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Lipid profiles of Helicobacter pylori and Helicobacter mustelae grown in serum-supplemented and serum-free media.

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Abstract

Many of Helicobacter species have been found to have novel cholesteryl glucosides (CGs). To study the biosynthetic mechanism of CGs, the lipid profiles of H. pylori and H. mustelae grown in serum-supplemented and cholesterol-restricted serum-free media were investigated. In contrast to the serum-supplemented state, helicobacters had less CGs in the serum-free state; a trace amount of CGs and no CG was detected in H. pylori and H. mustelae, respectively. The proportion of total and individual phospholipid also showed significant alteration. Unknown lipids which did not contain phosphate and sugar were detected in the serum-free state, but not in the serum-supplemented state. The CGs were found to be distributed mainly in the membrane fractions, and one of the unknown lipids was found exclusively in the cytosol fraction. Based on these data, it is apparent that the CGs of helicobacters are synthesized by de novo uptake of cholesterol from the media. The unknown lipids detected in the serum-free state may be storage lipids, appearing in response to depletion of nutrients, especially cholesterol, or other factors in the media.

KEYWORDS: Helicobacter, steryl glycoside, cholesteryl glucoside

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Lipid Profiles of *Helicobacter pylori* and *Helicobacter mustelae* Grown in Serum-Supplemented and Serum-Free Media

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Many of *Helicobacter* species have been found to have novel cholesteryl glucosides (CGs). To study the biosynthetic mechanism of CGs, the lipid profiles of *H. pylori* and *H. mustelae* grown in serum-supplemented and cholesterol-restricted serum-free media were investigated. In contrast to the serum-supplemented state, helicobacters had less CGs in the serum-free state; a trace amount of CGs and no CG was detected in *H. pylori* and *H. mustelae*, respectively. The proportion of total and individual phospholipid also showed significant alteration. Unknown lipids which did not contain phosphate and sugar were detected in the serum-free state, but not in the serum-supplemented state. The CGs were found to be distributed mainly in the membrane fractions, and one of the unknown lipids was found exclusively in the cytosol fraction. Based on these data, it is apparent that the CGs of helicobacters are synthesized by *de novo* uptake of cholesterol from the media. The unknown lipids detected in the serum-free state may be storage lipids, appearing in response to depletion of nutrients, especially cholesterol, or other factors in the media.

Key words: *Helicobacter,* steryl glycoside, cholesteryl glucoside

In view of the causal role of *Helicobacter pylori* in chronic gastritis, its strong association with peptic ulcer disease and the accumulating evidence of its role in gastric carcinoma and gastric B cell lymphoma, *Helicobacter* has become a widely studied genus (1-4). In the absence of a suitable animal model of *H. pylori*, the non-human helicobacters, such as *H. mustelae* and *H. felis* have been used in animal studies and have contributed significant insight on the pathogenesis of *H. pylori* infection (5, 6). To biochemically characterize *H. pylori* and to identify any lipids of special interest in *H. pylori* (because of its unique colonization site), we studied the lipid profile and observed the presence of three kinds of glycolipids (cholesteryl glucosides, CGs): cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside (G-1), cholesteryl-α-D-glucopyranoside (G-2), cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside (G-3) (7).

Subsequent investigation has revealed that these unique CGs are present in other *Helicobacter* species as well (7). The presence of CG in *H. pylori* and other *Helicobacter* species is a unique feature, since among the prokaryotes, the presence of CGs have been reported only in *Mycoplasma* (8), *Spiroplasma* (*S. citri*) (9), *Acholeplasma* (*A. laidlawii* and *A. axanthum*) (10) and spirochetes (*Borrelia hermsii*) (11).

To study the biosynthetic mechanism of these CGs, we made a comparative study on the lipid profile of *H. pylori* and *H. mustelae* grown in media with or without serum.

