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Characterization of novel *Helicobacter pylori* vaccine candidates

Yu Zhong

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1. Prof. Dr. Markus Gerhard

2. apl. Prof. Dr. Thilo M. Fuchs

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Abbreviations

Name	Abbreviations
°C	Degree celsius
AB	Antibody
AEBSF	AEBSF hydrochloride
BabA	*H. pylori* binding adhesin
BSA	Bovine serum albumin
CAG	Chronic atrophic gastritis
CagA	Cytotoxin-associated antigen A
CFU	Colony forming unit
CIP	Cleaning in place
CO_2	Carbon dioxide
CT	Cholera toxin
d	Day
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DYS	Dysplasia
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMA	Ethidium monoazide
ETOH	Ethanol
FliD	Flagellar hook-associated protein 2
GC	Gastric cancer
GeoMFI	Geometric mean of fluorescence intensity
gGpNA	L-γ-glutamyl-p-nitroanilide
gGT/HpgGT	*H. pylor* gamma-glutamyl transpeptidase
GroEL	Chaperonin GroEL
H. pylori/ HP	*Helicobacter pylori*
h	Hour
HCl	Hydrogen chloride
HcpC	*Helicobacter* cysteine-rich protein C
HEK293 cells	Human embryonic kidney 293
HP0231	*H. pylori* HP0231, a disulfide oxidoreductase
HpaA	Flagellar sheath adhesin A or Neuraminyllactose-binding hemagglutinin
HtrA	Serine protease A or High temperature requirement protein A
i.i.	Intranasal immunization
i.p.	Intraperitoneal immunization
i.v.	Intravenous immunization

Name	Abbreviations
IFNγ	Interferon gamma
IL	Interleukin
IM	Intestinal metaplasia
Jhp940	Serine/threonine-protein kinase CtkA
kDa	Kilodalton
LT	*E. coli* heat-labile enterotoxin
mA	Milliampere
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
MLN	Mesenteric lymph node
mM	Millimolar
MOI	Multiplicity of infection
MALT	Mucosa-associated tissue lymphoma
MW	Molecular weight
NaClO	Sodium hypochlorite
NaOH	Sodium hydroxide
NapA	Neutrophil activing protein A
NCBI	National center for biotechnology information
ng	Nanogram
Ni-NTA	Nickel-nitroloacetic acid
o.i.	Oral immunization
O_2	Oxygen
OD	Optical density
OMP	Outer membrane protein
P/S	Penicillin/Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Pondus hydrogenii
PMSS1	*H. pylori* pre-mouse Sydney Strain 1
PPI	Proton pump inhibitor
PP	Peyer's patch
PRRs	pathogen recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
rUreB	Recombinant UreB
S.D	Standard deviation

Name	Abbreviations
SabA	Sialic acid-binding adhesin
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SG	Superficial gastritis
Sodium phosphate	Dibasic sodium phosphate and monobasic sodium phosphate
SS1	*H. pylori* Sydney Strain 1
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th cell	T helper cell
Tig	Trigger factor
TNFα	Tumor necrosis factor alpha
Toll like receptors	TLRs
TRIS	Tris (hydroxymethyl) aminomethane
UreA	Urease subunit A
UreB	Urease B subunit
v/v	Volume per volume
VacA	Vacuolating toxin A
w/v	Weight per volume
WB	Western blot
WC Dent	Wilkins-Chalgren-*H. pylori* selection (Dent)
WT	Wild-type
µg	Microgram
µl	Microliter

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Abstract

Helicobacter pylori infects more than 50% of the world's population, leading to high incidence of gastric ulcers and cancer. Current antibiotic treatments of *H. pylori* infection cause high prevalence of antimicrobial resistance. Therefore, vaccination as an alternative approach against *H. pylori* was investigated in this dissertation.

H. pylori vaccine candidates were selected based on their sequence conservation and serological responses in *H. pylori*-infected patients. In this study, the outer membrane protein HpaA combined with NapA, gGT or HP0231 was selected for potential vaccine formulations. Prophylactic immunization against *H. pylori* revealed that NapA plus HpaA did not protect against *H. pylori* infection. However, HP0231 alone or HP0231 plus HpaA immunization significantly decreased *H. pylori* colonization, and this protection was associated with antigen-specific Th1 and Th17 responses, as well as IFNγ expression in the stomach.

Since HP0231 was an effective vaccine candidate, the functional role of HP0231 for *H. pylori* virulence was further investigated. HP0231 is an oxidoreductase that catalyzes disulfide bond formation. Thus, the effect of HP0231 on the functions of virulence factors containing disulfide bonds was analyzed by studying *hp0231*-deficient *H. pylori*. The absence of *hp0231* impaired CagA translocation into gastric epithelial cells, could not active NF-κB signalling pathway and IL-8 secretion upon *H. pylori* infection, and reduced VacA-induced cellular vacuolation by inhibiting VacA secretion. Moreover, *H. pylori* lacking *hp0231* was not able to colonize the gastric mucosa, while complementation of the gene reverted the phenotype. The inability of *hp0231*-deficient strains to colonize gastric mucosa was not due to impaired bacterial viability or binding to epithelial cells, but probably because of compromised motility.

This data demonstrates that HP0231 is an effective *H. pylori* vaccine candidate and seems to be a "master virulence factor" for *H. pylori*-induced pathogenesis.

Zusammenfassung

Mehr als 50% der Weltbevölkerung ist mit *Helicobacter pylor*i infiziert, was zu einer hohen Inzidenzrate von Magengeschwüren und Magenkrebs führt. Die aktuelle Antibiotika-Behandlung gegen *H. pylori* verursacht jedoch vermehrt Resistenzen. Daher wurden neue Vakzinierungsstrategien gegen *H. pylori* in der vorliegenden Dissertation als alternativer Therapieansatz untersucht. Die Selektion der Impfstoffkandidaten erfolgte anhand ihrer konservierten Proteinsequenz, sowie der serologischen Antwort in *H. pylori* infizierten Probanden. In dieser Arbeit wurden das Außenmembranprotein HpaA in Kombination mit NapA, gGT oder HP0231 für mögliche Vakzine ausgewählt. Die Kombination NapA mit HpaA induzierte keinen Schutz. Allerdings führte die Immunisierung mit HP0231 alleine oder in Kombination mit HpaA zu einer signifikanten Reduzierung der *H. pylori* Kolonisierung. Diese war mit einer Antigen-spezifischen Th1 und Th17 Immunantwort, sowie der Expression von IFNγ im Magen assoziiert.

Da HP0231 ein wirksamer Impfstoff-Kandidat war, wurde die funktionelle Rolle von HP0231 für die *H. pylori*-Virulenz näher untersucht. Da die Oxireduktase HP0231 die Disulfidbrückenbildung katalysiert, wurde der Einfluss von HP0231 auf die Funktionalität von Virulenzfaktoren, mit Disulfidbrücken mit Hilfe von *hp0231* defizientem *H. pylori* analysiert. Durch die Abwesenheit von HP0231 ist die CagA Translokation in Magenepithelzellen beeinträchtigt, findet keine NF-κB-Aktivierung und keine IL-8-Sekretion statt. Durch Hemmung der VacA Sekretion ist die VacA-induzierte zelluläre Vakuolisierung ebenfalls nicht zu beobachten. Des Weiteren, konnte *hp0231* defizienter *H. pylori* die Magenmucosa nicht besiedeln, während die Komplementierung des Gens den Phänotyp umkehrte. Die Unfähigkeit der *hp0231* defizienten Stämme die Magenschleimhaut zu kolonisieren war nicht aufgrund fehlender Lebensfähigkeit oder Bindung an Epithelzellen, sondern möglicherweise aufgrund beeinträchtigter Motilität des Bakteriums. Die Daten zeigen, dass HP0231 ein wirksamer *H. pylori* Impfstoffkandidat ist und scheinbar einen "Master Virulenz-Faktor" der *H. pylori*-Pathogenese darstellt.

Chapter 1: Introduction

1.1 *H. pylori* infection

1.1.1 *H. pylori* epidemiology

Helicobacter pylori (*H. pylori*) is a gram-negative, helical shaped (as its genus name implies) microaerophilic bacterium which resides in human stomach lining or in the upper part of the small intestine. Before its discovery, gastric diseases such as chronic gastritis, peptic ulcer and gastric adenocarcinoma were considered to be caused by stress, unhealthy lifestyle or bad sanitation. In 1984, *H. pylori* was found by two Australian researchers (Barry J. Marshall and Robin Warren) to be the essential factor causing a series of gastric diseases [1]. They were awarded the Nobel Prize because of their extraordinary discovery and deciphering of the process of the bacteria-induced pathology.

If not treated, an episode of *H. pylori* infection always turns into a lifelong event. The acute bacterial infection in humans usually occurs in childhood and occasionally in adulthood. *H. pylori* infection can be diagnosed by several tests such as a breath urea test and antigen serological test. *H. pylori* is the most prevalent member of the *Helicobacter* genus that can be found in human gastric mucosa biopsies [2]. More than 50% of the global population suffers from this bacterial infection, and the infection rate in developing countries is higher than that in developed countries: More than 70% of the total population is infected by *H. pylori* in developing countries [3, 4]. Potential reservoirs of *H. pylori* are naturally found in humans, houseflies, domestic cats and water sources [5-7]. Lack of good sanitation and organized hygiene management systems are associated with more than 70% of the total *H. pylori* infection cases [8-10]. This is a more prominent problem in highly populated areas where the infection can be easily transmitted among people. The transmission of *H. pylori* infection commonly occurs via oral to oral route cycles (through saliva or vomitus) and fecal to oral route cycles [11, 12]. Studies on epidemiology and

transmission cycles of *H. pylori* contribute greatly to the prevention and public management of this infection.

1.1.2 *H. pylori*-induced pathogenesis

Although *H. pylori* infection triggers recruitment of neutrophils, infiltration of macrophages and lymphocytes, and causes histological inflammation in the gastrointestinal tissues, the majority of the infections remain asymptomatic [13]. However, 90% of all gastric diseases are related to *H. pylori* infection [14]. For the majority of the patients, an acute *H. pylori* infection occurs in childhood and then develops into chronic infection, leading to the apoptosis of epithelial cells and disruption of the gastric mucosa (Figure 1) [15]. Approximately 10% to 20% of *H. pylori*-infected patients suffer from peptic ulcers. Peptic ulcers caused by *H. pylori* infection are termed duodenal ulcers if the damage has occurred in the duodenum (the upper part of the small intestine), or gastric ulcers if the damage has occurred in the stomach. In 1%-3% of the cases, gastritis further develops into gastric cancer, less than 1%, however, develops into mucosa-associated tissue (MALT) lymphoma [16].

H. pylori-induced gastric diseases have been shown to arise from changes in the gastric mucosa and lead to a multi-step development process of gastric lesions [17]. Changes at the cellular level in response to *H. pylori* infection may include gastric epithelial cell hypertrophy (changes in cell size), hyperplasia (changes in cell number), and atrophy (changes in cell shape) [18, 19]. As shown in Figure 1, patients initially suffer from an acute infection that subsequently develops into a chronic infection. Chronic infection can evolve into superficial gastritis (also referred to as simple gastritis) if there is slight inflammation in the upper part of the gastric mucosa. Once the inflammation becomes more extensive and spreads to the whole mucosa, superficial gastritis may become chronic atrophic gastritis. Gastric atrophy can develop into a gastric ulcer if the damage to columnar epithelial cells is sufficient to allow the penetration of gastric acid from lumen to the mucosa [20]. Additionally, there is a risk of developing intestinal metaplasia [21]. Intestinal metaplasia is the transformation of

gastric epithelium into an epithelium resembling the one found in the intestine. Intestinal metaplasia has the potential to develop into dysplasia. Dysplasia is a precancerous lesion, which may develop into gastric cancer [20, 22, 23].

The risk of gastric diseases related to *H. pylori* infection is different across populations and correlates with specific bacterial components and genotypes. Two *H. pylori* virulence factors have been reported to be associated with peptic ulcers and gastric cancer [24]. *H. pylori* cytotoxin-associated gene A (CagA), a carcinogenic protein translocated by *H. pylori* Type IV system (T4SS), is described as highly correlating with the incidence of gastric ulcers and gastric cancer [24-26]. Another virulence factor, Vacuolating toxin A (VacA), which induces vacuolation and cell apoptosis, correlates with high risk of gastric diseases, specifically peptic ulcers [24].

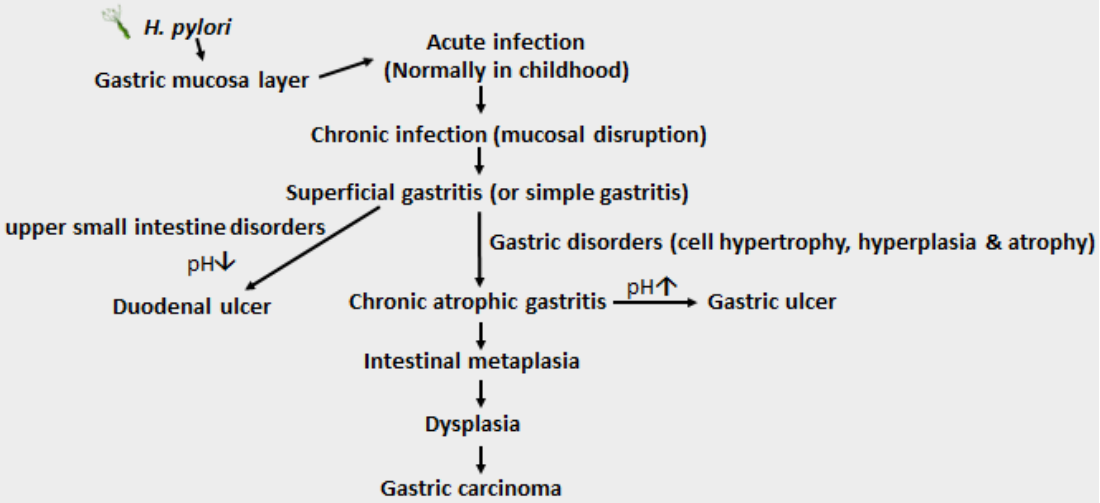


Figure 1 *H. pylori*-induced pathological processes

1.2 *H. pylori* virulence factors

The ability of lifelong colonization and infection of *H. pylori* in the gastric mucosa is determined by a combination of different virulence factors. These factors enable the bacteria to survive in the acidic mucosa and induce gastric diseases (Figure 2). For instance, *H. pylori* neutralizes the gastric acid with urease, increasing the pH in the gastric lumen and

therefore allowing the bacteria to survive in the stomach [27]. In order to cross the intercellular space, *H. pylori* is able to cleave E-cadherin to disrupt the tight junctions of the epithelial barrier by high-temperature requirement A (HtrA) [28]. Additionally, HP0569, tumor necrosis factor α inducing protein (Tipα), induces epithelial cell morphology changes, and promotes expression of TNFα and activation of NF-κB by binding to the cell nucleus [29, 30]. *H. pylori* heat shock protein (GroES) binds to toll-like receptor 4 (TLR4) via its C-terminal disulfide bonds, leading to NF-κB activation and IL-8 secretion [31]. The following sections give a detailed description of the major virulence factors contributing to *H. pylori*-induced pathology.

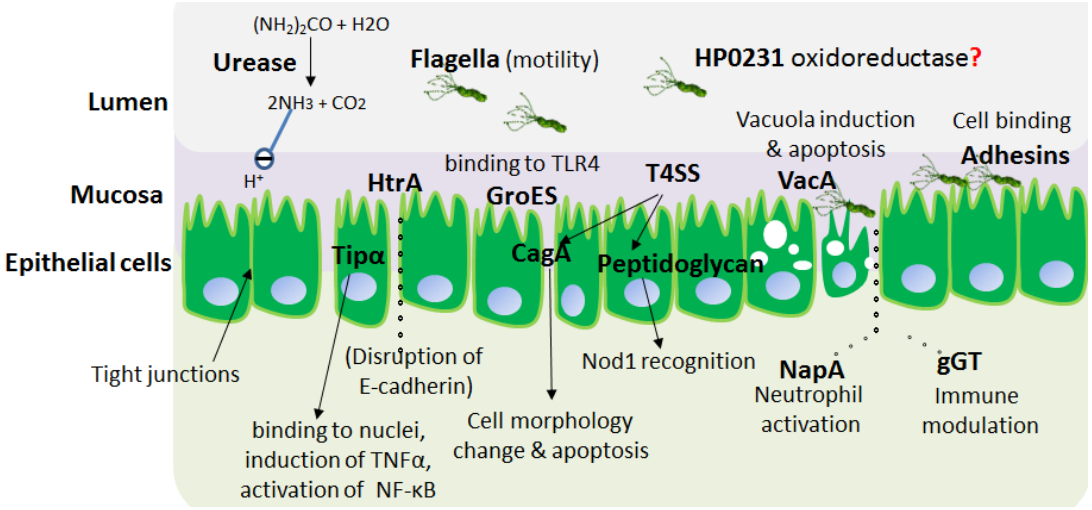


Figure 2 Important *H. pylori* virulence factors

1.2.1 The cag Pathogenicity Island

H. pylori cytotoxin associated gene Pathogenicity Island (*cag*-PAI) is an essential factor determining *H. pylori* virulence. *cag*-PAI is a 40 kDa macromolecular complex composed of approximately 28 conjugative proteins which share a single or a few operons [32]. It encodes a T4SS system that injects CagA into host cells. The T4SS system is exceptionally versatile in mediating protein secretion and uptake and release of DNA into the extracellular milieu [33]. In response to *H pylori* infection, *H. pylori* T4SS system activates NF-κB signalling pathway and thus induces IL-8 secretion by host epithelial cells [34, 35]. Notably, the activation of NF-κB

and the secretion of IL-8 are *H. pylori* T4SS system-dependent and CagA-independent [36, 37]. Mutations in *cag*-PAI components may result in a dysfunctional secretion apparatus. For instance, mutations of *cagL* or *cagE* impair the translocation of CagA and the secretion of IL-8 [38, 39]. The same is also observed in *H. pylori* strain SS1 which lacks a functional T4SS secretion apparatus [40]. Two important effectors, CagA and peptidoglycan, which are translocated by the T4SS system, are important for *H. pylori*-induced pathology.

CagA

CagA has been reported to highly correlate with gastric cancer [41, 42]. The oncogenic potential of CagA was shown after transgenic expression of CagA in mice. Thus, CagA transgenic mice developed gastric polyps and adenocarcinomas of the stomach and small intestine. [43]. CagA is translocated by *H. pylori* T4SS system, and contains a Glu-Pro-Ile-Tyr-Ala (EPIYA) motif in its C-terminal region. The tyrosine residues in the EPIYA motif are phosphorylated once CagA is injected into the cytoplasm of epithelial cells by host cell kinases Abl and Src [44]. Phosphorylated CagA binds to Src homology 2 domain phosphatase (SHP2) via its EPIYA motif, leading to host Src family kinase activation and host cell morphologic changes [45]. Notably, non-phosphorylated CagA can also induce morphologic changes in epithelial cells [46]. Non-phosphorylated CagA directly forms a complex with host cell cytoskeleton proteins, resulting in a disruption of both cellular tight junctions and cellular polarity, and causing an elongation of epithelial cells [47, 48]. In particular, non-phosphorylated CagA interacts with E-cadherin, leading to the disruption of the E-cadherin/β-catenin complex [49, 50].

Peptidoglycan

Peptidoglycan, another factor secreted by *H. pylori* T4SS system, can specifically bind to Nucleotide-binding oligomerization domain-containing protein 1 (NOD-1). Peptidoglycan/NOD complex has been reported to activate NF-κB signaling pathway in *H. pylori*-infected gastric epithelial cells, resulting in an induction of β-defensin-2 [51] and secretion of IL-8 [52].

Peptidoglycan is a component of the bacterial cell wall and has been shown to be important to maintain *H. pylori* helical shape and osmotic stability [53]. The mutation of genes involved in peptidoglycan modification can decrease bacterial colonization in the gastric mucosa; these include the mutation of peptidoglycan modification enzymes *csd1*, *csd3* and *csd4* [53, 54]. Moreover, the mutation of *H. pylori* peptidoglycan deacetylase A (*pgdA*) has been reported to reduce *H. pylori* colonization nine weeks post-infection in mice, and to increase the levels of IL-10 and TNFα expression in mice [55].

1.2.2 VacA

VacA is a protein auto-secreted through a bacterial Type V auto-transport system. During auto-transportation, the VacA precursor, a 140 kDa protein, was shown to undergo proteolytic cleavage to form a 33 amino acid signal peptide, a mature secreted 88 kDa VacA toxin and a carboxy-terminal β-barrel domain [56]. The mature 88 kDa VacA protein is composed of two smaller subunits, p33 and p55 [57, 58]. The carboxy-terminal β-barrel domain is responsible for proteolytic processing of the mature VacA toxin [59]. Although the majority of *H. pylori* strains express VacA, they show considerable differences in the vacuolation ability due to structural differences in the *vacA* gene sequence [60]. The *vacA* gene was shown to be composed of three parts, including the signal (*s*) region (localized in the p33 domain), the intermediate (*i*) region (localized between the *s* and *m* regions), and the middle (*m*) region (localized in the p55 domain). There are at least two polymorphic variations in each region [60, 61]. Two *s* region variants (*s*1 and *s*2) [62], three *i* region variants (*i*1, *i*2 and *i*3) [63], and two *m* region variants (*m*1 and *m*2) [64] are found in the *vacA* sequence. The diversity of these variations appears to be linked to the development of severe gastric ulcers and gastric cancers [60, 61, 65].

Part of the p55 domain and the whole p33 domain have been reported to be responsible for vacuole formation [66, 67]. The p33 domain helps VacA insertion into the epithelial cell membrane and induces vacuole formation [68], whereas p55 is crucial in binding for epithelial cells [69]. Besides

inducing vacuolation, VacA inhibits T cell proliferation by binding to β2 integrin receptor subunit (CD18) [70]. VacA interacts with additional cell surface receptors and activates mitogen-activated protein (MAP) kinase signaling, which can result in cytoskeletal rearrangements [71]. Additionally, VacA modulates the mitochondrial membrane potential and promotes cytochrome c release, thus leading to cell apoptosis [72, 73].

1.2.3 Adhesins

H. pylori outer membrane proteins (OMPs) include pro-inflammatory proteins, micropore proteins and adhesin proteins. Five major outer membrane protein families were identified in *H. pylori* [74]. Among them, the *Helicobacter* outer membrane protein (Hop) family includes the largest number of *H. pylori* adhesins, such as sialic acid-binding adhesin (SabA), blood group antigen-binding adhesin (BabA) and outer inflammatory protein (OipA) [75]. SabA and BabA are two of the best described *H. pylori* adhesins. SabA has been shown to bind to the carbohydrate structure sialyl-Lewis x (SLeX) on the surface of gastric epithelial cells [76]. BabA has been shown to bind to the surface receptor Lewis b on gastric epithelial cell and promotes *H. pylori* persistent colonization [77, 78].

1.2.4 Flagella

H. pylori utilizes three to five sheathed unipolar flagella as an “engine“ to assist its motility [79]. The flagellum is constituted of three fundamental structures: the basal body, the hook and the filament [80]. Flagellar proteins are transported across the cell membrane through a type III secretion system (T3SS) [81]. *H. pylori* are completely non-motile when filament protein *flaA* and *flaB* are double mutated [82, 83]. *H. pylori* flagellar hook-associated protein 2 (FliD) is a highly sensitive marker for *H. pylori* clinical diagnostics [84], and its mutation completely dampens *H. pylori* motility and colonization in mouse gastric mucosa [85]. Likewise, mutations of other flagella genes, such as *fliF*, *fliS*, *flhB*, *fliQ*, *fliG* or *fliI*, also result in flagellar misassembly and non-motile strains [86-88]. Another important flagellar protein HpaA is described below.

HpaA

H. pylori flagella sheath adhesin A (HpaA), also named neuraminyllactose-binding hemagglutinin, is a 29 kDa surface lipoprotein [89]. HpaA is recognized in *H. pylori*-infected human serum [90, 91]. Sequence alignment indicates that HpaA is a highly conserved protein among *H. pylori* strains [91] and shows no significant homologies with other proteins [92]. Moreover, HpaA is not involved in the attachment to epithelial cells [93], but is essential for *H. pylori* colonization; bacteria without *hpaA* fail to colonize mouse gastric mucosa [94]. The mechanism of this impairment on colonization is not fully understood, but is probably due to defects in motility, since HpaA is a flagella protein [95]. HpaA was also shown to induce IFNγ expression in human NK cells through the activation of TLR2 [96]. Therefore, HpaA appears to be an important protein for *H. pylori* infection.

1.2.5 NapA

Neutrophil-activating protein (NapA) is a 150 kDa dodecameric protein with a central cavity to which iron ions bind [97]. NapA monomer has been reported to consist of a four α helix bundles and seven residues of a fifth α helix oriented nearly vertical to the bundle [98]. The structure of NapA is similar to both dodecameric DNA-binding proteins (Dps) and ferritins proteins [99].

NapA induces IFNγ production in Th1 cells and suppresses IL-4 secretion in Th2 cells *in vitro* [100]. NapA directly binds to neutrophil glycosphingolipids [101] and mucins by interaction with carbohydrates on protein surfaces [102]. On one hand, NapA appears to induce oxidative stress in neutrophils by activating NADPH oxidase to increase reactive oxygen intermediates (ROI) production via its C-terminus ($NAP_{58–144}$) even without dodecamer formation [103, 104]. On the other hand, NapA appears to combat oxidative stress by binding with DNA to protect it from exposure to free radicals. Wang et al. [105] and Kottakis et al. [103] demonstrated this protection was dependent on the iron-sequestering ability of NapA, which helped *H. pylori* to reduce oxidative stress, while Ceci et al. [106]

described it was not dependent on iron binding, but dependent on the positively charged NapA surface.

The deficiency of *napA* reduces *H. pylori* survival in the presence of oxidative stress *in vitro* [107]. *In vivo*, mouse experiments showed that if mice were firstly inoculated with dead wild-type *H. pylori*, which could induce ROI production, and then infected with live wild-type or *napA*-isogenic mutant bacteria, *H. pylori* colonization was significantly lower in the absence of *napA*. However, if mice were first inoculated with dead *napA* deficient *H. pylori* which could not induce ROI production, and then infected with live wild-type or *napA*-isogenic mutant, no differences in colonization between mice infected with *napA* mutant and mice infected with wild-type bacteria were observed [105]. That indicates NapA promotes *H. pylori* survival in the stomach in the presence of oxidative stress.

1.2.6 gGT

H. pylori gamma-glutamyl transpeptidase (gGT) is a highly conserved virulence factor found in all *Helicobacter* isolates [108]. Bacterial gGT shares around 25% protein sequence homology with mammalian gGT but acts as a hydrolase rather than a transpeptidase when compared to mammalian gGT [109, 110]. gGT enzyme has been demonstrated to be synthesized as a 60 kDa precursor and is subsequently auto-catalytically cleaved to form two enzymatically active subunits: a 20 kDa small subunit and a 40 kDa large subunit [111]. gGT is responsible for transferring a γ-glutamyl moiety from γ-glutamyl containing proteins to other compounds, which can provide an energy source for *H. pylori* infection under microaerophilic conditions [112]. gGT catalyzes glutathione into glutamate and cysteinylglycine, and glutamine into glutamate and ammonia. The efflux of ammonia is toxic to host cells by inducing cell damage, while at the same time it increases the pH of *H. pylori* surrounding niches, which is more favorable for *H. pylori* growth. Moreover, *H. pylori* utilizes glutamate as a carbon and nitrogen source [113].

Apart from its important role in bacterial metabolism, *H. pylori* gGT has been described to have important immunomodulatory effects during

infection. Thus, gGT induces epithelial cell apoptosis [114] and inhibits T cell proliferation by induction of G1 phase cell cycle arrest [115]. gGT also induces inflammatory stress by upregulation of cyclooxygenase-2 [116] and IL-8 [117], and induces DNA damage by generating H_2O_2 [118] and reactive oxygen species (ROS) [117]. Furthermore, gGT induces immune tolerance by reprogramming dendritic cells in mouse [119] and human [120].

Previous studies in mice showed that the deletion of *ggt* affects the capacity of the bacterium to colonize the gastric mucosa abolished the capacity of bacterial colonization [121, 122]. Although different results on gastric colonization were observed in different studies, there is no doubt that gGT is important for the establishment and persistence of *H. pylori* infection. These crucial functions of gGT make it an ideal target for immunological treatments.

1.2.7 *H. pylori* disulfide oxidoreductases

H. pylori disulfide oxidoreductases are members of the disulfide bond (Dsb) enzyme family. The Dsb system in gram-negative bacteria is composed of proteins containing a Cys-XX-Cys motif and thioredoxin (TRX) domain. These proteins have been shown to contribute to the balance of oxidation and reduction state of disulfide bonds and assist in a wide range of protein folding [123]. The Cys-XX-Cys motif, the catalytic site of Dsb family, allows Dsb enzymes to either oxidize a pair of cysteines (forming an internal disulfide) or reduce the cysteines to free thiols. According to their functions, the Dsb system can be divided into two antagonistic pathways that are responsible for proper disulfide bond folding: 1) the oxidative pathway and 2) the isomerization/reduction pathway [124]. The balance between the oxidation and isomerization/reduction ensures the proper folding of proteins containing disulfide bonds and stabilizes their structures during bacterial growth.

In gram negative bacteria, disulfide bond formation occurs in the periplasm which is an oxidative environment [125]. For example, *E. coli* Dsb family contains five important Dsb proteins (DsbA, DsbB, DsbC, DsbD and DsbG).

DsbA and DsbB have been shown to catalyze disulfide bond formation while DsbC/DsbG and DsbD isomerases disrupt misfolded disulfide bonds [126]. DsbA is the primary oxidase that interacts with different substrates and exists only in a monomer form [126, 127], while DsbB is a very specific enzyme, which only oxidizes the reduced form of DsbA in conjunction with either menaquinone or ubiquinone in anaerobic conditions at the cytoplasmic membrane [128]. DsbC and DsbG are functionally similar [129, 130]. They are responsible for reducing misfolded disulfide bonds and maintaining their reduced forms through electron transfer provided by DsbD [126]. DsbD and its shortened homolog cytochrome c biogenesis protein (CcdA) are integral membrane proteins. They transfer electrons from the cytosol to the periplasm in the presence of thioredoxin [131].

In contrast to *E. coli*, *H. pylori* does not have the classical Dsb oxidation and isomerization pathways. 149 proteins with Cys-XX-Cys motif are found in the genome of *H. pylori* 26695, and four proteins (HP0231, HP0377, HP0824, and HP1458) contain TRX domains. One protein HP0595, which is structurally similar to *E. coli* DsbB, contains a Cys-XX-Cys motif, but does not have a TRX domain. Another protein, HP0625, which shows sequence similarity to other bacterial CcdAs, appears to be responsible for cytochrome c biogenesis [132].

HP0824 and HP1458 are two *H. pylori* thioredoxins carrying electrons derived from NADPH [133]. These electrons are transferred from the inner membrane to the periplasm by cytoplasmic membrane localized DsbD like proteins, which are predicted to be HP0861 and HP0265 in *H. pylori* [134]. However, the mechanism of electron transfer in *H. pylori* is unclear. HP0861 contains five cysteines in its structure, but its function is unclear to date. HP0265 contains six transmembrane domains with two cysteines localized in the first and the fourth transmembrane domains. HP0265 is thought to transfer electrons in a manner similar to other CcdA proteins [135-137]. *H. pylori* lacking *hp0265* grew very slowly in the absence of DTT, indicating the importance of HP0265 in the reduction pathway for bacterial growth [134]. HP0377 is one of the substrates of HP0265, since oxidized HP0377 was shown to exist in the absence of *hp0265* [134]. HP0377

usually exists in a reduced form in wild-type *H. pylori* and shows disulfide isomerase activity. HP0377 can catalyze the refolding of scrambled RNase, but cannot catalyze the reduction of insulin. Moreover, HP0377 does not appear to complement *E. coli dsbC* mutant or cooperate with *E. coli* DsbD [134]. This data indicates that HP0377/HP0256 may not act in a manner similar to DsbC/DsbD in the *H. pylori* oxidoreductase system.

Another putative *H. pylori* disulfide oxidoreductase, HP0595 (HpDsbI), has been predicted to contain five transmembrane fragments in the N-terminus and a β-propeller in the C-terminus [138]. HP0595 seems to influence bacterial gastric colonization, since the deficiency of the gene sharply reduced *H. pylori* colonization [139].

At present, few studies have elucidated the function of the *H. pylori* Dsb system. The role of HP0377 and HP0256 in *H. pylori* colonization and infection are still unclear. HP0231 is the most studied *H. pylori* oxidoreductase, which is responsible for disulfide bond formation [140]. The detailed function of HP0231 is summarized below.

H. pylori oxidoreductase HP0231

The oxidoreductase HP0231 was shown to be involved in the oxidation pathway in the *H. pylori* Dsb system [140], but its function is still far from being understood.

Structure of HP0231

HP0231 monomer is a 29 KDa protein which is highly conserved in *H. pylori* isolates. It is composed of a dimerization domain in the N-terminus, a catalytic TRX domain in the C-terminus and a long helical linker in between. HP0231 Cys-XX-Cys catalytic site localizes in the first helix of its TRX domain, which is identical to that of *E. coli* DsbA motif (CPHC), but different from that of *E. coli* DsbC motif (CGYC) and DsbG motif (CPYC) [141]. Only Cys_{159} of the two cysteines in the HP0231 Cys_{159}-XX-Cys_{162} motif has been predicted to be exposed in HP0231 crystal structure, and is expected to form a mixed disulfide bond with its substrates [140]. Two HP0231

monomers form a V-shaped dimeric protein like *E. coli* DsbC and DsbG [126, 142].

Function of HP0231

HP0231 was initially expected to be a reductase like *E. coli* DsbG [140]. However, functionally, it resembles *E. coli* oxidase DsbA. HP0231 displays a similar redox potential to *E. coli* DsbA *in vitro* and can compensate for *E. coli dsbA* mutant but not *E. coli dsbC* mutant [143]. Truncated HP0231 lacking its dimerization domain is able to rescue *E. coli dsbA* mutant, but unable to complement *E. coli dsbA* and *dsbB* double mutant or *H. pylori hp0231*-knockout strain [126]. In contrast, full length HP0231 compensates for *E. coli dsbA* and *dsbB* double mutant [143]. These results indicate that the dimeric form of HP0231 is essential for maintaining the oxidized state in *H. pylori* and the monomer HP0231 may require a DsbB like protein to re-oxidize HP0231 *in vivo*. HP0595, a DsbB like protein in *H. pylori*, promotes an oxidized state of HP0231. The deficiency of *hp0595* results in a noticeable accumulation of reduced HP0231 [143]. *H. pylori* lacking *hp0595*, however, still has partial oxidized HP0231 *in vivo* [306], indicating that there may be other proteins responsible for maintaining the oxidized state of HP0231.

Possible HP0231 substrates, proteins containing disulfide bonds, are reported in three studies. *H. pylori* cysteine rich (Hcp) family is composed of proteins with high number of cysteine residues [144]. HP0231 affects not only the secretion of HcpE and HcpC [306], but also the correct folding of HcpE *in vitro* [145]. Therefore, although HP0231 was initially named HpDsbG due to the structural similarity with *E. coli* DsbG [140], HP0231 was renamed DsbK due to its versatile functions [145].

In summary, the *H. pylori* Dsb system is different from the *E. coli* Dsb system, and the function of HP0231 on proper folding of *H. pylori* virulence factors with disulfide bonds is far from being understood. Whether HP0231 is important for proper functionality of the virulence factors containing disulfide bonds is still unclear.

