表　　題　　ヘリコバクター属細菌の
ホスファチジルエタノールアミンの特性

論 文 の 区 分　　博士課程

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2016 年 1 月 8 日申請の学位論文

表題:

ヘリコバクター属細菌のホスファチジルエタノールアミンの特性

1. 新規合成プロゲステロン誘導体のピロリ菌に対する殺菌活性
2. フェリス菌およびシネジ菌のホスファチジルエタノールアミン

Title:

Characterization of phosphatidylethanolamine of Genus *Helicobacter*

1. **Bactericidal activity of a novel synthetic progesterone derivative against *Helicobacter pylori***
2. **Phosphatidylethanolamine isolated from *Helicobacter felis* and *Helicobacter cinaedi***

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Dissertation Submission:

January 8th, 2016

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ABSTRACT

Helicobacter pylori, a pathogen responsible for gastroduodenal diseases in human, retains myristoyl phosphatidylethanolamine (MPE) in the cell membranes. *H. pylori* MPE is an important glycerophospholipid concerned with the intramembranal absorption of steroids such as cholesterol and pregnenolone with a 3β-hydroxyl. Meanwhile, progesterone with a 3-oxo induces the bacteriolysis to *H. pylori*, but the bactericidal mechanism of progesterone to the bacterial cells has not been still clarified. This study demonstrated that a novel synthetic progesterone derivative 17α-hydroxyprogesterone linoleate (17hPL) confers the effective bacteriolytic action against *H. pylori*. MPE of this bacterium turned out to exhibit higher binding affinity for 17hPL than palmitoyl PE of *Escherichia coli* used as a prevalent PE species of Gram-negative bacteria. As correlated with the selective binding affinity of 17hPL for *H. pylori* MPE, this progestin selectively killed *H. pylori* and had no influence on the viability of other commonplace bacteria. On this basis, 17hPL was considered to bind to MPE in the cell membranes of *H. pylori*, to induce the destabilization of the membrane structures, and to ultimately elicit the bacteriolysis. One of the hormonal effects of progesterone is the inhibition of nitric oxide (NO) production from macrophages stimulated with lipopolysaccharide (LPS). We,

therefore, examined the capability of 17hPL to inhibit the NO production in murine macrophage-like cells activated by LPS. As such, 17hPL turned out to be relatively weaker in its capability to inhibit NO production in LPS-activated cells than progesterone. This suggested that hormonal effect of progesterone is attenuated by the modification of its steroid molecule with a long-chain unsaturated fatty acid. In addition, 17hPL was conspicuously lower in cytotoxicity against murine macrophage-like cells than progesterone. These results, taken together with the results of antibacterial action of 17hPL, demonstrated the possibility that progesterone is a fundamental structure for designing new steroidal antibacterial drugs for the eradication of *H. pylori* in human stomach.

INTRODUCTION

Helicobacter pylori is a Gram-negative curved rod that possesses polar flagella as the motility organs [1]. This bacterium requires microaerophilic atmosphere composed with 5% O_2, 10% CO_2, and 85% N_2 for the growth. *H. pylori* causes gastroduodenal diseases such as chronic gastritis and peptic ulcers in human and is concerned with the development of the gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [2-9]. This pathogen was recognized as a first class carcinogen by the International Agency for Research on Cancer (IARC) in 1994. Stomach of people in more than half of the world population has been colonized by *H. pylori*. Almost all persons carrying *H. pylori* develop asymptomatic gastritis, and approximately 10% individuals in persons carrying this pathogen develop peptic ulcers. In addition, *H. pylori* leads to the

development of both gastric cancer and gastric MALT lymphoma to about 1% and less than 0.1% individuals, respectively, in persons infected with this pathogen. It is, therefore, important to eradicate *H. pylori* in human stomach in order to avoid the development of these gastroduodenal diseases.

Broad spectral antibiotics such as amoxicillin and clarithromycin are used for the eradication of *H. pylori* in human stomach. The broad spectral antibiotics, however, have influence on the survival not only of *H. pylori*, but also of normal bacterial flora. Therefore, persons infected with *H. pylori* often suffer from stomatitis and loose bowels resulted from collapse of the balance of normal bacterial flora due to the taking of such antibiotics. On this basis, we need to develop new antibacterial substances that selectively act on *H. pylori* without collapsing the balance of normal bacterial flora in human. In addition, we have to resolve the problem of drug-resistance of *H. pylori* by developing antibacterial substances that act on this pathogen with a novel antibacterial mechanism [10, 11].

The assimilation of cholesterol into the cell membranes is one of the unique features of *H. pylori* [12]. In sum, this bacterium selectively absorbs cholesterol into the bacterial cell membranes from a medium supplemented with serum, even though cholesteryl ester was higher concentration than cholesterol in serum [13]. A part of cholesterol absorbed into *H. pylori* cell membranes is glucosylated by the catalytic action of cholesterol-α-glucosyltransferase (CGT) that is encoded by the HP0421 gene on the chromosomal DNA [14, 15]. CGT of *H. pylori* plays an important role to acquire the resistance against host immune systems such as phagocytosis of macrophages [16]. A

recent study by our group has found out another function of CGT that detoxifies a direct cholesterol precursor (7-dehydrocholesterol) fatal to *H. pylori* in order to assimilate its toxic cholesterol into the bacterial cell membranes [17]. Meanwhile, several studies including our study demonstrated that cholesterol itself incorporated into *H. pylori* cell membranes serves to acquire the resistance against the antibacterial action of phosphatidylcholine, antibiotics, and bile salts [12, 18, 19].

No earlier investigations had, however, elucidated what components take part in the incorporation of cholesterol into the cell membranes of *H. pylori*. A recent study by our group has revealed that phosphatidylethanolamine (PE) in the membrane lipid compositions functions as a cholesterol-binding lipid in *H. pylori* [20]. PE of *H. pylori* thus exhibited more selective binding affinity for cholesterol than for cholesteryl ester. This finding corresponded to the selective absorption of cholesterol of *H. pylori* cells. In addition, *H. pylori* PE turned out to bind not only to cholesterol but also to other steroids such as pregnenolone and dehydroepiandrosterone, which retain a functional group at the carbon position 3 in the steroid backbone without esterification as seen in cholesterol. An earlier study by our group has demonstrated the possibility that female hormone progesterone is a fundamental structure for developing new steroidal antibacterial medicines for the treatment of *H. pylori* infectious disease [21]. Intriguingly, the modification by a short-chain saturated fatty acid ($C_{6:0}$) at the carbon position 17 in progesterone molecule turned out to enhance the bactericidal activity of progesterone against *H. pylori*. It had not been, however, elucidated whether progesterone interacts with *H. pylori* PE in the induction of the bacteriolysis. Apart from this, a number of

investigations have revealed that long-chain unsaturated fatty acid has the potential to kill various microorganisms including *H. pylori* [22-33]. On this basis, we were driven to modify the carbon position 17 in progesterone molecule with a long-chain unsaturated fatty acid.

In this study, we, therefore, synthesized a novel progesterone derivative (17α-hydroxyprogesterone linoleate) esterified with linoleic acid ($C_{18:2}$) at the part of the carbon position 17, to obtain further evidences that progesterone molecule serves as a basic structure to design new steroidal drugs for the eradication of *H. pylori*. Moreover, we examined whether the synthetic progesterone derivative targets *H. pylori* PE due to the induction of the bacteriolysis.

MATERIALS and METHODS

Bacterial strains and culture

Helicobacter pylori strains NCTC 11638 and ATCC 43504 were investigated in this study. Pleuropneumonia-like organisms (PPLO) broth from Difco Laboratories (MI, USA) was adopted as a medium for bacterial cultures. Bacteria were cultured in a microaerophilic chamber (Concept 400: Ruskinn, Technology, Leeds, UK). Apart from this, the two *H. pylori* strains acclimatized to PPLO broth containing cholesterol (30 μM: Wako Pure Chemical Industries Ltd., Tokyo, Japan) were prepared to use in the determination of minimum inhibitory concentrations (MICs) of the novel synthetic progesterone derivative. Other bacteria, *Escherichia coli* (strains NIH JC2 and ATCC 11775), *Pseudomonas aeruginosa* (strain ATCC 10145), *Staphylococcus aureus* (strain

FDA 209D), and *Staphylococcus epiderimidis* (strain sp-al-1), were cultured in PPLO broth under aerobic conditions at 37˚C.

Progestins

A novel progesterone derivative, 17α-hydroxyprogesterone linoleate (17hPL) was obtained from ChemGenesis Inc. (Tokyo, Japan). Progesterone (Preg4), 17α-hydroxyprogesterone (17hP4), and 17α-hydroxyprogesterone caproate (17hPC) obtained from Wako Pure Chemical Industries Ltd. were used as reference progestins. Progestins examined in this study were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored at 4˚C in the dark. The 0.1% DMSO used in this study had no effect on the growth of bacteria.

Colony-forming unit

Bacterial suspensions were serially diluted 10-fold using PPLO broth, spread on plates of 5% horse serum (Gibco, Auckland, NZ)-brain-heart infusion agar (Difco Laboratories), and cultured for 1 week in the microaerobic chamber. The colony-forming units (CFUs) were calculated based on the colony counts and the dilution factor of the bacterial suspension.

Bacterial cell densities

Bacterial cells were recovered from PPLO cultures (1 ml) by centrifugation (8000 g, 5 min) and re-suspended in PBS (1 ml) to measure the $OD_{660\ nm}$ of the bacterial cell

suspensions (200 μl) by a spectrophotometer.

Microscopic observation of bacteria

The bacteria in PBS solution (10 μl) was spread on a glass slide, stained with Coomassie brilliant blue (CBB) solution (0.05% CBB, 9% acetate, 45.5% methanol), and microscopically observed.

Organic solvent distribution method and thin-layer chromatography

A five-fold volume of chloroform-methanol (2:1) solution was added to the bacterial cell supernatant and vigorously mixed to ultimately separate into the chloroform phase and the water phase. The chloroform phase was then recovered, and the solvent vaporized under decompression to obtain lipids. To analyze the lipids via thin-layer chromatography (TLC), the lipids in chloroform-methanol (2:1) solution (40 μl) were dotted onto a Silica Gel 60 plate (Merck, Darmstadt, Germany) and separated on the plate surface with chloroform-methanol-water (70:30:5) solvents or chloroform-acetone-methanol (9:1:1) solvents. The plate was then sprayed with a 60% sulfuric acid solution or a 0.25% ninhydrin in ethanol solution and was heated at 180˚C to detect the spots of lipids on the TLC plate surface.

Defatted serum proteins

Serum proteins were obtained from 50 ml of heat-inactivated fetal calf serum (FCS: Gibco) via 80% ammonium sulfate precipitation and dialyzed against PBS using a

centrifugal filter device, Centriprep YM-30 (Millipore Co., MA, USA). The proteins in PBS (10 ml) were gradually dropped into methanol solution (20 ml), and then diethylether (10 ml) was added to the solution. Centrifugation (5000 g, 10 min) was carried out to obtain the precipitates. The precipitates were washed twice with methanol-diethylether (2:1) solution (30 ml) via centrifugation (5000 g, 10 min), and further washed twice with diethylether (30 ml) in order to obtain the defatted serum proteins. After the defatted serum proteins were dried under nitrogen airflow and the dry weight was measured, the proteins were dissolved in PBS to adjust to a 1% concentration, filtrated using a GDXS 25 syringe filter (Whatman, Buckinghamshire, UK), and stored at 4˚C.

Assay of the growth of *H. pylori*

H. pylori (10^6 to 10^8 CFU/ml) was incubated for 2 to 24 h with progestin (2 to 10 μM) in PPLO broth (1.5 ml) or PBS (1.5 ml) containing defatted 0.1% serum proteins, in the presence or absence of a 30 μM concentration of 2,6-di-*O*-methyl-β-cyclodextrin (dMβCD: Sigma-Aldrich Inc.) in the microaerobic chamber. After incubation, the CFUs were measured.

