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Relationship Between Steroid Hormones and *Helicobacter pylori*

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1. Introduction

Helicobacter pylori is a Gram-negative microaerobic curved-rod possessing polar flagella as the motility organ. This bacterium colonizes human gastric epithelium and causes chronic gastritis and peptic ulcers (Graham, 1991; Warren & Marshall, 1983; Wyatt & Dixon, 1988). Via longer periods of colonization in the human stomach, it also contributes to the development of gastric cancer and marginal zone B-cell lymphoma (Forman, Eurogast Study Group, 1993; Wotherspoon et al., 1991). Approximately half of population in the world is infected with *Helicobacter pylori*, and the majority of infected persons develop atrophic gastritis with or without symptoms. Among *Helicobacter pylori*-infected individuals, about 10% persons develop gastric and duodenal ulcers, 1% to 3% persons develop gastric adenocarcinoma, and 0.1% or less person develops gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Fukase et al., 2008; Peek & Blaser, 2002; Peek & Crabtree, 2006; Stolte et al., 2002; Uemura et al., 2001).

The bacterial species belonging to the genus *Helicobacter* have a unique feature of free-cholesterol (FC) assimilation into the membrane lipid compositions (Haque et al., 1995, 1996). *Helicobacter pylori* aggressively absorbs free-cholesterol supplemented to a medium, or extracts free-cholesterol from the lipid raft of epithelial cell membrane when the organism adhered onto the epithelial cell surface (Wunder et al., 2006). The free-cholesterol assimilated into the *Helicobacter pylori* membranes is glucosylated via the enzymatic action, and the organism utilizes as the membrane lipid components both free-cholesterol itself and the glucosylated cholesterols. Previous study by our group has identified the following three types of glucosyl cholesterols in the membrane lipid compositions of *Helicobacter pylori* (Hirai et al., 1995): cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (CPG). One of the enzymes involved in the biosynthesis of glucosyl cholesterols is HP0421 protein, a cholesterol α-glucosyltransferase encoded by HP0421 gene in *Helicobacter pylori* (Lebrun et al., 2006). The HP0421 protein adopts as the glucose source a uridine diphosphate-glucose (UDP-Glc) and catalyzes the dehydration condensation reaction between a 1α-hydroxyl (OH) group of D-glucose (Glc) molecule and a 3β-OH group of free-cholesterol (FC) molecule, and thereby CGL is synthesized. The other enzymes involved in the biosynthesis of CAG and CPG have still not been identified (Fig. 1).

Though it is almost special cases that bacterial species produce glucosyl sterols, plants and fungi universally produce various glucosyl sterols such as glucosyl sitosterol and glucosyl

ergosterol (Kim et al., 2002; Oku et al., 2003; Peng et al., 2002; Warnecke et al., 1994, 1997, 1999). As with *Helicobacter pylori*, the bacterial species that produce glucosyl cholesterol have been only reported in *Borrelia hermsi*, *Acholeplasma axanthum*, *Spiroplasma* spp., and *Mycoplasma gallinarum* (Livermore et al., 1978; Mayberry & Smith, 1983; Patel et al., 1978; Smith, 1971). Recent studies have shown that *Borrelia burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii* possess the galactosyl cholesterol that binds to the cholesterol a D-galactose as the sugar molecule in place of a D-glucose (Ben-Menachem et al., 2003; Schröder et al., 2003; Stübs et al., 2009). Plants and fungi carry out the biosynthesis of sterols by themselves, and thereafter attach a D-glucose molecule to the sterols biosynthesized via the catalytic action of sterol β-glucosyltransferase. In contrast, bacterial species including *Helicobacter pylori* do not have the anabolic pathway for cholesterol. Therefore, *Helicobacter pylori* must absorb free-cholesterol from the outside environments to biosynthesize glucosyl cholesterols. In addition, there is the structural difference between glucosyl cholesterols of *Helicobacter pylori* and the other glucosyl sterols. The D-glucose molecule in glucosyl cholesterols of *Helicobacter pylori* is attached to the cholesterol molecule with α-configuration, whereas the D-glucose molecule of phytogenic and fungal glucosyl sterols is attached to the sterol molecule with β-configuration. This structural difference is resulting from the enzymatic action catalyzing the binding of D-glucose into the sterol. In sum, the HP0421 protein of *Helicobacter pylori* catalyzes the α-glucosidic linkage between the 1α-OH group of D-glucose molecule and the 3β-OH group of free-cholesterol molecule, whereas the sterol β-glucosyltransferase of plants and fungi catalyzes the β-glucosidic linkage between the 1β-OH group of D-glucose molecule and the 3β-OH group of free-sterol molecule.

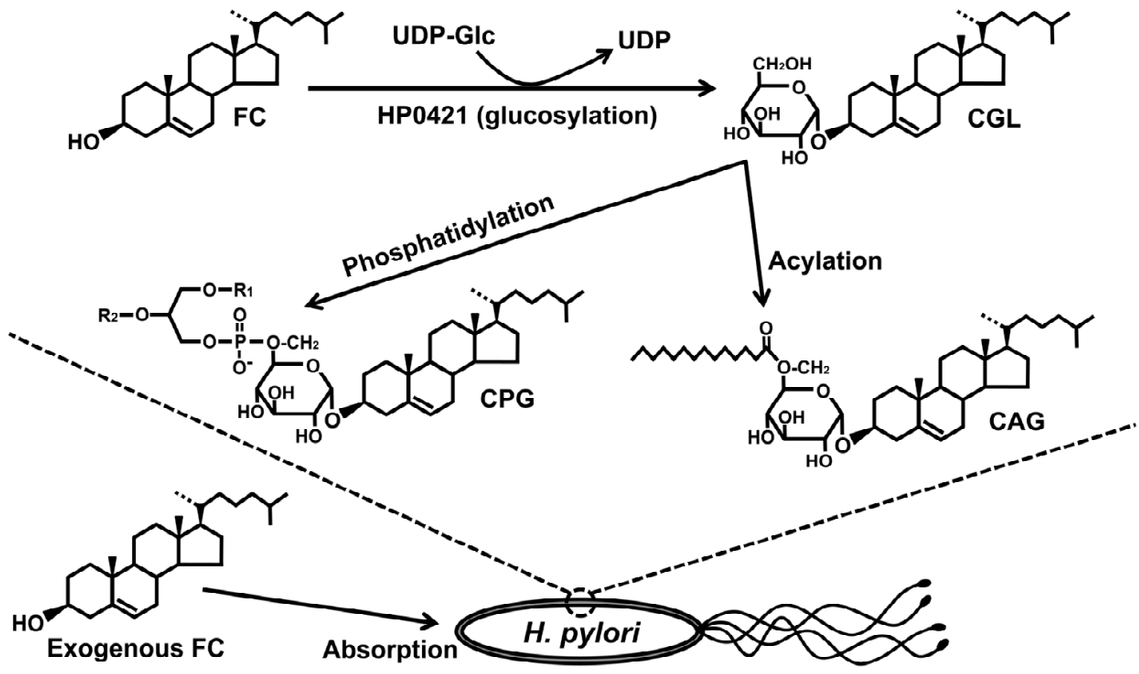

Fig. 1. The free-cholesterol (FC) assimilation of *Helicobacter pylori*

For many years, the biological significance of cholesterol glucosylation in *Helicobacter pylori* remained to be clarified. Recently, it has been, however, elucidated that *Helicobacter pylori*

induces the glucosylation of free-cholesterol absorbed into the membranes to evade the host immune systems (Wunder et al., 2006). The HP0421 gene-knockout *Helicobacter pylori* mutant, which lacks the capability to biosynthesize the glucosyl cholesterols and retains free-cholesterol without glucosylation, easily succumbs to the phagocytosis of macrophages, and strongly induces the activation of antigen-specific T cells, compared to the wild type *Helicobacter pylori*. In addition, when the HP0421 gene-knockout mutant is inoculated into the mouse via oral administration, the organism is promptly excluded from the murine gastric epithelium. The abnormal wild type *Helicobacter pylori*, which fails to induce the biosynthesis of enough amount glucosyl cholesterols by artificial insertion of excessive free-cholesterol into the membranes, also succumbs to the phagocytosis of macrophages and induces the activation of antigen-specific T cells, to similar to the cases of HP0421 gene-knockout mutant. In contrast, the normal wild type *Helicobacter pylori* and the HP0421 gene-reconstructed organism resist the phagocytosis of macrophages, control the induction of antigen-specific T cell activation, and colonize longer periods onto the gastric epithelium of mouse. These findings indicate that the glucosylation of free-cholesterol absorbed into the membranes of *Helicobacter pylori* plays an important role in survival and colonization of the organism in host.

However, it remains to be clarified about why *Helicobacter pylori* aggressively absorbs exogenous free-cholesterol into the membranes. If *Helicobacter pylori*, as with the general bacterial species, did not absorb free-cholesterol into the membranes, the organism will not need to glucosylate it. In addition, free-cholesterol is rather harmful for the survival of *Helicobacter pylori*, because the organism possessing free-cholesterol without glucosylation strongly activates the macrophages and the antigen-specific T cells, and is eradicated from the gastric epithelium by inducing the host immune responses. Moreover, whether the free-cholesterol is the only sterol absorbed into the membranes of *Helicobacter pylori* is also unsolved. Our group, thus, assumed that the free-cholesterol (or steroid) absorption in *Helicobacter pylori* has other some physiological role to maintain the viability of the organism.

