國立臺灣大學生命科學院生化科學研究所
博士論文

Graduate Institute of Biochemical Sciences
College of Life Science
National Taiwan University
Doctoral Dissertation

幽門桿菌感染造成人類六碳胺糖和岩藻糖水解酶的酵素活性提升：探討它們所扮演的角色

Role of Enhanced Human β-D-Hexosaminidase and α-L-Fucosidase Activities in *H. pylori* Infection

蔓茱菈
Manjula Nandakumar

指導教授：林俊宏 博士
Advisor: Chun-Hung Lin, Ph. D.

中華民國104年12月
December, 2015

中文摘要

胃幽門螺旋桿菌是一種感染胃上皮細胞的病菌，會造成許多上消化道疾病，包括胃潰瘍、十二指腸潰瘍、胃炎、胃癌和黏膜相關淋巴組織淋巴癌等等，但實際上致病的因子仍不甚清楚。在本篇論文中，我們利用分子探針去偵測當胃幽門螺旋桿菌感染時，表皮細胞所增加的岩藻糖水解酶和活性。以便了解宿主和致病菌之間的關係。此外我們同時偵測到岩藻糖水解酶和己醣胺水解酶，在細菌感染時，兩者活性大幅提升；這兩個酵素屬於醣水解酶，分別有非還原端水解岩藻糖和乙醯葡萄糖胺乙醯半乳糖胺。

在本實驗室先前的研究中，發現當胃幽門螺旋桿菌感染時，會促使宿主細胞釋放出第二型岩藻糖水解酶，並進而影響到細菌的貼附、生長及發病。利用產生醌甲基化物的活性探針，我們進一步地觀測到當胃幽門螺旋桿菌感染時，人類第一型岩藻糖水解酶的活性有增強的現象。在探討各種不同的細菌表面分子中，脂多醣為影響人類第一型岩藻糖水解酶活性增強的主因之一。

另外，第二型岩藻糖水解酶和己醣胺水解酶也會在胃幽門螺旋桿菌感染時分泌。其中，兩者產生共同作用，會降低胃幽門螺旋桿菌的生存力。在更進一步的實驗中，利用掃描式及穿越式電子顯微鏡，發現酵素的殺菌效果是藉由破壞細菌表面的脂多醣和肽聚醣。在表面受到破壞後，巨噬細胞呈現出更容易吞噬受到醣水解酶處理後的胃幽門螺旋桿菌。

Abstract

Helicobacter pylori, a Gram-negative bacterium found on the luminal surface of gastric epithelium, is the main cause to the development of three important upper gastrointestinal diseases: duodenal or gastric ulcers, gastric cancer, and gastric mucosa-associated lymphoid-tissue (MALT) lymphoma. Despite the known pathology, factors leading to disease progress still remain ambiguous. In this thesis we aim at examining how the pathogen interacts with gastric host, which includes the application of a synthetic probe to detect the enhanced α-L-fucosidase activity upon bacterial infection and the role of upregulated α-L-fucosidase and human β-D-hexosaminidase in *H. pylori* infection. The two glycosidases are in-charge of removal of fucose and GlcNAc/GalNAc residues from non-reducing termini of glycans, respectively.

We previously indicated that human fucosidase 2 (Fuca2) was secreted upon *H. pylori* infection, and that the enzyme is critical to bacterial adhesion, growth and pathogenesis. By using a quinone methide-generating, activity-based probe, we additionally observed enhanced activities of human fucosidase 1 (Fuca1, a lysosomal enzyme) upon bacterial infection. Further studying the effect of several bacterial stimulants on the enhanced Fuca1 activity, we identified the lipopolysaccharides (LPS) to be one major factor.

In addition to Fuca2, β-D-hexosaminidase is another major glycosidase secreted upon *H. pylori* infection. The enzyme was found to reduce viability of *H. pylori*, which was aggravated by the presence of Fuca2. Further studies, including the imaging analyses

of scanning electron microscopy and transmission electron microscopy, supported the idea that the bactericidal effect was due to the damage on the cell surface, and that LPS and the adjacent peptidoglycans appeared to be the targets of enzymatic degradation. Additionally, the released enzyme activities were shown to render *H. pylori* more vulnerable to the phagocytosis by macrophages.

Keywords: *Helicobacter pylori,* Glycosidase, Quinone methide, Lipopolysaccharide, Bactericidal activity.

Abbreviations

BabA	Blood group antigen binding adhesion
BODIPY	Boron-dipyrromethene
CagA	Cytotoxin-associated gene A
CagPAI	Cag pathogenicity island (PAI)
DMEM	Dulbecco's modified eagle medium
DU	Duodenal ulcer
DC	Dendritic cells
FI	Fucosidase Inhibitor
FNJ	Fuconojirimycin
FUCA2	α-L-Fucosidase 2
Gal	Galactose
GalNAc	*N*-acetyl-β-D-galactosamine
GlcNAc	*N*-acetyl-β-D-glucosamine
GC	Gastric cancer
HCC	Hepato-cellular carcinoma
HI	Hexosaminidase inhibitor
H. pylori	*Helicobacter pylori*
LacNAc	*N*-acetyl-lactosamine
Le^x	Lewis x
Le^y	Lewis y
Le^a	Lewis a

Le^b	Lewis b
LPS	Lipopolysaccharide
M6PR	Mannose-6-phosphate receptor
MALT	Mucosa-associated lymphoid tissue
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MUG	4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide
MUGS	4-Methylumbelliferyl *N*-acetyl-β-D-6-sulfo glucosaminide
NAP	Neutrophil-activating protein
NOD	Nucleotide-binding oligomerization domain
OA	Osteoarthritis
PPI	Proton pump inhibitor
PBS	Phosphate buffered saline
TFSS	Type IV secretion system
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
SabA	Sialic acid-binding adhesion
VacA	Vacuolating cytotoxin A

Table of Content

List of Figures

1.General Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori is the spiral, microaerophilic, flagellated, gram-negative bacteria. This bacteria whose ecological niche is human gut, currently is known to have infected over half of the total population. While most of the infected individuals remain asymptomatic, the infection is considered as a leading cause for gastrointestinal disorders such as gastritis, gastric ulcer, gastric cancer, or malignant lymphoma.(1) (**Figure 1**) During the past 20 years of research, the tentative association between persistent *H. pylori* infection and the development of gastric cancer has been well established, prompting the International Agency for Research on Cancer to classify *H. pylori* as a type I carcinogen.(2)

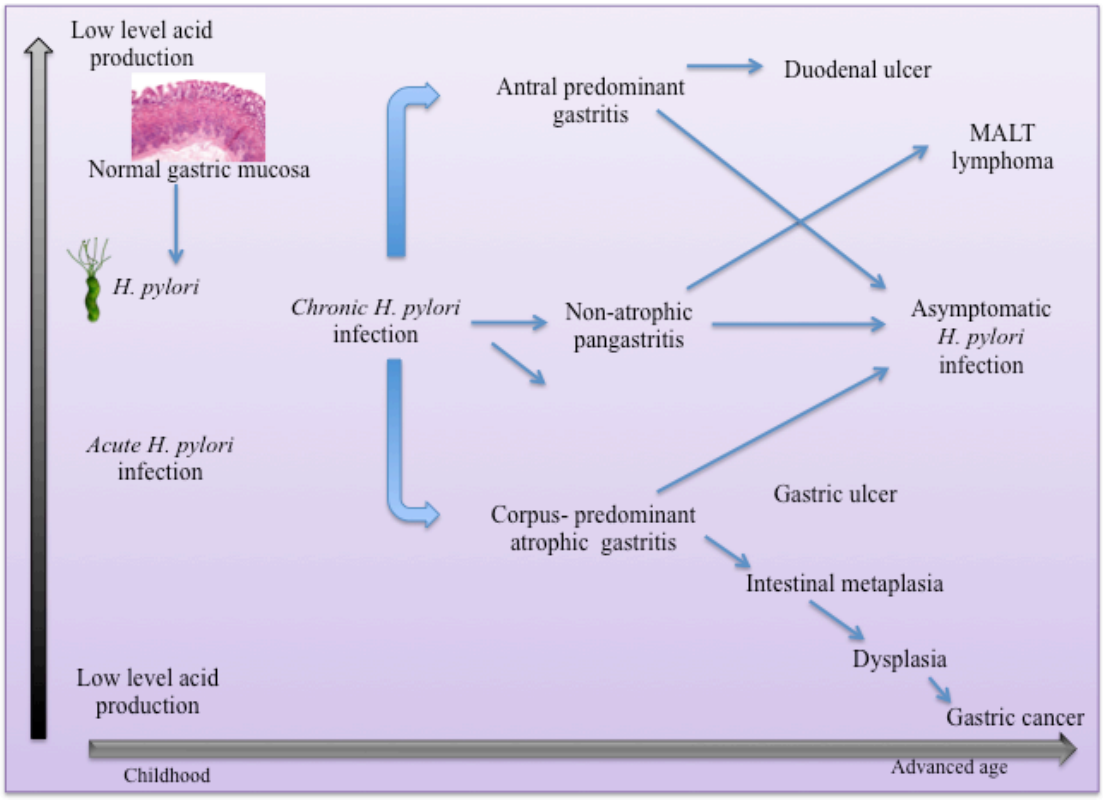


Figure 1. Pathophysiology of *H. pylori* on gastric mucosa

A number of virulence factors have been identified for this pathogen. The bacteria once into the host system requires flagella and urease to reach upto the site of colonization followed by adherence for which the adhesion molecules (ex: BabA, SabA,OipA) are the pre-requisite. The lipopolysaccharide on the outer surface of the bacteria not only maintains the bacterial membrane integrity but also is useful to evade the host immune system by blood group antigen mimicry, providing persistent infection. The exotoxins VacA and CagA secreted into the host cell initiate several signal transduction pathways leading to disease progression. (**Figure 2)**

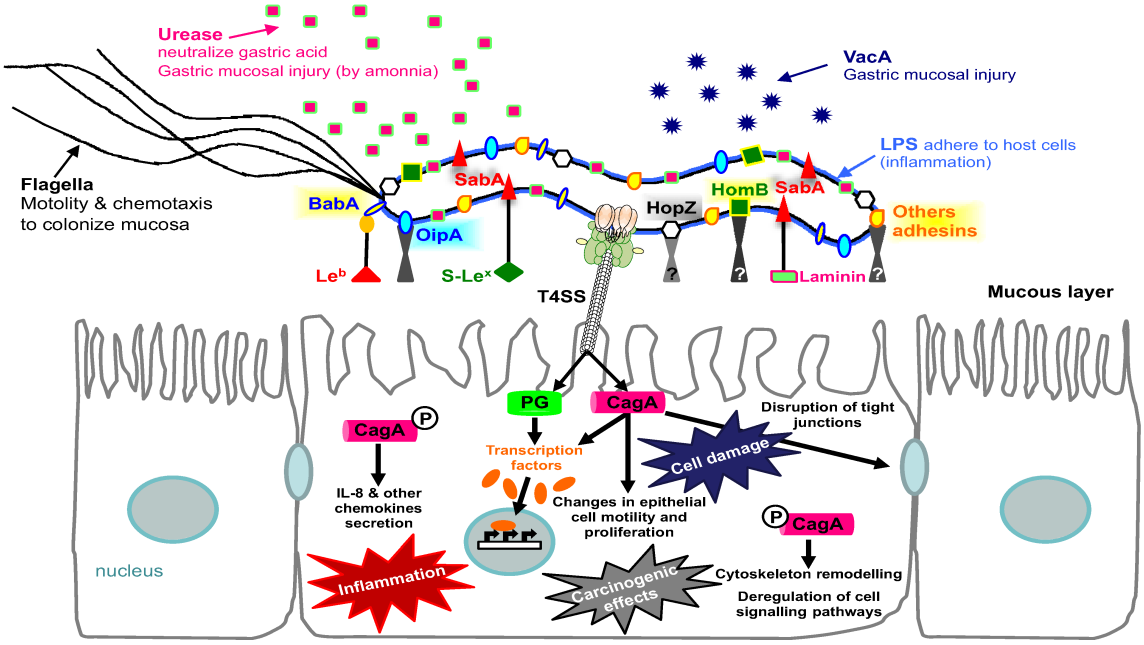


Figure 2. The role of *Helicobacter pylori* virulence factors in pathogenesis (3)

1.2 Virulence factors

Flagella: *H. pylori* usually have a unipolar bundle of two to six flagella bearing three structural elements, a filament, a hook and the basal body.(4) To date only components of filament and hook have been characterized to some extent. The flagella is composed of Falgellins FlaA and FlaB. Studies have shown both the subunits are essential for complete motility.(5) flaE gene encodes the protein of the hook and flaD encodes hook associated protein.

Adhesins: The bacterial cell surface proteins that help in adherence are adhesins. These adhesins belong to the largest outer membrane protein family called Hop family. The most studied adhesins of *H. pylori* are BabA, SabA, Hopz, Alp A/B and OipA.

Bab A: Lewis blood group antigen binding adhesin can bind to human Le^b and terminal fucoses of blood group antigen O, A and B on gastric epithelial cells.(6,7) The BabA mediated adherence to host cell aggravates the inflammatory response in stomach.(8) The BabA and lewis-b binding was also shown to induce DNA damage in host cell.(9) It is also shown that the BabA positive strains bind more densely compared to BabA mutant strains.

Sab A: Sialic acid binding adhesins bind to sialyl dimeric Le^x.(10) A number of *H. pylori* binding sialylated glycosphingolipids have been identified such as sialyl alpha 3 neolactohexaosylceramide, neolactooctaosyl ceramide, sialyl dimeric Le^x glycosphingolipid. It was shown that *H. pylori* could bind to sialic acid containing carbohydrates on granulocytes which causes oxidative burst in these cells.(11)

OipA: Outer inflammatory protein A is proinflammatory protein. Its exact role is unclear while it is said to increase secretion of IL8 secretion from gastric epithelial cells and along with cagPAI induces inflammation through phosphorylation of different signaling pathways.(12,13)

Lipopolysaccharide: Lipopolysaccharide is the major component on the bacterial cell wall of gram-negative bacteria. It is a complex molecule contributing to the membrane integrity of the bacteria. The LPS of *H. pylori* consists of O-specific polysaccharide chain, a core oligosaccharide and a lipid part called Lipid A. (14) The O-antigen of *H. pylori* contains several lewis antigens including Le^x, Le^y, Le^a, Le^b, which are also present on host cells providing molecular mimicry.(15) The molecular mimicry is considered pathogenic firstly because it can cause the induction of autoantibodies that cause tissue damage by fixing complement. Secondly, it might help in immune evasion by preventing the induction and binding of antibodies towards epitopes shared by itself and microorganism. This lack of response by the host towards the surface antigens leads to persistent infection. These O-antigens are hypothesized to help in bacterial adherence to host cells. The bacterial LPS undergoes phase variation(16),(17) providing dynamic adherent phenotype depending on the microenvironment around it.

Vac A: The 95KDa vacuolating cytotoxin injected by the bacteria into the host causes formation of vacuoles in the perinuclear area which grow in size leading to cell necrosis. (18) The mature toxin is suited to the gastric environment where the acids

activate it; this activated form causes significant changes in the epithelium. This toxin further becomes resistant to damage by acid and pepsin. (19)

CagPAI: A 40Kb gene containing about 31 coding regions, encoding components of Type IV secretion system such as CagT whose function is to translocate components through TFSS,(20) CagY whose function is to modulate host immune response in order to promote persistent bacterial infection.(21) On adhering at the site of infection the bacteria first forms the TFSS and pilus formation and translocates CagA into the host cell. SRC and ABL kinases phosphorylate the translocated CagA.(22) The phosphorylated CagA further interacts with several host proteins altering the host-signaling pathway. CagA translocation not only occurs in epithelial cells but also in immune cells such as B cells and dendritic cells. CagA translocation into epithelial cells activates host immune response whereas translocation into immune cells like DCs suppresses the immune response. In addition to CagA, peptidoglycan is also translocated into host cell inducing the proinflammatory cytokines through NOD1 activation.(23)

1.3 Host pathogen interplay

From bacterial colonization to infection, the microenvironments of the host are in constant flux as bacterial and host factors contribute to changes at the host-pathogen interface, with the host attempting to eliminate bacteria and the bacteria fighting to persist residency.