Assay of the binding of progestins to phosphatidylethanolamine

Phosphatidylethanolamine (PE) isolated from *H. pylori* or *E. coli* was fixed to a paper disk by the method described previously [20]. The PE (200 μg)-fixed paper disk was incubated for 2 h in the presence of progestin (100 μM) in a 50 mM Tris (pH 7.5)

buffer (2 ml) containing dMβCD (3 mM) using a 12-well cell culture plate on a shaker (100 rpm). After incubation, the PE-fixed paper disk was washed 6 times with distilled water (2 ml) on a shaker (100 rpm, 10 min). The paper disk was transferred into a Teflon tube, dried under decompression, and stirred in chloroform solution (800 μl) in order to extract the progestin from the paper disk. The progestin was then recovered via vaporization of the chloroform under decompression to analyze by TLC with chloroform-acetone-methanol (9:1:1) solvents. Visualization of progestin spot on the TLC plate surface was carried out using a 60% sulfuric acid solution.

Assay of the interaction of 17hPL with PE vesicles

Coomassie brilliant blue (CBB) inclusion vesicle of either dimyristoyl PE (DMPE: Sigma-Aldrich In.) or dipalmitoyl PE (DPPE: Sigma-Aldrich Inc.) was prepared by the method described previously [34]. In brief, a PE vesicle suspension (50 μl) was added into a 50 mM Tris (pH 7.5) buffer (1.45 ml) containing a 5 μM concentration of 17hPL, and shaken for 2 h at 37˚C. After the PE vesicles were removed via centrifugation (10000 xg, 5 min), the absorbance of the supernatant (200 μl) was measured at a wavelength of 590 nm to detect the elution of CBB from the PE vesicles.

Detection of PE in bacterial cell supernatant

The supernatant (1 ml) obtained from *H. pylori* (10^8 CFU/ml) incubated for 2 to 4 h with or without progestin (10 μM) in PBS containing defatted 0.1% serum proteins was recovered and subjected to organic solvent distribution to extract PE. The PE in the

supernatant was detected on a TLC plate surface sprayed with a 0.25% ninhydrin reagent after TLC analysis with chloroform-methanol-water (70:30:5) solvent system.

Minimum inhibitory concentration

H. pylori was acclimatized to either PPLO broth or PPLO broth containing 30 μM concentration of cholesterol. Each bacterial cell suspension (10 μl) adjusted to 10^8 CFU/ml was dropped onto either PPLO agar plates or cholesterol (30 μM)-dispersed PPLO agar plates and incubated for 1 week or for 24 h in the microaerobic chamber or under aerobic conditions at 37˚C. The minimum inhibitory concentrations (MICs) of the progestin were determined by confirming the growth of bacterial colonies on the agar plates. Three independent experiments were carried out to determine the MIC of progestin for each bacterial species.

Assay of cytotoxicity of progestins to mammalian cell

RAW 264.7 cell, a murine macrophage-like cell line, obtained from ATCC was cultured in RPMI 1640 medium (Sigma-Aldrich Inc.) supplemented with heat-inactivated FCS (10%), HEPES (10 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and $NaHCO_3$ (0.2%) in a CO_2 incubator. RAW 264.7 cell ($10^{5.7}$ cell/ml) suspensions (1 ml) were added into each well of a 24-well cell culture plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and were incubated for 4 h in the CO_2 incubator. Progestins dissolved in DMSO were dispersed into the media at various concentrations. The 0.1% concentration of DMSO had no influence on the viability of

RAW 264.7 cells. After the RPMI 1640 medium (1 ml) of RAW 264.7 cells was exchanged to RPMI 1640 medium (1 ml) containing progestins, the cells were incubated for further 24 h in the CO_2 incubator. An aliquot (100 µl) of MTT reagent (5 mg/ml in PBS: Sigma-Aldrich Inc.) was then added to each well at the last 4 h of 24-h incubation. To detect formazan blue crystals formed by viable cells [16], the supernatants were removed from the cells, and the cells were lysed in 5% formic acid-isopropanol solution (1 ml). The absorbance of the lysates (100 µl) was then measured at the wavelength of 540 nm.

Detection of nitric oxide produced by RAW 264.7 cells stimulated with lipopolysaccharide

RAW 264.7 cell ($10^{5.7}$ cell/ml) suspension (1.5 ml) was added into each well of a 12-well cell culture plate (Sumitomo Bakelite Co. Ltd.) and incubated overnight in the CO_2 incubator. The cells in each well were then washed three times with PBS (1 ml) to remove non-adherent cells and further incubated for 4 h in the presence or absence of progestin (1 µM or 10 µM) in the RPMI 1640 medium (1. 5 ml). After incubation, the cells were stimulated for 24 h with a 1 µg/ml concentration of *E. coli* (O55:B5) lipopolysaccharide (LPS) from Sigma-Aldrich Inc. The cell supernatant was then recovered, and the amount of nitric oxide (NO) in the cell supernatant was determined as nitrite using a Griess reagent. In brief, the cell supernatant (50 µl) was inoculated into the Griess reagent solution (50 µl) to measure the $A_{540\ nm}$. The NO standard solution was prepared using RPMI 1640 medium containing sodium nitrite (5 to 50 µM).

RESULTS

Anti-*H. pylori* activity of 17hPL

First, we compared the antibacterial activity of 17hPL against *H. pylori* with the antibacterial activity of 17hPC against the same bacterial species. When *H. pylori* was incubated for 24 h in the presence of 17hPL or 17hPC at concentrations ranging from 2 to 10 μM in PPLO broth, both progestins acted as an antibacterial substance to this bacterium. The CFU levels of *H. pylori* decreased along with the increase in the concentrations of 17hPL and 17hPC (Fig. 1B). The anti-*H. pylori* activity of 17hPL at the 2 μM concentration was obviously stronger than that of 17hPC at the same concentration, but the activity of 17hPL at the concentrations ranging from 4 to 10 μM was comparable to that of 17hPC at the same concentration ranges. In sum, a novel synthetic progesterone derivative 17hPL turned out to be a steroidal compound antibacterial to *H. pylori*.

Influence of dMβCD on anti-*H. pylori* action of 17hPL

An earlier study by our group has demonstrated that dMβCD, a steroid-solubilizer, inhibits the anti-*H. pylori* action of 17hPC via certain mechanisms [35]. We, therefore, investigated the influence of dMβCD on the anti-*H. pylori* action of 17hPL. When *H. pylori* was incubated for 24 h in the presence of 17hPL or 17hPC at concentrations ranging from 2 to 6 μM in PPLO broth with or without dMβCD, the dMβCD had no influence on the antibacterial effect of 17hPL to *H. pylori*. The decline curve of CFU in *H. pylori* incubated with 17hPL in the presence of dMβCD was similar to the decline curve of CFU in the bacteria incubated with 17hPL in the absence of dMβCD (Fig. 2A). In

contrast, the anti-*H. pylori* action of 17hPC was affected by dMβCD. In sum, the CFU decline curve of *H. pylori* incubated in the presence of 17hPC was more gently sloping in the presence of dMβCD than in the absence of it. The steroid-solubilizer dMβCD, thus, functioned as an inhibitor of 17hPC when it acts as the antibacterial substance on *H. pylori*, but did not affect the anti-*H. pylori* action of 17hPL.

Binding of 17hPL to *H. pylori* PE in the presence of dMβCD

A recent study by our group has demonstrated that myristoyl PE (MPE) of *H. pylori* plays an important role in the interaction of the cell membranes with steroids [20]. Next, we examined the binding of 17hPL and 17hPC to *H. pylori* MPE in the presence of dMβCD. After *H. pylori* PE-fixed paper disk or *E. coli* PE-fixed paper disk was incubated for 2 h with 17hPL or 17hPC in the buffer containing dMβCD, the 17hPL and 17hPC in the PE-fixed paper disks were analyzed by TLC. The TLC analysis demonstrated that the spot of 17hPL detected in the *H. pylori* PE-fixed paper disk was conspicuously denser than that of 17hPL detected in the *E. coli* PE-fixed paper disk, even though the binding of 17hPL to the PE was non-specific hydrophobic interaction between the steroid and the bacterial glycerophospholipid (Fig. 2B). This result means that 17hPL shows higher binding affinity for MPE of *H. pylori* than for palmitoyl PE (PPE) of *E. coli* used as a prevalent PE species of Gram-negative bacteria and is capable of interacting with *H. pylori* MPE even in the presence of dMβCD. In contrast, the binding of 17hPC to both PEs of *H. pylori* and *E. coli* was inhibited by dMβCD. The 17hPC was, therefore, undetectable in all paper disks fixed with *H. pylori* MPE or *E. coli* PPE. This result means

that 17hPC is incapable of interacting with any of the bacterial PE in the presence of dMβCD. The inhibition of the antibacterial action of 17hPC against *H. pylori* by dMβCD, thus, turned out to be closely correlated with the inhibition of the binding of 17hPC to *H. pylori* PE by dMβCD. In addition, dMβCD was concluded to have no capability to inhibit the antibacterial action of 17hPL against *H. pylori* and to obstruct the binding of 17hPL to *H. pylori* MPE.

Interaction of 17hPL with PE vesicles

A recent study by our group has revealed that dimyristoyl PE (DMPE) accounts for approximately 30% in total PE molecular species of *H. pylori* cells [20]. We next examined whether 17hPL has a higher binding affinity to DMPE than to dipalmitoyl PE (DPPE). A suspension of PE vesicles containing CBB prepared with either DMPE or DPPE was shaken for 2 h in the presence of 17hPL, and the absorbance of CBB eluted from the PE vesicles was measured in the supernatants. The level of CBB elution from DMPE vesicles incubated with 17hPL was statistically higher than the level of CBB elution from DPPE vesicles incubated with it (Fig. 3B). This tells us that 17hPL more strongly induces the structural destabilization of the DMPE vesicles than the DPPE vesicles, even though the molecular interaction between 17hPL and PE was non-specific hydrophobic bonding. Given that DMPE is one of the most predominant PE molecular species of *H. pylori* cells [20], we can assume that 17hPL binds at least to the DMPE on the *H. pylori* cells and exerts antibacterial action against this bacterium.

Bactericidal mechanism of 17hPL to *H. pylori*

A previous study by our group has demonstrated that *H. pylori* lyses in the presence of Preg4 or 17hPC [21]. Therefore, we examined whether 17hPL induces the bacteriolysis to *H. pylori*. When *H. pylori* was incubated for 4 to 24 h in the presence or absence of 17hPL in the broth, the $OD_{660\ nm}$ of the bacterial suspension without 17hPL was decreased at the 24-h incubation after the increases of $OD_{660\ nm}$ were observed in the bacterial suspensions incubated for 4 to 8 h, but the level of $OD_{660\ nm}$ in the 24-h incubated bacterial suspension was higher than that of $OD_{660\ nm}$ in the bacterial suspension (0 h) measured immediately after the incubation was started (Fig. 4A). This means that the *H. pylori* cell densities in the broth without 17hPL increased during the incubation. The $OD_{660\ nm}$ in the *H. pylori* cell suspensions incubated in the presence of 17hPL, however, reduced gradually along with the progression of the incubation time. This means that the *H. pylori* cell densities in the broth containing 17hPL were decreased during the incubation.

To confirm the bacterial cell bodies, we observed microscopically the state of *H. pylori* incubated for 24 h in the presence or absence of 17hPL in the broth. The bacterial cell suspension without 17hPL harbored a number of rod-shaped organisms (Fig. 4B). In contrast, the bacterial cell suspension with 17hPL included a large amount of objects, such as cellular debris, in place of the microorganisms. These results, taken together with Fig. 2, suggest that 17hPL induces the bacteriolysis to *H. pylori* via the interaction with PE in the bacterial cell membranes.