To elucidate these unsolved points, our group has initiated the investigations as to the capability of *Helicobacter pylori* to use steroid hormones. Steroid hormones, such as sex hormones and corticoids, are typical sterol analogues that are derived from free-cholesterol in mammals. A number of investigations have demonstrated that the enzymes involved in the biosynthesis and the activation of sex hormones are also expressed in human stomach tissue (Javitt et al., 2001; Miki et al., 2002; Takeyama et al., 2000; Turgeon et al., 2001). In addition, the expression of sex hormone receptors has been found in gastric cancer (Kominea et al., 2004; Matsuyama et al., 2002; Takano et al., 2002). These studies indirectly indicate that sex hormones exist in the human stomach environment. We know that *Helicobacter pylori* colonizes the human stomach. In sum, there is possibility that *Helicobacter pylori* has contact with sex hormones in the human stomach. No earlier studies, however, have investigated the assimilation of sex hormones in *Helicobacter pylori*, and/or the influence of sex hormones on the viability of the organism.

Based on the findings from our current basal research, this chapter summarizes the capability of *Helicobacter pylori* to assimilate various sex hormones, and the bactericidal activity of certain sex hormones targeting selectively the *Helicobacter pylori*.

2. The 3β-OH steroids and *Helicobacter pylori*

Of the steroid hormones including steroid pre-hormone, pregnenolone (PN), dehydroepiandrosterone (DEA), and epiandrosterone (EA) possess a hydroxyl group (3β-

OH) with β-configuration at the carbon-3 position of steroid framework, as with free-cholesterol (FC). First of all, our group has, therefore, examined the capability of *Helicobacter pylori* to assimilate the 3β-OH steroid hormones. After *Helicobacter pylori* (10^5 CFU, colony-forming unit/ml) was cultured for 24 hours with pregnenolone (50 μM concentration), dehydroepiandrosterone (50 μM concentration), or epiandrosterone (50 μM concentration) in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions (an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 at 37˚C), the membrane lipids of the organisms were purified via the Folch method (Folch et al., 1957) and analyzed by thin-layer chromatography (TLC). This paragraph summarizes the assimilation of 3β-OH steroid hormones in *Helicobacter pylori*.

2.1 Pregnenolone and *Helicobacter pylori*

Though the structure at the carbon-17 position of pregnenolone framework differs from that at the same carbon position of free-cholesterol framework, the membranes of *Helicobacter pylori* aggressively absorbed the exogenous pregnenolone, and the organism induced the glucosylation of this 3β-OH steroid pre-hormone: the TLC analysis detected the three spots of glucosyl pregnenolones equivalent to the three spots of glucosyl cholesterols (CGL, CAG and CPG) in the membrane lipid compositions of *Helicobacter pylori* (Hosoda et al., 2009).

The recombinant HP0421 protein expressed in *Escherichia coli* has been shown to catalyze α-glucosylation of various phytogenic and fungal sterols (Lebrun et al., 2006). Moreover, *Helicobacter pylori* lacks the gene that encodes sterol β-glucosyltransferase in plants and fungi. Therefore, the glucosyl pregnenolones detected in the membrane lipid compositions of *Helicobacter pylori* are easily guessed to be all α-glucosyl pregnenolones. As with free-cholesterol (FC), the functional group to which a D-glucose molecule can be attached via the catalytic action of HP0421 protein is the only 3β-OH group in the pregnenolone (PN) framework (Fig. 2). The spot of glucosyl pregnenolone corresponding to the CGL spot detected in the TLC analysis must, in sum, be a 3β-(α-D-glucosyl)-pregnenolone. In addition, the spot of glucosyl pregnenolone corresponding to the CAG spot is guessed to be a 3β-(6-*O*-tetradecanoyl-α-D-glucosyl)-pregnenolone, and the spot of glucosyl pregnenolone corresponding to the CPG spot is guessed to be a 3β-(6-*O*-phosphatidyl-α-D-glucosyl)-pregnenolone.

2.2 Dehydroepiandrosterone and *Helicobacter pylori*

As with pregnenolone, the structure at the carbon-17 position of dehydroepiandrosterone framework also differs from that at the same carbon position of free-cholesterol framework. *Helicobacter pylori*, however, absorbed the exogenous dehydroepiandrosterone into the membranes and induced the glucosylation of this androgen. Though the TLC analysis detected the three spots of glucosyl dehydroepiandrosterones, the detection level of the glucosyl dehydroepiandrosterone corresponding to the CGL was remarkably lower than the detection levels of the other glucosyl dehydroepiandrosterones corresponding to the CAG and CPG.

Our previous study has demonstrated that the detection level of CGL, a basic structure of glucosyl cholesterols, in the membrane lipid compositions of *Helicobacter pylori* undergoing the long-term cultures reduces via the conversion to both CAG and CPG that are produced by modifying the CGL molecule with an acyl group and a phosphatidyl group, respectively, and thereby the detection levels of CAG and CPG increase in the membrane lipid

compositions of organism (Shimomura et al., 2004). Our findings as to the assimilation of this androgen, in sum, suggest that *Helicobacter pylori* promptly converts the 3β-(α-D-glucosyl)-dehydroepiandrosterone, which is a fundamental structure of the glucosyl dehydroepiandrosterones, to those modified by an acyl group or a phosphatidyl group. However, the transferases that attach an acyl group or a phosphatidyl group to the CGL molecule have still not been identified in *Helicobacter pylori*. Investigations into the CGL acyltransferase and CGL phosphatidyltransferase will be, therefore, required to elucidate the anabolic pathway in glucosyl cholesterols and glucosyl steroid hormones.

2.3 Epiandrosterone and *Helicobacter pylori*

The only structural difference between dehydroepiandrosterone and epiandrosterone is in the part of double bond between the carbon-5 position and the carbon-6 position in the steroid molecule: dehydroepiandrosterone has the double bond between those carbon positions, while epiandrosterone lacks its double bond. The TLC analysis detected the three spots of glucosyl epiandrosterones in the membrane lipid compositions into which *Helicobacter pylori* assimilated this androgen, but the detection level of the glucosyl epiandrosterone corresponding to the CGL was remarkably lower than the detection levels of the other glucosyl epiandrosterones corresponding to the CAG and CPG, as with the case of dehydroepiandrosterone. These results also suggest that *Helicobacter pylori* promptly converts the 3β-(α-D-glucosyl)-epiandrosterone to those modified by an acyl group or a phosphatidyl group.

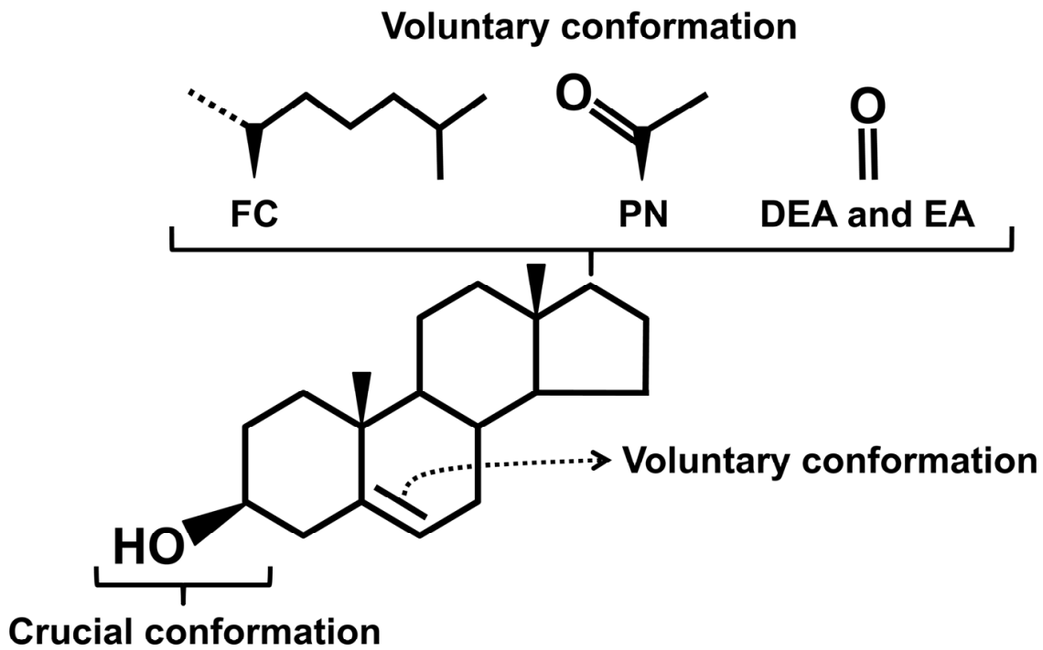

Fig. 2. The 3β-OH: a crucial conformation for steroid glucosylation by *Helicobacter pylori*

These findings from our recent study show that *Helicobacter pylori* glucosylates not only free-cholesterol, but also various 3β-OH steroid hormones. The only common structure among pregnenolone (PN), dehydroepiandrosterone (DEA), epiandrosterone (EA), and free-cholesterol (FC) is a 3β-OH group in the steroid framework (Fig. 2). This, thus, indicates that

the 3β-OH of steroids is a crucial conformation required for the steroid glucosylation by *Helicobacter pylori*. Further conformation analyses will be required to identify the chemical structures of glucosyl steroid hormones in more detail.
Our group has demonstrated the first report to describe the capability of *Helicobacter pylori* to glucosylate steroid hormones (Hosoda et al., 2009). In addition, no earlier studies have reported that the glucosyl sex hormones were detected in eukaryotes, prokaryotes, and/or environments. Further investigations will be necessary to analyze the hormonal actions or biological activities of these glucosyl sex hormones produced by *Helicobacter pylori*.

3. The 3-OH steroids and *Helicobacter pylori*

The three female hormones, namely, estrone (E1), estradiol (E2), and estriol (E3) possess a flat hydroxyl group (3-OH) at the carbon-3 position of the steroid framework. Our recent studies have revealed that these 3-OH steroid hormones have the different influences on the viability of *Helicobacter pylori*. This paragraph describes the relationship between the 3-OH steroid hormones and *Helicobacter pylori*.