The glycans present on the host and bacterial cell surface play important role in host pathogen interaction. While the glycans on host cell provide ligands for bacterial adhesion and source of energy at one end, the recognition of bacterial surface signature glycans activates host immune response on the other. In some instances the bacteria produces certain proteins as part of host pathogen interaction. For example *Aggregatibacter actinomycetemcomitans* produces dispersin B, which degrades the extracellular matrix around the bacteria. It has been reported that mycobacteria induces the expression of hyalurunon sythases in alveolar epithelial cells thereby synthesizing more hyaluronan on cell surface, which is cleaved by hyalurunidase like lyases. Further the cleaved hyaluronan is used as carbon source by the bacteria.(24) (**Figure 3**)

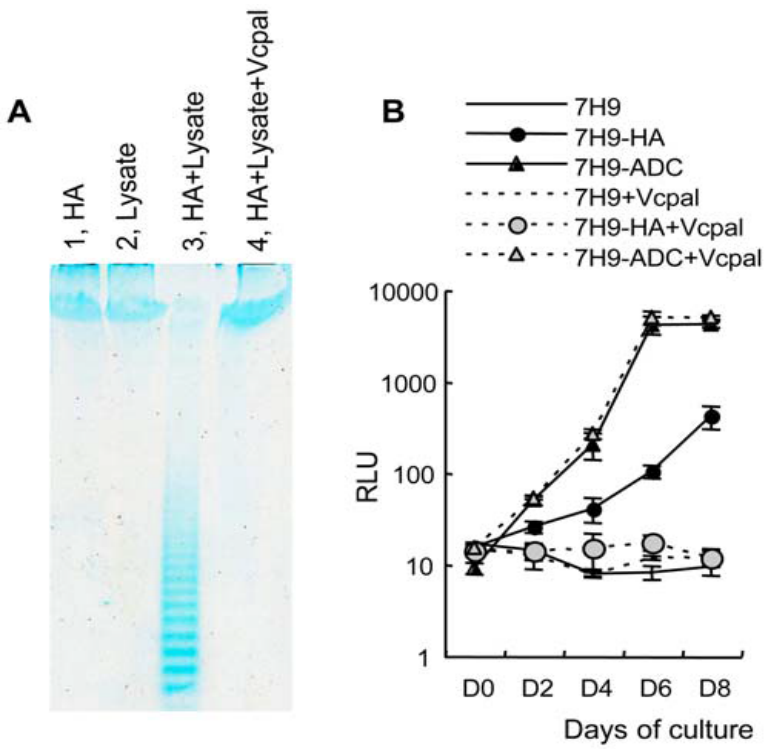


Figure 3. Hyaluronidase activity in mycobacteria and the effect of hyaluronidase inhibitor on hyaluronan-dependent growth of BCG and M. tuberculosis. (A), One mg/ml of hyaluronan and 700 mg/ml of BCG cell lysate was mixed and incubated for 3 days in the presence (HA+Lysate+Vcpal) or absence (HA+Lysate) of ascorbic palmitate (Vcpal), an inhibitor of hyaluronidase. As controls, hyaluronan alone (lane 1, HA) or BCG cell lysate alone (lane 2, Lysate) was treated in the same way. Hyaluronan was precipitated by ethanol after phenol extraction and resolved in water. Then hyaluronan was fractionated by PAGE gel electrophoresis and visualized by staining with alcian blue. (B), BCG-Luc (0.01 OD at 630 nm) was cultured in carbon-starved 7H9 media (7H9), media containing hyaluronan (500 mg/ml) as a sole carbon source (7H9-HA), or complete 7H9-ADC media (7H9-ADC) in the presence or absence of 25 mM Vcpal (+Vcpal), an inhibitor of hyaluronidase. The growth of bacteria was monitored by luciferase activity. RLU, relative luciferase unit (RLU).

In another example Spy1600 is the enzyme secreted by *Streptococcus pyogenes* which cleaves the β-O-linked *N*-acetylglucosamine from mammalian glycoproteins.(25)

In contrast to above listed examples there are reports where the host cells are known to secrete various proteins in response to bacterial infection as part of defense mechanism. Galectin 3 is expressed in macrophages, which is secreted out through a non-classical transport pathway and interacts with several pathogen surface glycans or LPS thereby negatively regulating the inflammatory response.(26). Lysozyme, lactoferrin, and collectins secreted by host cells act primarily by targeting the bacterial outer membrane (27). Epithelial cells in contact with *H. pylori*, release human defensin-2 and defensin-3, which is bactericidal not only for *H. pylori* but also to many gram-positive bacteria.(28) In many instances bacteria fights back to the host defense for survival and persist infection. Cathelicidin is produced in intestine in response to *H. pylori* infection and ablation of cathelicidin significantly increases the susceptibility of *H. pylori* colonization in mouse model.(29) However, the addition of phosphorylcholine (ChoP) to the oligosaccharide portion of NTHI (*Haemophilus influenzae* nontypeable strains lipooligosaccharide (LOS), results in increased resistance to the cathelicidin LL-37.(30) The compositions of OMVs in *V. cholerae* is altered by increase in Bap1 protein which binds to AMP leading to AMP resistance.(31) Through diversionary strategies like LPS and DNA secretion, AMP bacterial interactions are reduced, leading the bacterial biofilms to persist in the host and aid in disease progression.

We have previously shown the secretion of fucosidase2 in reponse to *H. pylori* infection in gastric epithelial cells. The released fucosidase cleaves the glycans on

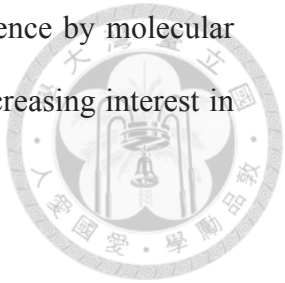
host cells to minimize the ligands for bacterial attachment. However, the bacteria takes up the cleaved fucose as energy source.(32) In another instance *Neisseria gonorrhoeae* resists to killing by human complement and cationic antimicrobial peptides by the addition of phosphoethanolamine to the phosphate group at the 4′ position using lipooligosaccharide phosphoethanolamine transferase A (LptA). Loss of this enzyme, increases bacterial sensitivity to killing by human complement and cationic antimicrobial peptides.(33)

Thus, host pathogen interactions at one end explains the host's effort to defend the infection on the other end explains bacterial strategies to evade host defense and its ways to persist infection benefitting from host, for survival.

1.4 Fucosylation

Specific terminal glycan modifications, including fucosylation, can confer unique functional properties to oligosaccharides and are often regulated during ontogeny and cellular differentiation.(34) Important roles for fucosylated glycans have been demonstrated in a variety of biological settings. It is found at the terminal or preterminal positions of many cell-surface oligosaccharide ligands that mediate cell-recognition and adhesion-signaling pathways. These include such normal events as early embryologic development and blood group recognition and pathologic processes including inflammation, infectious disease recognition, and neoplastic progression. They play an important role in cell recognition processes ranging from fertilisation and development through to pathological events and cell death (35). Certain bacteria decorate themselves with fucosylated glycans so as to mimic the

blood group antigens in host and thus escape the immune surviellence by molecular mimicry.(36,37) The important role played by fucoses led to an increasing interest in fucose related enzymes such as α-L-fucosidases.



1.5 Alpha-L-fucosidase

Alpha-L-fucosidase is one of the lysosomal hydrolases that catalyzes the hydrolytic removal of the L-fucose residues of glycoconjugates. Its clinical importance is exemplified by the disease fucosidosis, a lysosomal storage disorder caused by deficient fucosidase activity due to genetic defect.(38) It is also known that an abnormal increase in fucosidase activity is associated with several pathological conditions, such as inflammation,(39) carcinoma of the stomach, ovary,(40) and liver,(41) cystic fibrosis,(42) and bacterial infection (32). It has been proposed as a tumor marker since many studies reported increased fucosidase serum levels in patients with cirrhosis and HCC. (39) Mammalian fucosidases are multimeric, containing glycoprotein subunits of about 53kDa; they have a maximal activity between pH 4 and 6.5. They have a considerable degree of heterogeneity due to their glycosylation. The serum fucosidase activity in normal individuals is reported to be about 381 U/ml. (43) Currently, α-L-fucosidases are known to be classified under Glycoside hydrolase families GH29 and GH95, the latter are said to follow a strict substrate specificity to terminal Fuc α 1-2 Gal linkage and hydrolyze the linkage via inverting mechanism (44,45) whereas the former class follow relatively relaxed substrate specificities with hydrolysis proceeding via a retaining mechanism. (46)

1.6 *N*-Acetyl β-D-hexosaminidase

N-Acetyl β-D-hexosaminidase is a lysosomal hydrolase that cleaves the terminal beta linked GlcNAc or GalNAc residues from oligosaccharides, glycolipids, glycoproteins or Glycosminoglycans. It exist as an isoenzyme namely Hex A and Hex B formed by assembly of two subunits alpha and beta encoded by two closely related genes HEX A and HEX B. Another minor form Hex S that is also said to exist rarely is formed by two alpha subunits. A fully processed Hex A is said to be associated with plasma membrane lipid microdomains and lysosomal membrane. It has been found that, mutations in the α- and β-subunit coding genes lead to the development of Tay-Sachs and Sandhoff diseases, respectively, which are severe lysosomal storage disorders associated with neurodegeneration.(47) In addition, β-hexosaminidase altered expression has been often associated with cancer (48,49). Elevated levels of Hex B have been found in tumor tissues and serum samples in disease(50-52). The presence of Hex S has been observed in leukaemic cells but not in their normal counterparts. (53). β-hexosaminidase is often considered as biomarker for cancers. More recently increased activity of β-hexosaminidase was found in asthma patients (54) indicating a possible role of the enzyme in asthma related inflammations. β-hexosaminidase has been often used as a diagnostic marker to monitor the progression of different types of pathologic conditions such as rheumatoid arthritis,(55) osteoarthritis(56), arterial hypertension,(57) glomerulonephritis (58). β-hexosaminidase has been characterized as the dominant glycosidase released by chondrocytes(59) and was served as a glycosaminoglycan-degrading enzyme in the cartilage matrix. Therefore, inhibition of β-hexosaminidase activity might be a possible treatment to prevent the degradation of cartilage matrix glycosaminoglycan in the course of osteoarthritis (60)

It has been reported that β-hexosaminidase is secreted from immune cells such as mast cells and neutrophils in a process called degranulation. Increased secretion of β-hexosaminidase from mast cells on treatment with HP-NAP (*Helicobacter pylori* Neutrophil activating protein) has been reported. (**Figure 4**)

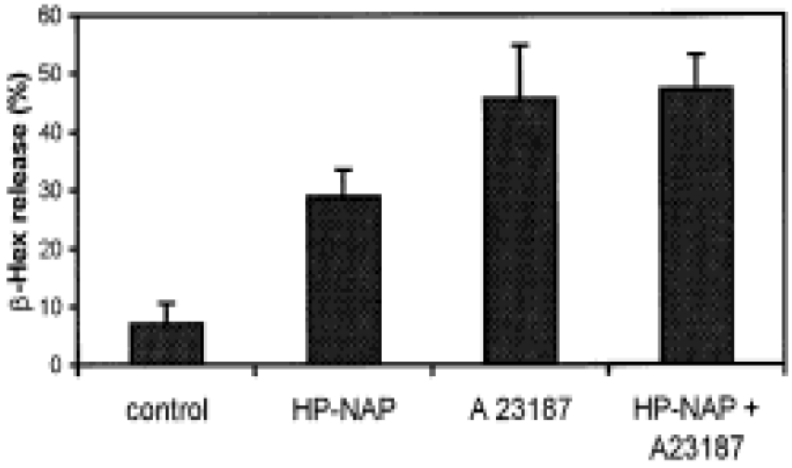


Figure 4. Effect of HP-NAP on peritoneal rat mast cells (PMC) degranulation. (A) PMC (1 Å~ 10^6 cells/ml) were incubated for 30 min at 37 °C with HP-NAP or with A23187 or with the two agonists together. The amounts of β-hexosaminidase in the supernatants were measured. (61)

The secreted β-hexosaminidase along with other cytokines is said to recruit the neutrophils and monocytes at the site of infection. Earlier reports show β-hexosaminidase has bactericidal effect in case of gram-positive bacteria. (62-64)

1.7 Macrophage-mediated phagocytosis

The different macrophage receptors recognise Gram-positive and Gram-negative bacteria through conserved structures on the bacterial surface and facilitate phagocytosis and the trigger for the adaptive immune response. Examples of such receptors include scavenger receptors, C-type lectins, integrins, Toll-like receptors and siglecs. These receptors recognize bacterial ligands ranging from lipopolysaccharides on Gram-negative bacteria to peptidoglycan and lipoteichoic acid on Gram-positive bacteria.(65) In histological sections of gastric mucosa from patients infected with *H. pylori*, an accumulation of lymphocytes, neutrophils, monocytes, and macrophages can be detected. Macrophages are involved in amplification of the inflammatory response by production of cytokines such as IL-1, TNF-α, and IL-6 (66-68) and IL-6 activation has been linked to activation of TLR4, MAPK, and NF-κB signaling events (69). Macrophages also function as effector cells in host defense. One such pathway involves generation of nitric oxide (NO) derived from the enzyme inducible NO synthase (iNOS, NOS2), which has been shown to be upregulated by *H. pylori* in macrophages *in vitro* (70) (71) (72) and *in vivo* (73). Co-culture studies demonstrate that *H. pylori* can be killed by macrophages even when physically separated from these effector cells by a filter support and that this antimicrobial defense is NO dependent (70,71). The arginase enzyme possessed by *H. pylori*, encoded by the gene *rocF* can compete sufficiently with macrophages for the iNOS substrate L-arginine (L-Arg) such that host NO production is impaired, leading to enhanced survival of the bacterium. (71) Bacterial arginase generates urea from L-Arg, which is then utilized by urease to synthesize ammonia that is required to neutralize gastric acid. However, attenuation of macrophage NO generation additionally benefits *H. pylori* by enhancing immune evasion. Another example of the ability of *H. pylori* to escape the macrophage response is via glucosylation of

cholesterol, and mutant strains that cannot process cholesterol have increased susceptibility to phagocytosis by macrophages and cannot colonize the mouse stomach. (74)

2. Research motivation

Many lysosomal hydrolases are upregulated during infections. From our previous report, as part of host defense, gastric epithelial cells release fucosidase 2 in response to *H. pylori* infection, which is used by bacteria as a beneficial strategy for adhesion and persistent infection (32). After successful adhesion, the bacteria paves its way for further infection. Literature shows that in an attempt to evade infection, that gastric epithelial cells internalize *H. pylori* where the bacteria enclosed in vacuoles induce autophagy. Upon induction of autophagy by *H. pylori* in gastric epithelial cells, internalized bacteria are able to multiply inside the autophagosome but are degraded after fusion of the autophagosome with lysosome. Having known the upregulation of secretory fucosidase (fuca2), we aimed to monitor the regulation of its intracellular counterpart, the lysosomal fucosidase during *H. pylori* infection.

Upon mycobacterial infection, β-hexosaminidase was characterized as a peptidoglycan hydrolase with mycobatericial effect when secreted from host lysosomes (62). (**Figure 5**)

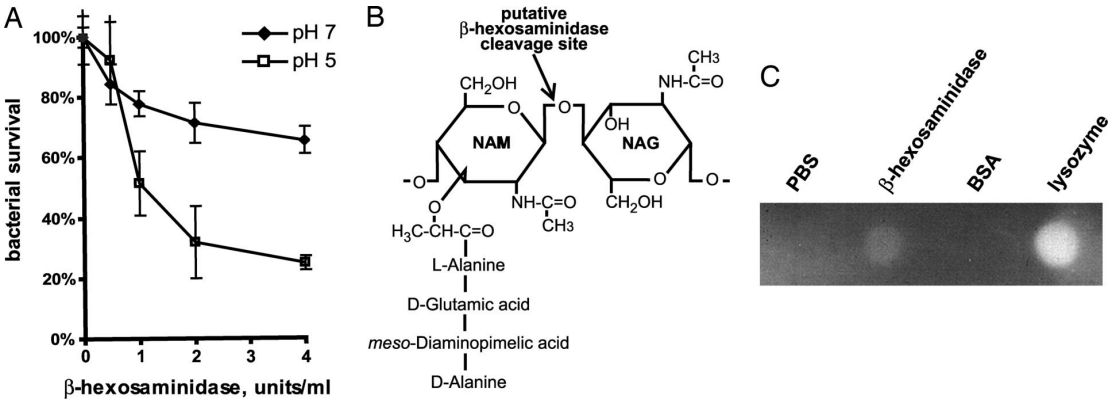


Figure 5. β-Hexosaminidase is a mycobactericidal peptidoglycan hydrolase. (A) Mm was incubated with 0, 0.5, 1, 2, and 4 units/ml human hexosaminidase. Bacterial survival was enumerated as colony-forming units on 7H10 agar. Assays were done in triplicate, at pH values of 5 and 7. (B) β-Hexosaminidase is predicted to hydrolyze the β-1,4-linked glycosidic bond between NAM and NAG, as shown. C) PG hydrolase activity of β-hexosaminidase was assessed by zymography. PBS and BSA were negative controls, and lysozyme was used as a positive control.

In another report β-hexosaminidase was said to effect the survival of some gram-positive bacteria including *S. aureus, S. epidermidis* and *B. subtilis* by degrading the PGN layer in the cell wall. These findings have revealed a role for β-hexosaminidase in host defense mechanism induced by bacterial infection. Both *M. tuberculosis* (Mtb) and *H. pylori* are successful commensal pathogens with high infection rates throughout the world. Once established, the infections would remain life-long. However, not all of the infected people develop same types of symptoms or diseases, highlighting the existence of possible host defense mechanisms in the pathogenesis of these bacteria. We thus hypothesized that induction of β-hexosaminidase may also occur in the pathogenesis of *H. pylori* infection, and possibly play a defensive role

In this thesis, as part of host pathogen interaction we study the regulation of intracellular human α-L-fucosidase and human β-D-hexosaminidase during *H. pylori* infection. We further explore bactericidal action of the secreted β-hexosaminidase and α-fucosidase during *H. pylori* infection. Finally investigate the effect of the action of these enzymes on macrophage-mediated phagocytosis.

3. Material and Methods

3.1 Cell lines and bacterial culture. AGS (ATCC® CRL-1739) and 293T (ATCC® CRL-11268) were grown to confluency using DMEM (Gibco) supplemented with 10 % FBS (Gibco), 1 % penicillin/streptomycin/ampicillin (Gibco) and 1 % sodium pyruvate (Gibco) and J774A.1 (ATCC® TIB-67™) were cultured using RPMI supplemented with 10 % FBS (Gibco) and 1 % sodium pyruvate (Gibco). Cells were cultured in an atmosphere of 5 % CO2 at 37 °C. *H. pylori* wild type (NTUH-C1) was obtained from National Taiwan University Hospital, *H. pylori* (26695) was obtained from BCRC, Taiwan. They were stored at -80 °C in sheep blood containing 15 % glycerol and cultured on 5 % sheep blood agar plates (BD) under anaerobic conditions.

