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☑成果報告
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探討胃幽門螺旋菌之cholesterol-α-glucosyltransferase酵素合成醣化膽固醇之致病性機制

Investigation of the roles of *Helicobacter pylori* cholesterol-α-glucosyltransferase in pathogenicity

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Title

Investigation of the roles of *Helicobacter pylori* cholesterol-α-glucosyltransferase in pathogenicity

Abstract

Helicobacter pylori (H. pylori) infection causes gastric diseases including gastritis, peptic ulcers, lymphoma, and/or carcinoma. The capability to evade host immune response by this peculiar microbe ensures its persistent colonization. *H. pylori* cholesterol-α-glucosyltransferase (CapJ) catalyzes the glycosylation of cholesterol to form the bacteria cell wall lipids cholesterol glucosides (CGs) that are major cell-wall components. This enzyme is found to play a crucial role in escaping phagocytosis, T cell activation, and bacterial growth. Importantly, mucus-associated *O*-glycans that inhibits the biosynthesis of cholesterol-α-glucopyranoside are natural antibiotics against *H. pylori* infection. We have expressed and purified the recombinant CapJ in the *Escherichia coli* expression system. *In vitro* enzyme activity assay showed recombinant CapJ is capable of catalyzing the formation of CGs. Our preliminary results also reveal an oligomeric state of CapJ in solution by electron microscopy. Furthermore, Structure determination of CapJ is in progression. We also investigated whether cholesterol-α-glucopyranosides mediate cholesterol absorption from host membranes.

Keywords: *Helicobacter pylori*; cholesterol α-glucosyltransferase; CapJ; CG; *O*-linked glycan.

Introduction

Infection by *Helicobacter pylori* (*H. pylori*) is a leading cause of gastritis diseases including peptic ulcer, mucosa-associated lymphoid tissue (MALT), and gastric cancer(1, 9). 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(2). Although the detailed mechanisms that lead to the transformation from normal mucosa to malignancy remain largely unknown, the bacterial virulence factors, host genetic susceptibility and environmental factors are now considered to contribute to the *H. pylori*-associated carcinogenesis (4, 7, 12-14). 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(5-7, 13). 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(2, 8). It is possible that mucin secreted from deeper layer of the gastric mucosa plays a protective role against *H. pylori(2)*.

Cholesterol is a major component of physiological membranes that is not only essential for their biophysical and biochemistry functions, but also mediates the formations of obesity and cardiovascular diseases(11). Recently, a *H. pylori's* gene (HP0421, *capJ*) encoded for cholesterol α-glucosyltransferase that converts host cholesterol into steryl glycosides (CGs) derivatives was found and suggested to be important to evade phagocytosis during infection(17). The function of CapJ is associated with the inhibition of T-cell activation and lipid-rafts formation(17). In addition, the alternation of the total amount of steryl glycosides and the relative proportions of cholesteryl-α-D-glucopyranoside (αCG), cholesteryl-6'-*O*-tetradecanoyl-α-D-glucopyranoside (αCAG), and cholesteryl-6'-*O*-phosphatidyl-α-D-glucopyranoside (αCPG) (Figure 1) are found to cause morphological changes of the bacterium or changes in colony variants(10). Moreover, inhibition of the cholesterol glucosyltransferase by *O*-glycans of the human gastric mucosa is found to suppress growth of the bacterium(3). Thus, cholesteryl-α-glucosides synthesized by the cholesterol

glucosyltransferase are important components of bacterial membranes to support the cellular morphology as well as pathogenicity of this organism.