3.1 Estrone and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estrone at the 50 μM concentration in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions, the membranes of organism efficiently absorbed the estrone (Hosoda et al., 2009). These findings indicate that *Helicobacter pylori* aggressively uses as the membrane lipid components not only 3β-OH steroids (including free-cholesterol), but also 3-OH steroid estrone. However, the organism has failed to induce the glucosylation of estrone absorbed into the membranes. In combination with the results of glucosylation observed in the 3β-OH steroid hormones, they strongly indicate that *Helicobacter pylori* recognizes only the 3β-OH conformation of steroid molecule and glucosylates only the 3β-OH steroids with or without the other structural differences.

3.2 Estradiol and *Helicobacter pylori*

Our group has recently clarified the inhibitory effect of estradiol on the growth of *Helicobacter pylori*. When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estradiol at the 50 and 100 μM concentrations in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the estradiol at these concentrations made the division and proliferation of *Helicobacter pylori* stagnate: the levels of colony-forming unit (CFU), which indicates the number of living bacterial cells in the meaning of the wide sense, at that time maintained the baseline CFU level (10^5 CFU/ml) immediately after the culture initiation (Hosoda et al., 2011). Estradiol has been, in sum, found to exhibit the bacteriostatic action to *Helicobacter pylori*. This estrogen seems to act to *Helicobacter pylori* by attaching to the cell surface of the organism, since it is contained in the membrane lipids purified from the *Helicobacter pylori* incubated in the presence of estradiol. Incidentally, estrone has no influence on the growth of *Helicobacter pylori*, and the proliferation capability of the organism even in the presence of estrone at the 100 μM concentration is comparable to that of the organism in the absence of its estrogen.
Earlier investigations (including our own) have shown that *Helicobacter pylori* morphologically converts from a bacillary form to a coccoid form when the organism

exposed to various stresses such as excessive oxygen, alkaline pH, or long-term culture (Benaïssa et al., 1996; Catrenich & Makin, 1991; Donelli et al., 1998; Shimomura et al., 2004). For many years, there is a controversy as to whether the coccoid-converted *Helicobacter pylori* cells are maintaining a viable state. Cells that had changed to a coccoid form lack the ability to form colonies on an agar plate, which it makes very difficult to accurately determine the CFU in coccoid-converted *Helicobacter pylori*. Our group has, therefore, examined whether estradiol confers the bacteriostatic action to *Helicobacter pylori* by promoting the induction of coccoid-conversion in the bacterial cells. Though the cell morphologies of *Helicobacter pylori* were observed with a differential interference microscope after the organisms (10^5 CFU/ml) were incubated for 24 hours with estradiol at the 100 μM concentration in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the coccoid *Helicobacter pylori* cells were unobserved. This indicates that estradiol inhibits the proliferation of *Helicobacter pylori* via a certain bacteriostatic mechanism but not the induction of coccoid-conversion against the bacterial cells.

Epidemiological studies and animal models have suggested that female hormones, particularly estrogens, play a protective role in gastric cancer (Campbell-Thompson et al., 1999; Freedman et al., 2007; Furukawa et al., 1982; Ketkar et al., 1978; Sipponen & Correa, 2002). *Helicobacter pylori* infection is also one of the risk factors in developing of gastric cancer in humans. Recent study by others has demonstrated that estradiol somehow protects against the development of *Helicobacter pylori*-induced gastric cancer in a mouse model (Ohtani et al., 2007). The bacteriostatic action of estradiol may play some role in mechanisms preventing the development of *Helicobacter pylori*-induced gastric cancer. Further investigations will be necessary to elucidate the relationship between estradiol and *Helicobacter pylori in vivo*.

3.3 Estriol and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estriol at the 50 and 100 μM concentrations in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions, estriol had no influence on the growth of *Helicobacter pylori*, and the CFU levels of the organism cultured in the presence of estriol (both 50 μM and 100 μM) were comparable to the control CFU level (10^8 CFU/ml) of the organism cultured in the absence of estriol. In addition, this estrogen was undetectable in the membrane lipid compositions of the organism in the TLC analysis. *Helicobacter pylori* has, thus, failed to use as the membrane lipid component estriol.

4. Membrane distribution of steroids assimilated by *Helicobacter pylori*

In general, Gram-negative bacteria including *Helicobacter pylori* have two membranes that are composed of the phospholipid bilayer, namely an inner membrane and an outer membrane. The phospholipid components constituting both the inner and outer membranes are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The outside lipid layer of the outer membrane also contains as the major glycolipid component a lipopolysaccharide (LPS) (Rietschel et al., 1994). LPS is composed of an *O*-polysaccharide chain, an outer core saccharide, an inner core saccharide, and a lipid A regions. The lipid A is composed of two phosphorylated glucosamine molecules linked by β (1→6)-glycosidic bond and plural fatty acid molecules (5 to 7 molecules) attached to the glucosamine molecules, and is buried in the outside lipid layer of the outer membrane. Meanwhile, the *O*-polysaccharide chain region has

direct contact with the outside of the bacterial cells and maintains the membrane permeability against exogenous lipophilic compounds. The outer core saccharide and the inner core saccharide regions are located between *O*-polysaccharide chain region and lipid A region.

In addition to LPS, *Helicobacter pylori* retains the glucosyl cholesterols (CGL, CAG and CPG) as another glycolipid components into the membranes when the organism assimilated exogenous free-cholesterol. Our previous study has demonstrated that the percentage of total glucosyl cholesterols is greater than 20% in the total lipids (except for LPS) composing the *Helicobacter pylori* membranes (Shimomura et al., 2004). Glycerophospholipids, such as phosphatidylethanolamine and phosphatidylglycerol, have been known in Gram-negative bacteria to be in both the inner membrane and the outer membrane. No earlier reports have, however, investigated the localization of glucosyl cholesterols (CGL, CAG and CPG) in *Helicobacter pylori* membranes.

To elucidate the membrane distribution of glucosyl cholesterols in *Helicobacter pylori*, we have divided the two membranes into the inner membrane fraction and the outer membrane fraction via sucrose-gradient centrifugation method, purified the lipids from each membrane fraction by the Folch method, and analyzed the lipid compositions obtained from these membrane fractions by thin-layer chromatography (TLC) (Shimomura et al., 2009). The TLC analysis detected the spot of free-cholesterol itself at a similar density in both the inner membrane fraction and the outer membrane fraction obtained from *Helicobacter pylori* ingested free-cholesterol from the medium. In contrast, the glucosyl cholesterols (CGL, CAG and CPG) produced via the absorption of free-cholesterol were detected at clearly higher levels in the outer membrane fraction than in the inner membrane fraction. In sum, the steroid itself was distributed into both the inner membrane and the outer membrane, whereas the glucosyl steroids were distributed into the outer membrane rather than into the inner membrane. We have also fractionated the inner membrane and the outer membrane of *Helicobacter pylori* that was cultured in the presence of estrone, and analyzed the lipids purified from each membrane fraction by TLC. As with the free-cholesterol itself, the spot of estrone absorbed by *Helicobacter pylori* was also detected at a similar density in both the outer and inner membrane fractions.

These findings indicate that *Helicobacter pylori* assimilates exogenous steroids into the outer and inner membranes and uses the glucosylated steroids as major lipid components constituting the outer membrane. This membrane distribution of steroids also suggests a possibility that *Helicobacter pylori* possesses a certain membrane transport system for the steroids: the steroids absorbed into *Helicobacter pylori* are shifted from the outer membrane to the inner membrane, and thereafter, the steroids glucosylated in the inner membrane are transported to the outer membrane. There is, however, no evidence that HP0421 protein catalyzing the α-glucosylation of steroids is distributed into the inner membrane of *Helicobacter pylori*. Therefore, it is important to ascertain the localization of HP0421 protein in the *Helicobacter pylori* membranes. In addition, further investigations will be necessary to clarify whether *Helicobacter pylori* possesses such a steroidal membrane transport system.

5. The physiological role of steroid assimilation in *Helicobacter pylori*

Though *Helicobacter pylori* aggressively assimilates various exogenous steroids into the membrane lipid compositions, the organism divides and proliferates even in the absence of steroids as well as the other bacterial species that have no ability to assimilate the exogenous steroids into the membranes. Our recent study has elucidated why *Helicobacter pylori*

physiologically requires steroids to survive (Shimomura et al., 2009). This paragraph describes an importance of steroid assimilation in maintaining the viability of *Helicobacter pylori*.
Phosphatidylcholine, the most prevalent phospholipid in mammals, is much higher in concentration than free-cholesterol in the blood plasma of humans; phosphatidylcholine exists at a concentration of approximately 144 mg/dl, whereas free-cholesterol exists at a concentration of approximately 60 mg/dl. The fundamental chemical structure of phosphatidylcholine is 1,2-diacyl-*sn*-glycero-3-phosphocholine (Fig. 3). In general, the carbon-1 (C1) position in the glycerol backbone of phosphatidylcholine carries a saturated fatty acid such as palmitic acid ($C_{16:0}$) or stearic acid ($C_{18:0}$), whereas the carbon-2 (C2) position in the glycerol backbone carries an unsaturated fatty acid such as oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), or arachidonic acid ($C_{20:4}$). Lyso-phosphatidylcholine (LPC) is a monoacyl-type phosphatidylcholine and generally lacks an unsaturated fatty acid at the carbon-2 (C2) position of the glycerol backbone. A number of investigations have demonstrated that unsaturated fatty acids and lyso-phosphatidylcholines have the potential to kill various microorganisms including *Helicobacter pylori* (Bruyn et al., 1996; Conley & Kabara, 1973; Constance et al., 1992; Kabara et al., 1972; Kanai & Kondo, 1979; Kanetsuna, 1985; Knapp & Melly, 1986; Kondo & Kanai, 1985; Nieman, 1954; Steel et al., 2002; Thompson et al., 1994). Thus, these individual lipophilic compounds constituting phosphatidylcholine act as antimicrobial agents against the various microorganisms. It remains unclear, however, whether the phosphatidylcholine itself also confers an antimicrobial action against the microorganisms.

