明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

計畫類別（單選）	■一般型研究計畫 □新進人員研究計畫 □其他				
研究型別	■個別型計畫 □整合型計畫				
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申請機構/系所（單位）	國立清華大學細胞與分子生物研究所				
本計畫主持人姓名	王雯靜	職稱	教授	身分證號碼	
本計畫名稱 中文	**探討胃幽門螺旋菌cholesterol α-glucosyltransferase之致病機制**				
本計畫名稱 英文	**Investigation of the roles of *Helicobacter pylori* choleaterol-α-glucosyltranferase in pathogenesis**				
整合型總計畫名稱					
整合型總計畫主持人				身分證號碼	
全程執行期限	自民國 98 年 01 月 01 日起至民國 98 年 12 月 31 日				
研究學門	學門代碼	學門名稱			
研究性質	■純基礎研究 □導向性基礎研究 □應用研究 □技術發展				

本年度申請主持國科會各類研究計畫(含預核案)共____件。(共同主持之計畫不予計入)
本件在本年度所申請之計畫中優先順序(不得重複)為第____。

本計畫是否為國際合作計畫 ■否； □是，合作國家：_____，請加填表I001～I003

本計畫是否申請海洋研究船 ■否； □是，請務必填寫表C014。

本計畫是否有進行下列實驗：（勾選下列任一項，須附相關實驗之同意文件）
□人體實驗/人體檢體 □人類胚胎/人類胚胎幹細胞 ■基因重組實驗/基因轉殖田間試驗 □動物實驗

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計畫主持人(申請人)簽章：_____ 日期：

Title: Investigation of the roles of *Helicobacter pylori* choleaterol-α-glucosyltranferase in pathogenesis

題目：探討胃幽門螺旋菌cholesterol α-glucosyltransferase之致病機制

中文摘要:

胃幽門螺旋菌(***Helicobacter pylori, H. pylori***)之**cholesterol-α-glucosyltransferase (CapJ)** 酵素能催化膽固醇的**glycosylation**以合成 **cholesteryl glucosides (CGs)**衍生物。此衍生物是***H. pylori***細胞壁脂質層的重要成分之一，功能上也具有逃避宿主免疫反應的功能。例如避免被宿主巨嗜細胞吞噬及抑制**T**細胞的活化。本提案將進一步研究**CapJ**的功能與其**CGs**衍生物在宿主與病原菌之間的交互作用。此研究將分別以不同研究方法包括：分子選殖、蛋白質純化、脂類分離與免疫螢光染色法等來進行。欲研究主題為：**(1)** 分析重組蛋白質**CapJ**的活性及純化其**CGs**衍生物，用以研究對**AGS**及**macrophage J774A1**細胞訊息傳遞；**(2)** 研究**CapJ**活性與***H. pylori***吸收宿主細胞膜膽固醇與**lipid-rafts**形成的關係。

Abstract:

Helicobacter pylori cholesterol-α-glucosyltransferase (CapJ) catalyzes the glycosylation of cholesterol to synthesize cholesteryl glucosides (CGs) that are the major cell-wall lipid components of *H. pylori.* This enzyme is found to play an important role for escaping phagocytosis by macrophage, T-cell activation and bacterial growth. In this proposal, we aim to study the detailed mechanisms of CapJ involved in host-pathogen interactions. Our specific aims include: (1) biochemical characterization of CapJ enzymatic function; (2) cellular responses (AGS cell and macrophage J774A1 cells) to the steryl glycosides synthesized by CapJ.

Background and Significance:

Infection by *Helicobacter pylori* (*H. pylori*) is a leading cause of gastritis diseases including peptic ulcer, mucosa-associated lymphoid tissue (MALT), and gastric cancer(3, 25). Half of the world's population is infected with *H pylori*, however, only a limited of those infected (approximately 3%) develop into severe clinical outcomes(8). Although the detailed mechanisms that lead to the transformation from normal mucosa to malignancy remain largely unknown, those factors including the bacterial virulence factors, host genetic susceptibility and environmental factors are now considered to contribute to the *H. pylori*-associated carcinogenesis (14, 19, 29-31). Several *H. pylori* virulence factors such as VacA (vacuolating toxin A), CagA (cytotoxin-associated gene A), the *cag* pathogenicity island (PAI), and the blood group antigen-binding adhesion (BabA), are closely associated with the progression of the gastritis diseases(15, 18, 19, 30). The gastric mucosa infected by *H. pylori* is largely associated with surface mucous cell–derived mucin and rarely found in the

deeper portion of the gastric mucosa, which suggesting a possibility that only a small fraction of infected persons will develop advanced stages of the gastric diseases such as peptic ulcer and gastric carcinoma (8, 24). It is possible that mucin secreted from deeper layer of the gastric mucosa plays a protective role against *H. pylori*.