Materials and Methods

Bacterial Strains and Culture Conditions

H. pylori (ATCC43504) and *H. mustelae* (HM 180), isolated from the ferret, were used for the study. *H. mustelae* was obtained through the courtesy of Dr. Adrian Lee, School of Microbiology and Immunology, University of New South Wales, New South Wales, Australia.

For the serum-supplemented culture, strains were grown on brain heart infusion agar supplemented with 5 % horse blood and for the serum-free culture, the strains were grown on PPLO agar without crystal violet (Difco

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Laboratories, Detroit, MI, USA) supplemented with 0.1 % dimethyl β-cyclodextrin (Teijin Limited, Tokyo, Japan) at 37°C under microaerophilic condition produced with Anaero Pack Campylo in a jar (Mitsubishi Gas Chemical Company, Inc., Osaka, Japan).

For the serum-supplemented liquid-culture, the strains were grown in brain heart infusion broth (BHIB, 200 ml in a 500-ml flask) supplemented with 5 % horse serum; for the serum-free liquid culture, the strains were grown in PPLO broth without crystal violet supplemented with 0.1 % dimethyl β-cyclodextrin. The flasks were placed in a gas jar containing Anaero Pak Campylo, incubated at 37°C and shaken at 100 rpm for 48 h. The broth cultures were harvested in the late exponential phase and washed three times with PBS (pH 7.2) by centrifugation at 10,000 × *g* for 10 min at 4°C, and the cell pellets were stored at −20°C until the extraction of lipids.

Fractionation of Cells

Accumulated frozen cell pellets were resuspended in Tris-HCl, pH 8.0 with 5 mM EDTA at a concentration of 100 mg/ml and were sonicated on ice with a sonicator (Heat-Systems Ultrasonic, model W380, Farmingdale, NY, USA) for 10 times with 30 s of sonication followed by 30 s of rest. The cell suspension was then centrifuged at 500 × *g* for 20 min to remove the whole cells, and the supernatant was centrifuged at 110,000 × *g* at 4°C for 1 h (55P-7 ultracentrifuge, Hitachi, Tokyo, Japan). The supernatant containing the cytosol fraction was collected and freeze-dried for the extraction of cytosol lipid; the pellet was resuspended in the above buffer, the centrifugation was repeated, and the pellet was used as the membrane fraction. The lipids from the cytosol and the membrane fractions were extracted as described below.

Lipid Analysis

Lipid extraction. Lipids were extracted by suspending and magnetically stirring the bacterial-cells successively in 20 volumes (w/v) of chloroform-methanol (1:1, v/v) for 1 h and chloroform-methanol (2:1, v/v) overnight followed by 1 h at room temperature. The filtrates from each step were accumulated together and evaporated to dryness under vacuum on a rotary evaporator.

The extracted lipids were purified by the method of Folch and Lees (12), which were used as total lipids. Briefly, the lipids were dissolved in chloroform-methanol (2:1, v/v) to a concentration of 2 mg/ml and equilibrated with 20 times (v/v) of 0.5 % (w/v) solution of sodium chloride in distilled water overnight in a glass tank. The upper water-methanol phase was removed by vacuum suction and the lower chloroform phase was taken in a flask, evaporated under vacuum on a rotary evaporator, dissolved in chloroform-methanol (2:1, v/v) to the desired concentration and stored at −20°C until use.

Thin-layer chromatography (TLC). The lipids were applied to the Silicagel 60 G (E Merck AG, Darmstadt, Germany) plates (0.35 mm thick) and were developed with chloroform-methanol-water (70:30:5, v/v) for one-dimensional TLC; with chloroform-methanol-water (65:25:4, v/v) in the first direction and chloroform-methanol-7N ammonia (60:35:4.5, v/v) in the second direction for the two-dimensional TLC. For the detection of glycolipid, orcin-sulfuric acid reagent (0.2 % orcinol in 2N sulfuric acid) was sprayed and charred at 120°C until the maximum purple color developed. Dittmer modified reagent was used to detect the phospholipids (13). On spraying the reagent, phospholipids appear as blue spots on a white background.