3.2 Co-culture conditions. The *H. pylori* clinical strain was cultured under microaerobic conditions. Bacterial cells were collected with a cell scraper in phosphate-buffered saline (PBS) and resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM). AGS cells were cultured in DMEM at 37 ^{0}C up to confluency and washed with PBS, then infected with *H. pylori* (MOI of 100 or 200) and incubated at 37 ^{0}C for 6–8 h in serum-free DMEM. To identify possible stimulants, the bacteria (MOI of 200), dead but intact bacteria (resuspended in PBS and heated at 60 ^{0}C for 1 h), LPS (10 mg/mL; obtained by hot phenol extraction), crude flagella, bacterial lysate, and pellet (bacteria were resuspended in PBS, lysed by repeated cycles of freezing and thawing, homogenized for 20 min, and centrifuged at 12000 g for 10 min at 4 ^{0}C to obtain the cell lysate and pellet) were all obtained from the same number of *H. pylori.* The resulting samples were suspended in PBS and added to serum-free DMEM to treat AGS cells. The control experiments were treated with PBS. For experiments with inhibitor FNJ, the cells were treated with FNJ (5 mm)

prior to infection with *H. pylori,* and colistin sulfate (10 mg/mL) was added to the cells for the LPS neutralization experiment. AGS cells were pretreated with Hexosaminidase inhibitor and or fucosidase inhibitor (5 μM) before and during infection for experiments to show the bactericidal effect of Hexosaminidase and fucosidase.

3.3 Confocal microscopy and Imaging. AGS cells (~1 X 10^5) seeded and cultured for 3 days on a cover slipped glass slides in 24-well plates. For the *H. pylori* infection experiment, the cells were infected as mentioned in co-culture procedure. Cells were treated with or without FNJ prior to the bacterial infection. Further the cells were incubated with 1 (10 μM) for 2 h. The cells were washed three times with PBS and then incubated with LysoTracker. Red DND-99 (*Invitro*gen) in serum-free medium at a concentration suggested by the manufacturer for the enzyme localization. For experiment to show effect of β-hexosaminidase and fucosidase on adherence and survival of bacteria, the cells were treated with β-hexosaminidase inhibitor (compound **4)** (5 μM) (75) or fucosidase inhibitor (compound **2)** (5 μM) (76) or both prior to and during infection. The cells were washed three times with PBS, fixed with formaldehyde (4 %). The fixed cells were labeled using rabbit anti-*H. pylori* antibody as primary and alexa fluor 488 donkey anti rabbit IgG as the secondary antibody. The cells were washed thrice with PBS and fixed by incubating cells with 3% formaldehyde solution for 15 min at 37 °C. The cells were further washed and stained for nucleus using Hoechst 33258 (Sigma) according to the guidelines given by the manufacturer. The cover glass was mounted using Prolong– antifade mounting solution (*Invitro*gen) and sealed. The images were acquired by using (objective 63 X) Zeiss confocal microscope. For experiment to show effect of β-hexosaminidase and fucosidase on adherence and survival of bacteria images were acquired by Z-

projection.

3.4 Labelling of Fuca1 in co-culture condition. For experiments involving co-culture with *H. pylori* or bacterial extracts (lysate, pellet, LPS, flagella and or colistin sulfate 10 μg/ml), co-culture was carried out as described under co-culture conditions. AGS cells were incubated with or without FNJ (5 μM) for 1 h prior to *H. pylori* infection for experiments involving inhibitor treatment. The cells were further treated with **1** (10 μM) for 2 h. The cells were washed thoroughly and scrapped using a cell scraper and lysed using RIPA lysis buffer (Sigma) supplemented with 1 % protease inhibitor cocktail (Calbiochem) and centrifuged at 12000 g for 15 min to obtain the lysate. Protein quantitation by Bradford assay and normalizations were performed. Slot blotted the samples onto PVDF membrane and the membrane was washed for 10 min, three times. The membrane was visualized under Fujifilm LAS-4000 Image acquisition System (with y515 blue filter) to obtain the fluorescence signal. The signals obtained were normalized against the signal obtained from control. The same membrane was blocked with 5 % BSA in Tris-buffered saline and Tween 20 (TBST, containing 0.1 % Tween 20) for 1 h at room temperature. Actin was detected using mouse anti-actin antibody (1:5000) (Sigma) as the primary antibody and Horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare) at a dilution of 1:5000, serving as the secondary antibody. Blots were imaged using ECL system (GE Healthcare) according to manufacturer's instruction. The signals obtained by immunoblotting were normalized against that of control.

3.5 Immunoblotting of Fuca1 in co-culture condition. AGS cells were infected with *H. pylori* (MOI of 100 or 200) for 6-8 h, washed, lysed and subjected to SDS-PAGE. The gel was transferred onto PVDF membrane, and blocked by BSA (5 %) for 1 h at RT. Fuca1 and actin (as the loading control) were detected by using mouse anti-

Fuca1 (Abgent) and anti-actin (Sigma) monoclonal antibodies as primary antibodies respectively and horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare) serving as the secondary antibody. Blots were imaged using ECL system (GE Healthcare) according to manufacturer's instruction.

3.6 LPS extraction. The extraction was carried out according to the procedure reported by Hildebrandt et al.(77) with slight modifications. After the cell culture and harvest, *H. pylori* was collected and washed with PBS. The bacterial cells were incubated in 60 mM Tris-HCl (pH 7.2) containing 2 % SDS at 95-98 °C for 10 min. The sample was further incubated at 60 °C for 2 h in 60 mM Tris-HCl (pH 7.2) that consists of 0.67 % SDS and 0.67 mg/ml proteinase K. Samples were extracted with 90 % hot phenol at 70 °C for 20 min, cooled down to 10 °C, and centrifuged at 12000 x g at 10 °C. The resulting aqueous layer was collected and the sample was re-extracted with water. The aqueous layers were pooled and adjusted to 0.5 M NaCl and treated with 10 volumes of ethanol and allowed to precipitate at -20 °C for overnight. The precipitant was collected by centrifugation at 20000x g for 20 min, air dried, resuspended in water, and lyophilized. The samples were dialysed in 3K MWCO dialysis bag against water to obtain a pure LPS. Quantitation of LPS was done by purpald assay.

3.7 Crude Flagella Extraction. The first step in the isolation of flagella as described by De Pamphilis et al. was followed with slight modifications. (78) The bacteria were suspended in PBS, washed and further processed in a sonicator (Branson) under low amplitude (32 %). The suspension was diluted in Tris buffer (pH 7.8) and centrifuged at 12000 x g for 10 min to sediment cell or cell debris and at 55000 x g for 1 h to sediment the flagella. The flagella pellet was dialyzed for a day and the crude extract

obtained was used for further studies.

3.8 Co-culture medium concentrate. AGS cells were cultured until 80 % confluency in DMEM medium. *H. pylori* was cultured and collected using a cell scrapper and suspended in PBS. AGS cells were washed and incubated with *H. pylori* at a multiplicity of infection of 200 for 8 h. The co-culture medium was collected filtered through 0.22 μm filter and concentrated using centricon 10KDa MWCO and washed with buffer 50 mM HEPES pH 5.6. The medium concentrate was used for assay or treatment with *H. pylori*.

3.9 *H. pylori* viability under co-culture condition. AGS cells were cultured in 12 well plates. The cells were infected with *H. pylori* at an MOI of 100 and incubated for 6-8 h. The cells were treated with β-hexosaminidase inhibitor or fucosidase inhibitor or both prior to and during infection [(5 μM) final concentration]. The cells were washed vigorously and the cells were treated with 0.01 % saponin solution (mild detergent). The lysate containing bacterial cells were plated onto blood agar plates and incubated for 5 days at 37 °C in humidified microaerobic chambers. The number of colonies formed was counted.

3.10 Bacterial survival assay. *H. pylori* was cultured and 1 x 10^5 cells were suspended in brucella broth in triplicate and treated with co-culture medium concentrate with or without β-hexosaminidase inhibitor (75) and fucosidase inhibitor and incubated at 37 °C for 3-5 days and OD at 600 nm was measured every 24 h using Flex station3 plate reader.

3.11 LPS degradation analysis. LPS was extracted as described above. LPS (1 μg) was treated with co-culture medium concentrate with or without β-hexosaminidase inhibitor (5 μM) or fucosidase inhibitor (5 μM) overnight followed by treatment with proteinase K (0.5 mg/ml) for 2 h at 37 °C. Analysis of bacterial LPS by SDS-PAGE was developed by modified silver-staining method. The fixation step was carried out by placing the gel into a 40 % ethanol-5 % acetic acid solution (200 ml) in a clean dish overnight. The fixing solution was then replaced with 0.7 % periodic acid in 40 % ethanol-5 % acetic acid solution, and incubated for 5 min to undergo LPS oxidation. After oxidation, the gel was washed three times with 500-1000 ml distilled water for at least 15 min each time. Freshly prepared staining reagent (150 ml) containing 2 ml concentrated ammonium hydroxide, 28 ml of NaOH (0.1 N), 5 ml of silver nitrate (20 % w/v) and 115 ml distilled water was poured and the gel was agitated vigorously (about 70 rpm) for 10 min. Then three 10-min washes were performed with distilled water. The water was then replaced with developer (200 ml) consisting of 50 mg citric acid and 0.5 ml of 37 % formaldehyde per liter. When the stain reached desired intensity, development was terminated by 10 % acetic acid.

3.12 Cell wall extraction. The procedure followed is as described by Wang et al. (79) with slight modifications. *H. pylori* cells were cultured and collected and washed with Tris-HCl pH 7.2 centrifuged at 8000 x g for 20 min at 4 ^{0}C and resuspended and treated with boiling 4 % SDS buffered with Tris-HCl pH 7.2 and boiled for 30 min. The samples were cooled overnight and centrifuged at 65000 rpm for 1h. The pellet was washed with water and resuspended in Tris-HCl pH 7.5 containing 10mM NaCl, DNase (10 μg/ml) and RNase (50 μg/ml) and incubated for 2 h at 37 °C. Followed by

addition of proteinase-K (50 μg/ml). The solution was re-extracted by boiling in 1 % SDS for 15 min and collected by centrifugation and washed with water 4-5 times. The cell wall pellet thus obtained was resuspended in distilled water and lyophilized.

3.13 Zymography analysis. Zymography analysis was performed as described by Koo et al. (62). Polyacryl amide gel containing cell wall extracted from *H. pylori* (~ 0.1 %) was spotted with co-culture medium concentrate, β-hexosaminidase (1.5 mU) or equivalent amount of BSA. The protein solutions were allowed to absorb into the gel for 1 h. The gel was washed and incubated for O/N in HBSS at 37 °C. The gel was then stained with methylene blue for 1 h and washed using water.

3.14 Scanning Electron microscopy. The *H. pylori* cells were cultured treated with co-culture medium with or without hexosaminidase inhibitor and fucosidase inhibitor for 4-6 h and bacterial cells were pelleted washed thrice with PBS. The samples were first fixed with glutaraldehyde 2 % washed and treated with 4 % OSO4. Then the samples were dehydrated using graded ethanol series. The final dehydration step included incubating sample in acetone for 5 min. The dehydrated samples were spread onto sample stud and coated with gold and images were acquired using NOVA 200 Nano SEM.

3.15 Transmission Electron Microscopy. Bacteria for TEM samples were grown and incubated with co-culture medium with or without inhibitors. Cell pellets were obtained from 10 ml of each control or treated cell suspension and fixed with

glutaraldehyde and OsO4 as described above. The same buffers and dehydration protocol as for SEM were used followed by graded acetone series and embedding in epoxy resin. Ultrathin sections were prepared on Formvar-coated grids and stained with 3 % uranyl acetate. The images were acquired by FEI Tecnai G2 F20 S-TWIN.

3.16 Macrophage mediated phagocytosis. J774A.1 cells were cultured and infected with *H. pylori* (MOI 100). The co-culture was carried out for 3 h. The cells were then washed, fixed and differential immunostaining using rabbit anti-*H.pylori* antibody (Abcam) and goat anti rabbit Alexa flour 647 antibodies to label the extracellular *H. pylori* was peformed. Cells were permeabilised with Triton-X (0.1 %) and intracellular and extracellular *H. pylori* were labeled with rabbit anti-*H.pylori* antibody (Abcam) and goat anti rabbit Alexa flour 488. Mounted slides were imaged using Leica TCS confocal microscopic system.

3.17 Glycosidase activities examined in co-culture medium. Glycoside hydrolase activities were determined by measuring the liberation of 4-methylumbelliferone (4-MU) from different glycosides (Sigma Aldrich). Each reaction contained 20 µl Hex assay buffer (50 mM HEPES buffer at pH 4.25, 100 mM NaCl, 0.1 % BSA), 15 µl of co-culture medium concentrate, and 15 µl 4-MU substrate (at a final concentration of ~300 µM for sialic acid, ~600 µM for other substrates). The reaction was incubated for 30 min at 37 °C, and then stopped by addition of 150 µl quenching buffer (200 mM glycine, pH 10.8). The fluorophore was excited at 365 nm, and the emission was measured at 465 nm.

4. Results

4.1 Upregulated activity of Fuca1 in *H. pylori* infection

The lysosomal enzymes have a critical role to play in the bacterial degradation during autophagy. Having known the upregulation of secretory fucosidase we aimed to monitor the activity of its intracellular counterpart that is fuca1, which is otherwise called as lysosomal fucosidase (Fuca1). We applied a quinomethide based activity based probe (compound **1**) [synthesized by Yu-Ling Hsu at Dr. L.C. Lo's laboratory, Dept. of chemistry, NTU] to monitor the activity of Fuca1 in cells upon *H. pylori* infection. (80) Compound **1** contains a-fluoromethylphenyl-α-L-fucopyranoside as the reacting group and recognition group along with fluorophore BODIPY as the reporter. (**Figure 6**) When the glycosidic bond is cleaved by α-L-fucosidase, the L-fucose-released intermediate undergoes 1,4- elimination with removal of a fluoride ion to generate a reactive quinone methide that reacts with a nearby nucleophile of the enzyme, thereby leading to the formation of a BODIPY-labeled enzyme adduct which can be visualized at excitation wavelength = 497 nm, emission wavelength = 520 nm. It was found to be cell permeable, non-toxic and specific to α-fucosidase. (81) AGS cells infected with *H. pylori* for 6 h were treated with **1** (10 μM) for 2 h. To validate the observed signals in cell to be α-L-fucosidase-specific, FNJ (5 μM) was added to the cultured AGS cells prior to the labeling step. The presence of FNJ was able to significantly abolish the signal of **1** (**Figure 7**), also evidenced by *in vitro* analysis (**Figure 8**). After several washes, the cells were further counterstained with a lysosome marker (Lysotracker) and a nucleus marker (Hoechst). The BODIPY signal of **1** was not only co-localized well with that of lysotracker, but also increased remarkably upon infection (**Figure 7**), indicating that there was an upsurge activity of the lysosomal Fuca1. The observation was also confirmed by

immunoblotting using anti-Fuca1 antibody (**Figure 9**). Thus the level of Fuca1 is upregulated along with Fuca2 during *H. pylori* infection.

