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Impact of L-proline transport and metabolism on osmotic stress tolerance and motility of the stomach pathogen *Helicobacter pylori*

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Publications and manuscripts related to this thesis

Henriquez T, Wirtz L, **Su D**, Jung H. [Review] Prokaryotic Solute/Sodium Symporters: Versatile Functions and Mechanisms of a Transporter Family. Int J Mol Sci. 2021 Feb 13;22(4):1880. doi: 10.3390/ijms22041880. PMID: 33668649; PMCID: PMC7918813.

Su D, Eder M, Hauke M, Jung H. Intracellular L-proline accumulation plays an important role in hyperosmotic environment tolerance of *Helicobacter pylori*. Manuscript in preparation.

Su D, Rivera-Ordaz A, Jung H. Intracellular L-proline accumulation is essential for flagellar-based motility of human gastric pathogen *Helicobacter pylori*. Manuscript in preparation

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Nomenclature

Gene deletions are marked by “Δ”. Complementary genes or plasmids are marked by “::”. Genes and proteins originated from *H. pylori* are marked by “Hp”, while genes and proteins originated from *E. coli* are marked by “Ec”.

Abbreviations

AA	Amino acid
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BB	Brucella broth
bp	Base pair
BSA	Bovine serum albumin
CTD	carboxyl terminal domain
cyclic AMP	Cyclic adenosine monophosphate
Da	Dalton
DNA	Deoxyribonucleic acid
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
Fe^{2+}	Ferrous ion
Fur	Ferric uptake regulator
FlaA	Major flagellar filament
FlaB	Minor flagellar filament
FMOC	Fluorenylmethyloxycarbonyl chloride
GFP	Green fluorescent protein
GK	γ-glutamyl kinase
Glu	Glutamate
GPR	γ-glutamyl phosphate reductase
HPLC	High performance liquid chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
K^+	Potassium ion
LB	Luria-Bertani (lysogeny broth)
MFS	Major facilitator superfamily of transporters
mRNA	Messenger RNA
Na^+	Sodium ion

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
nt	Nucleotide
OD_X	Optical density at the wavelength of x nm
ORF	Open reading frame
P5C	Δ^1-pyrroline-5-carboxylate
P5CDH	Δ^1-pyrroline-5-carboxylate dehydrogenase
PCR	Polymerase chain reaction
Pro	L-proline
PRODH	Proline dehydrogenase
PutA	Proline utilization A flavoenzyme
PutP	Proline permease
qRT-PCR	Real-time quantitative reverse transcription PCR
RHH	Ribbon-helix-helix
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SSS	Sodium/solute symporter
T4SS	Type IV secretion systems
UV	Ultraviolet
WHO	World Health Organization
wt	Wild-type
^{14}C	Carbon-14 carbon radioisotope
3D	Three-dimensional space

Summary

The Gram-negative microaerophilic bacterium *Helicobacter pylori* colonizes the gastric epithelium of about 50 % of the world's population and is the cause of gastritis and gastric cancer. Successful colonization of a host not only requires specific virulence factors, but is also linked to an adaptation of the metabolism to the conditions in the host. This includes adaptation to stress conditions such as pH and osmotic stress. In this context, it is interesting to note that L-proline is present in large amounts in the stomach of persons infected with *H. pylori*. Furthermore, previous analyses indicate that the genes *putP* (encoding a sodium/L-proline symporter) and *putA* (encoding a proline dehydrogenase) are essential for the colonization of the gastric epithelium by *H. pylori*. These findings suggest an important role of L-proline for the physiology and pathogenicity of *H. pylori*. This thesis now focuses on the mechanisms underlying the importance of proline and proline-specific systems (PutA, PutP, putative proline transporters ProP and ProVWX, putative proline synthesis enzyme ProC) in osmotolerance and motility of *H. pylori*.

The results of this thesis on osmotolerance of *H. pylori* revealed that individual deletion of both *putP* and *proP* significantly reduces cell tolerance to hyperosmotic stress. However, proline transport experiments showed that only PutP is able to accumulate proline in the cells, whereas evidence for a corresponding transport activity of ProP and ProVWX was not found. In contrast to *putP*, the expression of *proP* and *proVWX* was not stimulated by osmotic stress. ProP was also not affected at the activity level by osmotic stress. The latter result is consistent with the fact that ProP from *H. pylori* does not have the C-terminal helical extension that is responsible for osmosensing in ProP of *Escherichia coli*. For the putative proline synthetase gene *proC*, stimulation of gene expression by osmotic stress was shown only for cells in which *putP* was deleted. This suggests that proline biosynthesis is necessary to tolerate osmotic stress when proline uptake is disrupted. Analysis of the intracellular proline content revealed that *H. pylori* (wild type) not only exhibits an increase in intracellular proline under hyperosmotic stress conditions, but also a slight increase in the content of glutamate. The accumulation of glutamate is probably due to the activity of the proline dehydrogenase PutA. This observation is supported by the fact that the expression of *putA* is stimulated by hyperosmotic stress. The accumulation of glutamate under hyperosmotic stress is also known from other cells in which it compensates for the accumulation of potassium ions. Taken together, these results suggest that the proline-specific systems PutP, ProP, PutA, and ProC are involved in osmotolerance of *H. pylori* and that L-proline is important for *H. pylori* to cope with osmotic stress.

It was already known from a previous investigation that deletion of *putP* inhibits flagellar biogenesis and consequently *H. pylori* motility. These results were confirmed and extended in this thesis. In addition to the flagellar gene *flaA* (class III flagellar gene, encoding flagellin), the expression of *fliA* (encoding the sigma factor σ^{28} which

regulates class III flagellar genes) was also greatly reduced in the *putP* mutant compared with the wild-type strain. Consistent with these results, FlaA could be detected in Western Blots performed with protein extracts from *H. pylori* (wild type) but not from the *putP* mutant. Furthermore, analysis of DNA supercoiling levels revealed that the *putP* mutant had lower DNA supercoiling compared with the wild type, which is known to affect the expression of flagellar genes. In addition, the ATP content of the *putP* mutant was also significantly lower than that of the wild type. These results suggest that proline and the proline-specific proteins PutP and PutA systems are crucial for the energetic state of *H. pylori*. Alterations in DNA supercoiling and motility are most likely the consequence of a low energy state of the *putP* mutant.

1. Introduction

1.1 The pathogen *Helicobacter pylori*

1.1.1 A general introduction: from history to treatment

During millennia of the human civilization, gastric diseases have been noticed and recorded. Despite the unclear accurate time of the earliest medical record, people can find the descriptions of gastric diseases in antiquated writings from different cultures. In the ancient Chinese medical text *Huangdi Neijing*, which was continually written by many medical practitioners of then from the Warring States Period (481 BC to 403 BC) to the Western Han Dynasty (202 BC to 9 AD), the patients with gastric diseases were described as "they have stiffness on root of tongue, vomit after eating, feel abdominal distention and belch often…", it also indicated that the occurring of these symptoms can relate to the functional disorder of liver or spleen, and can be caused by pathogenic cold or improper diet and more (1). Another ancient text, which can be found in the temple of Aesculapius at Epidaurus in Greece (4th century BC), described a surgery for gastric ulcer as "…the god seemed to order his followers to seize and hold him, that he might incise his stomach. So, he fled, but they caught and tied him to the doorknocker. Then Asclepius opened his stomach, cut out the ulcer…" (2,3). Besides that, the first human who was determined to suffer from peptic ulcer, by archaeological autopsy, was a mummy of a man died in 167 BC from the Western Han Dynasty of China (4).

Although these diseases were recorded and investigated over the centuries, a key element of their etiological irritants was just found and proved four decades ago. For a long time, stress and lifestyle were believed to be the main causes of peptic ulcer disease in modern medicine. Even though microorganisms were already found to be present in the human stomach in late 19th century (5), people believed that they were just contaminants from ingested food due to the fact that many people without any clinical symptoms also carry the microorganisms in their stomach (6). In 1982, two Australian scientists, Barry J. Marshall and Robin Warren, announced that they discovered and isolated a bacterium, and elucidated its effect on human gastritis and peptic ulcer disease (7). However, their discovery was extensively doubted and criticized by the clinical community at that time. Owing to the unremitting efforts of the researchers who provided more and detailed experimental and clinical evidence (8-10), this finding finally became widely accepted. For instance, Marshall performed a self-ingestion experiment in 1985 to deliberately let the bacterium infect himself, which thereby did cause his subsequent acute gastric illness in truth (8). Originally, the bacterium was named as "*Campylobacter*-like organism", "gastric *Campylobacter*-like organism", "*Campylobacter pyloridis*" and "*Campylobacter pylori*", but now it is named as *Helicobacter pylori* because this microorganism actually differs from members of the genus *Campylobacter* (6, 11). In 2005, Marshall

and Warren were awarded the prestigious Nobel Prize in Physiology or Medicine on account of their ground-breaking work and revolutionary discovery on *H. pylori*.

H. pylori is categorized as ε subdivision of *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. This Gram-negative bacterium is 2 to 4 µm in length and 0.5 to 1 µm in width. It mostly appears as a helical rod (Figure 1.1), from which its name has been derived, and it can also be curved or straight-rhabdoid, but coccoid shapes emerge when cells are over cultured *in vitro* or under adverse environmental conditions (12, 13). The organism has up to seven unipolar, sheathed flagella of approximately 3 µm in length, which allow it to move fast in mucus layer overlying the gastric epithelial cells or other viscous solutions (13, 14). As a microaerophilic bacterium, *H. pylori* prefers the growth condition under oxygen level of 2 to 5 % and with additional 5 to 10 % of carbon dioxide at the optimal temperature of 37 °C with high humidity. Besides that, a pH range of 5.5 to 8.0 is required for the growth even though it can survive brief exposure to pHs of <4 (6, 15).

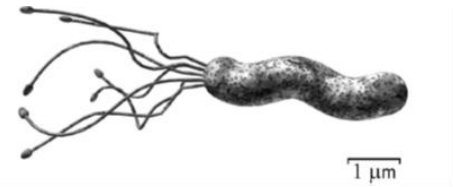

Figure 1.1 *Helicobacter pylori* 3-D image. *H. pylori* is named because of its spiral or helical shape. Figure was copied from Barry Marshall, 2002 (13).

As one of the most successful human pathogens, *H. pylori* has been co-evolving with humans for more than 60,000 years (16, 17), and now it colonizes over half of the population globally (18). The continuous and widespread colonization results in various phylogeographic patterns, which can not only reflect the ancient human migrations, but also the recent prevalence of *H. pylori* infection. Many researchers have been working on the systemic data analysis on the global distribution of *H. pylori* infection over the years, their conclusions suggest that usually the infection is more prevalent in developing countries in comparison with developed countries (Figure 1.2). In general, the infection rate correlates with socioeconomic status inversely, dietary mode or habit is involved in it as well (19-21). The exact mode of whereby *H. pylori* was initially obtained is still not fully understood yet because human stomach is the only known receptacle of *H. pylori* by far (22), and firm evidence for zoonotic transmission of *H. pylori* is still not found (23). New infections are deemed to proceed as an outcome of direct human-to-human transmission, which mainly includes gastro-oral, oral-oral and fecal-oral routes that occurring either within the same family or outside the family (24). Moreover, most acquisitions happen in early childhood and the infection usually retains lifelong if treatment is absent (6).

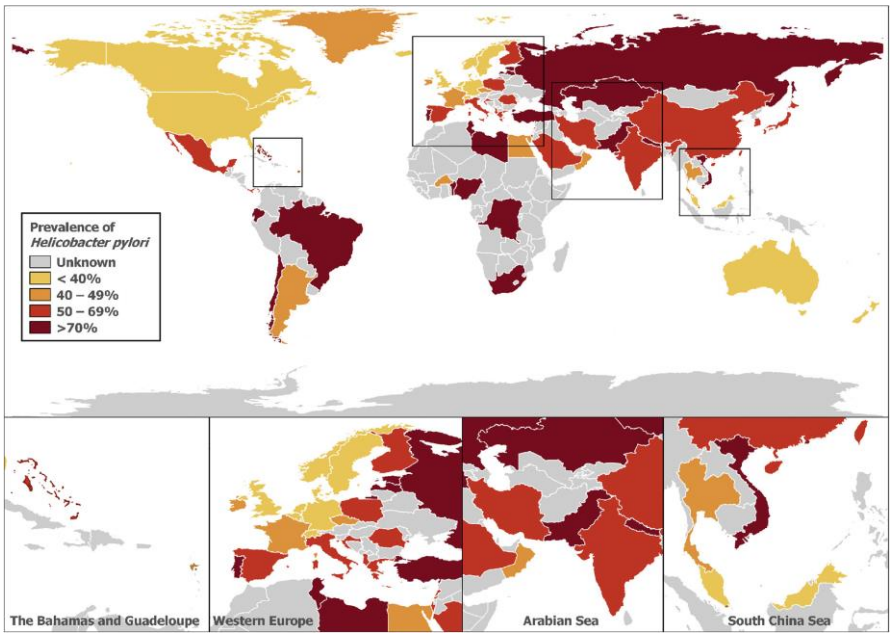

Figure 1.2 Global prevalence of *H. pylori* choropleth map. Certain regions are magnified to better display the smaller countries. The figure was copied from Hooi JKY, *et. al.*, 2017 (19).

Although only a minority of the *H. pylori* infected individuals can develop obvious clinical signs, the conclusive pathogenic role of the bacterium in development of different gastro-intestinal diseases is hard to be overstated. For example, *H. pylori* has been found to colonize the stomach of approximately 60 % and 90 % of patients with gastric adenocarcinoma and gastric lymphoma correspondingly (25). Many gastro-intestinal disorders such as acute/chronic gastritis, non-ulcer dyspepsia, peptic ulcers and gastric atrophy are highly related to *H. pylori* infection (6,26). Among all the infected people, when left untreated, around 1 to 2 % of them eventually develop to gastric cancer that has one of the worst survival rates and caused about 770,000 deaths and occupied 7.7 % of all cancer-caused deaths worldwide in 2020 (27). In addition, the World Health Organization categorized *H. pylori* as a type I carcinogen by reason of the actuality that *H. pylori* colonization increases the risk of gastric cancer approximately 10-fold (28). In recent years, more and more investigations have also indicated the association between *H. pylori* infection and extragastric diseases, including neurological diseases, dermatological diseases, hematologic diseases, cardiovascular diseases, metabolic diseases, hepatobiliary diseases, allergic diseases, ocular diseases and so on (29). These findings further reflect the significance and urgency of studies on *H. pylori*.

According to the ACG (American College of Gastroenterology) clinical guideline of treatment of *Helicobacter pylori* infection, all patients with a positive test of active infection with *H. pylori* should be offered treatment (30). However, the eradication of *H. pylori* is never an easy task due to the protection from stomach mucus gel layer that blocks the approach of antimicrobials to *H. pylori* (31). Presently, established treatment of *H. pylori* infection is the standard triple therapy with a proton pump

inhibitor (PPI), amoxicillin (AMO) and clarithromycin (CLA), which has been widely applied around the world (32). Even though the standard therapy has contributed to an eradication success rate of over 90 % in the earlier years (33), the increase of antibiotic resistance has destabilized the efficacy of it. Currently, it is necessary to assess the local prevalence of clarithromycin resistance and the previous use of macrolide for the choice of *H. pylori* eradication regimen. The recommended regimens for the first-line treatment are quadruple therapies (bismuth quadruple and concomitant), while levofloxacin-containing triple therapy and bismuth quadruple therapy are included in the second-line treatments (34). Besides that, although vaccine against *H. pylori* is a good option to prevent infection, most of the vaccine candidates under study were quitted after preclinical or phase I trials and thus no vaccine against *H. pylori* is available up till now (35-37). Challenging problems are the high genetic variability of *H. pylori* and its talent to escape from the immune system (38).

1.1.2 Bacterial response to stress conditions, in particular hyperosmotic condition

Microbes from dissimilar habitats constantly experience and challenge their exposure to fluctuating external environment that renders suboptimal conditions and stresses. Exist either *in vivo* or *in vitro*, these stresses are driven by the factors including but not limited to osmotic and acidic-alkaline perturbances, heat and cold shock, drought, metal exposure and nutrient deprivation (39, 40). In order to survive under stress conditions, bacteria have evolved multiple strategies to efficiently sense and rapidly adapt to the adverse perturbations, which includes the activation of alteration in the levels of cell membrane, ribosomes, proteins and nucleic acid (40). Despite the great complexity, it is important to investigate and thus understand the mechanisms behind bacterial stress response, in order to promote further study on potential applications related to probiotics utilization, pathogenic bacteria eradication and so on.

At present, for the microorganism *H. pylori*, studies regarding stress response have mostly focused on the aspects of oxidative and acidic stresses. As a microaerophilic bacterium, *H. pylori* is very sensitive to oxygen toxicity emanating from the aerobic environment and reactive oxygen species (ROS) (41). In the host's stomach, the source of ROS is mainly derived by the inflammatory response of the gastric epithelium (42). In addition, the phagocytic immune cells, neutrophils and macrophages are also able to produce abundant ROS by NADPH oxidase (43). In order to counter the stress generated by ROS, *H. pylori* has evolved numerous effective means. For example, the organism can prevent oxidant generation by minimizing the generation of peroxynitrite from superoxide (44). Importantly, it can also express multiple antioxidant enzymes including superoxide dismutase, catalase, peroxiredoxins and so on (45). Even so, comparing with other bacteria, *H. pylori* has a relatively underdeveloped antioxidant defence system, which may reflect specific adaptation to the stomach environment (45). Besides that, mechanisms of acidic stress response of *H. pylori*, which also partly correlate with the pathogenesis, have

also been well studied. The organism is capable of surviving in the harsh gastric environment characterized by high acidity in many ways. The role of urease in acidic stress defence will be introduced in the next section as an essential colonization factor. Apart from that, physiological acid adaptation (urease-independent survival) of *H. pylori*, which includes recombinational repair of DNA damage, urease-independent ammonia production, ferric uptake regulator (Fur) mediated iron-responsive regulation of amidases, histidine kinase protein mediated pH detection and response and so forth (46), is of significance in the acidic stress response. Additionally, mechanisms of other stress responses of *H. pylori*, such as serine protease HtrA regulated heat shock response (47), stringent response controlled nutrient deprivation response (48), Fur and NikR (Ni [II]-dependent transcriptional regulator) dominated metal stress sensing and response (49), have been investigated as well.

In addition to the above-mentioned conditions, osmotic stress is unquestionably one of the most important and most frequent types of environmental stress, which exists either in soil or in liquid. Resulting in a disruption in bacterial cell structure and function, the changes in extracellular osmotic pressure can lead to rapid water fluxes concentrating or diluting the cytoplasm of living cells (50). Commonly, in order to resist the loss of turgor pressure in hyperosmotic conditions, bacteria employ a short term "salt-import" strategy by uptaking K^+ and a long term "salt-export" strategy by accumulating compatible solutes (*e.g.,* proline, proline betaine, glycine betaine, glutamate, trehalose, taurine, ectoine, carnitine, *etc.*) in the cell (51, 52). The mechanism of osmotic stress response of *Escherichia coli* has been adequately studied. Briefly, in high osmotic pressure conditions, *E. coli* accumulates solutes via bio-synthesis or transport from external environment with the aid of its complex osmoregulatory systems (53). Firstly, the K^+ uptake is mediated by the low affinity TrkAEH(G) or high affinity KdpFABC acquisition systems. Secondly, following initial K^+ uptake, the membrane proteins including major facilitator superfamily (MFS) member ProP, ATP-binding cassette (ABC) transporter ProU, and betaine-carnitine-choline transporter (BCCT) family members BetT and BetU can mediate organic osmolyte uptake, among which ProP and ProU share similar range in substrate specificity including glycine betaine, proline betaine, proline and ectoine, while BetT is choline specific and BetU is betaine specific. BetA and BetB are responsible for the synthesis of trehalose and catalysation of choline into glycine betaine (54, 66). In addition to osmolality upshift conditions, mechanosensitive channels including MscS and MscL can release solutes from the cytoplasm of bacterial cells when hypoosmolality happens, and aquaporin AqpZ can increase osmotic stress by improving transmembrane water flux (55).

It is known that *H. pylori* is expert at survival in the turbulent gastric environment that expose the organism to different levels of osmolality changing along with the diversified diet of the host. During the process of colonization, osmotic pressure from the mucus layer of gastric epithelium is also an adverse threaten that *H. pylori* has to cope with. In addition, there is growing evidence that high dietary salt intake by the

host can increase the bacterial load and exacerbate *H. pylori*-induced gastric carcinogenesis (56, 57) by contributing to changes of the expression of genes encoding *H. pylori* virulence factors such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) *in vitro* (58, 59). By reason of the forgoing, the capacity of osmosensing and osmoregulation is crucial for *H. pylori*'s survival and colonization. However, the mechanisms of osmotic stress response of this bacteria, by far, are still poorly understood and require more investigation. Only some studies have investigated the potential osmoregulation mechanisms of *H. pylori*. For instance, Fur could act as an osmoregulatory factor because it was shown to be essential for the growth of *H. pylori* in the presence of high sodium chloride concentrations (60). The transcriptional repressor HspR might also be involved in osmoregulation of *H. pylori* since transcription from two promoters of the gene can be induced by salt treatment (61, 62). *H. pylori* deprived of the virulence factor protease high temperature requirement A (HtrA) also showed increased sensitivity to osmotic stress (63). Even so, we still understand too little about the role of K^+ and compatible solutes transport in the osmotic stress response of *H. pylori*. Unlike *E. coli* which employs complex and comprehensive solute transport systems, *H. pylori* does not contain many of the primary osmotic shock defence systems that are present in other bacteria. Even though a K^+ channel HpKchA has been found to be essential for the persistence of *H. pylori* in the gastric environment due to its function in K^+ passive transport (64), the systems involved in K^+ active uptake are barely known since the genome of *H. pylori* presents no genes encoding homologues of Kdp, Trk, Kup or Ktr (65). In addition, the role of other compatible solutes in the osmoregulation of *H. pylori* has not yet been defined (66), and therefore more interest should be devoted to this topic.

1.1.3 Pathogenesis and virulence factors

The human stomach is a hostile environment for bacteria to survive. In order to prevent the ingested bacteria from epithelium adhesion, the gastric mucosa secretes viscous mucus that arrests messes of bacteria, and the gastric acid eliminates over 90 % of ingested pathogens. Additionally, the gastric mucosa also produces antimicrobial peptides that facilitate the pathogen elimination. Importantly, the stomach mucosa is able to boost an intense innate and adaptive immune response to resist the colonization (69). *H. pylori*, however, is capable of overcoming these obstacles of human gastric environment to establish persistent infection and to deregulate host functions, which leads to gastric pathogenesis and cancer (67). In general, the pathogenesis of *H. pylori* infection can be concluded in the following four steps: firstly, survival under acidic stomach conditions; secondly, movement toward epithelium cells through flagella mediated motility; thirdly, attachment to host receptors by adhesins; lastly, causing tissue damage by toxin release (68). These steps are mediated by multitude virulence factors of *H. pylori* (Figure 1.3).

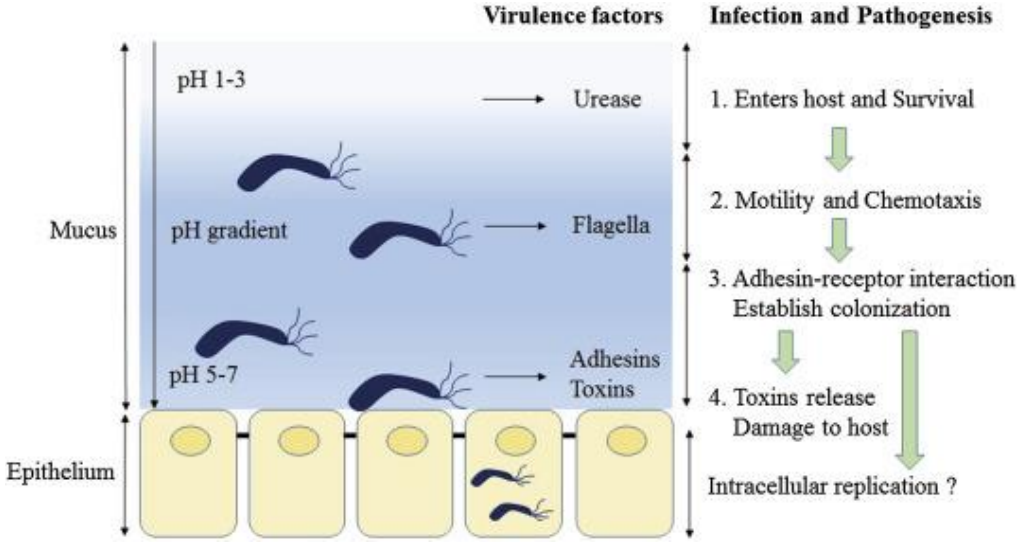

Figure 1.3 Schematic diagram of *H. pylori* infection and pathogenesis. The urease activity and flagella-mediated motility of *H. pylori* facilitate its survival and movement toward the lower mucus gel above the epithelium. Afterwards, adhesins and toxins promote the attachment to host receptors, damage of host tissue and intracellular replication. Figure was copied from Kao CY, *et. al*., 2016 (68).

Initially, when reaching host stomach, *H. pylori* survives under the acidic conditions mainly by virtue of its key virulence factor which is the enzyme urease catalysing the hydrolysis of urea to ammonia (NH_3) and carbon dioxide. Combining with water, ammonia immediately transforms to ammonium hydroxide that neutralizes the acidic micro-environment around the bacteria, which provides a buffering cloud that allows *H. pylori* to safely pass through the gastric juice (70,71). In addition to urease, other enzymes such as aminases AmiF and AmiE and gamma glutamyl transpeptidase are also employed by *H. pylori* to generate urea (72, 73). Along with the temporary "safety" by the buffering cloud, *H. pylori* is able to rapidly move through the mucosa layer to the neutral-pH basal layer, which is driven by the action of another important virulence factor, the flagella (74). More details regarding the flagella-mediated motility with be emphatically introduced in the next paragraph. With the help of flagella, *H. pylori* is able to penetrate the barriers of acid and mucus, afterwards it attaches to the gastric epithelium to establish long-term colonization and access to nutrition. In this step, a large number of proteins are employed by *H. pylori* to promote the attachment to the epithelial cells of the host, which includes the well-characterized adhesins blood-antigen binding protein A (BabA) and sialic acid-binding adhesin (SabA) that protect the bacteria from displacement promoted by peristalsis and gastric emptying (75, 76). Besides that, other proteins such as neutrophil-activating protein (NAP), heat shock protein 60 (Hsp60), adherence-associated proteins (AlpA and AlpB), outer membrane proteins (OipA, HopZ and HopQ), lacdiNAc-binding adhesin (LabA) and so on were also proven to be involved in the adhesion and attachment of *H. pylori* to the cellular surface receptors of host (77-83). In the last step of pathogenesis of *H. pylori* infection, tissue damage of host

is mediated by numerous bacterial toxins. Cytotoxin-associated pathogenicity island (cag PAI) that locates on the chromosome in *H. pylori* contains more than 30 genes, among which at least 6 genes are homologous with type IV secretion systems (T4SS) (84). Cag PAI is present in cagA positive *H. pylori* strains that exacerbate the severity of gastric diseases (85). Translocated by T4SS, cagA is able to enter the gastric cell cytoplasm upon contact with epithelium cells to disrupt host cell signalling and affect the host cell in many aspects including constitution of gastric epithelium cell pedestals, alternation of the cytoskeleton, influencing the proliferation of cells and motivating the secretion of IL-8 (86-88). Besides that, VacA induces vacuolization in epithelial cells of host, and is secreted by around 50 % of all *H. pylori* strains (89). VacA intervenes endosomal maturation of host cells and causes the formation of vacuole (90). Importantly, inflammatory response, cellular dysregulation and formation of anion-selective channels of host cells are also triggered by VacA that plays an important role in the pathogenesis of *H. pylori* infection (91, 92).

1.1.4 Flagellar-mediated motility of *H. pylori*

As mentioned above, flagella can mediate the motility of *H. pylori* and function as a crucial virulence factor in the process of colonization. *H. pylori* shows peculiar high velocity and movement in viscous substances due to its helical shape and polar flagella (93), thus even though *H. pylori* can merely survive in gastric lumen for minutes, it can rapidly migrate to mucus layer (94). Converting of urea into ammonia catalysed by urease can reduce the mucin viscoelasticity and thus promote the flagellar motility to cross the mucus and reach epithelial cells (95).

H. pylori possesses a tuft of up to 7 polar sheathed flagella, each single flagellum is a sophisticated structure that is comprised of 3 basic elements referred to as the basal body, hook, and filament (93). In a nutshell, the basal body is situated within the cell envelope and contains the flagellar rings, motor components, a rod and a type III secretion system (T3SS) that serves as the flagellar protein export apparatus (96-98). The hook is responsible for transmitting torque from the motor to the external helically shaped filament, which works as a propeller when rotated at its base and contains the minor flagellin FlaB and the major flagellin FlaA (99). More than 40 structural and regulatory genes are involved in the process of *H. pylori* flagellar biogenesis (100). These genes are divided into three classes according to their temporal order of expression. Class I genes, which encode components of the basal body in early flagellar assembly, are regulated by the housekeeping sigma factor σ^{80} (RpoD). σ^{54} (RpoN) regulates the transcription of class II genes encoding components of the hook and a minor flagellin that are required intermediately through flagellar biogenesis. The genes of class III, which encode the flagellins and other minor proteins of the filament, are needed late in the assembly process and governed by σ^{28} (FliA) (100-102). The flagellar biosynthesis is a complex process but its master regulator has not yet been found in *H. pylori*. In recent years, many studies have revealed that DNA supercoiling can influence the expression of flagellar genes in bacteria such as *H. pylori* and *Campylobacter jejuni* (103, 104).

1.2 Importance of L-proline for *H. pylori*

1.2.1 General impact of L-proline on pathogen-host interactions

L-proline, as an amino acid, plays complex roles in multiple cellular processes (Figure 1.4). Besides protein bio-synthesis and growth, proline also involves in the mechanisms of osmoregulation, redox signalling, unfolded protein responses, protein stability, cellular bioenergetics, stress resistance and biosynthesis of secondary metabolites of different organisms (105). Regarding pathogenic bacteria, the exact role of proline in their pathogenesis is depending on the physiology of certain host-pathogen interactions. Therefore, in order to develop novel strategies against bacterial infectious diseases, it is important to understand the mechanisms of how pathogenic bacteria utilize proline. The following part of this section will outline the previous and current findings or examples to demonstrate how proline facilitates the pathogenicity and affect pathogen-host interactions of several specific pathogens.