For the identification of each phospholipid, each spot which was positive with Dittmer modified reagent was characterized with ninhydrin reagent (14) for amino-lipids and Dragendorff reagent (15) for lipids containing choline. Finally, phospholipids on the TLC plate were identified by comparing their Rf values with those of authentic lipids on the two-dimensional TLC.

To study the neutral lipid profile, the Silicagel 60 G plate with the applied lipids was developed in petroleum ether-diethyl ether-acetic acid (41:18:2, v/v), sprayed with 40 % sulfuric acid and charred at 120°C for 1 h giving brown spots on a white background.

Fractionation of total lipids. The total lipids were fractionated into neutral lipid, glycolipid and phospholipid components by column chromatography using Iatrobeads 6RS-8060 (Iatron Lab. Inc., Tokyo, Japan) (7). Iatrobeads 6RS-8060 (1 g/25 mg of lipid) were packed in a glass column (1 × 30 cm) and the total lipids in chloroform solutions (10 mg/ml, total 50 mg of lipid) were applied. The lipids were eluted successively with 5 × column volumes of the following solvents: chloroform, chloroform-acetone (4:1, v/v), chloroform-acetone (1:1, v/v), acetone-methanol (3:1, v/v) and methanol. The individual lipids were further analysed by one-dimensional TLC.

TLC-purification of fractionated lipids. The CGs contained in the chloroform-acetone (4:1 and 1:1, v/v) fractions and in the acetone-methanol (3:1, v/v) fraction were further fractionated and purified by one-dimensional TLC using the Silicagel 60 G plates. The

fraction, concentrated under vacuum in a rotary evaporator, was applied to the plates as streaks and was developed in the chloroform-methanol-water (70:30:5, v/v) solvent system. The separated bands were visualized by exposing the plates to iodine vapour and were marked. After elimination of the iodine under vacuum suction, the delineated bands were scrapped off, crushed to powder form and applied to a filtration funnel so as to form a small column bed on application of the solvents. The column was eluted with chloroform-methanol (1:1, v/v). The eluant was concentrated and filtered with a 0.45-μm teflon-coated filter (Sartorius, Götingen, Germany), and then evaporated to dryness.

Estimation of phosphorous value. For estimation of the distribution of the individual phospholipid components, the phosphorus content of individual phospholipids and the total lipids were estimated by the method of Kates (16). Individual phospholipids were obtained by scraping off the corresponding area from the two-dimensional TLC plates of total lipids.

Nuclear Magnetic Resonance

The lipids were dissolved in $CDCl_3$-CD_3OD (1:1, v/v) at the concentration of *ca.* 5 mg/ml. ^{1}H-Nuclear magnetic resonance was recorded on a Varian (Palo Alto, CA, USA) VXR500 spectroscope (500 MH2 for ^{1}H) at ambient temperature. Chemical shifts were recorded with respect to internal tetramethylsilane.

Results

Lipid profile. The comparative distribution of lipids in *H. pylori* and *H. mustelae* grown in serum-supplemented or serum-free media is shown in Fig. 1. In the serum-supplemented state, *H. pylori* showed the presence of cholesteryl glucoside (G-1, G-2, G-3) (Fig. 1a), whereas in the serum-free state it did not demonstrate the G-1, G-2, G-3 (Fig. 1b), but showed 2 unknown lipids (NLip 1 and NLip 2) which were not detected in the serum-supplemented state. Similarly, *H. mustelae* had only G-2 in serum-supplemented state (Fig. 1c) and in the serum-free state *H. mustelae* did not have G-2, but had an unknown lipid (NLip 1) (Fig. 1d). The NLip 1 was, however, overshadowed by the neutral lipid (NL) fraction. The presence of the unknown lipids, distinct from the neutral lipid, is shown in Fig. 2.