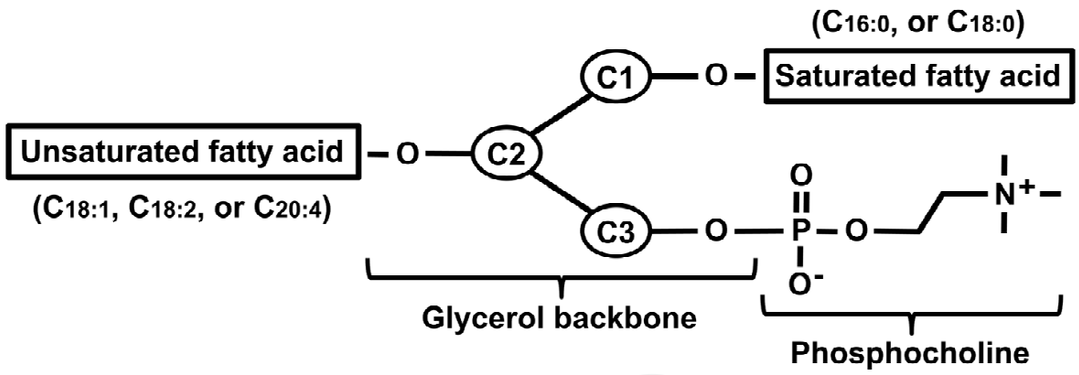

Fig. 3. The fundamental chemical structure of phosphatidylcholine

A previous study by others has shown that the concentration of phosphatidylcholine is 124.8 ± 62.6 μM in gastric juice from eight healthy volunteers (Berstad et al., 1992). We know that *Helicobacter pylori* colonizes the human gastric epithelium. In sum, this study indicates that *Helicobacter pylori* is surrounded by phosphatidylcholine *in vivo*. If phosphatidylcholine itself affects the survival of *Helicobacter pylori* that has failed to assimilate exogenous steroids such as free-cholesterol or steroid hormones, it may explain the importance of steroid assimilation in the organism. We have, therefore, investigated the antibacterial activity of phosphatidylcholine itself against *Helicobacter pylori* (Shimomura et al., 2009).

5.1 Antibacterial effects of phosphatidylcholine variants on the steroid-free *Helicobacter pylori*

When the steroid-free *Helicobacter pylori* (10^7 CFU/ml) that has no steroid in the membrane lipid compositions was incubated for 24 hours with various phosphatidylcholine variants

carrying different fatty acid molecules at the carbon-2 position of the glycerol backbone in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the phosphatidylcholine variants attaching either a linoleic acid ($C_{18:2}$) molecule or an arachidonic acid ($C_{20:4}$) molecule to the carbon-2 (C2) position in the glycerol backbone conferred an antibacterial action fatal to the steroid-free *Helicobacter pylori*. In contrast, the phosphatidylcholine variants attaching either an oleic acid ($C_{18:1}$) molecule or a palmitic acid ($C_{16:0}$) molecule to the carbon-2 (C2) position in the glycerol backbone had no influence on the viability of *Helicobacter pylori*.

To ascertain the antibacterial potencies of phosphatidylcholine-themselves, we have also investigated the antibacterial activity of compositional constituents of the two phosphatidylcholines that exhibited the anti-*Helicobacter pylori* action, a linoleic acid ($C_{18:2}$), an arachidonic acid ($C_{20:4}$), and a LPC (1-palmitoyl-*sn*-glycero-3-phosphocholine). When the steroid-free *Helicobacter pylori* (10^7 CFU/ml) was incubated for 24 hours with linoleic acid (10 μg/ml), arachidonic acid (10 μg/ml), or LPC (10 μg/ml) in the serum-free medium (3 ml) containing a 0.2% dMβCD (2,6-di-*O*-methyl-β-cyclodextrin) with continuous shaking under microaerobic conditions, the two fatty acids and LPC had no influence on the viability of the steroid-free organism. The dMβCD has, thus, completely counteracted the antibacterial action of these lipophilic compounds against the steroid-free *Helicobacter pylori*. Incidentally, the linoleic acid (10 μg/ml), arachidonic acid (10 μg/ml), and LPC (10 μg/ml) in the absence of dMβCD confer the antibacterial action fatal to the steroid-free *Helicobacter pylori*. Intriguingly, the dMβCD had no influence on the anti-*Helicobacter pylori* action of the phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, and these two phosphatidylcholine variants (concentrations ranging from 10 μg/ml to 100 μg/ml) conferred the bactericidal action to the steroid-free *Helicobacter pylori* even in the presence of the 0.2% dMβCD, as with had being done in the absence of dMβCD.

The dMβCD is a cyclic oligomer consisting of seven 2,6-di-*O*-methyl-α-D-glucose molecules linked by α (1→4)-glycosidic bonds and has the ability to solubilize lipophilic compounds through the formation of molecular inclusion complexes (Ohtani et al., 1989). To examine whether dMβCD inhibits the adsorption of unsaturated fatty acids, or phosphatidylcholines onto the steroid-free *Helicobacter pylori* cells, we carried out the following experiments. The heat-killed *Helicobacter pylori* cells that have no steroid in the membranes were incubated for 24 hours with a linoleic acid (100 μg/ml), an arachidonic acid (100 μg/ml), or phosphatidylcholine variants (100 μg/ml), to which either a linoleic acid molecule or an arachidonic acid molecule is attached as the acyl group, in the serum-free medium containing a 0.2% dMβCD with continuous shaking at 37˚C, and thereafter the membrane lipids were purified from the heat-killed cells recovered via the Folch method and analyzed by TLC. The membrane lipids obtained from the heat-killed cells incubated in the absence of dMβCD contained tremendous amounts of linoleic acid and arachidonic acid, whereas the membrane lipids obtained from the heat-killed cells incubated in the presence of dMβCD (0.2%) contained negligible amounts of those fatty acids. Surprisingly, the phosphatidylcholines contained in the membrane lipids of the heat-killed cells incubated in the presence of dMβCD (0.2%) were larger amount than those contained in the membrane lipids of the heat-killed cells incubated in the absence of dMβCD. In sum, dMβCD has inhibited the binding of the unsaturated fatty acids to the membranes of *Helicobacter pylori* but promoted the binding of the phosphatidylcholines to the membranes of the organism.

These results indicate that dMβCD obstructs the hydrophobic interaction between the unsaturated fatty acids and the *Helicobacter pylori* membranes by solubilizing those fatty acids, and thereby protects the organism from the bactericidal action of the unsaturated fatty acids, and probably LPC (Fig. 4). It is unclear why dMβCD promotes the adsorption of the two phosphatidylcholines onto the *Helicobacter pylori* membranes. These findings, however, indicate that the anti-*Helicobacter pylori* action originates in the potencies of phosphatidylcholine-themselves and that the unsaturated fatty acids (linoleic acid and arachidonic acid), and the LPC (1-palmitoyl-*sn*-glycero-3-phosphocholine), which result from the hydrolysis of the phosphatidylcholines, do not contribute to this action. We have, thus, elucidated the bactericidal activity of phosphatidylcholine (PC) variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone against the steroid-free *Helicobacter pylori*.

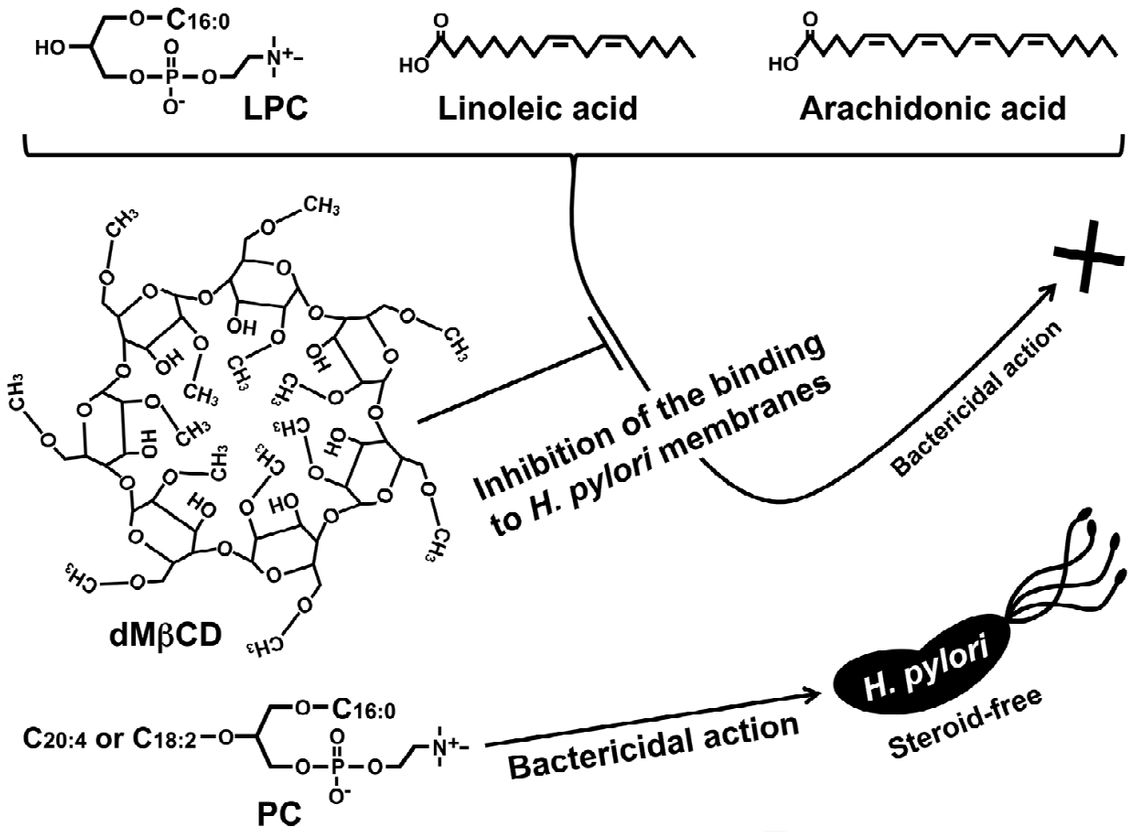

Fig. 4. The role of 2,6-di-*O*-methyl-β-cyclodextrin (dMβCD) on the anti-*Helicobacter pylori* action of lipophilic compounds