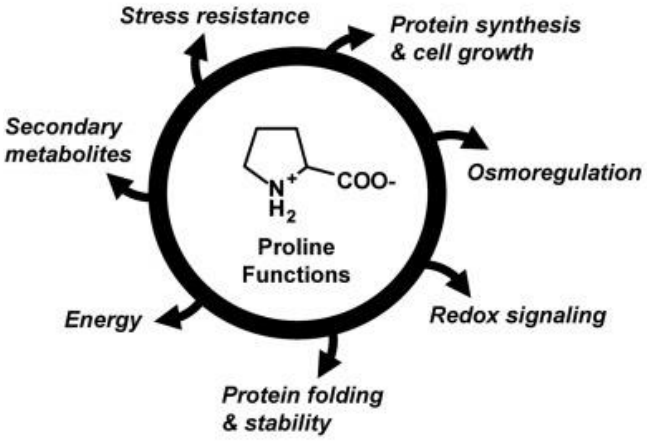

Figure 1.4 Potential biological functions of proline. The figure was copied from Christgen SL, *et. al.*, 2019 (105).

As introduced in section 1.1.2, proline is an osmolyte protectant employed extensively in organisms. Its role and mechanism in osmotic stress response has been widely investigated in bacteria. For instance, deprivation of proline transporter genes *proP* and *proU* can lead to a sluggish growth of *E. coli* in high-osmolality human urine (106); *Salmonella typhimurium* variant strains that characterized with higher osmotic stress tolerance have increased intracellular proline level (107); *Staphylococcus aureus* exposed to hyperosmotic conditions also has a higher proline level intracellularly, which were also confirmed by the result that expression of proline transporter gene *putP* is increased (108) and low-affinity proline transport system ProP is activated by high osmolality condition (109). Besides that, proline is proved to mediate other stress resistance of bacteria. In *E. coli*, proline catabolism can produce hydrogen peroxide that increases important antioxidant enzymes like catalase, thioredoxin and glutaredoxin (110), which may provide a pre-adaptive benefit by

lengthening cell survival, developing colonization efficiencies and enhancing virulence (111). Importantly, proline is important for the growth and colonization of pathogens under nutritional stressed conditions, which indicates that proline acts as carbon/nitrogen/energy source in bacteria. When *Mycobacterium* is cultivated under the stress caused by nitrogen limitation, expression of genes responsible for proline bio-synthesis is significantly upregulated (112, 113). *Ehrlichia chaffeensis* shows decreased infection of the host when proline transport is limited, and the mechanism is thought to be related to its utilization of proline as a carbon and energy source (114). The levels of glucose, a preferred carbon source of *S. aureus*, are limited on account of the host immune response, but proline can function as one of the major carbon sources during the growth of *S. aureus* (115).

E. coli is a very good example to explain the mechanisms of bacterial proline transport and accumulation on account of the adequate investigation focusing on its proline transport systems. It has been shown that three proline transport systems are employed by *E. coli*, which includes the high-affinity Na^+/proline symporter PutP, the proton/osmolyte symporter ProP and the ABC-type transporter ProU. PutP belongs to the Na^+/solute symporter (SSS) family and catalyses the symport of Na^+ and L-proline with a stoichiometry of 1:1. It exhibits a high specificity and affinity for proline with a $K_{d\ (Pro)}$ and $K_{m\ (Pro)}$ of 2 µM (127). Different from PutP, ProP and ProU of *E. coli* catalyse the uptake of a wide range of osmoprotective compounds including proline, glycine betaine, proline betaine and ectoine (129). Expression of the *proP* gene is activated by osmotic stress (132) and the transporter is post-translationally regulated as well (133) since ProP is almost inactive at low osmolality but maximally activated at high osmolality (130). This might result from binding of cations to the C-terminal domain of ProP that induces a conformational switch and increases transporter activity during osmotic stress (130, 134). ProU contains a membrane-bound ATPase (ProV), a permease (ProW) and a periplasmic binding protein ProX (135, 136). The expression of the *proU* operon encoding the three components of the ProU transport system (*proVWX*) is also activated by the osmotic stress (53, 137-139).

Apart from proline uptake, bacteria can also accumulate proline by bio-synthesis from glutamate, which is mediated by several enzymes in a train of steps (Figure 1.5). First of all, γ-glutamyl kinase (GK, encoded by *proB*) converts glutamate to γ-glutamyl phosphate that is afterwards reduced by γ-glutamyl phosphate reductase (GPR, encoded by *proA*) to glutamate-γ-semialdehyde (GSA), which then cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C) (140). After that, by utilizing nicotinamide adenine dinucleotide (NADH) and/or nicotinamide adenine dinucleotide phosphate (NADPH), the enzyme P5C reductase (P5CR, encoded by *proC*) reduces P5C to proline (140, 141).

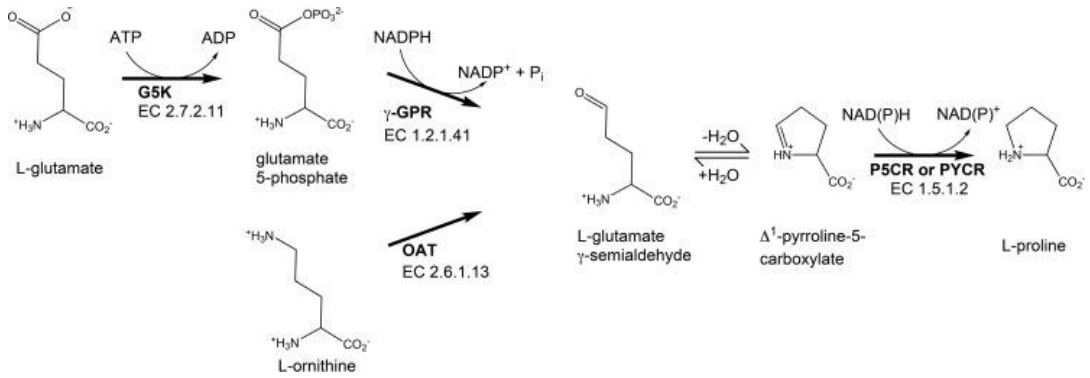

Figure 1.5 The reactions and enzymes of proline biosynthesis. G5K, glutamate-5-kinase; γ-GPR, γ-glutamate-phosphate reductase; OAT, ornithine-γ-aminotransferase. Figure was copied from Christensen EM, *et. al.*, 2017 (141).

The catabolism of proline to glutamate depends on the two-step oxidation catalysed by proline dehydrogenase (PRODH) and nicotinamide adenine dinucleotide (NAD^+)-dependent enzyme P5C dehydrogenase (P5CDH). In the first step, a flavoenzyme PRODH catalyses the oxidation of proline to P5C coupled with two-electron reduction of a noncovalently bound flavin adenine dinucleotide (FAD) cofactor to the membrane such as ubiquinone, which thus links proline catabolism with the respiratory chain (119, 142-144). P5C is then nonenzymatic hydrolysed to GSA that followed by P5CDH catalysed oxidation to glutamate with reduction of NAD^+ to NADH (145-147). Here what needs to be emphasized is that, the PRODH and P5CDH enzymes are expressed as two distinct monofunctional enzymes in eukaryotes and Gram-positive bacteria, whereas they are compounded into a single polypeptide called proline utilization A (PutA) in Gram-negative bacteria (148, 149). There are two types of PutAs classified based on their structure and functions, among which the trifunctional PutAs have an N-terminal ribbon-helix-helix (RHH) DNA binding domain and function as a transcriptional repressor of the *put* regulon that encodes PutP and PutA (148, 151, 152), whereas the bifunctional PutAs are missing the N-terminal extension for DNA binding in the oxidized state of the enzyme (119, 149, 150).

1.2.2 Known effects of proline uptake and metabolism in *H. pylori*

Regarding the roles of proline in the physiology and virulence of *H. pylori*, several studies have pointed out that proline can mediate oxidative stress resistance, function as carbon/nitrogen/energy source, and probably involve in the motility of the bacteria. Previously, gastric juice of patients infected with *H. pylori* has been shown to contain much higher amounts of proline compared with that of non-infected people (116). This is thought to result of the secretion of collagenase by *H. pylori*. The enzyme disintegrates collagen in the extracellular matrix of the stomach epithelium lifting the level of proline (117, 118), which has been found to be one of the preferred respiratory substrates of *Helicobacter* (116). The role of proline in *H.*

pylori infection has been revealed by investigating the importance of *putA* and *putP* in *H. pylori* colonization. Together with other 46 genes in a signature tagged mutagenesis screen of 960 mutants in *H. pylori*, *putP* encoding the Na^+/L-proline symporter was identified to be necessary for gastric colonization of Mongolian gerbil infection model (118, 121). Moreover, a *H. pylori putA* mutant strain that was incompetent to form flagella has been shown to lose the capacity to colonize mice, which is perhaps by reason of the loss of proline respiratory activity that causes lower virulence (122). The proline utilization A flavoenzyme (PutA) from *Helicobacter* species lacks the DNA binding domain that usually exists in other bacteria like *E. coli*, its major biological role should be empowering the bacterium to utilize proline as a carbon source, which is coupled with the formation of glutamate and reduction of the respiratory chain (Figure 1.6) (116, 119, 120). In comparison to the *Helicobacter hepaticus* wild type strain, the *putA* mutant strain showed lower expression of *katA* encoding catalase, which means that PutA mediated proline oxidation upregulates the expression of *katA* (123). In *H. pylori*, the expression of *katA* encoding catalase is upregulated to defence the oxidative stress caused by host neutrophil response which can lead to persistent chronic inflammation (124-126). All in all, the aforementioned results demonstrate that proline is favourable to the colonization and pathogenesis of *Helicobacter* species, and other potential feature of proline in the physiology and pathogenesis of *Helicobacter* species should be further explored.

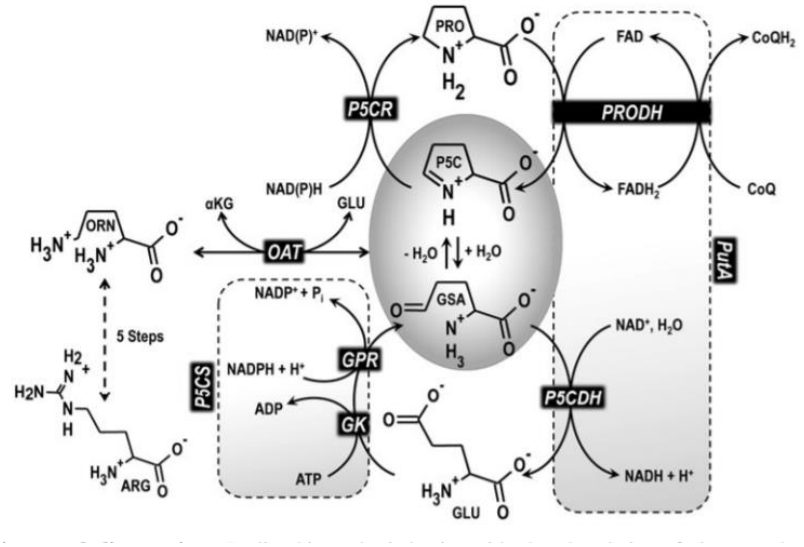

Figure 1.6 Proline metabolic reactions. Proline catabolism involves the two-step oxidation of proline to glutamate catalysed by PRODH and P5CDH. In gram-negative bacteria including *H. pylori*, the PRODH (proline dehydrogenase) and P5CDH (pyrroline-5-carboxylate dehydrogenase) activities are linked in the PutA bifunctional enzyme. Figure was copied from Christgen SL, *et. al.*, 2019 (105).

The proline uptake and metabolism in bacteria involve vast and complex systems on account of their intricate chemical and physical reactions. Therefore, to clearly understand the role of proline in physiology and pathogenesis of different microbes, these systems are supposed to be good targets and intermediaries in the process of research. Presently, the proline uptake and metabolism systems in *H. pylori* and their relative features are not fully studied yet. Based on the genome analysis of *H. pylori* P12 (GenBank: CP001217.1) (153), a reference strain investigated in this study, the proteins involved in the proline uptake and metabolism has been formerly predicted (Figure 1.7). There are three predicted proline transporters in *H. pylori* including PutP, ProP and ProVWX (homologous with ProU of *E. coli*), among which PutP has been quite well studied. As mentioned in the section 1.2.1, experimental evidences indicate that the SSS transporter PutP is essential for the colonization of *H. pylori* (118). Previously, Rivera-Ordaz, *et.al*. investigated the molecular basis of proline transport in *H. pylori* by PutP for the first time. Based on the results obtained from measurements of radiolabelled proline transport in *H. pylori* and *E. coli* heterologously expressing PutP of *H. pylori* as well as in proteoliposomes reconstituted with PutP of *H. pylori*, they demonstrated that the observed proline transport in *H. pylori* is indeed mediated by PutP with a high affinity for L-proline, which indicates the functional properties of PutP from *H. pylori* are almost identical to the *E. coli* ortholog (121). In addition to PutP, another part of *put* operon PutA has also been proved to be essential for colonization and motility for *H. pylori* as introduced above (119, 122, 123). The bifunctional PutA from *Helicobacter* species is different from the trifunctional PutAs from other bacteria that possesses *putP* and *putA* genes transcribed in opposite directions. In *H. pylori*, the *putA* and *putP* are transcribed in the same direction, and the bifunctional PutA from *H. pylori* does not contain the DNA binding domain; thus, its main function is to enable *H. pylori* to utilize proline as a carbon source (116, 119, 120). Besides proline transport, the bacterium may also accumulate proline by biosynthesis. According to the genome information of *H. pylori* P12 (153), only one gene is predicted to be responsible for the proline bio-synthesis, which is *proC* encoding the P5CR, whereas other genes such as *proA* and *proB* encoding GPR and GK respectively in other bacteria are missing. This indicates that P5CR encoded by *proC* may play a significant role in the proline synthesis of *H. pylori*. However, there is no reports available regarding the character and function of P5CR in *H. pylori* yet. Besides that, the putative proline transporter ProP and ProVWX in *H. pylori* have not been identified and investigated yet. Based on the results that proline transport in a *H. pylori putP* deletion mutant was not detected, it can be concluded that PutP is the sole proline transporter in *H. pylori* under the conditions tested (121). Therefore, further studies are needed on the role of ProP and ProVWX in the transport of proline or possible other substrates. Considering that *H. pylori* lacks the complex systems to transport K^+ and such to respond to hyperosmotic stress, the role of the potential substrates of these predicted transporters are of interest to this study. Overall, more research over the proline uptake and metabolism systems is imperative to help understand how exactly proline influence the pathogenesis and physiology of *H. pylori*.

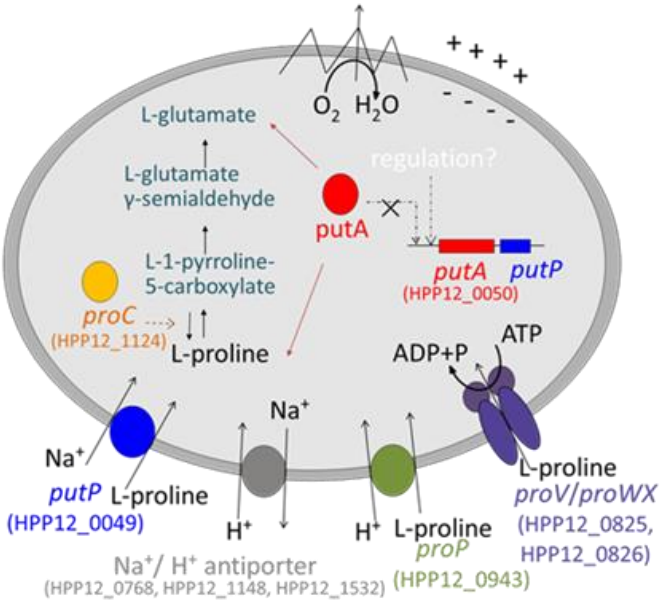

Figure 1.7 Proteins predicted to be involved in L-proline uptake and metabolism based on the genome analysis of *H. pylori* P12. Figure was modified based on data from Haas, *et. al.* (153).

1.3 Motivation and aim of the dissertation

As a type I carcinogen classified by the WHO, *H. pylori* persistently colonizes the stomach of half of the world's population. Even though several conventional therapies against *H. pylori* infection have been established, humans have to face the serious challenge caused by the rising antibiotic resistances that dramatically limit the therapeutic options. Furthermore, the current difficulties in the research and development of vaccines against *H. pylori* make the situation severe as well. The results of a number of studies indicate that proline is crucial for growth of *H. pylori* and stimulates colonization of the stomach by the bacterium. Therefore, to combat diseases caused by *H. pylori*, it is important to understand the mechanisms by which the bacterium transports and uses proline. The findings may lead to new therapeutic strategies, including the development of specific inhibitors of bacterial proline utilization. The general goal of this thesis is to further elucidate the molecular basis of proline transport and metabolism. In doing so, the work focuses on the following questions:

i. Is proline a key osmoprotectant of *H. pylori* to tolerate osmotic stress? If yes, which L-proline-specific transporters and enzymes are required to fulfil the task?

H. pylori can perfectly survive in the turbulent gastric environment that provides not only acidic or oxidative stress, but also osmotic changings. Therefore, the capacity of osmoregulation is important for survival and colonization of *H. pylori*. However, *H. pylori* does not possess genes encoding homologues of primary osmotic stress defence systems of other bacteria. As a compatible solute that is employed by many organisms, proline could be a preferred osmoprotectant utilized by *H. pylori*. To answer this question, a possible involvement of different proline transporters and enzymes in the adaptation of *H. pylori* to osmotic stress will be investigated.

ii. How dose proline accumulation and metabolism affect the motility of *H. pylori*?

Flagella-mediated motility is an undoubted crucial virulence factor of *H. pylori*. It has been shown that proline transport and metabolism can affect the motility of *H. pylori* by inhibiting the formation of flagella. However, there are more than 40 structural and regulatory genes involved in the process of flagellar biogenesis in *H. pylori*. In order to clarify the pathway by which proline affects flagella formation, it should be investigated which flagellar genes are involved in the effect and at what level. Likewise, the influence of different proline transporters and enzymes on flagellar biogenesis will be included in the investigations.

2. Results

Even though L-proline has been found to be important for the colonization by *H. pylori*, the mechanisms by which the bacterium transports and uses proline to obtain benefits are still not fully understood. Besides PutP and PutA, other proteins predicted to function as members in the proline transport and metabolism systems of *H. pylori* have not yet been studied. In this study, by using *H. pylori* P12 as a model strain, applying basic bacteriological, molecular biological and biochemical experiments, we investigated the predicted proline uptake and metabolism systems and revealed the roles of proline in the hyperosmotic stress tolerance and flagella-mediated motility of the organism. The results promote answering those questions and open new research pathway towards treatment of *H. pylori*-caused diseases development.

2.1 PutP and ProP contribute to osmotic stress tolerance of *H. pylori* P12

2.1.1 Creation of *H. pylori* derivatives

To explore whether the predicted proline uptake and metabolism systems are involved in the osmotic stress response of *H. pylori*, the genes encoding PutP, PutA, ProP, ProV and ProC were deleted, and plasmid-based complementation systems were developed.

2.1.1.1 *H. pylori* deletion mutants deprived of genes encoding proline uptake and metabolism systems

The *putP* and *putA* mutants of *H. pylori* P12 were previously generated and used to obtain information on basic functional properties of PutP (121, 155). In this study, the genes *proP*, *proV* and *proC* of the strain were deleted using the method described by Dailidiene (156) (Figure 2.1). In brief, a 1.5 kb two-gene cassette containing the *C. jejuni* gene *rpsL* that dominates streptomycin susceptibility and *erm* that confers erythromycin resistance was flanked by the up- and down-stream sequences of *proP*, *proV* or *proC* of *H. pylori*, and then cloned into the suicide plasmid pBlueScript II SK$^+$ (pBSK$^+$). With the help of *E. coli* WM3064, the recombinant plasmids were transformed into *H. pylori* P12 wild-type by conjugation (157) and thus the *H. pylori* mutants were generated via homologous recombination. To achieve this, the two flanking regions A and B of each selected gene were amplified using primers binding outside the genes (see Table 4.3). The PCR fragments A and B were designed to be overlapped by sharing the same restriction site. Therefore, an “A-B fragment” of each gene was generated by overlapping-PCR and cloned into the suicide plasmid pBSK$^+$ that does not replicate in *H. pylori*. The amplified *rpsLerm* cassette was then inserted

into the plasmid on the A-B fragment using the shared restriction site. The cloning procedure was done in *E. coli* DH5α, and the resulting plasmids were cloned into *E. coli* WM3064 that conjugates with *H. pylori* wild-type for transformation.

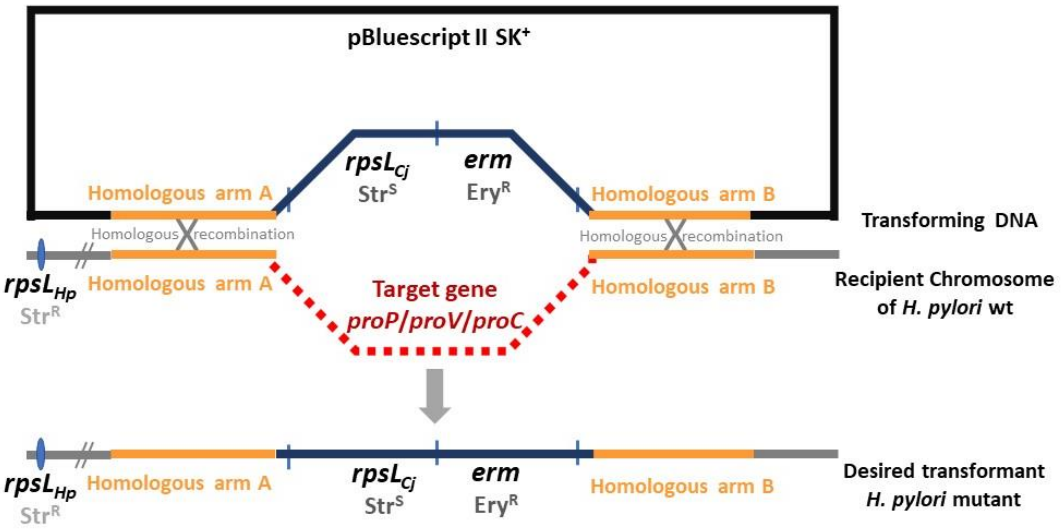

Figure 2.1 Contra-selection strategy for generation of the *H. pylori* deletion mutants. The flanking regions (Homologous arms A and B) of the selected genes were divided by the inserted *rpsLerm* cassette that confers erythromycin resistance and streptomycin susceptibility and cloned into the suicide vector pBluescript II SK$^+$. In regions of sequence homology, the transforming DNA was transferred to the recipient chromosome of *H. pylori* by homologous recombination, which results in the replacement of the original sequence for the *rpsLerm* cassette in the bacterial phenotype from Str^R to Str^S (156).

The colonies from conjugation were picked and striped on agar plates containing streptomycin or erythromycin to obtain the desired phenotype. The results of PCR test and DNA sequencing confirmed that the expected *rpsLerm* cassette was located at the correct position of the gene of *Str^{S}Erm^r* transformants (Figure 2.2).

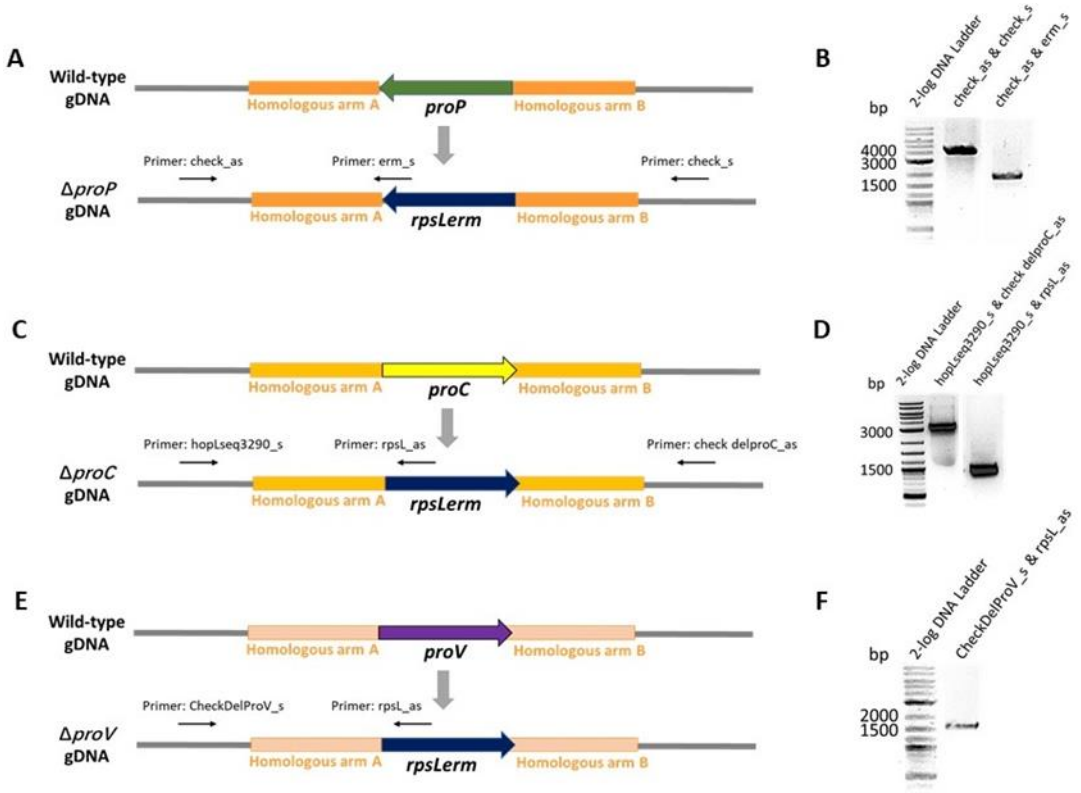

Figure 2.2 PCR tests confirming the *rpsLerm* cassette transformants of *H. pylori*. (A. C. E) Schematic illustration of the mutated region of *proP* mutant (A), *proC* mutant (C) and *proV* mutant (E) of *H. pylori*. PCR was performed to test the correct insertion of the *rpsLerm* cassette of *proP* mutant (using primers: check_as, check_s and erm_s), *proC* mutant (using primers: hopLseq3290_s, hopLseq3290_s and rpsL_as) and *proV* mutant (using primers: CheckDelProV_s and resL_as). **(B. D. F)** Analysis by gel electrophoresis shows the products of PCR testing inserted *rpsLerm* cassette in *proP* mutant (B), *proC* mutant (D) and *proV* mutant (F) of *H. pylori*. Fragments containing the sequences of both part of original genomic DNA and part of *rpsLerm* cassette confirmed the correct insertion.

2.1.1.2 Complementation of the *H. pylori* mutants

In order to test whether the deletion of the genes encoding the members of proline transport and metabolism systems can indeed result in the phenotype alternation regarding hyperosmotic tolerance and flagella-mediated motility, complementation of the mutants was necessary to generate. The complementary strains of the *putP* mutant and *putA* mutant of *H. pylori* have been generated by Rivera-Ordaz previously (121, 155) by using the shuttle-vector pIB6 that was provided by Prof. Dr. Rainer Haas (Max von Pettenkofer-Institut, LMU Munich). Similarly, in this study the complementary strains of the *H. pylori* Δ*proP*, Δ*proV* and Δ*proC* have also been created by using the shuttle vector pIB6 that can replicate in *H. pylori* autonomously and help cloning the genes from *H. pylori* in *E. coli* without expressing them heterologously, which is on account of the fact that its specific *alpA*-Promoter is inactive in *E. coli* (158).

Firstly, the *proP* gene from *H. pylori* P12 wild-type needed to be amplified. Previously, Menzel (159) has investigated the activity of the *H. pylori* ProP by heterologous expression according to the annotation of the gene. Unfortunately, neither the annotated gene nor the variants with different length by using putative start codons in front of the annotated gene start showed activity. In this study, we analysed the sequence of the *proP* gene region by using the ORFfinder (NCBI), which surprisingly showed a sole possibility of gene start resulting in a putative gene with 1383 bp encoding a protein with 460 AA in length with the alternative start codon AUA (Supplementary material 1). This prediction suggests that the original annotation of *proP* might have been incorrect. With this start codon, *proP* from *H. pylori* was amplified by PCR and cloned into the vector pIB6 with the restriction sites NdeI and SpeI. Secondly, the *H. pylori* genes *proV* and *proC* were cloned into pIB6 with the restriction sites NdeI & NotI and NdeI & SpeI respectively. *E. coli* WM3064 was transformed with the generated plasmids and used for conjugation with *H. pylori* corresponding mutants to generate the complementary strains and were confirmed by colony PCR (Supplementary material 2) and sequencing. At the same time, empty plasmid pIB6 was also transformed to *H. pylori* wild-type and mutants to act as control in further experiments.

2.1.2 Growth characterization of *H. pylori* derivatives under hyperosmotic conditions

As a very basic and important part in observable characteristics of bacterial cells, growth phenotypes define if and how fast a bacterium will grow. Their advantages are that they can be easily observed, scored, and measured without requiring expensive technology (159). Therefore, in this part the growth phenotype of *H. pylori* wild-type and aforementioned derivatives under hyperosmotic conditions in liquid medium and solid medium were analysed to investigate whether the members of proline uptake and metabolisms systems are involved in the hyperosmotic stress tolerance.

2.1.2.1 Growth of *H. pylori* in Brucella Broth liquid medium

Growth of *H. pylori* can be affected by hyperosmotic stress to varying degrees, the environment can be too harsh for *H. pylori* to live when the stress is very strong, whereas cells can also barely react in growth when the stress is too weak. Therefore, an appropriate hyperosmotic stress level, which will not be fatal for the bacterium but can cause obvious effects on physiology of cells, is very important for the further experiments in condition settings. Previously, PutP has been identified and characterized as a high-affinity proline transporter in *H. pylori* (121, 155). In order to find out the optimal osmotic stress condition for further experiments, the *H. pylori* wild-type and Δ*putP* were firstly cultivated in Brucella Broth liquid medium (BB medium) with different amounts of NaCl that creates different levels of osmolality. As shown in Figure 2.3, both *H. pylori* wild-type and Δ*putP* showed decreasing growth along with the increasing osmotic stress, and the OD_{600} values of *H. pylori* Δ*putP* were lower than that of wild-type under all the set osmolarities. These results were taken

as the first evidence that PutP contributes to the osmotic tolerance of *H. pylori*. An osmolality of 0.55 Osm / kg was selected for further studies on the osmotic tolerance of *H. pylori*, because the growth of Δ*putP* and wild-type were significantly different ($P < 0.01$) under the condition of 0.55 Osm / kg. Conditions with set osmolarities of 0.36 Osm / kg and 0.48 Osm / kg show relatively small influence on growth of the cells, whereas stronger stresses with osmolarities from 0.62 Osm / kg to 0.78 Osm / kg are too strong for mutant and wild-type so that a clear difference between the strains is not observed.