On fractionation of the total lipid with the column chromatography, solvents for elution and eluting lipids were as follows: a) chloroform-neutral lipids, b) chloroform:acetone (4:1, v/v)-G-1, c) chloroform:acetone (1:1, v/v)-G-2, NLip 1 & 2, d) acetone:methanol (3:1, v/v)-G-3 & phospholipids, e) methanol-phospholipids. The composition of lipids of *H. pylori* and *H. mustelae* is shown in Table 1. In the serum-supplemented state of *H. pylori* and *H. mustelae,* the CGs accounted for 25.5 % and 14.8 % of total lipid, respectively, and the phospholipids accounted for 67.5 % (75.7 %, including G-3 phosphoglycolipid) and 66.0 %, respectively. On the other hand, in the serum-free state, *H. pylori* and *H. mustelae* had 92.2 % and 93.4 % of phospholipids, respectively. The CGs were not observed in TLC of total lipids in the serum-free state of *H. pylori* and *H. mustelae*. However, TLC of concentrated lipids of the fractions (fractions b, c and e) of *H. pylori* showed trace amount of CGs. *H. mustelae* did not show any CG even in TLC of the fractionated lipids. The NL percentage showed a marked decline in the serum-free culture. In contrast to the NL from the serum-supplemented cultures, of which about 70 % was cholesterol and cholesterol ester, the NL from the serum-free cultures showed no detectable cholesterol or related compounds, but contained mostly fatty acids. The two unknown lipids in the serum-free state of *H. pylori* and *H. mustelae* accounted for 1.5 % and 1.6 % of total lipids, respectively. NLip 1, the major one, was phosphorous negative and showed purple coloration on charring with orcin-sulfuric acid reagent. However, on purification and ^{1}H-NMR analysis no sugar component was observed.

The composition of phospholipids in *H. pylori* and *H. mustelae* is shown in Table 2. Phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), diphosphatidyl glycerol (DPG) and phosphatidyl serine (PS) were the major

Table 1 Lipid composition of *Helicobacter pylori* and *Helicobacter mustelae* grown in serum-supplemented and serum-free media (% of total lipids)

Lipids	*H. pylori*		*H. musteleae*	
	With serum	Without serum	With serum	Without serum
Neutral lipids	7.0	6.3	19.2	5.0
Phospholipids	67.5(75.7*)	92.2	66.0	93.4
Cholesteryl glucosides	25.5	T	14.8	ND
Unknown lipids	ND	1.5	ND	1.6

*, Including clolesteryl phosphatidyl glucoside (G-3).
T, trace. ND, not detected.

phospholipids in the serum-supplemented state of *H. pylori*; the serum-free state had a similar pattern, however the proportions of PE and PG were increased, and the PS concentration was decreased. Phosphatidyl choline (PC) and other phospholipids were not observed.

In *H. mustelae*, PE and PG were the major phospholipids and DPG was not detected. In the serum-supplemented state the PE:PG ratio was 3:1, and in the