5.2 Bacteriolysis in the steroid-free *Helicobacter pylori* caused by the cell adsorption of phosphatidylcholines

To examine the antibacterial mechanism of phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, we performed the following experiments. After the steroid-free *Helicobacter pylori* (10^8 CFU/ml) was incubated for 8 hours with each phosphatidylcholine

(100 μg/ml) in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the supernatant recovered was subjected to the purification of proteins by an anion-exchange chromatography, and the proteins purified were analyzed by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A number of protein bands with tremendous high densities were detected in the supernatant obtained from the steroid-free *Helicobacter pylori* incubated with the phosphatidylcholines, in the SDS-PAGE analysis. Among those protein bands detected, the band for flavodoxin (FldA) protein was also contained. As FldA is an electron acceptor of the oxidoreductase that catalyzes acetyl-CoA synthesis in *Helicobacter pylori* cell (Hughes et al., 1995), we can assume that FldA is the intracellular protein. These results, in sum, indicate that the phosphatidylcholine variants attaching either a linoleic acid or an arachidonic acid as the acyl group to the carbon-2 position in the glycerol backbone exert deleterious effect on the cell membranes of steroid-free *Helicobacter pylori* and induce the bacterial cell lysis, resulting in abundant leakage of intracellular proteins (especially FldA protein) to outside of the bacterial cells. Incidentally, the SDS-PAGE analysis detected only negligible amount of proteins in the supernatant obtained from the steroid-free *Helicobacter pylori* incubated without either the phosphatidylcholines.

5.3 Acquirement of phosphatidylcholine resistance in *Helicobacter pylori* conferred by assimilating steroid

We next investigated whether the *Helicobacter pylori* with the assimilated steroid succumbs to the bactericidal action of the phosphatidylcholines, as with the steroid-free organism. When the steroid-free *Helicobacter pylori* is cultured for 24 hours in the serum-free medium containing free-cholesterol (50 μM concentration) with continuous shaking under microaerobic conditions, the organism recovered retains both free-cholesterol itself and glucosyl cholesterols (CGL, CAG and CPG) in the membranes. We, therefore, examined the anti-*Helicobacter pylori* action of the two phosphatidylcholine variants possessing the antibacterial action fatal to the steroid-free *Helicobacter pylori* by using the organism retaining the assimilated free-cholesterol that was prepared via the above culture procedure. When *Helicobacter pylori* (10^7 CFU/ml) with the assimilated free-cholesterol was incubated at various time points in a serum-free medium (3 ml) with shaking under microaerobic conditions in the presence or absence of each phosphatidylcholine (100 μg/ml), which carries either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, the *Helicobacter pylori* did not succumb to the antibacterial effects of the two phosphatidylcholine variants: the time-dependent growth-decline curve of *Helicobacter pylori* with each phosphatidylcholine roughly corresponded to the time-dependent growth-decline curve of the organism without either the two phosphatidylcholines, when the CFU values ($\log_{10}$ CFU/ml: vertical axis) and the incubation times (hour: horizontal axis) were plotted in a graph. *Helicobacter pylori* that had assimilated free-cholesterol (FC) has been, thus, found to resist the bactericidal action of phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone.

As described above, *Helicobacter pylori* retains free-cholesterol in the form of glucosyl cholesterols (CGL, CAG and CPG). This raises the question as to whether the glucosyl cholesterols are more important rather than the free-cholesterol itself in the expression of phosphatidylcholine resistance in *Helicobacter pylori*. To resolve this question, we examined

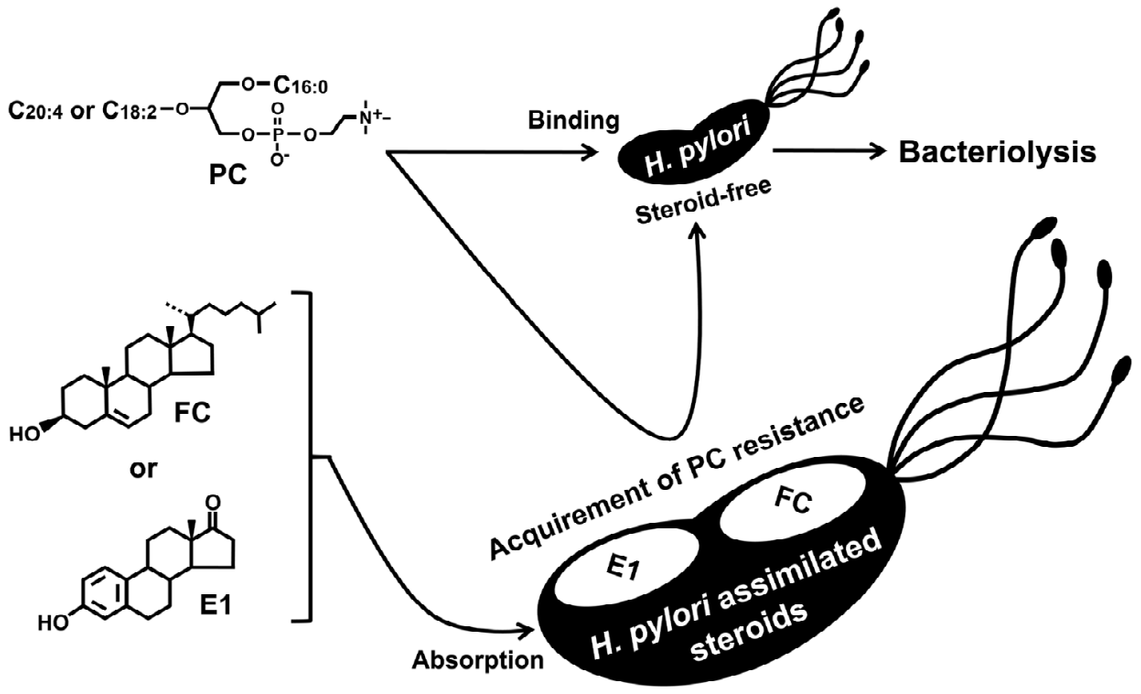

Fig. 5. The expression of phosphatidylcholine (PC) resistance in *Helicobacter pylori* with the assimilated steroids

the phosphatidylcholine resistance in *Helicobacter pylori* with another assimilated steroid. We have shown that *Helicobacter pylori* efficiently absorbs and retains the female hormone estrone into the membranes, but fails to glucosylate the estrogen (Hosoda et al., 2009). In addition, we have also found that other female hormone estriol is not absorbed into the membranes of *Helicobacter pylori*. Therefore, we decided to use estrone and estriol as steroid tools that are not glucosylated by *Helicobacter pylori*. After the steroid-free *Helicobacter pylori* was cultured for 24 hours with estrone (50 μM concentration) in a serum-free medium with continuous shaking under microaerobic conditions, the recovered organism (10^7 CFU/ml) that had assimilated estrone without glucosylation in the membranes was incubated for further 24 hours with each phosphatidylcholine (100 μg/ml), which carries either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, in a serum-free medium (3 ml) with continuous shaking under the same conditions. As with the *Helicobacter pylori* that assimilated free-cholesterol into the membranes, the organism with the assimilated estrone also resisted the bactericidal action of the two phosphatidylcholine variants, and the CFU was maintained to a high level (> 10^6 CFU/ml) comparable to the control CFU (10^7 CFU/ml) of *Helicobacter pylori* incubated for 24 hours without either the phosphatidylcholines. *Helicobacter pylori* has, in sum, expressed phosphatidylcholine resistance even when assimilated estrone (E1) without glucosylating it. In addition, this finding indicates that the glucosylation of steroid is so far not important in conferring resistance to the bactericidal action of phosphatidylcholine upon *Helicobacter pylori*, although the glucosylation of steroid is essential for *Helicobacter pylori* to evade the host immune systems. In contrast, the *Helicobacter pylori* treated for 24 hours with estriol (50 μM concentration) succumbed to the bactericidal action of the two phosphatidylcholine variants as with the steroid-free organism, and the CFU level reduced from 10^7 CFU/ml to <