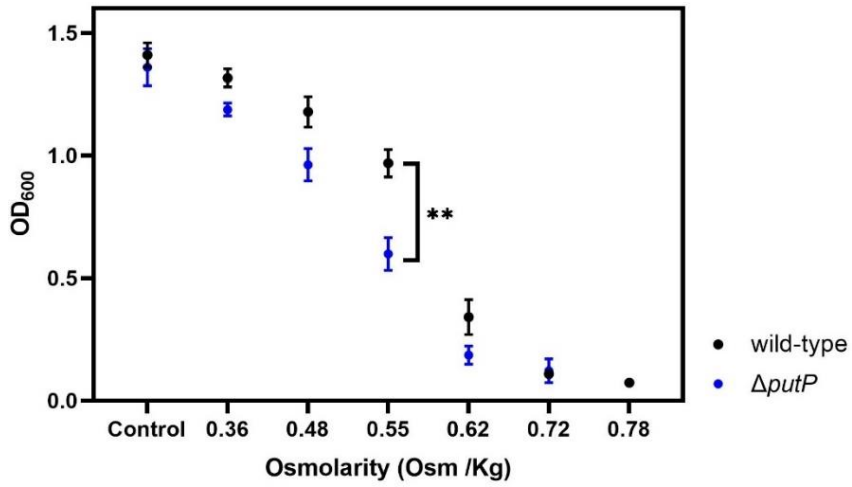

Figure 2.3 Growth of *H. pylori* wild-type and Δ*putP* in Brucella Broth liquid medium with different osmolarities after cultivating for 18 hours. OD_{600} of wild-type (black dots) and Δ*putP* (blue dots) after cultivation. Inocula were collected from GC-Agar plates and pre-cultured in BB for 24 hours. Cultures were adjusted to OD_{600} of 0.07 and amount of 1 ml in 24-well plates with indicated osmolarity of BBs and then shaken with 130 rpm and OD_{600} measurements were performed after 18 hours of cultivation. The experiment was done in triplicates. ** $P < 0.01$, as determined using the Student's *t*-test.

Next, all the aforementioned created *H. pylori* mutants along with wild-type were cultured in standard BB medium as control group and in BB medium with 0.55 Osm / kg as stress group for 18 hours and their OD_{600} values were measured and normalized. As shown in Figure 2.4 A, hyperosmotic stress caused growth inhibition can be observed in *H. pylori* wild-type and mutants. However, it was evident that the growth of Δ*putP* and Δ*proP* was more reduced under osmotic stress compared with that of wild-type, Δ*putA*, Δ*proV* and Δ*proC*. The estimation plot demonstrates that under stress conditions only the growth of Δ*putP* and Δ*proP* was reduced more strongly than the average difference (Figure 2.4 B). These results indicate that PutP and the putative proline transporter ProP might be involved in the hyperosmotic stress tolerance of *H. pylori*.

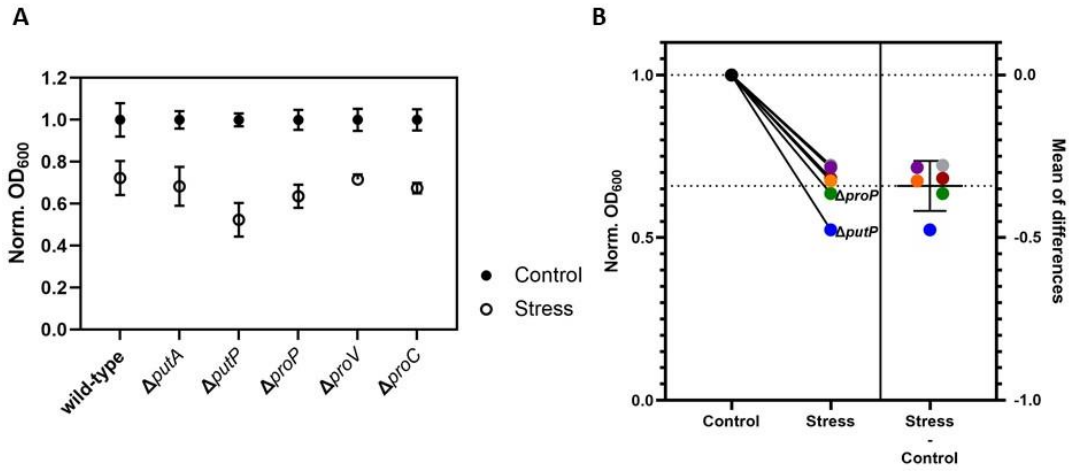

Figure 2.4 Growth of *H. pylori* wild-type and mutants in Brucella Broth liquid medium with and without hyperosmotic stress. (A) Normalized OD_{600} of wild-type and indicated mutants after 18 hours cultivation. Inocula were collected from GC-Agar plates and pre-cultured in BB for 24 hours. Each culture was adjusted to OD_{600} of 0.1 and amount of 0.5 ml in 24-well plates with standard BB medium (control, solid circles) and BB with 0.55 Osm / kg (stress, hollow circles). $OD6_{00}$ of cultures were measured after 18 hours of shaking at 130 rpm. The experiment was done in quadruplicates. **(B)** Estimation plot showing the normalized OD_{600} of tested cells (wild-type: grey dots, Δ*putA*: red dots, Δ*putP*: blue dots, Δ*proP*: green dots, Δ*proV*: purple dots, Δ*proC*: orange dots) and the mean difference of OD_{600} between the control group and stress group analysed by paired *t*-tests.

In order to further test the above conclusion, the growth of complements of *H. pylori* Δ*putP* and Δ*proP* was also assessed using the same assay. *H. pylori* Δ*putP* and Δ*proP* were transformed with the constructed plasmids pIB6-HpputP and pIB6-HpproPto express *putP* and *proP* respectively (Δ*putP*::pIB6-HpputP, Δ*proP*::pIB6-HpproP). Besides that, *H. pylori* wild-type and the mutants were also transformed with the empty plasmid pIB6 (wild-type::pIB6, Δ*putP*::pIB6, Δ*proP*::pIB6) to meet the simple variable principle. The experiment setting was the same as described in the previous paragraph, but 8 mg / L of kanamycin was added to the BB medium to keep the transformed plasmids in the cells. As Figure 2.5 shows, the defect of growth of the mutants under osmotic stress can be recovered by the complements expressing the corresponding genes, which further proved that PutP and ProP can play a role in osmotic stress tolerance of *H. pylori*.

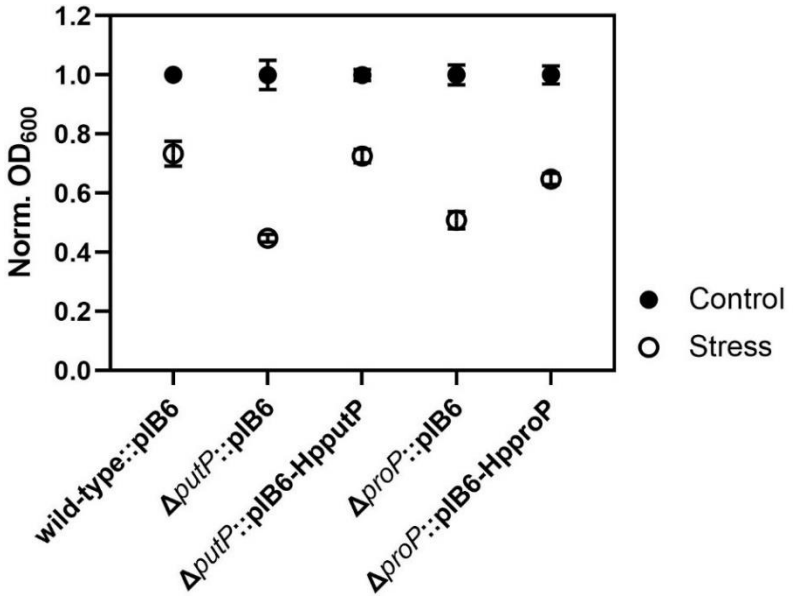

Figure 2.5 Growth of *H. pylori* wild-type, *putP* and *proP* mutants and their complements in Brucella Broth liquid medium with and without hyperosmotic stress. OD_{600} values of indicated *H. pylori* derivatives were measured and normalized after 22 hours of shaking at 130 rpm, before which the inocula were collected from GC-Agar plates and pre-cultured in BB or 24 hours. Each culture was adjusted to OD_{600} of 0.1 and amount of 0.5 ml in 24-well plates with standard BB medium (control, solid circles) and with 0.55 Osm / kg (stress, hollow circles). All BB medium and GC-Agar contains 8 mg / L of kanamycin, The experiment was done in triplicates.

2.1.2.2 Osmotolerance assay of *H. pylori*

To further test the disparity of how different *H. pylori* derivatives react to hyperosmotic stress more clearly and visually, an osmotic stress tolerance assay was also performed to show the survival on GC-Agar solid medium with and without osmotic stress. Similarly, an optimal stress condition is important for the experimental settings. However, the contents of GC-Agar and BB medium are apparently different, hence the inference of the osmolarity of GC-Agar is not available. Besides that, measurment of GC-Agar's osmolarity is also undoalbe on account of the fact that medium will turn to solid when temperature decreases to an unharmful level for the osmolality testing machine. In order to find out the most approperate stress condition, 4 g / L to 10 g / L of NaCl amounts were added in GC-Agar. Afterwards, *H. pylori* wild-type and *putP* deletion mutant in gradient dilutions were inoculated on all the agar plates added with different amount of NaCl. Plates with NaCl supplement lower than 6 g / L showed no apparent difference for growth of colonies of wild-type and *putP* mutant, whereas plates with NaCl supplement higher than 6 g / L could barely show any colonies for both wild-type and *putP* mutant, therefore 6 g / L of NaCl was found to be the optimal stress condition for osmotic stress tolerance assay.

With this condition setting, ten-fold dilutions of *H. pylori* wild-type and mutants were inoculated on the GC-Agar plates (Figure 2.6). After 7 days of incubation, it can be concluded that Δ*putP* and Δ*proP* exhibited increased sensitivity to osmotic stress since their colonies could be barely observed at dilution of 10^{-2} under osmotic stress, whereas colonies of wild-type and other mutants could still be seen at the dilutions of 10^{-5} to 10^{-6} under osmotic stress. At the same time, colonies of different dilutions from all the cultures on the standard agar plate keeps the same order of magnitudes and visible, which means these samples were originally equivalent to each other on amount and activity and the result on the stress group is credible.

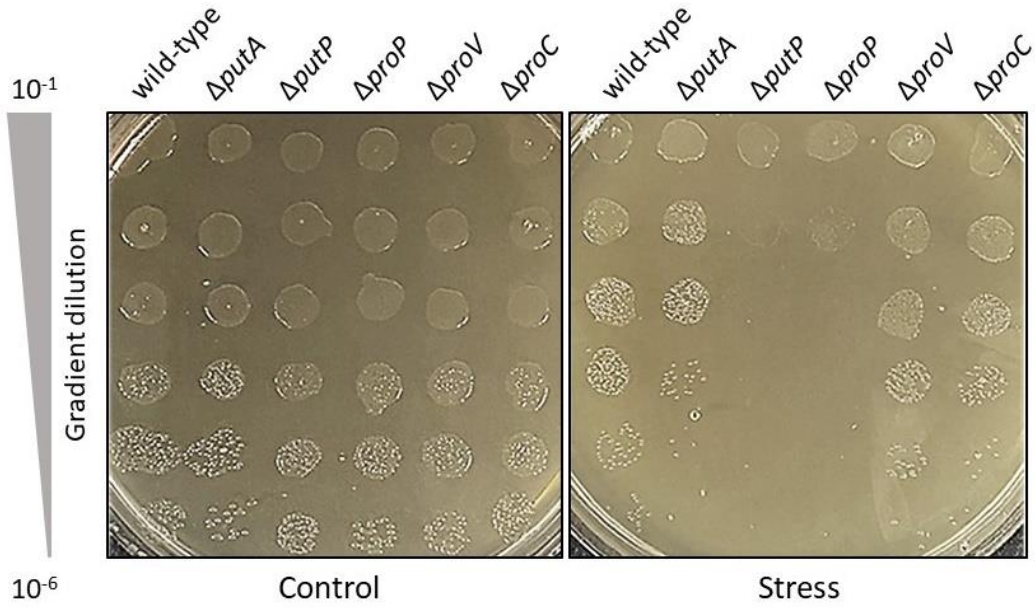

Figure 2.6 Osmotic stress tolerance assay of *H. pylori* wild-type and mutants on GC-Agar plates. *H. pylori* wild-type and mutants were pre-cultured in BB medium for 24 hours, afterwards each culture was adjusted to OD600 of 1.0 and then diluted in fresh BB medium ten-fold gradationally (5 gradients were taken: 10^{-1} to 10^{-6}). 5μL of each dilution from each culture was dropped on standard GC-Agar plate (Control group) and GC-Agar plate with 6 g / L NaCl (Stress group) in the order as indicated in the figure. The plates were incubated under 37 °C for one week. This experiment was repeated for at least three times.

The same assay was also performed to confirm the role of PutP and ProP in osmotic stress tolerance of *H. pylori* by assessing the tolerance of complements of Δ*putP* and Δ*proP* under the stress. As shown in Figure 2.7 A and B, the *H. pylori putP* and *proP* mutants containing empty plasmid both exhibit a higher sensitive to hyperosmotic stress, whereas the tolerance of the stress was recovered when *putP* and *proP* were expressed from the constructed plasmids in the corresponding mutants, which proved that exist of PutP and ProP is important for the hyperosmotic stress tolerance of *H. pylori*.

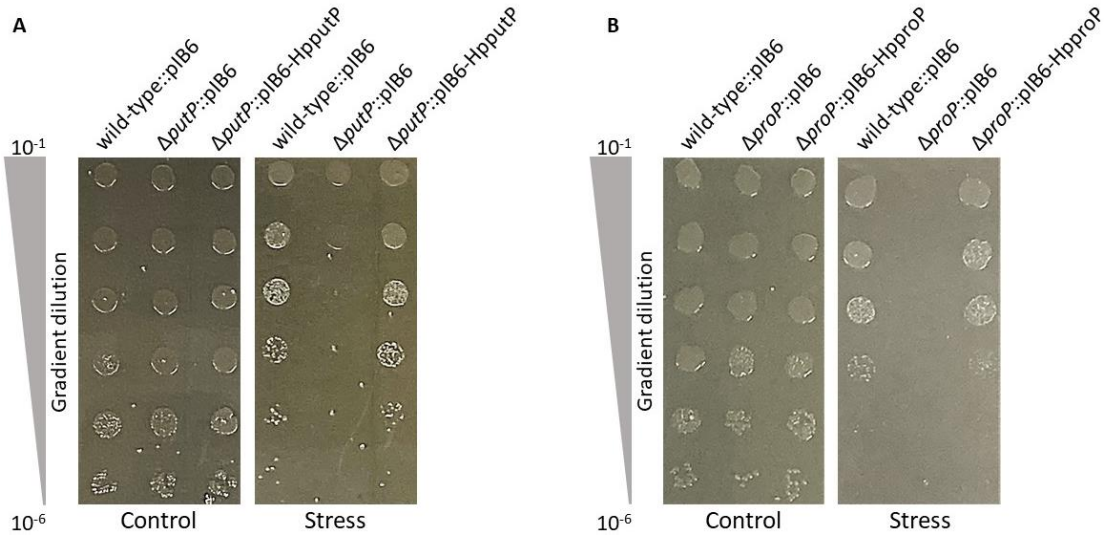

Figure 2.7 Osmotic stress tolerance assay of *H. pylori* Δ*putP* and Δ*proP* and their complements on GC-Agar plates. (A) Osmotic stress tolerance assay of *H. pylori* wild-type (wild-type::pIB6), *putP* mutant (Δ*putP*::pIB6) and its complement (Δ*putP*::pIB6-HpputP). **(B)** Osmotic stress tolerance assay of *H. pylori* wild-type (wild-type::pIB6), *proP* mutant (Δ*proP*::pIB6) and its complement (Δ*proP*::pIB6-HpproP). Bacteria were pre-cultured in BB medium for 24 hours and each culture was adjusted to OD600 of 1.0 and then diluted in fresh BB medium ten-fold gradationally (5 gradients were taken: 10^{-1} to 10^{-6}). 5µL of each dilution from each culture was dropped on standard GC-Agar plate (Control group) and GC-Agar plate with 6 g / L NaCl (Stress group) in the order as indicated in the figure. All GC-Agar contains 8 mg / L of kanamycin. The plates were incubated under 37 °C for one week. This experiment was repeated for at least three times.

2.1.3 Transcription of genes encoding the members of proline uptake and metabolism systems

Bacteria have to respond to the changing environmental conditions including osmotic stress by adapting their physiology through sometimes drastic changes in gene expression for the purpose of survival, which also constitute the main component of the bacterial response to stress (160). Admittedly, there are a myriad of different known mechanisms involved in the changes of gene expression to cope with stress adaptation, but endless variations and new elements of gene regulation on this theme are also constantly being discovered (160).

In this study, since *H. pylori* deprived of *putP* and *proP* showed increased sensitivity to osmotic stress, we therefore further examined the transcriptional levels of these genes and also *putA*, *proV*, *proC* using real-time reverse transcriptase PCR to investigate whether these systems are involved in the osmotic stress response through the changes in gene expression. To induce a change of gene expression, *H. pylori* wild-type was cultured in the BB medium with osmolality of 0.55 Osm / kg for 2 hours after a standard cultivation. As shown in Figure 2.8, with osmotic stress, only the transcription of *putA* and *putP* showed significant upregulation, which were

approximately 2- and 2.5-times fold respectively. Since PutA in *H. pylori* is involved in the formation of glutamic acid from proline (116, 119, 120), and glutamic acid is another important osmoprotectant for bacterium, the upregulation of *putA* under osmotic stress thus also makes sense. The expression of *proV* was upregulated very slightly, the difference was not significant. Surprisingly, *proP* that proved to be crucial in the osmotic stress tolerance from last section also showed unsignificant upregulation. Besides that, expression of *proC* was about 2fold upregulated, which was however not significant due to the big variation regarding the results from repeated experiments.

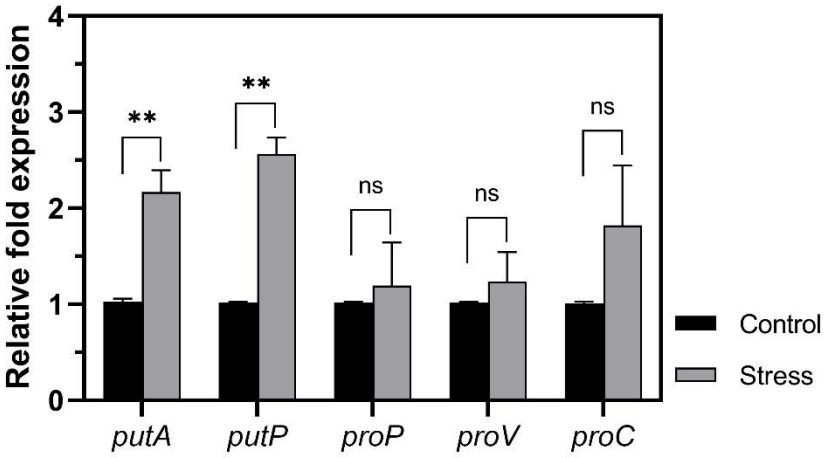

Figure 2.8 Expression of genes encoding the proline uptake and metabolism systems of *H. pylori* when treated by osmotic stress. *H. pylori* wild-type was pre-cultured for 24 hours and then re-inoculated and cultured for 12 hours in standard BB medium. Afterwards the cells were collected, washed by fresh BB and inoculated to standard fresh BB medium (Control) and BB medium with osmolality of 0.55 Osm / kg (Stress) with adjusted OD_{600} of 1.0 respectively. After 2 hours of cultivation and induction at 130 rpm, the cells were collected and their mRNA samples were extracted. With the cDNA required from reverse transcription, the expression of the indicated genes was tested using qRT-PCR. Fold expression of the genes in stress group compared with control group was calculated using the housekeeping gene *era* as reference gene. Expression values are representative of four independent biological experiments. **P < 0.01; ns, not significant, as determined using the Student's *t*-test.

In *H. pylori*, PutP is the identified and confirmed proline transporter, whereas ProP and ProVWX are the predicted but unconfirmed proline transporters. To test if the predicted proline transporters can take over the function when *putP* is absent and involved in osmotic stress tolerance of *H. pylori*, the expression of *proP* and *proV* of *H. pylori* Δ*putP* was tested using qRT-PCR as well. The results showed that neither *proP* (Figure 2.9 A) nor *proV* (Figure 2.9 B) exhibits significant changes on expression in *putP* deletion mutant under the set conditions.

Figure 2.9 Expression of genes encoding the predicted proline transporter of *H. pylori* when *putP* was absent and treated by osmotic stress. *H. pylori* wild-type and Δ*putP* was pre-cultured for 24 hours and then re-inoculated and cultured for 12 hours in standard BB medium. Treatment and sample acquisition were performed as described. Fold expression of *proP* **(A)** and *proV* **(B)** under different conditions was calculated using the housekeeping gene *era* as reference gene. Expression values are representative of three independent biological experiments. Statistical significance was not found according to the determination using the Student's *t*-test.

In *H. pylori*, ProC is responsible for proline bio-synthesis, which is another pathway for acquisition of intracellular proline different from the proline-transport. We thus analysed the gene expression of *proC* when the proline transporter PutP was absent and the osmotic stress was present. The result of qRT-PCR indicates that when *putP* was deleted and stress was present, the upregulation of *proC* expression was the most conspicuous compared to that of other conditions (Figure 2.10). However, t-test of these data did not show a significant difference due to the big variation regarding the results from repeated experiments.

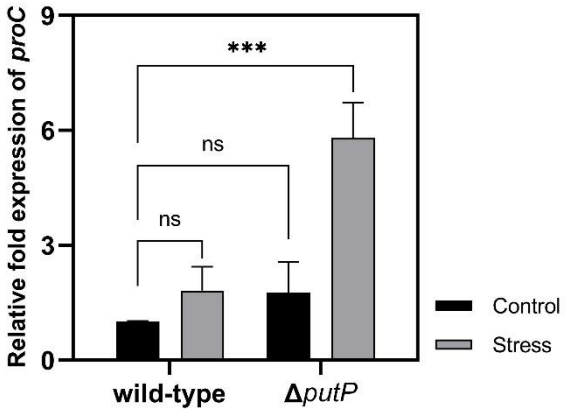

Figure 2.10 Expression of *proC* of *H. pylori* when *putP* was absent and treated by osmotic stress. *H. pylori* wild-type and Δ*putP* was pre-cultured for 24 hours and then re-inoculated and cultured for 12 hours in standard BB medium. Treatment and sample acquisition were performed as described. Fold expression of *proC* under different conditions was calculated using the housekeeping gene *era* as reference gene. Expression values are representative of three independent biological experiments. Statistical significance was not found according to the determination using the Student's *t*-test.

2.1.4 Proline transport of *H. pylori* derivatives lacking predicted proline transporters

Regarding proline transporters in *H. pylori*, the results of *H. pylori* growth under osmotic stress (section 2.1.2) revealed that PutP and ProP but not ProVWX are essential for the osmotic stress tolerance of *H. pylori*. However, the results of transcription analysis (section 2.1.3) show that only the expression of *putP* responded to the hyperosmotic stress and upregulated significantly, but genes of other predicted proline transporters could not respond to the stress even when *putP* was absent. These results were kind of inconsistent. Therefore, we further tested the proline uptake of *H. pylori* derivatives that deprived of the putative proline transporters by using the ^{14}C L-proline as an indicator.

The results indicate that proline uptake could barely be observed when *putP* was deleted, whereas deprivation of other predicted proline transporters had almost no influence on the proline uptake (Figure 2.11). Furthermore, since osmotic stress can drive and stimulate the proline transport by ProVWX or ProP, additional osmotic stress was also applied before proline uptake measurements, but no proline uptake stimulation was observed. Instead, an inhibition of proline uptake could be detected, which might be due to the adverse effect caused by the stress. Besides that, previous data showed that *putP* deletion mutant of *H. pylori* could regain the proline transport when complementation of the *putP* expression was applied (125, 155). These results revealed that PutP seemed to be the sole active proline transporter of *H. pylori*, whereas ProVWX could be inactive in proline transport and ProP might play a role in osmotic stress tolerance by transporting other osmoprotectant but not proline.

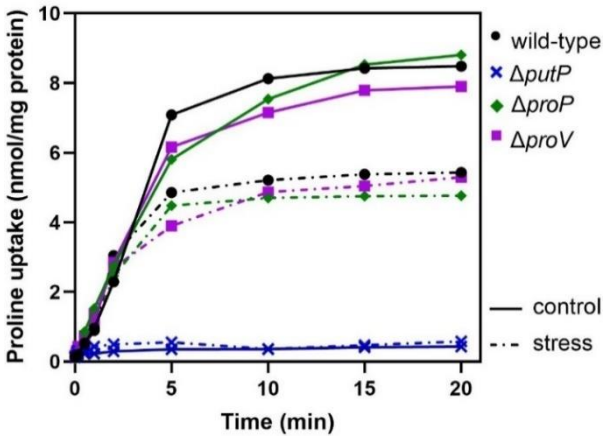

Figure 2.11 Time course of active proline uptake in *H. pylori*. Measurements were performed as described as in section 4.2.2.5. The transport data corresponds to 200 µL of a cell suspension which was afterwards incubated at 37 °C shortly before use. Reactions were performed with simultaneous addition of 50 mM NaCl and 10 µM of ^{14}C-L-proline (26 µCi / µmol) and stopped after different time intervals (0 s, 5 s, 10 s, 30 s, 1 min, 2 min, 5 min, 10 min, 15 min, 20 min) with a total of 6 ml of potassium-lithium-Buffer. From the filters used, the accumulated proline in the cells was determined in a β-Counter. All samples were divided to control group (no osmotic stress stimulation, full line) and stress group (treated by 5.5 Osm / kg by NaCl for 30 min, dotted line) before proline uptake experiment.

In order to test if ProP protects *H. pylori* from the adverse impact caused by osmotic stress by transporting other osmoprotectants, growth of *H. pylori* was measured with addition of different substrates of ProP. However, Figure 2.12 B shows that none of the additions (40 mM of either proline, glycine betaine, ectoine, carnitine or trehalose) resulted in a significant growth benefit under osmotic stress. Even though Figure 2.12 B shows a slight increasing growth for *proP* deletion mutant when proline was added under control condition comparing that without proline addition, the difference is too small to make a conclusion. These data could not tell if ProP of *H. pylori* transports other substrate to play a role in osmotic stress, which might be due to the super complexity of the BB medium.

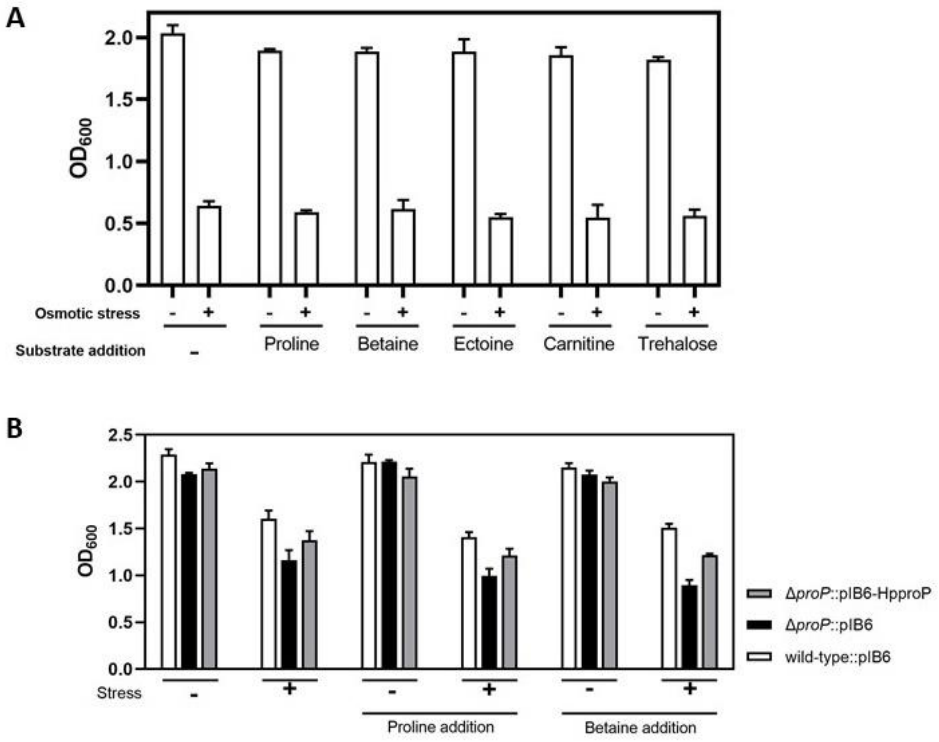

Figure 2.12 Growth of *H. pylori* with addition of potential substrates of ProP under osmotic stress. (A) Growth of *H. pylori* wild-type with 40 mM of indicated substrates with and without osmotic stress. **(B)** Growth of *H. pylori* wild-type (wild-type::pIB6), *proP* deletion mutant (Δ*proP*::pIB6) and its complement (Δ*proP*::pIB6-HpproP) added 40 mM of proline and glycine betaine with and without osmotic stress. For all measurements, OD_{600} values of indicated *H. pylori* derivatives were measured after 22 hours of shaking at 130 rpm, before which the inocula were collected from GC-Agar plates and pre-cultured in BB or 24 hours. Each culture was adjusted to OD_{600} of 0.1 and amount of 0.5 ml in 24-well plates with standard BB medium and with 0.55 Osm / kg. All BB medium and GC-Agar contains 8 mg / L of kanamycin only for Figure 2.12 B. The experiment was done in triplicates. Statistical significance was not found according to the determination using the Student's *t*-test.

2.1.5 Structural modelling of HpProP

According to the time course of active proline uptake in *H. pylori* cells (Figure 2.11), we assume that PutP seemed to be the sole active proline transporter of *H. pylori*. Besides PutP, however, ProP has also been shown to be involved in osmotolerance of *H. pylori* (Section 2.1.2). In order to get more insights into the relationship between the structure and function of the ProP transporter, we used AlphaFold2 (258) to predict the structure of HpProP according to its sequence, which is 1383 bp and encodes a protein with 460 AA size. At the same time, structure of EcProP was also shown as a reference.