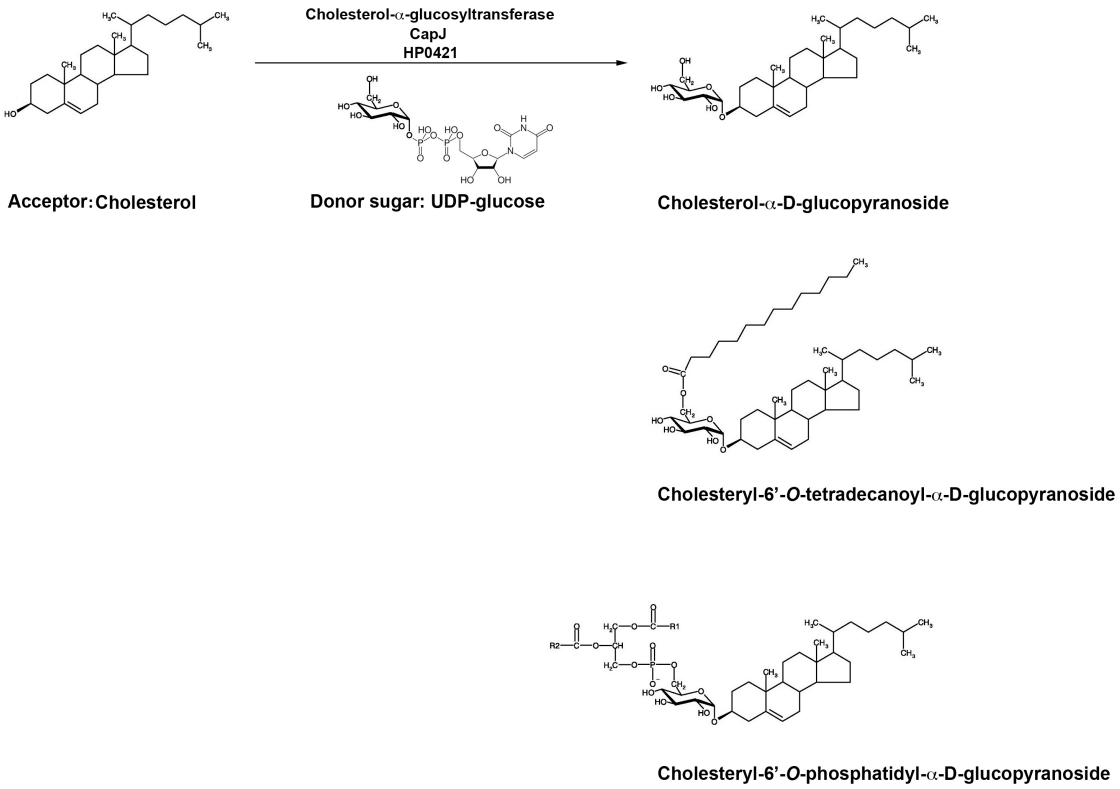

Figure 1. The reaction of CapJ. Structures of cholesterol and CGs are showed.

Results and Discussion

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

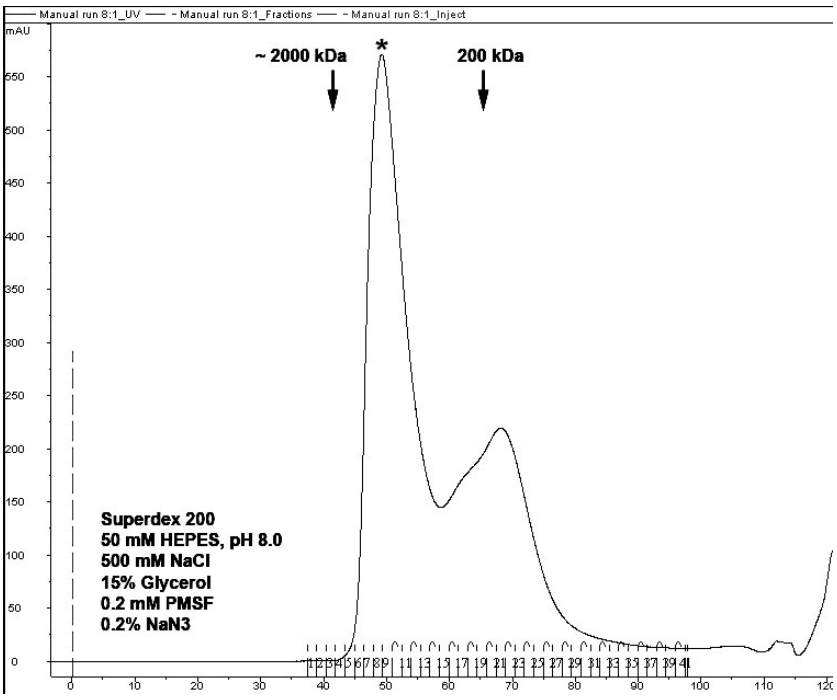

Figure 2. Size exclusion chromatography of the recombinant cholesterol α-glucosyltransferase by a Superdex 200 column. Arrows on the top of the figure indicate molecular weight. Asterisk denotes the presence of CapJ.