1.3 Immune response to *H. pylori* infection

H. pylori elicits a range of innate and adaptive immune responses during both acute and chronic infection through antigen-independent and antigen-dependent mechanisms. Innate immunity is mediated by different innate immune cells such as dendritic cells and neutrophils, which mediate antigen recognition and activate adaptive immune responses. Adaptive immunity offers an antigen-dependent protection and is mediated by T and B cells [146, 147]. Compared to non-infected gastric mucosa, *H. pylori*-infected mucosa shows infiltration with various types of leukocytes, including dendritic cells, neutrophils, macrophages, eosinophils, mast cells, natural killer cells (NK cells), T cells and B cells [148, 149].

1.3.1 Innate immunity to *H. pylori* infection

The innate immune system is the first line of defense against *H. pylori* infection. The main innate immune cells involved in *H. pylori* infection have been shown to be epithelial cells, professional antigen-presenting cells (APCs), neutrophils, and mast cells.

1.3.1.1 Role of epithelial cells in *H. pylori* infection

The gastric epithelium is the first line of defense against *H. pylori* infection. It is composed of a single layer of columnar epithelial cells (also named foveolar cells). Columnar epithelial cells secrete mucus acting as lubricant [150]. *H. pylori* infection induces the secretion of IL-8 in epithelial cells, leading to the recruitment of other innate immune cells, such as neutrophils [151]. Additionally, *H. pylori* infection disrupts the epithelium line through altering the morphology of epithelial cells by injecting CagA [152], inducing vacuolation and apoptosis by VacA [153, 154] and breaking epithelial tight junctions by HtrA [155]. The disruption of the epithelium causes a penetration of hydrochloric acid (secreted by parietal cells) and pepsin (secreted by chief cells) from the gastric lumen to the lamina propria, resulting in gastritis and peptic ulcer [156].

1.3.1.2 Role of professional APCs in *H. pylori* infection

Professional APCs, dendritic cells and macrophages, recognize *H. pylori* components such as LPS, OMP and secreted antigens. These APCs play a major role in antigen presenting for connecting the innate and adaptive immunity, and present signals to lymphocytes in different lymphoid organs.

Dendritic cells

Dendritic cells (DCs) mainly act as antigen presenting cells. DCs deliver digested exogenous peptide fragments to downstream lymphocytes by major histocompatibility complex (MHC) class molecules on their cell surface, and subsequently activate the adaptive immune response [157].

Mature DCs are responsible for antigen presentation and T cell stimulation. Once stimulated via pattern recognition receptors (PRRs), DCs begin to migrate to lymph nodes and then interact with T cells [158]. DCs are found in the gastric lamina propria, isolated lymphoid follicles (ILF), Peyer's Paches and mesenteric lymph nodes [159]. Several studies have described the effects of *H. pylori* infection on DCs. Intraepithelial DCs were detected in gastric mucosa in *H. pylori* induced gastritis in patients [160]. In mice, CD11c+ DCs were recruited to gastric lamina propria upon *H. pylori* infection [161, 162]. Adoptive transfer of *H pylori*-pulsed DCs decreased the ratio of gastric IL-17/Foxp3 mRNA level in mice, resulting in a skewed Treg immune response [163]. The tolerogenic effect of *H. pylori* on DCs was further investigated in human cells. CagA and gGT were found to be the main bacterial virulence factors inducing tolerance by very diverse mechanisms. Thus, CagA was described to induce semi-maturation of DCs via IL-10-induced STAT3 activation [164], while gGT induced tolerogenic DCs by activating glutamate receptors, thereby inhibiting cAMP signaling and dampening IL-6 secretion in response to the infection [120]. Moreover, the depletion of DCs improves the vaccine-mediated protection against *H. pylori* infection [165].

Macrophages

Macrophages are a subtype of white blood cells that play an essential role

in eliminating microbes, apoptotic cells, and foreign components by engulfment and digestion (so-called phagocytosis) [166]. Macrophages recruit lymphocytes to trigger antigen-specific adaptive immunity, and play a role in the pro-inflammatory process through the release of related cytokines. For instance, M1 macrophages cause inflammation by promoting high level of IL-12 and low level of IL-10. M2 macrophages inhibit inflammation and promote tissue repair by releasing high levels of IL-10, TGFβ and low level of IL-12 [167].

A Previous study showed that *H. pylori* could replicate in certain macrophage cell lines, and the prolongation of *H. pylori* survival in macrophages promoted apoptosis [168]. This process is mainly dependent on the *H. pylori* virulence factors CagA and VacA [169]. *H. pylori cagA*- or *vacA*-deficient strains are completely degraded in macrophage phagolysosome [169]. *H. pylori* infection induces an increase of cytochrome c expression in cytosol and promotes the permeability of the mitochondrial membrane [170]. *H. pylori* infection also stimulates the expression of polyamine oxidase 1 [171] and spermine oxidase [172] in macrophages, leading to the production of nitrogen oxide and the release of H_2O_2. Apoptosis of macrophages induced by *H. pylori* is a mechanism by which *H. pylori* evades the host immune response and survives in the harsh gastric environment.

1.3.1.3 Role of neutrophils in *H. pylori* infection

Neutrophils, the most abundant granulocytes in the majority of mammals, play an important role in innate immunity. Neutrophils are short-living cells, and can be quickly mobilized to the sites of inflammation or infection via chemotaxis [173]. Neutrophils express cell surface receptors for a wide range of signals, including cytokines (such as IFNγ), chemokines, lectins, opsonin, and receptors for the attachment to endothelium [173].

Neutrophils are activated in the acute phase of *H. pylori* infection. They infiltrate from the foveolar epithelium to the lamina propria [148, 174] when IL-8 is secreted by epithelial cells or NapA is secreted by *H. pylori* [104, 175, 176]. *H. pylori*-induced neutrophilic infiltration generates ROS, which

induces DNA damage in nearby proliferating cells [177, 178]. The DNA damage caused by neutrophils is thought to contribute to gastric carcinogenesis [179].

1.3.1.4 Role of mast cells in *H. pylori* infection

Mast cells are white blood cells derived from myeloid progenitor cells. They are involved in allergy, anaphylaxis, immune defense and tolerance against pathogens [180]. Mast cells are located near the vessels in the connective tissues and promote the recruitment of leukocytes such as neutrophils [181].

The role of mast cells during *H. pylori* infection is not widely studied. However, a high frequency of mast cells was shown to exist in *H. pylori*-infected human gastric mucosa, and the frequency of these mast cells was decreased after *H. pylori* eradication therapy [182]. The increased density of mast cells in gastritis sites is highly dependent on *H. pylori* virulence factors. For instance, an increased number of mast cells was observed in the gastric mucosa of patients who were infected with *cagA*, *babA2* or *vacAs1/m1* positive strains [183]. The level of vaccine-induced protection generated by the immunization with *H. pylori* lysate partially decreased in mast cell-deficient mice compared to wild-type mice [184]. Additionally, the expression of gastric IL-17 and tumor necrosis factor α (TNFα) were significantly reduced in mast cell-deficient mice immunized with *H. pylori* lysate [184]. These results indicate an important role of mast cells during *H. pylori* infection.

1.3.2 Adaptive immunity to *H. pylori* infection

In contrast to innate immunity, adaptive immunity is an acquired and highly specific immune response. It sets up an immunological memory to a specific antigen, leading to an antigen-specific immune response.

The adaptive immune response against *H. pylori* infection has been investigated in human volunteers. An increased number of CD4+ T cells, CD8+ T cells and elevated expression of cytokines are detected in the gastric mucosa of *H. pylori*-infected patients [185]. Moreover, in

lymphocyte-deficient mice (recombination-activating gene knockout, RAG -/-) or SCID mice (severe, combined, immuno-deficient), higher levels of *H. pylori* colonization were observed compared to wild-type infected mice [186, 187]. Although *H. pylori* colonized the stomach, these deficient mice did not develop mucosal inflammation. These results address the essential role of adaptive immunity during *H. pylori*-induced pathogenesis.

1.3.2.1 Role of T cells in *H. pylori* infection

Antigens presented by APCs interact with specific T cell receptors (TCRs), leading to the activation and differentiation of T cells. Specifically, MHCII peptides interact with CD4+ T cells and MHCI peptides interact with CD8+ T cells. Effector CD8+ T cells (cytotoxic CD8+ T cells) help to kill infected target cells and activate macrophages [188]. Effector CD4+ T cells can diversify into T helper cells (Th1, Th2, and Th17) and regulatory T cells (Tregs) [189]. Th1 cells and cytotoxic CD8+ T cells mainly induce cellular immune responses and Th2 cells promote humoral immune responses through B cell stimulation [190]. Th17 cells secrete IL-17 and facilitate bacterial clearance at the mucosal surface. Tregs, also known as suppressor T cells, suppress T helper cell proliferation and regulate antigen-specific immune tolerance [191].

During *H. pylori* infection, CD4+ T cells, but not CD8+ T cells, are important for the development of gastritis. Although an increased number of CD8+ T cells are observed in *H. pylori* positive mucosa [185], CD8+ T cells may not participate in *Helicobacter*-induced gastritis, since the colonization and the inflammation degree did not change in *Helicobacter* felis (*H. felis*)-infected RAG1 -/- mice after adoptive transfer of CD8+ T cells derived from *H. felis*-infected wild-type donors [192]. In contrast, *H. felis*-infected RAG1 -/- mice, only developed severe gastritis and showed significant bacterial clearance after adoptive transfer of CD4+ T cells from *H. felis*-infected wild-type mice [192]. Similar results were also observed in *H. pylori*-infected SCID mice after adoptive transfer of splenocytes or CD4+ T cells from wild-type mice [186, 187], indicating an essential role of CD4+ T cells in *H. pylori*-induced gastric inflammation. Furthermore, a significant decrease of bacterial colonization was detected in *H. felis*-infected TCRβ -/- mice (lacking the

TCRα/β+ subset of T cells) after adoptive transfer of CD4+ T cells from *H. felis*-infected wild-type mice, while no differences in bacterial colonization between *H. felis*-infected TCRβ -/- mice and *H. felis*-infected wild-type mice after both adoptive transfer of IFNγ -/- CD4+ T cells derived from *H. felis*-infected IFNγ -/- mice [192]. These results suggest that IFNγ produced by CD4+ T cells is important for *Helicobacter* clearance.

H. pylori infection induces a Treg-skewed response, and Tregs play a regulatory role that suppresses *H. pylori*-induced T cell proliferation and IFNγ production upon *H. pylori* infection [193]. Gray et al. observed that gastritis was significantly suppressed in *H. pylori*-infected mice after adoptive transfer of high dose of Tregs, providing strong evidence for an essential role of Tregs in the regulation of the host immune response to *H. pylori* and development of subsequent disease [194].

Both, Th1 and Th17 cells are involved in *H. pylori*-induced gastritis. Gastritis was slightly reduced but still developed in *H. pylori*-infected IL-17A-deficient mice [195]. Moreover, gastritis cannot develop in *H. pylori*-infected SCID mice, but it can develop in *H. pylori*-infected SCID mice after adoptive transfer with CD4+ T cell derived from T-bet KO mice (T-bet knockout, failing to express IFN-γ in Th1 cells, NK cells, or NK T cells) [196]. These results indicate that IFNγ-secreting Th1 cells are not essential for the induction of gastritis. In fact, IFNγ secreted from non-T helper cells are important for *H. pylori*-induced gastritis. Gray et. al demonstrated that IFNγ produced by innate immune cells was necessary for *H. pylori*-induced gastritis and IFN-γ secreted by Th1 cells alone was not sufficient [194]. They found that *H. pylori*-infected RAGγc -/- mice did not develop gastritis even after adoptive transfer of CD4+ T cells derived from wild-type mice. RAGγc -/- mice are deficient for NK cells, T cells and B cells as well as for other cytokines with the common cytokine gamma chain [197]. In RAGγc -/- mice, IFN-γ was secreted by the adoptively transferred CD4+ Th1 cells but was not secreted by the innate immune cells of these mice. In addition, gastritis did not develop after adoptive transfer, indicating that Th1 cells are insufficient for *H. pylori*-induced gastritis.

Th2 cells play a minor role in *H. pylori*-induced gastritis, although Th2 cells are considered to mainly mediate host immunity against extracellular pathogens [198]. Th2 cells promote antibody (AB) production and can be characterized by the secretion of IL-4, IL-5, IL-9, IL-10, and IL-13. It has been reported that the expression of IL-4 in the gastric mucosa is similar between *H. pylori*-infected and non-infected individuals [198]. Moreover, similar colonization levels were observed in IL-4-deficient mice and wild-type mice after *H. pylori* infection [199].

In summary, CD4+ T cells are essential for *H. pylori*-induced gastritis. Gastritis is partially related to Th1 cells, Tregs and Th17 cells, and has little to do with Th2 cells and CD8+ T cells.

1.3.2.2 Role of B cells in *H. pylori* infection

B cells are derived from bone marrow stem cells, and differentiate to memory B cells and plasma cells under the activation of B cell receptors (BCRs). Plasma cells are responsible for the synthesis of antibodies, including IgM, IgG, IgA, and IgE [200].

H. pylori infection induces a systemic wave of antibody production. Serological IgM production was detected two to four weeks after *H. pylori* infection [185]. Mucosal and serological *H. pylori*-specific IgA and IgG antibodies were also detected in *H. pylori*-infected patients [201-203]. Analysis of the sera from infected patients is routinely performed as part of *H. pylori* clinical diagnosis and includes identification of *H. pylori* antigens such as CagA and FliD [84, 204]. Surprisingly, the production of *H. pylori*-specific antibodies was reported to play a minor role in the development of gastritis in mice since similar inflammation levels were shown in *H. pylori*-infected IgA-deficient and immunoglobulin-deficient mice compared to wild-type mice [205]. Similar gastric inflammation levels were also detected between *H. pylori*-infected B cell-deficient mice and wild-type mice [206]. Interestingly, similar levels of *H. pylori* colonization were observed in both B cell-deficient and wild-type mice at the initial phase of infection, but the colonization sharply decreased after 8 weeks and 16 weeks of infection in B cell-deficient mice compared to wild-type mice [206]. Therefore, although

B cells may not contribute directly to the development of *H. pylori*-induced gastritis, they may contribute to *H. pylori* colonization in the stomach.

1.3.3 Cytokines involved in *H. pylori* infection

Generally, cells involved in both innate and adaptive immunity produce cytokines upon stimulation. *H. pylori* infection induces the production of many pro-inflammatory cytokines. For instance, the expression levels of IL-1β, IL-6, IL-8, IL-10, IL-12, IL17, IL-21, IL23, IFNγ and TNFα were found to be significantly higher in *H. pylori*-infected stomachs compared to uninfected stomachs [199, 207-210].

Pro-inflammatory cytokines induced by *H. pylori* contribute to the development of gastric lesions. The level of IFNγ correlates well with the development of gastritis. *H. pylori* was able to colonize IFNγ-deficient mice without inducing gastritis or inflammation [194, 199, 211-213]. Gastritis was completely abolished when treated *H. pylori*-infected mice with specific IFN-γ AB [194]. These results indicate that IFNγ is required for *H. pylori*-induced gastritis. Colonization of *H. pylori* in IFNγ-deficient mice has shown variations across different studies. In one study, *H. pylori* colonization decreased four or eight weeks after infection, but no changes after long-term infection in IFNγ-deficient mice were observed [211]. In another study, *H. pylori* colonization in IFNγ-deficient mice increased four weeks after infection [212]. Other studies showed that the lack of IFNγ did not impact *H. pylori* colonization neither after short-term nor long-term infection [199, 213]. Therefore, the influence of IFNγ in *H. pylori* colonization is inconclusive. However, the function of IL-17 has been more clearly revealed. *H. pylori* colonization in IL17-deficient mice was significantly lower than in wild-type mice [195, 214]. Likewise, inhibition of IL-17 with anti-IL17A AB treatment before infection significantly reduced *H. pylori* gastric colonization and inflammation [195].

TNFα might play a limited role in *H. pylori*-induced pathogenesis, since TNFα-deficient mice developed the same degree of gastric inflammation as wild-type mice, although higher *H. pylori* colonization was observed [215, 216]. TGFβ is secreted by gastric epithelial cells and was reported to

prevent CD4+ T cell proliferation and promote the proliferation of Tregs upon *H. pylori* infection [217].

Interleukins play diverse roles in *H. pylori*-induced inflammation. Deficiency of IL-10 led to more severe inflammation compared to that in wild-type mice in response to *H. pylori* infection [186, 218]. Additionally, IL-12, secreted by neutrophils, macrophages and DCs, promoted a Th1 response upon *H. pylori* infection [219], although no significant changes in inflammation were observed in IL-12-deficient mice compared to wild-type mice [212, 213]. Thus, IL-12 may play a role in regulating Th1 responses rather than a role in gastric inflammation. However, another cytokine, IL-21, contributes to gastric inflammation development. IL-21 deficiency increased *H. pylori* colonization but sharply decreased the degree of the inflammation in mice. Its deficiency resulted in lower levels of IL-17 and IFNγ, indicating a significant role of IL-21 in mediating Th1/Th17 responses [220].

1.4 *H. pylori* treatment

1.4.1 Antibiotic treatment

1.4.1.1 Antibiotic therapy regimens

Despite the strong inflammatory response elicited by *H. pylori*, the host immune system is not able to clear the infection, leading to chronic gastric inflammation and other serious gastric lesions that persist for decades.

Current treatment regimens, including triple therapy (initial therapy), sequential therapy, quadruple therapy, concomitant therapy and hybrid therapies, are combinations of common and new antibiotics to ensure a more effective eradication of *H. pylori*. Three treatment stages (first-line treatment, second-line treatment, and third-line treatment) are usually required for completing *H. pylori* eradication. A typical therapy normally starts with the first-line treatment such as typical triple therapy, sequential and hybrid therapy. The initial recommended regimen is the triple therapy, including amoxicillin, clarithromycin and a proton pump inhibitor (PPI) [221]. Theoretically, a PPI blocks the hydrogen/potassium adenosine triphosphatase enzyme system and neutralizes the hydrogen (H^+) secreted

by parietal cells in the gastric mucosa, resulting in a reduction of gastric acid [222]. Antibiotics, like amoxicillin and clarithromycin, kill the bacteria through the inhibition of cell wall formation or bacterial protein biosynthesis [223]. However, bacteria growing under antibiotic pressure evolve to generate mutations in genes that enhance their survival and growth.

If the first line therapy fails to eradicate *H. pylori* or patients have high dual resistance to clarithromycin and metronidazole, second-line treatment is suggested. Normally, high eradication rates (exceeding 90%) of *H. pylori* are achieved after second-line treatment [188]. If not, third-line treatment is recommended for eradication after testing antibiotic susceptibility. Despite a number of well-designed studies, the optimal anti-*H. pylori* therapeutic regimen has not been defined due to the complex stomach microenvironment and antimicrobial resistances in different individuals [221]. Nevertheless, effectiveness, cost, ease of administration and side effects should be taken into consideration when selecting the treatment regimen for patients.

1.4.1.2 Antibiotic resistance

Antibiotic treatment has become a controversial issue when people are paying more attention to their side effects, high recurrence rates and development of multidrug resistance [224]. For typical *H. pylori* treatment, three antibiotics (clarithromycin, metronidazole and amoxicillin) are recommended in the initial triple therapy. Antibiotic resistance rates against these three drugs in different regions are presented in Figure 3, including areas from America [225], Sweden [226], Italy [227], Poland [228], France [229], Germany [230], Netherlands [231], Turkey [232], Japan [233], South Korea [234], China [235], Saudi Arabia [236], Iran [237] and Africa [238].

As shown in Figure 3, the prevalence of *H. pylori* primary drug resistance rates are different between countries and continents due to the choices of antibiotic concentrations used in specific regions. The high incidences of antibiotic resistance are present in countries where clarithromycin and metronidazole are widely administrated for therapeutic treatment of *H. pylori* infection (Figure 3). However, in general, countries that advocate

prudent consumption of antibiotics present lower tendency of antibiotic resistance, such as lower metronidazole resistance rates in most of the developed countries (Figure 3). In spite of high susceptibility to amoxicillin in most areas, Africa presents the highest resistance rate, which may correlate with the bad hygiene conditions and antibiotic excessive use. In general, the worldwide tendency of *H. pylori* antibiotic resistance has increased significantly over the past several years. For example, the prevalence of clarithromycin and amoxicillin resistance increased by 17% and 13% respectively from 1997 to 1999 [239]. Gao et al. monitored *H. pylori* resistance over 10 years in Beijing and observed a significant increment of clarithromycin resistance (from 14.8 to 65.4%), metronidazole (from 38.9 to 78.8%,), and fluoroquinolone resistance (from 27.1 to 63.5%) [240]. A similar tendency toward metronidazole resistance was also reported in the United States [241]. A systematic meta-analysis of global prevalence of *H. pylori* antibiotic resistance from 1993 to 2009 revealed an overall diversity of antibiotic resistance in geography, including the antibiotic resistance to amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, and multi-drugs [238]. The divergence of antibiotic resistance was also observed in different age groups. Japanese youth presented a higher clarithromycin resistance rate than Japanese middle-aged and elderly people [242]. In addition to antibiotic resistance, the side effects caused by antibiotic therapies need to be seriously addressed. Around 35.5% of patients in America who received bismuth quadruple therapy or a clarithromycin triple therapy against *H. pylori* infection developed side effects [243]. Therefore, developing novel preventive strategies against *H. pylori* infection such as vaccines should be undertaken for controlling the high incidence of antibiotic resistance.

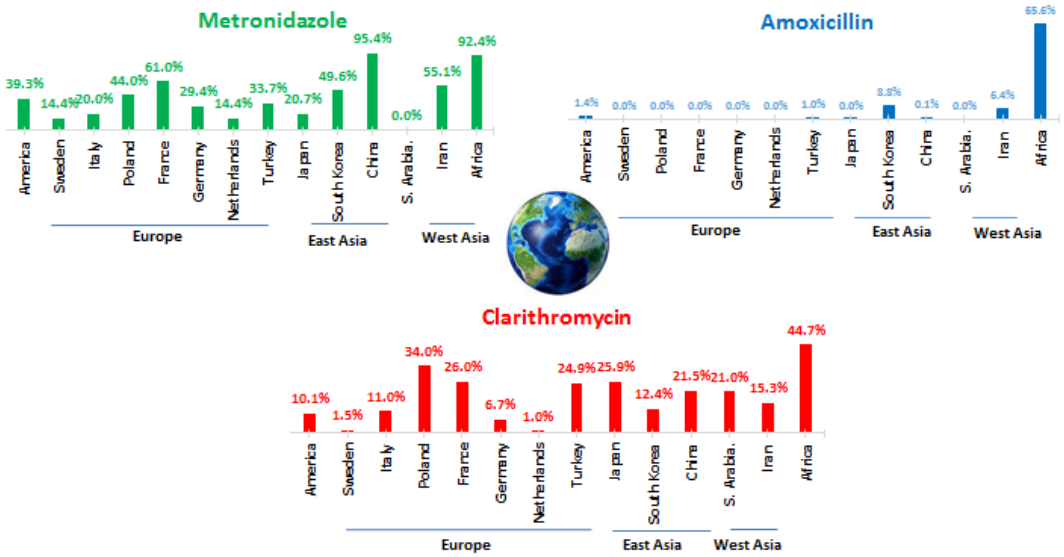


Figure 3 Global primary antibiotic resistance rates. Data shows the prevalence of primary drug resistance rates (%) in different countries and continent between 2000-2010, including America [225], Sweden [226], Italy [227], Poland [228], France [229], Germany [230], Netherlands [231], Turkey [232], Japan [233], South Korean [234], China [235], Saudi Arabia [236], Iran [237] and Africa [238]. Resistance to clarithromycin, metronidazole or amoxicillin is listed.

1.4.2 Vaccinations against *H. pylori* infection

1.4.2.1 Vaccine-induced immunity against *H. pylori* infection

The vaccine-induced immune response against *H. pylori* infection is mainly dependent on cytokines and CD4+ T helper cells, but not on CD8 cells or B cells. A dominant Th1 response and a Th1/Th17 modulatory mechanism contribute to vaccine-induced protection against *H. pylori* (see below). The immune responses generated by different vaccine strategies are compared and shown in Tables 1 to 4.

Role of cytokines in vaccine-induced protection

IL-12, IL-18, IL-23, IFNγ and IL-17A are reported to contribute to the protective immunity against *H. pylori* infection.

IL-12 (p35/p40) is a heterodimeric cytokine composed of the p35 and p40 subunits. IL-23 (p19/p40) also contains a p40 subunit that forms a complex with a different subunit, p19. Because both IL-12 and IL-23 share a p40 unit, they can be neutralized by anti-p40 antibodies or inactivated by the deletion

of p40 [219]. IL-12 and IL-23 double knockout mice (p40-deficient mice) showed no vaccine-induced protection after prophylactic immunization against *H. felis* and developed less inflammation when compared to wild-type mice [212]. Meanwhile, prophylactic immunization against *H. felis* followed by the blockage of p40 with anti-p40 AB induced an increased colonization compared to non-anti-p40 AB treated mice [244], indicating a protective role of IL-12 and IL-23 recombination (p40) upon vaccination.

Interestingly, similar colonization levels were observed in both p35(IL-12)-deficient mice and p19(IL-23)-deficient mice compared to wild-type mice when mice were immunized with *H. pylori* lysate and then infected with *H. pylori* [244], indicating that a lack of IL-12 or IL-23 as a single deficiency does not alter protection upon vaccination. However, other studies showed that IL-12 does play a protective role upon vaccination. The therapeutic treatment with IL-12 in *H. felis*-infected wild-type mice reduced bacterial colonization and increased the degree of gastric inflammation compared to non-IL-12 treated wild-type mice [244]. Akhiani et al. reported that the colonization was significantly increased in IL-12- or IFNγ-deficient mice compared to wild-type mice when mice were prophylactically immunized with *H. pylori* lysate and infected with *H. pylori* [213]. Thus, IL-12 and IFNγ play an important role in vaccine-induced protection.

Another cytokine, IL-18, is a regulator of Th1 cell differentiation and a cytokine that interacts with IL-12 to promote AB production. IL-18 was suggested not to confer protection upon vaccination against *H. pylori* in IL-18-deficient mice compared to wild-type mice [245]. However, another study found that IL-18 deficiency led to a lack of vaccine-induced protection against *H. pylori* [206]. Therefore, IL-18 may also contribute to vaccine-induced protection.

Role of CD4+ T cells and CD8+ T cells in vaccine-induced protection

CD4+ T cells and CD8+ T cells are activated by MHCII and MHCI molecules, respectively. Immunization studies on MHCI- and MHCII-deficient mice revealed that CD4+ T cells, but not CD8+ T cells, protect the host against *H. pylori* infection. Pappo et al. reported that the colonization

in both MHCI-deficient and wild-type mice was significantly reduced compared to unimmunized mice upon bacterial lysate-based prophylactic immunization against *H. pylori*, but the colonization level in MHCII-deficient mice was the same as that observed in non-immunized mice [246]. These findings indicate that CD4+ T cells, but not CD8+ T cells, are responsible for vaccine-induced protection. Moreover, serum IgG1 response was not detectable in MHCII-deficient mice, indicating a role of CD4+ T cells in regulating antibody production [246]. A similar protective role of CD4+ T cells was observed when urease was used as an antigen in vaccination experiments [247]. These results suggest a major role of CD4+ T cells in vaccine-induced protection against *H. pylori* infection.

Role of Th1, Th2 and Th17 cells in vaccine-induced protection

Since Th2 cells are characterized by the secretion of IL-4, effects of altered levels of IL-4 can reflect the role of Th2 cells in the adaptive immune response against *H. pylori* infection. The deficiency of IL-4 did not influence bacterial colonization when mice were prophylactically immunized with bacterial lysate and infected with *H. pylori* [213, 248], indicating Th2 cells may not play a role in the process of the vaccine-induced protection against *H. pylori* infection.

As mentioned before, *H. pylori* induces strong Th1 and Th7 responses, which have been also implicated in vaccine-induced protection. IFNγ deficiency has been shown to result in insufficient or a complete inability to develop protection against *H. pylori* infection [192, 212, 213], which suggests an essential role of IFNγ in regulating vaccine-induced protection. Since Th1 immune response is characterized by the production of IFNγ, the role of IFNγ in bacterial clearance can be used as surrogate of Th1 response in *H. pylori* infection. Interestingly, Th1 immune responses could compensate for the lack of Th17 cells upon vaccination against *H. pylori* [244]. At the same time, the deficiency of IL-17 affected Th1 cell-mediated immune response against *H. pylori* infection [195], indicating that Th1 and Th17 responses modulate each other during vaccine-induced protection against *H. pylori* infection.

As shown in Table 3, neutralization of IL-17 with anti-IL-17A AB increased *Helicobacter* colonization after prophylactic immunization and infection [249, 250], but neutralization of TNF [250] and IFNγ [250, 251] did not affect colonization. These results indicate that IL-17 pre-depletion before infection decreases the subsequent *H. pylori* colonization [195, 214], while IL-17 depletion after prophylactic immunization and infection enhances *H. pylori* colonization [250]. In contrast, the depletion of IFNγ and TNF after immunization followed by infection dose not impact *H. pylori* colonization [250].

However, the deficiency of IL-17A did not impact the vaccine-induced protection against *H. pylori* when mice were immunized with either *H. pylori* lysate [252], ureB or UreB epitope fragments [249], since the colonization levels did not change in IL-17A-deficient mice compared to wild-type mice upon prophylactic vaccination against *H. pylori* [249, 252]. These studies indicate that the vaccine-induced protection in the context of IL-17 deficiency is generated by a Th1 response. This hypothesis was verified by the adoptive transfer of Th1 and Th17 cells. Adoptive transfer of UreB epitope-specific Th1 cells reduced *H. pylori* colonization [249]. In contrast, the transfer of UreB epitope-specific Th17 cells did not influence *H. pylori* colonization when compared to non-transferred mice (Table 2). Therefore, although prophylactic immunization induces Th1 and Th17 responses, the vaccine-induced protection is generated by Th1 response but not Th17 response. A dominant Th1 response and a Th1/Th17 modulatory mechanism contribute to vaccine-induced protection against *H. pylori*.

Role of B cells in vaccine-induced protection

Although the vaccination studies against *H. pylori* generate stable antibody production, B cells may not play an important role in vaccine-induced protection against *H. pylori* infection. The deficiency of B cells did not change the vaccine-induced protection upon *H. pylori* infection [206]. Therapeutic immunization against *H. pylori* in B cell-deficient mice showed that B cell deficiency and the vaccine-induced immune protection were independent [253]. The same result was observed upon vaccination following *H. felis* infection [205]. Therefore, B cells may not participate in

the process of vaccine-induced protection. However, antibodies secreted from plasma cells in the gastric mucosa induced neutrophil infiltration, allowing recruitment of T cells and DCs, and facilitating the clearance of *H. pylori* [254].

Table 1 Prophylactic Immunization/Challenge in knockout mice

Mouse strains	Immunization	Infection	Pathology		Conclusions
			Colonization	Inflammation	
MHC II -/- C57BL/6	Urease + LT, o.i.	SS1	↑	not reported	CD4 T helper cells, but not CD8 cells or B cells, contributed to the clearance of *H. pylori* infection [247].
MHC I-/- C57BL/6, B cell -/- C57BL/6	Urease + LT, o.i. Urease + LT, o.i.	SS1	both ↔	not reported	
MHC I -/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↔	not reported	The deficiency of MHCI, but not MHCII, contributed to vaccine-induced protection against *H. pylori* [246].
MHC II-/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↑	not reported	
B cell -/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↗	similar CD4+ T cell infiltration	The deficiency of B cells did not influence *H. pylori* clearance upon prophylactic vaccination [206].
IgA -/- C57BL/6, B cell -/- C57BL/6	*H. felis* lysate + CT, o.i.	*H. felis*	both ↗	both similar	The deficiency of IgA or B cells had minor impact in *H. pylori* clearance upon prophylactic vaccination against *H. felis* [205].
IL18 -/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↔	not reported	IL-18 deficiency did not impact the protection upon vaccination against *H. pylori* [245].
IL18 -/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↗	less gastritis	IL-18 deficiency failed to develop protection upon vaccination against *H. pylori* [206].
IL4 -/- C57BL/6	SS1 lysate + CT, o.i.	SS1	↔	significantly lower	Th2 response displayed no effect on immune protection against *H. pylori*, and the immune protection did not directly relate with the degree of stomach inflammation [213].
IL4 -/- C57BL/6	urease carried by Salmonella strain o.i.	*H. pylori*	↔	not reported	Vaccine-induced protection was independent of IL-4 and IL-13 [248].

Mouse strains	Immunization	Infection	Pathology		Conclusions
			Colonization	Inflammation	
IL-12 p35-/-C57BL/6, IL-23 p19 -/- C57BL/6	*H. felis* lysate + CT,i.i	*H. felis*	both ↔	not reported	Either IL-12 or IL-23 single deficiency did not impact vaccinated protection against *H. felis* [244].
p40 -/- C57BL/6	SS1 lysate + CT, i.i.	SS1	↑	significantly lower	p40-deficient mice showed no protection in prophylactic immunization against *H. pylori* and develop less inflammation after immunization [212].
IFNγ -/- C57BL/6	SS1 lysate + CT, i.i.	SS1	↗	similar gastritis	IFN-γ deficient mice developed an insufficient protection against *H. pylori* than wild-type mice [212].
IFNγ -/- C57BL/6	SS1 lysate + CT, i.i.	SS1	↑	significantly lower	CD4 mediated IFN-γ was essential for *H. pylori* clearance upon vaccination against *H. pylori* [192].
IFNγ -/- C57BL/6, IL12 -/- C57BL/6	SS1 lysate + CT, i.i.	SS1	↑	both significantly reduced	Both IFNγ and IL-12 deficient mice failed to develop protection in prophylactic immunization against *H. pylori* [213].
IL-17-/- BALB/c	SS1 lysate + CT, i.i	SS1	↔	similar gastritis	Deficiency of IL-17A did not impact the vaccine-induced protection against *H. pylori* [252].
IL-17-/- BALB/c	UreB or UreB epitopes + CpG, i.i	*H. pylori*	↔	not reported	Deficiency of IL-17A did not impact the vaccine-induced protection, while Th1 cells protected the host against *H. pylori* infection in the absence of IL-17A [249].

Arrows refer to colonization level compared to wild-type immunized mice. ↔(no differences), ↑(significantly increased), ↗(comparable higher but not statistically different), ↓ (significantly decreased).

Abbreviations: i.i. (intranasal immunization), i.p. (intraperitoneal immunization), o.i. (oral immunization), CT (cholera toxin), LT (E. coli heat-labile enterotoxin).

Table 2 Adoptive transfer/ AB treatment against *Helicobacter* infection

Mouse strains	Adoptive transfer/AB treatment	Infection	Clonization	Conclusions
SCID C57BL/6	wild-type spleen lymphocytes i.p. [187]	SS1	↓	Adaptive transfer of wild-type lymphocytes decreased the colonization and induced severe gastritis in *H. pylori*-infected SCID mice [186, 187]
SCID C57BL/6	wild-type spleen lymphocytes i.p. [186]	SS1	↓	
BALB/c	UreB epitope-specific Th1 cell, i.v.	*H. pylori*	↓	The adoptive transfer of UreB epitope-specific Th1 cell, but not Th17 cell, reduced *H. pylori* colonization [249].
BALB/c	UreB epitope-specific Th17 cell, i.v.	*H. pylori*	↔	
C57BL/6	anti-IL-17 AB, i.p.	SS1	↓	Blockage of IL-17 with anti-IL17 AB significantly reduced *H. pylori* gastric colonization and inflammation [195].

Arrows refer to colonization level compared to non-cell transferred mice or non-AB treatment mice. ↔(no differences), ↓(significantly decreased).