As shown in Figure 2.13, the tertiary structures of HpProP and EcProP are quite similar and both of them have 12 transmembrane domains. However, the long cytoplasmic carboxyl terminal coiled coil seen in EcProP is missing in HpProP. It is well-known that the C-terminal helical structure is a key for osmosensing and activation of ProP in bacteria (229, 230). For the ProP proteins lacking the C-terminal helix, conditions with much higher osmotic stress are necessary for their activation (229, 231). These facts and our result might explain why ProP of *H. pylori* could not be activated by osmotic lift in the experiments of section 2.1.4.

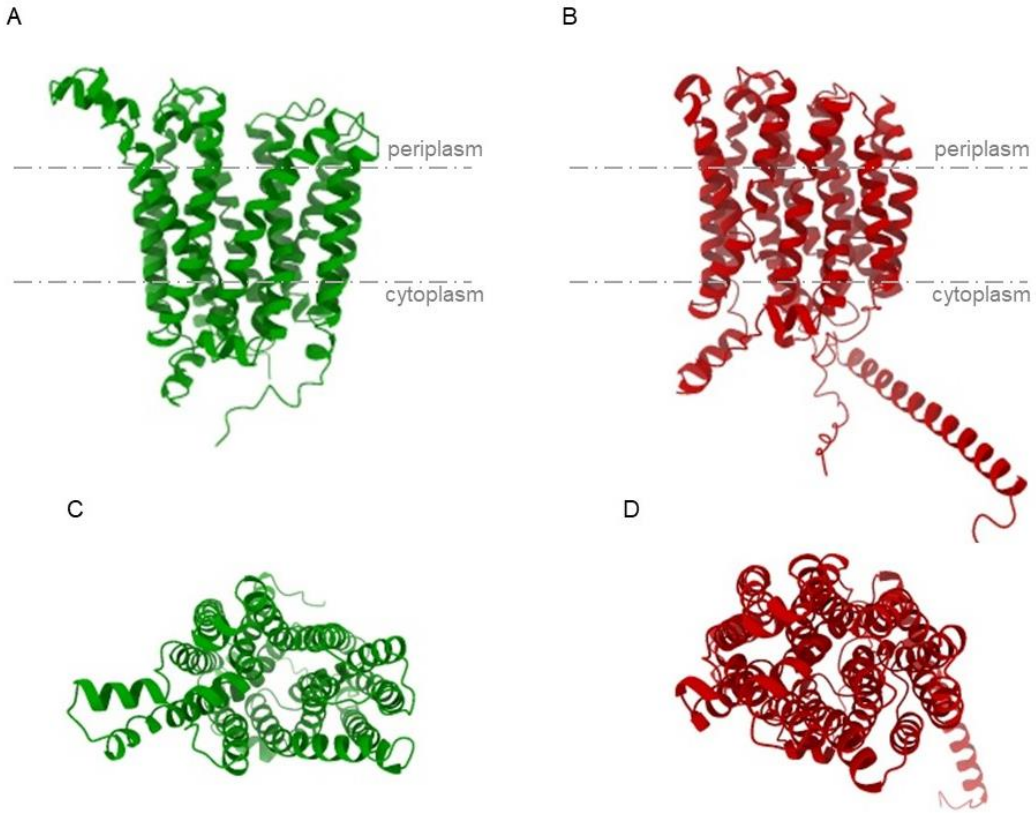

Figure 2.13 Tertiary structure model of ProP transporters. Front view of **(A)** HpProP and **(B)** EcProP with periplasmic and cytoplasmic sides indicated. Periplasmic view of **(C)** HpProP and **(D)** EcProP. These images were made with the program Alphafold2 (258).

2.1.6 Intracellular proline and glutamic acid amounts in *H. pylori*

According to our conclusion from previous sections, intracellular proline acts as an important osmoprotectant for *H. pylori*. To further test this hypothesis, the high-performance liquid chromatography (HPLC) was performed to analyze the amount of intracellular amino acid of *H. pylori* under osmotic stress. In order to obtain more accurate data, derivatization of amino acids using 9-fluorenylmethyloxycarbonyl chloride (FMOC) was necessary (161).

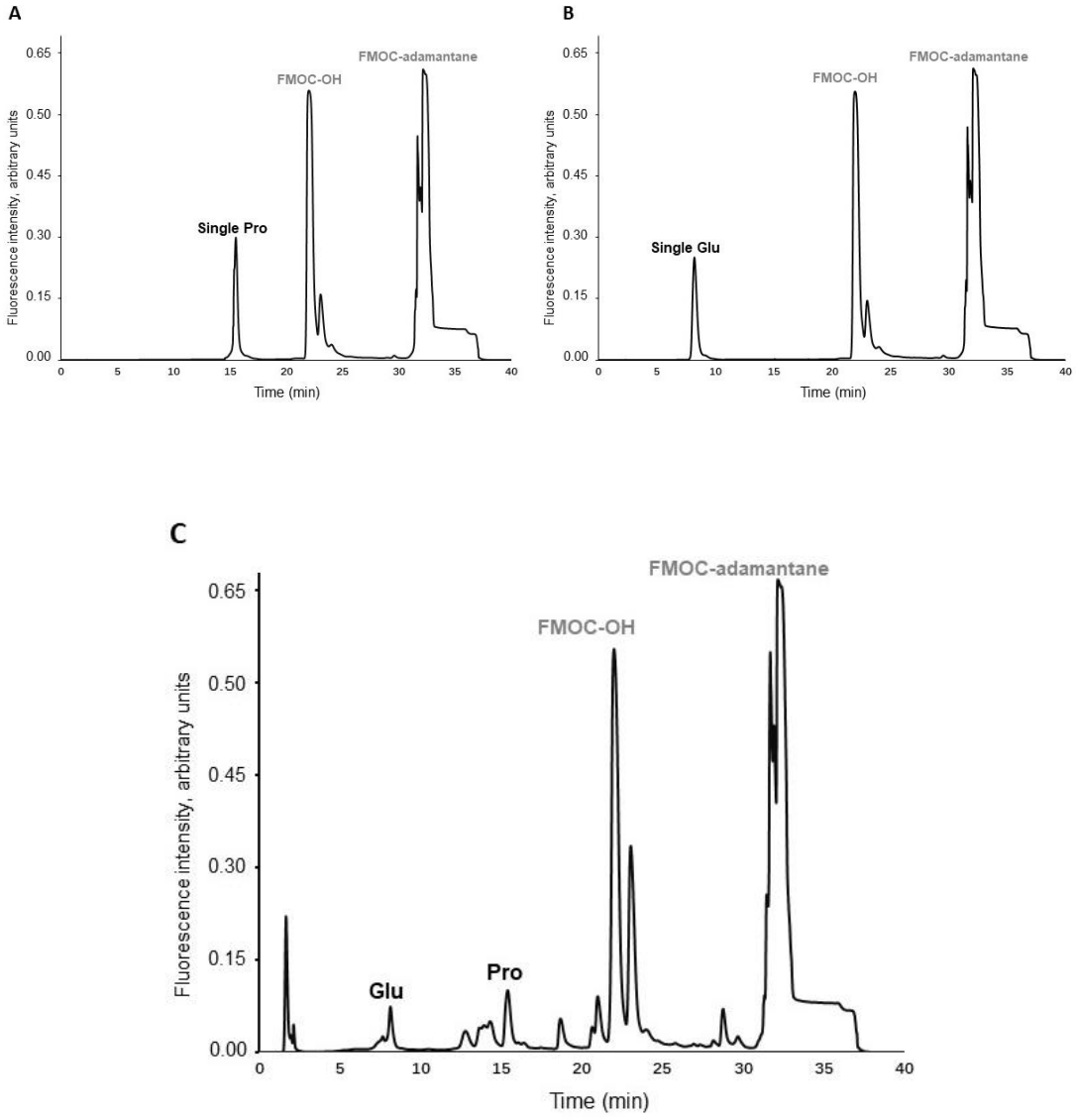

Figure 2.14 Elution shape of the FMOC derivatives of amino acids (continuous line), obtained with fluorescence detection. (A) Reference elution shape of proline derivatised by FMOC. **(B)** Reference elution shape of glutamic acid derivatised by FMOC. **(C)** Elution shape of the FMOC derivatives of amino acids of *H. pylori* wild-type. Peak of detected proline and glutamic acid are indicated. FMOC derivatization and HPLC were performed as described in section 4.2.2.1.

Total amino acid samples of *H. pylori* wild-type with osmotic treatment and control were obtained after ultrasonication and quantity normalization and the FMOC mediated derivatization was performed according the protocol introduced by Jámbor and Molnár-Perl (161). According to the elution shape of the FMOC derivatives of amino acids from the mentioned publication (161) and single proline and glutamic acid as standard substance in this study (Figure 2.14 A, B), our elution shape of FMOC derivatized total amino acids of *H. pylori* shows the time points of the peak of proline and glutamic acid as indicated in Figure 2.14 C, based on which the amounts of intracellular proline and glutamic acid from *H. pylori* wild-type of control and stress group were calculated (Figure 2.15). The data shows that the amounts of both intracellular proline and intracellular glutamic acid were increased slightly after *H. pylori* was treated by 0.55 Osm / kg of osmotic stress for 2 hours, which further confirmed the involvement of proline in hyperosmotic stress of *H. pylori*. Besides that, the increase of glutamic acid further verified the result of section 2.1.3, which showed the expression of *putA* that is involved in the conversion of proline to glutamic acid was upregulated by osmotic stress. However, statistics analysis of these data does not show significant differences due to the big error bars, which might be caused by the complex medium used for cultivating the *H. pylori* cells. Besides that, triplicated experiments might be not enough to gain a more stable result, thus more repeats of the experiments should be necessary.

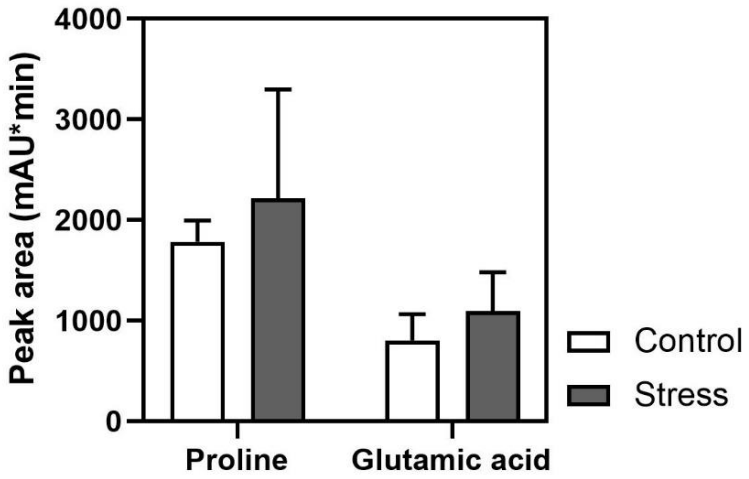

Figure 2.15 Relative amount of intracellular proline and glutamic acid of *H. pylori* detected by HPLC after derivatization with FMOC. Total amino acid samples of *H. pylori* wild-type treated with osmotic stress (0.55 Osm / kg, 2 hours) (stress group) and standard BB medium (control group) were obtained after ultrasonication and quantity normalization. The FMOC mediated derivatization of samples was performed as described in section 4.2.2.1. The peak area that is proportional to amount of the tested amino acid was calculated by the multiplying the height of the peak times its width at half height. The experiments were performed in triplicates. Statistical significance was not found according to the determination using the Student's *t*-test.

2.1.7 Fur and *put* operon regulation

As introduced in section 1.1.2, PutA of *H. pylori* is different from that of *E. coli* or other bacteria with respect to its domain composition. The HpPutA is missing the N-terminal extension for DNA binding in the oxidized state of the enzyme, which means that HpPutA is not able to function as a transcriptional repressor of the *H. pylori put* operon. It is known that the ferric uptake regulator (Fur) superfamily proteins play a pivotal role for microbial survival under adverse conditions and in the expression of virulence in most pathogens (60, 177). Moreover, Fur could act as an osmoregulatory factor because it is essential for the growth of *H. pylori* in the presence of high sodium chloride concentrations (60). Therefore, we wondered if Fur could play a role in regulating expression of the *put* operon in *H. pylori*.

Fur usually depresses the transcription of Fur regulated genes by combining with iron and binding to the specific DNA sequence called Fur box that is in the promoter region when the iron is abundant (178). The plasmid pBBR1MCS5-lux containing a *lux* cluster was used to analyze the regulation of *put* operon. The *putA* promoter region from *H. pylori* (HpputA_prmt) was inserted in to this plasmid upstream of the *lux* cluster, yielding the plasmid pBBR1MCS5-lux-EcputA_prmt to show the expression of the operon in real time visually. At the same time, another plasmid pTrc99A-HpFur was also created to express the *Hpfur* that can be induced by IPTG. The expression of induced *Hpfur* in *E. coli* JM109 was preliminarily tested by SDS-PAGE of total protein of the bacterium (Figure 2.16 A). Growth and luminescence of *E. coli* JM109 transformed with the two plasmids pBBR1MCS5-lux-HpputA_prmt and pTrc99A-HpFur was tested by Tecan microplate reader with the conditions as indicated in Figure 2.16. The growing states of *E. coli* cultivated under different conditions basically kept the same during 5 hours (Figure 2.16 B). As shown in Figure 2.16 C, when IPTG was added, which means HpFur was induced, the expression of *lux* cluster fused with *putA* promoter region was always lower than that without IPTG added, and the difference showed 3-folds at time point of 2 hours. This result could indicate that the overproduced HpFur might bind to the *putA* promoter region and inhibit the expression of the fused *lux* cluster. However, no matter if the iron was supplimented or chelated, no obvious difference could be observed in gene expression, which might be caused by the already sufficient quantity of iron in the LB medium. Nevertheless, these results can provide a first hint on a role of Fur in regulating the gene expression in *H. pylori*, which can be involved in the mechanism of osmotic stress tolerance of *H. pylori*. But further analyses are absolutely necessary to prove or confute this idea.

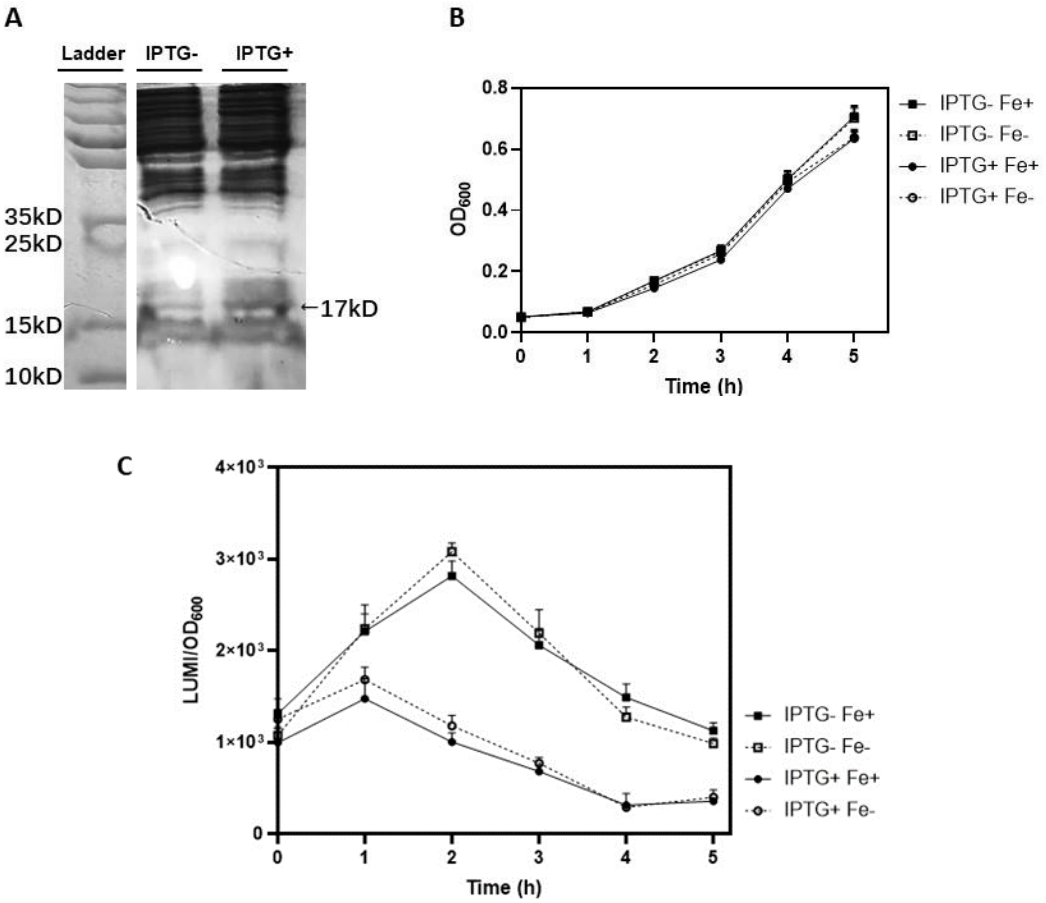

Figure 2.16 Investigation of *put* operon regulation of *H. pylori* by overexpression of Fur. (A) SDS-PAGE testing the induction of *fur* expression by IPTG. *E. coli* JM109 was transformed with the created plasmid pTrc99A-HpFur and cultivated in LB medium at 37 °C until OD_{600} of 0.8. Afterwards, 1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to induce expression of *fur*. Culture without IPTG addition was used as control. Cells were collected when OD_{600} reached to 1.5 for whole protein extraction. HpFur with molecular weight of 17 kD is indicated in the figure. Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder was used as ladder. Procedure of SDS-PAGE was performed as described in Section 4.2.2.3. **(B)** Growth curves of *E. coli* JM109 transformed with plasmids pBBR1MCS5-lux-HpputA_prmt and pTrc99A-HpFur with HpFur overproduced (squares) and not overproduced (circles) under conditions of iron supplement (solid) and iron chelator (hollow). **(C)** Luciferase luminescence of the *put* operon of *H. pylori* with HpFur overproduced (squares) and not overproduced (circles) under conditions of iron supplement (solid) and iron chelator (hollow) in *E. coli* JM109 cells. Data of (B) and (C) were detected by a Tecan plate reader at 37 °C over five hours.

2.2 L-proline is essential for motility of *H. pylori*

Previous work has indicated a link between proline uptake and metabolism and flagella-mediated motility of *H. pylori*. For example, a mutant with an inactivated *putA* gene (encoding proline dehydrogenase) showed no respiratory activity in the presence of L-proline, no amino acid stimulated motility, and no swarming activity. This mutant was ultimately unable to colonize nude mice (122). In addition, using signature tagged mutagenesis in the gerbil model, it was shown that *putP* (encoding the sodium/proline symporter) is another gene essential for *H. pylori* colonization of the stomach (118). In the past, Rivera-Ordaz from our lab also observed the loss of motility and deficiency of flagella formation of a *H. pylori putP* deletion mutant by motility assay and electron microscopy (155). However, insights into the mechanisms linking proline metabolism to bacterial motility are lacking and need further study. As stated in Section 1.1.3, it is known that many genes are involved in the formation and function of bacterial flagella. To better understand the influence of proline and proline-specific genes on motility, we have paid more attention to flagellar genes.

2.2.1 Motility of *H. pylori* derivatives

To investigate the involvement of proline uptake and metabolism systems in motility of *H. pylori*, a motility assay was performed. By using the soft agar plates, the swarming activity of *H. pylori* mutants deprived of *putA* (encoding proline utilization A flavoenzyme), *putP* (encoding major proline transporter PutP), *proP* (encoding predicted proline transporter ProP), *proV* (encoding the subunit ProV of predicted proline transporter ProVWX) and *proC* (encoding Δ^1-pyrroline-5-carboxylate reductase) were detected. As shown in Figure 2.16 A, compared to *H. pylori* wild-type, Δ*putA* and Δ*putP* were not motile, which is consistent with the observation from the aforementioned previous studies (118, 122, 155). In addition, the Δ*proP* strain did not show motility either, which indicates that ProP could also somehow play a role in the motility of *H. pylori*. At the same time, Δ*proV* and Δ*proC* did not show significant deficiency on motility, which means that they are not important for the motility of *H. pylori* under the test conditions. In this study, we mainly focus on the influence of intracellular proline accumulation on the motility of *H. pylori*. Therefore, we looked more closely at the link between the predicted proline transporters and motility. Thus, a sedimentation assay of *H. pylori* deprived of genes encoding putative proline transporters was also performed to test the results from motility assay on soft agar (Figure 2.17 B). In addition, the complemented mutants were also characterized by the soft agar assay and results showed that motility was reestablished (Figure 2.18).

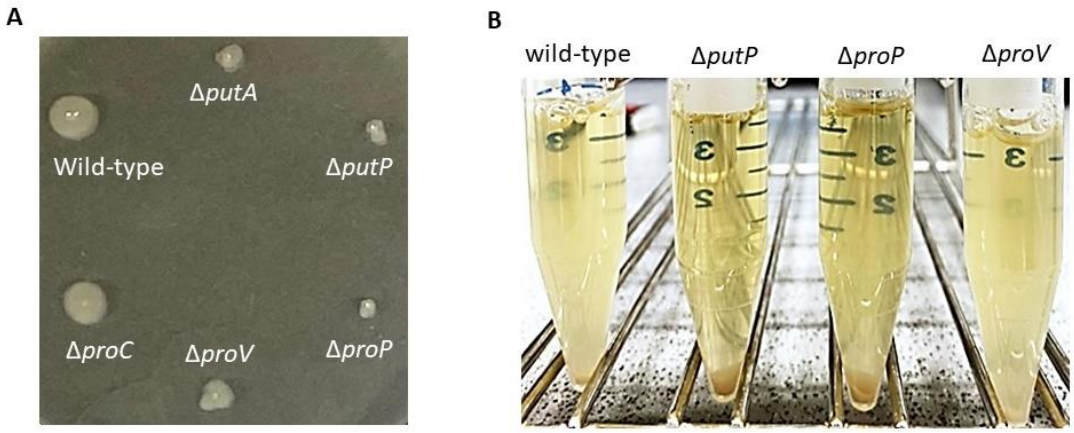

Figure 2.17 Assessment of motility of *H. pylori* wild-type and derivatives. *H. pylori* wild-type and indicated derivatives were pre-cultured in BB medium for 24 hours and then re-cultured in BB medium overnight. Cells were collected, washed and adjusted with fresh BB medium to OD_{600} of 1.0. **(A)** Motility assay using soft-agar. The adjusted cultures were stabbed with toothpicks into plates containing brucella broth with 0.3 % agar and incubated micro aerobically at 37 °C for 5 days. **(B)** Sedimentation assay of *H. pylori* Δ*putP*, Δ*proP* and Δ*proV*. The adjusted cultures were directly set to stand for 24 hours under 37 °C and micro aerobically.

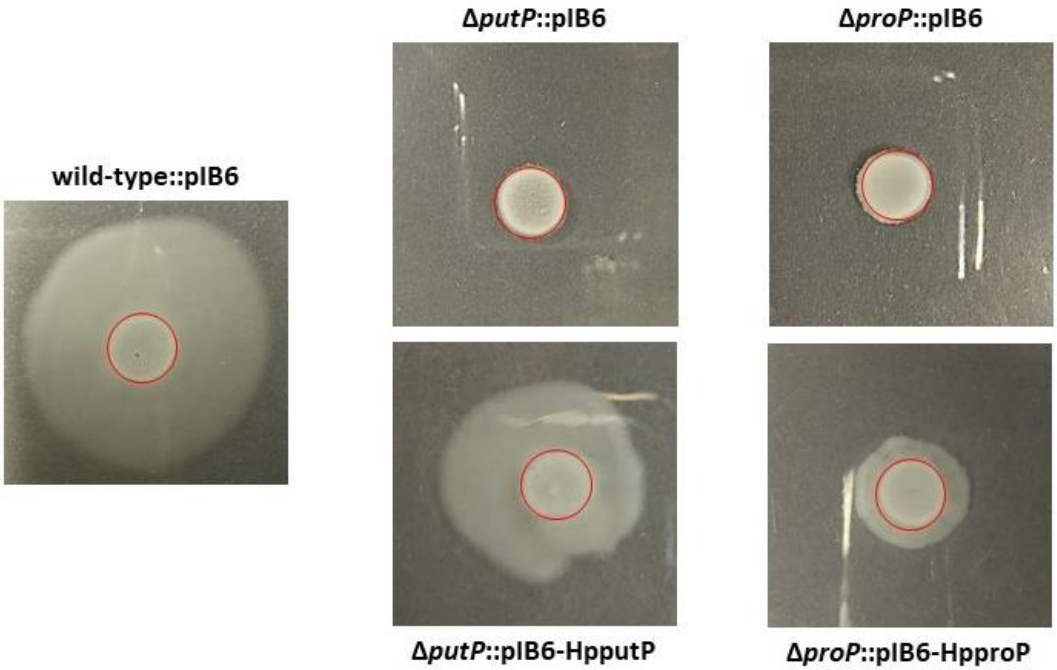

Figure 2.18 Assessment of motility of *H. pylori* wild-type and complemented mutants. *H. pylori* wild-type containing empty plasmid pIB-6 and the indicated derivatives were pre-cultured in BB medium for 24 hours and then re-cultured in BB medium overnight. Cells were collected, washed and adjusted by fresh BB medium to OD_{600} of 1.0, afterwards 5 µL of the adjusted cultures were carefully inoculated onto the plates containing brucella broth with 0.3 % agar and 8 mg / L kanamycin, and incubated micro aerobically at 37 °C for 7 days. Red circles indicate the area of initial inocula (day 0) on plates.

2.2.2 Impact of the deletion of *putP* gene on *flaA* expression.

In section 1.1.3, it has been expounded that the bacterial flagellar genes are divided into three classes according to their temporal order of expression and are regulated by sigma factors σ^{80} (RpoD), σ^{54} (RpoN) and σ^{28} (FliA) respectively. To further investigate the mechanisms behind our finding that *H. pylori* proline transporter deficient mutants lost their motility, the analysis regarding the expression of flagellar genes are necessary. There are more than 40 structural and regulatory genes involved in the process of *H. pylori* flagellar biogenesis. Previously, Rivera-Ordaz from our lab has selected several genes (*flaA*, *flaB*, *flgE*, *flgM*, *flhA*, *flhF* and *rpoN*) from each class and tested their expression in *putP* mutant. The result showed that only *flaA* encoding the major flagellin from class III were severely down-regulated when *putP* was absent (155). In this study, a deeper investigation regarding the flagellar genes, especially the class III related genes, was carried out.

Since there are signs that PutP should be the only active proline transporter in *H. pylori*, and a *putP* mutant showed clear and significant effects in both the osmotic stress and motility assays, we analysed the expression of flagellar genes by comparing their changes in wild-type, Δ*putP* and its complement. Besides *flaA* that encodes the major flagellin in *H. pylori*, we were also curious about the expression of *fliA* encoding σ^{28} and another class III gene *flaG* that encodes polar flagellin (162) in *H. pylori putP* mutant and complement. By analysing the expression of *flaA*, *fliA* and *flaG* using qRT-PCR, it showed that the expression of *flaA* in *putP* mutant was down-regulated around 400-folds, which was a severe difference and confirmed our previous result. Besides that, expression of *fliA* and *flaG* also exhibited 550- and 13-folds, respectively, down-regulation. Expression of all the three selected genes can be restored by the *putP* complementation partly (Figure 2. 19 A). Besides that, the result of semi-quantitative PCR analysing the expression of these genes in *H. pylori* wild-type and *putP* deletion mutant also confirmed this result (Figure 2.19 B). These results indicate that a defect in proline uptake into cells caused by *putP* deprivation may reduce the motility of *H. pylori* by influencing the expression of class III flagellar genes and their regulator σ^{28}, directly or indirectly.

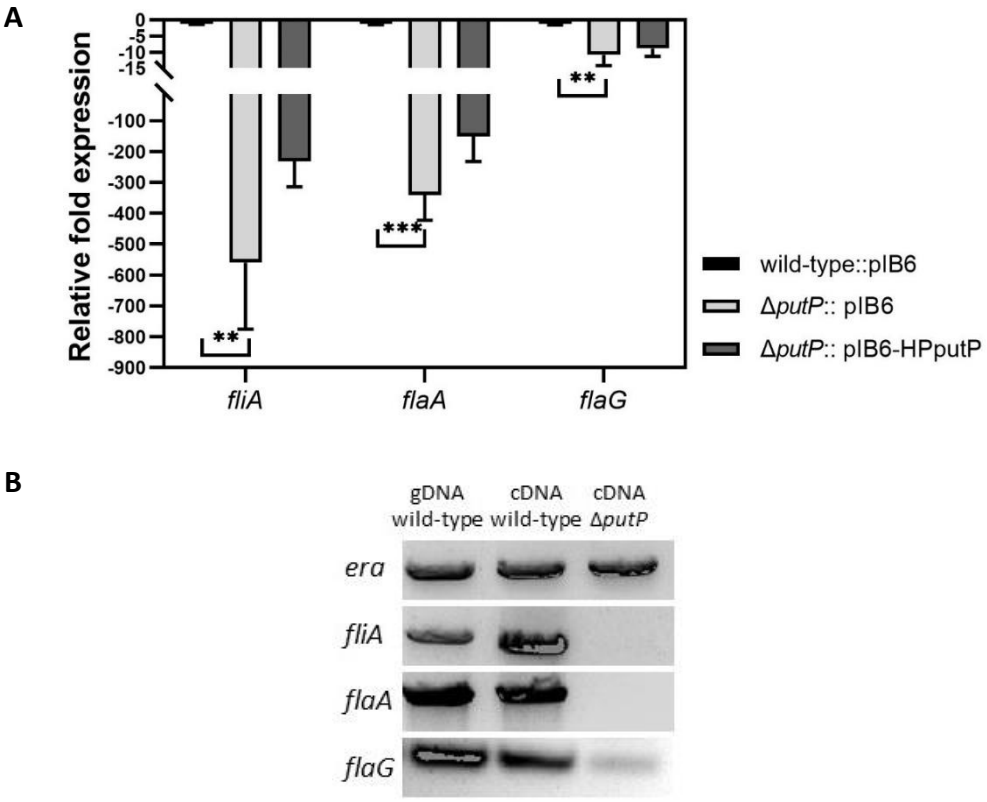

Figure 2.19 Expression of selected class III flagellar genes of *H. pylori.* (A) qRT-PCR analysing the expression of *fliA*, *flaA* and *flaG* of *H. pylori* wild-type (wild-type::pIB6, black column), Δ*putP* (Δ*putP*::pIB6, light-grey column) and the complement (Δ*putP*::pIB6-HpputP, dark-grey column). Cells were pre-cultured for 24 hours and then re-inoculated and cultured for 12 hours in standard BB medium. Sample acquisition were performed as described. Relative fold expression of the genes from different cultures was calculated using the housekeeping gene *era* as reference gene. Expression values are representative of three independent biological experiments. ** $P < 0.01$; *** $P < 0.001$, as determined using the Student's *t*-test. **(B)** Semi-quantitative PCR analysing the expression of *fliA*, *flaA* and *flaG* of *H. pylori* wild-type and *putP* deletion mutant. PCR was performed with the equivalent amount of template from cDNA of wild-type, Δ*putP* and gDNA as control. Housekeeping gene *era* was amplified as a reference.