Next, we examined the bactericidal mechanism of 17hPL to *H. pylori*. When *H.*

pylori cells were incubated with 17hPL in PBS containing defatted serum proteins, the CFU levels of the bacteria fell gradually along the time axis of the incubation regardless of the presence of 17hPL, but the degree of the CFU reduction was greater in *H. pylori* incubated in the presence of 17hPL than in the absence of it (Fig. 5A).

After *H. pylori* was incubated in the presence or absence of 17hPL from 2 to 4 h in PBS containing defatted serum proteins, PE in the supernatant was detected by TLC analysis. The spots of PE detected in the cell supernatants of *H. pylori* incubated in the presence of 17hPL turned out to be consistently denser than those of PE detected in the supernatants of the bacteria incubated in the absence of 17hPL (Fig. 5B). In sum, 17hPL induced the release of PE from *H. pylori* cells. Given that *H. pylori* incorporates a number of steroids regardless of the differences of structures into the cell membranes and adopts as the membrane lipid compositions only the steroids beneficial to survive [12-14], [17], [35-37], we can assume that 17hPL induces the destabilization of the membrane structures by being embedded into the lipid layers after the binding to PE on the outer membrane and injures the bacterial cell membranes and thereby PE, the most predominant composition in the membrane lipid layers, is ultimately released from the membranes. 17hPL was, thus, found to be disadvantageous steroid on the survival of *H. pylori*.

Selective antibacterial action of 17hPL against *H. pylori*

H. pylori ingests cholesterol from the outside, and retains the cholesterol as membranal lipid constituents. Earlier investigations by our group and others have

revealed that the cholesterol incorporated into the membranes serves for *H. pylori* to acquire the resistance to the antibacterial action of phosphatidylcholine, antibiotics and bile salts [12, 18, 19]. We therefore examined the antibacterial activity of 17hPL against *H. pylori* absorbed cholesterol into the membranes. The minimum inhibitory concentrations (MICs) of 17hPL to the growth of *H. pylori* without cholesterol were the 1 μM and 2 μM for the strains NCTC 11638 and ATCC 43504, respectively (Table 1). Meanwhile, the MICs of 17hPL to the growth of *H. pylori* with retained cholesterol were the 9 μM and 10 μM for the strains NCTC 11638 and ATCC 43504, respectively. We next examined the antibacterial action of 17hPL against other bacteria. Intriguingly, 17hPL did not affect the growth of the four bacterial species, *E. coli* (strain NIH JC2), *P. aeruginosa* (strain ATCC 10145), *S. aureus* (strain FDA 209D), and *S. epiderimidis* (strain sp-al-1). In sum, all four bacterial species formed bacterial colonies, even on the agar plate containing 17hPL at the highest concentration (100 μM) examined. These results indicate that 17hPL is a selective bactericidal substance to *H. pylori*.

Cytotoxicity of 17hPL to RAW 264.7 cells

Next, we investigated the influence of 17hPL on the viability of murine macrophage-like cells (RAW 264.7 cells). After RAW 264.7 cells were incubated for 24 h in the presence of each progestin at the 100 μM concentrations, the viable cells were estimated by MTT assay. Of the four progestins examined, progesterone (Preg4) and 17α-hydroxyprogesterone (17hP4) elicited the toxic effect to the cells. Hence, Preg4 and 17hP4 at the 100 μM concentration were decreased the ratio of viable RAW 264.7 cells to

53% and 62%, respectively compared to the 100% survival of the cells incubated in the absence of any progestins. Meanwhile, 17hPC and 17hPL did not affect the viability of RAW 264.7 cells, and therefore a decrease of viable cells was not observed even in the presence of the 100 μM concentration. Incidentally, the above four progestins had no toxicity to RAW 264.7 cells at less than 10 μM concentration. These results suggest that 17hPL at the μM concentration range that is necessary to lyse *H. pylori* has no influence on the viability of mammalian cells.

Inhibition effect of 17hPL on the NO production of RAW 264.7 cells stimulated with LPS

The inhibition of NO production from mouse macrophages activated by LPS is one of the hormonal effects of Preg4 [38-43]. We next examined whether 17hPL has the capability to inhibit NO production in LPS-stimulated RAW 264.7 cells. After RAW 264.7 cells were pre-incubated for 4 h with each progestin, the cells were incubated for further 24 h in the presence of LPS to stimulate RAW 264.7 cells and to measure the NO amount in the supernatant of the LPS-activated cells. The production of NO from RAW 264.7 cells activated by LPS was suppressed to approximately 49%, 54%, and 62% in the presence of Preg4, 17hPC, and 17hPL, respectively, when the NO production in RAW 264.7 cells activated by LPS alone was 100% (Fig. 6B). Thus, of the three progestins investigated, 17hPL turned out to exhibit the weakest inhibition effect on the NO production of RAW 264.7 cells stimulated with LPS. In contrast, the inhibition effect of 17hP4 was negligible on the NO production of the LPS-stimulated RAW 264.7 cells.

Incidentally, none of the progestins induced NO production from RAW 264.7 cells without LPS-stimulation.

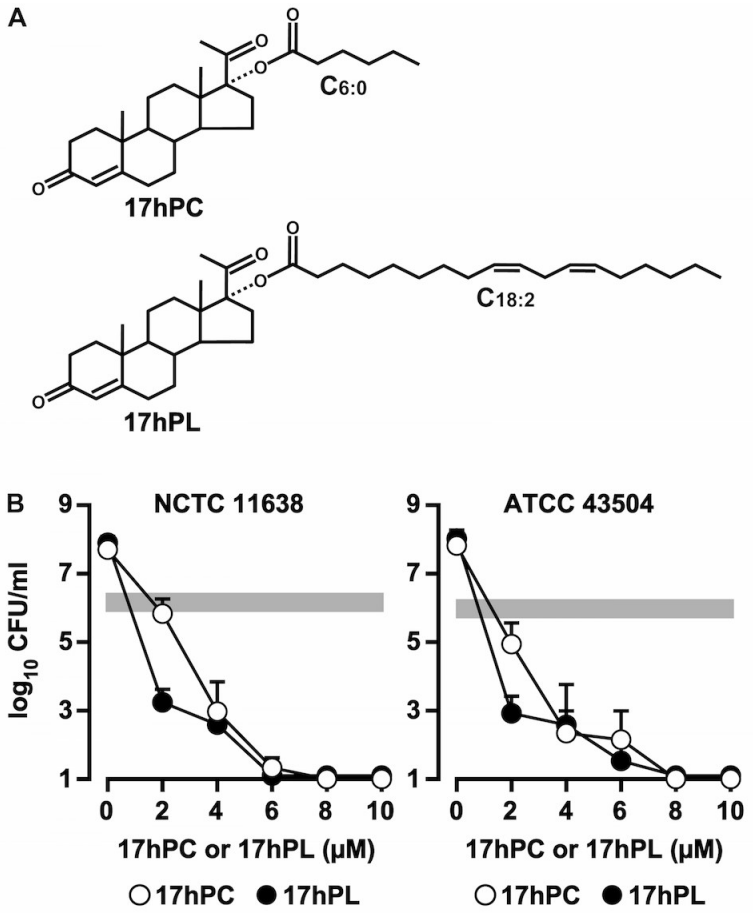

Fig. 1. Anti-*H. pylori* activity of 17hPL and 17hPC

(A) Chemical structures of 17α-hydroxyprogesterone caproate (17hPC) and 17α-hydroxyprogesterone linoleate (17hPL). (B) After *H. pylori* strains NCTC 11638 and ATCC 43504 were incubated for 24 h in the presence of either 17hPL or 17hPC, the CFUs were measured. The gray bars in the graphs indicate CFU levels measured immediately after incubation was started.

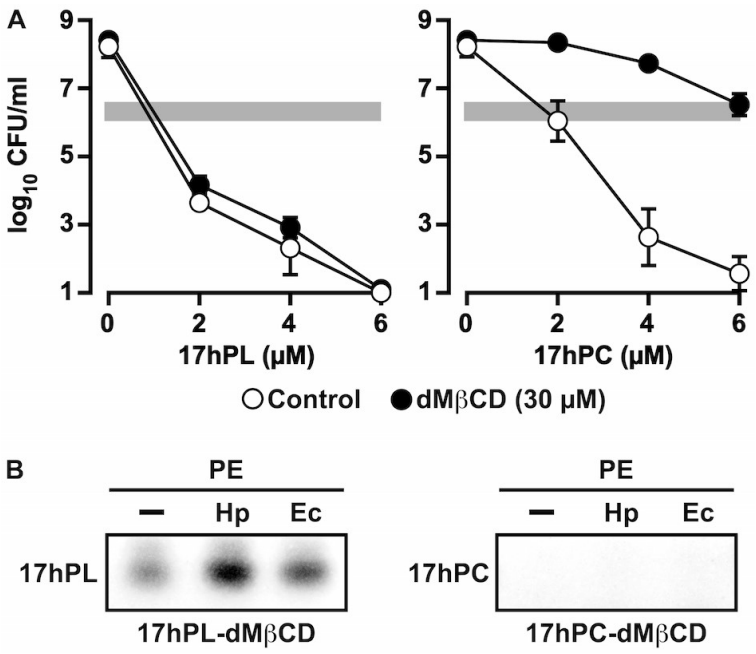

Fig. 2. The influence of dMβCD on anti-*H. pylori* action of 17hPL and 17hPC, and the binding affinity of 17hPL and 17hPC for *H. pylori* PE in the presence of dMβCD

(A) *H. pylori* strain NCTC 11638 was incubated for 24 h in the presence or absence of dMβCD (30 μM) in the broth containing 17hPL or 17hPC, and then the CFUs were measured. The grey bars in the graphs indicate CFU levels measured immediately after incubation was started. (B) PE (200 μg) obtained from *H. pylori* strain NCTC 11638 (Hp) or *E. coli* strain ATCC 11775 (Ec) was dotted into a paper disk and then the PE-fixed paper disk was soaked for 2 h in the presence of dMβCD (3 mM) in the buffer containing 17hPL (100 μM) or 17hPC (100 μM). After 2 h, the 17hPL and 17hPC contained in the PE-fixed paper disks were detected by TLC analysis. The minus lanes show the detection levels of 17hPL and 17hPC contained in the paper disks without either HpPE or EcPE.

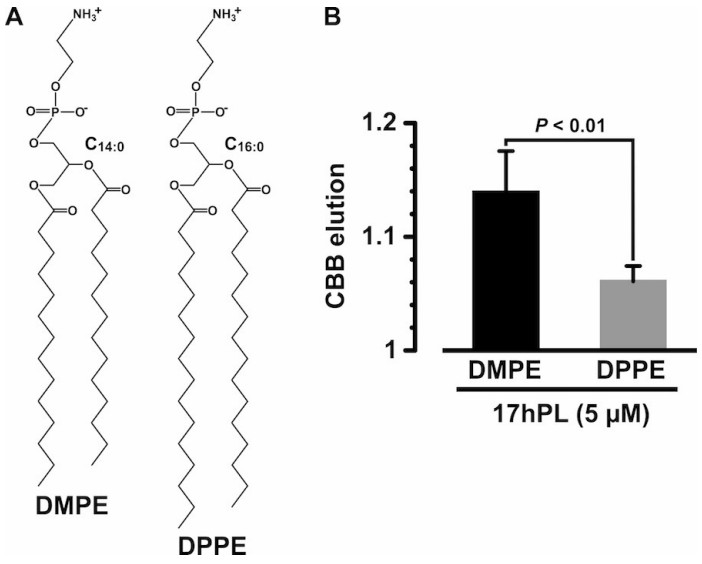

Fig. 3. Interaction of 17hPL with the PE vesicles

(A) Chemical structures of dimyristoyl PE (DMPE) and dipalmitoyl PE (DPPE). (B) PE vesicle suspensions containing CBB were shaken for 2 h in the presence of 17hPL, and $A_{590\ nm}$ was measured in the supernatant. CBB elution was denoted by the relative $A_{590\ nm}$ to the $A_{590\ nm}$ measured as a value of 1 in the supernatant of the PE vesicles incubated without 17hPL. The statistical significance of differences between the CBB elutions from the DMPE vesicles and DPPE vesicles was evaluated by the *t*-test based on data obtained from four independent pair-experiments.