Abbreviations: i.p. (intraperitoneal immunization), i.v. (intravenous immunization).

Table 3 AB neutralization upon immunization/challenge in mice

Mouse strains	Immunization	Infection	AB neutralized	Colonization	Conclusions
C57BL/6	*H. felis* lysate + CT, i.i	*H. felis*	anti- p40 AB, i.p.	↑	Blockage of p40 (IL-12 and IL-23) with anti-p40 AB resulted in a loss of protection against *H. pylori* [244].
C57BL/6	SS1 lysate + CT, sublingually	SS1	anti-IL17A AB, i.p.	↑	Neutralization of IL-17 with anti-IL-17A AB increased the colonization upon vaccine-induced protection against *H. pylori* [250].
C57BL/6			anti-TNF AB, or anti-IFNγ AB, i.p.	both ↔	
C57BL/6	Urease + CT, i.p.	*H. felis*	anti-IL17 AB, i.p.	↑	Blockage of IL-17 with anti-IL-17A AB resulted in a loss of protection upon vaccine-induced protection against *H. felis* [251].
BALB/c	SS1 lysate + CT, i.i	SS1	anti-IFNγ AB, i.p.	↔	AB blockage of IFNγ did not impact the vaccine-induced protection [252].

Arrows refer to colonization level compared to non-AB treated mice or control IgG treated mice. ↔ (no differences), ↑(significantly increased).
Abbreviations: i.i. (intranasal immunization), i.p. (intraperitoneal immunization), CT (cholera toxin).

Table 4 Therapeutic Challenge/Immunization against *H. pylori*

Mouse strains	Infection	Immunization	Protection	Conclusions
BALB/c	*H. felis*	IL-12 i.p.	yes	Therapeutic treatment with IL-12 against *H. felis* infection reduced bacterial colonization and increased the degree of gastric inflammation [244].
B cell -/- C57BL/6,	SS1	SS1 lysate + CT, o.i.	no	B cell-deficient mice showed the same protection level as wild-type mice in therapeutic immunization against *H. pylori* infection [253].
WT C57BL/6	SS1	SS1 lysate + CT, o.i.	yes	
BALB/c	*H. pylori* B6	CagA-VacA-UreB fusion through *Salmonella* system, o.i.	yes	Therapeutic treatment with CagA-VacA-UreB fusion protein against *H. pylori* infection related to Th1 response, serum IgG and mucosal sIgA antibody responses [255] .
C57BL/6	*H. pylori*	*Salmonella*-delivered OipA, o.i.	yes	Protection against *H. pylori* with *Salmonella*-delivered OipA was associated with an abundant Th1/Th2 immune responses [256]

Abbreviations: i.i. (intranasal immunization), i.p. (intraperitoneal immunization), o.i. (oral immunization), CT (cholera toxin)

1.4.2.2 Adjuvants used for *H. pylori* vaccination

H. pylori vaccines can be administered intranasally, intraperitoneally or orally. Intranasal immunization showed similar effects as oral immunization, but with lower dosage requirements for immunization [257, 258]. Common adjuvants for boosting host immunity include cholera toxin (CT), chitosan, *E. coli* heat-labile enterotoxin (LT), and CpG oligodeoxynucleotides (CpG ODN). Among these adjuvants, cholera toxin is the most prevalent adjuvant for *H. pylori* vaccination used in mice [259]. This adjuvant increases the vascular permeability and mucosal IgA production. Another adjuvant, CTA1-DD, which was derived from cholera toxin, showed similar protection levels as cholera toxin, but it did not induce inflammation in mouse stomach [260]. The adjuvant chitosan eliminated more bacteria than cholera toxin in the therapeutic treatment against *H. pylori* infection [261]. Nevertheless, the choice of suitable adjuvants is one important aspect to achieve the optimal immune protection.

1.4.2.3 The development of *H. pylori* vaccines in mice

Due to the high prevalence of antimicrobial resistance, new therapeutic options and vaccinations seem to be profitable approaches. The choice of the appropriate vaccine antigens is determined by the antigen-induced immunogenic protection rather than antigen immune recognition. So far, some vaccination approaches against *H. pylori* infection have demonstrated effectiveness when mice were immunized with specific antigens together with an adjuvant. Many studies have tested either a single antigen or a mixture of recombinant antigens (Table 5).

In the past few decades, around 1700 *Helicobacter* predicted proteins have been elucidated through genomic sequencing of *H. pylori* strains [92], and some of these proteins have been applied for immunization in mice. As shown in Table 5, many proteins, such as catalase, CagA, VacA, HpaA, gGT, HspA and urease, have been tested in mice and show protective effect against *H. pylori* infection. Among these proteins, urease is the most tested antigen, which has been demonstrated to be an effective vaccine not

only for mice but also the other animal models, such as rhesus monkeys [262].

Meanwhile, compared to single antigens, multi-component-vaccines have been tested to show higher vaccine-induced protection against *Helicobacter* infection, such as UreA plus HpaA [263], omp22 plus HpaA [264], gGT plus UreB [265], SOD plus catalase [266], Tpx plus catalase [266], GreES plus UreB [267], and HspA plus gGT [268]. Additionally, fusion epitopes are also promising strategies for vaccination based on *Salmonella* expression system. The immunization of CagA-VacA-UreB fusion or optimized OipA delivered by *Salmonella*-based expression system significantly reduces *H. pylori* colonization in the mouse gastric mucosa [255].

However, in most cases, vaccine-based immunizations do significantly reduce, but seldom completely eradicate *H. pylori* colonization in the gastric mucosa, either in murine, nonhuman primates or human beings. Until now, only a few studies have reported complete sterilizing protection in mice [269-271]. This raises the question about what kind of vaccine formulations would induce a high rate of *H. pylori* eradication or even complete protection. Actually, any of the *H. pylori* proteins are potential vaccine candidates. However, the challenge is in selecting efficient *H. pylori* vaccine formulations. There is no doubt that multi-component-antigen vaccines are better than single antigen formulations. However, the identification of efficient vaccine formulations for *H. pylori* still requires further investigation.

Table 5 Properties of common single antigen administration in mice

Single antigen	Surface exposed [a]	Seroreactive in humans[b]	Protection
AhpA (alkyl hydroperoxide reductase)	+	+	yes [254]
AhpC (alkyl hydroperoxide reductase)	+	+	yes [272]
CagA	secreted	+	yes [273]
Catalase	secreted	+	yes [274]
Citrate synthase	secreted	+	yes [275]
Epitope UreB317-329	a.c.	+	yes [249]
Epitope UreB409-421	a.c.	+	yes [249]
Flagellin	+	+	yes [276]
gGT	secreted	+	yes [265]
GroES	secreted	+	yes [267]
Hemolysin secretion protein precursor	secreted	+	yes [277]
HP0175	secreted	+	n.d.
HP0231	secreted	+	yes [278]
HP0410	+	+	yes [278]
HpaA	+	+	yes [94, 279]
HspA (heat shock protein A)	secreted	+	yes [268]
HspB	secreted	+	yes [265]
HtrA	+	+	n.d.
Lipoprotein Lpp20	+	+	yes [280]
NapA	secreted	+	yes [104], no[281]
OipA (outer inflammatory protein A)	+	+	yes [256]
Omp22	+	n.d.	yes [264]
SOD (Superoxide dismutase)	secreted	+	yes [282]
Tip-α	secreted	n.d.	yes [283]
Tpx (Thiolperoxidase)	secreted	+	n.d.
UreA	secreted	+	yes [284]
UreB	secreted	+	yes [249, 285]
VacA	secreted	+	yes [286]

Abbreviations: a (data from [29, 254, 287-289]), b (data from [290-292]), n.d. (not detected), a.c. (artificial cloned)

1.4.2.4 Clinical trials of *H. pylori* vaccines

Based on studies in mice, vaccination trials in humans have been carried out for the treatment of *H. pylori* infection. These clinical studies aim to prevent an experimental challenge, or eliminate a natural *H. pylori* infection, or test the immunogenicity and safety for several vaccine formulations (Table 6). The first human trial was conducted to eradicate the natural infection with orally immunized urease plus *E. coli* heat-labile enterotoxin (LT), and led to a significant decrease of bacterial colonization but serious diarrhea in 66% of the volunteers [284]. The incidence and severity of the side effects was not linked to urease, but to the adjuvant, LT, which induced diarrhea even at a low dose (2.5 µg/person) in many individuals [293]. Thereafter, another clinical trial showed similar side effects induced by LT and failed to promote *H. pylori* eradication in infected volunteers through oral administration of a formalin-inactivated *H. pylori* whole-cell vaccine [294]. Thus, clinical trials indicate the antigens used for *H. pylori* vaccine are immunogenic but choice of adjuvants for oral administration is problematic.

Therefore, several immunogenicity and safety tests were carried out with different vaccine formulations. Low LT doses were immunogenic after rectal immunization, but urease failed to induce either humoral or T cell mediated responses [295]. The intramuscular administration of recombinant VacA, CagA and NapA with aluminium hydroxide resulted in antigen-specific T cell memory, antigen immunogenicity, and mild side effects in vaccinated volunteers [296]. This study indicates that aluminium hydroxide is a better adjuvant than LT for human trials. Additionally, oral administration of phoP/phoQ-deleted *Salmonella*-based urease in human trials showed an undetectable or a very low level of urease-specific immune response, this was a dramatic difference compared to results in mice [297, 298]. Another *Salmonella* strain, Ty21a, carrying a urease based vaccine, showed mild side effects and induced antigen-specific humoral and cellular responses [299]. However, Ty21a *Salmonella* expressing urease or HP0231 did not show satisfactory protection when compared to Ty21a *Salmonella*-treated volunteers, although milder

dyspeptic symptoms and 69% *H pylori*-specific T helper cells were detected [300]. Although it is a safe system and induces only very mild adverse reactions, the results indicate that the *Salmonella* expressing system for antigen delivery in humans is insufficient. In conclusion, the inconclusive results of the clinical trials in eradication of *H. pylori* still encourage the development of novel vaccines.

Table 6 Clinical trials for *H. pylori* vaccination

Volunteers	Antigens, Adjuvant and route	Purpose	Conclusions
H. pylori infected	Urease, LT, oral immunization	Therapeutic administration for infected individuals	Significant decrease of *H. pylori* density following urease administration, but high incidence of diarrhea due to the adjuvant [234].
H. pylori infected	Inactivated *H. pylori* whole-cell vaccine, LT or mutant LT (LTR192G), oral immunization	Therapeutic administration for infected individuals	Oral administration with whole-cell formalin-inactivated *H. pylori* showed no effect on reducing *H. pylori* colonization in infected volunteers [294].
non-infected	recombinant VacA, CagA, and NapA, aluminium hydroxide intramuscular	Immunogenicity and safety test	Antigen-specific T cell memory, antigen immunogenicity and mild side effects [296].
non-infected	Urease, LT, rectal	Antigen safety and LT efficacy test	LT was immunogenic, urease failed to induce either humoral or T cell-mediated responses [295].
non-infected	phoP/phoQ-deleted *Salmonella* expressing urease, oral immunization	Immunogenicity and safety test	Undetectable or very low levels of urease-specific immune response [297, 298].
non-infected	Ty21a *Salmonella* strain expressing *H pylori* urease, orally immunization	Immunogenicity and safety test	Slight side effects and induction of antigen-specific humoral and cellular responses [299].
non-infected	Ty21a expressing *H. pylori* urease or HP0231, oral immunization	Immunization followed by *cag*PAI negative *H pylori* infection	No protection observed [300].

1.5 Aim of the present study

General objectives:

H. pylori infection is the most prevalent bacterial infection around the world. Billions of euros are spent each year on the treatment of *H. pylori*-induced gastric diseases, especially gastric cancer and ulcers [22, 301]. However, current antibiotic treatments are not effective because of the high prevalence of antimicrobial resistance. Therefore, novel vaccination strategies are urgently needed to offer protection against this pathogen. For this purpose, identifying potential vaccine candidates and elucidating their functional roles are extremely important in developing novel vaccines for the treatment of *H. pylori* infection. This will not only contribute to our understanding of host-pathogen interactions and vaccine-induced immunity, but will help to decipher the mechanisms of effective vaccinations to successfully treat *H. pylori*.

Specific objectives:

Multiple pathogen-derived virulence factors contribute to *H. pylori* infection. Identifying the immunogenicity of these antigens in the serum of *H. pylori*-infected patients is an important step to evaluate potential vaccine candidates. Therefore, the first objective of this dissertation was to select potential vaccine candidates based on their protein sequence conservation and protein seroreactivity in *H. pylori*-infected patients with different *H. pylori*-induced diseases. After identifying the vaccine candidates, the influence of the selected vaccine candidates on *H. pylori* colonization was examined. Moreover, the vaccine efficacies of these antigens against *H. pylori* infection were evaluated in mice, and the vaccine induced-protection was also investigated. Finally, the functional role of the effective candidate was studied.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Bacteria, cells and mouse strains

2.1.1.1 Bacteria

Table 7 List of bacterial strains

Bacterium	Description	Reference
PMSS1/SS1	Human clinical isolate, can colonize in mouse stomach	[302, 303]
G27	Human clinical isolate	[304]
P12	Human clinical isolate	[305, 306]
PMSS1Δ*hp0231* G27Δ*hp0231* P12Δ*hp0231* SS1Δ*hp*0231	The *hp0231* gene was replaced by a chloramphenicol resistance cassette	In this study
J99Δ*napA*	The *napA* gene was replaced by a kanamycin resistance cassette	[107]
PMSS1Δ*napA*	The *napA* gene was replaced by a kanamycin resistance cassette	In this study
PMSS1Δ*ggt* SS1Δ*ggt* G27Δ*ggt* P12Δ*ggt*	*The ggt* gene was replaced by a kanamycin resistance cassette	[307]
PMSS1*hp0231*+ P12*hp0231*+	Complemented strain of PMSS1Δ*hp0231* and P12Δ*hp0231*	In this study
PMSS1Δ*cagA* G27Δ*cagA* P12Δ*cagA*	The *cagA* gene was replaced by a chloramphenicol resistance cassette	[308]
PMSS1Δ*cagE* G27Δ*cagE*	The *cagE* gene was replaced by a chloramphenicol resistance cassette	[309]
G27Δ*sabA*	The *sabA* gene was replaced by a chloramphenicol resistance cassette	[310]

Bacterium	Description	Reference
G27Δ*babA*	The *babA* gene was replaced by a kanamycin resistance cassette	[310]
G27Δ*babA/sabA*	The *sabA* and *babA* genes were replaced by chloramphenicol resistance cassette and kanamycin resistance cassette respectively	[311]
P12Δ*vacA*	The *vacA* gene was replaced by a chloramphenicol resistance cassette	[312]
DH5α	*E. coli* competent cells	NEB company
DE3	*E. coli* competent cells	[313]

2.1.1.2 Cell lines

Name	Description	ATCC Nr /reference
AGS	Human gastric cancer cell line, derived from a female patient with gastric adenocarcinoma with no prior anti-cancer treatment	ATCC CRL-1739
MKN45	Human gastric cancer cell line, derived from a poorly differentiated adenocarcinoma from the stomach of a female patient	[314]

2.1.1.3 Mouse strains

Name	Description	Source
BALB/c	6 to 8 weeks age old BALB/c congenic mice	Harlan Laboratories, USA
C57BL/6	6 to 8 weeks age old C57BL/6 congenic mice	Harlan Laboratories, USA

2.1.2 Primers and Plasmids

2.1.2.1 Primers

Table 8 Primers

Name	Primer sequence 5'-3'
V1HP231f	AAG CGA AGA TGT GAA ATT AG
V2HP231r	GAG CGT TAA TAT CGT ATT GG
V3NapAf	GGA ATT CCA TAT GAA AAC ATT TGA AAT TCT AAA ACA T
V4NapAr	GCC CTC GAG AGC CAA ATG GGC TTG CAG
P10231f	GTTTAACTTTAAGAAGGAGATATACAT ATG AAT GAC AAA CGG ATG CAG GAT AA
P20231r	ATC TCA GTG GTG GTG GTG GTG GTG CTC GAG TGC CTT ATA ATG GTA TAA AAA AGG C
P3NapAf	G GAATTC CAT ATG AAA ACA TTT GAA ATT CTA AAA CAT
P4NapAr	CCG CTC GAG AGC CAA ATG GGC TTG CAG
P5HpaAf	CAT ATG GAA ACC AAT GAA GTC GCT TTG
P6HpaAr	AAG CTT CTA GTG ATG GTG ATG GTG ATG
GAPDHf	GCC TTC TCC ATG GTG GTG AA
GAPDHr	GCA CAG TCA AGG CCG AGA AT
IL17f	GCT CCA GAA GGC CCT CAG A
IL17r	AGC TTT CCC TCC GCA TTG A
IFNγf	TCA AGT GGC ATA GAT GTG GAA GAA
IFNγr	TGG CTC TGC AGG ATT TTC ATG

2.1.2.2 Plasmids

Table 9 Plasmids

Name	Description	Source
pET−30b+	Prokaryotic protein expression vector	AddGene Catalog Nr: 69909-3
pGMTeasy-Δ231	Cloning vector for *hp0231*-knockout strain construction	Provided by Prof.Krynicka [143]
pHeL3-231+	Shuttle vector for *hp0231*-complemented strain construction	Provided by Prof.Krynicka

2.1.3 Buffer, solutions and media

2.1.3.1 Cell culture media

AGS and MKN45 cell culture medium:

DMEM, 10% FCS, P/S (Penicillin/Streptomycin, 100x), 2 µg/ml ciprofloxacin

Lymphocyte culture medium:

RPMI 1640 Medium, 10% FCS, P/S (100x)

EDTA HBSS solution:

20 mM EDTA in Ca/Mg free HBSS buffer

Primary cell culture medium:

DMEM/ F12 1:1, 10% FCS, 100 µg/ml primocin, 0.5 µg/ml gentamycin, 4 µg/ml ciprofloxacin

Red blood cell lysis buffer:

155 mM NH_4Cl, 10 mM $KHCO_3$ or $NaHCO_3$, 0.1 mM EDTA, pH 7.3,

Rat tail collagen stock solution:

3 mg/ml, 4°C in dark

Pre-coating solution for primary cell isolation:

10 µg/cm^2 rat tail collagen in 0,02 N acetic acid

2.1.3.2 Bacterial culture media

DENT antibiotic (250x):

5 µg/ml trimethoprim, 5 µg/ml amphotericin B, 10 µg/ml vancomycin, 5 µg/ml cefsulod in ddH_2O

BHI DENT medium:

3.7% (w/v) brain heart infusion (BHI) broth, 20% FCS, DENT antibiotic

H. pylori liquid culture medium:

10% FCS, DENT antibiotic (250x), 3.7% (w/v) Brucella broth

LB medium:

1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1% (w/v) tryptone, pH 7.4

SOC medium:

2% w/v tryptone, 0.5% w/v yeast extract, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose

TB medium:

12 g tryptone, 24 g yeast extraction, 4 ml glycerol, filled up with ddH_2O to 900 ml.

Protein auto-induction medium:

100 ml potassium phosphate buffer (pH 7.0), 2 ml 1M MgSO4, 2g lactose, 0.2 ml poly propylene glycol, 900 ml TB medium.

WC DENT agar plates:

21.5 g WC-agar, 0.2 g KNO_3, 50 ml defibrinated horse blood, DENT antibiotic (250x) in 500 ml ddH_2O

WC DENT special plates:

WC DENT agar plates containing 200 µg/ml bacitracin, 10 µg/ml nalidixic acid, and 3 µg/ml polymyxin B. NaOH was used to adjust pH to dissolve the chemicals and HCl was then used to adjust the buffer to pH 7.

H. pylori mobility assay plates:

0.35% (w/v) agar, 10% (v/v) FCS, 2.8% (w/v) Brucella broth base, DENT antibiotic (250x), pH 7,4.

LB agar plates:

0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1.5% (w/v) agar, pH 7.4

All the agar plates were autoclaved and cooled to around 60 °C before adding antibiotic, FCS or blood. After cooled down to room temperature, plates were stored at 4 °C for long term usage.

2.1.3.3 Protein purification solutions

Lysozyme stock:

50 mg/ml (50x), -20°C

Protease inhibitor AEBSF hydrochloride stock (100x):

100 mM AEBSF stock

DNase stock:

15,000 U/ml (1000x), -20°C

Ni-NTA binding buffer:

20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4, 4°C

Ni-NTA elution buffer:

20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4, 4°C

NapA SEC buffer:

20 mM sodium phosphate, 500 mM NaCl, 2.5% glycerol, pH 5.5, 4°C

HP0231 SEC buffer:

50 mM sodium phosphate, 150 mM NaCl, 2.5% glycerol, pH 7.4, 4°C

HpaA SEC buffer:

1 M arginine, 250 mM NaCl, 10 mM N-acetyl cysteine, pH 7.4, 4°C

2.1.3.4 Buffers for protein or DNA biochemistry

Nitrocefin stock solution:

290 mM in DMSO, -20°C in dark

NapA activity assay solution I:

450 mM sodium acetate buffer (pH 5.8), RT

NapA activity assay solution II:

130 mM thiourea, 380 mM ferrous ammonium sulfate (Fe $(NH4)_2(SO4)_2$), in ddH_2O, 4°C.

Since the ferrous ions are easily oxidized, 130 mM thiourea solution was firstly prepared. Ferrous ammonium sulfate was then solved in thiourea solution to avoid oxidation.

NapA activity assay solution III:

1.8 mM ferrozine in solution I

GlyGly stock solution:

100 mM GlyGly in Tris-HCl buffer (pH 8.0), 4°C

gGpNA stock solution:

5 mM L-γ-glutamyl-p-nitroanilide, -20°C

Cell lysis buffer:

25 mM Tris pH 6.8, 6% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue

4x SDS buffer:

240 mM Tris/HCl pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 5% ß-mercaptoethanol, 0.004% (w/v) bromophenol blue

FACS buffer:

0.5% BSA in PBS, filtered, 4°C

ELISA blocking buffer:

1% BSA in PBS, filtered, 4°C

ELISA stop buffer:

1 M H_2SO_4, RT

100 mM PMX464 stock solution:

PMX464 (Pharminox Isolation Ltd) was solved in DMSO according to the instructions. Small aliquots were prepared and stored at -20°C in the dark.

Agarose gel electrophoresis buffer 1x TAE:

40 mM Tris, 20 mM acetic acid, and 1 mM EDTA

SDS-PAGE gel wash buffer:

10% glacial acetic acid in ddH_2O

SDS-PAGE running buffer:

25 mM Tris, 0.2 M glycine, 0.1% (w/v) SDS

1xPBS:

1.4 mM KH_2PO4, 4.3 mM $Na_2HPO4{\cdot}2H_2O$, 0.137 M NaCl, 2.7 mM KCl, pH 7.4

1x TBS:

50 mM Tris, 150 mM NaCl, pH 7.5

1x TBS-T:

1x TBS, 0.1% Tween 20

WB blocking buffer:

5% milk powder in TBS-T

Antibody dilution buffer:

5% BSA in TBS-T

Semi-dry transfer buffer:

5.8 g/L Tris base, 2.9 g/L glycine, 0.37 g/L SDS, 200 ml/L methanol

SDS-PAGE gel recipe:

Separating gel (1x):

1.5 M Tris, 0.4& SDS. pH 8.8		1.5 ml
10% APS		30 µl
TEMED		6 µl
6% Separating Gel	40% Acrylamide	0.9 ml
	ddH_2O	3.564 ml
8% Separating Gel	40% Acrylamide	1.2 ml
	ddH_2O	3.264 ml
10% Separating Gel	40% Acrylamide	1.5 ml
	ddH_2O	2.964 ml
12% Separating Gel	40% Acrylamide	1.8 ml
	ddH_2O	2.664 ml
15% Separating Gel	40% Acrylamide	2.25 ml
	ddH_2O	2.214 ml

Stacking gel (1x)

0.5 Tris, 0.4% SDS, pH 6.5	0.5 ml
ddH_2O	1.288 ml
40% Acrylamide	0.2 ml
10% APS	10 µl
TEMED	2 µl

Coomassie blue solution:

10% glacial acetic acid, 40% ddH_2O, 50% ethanol, 0.25% coomassie blue

2.1.4 Line blot

2.1.4.1 Strips for line blot

RecomLine helicobacter IgG strips for line blot were produced by Mikrogen GmbH. 15 *H. pylori* proteins were individually fixed and settled on one nitrocellulose strip. The proteins immobilized on one strip were CagA, VacA, VacAm1, VacAm2, GroEL, UreA, HcpC, gGTmut2, FlgE, HtrA, HpaA, Tig, Jhp940, NapA, and HP0231.

2.1.4.2 Serum samples for line blot

Serum samples for line blot were taken from the *H. pylori*-infected Chinese and German patients with different gastric diseases, including superficial gastritis (SG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM), dysplasia (DYS), and gastric cancer (GC) (Table 10).

Table 10 Patients' serum information

Country	Provided by	Pathology	Cases
China Average age : 51.8	Prof. Pan, Beijing Cancer Hospital, Peking University	Superficial gastritis	86
		Chronic atrophic gastritis	88
		Intestinal metaplasia	88
		Dysplasia	86
		Gastric Cancer	166
Germany Average age: 59.4	Michael Geppert, Doctor's Practice for Gastroenterology, Bayreuth, Germany; Michael Vieth, Department of Pathology, Klinikum Bayreuth, Bayreuth, Germany	Superficial gastritis	243
		Chronic atrophic gastritis	15
		Intestinal metaplasia	32
		Dysplasia	12
		Gastric Cancer	0

2.1.5 Antibodies

Table 11 Antibody list

Antibody	Manufacturer	Dilution
Anti-mouse CD3 PECy7	eBioscience	1:100
Anti-mouse CD3 purified	eBioscience	1:1000
Anti-mouse CD4 Pacific Orange	eBioscience	1:50
Anti-mouse CD45 APC	eBioscience	1:100
Anti-mouse CD8α eF780	eBioscience	1:100
Anti-mouse CD8β PE	eBioscience	1:100
Anti-mouse IFNγ FITC	eBioscience	1:100
Anti-mouse IL17 APC	eBioscience	1:100
Anti-mouse Foxp3 eF450	eBioscience	1:100
Anti-mouse foxp3 eF450	eBioscience	1:100
Anti-mouse IL2 PE	eBioscience	1:100
Anti-mouse TCRΥ/δ- FITC	eBioscience	1:100
Anti-mouse TNFα PE-Cy7	eBioscience	1:100
Anti-mouse gGT multi-clonal AB	In this study	1:1000
Anti-mouse His-tag	AbDSerotec	1:10000
Anti-mouse HP0231 multi-clonal AB	In this study	1:1000
Anti-mouse NapA multi-clonal AB	In this study	1:1000
Anti-mouse NFkB p65 (D14E12)	Cell Signaling	1:1000
Anti-mouse pNFkB p65 (Ser536)	Cell Signaling	1:1000
Anti- mouse pTyrosine	Millipore	1:1000
Anti-mouse VacA (AK197)	Provided by Dr. Fischer	1:2000
Anti-mouse β-actin	Sigma-Aldrich	1:10000
Anti-rabbit CagA	Provided by Dr. Vogelmann	1:3000

2.1.6 Enzymes

Table 12 Enzymes

Name	Manufacturer
DNase	Invitrogen
Herculase II Fusion DNA Polymerase	Agilent Technologies
Proteinase K	Invitrogen
Reverse transcriptase	Promega
RNase	Invitrogen
SYBR Green Mix (2x)	Promega
T4 DNA ligase	New England Biolabs
NdeI	Promega
XhoI	Promega

2.1.7 Kits

Table 13 Kits

Name	Manufacturer
Ammonia assay kit (Rapid)	Megazyme International Ireland
Foxp3 intracellular staining kit	eBioscience
GenElute mammalian total RNA Miniprep kit	Sigma-Aldrich
Gibson Assembly Cloning kit	New England Biolabs
IL-8 ELISA kit	eBioscience
PureLink Genomic DNA Mini kit	Invitrogen
PureYield™ Plasmid Midiprep System	Promega
PureYield™ Plasmid Miniprep System	Promega

2.1.8 Consumables

Table 14 Chemicals

Name	Manufacturer
0,05% Trypsin-EDAT	Gibco Life Technologies
AEBSF	Panreac AppliChem
Ampicillin	Sigma-Aldrich
Bacitracin	Sigma
Bench Top DNA ladder	Promega
BHI (Brain heart infusion)	Oxoid
Brucella broth Base	Fluka
BSA	Panreac AppliChem
CFDA-SE	Life Technologies
Chloramphenicol	Roth
Cholera toxin	Sigma
Ciprofloxacin	KabiPac
Clarity™ western ECL substrate	Bio-Rad
Collagenase IV	Sigma
Coomassie brilliant blue	Thermo Scientific
DAPI	Thermo Fisher Scientific
Deep Red	Thermo Fisher Scientific
DENT antibiotics / supplement	Oxoid
Defibrinated horse blood	Oxoid
DMEM	Gibco Life Technologies
DMEM/ F12	Gibco Life Technologies
DMSO	Sigma-Aldrich

Name	**Manufacturer**
DNase	Sigma
dNTP	Agilent Technologies
DPBS	Gibco Life Technologies
DTT	Applichem
EDTA	Sigma
ELISA substrate reagent	BD Bioscience
Ethanol, absolute	AppliChem
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO4)_2 \cdot 6H_2O$)	Sigma
Ferrozine	Sigma
Formaldehyde	Otto Fischar
Gentamycin	InvivoGen
Glycerol	Sigma-Aldrich
Gly-Gly	Sigma
Golgi Plug, protein transport inhibitor	BD Bioscience
HBSS	Thermo Scientific
Insulin	Sigma
IPTG	Roth
Kanamycin	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Milk Powder	Roth
Nalidixic acid	Sigma
Nitrocefin	Sigma-Aldrich
Pefa block	Sigma-Aldrich
Penicillin/Streptomycin (P/S) 100x	Gibco Life Technologies

Name	Manufacturer
PFA	Sigma
PMX464 (Thioredoxin inhibitor)	Pharminox Isolation Ltd
Polymyxin B	Sigma
Primocin	InvivoGen
Pefa block	Sigma-Aldrich
Protein marker	Bio-Rad
Rat tail collagen	Sigma-Aldrich
Restore Western blot stripping buffer	Thermo Scientific
RPMI medium	Gibco Life Technologies
ROTI safe	Carl Roth
SDS	Sigma
Snake skin@ Dialysis tubing, 10 MWCO, 35 mm dry	Thermo Scientific
Sodium acetate	Sigma
Sodium hypochlorite (NaClO)	Roth
ß Mercaptoethanol	Sigma
SYBR Green Mix	Promega
TCA	Sigma
TEMED	AppliChem
Thiourea	Roth
Tryptone	Sigma-Aldrich
Tween 20	Sigma-Aldrich

Table 15 Consumables

Name	Manufacturer
15 ml centrifuge tubes	Greiner Bio-one
4-well cell culture chamber slide	Corning
70 µm cell strainers	BD Bioscience
50 ml centrifuge tubes	Greiner Bio-one
96-well white polystyrene, flat bottom	Corning
96-well white polystyrene, round bottom	Corning
Amicon® Ultra-15 ml centrifugal filters	Millipore
Bacteria scraped loop	VWR International
Cell culture flasks 75 cm^2	VWR International
Cover slips	Menzel-Gläser
Filter pipette tips	Starlab
Inoculation spreader	VWR International
Line blot strips	Mickrogen
Novax® Gel cassettes	Invitrogen
Pipette tips	VWR International
Safe lock microcentrifuge tubes	Eppendorf
Tissue culture dishes	BD Bioscience
Tissue culture flasks	BD Bioscience
Tissue culture plates	BD Bioscience

2.1.9 Laboratory equipment

Table 16 Laboratory equipment

Name	Manufacturer
HisTrap FF crude (1 & 5 ml)	GE Healthcare
NanoDrop 1000	Thermo
pH-meter	Biometra
Agar gel chambers	Thermo
Agarose gel scanner	Bio-Rad
SDS gel chambers	Thermo
PCR Thermocycler	Thermo
Western blot membrane scanner	Invitrogen
Centrifuge rotor SLA-3000	Thermo
Centrifuge rotor SS-34	Thermo
CyAn flow cytometry	Beckman Coulter
Homogenizer (Homo genius)	GEA Niro Soavi
Homogenizer (Ultra TURRAX)	IKA
Confocal microscopy	Olympus Life Science, Japan
Automation of strip assays	Microgen
Mithras multimode microplate reader	Berthold technologies
ELISA reader	Berthold technologies
Semi-Dry transfer cell	Bio-Rad
ChemoCam imager	INTAS Science Imaging
CFX384™ real time system	Bio-Rad
Superdex 200 HR 10/30	GE Healthcare

2.1.10 Software

Table 17 Software

Software	Description
FlowJo	Treestar Ashland, USA
Graphpad Prism 5	Graph Pad Software, San Diego, USA
NCBI/BLAST	National Center for Biotech
OligoCalc	Northwestern University Chicago
Photoshop CS	Adobe systems
PrediSI	Signal peptide prediction
Quantity One, 4.2.1	BIO-RAD
SignalP 4.1	Signal peptide prediction
Software recomScan 3.4	Line blot scanner
UniRef90	Uniprot protein alignment with at least 90% similarity

2.2 Methods

2.2.1 Protein purification

2.2.1.1 Plasmid construction

The signal peptides of target proteins were predicted by PrediSI and SignalP 4.1, and their sequences were excluded from the protein open reading frame (ORF). All the candidate proteins were designed to contain a His-tag in the end of their C-terminus. The candidate gene was cloned from *H. pylori* G27 genome by Polymerase Chain Reaction (PCR). *Hp0231*, *napA* and *hpaA* were amplified by primer pairs P10231f & P20231r, P3NapAf & P4NapAr, and P5HpaAf & P6HpaAr respectively. The PCR fragment of *Hp0231* was inserted into the expression vector pET-30b+ by Gibson Assembly Cloning kit, and transferred into DH5α competent cells (NEB) for single colony selection on kanamycin plates. The PCR fragments of *napA* and *hpaA* were digested by Ndel and Xhol, and linked to pET-30b+ vector by T4 DNA ligase (New England Biolabs), and then transferred into DH5α competent cells for single colony selection on kanamycin plates. The plasmid was extracted by PureYield™ Plasmid Miniprep System (Promega) and was sequenced (Promega). The concentration of the vector was measured by Nanodrop (Thermo Scientific).

2.2.1.2 Protein expression

The expression vector containing the protein of interest was transformed into *E. coli* DE3 competent cells on an antibiotic selection plate. Single colonies were inoculated from the plate and cultured overnight in 200 ml LB medium in an Erlenmeyer flask at 37°C and 200 rpm rotation. The next day, the bacteria culture was added into 1 L protein auto-induction TB medium at a starting OD 600 of 0.1. The plasmid-encoded protein was induced at 30°C for 24h at 200 rpm. After growth, the bacterial pellets were spun down at 5000 rpm for 10 min at 4°C. The pellets were weighted and frozen at -80°C before protein purification.

2.2.1.3 Sample homogenization

Bacterial pellets were thawed and suspended in Ni-NTA binding buffer (1 g/10 ml buffer) with 1 mg/ml lysozyme, 1 mM AEBSF, 150 U/L DNase and 1 mM $MgCl_2$ for 30 min at 4°C. The pellets were homogenized using a homogenizer (GEA Niro Soavi). Briefly, bacterial solution was added into the homogenizer inlet, and the pressure of the homogenizer was increased up to 800 to 1000 bar. The pressure was then released to 200 bar, and the homogenized solution containing candidate protein was flowed out into outlet tube. After homogenization, samples were equilibrated and centrifuged in the SS-34 rotor at 15,000 rpm for 10 min at 4°C. The supernatants were collected for Ni-NTA chromatography.