Results from qRT-PCR and semi-quantitative PCR revealed a significant down-regulation of *flaA* expression ($P<0.001$) in the *putP* mutant of *H. pylori*. In order to strengthen and confirm the evidence for this result, one additional method was deployed in this study in form of promoter activity analysis of *flaA* through transcriptional fusion to the GFP reporter gene. In general, the promoter region of *flaA* was detected, amplified, fused to the upstream of *gfp* gene and cloned into the plasmid pIB6. The generated plasmid (pIB6-HpflaA^{Prmt}gfp) was then set into *H. pylori* wild-type and Δ*putP* using conjugation aided by *E. coli* WM3064 and then analysed by microscopy. Figure 2.20 shows that *gfp* gene fused with *flaA* promoter was successfully expressed in *H. pylori* wild-type thus fluorescence can be observed (Figure 2.20 B), whereas cells of the Δ*putP* mutant were not fluorescent (Figure 2.20 E). This result confirmed the result from qRT-PCR that expression of *flaA* was extremely down-regulated in *putP* deletion mutant comparing with that of wild-type.

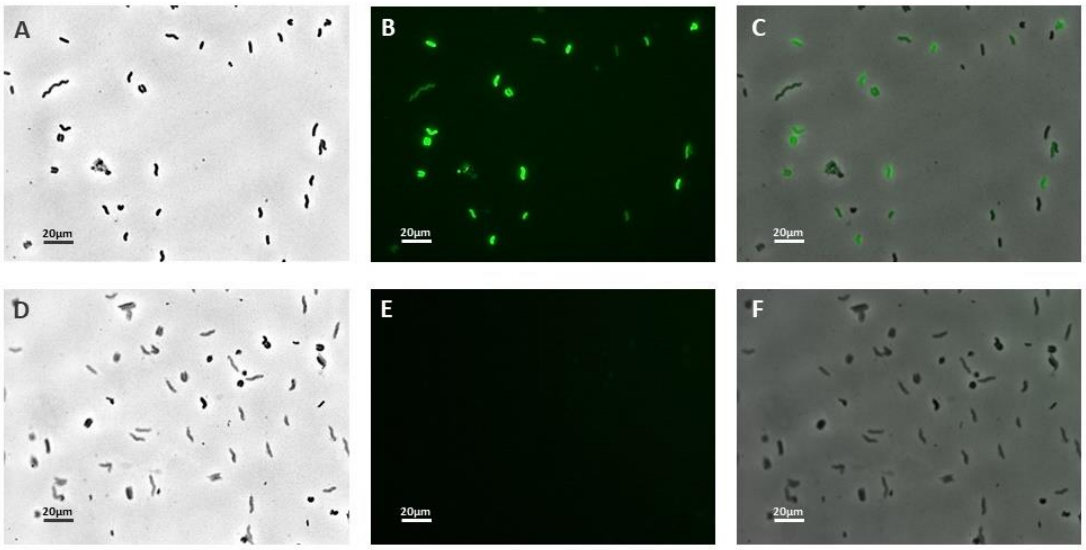

Figure 2.20 Fluorescent microscopy of *H. pylori* wild-type and *putP* deletion mutant. *H. pylori* wild-type (wild-type::pIB6-HpflaA^{Prmt}gfp) in **(A)** phase contrast; **(B)** GFP; **(C)** overlay. *H. pylori* Δ*putP* (Δ*putP*::pIB6-HpflaA^{Prmt}gfp) in **(D)** phase contrast; **(E)** GFP; **(F)** overlay. *H. pylori* cells were pre-cultured for 24 hours, re-inoculated and cultivated overnight in fresh BB medium containing 8 mg / L of kanamycin. Cultures were then adjusted to OD_{600} of 1.0 and 5 µL of each culture was imaged under light and fluorescent microscopy.

Strong evidence from above results has proved the extreme down-regulation of the *flaA* expression in *H. pylori putP* deletion mutant on transcriptional level. A further investigation regarding FlaA expression was performed using the method Western Blot. The antibody against HpFlaA was provided by Prof. Dr. Rainer Haas (Max von Pettenkofer-Institut, LMU Munich). Whole protein samples were extracted from *H. pylori* cultures and then adjusted to equivalent amount. Figure 2.21 A shows that the protein FlaA could be detected in *H. pylori* wild-type but not in the Δ*putP* strain, which was perfectly in accord with the result of transcriptional expression of gene *flaA*. However, when western blot was applied using the samples from the complement of *H. pylori* Δ*putP*, expression of FlaA surprisingly could not be detected (Figure 2.21 B).

In order to guarantee that the *H. pylori* derivatives harbouring the generated pIB6- plasmids could work well for FlaA detection, a series of experiments were carried out. Another complement of *putP* mutant was created using the constructed plasmid harbouring a fusion of *putP* and *gfp* (pIB6-HpputPgfp) that expresses *putP* and *gfp* at the same time. Figure 2.22 A shows that proline uptake was regained in complements of *putP* mutant, and the fluorescence in Figure 2.22 B indicates that *putP* was expressed and the protein was located on the cell membrane correctly. Furthermore, western blot could detect the expression of FlaA in *H. pylori* wild-type overproducing PutP (Figure 2.22 C), this means that overproduction of PutP was not toxic for *H. pylori* and the expression of *flaA* was not influenced. Even so, FlaA still could not be detected in complements of *putP* mutant (Figure 2.22 D).

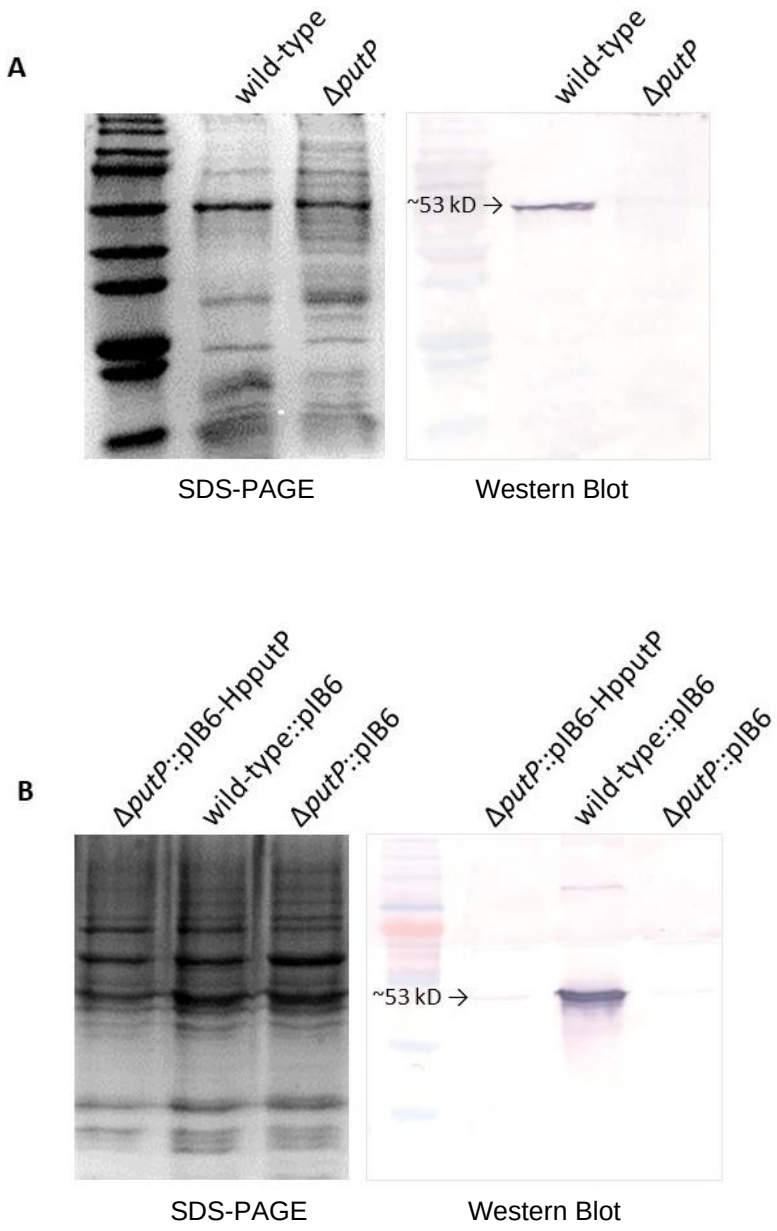

Figure 2.21 Western blot analysis of FlaA in *H. pylori*. (A) Samples of *H. pylori* wilt-type and Δ*putP* were prepared from liquid culture in the log-phase. Cells were distributed by sonification and whole protein samples were loaded on a SDS-PAGE gel (left). Polyacrylamide gel was transferred to a nitrocellulose membrane (right) and then incubated with anti-HpFlaA antibody and then developed by Alkaline phosphatase for western blot assay. **(B)** Samples of *H. pylori* wild-type (wild-type::pIB6), Δ*putP* (Δ*putP*::pIB6) and the complement (Δ*putP*::pIB6-HpputP) were prepared from liquid culture containing 8 mg / L of kanamycin in the log-phase. SDS-PAGE (left) and western blot (right) were performed exactly the same as described in (A). The molecular weight of HpFlaA is around 53 kD and has been indicated on the figures.

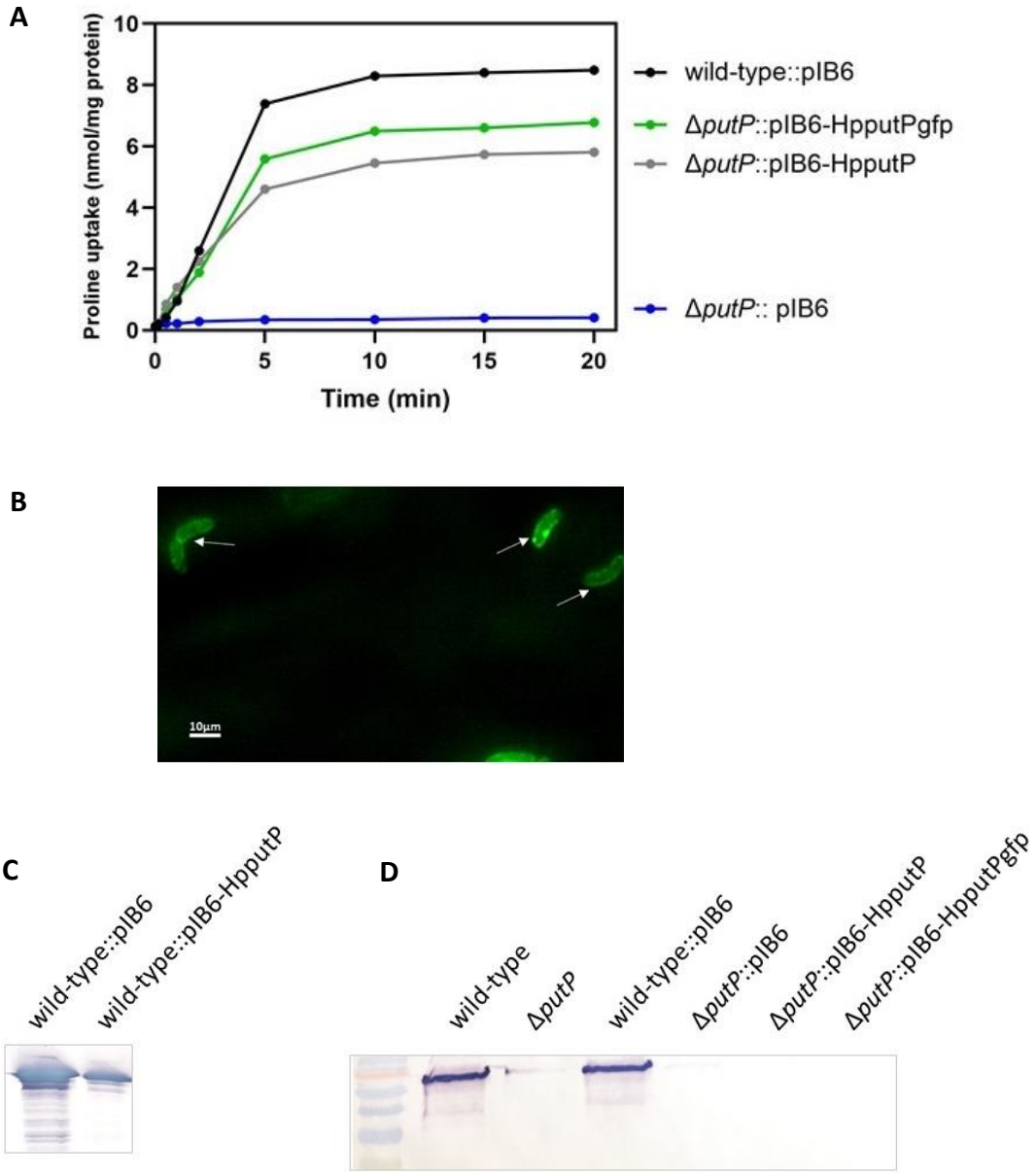

Figure 2.22 Analysis of *putP* and *flaA* expression on *H. pylori* wild type and Δ*putP* mutant expressing plasmid-encoded *putP*. (A) Time course of active proline uptake in *H. pylori* wild-type::pIB6 (black line), Δ*putP*::pIB6-HppuPgfp (green line), Δ*putP*::pIB6-HppuP (grey line) and Δ*putP*::pIB6 (blue line). **(B)** Fluorescent microscopy of *H. pylori* Δ*putP*::pIB6-HppuPgfp, expressed PutP-GFP proteins on cell membrane were indicated by arrows. **(C)** Western blot analysis of FlaA in *H. pylori* wild-type::pIB6 and wild-type::pIB6-HpputP that overproduced PutP. **(D)** Western blot analysis of FlaA in *H. pylori* wild-type, Δ*putP*, wild-type::pIB6, Δ*putP*::pIB6-HppuP and Δ*putP*::pIB6-HppuPgfp. All the experiments were performed as described previously.

2.2.3 DNA supercoiling of *H. pylori* wild type and *putP* deletion mutant

It is widely known that all living cells can supercoil their DNA, as a result of which the changes in DNA structure allows the compaction of DNA so that it fits within the limited space of a cell (163, 164). Recently, numbers of studies have revealed that DNA supercoiling plays a key role in gene regulation in a variety of pathogens (165-169). In 2016, Shortt, *et. al.* revealed a direct correlation between DNA supercoiling and motility in *H. pylori*'s closely related bacterium *C. jejuni* (104). In 2007, Ye, *et. al.* reported that a variety of flagellar genes were shown to be regulated by changes in DNA supercoiling (103).

In this study, we thus analysed the DNA supercoiling in *H. pylori* wild-type and Δ*putP* by using pIB6 as a reporter plasmid. Based on the protocol described by Liu, *et. al.*, a giant agarose gel containing 10 % of chloroquine was prepared to detect the DNA supercoiling of the reporter plasmid isolated from *H. pylori* (170). Chloroquine gel analysis revealed that the plasmid isolated from *H. pylori* wild-type was markedly more negatively supercoiled than the plasmid isolated from the *putP* deletion mutant (Figure 2.23 A). Besides that, changes of the DNA topology of the bacterial cell are predominantly a result of two competing enzymes, which are DNA topoisomerase I (TopA) that relaxes DNA and DNA gyrase (GyrA/GyrB) that is responsible for introducing negative supercoiling (104, 171). Therefore, the transcription of these genes (*topA*, *gyrA* and *gyB*) in wild-type and *putP* deletion mutant were also tested by qRT-PCR. However, even though *putP* deletion mutant showed a slight down-regulation in expression of *gyrA* and *gyrB*, and a slight up-regulation can be seen in expression of *topA*, the difference was not significant (Figure 2.23 B).

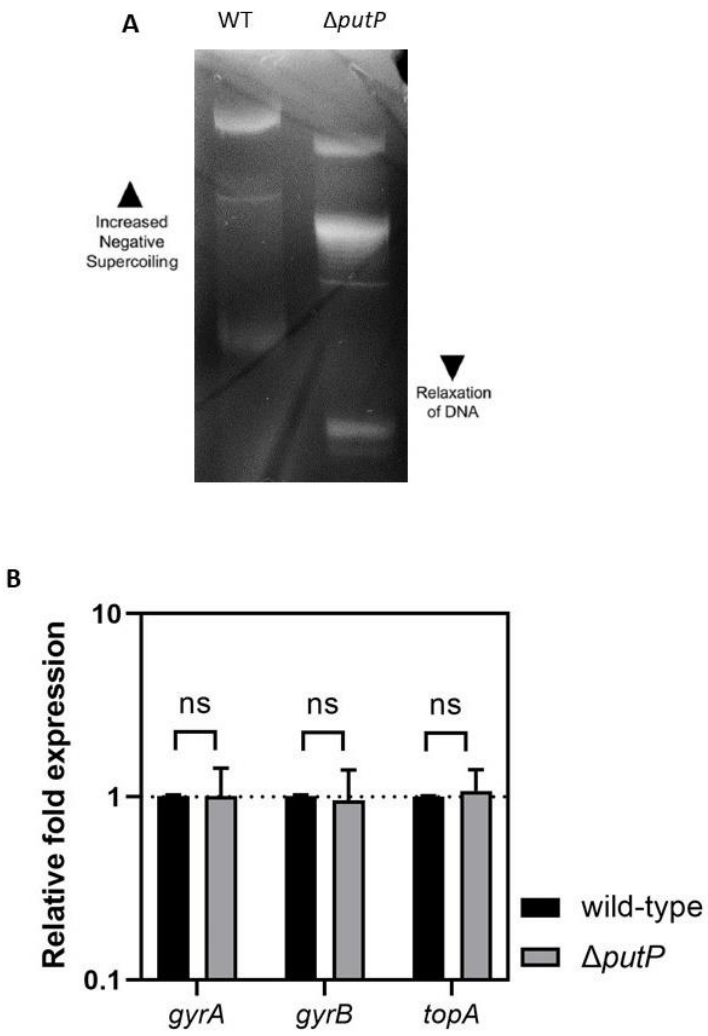

Figure 2.23 DNA supercoiling level and related gene expression in *H. pylori*. (A) Changes of plasmid pIB6 DNA supercoiling shown by chloroquine gel electrophoresis. *H. pylori* wild-type and *putP* deletion mutant harbouring pIB6 plasmid were cultivated and collected at log phase from BB medium containing 8 mg / L of kanamycin. The plasmids were extracted, adjusted to equivalent amount and loaded into the agarose gel containing 10 % of chloroquine followed by an electrophoresis. **(B)** qRT-PCR analysing the expression of *gyrA*, *gyrB* and *topA* of *H. pylori* wild-type and the Δ*putP* strain. Cells were pre-cultured for 24 hours and then re-inoculated and cultured for 12 hours in standard BB medium. Sample acquisition were performed as described. Relative fold expression of the genes from different cultures was calculated using the housekeeping gene *era* as reference gene. Expression values are representative of three independent biological experiments. ns, not significant, as determined using the Student's *t*-test.

2.2.4 ATP-level of *H. pylori* wild type and *putP* deletion mutant

The above results suggested that absence of proline transporter PutP in *H. pylori* could reduce 1) the DNA supercoiling, 2) expression of class III flagellar genes and the gene encoding their regulator σ^{28}, and thus 3) the flagella-mediated motility. To further investigate the connection between these phenomena and the mechanism underling them, ATP levels of *H. pylori* needed to be measured because flagellar assembling is a high-energy costing action. Besides that, DNA supercoiling in bacteria is also related to the intracellular energy level due to the fact that gyrase is unique in using ATP to drive the energetically unfavourable negative supercoiling of DNA (172). Therefore, in this study the intracellular energy level of *H. pylori* wild-type and *putP* deletion mutant was analysed by the measurement of ATP level using BacTiter-Glo™ Microbial Cell Viability Assay (Promega). As shown in Figure 2.24, the ATP level was reduced in *putP* deletion mutant of *H. pylori*, and it could restore in the complement.

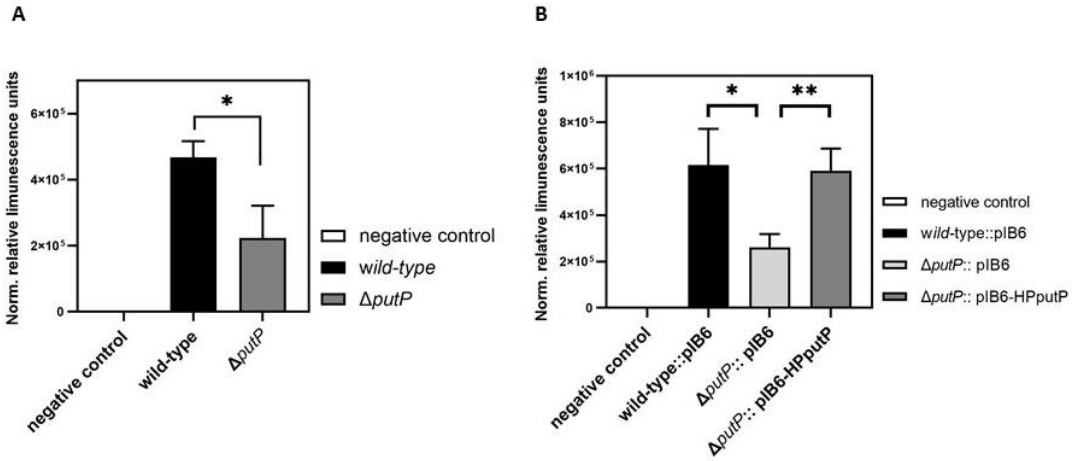

Figure 2.24 Intracellular ATP levels in *H. pylori*. (A) ATP levels of *H. pylori* wild-type and *putP* deletion mutant. * $P < 0.05$, as determined using the Student's *t*-test. **(B)** ATP levels of *H. pylori* wild-type (wild-type::pIB6), *putP* deletion mutant (Δ*putP*::pIB6) and complement (Δ*putP*::pIB6-HpputP). * $P < 0.05$; ** $P < 0.01$, as determined using the Student's *t*-test. Measurements were performed using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega). Samples were collected from liquid cultures in log phase, adjusted to an OD_{600} of 1.0 and 100 µL of each sample was added in a 96-well plate in BB medium. Control wells containing medium without inocula were prepared to obtain a value for background luminescence. The reagent of BacTiter-GloTM was added in an equal volume of cell culture in each well. The content was mixed and incubated for 5 min and luminescence was recorded and normalized.

3. Discussion

According to GLOBOCAN 2020 data, the worldwide incidence rates of gastric cancer is 1.58 % in males and 0.7 % in females in 2020 (27). Even though the overall incidence rates for gastric cancer have decreased markedly over the past 75 years, it still remains the fifth most commonly diagnosed cancer, and the third most deadly (179). Classified as a "type I carcinogen", *H. pylori* is a particularly important risk factor in the development of gastric cancer (28). The use of antibiotics for eradication of *H. pylori* can reduce the incidence of precancerous lesions only in the early stages of gastric carcinogenesis (180). In recent years, the development of clarithromycin resistance of *H. pylori* resulted in decreased *H. pylori* eradication rates worldwide (181). Therefore, new strategies for eradication of *H. pylori* and treatment of gastric cancer are urgently needed. Although the effects of *H. pylori* on gastric cancer appear to be multifactorial and the mechanism that leads to gastric cancer is not completely understood yet (182), it is important to explore more about the relationships between metabolism, physiology, and virulence of *H. pylori* to provide ideas for the development of new therapeutic strategies.

High saline intake is known to exacerbate gastric carcinogenesis triggered by *H. pylori*, and the gastric environment is also turbulent and has variable osmolality values. (57). However, the mechanisms of how *H. pylori* respond and adapt to the hyperosmotic environment are not fully understood yet. In fact, the genome of the bacterium does not contain genes encoding homologues of most of the primary osmotic shock defence systems that are present in other bacteria (65). A number of studies have demonstrated that proline is an osmolyte protectant employed by different bacteria, and the proline transport systems of which are involved in osmotic stress response (106-109). These findings inspired this study on the physiology of *H. pylori*, focusing on the role of proline uptake and metabolism in adaptation to hyper-osmotic stress. In addition, previous studies have shown that the sodium/proline transporter PutP and the bifunctional protein PutA are involved in *H. pylori* colonization by affecting flagella-mediated motility (122, 155), which in turn is essential for the pathogenesis of *H. pylori*. However, the mechanism of behind these findings and the involvement of other predicted proline uptake and metabolism systems had not been investigated. In this study, we aimed to gain more detailed insights into these issues.

3.1 PutP and ProP contribute to osmotic stress tolerance of *H. pylori* P12

3.1.1 The key proline transporter PutP is vital for osmotolerance of *H. pylori*

Previously, the proline transporter PutP of *H. pylori* was experimentally characterized as a high-affinity Na^+/proline symporter (121, 155). Based on this conclusion, we started our investigation by comparing the growth of wild-type and *putP* deletion mutant under high salinity to check if deprivation of proline uptake can influence the osmotolerance of this bacterium. Our results from section 2.1.2 indicate that the

osmotolerance of *H. pylori* is highly related to the presence of functional PutP, which also provides the first evidence that proline can be used as an important compatible solute by this microorganism. Our results of transcriptional expression of *putP* from section 2.1.3 and intracellular proline amount detected by HPLC from section 2.1.5 further confirmed this conclusion.

PutP was shown to be involved in osmotolerance also for other bacteria. For instance, expression of the *putP* gene of *S. aureu*s increases in a low-proline and high-osmolality environment both in growth media and in murine or human clinical specimens (108), indicating that proline import is required for osmotic stress protection. Besides that, *Jeotgalibacillus malaysiensis*, a moderate halophilic bacterium isolated from a pelagic area, appears to preferentially use proline as an osmoprotectant during prolonged osmotic stress. The expression of its *putP* gene increases 8-fold under osmotic stress, suggesting an increase in proline uptake under these conditions (222). Osmoadaptation of another halophilic bacterium *Vibrio cholerae* has also been studied. Together with OpuD, PutP is responsible for most of the proline uptake by *V. cholerae* under the osmotic stress condition. Distinctively, PutP of *V. cholerae* is also involved in uptake of glycine betaine in the study (223). In fact, studies of osmoregulatory mechanisms in *E. coli* have provided a foundation for understanding general prokaryotic osmotic stress responses. PutP of *E. coli* that shares 50 % identical amino acids with PutP of *H. pylori* has been intensively studied (155). Since a long period of time, it is known that ProP and ProU transporters of *E. coli* are responsible for accumulation of proline (and glycine betaine) to make it possible for the cells to adapt to higher osmolality (212), whereas PutP of *E. coli* is required for the transport of proline as a carbon or nitrogen source (213). In 2016, Sasaki, *et. al.* reported that an *E. coli* strain with high expression of *putP* exhibits increasing tolerance to high salinity, which suggested that the extent of *putP* expression plays an important role in the adaptation of *E. coli* cells to hyperosmotic conditions (214). By comparison, we found that PutP seems not to be the major proline transporter operating in osmotolerance in *E. coli*, whereas PutP is pivotal in osmotolerance in *H. pylori*. Furthermore, proline is also a critical osmolyte in *B. subtilis* with intracellular proline concentrations increasing up to 700 mM after osmotic shock (216). In *B. subtilis*, proline transporters PutP and OpuE share 61 % amino acid sequence identity and both of them are high-affinity sodium/proline symporters (188). In this case, PutP is critical for use of proline as a nutrient source, while OpuE is important for using proline as an osmotic stress protectant (217). As is seen from the above examples, bacteria usually contain different types of proline uptake systems, and they transport proline into cells to achieve different physiological objectives. In the case of *H. pylori*, apart from PutP that has proved to transport proline into cells and is involved in osmotolerance, ProP and ProVWX were only predicted as proline transporters, more functional details were still missing.

3.1.2 ProP is involved in the osmotolerance of *H. pylori*

To clarify whether ProP or ProVWX also plays a role in osmotolerance or proline transport in *H. pylori*, growth of *proP* and *proV* deletion mutants under hyperosmolality was also analysed as a priority in this study. The results in section 2.1.2 show that ProP can also contribute to the tolerance of *H. pylori* to high osmolality, while ProVWX seems not to be involved in osmotolerance.

3.1.2.1 ProP contributes to stress tolerance in microorganisms

It is well-known that the MFS superfamily member ProP can be employed by multiple bacteria as an osmoprotectant transporter that is broad in substrate specificity (105). The important role of ProP in stress tolerance for microorganisms has been shown frequently. Apart from ProP of *E. coli* that has been intensively studied, ProP of the food-borne pathogen *Yersinia enterocolitica* was found to play a role in betaine uptake during osmotic and chill stress responses (224). In *Corynebacterium glutamicum*, high redundancy of uptake systems and biosynthesis pathways for compatible solutes were employed to tolerate hyperosmotic stress, among which ProP is the most strongly regulated compatible solute uptake system catalysing the transport of proline and betaine (225). Previously, an in-silico analysis of osmotolerance in the gastrointestinal pathogen *Cronobacter sakazakii* showed that this microorganism contains seven copies of the *E. coli proP* homolog. Thus, it was hypothesised that the six additional ProP homologs may contribute to explaining the significantly increased osmotolerance of *C. sakazakii* relative to *E. coli* which possesses only a single transporter (226). Subsequently, heterologous expression against an osmotically sensitive *E. coli* host revealed that each homologue conferred an osmotolerance phenotype in varying degrees, in growth media with differences in osmolyte availability (227). Our results that ProP contributes to osmotolerance in growth are consistent with the above examples.