Cholesterol is a ubiquitous component of all eukaryotic membranes that influences their biophysical properties, and is crucial to bilayer organization, dynamics, function and sorting *in vivo* (27). Cholesterol distributes non-uniformaly among the plasma mamebrae. . In couple with sphingolipids, these lipids constitute dynamic lateral heterogeneous microdomains (also termed lipid rafts) that are implicated in cell signalling and trafficking (9, 27). Notably, membrane cholesterol is a critical early determinant for many initial pathogen–host contacts taking place at the plasma membrane. For example, cholesterol-rich microdomains act as concentration platforms for bacterial pore-forming exotoxins (2, 10, 15) and also appear as preferential entry portals for many pathogens(6, 16, 17).

Recently, a hypothetical gene (*capJ*) of *H. pylori* was found and characterized as a cholesterol α-glucosyltransferase that converts the host cholesterol into steryl glycosides derivatives (CGs) (21). CapJ has been suggested to be important to evade phagocytosis of macrophages and T-cell activation during infection (33). Lipid rafts are also indicated to be vital for the CapJ enzymatic activity (33). Three major steryl lipids from *H. pylori* include cholesteryl-α-D-glucopyranoside (αCG), cholesteryl-6'-*O*-tetradecanoyl-α-D-glucopyranoside (αCAG), and cholesteryl-6'-*O*-phosphatidyl-α-D-glucopyranoside (αCPG) (Figure 1). It is noted that αCAG and αCPG are derivatives of αCG. Deletion of the *capJ* gene led to the loss of cholesteryl α-glucoside and its derivatives in the cell wall, suggesting that CapJ is indeed a stryl α-glucosyltransferase that acts on cholesterol. Inhibition of the CapJ by *O*-glycans of the human gastric mucosa that suppresses the growth of *H. pylori* suggests that CapJ is a new target for anti-H. pylori therapy (12). The knock-out *H. pylori* mutant lacking cholesterol α-glucosyltransferase greatly lost the capability to escape phagocytosis and subsequent immune clearance responses (28). One hypothesis is that cholesteryl α-glycosides may influence the membrane fluidity directly or indirectly, hence disturbing subsequent phagocytosis or downstream trafficking/signaling.