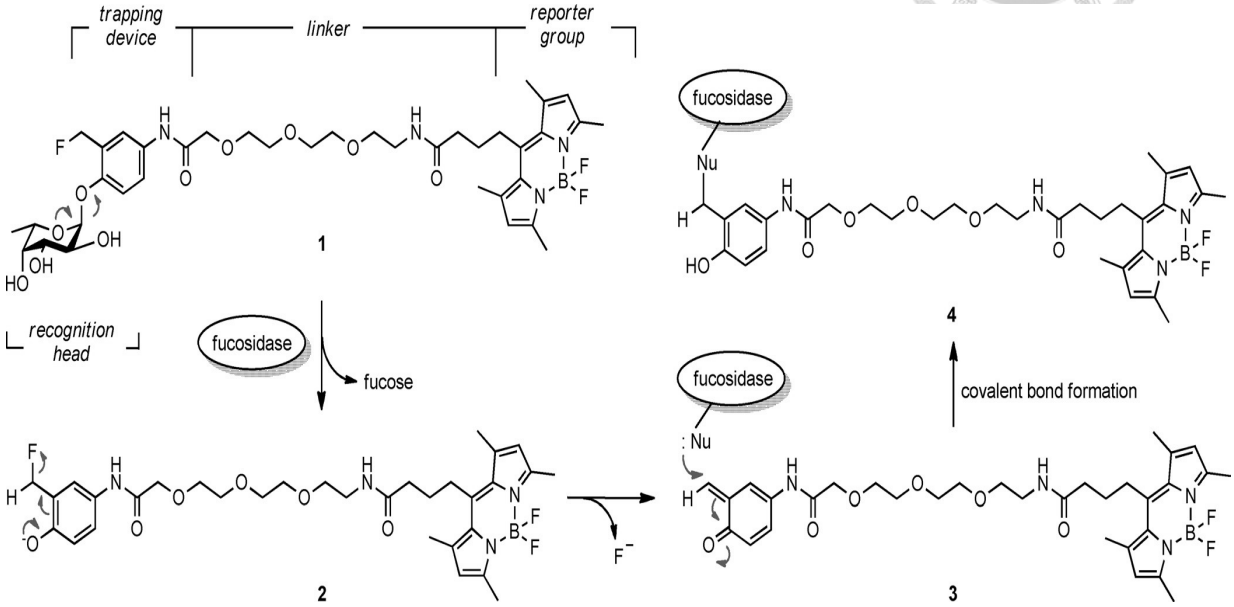


Figure 6: Structure and mechanism of compound **1**

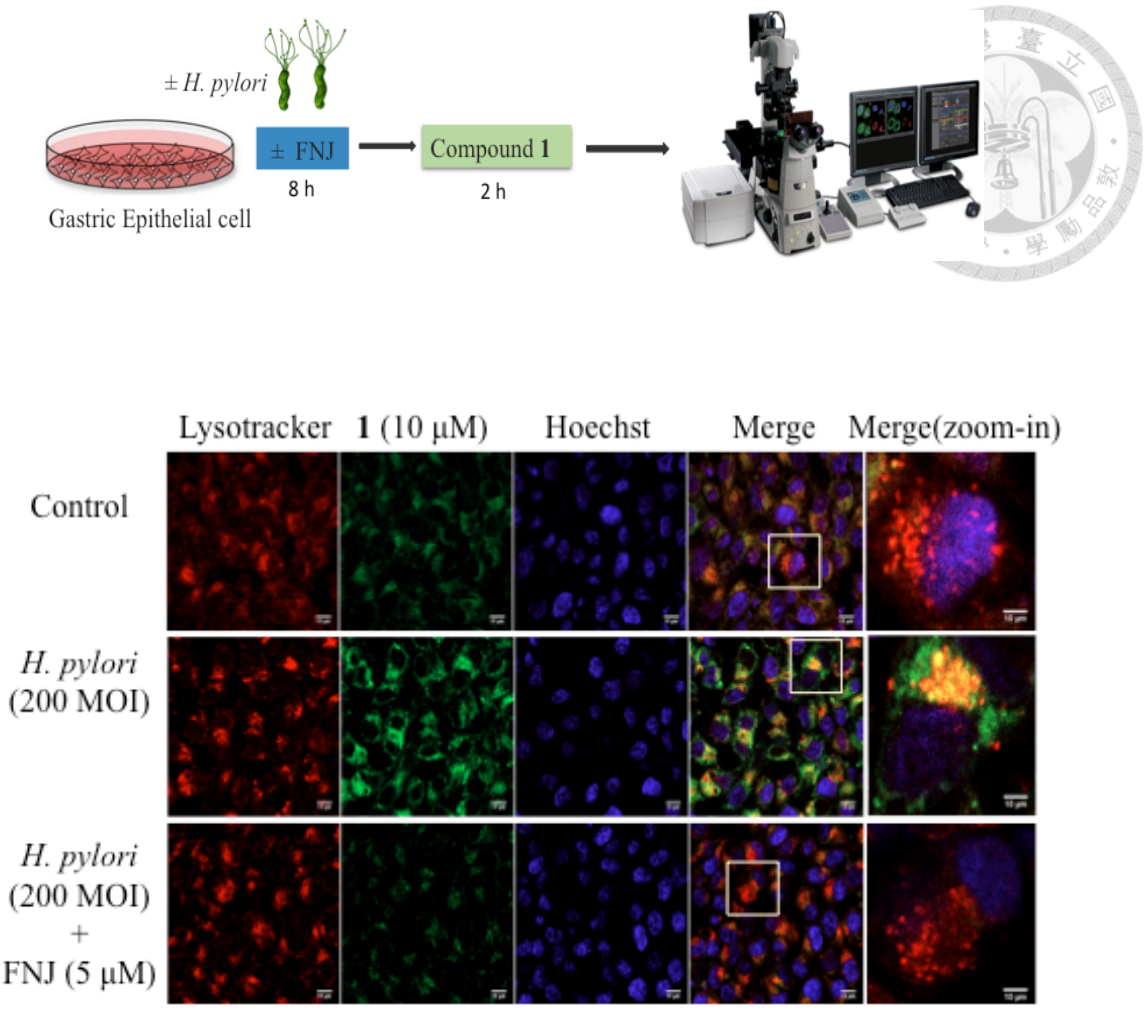


Figure 7. Compound **1**- derived labeling of Fuca1 and Fuca2 in cell during *H. pylori* infection confocal imaging of AGS cells infected with *H. pylori* (200 MOI; 2 h) with/without FNJ (5 μM), followed by labeling with **1** (10 μM, 2 h) and staining with LysoTracker DND99 red and Hoechst. An increased signal was observed upon *H. pylori* infection. Co-localization of Fuca1 and lysosomes is indicated in yellow.

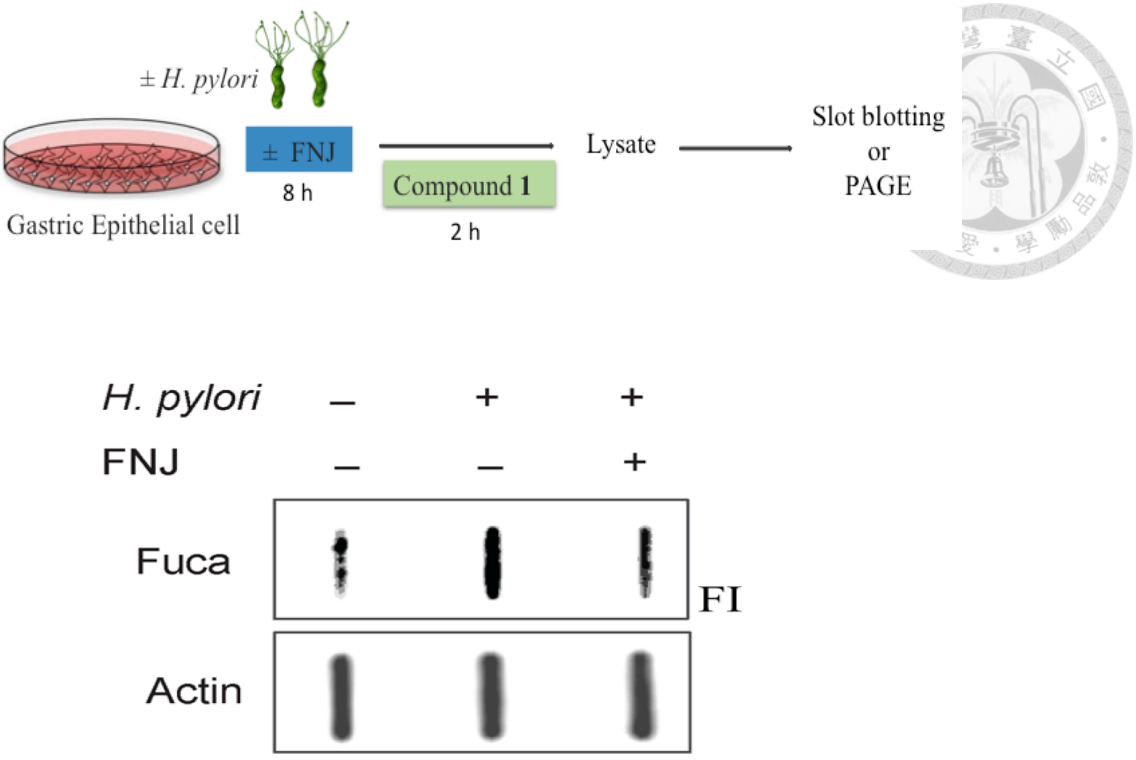


Figure 8. *In vitro* analysis to display the enhanced intracellular α-fucosidase activity using **1**. AGS cells were first infected with *H. pylori* (MOI of 200) for 6 h, followed by treatment with **1** for 2 h and the resulting lysates were transferred onto PVDF membrane by slot blotting. The fluorescence signal indicated the existence of **1**-conjugated α-fucosidase. The signal was diminished in presence of FNJ, supporting the specificity of **1**. The same membrane was subjected to immunoblotting using anti-actin antibody.

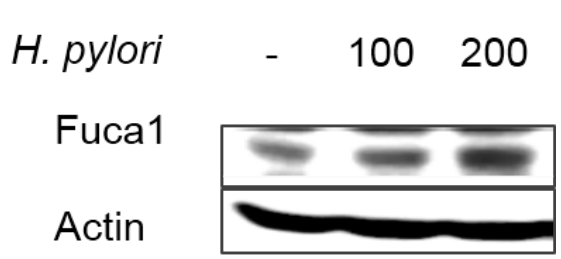
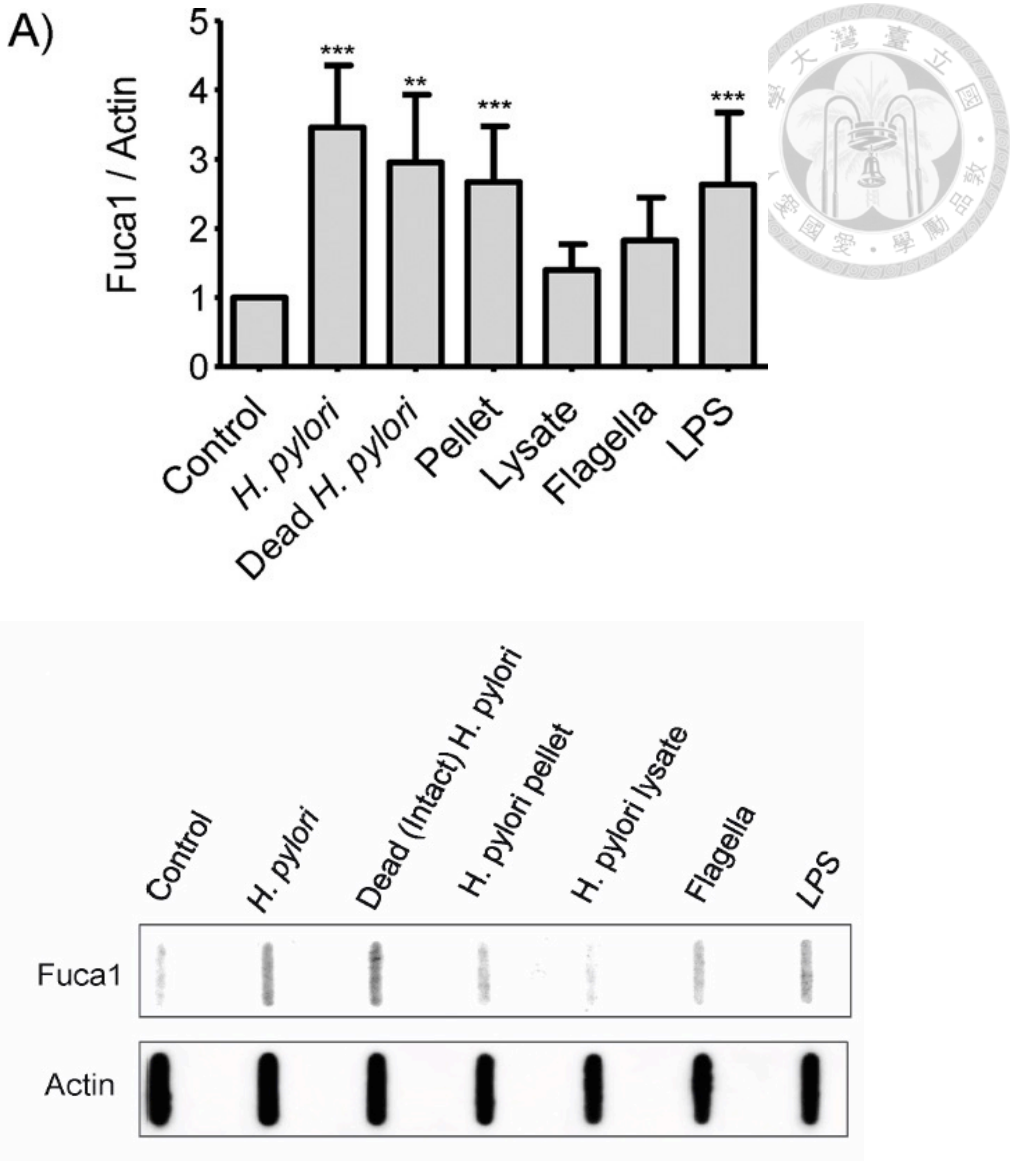


Figure 9. Up-regulation of Fuca1 in AGS cells owing to *H. pylori* infection by immunoblotting. The *H. pylori*-infected AGS cells (MOI of 100 or 200) were cultured for 6-8 h, washed, lysed and analyzed with SDS-PAGE. The gel was transferred onto PVDF membrane, and blocked by 5 % BSA for 1 h at RT. Fuca1 and actin (as the loading control) were detected by using mouse anti-Fuca1 and anti-actin monoclonal antibodies, respectively.

4.2 Stimulant for upregulation of Fuca

As the *H. pylori* adhesion pulls the trigger to enhance the fucosidase activities, it is thus worth identifying possible molecules or components behind the scenes. AGS cells were treated with each of several stimulants for 6 h, including live *H. pylori*, dead but intact *H. pylori,* the bacterial cell pellet and lysate, all resulting from the same number of bacteria. The cells were further treated with **1** (10 μM) for 2 h, washed, lysed and subjected to SDS-PAGE. The fluorescence signals of labelled Fuca1 were monitored. We observed enhanced Fuca1 activity in the cells treated with dead but intact *H. pylori* or the bacterial cell pellet, which is comparable to *H. pylori*-treated cells (the left four bars in **Figure 10A**), implying that the bacterial cell surface appears to be essential for the elevated enzyme activities. Being the major virulence factors on the cell surface of bacteria, (82), (83) the lipopolysaccharides (LPS) and flagella were purified and obtained for further incubation with AGS cells to monitor the Fuca1 activity. We observed a significant increase in the Fuca1 activity with the LPS treatment (the right two bars in **Figure 10A**) and this signal was abrogated on the co-treatment with colistin sulfate (**Figure 10B**), a polymyxin antibiotic known for LPS binding and neutralizing activity.(84) This result indicated LPS could be majorly but not solely responsible for the up-regulation of Fuca1 caused by the infection, as we cannot rule out other factors present on the bacterial surface


A)
Fuca1 / Actin
5
4
3
2
1
0
***
**
***
***
Control
H. pylori
Dead H. pylori
Pellet
Lysate
Flagella
LPS
Control
H. pylori
Dead (Intact) H. pylori
H. pylori pellet
H. pylori lysate
Flagella
LPS
Fuca1
Actin

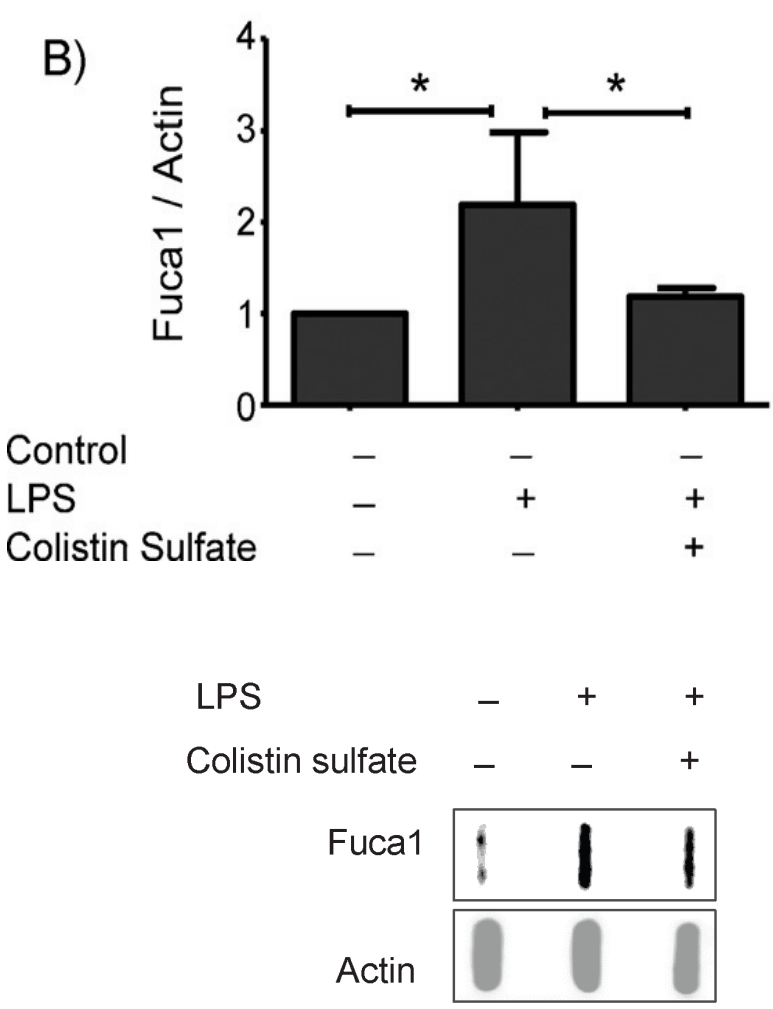
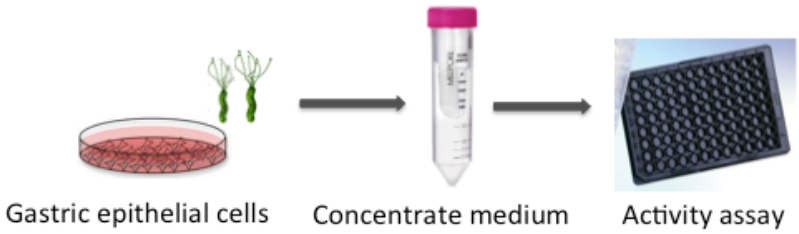
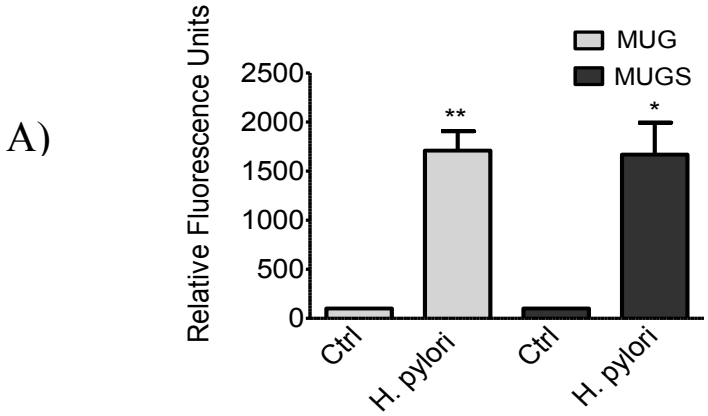


Figure 10. Stimulants for enhanced fucosidase activity. A) AGS cells treated with *H. pylori*, dead (intact) *H. pylori*, bacterial pellet and lysate, LPS, crude flagella extract, or PBS. Fluorescence intensities measured were normalized against the control. The signals obtained for actin by immunoblot served as the loading control. Data represents the ratio of signals obtained from labeled fucosidase to the signals obtained from actin. Data were compared by using Student's t-test. Bars represent the mean±.SD of three independent experiments with **P.0.01, ***P.0.001 for each treatment compared to the control. Representative of one of the three experiments is shown under the graph B) AGS cells wereincubated with LPS (10 μg/mL) with or without colistin sulfate (10 μg/mL) for 6–8 h and further treated with **1** (10 μM) for 2 h. The cell lysates were blotted onto PVDF membrane by slot blotting. The elevated fluorescence signals corresponding to Fuca1 activity due to LPS treatment were abrogated in the presence of colistin sulfate. Data were compared by using Student's t-test. Bars represent the mean ± SD of three independent experiments with *P.0.05 Representative of one of the three experiments is shown under the graph.