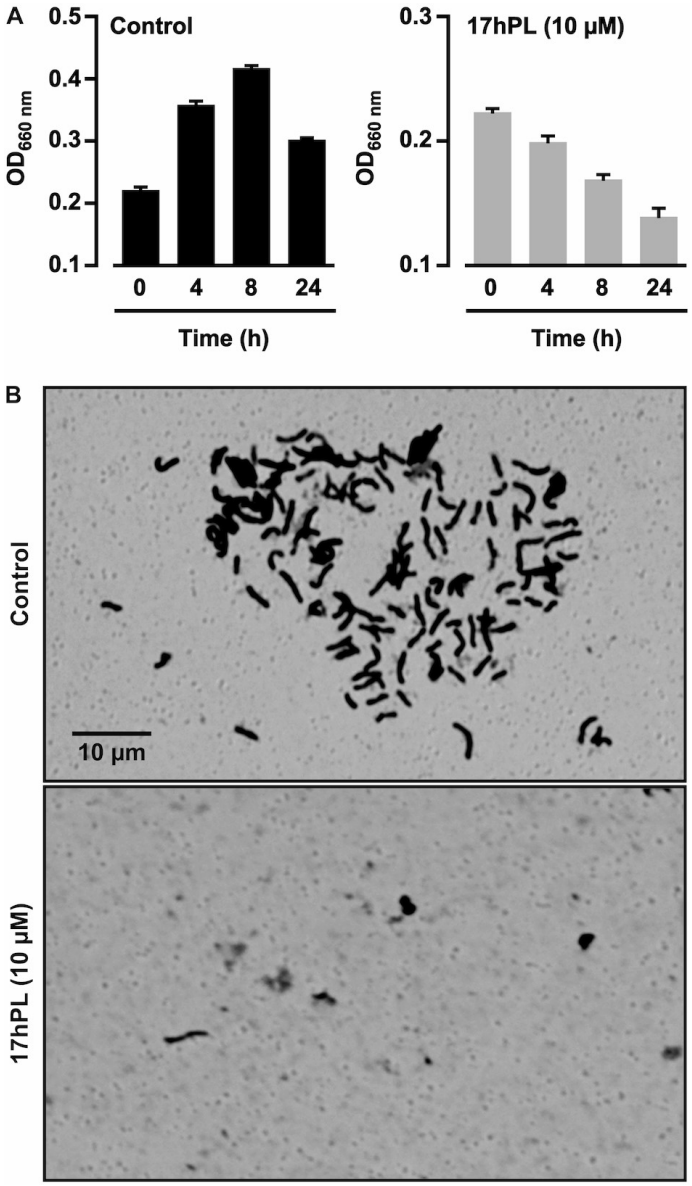

Fig. 4. Bacteriolytic action of 17hPL against *H. pylori*

(A) After *H. pylori* strain NCTC 11638 (10^8 CFU/ml) was incubated in the presence or absence of 17hPL (10 μM) in broth (1 ml) at various time points and re-suspended in PBS (1 ml), the $OD_{660\ nm}$ of the bacterial suspensions (200 μl) was measured. (B) After *H. pylori* strain NCTC 11638 (10^8 CFU/ml) incubated for 24 h in the presence or absence of 17hPL (10 μM) in broth (1 ml) and re-suspended in PBS (1 ml), the specimens of the bacterial suspension (10 μl) stained with CBB were observed microscopically.

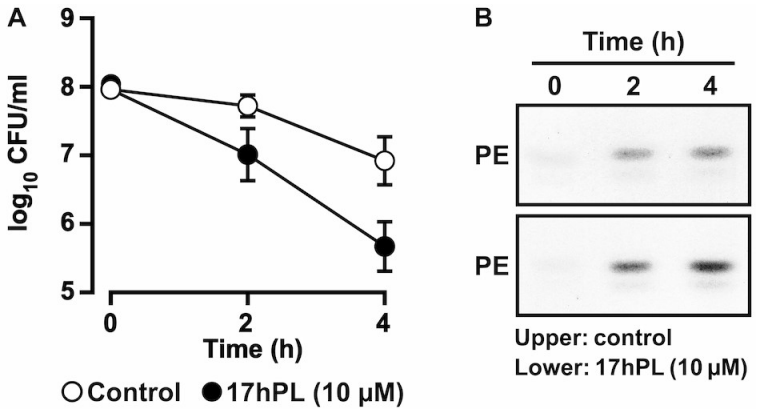

Fig. 5. Release of PE from *H. pylori* via the interaction with 17hPL

(A) *H. pylori* strain NCTC 11638 with the CFU level shown at 0-h in the graph were incubated for 2 to 4 h in the presence or absence of 17hPL (10 μM) in PBS (1.5 ml) containing defatted serum proteins (0.1%), and then the CFUs were measured. (B) PE was detected in the supernatants (1 ml) of *H. pylori* (10^8 CFU/ml) incubated for 2 to 4 h in the presence or absence of 17hPL (10 μM) in PBS (1.5 ml) containing defatted serum proteins (0.1%) by TLC analysis.

Table 1. MIC (μM) of 17hPL for *H. pylori* and other bacteria

Bacterial strain	Bacterial strain without cholesterol	Bacterial strain with retained cholesterol
***H. pylori* NCTC 11638**	**1**	**9**
***H. pylori* ATCC 43504**	**2**	**10**
***E. coli* NIH JC2**	**> 100**	**ND**
***P. aeruginosa* ATCC 10145**	**> 100**	**ND**
***S. aureus* FDA 209D**	**> 100**	**ND**
***S. epiderimidis* sp-al-1**	**> 100**	**ND**

H. pylori strains without cholesterol were acclimatized to PPLO broth.

H. pylori strains with retained cholesterol were acclimatized to PPLO broth containing cholesterol at the 30 μM concentration.

ND, not determined

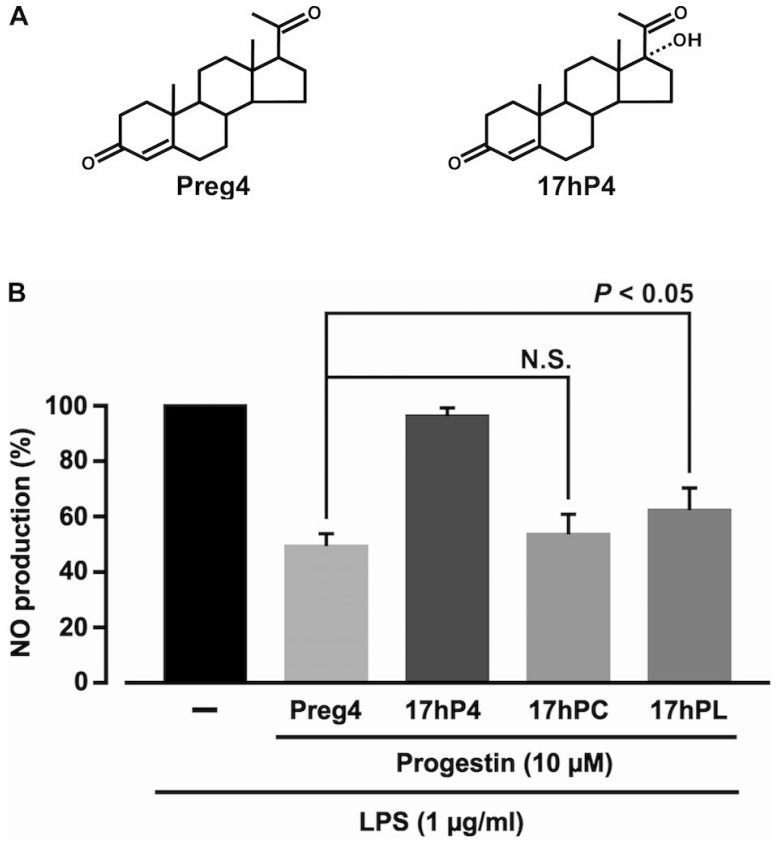

Fig. 6. Inhibitory effect of 17hPL on the NO production of RAW 264.7 cells stimulated with LPS

(A) Chemical structures of progesterone (Preg4) and 17α-hydroxyprogesterone (17hP4). (B) After RAW 264.7 cells (10^6 cell/ml) were incubated for 4 h in the presence or absence of Preg4, 17hP4, 17hPC, and 17hPL at the 10 μM concentration in RPMI 1640 medium (1.5 ml), the cells was further incubated for 24 h with LPS (1 μg/ml). The amount of NO in the cell supernatants was measured using a Griess reagent. The production of NO is indicated with a ratio calculated by comparing the NO amount of the cells stimulated with LPS in the presence of each progestin to the NO amount of the cells activated by LPS without any progestins. The statistical significance of the inhibition effect of 17hPL or 17hPC versus Preg4 on NO production in the LPS-activated cells was evaluated via t-test ($P< 0.05$). N.S. stands for "no statistical significance" in the t-test ($P> 0.1$).

DISCUSSION

A recent study by our group has demonstrated that *H. pylori* glucosylates 7-dehydrocholesterol, a toxic steroid for the survival of this bacterium, via the catalytic action of CGT in order to detoxify it and to use the glucosylated 7-dehydrocholesterol as membrane components [17]. The crucial structure of steroid compounds glucosylated by the enzymatic action of CGT is a 3β-hydroxyl (3β-OH) attached to the carbon position 3 of a steroid molecule [15, 35, 37]. The 17hPL investigated in this study possesses a 3-oxo but not a 3β-OH. Hence, we understand that 17hPL is not detoxified by the action of CGT and acts as the bactericidal substance against *H. pylori*.

A recent study by our group has demonstrated that the fatty acid compositions of *H. pylori* PE significantly differ from the fatty acid compositions of PE of other commonplace Gram-negative bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* and suggested that the myristic acid ($C_{14:0}$) in *H. pylori* PE plays an important role in the selective binding of non-esterified steroids to its PE [20], [44-47]. PE is the most predominant glycerophospholipid for constituting bacterial cell membranes, and the most prevalent saturated fatty acid constituents of PE are consistently unchanged in each bacterial species. In sum, the appearance ratio of PE mutant strains is considered to be tremendously low. This study concluded that 17hPL binds to the myristoyl PE in the membranes of *H. pylori*, induces the release of the PE from its membranes, and ultimately lyses the bacterial cells. These results suggest that resistant strains of *H. pylori* against 17hPL may rarely develop because this steroidal substance targets the PE which accounts

for greater than 60% of the membrane lipid compositions (except for LPS) of *H. pylori* undergoing the logarithmic growth phase [18].