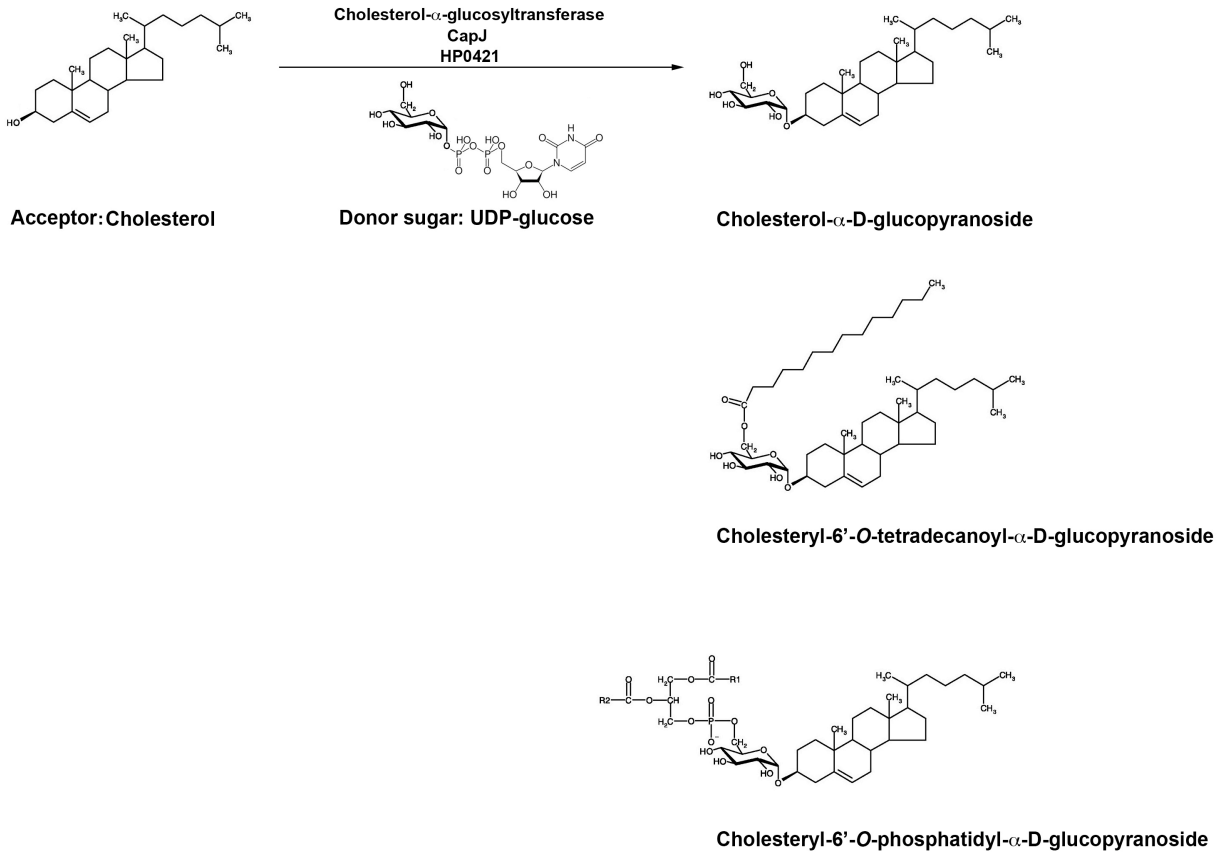

Figure 1. The reaction of CapJ. Structures of CG and its derivatives are showed.

In this proposal, we will focus on the host-pathogen interactions, regarding CapJ functions and the molecular mechanisms that lead to immune evasion and morphological alternation of the host cells during *H. pylori* infection. We will purify both CapJ and its enzymatic products to study the cellular responses and signal transduction of the host cells (AGS and macrophage cells J774A1) when treated with CGs.

Specific Aims

Aim 1: Biochemical characterization of CapJ enzymatic function

1.1 Cloning, overexpression and purification of CapJ

1.2 Characterization of recombinant CapJ enzymatic activity by photospectrometry and thin-layer chromatrography.

Aim 2. Cellular responses (AGS cell and macrophage J774A1 cells) to the steryl glycosides synthesized by CapJ.

2.1 Construction of capJ knockout mutant by allelic exchange mutagenesis.

2.2 Investigation of cholesterol/GM1 on AGS cells infected with wild-type H. pylori or knock-out capJ H. pylori.

Research design:

CapJ catalyzes the glycosylation of cholesterol to produce cholesteryl-α-glucopyranoside (CG) and other steryl glycosides (see Figure 1), which may help to evade host phagocytosis and subsequent clearance (33). Thus, the first aim is to investigate the host-pathogen interactions related with CapJ. First, we will use genetic approach, which *capJ* knockout mutant will be constructed and cell responses will be compared with the wild-type strain. In order to investigate the relationship of CapJ enzyme products and the CapJ-related host cellular responses *in vitro*, we have overexpressed the CapJ with glucosyltransferase activity. Then, we will isolate its enzymatic products, the CGs. Furthermore, the biochemical and fluorescence immunostaining approaches will be performed to identify the cholesterol absorption and the lipid raft formmattion.

Aim 1: Biochemical characterization of CapJ enzymatic function

In this proposal, we will focus on CapJ given its unique enzymatic activity to glycosylate cholesterol, producing cholesteryl-α-D-glucopyranosides (35,39). Since our laboratory has been working on H. pylori for years, we have cloned and expressed this enzyme. Initial attempts to express the recombinant CapJ were not quite successful since a large of proportion of the expressed proteins were not in the soluble fraction but in inclusion bodies or in the sediment of membrane fractions (data not shown). After extensive efforts, we finally worked out a procedure to obtain a soluble form of cholesterol α-glucosyltransferase (Figure. 2). In this specific aim, we will continue our efforts to investigate the enzymatic properties of CapJ. As the acceptor of cholesterol α-glucosyltransferase, cholesterol, is preferentially present in unique microdomains of the plasma membrane, it will be of interest to delineate whether the synthesized products including cholesterol α-glucoside and its derivatives influence membrane dynamics. A straightforward structural and biochemical approach is pursued as follows.