3.1.2.2 Substrates of ProP

However, ProP of *H. pylori* has not been experimentally characterized yet. Even though ProP has exhibited an important role in osmotolerance of *H. pylori* in this study, we could not deduce directly that proline is the substrate of ProP. In order to further understand how ProP affects osmotolerance of *H. pylori*, more experimental evidences regarding functions of ProP were needed. It is known that osmotic stress activates expression of the *proP* gene in *E. coli* (132, 139, 193) and ProP is regulated at the protein level with ProP being nearly inactive at low osmolality but becoming maximally activated in a highly osmolality medium (130, 133). Based on these facts, ^{14}C L-proline uptake of *H. pylori* mutants lacking different putative proline transporters were analysed with and without osmotic stress. The results from section 2.1.4 show that *putP* deletion mutant had almost no proline uptake at all. The proline uptake also did not increase by high osmolality in any of the mutants. Apparently, the other putative proline transporters of *H. pylori* could not take over the proline uptake of *putP* mutant even under higher osmolality. Moreover, ^{14}C L-proline uptake via

HpProP heterogeneously expressed in an *E. coli* strain defective in proline transport was not observed, and *H. pylori* Δ*proP* did not lose the ability of proline transport. All these findings indicate that ProP is not active in proline transport in *H. pylori*. In fact, many studies have revealed that ProP-mediated uptake of substrates such as glycine betaine and ectoine can provide more effective osmo-protection than that of proline (218-221). Considering the inactivity of ProP for proline transport in *H. pylori*, it is logical to assume that ProP may play a role in osmotolerance of *H. pylori* by transporting other substrates instead of proline. Unfortunately, the missing of defined medium or minimum medium specially for *H. pylori* limited the accuracy and credibility of the results from section 2.1.4, in which growth of *H. pylori* were tested with addition of different potential substrates of ProP and no obvious difference was observed. Therefore, more experiments testing the substrates of ProP in *H. pylori* should be performed with radioactivity labelling technique or so.

3.1.2.3 C-terminal domain is important for the function of ProP

In this study, we also predicted the structure of ProP of *H. pylori* to better analyse the function of ProP. Checking the annotation of the *proP* gene (locus tag: HPP12_0933, GenBank: ACJ08085.1), we suspect that the initial annotation of *proP* gene might be erroneous due to its small size (391 AA) comparing to that of *E. coli* (500 AA). Even though the annotated start codon "GUG" is an alternative start codon that can be utilized for translation initiation with non-negligible frequencies in prokaryotes (183), we re-analysed the *proP* gene region by using the ORFfinder from NCBI and attempted to find a more reliable and reasonable start codon of the gene. Surprisingly, a sole possibility of gene start codon "AUA" was predicted 210 bp upstream of the annotated start codon "GUG". This prediction suggests that the *proP* gene of *H. pylori* P12 has 1383 bp and encodes a protein with size of 460 AA. In prokaryotes, other than the canonical start codon "AUG", some near-cognates start codons "GUG" and "UUG" are also quite common for translation initiation (184), whereas an "AUA" start codon is rarely found naturally in bacteria. However, studies in recent years have proved the initiation of translation at "AUA" codon in *E. coli* (184-186), which indicates the prediction of *proP* start codon of "AUA" in *H. pylori* P12 could be possible. Based on the DNA sequence of *proP* gene that translates to a 460 AA size protein, 3D structure of ProP protein from *H. pylori* P12 was analysed. Results in section 2.1.5 show that the 3D structures of ProP proteins from both *H. pylori* and *E. coli* are quite similar with the exception that the carboxyl terminal coiled-coil seen in EcProP is missing in HpProP. In general, there are two groups of ProP orthologs that are differentiated by their carboxyl terminal domain (CTD) structures (228). CTDs of group A transporters such as EcProP form intermolecular, antiparallel, α-helical coiled coils *in vivo* and *in vitro*, whereas the shorter CTDs of group B transporters lack coiled-coils (229, 230). Therefore, HpProP should belong to the group B on account of its missing coiled-coil structure. In the case of *E. coli*, when amino acid changes were introduced to the coiled-coil, ProP required a larger osmotic upshift to become activated, suggesting that the C-terminal domain plays a role in osmosensing (229). Besides that, another research indicated that the six ProP homologues in *C. sakazakii* lacking the C-

terminal coiled-coil are significantly less osmoprotective than the ProP with the coiled-coil of this bacterium (227). When the C-terminal domain containing the coiled-coil of ProP in *C. sakazakii* was spliced onto the C-terminal end of one of the six short ProP homologues, a chimeric protein was created and it exhibited increased osmotolerance relative to the wild type (231). The important role of C-terminal structure in osmosensing might explain why ProP of *H. pylori* could not be activated by osmotic lift in this study. Besides that, many studies have demonstrated that the ProP transporters lacking the coiled coil domain still can be activated, but requires an even higher osmolality (229,231). In the case of our study, a higher osmotic stress might be necessary to set for better characterization of ProP in the future. Additionally, some studies also revealed that the activity of ProP is related to its localization on the membrane and the phospholipid composition of membrane of *E. coli* (242, 243), which might be a valuable perspective to research for HpProP in the future as well.

3.1.2.4 Regulation of ProP expression

We also analysed the transcriptional level of *proP* gene in response to osmotic upshift in *H. pylori* wild-type and *putP* deletion mutant, but no significant difference was seen between different test groups. In this case, it seems that ProP of *H. pylori* needs to be regulated at the translational level at a large extent to achieve the activation under different conditions. In *E. coli*, a cytoplasmic protein ProQ was recently clarified as a translational regulator of *proP* mRNA. ProQ was shown to play a role as an RNA chaperone in post-translational modification and osmotic activation of ProP (193, 232, 233). Furthermore, *proP* transcript levels are not changed in a *proQ* mutant, ProP expression is affected by deletion of *proQ* and no physical interaction was detected between ProP and ProQ (234). Similar translational regulation of ProP in *H. pylori* might also occur, especially considering the fact that few transcriptional regulators are encoded by the small *H. pylori* genome and post-transcriptional regulation has been proposed as a major level of control of gene expression in this pathogen (235). However, whole genome analysis revealed that the genome of *H. pylori* does not encode ProQ (236). This means that the mechanism of translational regulation of ProP in *H. pylori* does not follow the same rules that were established in the model bacterium *E. coli*, thus it needs further study for a deep understanding. In fact, the mechanism of transcriptional regulation for *proP* in *E. coli* has also been studied and established in detail, and this could provide possible ideas for the exploration of *proP* gene expression regulation in *H. pylori* as well. In *E. coli*, *proP* is transcribed from two different promoters. The *rpoD*-dependent promoter P1 is upregulated under hyperosmotic conditions and is transiently induced upon subculture, while the *rpoS*-dependent promoter P2 is induced by the stationary phase in the presence of a small and nucleoid-associated protein named Fis (139, 237, 28). Besides that, the stability of the P1 promoter-derived *proP* mRNA is post-transcriptionally regulated by RNase III whose activity on *proP* mRNA degradation is downregulated under osmotic stress (132). In contrast, no *rpoS* was found in the genome of *H. pylori*, whereas *rpoD* encoding σ^{80}, which is one of the three sigma factors in the bacterium, has been found

to regulate the expression of multiple genes (239). In addition, although *H. pylori* presents a remarkably low number of predicted conserved RNases comparing with other bacteria, RNase III is an important ribonuclease employed by this microorganism to degrade target mRNA (240). Therefore, mechanisms of transcriptional regulation of *proP* in *H. pylori* might be connected with the *rpoD*-dependent promoter P1 regulation of *proP* in *E. coli*, which would be interesting to investigate in the future.

3.1.3 Role of ProVWX system in bacteria

Apart from PutP and ProP, ProVWX is another predicted proline transport system in *H. pylori* P12, but more details about HpProVWX are still unknown. In *E. coli*, ProU (ProVWX) system efficiently scavenges glycine betaine as well as proline betaine to cope extreme osmotic stress (131). This system is composed of three proteins including ProV, ProW and ProX, which are encoded by an operon *proVWX* (244). ProV belongs to the ATP-binding cassette (ABC) superfamily and hydrolyses ATP providing energy for transporting substrates against the concentration gradient, ProX is a periplasmic soluble substrate-binding protein which can bind and deliver glycine betaine to the inner membrane protein ProW (212, 245). As mentioned above, in this study the results show that deletion of *proV* did not influence the osmotolerance and proline transport of *H. pylori*, the transcription of *proV* did not change under osmotic stress either. Thus, we estimate preliminarily that the ProVWX system seems not involved in osmotolerance of *H. pylori*. Certainly, in order to draw a precise conclusion about the roles of ProVWX in *H. pylori*, more researches focusing on areas such as the characterization and expressional regulation of ProVWX are still necessary. Besides osmotic stress, connection between ProVWX and other possible aspects of physiology of the bacterium should not be excluded. For instance, ProVWX is involved in the stationary phase-dependent accumulation of ectoine, which is an efficient adaptation strategy in *Vibrio anguillarum* against cold stress (241). In 2017, Álvarez and Toledo reported that the histone-like protein HU has a role in gene expression during the acid adaptation response in *H. pylori* (246). On the other hand, HU has been reported to regulate the overexpression of the *proVWX* operon in *E. coli* exposed to hyperosmolar environments (247), which shows a possible relation of ProVWX and acid adaptation for *H. pylori*.

3.1.4 PutP functions as the sole active proline transporter of *H. pylori*

In this study, what should not be ignored is that PutP is very likely to function as the sole active proline transporter of *H. pylori*. As mentioned above, *putP* deletion mutant showed almost no proline uptake at all, no matter the osmotic stress was applied or not. Apparently, neither ProP nor ProVWX of *H. pylori* could take over the proline uptake of *putP* mutant. This character of *H. pylori* is of significant different from the proline uptake systems of other bacteria. As described in above paragraphs, bacteria such as *E. coli*, *B. subtilis* and *V. cholerae* employ different types of proline transporter to uptake proline for different purposes such as osmoadaptation and acquisition of

carbon or nitrogen source (212, 213). If PutP is indeed the sole proline transporter of *H. pylori*, it would undertake the proline uptake for multiple physiological objectives, but this is actually not surprising due to the compact genome and reduced redundancy of genes in *H. pylori* (65, 248). Therefore, it is also meaningful to detect in what extent the PutP of *H. pylori* would mediate proline uptake for purpose of acquisition carbon/nitrogen source and osmotolerance respectively.

3.1.5 PutA and regulation of *put* operon in *H. pylori*

3.1.5.1 Role of PutA in osmotolerance of *H. pylori*

Although the proline uptake and metabolism systems in *H. pylori* have not been investigated adequately, *putA* encoding the proline utilization A flavoenzyme has already been reported to be involved in the colonization and oxidative stress tolerance of the bacterium (119, 122, 123). In this study, we also investigated the connection between *putA* and osmotolerance of *H. pylori*. Our results show that even deletion of *putA* did not weaken the osmotolerance of *H. pylori*, *putA* gene has a significant higher transcriptional level under hyperosmotic condition in *H. pylori* wild-type. Glutamate, the product of the reaction catalysed by PutA, acts as a compatible solute as well (105, 190). As shown in section 2.1.5, considering the amount of glutamate in *H. pylori* cells was also increased slightly under hyperosmotic stress, it is possible that glutamate could also be involved in the osmotolerance of *H. pylori*. The reason why deletion of *putA* did not influence the osmotolerance might be that glutamate is not a main and efficient osmoprotectant for the bacterium to resist osmotic stress. Besides that, the *putA* and *putP* genes are arranged in a *putAP* operon in *Helicobacter* (119), which could also explain the increasing expression of *putA* together with *putP* in response to hyperosmotic conditions.

3.1.5.2 Possible involvement of Fur in regulation of *put* operon of *H. pylori*

Speaking of *putAP* operon, its regulation is also an interesting topic to explore for better understanding the mechanism of osmotolerance and osmoadaptation in bacteria. Regulation of *putAP* operon has been established in bacterial models such as *E. coli*, *S. typhimurium*, *P. aeruginosa*, *Rhodobacter capsulatus* and *Vibrio vulnificus*. For example, the PutA from *E. coli* and *S. typhimurium* is not only an enzyme for proline utilization, but also a transcriptional repressor regulating the *putAP* operon that contains *putA* and *putP* transcribed in opposite directions from the *put* control intergenic DNA. The N-terminal ribbon-helix-helix (RHH) DNA binding domain of PutA is essential for the *putAP* operon regulation (148, 151, 152). In contrast, the PutA of bacteria such as *P. aeruginosa* and *R. capsulatus* does not function as an autogenous transcriptional regulator. These bacteria use another mechanism to regulate *putAP* operon that contains *putA* and *putP* transcribed in the same direction. In this case, the regulation needs regulatory proteins such as Lrp-type transcriptional repressor PutR (251) and AraC/XylS family regulator PruR (252). Besides that, the cyclic AMP receptor protein has been deduced to regulate the *putAP* genes of the food-borne pathogen *V. vulnificus* (190).

Genome analysis of *H. pylori* suggests that the *putA* and *putP* genes are arranged in an operon similar to those of *P. aeruginosa* and *V. vulnificus*, and PutA of *H. pylori* lacks the DNA binding domain for gene regulation. However, potential homologues of PutR and PruR in the genome of *H. pylori* are not found, the cyclic AMP receptor protein binding site is not present in the promoter regions of the *putAP* operons of *H. pylori* either (119). These findings indicate a wide divergence of mechanisms for the regulation of *putAP* genes between *H. pylori* and other bacteria. In *H. pylori*, Fur regulates the transcription of genes involved in iron acquisition and storage in response to changes in iron availability (197, 253, 254). Other than that, however, Fur also regulates gene expression in response to other environmental change such as low pH (255), oxidative stress (256) and salt (60), which is interesting for our research of osmotolerance of *H. pylori*. Fur box is a specific DNA sequence in Fur-regulated genes that can be recognised by the Fur-Fe^{2+} homodimer. Under Fe-replete conditions, Fur-Fe^{2+} homodimer binds to DNA at Fur box position and blocks access of the −35 and −10 sites by RNA polymerase, which represses expression of Fur-regulated genes (178). Previously, Pich and co-workers identified a Fur box core sequence of *H. pylori* G27 and analysed the occurrence of the proposed Fur box in the chromosome of *H. pylori* G27 (198). Using bioinformatics method, they found 18 intergenic regions and 15 coding regions harbouring the Fur box like sequence, one of which is located upstream of *putA* gene (198). Afterwards, the repression of *putA* expression by Fur-Fe^{2+} was then preliminarily proved by RNA dot blot hybridization (198), and our research using luciferase luminescence assay further confirmed this result. As shown in section 2.1.6, when induction of HpFur in *E. coli* was applied for 2 hours, the expression of *lux* cluster fused with *HpputA* promoter region was only 1 / 3 of that in *E. coli* with no HpFur induction. This indicates that the expression of HpFur might have a repression effect on gene transcription initiated by *HpputA* promoter region. Although the difference of *lux* expression between the set experimental groups were going smaller following the induction time, *E. coli* cells with HpFur induction always expressed lower *lux* than that without induction. In this luciferase luminescence assay, pTrc99A plasmid containing *trc* promoter and *E. coli* JM109 strain containing *lacI*^{q} expressing more lacI repressor of *trc* promoter were used to express Hp*fur*. In theory, the expression of HpFur should be tightly controlled by IPTG due to the overexpression of *lacI* (257). However, in our experiments, it seems the expression of *lacI* repressor in *E. coli* was not enough to control the induction of HpFur mediated by IPTG in pTrc99A, especially when cultivation time was longer. This might be an explanation for the decreasing difference of *lux* expression in *E. coli* between groups with and without HpFur induction following with time going.

3.1.6 Role of ProC in osmotolerance of *H. pylori*

Besides proline transport, bacteria also accumulate proline by bio-synthesis. In *H. pylori* P12, only *proC* gene encoding Δ^1-pyrroline-5-carboxylate reductase is predicted to be responsible for the proline bio-synthesis, other genes such as *proA* encoding γ-glutamyl phosphate reductase and *proB* encoding γ-glutamyl kinase in other bacteria

are missing (153). This indicates that *proC* may play a key role in the proline synthesis of *H. pylori*. In this study, deletion of the *proC* did not cause osmotic intolerance of *H. pylori*, while the expression of *proC* showed up-regulation only under the combined condition of osmotic stress and absence of *putP*, though the increase was not significant enough due to a big error bar caused by lacking enough experimental repeats. The uptake of compatible solutes by microorganisms is typically preferred over their de novo synthesis, which is probably for energetic reasons (191, 196). Therefore, the proline uptake from extracellular environment should dominate the accumulation of proline of bacteria under osmotic stress (105, 192). This might explain why deletion of single *proC* did not influence the osmotolerance of *H. pylori*. At the same time, since PutP has been deduced as the sole proline transporter of *H. pylori*, the up-regulation of *proC* in *putP* deletion mutant under hyperosmotic condition is a sign that *H. pylori* cells need more proline synthesis when the proline uptake from extracellular environment is cut off, which further suggests the significant role of proline in osmotolerance in *H. pylori*. Similar effect has been observed in *C. glutamicum* that takes up only ectoine, proline, glycine betaine and its precursor dimethyl glycine as compatible solutes via five secondary carriers, namely, PutP, BetP, EctP, LcoP, and ProP (225). The *C. glutamicum* strain DHPF carrying deletions in all five gene loci is completely deficient in the uptake of the mentioned compatible solutes, but is still able to survive a strong hyperosmotic stress due to the *de novo* synthesis of proline, glutamine, and trehalose (225, 249, 250).

3.1.7 Technical challenges and improvements in osmotolerance study of this thesis

During the process of investigating the osmotolerance of *H. pylori* in this study, doable and reasonable technical strategies are for sure essential to guarantee the succeed of the research. However, there would be certainly a room to improve the technical settings to further refine the study in the future. Here in this research, we started the experiments with only *H. pylori* wild-type and *putP* deletion mutant because PutP of the bacterium has been studied and characterized previously (121). The PutP deletion mutant was first used together with the wild-type strain to establish optimal experimental conditions for the for the analysis of adaptation to osmotic stress and to show possible growth differences between both strains. As described in section 2.1.2.1, an osmolality of 0.55 Osm / kg asserted by NaCl was found to be the optimal stress condition for the *H. pylori* growth analysis in liquid medium. In natural environment, osmotic stress can be asserted not only by ionic substances like NaCl, but also by non-ionic substances like sucrose. For the same osmotic pressure asserted by NaCl and sucrose, sucrose was added in twice the concentration, as osmolarity is measured in moles of solute particles (187). During the procedure of searching for optimal stress condition, sucrose was also tested and used to make osmotic stress in medium, and a very similar optimal osmolality of stress condition was found. But the following experiments were not performed with sucrose and data are not presented in this work, as the high viscosity of medium caused by addition of massive sucrose led to unwieldy operation in the following experiments. Besides the Brucella broth

liquid culture, we also tested the osmotolerance of *H. pylori* by using GC-Agar plates with different osmolarities. However, the liquid GC-Agar was too hot to perform the osmolarity measurement, whereas GC-Agar would be solidified when its temperature decreased, hence the osmolarity measurement for GC-Agar was impossible to perform. In order to find out the optimal amount of NaCl supplement in GC-Agar for stress condition, a mountain of work has been done by inoculating series of gradient dilutions of *H. pylori* wild-type and *putP* deletion mutant on GC-Agar plates supplemented with different amounts of NaCl. Besides that, we detected the intracellular amount of proline and glutamate of *H. pylori* wild-type and *putP* deletion mutant using HPLC in this study. Amino acids are zwitterions, they have a low solubility near isoelectric point and most of them have poor UV absorbance (194). It is necessary to derivatize the samples to impart desirable properties such as the reduction of polarity and improvement of sensitivity to UV and fluorescence (194). In this study, we used FMOC instead of ortho phthalaldehyde (OPA) to perform the pre-column derivatization, because OPA cannot be used to derivate secondary amino acids like proline, hydroxyproline and so on (161, 195). The result indicated that the amounts of both proline and glutamate in *H. pylori* wild-type increased slightly after osmotic stress treatment. However, this increase was not significant according to statistical analysis. This might be resulted by the uncertainty of complex medium used for *H. pylori* cultivation and treatment, which led to the big error bars of the data from triplicate experiments. During cultivation, the extracellular proline amount in complex medium could be too abundant or too insufficient for *H. pylori* to uptake and cope the stress, which could also influence the amount of glutamate as a metabolite of proline metabolism. Problems caused by the uncertainty of complex medium might also happen in our experiments detecting the growth of *H. pylori* with addition of potential substrates of ProP under osmotic stress. Therefore, the invention of minimum medium for *H. pylori* in the future should be extraordinarily important for a series of study on *H. pylori*.

Overall, in this part we found that PutP is vital for the osmotolerance of *H. pylori* by function as the sole proline transporter of the bacterium. ProP of *H. pylori* lacking the C-terminal coiled-coil is also important for *H. pylori* to tolerate osmotic stress, but its mechanism needs further research. Besides that, transcriptional change of *proC* and *putA* under osmotic stress indicates the important role of proline synthesis and metabolism in osmotolerance of *H. pylori* (Figure 3.1).

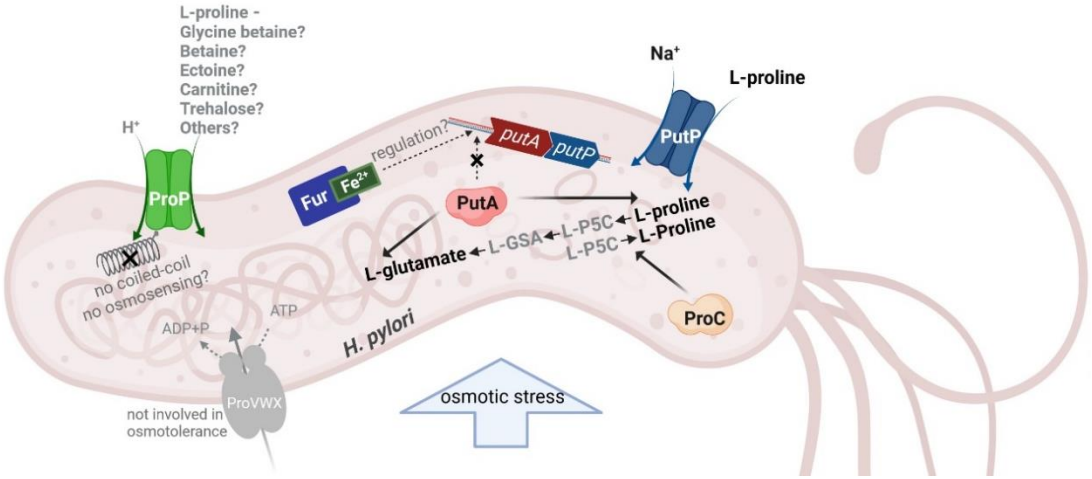

Figure 3.1 Proline uptake and metabolism specific systems involved in osmotolerance of *H. pylori*. Results showed that PutP is the sole active proline transporter, which is vital for osmotolerance of *H. pylori*. Even though the loss of C-terminal coiled-coil in HpProP might lead to a no osmosensing of this protein, HpProP is involved in osmotolerance of *H. pylori* despite its substrates are indeterminate by far. Up-regulation of *putA* expression in *H. pylori* wild-type under hyperosmotic stress reveals the possible role of glutamate in osmotolerance of *H. pylori*. The regulation of *putAP* operon might be mediated by Fur. Up-regulation of *proC* expression in *putP* deletion mutant under hyperosmotic stress indicates the role of proline bio-synthesis in osmotolerance of *H. pylori*. Putative transporter system ProVWX seems not involved in the osmotolerance or proline uptake of *H. pylori*. Overall, L-proline is of significance for *H. pylori* to cope hyperosmotic stress. Created with BioRender.com.

3.2 L-proline is essential for *H. pylori*

3.2.1 Motility of *H. pylori* and its proline uptake and metabolism systems

Several previous studies have demonstrated that *putA* and *putP* can affect the motility and colonization of *H. pylori* (118, 121, 122, 155). Considering the role of proline in this effect, the connection between other putative genes encoding proline uptake and metabolism systems and flagella-mediated motility of *H. pylori* was also investigated in this study. Initially, we performed the motility assay of *H. pylori* mutants that were deprived of these genes (*putP*, *putA*, *proP*, *proV* and *proC*). As expected, *putP* deletion mutant showed a deficiency in motility, which is in accordance with the results in the previous studies (118, 155), and the motility of *putP* deletion mutant could recover when *putP* expression was complemented. Similarly, *putA* deletion mutant also lost part of the swimming ability. However, our experiment showed that the motility of the *putA* deletion mutant was not as impaired as in previous studies reported by Nakajima (122). In that study, however, the *H. pylori putA* mutant also did not import proline from the surrounding media. The authors thus conjectured that the *putA* mutant may also have loss of *putP* expression due to the fact that *putA* and *putP* genes are arranged in a *putAP* operon (122). Therefore, the loss of *putP* also contributed to the deficiency of motility in that study. Differently, in our research, integrality of *putP* gene was already guaranteed by the DNA sequencing after creating *putA* mutant. In addition, surprisingly, *proP* also seemed to be involved in motility of *H. pylori* according to the motility and sedimentation assays (Figure 2.17, Figure 2.18). However, in this study, we did not further analyse this phenomenon in molecular levels, because we did not find any experimental evidence that ProP is active in proline uptake of *H. pylori*. In order to take a straightforward direction on proline-based studies, we placed more emphasis on the relationship between PutP and motility of *H. pylori* in the following experiments.

3.2.2 Flagellar genes influenced by *putP* deletion in *H. pylori*

There are more than 40 proteins involved in the biosynthesis and operation of flagella and their control of *H. pylori* (100), hence motility is one of the most complex processes in the bacterial cell. Despite intensive study of the role of motility in the pathogenesis of *H. pylori*, the transcriptional network controlling expression of flagella genes in *H. pylori* is still not fully understood (100). Based on the data of a previous study, *flaA* of Class III flagellar genes showed the most significant down-regulation among the selected genes from different classes in *putP* deletion mutant (155). In this study, we thus focused on the analysis of expression of some representative genes from Class III, also including the *fliA* gene encoding their regulator. Besides the expected down-regulation of *flaA*, the result also showed a great down-regulation of *fliA* and a significant down-regulation of *flaG* in the *putP* deletion mutant. Even though the polar flagellin FlaG was thought to control the flagellar filament length, inactivation of *flaG* in different bacteria has resulted in conflicting outcomes (162). So, more studies regarding *flaG* of *H. pylori* are needed.

Since the regulatory gene *fliA* governing expression of Class III genes showed a great down-regulation here, it is in all probability that most genes of Class III could be influenced by deletion of *putP*. But this deduction needs to be verified in the future by more exhaustive investigations using techniques like whole-genome microarray or even transcriptome analysis (100).

The gene *flaA* encoding the dominant flagellin subunit is also a potent immunogenic factor of *H. pylori* (204). Recent research indicates that a DNA vaccine containing *flaA* gene can induce significant immune responses against *H. pylori* infection in mice (205). Considering the particular important roles of *flaA* in pathogenesis of *H. pylori*, we further analysed its expression by Western Blot. With the antibody against HpFlaA, it was as expected and very obvious that Δ*putP* has almost no *flaA* expression at all. However, expression of *flaA* could not be detected in the complement of *putP* mutant by Western Blot, even though the proline uptake experiment and fluorescent microscopy have shown that *putP* was successfully expressed (Figure 2.22). Besides that, FlaA could be detected by Western Blot in the *H. pylori* wild-type overexpressing *putP*, which means overproduced PutP in the complement of *putP* mutant was not toxic for *flaA* expression. In previous study of Rivera-Ordaz (155), flagella of *putP* complementation showed small structures at the base localization but not with in a typical morphology of full length. Additionally, our results showed that motility of *H. pylori* Δ*putP* could be restored by *putP* complementation, but the restoration was not fully achieved (Figure 2.18). Indeed, the assembling of flagella is a huge and complex process that needs precise regulation (100). Therefore, restoration of full-length flagella and FlaA expression in mutant might not be simply obtained by just a *putP* complement in a plasmid. In principle, the expression of *putP* in the shuttle vector pIB6 should be ten times higher than that of the chromosomal *putP* in *H. pylori* (206), the distinction of complemented *putP* expression might be one of the factors affecting *flaA* expression. The *flaA* transcription was also analysed in the Δ*putP* complemented strain and compared with that of wild-type and *putP* deletion mutant containing empty pIB6 (Figure 2.19 A), the result showed that expression of *flaA* could be partly restored but with relatively large data variations in repeats. If the restoration of *flaA* transcription was reliable, the negative result of FlaA expression in Western Blot might mean a deviate translational or post-transcriptional process in *putP* complementation. Besides that, the loss of *putP* presumably can influence many other Class III flagellar genes due to the down-regulation of *fliA* expression, their expression might recover to a functional level in the complement, which could explain the partial restoration of motility and the incomplete flagella formation in *putP* complementation.

3.2.3 The connections between DNA supercoiling, ATP-level, *putP* deletion and motility of *H. pylori*

Many studies have revealed that the variation in DNA supercoiling provides an indirect way for gene expression to respond to changes in environment (171, 207-209). The DNA supercoiling level of the chromosome is finely controlled by the cell in response to environmental conditions, and almost all types of environmental challenges have been associated with DNA supercoiling variations (210). DNA supercoiling has also been proved to be an essential factor for motility of bacteria. For instance, when novobiocin was added into *H. pylori* culture, the DNA supercoiling was relaxed and motility was inhibited with down-regulation of related flagellar genes expression. In addition, expression of genes related to topoisomerase and gyrase also showed down-regulation (103). However, the relaxation of DNA supercoiling was artificially imposed and induced by novobiocin in the mentioned research. In this study, we found a relaxation of DNA supercoiling directly in the *putP* mutant at log phase without inductive agent, which indicated that limitation of proline uptake can cause the relaxation the DNA supercoiling in *H. pylori*. Differently from the previous research, expression of *gyrA*, *gyrB* and *topA* did not show any significant change in *putP* deletion mutant. Despite the possibility that only the activity but not expression of these enzymes is influenced by the absence of *putP*, it should be considered that the DNA supercoiling level, flagella assembling and even the expression of gyrase and topoisomerase in *H. pylori* are closely associated with growth phase of the bacterium with no anthropogenic intervention (103). The supercoiling oscillates from a high value at early log phase through a low value in mid-log phase to a high value at late log and finally to a low value in stationary phase (103). Regarding flagella, the “early”, “middle”, and “intermediate” flagellar genes are predominantly expressed in early growth phases, and production of FlaA occurs at very high levels in late log phase (100). In respect of gyrase and topoisomerase, it is known that gyrase genes were expressed at a steady level during log growth and topoisomerase I transcript was most abundant during early to mid-log phase (103). In this study, we tested the gene expression and DNA supercoiling only at log phase. In order to obtain more information regarding this topic, a comprehensive analysis should be performed during all growth phases of *H. pylori* wild-type and *putP* deletion mutant in the future.

