Function and Significance of the Putative Na^+/Solute Symporter PutP of *Helicobacter pylori*

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ABBREVIATIONS

BSA	Bovine serum albumin
cag-PAI	Cag pathogenicity island
CagA	protein CagA
CFU	Colony forming units
DDM	n-Dodecyl-ß-D-maltopyranoside
EM	Extracellular matrix
FlaA	Major flagellar filament
FlaB	Minor flagellar filament
GFP	green fluorescent protein
GSA	Glutamate semialdehyde.
LPS	Lipopolysaccharide
mEq	Milliequivalents
Ni^{2+}-NTA	Ni^{2+}-nitrilotriacetic acid
P5C	Δ1-pyrroline-5-carboxylate
P5CDH	1-pyrroline-5-carboxylate (P5C) dehydrogenase
PRODH	Proline dehydrogenase
PutA	Proline utilization A flavoenzyme
PutP	proline permease
ROS	Reactive oxigen species
SEM	Scanning electron microscopy
SHP-2	tyrosine phosphatase,
SMF	Na^+ motive force
SSS family	Na^+/solute symporter family
STM	Signature tagged mutagenesis
Tn	Transposon
VacA	Vacuolating cytotoxin A
vSGLT	Na^+-dependent galactose symporter of *Vibrio parahaemolyticus*

SUMMARY

Helicobacter pylori is a Gram-negative, pathogenic, microaerobic bacterium colonizing the gastric epithelium of about 50% of the world population. It is responsible for type B gastritis, peptic ulcers, and for increasing the risk of gastric carcinoma. Successful interaction of the pathogen with its host does not only require specific virulence factors, but depends also on its capability to cope with nutrient supply and stress conditions found in the host. Previous genome analyses revealed that genes encoding L-proline transport (*putP*) and metabolizing proteins (*putA*) are essential for *H. pylori* for gastric colonization. Furthermore, it has been shown that L-proline is present in high amounts in humans infected with *H. pylori*, which can be used as energy source by the bacteria.

This research focuses on the mechanisms underlying the particular physiological significance of L-proline and L-proline-specific systems for *H. pylori.* First, the putative proline transporter PutP of *H. pylori* (HpPutP) was functionally characterized. The gene *HpputP* was cloned from strain P12 and heterologously expressed in *E. coli*. *HpputP* was shown to complement an *E. coli putP* deletion mutant. HpPutP was purified by affinity chromatography and reconstituted into proteoliposomes. Functional analyses with proteoliposomes demonstrated that the activity of HpPutP depends on an electrochemical sodium gradient. Kinetic parameters of the Na^+/proline symport process were determined and found to be in the same order of magnitude as the parameters of PutP of *E. coli* (EcPutP). Furthermore, sites known from EcPutP to be of functional significance were investigated in HpPutP. By this means, residues potentially involved in sodium or proline binding and/or translocation were identified in HpPutP. Analysis of proline transport in *H. pylori* wild type showed accumulation of extracellularly applied L-proline, and deletion of the putative transporter gene inhibited transport completely. Kinetic parameters for proline and sodium measured in *H. pylori* differed from those measured in *E. coli*. This phenomenon is explained by differences in membrane composition of the strains. Deletion mutants for *HpputP, HpputA* and *HpputAP* showed an altered energy status, and contrary to the wild type were non-motile. *FlaA* gene expression was found to be impaired, and phenotypic characterization demostrated the absence of flagella. Since motility is essential for virulence, these phenomena may represent the reason for the previously reported loss of infectivity. Complemented mutants were able to restore L-proline transport, ATP levels and partially flagella structures. Therefore, proline availability is crucial for the course of *H. pylori* infection, and HpPutP may represent a new drug target.

1. INTRODUCTION

1.1 *Helicobacter pylori*: History and general description

By the late 19th and early 20th centuries, several investigators reported the presence of spiral microorganisms in the stomachs of animals. The Italian Guilio Bizzozero was the first one who observed and described spiral organisms in the stomach of dogs (Bizzozero, 1893). Soon afterwards, the German Hugo Salomon confirmed Bizzozero's work (Salomon H, 1896).

In the beginning of the twentieth century another German, W. Krienitz, detected spiral germs in the stomachs of patients with gastric carcinoma (Krienitz W, 1906). These bacteria, however, were thought to be contaminants from digested food rather than true gastric colonizers until the early 1980s.

In 1983, Barry Marshall and Robin Warren described the successful isolation and culture of a spiral bacterial species present in the stomach of 58 of 100 patients analyzed in which bacteria colonization was strongly associated to histological changes. This bacterium was later known as *Helicobacter pylori* (Warren J R and B J Marshall 1983). Self-ingestion experiments by Marshall (Marshall B J, *et al.,* 1985) and Morris (Morris A and G Nicholson, 1987) and later with volunteers (Morris A J., *et al.,* 1991) demonstrated that these bacteria can colonize the human stomach, thereby inducing inflammation of the gastric mucosa. These initial data strongly stimulated further research, which showed that gastric colonization with *H. pylori* can lead to several upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa associated lymphoid tissue (MALT), and gastric cancer (Ernst P B and B D Gold, 2000). This discovery resulted in the awarding of the 2005 Nobel Prize in Physiology or Medicine to Robin Warren and Barry Marshall for their "Discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease."

The organism was initially named "*Campylobacter*-like organism," "gastric *Campylobacter*-like organism," "*Campylobacter pyloridis*," and "*Campylobacter pylori*" but is now named *Helicobacter pylori* in recognition of the fact that this organism is distinct from members of the genus *Campylobacter* (Goodwin C S, *et al.,* 1989).

The genus *Helicobacter* belongs to the ε subdivision of the Proteobacteria, order *Campylobacterales,* family *Helicobacteraceae*. To date, the genus *Helicobacter* consists of over 20 recognized species, with many species awaiting formal recognition (Fox J G, 2002). Members of the genus *Helicobacter* are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are urease positive. *Helicobacter* species can be subdivided into two major lineages, the gastric *Helicobacter* species and the enterohepatic (nongastric) *Helicobacter* species.