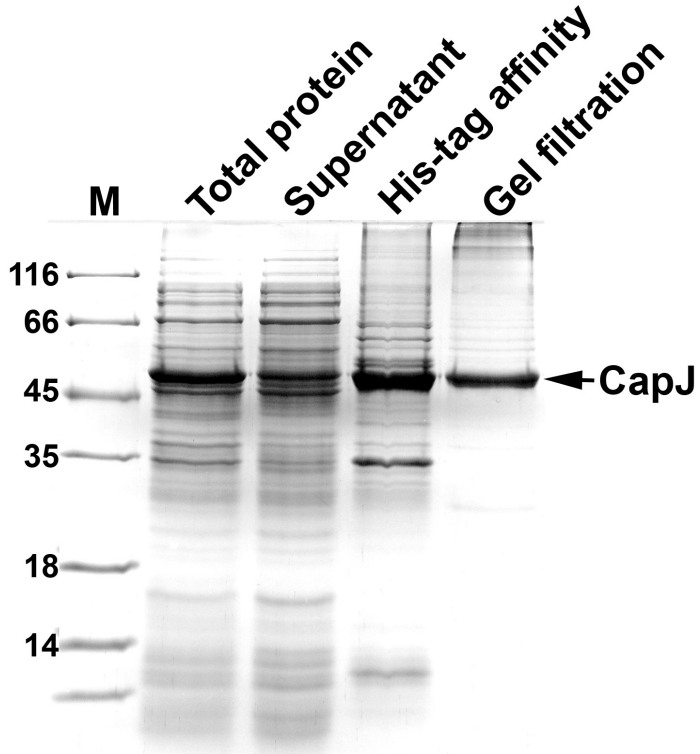

Figure 3. SDS-PAGE analysis of the purified CapJ recombinant proteins. M, size markers; total protein, total lysates from the expressed *E. coli* cells; supernatant, supernatants of the total lysates from the expressed *E. coli* cells; His-tag affinity, proteins purified from the His-tag affinity chromatography;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

Construction of CapJ knock-out mutant. 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 (15) into *capJ* locus through allelic replacement and selection of chloramphenicol-resistant clones(16). 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.

Enzymatic characterization of CapJ. We have established the enzymatic assay for CapJ. Two different methods were developed. (1) Spectrophotometric method: In couple with three enzymes (CapJ, pyruvate kinase and lactate dehydrogenase), the consumed NADH that is theoretically proportional to UDP-glucose can be measured by OD340 nm. (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 4). Our preliminary results indeed showed that the recombinant CapJ synthesized α-cholesterol glucoside in a time-dependent manner (Figure 5).

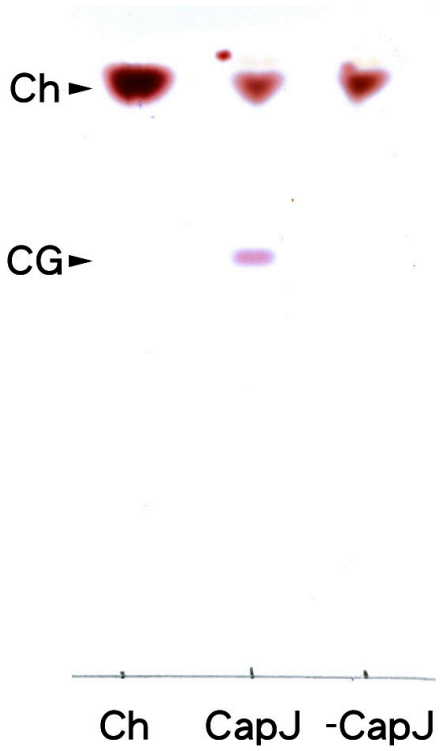

Figure 4. *In vitro* activity assay of recombinant CapJ expressed in E.coli. The assay mixtures contained UDP-glucose and cholesterol. The lipophilic products were separated by TLC. Ch, cholesterol; CapJ, the assay mixture contained 10 ug of CapJ; -CapJ, the assay mixture did not contain CapJ; CG, cholesteryl-glucoside.