10^3 CFU/ml, when the estriol-treated organism was incubated for 24 hours in the serum-free medium containing the respective phosphatidylcholine variants (100 μg/ml) to which either a linoleic acid or an arachidonic acid is attached as the acyl group. These results, together with our findings on the free-cholesterol assimilation in *Helicobacter pylori*, indicate that bacteria of this species acquire a resistance against the bacteriolytic activity of phosphatidylcholine by assimilating the exogenous steroids into the membranes (Fig. 5).
Phosphatidylcholine is not a single molecule, but a family of variants with different fatty acid compositions attached to the glycerol backbone of the phosphatidylcholine (Fig. 3). The predominant phosphatidylcholine in human serum has been known to carry a palmitic acid ($C_{16:0}$) molecule and a linoleic acid ($C_{18:2}$) molecule, and recently, the predominant phosphatidylcholine in the human gastric mucus has also been shown to carry the same two fatty acids (Orihara et al., 2001). One of the two phosphatidylcholines investigated as to the anti-*Helicobacter pylori* effect by our group is exactly its variant carrying a palmitic acid molecule and a linoleic acid molecule: 2-linoleoyl-1-palmitoyl-*sn*-3-phosphocholine. In sum, the phosphatidylcholine attaching a palmitic acid and a linoleic acid to the carbon-1 position and to the carbon-2 position in the glycerol backbone is the most prevalent phosphatidylcholine in humans. *Helicobacter pylori* colonizes the human gastric epithelium and inhabits the human stomach for many years. On this basis, we can assume that *Helicobacter pylori* is constantly exposed to various phosphatidylcholine variants, particularly the phosphatidylcholine carrying a palmitic acid molecule and a linoleic acid molecule. Our recent study, in sum, indicates that the steroid assimilation in *Helicobacter pylori* plays an important role in reinforcing the membrane lipid barrier and conferring resistance to the bacteriolytic action of hydrophobic compounds such as phosphatidylcholine.

6. The 3=O steroids and *Helicobacter pylori*

Testosterone, androstenedione and progesterone possess an oxo (3=O) group at the carbon-3 position of the steroid framework. Our recent studies have revealed that *Helicobacter pylori* cannot use as the membrane lipid components these 3=O steroids and rather succumbs to the antibacterial action of certain 3=O steroids. This paragraph describes the 3=O steroids as bactericidal agents to *Helicobacter pylori*.

6.1 Testosterone and *Helicobacter pylori*

Like estriol, testosterone also was not utilized as the membrane lipid component of *Helicobacter pylori*: the TLC analysis did not detect testosterone in the membrane lipid compositions of *Helicobacter pylori* cultured for 24 hours with this androgen at the 50 μM concentration (Hosoda et al., 2009). Testosterone did not, therefore, contribute to the phosphatidylcholine resistance upon *Helicobacter pylori* (Shimomura et al., 2009). In addition, this 3=O steroid at the 50 μM concentration hardly affected the growth of *Helicobacter pylori*.

6.2 Androstenedione and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours in the serum-free medium (3 ml) containing androstenedione at concentrations ranging from 10 to 100 μM with continuous shaking under microaerobic conditions, this 3=O steroid exhibited inhibitory effect on the growth of *Helicobacter pylori* at concentrations grater than 50 μM. Androstenedione was, however, relatively low potency in inhibiting the growth of *Helicobacter pylori*. The decrease in CFU (10^4 CFU/ml) of *Helicobacter pylori* cultured with

androstenedione at the 100 μM concentration was slight compared to the baseline CFU (10^5 CFU/ml) immediately after the culture initiation (Hosoda et al., 2011).

6.3 Progesterone and *Helicobacter pylori*

Of the three 3=O steroid hormones (testosterone, androstenedione, and progesterone) investigated, the progesterone has demonstrated the most effective anti-*Helicobacter pylori* action. Progesterone efficiently inhibited the growth of *Helicobacter pylori* by a manner dependent on the greater doses added into the medium, and the CFU of the organism in the presence of progesterone at the 100 μM concentration was below the limits of detection (< 10 CFU/ml), when *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours in the serum-free medium (3 ml) containing progesterone at concentrations ranging from 10 to 100 μM with continuous shaking under microaerobic conditions (Hosoda et al., 2011).

6.4 Progesterone derivatives and *Helicobacter pylori*

We have discovered the effective anti-*Helicobacter pylori* action of progesterone. Progesterone has at least two derivatives, namely, 17α-hydroxyprogesterone and 17α-hydroxyprogesterone caproate. The derivatives, 17α-hydroxyprogesterone and 17α-hydroxyprogesterone caproate are modified by a hydroxyl group and an acyl group (caproic acid), respectively, at the carbon-17 position of the progesterone framework. 17α-hydroxylprogesterone is a natural progesterone derivative, while 17α-hydroxyprogesterone caproate is a synthetic progesterone derivative. Noting this, we have examined the anti-*Helicobacter pylori* action of these progesterone derivatives. When *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours in a serum-free medium (3 ml) containing 17α-hydroxyprogesterone with continuous shaking under microaerobic conditions, surprisingly, this natural progesterone derivative had no influence on the growth of *Helicobacter pylori*: even in the presence of 17α-hydroxyprogesterone at the 100 μM concentration, the CFU was comparable to the control CFU (10^8 CFU/ml) of *Helicobacter pylori* cultured for 24 hours without steroid. In contrast, 17α-hydroxyprogesterone caproate had a stronger anti-*Helicobacter pylori* action than progesterone, and the CFU was below the limits of detection (< 10 CFU/ml), when the organism (10^6 CFU/ml) was cultured for 24 hours with 17α-hydroxyprogesterone caproate at the 10 μM concentration in a serum-free medium with continuous shaking under microaerobic conditions. Incidentally, caproic acid ($C_{6:0}$), a constituent of 17α-hydroxyprogesterone caproate, did not affect the viability of *Helicobacter pylori* even when added into the bacterial cell suspension at a 100 μM concentration (Hosoda et al., 2011). These findings suggest that the acylation at the carbon-17 position in the progesterone framework plays an important role in reinforcing the anti-*Helicobacter pylori* action of progesterone.

6.5 Antibacterial effects of progesterone and its derivative on *Helicobacter pylori*

To ascertain the antibacterial potencies of progesterone and 17α-hydroxyprogesterone caproate, we investigated the time-dependent antibacterial effects of these two gestagens on *Helicobacter pylori* (Fig. 6A). When *Helicobacter pylori* (approximately 10^7 CFU/ml) was incubated with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) in a serum-free medium (3 ml) at various time points with shaking under microaerobic conditions, the CFUs of the organism incubated with progesterone (PS) moved along a

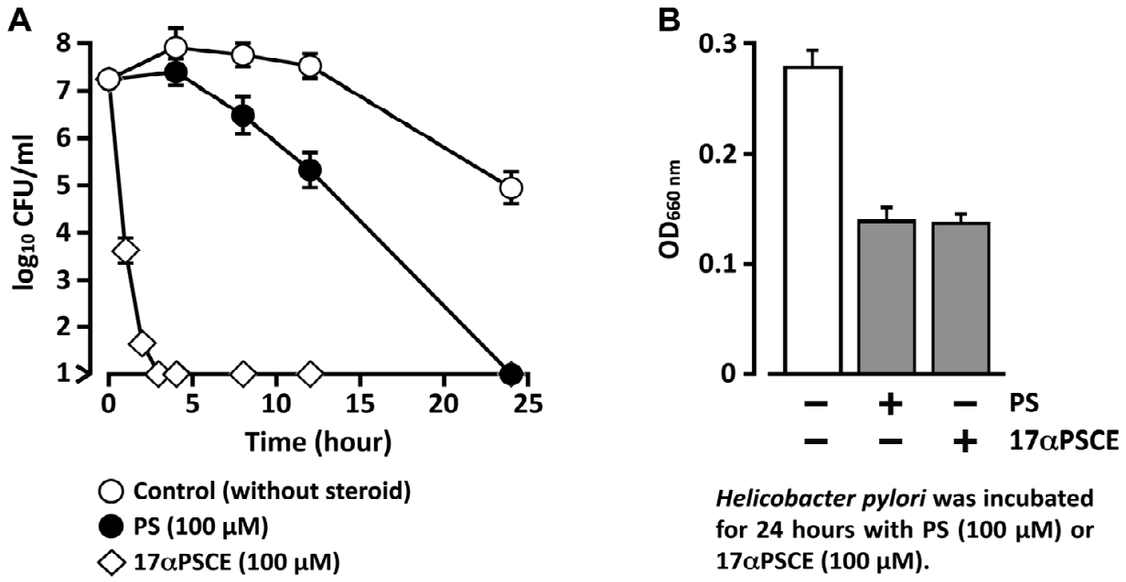

Fig. 6. Antibacterial effects of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE) on *Helicobacter pylori*

gently-sloping curve, falling below the limits of detection (< 10 CFU/ml) by 24 hours after the start of incubation. In contrast, the CFUs of *Helicobacter pylori* incubated with 17α-hydroxyprogesterone caproate (17αPSCE) dropped off sharply, falling the limits of detection within 4 hours after the start of incubation. In sum, 17α-hydroxyprogesterone caproate (17αPSCE) has been found to be much more prompt in conferring the antibacterial action fatal to *Helicobacter pylori* than progesterone (PS).

6.6 Bacteriolysis in *Helicobacter pylori* caused by the cell surface binding of progesterone and its derivative

To clarify the antibacterial mechanism of progesterone and 17α-hydroxyprogesterone caproate against *Helicobacter pylori*, we measured an optical density ($OD_{660\ nm}$) in the bacterial cell suspensions after *Helicobacter pylori* (10^8 CFU/ml) was incubated for 24 hours with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions. The decline of $OD_{660\ nm}$ means that the bacterial cells in suspension had been lysed via certain physical or chemical actions. As it turned out, the $OD_{660\ nm}$ of the bacterial cell suspension incubated with progesterone or 17α-hydroxyprogesterone caproate declined to half value of that of the control cell suspension of *Helicobacter pylori* incubated for 24 hours in the absence of steroid (Fig. 6B).