The organism measures 2 to 4 µm in length and 0.5 to 1 µm in width. Although usually spiral-shaped (Figure 1), it can appear also as a rod, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment (Kusters J G, *et al.,* 1997). It has been suggested that coccoid forms may represent a viable, nonculturable state (Enroth H, *et al.*, 1999). The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 µm in length, which often carry a distinctive bulb at the end (O'Toole P W, *et al.,* 2000).

Figure 1. ***Helicobacter pylori*** **morphology** (A) Scanning electron micrograph of *H. pylori* shows the gently curved morphology, multiple bipolar flagella, and absence of periplasmic fibers. Bar, 1 µm. Photo courtesy of Adrian Lee, Jani O'Rourke, and Lucinda Thompson. "Reproduced with permission from the American Society for Microbiology". [Clin. Microbiol. Rev. 14(1):59.DOI:10.1128/CMR.14.1.59-97.2001.] (Solnick JV, Schauer DB), copyright (2010)

The size of the sequenced *H. pylori* genomes is approximately 1.7 Mbp, with an average G+C content of 39% (Tomb J F *et al.,* 1997). *H. pylori* is genetically heterogeneous, suggesting a lack of clonality. It has been estimated that every *H. pylori*-positive subject carries a distinct strain (Kansau I, *et al.,* 1996), although differences within relatives may be small. The genetic heterogeneity is possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers E J *et al.,* 2000)

H. pylori is a microaerophilic bacterium that does not tolerate high oxygen conditions, but it requires a minimum of 2% O_2 (Mendz G L, *et. al.,* 1997). This is because *H. pylori* uses oxygen as a terminal electron acceptor. *H. pylori* cannot utilize alternative electron acceptors, such as nitrate or formate, although there is a single report on anaerobic growth of *H. pylori* using fumarate (Smith M A and D I Edwards, 1995). *H. pylori* requires complex growth media which are often supplemented with blood or serum and the cultures require from 5 to 10% CO_2 and high humidity.

H. pylori has a stripped-down metabolic route with very few redundancies and lacks biosynthetic pathways for some amino acids. Therefore, *H. pylori* is auxotrophic for several amino acids, supporting the idea that its growth in vivo is strictly dependent on the gastric environment. The minimal amino acid requirements of this bacterium are arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine, with some strains also requiring alanine or serine (Nedenskov P, 1994; Reynolds D J and Penn C W, 1994). Besides, *H. pylori* is urease, catalase, and oxidase positive, characteristics which are often used in identification of *H. pylori.* The bacterium can catabolize glucose, and both genomic and biochemical information indicates that other sugars cannot be catabolized by *H. pylori* (Berg D E, *et al.,* 1997; Marais A, *et al.,* 1999; Nedenskov P, *1994).*

1.1.1 Epidemiology and Transmission

H. pylori infection affects approximately one half of the world's population but the prevalence varies greatly among countries and among population groups within the same country (Feldman R A, 2001). A steady decrease in the prevalence of *H. pylori* infection and the incidence of gastric cancer has been observed in most populations in recent decades, more accentuated in wealthy western societies. Despite a general decline in the incidence of gastric cancer, it remains the fourth most common cancer and second leading cause of cancer-related deaths worldwide.

Several factors must be considered to explain the cause of the geographic differences in the disease pattern, like host-related factors (host genetics), the duration of infection (age at acquisition) and/or environmental factors (poor nutrition in childhood, high salt intake and nitrate consumption) (Yamaoka Y, 2010). Besides, *H. pylori* is a highly heterogeneous bacterium which virulence has also changed geographically since it has co-evolved with humans at least since they migrated out of Africa approximately 58,000 years ago and probably throughout their evolution (Figure 2).

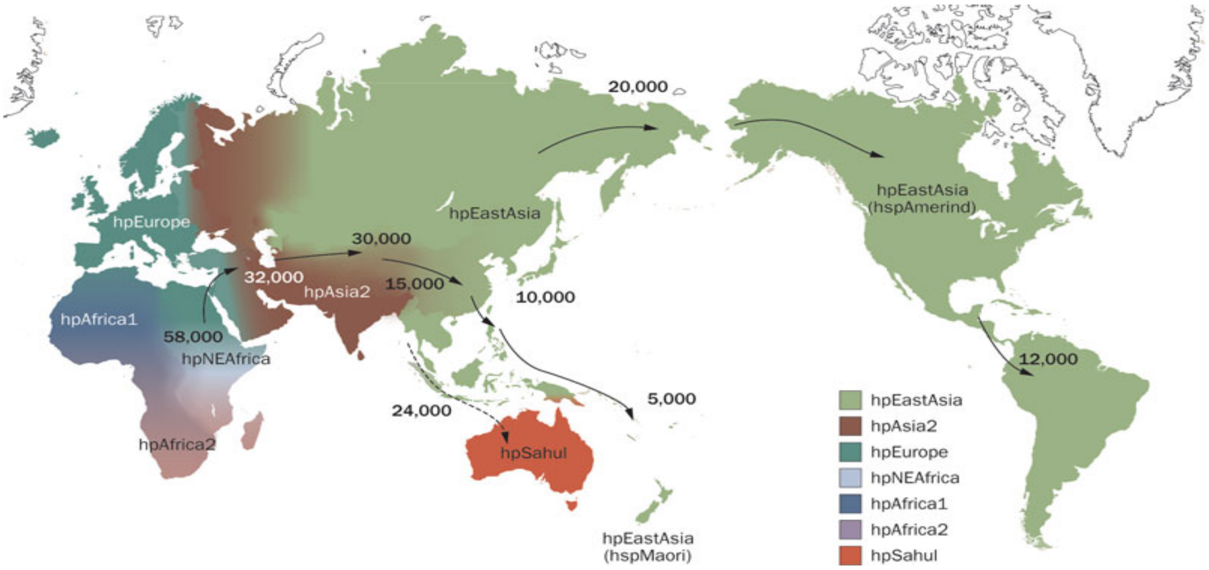


Figure 2. Distribution of *Helicobater pylori* genotypes. *H. pylori* is predicted to have spread from East Africa over the same time period as anatomically modern humans (~58,000 years ago), and has remained intimately associated with their human hosts ever since. Estimated global patterns of *H. pylori* migration are indicated by arrows and the numbers show the estimated time since they migrated (years ago). The broken arrow indicates an unconfirmed migration pattern. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Gastroenterol Hepatol] (Yamaoka, Y.), copyright (2010)