Beta-cyclodextrins (βCDs) including dMβCD are a cyclic oligomer composed of seven glucose molecules linked by α-glucosidic bonds and capable of solubilizing into water solvents a number of hydrophobic compounds through the formation of molecular inclusion complexes [48-52]. Therefore, βCDs are often added as a solubilizer, termed a nano capsule, to nutrition-supplement tablets or oral medicines containing hydrophobic compounds. Recent studies by our group have demonstrated that dMβCD functions as a cholesterol-carrier molecule for promoting the assimilation of cholesterol and 7-dehydrocholesterol into *H. pylori* cell membranes [17, 20]. In contrast, the incorporation of steroid hormones such as pregnenolone and dehydroepiandrosterone into *H. pylori* cell membranes has turned out to be inhibited by the action of dMβCD. In addition, dMβCD counteracts the bacteriolytic action of progesterone and 17hPC against *H. pylori* [21]. Taken together, only the cholesterol and 7-dehydrocholesterol in the steroids examined until this time have been capable of interacting with *H. pylori* even in the presence of dMβCD. This study demonstrated that the bactericidal activity of 17hPL against *H. pylori* is not affected by the action of dMβCD, although this agent did not augment the anti-*H. pylori* action of 17hPL as it promoted the assimilation of cholesterol and 7-dehydrocholesterol into the bacterial membranes. This suggests the possibility that βCDs including dMβCD function as nano capsules for delivering 17hPL to *H. pylori* on the gastric epithelium when a pill of 17hPL is administrated orally to patients infected with this pathogen.

An earlier study by another group has revealed that Preg4 at a high concentration (200 μM) induces the cell death of Chinese hamster ovary (CHO) cells through the inhibition of intracellular cholesterol biosynthesis and that the inhibition effect of Preg4 on the cholesterol biosynthesis is observed even in other mammalian cell lines such as the HeLa and Caco-2 cells [53]. This study demonstrated that Preg4 and 17hP4 have influence on the viability of RAW 264.7 cells at the 100 μM concentrations. To confirm whether Preg4 and 17hP4 at high concentrations kill RAW 264.7 cells via the inhibition of cholesterol biosynthesis, further investigations will be necessary in another study of the biological activity of progesterone.

This study demonstrated that 17hPL at the μM concentration range required for completely killing *H. pylori* (10^6 to 10^7 CFU/ml) is relatively weaker in the hormonal activity than Preg4 and 17hPC at the same concentration range. However, further experiments are waiting in the future to examine whether a novel synthetic progestin 17hPL confers the attenuated progestational effects to other mammalian cells, as with the case of macrophages. As the next stage, experiments using animal models will be essential to estimate the treatment effect of 17hPL on hosts infected with *H. pylori*, and to investigate the side effects of its steroid on mammals.

ACKNOWREGEMENTS

This work was supported by JSPS KAKENHI Grant Number 25460544 and, in part, by JKA promotion funds from KEIRIN RACE.

REFERENCES

1. B. Marshall, J.R. Warren, Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1 (1983) 1273-1274.
2. D.Y. Graham, *Helicobacter pylori*: its epidemiology and its role in duodenal ulcer disease. *J. Gastroen. Hepatol.* 6 (1991) 105-113.
3. A.C. Wotherspoon, C. Oriz-Hidalgo, M.R. Falzon, P.G. Isaacson, *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 338 (1991) 1175-1176.
4. D. Forman, the Eurogast Study Group, An international association between *Helicobacter pylori* infection and gastric cancer. *Lancet* 341 (1993) 1359-1363.
5. N. Uemura, S. Okamoto, S. Yamamoto, M. Matsumura, S. Yamaguchi, M. Yamakido, K. Taniyama, N. Sasaki, R.J. Schlemper, *Helicobacter pylori* infection and the development of gastric cancer. *N. Engl. J. Med*. 345 (2001) 829-832.
6. R.M. Peek Jr., M.J. Blaser, *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer*. 2 (2002) 28-37.
7. M. Stolte, E. Bayerdorffer, A. Morgner, B. Alpen, T. Wundish, C. Thiede, A. Neubauer, *Helicobacter* and gastric MALT lymphoma, *Gut* 50 (2002) III19-III24.
8. R.M. Peek Jr, J.E. Crabtree, *Helicobacter* infection and gastric neoplasia. *J. Pathol*. 208 (2006) 233-248.
9. K. Fukase, M. Kato, S. Kikuchi, K. Inoue, N. Uemura, S. Okamoto, S. Terano, K. Amagai, S. Hayashi, M. Asaka, Japan Gast Study Group, Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* 372 (2008) 392-397.
10. I. Kobayashi, K. Murakami, M. Kato, S. Kato, T. Azuma, S. Takahashi, N. Uemura, T. Katsuyama, Y. Fukuda, K. Haruma, M. Nasu, T. Fujioka, Changing antimicrobial susceptibility epidemiology of *Helicobacter pylori* strains in Japan between 2002 and 2005. *J. Clin. Microbiol*. 45 (2007) 4006-4010.

11. V. De Francesco, F. Giorgio, C. Hassan, G. Manes, L. Vannella, C. Panella, E. Ierardi, A. Zullo, Worldwide *H. pylori* antibiotic resistance: a systematic review. *J. Gastrointestin*. *Liv. Dis.* 19 (2010) 409-414.
12. H. Shimomura, K. Hosoda, S. Hayashi, K. Yokota, K. Oguma, Y. Hirai, Steroids mediate resistance to the bactericidal effect of phosphatidylcholines against *Helicobacter pylori*. *FEMS Microbiol. Lett*. 301 (2009) 84-94.
13. M. Haque, Y. Hirai, K. Yokota, K. Oguma, Steryl glucosides: a characteristic feature of the *Helicobacter* spp.?. *J. Bacteriol*. 177 (1995) 5334-5337.
14. Y. Hirai, M. Haque, T. Yoshida, K. Yokota, K. Oguma, Unique cholesteryl glucosides in *Helicobacter pylori*: composition and structural analysis. *J. Bacteriol*. 177 (1995) 5327-5333.
15. A. Lebrun, C. Wunder, J. Hildebrand, Y. Churin, U. Zähringer, B. Lindner, T.F. Meyer, E. Heinz, D. Warnecke, Cloning of a cholesterol-α-glucosyltransferase from *Helicobacter pylori*. *J. Biol. Chem*. 281 (2006) 27765-27772.
16. C. Wunder, Y. Churin, F. Winau, D. Warnecke, M. Vieth, B. Lindner, U. Zähringer, H.J. Mollenkopf, E. Heinz, T.F. Meyer, Cholesterol glucosylation promotes immune evasion by *Helicobacter pylori*. *Nat. Med*. 12 (2006) 1030-1038.
17. H. Shimomura, K. Hosoda, D.J. McGee, S. Hayashi, K. Yokota, Y. Hirai, Detoxification of 7-dehydrocholesterol fatal to *Helicobacter pylori* is a novel role of cholesterol glucosylation. *J. Bacteriol*. 195 (2013) 359-367.
18. D.J. McGee, A.E. George, E.A. Trainor, K.E. Horton, E. Hildebrandt, T.L. Testerman, Cholesterol enhances *Helicobacter pylori* resistance to antibiotics and LL-37. *Antimicrob*. *Agents Chemother*. 55 (2011) 2897-2904.
19. E.A. Trainor, K.E. Horton, P.E. Savage, T.L. Testerman, D.J. McGee, Role of the HefC efflux pump in *Helicobacter pylori* cholesterol-dependent resistance to ceragenins and bile salts. *Infect. Immun*. 79 (2011) 88-97.
20. H. Shimomura, K. Hosoda, S. Hayashi, K. Yokota, Y. Hirai, Phosphatidylethanolamine of *Helicobacter pylori* functions as a steroid-binding

lipid in the assimilation of free cholesterol and 3β-hydroxyl steroids into the bacterial cell membrane. *J. Bacteriol*. 194 (2012) 2658-2667.

21. K. Hosoda, H. Shimomura, S. Hayashi, K. Yokota, Y. Hirai, Steroid hormones as bactericidal agents to *Helicobacter pylori*. *FEMS Microbiol. Lett*. 318 (2011) 68-75.
22. O. Wyss, B.J. Ludwig, R.R. Joiner, The fungistatic and fungicidal action of fatty acids and related compounds. *Arch. Biochem*. 7 (1945) 415-425.
23. C. Nieman, Influence of trace amount of fatty acids on the growth of microorganisms. *Bacteriol. Rev*. 18 (1954) 147-163.
24. J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, Fatty acids and derivatives as antimicrobial agents. *Antimicrob. Agents Ch*. 2 (1972) 23-28.
25. A.J. Conley, J.J. Kabara, Antimicrobial action of esters of polyhydric alcohols. *Antimicrob. Agents Ch.* 4 (1973) 501-506.
26. K. Kanai, E. Kondo, Antibacterial and cytotoxic aspects of long-chain fatty acids as cell surface: selected topics. *Jpn. J. Med. Sci. Biol*. 32 (1979) 135-174.
27. F. Kanetsuna, Bactericidal effect of fatty acids on mycobacteria, with particular reference to the suggested mechanism of intracellular killing. *Microbiol. Immunol*. 29 (1985) 127-141.
28. E. Kondo, K. Kanai, Mechanism of bactericidal activity of lysolecithin and its biological implication. *Jpn. J. Med. Sci. Biol*. 38 (1985) 181-194.
29. H.R. Knapp, M.A. Melly, Bactericidal effect of polyunsaturated fatty acids. *J. Infect. Dis*. 154 (1986) 84-94.
30. E. Constance, J. van Rensburg, G.K. Joone, J.F. Osullivan, R. Anderson, Antibacterial activities of clofazimine and B669 are mediated by lysophospholipids. *Antimicrob. Agents Ch*. 36 (1992) 2729-2735.
31. L. Thompson, A. Cockayne, R.C. Spiller, Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori*: a possible explanation of the effect of diet on peptic ulceration. *Gut*. 35 (1994) 1557-1561.
32. E.E.D. Bruyn, H.C. Steel, C.E.J. van Rensburg, R. Anderson, The riminophenazines,

clofazimine and B669, inhibit potassium transport in gram-positive bacteria by a lysophospholipid-dependent mechanism. *J. Antimicrob. Chemoth*. 38 (1996) 349-362.

33. H.C. Steel, R. Cockeran, R. Anderson, Platelet-activating factor and lyso-PAF possess direct antimicrobial properties *in vitro*. *APMIS*. 110 (2002) 158-164.
34. K. Hosoda, H. Shimomura, K. Wanibuchi, H. Masui, A. Amgalanbaatar, S. Hayashi, T. Takahashi, Y. Hirai, Identification and characterization of a vitamin D3 decomposition product bactericidal against *Helicobacter pylori*. *Sci. Rep*. 5 (2015) 8890; DOI:10.1038/srep08860.
35. H. Shimomura, Relationship between steroid hormones and *Helicobacter pylori*. *In* Hassan A (ed), *Steroids-clinical aspect* (2011). InTech, Rijeka, Croatia. http://www.intechopen.com/books/steroids-clinical-aspect/relationship-between-steroid-hormones-and-helicobacter-pylori.
36. H. Shimomura, S. Hayashi, K. Yokota, K. Oguma, Y. Hirai, Alteration in the composition of cholesteryl glucosides and other lipids in *Helicobacter pylori* undergoing morphological change from spiral to coccoid form. *FEMS Microbiol. Lett*. 237 (2004) 407-413.
37. K. Hosoda, H. Shimomura, S. Hayashi, K. Yokota, Y. Hirai, Anabolic utilization of steroid hormones in *Helicobacter pylori*. *FEMS Microbiol. Lett*. 297 (2009) 175-179.
38. L. Miller, E.W. Alley, W.J. Murphy, S.W. Russell, J.S. Hunt, Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. *J. Leukocyte Biol*. 59 (1996) 442-450.
39. Y. Kohmura, T. Kirikae, F. Kirikae, M. Nakano, I. Sato, Onapristone (ZK299) blocks the suppressive effect of progesterone, but not that of dexamethasone, on inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. *Int. J. Immunopharmacol*. 22 (2000) 765-774.
40. L.A. Jones, J.P. Anthony, F.L. Henriquez, R.E. Lyons, M.B. Nickdel, K.C. Carter, J.