In addition, the ATP level of *H. pylori* was decreased when *putP* was deleted and this could be restored by *putP* complementation (Figure 2.24). This result is not surprising due to the well-known fact that flagellar mediated motility of bacteria is a high energy cost process (172). In bacteria, a complex signalling pathway including a number of stimulus-specific receptors controls the motility, which is based on rotating flagellar filaments powered by the proton motive force. The motility spends several percent of total cellular energy and protein budgets, which are primarily consumed for biogenesis of flagella, powering of flagellar rotation and chemotactic signalling (259). Besides that, when ATP/ADP ratio is increased, gyrase introduces supercoils in the chromosomal DNA more actively and its supercoiling level increases (211). According to the above-mentioned fact that proline might be used as alternative

energy source (116), we assume that deletion of *putP* might limit the energy consumption of *H. pylori* and reduce the ATP level in cells. Therefore, it is likely that DNA supercoiling, ATP level and motility form a complicated network in which they regulate and influence each other in elaborate ways, and this network has connections to intracellular proline of *H. pylori*.

3.2.4 Possible involvement of ProP in motility of *H. pylori*

Last but not least, considering the role of ProP in osmotolerance *H. pylori*, we still could make some assumption about how it may influence motility. It is well-known that flagella formation and the motility are high-level energy consumption actions (172). Some evidences have shown that osmoprotectants like glycine betaine, choline and carnitine can also serve as both carbon and nitrogen sources for some bacteria (199-203). Therefore, perhaps some solutes of ProP can be alternative energy source of *H. pylori* and thus influence the motility. Besides that, the deletion of *proP* might directly or indirectly affect some of the flagellar genes or regulations. These assumptions need to be further investigated in the future to better understand the mechanisms behind this observation.

In general, in this part we found that the proline transporter PutP is essential for motility of *H. pylori*. Class III flagellar genes, DNA supercoiling and ATP level of the bacteria are involved in this effect (Figure 3.2). Besides that, ProP and PutA might also play a role in motility of *H. pylori* in different ways.

Taken together, proline has been shown to play a significant role in physiology and pathogenesis of *H. pylori* via the study focused on proline uptake and metabolism systems of the bacterium. PutP, a likely sole active proline transporter of *H. pylori*, is involved in both osmotolerance and motility of *H. pylori* in very large extend.

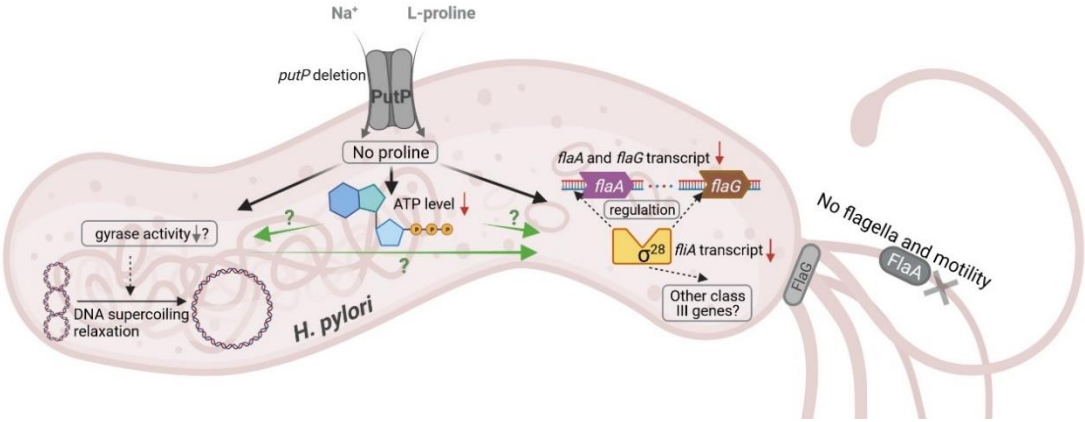

Figure 3.2 Influence of *putP* deletion on motility of *H. pylori*. Results showed that deletion of *putP* encoding proline transporter cause the loss of motility of *H. pylori*. Firstly, when PutP is absent, expression of Class III flagellar genes *flaA* encoding the filament and *flaG* encoding the polar flagellin is strongly decreased and no flagellum can be observed. The *fliA* gene encoding the σ^{28} that regulates Class III flagellar genes also shows a significant transcriptional decrease in *putP* deletion mutant, indicating that expression of other Class III flagellar genes might also be influenced by deletion of *putP*. Secondly, *putP* deletion mutant cells have lower ATP-level comparing that of wile-type. Thirdly, deletion of *putP* leads to relaxation of DNA supercoiling, but no change in expression of genes encoding gyrases. A complicated network formed by the three factors of 1. flagella formation, 2. ATP level and 3. DNA supercoiling has connection to proline uptake, the three factors can influence each other in elaborate ways as well. Proline is considered as an energy source of *H. pylori*; the limit of proline uptake might lead to the decrease of ATP level. Flagella formation that needs high level of energy is thus influenced by lower ATP level. At the same time, the ATP-dependent gyrase activity might be reduced either. Additionally, the relaxed DNA supercoiling might also contribute to the decrease of motility since DNA supercoiling was proved to be a key regulator of motility (104). Created with BioRender.com.

3.3 Outlook

Derivatives of *H. pylori* with defects in proline uptake and metabolism systems have been created and studied. More features of these derivatives need to be investigated, such as the uptake of possible compatible solutes other than proline. Besides that, double deletions or even triple deletions of these genes should be accomplished to better investigate of role of different genes in *H. pylori*.

Regarding the cultivation of *H. pylori*, it is important to invent a feasible minimal and defined medium to improve the assays such as growth, HPLC and so on. Besides that, it would be more rigorous to prepare the hyperosmotic stress by adding other alternative substance. Although the difference of osmotolerance in different derivatives were obvious enough, it would be better to further quantize the difference such as calculation of survival under different conditions.

The function of ProP needs to be well studied in detail. For example, the substrates of ProP of *H. pylori* need to be identified and thus the mechanism of how ProP effect osmotolerance and motility could be further investigated.

Regulation of *put* operon of *H. pylori* is important for better understanding the mechanism related to the involvement of proline in its physiology and pathogenesis. A direct relation between Fur and *put* operon regulation is very necessary to be provides.

Transcription of genes related to flagella and DNA-supercoiling in *H. pylori* together with energy level could be analysed at different phases of growth. Afterwards, a systematic analysis of these information could be performed to make the connection clearer.

Besides *flaA*, *flaG* and *fliA* that were studied in this thesis, expression of all the other flagellar genes also needs to be analysed in different *H. pylori* derivatives. An overall analysis of these genes in the level of protein would be helpful for this study.

In the end, tests of colonization, pathogenicity and lethality of these mentioned derivatives of *H. pylori* in animal models are essential to test the upstream analysis under *in vivo* conditions.

4. Material and Methods

4.1 Material

4.1.1 Bacterial strains

Escherichia coli and *Helicobacter pylori* strains used in this study are listed in Table 4.1.

Table 4.1 Strains used in this study

Strain	Description
E. coli DH5α	F⁻ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ⁻*thi*-1 *gyr*A96 *rel*A1 (173)
E. coli WM3064	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)*567*Δ*dapA1341*::[*erm pir*(wt)] (174)
E. coli WM3064::pIB6	*E. coli* WM3064 containing the plasmid pIB6 for conjugation with *H. pylori*
E. coli WM3064:: pIB6-HpputP	*E. coli* WM3064 containing the plasmid pIB6-HpputP for conjugation with *H. pylori*
E. coli WM3064:: pIB6-HpputA	*E. coli* WM3064 containing the plasmid pIB6-HpputA for conjugation with *H. pylori*
E. coli WM3064:: pIB6-HpproP	*E. coli* WM3064 containing the plasmid pIB6-HpproP for conjugation with *H. pylori*
E. coli WM3064:: pIB6-HpproV	*E. coli* WM3064 containing the plasmid pIB6-HpproV for conjugation with *H. pylori*
E. coli WM3064:: pIB6-HpproC	*E. coli* WM3064 containing the plasmid pIB6-HpproC for conjugation with *H. pylori*
E. coli WM3064:: pBSK-HpproP-rpsLerm	*E. coli* WM3064 containing the plasmid pBSK-HpproP-rpsLerm for conjugation with *H. pylori*
E. coli WM3064:: pBSK-HpproV-rpsLerm	*E. coli* WM3064 containing the plasmid pBSK-HpproV-rpsLerm for conjugation with *H. pylori*
E. coli WM3064:: pBSK-HpproC-rpsLerm	*E. coli* WM3064 containing the plasmid pBSK-HpproC-rpsLerm for conjugation with *H. pylori*

E. coli WM3064:: pIB6-HpflaA^{Prmt}gfp	*E. coli* WM3064 containing the plasmid pIB6-HpflaA^{Prmt}gfp for conjugation with *H. pylori*
E. coli JM109	F' *traD36 proA*$^{+}$*B*$^{+}$*lacI*^{q}Δ *(lacZ)*M15*/* Δ*(lac-proAB) gln*V44 *e14*$^{-}$ *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi hsd*R17 (175)
E. coli JM109:: pTrc99A-HpFur	*E. coli* JM109 containing the plasmid pTrc99A-HpFur
E. coli JM109::[(pTrc99A-HpFur) (pBBR1MCS5-lux-HpputA_prmt)]	*E. coli* JM109 containing the plasmids pTrc99A-HpFur and pBBR1MCS5-lux-HpputA_prmt
H. pylori P12	Clinical Isolate (888-0) from a patient with duodenal ulcer, Department of Medical Microbiology and Immunology, University of Hamburg, Germany
H. pylori P12::pIB6	*H. pylori* P12 wild-type containing the empty plasmid pIB6
H. pylori P12::pIB6-HpputP	*H. pylori* P12 wild-type containing the plasmid pIB6-HpputP
H. pylori P12 Δ*putA*	*H. pylori* P12 Δ*putA*::rpsLerm, Amp^{R} Erm^{R} Strep^{S} (155)
H. pylori P12 Δ*putA*::pIB6-HpputA	*putA* complement of *H. pylori* P12 Δ*putA* containing the plasmid pIB6-HpputA (155)
H. pylori P12 Δ*putA*::pIB6	*H. pylori* P12 Δ*putA* containing empty plasmid pIB6
H. pylori P12 Δ*putP*	*H. pylori* P12 Δ*putP*::*rpsLerm*, Amp^{R} Erm^{R} Strep^{S} (121, 155)
H. pylori P12 Δ*putP*::pIB6-HpputP	*putP* complement of *H. pylori* P12 Δ*putP* containing the plasmid pIB6-HpputP (121, 155)
H. pylori P12 Δ*putP*::pIB6	*H. pylori* P12 Δ*putP* containing the empty plasmid pIB6 (155)
H. pylori P12 Δ*putP*::pIB6-HppuPgfp	*H. pylori* P12 Δ*putP* containing the plasmid pIB6-HpputPgfp (155)
H. pylori P12 Δ*proP*	*H. pylori* P12 Δ*proP*::rpsLerm, Amp^{R} Erm^{R} Strep^{S}

H. pylori P12 Δ*proP*:: pIB6-HpproP	*proP* complement of *H. pylori* P12 Δ*proP* containing the plasmid pIB6-HpproP
H. pylori P12 Δ*proP*::pIB6	*H. pylori* P12 Δ*proP* containing the empty plasmid pIB6
H. pylori P12 Δ*proV*	*H. pylori* P12 Δ*proV*::rpsLerm, Amp^R Erm^R $Strep^S$
H. pylori P12 Δ*proV*:: pIB6-HpproV	*proV* complement of *H. pylori* P12 Δ*proV* containing the plasmid pIB6-HpproV
H. pylori P12 Δ*proV*::pIB6	*H. pylori* P12 Δ*proV* containing the empty plasmid pIB6
H. pylori P12 Δ*proC*	*H. pylori* P12 Δ*proC*::rpsLerm, Amp^R Erm^R $Strep^S$
H. pylori P12 Δ*proC*:: pIB6-HpproC	*proV* complement of *H. pylori* P12 Δ*proC* containing the plasmid pIB6-HpproC
H. pylori P12 Δ*proC*::pIB6	*H. pylori* P12 Δ*proC* containing the empty plasmid pIB6
H. pylori P12:: pIB6-Hp$flaA^{Prmt}$gfp	*H. pylori* P12 wild-type containing the plasmid pIB6-Hp$flaA^{Prmt}$gfp
H. pylori P12 Δ*putP*:: pIB6-Hp$flaA^{Prmt}$gfp	*H. pylori* P12 Δ*putP* containing the plasmid pIB6-Hp$flaA^{Prmt}$gfp

4.1.2 Plasmids

Plasmids used in this study are listed in Table 4.2.

Table 4.2 Plasmids used in this study

Plasmid	Description	Use
pBlueScript II SK (+)	Amp^R, *ori*colE1, *ori*f1(+), *lacZ*, M13 forward-/reverse (176)	Empty plasmid
pBSK-HpproP-rpsLerm	pBlueScript II SK (+) harbouring *HpproP* and the resistance cassette *rpsLerm*	Creation of *H. pylori proP* deletion mutant
pBSK-HpproV-rpsLerm	pBlueScript II SK (+) harbouring *HpproV* and the resistance cassette *rpsLerm*	Creation of *H. pylori proV* deletion mutant
pBSK-HpproC-rpsLerm	pBlueScript II SK (+) harbouring *HpproC* and the resistance cassette *rpsLerm*	Creation of *H. pylori proC* deletion mutant

pIB6	*alpA* promotor-region in shuttle vector pHel3 (158)	Empty plasmid
pIB6-HpputP	Shuttle vector pIB6 harbouring *HpproP* (155)	Over expression of *HpputP* in *H. pylori* wt
pIB6-HpputPgfp	Shuttle vector pIB6 harbouring *HpproP* and *gfp* (155)	Verification of *HpputP* expression from plasmid
pIB6-HpproP	Shuttle vector pIB6 harbouring *HpproP*	Complementation of *proP* deletion mutant
pIB6-HpproV	Shuttle vector pIB6 harbouring *HpproV*	Complementation of *proV* deletion mutant
pIB6-HpproC	Shuttle vector pIB6 harbouring *HpproC*	Complementation of *proC* deletion mutant
pIB6-HpflaA^{Prmt}gfp	Shuttle vector pIB6 harbouring *HpflaA* promoter region and *gfp*	Reporter of *flaA* expression in *H. pylori*
pTrc99A	IPTG-inducible expression vector, Amp^R, *Trc*/*lac* (266)	Empty plasmid
pTrc99A-HpFur	pTrc99A harbouring *HpFur*	Expression of HpFur in *E. coli*
pBBR1MCS5	Broad-host-range cloning vector, Gm^R, *lacZ* (267)	Empty plasmid
pBBR1MCS5-lux-HpputA_prmt	pBBR1MCS5 harbouring *lux* cluster and *HpputA* promoter region	Reporter of Fur-mediated regulation of *put* operon

4.1.3 Oligonucleotides used in this study

Oligonucleotides were designed according to the standard parameters and produced by Sigma-Aldrich (Germany), which are listed in Table 4.3. The restriction sites are highlighted with underline.

Table 4.3 Oligonucleotides used in this study

Name	Sequence (5'→3')	Information
putA_s	AAGGCACGCTCATATCAGGGAG	qRT-PCR testing *HpputA*
putA_as	GCCCGCTTTGAAATGATTGAGC	
putP_s	GCGATCGTTCTATGGGCCCT	qRT-PCR testing *HpputP*
putP_as	TGCGAGTTGATTAAGCCTCCCA	
proP_s	TGGAGGGGTTTTTGGTAT	qRT-PCR testing *HpproP*
proP_as	GCGGGAATTTGACTAAGG	
proV_s	GAGACCCTTTCAAACAACGC	qRT-PCR testing *HpproV*
proV_as	GGCTTCTTTATCCATGCCTTG	
proC_s	GCCCCGAGATGATTATAGA	qRT-PCR testing *HpproC*
proC_as	CCTAACCCCCTTTTTTTC	
era_s	AAGAAGAACGCATAGACAAG	qRT-PCR testing *Hpera*
era_as	ATTCACCCCATTTTTGCC	
flaA_s	ACTATGGGCGGCTTTCTCT	qRT-PCR testing

flaA_as	ATTCCCCAAAACCAATCGCT	*HpflaA*
fliA_s	CAACAACACCACCATCAA	qRT-PCR testing *HpfliA*
fliA_as	ATCAATAGAGCTGGGCAA	
flaG_s	CAAACAACCCCCACAAAA	qRT-PCR testing *HpflaG*
flaG_as	GGTTTGTATTGATCAGGGTC	
gyrA_s	GTGCATAGGCGTATTTTGT	qRT-PCR testing *HpgyrA*
gyrA_as	ACCAATCACATCACCCAC	
gyrB_s	GGCAAAACCAAAAGCACTAA	qRT-PCR testing *HpgyrB*
gyrB_as	TTGCAAAATACCCGCTTGA	
topA_s	TTGGGGGGAAATTAGAGAG	qRT-PCR testing *HptopA*
topA_as	TTTGTCGTGGGGTTTTTAG	
HpProP_s	CCGCTTGGCGGATCCGTGATGGCCCAC	Amplification of *HpproP*
HpProP_as	AGGAAAGGAGGATCCATGGCCCACTTTGG	
delproP_A_s	ACAACATAGAAGCTTGCATGAAGGATGCGC	Construction of plasmids for *HpproP* deletion
delproP_A_as	AGATTTATTGGATCCATGTTTTTACGACCGAATC	
delproP_B_s	TAAAAACATGGATCCAATAAATCTAATACCCAAC	
delproP_B_as	GTTTCAGGCGCGGCCGCAGGCTTCATATC	
proP_NdeI_s	GTGGAAGGATAACATATGAAACATTTAGGC	Complementation of *HpproP* in plasmid pIB6
proP_SpeI_as	AGGTTTGCGACTAGTTCATGCTGTGG	
delproV_A_s	GCAGGCGAGAATTCGAGGGCTAAAC	Construction of plasmids for *HpproV* deletion
delproV_A_as	GATTTCTAGGGTGGATCCCACATTCTCTATTTTGAC	
delproV_B_s	GAGAATGTGGGATCCACCCTAGAAATCAAAG	
delproV_B_as	GAATGTTGTCTAGAGGCTTGAGCTGC	
proV_NdeI_s	GGGTTATAACATATGAAAGAAATCGTC	Complementation of *HpproV* in pIB6
proV_NotI_as	CAATAACACCACGCGGCCGCTTTTAAACG	
delproC_A_s	GTAGTGGTACTGCAGCTACTTGCACCG	Construction of plasmids for *HpproC* deletion
delproC_A_as	ACGCTTTCATGGGATCCTGAATTGTAAGAT	
delproC_B_s	TTACAATTCAGGATCCCATGAAAGCGT	
delproC_B_as	CGCAAATGTTCTAGACCCCACTCAAATC	
proC_NdeI_s	GGGTGTCATATGGAAATCTTAC	Complementation of *HpproC* in plasmid pIB6
proC_SpeI_as	GAGTGACTAGTTTAGAGGTGC	
HputAprmt_s	CTGCGGATCCAAAAAAGCCCTTAGGCGT	*HpputA* promoter region in pBBR1MCS5-lux
HputAprmt_as	CCGCTCGAGCTTACCTTTTATTTAAGAAT	
HpFur_s	GCATGCCATGGATGAAAAGATTAGAAACTT	Amplification of *Hpfur* in plasmid pTrc99A
HpFur_as	CCAATGCATTGGTTCTGCAGTTAACATTCACTCT	
flaAprom_s	CCGCTCGAGGCAATAAGATTTGG	*HpflaA* promoter region in pIB6 with *gfp*
flaAprom_as	GGGAATTCCATATGTGTTGTAACTCCTTG	
gfpXho_s	GAGGCCTCGAGCATGGTACCAAG	Amplification of *gfp* in pIB6
gfpXho_as	AAAACAGCCGCTCGAGGCTTTGTATAGTTC	
pIB6_s	AGCTCTGATGTAATTTACAAGC	Check-primer in pIB6
pIB6_as	TTGGATAAGGGTTTTGGGTTTG	

4.1.4 Antibodies

The antibodies used in this study are listed in Table 4.4.

Table 4.4 Antibodies used in this study

Antibody	Source
Monoclonal rabbit anti-HpFlaA antibody	Prof. Dr. Rainer Haas (Max von Pettenkofer-Institut, LMU Munich)
HRP-conjugated goat anti-rabbit IgG antibody	Sigma-Aldrich

4.1.5 Chemicals and reagents

Tetraacetylethylenediamine (TEMED) (Merk), Acrylamide/Methylenbisacrylamide 29:1 (30%) (Roth), polysorbate 20 (Tween 20) (Serva), deoxynucleotide triphosphate mix (dNTPs) (Roche), dimethyl sulfoxide (DMSO) (Sigma), ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), ethidium bromide (EB) (Sigma-Aldrich), Midori Green Advance (NIPPON Genetics Europe), agarose (Serva), gentamicin (Sigma-Aldrich), ampicillin (Sigma-Aldrich), trimethoprim (Sigma-Aldrich), trypan blue (Invitrogen), erythromycin (Roth), vancomycin (Sigma-Aldrich), nystatin (Sigma-Aldrich), trimethoprim (Sigma-Aldrich), kanamycin sulphate (Roth), Scintillation liquid (EcoLite; MP Biomedicals), 2-(N-Morpholino)-ethane sulphonic acid (MES) (Roth), carbenicillin (Roth), hydroxyproline (AppliChem), tryptone (Roth), agar (BD Difco), yeast extract (BD Difco), sodium chloride (Prolabo), TriPure™ Isolation Reagent (Roche), diaminopimelic acid (DAP) (Sigma-Aldrich), fluorenylmethyloxycarbonyl chloride (FMOC) (Sigma-Aldrich), sodium deoxycholate (Sigma-Aldrich), Rubidium chloride (Sigma-Aldrich), boric acid (Sigma-Aldrich), 1-adamantanamine·HCl (ADAM) (Sigma-Aldrich), 4-Morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich), trichloroacetic acid (TCA) (Sigma-Aldrich), ammonium persulfate (APS) (Sigma-Aldrich), bathophenanthroline (BP) (Sigma-Aldrich), Brucella Broth (Thermo Scientific), GC-Agar base (Oxoid), horse serum (Gibco), Fetal bovine serum (FBS) (Gibco), glycerine (Roth), isopropyl β-D-1-thiogalactopyranoside (IPTG) (AppliChem), adenosine triphosphate (ATP) (Roche), bromophenol blue (Sigma-Aldrich), chloroform (Sigma-Aldrich), chloroquine (Sigma-Aldrich), ethanol (Roth), acetonitrile (ACN) (Roth), Incidin™ Plus (Ecolab).

4.1.6 Antibiotics

The antibiotics used in this study are listed in Table 4.5.

Table 4.5 Antibiotics used in this study

Antibiotic	Abbreviation	Dissolved in	Final concentration (in medium)
Ampicillin	Amp	H_2O	100 mg / L (LB-liquid/-solid medium)
Diaminopimelic Acid	DAP	H_2O	14 mg / L (LB-liquid / solid medium / GC-Serum solid medium)
Kanamycin	Kan	H_2O	50 mg / L (LB-liquid / solid medium); 8 mg / L (BB liquid / GC-Serum solid medium)
Erythromycin	Erm	EtOH	250 mg / L (LB-liquid / solid medium) 10 mg / L (GC-Serum solid medium)
Nystatin	Nys	DMSO	1 mg / L (BB liquid / GC-Serum solid medium)
Streptomycin	Str	H_2O	250 mg / L (LB-liquid / solid medium); 10 mg / L (GC-Serum solid medium)
Trimethoprim	Tri	DMSO	5 mg / L (BB liquid / GC-Serum solid medium)
Vancomycin	Van	H_2O	10 mg / L (BB liquid / GC-Serum solid medium)
Gentamicin	Gm	H_2O	10 mg / L (LB-liquid / solid medium)

4.1.7 Cultural media

Cultural media used in this study are listed in Table 4.6.

Table 4.6 Cultural media used in this study

Bacterium	Solution	Recipe
For *E. coli*	LB-liquid medium	Luria Bertani-Medium (260). 1 % (w / v) bacto tryptone, 0.5 % yeast extract and 1 % (w / v) sodium chloride. Autoclaved
	LB-solid medium	1.5 % BD Difco™ agar was added to the LB-liquid medium and autoclaved
	Glycerol liquid medium	LB-liquid medium, 20 % glycerin (v / v)
For *H. pylori*	GC-Agar solid medium	36 g / L GC-Agar Base, autoclaved. 10 mL / L vitamin mix, 80 mL / L horse serum, 10 mg / L vancomycin, 1 mg / L nystatin, 5 mg / L trimethoprim

For *H. pylori*	Vitamin mix (For GC-Agar)	100 g / L α-D-glucose, 10 g / L L-glutamine, 26 g / L L-cysteine, 0.1 g / L cocarboxylase, 20 mg / L Fe (III)-nitrate, 3 mg / L thiamine, 13 mg / L 4-aminobenzoic acid, 0.25 g / L NAD, 10 mg / L vitamin B12, 1.1 g / L L-cystine, 1 g / L adenine, 30 mg / L guanine, 0.15 g / L L-arginine, 0.5 g / L uracil, sterilized by filtration
	Brucella broth liquid medium	28 g / L Brucella-Broth autoclaved, supplemented with 80 mL / L FBS
	Glycerol liquid medium	Brucellar-broth liquid medium, 20 % glycerin (v / v), 10 % FBS (v / v)

4.1.8 Buffers and solutions

4.1.8.1 Buffers for transformation of competent cells in *E. coli*

Buffers used for transformation of competent cells in *E. coli* in this study are listed in Table 4.7.

Table 4.7 Buffers for transformation of competent cell in *E. coli*

Buffer	Recipe
Buffer I	10 mM MOPS, 10 mM RbCl, pH 7, sterilized by filtration
Buffer II	10 mM MOPS, 10 mM RbCl, 50 mM CaCl2, pH 6.5 adjusted with KOH, sterilized by filtration

4.1.8.2 Buffers for standard agarose gel electrophoresis

Buffers used for standard agarose gel electrophoresis in this study are listed in Table 4.8.

Table 4.8 Buffers for standard agarose gel electrophoresis

Buffer	Recipe
TAE-Buffer	40 mM Tris, 40 mM acetic acid, 1 mM EDTA
Loading Buffer	50 % (v / v) glycerine, 0.1 M EDTA, 1 % (w / v) SDS, 0.2 % bromophenol blue dye

4.1.8.3 Buffers and solutions for sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Buffers and solutions of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are listed in Table 4.9.

Table 4.9 Buffers and solutions for SDS-PAGE

Buffer or Solution	Recipe
Spacer buffer	0.4 % SDS, 0.5 M Tris / HCl (pH 6.76), 10 % APS, 0.1 % TEMED
Stacking buffer	0.4 % SDS (w / v), 1.5 M Tris / HCl (pH 8.8), 10 % APS, 0.1 % TEMED
Electrophoresis Buffer	25 mM Tris-HCl, 250 mM glycine, 0.1 % (w / v) SDS, pH 8.3
Coomassie-Solution	0.275 % Coomassie Brilliant Blue R250 (w / v), 50 % methanol (v / v), 10 % acetic acid (v / v)
Destaining solution	10 % methanol (v / v), 10 % ethanol (v / v), 7.5 % acetic acid (v / v)

4.1.8.4 Buffers for Western-Blot

Buffers for Western-Blot are listed in Table 4.10.

Table 4.10 Buffers for Western-Blot

Buffer	Recipe
Transfer Buffer	15.6 mM Tris, 120 mM glycine, 20 % (v / v) methanol, 0.02 % (w / v) SDS
Tris-buffered saline (abbreviated TBS)	150 mM NaCl, 20 mM Tris-HCl, pH 7.5
TBS-T	TBS, 0.5 % Tween 20 (v / v)
TBS-TT-Buffer	TBS-T Buffer with 0.1 % (v / v) Tween
Blocking Buffer	TBS, 3 % BSA (w / v)

4.1.8.5 Solutions for protein quantification

For protein quantification, the method of Peterson was used and the solutions used for each are listed in Table 4.11.

Table 4.11 Solutions for Peterson

Solution	Recipe
CTC solution	0.22 % KNa-tartrate $4H_2O$, 0.1 % CuSO4 $5H_2O$; dissolved in 10 % Na_2CO_3
Solution A	H_2O_{dest} / CTC / 0.8 N NaOH / 10 % SDS = (1 : 1 : 1 : 1, v / v / v / v)
Solution B	Folin-Ciocalteu reagent (FCR) / H_2O_{dest} = (1 : 5, v / v)

4.1.8.6 Buffers for transport measurements in *H. pylori*

Buffers for transport measurements in *H. pylori* are listed in Table 4.12.

Table 4.12 Buffers for transport measurements in *H. pylori*

Buffer	Recipe
Transport Buffer	100 mM Tris / MES Buffer, 150 mM KCl, pH 7.0
Stop Buffer	100 mM LiCl, 100 mM KH_2PO_4

4.1.8.7 Buffers and solutions for high-performance liquid chromatography

Buffers and solutions for high-performance liquid chromatography (HPLC) are listed in Table 4.13.

Table 4.13 Buffers and solutions for HPLC

Buffer or solution	Recipe
Borate buffer	25 mL 0.8 M boric acid dissolved in 0.8 M potassium chloride solution, pH 8.0 to pH 9.0, diluted with distilled water up to 50 mL
FMOC stock solution	FMOC dissolved in 5.0 mL acetonitrile (ACN), 20 mM; further diluted before use
ADAM solution	ADAM dissolved in ACN / 0.2 M HCl (1 : 1, v / v), 80 mM
Standard solutions	Free amino acids (L-proline and glutamate) dissolved in distilled water, 0.03 M; further diluted before use, in every second day
Eluent A	0.05M sodium acetate, pH 7.2
Eluent B	0.1 M sodium acetate / ACN = (23 : 22, v / v), pH 7.2,
Eluent C	ACN

4.1.9 Enzymes used in this study

Enzymes used in this study are listed in Table 4.14.

Table 4.14 Enzymes used in this study

Enzyme	Company
Taq DNA Polymerase	New England Biolabs, Schwalbach
Phusion High-Fidelity DNA Polymerase	New England Biolabs, Schwalbach
T4 DNA Ligase	New England Biolabs, Schwalbach
Restriction enzymes	New England Biolabs, Schwalbach
Proteinase K	New England Biolabs, Schwalbach
Pierce™ Alkaline Phosphatase	Thermo Scientific
DNase I	Thermo scientific

4.1.10 Molecular markers

Molecular markers used in this study are listed in Table 4.15.

Table 4.15 Molecular markers used in this study

Molecular markers	Company
DNA-Gel electrophoresis 2 log DNA Ladder	New England Biolabs
PageRuler™ Prestained Protein Ladder standard	Thermo Scientific

4.1.11 Commercial kits

Commercial kits used in this study are listed in Table 4.16.