The infection is acquired by oral ingestion of the bacterium and is mainly transmitted within families in early childhood (Feldman R A, 2001; Rowland M, 1999). It seems likely that in industrialized countries direct transmission from person to person by vomitus, saliva, or feces predominates; additional transmission routes, such as water, may be important in developing countries (Parsonnet J, 1999; Goodman K J, 1996)

1.1.2 Pathogenesis of Infection

Normally gastric mucosa is well protected against bacterial infections but *H. pylori* is highly adapted to this ecologic niche, with a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response, and, as a result, persistent colonization and transmission. There is very strong evidence that *H. pylori* increases the risk of gastric cancer. Therefore, *H. pylori* has been classified as a type I (definite) carcinogen since 1994, mainly on the basis of large seroepidemiologic case–control studies (Parsonnet J, *et al.* 1991; Nomura A, *et al.* 1991; Forman D*, et al.* 1991). The clinical course of *H. pylori* infection is highly variable and is influenced by both microbial and host factors (Figure 3).

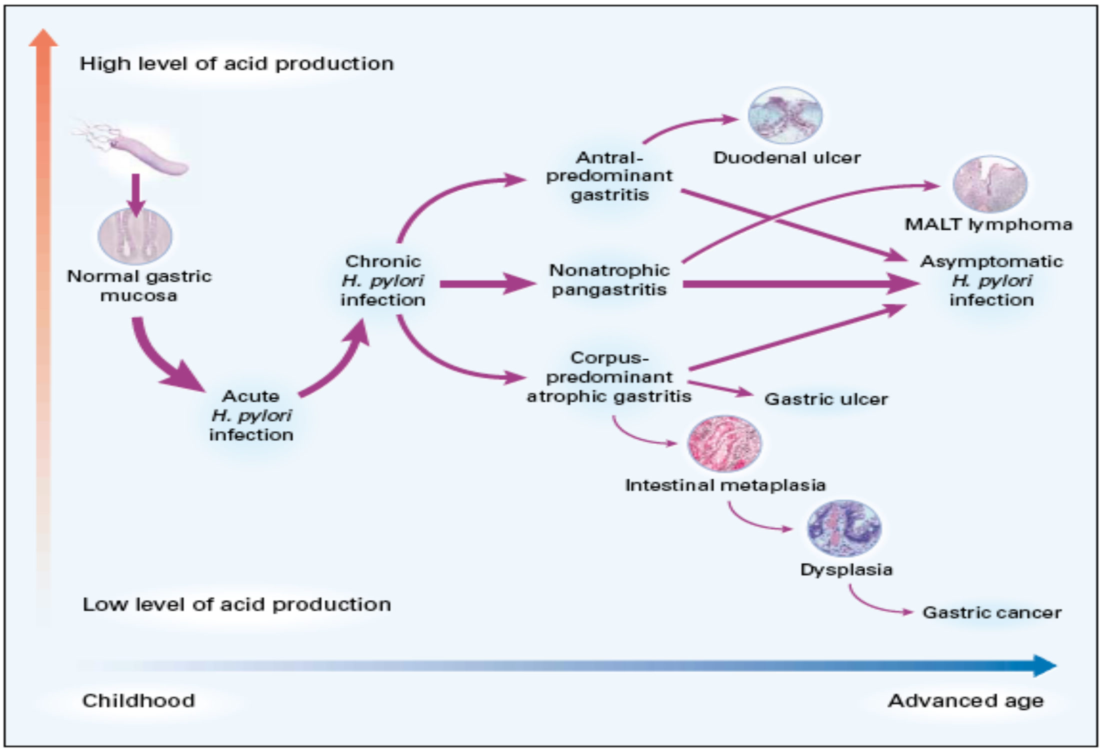


Figure 3. Natural progression of *H. pylori* infection. Infection usually occurs during childhood and causes symptomatic acute gastritis. Because the symptoms of acute gastritis are non-specific and transient, a diagnosis is rarely made at this stage. Acute infection transforms to chronic active gastritis in most patients and persists for decades or is life-long. The infection can take multiple courses. Most people that are infected with *H. pylori* will never develop symptomatic disease. 10–15% will develop ulcer disease (gastric or duodenal ulcer), approximately 1% will develop gastric adenocarcinoma, and a small group of patients will develop gastric MALT lymphoma. Reproduced with permission from (Suerbaum S, Michetti P.2001), Copyright Massachusetts Medical Society.

Production of urease and motility are essential for the first step of infection. In one hand, urease hydrolyzes urea into carbon dioxide and ammonia, thereby permitting *H. pylori* to survive in an acidic environment (Mobley H L T. *et al,* 2001). On the other, flagella enable the bacterium to move in the highly viscous mucous layer of the gastric epithelium. Once *H. pylori* reaches the epithelial surface, it can bind tightly to the epithelial cells by multiple bacterial-surface components (Gerhard M, 2001). The majority of *H. pylori* strains express the 95-kD vacuolating cytotoxin VacA, a secreted exotoxin (Montecucco C, 2001). The toxin inserts itself into the epithelial-cell membrane and forms a hexameric anion-selective, voltage-dependent channel through which bicarbonate and organic anions can be released, possibly providing the bacterium with nutrients (Szabo I, 1999). VacA is also targeted to the mitochondrial membrane, where it causes release of cytochrome *c* and induces apoptosis (Galmiche A, *et al.* 2000). Most strains of *H. pylori* possess the Cag pathogenicity island (*cag*-PAI), a 37-kb genomic fragment containing 29 genes (Censini S, *et al.* 1996). Several of these encode components of a predicted type IV secretion apparatus that translocates the 120-kD protein CagA into the host cell (Odenbreit S, *et al.* 2000; Segal E D, 1999). After entering the epithelial cell, CagA is phosphorylated and binds to SHP-2 tyrosine phosphatase, leading to a growth factor–like cellular response and cytokine production by the host cell (Figure 4).