2.2.1.4 Protein Ni-NTA chromatography

Since the candidate proteins were constructed with a His-tag at their C-terminus, target proteins could be purified by separation on a Ni-NTA column. His-tag has the ability to bind nickel (Ni) which chelates to the nitroloacetic acid (NTA) bead. The general principle of the method is to absorb the His-tag labeled protein onto the NTA column at a low concentration of imidazole (20 mM), while non-specific binding proteins are washed away. Bounded candidate proteins can be eluted with high concentration imidazole (500 mM) via the competition to His-tag affinity. The entire purification was performed at 4°C. The column and the injection loop were cleaned with Ni-NTA binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7,4) according to the manual (GE Healthcare) before purification. One sample inlet for Ni-NTA binding buffer and one inlet for Ni-NTA elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7,4) were setup. The purification protocol was setup according to the manual (GE Healthcare). Samples were loaded with 1 ml/min flow rate. After purification, the eluted proteins were monitored by measuring absorbance at 280 nm. Further analysis of the eluted fractions was done by SDS-PAGE. The eluted proteins were then dialyzed and subjected to size exclusion chromatography (SEC, also named gel filtration).

2.2.1.5 Protein dialysis

Target proteins were dialyzed in their specific size exclusion chromatography buffer with a dialysis tubing (Thermo) at 4°C. Ten times of the protein sample volume of SEC buffer was taken for dialysis. Samples were added into membrane tubing and sealed with brackets in both sides. SEC buffer was changed three times per hour for three cycles, and protein samples were finally dialyzed overnight for SEC.

2.2.1.6 Protein size exclusion

Before applying the protein samples to SEC, the target protein samples were sterile filtered and sample load was calculated for SEC (no more than 2.5 mg for one round SEC). The sample volume was adjusted to 5 ml for one round SEC (normally it should be in a range of 0.5% - 3% of the column volumes). The SEC column (Superdex 200 HR 10/30, GE Healthcare) was equilibrated with the SEC buffer before introducing the samples to the column and the purification protocol was setup according to the SEC manual (GE Healthcare). Sample was run with 1 ml/min flow rate. The eluted fractions containing purified proteins were detected by SDS-PAGE and the proteins in the range of the same peaks were pooled together.

After SEC, pooled fractions containing target protein were concentrated by a centrifuge filter (Millipore). The filter used for protein concentration depended on the size of the target protein (Table 18). After concentration, proteins were frozen in liquid nitrogen and stored at -80°C until use.

Table 18 Candidate protein size and filter choice information

Name	Molecular weight	Ext. coefficient (1000x)	Centrifugal Filters
NapA	17.9 KDa	15.5	Amicon® Ultra 10 K device
HpaA	29.2 KDa	14.6	Amicon® Ultra 30 K device
HpgGT	58.5 KDa	49.3	Amicon® Ultra 50 K device
HP0231	28.17 KDa	15.9	Amicon® Ultra 30 K device

2.2.2 Protein activity assays

2.2.2.1 Activity assay of NapA

The enzymatic activity of purified NapA was measured by the catalysis of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions according to the procedure of Ozcan Erel with a slight modification [103, 315]. Ferrous ions were catalyzed to ferric ion in the presence of NapA. The remaining ferrous ions were neutralized by ferrozine to form chromogenic compounds and absorbance was measured at 570 nm. Briefly, serial dilutions of NapA proteins were prepared in 50 ml NapA activity assay solution I (sodium acetic buffer, pH 5.8) and then mixed with NapA activity assay solution II (48 μM ferrous ammonium sulfate), and incubated at 37˚C for 30 min. The reaction was then quenched by addition of NapA activity assay solution III (90 mM ferrozine) and measured at 570 nm in a plate reader.

2.2.2.2 Activity assay of HP0231

HP0231 activity was measured by its ability to reduce insulin α and β chains that trigger insulin aggregation. Serial dilutions of HP0231 were added to 100 mM sodium phosphate buffer (pH 7) with 2 mM EDTA and 400 μM insulin. The reaction started after addition of 1 mM DTT for each reaction. Insulin aggregation was measured at 650 nm for 2h using a Mithras multimode microplate reader (Berthold technologies).

The effect of thioredoxin inhibitor PMX464 on HP0231 activity was also detected by insulin reduction. PMX464 was diluted in DMSO according to manufacturer's instruction (Pharminox Isolation Ltd). 10 μM HP0231 was pre-incubated with different concentrations of PMX464, or with the same amount of DMSO as control. After 30 min incubation at 37˚C, the incubated samples were added into 100 mM sodium phosphate buffer (pH 7) with 2 mM EDTA, 172 μM insulin. The reaction started after adding 1 mM DTT for each reaction. Insulin aggregation was then measured at 650 nm for 2 to 3 h using a Mithras multimode microplate reader. The effect of PMX464 on HP0231 activity was also measured by insulin reduction without pre-incubation with HP0231.

2.2.2.3 Activity assay of gGT

H. pylori gGT activity was monitored by its ability to cleave γ-glutamyl-p-nitroanilide (gGpNA). gGT can catalyze the hydrolysis of gGpNA into p-nitroanilide and γ-glutamyl. p-nitroanilide is a colorimetric compound and can be measured by spectrophotometry at 405 nm. The residual γ-glutamyl is neutralized by the receptor GlyGly to form a new γ-gultamy compound. Briefly, 10^8 *H. pylori* cells (calculated by OD, OD1 in 1ml contained $2x10^8$ bacterial cells) were suspended in 20 mM glycyl-glycine, 2.5 mM L-γ-glutamyl-p-nitroanilide in 200 µl Tris-buffer (pH 8.0), and incubated at 37°C for 10 min. gGT protein was used as a positive control. The release of colorimetric p-nitroanilide was then monitored at 405 nm.

2.2.3 Generation of *H. pylori* mutant strains

2.2.3.1 *H. pylori* natural transformation

10 µg plasmid DNA or 50 µg genomic DNA were introduced into around $4x10^8$ wild-type exponential phase *H. pylori*. After 24 h incubation on a WC DENT plate in a microaerophilic incubator, *H. pylori* bacteria were transferred onto a WC DENT plate with the specific antibiotic for selection. Single colonies were then inoculated into liquid culture. After 4 to 6 days culturing, the correct knockout and complemented *H. pylori* bacteria were identified by PCR and Western blot.

2.2.3.2 Generation of *H. pylori napA* knockout strain

H. pylori SS1 lacking *napA* strain was generated by genomic DNA natural transformation. Briefly, *H. pylori* J99Δ*napA* [107] genomic DNA was extracted by PureLink Genomic DNA Mini kit (Invitrogen). 50 µg genomic DNA were introduced into SS1 by natural transformation. After 4 to 6 days cultured on WC DENT kanamycin plates, single colonies were picked and cultured. Genomic DNA from single colonies was extracted and tested by PCR with primers V3NapAf and V4NapAr. Correct knockout strains were also validated by Western blot.

2.2.3.3 Generation of *H. pylori hp0231*-knockout strain

H. pylori hp0231-knockout strains were generated by introducing pGMTeasy-Δ231 vector to wild-type strains PMSS1, G27, P12 or SS1 via natural transformation. The PCR fragment for nature transformation was composed of the upstream region of *hp0231* gene (around 500 bp), a chloramphenicol cassette and the downstream region of *hp0231* gene (around 500 bp). This PCR fragment was inserted into the pGEM-T easy vector to generate the knockout plasmid pGMTeasy-Δ231. 10 µg knockout plasmid DNA were introduced into $4x10^8$ wild-type *H. pylori* bacteria. After culturing on WC DENT chloramphenicol plates, right colonies were detected by PCR with primers V1HP231f & V2HP231r and Western blot.

2.2.3.4 Generation of *H. pylori hp0231*-complemented strain

H. pylori hp0231-complemented strains were generated in PMSS1Δ*hp0231* and P12Δ*hp0231* strains. *Hp0231* ORF was cloned from *H. pylori* 26695 genomic DNA and introduced into the shuttle vector pHeL3 for constructing the complemented vector pHeL3-231+. The shuttle plasmid was then introduced into P12Δ*hp0231* and PMSS1Δ*hp0231* via natural transformation. After cultured on WC DENT kanamycin plates, the correct colonies were detected by PCR and Western blot.

2.2.4 Cultivation of bacteria and cells

2.2.4.1 Cultivation of *H. pylori*

H. pylori bacteria were cultured under microaerophilic conditions on WC DENT plates at 37°C, 5% CO_2. All *H. pylori* strains were sub-cultured no more than three generations to avoid phenotypic or genotypic changes.

2.2.4.2 Cultivation of mammalian cell lines

Mammalian cell lines AGS (ATCC CRL-1739) and MKN45 [314] used in this study were cultured at 37°C, 5% CO_2 in serum containing medium (100 units/ml penicillin, 100 g/ml streptomycin, 10% inactive FCS DMEM

medium). Cells used for experiments were routinely checked for mycoplasma contaminations.

2.2.4.3 Infection of epithelial cells with *H. pylori*

For *H. pylori* infection experiments, mammalian gastric cells (AGS or MKN45) were seeded on cell culture plates. After 24h, gastric cells were infected with different *H. pylori* strains at MOI 10, 20, 50 or 100. After infection at indicated time, photos were taken and cells were lysed by cell lysis buffer for Western blot. Cell culture supernatants were harvested for Western blot, measurement of IL-8 secretion or ammonia concentration by (ammonia assay kit, Megazyme International Ireland).

2.2.5 *H. pylori* based assays

2.2.5.1 *H. pylori* mobility assay

H. pylori mobility assay was performed on a soft WC DENT agar plate as previously described with a slight modification [143, 316]. The soft WC DENT agar plates were composed of 5 µg/ml amphotericin B, 5 µg/ml cefsulod, 5 µg/ml trimethoprim, 10 µg/ml vancomycin, 2.8% (w/v) Brucella broth base, 10% (v/v) FCS, and 0.35% (w/v) agar. *H. pylori* strains were inoculated into the soft agar plate with a sterile pipette tip. Pictures were taken after two to three days.

2.2.5.2 *H. pylori* viability assay

Log phase *H. pylori* bacteria on WC DENT plates were suspended in PBS and inoculated in Brucella broth DENT liquid culture medium with 10% FCS. Bacterial cells were cultured by starting at OD 0.1 or OD 0.5. *H. pylori* growth was monitored by measuring cell density at OD600 at different time points (6h, 8h or 14h).

2.2.5.3 *H. pylori* binding assay to gastric cancer cell lines

AGS and MKN45 cells were counted and seeded in 96-well plates at a concentration of $1.5x10^5$ cells per well. Different *H. pylori* strains were labeled by CFDA-SE (cell tracer, Life Technologies) in sterile PBS at 37°C for 30 min. After washing the bacteria three times with PBS, AGS and

MKN45 cells were incubated with labeled *H. pylori* at MOI 10 for 1 h at 37°C. Samples were then washed with FACS buffer three times and stained with EMA (1:1000) for live-dead staining. After washing three times with FACS buffer, samples were fixed in 100 µl 4% paraformaldehyde (PFA) for 5 min and suspended in 200 µl FACS buffer for flow cytometry. The Log geometric mean fluorescence intensity (GeoMFI) was calculated for the binding assay.

H. pylori binding to gastric epithelial cells was also detected by confocal microscopy. Briefly, $0.5x10^5$ AGS cells were plated on one 4-well chamber slide (Corning). After 24 h, AGS cells were infected with CFDA-SE labeled *H. pylori* at MOI 5, and incubated for 1 h at 37°C. Samples were washed five times with PBS to make sure the non-binding bacteria were washed away. Then, samples were fixed with 4% PFA for 10 min at room temperature. After washing once with PBS, samples were stained with 250 µl pre-warmed cell plasma stain deep red solution (1:1000 dilution with PBS) for 15 min at 37°C. After washing two times with PBS, the chamber slide was separated and samples were stained with DAPI. The samples were then covered with a cover slide and photos were taken by confocal microscopy (Fluoview FV101, Olympus Life Science).

2.2.5.4 *H. pylori* binding assay to mouse primary gastric epithelial cells

6 to 8 weeks old C57BL/6 mice were used for gastric epithelial cell isolation. A 6-well plate was pre-coated with coating buffer (10µg/cm^2 rat tail collagen in 0.02 N acetic acid) one day before isolation. The coating solution was removed and the plate was dried under the laminar flow cabinet overnight. The next day, mice glandular stomachs were taken. Stomach tissue was washed with PBS three times and incubated in 0.04% sodium hypochlorite (NaClO) buffer for 15 min for disinfection. After three times wash with PBS, tissue samples were put in 3 ml of 20 mM EDTA HBSS solution and incubated for 7 min at 100 rpm at 37°C. Samples were transferred to a petri dish and smashed using two glass slides. The smashed debris was transferred into a 50 ml falcon and centrifuged at 1000 rpm for 5 min. After

centrifugation, supernatants were discarded and the pellets were suspended up and down at least 15 times in primary cell culture medium to make sure the pelleted cells were isolated completely. Pelleted cells in culture medium were then transferred into the 6 well pre-coated plate and centrifuged at 1000 rpm for 1 min to let the cells attach easily to the plate. The extracted epithelial cells were incubated at 37°C before the binding assay.

After 24 h, the primary epithelial cells were washed with PBS three times and digested with trypsin-EDTA (Gibco Life Technologies). The number of primary epithelial cells was counted and infected with labeled *H. pylori* at MOI 10 in a round-bottom 96 well plate. Samples were stained with EMA (1:1000) for live-dead staining and anti-mouse CD45 APC to exclude T cells which might be present during cell isolation. Samples were fixed in 4% PFA and suspended in FACS buffer for flow cytometry.

2.2.5.5 Quantification of VacA secretion

H. pylori wild-type and *hp0231*-isogenic mutant strains were cultured by starting an OD 2 overnight culture in 10% FCS Brucella broth DENT medium with the antibiotic required for selection (wild-type in normal medium, *hp0231*-knockout strain in medium supplemented with chloramphenicol). Thereafter, the bacterial supernatants were centrifuged at 13000 rpm for 10 min and collected for Western blot. The same volume of supernatants from different strains were diluted in 4x SDS lysis buffer and boiled at 95℃ for 10 min to detect VacA expression level. gGT was used as an internal control for quantification.

2.2.6 Mouse infection and immunization

2.2.6.1 Mouse infection

Log phase *H. pylori* bacteria were scraped from agar plates using bacterial inoculating loops and then added into BHI DENT medium. Bacterial density was measured at OD600. Mice were starved 5h before infection, and challenged with 200 µl of $6x10^8$ *H. pylori* suspension in BHI DENT medium. Mice were infected with *H. pylori* strains every two days for three times by

oral administration through a feeding needle (Figure 4). Mice were sacrificed at different time points, and mouse glandular stomachs were taken and homogenized. Colony forming units (CFU) were calculated by plating out homogenized stomach pieces on WC DENT special agar plates at serial dilutions (1:10, 1:100, 1:1000, 1:10,000).

2.2.6.2 Mouse prophylactic immunization against *H. pylori* infection

The vaccination experiments in mice were carried out in two different mouse strains, C57BL/6 and Balb/c, accounting for known differences in the vaccine-induced protection against *H. pylori* [317]. Cholera toxin was used as adjuvant. Oral and intraperitoneal immunizations were performed. Briefly, 1 µg cholera toxin and 30 µg of each protein were diluted in 200 µl PBS for one immunization. Two weeks after the last immunization, mice were infected with *H. pylori* strain SS1 and were sacrificed 4 weeks after infection. Serum was taken at different time points. The experiments were performed as depicted in Figure 4.

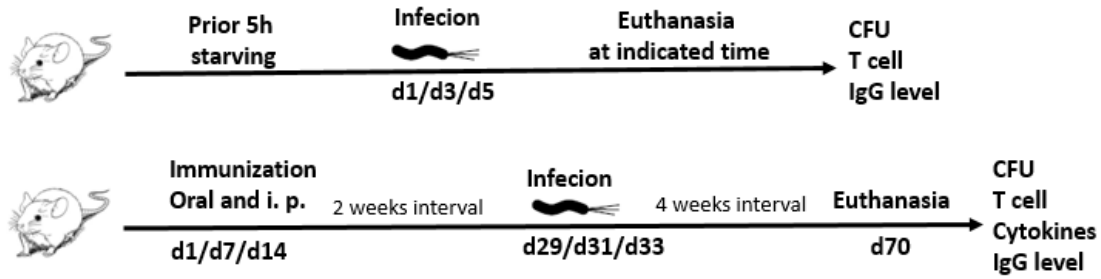


Figure 4 Infection or prophylactic vaccination strategy in mice

2.2.7 Flow cytometry

2.2.7.1 Staining of lymphocytes for flow cytometry

MLN and PP lymphocytes were isolated by rubbing the tissue and filtered through 70 µm cell strainers (BD Bioscience). The cells were centrifuged at 1200 rpm for 5 min at 4°C and suspended in RPMI medium for staining on ice. Lymphocytes from MLN or PP were firstly stained with EMA for 20 min under the light. After three times wash with FACS buffer, samples were

stained with anti-mouse CD45 APC (1: 100), anti-mouse CD3 PECy7 (1: 100), anti-mouse CD4 PO (1: 50), anti-mouse CD8α eF780 (1: 100), anti-mouse CD8β PE (1: 100) for 20min in the dark. Thereafter, samples were stained with anti-mouse foxp3 eF450 (1: 100) for intracellular staining according to the manufacturer's instructions (eBioscience). After staining, samples were analyzed on a CyAn flow cytometer (Beckman Coulter). The FACS data was analyzed using FlowJo software (Treestar).

2.2.7.2 Intracellular cytokine restimulation and staining

Spleen lymphocytes were used for intracellular cytokine restimulation. Lymphocytes were isolated by disrupting the spleens and filtered through 70 µm cell strainers. Then, samples were transferred into a 15 ml falcon tube. Cells were centrifuged at 1200 rpm for 5 min and suspended with 7 ml freshly prepared red blood cell lysis buffer for 10 min to remove the red cells. 5 ml RPMI medium was added into the falcon tube to stop the reaction and samples were centrifuged at 1200 rpm for 5 min at 4°C. Pellets were suspended in 8 ml RPMI medium and 1 ml sample was taken for FACS staining. 75 µg/ml proteins (HpaA, HP0231 or SS1 sonicated lysate) were used to restimulate each lymphocyte sample at 37°C overnight. Anti-mouse CD3 (1:1000) was used as a positive control and Golgi Plug (1: 500) was added to the medium after 2 h restimulation to block cytokine secretion into the medium. Samples were stained with anti-mouse CD4 eF450 APC (1: 100), anti-mouse IL2 PE (1: 100), anti-mouse TNFα PECy7 (1: 100), anti-mouse IL17 APC (1: 100), and anti-mouse IFNγ FITC (1: 100). After staining, samples were analyzed by flow cytometry. The FACS data was analyzed using FlowJo software.

2.2.8 Line blot assay

H. pylori line blot assay allows the identification of specific antibody responses to different *H. pylori* virulence factors. Highly purified *H. pylori* proteins were individually immobilized on a nitrocellulose membrane strip (Mikrogen). The nitrocellulose strips were first incubated with different human serum samples (1:20 dilution), and then incubated with anti-human IgG (1:100 dilution). Finally, a peroxidase-based staining reaction (TMB

substrate, Mikrogen) was used to detect the individual antigen reactions, resulting in a dark band appearing on the position of the corresponding antigen lane. The program was automatically run by the Mikrogen recomLine machine. The test strips were scanned by recomScan 3.4 and the intensity of the bands was measured and set in relation to the cutoff (control).

2.2.9 Molecular and biochemical methods

2.2.9.1 DNA extraction

Genomic DNA was extracted using PureLink Genomic DNA Mini kit (Invitrogen). Plasmid DNA was isolated by PureYield™ Plasmid Midiprep System (Promega) or PureYield™ Plasmid Miniprep System (Promega).

2.2.9.2 RT-PCR

In order to check the expression level of IFNγ and IL-17A in stomach tissues, quantitative reverse transcription PCR (RT-PCR) was conducted. The protocol for RT-PCR is as below.

RNA extraction

Stomach tissue RNA was extracted by GenElute mammalian total RNA Miniprep kit according to the manufacturer's instructions (Sigma-Aldrich).

Reverse transcription

6 µg of RNA were taken for each reverse transcription reaction. Samples were prepared according to the manufacturer's instructions (Promega), and incubated at 65°C for 5 min in a PCR machine (Thermo). After keeping samples for 5 min on ice, PCR products were prepared for cDNA synthesis according to the manufacturer's instructions (Promega). PCR was set as: 20°C for 10 min; 42°C for 50 min; 70°C for 15 min. After reverse transcription, samples were 1 to 5 diluted and kept at -20°C for RT-PCR.

Quantitative PCR

Quantitative PCR (qPCR) was performed using CFX384™ Real time System (Bio-Rad). The housekeeping gene GAPDH was used as control. For each reaction a master mix was prepared as follows:

SYBR Green Mix (2x)	5 µl
Pimer pair mix (5 pmol/µl)	1 µl
cDNA	4 µl

The PCR conditions were: 95°C for 3 min; 40 cycles of 95°C for 15s and 60°C for 45s; 65°C to 95°C for melting curve. The melting curve was checked to detect the amplification plot or bimodal melting curve. The expression levels of IFNγ and IL-17A were normalized to GAPDH.

2.2.9.3 Agarose gel electrophoresis

Amplified DNA was loaded in 1% agarose gel electrophoresis. 1 g agarose was boiled in 100 ml TAE buffer and 5 µl ROTI safe (Carl Roth) was added for DNA staining. The agarose gel was run under 100V voltage for around 40 min and was scanned using an agarose gel scanner (Bio-Rad).

2.2.9.4 Western blot

SDS-PAGE gels were prepared as indicated in page 48. Percentage of acrylamide used depended on the size of the proteins analyzed. Electrophoresis was performed for 60 min at 150V. Gels were transferred to a nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad) at 90 mA for 100 min. After transfer, the membranes were washed three times with TBS-T for 5 min, blocked with 5% milk powder for 1 h at RT, washed three times with TBS-T for 10 min, and incubated with primary antibody diluted in 5% BSA of TBS-T at 4°C overnight. Afterwards, the membranes were washed three times with TBS-T for 10 min, incubated with secondary antibody diluted in 5% BSA of TBS-T for 1 h at RT, and washed three times with TBS-T for 10 min. Finally, the membranes were developed using Clarity™ western ECL substrate (Bio-Rad) for 5 min at RT and were scanned using the ChemoCam Imager (INTAS Science Imaging).

2.2.9.5 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was used to detect the serum IgG responses to target proteins. 20 µg/ml of target protein (HpaA,

HP0231 or SS1 sonicated lysate) in PBS was coated in a 96 well plate for 2 h at 37°C. The plates were washed three times with ELISA blocking buffer (1% BSA in PBS) and blocked at 37°C for 2 h or 4°C overnight. Plates were washed three times and probes were added at different dilutions in blocking buffer for 1 h at 37°C. After washing three times, HPR-conjugated anti-IgG antibody (1:10,000 in blocking buffer) was added and incubated for 1h at 37°C. After washing four times, 100 µl/well of ELISA substrate reagent (BD Bioscience) were added and incubated for 1 to 5 min at RT. At the end, 50 µl of 1 M H_2SO4 were added to stop the reaction, and the absorbance was measured at 450 nm.

2.2.10 Statistics

Statistical analysis was performed using GraphPad Prism 5 (Graph Pad Software, La Jolla, CA). T test was performed for two group comparisons as indicated in the figure. Non-parametric Mann-Whitney U test was used to compare differences of mouse colonization levels. Kruskal-Wallis test with Dunn's correction or one way ANOVA followed by Bonferroni's multiple comparison test was used to establish differences in multiple comparisons as indicated in the figure. Statistical significance was established when $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Chapter 3: Results

3.1 Candidate protein vaccination in mice

3.1.1 HpaA combined with NapA, gGT or HP0231 is selected as potential vaccine formulation

Currently, there is no gold standard for *H. pylori* vaccine candidate selection. However, four essential requirements could be considered for the selection of suitable *H. pylori* vaccine candidates: 1) protein function during *H. pylori*-induced pathogenesis; 2) serological immunity in *H. pylori*-infected patients; 3) high conservation in *H. pylori* strains; and 4) high specificity compared to other species, especially to mammalian proteins.

3.1.1.1 Candidate protein selection based on serological responses in *H. pylori*-infected individuals

In order to select *H. pylori* vaccine candidates, a line blot assay was established to evaluate the seroreactivity to various antigens of *H. pylori*-infected patients. It is necessary to investigate the seroreactivity for each vaccine candidate, since the level of the serological response for each antigen may affect the efficiency of the vaccination. Fifteen *H. pylori* proteins of interest were individually immobilized on a nitrocellulose strip. These antigens included CagA, VacA, VacAm1, VacAm2, GroEL, UreA, HcpC, gGT, FliD, HtrA, HpaA, Tig, Jhp940, NapA, and HP0231. The function of the candidate proteins is listed in Table 19. The serum samples from *H. pylori*-infected patients were collected from East Asia (China) and Europe (Germany). These serum samples were from patients with different gastric diseases, including superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia and gastric cancer. These gastric lesions were analyzed on histological sections (Figure 5). An example of a line blot screening result is depicted in Figure 6. The intensity of each antigen band was scanned by the software recomScan 3.4 and set in relation to the control band (cut off). An individual antigen band stronger than the cut off was considered as a positive reaction. The prevalence of the IgG

responses to individual antigen is shown as a frequency of positive reaction in screened samples from different categories.

The line blot results revealed that the serological responses to these 15 antigens in *H. pylori*-infected patients were different in the German and the Chinese populations (Figure 7). In the German population, a high prevalence of IgG responses to CagA, HpaA, GroEL and FliD were observed. Similarly, a high prevalence of IgG responses to CagA, HpaA and FliD were detected in the Chinese population (Figure 7). These results indicate that CagA, HpaA, GroEL and FliD for the German population, and CagA, HpaA and FliD for the Chinese population, could be considered as highly sensitive diagnostic markers for *H. pylori* infection. Moreover, a higher prevalence of IgG responses to CagA and VacAm2 were detected in the Chinese patients compared to the German patients. Since CagA has been reported to increase the risk of gastric cancer [318-320], this observation may provide an explanation for the higher prevalence of gastric cancer in the Chinese population. When characterizing patient samples by disease types (Table 10), higher frequencies of IgG responses to CagA, FliD and HpaA seemed to correlate with gastric lesion progression since the IgG responses increased from superficial gastritis to intestinal metaplasia (Figure 8). Actually, CagA has been reported to correlate well with *H. pylori*-induced gastric pathological changes in previous studies [321-323]. Because there were few cases of metaplasia and dysplasia in the German cohort, no further statistical analysis in disease categorization could be obtained.

Strong serological responses to the outer membrane proteins, FliD and HpaA, were observed in both the German and Chinese patients, while lower serological responses to proteins UreA, HtrA, Jhp940, NapA and HP0231 were observed when compared to FliD and HpaA in these two populations (Figure 7). Considering the low efficiency of vaccine-induced protection induced by a single antigen (see 1.4.2.3), two *H. pylori* proteins (one OMP and one protein possessing a relevant function in bacterial colonization and survival, inducing low serological response) were chosen for the vaccine formulation. Ideally, surface exposed OMP is easily

recognized by the immune system, and protein inducing low serological response with relevant function might be able to boost the host immunity upon immunization to prevent *H. pylori* colonization. Candidate proteins were then further selected based on both sequence conservation and sequence specificity. Protein sequence conservation among *Helicobacter* strains was predicted by UniRef90 (Uniprot protein alignment with at least 90% similarity). Sequence specificity was established by comparing the candidate protein sequence (NCBI blast) to protein sequence from other species (excluding *Helicobacter*). Proteins with high conservation in *Helicobacter* and low similarity compared to other species were selected for potential vaccine candidates.

Based on this standard, HpaA was selected as the OMP candidate since the protein alignment of HpaA showed no homologs with proteins in other species, while FliD showed up to 38% sequence similarity with one *Campylobacter* protein (Table 20). Furthermore, another protein in the vaccine formulation was chosen among antigens showing low immunity; these included UreA, Jhp940, HP0231, NapA and gGT. Among these candidates, Jhp940 was not conserved in *H. pylori* strains. UreA plus HpaA immunization against *H. pylori* has been described in a previous study [263]. NapA and HP0231 were good candidates based on sequence alignment results (Table 20). *H. pylori* gGT sequence was not highly specific compared to *Campylobacter* gGT (Table 20), and it displayed 25% homology with mammalian gGT [109, 110]. However, gGT has been reported to be an important enzyme for *H. pylori* metabolism that can inhibit T cell proliferation and induce cell-cycle arrest and apoptosis in gastric epithelial cells [108, 324]. Thus, gGT was also chosen as a vaccine candidate. Therefore, HpaA combined with NapA, gGT or HP0231 was selected as a vaccine formulation in this study. The immunization using HpaA and gGT was part of another study which is not included here.

Table 19 Function of candidate proteins

OMP	Function
FliD	Flagella protein, essential for functional flagella assembly [84, 85].
HpaA	Outer membrane protein, essential for *H. pylori* colonization in mice [94]
Other proteins	**Function**
NapA	Neutrophil-activating protein, induces neutrophil recruitment and influences oxidative stress [105].
gGT	Gamma-glutamyl transpeptidase, catalyzes glutamine and glutathione, inhibits T cell proliferation, [108, 324].
HP0231	Disulfide oxidoreductase, important for disulfide formation [134].
CagA	Essential *H. pylori* toxin, oncogenic protein, induces cell apoptosis [325].
GroEL	Chaperonin, secreted iron-binding protein [326].
HcpC	*Helicobacter* cysteine-rich protein C, Sel1-like multi-repeat protein which contains TPR and SEL1-like repeat motifs [327].
HtrA	Serine protease, cleaves E-cadherin to disrupt intercellular adhesion [155].
Jhp940	Serine/threonine-protein kinase CtkA, induces human macrophages TNFα and IL-8 secretion [328].
Tig	Trigger factor, involved in protein export and acts as a protein folding chaperone [329].
UreA	Urease subunit A, hydrolyzes urea and neutralizes pH in the stomach [330].
VacA	Vacuolating toxin, induces host cell vacuolation and cell apoptosis [331].

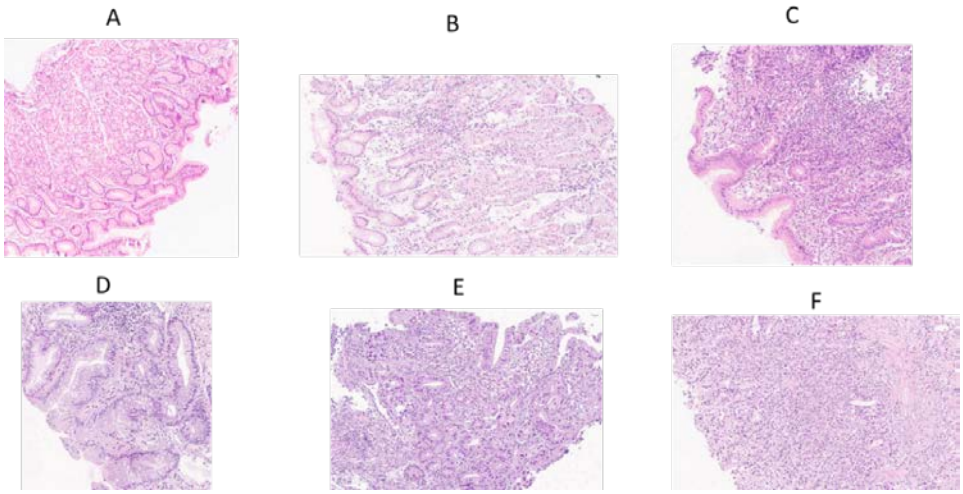


Figure 5 Histology of different *H. pylori*-induced gastric diseases. *H. pylori* negative stomach (or healthy stomach, A), superficial gastritis (B), chronic atrophic gastritis (C), intestinal metaplasia (D), dysplasia (E), and gastric cancer (F) are shown.

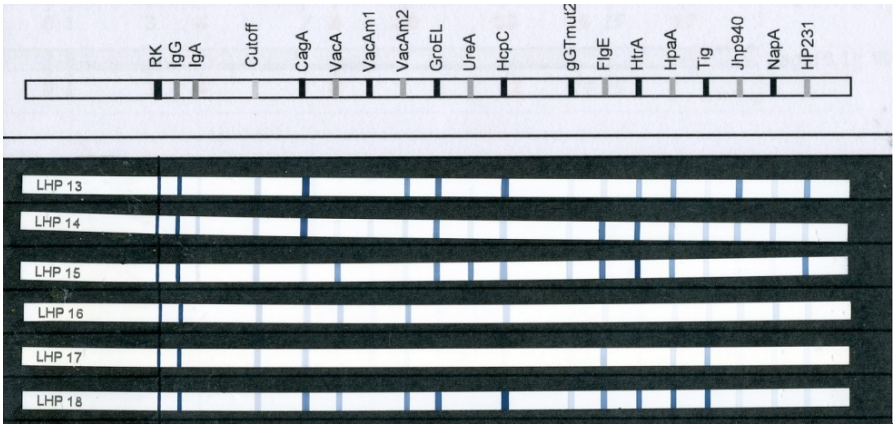


Figure 6 Line blot for *H. pylori* antigens in human sera. Representative image of a line blot assay. The presence of antibodies against the indicated *H. pylori* virulence factors led to the appearance of a dark band.

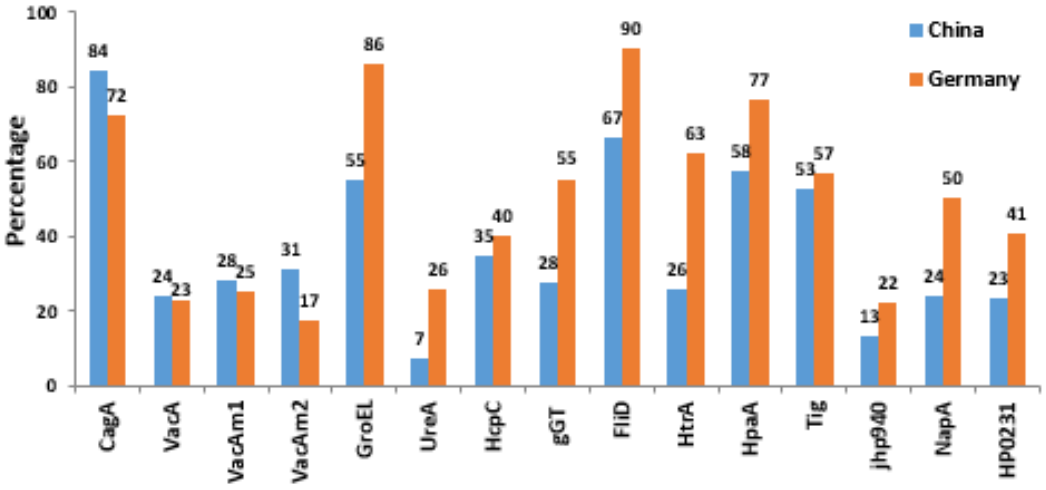


Figure 7 Prevalence of IgG responses to individual antigen in *H. pylori*-infected Chinese and German cohorts. 414 samples in the Chinese cohort and 307 samples in the German were analyzed. The frequency of positive reaction for each antigen in the Chinese or German cohorts is shown.