Alexander, C.W. Roberts, Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors. *Immunol*. 125 (2008) 59-69.

41. F.M. Menzies, F.L. Henriquez, J. Alexander, C.W. Roberts, Selective inhibition and augmentation of alternative macrophage activation by progesterone. *Immunol*. 134 (2011) 281-291.
42. L. Miller, J.S. Hunt, Regulation of TNF-α production in activated mouse macrophages by progesterone. *J. Immunol*. 160 (1998) 5098-5104.
43. L. Su, Y. Sun, F. Ma, P. Lii, H. Huang, J. Zhou, Progesterone inhibits Toll-like receptor 4-mediated innate immune response in macrophages by suppressing NF-κB activation and enhancing SOCS1 expression. *Immunol. Lett*. 125 (2009) 151-155.
44. J.K. Dunnick, W.M. O'Leary, Correlation of bacterial lipid composition with antibiotic resistance. *J. Bacteriol*. 101 (1970) 892-900.
45. D.B. Kearns, J. Robinson, L.J. Shimkets, *Pseudomonas aeruginosa* exhibits directed twitching motility up phosphatidylethanolamine gradients. *J. Bacteriol*. 183 (2001) 763-767.
46. A. Shokri, G. Larsson, Characterisation of the *Escherichia coli* membrane structure and function during fed batch cultivation. *Microb*. Cell Fact. 3 (2004) 9.
47. A. Amine, K.S. Saloua, M. Mouadh, E.I.M. Alya, I. Ahmed, The absence of the "GATC-binding protein SeqA" affects DNA replication in *Salmonella enterica* serovar Typhimurium. *In* Kusic-Tisma J (ed), *DNA replication and related cellular processes* (2011), InTech, Rijeka, Croatia. http://www.intechopen.com/book/dna-replication-and-related-cellular-processes/the-absence-of-the-gatc-binding-protein-seqa-affects-dna-replication-in-salmonella-enterica-serovar-typhimurium.
48. E. Junquera, L. Peña, E. Aicart, Binding of sodium salicylate by β-cyclodextrin or 2,6-di-*O*-methyl-β-cyclodextrin in aqueous solution. *J. Pharmaceutical Sci*. 87 (1998) 86-90.

49. M. Fukasawa, M. Nishijima, H. Itabe, T. Takano, K. Hanada, Reduction of sphingomyelin level without accumulation of ceramide in Chinese hamster ovary cells affects detergent-resistant membrane domains and enhances cellular cholesterol efflux to methyl-beta-cyclodextrin. *J. Biol. Chem*. 275 (2000) 34028-34034.
50. T.G. Anderson, A. Tan, P. Ganz, J. Seelig, Calorimetric measurement of phospholipid interaction with methyl-β-cyclodextrin. *Biochemistry* 43 (2004) 2251-2261.
51. H. Arima, K. Yunomae, T. Morikawa, F. Hirayama, K. Uekama, Contribution of cholesterol and phospholipids to inhibitory effect of dimethyl-β-cyclodextrin on efflux function of P-glycoprotein and multidrug resistance-associated protein 2 in vinblastine-resistant Caco-2 cell monolayers. *Pharmaceutical Res*. 21 (2004) 625-634.
52. Z. Yu, M. Cui, C. Yan, F. Song, Z. Liu, S. Liu, Investigation of heptakis (2,6-di-*O*-methyl)-β-cyclodextrin inclusion complexes with flavonoid glucosides by electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom*. 21 (2007) 683-690.
53. J.E. Metherall, K. Waugh, H. Li, Progesterone inhibits cholesterol biosynthesis in cultured cells: accumulation of cholesterol precursors. *J. Biol. Chem*. 271 (1996) 2627-2633.

2. フェリス菌およびシネジ菌のホスファチジルエタノールアミン

2. Phosphatidylethanolamine isolated from *Helicobacter felis* and *Helicobacter cinaedi*

ABSTRACT

Several studies by our group have considerably revealed the relationship between cholesterol and *Helicobacter pylori*. This bacterial species aggressively incorporates exogenous cholesterol into the cell membranes at least by the intermediation of phosphatidylethanolamine (PE) in the membrane lipid constituent. PE of *H. pylori* contains myristic acid ($C_{14:0}$) as the most prevalent saturated fatty acid and is easily distinguishable from PE of commonplace Gram-negative bacteria that contains palmitic acid ($C_{16:0}$) as the most prevalent saturated fatty acid. *H. pylori* PE is considered to bind more selectively to cholesterol than to cholesteryl ester by the myristic acid in its PE molecule. Therefore, the bacterial cell membranes of *H. pylori* selectively absorb cholesterol from a medium supplemented with serum, even though the concentration of cholesterol is conspicuously lower than that of cholesteryl ester in serum. As observed in *H. pylori*, the same bacterial Genus, *H. felis* and *H. cinaedi* likewise selectively absorb cholesterol into the cell membranes, when the bacteria were incubated using serum-supplemented medium. No earlier investigations have, however, elucidated as for whether PE of the two *Helicobacter* bacterial species takes part in the selective absorption of cholesterol into the cell membranes. We, therefore, isolated the PE from the

two *Helicobacter* species to analyze the hydrophobic interaction between cholesterol and its glycerophospholipid. PE of both *Helicobacter* bacteria interacted more selectively with cholesterol than with cholesteryl ester, and the degree of the selective binding of cholesterol was higher in the PE than in the phosphatidylglycerol-cardiolipin of the same bacteria. These results suggested the possibility that the bacterial cells of *H. felis* and *H. cinaedi* may abundantly contain PE with myristic acid. On this basis, we analyzed the PE molecular species of the *Helicobacter* bacteria and demonstrated that the PE containing myristic acid accounts for more than 35% in the total PE. These results suggest that the myristoyl PE takes part in the absorption of cholesterol in *H. felis* and *H. cinaedi*.

INTRODUCTION

Helicobacter felis is a Gram-negative spiral rod equipped with bipolar tufts of flagella. This bacterium is isolated from the gastric mucosa of cats and dogs [1-3]. *H. felis* also causes chronic gastritis and gastric MALT lymphoma in mouse when it colonizes the mouse stomach [4, 5]. As such, mice infected with *H. felis* are often used as the substitution animal models to examine the clinical pathology of *H. pylori* infectious disease in human.

H. cinaedi is a Gram-negative rod equipped with bipolar flagella. This bacterium is isolated from the intestinal tracts and livers of various animals such as human, dog, cat, and hamster [6, 7]. In hamsters, *H. cinaedi* is considered to commonly inhabit the intestine [8]. Most persons infected with *H. cinaedi* show no clinical symptoms, though some individuals suffer from systematic inflammations, such as phlegmone, arthritis, and

meningitis, due to the bacteriaemia [9].

An earlier study by our group has demonstrated that *H. felis* and *H. cinaedi* absorb cholesterol rather than cholesteryl ester in the medium supplemented with serum and the former induces the glucosylation of the absorbed cholesterol [10]. Recent studies by our group have revealed that *H. pylori*, a pathogen responsible for gastroduodenal diseases in human [11], retains phosphatidylethanolamine (PE) as the most prevalent glycerophospholipid, and have suggested that the PE of *H. pylori* plays an important role in the assimilation of exogenous cholesterol into the bacterial cells [12-14]. No earlier investigations have, however, elucidated as for whether the PE of *H. felis* and *H. cinaedi* exhibits the selective binding to cholesterol compared to cholesteryl ester.

This study, therefore, examined the hydrophobic interaction between cholesterol and the two *Helicobacter* bacterial PE, and analyzed the PE molecular species of the two *Helicobacter* bacterial species.

MATERIALS and METHODS

Bacterial strains and culture

Two bacterial species *H. felis* strain ATCC 49179, an isolate from a cat, and *H. cinaedi* strain 1193, an isolate from a hamster were investigated in this study. We cultured the bacteria using a pleuropneumonia-like organisms (PPLO) broth (Difco Laboratories) without serum to avoid the contaminations of hydrophobic materials derived from serum. For the investigation of the cholesterol absorption of bacteria, the bacterial cells were cultured using a PPLO broth containing both 30 μM cholesterol (Wako Pure Chemical

Industries Ltd.) and 0.2% 2,6-di-*O*-methyl-β-cyclodextrin (dMβCD: Sigma-Aldrich Inc.). For the purification of PE and phosphatidylglycerol-cardiolipin (PG-CL), the bacterial cells were cultured using PPLO broth without either cholesterol or dMβCD. The cultures were carried out on a shaker under microaerophilic conditions at 37˚C (Concept 400: Ruskinn Technology), and the bacteria were harvested by centrifugation (10000 xg, 10 min) after the cultures from 24 to 48 h. The bacterial pellets were washed three times with PBS by centrifugation (10000 xg, 10 min).

Extraction of lipids

The bacterial cell pellets were thoroughly sonicated with PBS (16 ml) in order to prepare the lysates. Chloroform-methanol (2:1) solvents (80 ml) were then added into the lysates (16 ml) in a glass bottle, vigorously shaken, and incubated overnight at 4˚C. After incubation, the lower liquid phase was recovered, and the solvent was vaporized at 60˚C using a rotary-evaporator to obtain the whole bacterial cell lipids. A thin-layer chromatography (TLC) was carried out to analyze the lipid profiles using chloroform-methanol-water (70:30:5) solvents.

Quantification of cholesterol in bacterial lipids

Cholesterol in the bacterial lipids was quantified by ferrous chloride-sulfuric acid method. In brief, the lipid specimen dissolved in an acetic acid solution (600 µl) was mixed with a 400 µl of ferrous chloride-sulfuric acid reagent [phosphoric acid-sulfuric acid (2:25) solution containing 0.2% $FeCl_2$-$6H_2O$], thoroughly stirred, and incubated for

15 min at room temperature. After color reaction, the absorbance of the mixture (200 µl) was measured at wavelength of 550 nm. The amounts of cholesterol were quantified based on a cholesterol standard curve, and the cholesterol content was calculated as a ratio to the dry weight of total lipid.

Isolation of PE and PG-CL from bacterial lipids

PE and PG-CL in the bacterial lipids were purified via the method our group reported earlier [14]. In brief, the lipids were stepwisely eluted in turn of acetone-methanol (7:3) solvents (10 ml), acetone-methanol (4:6) solvents (10 ml) and acetone-methanol (2:8) solvents (10 ml) from a column (1 cm-diameter; 5 cm-height) filled with chloroform-activated Iatrobead 6RS-8060 (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). To confirm the purity, the fractions of the PE and PG-CL were analyzed by TLC using chloroform-methanol-water (70:30:5) solvents.

Analysis of the hydrophobic interaction between cholesterols, PE, and PG-CL

The hydrophobic bonding of cholesterol to either PE or PG-CL was evaluated by the method our group reported earlier [14]. In brief, the PE(200 µg)-fixed paper disk or PG-CL(200 µg)-fixed paper disk was soaked in a 50 mM Tris (pH 7.5) buffer (2 ml) containing both 30 µM cholesterol and 3 mM dMβCD, and shaken for 2 h at 37˚C using a well of a 12-well micro-plate. The paper disk was then washed six times with distilled water (2 ml) by shaking, dried using a centrifugal concentrator, and soaked in a chloroform solvent (800 µl) in a Teflon tube in order to elute the lipid components from

the paper disk. After the paper disk was removed, chloroform solvent was vaporized using a centrifugal concentrator to quantify the cholesterol contained in the paper disk by the ferrous chloride-sulfuric acid method. The paper disk without either PE or PG-CL was used as a negative control. Cholesteryl ester (cholesterol hexanoate: Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was used in place of cholesterol to carry out the same experiment.