Patients with antral-predominant gastritis, the most common form of *H. pylori* gastritis, are predisposed to duodenal ulcers, whereas patients with corpus-predominant gastritis and multifocal atrophy are more likely to have gastric ulcers, gastric atrophy, intestinal metaplasia, and ultimately gastric carcinoma.

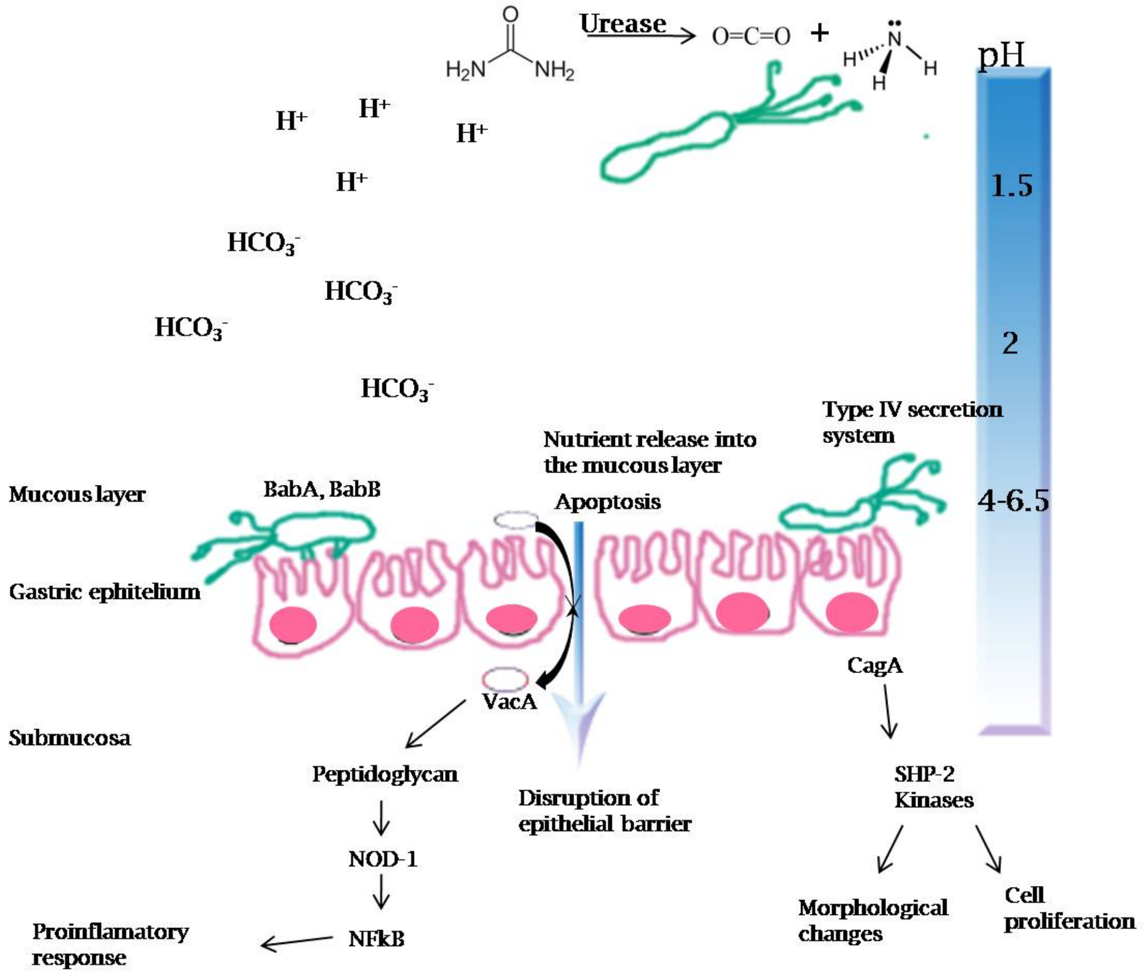


Figure 4. Persistent *Helicobacter pylori* infection. Interplay between *H. pylori* factors and the host response leads to chronic gastritis and persistent colonization. *H. pylori* survives the gastric acid by producing urease, which converts urea into ammonia and carbamate, which spontaneously decomposes into another ammonia molecule and carbon dioxide (Burne R A, and Y M Chen. 2000). The ammonia produced by this reaction increases the pH and is thought to have a cytotoxic effect on gastric epithelial cells (Smoot, D. T. *et al.* 1990), whereas bicarbonate is thought to suppress the bactericidal effect of peroxynitrite, a nitric oxide metabolite (Kuwahara H *et al*. 2000). *H. pylori* reaches the stomach mucous layer using the flagella and binds to gastric epithelial cells through BabA and other adhesins (Monack D M, *et al.,* 2004). Secretion of VacA induces membrane channel formation, disruption of endosomal and lysosomal activity, effects on integrin receptor-induced cell signaling, interference with cytoskeleton-dependent cell functions, induction of apoptosis, and immune modulation (Kusters J G, 2006). Strains carrying the *cag* PAI (CagA$^+$ strains) can build a type IV secretion apparatus, which forms a syringelike structure capable of penetrating the gastric epithelial cells and facilitating the translocation of CagA, peptidoglycan, and possibly other bacterial factors into host cells. (Asahi M, *et al.* 2000). Once delivered inside the cell, the CagA protein is phosphorylated by Src family kinases and then interacts with a range of host signaling molecules, such as the tyrosine phosphatase SHP-2, which results in morphological changes in the epithelial cells (Higashi H *et al.* 2002).