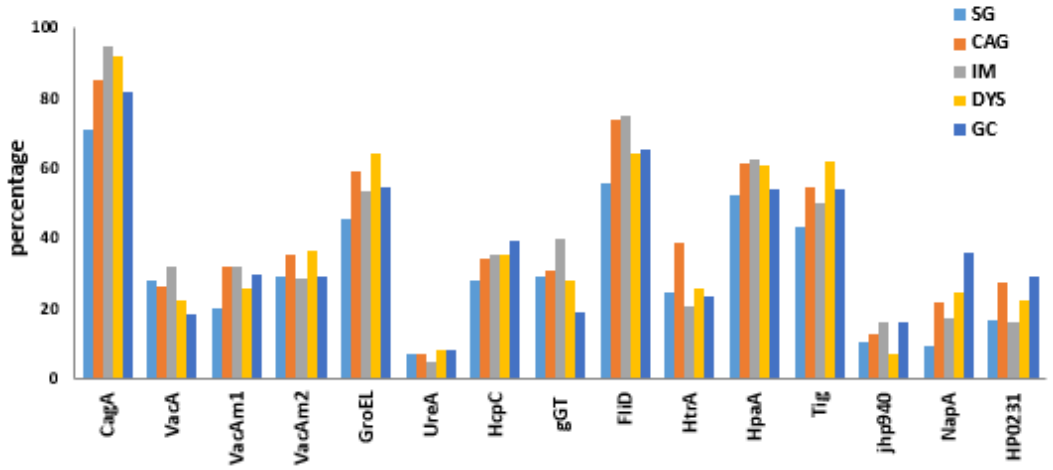


Figure 8 Prevalence of IgG responses to individual antigen in different gastric diseases. 86 superficial gastritis (SG), 88 chronic atrophic gastritis (CAG), 88 intestinal metaplasia (IM), 86 dysplasia (DYS), and 166 gastric cancer (GC) samples were screened. Frequencies of positive reaction for each antigen in different diseases in the Chinese population are shown.

Table 20 Candidate protein conservation and specificity

Candidate Protein	Conservation (Nr of proteins showing 90% similarity among *Helicobacter*)	Specificity (highest similarity excluding *Helicobacter*)
NapA	230	48% with *Campylobacter*
gGT	333	67% with *Campylobacter*
HP0231	303	38% with *Campylobacter*
Jhp940	34	53% with *Campylobacter*
HpaA	323	0%
FliD	360	38% with *Campylobacter*

Conservation: the protein sequences were compared using UniRef90 online. Data shows the number of proteins which share at least 90% similarity in protein sequence among *Helicobacter* strains. A value above 200 is considered as high similarity in protein sequence.

Specificity: protein sequences were compared using nucleotide collection (nr/nt) database excluding *Helicobacter* species (NCBI tblastn). Data shows the highest sequence similarity rate. Rate below 50% is considered as low similarity in protein sequence.

3.1.1.2 *H. pylori* SS1 is suitable for vaccination studies in mice

Few of the clinically isolated *H. pylori* strains harbor the ability to colonize the mouse stomach [332]. *H. pylori* PMSS1 and *H. pylori* SS1 are two

strains among them. Before studying the protective effect induced by the candidate proteins, the colonization abilities of these two bacterial strains in mouse stomach were confirmed. C57BL/6 mice were infected with the same dose of PMSS1 or SS1. Mouse stomach tissues were homogenized and CFU were counted at different time points pose-infection. Higher CFU were observed in *H. pylori* SS1-infected mice than PMSS1-infected mice, and the final bacterial load of SS1 showed less variation compared to the CFU of mice infected with PMSS1 (Figure 9). Moreover, SS1 could colonize in different mouse strains (both C57BL/6 mice and BALB/c mice), while PMSS1 could only colonize in C57BL/6 mice. In addition, Treg and CD8+ T cell populations in mesenteric lymphocytes and Peyer's Patches were analyzed by FACS (Figure 10A). Tregs play an immune regulatory role by inhibiting T helper cell proliferation in response to *H. pylori* infection [193], and cytotoxic T cells have been shown to activate macrophages and support the killing of infected target cells [188]. Thus, the percentages of Tregs (foxp3+ T cells) and cytotoxic T cells (CD8αβ+ T cells) in the total T cells (CD3+ T cells) were calculated. No significant differences on Treg and cytotoxic T cell distributions were observed in MLN and PP during chronic infection in SS1-infected and PMSS1-infected mice (Figure 11B). Thus, based on these results, SS1 was used for vaccination experiments in this study.

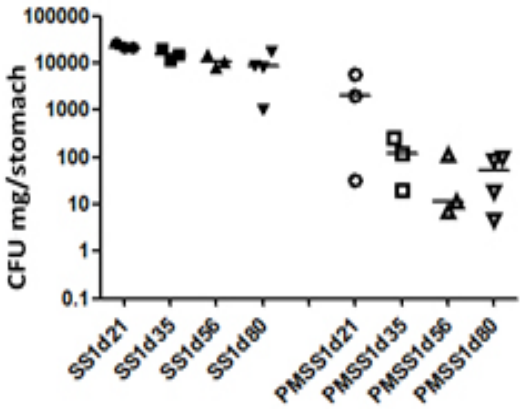


Figure 9 SS1-infected mice show higher CFU than PMSS1-infected mice. C57BL/6 mice were infected with $6x10^8$ *H. pylori* PMSS1 or SS1. CFU were calculated at different time points after infection. Each dot represents one mouse. 0.1 on the Y axis indicates no colony formation detected. Horizontal bars indicate mean.

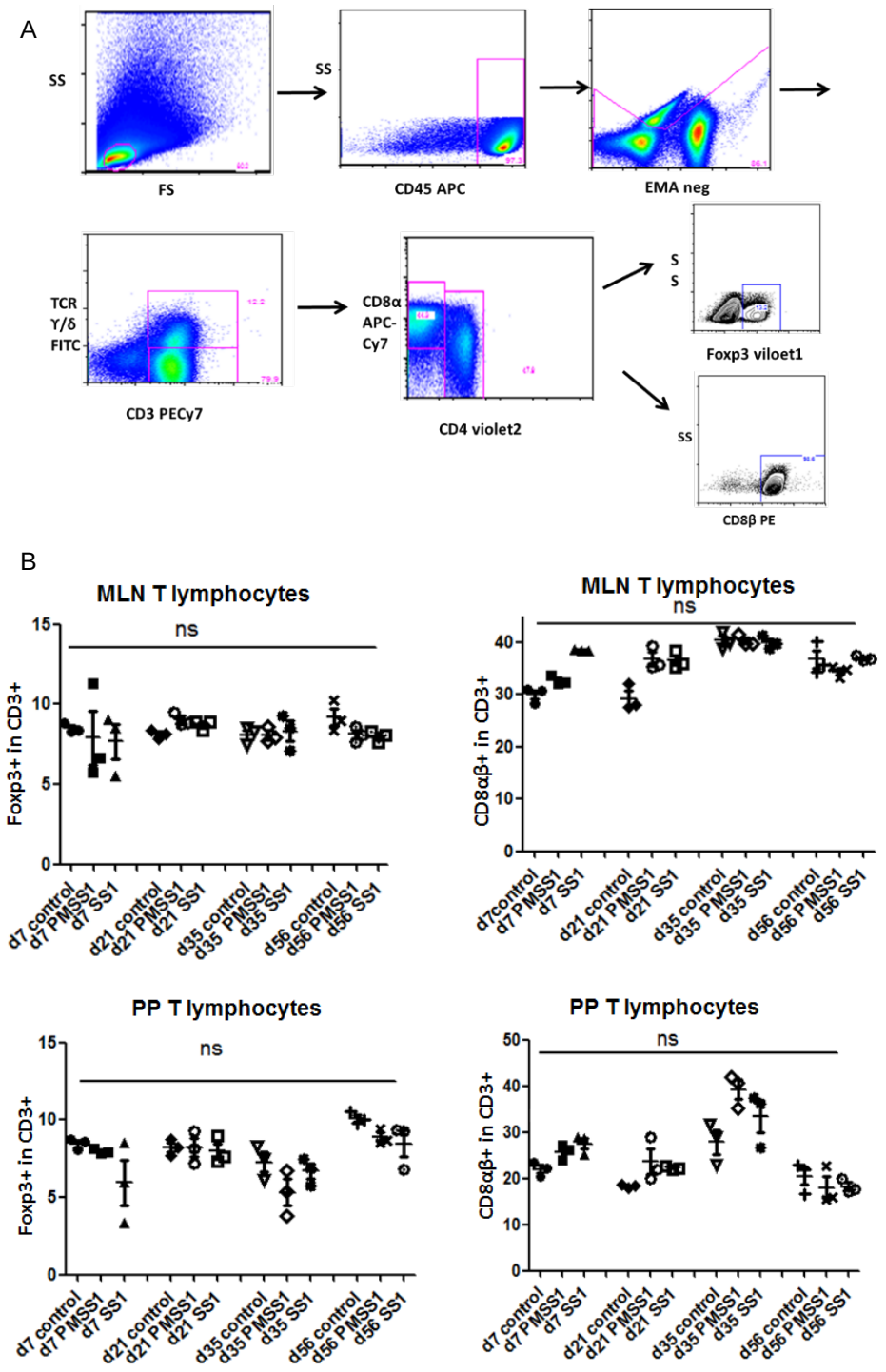


Figure 10 No differences in T cell distribution in MLN and PP between SS1-and PMSS1-infected mice. (A) Representative FACS staining panel for T lymphocytes. MLN and PP lymphocytes were extracted for FACS analysis. (B) Treg and Cytotoxic T cell population in MLN and PP. The proportion of Foxp3+ cells or CD8αβ+ cells in total CD3+ cell population was calculated.

3.1.1.3 The serological response to HpaA in mice is not dependent on the final colonization density but on the initial infection phase

Next, serum immune responses to candidate proteins were investigated in *H. pylori*-infected mice. C57BL/6 mice were infected with *H. pylori* PMSS1 or SS1 and serum was taken at regular intervals for ELISA analyses. CFU were counted 80 days after infection (Figure 11A). No specific IgG responses to HP0231, NapA and gGT were detected in the serum of *H. pylori*-infected mice (Figure 11B), while anti-HpaA IgG was detectable in infected mice (Figure 11C). This indicates that HpaA can be recognized in the serum of *H. pylori*-infected mice. Moreover, the line blot results revealed that HpaA was highly recognized in *H. pylori*-infected human sera (Figure 7). Thus, HpaA is a highly sensitive diagnostic marker for *H. pylori* infection in both mice and humans.

Notably, the serological response to HpaA did not depend on the final colonization, since a significant IgG response to HpaA was detected in mice infected with *ggt*-knockout strains (Figure 11C), while no colonization was observed in the absence of *ggt* (Figure 14). A similar result was observed in mice infected with a *hp0231*-deficient strain. No bacterial colonization in mice infected with *H. pylori* lacking *hp0231* was detected (Figure 15A), while a significant IgG response to HpaA was detected 28 days post-infection. Serological levels were similar to mice infected with wild-type bacteria (Figure 11D). Thus, these results indicate that the serological response to HpaA in mice is not dependent on the final colonization density but on the initial infection phase.

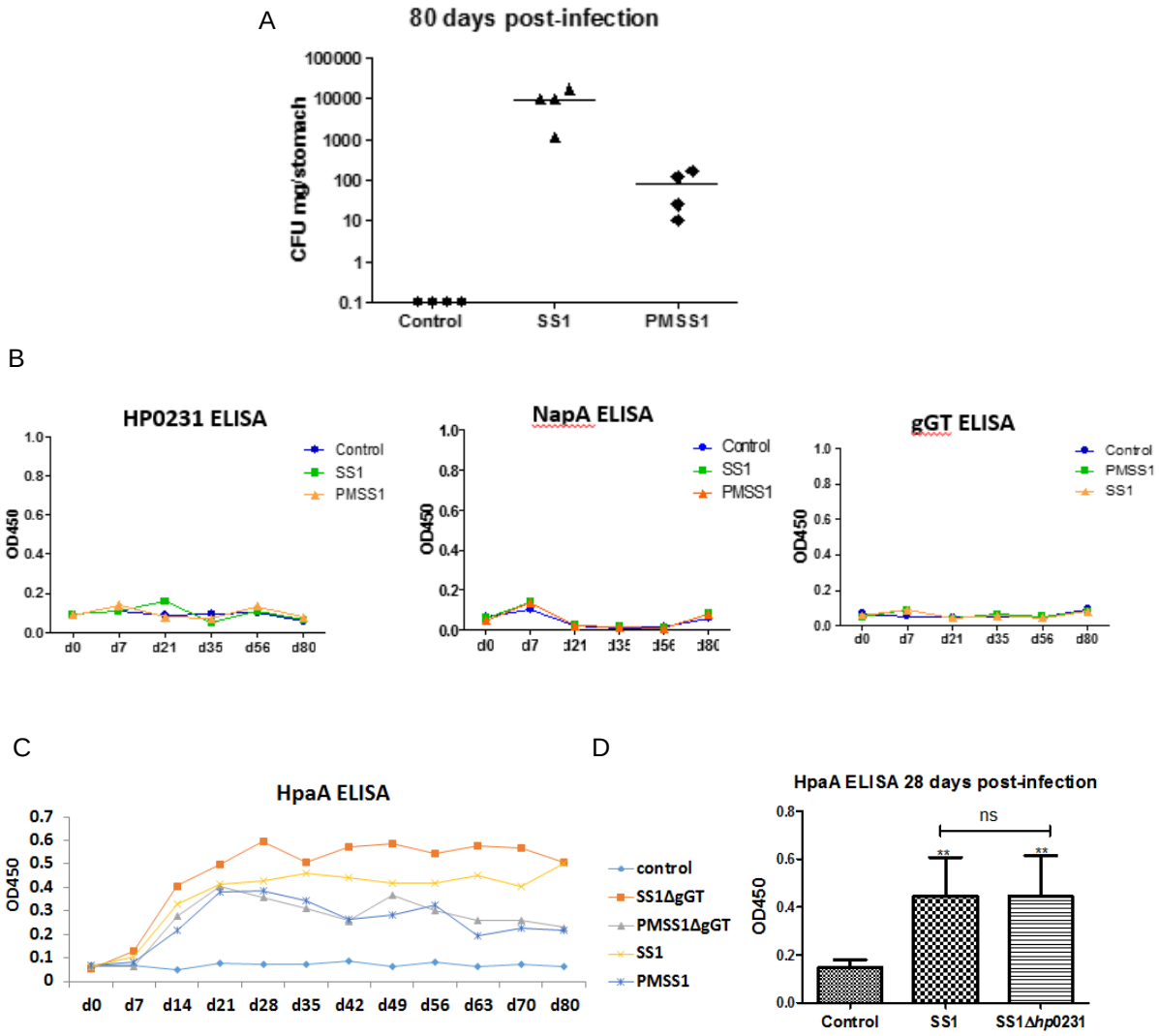


Figure 11 Serological response to HpaA in mice is not dependent on the final colonization density but on the initial infection phase. (A) C57BL/6 mice were infected with 6x10^8 *H. pylori* PMSS1 or SS1. CFU were analyzed 80 days post-infection. Control is CFU from non-infected mice. Each dot represents one mouse. 0.1 on the Y axis indicates no colony formation detected. Horizontal bars indicate mean. (B) Serum IgG response to NapA, gGT, and HP0231. Serum was taken at different time points before or after infection. Serum dilution 1:900. IgG dilution 1:10,000. (C) Serum IgG responses to HpaA. Serum was taken regularly from 6x10^8 SS1Δ*ggt*, SS1, PMSS1 and PMSS1Δ*ggt*-infected mice. Control is the serum response in non-infected mice. Data shows HpaA ELISA in pooled serum. Four mice were in each group. Serum dilution 1:900. IgG dilution 1:10,000. (D) Serum IgG responses to HpaA. Mice were infected with the same dose of SS1 or SS1Δ*hp*0231 and serum was taken at 28 days post-infection. Control is the serum derived from non-infected mice and asterisks on top indicate significances relative to control. ANOVA followed by a Bonferroni's multiple comparison test was performed. *** P < 0.001.. Eight mice per group. Serum dilution 1:900, IgG dilution 1:10,000.

3.1.2 Role of candidate proteins in *H. pylori* colonization

3.1.2.1 Lack of *napA* does not affect *H. pylori* colonization

To investigate the role of NapA in *H. pylori* colonization, *napA*-isogenic knockout strain was generated by natural transformation of J99 Δ *napA* genomic DNA. The knockout strain of *napA* was confirmed by PCR and Western blot (Figure 12). C57BL/6 mice were orally infected with the same dose of *napA*-knockout or wild-type *H. pylori* three times every two days. *NapA* deletion did not affect *H. pylori* colonization seven days post-infection (Figure 13). Therefore, *napA* is not required for *H. pylori* colonization.

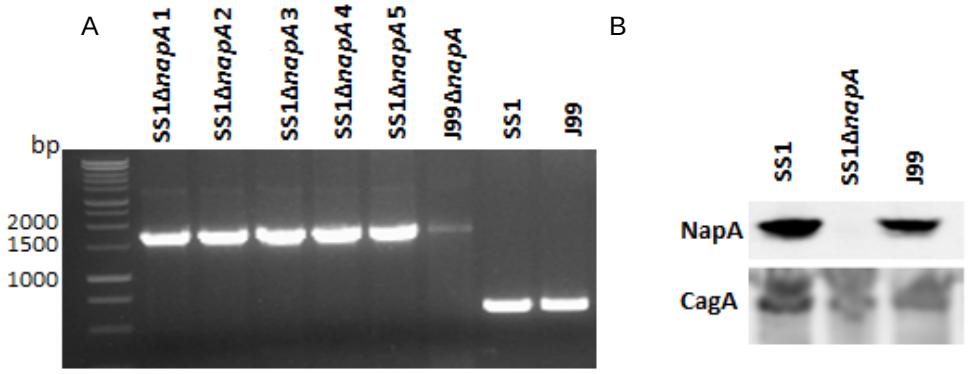


Figure 12 Detection of *napA* by PCR and Western blot. SS1Δ*napA* strains were selected from a kanamycin agar plate. (A) *NapA* detected by PCR in five single colonies. J99Δ*napA* was used as negative control, SS1 and J99 as positive control. (B) NapA detected by Western blot in one selected colony. CagA was used as a control.

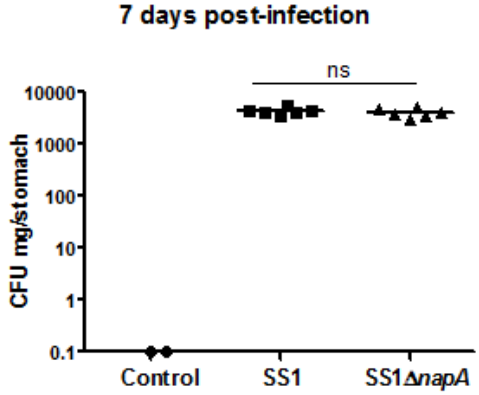


Figure 13 Lack of *napA* does not affect *H. pylori* colonization. C57BL/6 mice were infected with $6x10^8$ *H. pylori* SS1 or SS1Δ*napA*. CFU were calculated 7 days after infection. Each dot represents one mouse. 0.1 on the Y axis indicates no colony formation detected. Horizontal bars indicate mean.

3.1.2.2 The deficiency of *ggt* hampers *H. pylori* colonization

The ability of *H. pylori* to colonize mouse stomach after deleting *ggt* was also investigated. C57BL/6 mice were infected with the same dose of *ggt*-knockout strain or wild-type *H. pylori* strain. Serum was taken regularly at different time points and mice were euthanized for CFU analysis at the indicated time points post-infection. The deficiency of *ggt* hampered *H. pylori* colonization after short term as well as long term infection (Figure 14). Moreover, the defect in colonization lacking *ggt* was not strain specific (Figure 14). This data indicates that gGT is an important virulence factor for *H. pylori* colonization of the stomach.

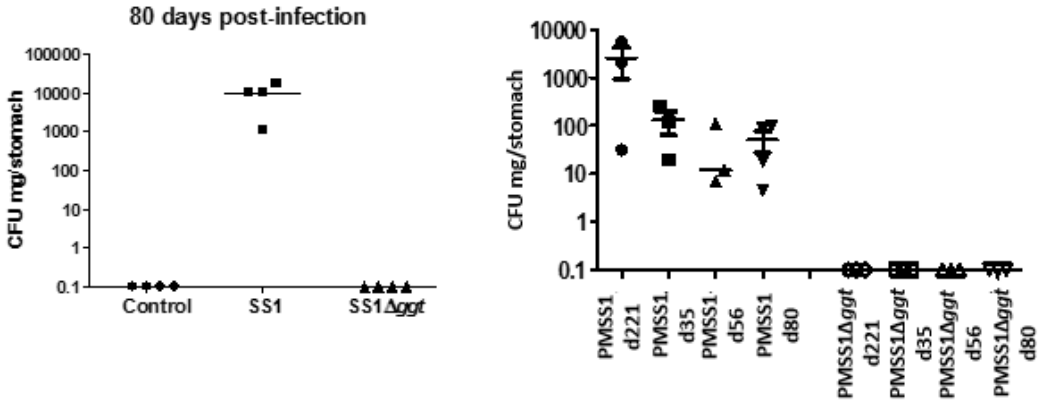


Figure 14 Deficiency of *ggt* hampers *H. pylori* colonization. C57BL/6 mice were infected with $6x10^8$ *H. pylori* PMSS1, PMSS1Δ*ggt*, SS1 or SS1Δ*ggt*. CFU were calculated at different time points. 0.1 on the Y axis indicates no colony formation detected. Each dot represents one mouse. Horizontal bars indicate mean.

3.1.2.3 HP0231 is required for gastric colonization

To investigate the role of HP0231 in bacterial colonization, *hp0231*-deficient or complemented strains were constructed in different *H. pylori* backgrounds. *Hp0231* was deleted by inserting pGMTeasy-Δ231 and restored by complementation with pHeL3-231+. *Hp0231*-knockout strains or complemented strains were selected from chloramphenicol or kanamycin agar plates, and expression of HP0231 was analyzed to confirm its deletion by PCR and Western blot (Figure 15).

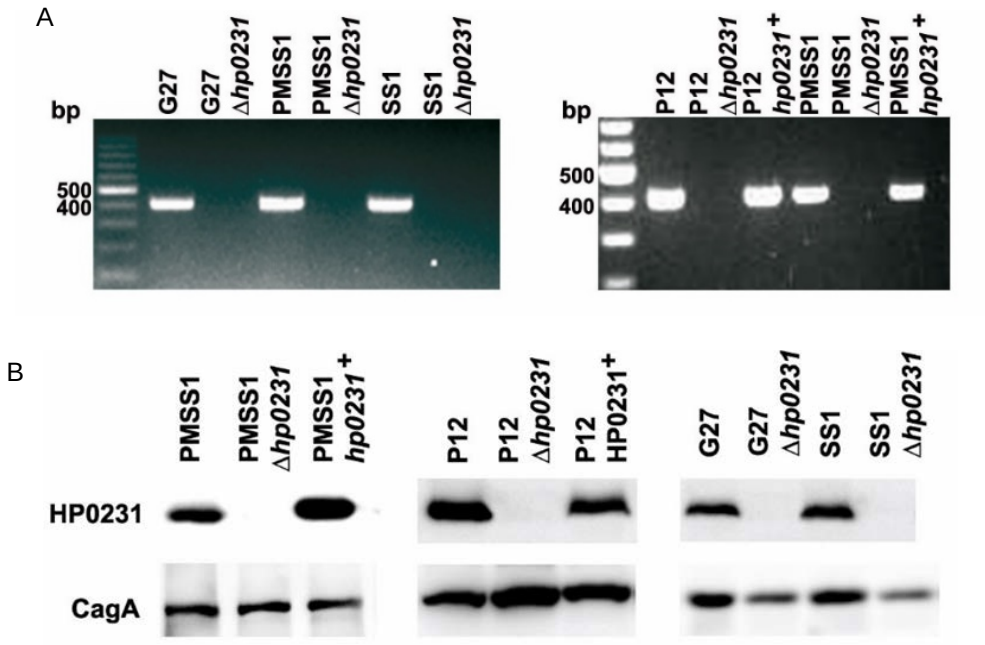


Figure 15 Detection of *hp0231* by PCR and Western blot. *Hp0231*-deficient or complemented strains were selected from chloramphenicol or kanamycin agar plates. (A) PCR analysis of *hp0231* expression. *Hp0231*-deficient and complemented strains were verified by PCR with primers V1HP231f and V2HP231r. (B) Western blot detection of HP0231. CagA was used as control.

C57BL/6 mice were infected with wild-type *H. pylori* or *hp0231*-isogenic mutant strains for seven days or one month. The lack of *hp0231* hampered *H. pylori* colonization at both time points of analysis, (Figure 16A), while complementing the gene in *hp0231*-isogenic deficient strain reverted the phenotype (Figure 16B). Additionally, the impact of HP0231 on *H. pylori* colonization was not strain specific. Thus, HP0231 is required for gastric colonization.

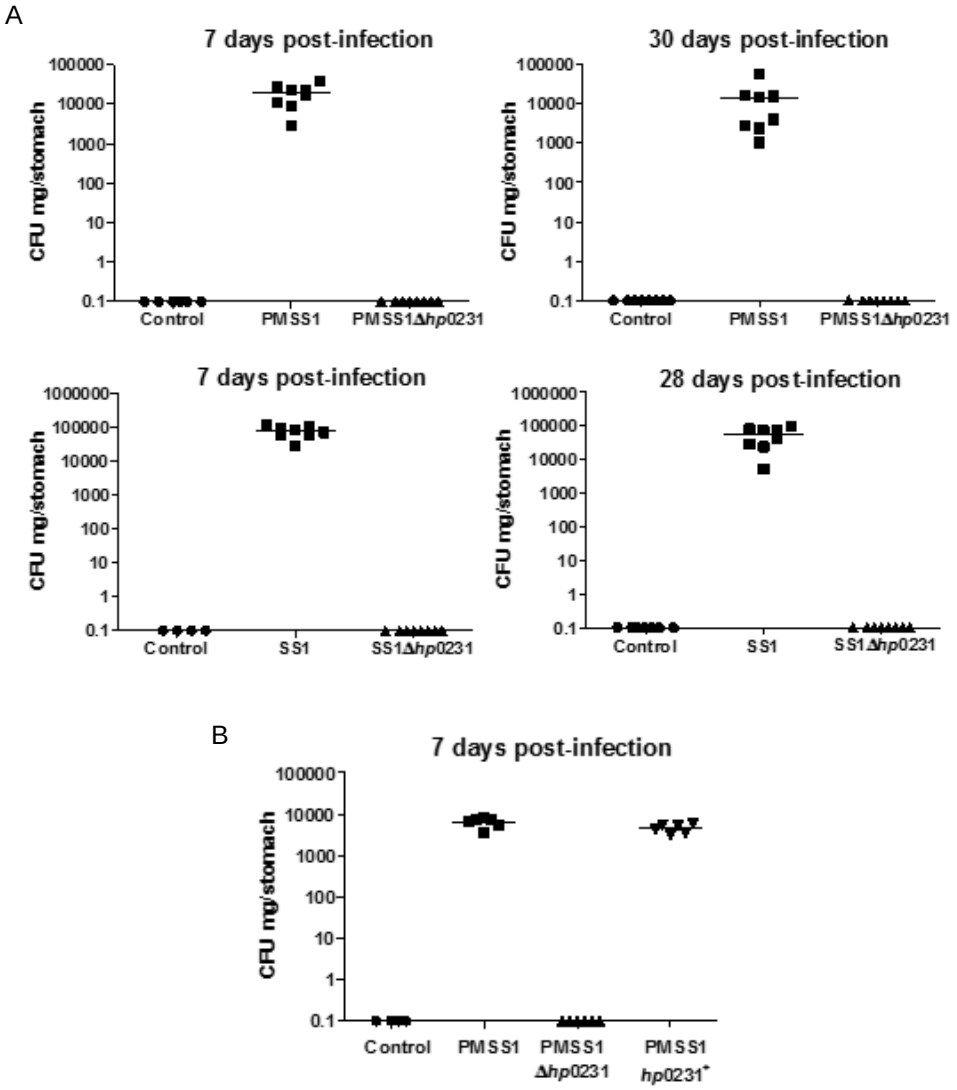


Figure 16 *H. pylori hp0231*-deficient bacteria cannot colonize in mouse gastric mucosa. C57BL/6 mice were infected with $6x10^8$ *H. pylori* PMSS1, SS1 wild-type or *hp0231*-deficient strains (A) or *hp0231*-complemented strain (B). CFU was examined after plating serial dilutions of stomach homogenates. Each dot represents one mouse. 0.1 on the Y axis indicates no colony formation detected. Horizontal bars indicate mean.

3.1.3 Prophylactic immunization of candidate proteins against *H. pylori* infection

3.1.3.1 NapA plus HpaA is not an effective vaccine against *H. pylori* infection

3.1.3.1.1 NapA and HpaA purification

To test the efficiency of vaccine candidates, target proteins were first purified by two-step chromatography: Ni-NTA affinity chromatography, dialysis and size exclusion chromatography (Figure 17). His-tagged HpaA protein was overexpressed in *E. coli* and purified by the Ni-NTA chromatography. Purified samples were dialyzed, concentrated and loaded for SEC. Very few HpaA protein was observed in the range of the first peak of SEC, probably due to protein aggregation (Figure 18B). HpaA protein in the first peak was the same size as the protein in the second peak detected by SDS-PAGE (Figure 18C). NapA was purified in the same way. Like HpaA, two peaks also appeared in NapA SEC (Figure 19B) and protein samples showed the same size when analyzed by SDS-PAGE (Figure 19C). This indicates that NapA protein aggregated during SEC. Although the protein aggregation does not affect antibody production, the aggregated proteins are more toxic than normal folded proteins [333, 334]. Therefore, HpaA and NapA in the second peaks were used for the immunization studies. In addition, the enzymatic activity of purified NapA was measured by the catalyzation of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions. Ferrous ions were catalyzed to ferric ions in the presence of NapA, and the remaining ferrous ions in the end were neutralized by ferrozine to form chromogenic compounds. Then, the absorbance was measured at 570 nm. Purified NapA showed a high catalytic activity (Figure 20), suggesting its biological function is preserved after the purification process.

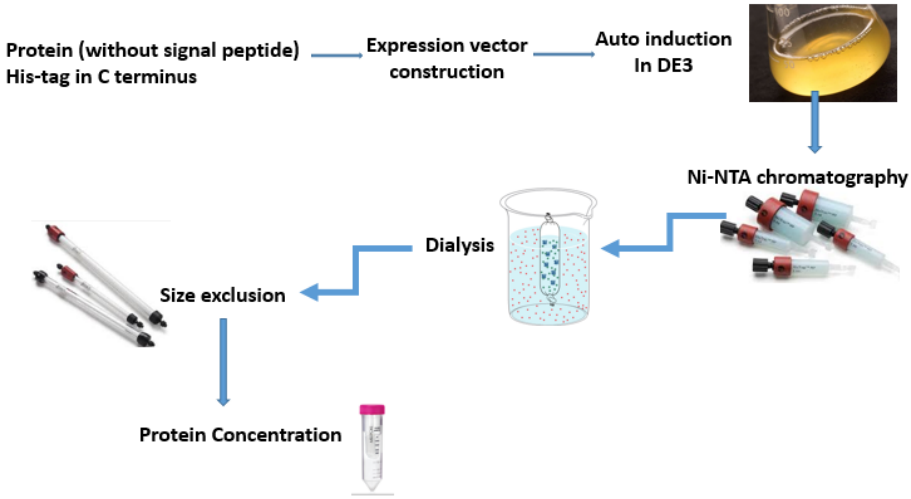


Figure 17 Protein purification scheme. Target protein with a His-tag in the C-terminus was first introduced into pET30b vector for overexpression. Protein was auto-inducted in TB medium. Bacterial pellets were homogenized and purified by Ni-NTA chromatography. After dialysis three times, protein samples were further purified by SEC chromatography. Finally, purified protein samples were concentrated and stored at -80℃.

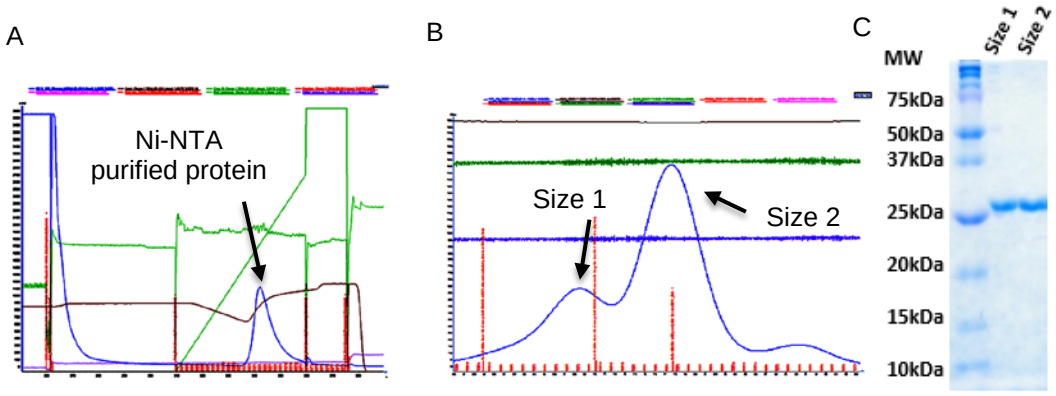


Figure 18 Purification of HpaA. (A) HpaA Ni-NTA chromatography. The protein UV curve (blue) at 280nM for HpaA purification is shown. A clear peak of protein was observed after His affinity purification (arrow pointed). (B) HpaA SEC. The protein UV curve (blue) at 280nM for HpaA SEC is shown. Two peaks were observed after SEC and proteins in the range of the same peaks were pooled (arrow pointed). (C) Analysis of purified HpaA by SDS-PAGE. An SDS-PAGE gel stained by coomassie blue is shown. Size 1 and size 2 stand for the samples from the two ranges of HpaA SEC fractions.

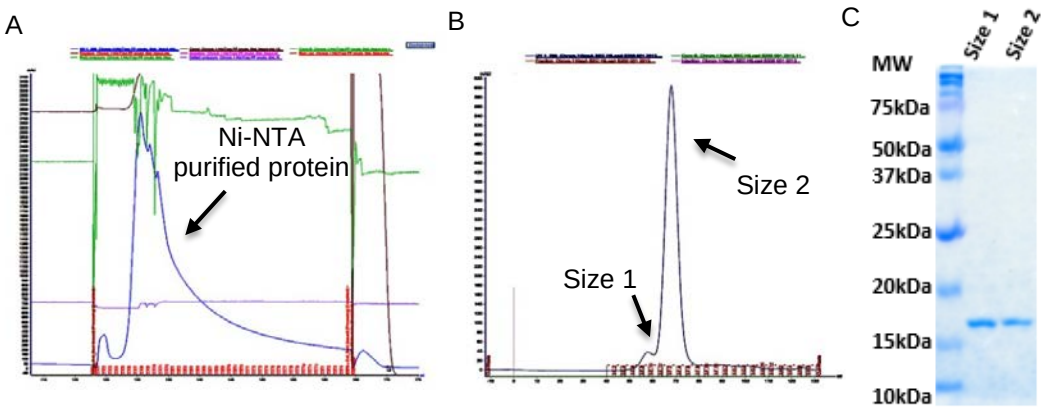


Figure 19 Purification of NapA. (A) NapA Ni-NTA chromatography. The protein UV curve (blue) at 280nM for NapA purification is shown. A clear peak of protein was observed after His affinity purification (arrow pointed). (B) NapA SEC. The protein UV curve (blue) at 280nM for NapA SEC is shown. Two peaks were observed after SEC and proteins in the range of the same peaks were pooled (arrow pointed). (C) Analysis of purified NapA by SDS-PAGE. An SDS-PAGE gel stained by coomassie blue is shown. Size 1 and size 2 stand for the samples from the two ranges of SEC fractions.