Analysis of the fatty acid compositions of PE

After methanolysis of the purified PE (20 μg), the specimen (1 ml) was mixed with hexane (1 ml) to prepare a fatty acid methyl ester [14]. The fatty acid methyl ester (1 μl) was then applied to a GCMS-QP2010 device (Shimadzu Techno-Research Inc., Kyoto, Japan) to analyze the fatty acid compositions of the PE by gas chromatogram-mass spectrometry (GC-MS). The fatty acids were identified based on a database library of the spectral peaks of the fatty acid methyl ester molecules. The ratios of the fatty acid components of the PE were calculated from the magnitude of the peak area of each fatty acid spectrum. Palmitic acid-d_{31} (Wako Pure Chemical Industries Ltd.) as an internal standard was injected together with the specimens onto the GC column.

Analysis of PE molecular species

The purified PE (100 μg) was dissolved in chloroform-methanol (1:1) solvents (1 ml), diluted to a 1 μg/ml concentration with methanol, and applied as diluent (5 μl) to the Asahipak ODP-40 2D column of the LC-20A system at 40˚C (Shimadzu

Techno-Research Inc.) for analysis by high-performance liquid chromatography (HPLC). After the HPLC, the mass spectra of the PE molecular species were detected by a negative mode of electrospray ionization mass spectrometry using an API3000 mass spectrometry device (AB Sciex Co., CA). The PE molecular species were identified with the spectra resulting from the combinations of the fatty acid pairs attached to each PE. The fatty acid combinations of the PE molecular species were determined based on the GC-MS analysis of the fatty acid compositions of PE. The ratios for each PE molecular species were calculated from the magnitude of the peak area of each mass spectrum.

RESULTS

Cholesterol absorption of *H. felis* and *H. cinaedi*

Our first step in this study was to examine the absorption of cholesterol in the two *Helicobacter* bacteria. When *H. felis* and *H. cinaedi* were cultured in the presence of cholesterol and dMβCD, the bacterial cells of *H. felis* and *H. cinaedi* obviously absorbed the cholesterol (Fig. 1A). Therefore, the ratios of cholesterol contained in the total lipid extracted from *H. felis* and *H. cinaedi* were approximately 20% and 1.3%, respectively. The TLC analysis detected the spots of two types of glucosylated cholesterols CGL and CAG and cholesterol in the lipid compositions of *H. felis* (Fig. 1B). Meanwhile, the spot of cholesterol was detected in the lipid compositions of *H. cinaedi* by TLC analysis.

Purification of PE and PG-CL of *H. felis* and *H. cinaedi*

As seen in Fig. 1B, *H. felis* and *H. cinaedi* retained PE and PG-CL as the

predominant glycerophospholipids. We next purified the bacterial PE and PG-CL for use in the experiments below. The TLC analysis was carried out to confirm the purity of the PE and PG-CL isolated from the bacterial cells of *H. felis* and *H. cinaedi*. The PE and PG-CL of both *Helicobacter* bacteria were recovered with a high purity in the fractions eluted with acetone-methanol (2:8) solvent and acetone-methanol (7:3) solvent, respectively, from the Iatrobead-column (Fig. 2). On this basis, we decided to use the fractions obtained from the acetone-methanol (2:8) eluate and acetone-methanol (7:3) eluate as the preparation of PE and PG-CL, respectively.

Hydrophobic bonding of cholesterols to the *Helicobacter* bacterial PE

A recent study by our group has demonstrated that *H. pylori* PE functions as a cholesterol-binding lipid in the assimilation of its cholesterol [14]. We next analyzed the binding of either cholesterol or cholesteryl ester to the *Helicobacter* bacterial PE in the comparison with the binding of either cholesterol or cholesteryl ester to the same bacterial PG-CL. Both *Helicobacter* bacterial PE in the paper disks bound more selectively cholesterol than cholesteryl ester (Fig. 3). Hence, the amount of cholesterol detected in the PE fixed into a paper disk was conspicuously larger than that of cholesteryl ester detected in the same glycerophospholipid fixed into a paper disk. Though the PG-CL of the two *Helicobacter* bacteria fixed into a paper disk showed somewhat selective binding to cholesterol in the comparison to cholesteryl ester, the degree of the selective binding of cholesterol to the PG-CL was obviously lower than that of the selective binding of cholesterol to the PE. These results indicate that PE of *H. felis*

and *H. cinaedi* is higher in the potential to selectively bind cholesterol than PG-CL of the same bacteria, even though the number of PE molecules fixed into a paper disk was significantly different from the number of PG-CL molecules fixed into a paper disk.

Fatty acid composition of PE of *H. felis* and *H. cinaedi*

Next, we analyzed the fatty acid compositions of the purified PE from *H. felis* and *H. cinaedi*. Palmitic acid ($C_{16:0}$) and myristic acid ($C_{14:0}$) were the most abundant and second most abundant saturated fatty acid components in the PE from the two *Helicobacter* bacteria, respectively (Table 1). Intriguingly, lauric acid ($C_{12:0}$) accounted for a relatively high ratio of the fatty acid composition of each bacterium. Only one unsaturated fatty acid component, oleic acid ($C_{18:1}$), was detected in the PE from both *H. felis* and *H. cinaedi*. Low amounts of three fatty acids, tridecanoic acid ($C_{13:0}$), pentadecanoic acid ($C_{15:0}$) and heptadecanoic acid ($C_{17:0}$) were detected in the PE from *H. cinaedi*.

PE molecular species of *H. felis* and *H. cinaedi*

We identified the PE molecular species of *H. felis* and *H. cinaedi* based on the results on the fatty acid compositions. More than 75% of the purified *H. felis* PE was composed of PE molecular species containing two saturated fatty acid: one was a PE molecular species with a lauric acid ($C_{12:0}$) and a palmitic acid ($C_{16:0}$); the other was a PE molecular species with a myristic acid ($C_{14:0}$) and a palmitic acid ($C_{16:0}$) (Table 2). The same two PE molecular species accounted for approximately 64% of the purified *H. cinaedi* PE. In addition, a PE molecular species containing two unsaturated fatty acids ($C_{18:1}$) was

detected at a relatively high ratio in the purified PE from these bacteria.

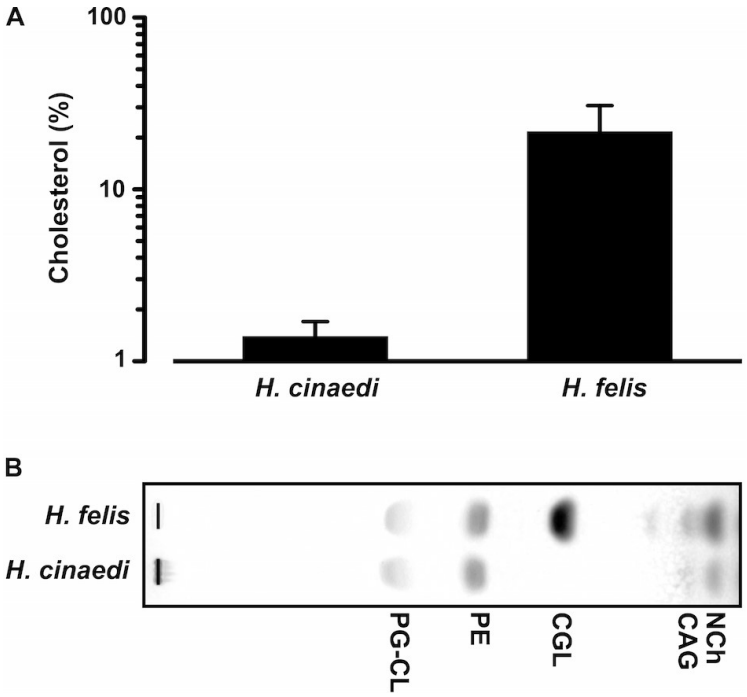

Fig. 1. Cholesterol content in the lipid compositions extracted from the *Helicobacter* species

(A) *H. felis* and *H. cinaedi* were cultured in the presence of cholesterol (30 μM) and dMβCD (0.2%), and the lipids were extracted from the cells by the organic solvent distribution method. The amounts of cholesterol contained in the lipids (200 μg) were measured by the ferrous chloride-sulfuric acid method. Results show a percentage of cholesterol in the total lipid. (B) The lipids (200 μg) extracted from *H. felis* and *H. cinaedi* cultured in the presence of cholesterol (30 μM) and dMβCD (0.2%) were analyzed by TLC using chloroform-methanol water (70:30:5) solvents, and the lipid spots were detected using a 60% sulfuric acid solution. CGL, cholesteryl-α-D-glucopyranoside; CAG, cholesteryl-6-*O*-acyl-α-D-glucopyranoside; NCh, chole- sterol

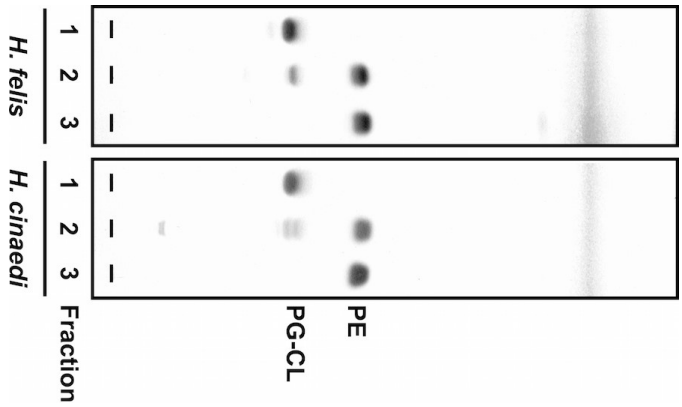

Fig. 2. Lipid profiles of *H. felis* and *H. cinaedi* fractionated by Iatrobead-column chromatography

H. felis and *H. cinaedi* were cultured without either cholesterol or dMβCD, and the lipids were extracted from the cells by the organic solvent distribution method in order to isolate PE and PG-CL by an Iatrobead-column chromatography. Fractions 1, 2, and 3 denote the lipid (200 μg/lane) profiles obtained from eluates of acetone-methanol (7:3), acetone-methanol (4:6), and acetone-methanol (2:8), respectively. The lipid spots were detected with a 60% sulfuric acid solution after TLC with chloroform-methanol-water (70:30:5) solvents.