Six complete *H. pylori* genome sequences are currently available (Tomb J F and White O *et al.* 1997; Alm, R A, *et al.* 1999; Oh JD *et al.* 2006). Of the 1590 predicted genes in *H. pylori* two thirds were assigned biological roles, but about one third did not show any database match. Our understanding of the genes necessary for the specific adaptation of the pathogen to the gastric mucosa is still very limited despite the description of essential and nonessential genes *in vitro* (Chalker A F 2001; Jenks P J, 2001). Therefore we need to exploit the information from the *H. pylori* genome sequences as performed for other pathogens (Hutchison C A, *et al.* 1999; Akerley B J *et al.* 2002).

1.1.3 Motility as an important virulent factor

Motility is an essential colonization factor for *H. pylori* in experimental infection models (Eaton K, *et al.,* 1992; Eaton K A, *et al.,* 1996; Mankoski R, *et al.,* 1999). Because of the contribution of flagella to virulence, the genomic basis of *H. pylori* flagellum production is therefore of considerable interest. As with enteric bacteria, the *H. pylori* flagella are composed of three structural elements: a basal body which serves as a cell anchor and contains the proteins required for rotation and chemotaxis, a curved hook structure composed of the FlgE protein (O'Toole P W, *et al.,* 2000), and the helically shaped flagellar filament. Thus far, only a few of the numerous proteins involved in the formation of this complex structure have been characterized in some detail; these include the components of the flagellar filament FlaA (Leying H, *et al.,* 1992) and FlaB (Suerbaum S *et al.,* 1993), which are expressed by cultured cells to very different levels, FlaA being the predominant subtype.

As with other bacteria, flagellar biosynthesis is a hierarchical process that is subject to temporal and growth phase regulation (Spohn and Scarlato, 1999; Niehus E *et al.,* 2004; Josenhans C *et al.*, 2002) involving sequential activation of approximately 40 genes (Niehus E *et al.,* 2004; Macnab R M, 2003). Expression of flagellar genes is controlled by at least three RNA polymerase sigma factors, σ^{80}, σ^{54}, and σ^{28} (Alm R A, 1999; Beier D;1998 Tomb J F, 1997), and a two-component system for σ^{54}-regulated genes (Spohn G and V Scarlato, 1999).

A whole-genome microarray analysis of strains mutated in flagellar regulatory genes (*rpoN, flgR*, *flhA*, *flhF*, HP0244) was performed to elucidate the complex transcriptional circuitry of flagellar biosynthesis and therefore the establishment of a model for flagellar gene regulation in *H. pylori*. This study revealed three different classes of flagella genes that are regulated by three different σ factors. Class 1 gene expression is controlled by the σ^{80} factor, while Class 2 genes are controlled by the σ^{54} factor (RpoN), and class 3 genes are expressed by the σ^{28}-FlgM-controlled system. Seven novel genes dependent on σ^{54} were identified (Niehus E *et al.,* 2004) and no master regulator of flagellar biosynthesis (such as the enterobacterial flhCD genes) was found (Niehus E *et al.,* 2004) (Figure 5).