4.3 Secretion of β-hexosaminidase from gastric epithelial cells upon *H. pylori* infection

H. pylori infection in mast cells induces secretion of β-hexosaminidase,(61) However little is known about β-hexosaminidase in epithelial cells. Also since fuca2 is secreted on *H. pylori* infection we were interested to learn about other glycosidases and since β-hexosaminidase was reported to be secreted on treatment with HP-NAP we chose to study the regulation of β-hexosaminidase during *H. pylori* infection. Since the gastric epithelial cells are the primary site of infection we investigated the effect of *H. pylori* infection on the levels of β-hexosaminidase in gastric epithelial cells. AGS and Capan 1 cells were infected with *H. pylori* at an MOI of 200 for 6-8 h. The co-culture medium was collected filtered through 0.22 μm filters, concentrated and assayed for presence of β-hexosaminidase. Increased β-hexosaminidase activity was found in cells infected with *H. pylori* when compared with untreated cells. (**Figure 11 A,B**) The secretion was dependent on period of infection (**Figure 12A**) and multiplicity of infection (**Figure 12B**). To know if the enzyme released from the cells is due to cell damage/leakage or cell death on infection, we performed cell viability assay. The result confirms that the release of enzyme was not due to apoptosis or cell damage as the cells are viable on infection under specified time (**Figure 13**). β-hexosaminidase is known to secrete in small vesicles from immune cells in a process called degranulation as part of host defense. Our results show that gastric epithelial cells also secrete β-hexosaminidase in an attempt to defend against infection.





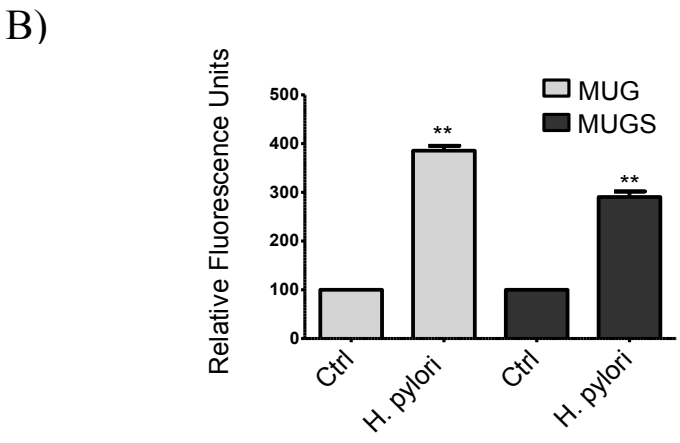
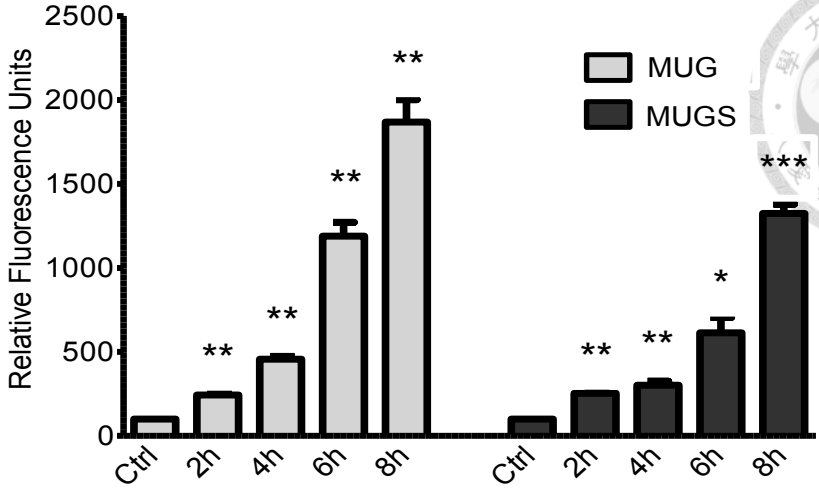


Figure 11. Increased β-hexosaminidase activity in co-culture medium upon *H. pylori* infection: (A) AGS cells (B) CAPAN1 cells were treated with *H. pylori* (MOI of 200) for 8 h and the co-culture medium was collected and concentrated. β-Hexosaminidase activity assay was performed using MUG (4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide) and MUGS (4-Methylumbelliferyl-*N*-acetyl-beta-D-glucosaminide-6-sulfate) as the substrates. Several fold increase in β-hexosaminidase activity in medium was observed. The bars represent mean ± SD with P values * ≤ 0.05, ** ≤ 0.005

A)



B)

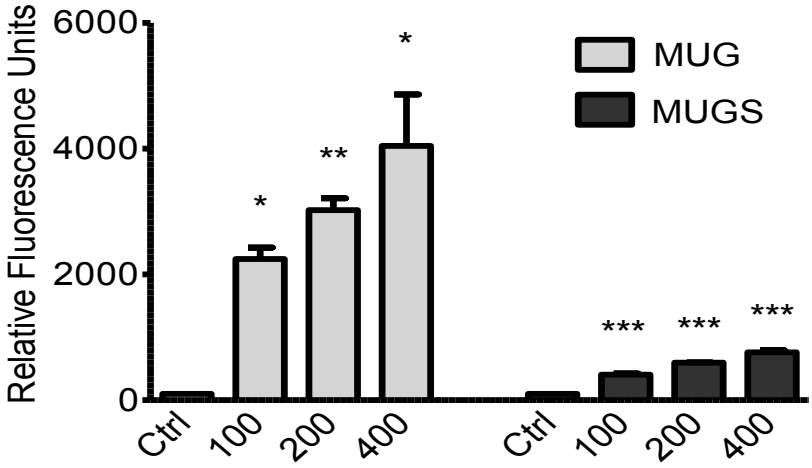


Figure 12. Time- and MOI-dependent increase of β-hexosaminidase activity in co-culture medium on *H. pylori* infection: AGS cells were treated with (A) *H. pylori* (MOI of 200) (B) *H. pylori* (MOI of 100, 200, 400) for 8 h and the co-culture medium was collected concentrated. β-Hexosaminidase activity assay was performed using MUG and MUGS substrates. The bars represent mean ± SD with P values * ≤ 0.05, ** ≤ 0.005, *** ≤ 0.001

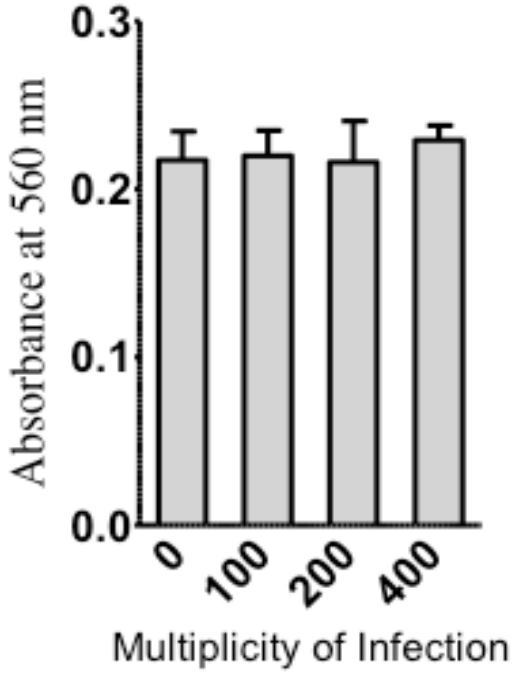
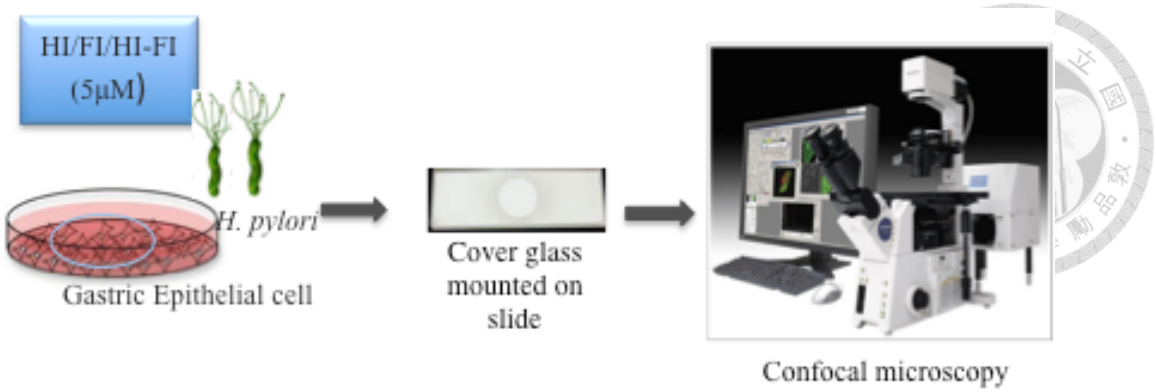


Figure 13. Viability of AGS cells was measured upon *H. pylori* infection. AGS cells were treated with *H. pylori* (MOI of 100, 200, 400) for 8 h and the cells were treated with MTT for 2 h, followed by solubilizing buffer (acidified isopropanol). The absorbance at 570 nm was measured indicating the cell viability. The bars represent mean ± SD

4.4 Bactericidal effect of β-hexosaminidase.

Although it has been reported that the surface protein HP-NAP in mast cells induces β-hexosaminidase, the role of the upregulated enzyme during *H. pylori* infection is not known. Since β-hexosaminidase affects the survival of gram-positive bacteria, we aimed to investigate the possible role of β-hexosaminidase released by gastric epithelial cells in *H. pylori* viability. AGS cells were infected with *H. pylori* (MOI of 200). We made use of β-hexosaminidase inhibitor (*Ki* = 0.69 ± 0.077 nM*)* to nullify the effect of β-hexosaminidase by treatment of AGS cells with β-hexosaminidase inhibitor before and during *H. pylori* infection. Infection was carried out for 8 h and the cells were washed thoroughly to remove unattached and / or dead and detached *H. pylori.* Immunocytochemistry was performed to label *H. pylori*. The fluorescence indicating the number of bacterial cells attached onto host cells was monitored. The results showed increased fluorescence in wells treated with β-hexosaminidase inhibitor indicating the inhibitory effect of β-hexosaminidase on bacterial attachment and survival (**Figure 14**). Further to know if the secreted α-fucosidase has any effect on bacterial viability we treated the cells with α-fucosidase inhibitor. The cells treated with both β-hexosaminidase inhibitor and α-fucosidase inhibitor showed further increase in number of bacteria adhered to the cell indicating synergistic role of α-fucosidase along with β-hexosaminidase. To further confirm the viability of bacteria, the co-culture was treated with saponin 0.01 %. The total lysate containing bacteria was plated on to sheep blood agar plates and incubated for 3-5 days and colony forming units were counted showing increased number of colonies formed on treatment with β-hexosaminidase inhibitor compared to untreated cells (**Figure 15**). The number of colonies formed in the case of co-treatment of α-fucosidase inhibitor and β-hexosaminidase inhibitor indicates a synergistic

bactericidal activity of the two enzymes. Thus confirming the inhibitory effect of β-hexosaminidase on bacterial survival. It was observed that α-fucosidase alone could not present a significant bactericidal effect shown in cells treated with α-fucosidase inhibitor alone. A similar result was observed in order to see the effect through the growth curve in an alternate approach wherein the bacteria were directly treated with co-culture medium concentrate with either β-hexosaminidase inhibitor or α-fucosidase inhibitor or co-treatment of the two, and the bacterial growth curve was monitored (**Figure 16**).



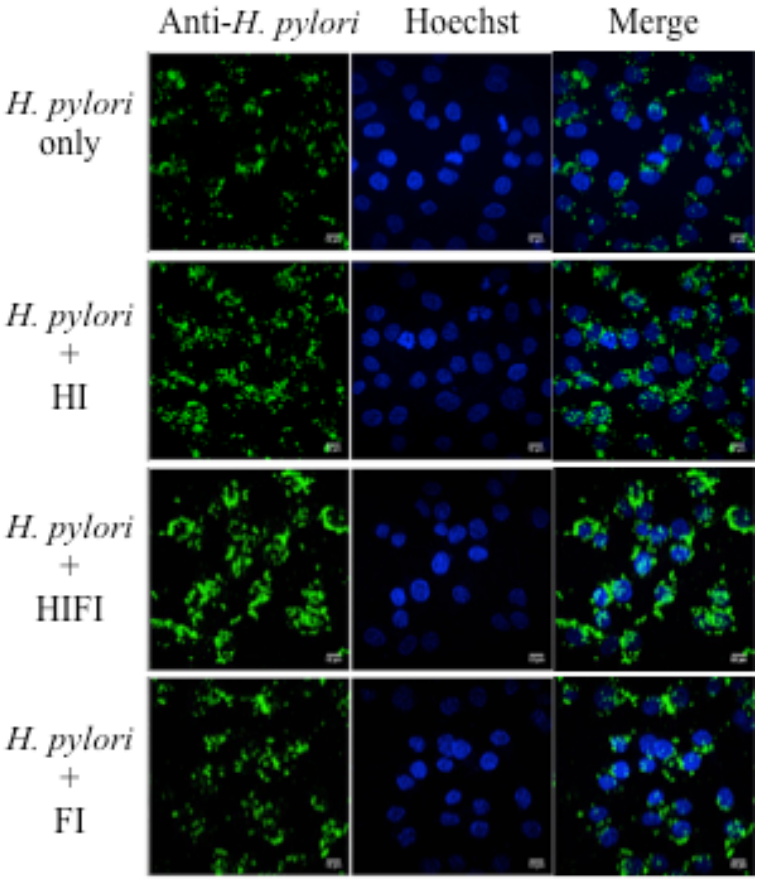


Figure 14. β-Hexosaminidase in co-culture medium was found to affect bacterial viability: (A) AGS cells were treated with *H. pylori* (MOI of 200) for 8 h. The cells were treated with β-hexosaminidase inhibitor (HI) and α-fucosidase inhibitor (FI) or both before and during the infection. The cells were washed several times and fixed, followed by incubation with anti-HP antibody and then by secondary antibody with Alexa Flour 488. The cells were further counterstained for nuclei using Hoechst. Confocal microscopy images were acquired.

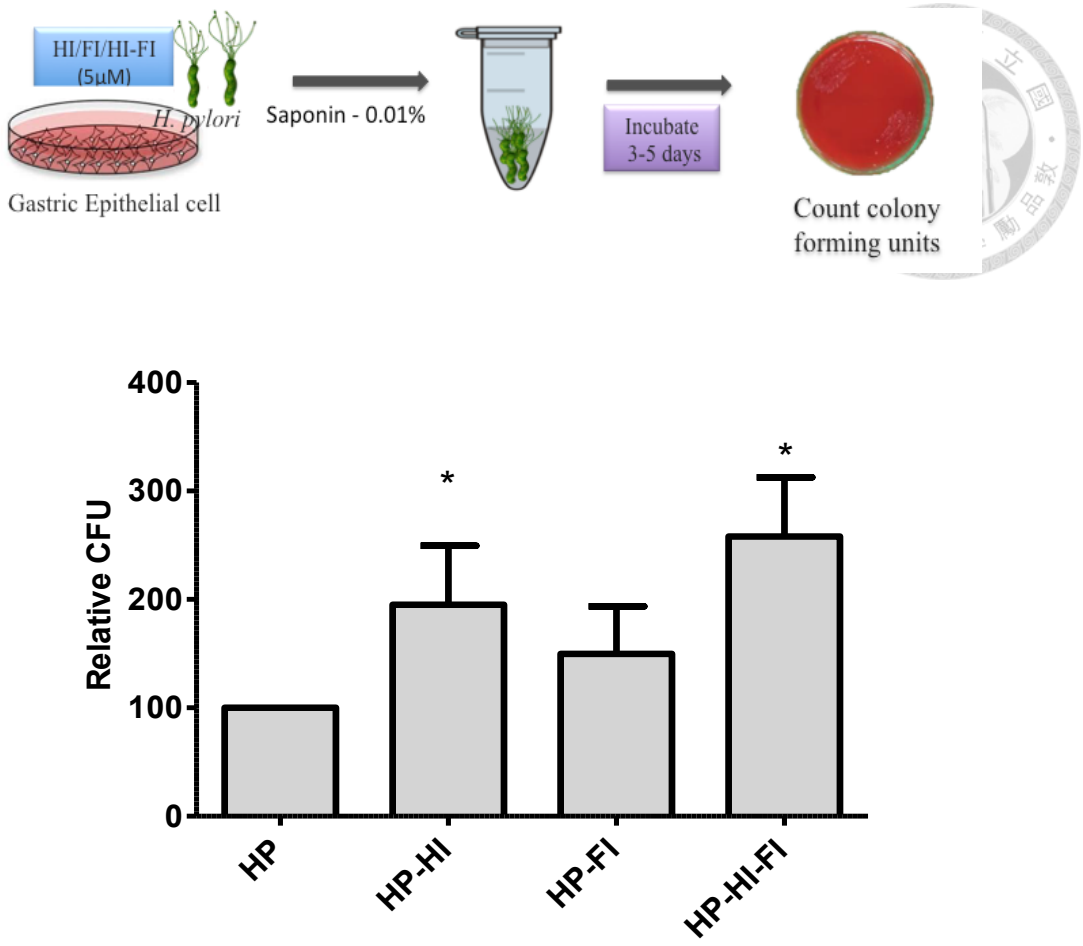


Figure 15. β-Hexosaminidase in co-culture medium affects bacterial viability by CFU enumeration method: AGS cells were treated with *H. pylori* (MOI of 200) for 8 h. The cells were treated with HI and FI or both before and during the infection. The cells were washed several times and lysed using saponin 0.01 %, followed by plating the lysate onto CDC plates. The resulting colonies formed were ennumerated. The bars were plotted with values obtained from three independent experiments. The bars represent mean ± SD. with P values * ≤ 0.05