1.1. Cloning, overexpression and purification of CapJ—This work is straightforward in our laboratory given the established *E. coli* expression and protein purification systems. The *capJ* gene obtained by polymerase chain reaction (PCR) amplification was inserted into pET30a for overexpression (Figure 2). The expressed protein was purified by affinity chromatography followed by gel filtration column. Indeed, we have purified CapJ with over 95% of purity (Figure 3).

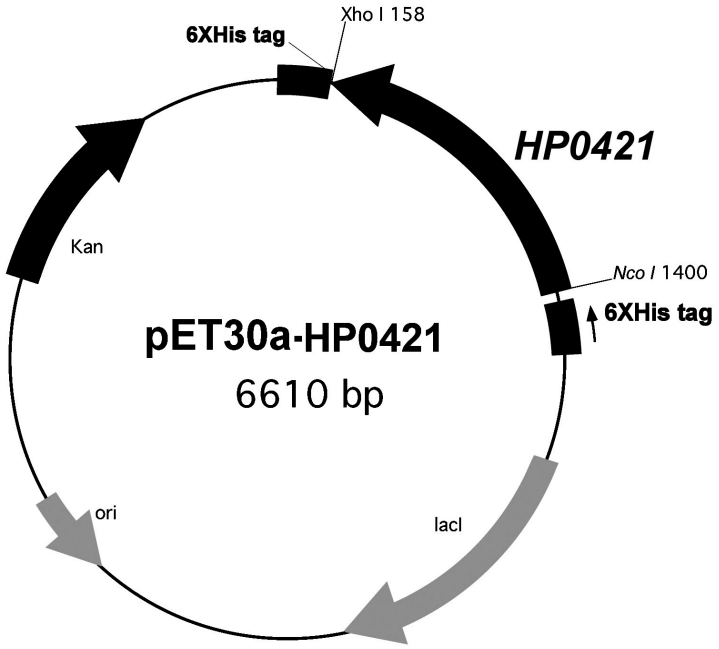

Figure 2. Scheme of CapJ expression vector.

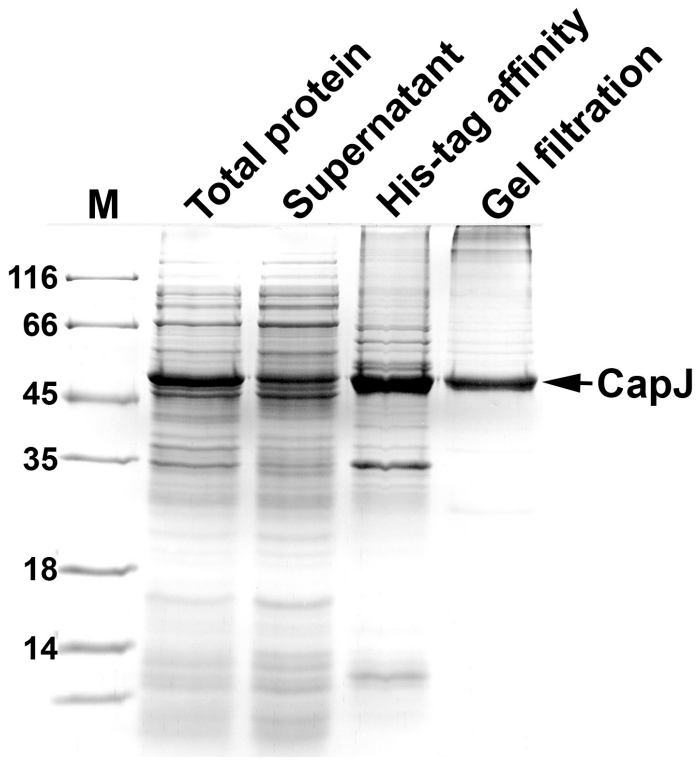

Figure 3. SDS-PAGE analysis of the purified cholesterol α-glucosyltransferase recombinant proteins. M, size markers; total protein, total lysates from the expressed *E. coli* cells; supernatant, supernatants of the total lysates from the expressed *E. coli* cells; His-tag affinity, proteins purified from the His-tag affinity chromatography; Gel-filtration, proteins further purified from the gel filtration chromatography.