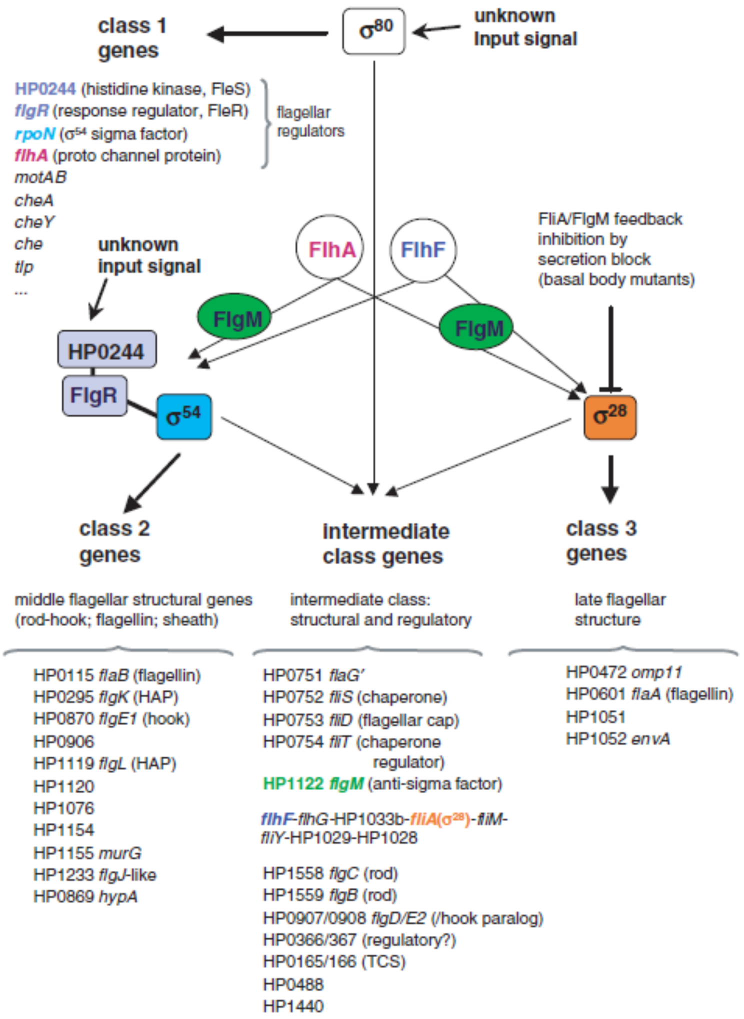


Figure 5. Current model of regulation pathways in flagellar biosynthesis of *H. pylori.* Three different classes of flagellar genes are governed by the housekeeping sigma factor σ^{80} (class 1) and the alternative sigma factors σ^{54} (class 2) and σ^{28} (class 3). A number of intermediate genes controlled by more than one promoter are grouped in a separate category. Class 1 flagellar genes comprise, among others, most of the major regulatory genes of the flagellar system. The list of putative class 1 genes is not complete, as these regulons have not been investigated in detail. Transcription of class 2 genes is governed by RpoN, assisted by the histidine kinase HP0244 (FlgS) and the response regulator FlgR. The class of RpoN-dependent genes could be extended by a number of novel genes indicated by HP numbers. FlhA and FlhF are both necessary for full transcription of flagellar classes 2 and 3 and the intermediate class. FlgM plays a role in the transcriptional block of class 2 and 3 genes in *flhA* mutants, but only in the feedback block of class 3 genes in *flhF* mutants. A common mechanism of feedback inhibition on class 3 might be mediated by a deficient basal body via FlgM. Reproduced with permission from (Niehus, E. et al.,.2004), Copyright© 2004 John Wiley and Sons.

1.2 L-proline significance for *H. pylori*

To unravel the bacterial factors necessary for the process of gastric colonization, Kavermann, *et al.* 2003 improved and adapted the signature tagged mutagenesis (STM) method to *H. pylori,* in which 960 independent *H. pylori* transposon (Tn) insertion mutants were analyzed and 47 genes proved to be absolutely essential for gastric colonization of *H. pylori* in the well suited gerbil model. Among these essential genes, HPP12_0049, which encodes the putative proline permease PutP, was found to be essential for *H. pylori* to colonize *in vivo*, indicating that *H. pylori* apparently relies on the exogenous uptake of these amino acid from the stomach mucosa. Such an amino acid exchange between bacteria and host is frequently observed in primary and secondary symbionts of plants and animals (Graf J and E G Ruby, 1998).

The carbohydrate utilized by *H. pylori* as the energy source has originally been reported to be only glucose (Mendz G L, *et al.,* 1993). The whole-genome analysis of *H. pylori* has supported these findings (Tomb J F *et al.,* 1997). However, more recent investigations indicate that glucose is not the preferred energy substrate of *H. pylori*. This was shown in experiments where glucose added to the culture medium composed of a mixture of amino acids was not utilized until the amino acids were significantly depleted (Mendz G L *et al*., 1993; Mendz G L and S L Hazell, 1995). These results suggested that *H. pylori* can grow employing amino acids as main energy source. Therefore, Nagata and coworkers in 2003 investigated the respiratory activity of intact *H. pylori* cells with alanine, serine and proline, and other amino acids of which both D- and L-isomers serve as respiratory substrates. They described a high rate of utilization of L-serine and L-proline as respiratory substrates, an unusual high content of L-proline in *H. pylori* cells, that L-proline predominated in samples of human gastric juice and that the content of L-proline in infected specimens was significantly higher than that in uninfected ones (up to 10mg per g of gastric juice) (Nagata K *et al.,* 2003).

A possible explanation for the high L-proline content in the stomach of infected persons is the secretion of a collagenase by *H. pylori*. Infection studies in the Mongolian gerbil (*Meriones unguiculatus*), a well-established *H. pylori* animal model, was used to characterize this gene (HP0169), which was found to be essential for *H. pylori* to colonize in the gerbil stomach. This collagenase was verified as a novel virulence factor of *H. pylori* for stomach colonization (Kavermann, *et al.* 2003). An abundant reservoir for proline is the collagen present in the extracellular matrix (EM), connective tissue, and bone (Phang J M, *et al.* 2010). Therefore the collagen present in the EM in the gastric tissue could be the source of proline in the gastric juice. Besides, there is an enhancement of collagen turnover in stomach cancer that can be induced by the collagenase secretion during the *H. pylori* infection (Phang J M, *et al*. 2010).

1.2.1 Known effects of L-proline transport and metabolism on bacteria-host interactions

In general, L-proline can be used for most of the living cells as a source of carbon, nitrogen and energy; can be a compatible solute during adaptation to osmotic stress; a modulator of the intracellular redox environment and scavenger of reactive oxygen species (Rodriguez R and R Redman, 2005). In this way, previous studies have suggested that under high-osmolarity growth conditions, proline accumulates and serves as an osmoprotectant for *Staphylococcus aureus* (Graham J E, and B J Wilkinson 1992; Bae J-H, and K J Miller 1992.) and *Bacillus subtilis* (von Blohn *et al.,* 1997) as well as Gram-negative bacteria (Csonka L N, and A D Hanson 1991; H Jung, 2002).

S. aureus is also a pathogenic microorganism and is responsible for a variety of distinct and divergent diseases, including osteomyelitis, endocarditis, bacteremia, wound and skin infections, abscess formation and a host of other afflictions, in humans and other animals (Easmon C S F, and C Adlam 1983). Schwan W R *et al.* in 1998 showed that disruption of the proline permease PutP by transposon insertion has a deleterious effect on proline uptake, which in turn affects the in vivo survival of *S. aureus*.

Another example is *Vibrio vulnificus,* the causative agent of food-borne deseases, which needs the gene product of *putP* for the adaption to changing osmolalities during host infection (Kim H J, *et al.,* 2002). As mentioned before, L-proline transport and metabolism is also important for interactions between Gram-negative bacterial pathogens and hosts. In *Francisella novicia* proline is required for pulmonary and systematic infection of mice (Kraemer P S, *et al.,* 2009). In *Vibrio cholera* it is important for initial steps in biofilm formation (monlayer formation) (Kapfhammer D *et al.,* 2005). Also in *E. coli* and *Salmonella typhimurium*, the *putP* gene product serves as a major carrier, bringing L-proline across the cytoplasmic membrane. Several proline transporters within the bacteria serve to bring proline into the bacterial cell. At least three proline transporters are able to transport proline into the bacterial cell; these include the low-affinity ProP and ProU proteins as well as the high-affinity PutP protein (Wood J M, 1988; Jung H, 2002).