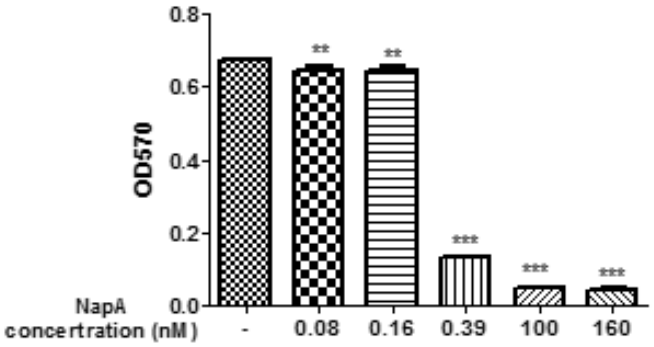


Figure 20 Purified NapA activity assay. Assays were performed in 50 mM sodium acetate buffer (pH 5.8), 48μM ferrous ammonium sulfate, incubated at 37˚C for 30 min. The reactions were quenched by adding 90 mM ferrozine and absorbance was measured at 570 nm. Data from one experiment with 3 repeats per group is shown. ANOVA followed by Bonferroni's multiple comparison test was performed. The bars indicate the mean ± SD. ** p <0.01, *** p <0.001. Asterisks on top of the bars indicate significances relative to control.

3.1.3.1.2 NapA plus HpaA immunization does not protect against *H. pylori* infection

Once NapA and HpaA were purified, prophylactic immunizations against *H. pylori* infection were carried out in mice. To effectively induce mucosal immune responses, cholera toxin was chosen as the adjuvant in this study. Prophylactic immunization and infection were performed as depicted in Figure 21. Six to eight week-old female C57BL/6 mice were immunized with NapA alone or NapA plus HpaA three times weekly. Each mouse was

both orally and intraperitoneally immunized. Two weeks after the last immunization, the same mice in each group were orally infected with the *H. pylori* strain SS1 and euthanized to isolate stomach tissue for bacterial colonization evaluation four weeks after infection (Figure 4). As shown in Figure 21, all immunized mice displayed no significant differences in gastric bacterial load compared to mice only treated with adjuvant. This indicates that neither NapA alone nor NapA plus HpaA are effective as *H. pylori* vaccine.

Group	**Phase I Immunization**	**Phase II Infection**
Control	o.i. 1µg CT/mouse i.p. 1µg CT/mouse	$1X10^7$ SS1 per mouse, three times every two days
NapA	o.i. 1µg CT/mouse +30µg NapA/mouse i.p. 1µg CT/mouse +30µg NapA/mouse	
NapA&HpaA	o.i. 1µg CT/mouse +30µg NapA/mouse + 30µg HpaA/mouse i.p. 1µg CT/mouse +30µg NapA/mouse + 30µg HpaA/mouse	

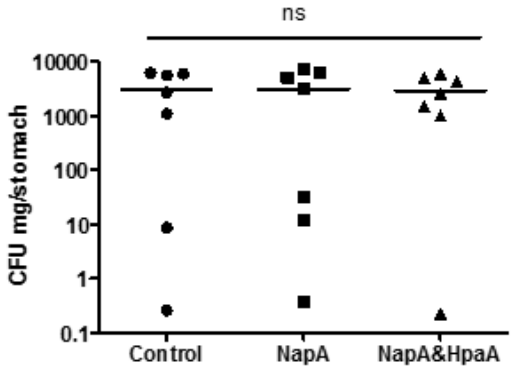


Figure 21 NapA plus HpaA immunization does not protect against *H. pylori* infection. C57BL/6 mice were immunized orally and intraperitoneally three times weekly. All animals were orally challenged with $1x10^7$ SS1 two weeks after immunization and sacrificed four weeks after infection. CFU were examined after plating serial dilutions of stomach homogenates. 0.1 on the Y axis indicates no colony formation detected. Each dot represents one mouse. Horizontal bars indicate mean.

3.1.3.2 HP0231 alone or HP0231 plus HpaA is an effective vaccine against *H. pylori*

3.1.3.2.1 HP0231 purification

To study the efficiency of HP0231 as a vaccine, His-tagged HP0231 was purified in the same manner as NapA and HpaA. Size exclusion purification of HP0231 also yielded two major peaks (Figure 22). The samples in the first peak probably were proteins aggregated during SEC. When analyzed by SDS-PAGE, protein samples showed very high purity in both peaks (Figure 22C). Moreover, an insulin reduction assay was performed to test the catalytic activity of purified HP0231 on disulfide formation. Insulin was aggregated by its β chain precipitation in the presence of HP0231. HP0231 was able to catalyze the reduction of insulin in the presence of DTT (Figure 23), suggesting a correctly purified protein.

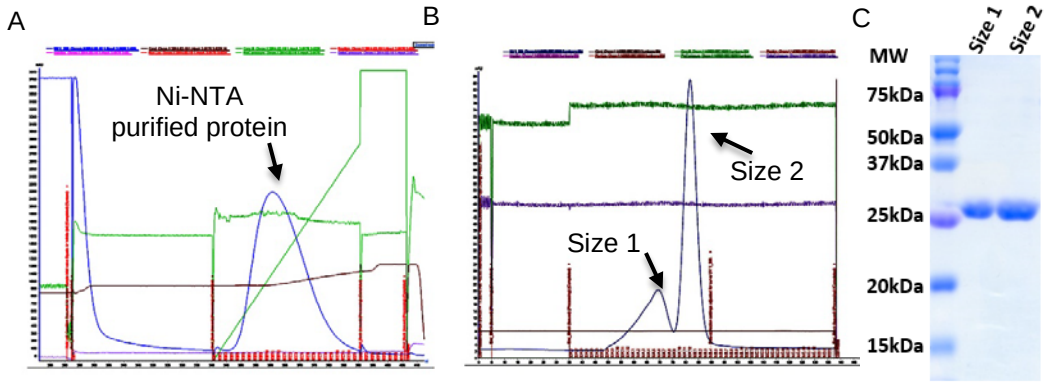


Figure 22 Purification of HP0231. (A) HP0231 Ni-NTA chromatography. The protein UV curve (blue) at 280nM for HP0231 purification is shown. A clear peak of protein was observed after His affinity purification (arrow pointed). (B) HP0231 SEC. The protein UV curve (blue) at 280nM for HP0231 SEC is shown. Two peaks were observed after SEC and proteins in the range of the same peaks were pooled (arrow pointed). (C) Analysis of purified HP0231 by SDS-PAGE. The SDS-PAGE gel stained by coomassie blue is shown. Size 1 and size 2 stand for the samples from the two ranges of SEC fractions.

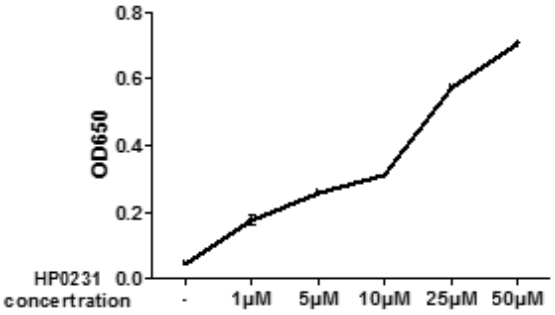


Figure 23 Insulin reduction assay. Assays were performed in PBS, 2 mM EDTA, 1 mM DTT, 400 μM insulin, and absorbance was measured at 650 nm for insulin aggregation. The reaction started by adding DTT. Data from one experiment with 3 repeats per group is shown.

3.1.3.2.2 HP0231 alone or HP0231 plus HpaA immunization significantly decreases *H. pylori* colonization

In order to test whether HP0231 or HP0231 plus HpaA immunization could confer protection against *H. pylori*, C57BL/6 mice were orally and intraperitoneally immunized with HP0231 alone or HP0231 plus HpaA. Control mice only received the adjuvant (CT) (Figure 24A). Mouse serum was collected at different time points to analyze specific IgG antibody responses. Spleen lymphocytes were isolated for antigen-specific immune response analysis.

After mice were challenged with *H. pylori*, vaccine-induced protection was detected by analyzing CFU in mouse stomachs. HP0231 alone and HP0231 plus HpaA immunization significantly decreased *H. pylori* colonization in both C57BL/6 mice (Figure 24B) and BALB/c mice (Figure 24C). HP0231 immunization resulted in an approximately 6.1 fold reduction of bacterial load and HP0231 plus HpaA immunization resulted in an average 6.3 fold reduction in C57BL/6 mice (Figure 24B). In BALB/c mice, HP0231 alone led to an average 3.4 fold reduction in CFU and HP0231 plus HpaA led to a 7.5 fold bacterial reduction compared to the adjuvant control group (Figure 24C). The bacterial load in HP0231 plus HpaA immunization group was slightly but not significantly lower than HP0231 alone immunization group in both C57BL/6 mice (Figure 24B) and BALB/c mice (Figure 24C), suggesting HP0231 plays a major role in the HpaA and

HP0231 vaccine formulation. However, the reduction in bacterial load observed after HP0231 immunization was not higher than that of mice immunized with *H. pylori* lysate, although the colonization was significantly reduced compared to control group (Figure 25A). Nevertheless, these results indicate that HP0231 or HP0231 plus HpaA immunization confers effective protection in different mouse models upon *H. pylori* infection.

To determine antigen humoral immune responses, serum samples were collected to test specific antigen IgG responses. Sera were taken at different time points (d0-before infection, d28-two weeks after immunization, d56-two weeks after infection, and d70-four weeks after infection). Both anti-HP0231 IgG antibodies and anti-HpaA IgG antibodies were significantly increased in antigen-specific immunization groups compared to the control group, and the IgG titers were stable over time (Figure 24D,), revealing that HP0231 and HpaA are able to induce strong and continuous humoral immune responses in immunized mice. In addition, when using *H. pylori* SS1 sonicated lysate as the positive control, high levels of anti-HP0231 IgG antibodies were detected in the SS1 lysate immunized group, and anti-SS1 lysate IgG were detected in the HP0231 immunized group (Figure 25B), indicating that HP0231 is an important immunogenic component in *H. pylori* sonicated extracts. Moreover, the levels of anti-HpaA IgG increased in non-HpaA immunized mice after *H. pylori* infection, indicating that HpaA is an important marker for detecting *H. pylori* infection (Figure 24D). These results demonstrate that HP0231 and HpaA immunizations are able to induce strong and continuous humoral immune responses in mice.

A

Group	Phase I Immunization	Phase II Infection
Control	o.i. 1μg CT/mouse i.p. 1μg CT/mouse	$1X10^8$ SS1 per mouse, three times every two days
HP0231	o.i. 1μg CT/mouse +30μg HP0231/mouse i.p. 1μg CT/mouse +30μg HP0231/mouse	
HP0231&HpaA	o.i. 1μg CT/mouse +30μg HP0231/mouse + 30μg HpaA/mouse i.p. 1μg CT/mouse +30μg HP0231/mouse + 30μg HpaA/mouse	

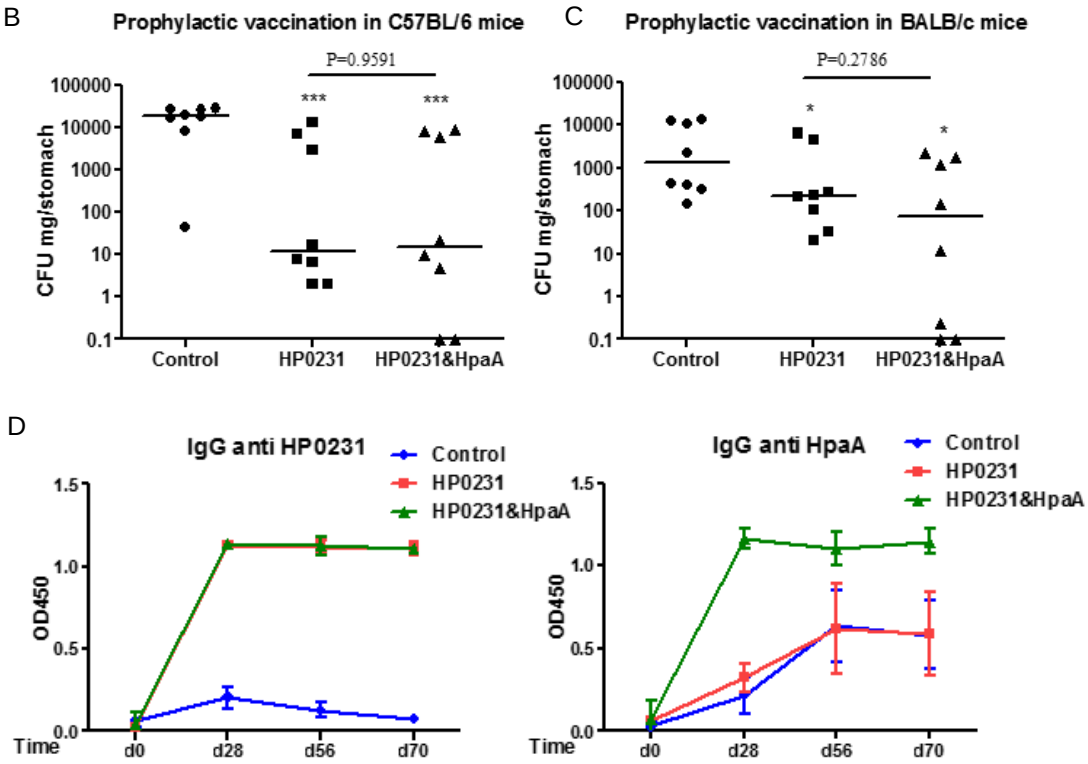
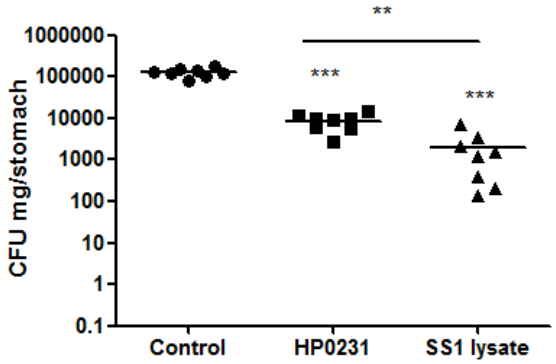


Figure 24 HP0231 alone or HP0231 plus HpaA immunization significantly decreases *H. pylori* colonization. (A) Prophylactic immunization strategy in C57BL/6 mice and BALB/c mice. Mice were immunized orally and intraperitoneally three times at one week interval. All animals were orally challenged with SS1 two weeks after immunization and then were euthanized 4 weeks after infection. CFU were examined after plating serial dilutions of stomach homogenates. (B) CFU in stomach homogenates of C57BL/6 mice; (C) CFU in stomach homogenates of BALB/c mice. 0.1 on the Y axis indicates no colony formation detected. Each dot represents one mouse. Horizontal bars indicate mean. * $P < 0.05$; *** $P < 0.001$. (D) Serum IgG responses to HP0231 or HpaA. C57BL/6 mice naive (d0), two weeks after immunization (d28), two weeks after infection (d56) and 4 weeks after infection (d70) serum. Eight mice per group. Data with mean ± SD is shown. Serum dilution 1:900. IgG dilution 1:10,000

A

Group	Phase I Immunization	Phase II Infection
Control	o.i. 1µg CT/mouse i.p. 1µg CT/mouse	
HP0231	o.i. 1µg CT/mouse +30µg HP0231/mouse i.p. 1µg CT/mouse +30µg HP0231/mouse	$6X10^8$ SS1 per mouse, three times every two days
SS1 lysate	o.i. 1µg CT/mouse + 450µg SS1 lysate/mouse i.p. 1µg CT/mouse + 50µg HP0231/mouse	



B

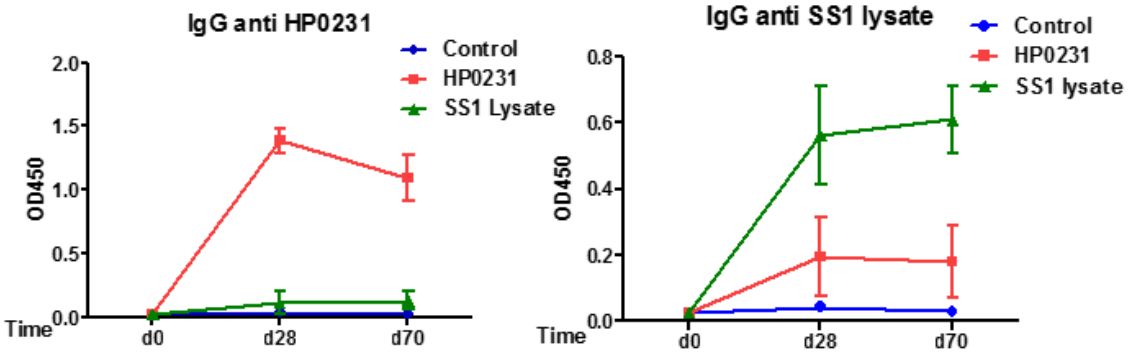


Figure 25 HP0231 immunization confers protection in C57BL/6 mice. (A) CFU in stomach homogenates of C57BL/6 mice immunized with HP0231. SS1 lysate was taken as positive control. Each dot represents one mouse. Horizontal bars indicate mean. ** P < 0.01; *** P < 0.001. (B) Serum IgG responses to HP0231 or SS1 lysate. C57BL/6 naive (d0), two weeks after immunization (d28), two weeks after infection (d56) and 4 weeks after infection (d70) mouse sera was analyzed. Serum dilution 1:2700. IgG dilution 1:10,000. Eight mice per group.

3.1.3.2.3 Vaccine-induced protection is associated with antigen-specific Th1 and Th17 responses as well as IFNγ expression in the stomach

Previous data shows that CD4+ T cells and IFNγ play a dominant role in vaccine-induced protection against *H. pylori* infection (see introduction 1.4.2.1). Thus, the vaccine-induced protection based on cytokine expression and antigen-specific immune responses induced by CD4+ T cells were investigated in this study. Lymphocytes isolated from spleen were restimulated with HP0231 or HpaA. Golgi Plug was added to block cytokine release into the medium, and cytokine levels were determined by flow cytometry. A representative cytokine restimulation FACS profile is shown in Figure 26. The expression levels of TNFα and IFNγ in CD4+ T cells were significantly elevated in the HP0231 or HpaA antigen-specific immunized groups compared to the control group (Figure 27A). When SS1 lysate extract was used as a positive control, similar results were observed (Figure 28). As the expression of TNFα and IFNγ in CD4+ T cells is a hallmark of Th1 cells, these results indicate an antigen-specific Th1 response to the immune protection against *H. pylori*.

In HP0231 and HpaA immunized C57BL/6 mice, the IL-17 level in CD4+ T cells was not significantly different compared to the control group (Figure 27A). However, increased levels of IL-17 were detected in the HP0231 and the SS1 lysate immunization groups in C57BL/6 mice (Figure 28) as well as in BALB/c mice (Figure 29). Thus, these results indicate that a Th17 response is also induced during vaccination.

When measuring cytokine expression levels in the stomach, IFNγ levels in the antigen vaccination groups were significantly increased compared to the adjuvant control group ($P < 0.05$), while no significant changes in IL-17A production were observed, revealing the important role of IFNγ upon vaccination (Figure 27B). Together, these results indicate that vaccine-induced protection against *H. pylori* is associated with antigen-specific Th1 and Th17 responses as well as IFNγ expression in the stomach.

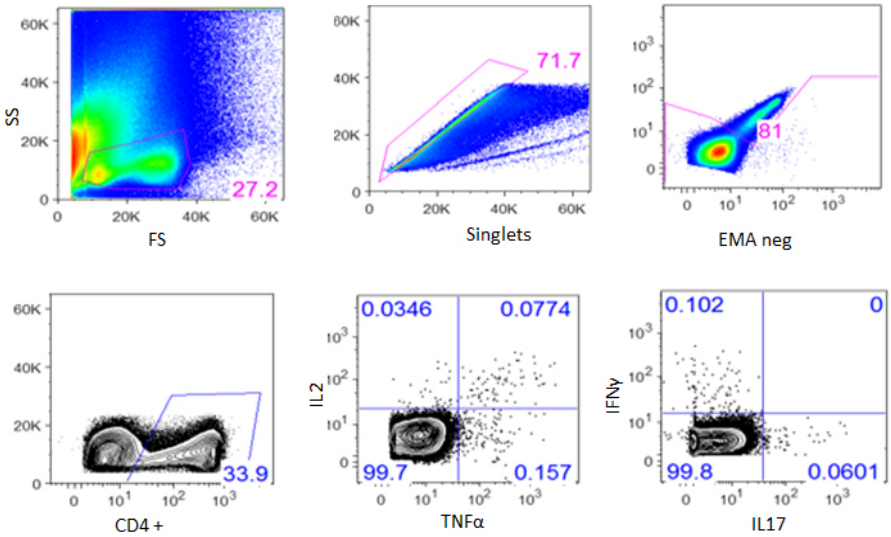
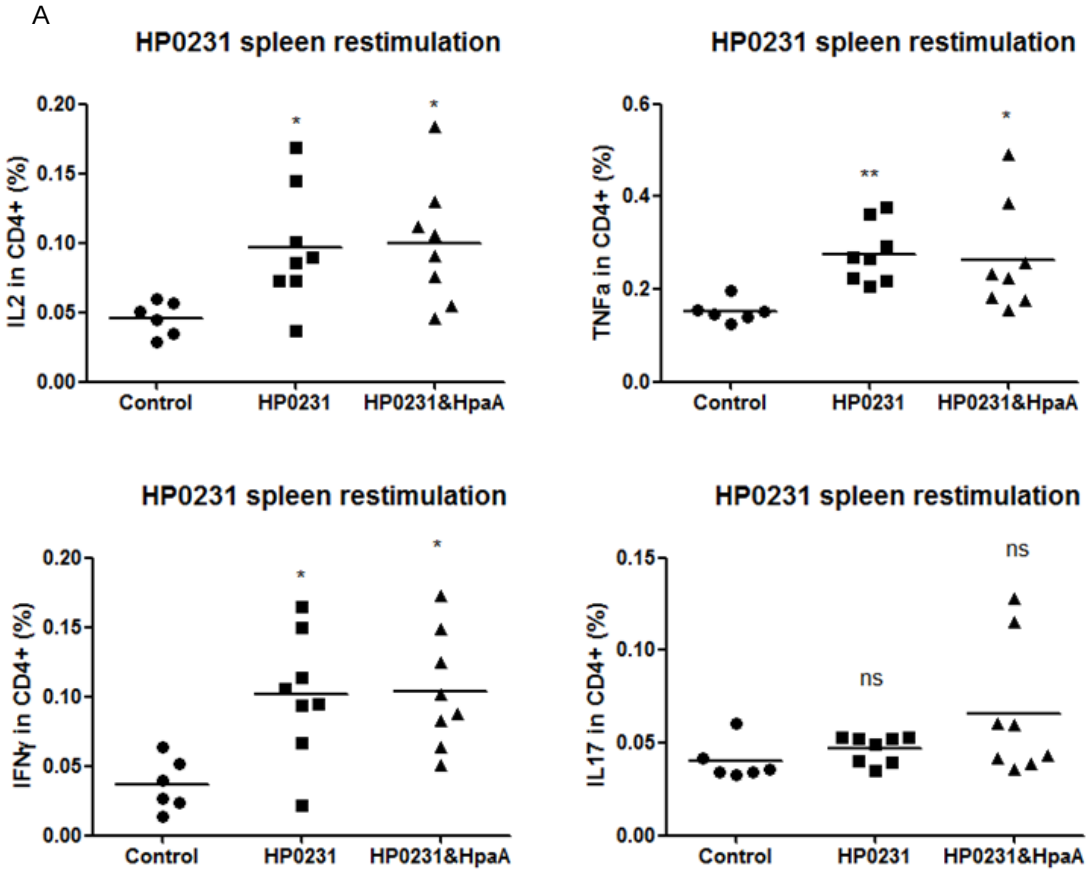


Figure 26 Representative cytokine restimulation pattern in CD4+ T cells. Spleen lymphocytes were stimulated overnight with 75 µg/ml HP0231 or HpaA protein. Golgi Plug (1: 500) was added to the medium after 2 h restimulation. Samples were stained by anti-mouse IL2 PE (1: 100), anti-mouse TNFα PECy7 (1: 100), anti-mouse IL17 APC (1: 100) and anti-mouse IFNγ FITC (1: 100).



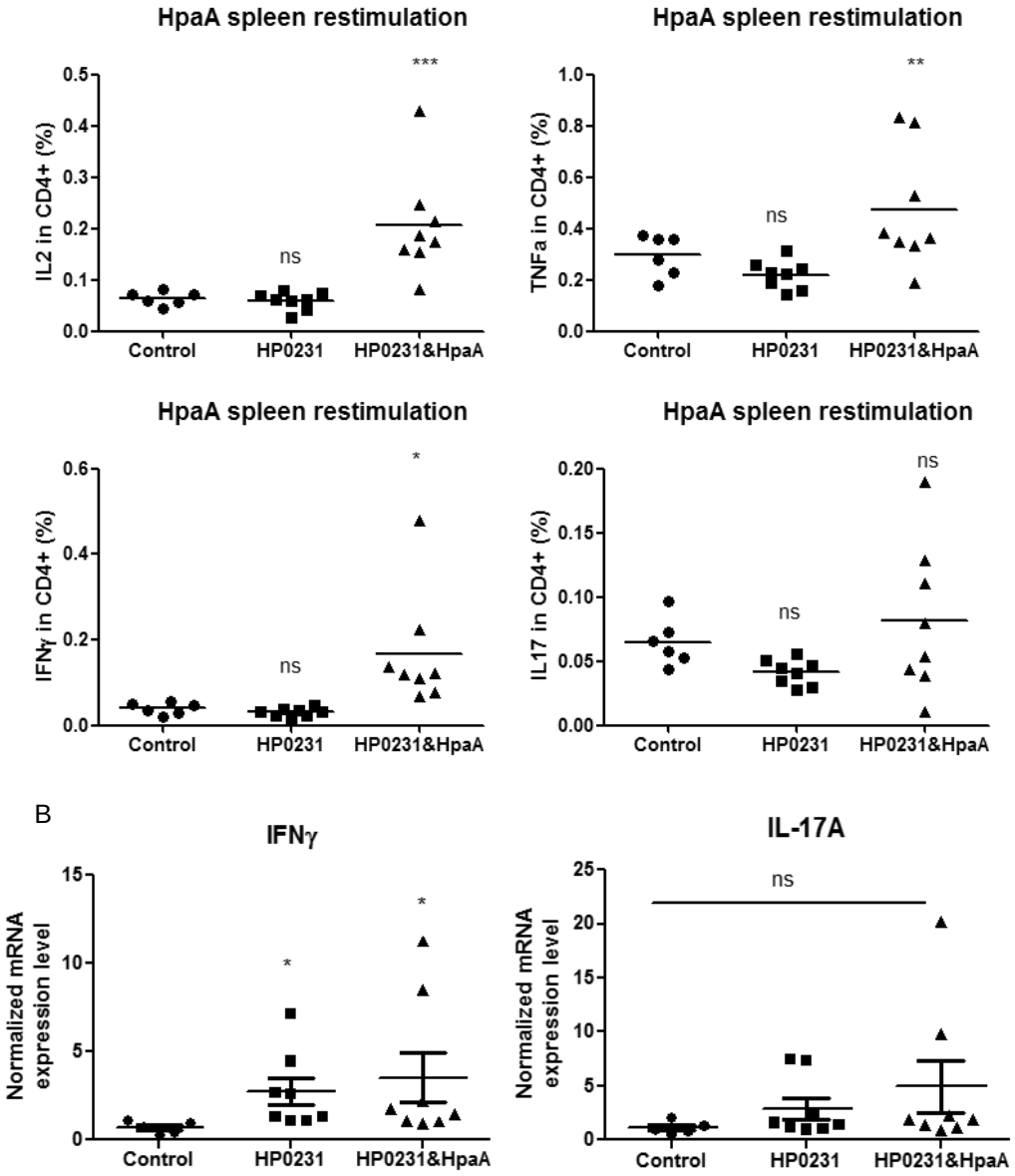


Figure 27 Antigen restimulation in spleenocytes and gastric cytokine expression in C57BL/6 mice. (A) HpaA and HP0231-specific restimulation in spleenocytes. Experiments were conducted with cells from control, HP0231 alone or HP0231 & HpaA immunized C57BL/6 mice. Lymphocytes were restimulated with 75 µg/ml HP0231 or HpaA proteins. Percentages of the intracellular cytokines in CD4+ T cells were calculated. The bar indicates the mean of the group and asterisks on top indicate significances relative to control. Each dot represents one mouse. Kruskal-Wallis test with Dunn's correction was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) IFNγ and IL-17A expression in C57BL/6 mouse stomach detected by RT-PCR. Result was normalized to housekeeping gene GADPH. Asterisks on top indicate significances relative to control. Kruskal-Wallis test with Dunn's correction was performed. * $P < 0.05$. The bar indicates the mean ± SD.

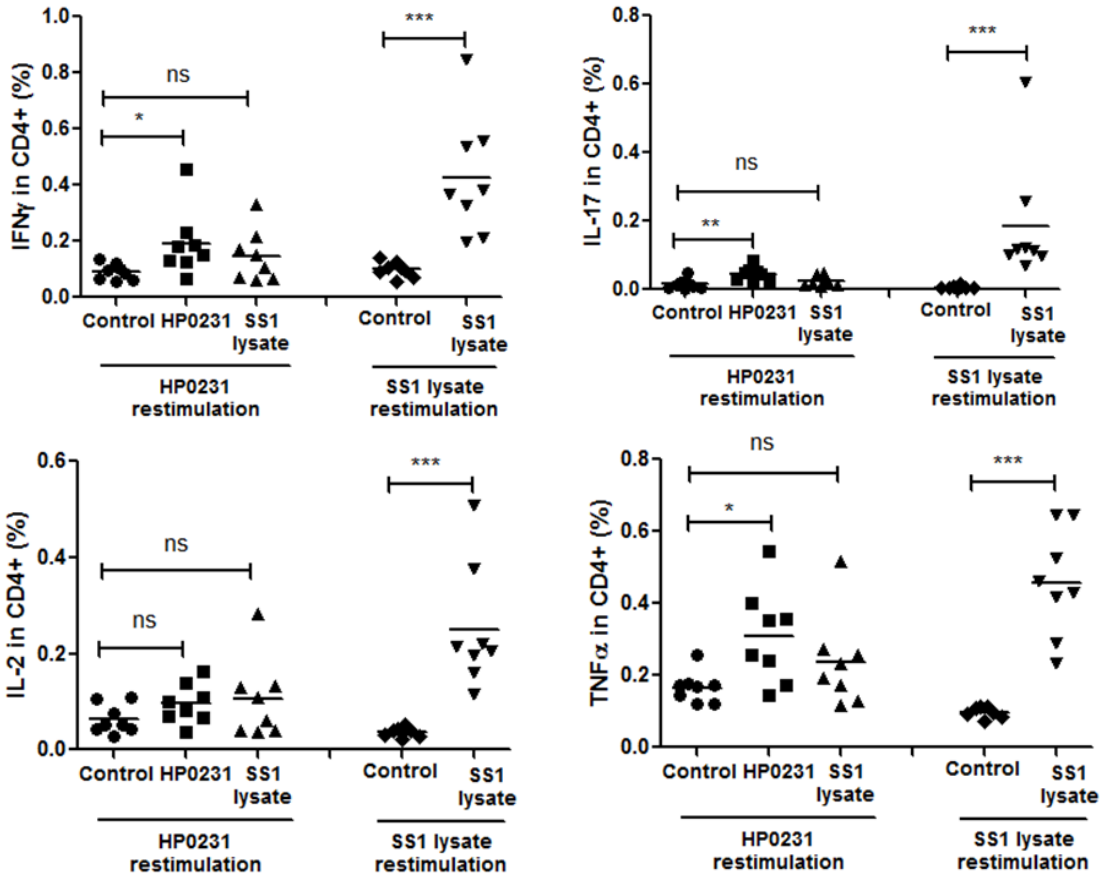


Figure 28 HP0231 or SS1 lysate-specific restimulation in spleenocytes. Antigen-specific restimulation was conducted on C57BL/6 mice spleen lymphocytes. Control group, HP0231 group and SS1 lysate group spleen lymphocytes were stimulated with 75 µg/ml of HP0231, or control group and SS1 lysate group were stimulated with 75 µg/ml SS1 sonicated lysate. Percentages (%) of the intracellular cytokines in CD4+ T cells were calculated. The horizontal bar indicates the mean of the group. Each dot represents one mouse. Kruskal-Wallis test with Dunn's correction was performed.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

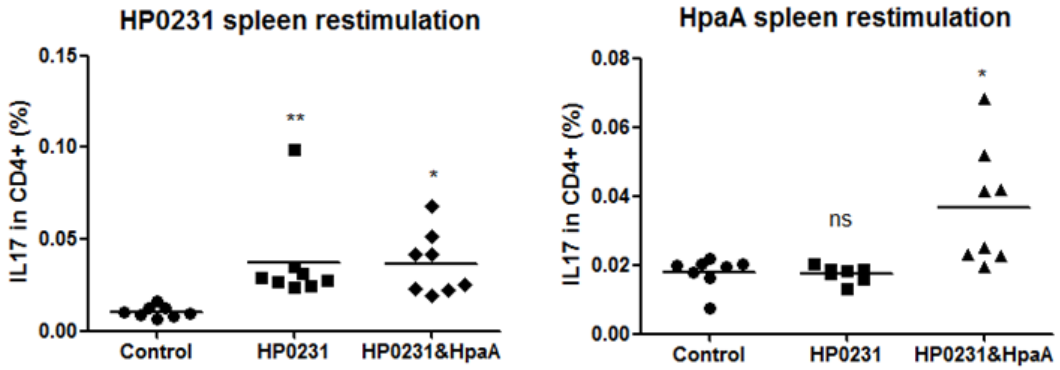


Figure 29 IL-17 levels in BALB/c splenocytes upon antigen-specific restimulation. Cytokine restimulation in lymphocytes was conducted on the spleen lymphocytes of BALB/c mice. Control group, HP0231 group and HP0231& HpaA group lymphocytes were stimulated with 75 µg/ml HP0231 and HpaA. Percentages of the intracellular cytokines in CD4+ T cell were calculated and shown. The horizontal bar indicates the mean of the group and asterisks on top of the bars indicate significances relative to control. Each dot represents one mouse. Kruskal-Wallis test with Dunn's correction was performed.*, $P < 0.05$; ** $P < 0.01$.

3.2 Functional role of HP0231

3.2.1 Defects in motility may impair the gastric colonization of *hp0231*-deficient bacteria

3.2.1.1 Lack of *hp0231* does not alter bacterial viability

Since bacteria lacking *hp0231* were not able to colonize the gastric mucosa, the potential mechanism behind this fact was investigated. Several reasons may explain the defects in *H. pylori* colonization in the context of *hp0231* deficiency. Loss of *hp0231* could have a major impact on bacterial growth, bacterial binding to host cells or bacterial motility [335]. To exclude defects in bacterial growth, exponential phase wild-type *H. pylori* strains and *hp0231*-deficient strains were incubated in Brucella broth DENT medium with 10% FCS for two to four days, and bacterial growth was determined by measuring optical density at the indicated time points. The lack of *hp0231* did not alter bacterial viability in different *hp0231*-deficient strains *in vitro* (Figure 30).