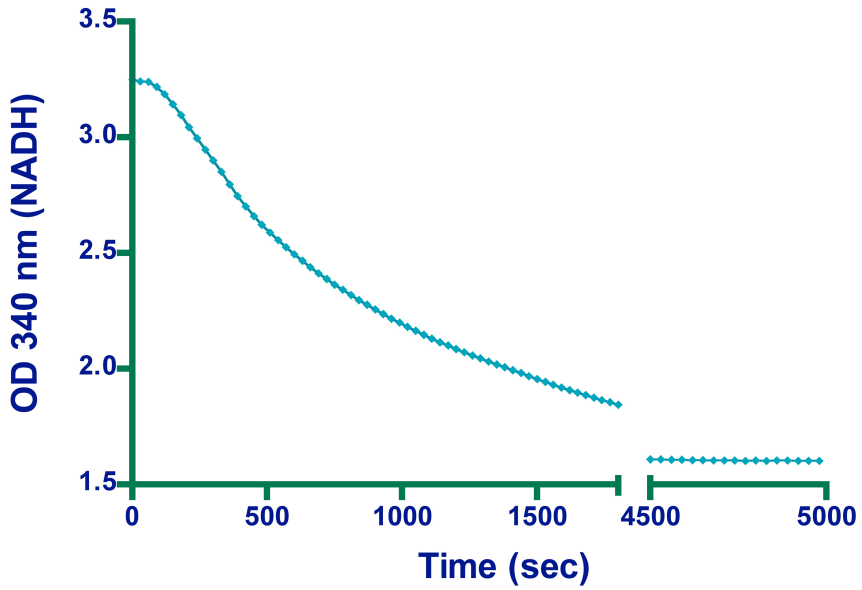

Figure 5. Coupling assay for CapJ. CapJ is incubated with UDP-glucose and cholesterol together with two enzymatic systems that convert formed UDP to UTP and then to NAD+. The amount of NADH can be measured at 340 nm. The decrease of NADH is the measurement of the CapJ activity.

Electron microscopic visualization of cholesterol α-glucosyltransferase. Recombinant CapJ purified from the affinity chromatography was characterized with respect to its oligomerization in solution using a Superdex-200 column. As showed in Figure 2, CapJ was primarily detected in the first peak corresponding to a position of ~850 kDa during the process of purification, suggesting that CapJ is present as oligomeric forms in solution. We have collaborated with Dr. Gan-Guang Liou to visualize CapJ using electron microscopic analysis. Indeed, our preliminary results showed an image of oligomers for the purified CapJ (Figure 6). We will continue this direction and collect significant amounts of images to build the 3-D map of CapJ in collaboration with Dr. Liou. We have recently prepared mouse poly-antisera against CapJ. Transmission EM or cryo EM will then be conducted to examine the assembly and the distribution of cholesterol α-glucosyltransferase in bacteria or upon contact with AGS cells.

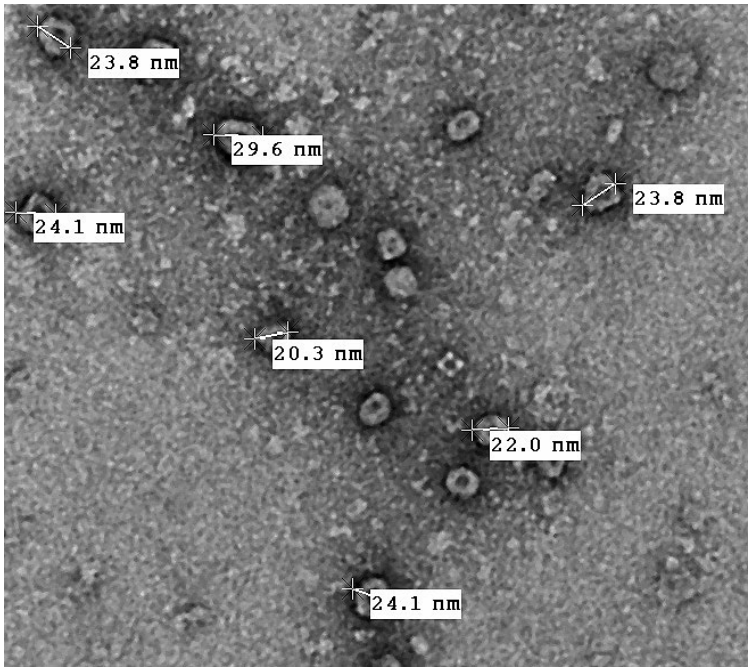

Figure 6. Electronic microscopic analysis of cholesterol α-glucosyltransferase. The diameter of an oligomer particles is marked.

Cholesterol absorption by H. pylori. AGS cells are infected with wild-type *H. pylori* or knock-out CapJ mutant [multiplicity of infection (MOI) = 50] at 37 °C for different time intervals followed by confocal microscopic visualization. Non-infected AGS cells are used as a negative control. Cells are then stained with cholera toxin subunit B (CTB) to visualize the distribution of GM1 with or without infection. Alternatively, AGS cells are stained by FITC-labeled CTB that binds to GM1 on rafts at 4 °C [or Macrophage cells] (at that temperature, membrane is rigid). The labeled cells are then infected with wild-type H. pylori or knock-out CapJ mutant [multiplicity of infection (MOI) = 50] at 37 °C for different time intervals followed by confocal microscopic visualization. Non-infected AGS cells are used as a negative control. Similarly, the distribution of NBD-cholesterol for NBD-cholesterol-loaded AGS cells that are infected with wild-type bacteria or knock-out *capJ* mutant has been initially observed (Figure 7).