1.2.2 PutP of *E. coli* and PutP of *H. pylori*

There is only very limited experimental evidence on the mechanism of L-proline accumulation in *H. pylori*. The genome analysis predicts the existence of minimum three putative L-proline transporters: PutP, ProP, and ProV/ProWX (Tomb J F *et al.,* 1997). And the deletion of the putative *putP* gene prevents gastric colonization of the stomach (Kavermann H *et al.,* 2003).

To date, most of the knowledge on the L-proline metabolism in bacteria is from *E. coli.* PutP of *E. coli* is the best characterized bacterial L-proline transporter of the Na^+/solute symporter family (SSS family), which contains several hundred proteins of pro- and eukaryotic origin (Wright E M and E Turk, 2004; Reizer J, 1994). It catalyzes the symport of Na^+ and L-proline with a stoichiometry of 1:1 (Yamato I and Y Anraku, 1993). Na^+ can be substituted by Li^+, and proton-driven L-proline uptake by PutP could not be demonstrated (Chen C C, *et al.* 1985; Cairney J, 1984). PutP was also identified as a high affinity L-proline transporter with a kd(Pro) and K_m (Pro) of 2 μM, by using kinetic and ligand binding analyses (Jung H, *et al.* 1998a; Yamato I and Y Anraku, 1993). A new secondary structure model is proposed according to which the protein consists of 13 transmembrane domains (TMs) with the N terminus on the outside and the C terminus facing the cytoplasm (Jung H, *et al.* 1998a) (Figure 6).

Differing from the organization of the *put* operon in *E. coli*, *putP* and *putA* of *H. pylori* (*HpputP, HpputA*) are transcribed in the same direction.

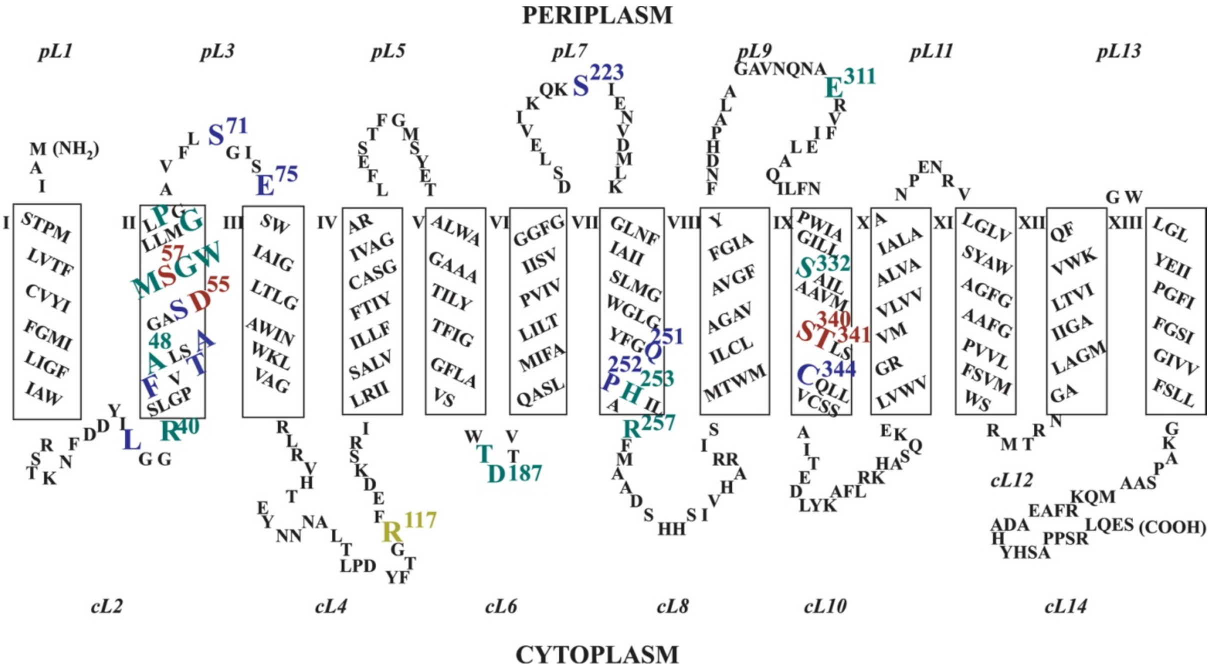


Figure 6. Secondary structure model of PutP of *E. coli* highlighting functional important residues. Gene fusion, cysteine accessibility, and EPR spectroscopic analyses of EcPutP suggest a secondary structure model according to which PutP contains 13 transmembrane domains (TMs) with the N terminus located on the periplasmic side of the membrane and the C terminus facing the cytoplasm.“Reprinted from FEBS Letters, Vol 529/ Issue 1, Heinrich Jung, The sodium/substrate symporter family:structural and functional features, Pages No. 73–77, Copyright (2002), with permission from Elsevier.”

1.2.3 Proline metabolism of *H. pylori*

Proline catabolism has been shown to be an important energy pathway in *H.pylori*, with proline serving as a preferred respiratory substrate (Nagata K, *et al.,* 2003). All organisms convert proline to glutamate in two successive steps catalyzed by proline dehydrogenase (PRODH) and 1-pyrroline-5-carboxylate (P5C) dehydrogenase (P5CDH). In gram-negative bacteria, PRODH and P5CDH activities are combined within the proline utilization A (PutA) flavoenzyme. In the first step, proline is oxidized to Δ1-pyrroline-5-carboxylate (P5C) by the FAD-dependent enzyme proline dehydrogenase. P5C forms a nonenzymatic equilibrium with glutamate semialdehyde (GSA). The P5C/GSA equilibrium is strongly pH-dependent with P5C favored at pH greater than 6.5 (Lewis *et al.* 1993; Bearne and Wolfenden 1995). P5C dehydrogenase (P5CDH, EC 1.5.1.12) completes the transformation of proline to glutamate by catalyzing the oxidation of GSA, utilizing NAD$^+$ as the electron acceptor. PRODH and P5CDH are highly conserved throughout eukaryotes and bacteria (Abrahamson J L A, *et al.,* 1983; Krishnan N and D F Becker, 2005; Menzel R and J Roth 1981; Scarpulla R C and R L Soffer 1978).