1.2 Characterization of recombinant CapJ enzymatic activity by photospectrometry and thin-layer chromatrography—We have established the enzymatic assay of CapJ. Two different methods are developed. (1) Spectrophotometric method: In couple with three enzymes (cholesterol α-transferase, pyruvate kinase and lactate dehydrogenase), the consumed NADH that is theoretically proportional to UDP-glucose can be measured by OD340 nm (Figure 4). (2) Thin layer chromatography (TLC) method: The assay mixtures contained UDP-glucose and cholesterol. The reaction products were separated by thin-layer chromatography in chloroform/methanol (90:10) and detected by dehydration with 10% of H_2SO_4 at 160°C (Figure 5). Our preliminary results showed that the CapJ synthesized α-cholesterol glucoside (CG) in a time-dependent manner (Figure 4). We will conduct *in vitro* assays (described in 1.4) with various sugar donors (NDP-sugars: UDP-glucose, UDP-galactose, UDP-glucuronic acid, UDP-mannose, and GDP-mannose) and acceptors (sterols: cholesterol, ergosterol, β-sitosterol, stigmasterol, the steroidal alkaloid tomatidine, and ceramide). After thin layer chromatography (TLC), the presence of products in the lipophilic reaction products will be determined. A comparison of the substrate specificities of the recombinant enzymes will then be made. Results from these experiments will reveal the substrate specificity and the presumed enzymatic activity and also provide a basis to prepare various glycolipids.

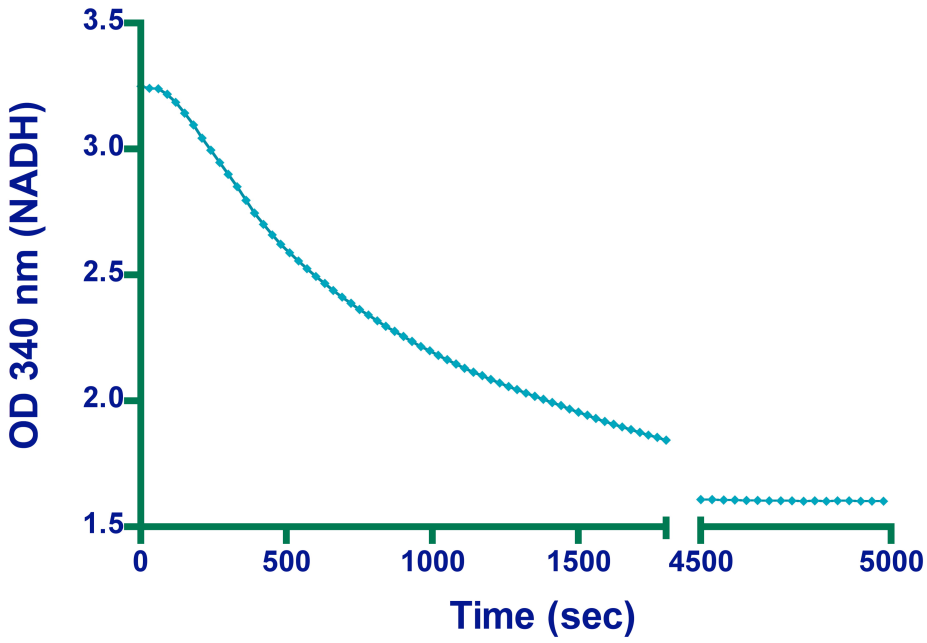

Figure 4. Coupling assay for CapJ. CapJ is incubated with UDP-glucose and cholesterol together with two enzymatic systems that convert formed UDP to UTP and then to NAD+. The amount of NADH can be measured at 340 nm. The decrease of NADH is the measurement of the CapJ activity.