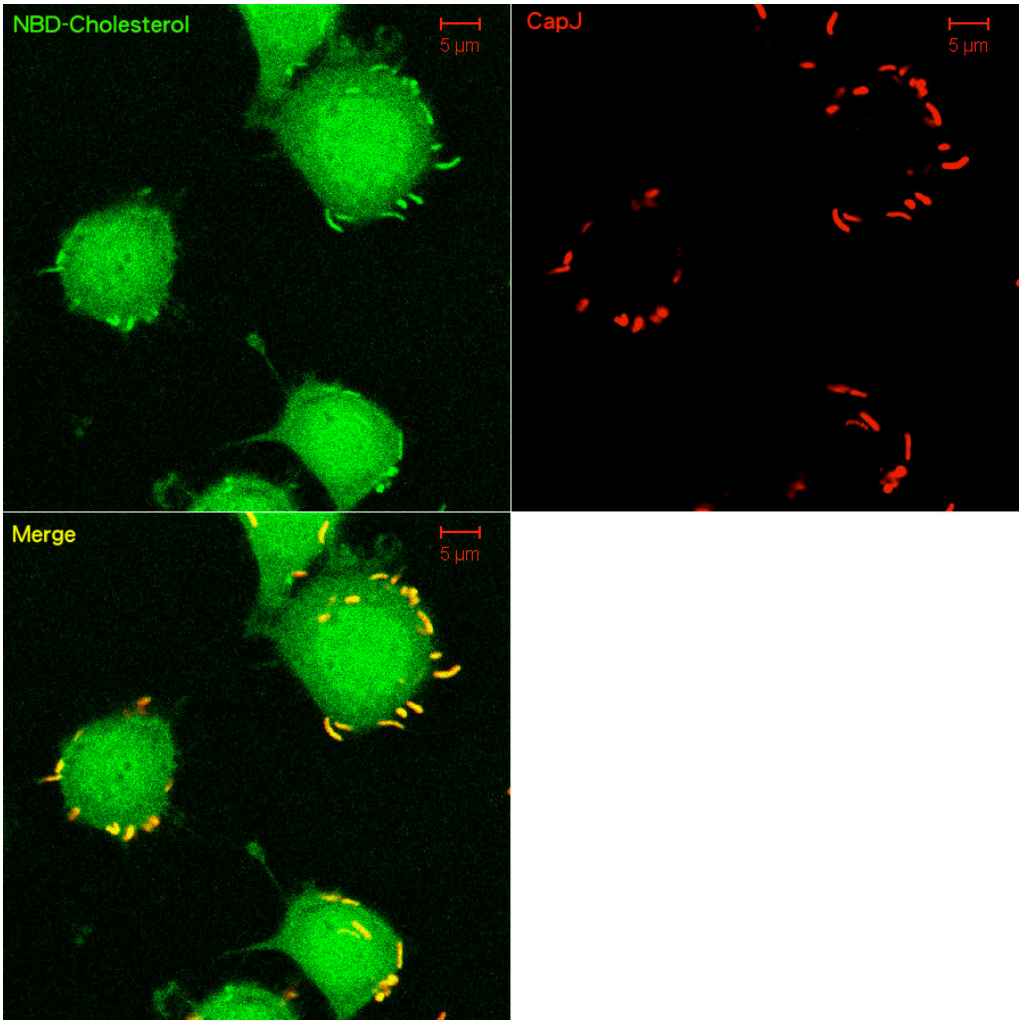

Figure 7. Images of confocal microscopy. NBD-cholesterol-loaded AGS cells (green) were infected with wild-type *H. pylori* (red, visualize by Cy5-conjuged anti-mouse antibody that recognizes anti-CapJ serum from immunized mouse). Merged (yellow) regions indicated NBD-cholesterol is absorbed by *H. pylori*.

Conclusion

To study CapJ-related host-pathogen interactions and structure-function relationship, first, our results showed that the recombinant CapJ protein can be expressed as homogenous and activity forms. CapJ activity was successfully measured with spectrophotometry and thin-layer chromatography, respectively. We also observed the oligomeric forms of CapJ in solution. The molecular weight of the oligomer is characterized as around 850 kDa by gel

filtration chromatography. Accordingly, oligomeric ring-form CapJ was also visualized with electron microscopy. We have clearly showed that wild type *H. pylori* can absorb cholesterol from the cell membrane of AGS cells that were pre-loaded with fluorescence dye-labeled cholesterol.

Material and methods

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.(14).

Heterologous 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 A *vacA*::*cat* mutant is then generated using a natural transformation method described in Wang et al(15).

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.

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.

(2)

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