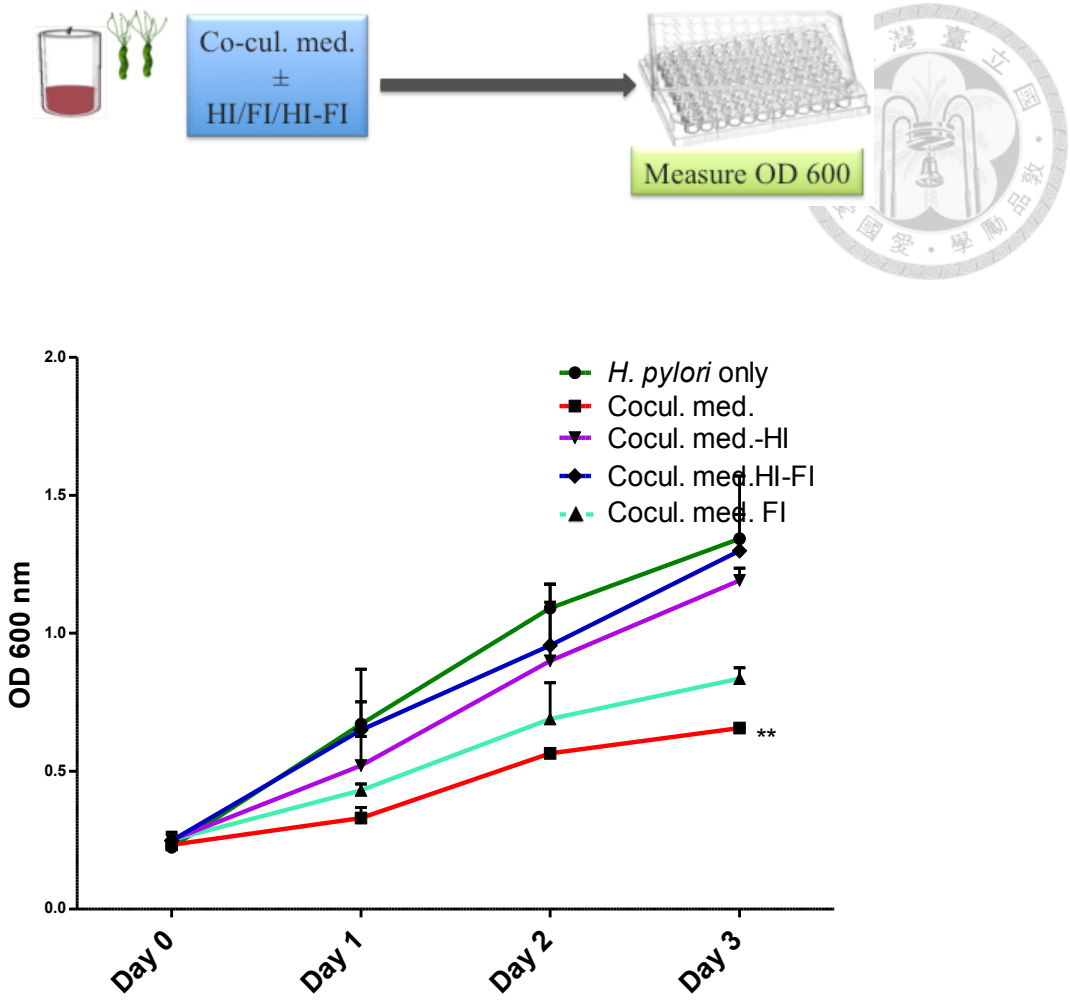


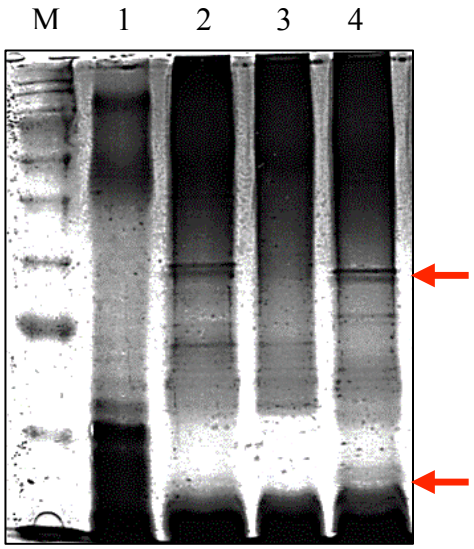
Figure 16. Effect of β-hexosaminidase and α-fucosidase in co-culture medium on bacterial growth curve: *H. pylori* ($1x10^6$) cells were treated with co-culture medium concentrate with or without HI, FI or both in brucella broth and cultured under microareobic conditions for 3-5 days. OD 600 nm was measured for every 24 h and the growth was plotted. The error bars represent mean ± SD with P values * ≤ 0.05, ** ≤ 0.001.

4.5 Degradation of bacterial surface structures by β-hexosaminidase and α-fucosidase.

β-hexosaminidase has been shown to cleave the glycosidic bonds in the biofilm and cell walls,(62) we therefore tested if β-hexosaminidase has a target site on *H. pylori* surface. Since *H. pylori* is a gram-negative bacteria we considered checking if it has any effect on LPS. We extracted LPS from *H. pylori* and treated with co-culture medium with or without inhibitors and run the sample on SDS-PAGE followed by modified silver staining to see the pattern of the LPS. We found a significant change in pattern on treatment with co-culture medium and in the samples treated with β-hexosaminidase and α-fucosidase inhibitors there was rescue of degradation of major bands as indicated by the arrow (**Figure 17A**). β-hexosaminidase has been shown to degrade the cell wall in some gram positive bacteria and therefore we further extracted bacterial cell wall and performed zymography to show β-hexosaminidase degrades the cell wall (peptidoglycan) of *H. pylori* (**Figure 17B**). Thus the above results imply that β-hexosaminidase shows antibacterial effect by degrading the LPS and further making the cell wall leaky. This was further confirmed by the electron microscopy where in the above panel shows the SEM images with intact/normal bacterial surface in untreated groups; the cells are aggregated and the surface is distorted in case of co-culture treated groups and the surface integrity is rescued majorly on treatment with the β-hexosaminidase and α-fucosidase inhibitor. (**Figure 18**). The lower panel showing the TEM images indicates a similar result wherein the co-culture medium concentrate treated bacteria shows the damaged cell wall which is rescued by the co-treatment of β-hexosaminidase and α-fucosidase inhibitor (**Figure 18**) Therefore β-hexosaminidase and α-fucosidase degrade LPS followed by

degradation of peptidoglycan in cell wall by β-hexosaminidase thus exhibiting bactericidal activity.

A)



1- *E. coli* LPS
2- Hp LPS ctrl
3- Hp LPS + co-culture medium
4- Hp LPS + co-culture medium + HI-FI

B)

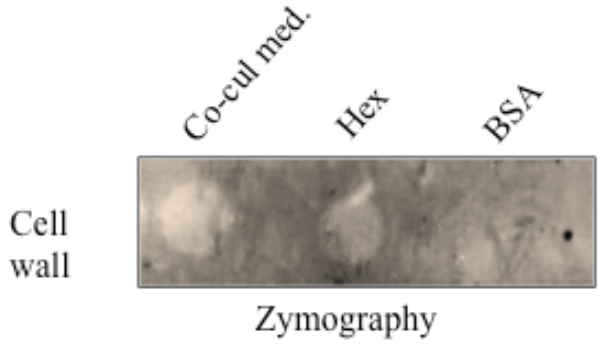


Figure 17. Degradation of *H. pylori* LPS and cell wall by secreted β-hexosaminidase and α-fucosidase. (A) *H. pylori* LPS (1 μg) was treated with co-culture medium concentrate with or without HI and FI for 8 h, and analysed by SDS-PAGE. The gel was stained by modified silver stain method. The result shows significant pattern change on treatment with co-culture medium concentrate and the degradation was rescued on treatment with the inhibitors. B) *H. pylori* cell wall was extracted and embedded in 8 % polyacryl amide gel and co-culture medium, 1.5 mU β-hexosaminidase and BSA were spotted onto the membrane. Staining was performed using methylene blue.

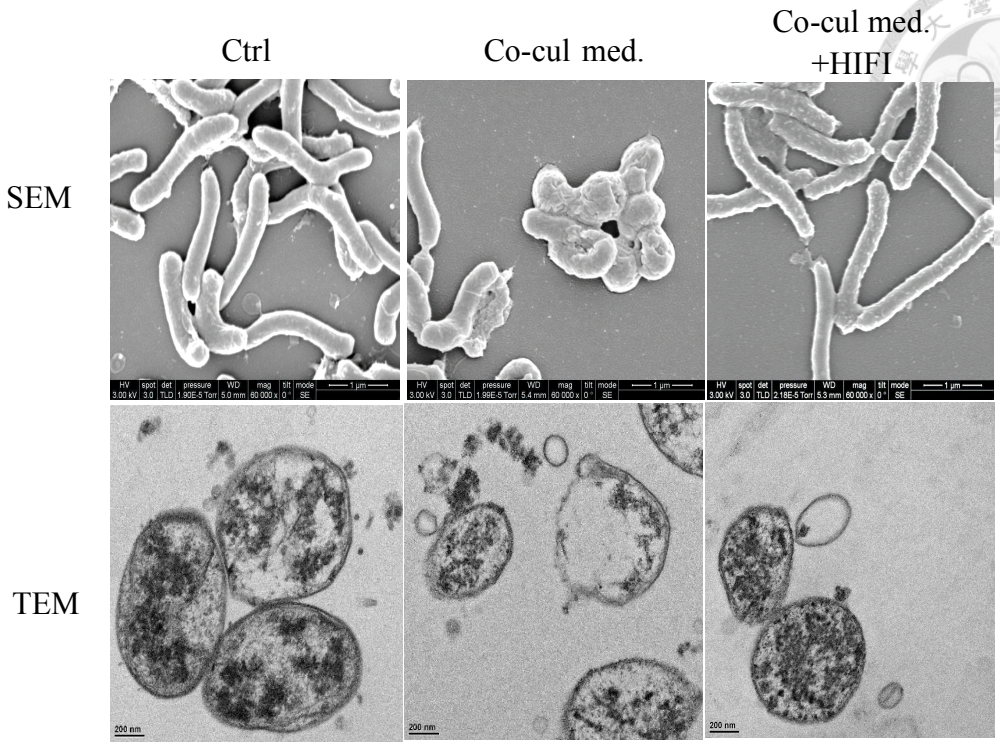
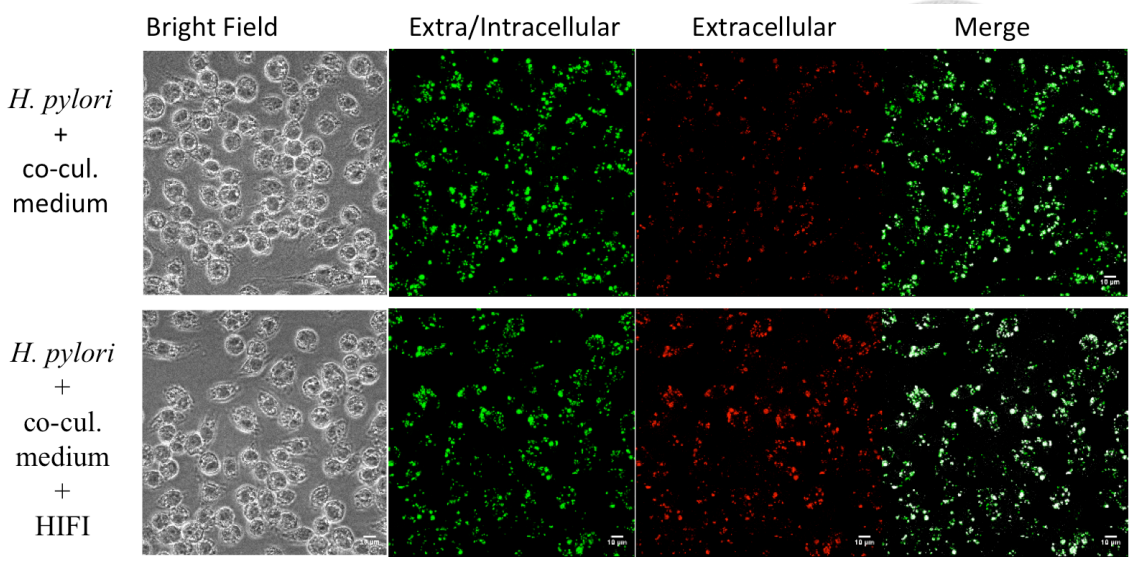


Figure 18. Degradation of *H. pylori* cell surface shown by SEM and TEM. *H. pylori* was treated with co-culture medium with or without HI and FI. The samples were processed for SEM and TEM as described (see Material and Methods, section 3.14 and 3.15) and the images were acquired.

4.6 Effect of bactericidal action of β-hexosaminidase and α-fucosidase on macrophage mediated phagocytosis.

The bacteria, upon infection, resist immune surveillance by molecular mimicry. Thus the immune cells cannot differentiate bacterial surface from host due to this phenomena of molecular mimicry. The action of these secreted glycosidases damage the surface glycans and disturb the molecular mimicry. Thus, we wanted to see the effect of degradation of *H. pylori* surface by β-hexosaminidase and α-fucosidase on macrophage-mediated phagocytosis. We treated *H. pylori* with co-culture medium concentrate with or without β-hexosaminidase and α-fucosidase inhibitor and incubated these bacteria with macrophage for about 3 h. The cells were then washed fixed and subjected to differential immunostaining using anti-*H. pylori* antibody to label the extracellular and intracellular bacteria. The number of bacteria phagocytosed when the bacteria were treated with co-culture medium concentrate with or without the co-treatment of β-hexosaminidase and α-fucosidase inhibitor was assessed by merging the confocal microscopy images obtained by differential staining. As shown in the images, extracellular bacteria are shown in red and extracellular and intracellular bacteria stained upon permeabilisation are shown in green. The merge image shows the white (pseudocolor) obtained by overlap of red with green that indicates the extracellular bacteria and the green dots indicates the intracellular bacteria. The result obtained shows an increase in number of phagocytosed *H. pylori,* which has been treated with co-culture medium concentrate compared to the group which was co-treated with inhibitors (**Figure 19 A,B**) indicating that the surface degradation by the secreted enzymes lends bacteria more vulnerable to macrophage-mediated phagocytosis.

A)



B)

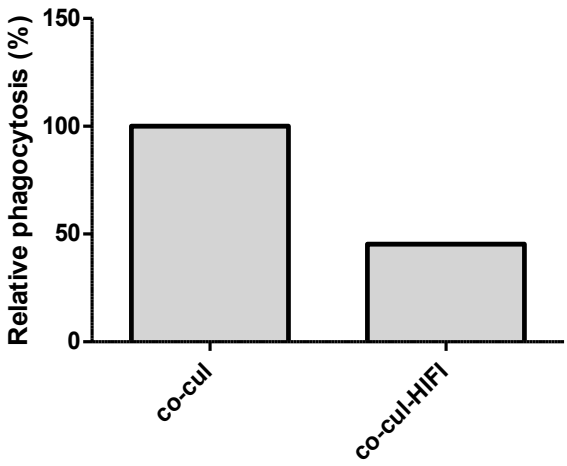


Figure 19. Effect of surface degradation on macrophage-mediated phagocytosis. **(A)** J774A.1 cells were cultured and infected with *H. pylori* (MOI 100) pretreated with co-culture medium with or without addition of HI and FI. Incubation was carried out for 3 h. The cells were fixed and differential immunostaining was performed. Merge panel showing green dots indicate phagocytosed bacteria. (B) Quantitation of phagocytosis from confocal images using Image J.

5. Discussion and conclusion

When gastric epithelial cells were infected with *H. pylori,* there was an increased activity of intracellular fucosidase (Fuca1) along the secretory type of fucosidase (Fuca2). We made use of Compound **1**, to visualize the enzyme activity. Since the signal obtained from the probe is colocalised with the lysotracker, it can be said that the majority of the signal is from the lysosomal fucosidase.

This upregulation in the activity of enzyme could be the reflection of a host defense mechanism arising from the microbial infection. The extracellular release of Fuca2 is believed to alter the surface glycan structures of the host in response to bacterial adhesion. (32) However the bacteria makes use of this scenario by feeding on the cleaved fucoses. In an attempt to overcome the infection, host cells come with alternate approaches such as elevating lysosomal killer enzymes. *H. pylori* is internalized by epithelial cells through receptor mediated endocytosis, pinocytosis and actin polymorphism (85,86). It is not surprising that the observed increase of lysosomal Fuca1 aids in digesting the invading bacteria or bacterial components, in agreement with the host defense strategies of other lysosomal enzymes. (87,88). However, more experimental evidence is required to confirm this. The escalation of human Fuca1 and Fuca2 activities could be also considered as a sign of cell inflammation as the consequence of bacterial infection.

