria such as the campylobacters, species of *Wolinella*, and members of the *Vibrionaceae* do not have CGs (personal observations). The few other prokaryotic species demonstrating CGs have genotypic and phenotypic characteristics very different from those of the helicobacters. It may be mentioned that among the prokaryotes steryl glycosides have been reported only for mycoplasmas (*Mycoplasma gallinarum* [39]), spiroplasmas (*Spiroplasma citri* [36]), acholeplasmas (*Acholeplasma laidlawii* [32] and *Acholeplasma axanthum* [32]), and spirochetes (*Borrelia hermsii* [29]). The CGs, therefore, can be of assistance in the confirmation and differentiation of the *Helicobacter* species. This is particularly important because *Helicobacter*, *Arcobacter*, *Campylobacter*, and *Wolinella* spp. are inert to most of the biochemical tests used for the identification and differentiation of bacteria and there is a paucity of such tests for phenotypic differentiation. This makes it increasingly difficult to identify the species by traditional biochemical tests (5). In this report, a simple and rapid TLC-based test for the direct detection of CGs from whole cells, taking only about 30 min, has been described. As CGs have been found to be a characteristic feature of the *Helicobacter* spp., their presence and profiles can be used as an additional test for the biochemical differentiation of these species.

High levels of CGs have been found in all the investigated species colonizing the stomach. Hemolytic activities have been observed with the CGs of *H. pylori* (18), and these are likely to have some cytopathic effect on gastric parietal cells. Besides this, 14:0, having antisecretory effects on parietal cells, has been selectively accumulated in the CAGs of *H. pylori*, *H. felis*, and *H. nemestrinae* primarily inhabiting the stomach (16, 18). The CGs may therefore have some supportive role in the adaptation of the *Helicobacter* spp. to the unique gastric microenvironment. Because of the presence of a glucose utiliza-

tion mechanism in *H. pylori* (33) the CGs may also be involved in some way with its metabolism.

There are large accumulations of free cholesterol (70% and above) in the neutral lipids of the helicobacters studied, whereas the neutral lipid fraction of horse serum added to the culture media has about 25% free cholesterol and 75% cholesterol esters (16). Similar high percentages of free cholesterol were observed in other *Helicobacter* spp., compared with only about 17% free cholesterol in *Escherichia coli* (16), which clearly shows a selective accumulation of free cholesterol in the helicobacters. Such a selective accumulation of free cholesterol was also observed in some of the parasitic mycoplasmas (2). The reason behind this is not clear. However, it may have something to do with the biosynthesis of the CGs present in high percentages in the bacterial membrane (15).

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Takashi Yoshida, Department of Pharmacognosy, Faculty of Pharmaceutical Science, Okayama University, for the nuclear magnetic resonance analysis. We also thank Tatsuji Yasuda and Shinobu Watarai, Department of Cell Chemistry, Okayama University Medical School, for their valuable suggestions during the study. Thanks are due to Yukiko Nishiuchi, Department of Bacteriology, Osaka City Medical School, for her technical assistance. We also thank Anwarul Haq, Department of Microbiology, University of Maryland, College Park, Md., for his assistance in obtaining strains from the American Type Culture Collection.

This research was supported in part by grants from Ohyama Health Foundation and the Ministry of Education, Science and Culture of Japan. M.H. was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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