To confirm the cell lysis of *Helicobacter pylori*, we examined the bacterial morphologies using a differential interference microscope (Fig. 7). When *Helicobacter pylori* (10^7 CFU/ml) was incubated for 24 hours in a serum-free medium in the presence or absence of the two 3=O steroids, the control cell suspension of *Helicobacter pylori* incubated without the steroids harbored the organisms in both mixed rod and coccoid forms. In contrast, the cell suspension of the *Helicobacter pylori* incubated with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) harbored hardly any organisms, although objects

such as cellular debris were observed. These results, together with the findings from the measurement of $OD_{660\ nm}$ in the bacterial cell suspension, suggest that *Helicobacter pylori* cells are lysed by a certain action of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE).

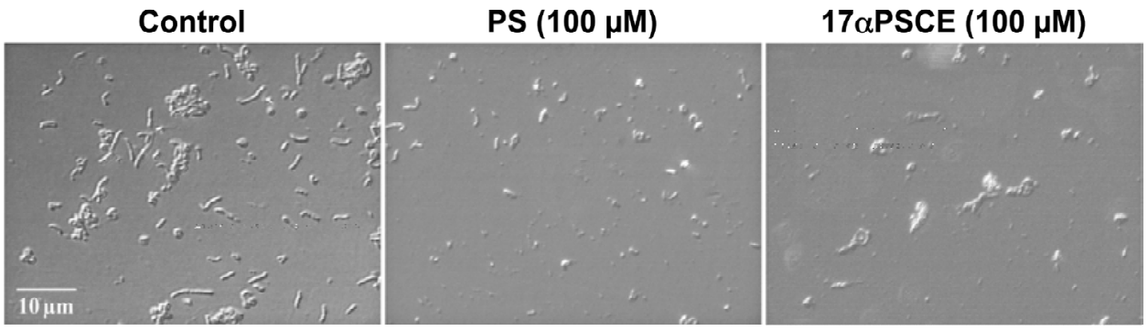

Fig. 7. Cell lysis on *Helicobacter pylori* induced by progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE)

Next, we carried out a series of experiments to examine whether progesterone and 17α-hydroxyprogesterone caproate induce the cell lysis of *Helicobacter pylori* via membrane injury. After *Helicobacter pylori* (10^9 CFU/ml) was incubated for 5 hours with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) using phosphate-buffered saline (PBS: 10 ml), in place of the serum-free medium, with continuous shaking under microaerobic conditions, the proteins in the bacterial cell supernatant were analyzed by SDS-PAGE. The protein bands detected in the cell supernatant of *Helicobacter pylori* incubated with progesterone or 17α-hydroxyprogesterone caproate were considerably denser than the protein bands detected in the control cell supernatant of *Helicobacter pylori* incubated for 5 hours without steroid. A band for flavodoxin (FldA), an intracellular protein, was also found among the other protein bands. Though progesterone conferred the remarkable antibacterial effect to *Helicobacter pylori* suspended into the PBS, the potency of progesterone to decrease the CFU of *Helicobacter pylori* was somewhat lower than that of 17α-hydroxyprogesterone caproate. In addition, the control CFU of *Helicobacter pylori* suspended into PBS without steroid was also decreased, but the decrease magnitude in the CFU was slight. The amount of FldA protein detected in the bacterial cell supernatant correlated closely with the decreases of CFU: the FldA protein band became more noticeable when the CFU decreased by a greater magnitude. In sum, a large amount of FldA protein has leaked from the *Helicobacter pylori* cells to outside, when the organism was exposed to progesterone and 17α-hydroxyprogesterone caproate. These results indicate that progesterone and 17α-hydroxyprogesterone caproate injure the membranes of *Helicobacter pylori* and thereby induce the cell lysis more promptly than autolysis (Hosoda et al., 2011).

6.7 Antibacterial effects of progesterone and its derivative on other Gram-positive and Gram-negative bacteria

To estimate the antibacterial effects of progesterone and 17α-hydroxyprogesterone caproate against other representative Gram-positive and Gram-negative bacteria, we have

determined the minimum inhibitory concentrations (MICs) of these 3=O steroids by the following method. Progesterone or 17α-hydroxyprogesterone caproate was serially diluted 2-fold with a dimethyl sulfoxide (DMSO) solution and added to agar plates of serum-free medium. Bacterial cell suspension (10 μl) adjusted to approximately 10^7 CFU/ml was dotted onto agar plates containing progesterone or 17α-hydroxyprogesterone caproate (from 1.6 μM to 100 μM) and cultured for 1 week under microaerobic conditions. The MICs (μM) of progesterone and 17α-hydroxyprogesterone caproate for the four *Helicobacter pylori* strains (NCTC 11638, ATCC 43504, the clinical isolates A-13 and A-19), *Escherichia coli* strain NIH JC-2, *Pseudomonas aeruginosa* strain ATCC 10145, *Staphylococcus aureus* strain FDA 209D, and *Staphylococcus epiderimidis* strain sp-al-1 were determined by confirming the growth of colonies from the organisms on the agar plates. As it turned out, the MICs of progesterone and 17α-hydroxyprogesterone caproate for the four *Helicobacter pylori* strains were 50 μM and 3.1 μM, respectively (Table 1). Intriguingly, progesterone and 17α-hydroxyprogesterone caproate had no influence on the growth of the other four bacterial species, namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epiderimidis*: all four species grew even in the presence of progesterone or 17α-hydroxyprogesterone caproate at 100 μM (the highest concentration examined). The antibacterial spectra of progesterone and 17α-hydroxyprogesterone caproate have, thus, been remarkably narrow. The four bacterial species, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epiderimidis* have no capability to incorporate exogenous steroids into the membranes. Given the unique feature of *Helicobacter pylori* as an aggressive assimilator of exogenous steroids, we can assume that progesterone and 17α-hydroxyprogesterone caproate attacked *Helicobacter pylori* without targeting the other four bacterial species.

Bacterial species	MIC (μM)	
	PS	17αPSCE
Helicobacter pylori	**50**	**3.1**
Escherichia coli	**> 100**	**> 100**
Pseudomonas aeruginosa	**> 100**	**> 100**
Staphylococcus aureus	**> 100**	**> 100**
Staphylococcus epiderimidis	**> 100**	**> 100**

Table 1. MICs of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE) for various bacterial species

7. Investigation of the steroid-binding site on *Helicobacter pylori*

As described above, we have demonstrated the relationship between *Helicobacter pylori* and steroids. Certain steroids such as free-cholesterol and estrone have been found to be beneficial for the survival of *Helicobacter pylori*. Conversely, other steroids such as estradiol and progesterone have been found to impair the viability of *Helicobacter pylori*. From these findings, in sum, *Helicobacter pylori* seems to bind various steroids to the identical regions on the cell surface. In light of this, we hypothesized that progesterone and free-cholesterol act

to steroid-binding sites existing on the *Helicobacter pylori* cell surface. To verify this hypothesis, we carried out the following experiments (Hosoda et al., 2011). After a 24-hour preculture of *Helicobacter pylori* (10^6 CFU/ml) with progesterone (5 μM or 10 μM) in a serum-free medium (30 ml), the *Helicobacter pylori* cells (10^8 CFU/ml) recovered were incubated for 4 hours in a serum-free medium (30 ml) containing free-cholesterol fixed-beads (free-cholesterol concentration: 250 μM). Thereafter, the amount of free-cholesterol absorbed into the *Helicobacter pylori* cells was quantified via the ferric chloride-sulfuric acid reagent method. The amount of free-cholesterol per CFU obviously tended to reduce by preculturing *Helicobacter pylori* with progesterone. These results suggest that progesterone strongly binds to the *Helicobacter pylori* cell surface and thereby obstructs the free-cholesterol absorption of *Helicobacter pylori* by inhibiting the cell surface binding of free-cholesterol. Incidentally, progesterone had no influence on the viability of *Helicobacter pylori* at the 5 and 10 μM concentrations: the CFUs of the *Helicobacter pylori* cultured for 24 hours with progesterone were similar to the control CFU (10^8 CFU/ml) of the *Helicobacter pylori* cultured for 24 hours without progesterone.

Helicobacter pylori glucosylates the absorbed free-cholesterol and synthesizes glucosyl cholesterols (CGL, CAG and CPG). With this in mind, we decided to examine the influence of progesterone on the glucosylation of free-cholesterol. After a 24-hour preculture of *Helicobacter pylori* (10^6 CFU/ml) in the presence or absence of progesterone (10 μM) in a serum-free medium (30 ml), the *Helicobacter pylori* cells (10^8 CFU/ml) recovered were incubated for 4 hours with free-cholesterol fixed-beads (free-cholesterol concentration: 250 μM) in a serum-free medium (30 ml), and the membrane lipids were purified to analyze the glucosyl cholesterol levels in the membrane lipid compositions by TLC. The TLC analysis detected the glucosyl cholesterols (CGL, CAG and CPG) in the membrane lipids of *Helicobacter pylori* precultured with progesterone, although no free-cholesterol was found to have accumulated within the lipids. Meanwhile, the glucosyl cholesterol levels detected in the membrane lipids of *Helicobacter pylori* precultured with progesterone were similar to the glucosyl cholesterol levels detected in the membrane lipids of *Helicobacter pylori* precultured without progesterone. Progesterone has been found to exert no inhibitory effects on the enzymes involved in the glucosyl cholesterol synthesis.