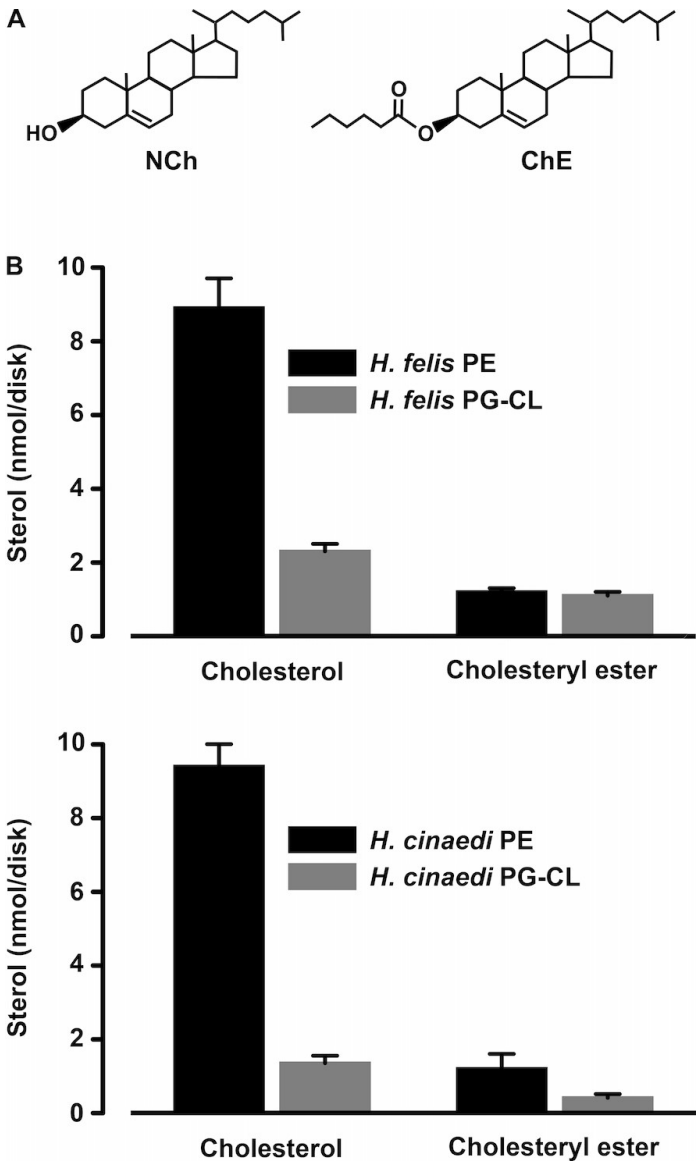

Fig. 3. Binding of cholesterols to PE and PG-CL of *H. felis* and *H. cinaedi*

(A) Chemical structures of cholesterol (NCh) and cholesteryl ester (ChE). (B) A 200 μg of either the PE or PG-CL fixed into a paper disk was soaked in a buffer (2 ml) containing dMβCD (3 mM) and shaken for 2 h in the presence of cholesterol (30 μM) or cholesteryl ester (30 μM) in the buffer at 37˚C. After wash of the paper disk, the amounts of cholesterol and cholesteryl ester contained in the paper disks were quantified by the ferrous chloride-sulfuric acid method.

Table 1. Fatty acid composition (%) of PE

Fatty acid	*H. felis* PE	*H. cinaedi* PE
$C_{12:0}$	11.2	7.4
$C_{13:0}$	UD	1.0
$C_{14:0}$	16.2	12.8
$C_{15:0}$	UD	1.6
$C_{16:0}$	54.8	53.2
$C_{17:0}$	UD	0.9
$C_{18:1}$	17.7	23.1
Total	99.9	100

UD, undetected

Table 2. PE molecular species (%) of Genus *Helicobacter*

Combination of fatty acid	*H. felis* PE	*H. cinaedi* PE
$C_{12:0}$-$C_{15:0}$	UD	1.5
$C_{12:0}$-$C_{16:0}$	40.4	31.2
$C_{13:0}$-$C_{16:0}$	UD	5.2
$C_{14:0}$-$C_{15:0}$	UD	1.5
$C_{14:0}$-$C_{16:0}$	36.9	32.9
$C_{14:0}$-$C_{18:1}$	UD	1.5
$C_{16:0}$-$C_{16:0}$	3.7	4.9
$C_{16:0}$-$C_{18:1}$	4.3	7.4
$C_{18:1}$-$C_{18:1}$	10.4	7.8
Total	95.7	93.9

Less than 1% of PE molecular species is not shown.

UD, undetected

DISCUSSION

A representative *Helicobacter* species *H. pylori* is known to assimilate the exogenous cholesterol into the bacterial cells [12-19]. This study demonstrated that the cells of *H. felis* and *H. cinaedi* absorb cholesterol added into the medium, as observed in the cells of *H. pylori*. A recent study by our group has suggested that *H. pylori* interacts with exogenous cholesterol by the intermediation of the PE containing myristic acid [14]. This study revealed that *H. felis* and *H. cinaedi* retain the PE containing myristic acid at a relatively high ratio. These results suggest the possibility that the myristoyl PE takes part in the incorporation of cholesterol into the cells of *H. felis* and *H. cinaedi*. The goal of our study will be to chemically synthesize the individual PE molecular species of *H. felis* and *H. cinaedi*, and to clarify which PE molecular species exhibit the highest selectivity in the binding of cholesterol.

Recent studies by our group have suggested that the outermost layer of the outer membrane of *H. pylori* retains PE in relatively abundant amounts whereas the outermost layer of the outer membrane of *Escherichia coli* retains PG-CL in relatively abundant amounts [14, 20]. This study demonstrated that *H. cinaedi* cells are lower in the capability to absorb the exogenous cholesterol than *H. felis* cells, even though *H. cinaedi* PE is comparable to *H. felis* PE in the potential to bind the cholesterol. These results suggest that PE may be infrequent in the outermost layer of the outer membrane of *H. cinaedi*. Apart from this, Gram-negative bacteria generally possess a lipopolysaccharide (LPS) as the lipid composition in the outermost layer of the outer membrane. LPS is a glycolipid consisting of a long polysaccharide chain and fatty acid residues, and the part of the

polysaccharide chain directly faces to the outsides of the bacterial cells [21]. One of the functions of LPS is to limit the membrane permeability of hydrophobic compounds to the bacterial cells. This may mean that the LPS content in the lipid compositions of the outer membrane is less in *H. felis* than in *H. cinaedi* and therefore the former bacterial cells easily contact the exogenous cholesterol via the intermediation of the PE. Further investigations will be necessary to clarify the difference of the lipid compositions (including LPS) in the outermost layer of the outer membrane between the two *Helicobacter* bacterial species.

ACKNOWLEDGMENTS

This work was supported in part by JKA Promotion Funds from Keirin Race.

REFERENCES

1. A. Lee, S.L. Hazell, J. O'Rourke, S. Kouprach, Isolation of a spiral-shaped bacterium from the cat stomach. *Infect. Immun*. 56 (1988) 2843-2850.
2. B.J. Paster, A. Lee, J.G. Fox, F.E. Dewhirst, L.A. Tordoff, G.J. Fraser, J.L. O'Rourke, N.S. Taylor, R. Ferrero, Phylogeny of *Helicobacter felis* sp. Nov., *Helicobacter mustelae*, and related bacteria. *Int. J. Sys. Bacteriol*. 41 (1991) 31-38.
3. T.K. Hansen, P.S. Hansen, A. Nørgaard, H. Nielsen, A. Lee, L.P. Andersen,*Helicobacter felis* does not stimulate human neutrophil oxidative burst in contrast to '*Gastrospirillum hominis*' and *Helicobacter pylori*. *FEMS. Immunol. Med. Microbiol*. 30 (2001) 187-195.
4. A. Enno, J.L. O'Rourke, C.R. Howlett, A. Jack, M.F. Dixon, A. Lee, MALToma-like lesions in the murine gastric mucosa after long-term infection with *Helicobacter felis*. *Am. J. Pathol*. 147 (1995) 217-222.

5. M. Mohammadi, R. Redline, J. Nedrud, S. Czinn, Role of the host in pathogenesis of *Helicobacter*-associated gastritis: *H. felis* infection of inbred and congenic mouse strains. *Infect. Immun*. 64 (1996) 238-245.
6. J.A. Kiehlbauch, D.J. Brenner, D.N. Cameron, A.G. Steigerwalt, J.M. Makowski, C.N. Baker, C.M. Patton, I.K. Wachsmuth, Genotypic and phenotypic characterization of *Helicobacter cinaedi* and *Helicobacter fennelliae* strains isolated from humans and animals. *J. Clin. Microbiol*. 33 (1995) 2940-2947.
7. J.G. Fox, L. Handt, B.J. Sheppard, S. Xu, F.E. Dewhirst, S. Motzel, H. Klein, Isolation of *Helicobacter cinaedi* from the colon, liver, and mesenteric lymph node of a rhesus monkey with chronic colitis and hepatitis. *J. Clin. Microbiol*. 39 (2001) 1580-1585.
8. C.J. Gebhart, C.L. Fennell, M.P. Murtaugh, W.E. Stamm,*Campylobacter cinaedi* is normal intestinal flora in hamsters. *J. Clin. Microbiol.* 27 (1989) 1692-1694.
9. Y. Kawamura, J. Tomida, Y. Morita, S. Fujii, T. Okamoto, T. Akaike, Clinical and bacteriological characteristics of *Helicobacter cinaedi* infection. *J. Infect. Chemother*. 20 (2014) 517-526.
10. M. Haque, Y. Hirai, K. Yokota, K. Oguma, Steryl glucosides: a characteristic feature of the *Helicobacter* spp.? *J. Bacteriol*. 177 (1995) 5334-5337.
11. B. Marshall, J.R. Warren, Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1 (1983) 1273-1274.
12. H. Shimomura, S. Hayashi, K. Yokota, K. Oguma, Y. Hirai, Alteration in the composition of cholesteryl glucosides and other lipids in *Helicobacter pylori* undergoing morphological change from spiral to coccoid form. *FEMS. Microbiol. Lett* .237 (2004) 407-413.
13. H. Shimomura, K. Hosoda, S. Hayashi, K. Yokota, K. Oguma, Y. Hirai, Steroids mediate resistance to the bactericidal effect of phosphatidycholines against *Helicobacter pylori*. *FEMS. Microbiol. Lett*. 301 (2009) 84-94.
14. H. Shimomura, K. Hosoda, S. Hayashi, K. Yokota, Y. Hirai,

Phosphatidylethanolamine of *Helicobacter pylori* functions as a steroid-binding lipid in the assimilation of free cholesterol and 3β-hydroxyl steroids into the bacterial cell membrane. *J. Bacteriol*. 194 (2012) 2658-2667.

15. Y. Inamoto, S. Hamanaka, Y. Hamanaka, T. Nagate, I. Kondo, T. Takemoto, K. Okita, Lipid composition and fatty acid analysis of *Helicobacter pylori*. *J. Gastroenterol*. 30 (1995) 315-318.
16. Y. Hirai, M. Haque, T. Yoshida, K. Yokota, K. Oguma, Unique cholesteryl glucosides in *Helicobacter pylori*: composition and structural analysis. *J. Bacteriol*. 177 (1995) 5327-5333.
17. C. Wunder, Y. Churin, F. Winau, D. Warrecke, M. Vieth, B. Lindner, U. Zähringer, H.J. Mollenkopf, E. Heinz, T.F. Meyer, Cholesterol glucosylation promotes immune evasion by *Helicobacter pylori*. *Nat. Med*. 12 (2006) 1030-1038.
18. D.J. McGee, A.E. George, E.A. Trainor, K.E. Horton, E. Hildebrandt, T.L. Testerman, Cholesterol enhances *Helicobacter pylori* resistance antibiotics and LL-37. *Antimicrob*. *Agents Chemother*. 55 (2011) 2897-2904.
19. E.A. Trainor, K.E. Horton, P.E. Savage, T.L. Testerman, D.J. McGee, Role of HefC efflux pump in *Helicobacter pylori* cholesterol-dependent resistance to ceragenins and bile salts. *Infect*. *Immune*. 79 (2011) 88-97.
20. A. Amgalanbaatar, H. Shimomura, K. Hosoda, S. Hayashi, K. Yokota, Y. Hirai Antibacterial activity of a novel synthetic progesterone species carrying a linoleic acid molecule against *Helicobacter pylori* and the hormonal effect of its steroid on a murine macrophage-like cell line. *J. Steroid. Biochem. Mol. Biol*. 140 (2014) 17-25.
21. E.T. Rietschel, T. Kirikae, F.U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A.J. Ulmer, U. Zähringer, U. Seydel, F.D. Padova, M. Schreier, H. Brade, Bacterial endotoxin: molecular relationship of structure to activity and function. *FESEB. J.* 8 (1994) 217-225.

ACKNOWLEDGMENTS

I’m deeply grateful to Dr. Yoshikazu Hirai who was the previous professor of Division of Bacteriology in Jichi Medical University and considerately conducted me to a preferable bacteriological researcher. I’m also thankful to Dr. Hirofumi Shimomura who is an associated professor of Division of Bacteriology in Jichi Medical University and to Dr. Kouichi Hosoda who is a researcher belonging to Civil International Corporation.