Literature shows bacterial surface can stimulate host cell proteins or host cell defense system. Heat killed intact bacteria was shown to stimulate immunoglobulins in Xenopus B cells. (89) Bacterial LPS has been found to stimulate antigen processing,

cytokine production, lysosomal enzyme upregulation (lysosomal thiol reductase) in immune cells. (90). Bacterial flagella, and LPS are known to upregulate chemokine expressions. Thus indicating the bacterial surface or surface structures stimulates host proteins or trigger host immune system. Even in the case of Fuca1, upregulation was observed in cells treated with dead intact bacteria and pelleted bacterial surface structures compared to untreated or bacterial lysate treated groups. Further we found significant increase in Fuca1 on treatment with LPS, and the presence of LPS neutralizing molecule significantly abrogated the effect. We did consider some of the bacterial surface adhesins such as babA and sabA by using babA mutant and sab A mutants. However sabA and babA mutants' binding ability was reduced by about 30 % and as maintaining the same number of bacteria during infection was critical for comparing the different factors, we ruled out the adhesins in the experiment.

It is believed that in a strategy to evade the phagolysosomal degradation, the pivotal lysosomal components such as CTSD Cathapsin D are made inactive by unknown mechanism, leading to reduced efficiency of lysosome mediated degradation.(88) Thus in order to overcome this strategy, host cells might elevate other lysosomal enzymes to carry out the degradation. Elevated Fuca1 activity might also be a part of this process. Study of other lysosomal enzyme activities in such a scenario can give a clear view on degradation of internalized bacteria in epithelial cells.

We also report the elevated activity of β-hexosaminidase secreted by the host cells into the medium on *H. pylori* infection. This released enzyme affected bacterial survival. We screened for the activity of various other glycosidases upon *H. pylori*

infection and observed an increased activity of many of them in the co-culture medium. (**Figure 20**)

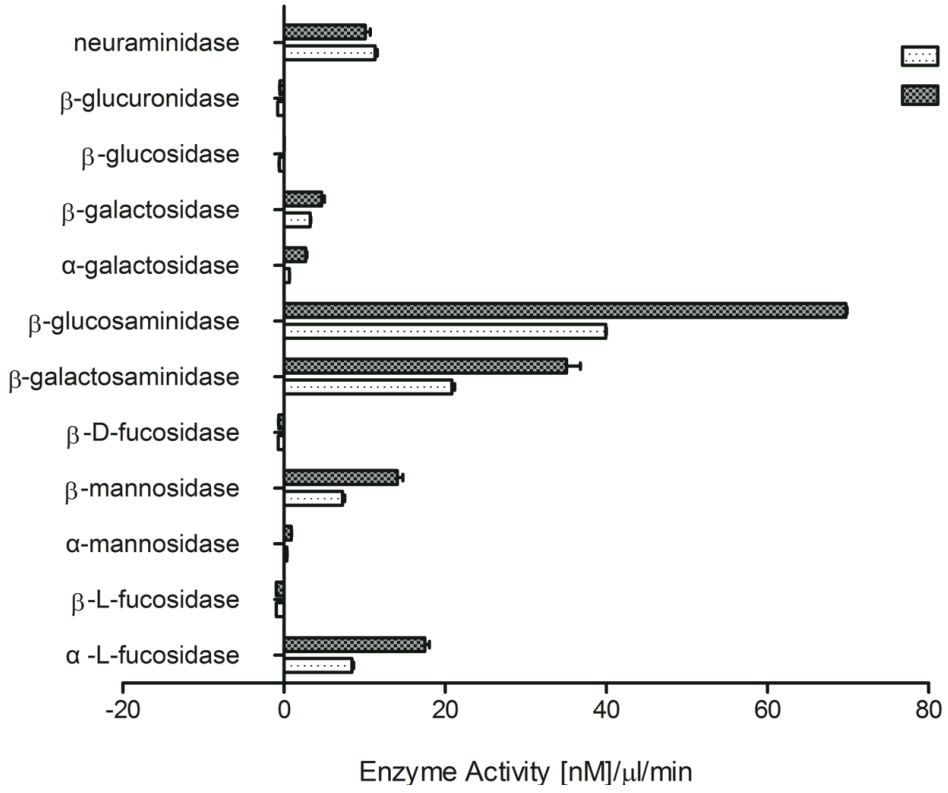


Figure 20. Glycosidase activities examined in co-culture medium. AGS cells (~8×10^7) were infected with *H. pylori* at MOI of 200. Glycosidase activities in co-culture medium were measured after 8 h infection. The co-culture media from PBS only (ctrl.) and *H. pylori* (26695) (inf.) were concentrated by 10-kDa centricon before activity assay. Enzyme activities were evaluated by corresponding substrates consumed per minute per micro liter of co-culture medium. Each measurement was done in triplicates.

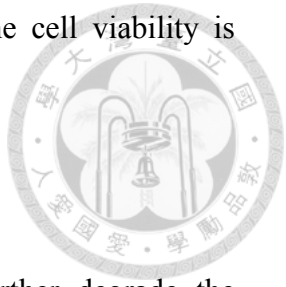
We hypothesize that the released glycosidases together play a role in the sequential degradation of bacterial surface glycans thereby disrupting the bacterial outer surface and affecting its survival. As *H. pylori* is a gram-negative bacteria its outer surface is covered by a dense lipopolysaccharide layer. The structure of O-antigen in the 26695 shows that its outer most layer is decorated with fucoses more in the form of le^x and lesser as le^y. (15,91) The α-fucosidase2 and hexosaminidase released into the medium together involve in the degradation process by cleaving the

terminal fucoses and underlying hexoses. Traces of galactosidases probably aid in cleaving the galactoses present. Thus the enzyme enters further into the deeper layers and act on the underlying peptidoglycan layer. Degradation of the bacterial cell wall by the enzyme lends it distorted and leaky.

β-Hexosmaninidase is the enzyme, which has earlier shown to have mycobactericidal effect.(62) It acts by degradation of bacterial cell wall in gram-positive mycobacterial species. β-hexosaminidase has also been shown to disrupt the bacterial biofilm to disperse bacterial cells. Reports indicating that β-hexosaminidase can degrade the peptidoglycan layer in the Bacillus anthrax spore to release the active form of anthrax.(63)

It was observed that addition of potent β-hexosaminidase inhibitor abrogated the activity of β-hexosaminidase secreted in the co-culture medium. Thus the number of live bacteria in β-hexosaminidase inhibitor treated co-culture condition was higher than the control well (only *H. pylori* / no inhibitor treated) and the live bacteria were further increased on co-treatment of β-hexosaminidase inhibitor and α-fucosidase inhibitor. This indicates that β-hexosaminidase has bactericidal effect and Fuca2 along with β-hexosaminidase shows synergistic effect. The similar effect was observed when the bacteria were directly treated with medium concentrate *in vitro* with or without inhibitors treatments. However it was observed that α-fucosidase alone showed no or minimal effect on the viability of bacteria indicating loss of fucose units alone doesn't affect the viability of bacteria where as when the deeper

layers are degraded and the membrane backbone is disrupted the cell viability is significantly affected.



The secreted enzymes cleave the surface glycans and further degrade the peptidoglycan layer in the cell wall and render the cell wall leaky shown by modified silver stain of LPS treated with co-culture medium with/without co-treatment of β-hexosaminidase inhibitor and α-fucosidase inhibitor and by zymography of cell wall. This is further confirmed by transmission electron microscopy. The bacterial surface is damaged and the molecular mimicry is lost and the bacteria now tend to become more vulnerable to immune cell surveillance.

The bacteria interacts with immune cells either by passing through the disrupted tight junctions of the epithelial cells or through the M cells present in the lining of the epithelium. The process of recognition of *H. pylori* by the macrophages involves the interaction of glycosaminoglycans expressed on the surface of macrophage and the surface bacterial protein binding heparin. Covering with hyaluronic acid inhibited phagocytosis of *H. pylori*. Literature reports suggest bacterial adhesion or surface protein dependent interaction of *H. pylori* with macrophages. (92) In this study we tried to explore the bacterial surface glycan dependent phagocytosis. We hypothesize, on degradation of surface glycans by the secreted β-hexosaminidase and α-fucosidase the molecular mimicry is lost and phagocytosis of the surface degraded bacteria is more compared to the bacteria whose surface degradation is rescued by the presence of β-hexosaminidase inhibitor and α-fucosidase inhibitors indicated by differential immunostaining data. The fate of bacteria with loss of surface fucoses alone by the

secreted α-fucosidase and the effect of presence of fucose with different linkages on the o-antigen is under investigation.

Thus, overall it can be said that on bacterial infection, host cells secrete the glycosidases as part of host defense mechanism and at certain instances the bacteria smartly utilizes the situation for example it uses the cleaved host fucoses as energy source but as infection progresses and the enzymes secreted further target bacterial glycans to protect the host cells from infection. In the due course the molecular mimicry on bacterial surface is lost and the bacteria becomes vulnerable to immune surveillance.

6. Summary:

In this thesis, we show that gastric epithelial cells exhibit enhanced intracellular α-fucosidase activity along with secretory type of α-fucosidase and bacterial surface, majorly bacterial lipopolysaccharide is the stimulant, which induces the enhanced activity of the enzyme. Besides α-fucosidase, we have observed increased activity of β-hexosaminidase secreted into the co-culture medium. The secreted β-hexosaminidase and α-fucosidase together show bactericidal effect by degrading bacterial surface structure and lending the bacteria more vulnerable to macrophage mediated phagocytosis.

7. References

1. Yamaoka, Y. (**2010**) Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nature reviews. Gastroenterol & hepatol* **7**, 629-641
2. Kalali, B., Mejias-Luque, R., Javaheri, A., and Gerhard, M. (**2014**) H. pylori virulence factors: influence on immune system and pathology. *Mediators Inflamm.* 2014, 426309
3. Oleastro, M., and Menard, A. (**2013**) The Role of *Helicobacter pylori* Outer Membrane Proteins in Adherence and Pathogenesis. *Biol.* **2**, 1110-1134
4. Alm, R. A., Ling, L. S., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelsen, M., Mills, D. M., Ives, C., Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trust, T. J. (**1999**) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature.* **397**, 176-180
5. Josenhans, C., Labigne, A., and Suerbaum, S. (**1995**) Comparative ultrastructural and functional studies of *Helicobacter pylori* and *Helicobacter mustelae* flagellin mutants: both flagellin subunits, FlaA and FlaB, are necessary for full motility in Helicobacter species. *J Bacteriol.* **177**, 3010-3020
6. Aspholm-Hurtig, M., Dailide, G., Lahmann, M., Kalia, A., Ilver, D., Roche, N., Vikstrom, S., Sjostrom, R., Linden, S., Backstrom, A., Lundberg, C., Arnqvist, A., Mahdavi, J., Nilsson, U. J., Velapatino, B., Gilman, R. H., Gerhard, M., Alarcon, T., Lopez-Brea, M., Nakazawa, T., Fox, J. G., Correa, P., Dominguez-Bello, M. G., Perez-Perez, G. I., Blaser, M. J., Normark, S., Carlstedt, I., Oscarson, S., Teneberg, S., Berg, D. E., and Boren, T. (**2004**)

Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin. *Science.* **305**, 519-522

7. Boren, T., Falk, P., Roth, K. A., Larson, G., and Normark, S. (**1993**) Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science.* **262**, 1892-1895

8. Prinz, C., Schoniger, M., Rad, R., Becker, I., Keiditsch, E., Wagenpfeil, S., Classen, M., Rosch, T., Schepp, W., and Gerhard, M. (**2001**) Key importance of the *Helicobacter pylori* adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Res.* **61**, 1903-1909

9. Toller, I. M., Neelsen, K. J., Steger, M., Hartung, M. L., Hottiger, M. O., Stucki, M., Kalali, B., Gerhard, M., Sartori, A. A., Lopes, M., and Muller, A. (**2011**) Carcinogenic bacterial pathogen *Helicobacter pylori* triggers DNA double-strand breaks and a DNA damage response in its host cells. *Proc Natl Acad Sci U S A*, **108**, 14944-14949

10. Mahdavi, J., Sonden, B., Hurtig, M., Olfat, F. O., Forsberg, L., Roche, N., Angstrom, J., Larsson, T., Teneberg, S., Karlsson, K. A., Altraja, S., Wadstrom, T., Kersulyte, D., Berg, D. E., Dubois, A., Petersson, C., Magnusson, K. E., Norberg, T., Lindh, F., Lundskog, B. B., Arnqvist, A., Hammarstrom, L., and Boren, T. (**2002**) *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science.* **297**, 573-578

11. Unemo, M., Aspholm-Hurtig, M., Ilver, D., Bergstrom, J., Boren, T., Danielsson, D., and Teneberg, S. (**2005**) The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *J Biol Chem.* **280**, 15390-15397

12. Yamaoka, Y., Kwon, D. H., and Graham, D. Y. (**2000**) A M(r) 34,000 proinflammatory outer membrane protein (oipA) of *Helicobacter pylori*. *Proc Natl Acad Sci U S A.* **97**, 7533-7538

13. Tabassam, F. H., Graham, D. Y., and Yamaoka, Y. (**2008**) OipA plays a role in *Helicobacter pylori*-induced focal adhesion kinase activation and cytoskeletal re-organization. *Cell Microbiol.* **10**, 1008-1020

14. Muotiala, A., Helander, I. M., Pyhala, L., Kosunen, T. U., and Moran, A. P. (**1992**) Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infect Immun.* **60**, 1714-1716

15. Monteiro, M. A., Appelmelk, B. J., Rasko, D. A., Moran, A. P., Hynes, S. O., MacLean, L. L., Chan, K. H., Michael, F. S., Logan, S. M., O'Rourke, J., Lee, A., Taylor, D. E., and Perry, M. B. (**2000**) Lipopolysaccharide structures of *Helicobacter pylori* genomic strains 26695 and J99, mouse model H. pylori Sydney strain, *H. pylori* P466 carrying sialyl Lewis X, and H. pylori UA915 expressing Lewis B classification of *H. pylori* lipopolysaccharides into glycotype families. *Eur J Biochem. / FEBS* **267**, 305-320

16. Appelmelk, B. J., Shiberu, B., Trinks, C., Tapsi, N., Zheng, P. Y., Verboom, T., Maaskant, J., Hokke, C. H., Schiphorst, W. E., Blanchard, D., Simoons-Smit, I. M., van den Eijnden, D. H., and Vandenbroucke-Grauls, C. M. (**1998**) Phase variation in *Helicobacter pylori* lipopolysaccharide. *Infect Immun.* **66**, 70-76

17. Appelmelk, B. J., Martin, S. L., Monteiro, M. A., Clayton, C. A., McColm, A. A., Zheng, P., Verboom, T., Maaskant, J. J., van den Eijnden, D. H., Hokke, C. H., Perry, M. B., Vandenbroucke-Grauls, C. M., and Kusters, J. G. (**1999**) Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in

the lengths of poly(C) tracts in alpha3-fucosyltransferase genes. *Infect Immun.* **67**, 5361-5366

18. Figura, N., Guglielmetti, P., Rossolini, A., Barberi, A., Cusi, G., Musmanno, R. A., Russi, M., and Quaranta, S. (**1989**) Cytotoxin production by Campylobacter pylori strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. *J Clin Microbiol.* **27**, 225-226
19. Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J. L., Rappuoli, R., and Montecucco, C. (**1998**) Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *The J Clin Invest.* **102**, 813-820
20. Ding, H., Zeng, H., Huang, L., Dong, Y., Duan, Y., Mao, X., Guo, G., and Zou, Q. (**2012**) *Helicobacter pylori* chaperone-like protein CagT plays an essential role in the translocation of CagA into host cells. *J Microbiol Biotechnol* **22**, 1343-1349
21. Barrozo, R. M., Cooke, C. L., Hansen, L. M., Lam, A. M., Gaddy, J. A., Johnson, E. M., Cariaga, T. A., Suarez, G., Peek, R. M., Jr., Cover, T. L., and Solnick, J. V. (**2013**) Functional plasticity in the type IV secretion system of *Helicobacter pylori*. *PLoS Pathog.* **9**, e1003189
22. Hayashi, T., Morohashi, H., and Hatakeyama, M. (**2013**) Bacterial EPIYA effectors--where do they come from? What are they? Where are they going? *Cell Microbiol.* **15**, 377-385
23. Allison, C. C., Kufer, T. A., Kremmer, E., Kaparakis, M., and Ferrero, R. L. (**2009**) *Helicobacter pylori* induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism. *J Immunol.* **183**, 8099-8109
24. Hirayama, Y., Yoshimura, M., Ozeki, Y., Sugawara, I., Udagawa, T., Mizuno, S., Itano, N., Kimata, K., Tamaru, A., Ogura, H., Kobayashi, K., and

Matsumoto, S. (**2009**) Mycobacteria exploit host hyaluronan for efficient extracellular replication. *PLoS Pathog.* **5**, e1000643

25. Sheldon, W. L., Macauley, M. S., Taylor, E. J., Robinson, C. E., Charnock, S. J., Davies, G. J., Vocadlo, D. J., and Black, G. W. (**2006**) Functional analysis of a group A streptococcal glycoside hydrolase Spy1600 from family 84 reveals it is a beta-N-acetylglucosaminidase and not a hyaluronidase. *Biochem. J.* **399**, 241-247

26. Li, Y., Komai-Koma, M., Gilchrist, D. S., Hsu, D. K., Liu, F. T., Springall, T., and Xu, D. (**2008**) Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. *J Immunol.* **181**, 2781-2789

27. Finlay, B. B., and Hancock, R. E. (**2004**) Can innate immunity be enhanced to treat microbial infections? *Nat Rev Microbiol.* **2**, 497-504

28. Uehara, N., Yagihashi, A., Kondoh, K., Tsuji, N., Fujita, T., Hamada, H., and Watanabe, N. (**2003**) Human beta-defensin-2 induction in *Helicobacter pylori*-infected gastric mucosal tissues: antimicrobial effect of overexpression. *J Med Microbiol.* **52**, 41-45