Next, we examined whether free-cholesterol conversely inhibits the anti-*Helicobacter pylori* action of progesterone. When the *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours with free-cholesterol fixed-beads at various volumes (free-cholesterol concentration: 30 to 90 μM) in a serum-free medium (15 ml) containing progesterone (30 μM), the free-cholesterol did not inhibit the anti-*Helicobacter pylori* action of progesterone: the CFU increase was not observed in any concentrations of free-cholesterol, and the CFU levels hardly altered from the control CFU (10^6 CFU/ml) of *Helicobacter pylori* cultured for 24 hours with progesterone in the absence of free-cholesterol fixed-beads. These results, at least, indicate that free-cholesterol does not competitively inhibit the anti-*Helicobacter pylori* action of progesterone. This compelled us, in sum, to examine the inhibitory effect of a high concentration of free-cholesterol on the anti-*Helicobacter pylori* action of progesterone. When the *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours with progesterone at concentrations ranging from 10 to 30 μM in a serum-free medium (15 ml) containing free-cholesterol fixed-beads (free-cholesterol concentration: 500 μM) or simple-beads (the volumes similar to the free-cholesterol fixed-bead volumes), free-cholesterol at the highest concentration (500 μM) had a noticeable influence on the anti-*Helicobacter pylori* action of the

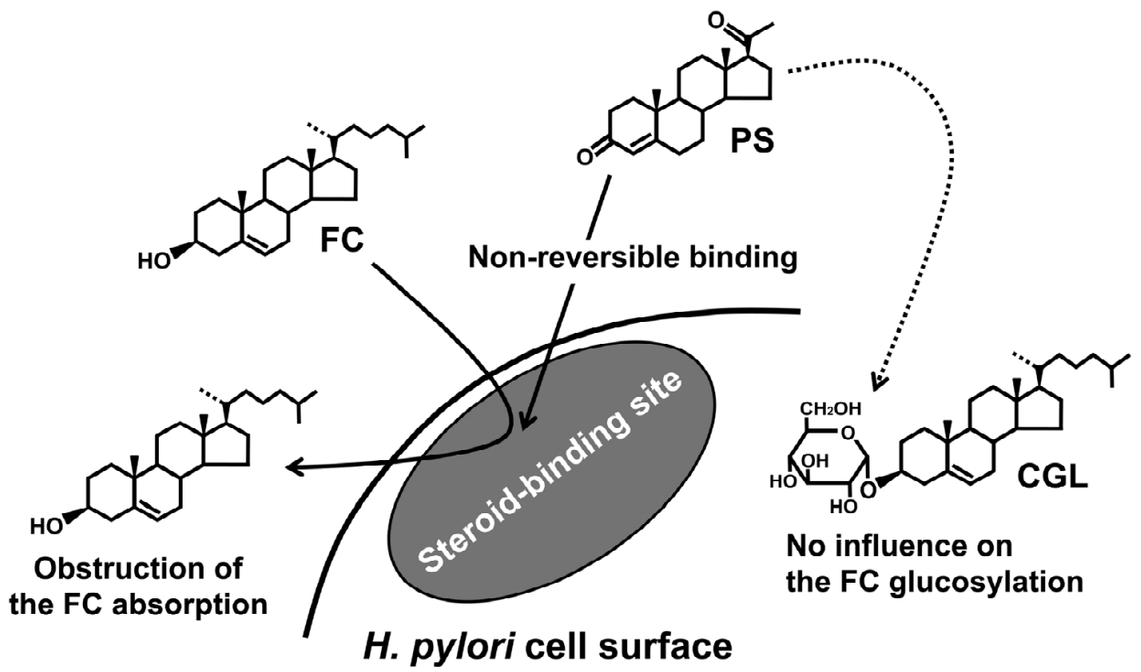

Fig. 8. The obstruction of free-cholesterol (FC) absorption in *Helicobacter pylori* by progesterone (PS)

progesterone: the growth-inhibitory curve of *Helicobacter pylori* cultured with progesterone in the presence of free-cholesterol fixed-beads shifted from the control growth-inhibitory curve of *Helicobacter pylori* cultured with progesterone in the presence of simple-beads to the right side, when the CFU values ($\log_{10}$ CFU/ml: vertical axis) and the progesterone concentrations (μM: horizontal axis) were plotted in a graph. These results indicate that free-cholesterol noncompetitively inhibits the anti-*Helicobacter pylori* action of progesterone. In combination with the results of the inhibitory effect of progesterone on the binding of free-cholesterol onto the *Helicobacter pylori* cells, they also strongly suggest that progesterone non-reversibly binds to the *Helicobacter pylori* cells and thereby induces the cell lysis, and/or inhibits the free-cholesterol absorption of the organism.

Our recent study has shown that progesterone inhibits the free-cholesterol absorption of *Helicobacter pylori*, and conversely, that a relatively high concentration of free-cholesterol inhibits the anti-*Helicobacter pylori* action of progesterone. Progesterone and free-cholesterol, in sum, seem to bind to identical sites on the *Helicobacter pylori* cell surfaces and thereby obstruct each other's effects (Fig. 8). This suggests that *Helicobacter pylori* may express a certain component, such as a steroid-binding protein, on the cell surface. Further investigations will be required to elucidate whether such a steroid-binding protein does indeed exist in *Helicobacter pylori*.

8. Conclusion

Our current basal research has revealed the following relationship between *Helicobacter pylori* and steroid hormones: pregnenolone (PN), dehydroepiandrosterone (DEA), epiandrosterone (EA), and estrone (E1) are absorbed into the membranes of *Helicobacter pylori* and play an important role to reinforcing the membrane lipid barrier, and thereby

Helicobacter pylori acquires the phosphatidylcholine resistance. Conversely, estradiol, androstenedione, and progesterone are harmful for the survival of *Helicobacter pylori*, and especially progesterone (PS) exhibit more effective antibacterial action to *Helicobacter pylori* than the other steroid hormones (Fig. 9). In addition, we have discovered that the acylation at the carbon-17 position of progesterone framework considerably augments the anti-*Helicobacter pylori* action of progesterone and that the hydroxylation at the same carbon position of progesterone framework conversely cancels out this action of progesterone. In sum, 17α-hydroxyprogesterone caproate (17αPSCE) exhibits much stronger anti-*Helicobacter pylori* action than progesterone, whereas 17α-hydroxyprogesterone has no anti-*Helicobacter pylori* action. These findings are expected to contribute to the development of a novel antibacterial steroidal medicine that targets *Helicobacter pylori* as an aggressive assimilator of exogenous steroids. Particularly, progesterone may be useful as a fundamental structure for designing new anti-*Helicobacter pylori* steroidal agents.

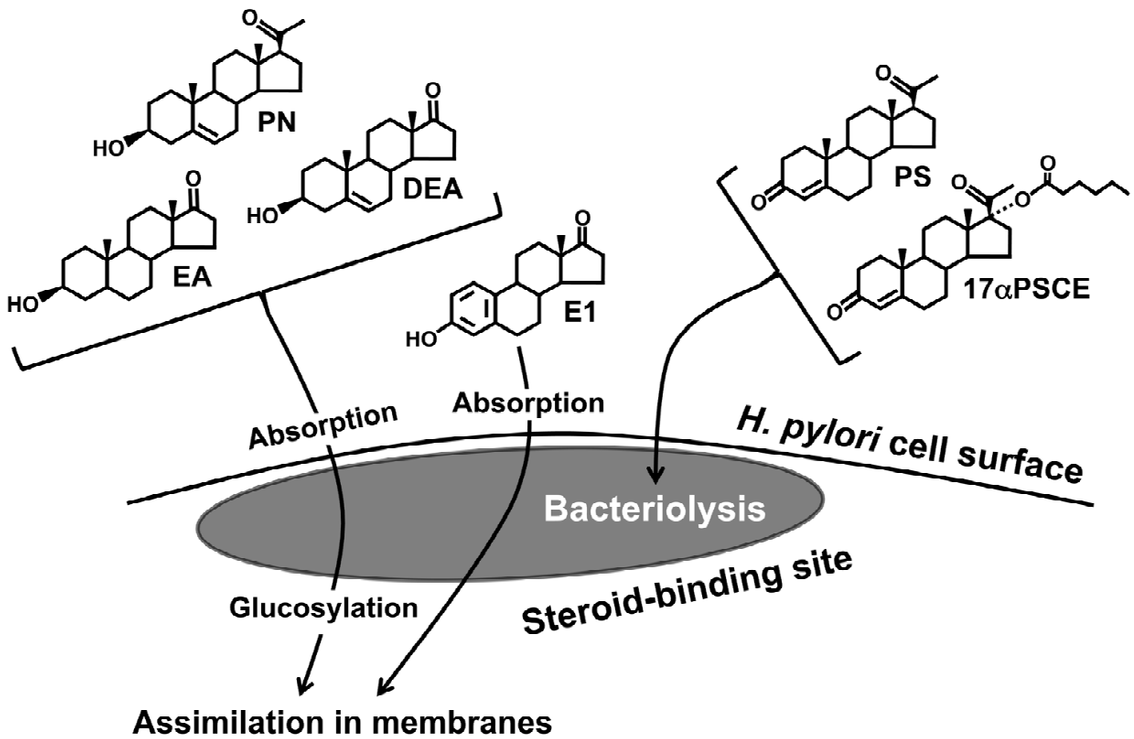

Fig. 9. The relationship between *Helicobacter pylori* and steroid hormones

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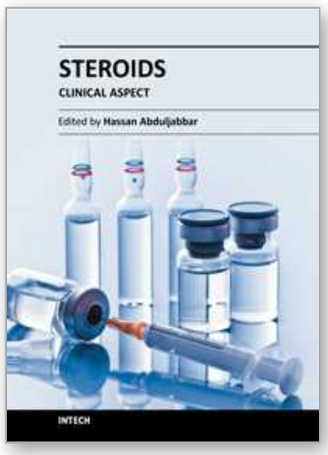

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