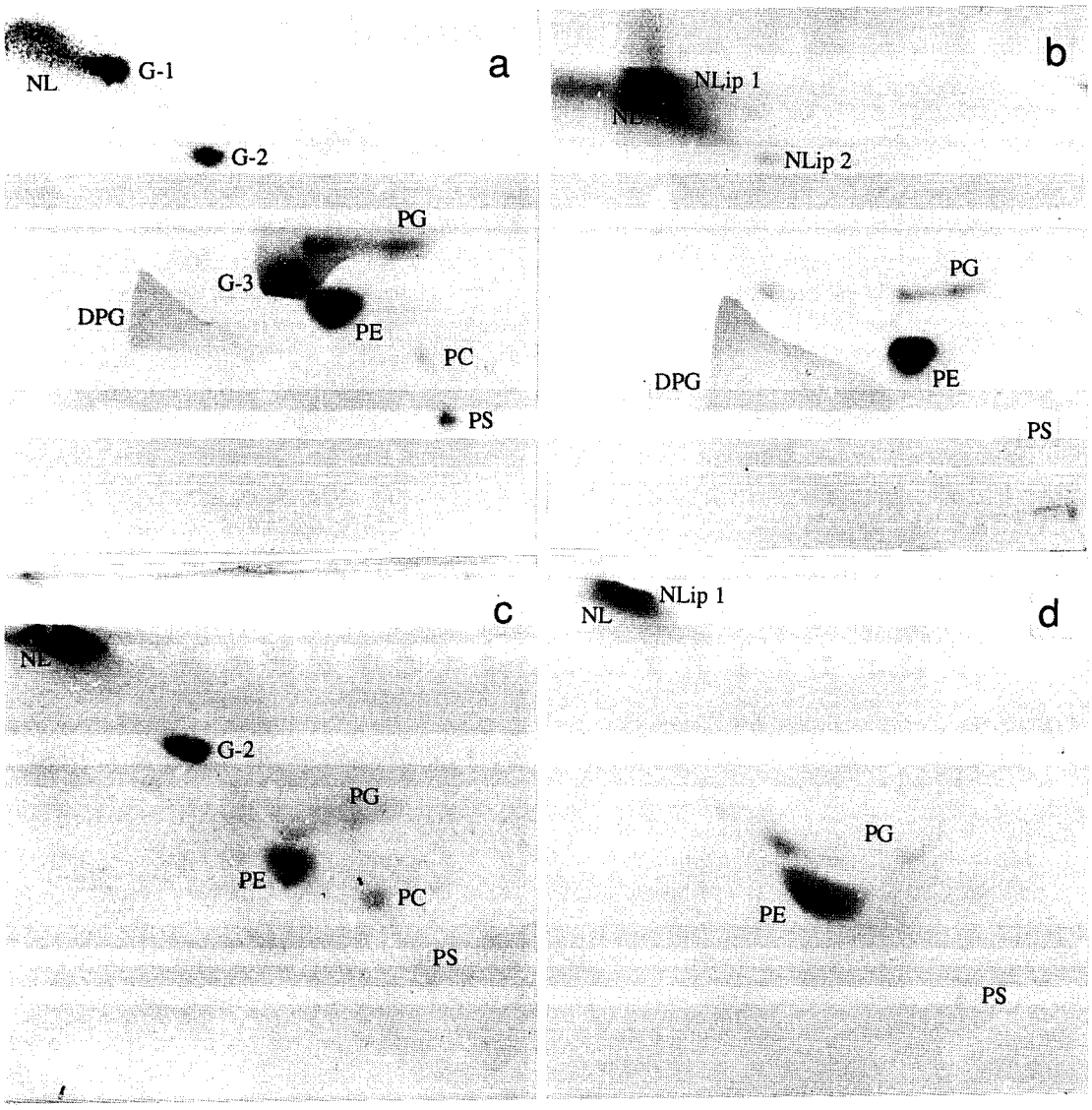

Fig. 1 Two-dimensional thin-layer chromatography of total lipids from *H. pylori* and *H. mustelae* grown in serum-supplemented and serum-free media. The spots were visualized by 40% sulfuric acid.

a, *H. pylori* grown in serum-supplemented media; **b**, *H. pylori* grown in serum-free media; **c**, *H. mustelae* grown in serum-supplemented media; **d**, *H. mustelae* grown in serum-free media; NL, neutral lipid; NLip 1, unknown lipid 1; NLip 2, unknown lipid 2; G-1, cholesteryl acyl glucoside; G-2, cholesteryl glucoside; G-3, cholesteryl phosphatidyl glucoside; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; PS, phosphatidyl serine; and PC, phosphatidyl choline.