29. Zhang, L., Yu, J., Wong, C. C., Ling, T. K., Li, Z. J., Chan, K. M., Ren, S. X., Shen, J., Chan, R. L., Lee, C. C., Li, M. S., Cheng, A. S., To, K. F., Gallo, R. L., Sung, J. J., Wu, W. K., and Cho, C. H. (**2013**) Cathelicidin protects against *Helicobacter pylori* colonization and the associated gastritis in mice. *Gene Ther.* **20**, 751-760

30. Lysenko, E. S., Gould, J., Bals, R., Wilson, J. M., and Weiser, J. N. (2000) Bacterial phosphorylcholine decreases susceptibility to the antimicrobial peptide LL-37/hCAP18 expressed in the upper respiratory tract. *Infect Immun.* **68**, 1664-1671

31. Duperthuy, M., Sjostrom, A. E., Sabharwal, D., Damghani, F., Uhlin, B. E., and Wai, S. N. (**2013**) Role of the Vibrio cholerae matrix protein Bap1 in cross-resistance to antimicrobial peptides. *PLoS Pathog.* **9**(10): e1003620

32. Liu, T. W., Ho, C. W., Huang, H. H., Chang, S. M., Popat, S. D., Wang, Y. T., Wu, M. S., Chen, Y. J., and Lin, C. H. (**2009**) Role for alpha-L-fucosidase in the control of *Helicobacter pylori*-infected gastric cancer cells. *Proc Natl Acad Sci U S A*. **106**, 14581-14586

33. Handing, J. W., and Criss, A. K. (**2015**) The lipooligosaccharide-modifying enzyme LptA enhances gonococcal defence against human neutrophils. *Cell Microbiol.* **17**, 910-921

34. Becker, D. J., and Lowe, J. B. (**2003**) Fucose: biosynthesis and biological function in mammals. *Glycobiol.* **13**, 41R-53R

35. Staudacher, E., Altmann, F., Wilson, I. B., and Marz, L. (**1999**) Fucose in N-glycans: from plant to man. *Biochim Biophys Acta.* **1473**, 216-236

36. Moran, A. P. (**2008**) Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydr Res* **343**, 1952-1965

37. Moran, A. P., and Prendergast, M. M. (**2001**) Molecular mimicry in *Campylobacter jejuni* and *Helicobacter pylori* lipopolysaccharides: contribution of gastrointestinal infections to autoimmunity. *J Autoimmun* **16**, 241-256

38. Cragg, H., Williamson, M., Young, E., O'Brien, J., Alhadeff, J., Fang-Kircher, S., Paschke, E., and Winchester, B. (**1997**) Fucosidosis: genetic and biochemical analysis of eight cases. *J Med Genet.* **34**, 105-110

39. Wang, K., Guo, W., Li, N., Shi, J., Zhang, C., Lau, W. Y., Wu, M., and Cheng, S. (**2014**) Alpha-1-fucosidase as a prognostic indicator for hepatocellular

carcinoma following hepatectomy: a large-scale, long-term study. *Br J Cancer.* **110**, 1811-1819

40. Abdel-Aleem, H., Ahmed, A., Sabra, A. M., Zakhari, M., Soliman, M., and Hamed, H. (**1996**) Serum alpha L-fucosidase enzyme activity in ovarian and other female genital tract tumors. I*nt J Gynaecol Obstet.* **55**, 273-279

41. Bukofzer, S., Stass, P. M., Kew, M. C., de Beer, M., and Groeneveld, H. T. (**1989**) Alpha-L-fucosidase as a serum marker of hepatocellular carcinoma in southern African blacks. *Br J Cancer* **59**, 417-420

42. Scanlin, T. F., and Glick, M. C. (**1999**) Terminal glycosylation in cystic fibrosis. *Biochim Biophys Acta.* **1455**, 241-253

43. Ali, S., Jenkins, Y., Kirkley, M., Dagkalis, A., Manivannan, A., Crane, I. J., and Kirby, J. A. (**2008**) Leukocyte extravasation: an immunoregulatory role for alpha-L-fucosidase? *J Immunol.* **181**, 2407-2413

44. Katayama, T., Sakuma, A., Kimura, T., Makimura, Y., Hiratake, J., Sakata, K., Yamanoi, T., Kumagai, H., and Yamamoto, K. (**2004**) Molecular cloning and characterization of Bifidobacterium bifidum 1,2-alpha-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). *J Bacteriol.* **186**, 4885-4893

45. Nagae, M., Tsuchiya, A., Katayama, T., Yamamoto, K., Wakatsuki, S., and Kato, R. (**2007**) Structural basis of the catalytic reaction mechanism of novel 1,2-alpha-L-fucosidase from Bifidobacterium bifidum. *J Biol Chem.* **282**, 18497-18509

46. Sulzenbacher, G., Bignon, C., Nishimura, T., Tarling, C. A., Withers, S. G., Henrissat, B., and Bourne, Y. (**2004**) Crystal structure of *Thermotoga maritima* alpha-L-fucosidase. Insights into the catalytic mechanism and the molecular basis for fucosidosis. *J Biol Chem.* **279**, 13119-13128

47. Jones, D. T., Lechertier, T., Mitter, R., Herbert, J. M., Bicknell, R., Jones, J. L., Li, J. L., Buffa, F., Harris, A. L., and Hodivala-Dilke, K. (**2012**) Gene expression analysis in human breast cancer associated blood vessels. *PloS One.* **7**, e44294

48. Tancini, B., Magini, A., Latterini, L., Urbanelli, L., Ciccarone, V., Elisei, F., and Emiliani, C. (**2010**) Occurrence of an anomalous endocytic compartment in fibroblasts from Sandhoff disease patients. *Mol Cell Biochem.* **335**, 273-282

49. Pang, J., Liu, W. P., Liu, X. P., Li, L. Y., Fang, Y. Q., Sun, Q. P., Liu, S. J., Li, M. T., Su, Z. L., and Gao, X. (**2010**) Profiling protein markers associated with lymph node metastasis in prostate cancer by DIGE-based proteomics analysis. *J Proteome Res.* **9**, 216-226

50. Lo, C. H., and Kritchevsky, D. (**1978**) Human serum hexosaminidase: elevated B form isozyme in cancer patients. *J Med.* **9**, 313-336

51. Pretlow, T. G., 2nd, Harris, B. E., Bradley, E. L., Jr., Bueschen, A. J., Lloyd, K. L., and Pretlow, T. P. (**1985**) Enzyme activities in prostatic carcinoma related to Gleason grades. *Cancer Res.* **45**, 442-446

52. Waszkiewicz, N., Zalewska-Szajda, B., Szajda, S. D., Kepka, A., Waszkiewicz, M., Roszkowska-Jakimiec, W., Wojewodzka-Zelezniakowicz, M., Milewska, A. J., Dadan, J., Szulc, A., Zwierz, K., and Ladny, J. R. (2012) Lysosomal exoglycosidases and cathepsin D in colon adenocarcinoma. *Pol Arch Med Wewn* **122**, 551-556

53. Martino, S., Emiliani, C., Tabilio, A., Falzetti, F., Stirling, J. L., and Orlacchio, A. (1997) Distribution of active alpha- and beta-subunits of beta-N-acetylhexosaminidase as a function of leukaemic cell types. *Biochim Biophys Acta* **1335**, 5-15

54. Tomasiak, M. M., Tomasiak, M., Zietkowski, Z., Skiepko, R., and Bodzenta-Lukaszyk, A. (**2008**) N-acetyl-beta-hexosaminidase activity in asthma. *Int Arch Allergy Immunol.* **146**, 133-137

55. Popko, J., Zalewska, A., Golaszewska, Z., Marciniak, J., Sierakowski, S., Worowski, K., and Zwierz, K. (**2005**) Comparative analysis of hexosaminidase and cathepsin D expression in synovial fluid of patients with rheumatoid arthritis and traumatized joints. *Clin Exp Rheumatol.* **23**, 725-726

56. Liu, J., Numa, M. M., Liu, H., Huang, S. J., Sears, P., Shikhman, A. R., and Wong, C. H. (**2004**) Synthesis and high-throughput screening of N-acetyl-beta-hexosaminidase inhibitor libraries targeting osteoarthritis. *J Org Chem.* **69**, 6273-6283

57. Perez-Blanco, F. J., Ruiz-Martin, A., Moreno-Terribas, G., and Cantero-Hinojosa, J. (**1996**) Urinary activity of N-acetyl-beta-glycosaminidase (NAG) in arterial hypertension. *Clin Nephrol.* **45**, 65-66

58. Olszewska, E., Olszewski, S., Borzym-Kluczyk, M., and Zwierz, K. (**2007**) Role of N-acetyl-beta-d-hexosaminidase in cholesteatoma tissue. *Acta Biochim Pol.* **54**, 365-370

59. Shikhman, A. R., Brinson, D. C., and Lotz, M. (**2000**) Profile of glycosaminoglycan-degrading glycosidases and glycoside sulfatases secreted by human articular chondrocytes in homeostasis and inflammation. *Arthritis Rheum.* **43**, 1307-1314

60. Liu, J., Shikhman, A. R., Lotz, M. K., and Wong, C. H. (**2001**) Hexosaminidase inhibitors as new drug candidates for the therapy of osteoarthritis. *Chem Biol.* **8**, 701-711

61. Montemurro, P., Nishioka, H., Dundon, W. G., de Bernard, M., Del Giudice, G., Rappuoli, R., and Montecucco, C. (**2002**) The neutrophil-activating protein

(HP-NAP) of *Helicobacter pylori* is a potent stimulant of mast cells. *Eur J Immunol.* **32**, 671-676

62. Koo, I. C., Ohol, Y. M., Wu, P., Morisaki, J. H., Cox, J. S., and Brown, E. J. (**2008**) Role for lysosomal enzyme beta-hexosaminidase in the control of mycobacteria infection. *Proc Natl Acad Sci U S A.* **105**, 710-715

63. Lambert, E. A., and Popham, D. L. (**2008**) The Bacillus anthracis SleL (YaaH) protein is an N-acetylglucosaminidase involved in spore cortex depolymerization. *J Bacteriol.* **190**, 7601-7607

64. Roy, S., Phansopa, C., Stafford, P., Honma, K., Douglas, C. W., Sharma, A., and Stafford, G. P. (**2012**) Beta-hexosaminidase activity of the oral pathogen Tannerella forsythia influences biofilm formation on glycoprotein substrates. *FEMS Immunol Med Microbiol.* **65**, 116-120

65. Pluddemann, A., Mukhopadhyay, S., and Gordon, S. (**2006**) The interaction of macrophage receptors with bacterial ligands. *Expert Rev Mol Med.* **8**, 1-25

66. Gobert, A. P., Bambou, J. C., Werts, C., Balloy, V., Chignard, M., Moran, A. P., and Ferrero, R. L. (**2004**) *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol Chem.* **279**, 245-250

67. Harris, P. R., Ernst, P. B., Kawabata, S., Kiyono, H., Graham, M. F., and Smith, P. D. (**1998**) Recombinant *Helicobacter pylori* urease activates primary mucosal macrophages. *J Infect Dis.* **178**, 1516-1520

68. Mai, U. E., Perez-Perez, G. I., Wahl, L. M., Wahl, S. M., Blaser, M. J., and Smith, P. D. (1991) Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. *J Clin Invest.* **87**, 894-900

69. Pathak, S. K., Basu, S., Bhattacharyya, A., Pathak, S., Banerjee, A., Basu, J., and Kundu, M. (**2006**) TLR4-dependent NF-kappaB activation and mitogen- and stress-activated protein kinase 1-triggered phosphorylation events are central to *Helicobacter pylori* peptidyl prolyl cis-, trans-isomerase (HP0175)-mediated induction of IL-6 release from macrophages. *J Immunol.* **177**, 7950-7958

70. Bussiere, F. I., Chaturvedi, R., Cheng, Y., Gobert, A. P., Asim, M., Blumberg, D. R., Xu, H., Kim, P. Y., Hacker, A., Casero, R. A., Jr., and Wilson, K. T. (**2005**) Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. *J Biol Chem.* **280**, 2409-2412

71. Gobert, A. P., McGee, D. J., Akhtar, M., Mendz, G. L., Newton, J. C., Cheng, Y., Mobley, H. L., and Wilson, K. T. (**2001**) *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci U S A* **98**, 13844-13849

72. Wilson, K. T., Ramanujam, K. S., Mobley, H. L., Musselman, R. F., James, S. P., and Meltzer, S. J. (**1996**) *Helicobacter pylori* stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. *Gastroenterol.* **111**, 1524-1533

73. Fu, S., Ramanujam, K. S., Wong, A., Fantry, G. T., Drachenberg, C. B., James, S. P., Meltzer, S. J., and Wilson, K. T. (**1999**) Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterol.* **116**, 1319-1329

74. Rad, R., Brenner, L., Bauer, S., Schwendy, S., Layland, L., da Costa, C. P., Reindl, W., Dossumbekova, A., Friedrich, M., Saur, D., Wagner, H., Schmid, R. M., and Prinz, C. (**2006**) CD25+/Foxp3+ T cells regulate gastric

inflammation and *Helicobacter pylori* colonization in vivo. *Gastroenterol.* **131**, 525-537

75. Ho, C. W., Popat, S. D., Liu, T. W., Tsai, K. C., Ho, M. J., Chen, W. H., Yang, A. S., and Lin, C. H. (**2010**) Development of GlcNAc-inspired iminocyclitiols as potent and selective N-acetyl-beta-hexosaminidase inhibitors. *ACS Chem Biol.* **5**, 489-497

76. Wu, H. J., Ho, C. W., Ko, T. P., Popat, S. D., Lin, C. H., and Wang, A. H. (**2010**) Structural basis of alpha-fucosidase inhibition by iminocyclitols with K(i) values in the micro- to picomolar range. *Angew Chem Int Ed Engl.* **49**, 337-340

77. Hildebrandt, E., and McGee, D. J. (**2009**) *Helicobacter pylori* lipopolysaccharide modification, Lewis antigen expression, and gastric colonization are cholesterol-dependent. *BMC Microbiol.* **9**, 258

78. DePamphilis, M. L., and Adler, J. (**1971**) Purification of intact flagella from Escherichia coli and Bacillus subtilis. *J Bacteriol.* **105**, 376-383

79. Wang, G., Olczak, A., Forsberg, L. S., and Maier, R. J. (**2009**) Oxidative stress-induced peptidoglycan deacetylase in *Helicobacter pylori*. *Biol Chem.* **284**, 6790-6800

80. Hsu, Y. L., Nandakumar, M., Lai, H. Y., Chou, T. C., Chu, C. Y., Lin, C. H., and Lo, L. C. (**2015**) Development of Activity-Based Probes for Imaging Human alpha-l-Fucosidases in Cells. *J Org Chem.* **80**, 8458-8463

81. Nandakumar, M., Hsu, Y. L., Lin, J. C., Lo, C., Lo, L. C., and Lin, C. H. (**2015**) Detection of Human alpha-L-Fucosidases by a Quinone Methide-Generating Probe: Enhanced Activities in Response to *Helicobacter pylori* Infection. *Chembiochem.* **16**, 1555-1559

82. Lee, J. S., Paek, N. S., Kwon, O. S., and Hahm, K. B. (**2010**) Anti-inflammatory actions of probiotics through activating suppressor of cytokine signaling (SOCS) expression and signaling in *Helicobacter pylori* infection: a novel mechanism. *J Gastroenterol Hepatol.* **25**, 194-202

83. Smith, M. F., Jr., Mitchell, A., Li, G., Ding, S., Fitzmaurice, A. M., Ryan, K., Crowe, S., and Goldberg, J. B. (**2003**) Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem.***278**, 32552-32560

84. Bruschi, M., Pirri, G., Giuliani, A., Nicoletto, S. F., Baster, I., Scorciapino, M. A., Casu, M., and Rinaldi, A. C. (**2010**) Synthesis, characterization, antimicrobial activity and LPS-interaction properties of SB041, a novel dendrimeric peptide with antimicrobial properties. *Peptides* **31**, 1459-1467

85. Evans, D. G., Evans, D. J., Jr., and Graham, D. Y. (**1992**) Adherence and internalization of *Helicobacter pylori* by HEp-2 cells. *Gastroenterol.* **102**, 1557-1567

86. Birkness, K. A., Gold, B. D., White, E. H., Bartlett, J. H., and Quinn, F. D. (**1996**) In vitro models to study attachment and invasion of *Helicobacter pylori*. *Ann N Y Acad Sci.* **797**, 293-295

87. Benjamin, J. L., Sumpter, R., Jr., Levine, B., and Hooper, L. V. (**2013**) Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe.* **13**, 723-734

88. Deen, N. S., Huang, S. J., Gong, L., Kwok, T., and Devenish, R. J. (**2013**) The impact of autophagic processes on the intracellular fate of *Helicobacter pylori*: more tricks from an enigmatic pathogen? *Autophagy* **9**, 639-652

89. Morales, H., Muharemagic, A., Gantress, J., Cohen, N., and Robert, J. (**2003**) Bacterial stimulation upregulates the surface expression of the stress protein gp96 on B cells in the frog Xenopus. *Cell Stress Chaperones.* **8**, 265-271

90. Lackman, R. L., and Cresswell, P. (2006) Exposure of the Promonocytic Cell Line THP-1 to Escherichia coli Induces IFN- -Inducible Lysosomal Thiol Reductase Expression by Inflammatory Cytokines. *J Immunol.* **177**, 4833-4840

91. Altman, E., Chandan, V., Li, J., and Vinogradov, E. (**2011**) Lipopolysaccharide structures of *Helicobacter pylori* wild-type strain 26695 and 26695 HP0826::Kan mutant devoid of the O-chain polysaccharide component. *Carbohydr Res.* **346**, 2437-2444

92. Chmiela, M., Czkwianianc, E., Wadstrom, T., and Rudnicka, W. (**1997**) Role of *Helicobacter pylori* surface structures in bacterial interaction with macrophages. *Gut* **40**, 20-24