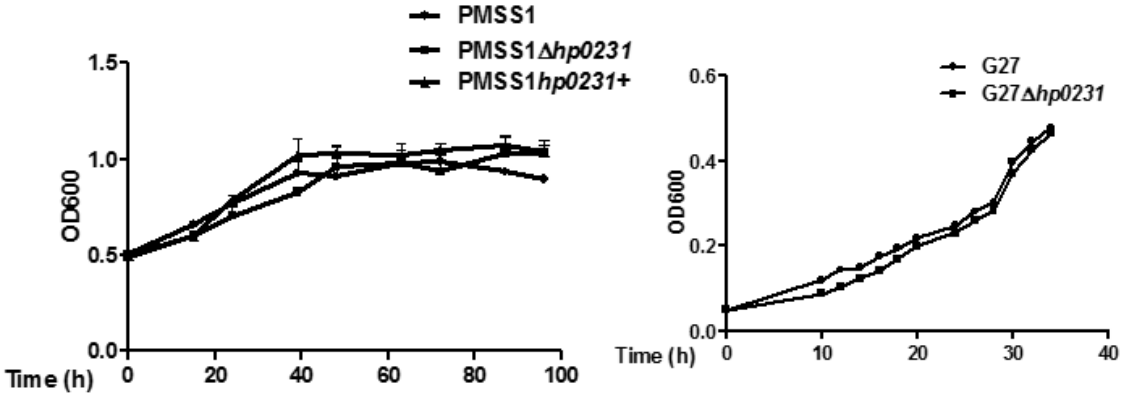
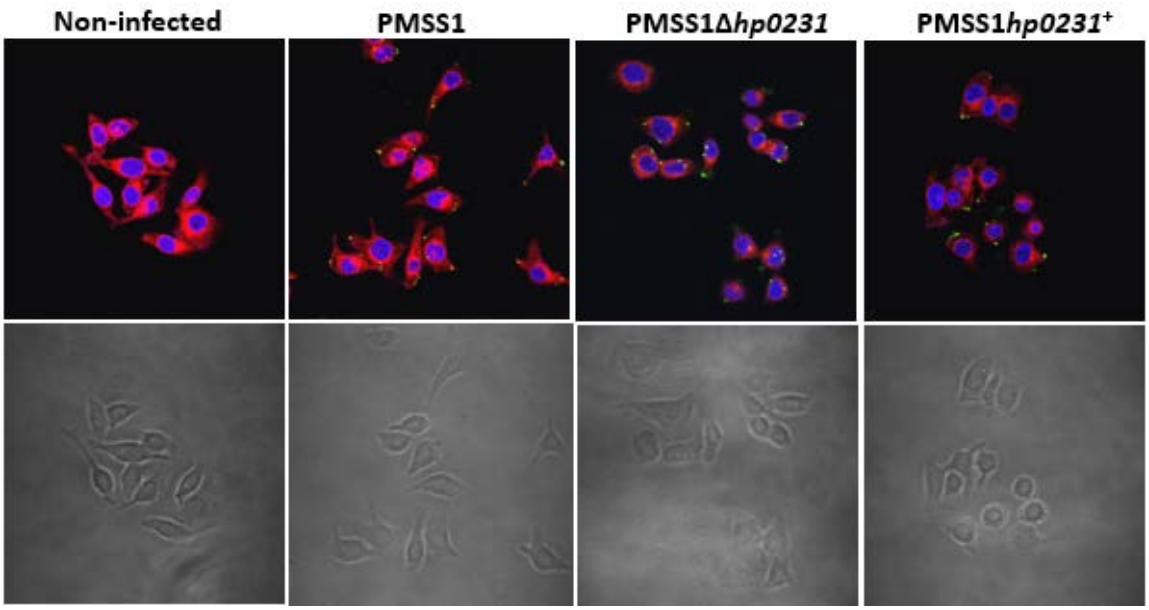


Figure 30 Lack of *hp0231* does not alter bacterial viability. Bacterial growth was determined by measuring optical density at the indicated time points in Brucella broth DENT medium with 10% FCS. Results from PMSS1, PMSS1Δ*hp0231* and PMSS1*hp0231*$^{+}$ (three repeats per group), and results from G27 and G27Δ*hp0231* (two repeats per group) are shown.

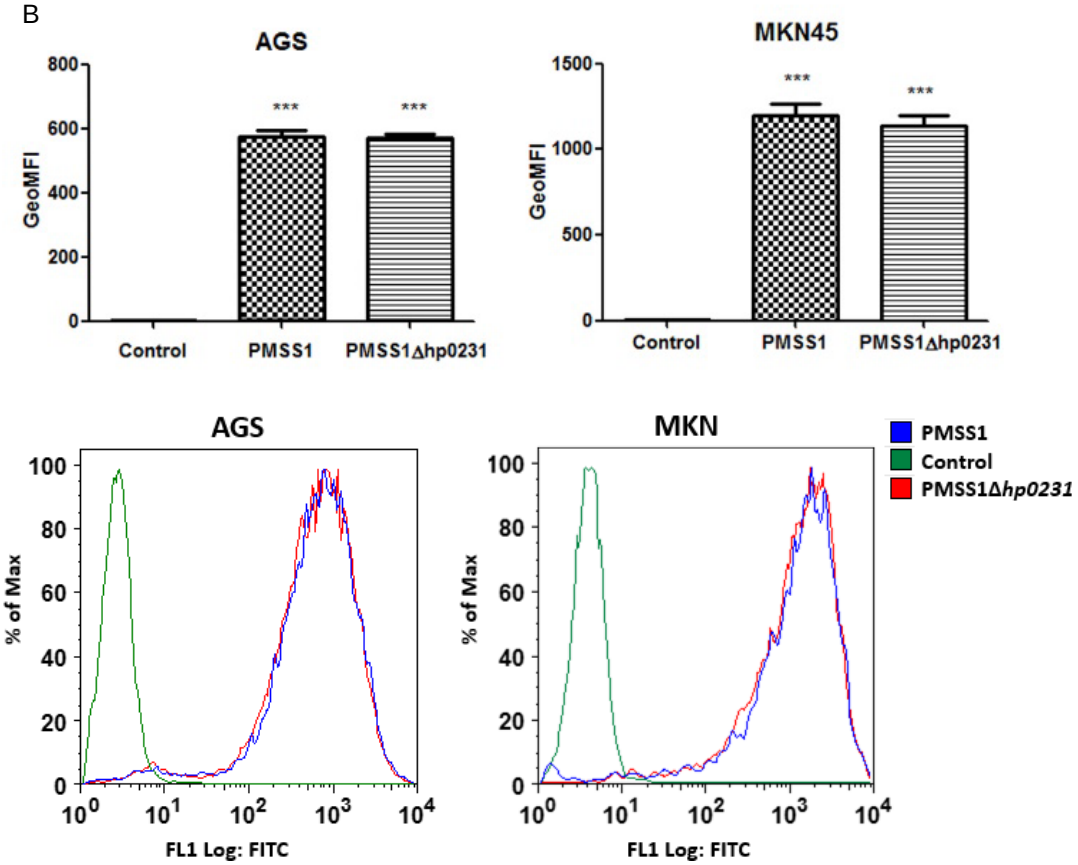
3.2.1.2 Deletion of *hp0231* does not induce changes in *H. pylori* binding

Another possible factor impairing *H. pylori* colonization in the context of *hp0231* deficiency could be reduced bacterial binding to epithelial cells. *H. pylori* requires different adhesins to bind to the host epithelial cells such as SabA and BabA. SabA [336] and BabA [337] are predicted to contain a disulfide bond in their extracellular domain. The lack of *hp0231* could therefore affect *H. pylori* binding status to gastric epithelial cells by altering the conformation of bacterial adhesins containing disulfide bonds. To study this, human gastric epithelial cancer cells were incubated with fluorescence-labeled *H. pylori in vitro*, and the bacterial binding status was analyzed by confocal microscopy [335]. *Hp0231*-deficient bacteria were able to bind to gastric epithelial cells (Figure 31A). To further quantify bacterial binding, human AGS cells were co-incubated with fluorescence-labeled *H. pylori*, and the binding was determined by flow cytometry. The absence of *hp0231* did not change the bacterial binding to gastric epithelial cells, since no differences in *H. pylori* binding were detected between wild-type and *hp0231*-deficient bacteria (Figure 31B). In contrast, lack of *sabA* and *babA* slightly reduced *H. pylori* binding (Fig 31C). Since the deletion of *hp0231* impaired bacterial colonization in mice, mouse primary gastric epithelial cells were isolated, infected with fluorescence-labeled *H. pylori*, and the total binding rate was determined by flow cytometry. The same results were observed when mouse gastric epithelial cells were used for the assays (Fig 31D), indicating that the deletion of *hp0231* does not induce changes in *H. pylori* binding to host gastric epithelial cells.

A


Non-infected
PMSS1
PMSS1Δhp0231
PMSS1hp0231+

B


AGS
GeoMFI
800
600
400
200
0
***
***
Control
PMSS1
PMSS1Δhp0231
MKN45
GeoMFI
1500
1000
500
0
***
***
Control
PMSS1
PMSS1Δhp0231
AGS
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
FL1 Log: FITC
MKN
% of Max
FL1 Log: FITC
PMSS1
Control
PMSS1Δhp0231

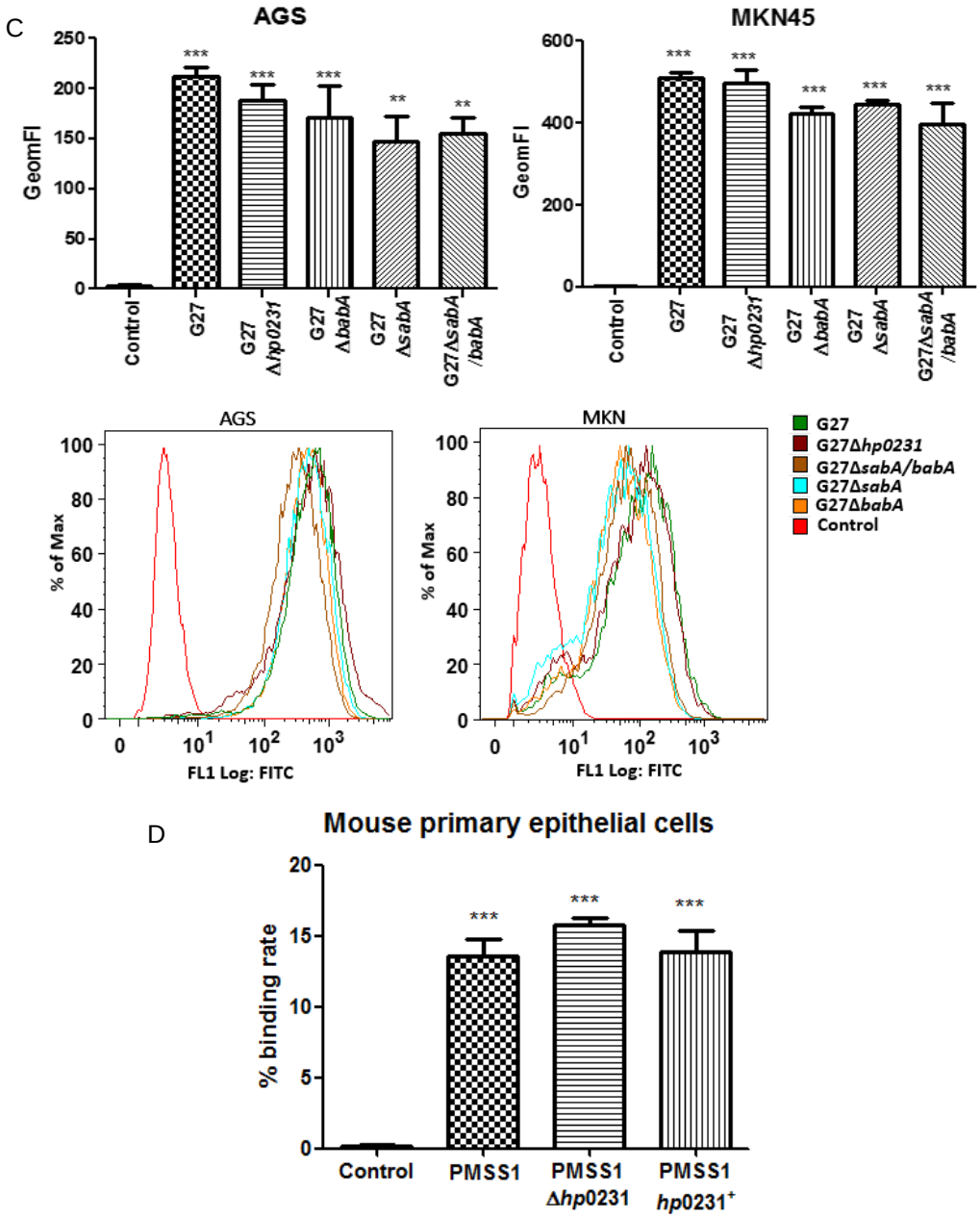


Figure 31 Lack of *hp0231* does not induce changes in *H. pylori* binding. (A) Immunofluorescence and corresponding bright field pictures of AGS cells co-incubated for 1 hour with the indicated *H. pylori* strains (green, MOI 5). Nuclei were stained with DAPI (blue) and cell membranes with deep red (red). Magnification 600x. (B) Human gastric cancer epithelial cells were infected with CFDA-SE-labeled *H. pylori* PMSS1 or the PMSS1*hp0231* mutant strain (MOI 10), and binding was analyzed by FACS. (C) Human gastric cancer epithelial cells were infected with CFDA-SE-labeled *H. pylori* (MOI 10). Samples were stained with EMA for live/dead gating and binding was analyzed by flow cytometry. GeoMFI from three independent experiments and a representative histogram are shown. ANOVA followed by Bonferroni's multiple comparison test was performed. $P < 0.01$; *** $P < 0.001$. Asterisks on top of the bars indicate significances relative to uninfected cells. (D) Mouse primary gastric epithelial cells were isolated, and infected with CFDA-SE-labeled *H. pylori* (MOI 10). Samples were stained with EMA for live/dead discrimination and CD45 APC to exclude T cells. Binding rate was analyzed by flow cytometry. The binding rates from three independent experiments are shown. ANOVA followed by Bonferroni's multiple comparison test was performed. *** $P < 0.001$. Asterisks on top of the bars indicate significances relative to uninfected cells.

3.2.1.3 Lack of *hp0231* reduces *H. pylori* motility

Several essential proteins, such as cell shape-determining protein 3 (CSD3) [338] and flagellar biosynthesis protein (FlhF) [339] involved in *H. pylori* helical shape maintenance and flagella composition, contain disulfide bonds. The lack of *hp0231* may interfere with the proper folding of these proteins, thereby damaging *H. pylori* rotary movement and preventing bacterial attachment to the gastric mucosa. Therefore, the motility of *hp0231*-mutant strain was examined as one possible factor preventing colonization [335]. Bacterial motility was assessed after two days culture on WC DENT soft agar plates. The lack of *hp0231* severely affected bacterial motility, which could probably lead to impaired gastric bacterial colonization (Figure 32).

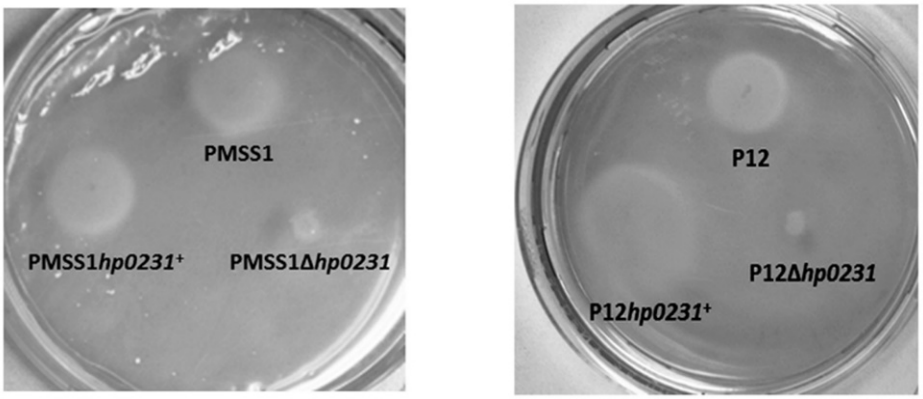


Figure 32 Lack of *hp0231* reduces *H. pylori* motility. *H. pylori* motility was assessed after 2 days incubation on soft agar plates. Data shows PMSS1, P12, *hp0231*-deficient or *hp0231*-complemented strains.

3.2.2 The absence of *hp0231* alters the function of *H. pylori* T4SS system

3.2.2.1 HP0231 is essential for CagA translocation

Since some proteins in the *H. pylori* T4SS system contain disulfide bonds to maintain their structure [340], it is questionable as to lack of *hp0231* could change T4SS function. Thus, this was investigated by analyzing CagA translocation and phosphorylation in gastric epithelial cells. CagA is a

typical effector translocated by *H. pylori* T4SS and the injection of CagA leads to a morphologic change of host cells known as a hummingbird phenotype [46]. Therefore, AGS cells were infected with different *H. pylori* strains at MOI 20 for 6 h [335]. *H. pylori* wild-type bacteria induced the hummingbird phenotype in infected cells (Figure 33). No morphologic changes were observed in cells infected with *hp0231*-deficient bacteria or when using a *cagA*-isogenic deficient strain, while cells infected with the complemented bacteria showed again the hummingbird phenotype (Figure 33A). This data indicates that CagA translocation was blocked in the context of *hp0231* deficiency. To further analyze CagA translocation after deleting *hp0231*, different *H. pylori* lysates from infected cells were subjected to Western blot in order to detect CagA phosphorylation. CagA tyrosine phosphorylation was detected in cells infected with wild-type *H. pylori*, while phosphorylation of CagA could not be detected in the absence of *hp0231* (Figure 34A). As expected, CagA was not phosphorylated in cells infected with bacteria lacking *cagE* or infected with SS1 due to the inability of these two strains to translocate CagA [38, 341] (Figure 34A).

Since genetic manipulation of bacteria often causes secondary genetic changes leading to an impairment of CagA translocation and phosphorylation, cells were infected with *H. pylori* in which the *hp0231* gene had been complemented [335]. In contrast to *hp0231*-knockout strains, CagA phosphorylation was detected in cells infected with *H. pylori* strains once *hp0231* was complemented (Figure 34B). Therefore, the impaired CagA phosphorylation is not due to off-target effects after deleting *hp0231*.

Impaired CagA translocation was further checked by analyzing CagA translocation in infected cells using a TEM-1-CagA β-lactamase activity reporter system [342]. β-lactamase activity was observed when using CagA from wild-type *H. pylori*, while no β-lactamase activity was detected when CagA from *hp0231*-isogenic knockout strain was analyzed (Figure 34C) [335]. This data indicates that HP0231 is essential for CagA translocation.

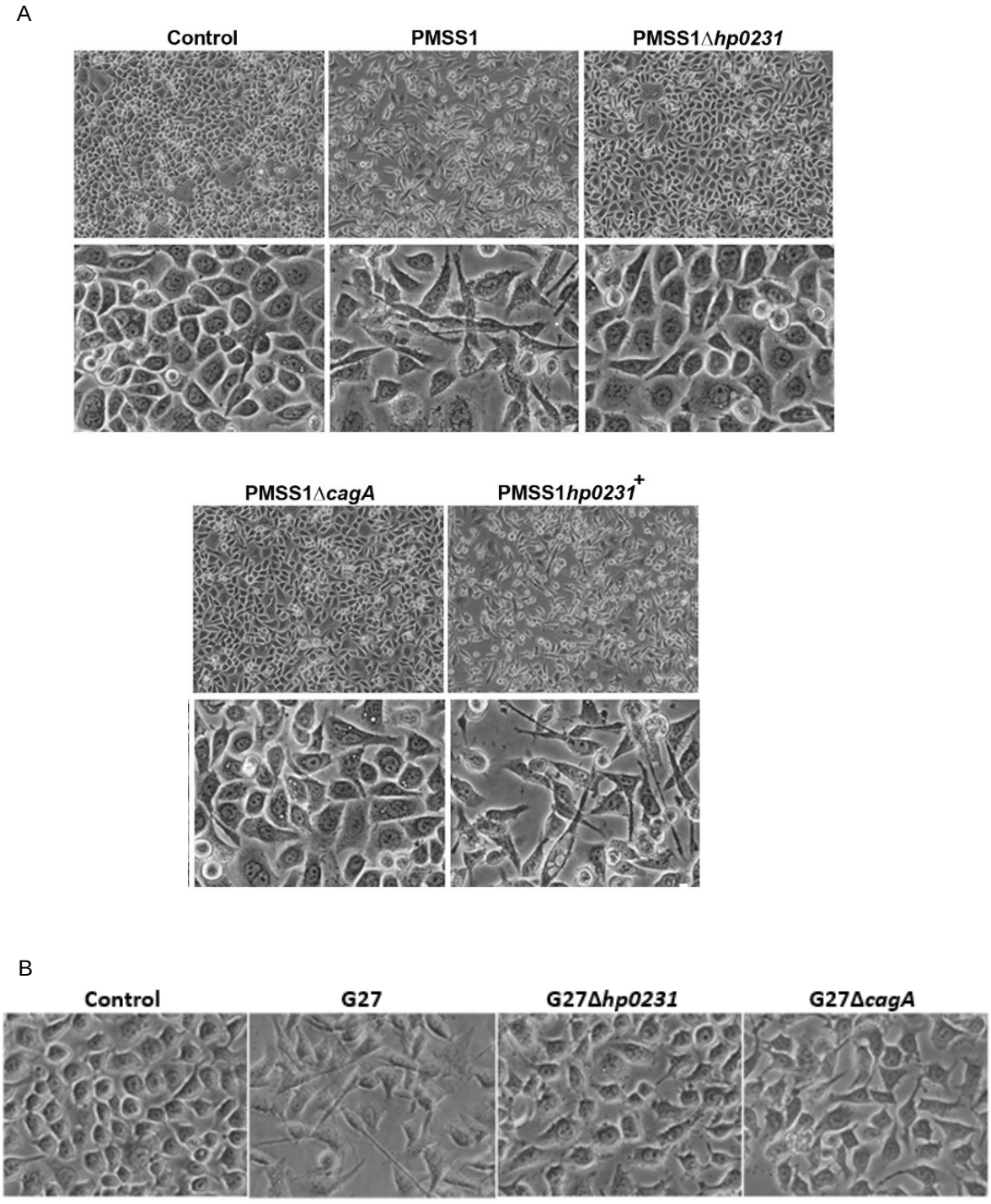


Figure 33 Lack of *hp0231* prevents *H. pylori*-induced hummingbird phenotype. (A) Representative pictures of AGS cells infected with the *H. pylori* strain PMSS1 and the indicated isogenic mutant strains at MOI 20. Pictures of the cultures were taken after 6 hours of infection at 100x (upper panel) and 400x (lower panel) magnification. (B) Representative pictures of AGS cells infected with the *H. pylori* strain G27 and the indicated isogenic mutant strains at MOI 20. Pictures of the cultures were taken after 6 hours of infection at 400x magnification.

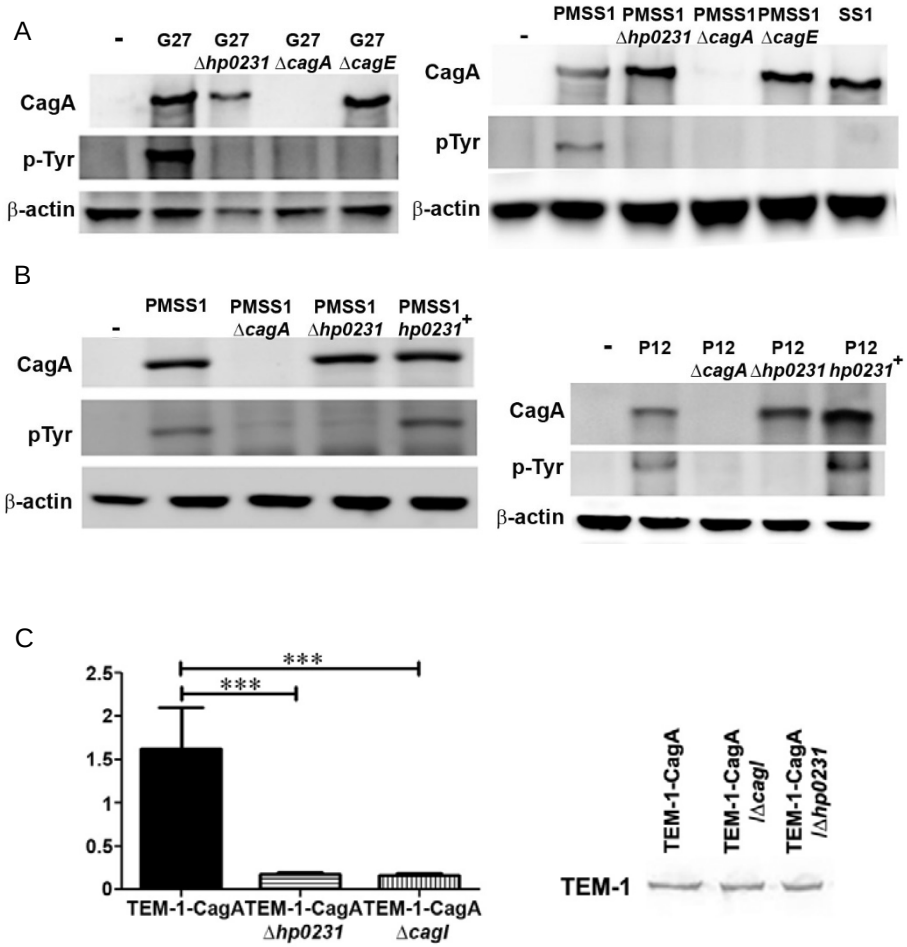


Figure 34 Absence of *hp0231* prevents CagA translocation. (A) and (B) CagA phosphorylation detected by Western blot after infection of AGS cells with wild-type *H. pylori* or *H. pylori* isogenic mutant strains for 6 hours at MOI 20. β-actin was used as a loading control. One representative blot from three independent experiments is shown. (C) CagA translocation measured by β-lactamase activity in cells infected with *H. pylori* strains producing TEM-1-CagA. Mean ± SD of blue-to-green fluorescence ratios obtained from four independent experiments performed in duplicate are shown. ANOVA followed by Bonferroni's multiple comparison test was performed. *** p<0.001.

3.2.2.2 Absence of *hp0231* cannot active NF-κB signaling pathway and induce IL-8 secretion

Since CagA was not translocated into gastric epithelial cells in the absence of *hp0231*, lack of *hp0231* was expected to impair the activation of signaling pathways by the T4SS, such as NF-κB [335]. p65 is one member of NF-κB signaling pathway, and its phosphorylation induces the activation of NF-κB-target genes [343]. The activation of NF-κB signaling pathway is T4SS system-dependent and CagA-independent [36, 37]. Phosphorylation of p65 was induced when AGS cells were infected with wild-type bacteria (Figure 35), indicating the activation of NF-κB upon *H. pylori* infection. Notably, infection of cells with bacteria lacking *hp0231* did not induce the phosphorylation of p65 (Figure 35). Similar results were detected when infecting the cells with *cagE*-deficient bacteria, as expected, while the lack of *cagA* still resulted in p65 phosphorylation since the phosphorylation of p65 is CagA-independent (Figure 35). Complementation of the *hp0231* gene in the knockout strain fully induced the phosphorylation of p65 similarly to the levels observed when cells were infected with the wild-type bacteria (Figure 35), confirming that HP0231 is essential for T4SS function.

As CagA translocation and activation of NF-κB initiate a pro-inflammatory response mainly inducing IL-8 expression in epithelial cells, the secretion of IL-8 in the absence of *hp0231* was assessed. AGS cells infected with wild-type *H. pylori* and the *cagA*-deficient strain secreted IL-8 in response to the bacteria infection, while only slight levels of IL-8 were detected in the absence of *hp0231*, similar to the levels observed after infection with *cagE*-deficient bacteria (Figure 36). Complementation of *hp0231* restored the secretion of IL-8 (Figure 36). Together, these results suggest that the deficiency of *hp0231* alters *H. pylori* T4SS function, thus impairing CagA translocation and activation of NF-κB signaling pathway and IL-8 secretion.

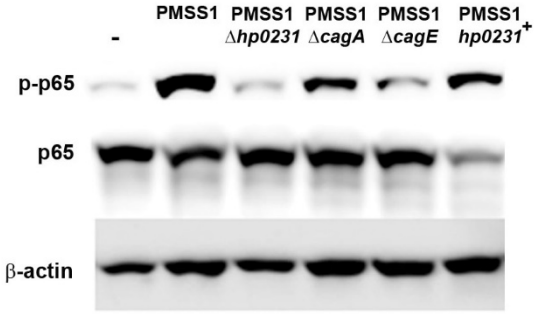


Figure 35 *hp0231*-deficient *H. pylori* does not activate NF-κB signaling pathway. Levels of phosphorylated and total p65 detected by Western blot in lysate from AGS cells infected for 6 h with the indicated *H. pylori* strains at MOI 50. A representative Western blot of three independent experiments is shown.

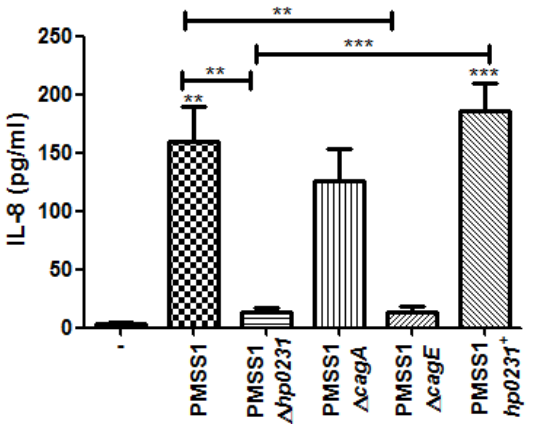


Figure 36 Lack of *hp0231* impairs *H. pylori*-induced IL-8 secretion by gastric epithelial cells. IL-8 secretion in cell culture supernatants after infection of AGS cells for 6 h with the indicated *H. pylori* strains at MOI 50. Results of three independent experiments expressed as mean ± SD are shown. ANOVA followed by Bonferroni's multiple comparison test was performed. ** $P < 0.01$, *** $P < 0.001$. Asterisks on top of the bars indicate significances relative to uninfected cells.

3.2.3 *Hp0231* deficiency reduces cellular vacuolation by inhibiting VacA secretion

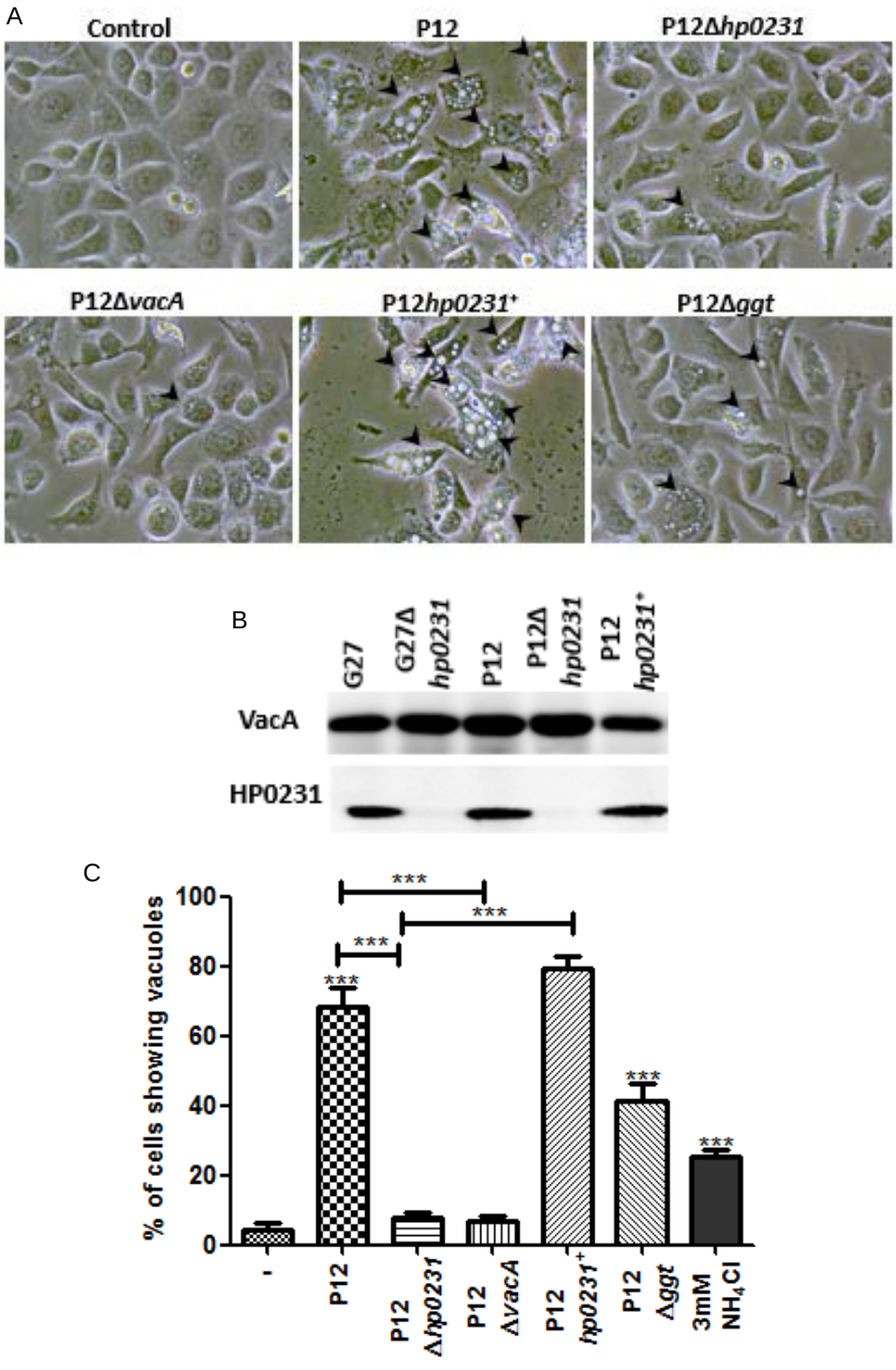
3.2.3.1 Deletion of *hp0231* reduces VacA-induced vacuole formation

VacA is another major *H. pylori* virulence determinant, containing disulfide bonds [307]. Whether VacA function was influenced in the absence of *hp0231 in vitro* was investigated by comparing cellular vacuole formation

upon infection with wild-type and mutant *H. pylori* [335]. Infection with wild-type bacteria led to cellular vacuolation in the host cells, while vacuolation was sharply reduced in cells infected with *hp0231*-deficient bacteria, which showed vacuolation to a similar extent as cells infected with *vacA*-deficient bacteria (Figure 37A). Vacuolation was restored when *hp0231* was complemented in the knockout strain (Figure 37A). Further quantification demonstrated that the percentage of cells showing vacuole formation in *hp0231*-deficient *H. pylori*-infected cells was similar to those in *vacA* deficient *H. pylori*-infected cells (Figure 37C). Interestingly, VacA expression levels were not affected by *hp0231* deletion, indicating that HP0231 altered VacA function but not its expression (Figure 37B).

Ammonia is another factor inducing vacuole formation, and its effect on vacuolation is VacA-independent [344]. To exclude the influence of ammonia on vacuolation, ammonia concentrations were analyzed in the supernatants of cells infected by different *H. pylori* strains. The ammonia level was significantly decreased in the absence of *ggt* as expected (Figure 37E), since gGT hydrolyzed glutamine to glutamate and ammonia. Decreased ammonia concentration and reduced vacuole formation in the absence of *ggt* were also reported in a previous study [344]. However, ammonia levels did not change when cells were infected with bacteria lacking *vacA* or *hp0231*, indicating that lack of *hp0231* does not change the ammonia concentration, and the effect of vacuolation in the absence of *hp0231* is specifically due to the effect on VacA.