Table 4.16 Commercial kits used in this study

Use	Kit	Company
DNA extraction	ReliaPrep™ gDNA Tissue Miniprep System	Promega
Plasmid purification	Hi Yield® Plasmid Mini DNA Extraction kit	SLG
DNA gel extraction	Hi Yield® Gel / PCR DNA Fragment Extraction Kit	SLG
Reverse transcription	RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific
Real-time PCR	iQ™ SYBR® Green Supermix	Bio-Rad
ATP measurement	BacTiter-Glo™ Microbial Cell Viability Assay	Promega

4.1.12 Main instruments and equipment

Mastercycler personal thermal cycler (Eppendorf), CFX96 Touch Real-Time PCR Detection System (Bio-Rad), Nanodrop ND-100 Spectrophotometer (Peqlab), Sunrise absorbance microplate reader (TECAN), anaerobic jar (Schuett Biotech GmbH), microflow biological safety cabinet (Nunc, Innova™ 4330), DM IRE2 microscope (Leica), Ecotron table-top incubation shaker for microorganisms (Infors HT), MI21N anaerobic chamber (SCHOLZEN Microbiology Systems AG), Dionex™ UltiMate™ 3000 HPLC System (Thermo Scientific).

4.2 Methods

4.2.1 Bacteria cultures

4.2.1.1 Cultures for *E. coli*

4.2.1.1.1 Normal cultivation for *E. coli*

E. coli strains were grown on LB agar plates or in LB-liquid medium at 37 °C (260). For the selection of plasmids in *E. coli*, media was supplemented with erythromycin (10 mg / L), ampicillin (100 mg / L), gentamicin (10 mg / L) or kanamycin (50 mg / L). For the strain collection, the cell material was taken from an overnight liquid culture with glycerine (3:1) and stored at - 80 °C. Recombinant plasmids were transformed and maintained in *E. coli* strain DH5α, WM3064 or JM109.

4.2.1.1.2 Microwell plate reader assay

Microwell plate reader assay was used to detect the *putAP* operon regulation in this study. *E. coli* JM109 containing plasmids of both pTrc99A-HpFur and pBBR1-MCS5-lux-HpputA_prmt was inoculated into LB-liquid medium containing gentamicin (10 mg / L) and ampicillin (100 mg / L), followed by overnight cultivation with shaking at 37 °C. Afterwards, several aliquots of 100 µL of diluted culture (10 ^ -1) was mixed with 100 µM of FeCl3, 0.5 mM of bathophenanthroline (BP) or 0.5 mM of IPTG according to experimental design, and fresh LB was added to each aliquot till to 1000 µL. Then 150 µL of each mixed aliquot was added into each well of a black 96-well plate according to the design. Besides that, *E. coli* JM109 containing empty plasmids was used as negative control. The 96-well plate was then set into a thermomixer and *E. coli* was cultivated under 37 °C. The OD_{600} and luminescence of each mixture was measured per hour for 5 hours by the Tecan Sunrise spectrometer.

4.2.1.1.3 Transformation of *E. coli*

Chemically competent cells were prepared according to Rubidium Chloride Method. A single colony from *E. coli* was used to inoculate 5 mL of LB medium and incubated overnight at 37°C with vigorous shaking. 100 µL from this overnight culture was used to prepare a new 10 mL culture which was incubated 2 hours at 37 °C until an OD_{600} of 0.3 - 0.5 was reached. Cells were harvest by centrifugation 5 min at 5000 rpm 4 °C and resuspended in 5 mL of Buffer I, washed again and resuspended in 5 mL of Buffer II. Cells were incubated 30 min on ice, centrifuged for 5 min at 5000 rpm and under 4 °C, and then resuspended in 1 mL of Buffer II.

For heat shock transformation of *E. coli* cells, 200 µL of competent cells were inoculated with 1 µL of Plasmid-DNA and incubated for 30 min at 4 °C. A thermal shock was applied for 20 sec at 37 °C and then incubated for 2-3 min on ice. 800 µL of LB medium was added followed by incubation for 45 min under 37 °C at 750 rpm. In the end, 100 µL of aliquots of the cell suspension was spread on LB selective-plates and incubated at 37 °C overnight.

4.2.1.2 Cultures for *H. pylori*

4.2.1.2.1 Normal cultivation for *H. pylori*

H. pylori strains were cultured on GC agar serum plates or brucella broth (BB) for liquid cultures supplemented with 10 % horse serum (HS) and the following antibiotics: vancomycin (10 mg / L), nystatin (1mg / L), trimethoprim (5 mg / L). Cultures were incubated at 37 °C under microaerobic conditions (85 % of N_2, 10 % of CO_2, 5 % of 0_2) (158). When necessary for selection of *H. pylori* allelic exchange mutants, erythromycin (10 mg / L) or streptomycin (10 mg / L) was used in addition to the up-mentioned antibiotics. For the strain collection, the cells from one plate were resuspended in 1 mL of brucella-medium with 20 % glycerine and 10 % HS and stored at - 80°C.

4.2.1.2.2 Hyperosmotic stress tolerance assay

For detecting the OD_{600} of *H. pylori* cells in BB liquid medium with and without hyperosmotic stress, osmolarities of standard fresh BB medium and fresh BB media added with different amount of NaCl were tested by osmometer. *H. pylori* Inocula were collected from GC-Agar plates and pre-cultured in BB liquid medium for 24 hours. Cultures were then adjusted to OD_{600} of 0.07 by different osmolarities of fresh BB medium. The adjusted cultures were cultivated in 24-well plates by shaking at 37 °C and 130 rpm under microaerobic conditions, the OD_{600} measurements were performed after 18 hours of cultivation.

For detecting the colony growth of *H. pylori* on GC-Agar plates with and without hyperosmotic stress, optimal amount of NaCl supplement in GC-Agar for stress condition was found out by a series of work. In general, 4 g / L to 10 g / L of NaCl amounts were added in GC-Agar, then *H. pylori* wild-type and *putP* deletion mutant in gradient dilutions were inoculated on all the agar plates added with different amount of NaCl. The optimal stress condition was found based on the GC-Agar plate with the most obvious difference between the colony amounts of wild-type and *putP* mutant. Afterwards, *H. pylori* wild-type and all mutants were pre-cultured in BB medium for 24 hours, afterwards each culture was adjusted to OD_{600} of 1.0 and then diluted in fresh BB medium ten-fold gradationally (5 gradients were taken: 10^{-1} to 10^{-6}). 5 µL of each dilution from each culture was dropped on standard GC-Agar plate (Control group) and GC-Agar plate with 6 g / L NaCl (Stress group) in a designed order. The plates were incubated under 37 °C for one week.

4.2.1.2.3 Hyperosmotic stress treatment

To prepare the samples for qRT-PCR in section 2.1, *H. pylori* cells were treated by hyperosmotic stress before the RNA extraction. Briefly, *H. pylori* wild-type or derivative strains were pre-cultured in BB-liquid medium overnight. The cells were then re-inoculated in fresh BB-liquid medium and adjusted to OD_{600} of 0.1 and cultivated overnight. Cultures were then divided into two equal portions and centrifuged at 3,000 rpm for 10 min, the old media were removed and discarded. For the two portions of each culture, pellet of the first portion was added with standard

fresh BB-liquid medium and suspended, while pellet of the second portion was added with same amount of BB-liquid medium that supplemented by NaCl making 0.55 Osm / kg. All the resuspended cells of *H. pylori* were then cultivated for 2 hours followed by cell collection with centrifugation. The pellets were either directly used for RNA extraction or stored at - 80 °C after a liquid nitrogen processing.

4.2.1.2.4 Motility assay

H. pylori cells were cultured in plates for 48 h, resuspended in BB medium supplemented with 10 % of horse serum and adjusted to bacterial concentrations of 1x 10^8 cells per mL (OD_{600} of 0.2). Bacterial cells were stabbed with toothpicks into plates containing BB with 0.3 % agar and incubated micro aerobically at 37 °C for 5 days (116).

4.2.1.2.5 Sedimentation assay

H. pylori cells were pre-cultured and then re-cultured in BB medium overnight. The cultures grown to similar OD_{600} were all adjusted to OD_{600} of 1.0 and to amount of 30 mL in 50 mL-falcon tubes. The tubes were set to stand under 4 °C for 1 day to 2 days and then the sedimentation of culture in each tube was observed.

4.2.1.2.6 Conjugation of *H. pylori*

E. coli WM3064 was transformed with the plasmids of interest by the rubidium chloride method already mentioned above. The donor *E. coli* WM3064 was cultivated in LB medium supplemented with 0.3 mM diaminopimelic acid (DAP) and selective antibiotic overnight. At the same time, the recipient *H. pylori* strain was also cultivated overnight in BB medium with selective antibiotic. 100 µL of the donor strain and 300 µL of the recipient strain were spin down, washed once with medium and resuspended in 100 µL of fresh BB medium. The entire volume of cells (100 µL of each strain) was added to a serum DAP plate (without antibiotic). Cells were mixed and swirled in the middle of the plate and then incubated at 37 °C for about 8 hours. Afterwards, cells were washed with medium and centrifuged at 3500 g for 5 min and plated in serum-selective plates (without DAP). The plates were incubated under 37 °C and microaerophilic conditions for 3 to 4 days (157).

4.2.2 Biochemical methods

4.2.2.1 High-performance liquid chromatography

In this study, amounts of intracellular proline and glutamic acid in *H. pylori* derivative strains under different conditions were tested by high-performance liquid chromatography (HPLC) after derivatization with 9-fluorenylmethoxycarbonyl (FMOC) based on the method established by Jámbor, *et. al.* (161). FMOC was used for derivatization since FMOC can derivate secondary amino acids like proline and so on (161, 195).

Derivatizations were performed by mixing (in the autosampler vial), in order of listing 150 µL of amino acid solutions with 150 µL of borate buffer and with 300 µL

FMOC stock solution. After 20 min of standing at room temperature, the reaction was stopped by adding 50 µL ADAM solution. For chromatography, the system was Thermo Scientific™ Dionex™ UltiMate™ 3000. Blank runs were performed with reagent solutions every day at least twice. Eluent A, B and C were prepared as Table 4.14 shown. The gradient program for the amino acids was set according to the method established by Jámbor, *et. al.* (161). Blank and test samples were evaluated at 262 nm.

4.2.2.2 Protein quantification

Determination of protein was performed according to a modified Lowry method (261) for total membrane protein. In general, 1 of each bacterial sample was added to 499 µL of H_2O_{dest} and mixed with 50 µL of 0.15 % sodium deoxycholate. The samples were vortexed and then let stand for 10 min at room temperature. Afterwards, 50 µL of 90 % trichloroacetic acid (TCA) was added to each sample, vortexed and then let stand for 10 min at room temperature again. The samples were centrifuged for 10 min with 14000 rpm and the supernatants were removed with pipette carefully. Then 500 µL of H_2O_{dest} was added to the pellet of each sample, followed by addition of 500 µL of Solution A and vortex. After 10 min of incubation at room temperature, 250 µL of Solution B was added to each sample. The samples were vortexed and then incubated for 30 min at room temperature. In the end, all the samples were transferred to cuvettes and the optical density at 750 nm was measured. 0.5 mg / mL BSA was used to make calibration series.

4.2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The protein separation was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Lämmli (262). Proteins were separated according to their electrophoretic mobility. SDS which is an anionic detergent was applied to the protein samples to linearize the proteins and to impart a negative charge to them. Besides the addition of SDS, β-mercaptoethanol, a reducing agent was added, which further denatures the proteins by reducing disulphide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits).

The protein was mixed with SDS-Buffer [2 % (w / v) SDS, 2 % (v / v) glycerine, 0,005 % (w / v) bromophenol blue and 25 mM Tris / HCl, pH 6,76] and 5 % (v / v) β-mercaptoethanol and then loaded on the gel. The spacer and stacking gel were prepared according to Sambrook *et. al.* (263). The acrylamide concentration in the gel was 12 % (Table 4.17).

Table 4.17 SDS-Gel preparation used in this study

Reagent	Spacer gel	Staking gel
Acrylamide (%)	4.9	12.5
5 x Spacer buffer [0.4 % (w / v) SDS, 1.5 M Tris / HCl, pH 8.8]	-	6 mL
5 x Stacking buffer [0.4 % (w / v) SDS, 0.5 M Tris / HCl, pH 6.6]	2.5 mL	-
APS (10 %)	45 µL	160 µL
TEMED	15 µL	16 µL
H_2O_{bidest}	5.86 mL	8 mL

The PageRuler™ Prestained Protein Ladder Standard (Thermo Scientific) was used as protein standard. And the electrophoresis was performed at 200 V for 1 h in SDS 1x buffer.

4.2.2.4 Western-blot and protein immunological analysis

For Western-blot experiments in the complemented mutants in *H. pylori*, strains were first amplified on plates for 2 days, afterwards grown for 24 h in BB-liquid medium supplemented with 10 % HS and kanamycin (8 mg / L) if necessary. Cells were spin down at 3500 rpm 4 °C and a new fresh culture was inoculated from the pre-culture. Cells were then harvested, resuspended in a mixture of PBS, completed and disrupted by sonification at 3 x 30s with 35 % amplitude at the Branson cell disruptor B 15. The samples were centrifuged for 10 min at 10,000 rpm to remove the cell debris; the supernatant of each sample was recovered and ultracentrifuged for 60 min at 80,000 rpm. The resulting pellet was resuspended in 50 µL of PBS and stored at -20 °C before use. The protocol is modified from Quick M S, *et. al.* (264).

4.2.2.5 ^{14}C L-proline transport experiments

The procedure was adapted from that used by Leduc D, *et. al.* (265). *H. pylori* parental strain and the isogenic knockout mutants or complements were amplified on GC-Agar plates for 2 days, grown in 25 mL BB-liquid medium and the corresponding antibiotic if necessary for 24 h. Afterwards, cells were spin down at 3500 rpm 4 °C and a fresh 50 mL culture was inoculated from the pre-culture. After 12 hours, the cells were collected by centrifugation and washed with 100 mM Tris-morpholine ethane sulfonic acid (MES) buffer, pH 7.0, with 150 mM KCl. Cells were then adjusted to an OD_{600} of 0.8 and from the prepared suspension, osmotic stress was applied by using NaCl if necessary. Aliquots of 200 µL were added to the reaction tubes and kept at 37 °C until use. Directly before starting the reaction 50 mM NaCl and 2 µL of ^{14}C L-proline 1 mM were added and mixed quickly. The samples were incubated depending of the required time interval and immediately filtrated on Durapore 0.45-µm membrane filters (Millipore) that were abundantly washed with 3 mL of cold Stop Buffer. Finally, filters were transferred into scintillation vials with 4.5 mL of scintillation liquid (EcoLite; MP Biomedicals). Radioactivity of bacteria retained on the filters was quantified by liquid scintillation counting.

4.2.2.6 ATP measurements

To determine the ATP levels in *H. pylori* cells, the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) was used. In a nutshell, samples were collected from liquid cultures in logarithmic phase, adjusted to an OD_{600} of 1.0 in an opaque-welled multiwell plate in culture medium (*e.g.*, 100 µL for each well of a 96-well plate). Control wells containing medium without cells were prepared to obtain a value for background luminescence. The BacTiter-Glo™ Reagent was added in an equal volume of cell culture medium present in each well (*e.g.*, add 100 µL of reagent to 100 µL of medium containing cells for the 96-well plate format). The content was mixed briefly on a shaker and incubated for five minutes and luminescence was recorded. For the calculations, a standard curve was prepared with 10-fold serial dilutions of ATP in culture medium (1µM to 10 pMof ATP).

4.2.3 Molecular methods

4.2.3.1 Extraction of plasmid-DNA

Plasmid-DNA of *E. coli* or *H. pylori* was extracted using the HiYield Plasmid Mini DNA-Kit (SLG) according to the manufacturer's instructions.

4.2.3.2 Extraction of genomic-DNA

Genomic-DNA from 2-day-old *H. pylori* plates or late-log phase liquid cultures was extracted by following the ReliaPrep™ gDNA Tissues Miniprep System (Promega). The genomic DNA was then quantified using the Nanodrop ND-100 (Nanodrop).

4.2.3.3 Polymerase-chain reaction

Polymerase-chain reactions (PCRs) were performed in Mastercycler personal thermal cycler (Eppendorf). In each reaction mixture, target DNA, reaction buffer, 5 µmol of each primer, Taq or Phusion polymerase according to the sample and standard concentrations of deoxynucleotides were included. As target DNA, purified genomic DNA or plasmid DNA preparations were used. When using Taq polymerase, the PCR mixtures were denatured at 94 °C for 1 min, annealed at temperatures between 50 °C and 60 °C (depending on the calculated melting temperatures of the primers) for 0.5 to 1 min, and extended at 72 °C for 1 to 3 min. A total of 35 cycles was performed. When using Phusion polymerase, the PCR mixtures were denatured at 98°C for 10 sec, annealed at temperatures between 50 °C and 60 °C (depending on the calculated melting temperatures of the primers) for 30-45 sec, and extended at 72 °C for 1 to 3 min. A total of 35 cycles was performed.

4.2.3.4 DNA agarose-gel electrophoresis

For the analysis of the separation of DNA fragments, agarose gels from 1-2 % were used. Agarose was dissolved in 1x TAE Buffer and run at 100 V for 40 min. To make DNA or RNA bands visible for agarose gel electrophoresis, Midori Green or ethidium bromide (EB) was used (1 mg / L). They fluoresce under UV light when intercalated into the major groove of DNA (or RNA). By running DNA through Midori Green or EB-

treated gel and visualizing it with UV light, any band containing more than around 20 ng DNA becomes distinctly visible.

4.2.3.5 DNA supercoiling analysis

The DNA supercoiling detection in this study was performed based on the method described by Liu, *et. al.* (170). Typically, plasmid DNA molecules are used to measure DNA supercoiling status inside bacterial cells. In this study, empty plasmid pIB6 was extracted from *H. pylori* wild-type and *putP* deletion mutant at log phase as reporter plasmids. 1 % chloroquine was added to a giant agarose gel [18.5 cm (W) × 40 cm (L) × 0.5 cm (H)] and to 1× TAE to resolve the DNA topoisomers of reporter pIB6 plasmids during the electrophoresis, besides that, EB was added to the gel to show the DNA supercoiling status.

4.2.3.6 DNA restriction by restriction enzymes

Restriction of the desired PCR fragments was performed according to the manufacturer's instructions of the restriction enzymes (New England Biolabs, Schwalbach). Restriction was performed for 2 h at 37 °C.

4.2.3.7 DNA ligation

Ligation was performed at 16 °C for 2 h or overnight. For cohesive (sticky) ends, 1 µL of T4 DNA Ligase in a 20 µL reaction was used. To avoid self-ligation of the restricted vector and decrease the vector background in the cloning, 1 µL alkaline phosphatase was added to remove the 5'-phosphate groups from DNA.

4.2.3.8 Construction of *H. pylori* mutants and complemented mutants

The construction of the *H. pylori* mutants was based on the contra-selection procedure established by Dailidiene, *et. al.* in 2006 (156). Briefly, chromosomal inactivation of the genes (*proP*, *proV* and *proC*) was performed in *H. pylori* strain P12. Deletions were introduced by allelic exchange using a suicide plasmid pBluescript II SK (+) in which around 1000 bp of the 5'-end and the 3'-end regions flanking the open reading frame of the target gene and an antibiotic resistance cassette (*rpsLerm* cassette) were cloned. These plasmids were constructed and amplified in *E. coli* DH5α and used as suicide plasmids in *H. pylori*. *H. pylori* mutants were obtained via conjugation mediated by *E. coli* WM3064 containing these suicide plasmids as previously described. Correct chromosomal insertion of the cassette and correct allelic exchange were verified by PCR and DNA sequencing.

For the construction of the complementation mutants, the genes *proP*, *proV* and *proC* were amplified and cloned into the NotI and NdeI sites of pIB6 to obtain the reconstructed plasmids. These plasmids were used for the complementation of the *H. pylori proP*, *proV* and *proC* mutants by conjugation.

4.2.3.9 Extraction of RNA

RNA was extracted from the *H. pylori* P12 cells grown in BB-liquid culture at the late-log phase using the TriPure™ Isolation Reagent (Roche) according to the

manufacturer's instruction. In general, the cells were harvested in 10-20 mL of culture using 50 mL falcon tubes by centrifuging at 4550 rpm for 10 min at room temperature. 1 mL of TriPure™ Isolation Reagent was added and then samples were suspended and incubated at room temperature for 5 min. Each sample was removed into a 2 mL-Eppendorf tube and 0.2 mL of cold chloroform was added. All the samples were then shaken violently for 15 sec and let them stand at room temperature for 2-15 min. The phases of samples were separated by centrifuging at 10,000 rpm for 15 min under 4 °C. The upper phase of each sample was taken and removed to a new 2 mL-Eppendorf tube. Afterwards, 0.5 mL of cold isopropanol was added to each sample and mixed slightly. After 5-10 min of incubation at room temperature, the samples were centrifuged at 10,000 rpm for 10 min under 4 °C, the liquid was then discarded. 1 mL of 75 % ethanol was added to each sample and then discarded after centrifuging at 7,500 rpm for 5 min under 4 °C. The pellets were then dried out by standing at room temperature for 20-60 min. 25-200 µL of DEPC treated H_2O_{dest} was added to dissolve the pellet followed by heating under 55-60 °C for 10 min. The quality and quantity of each RNA sample was then determined by agarose gel analysis and Nanodrop ND-100 Spectrophotometer. All the tips, tubes and so on used in RNA extraction were RNase-free and all the operations were taken in a biological safety cabinet.

4.2.3.10 DNase I digestion

DNA was removed from RNA preparations by DNase I digestion with 1 U RNase-free DNase I recombinant (Thermo scientific) for 30 min at 37 °C. To stop the reaction 1 µL of 50 mM EDTA was added and the reaction was incubated for 10 min at 65 °C.

4.2.3.11 Retro-transcription

A minimum of 0.5 µg of RNA was reverse transcribed using random primer and the RevertAid First Strand cDNA synthesis kit (Thermo scientific) according to the manufacturer's recommended protocol.

4.2.3.12 Semi-quantitative polymerase chain reaction

The operation of semi-quantitative PCR was mostly as same as the normal PCR. The DNA templates of semi-quantitative PCR in this study were cDNA obtained from retro-transcription of RNA extracted from *H. pylori* wild-type and putP deletion mutant. All the cDNA samples were adjusted to the same amount and the housekeeping gene *era* (HPP12_0523) was used as reference.

4.2.3.13 Real-time polymerase chain reaction

Real-time PCR primers for the tested genes and *era* were evaluated. qRT-PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Each 12.5 µL reaction contained 400 nM of each primer and 12.5 µL of iQ™ SYBR® Green Supermix (Bio-Rad). Individual amplification reactions were optimized for single-band specificity and verified by gel analysis of the pilot reaction products. Fold change in expression was calculated according to the standard formula 2 (-ΔΔCt), where ΔΔCt = (Ct_{target} - $Ct_{reference}$) calibrator - (Ct_{target}-$Ct_{reference}$) sample. The gene *era* was used as

reference. We refer to the calibrator as the normal *H. pylori* P12 wild-type strain at standard condition, while the sample to the knockout strain or wild-type strain under osmotic stress as necessary. qRT-PCRs were repeated on three different sets of cultures collected on separate days.

4.2.3.14 Fluorescent microscopy

H. pylori samples were taken from BB-liquid cultures in the logarithmic phase and bacterial suspensions were fixed to the glass slides with poly-L-lysine to allow adhesion. A stock solution of poly-L-lysine (1 mg / mL, in bidistilled water filtrated) was diluted 1: 20 with bidistilled water to get the working solution. A slide was covered with 200 µL and 500 µL of the working solution and incubated for 2 h at 37 °C, the slides were put in petri dishes to prevent that dust particles set down on the slides. Afterwards, the excess solution was removed and slides were let overnight at 37 °C. Slides were kept at room temperature until used. Samples were put on the slides and let it stand for 20 min, washed for 2 times with PBS to remove unfixed cells. Fluorescence pictures were obtained using a fluorescent Leica DM IRE2 microscope.

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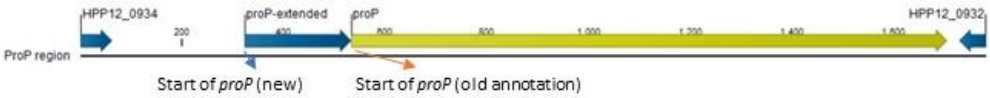
Supplementary materials

Supplementary material 1

A



B

ATAAAACATTTAGGCAAAAAAGAGGTAAGAACCTTAGGGTTATCTTCGCTTGGGGGGACTTTAGAATTTTATGATTTTATTATCTTT
I K H L G K K E V R T L G L S S L G G T L E F Y D F I I F
Start of *proP* (new)

GTATTTTTTACGAGTATCATTGCCAAACACTTTTTCCCAAACACGCTCAGCCCTATTTGGTCTGAAATCAACACTTATGGTATCTTTGCT
V F F T S I I A K H F F P N T L S P I W S E I N T Y G I F A

GCAGGTTATCTAGCACGCCCGCTTGGCGGCATAGTGATGGCCCACTTTGGGGATAGATTCGGTCGTAAAAACATGTTCATGCTCTCTATT
A G Y L A R P L G G I V M A H F G D R F G R K N M F M L S I
Start of *proP* (old annotation)

TTATTGATGGTGATCCCAACCTTTGCGCTCGCTTTGATGCCAACTTTTAATCATTTGGTGGGTTTTGGCGTTGATAGCATGGGGCTTACC
L L M V I P T F A L A L M P T F N H L V G F G V D S M G L T

CCTAAAAACGCTCATTATCTTGGTTACATAGCTCCTGTTTTTTGGTGTTTGTTAGGATTTGTCAAGGCGTTGCTGTGGGTGGTGAATTG
P K N A H Y L G Y I A P V F L V F V R I C Q G V A V G G E L

CCTGGCGCTTGGGTTTTTGTCCATGAACATGCCCCGCAAGGCCAAAAAAACACTTATATCGGTTTTTTAACCGCTTCTGTAGTTTCTGGG
P G A W V F V H E H A P Q G Q K N T Y I G F L T A S V V S G

ATTTTGCTTGGGAGTTTGGTTTATATAGGGATTTATATGGTTTTTGACAAGCCTGTTGTTGAAGATTGGGCTTGGAGGGTTGCTTTTGGG
I L L G S L V Y I G I Y M V F D K P V V E D W A W R V A F G

CTTGGAGGGGTTTTTGGTATCATTTCTGTCTATTTGAGGCGCTTTTTAGAAGAAACTCCTGTTTTTCAGCAAATGAAGCAAGACGATGCC
L G G V F G I I S V Y L R R F L E E T P V F Q Q M K Q D D A

TTAGTCAAATTCCCGCTTAAAGAGGTGTTTAAAAATTCCCTCTTTGGTATATCAATCTCCATGCTTATCACTTGGGTTTTAACCGCTTGT
L V K F P L K E V F K N S L F G I S I S M L I T W V L T A C

ATTTTGATTTTTATCCTTTTTGTTCCCAATTTTACCCTCATGCATCCTAATTTTAATTTCACTCCGTTTGAAAAAACCTATTTTCAAATT
I L I F I L F V P N F T L M H P N F N F T P F E K T Y F Q I

TTAGGACTTGTTGGTATTGTAAGTTCTATTATTTTAACCGGGTTTTTGGCCGATAAAATCAAACCGCACAAAGTTTGCATGGCTTTTAGC
L G L V G I V S S I I L T G F L A D K I K P H K V C M A F S

GTGGCCTTTGCCTTTTTTGGCTTTTTATTCTTTAAGGAATTTTATTCTAACGCGCCAAGTTTAATCAATACCATAGTTTTATACTTTTTA
V A F A F F G F L F F K E F Y S N A P S L I N T I V L Y F L

GCTTGCTTTTGCGCAGGCATTATGAATTTTTGCCCCATTTTCATGAGCGATGTGTTTAGCGCTAGAATCCGTTTTAGCGGGATTTCCTTT
A C F C A G I M N F C P I F M S D V F S A R I R F S G I S F

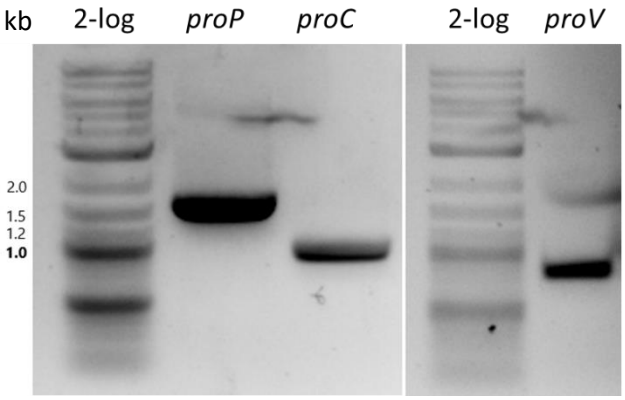
GCCTATAACATAGCCTATGCTATAACCGCTGGCTTTACCCCTCAACTTTCAAGCTGGTTAAACGCAAAAGCTATAGCAGCGCCTGAAAGT
A Y N I A Y A I T A G F T P Q L S S W L N A K A I A A P E S

TTGCAAAGTTATGGTTTGAACTTTTATATCCTCATAGTTTCTTTAATTGCTTTTATTACATCGCTTTTAATGGCGCCAATTTACAATAAA
L Q S Y G L N F Y I L I V S L I A F I T S L L M A P I Y N K

TCTAATACCCAACACGAAGTGTCGCCCACAGCATGA
S N T Q H E V S P T A *

Supplementary material 1 (A) Diagrammatic figure of the *HpproP* region showing the start of *proP* gene (new and old annotation are indicated). **(B)** Nucleotide sequence of *HpproP* gene and protein sequence of HpProP. Start codon from new and old annotation of the gene are underlined and indicated.

Supplementary material 2



Supplementary material 2 Identification of complementary plasmids with correct insertion. Colony PCR was performed using primers pIB6_s and pIB6_as. Correct sizes of amplification of the plasmids pIB6-HpproP, pIB6-HpproC and pIB6-HpproV were around 1.4 kb, 1.0 kb and 0.8 kb respectively (indicated as "*proP*", "*proC*" and "*proV*" in the figure). 2 log DNA Ladder was used as marker here.

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"雄关漫道真如铁，而今迈步从头越。"

However difficult it might seem; the challenge will be overcome.

In the end, I appreciate all the difficulties I have met and overcome in research and life during my years in Munich, which raised me to be a better me and truly contributed to the accomplishment of this work.

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Su D, Eder M, Hauke M, Jung H. Intracellular L-proline accumulation plays an important role in hyperosmotic environment tolerance of *Helicobacter pylori*. (Manuscript in preparation)

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* Equal contribution in each indicated publication

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