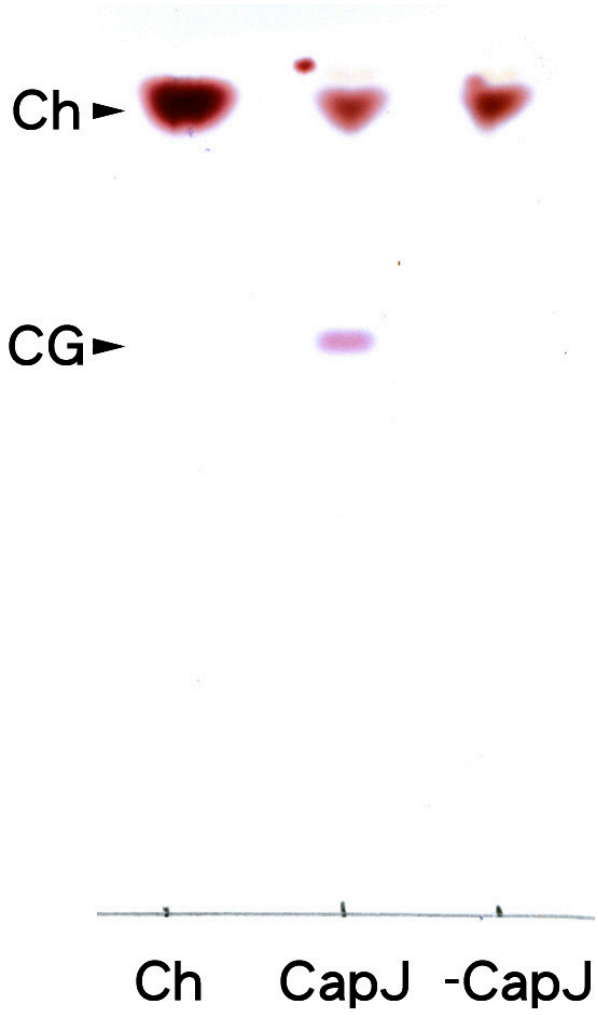

Figure 5. *In vitro* activity assay of recombinant CapJ expressed in E.coli. The assay mixtures contained UDP-glucose and cholesterol. The lipophilic products were separated by TLC. Ch, cholesterol; CapJ, the assay mixture contained 10 ug of CapJ; -CapJ, the assay mixture did not contain CapJ; CG, cholesteryl-glucoside.

Aim 2. Cellular responses (AGS cell and macrophage J774A1 cells) to the steryl glycosides synthesized by CapJ.

2.1 Constructino of capJ knockout mutant by allelic exchange mutagenesis—In order to investigate whether CapJ is engaged in absorbing cholesterol from lipid rafts, we have established a means of allelic-exchange mutagenesis to generate knock-out strains that encodes capJ from *H. pylori*. The *capJ* knock-out mutants was generated by insertion of the *cat* fragment derived from pUOA20 into *capJ* locus through allelic replacement and selection of chloramphenicol-resistant clones. The *capJ* gene fragment was amplified from strain 26695 chromosome by PCR and cloned into plasmid pGEMT (Promega), yielding the plasmid pGEM-*capJ*. The *cat* fragment was amplified by PCR and is inserted into the pGEM-*capJ* plasmid. The *capJ*::*cat* mutant was then obtained by natural transforming the resultant plasmid into the parental strain *H. pylori* 26695 through allelic replacement and selection of chloramphenicol-resistant clones (31).

2.2 Investigation of cholesterol/GM1 on AGS cells infected with wild-type H. pylori or knock-out capJ H. pylori—We will first evaluate whether cholesterol α glucosides partition into regions of lipid rafts in membranes. Two reagents are utilized to reduce the level of cholesterol in the plasma membranes: methyl-β-cyclodextrin (MCD) and lovastatin that reduce cellular cholesterol from AGS cells without influencing cell viability (47). We have previously shown that MCD extracted GM1 from lipid rafts (47). αCG will be prepared as described above, followed by adding it into the untreated AGS cells or cholesterol-depleted AGS cells (treated with MCD or lovastatin). After washes, three means will be performed to evaluate the distribution of αCG in rafts or non-rafts. (1) A cold detergent insolubility assay as described in (47) is used to investigate whether αCG partitions into regions of lipid rafts. Co-migration of αCG with a given marker such as GM1 will hint its residence; GM1 is enriched in detergent resistant membrane fractions or rafts, while the transferrin receptor is not. (2) The TLC method will be used to detect the presence of αCG in the membrane fraction. (3) Confocal microscopic visualization will be performed to inspect the distribution of αCG (fluorescently labeled αCG) in the membrane of AGS cells. Whether αCG is colocalized with the raft-associated GM1 can be evaluated.