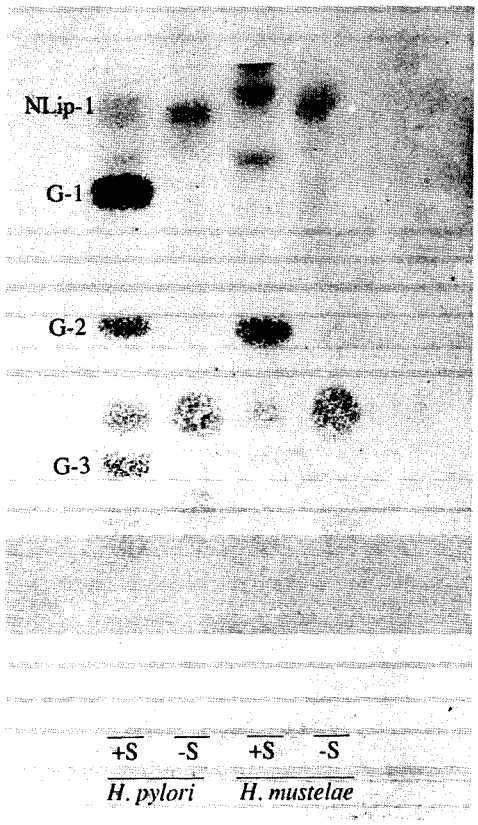

Fig. 2 One-dimensional thin-layer chromatography of total extractable lipids from *H. pylori* and *H. mustelae* grown in serum-supplemented and serum-free media. The spots were visualized by orcin-sulfuric acid reagent. NLip 1, unknown lipid 1; G-1, cholesteryl acyl glucoside; G-2, cholesteryl glucoside; G-3, cholesteryl phosphatidyl glucoside; + S, serum-supplemented media; and − S, serum-free media.

serum-free state, because of the marked increase in PE and the drop in PG, this was altered to 15:1. PS was also decreased, and PC and other lipids were not observed.

Cellular distribution of lipids. An attempt was made to study the cellular distribution of lipids of special interest (the CGs and the NLip 1) in *H. pylori*. The membrane and the cytosol fractions were separated. The cytosol accounted for only 2.0 and 2.9 % of total lipids in the serum-supplemented and serum-free states, respectively; the rest were membrane lipids. In the serum-supplemented state G-1, G-2 and G-3 were detected mainly in the membrane fraction, but a small amount was detected in the cytosol fraction. In the serum-free state, the NLip 1 was detected exclusively in the cytosol and trace amounts of CGs (G-1, G-2 and G-3) also became apparent in the membrane fraction.

Table 2 Phospholipid composition of *Helicobacter pylori* and *Helicobacter mustelae* grown in serum-supplemented and serum-free media (phosphorus %)

Phospholipids	*H. pylori*		*H. musteleae*	
	With serum	Without serum	With serum	Without serum
PE	55.0	61.6	69.0	92.7
PG	12.7	15.9	23.6	6.1
DPG	19.3	20.4	ND	ND
CPG	6.2	T	ND	ND
PC	2.2	ND	4.0	ND
PS	2.8	1.5	2.7	0.8
Other	1.8	0.6	0.7	0.4

PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; CPG, cholesteryl phosphatidyl glucoside (G-3); PC, phosphatidyl choline; and PS, phosphatidyl serine.
T, trace. ND, not detected.

Discussion

The presence of a significant amount of CGs in *H. pylori* and *H. mustelae* is noteworthy because of the likelihood that they are somehow involved in the colonization of the gastric mucosa, either in terms of the growth and metabolism of the bacteria or the adaptation of the bacteria to the environment of gastric mucosa. We have found hemolytic activity in the CGs of *H. pylori* (7). On the other hand, a detergent-like effect of C14:0 and C19:0 cyclopropane fatty acids have been shown to affect the parietal cell activities (17). The presence of hydrophilic and hydrophobic moieties on the CGs sugests that they may have some detergent-like activity, and it is conceivable that the mechanism of adaptation to gastric microenvironment could involve detergent-mediated modification of pariental cell activity. In view of the above, the CGs may provide an adaptational advantage to the helicobacters.

To study the biosynthetic mechanism of the CGs in *H. pylori*, because of its probable importance in adaptation to the gastric mucosa, we carried out the present comparative study of *H. pylori* and *H. mustelae* lipids from cells grown in serum-supplemented and serum-free media.