To exclude non-specific effects of the lack of *hp0231*, whether HP0231 changed the functions of other virulence factors was also analyzed. Specifically, gGT was the focus, since no disulfide bonds are contained in its structure [344]. No changes in gGT activity were observed in the absence of *hp0231* (Figure 37D), confirming that the effects induced by HP0231 are specific for bacterial proteins forming disulfide bonds.


A
Control
P12
P12Δhp0231
P12ΔvacA
P12hp0231+
P12Δggt
B
G27
G27Δ hp0231
P12
P12Δ hp0231
P12 hp0231+
VacA
HP0231
C
% of cells showing vacuoles
100
80
60
40
20
0
***
-
P12
P12 Δhp0231
P12 ΔvacA
P12 hp0231+
P12 Δggt
3mM NH4Cl

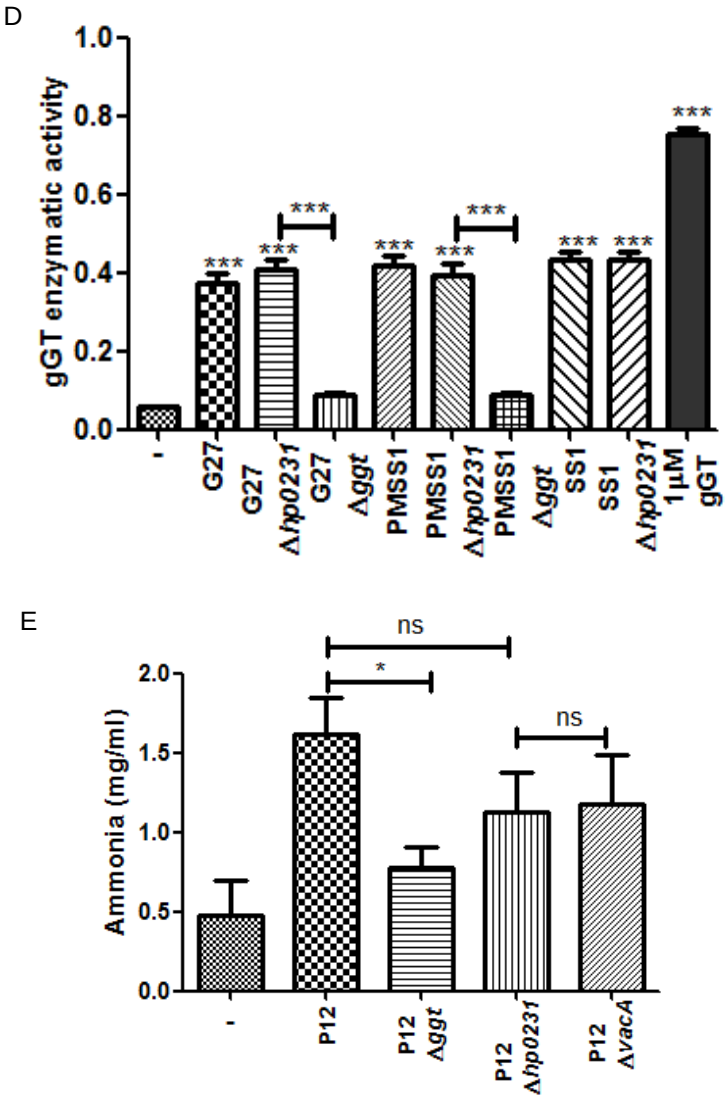


Figure 37 Deletion of *hp0231* reduces VacA-induced vacuole formation. (A) Representative pictures of AGS cells infected with indicated *H. pylori* strains for 24 hours at MOI 100. Arrows indicate vacuoles (400 x magnifications). (B) VacA and HP0231 expression detected by Western blot (C) Percentage of cells showing vacuoles in response to *H. pylori* infection at MOI100 for 24h. 3 mM NH_4Cl was used as positive control. Five high power fields (400x) were counted for each experiment. Results (mean ± SD) of three independent experiments are shown. ANOVA followed by a Bonferroni's multiple comparison test was performed. *** $p<0.001$. Asterisks on top of the bars indicate significances relative to uninfected cells. (D) *H. pylori* gGT activity assay. *H. pylori ggt*-knockout strains were used as negative controls, while gGT recombinant protein was used as a positive control. Results from three independent experiments (mean ± SD) are shown. ANOVA followed by Bonferroni's multiple comparison test was performed. *** $p<0.001$. Asterisks on top of the bars indicate significances relative to PBS control. (E) Ammonia concentration in cell culture supernatant after 24h infection at MOI100. Three independent experiments are shown. Kruskal-Wallis test with Dunn's correction was performed, * $p<0.05$.

3.2.3.2 Absence of *hp0231* reduces VacA secretion

Since the role of HP0231 on vacuolation in host cells was VacA-dependent, the mechanism of HP0231 affecting VacA function was analyzed. Mature VacA, a 88 kDa toxin with disulfide bonds has been shown to be auto-secreted through a bacterial Type V auto-transport system [307]. It interacts with multiple cell surface receptors, and induces vacuole formation and cell apoptosis [153]. The cysteine residues on VacA p55 domain were reported to be essential for VacA secretion [345], therefore, HP0231 might influence VacA secretion by altering disulfide bonds. As shown in Figure 38 A and B, VacA secretion levels were sharply reduced in different *hp0231*-isogenic deficient strains compared to wild-type bacteria. *H. pylori* gGT was used as a quantification control since the expression of gGT in both bacterial pellets and culture supernatants was not affected in the absence of *hp0231* (Figure 38 C and D). These results indicate that impaired induction of vacuolation in the absence of HP0231 might probably be due to an effect on disulfide bond formation in VacA. This may lead to subsequent defects in VacA auto-transportation and a reduction on its secretion. Further, they support the likelihood that HP0231 plays a critical role in *H. pylori* virulence via specifically facilitating disulfide bond formation of essential virulence factors.

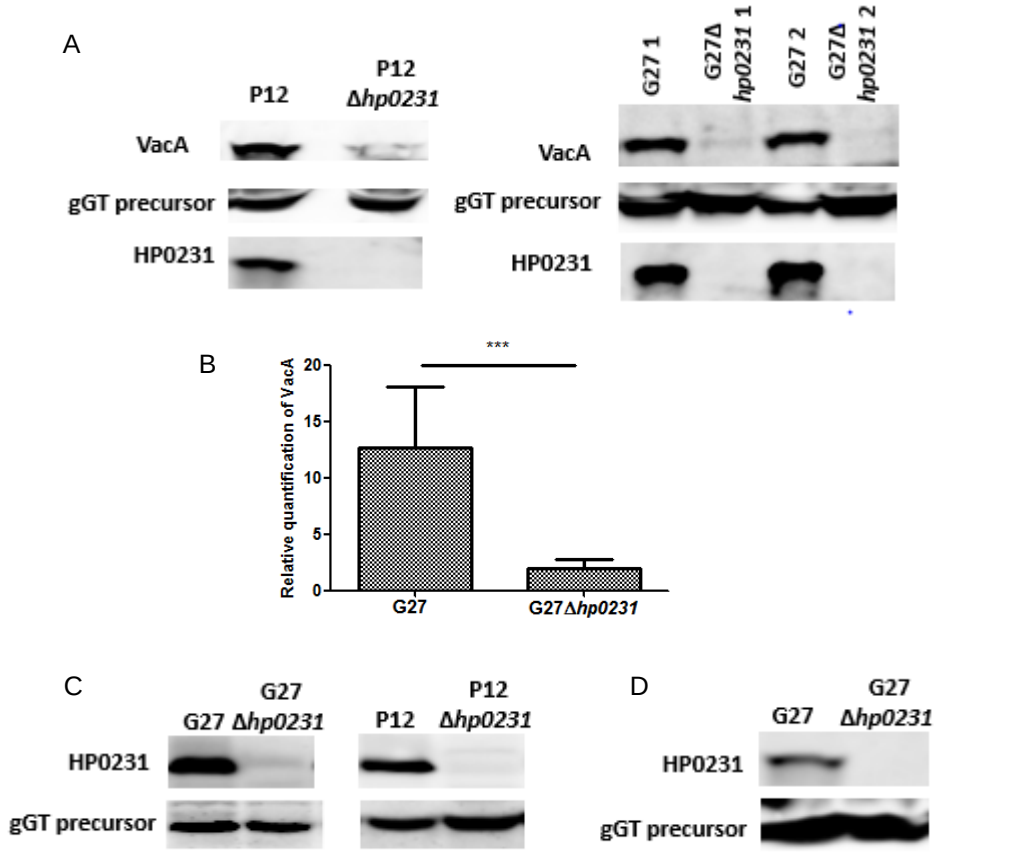


Figure 38 Lack of *hp0231* decreases VacA secretion. (A) VacA detected by Western blot in supernatants of *H. pylori* wild-type and *hp0231*-isogenic mutant culture medium. The indicated *H. pylori* strains were cultured in Brucella broth DENT medium overnight. The same amounts of supernatants were taken for Western blot. gGT was used as a positive control. (B) Quantification of VacA secretion levels in G27 and G27Δ*hp0231* bacterial culture supernatants. Secreted gGT was used as control for quantification. Results (mean ± SD) of three independent experiments are shown. T test; ***, p<0.001. (C) gGT expression was not affected by the absence of *hp0231*. The same amount of pellet samples from *H. pylori* wild-type and *hp0231*-deficient strains were taken for experiments. One representative blot is shown. (D) HP0231 protein detected by Western blot. Same amount of supernatant samples from G27 or G27Δ*hp0231* bacterial overnight culture in Brucella broth DENT medium were collected for Western blot. gGT precursor was used as a loading control.

3.2.4 PMX464 is not a specific inhibitor of HP0231

The discovery of HP0231 as an essential virulence factor for *H. pylori* colonization demonstrates the potential value of developing an inhibitor for HP0231 to eradicate *H. pylori* infection. An effective HP0231 inhibitor would potentially disable the function of the T4SS system; reduce VacA secretion and block *H. pylori* motility, therefore, not only reducing *H. pylori* virulence but also impairing bacterial colonization. Thus, the potential molecules

which can effectively inhibit the activity of HP0231 are worth investigating. PMX464, an effective and promising inhibitor of thioredoxin, was tested for the effect on inhibiting HP0231 activity in insulin reduction assays. PMX464 did not inhibit the activity of HP0231 without pre-incubation with HP0231 at 37℃ (Figure 39A). When pre-incubating PMX464 with HP0231 for 30 min, protein activity was inhibited to some extent but not completely (Figure 39B). Therefore, PMX464 is not specific for inhibiting HP0231 activity. In view of the essential function of HP0231, developing a specific inhibitor for HP0231 should be undertaken to block *H. pylori* motility and bacterial virulence.

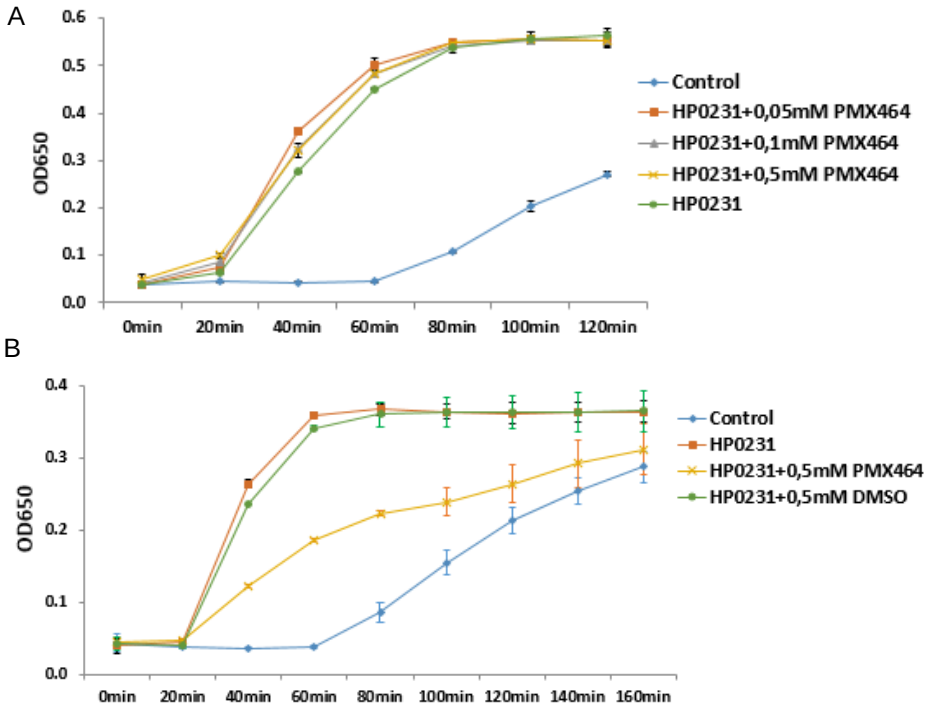


Figure 39 PMX464 is not a specific inhibitor for HP0231. Insulin reduction was tested in PBS (control) or 10 μM HP0231 or 10 μM HP0231 plus PMX464, 2 mM EDTA, 172 μM insulin and 1 mM DTT. Insulin aggregation was measured at 650 nm at RT. Reaction started by adding DTT. Three independent experiments were performed and data from one experiment with 3 repeats per group is shown. (A) Insulin reduction induced by HP0231 without pre-incubation with PMX464. Different concentrations of PMX464 were added together with 10μM of HP0231 protein directly to measure insulin reduction. The reaction was measured at 650 nM. (B) Insulin reduction induced by HP0231 with pre-incubation with PMX464. 10μM HP0231 was pre-incubated with 0.5 mM PMX464 or 0.5 mM DMSO at 37℃ for 30 min, and insulin reduction was measured at 650 nM.

Chapter 4: Discussion

Since traditional antibiotic treatment leads to a high incidence of resistance, investigating alternative means to prevent or treat *H. pylori* infection is necessary. In this dissertation, HP0231 alone or HP0231 plus HpaA immunization is shown to significantly decrease *H. pylori* colonization in mouse stomachs. HP0231 is important for the function of *H. pylori* virulence factors that contain disulfide bonds.

4.1 Vaccine approaches for *H. pylori*

4.1.1 Criteria for promising vaccine candidates

The interventions to prevent bacterial infection via vaccination have been proven to be safe and effective from both economic and public health perspectives [346]. However, choosing the most effective antigens from abundant proteins remains a problem for *H. pylori* vaccine selection. With the help of recent development in genomics and proteomics, the evaluation process of potential antigens has been greatly accelerated.

First, the appropriate antigens for vaccination should be determined based on their functions. A specific functional antigen such as urease is expected to protect the host efficiently against *H. pylori* infection [249, 284, 285]. Second, *H. pylori* clinical isolates are quite distinct with marked geographic variances [347]. Therefore, the sequence of candidate antigens should be highly conserved in most *Helicobacter* strains and different relative to other species. Protein sequence alignments of candidate antigens against proteins across different bacterial species can be used to determine the potential functions of the antigens and to evaluate their sequence conservation and specificity. The vaccine candidates HpaA, NapA, gGT and HP0231 fulfil these requirements.

In addition, seroreactivity is a useful parameter to evaluate appropriate antigens, because positive serological recognition suggests that the antigens are immunogenic and will be able to provoke immune responses in the host immune system. The seroreactivity of individual antigen was

evaluated by line blot in this study. The line blot setup is a useful method not only for comparing the antibody responses to different antigens in individual patients, but also for the fast comparison of specific antigens across samples from different geographic sites or samples showing different degree of pathology. The data from the line blot screening revealed that the immunogenicity of *H. pylori* virulence factors in human patients varies significantly by geography: CagA, HpaA, GroEL and FliD in the German population or CagA, HpaA and FliD in the Chinese population were shown to be highly sensitive diagnostic markers for *H. pylori* infection. The serological data also indicates that CagA may increase the risk of gastric cancer, which is in line with previous studies [318-320]. Furthermore, line blot results provided a reference for vaccine selection. One OMP (HpaA) and one antigen showing low seroreactivity were chosen for vaccine formulation. The surface exposed HpaA was easily recognized by the immune system, and the other secreted protein showing low seroreactivity, such as HP0231, was able to prevent *H. pylori* colonization due to its function. The vaccination results indicated that a protein inducing low serum reactivity with essential function was important for the eradication of the bacteria, since HP0231 but not NapA conferred a significant protection in different vaccine formulations (see 4.1.2). A protein showing low humoral response in human sera and an essential function in *H. pylori* such as UreA has been proven to be an effective *H. pylori* vaccine candidate [263, 284]. Nevertheless, protein function, conservation, and seroreactivity might represent the basic criteria for selection of additional promising vaccine candidates.

4.1.2 HP0231 and HpaA, but not NapA, are good candidates for an *H. pylori* vaccine

To evaluate the role of vaccine candidates during *H. pylori* infection, the impact of the candidate antigens on *H. pylori* colonization was evaluated. The data showed that the deficiency of *ggt* hampered *H. pylori* colonization after short term and long term infection, a finding that is similar to other studies [121, 122]. This suggests that gGT is an important virulence factor

for *H. pylori* survival in the stomach. Initially, gGT plus HpaA was suggested to be used in a vaccine formation, but this vaccination data was part of another study which was not included here.

HpaA plus NapA or HpaA plus HP0231 were investigated for vaccination studies. The deletion of *napA* did not affect *H. pylori* colonization seven days after infection. A similar result was shown in a previous study [105]. NapA is important for *H. pylori* to combat the oxidative stress in the stomach. The previous study showed that the lack of *napA* did not hamper *H. pylori* colonization without prior induction of oxidative stress. However, if oxidative stress was induced, the colonization of *napA*-mutant was sharply decreased [105]. This indicates that NapA may play a role in *H. pylori* chronic colonization in the presence of oxidative stress. Nevertheless, NapA is not important for *H. pylori* colonization of the mouse stomach under normal conditions. The vaccination results demonstrate that immunization with NapA alone or NapA plus HpaA do not protect the mice against *H. pylori* infection. Recent studies have shown that NapA plays a protective role against *H. pylori* infection [104] but does not play a protective role against *H. suis* infection [281]. The contrasting results from these studies may be due to several factors, such as the techniques used to assess the colonization, the different genetic backgrounds of the mouse models, the experimental protocols, and the dose of antigens used for immunization. Nevertheless, NapA does not seem to be a good vaccine candidate for *H. pylori*.

However, HP0231 turned out to be a suitable vaccine candidate. HP0231 is required for gastric colonization, since *H. pylori* lacking *hp0231* did not colonize in the stomach. Likewise, bacteria without *hpaA* failed to colonize the mouse gastric mucosa [94]. The prophylactic immunization experiments showed that either HP0231 alone or HP0231 plus HpaA immunization effectively protected mice from *H. pylori* infection after challenge with either a high dose or low dose of bacteria. Moreover, the efficiency of this formulation was not limited to a specific mouse strain. The bacterial load in HP0231 and HpaA double-immunized mice was slightly but not significantly lower than that of mice immunized only with HP0231, indicating that

HP0231 plays a major role in the HpaA and HP0231 vaccine formulation. However, some mice showed complete eradication of *H. pylori* infection in the HP0231 plus HpaA double-immunized group. This suggests HpaA still plays a role in this formulation. In fact, the protective immune responses induced by antigens are generally dose-dependent. Intragastric immunization normally requires higher antigen doses compared to other immunization methods, such as intranasal immunization [212]. The doses used for intragastric immunization in this study were much lower than many oral immunizations [90, 205]. Thus, a more effective protection or even a sterilizing protection after HP0231 and HpaA immunization could be expected with higher doses.

The serological data suggests that HpaA is not only a highly sensitive diagnostic maker for *H. pylori* infection in human, but also a marker for detection of *H. pylori* infection in the initial infection phase in mice. *H. pylori* lacking *gGT* or *hp0231* were unable to colonize the mouse stomach, but high IgG responses to HpaA were detected in mice that were infected with these two mutant strains. The antibody response to HpaA was detected both after short term and long term infection, which indicates the serological response to HpaA in mice is not dependent on the final colonization density but on the initial infection phase. Thus, HpaA is an important marker to detect *H. pylori* infection in both human and mice. In addition, HP0231 is an important immunogenic component in *H. pylori* whole cell extracts since HP0231 is recognized in the serum of mice immunized with *H. pylori* lysate. Moreover, HP0231 and HpaA are able to induce strong and continuous humoral immune responses in the HP0231 and HpaA immunized mice. Therefore, HP0231 and HpaA are promising vaccine candidates for *H. pylori*, though the optimum dosage and route of vaccine application requires further investigation. Meanwhile, development of a multi-component-vaccine formulation that also includes additional *H. pylori* proteins such as urease, gGT, or Tipα combined with HpaA and HP0231 may represent a more powerful vaccine.

4.1.3 Vaccine-induced protection is associated with antigen-specific Th1 and Th17 responses as well as IFNγ expression in the stomach

Although the mechanism for vaccine-induced protective immunity has been proposed in many studies, it remains incompletely understood. It is widely accepted that the vaccine-induced protection against *H. pylori* is mainly dependent on cytokines and CD4+ T cells, but not on CD8+ T cells or B cells. Specifically, Th1 and Th17 cells are the dominant CD4+ T cells involved in vaccine-induced protection (see 1.4.2.1). Therefore, the role of Th1 and Th17 cells and associated cytokines expression on vaccine-induced protection were investigated in this study.

Increased expression of IFNγ, but not IL-17A, was detected in immunized mice, in agreement with previous studies [212]. HP0231 and HpaA immunization induces antigen-specific Th1 and Th17 responses, which has been reported in previous studies [212, 249]. Notably, previous studies concluded that, although prophylactic immunization induces both Th1 and Th17 responses, the vaccine-induced protection is mainly generated by the Th1 response but not the Th17 response. The deficiency of IL-17A does not affect vaccine-induced protection against *H. pylori* [249, 252], while it is highly reduced or abrogated in the absence of IFNγ [192, 212, 213], suggesting an essential role of IFNγ for vaccine-induced protection. In addition, the adoptive transfer of UreB epitope-specific Th1 cells reduces *H. pylori* colonization [249], but the adoptive transfer of UreB epitope-specific Th17 cells does not affect *H. pylori* colonization compared to control mice [249]. These results further indicate that Th1 cells, but not Th17 cells, contribute to vaccine-induced protection. Additionally, Th1 cells can compensate for the lack of Th17 cells upon vaccination [244].

4.2 HP0231 seems to be a “master virulence factor” for *H. pylori*-induced pathogenesis

Disulfide bond formation is an essential post-translational modification to maintain the proper folding of many proteins. Many bacterial virulence factors, including secreted toxins and surface components, contain disulfide bonds. Therefore, various disulfide oxidoreductases are involved in maturation of the virulence factors [141]. In gram-negative bacteria, the Dsb system is responsible for reducing or introducing disulfide bonds to their substrates. However, minimal data on *H. pylori* Dsb proteins has been reported to date. Recently, HP0231, a *H. pylori* Dsb protein, has been described as a dimeric oxidoreductase that catalyzes the formation of disulfide bonds in the bacterial periplasm [348]. Since many *H. pylori* virulence factors contain disulfide bonds, a role for HP0231 in maintaining their functions was analyzed by analyzing the impact of *hp0231* deficiency on *H. pylori* colonization, T4SS system functionality and VacA secretion [335].

4.2.1 Defects in motility impair gastric colonization of *hp0231*-deficient bacteria

The data presented here shows that HP0231 is required for gastric colonization. *H. pylori* lacking *hp0231* was unable to colonize in the mouse stomach, while complementation of the gene restored the ability of the bacterium to colonize. Therefore, several possibilities were investigated that could explain this observation, including the impact on bacterial viability *in vitro*, bacterial binding to epithelial cells and bacterial motility.

Since it is very challenging to investigate bacterial growth inside the mouse stomach, bacterial viability was evaluated *in vitro*. Lack of *hp0231* did not alter bacterial growth of different *H. pylori* strains. However, the growth of *H. pylori* lacking *hp0231* was reduced in the presence of DTT *in vitro* [143], indicating that the function of HP0231 might be more important for balancing the redox rate when bacterial cells are growing in a reducing environment. Since *H. pylori* has multiple proteins involved in the bacterial

redox balance, such as thioredoxins, SOD and catalase [108, 349], the way in which HP0231 cooperates with other proteins to maintain the redox homeostasis is unclear.

Although some *H. pylori* adhesins have been predicted to contain disulfide bonds in their crystal structures, like SabA [336] and BabA [337], the deficiency of *hp0231* does not induce changes in *H. pylori* binding to epithelial cells. Notably, although BabA contains eight cysteines, it does not form disulfide bonds in non-reducing conditions and its natural state is in an oxidized status in the periplasm of *H. pylori* [350]. This indicated that the predicted biochemical structures do not always mirror their physiological forms. That is one possible explanation that *hp0231* deletion does not change bacterial binding to epithelial cells. Another possibility is that *hp0231* deletion does affect the proper conformation of adhesins containing disulfide bonds. However, other adhesins without disulfide bonds whose functions are not affected by HP0231 can supplement for the lack of the binding of the affected adhesins.

Compromised motility hampered gastric colonization of the *hp0231*-deficient bacteria. Defects in motility have previously been reported for the *H. pylori* strain N6 when *hp0231* is deleted [351]. These results indicate that this is not a strain specific effect, and HP0231 is important for maintaining bacterial motility in general. Notably, the defects in motility observed in *hp0231*-deficient bacteria were not attributed to defects in the structure or distribution of the flagella, but to morphological changes of the cells. *Hp0231*-knockout bacteria showed straight-rod or curved morphologies and were approximately 50% longer than wild-type bacteria showing helical morphology [351]. The morphological changes concomitant to a lack of motility would significantly hamper colonization and thus the bacteria would be rapidly cleared. Studies have shown that the movement of *H. pylori* in the gastric mucosa is a flagella-mediated, spiral shape-required and chemosensory-directed process [53]. HP0231 probably regulates disulfide bond formation in proteins involved in *H. pylori* movement and bacterial shape maintenance, such as cell shape-determining protein CSD3 [338].

4.2.2 The absence of *hp0231* alters the function of the T4SS system

H. pylori T4SS is an essential virulence factor for bacteria-induced pathology since the oncogenic protein CagA is translocated by the T4SS [36, 37]. CagA translocation was observed to be impaired in the absence of *hp0231*, suggesting that the lack of *hp0231* might destabilize the components of the T4SS system to prevent CagA from being injected into host cells. This result is further verified by observing no activation of NF-κB and no secretion of IL-8 by cells infected with *hp0231*-deficient strains, since NF-κB activation and IL-8 secretion are *H. pylori* T4SS-dependent [36, 37]. Several proteins in the T4SS system are identified to contain disulfide bonds. For instance, T4SS pilus surface-localized protein CagL contains disulfide bonds in the α helix of its RGD motif [352]. The lack of the RGD motif in *cagL* disables CagA injection and reduces the binding to host cell integrins [353]. The expression level of CagL has been reported to depend on the correct assembly of other components within the *H. pylori* cag-PAI secretion apparatus [354]. Indeed, CagL expression level was sharply reduced in the deficiency of *hp0231* [335]. Another protein containing a disulfide bond is CagY, a surface filament structure in the T4SS, which is required for CagA translocation [355, 356]. Therefore, lack of HP0231 could also affect CagY structure and thereby impair CagA translocation into gastric epithelial cells. Together, the data presented here suggests that HP0231 is involved in the correct assembly of the T4SS secretion apparatus.

4.2.3 HP0231 affects VacA secretion

H. pylori VacA inhibits T cell proliferation and induces cell apoptosis when it is auto-secreted outside of the cell wall [70, 72, 73]. The vacuolation induced by bacteria lacking *hp0231* was similar to vacuolation induced by *vacA*-mutant bacteria, while vacuolation was restored when *hp0231* was complemented. In addition, the reduction of host cell vacuole formation was not due to changes in ammonia concentration but to the specific effect of HP0231 on proper secretion of VacA. Significantly reduced VacA secretion

into extracellular medium was observed in the absence of *hp0231*. The VacA p55 domain contains a disulfide bond in its C-terminus [307], and a previous study showed that the mutation of any cysteine residue on the p55 disulfide bond led to a significantly decrease in VacA secretion [345]. Therefore, disulfide bond formation in VacA might be influenced by HP0231, thereby reducing the level of VacA secretion.

In conclusion, the results for HP0231 not only support the crucial role previously suggested for HP0231 in reducing bacterial virulence, but also demonstrate that the injection of CagA and the secretion of VacA are influenced by HP0231. CagA and VacA are two major toxins which determine *H. pylori* virulence [154, 357]. The results presented in this study indicate that *H. pylori* cannot compensate for the lack of *hp0231* when concerned with the functions of the T4SS and VacA, although *H. pylori* might possess other proteins involved in disulfide bond formation [348]. Nevertheless, structural studies are necessary to identify specific conformational changes in those proteins upon deletion of *hp0231*. Because of its essential role in *H. pylori* gastric colonization and virulence, HP0231 seems to be a “master virulence factor” for *H. pylori*-induced pathogenesis.

4.2.4 Potential *H. pylori* factors influenced by HP0231

Potential *H. pylori* factors influenced by HP0231 are shown in Figure 42. Besides its role in motility, T4SS function and VacA secretion, HP0231 was reported to affect the secretion of two members of *H. pylori* cysteine rich family: HcpC [306] and HpcE, as well as the correct folding of HcpE [145]. Therefore, the reduced secretion of the other Hcp family proteins would be expected in the absence of *hp0231*.

Other proteins that lose their functions without disulfide bond formation may also be the targets of HP0231. For instance, HP0569, also known as Tipα, contains a disulfide bond in its N-terminus which is essential for homo-dimerization. The point mutations of either one cysteine or both cysteines affect Tipα penetration into the cell nucleus and thus reduce TNFα

expression and activation of NF-κB signalling pathway [30]. In addition, *H. pylori* GroES, which binds to TLR4 via its two disulfide bonds in the C-terminus, is dysfunctional if its four cysteines are mutated [31]. Thus, it would be interesting to test the relationship between HP0231 and these proteins by checking their secretion status and function in the context of *hp0231* deficiency. Moreover, since HP0231 is an abundantly secreted protein and its secretion level is 50 times more than urease [289], HP0231 may also modify extracellular proteins or proteins in host cells infected by *H. pylori*.

In addition, HP0231 plays an oxidative role in the *H. pylori* Dsb family. To maintain its physiological form, HP0595, a DsbB like protein in *H. pylori*, promotes the oxidization of HP0231 for proper enzymatic activity [143]. However, there may be other enzymes oxidizing HP0231, since some oxidized HP0231 is still present in the absence of *hp*0595 [143]. It is unclear which of the other Dsb proteins helps to maintain HP0231 in an oxidized form. It is also unclear whether HP0231 misfolds its substrates and which Dsb helps to reduce the extent of misfolded proteins. One possible candidate is HP0377. HP0377 acts as in the reduction pathway [134] and HP0377 may contribute to the reduction of disulfide bonds of misfolded proteins in *H. pylori*. In fact, HP0377 has been reported to catalyze the refolding of scrambled RNase [134]. Nevertheless, the entire *H. pylori* Dsb network is still far from being understood and more studies should be conducted to complete the Dsb network.

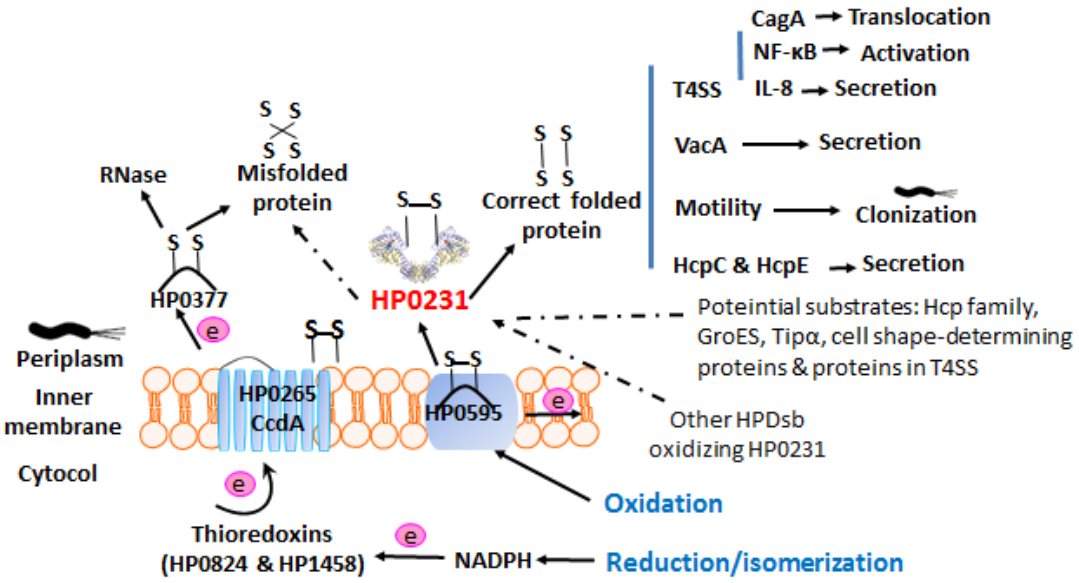


Figure 40 Potential *H. pylori* factors influenced by HP0231

4.2.5 Development of HP0231 specific inhibitors may be a valuable approach for *H. pylori* eradication

H. pylori infection remains a highly prevalent infection worldwide. Current therapeutic strategies based on antibiotic treatment have failed mostly due to an increased rate of antibiotic resistance. In addition, vaccine-based immunizations so far cannot completely eradicate *H. pylori* colonizing the gastric mucosa in most cases. Thus, the search for novel therapies based on specific inhibitors represents an interesting alternative to antibiotics and vaccines. The function of HP0231 suggests that it is a good candidate for developing inhibitors for the treatment of *H. pylori* infection.

In this dissertation, the role of a thioredoxin inhibitor on HP0231 was evaluated. However, this substance displayed a limited inhibition of HP0231 activity. Therefore, the development of a specific inhibitor for HP0231 might be a valuable approach for *H. pylori* treatment. Blocking HP0231 in bacteria, on the one hand, would contribute to bacterial eradication by altering the mobility of *H. pylori*; on the other hand, it would reduce bacterial virulence once the bacterium has colonized the stomach by blocking CagA translocation and VacA secretion.

Chapter 5:
Conclusion and Future prospects

5.1 Conclusion

Regarding the costly treatment of *H. pylori*-induced gastric diseases and high antimicrobial resistance elicited by antibiotic treatment, the development of a *H. pylori* vaccine is a feasible and economical alternative to prevent *H. pylori* infection. HpaA plus HP0231 is shown to be a valuable formulation for a *H. pylori* vaccine.

In addition, this is the first time that *H. pylori* HP0231 has been found to show a very important function. HP0231 is not only required for *H. pylori* colonization but is also essential for regulating bacterial virulence. On one hand, HP0231 impacts *H. pylori* colonization probably due to the regulation of bacterial motility. On the other hand, the functions of two essential toxins (CagA and VacA) have been found to be influenced by HP0231. CagA and VacA are the most described *H. pylori* toxins correlating with *H. pylori*-induced gastric diseases [24]. The injection of CagA is blocked via the alteration of *H. pylori* T4SS function in the context of *hp0231* deficiency. The secretion of VacA is sharply reduced in the absence of *hp0231*; thereby reducing VacA-induced cellular vacuolation. These results indicate the ability of HP0231 to affect the proper functions of virulence factors containing disulfide bonds.

5.2 Future prospects

Based on its essential effects in influencing *H. pylori* colonization and the functions of two essential toxins, HP0231 appears to be a promising drug target. A specific inhibitor of HP0231 would most likely prevent *H. pylori*-induced pathology and impair bacterial colonization. In addition, the effects of HP0231 on the other virulence factors containing disulfide bonds need to be understood by mapping the entire Dsb system and its substrates.

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