Experimental procedures:

H. pylori culture—Clinical isolates were recovered from frozen stocks on Wilkins-Chalgren agar with 10% sheep blood, 0.2% β-cyclodextrin, 1% IsoVitaleX, 10 mg/L nalidixic acid and 8 U/ml polymixin B in a microaerophilic atmosphere for 3–7 days at 37°C until colonies were visible. After passages onto fresh plates, the bacteria were routinely cultured on Brucella agar plates (Becton Dickinson, NY, USA) with 6 mg/L vancomycin and 2 mg/L amphotericin B. For liquid culture, the bacteria harvested from plates were cultured in Brucella broth containing 2% fetal bovine serum, 0.2% β-cyclodextrin, 1% IsoVitaleX, 6 mg/L vancomycin and 2 mg/L amphotericin B for 2 days.

Construction of H. pylori isogenic mutants—An isogenic mutant *H. pylori* is generated by insertion of the *cat* fragment into the gene of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones (45). A *capJ*::*cat* mutant is then generated using a natural transformation method described in Wang et al.(31).

Expression of CapJ—Molecular cloning experiments are performed by standard methods. For structural studies, expression and purification large amounts of target protein in a soluble, active form are required. To optimize the yield of protein expression, various conditions including vectors, hosts, and culture conditions, inducing conditions will be tested. We will first test *E. coli* expression system. Cultures of *E. coli* are grown overnight at 37ºC. Expression cultures start at OD600 = 0.05 and are grown to an OD of 0.8. Induction is performed by adding isopropyl thio-β-D-galactoside (IPTG) and further incubation for an appropriate time at 37ºC. If the protein can be expressed at a sufficiently high level and in soluble form, the clone will be used for large-scale preparation (1-5 liters). The appending tag in the expressed polypeptide makes it useful for an efficient

purification of the expressed protein. The tag will then be cleaved by the designed protease and the protein will be further purified by a suitable chromatography to achieve a single homogeneous species.

Construction of H. pylori isogenic mutants—An isogenic mutant *H. pylori* is generated by insertion of the *cat* fragment into the gene of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones [17]. A *vacA*::*cat* mutant is then generated using a natural transformation method described in Wang et al (32)

Size exclusion chromatography—Samples (0.2-5 mg per ml) are separated using the Superdex-200 column equilibrated with HEPES buffer containing 500 mM NaCl and 15% Glycerol.

In vitro CapJ enzyme assays—The CapJ activity that requires UDP-glucose can be measured by coupling assay and spectrophotometer based on the generation of UDP from UDP-glucose. Three enzymes are included: GT, pyruvate kinase, and lactate dehydrogenase. UDP that is produced by GT from UDP-glucose is used by pyruvate kinase and lactate dehydrogenase that requires NADH. The consumption of NADH can be determined by OD340 nm.

Gentamicin protection assay—J774A1 cells were added to mid-logarithmic phase grown bacteria at an MOI of 50 and incubated at 37°C for various periods. To determine the number of cell-associated bacteria, infected cells were washed three times to remove unbound bacteria and then lysed with distilled water for 10 min. Lysates were then diluted in PBS, plated onto Brucella blood agar plates and cultured for 4–5 days, after which the colony-forming units (CFUs) were counted (18).

Lipid extraction and analysis—Lipids of cells are fractionated. After pre-elution with chloroform, glycolipids are eluted with acetone:isopropanol 9:1. Lipid bands on TLC plates are visualized by dipping the TLC plates into a solution of 10% copper sulfate in 10% (v/v) H_2SO_4, drying at 100ºC and heating up 160ºC for 10 min. For NMR spectroscopy and MS, the purified glycolipids are acetylated with acetic anhydride in pyridine and subjected to preparative TLC in diethyl ether.

Anticipated results

We have previously accumulated experiences on investigating *H. pylori* pathogenesis among Taiwanese patients and characterizing several important virulence factors of *H. pylori*. Used our established approaches, we are confident in the execution of this proposal. We anticipate obtaining research results as in the following.

1. We anticipate obtaining milligram quantities of recombinant CapJ. This protein will be further used for

various applications such as enzymatic assay and substrate specificity testing.

2. We will measure CapJ enzyme activity and purify the enzymatic products (CGs). Thus, we anticipate understanding the cellular responses (AGS and J774A1 cells) to CGs.

3 We anticipate understanding the formation of lipid rafts by investigating of cholesterol/GM1 on AGS cells infected with wild-type *H. pylori* or knock-out *capJ H. pylori*.

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