Usually, for the culture of helicobacters, an enriched media such as BHIB, brucella broth (BB), Trypticase Soy Broth (TSB) *etc.* supplemented with 3–5 % serum has been used. We screened the lipids extracted from these media and found the presence of cholesterol and cholesterol ester in BHIB, and cholesterol only in BB and TSB. In addition, serum is also a very rich source of cholesterol. Therefore, with these media it is difficult to see whether cholesterol is taken up *de novo* from the media or synthesized by the helicobacters. PPLO broth contained no detectable cholesterol or cholesterol esters. Furthermore, it has been reported that *H. pylori* can grow well in serum-free media supplemented with dimethyl β-cyclodextrin (18). Therefore, we used PPLO broth with 0.1 % dimethyl β-cyclodextrin as a serum-free media.

In the serum-free culture of *H. pylori*, trace amounts of G-1, G-2 and G-3 were detected, but not in that of *H. mustelae*. Although there was no detectable cholesterol in the PPLO broth, the detection of trace amounts of CGs in *H. pylori* indicates that it may be having a highly efficient cholesterol uptake system which can take up even trace amounts of cholesterol. *H. mustelae*, however, may be less efficient than *H. pylori* in this regard. The absence of a significant amount of CGs in lipids from serum-free culture shows that the CGs are synthesized by the *de novo* uptake of cholesterol from the media and that the bacteria do not have cholesterol-synthesizing machinery. Furthermore, CGs may not be essential for the growth of *H. pylori* and in the absence of these, *H. pylori* also possess sufficient proliferating potential.

Besides the noteworthy absence of detectable amounts of CGs in the total lipids of serum-free state, two unknown lipids, which were not detected in the serum-supplemented state, have been observed in *H. pylori*. It was suggested that one of the unknown lipids (NLip-1) may contain a sugar component because of the positive reaction with orcin-sulfuric acid reagent. However, no sugar component was observed on NMR analysis. Further structural analysis of this lipid is needed.

The phospholipid pattern of *H. mustelae* is reported for the first time. Unlike *H. pylori*, DPG could not be detected in *H. mustelae*. In the absence of serum, phospholipid is the major lipid (93.4 %) and a unknown lipid accounts for about 1.6 % of the extractable lipid.

PE showed an increase in both the serum-free culture, however, it was marked in the case of *H. mustelae*. PG showed an increase in *H. pylori* and a decrease in *H. mustelae*. Variation in the lipid composition in response to the variation in culture media has been reported previously (19–21). However, the increase in PE in the serum-free state is particularly noteworthy. PE has been shown to play a key role in the virulent characteristics of the strains of *Bacillus anthrax* and in the activation of enzymes in the biosynthesis of lipopolysaccharides of Gram-negative bacteria. Therefore, the increase in PE in the serum-free media may have something to do with the activation of the biosynthetic processes in the absence of nutrients from the serum.

To identify the pathophysiologic role of CGs and NLip 1, the cellular distribution of these two lipids were investigated in *H. pylori*. In the serum-supplemented culture CGs were found to be present mainly in the membranes. We tried to separate the membrane fraction into the inner and the outer membranes to investigate the localization of CGs in the membranes. However, as both the membranes remained closely adherent to each other even after density gradient centrifugation, it was not possible to separate the membrane fraction. Therefore, further localization of the CGs could not be done. Like us, another group has also found it difficult to separate the membrane fraction (22).

NLip 1 was distinctly found to be localized in the cytosol. The presence of this lipid only in the cytosol suggests that the compound may be one of the storage lipids, appearing in response to depletion of nutrients or other factor in the media.

In conclusion, it is apparent that the CGs of the helicobacters are synthesized by the selective *de novo* uptake of cholesterol from the media. These CGs may not be essential for the bacteria, but may provide adaptational advantage for colonization in the unique gastric microenvironment. The unknown lipids, seen in the serum-free cultures, may have arisen in response to its adaptation to the serum-deficient state. Studies are underway to characterize the structure of the unknown lipid.

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