Primary structure analysis of PutA from *H. pylori* (HpPutA) predicts that PutA is missing the N-terminal extension used by EcPutA for DNA binding in the oxidized state of the enzyme. Therefore, we could infer that HpPutA is not able to function as a transcriptional repressor of the *H. pylori put* operon. HpPutA showed reactivity with molecular oxygen during catalytic turnover with proline that is much higher than that of EcPutA, leading to the formation of reduced oxygen species such as hydrogen peroxide

(H_2O_2). The impact of *Helicobacter* PutA reactivity with oxygen was assessed by oxidative stress studies in an *E. coli* model system that demonstrated that the enzyme action of HpPutA is toxic to *E. coli* (Krishnan N and D F Becker, 2006).

There is also evidence that the bifunctional proline dehydrogenase HpPutA appears to be important for infection. A *putA* mutant did not display respiratory activity for L-proline, had little motility and did not show swarming activity. Flagella seem to exist but full length sheathed flagella are rarely observed. Finally, the *putA* mutant proved incapable to colonize the stomach of mice (Nakajima K *et al.,* 2008).

1.2.4 Aim of this study

Many organisms accumulate proline far in excess of the demands of protein synthesis to cope with environmental stress as well as an energy source.
The conditions of the ecological niche of *H. pylori* are dominated by the gastric H^+, K^+-ATPase, which secretes acid into the lumen of the stomach (Marais A *et al.* 1999). Besides, the host inflammatory response can contribute to changes in the gastrointestinal environment trough the production of reactive oxygen species (ROS). Thus, *H. pylori* requires to cope with an onslaught of ROS during infection to survive (Alamuri P and R J Maier 2004; Kelly D J, 1998; Wang G and R J Maier, 2004). The bacterium must have developed physiological strategies to survive in this environment. One of these strategies could be related to the proline metabolism because as mentioned before, in a genome wide search, the putative proline transporter in *H. pylori putP* was among the genes essential for gastric colonization in the Mongolian gerbil infection model (Kavermann H *et al.,* 2003).
This research proposal attempts to unravel the mechanisms underlying the particular significance of L-proline and L-proline-specific systems for *H. pylori* physiology.

Specific goals of this study were:

I. The molecular characterization of HpPutP of *H. pylori*.

This part of the project focused on the identification of the transporter required to accomplish transport of L-proline in *H. pylori.* In the first place, energetics of transport activity were analyzed. For this, the dependence of a proton motive force or sodium motive force was assessed. Afterwards kinetics of the transport activity of HpPutP was evaluated. And finally, the identification of the functional important amino acids involved in binding either to sodium or proline was performed.

II. The significance of L-proline in the energy metabolism of *H. pylori*

The energy balance of a cell is crucial for numerous cellular functions including flagella motility and the synthesis of virulence factors; and L-proline is proposed to constitute an energy source during gastric colonization. Therefore, characterization of HpPutP, HpPutA and HpPutAP deletion mutants was performed by measuring growth, L-proline uptake and ATP levels in the mutants compared to the wild type.

III. Does L-proline accumulation and metabolism affect the biogenesis and function of virulence factors and if so, what are the molecular mechanisms underlying these effects?

Since a *putA* deletion was reported to cause flagella deformation (Nakajima K *et al.,* 2008). Effects of *putP* and *putA* deletions on the flagella morphology were investigated by electron microscopy.

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Bacterial strains

Escherichia coli strains and *Helicobacter pylori* strains used in this work are listed in Table 1.

Table 1. Strains used in this study

Strain	Genotype
E. coli DH5α	F-Φ80d lacZ ΔM15 Δ(lacZYA-argF) *U169 deoR recA1 endA1 hsdR17* (rK- mK+) *phoA supE44 λ-thi-l gyr A96 relA1* (Invitrogen) (Hanahan, 1983)
E. coli WG170	(F_ trp *lacZ rpsL thi* _(*putPA*)101 *proP*219) (Stalmach et al., 1983)
E. coli WM3064	(thrB1004 *pro thi rpsL hsdS* lacZΔM15 RP4-1360 Δ(*ara*BAD)567 Δ*dapA*1341::[erm pir])
Helicobacter pylori P12	Clinique Isolate (888-0) Division of „Medizinische Mikrobiologie und Immunologie“ der Universität Hamburg (Schmitt *et al.*, 1994)

2.1.2 Plasmids used in this study

Plasmids used in this study are listed in Table 2.

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pT7-5	Amp^{R}, expression vector	Tabor & Richardson, 1990
pTMH6FH	Amp^{R}, *putP*- wildtype in pt7-5	Jung, 1998
pBluescript II SK+	Amp^{R}, *ori*colE1, *ori*f1(+), *lacZ*, M13 forward-/ reverse	Stratagene
pTHpputP6H	Amp^{R}, *HpputP*- wildtype in pt7-5	This work
pRHpputP6H	Amp^{R}, *HpputP*- wildtype in pTrc99a	This work
pBSK HpputP rpsLerm	pBluescript II SK+ harboring *HpputP* and the resistance cassette *rpsLerm*	This work
pBSK HpputA rpsLerm	pBluescript II SK+ harboring the resistance cassette *rpsLerm* inserted between the flanking regions of *HpPutA*	This work
pBSK HpputP/HpputA rpsLerm	pBluescript II SK+ harboring the resistance cassette *rpsLerm* inserted between the flanking regions of *HpputP* and *HpputA*	This work
pBAD24 GFP	pBAD *araC* Amp^{r}	Guzman L M, *et al.* 1995.

pIB6	*alpA* Promotor-Region in Shuttle-Vector pHel3	Barwig I, 2009
pIB6 alpA GFP	Shuttle-Vector pIB6 harboring *GFP*	This work
pIB6 alpA HpputP	Shuttle-Vector pIB6 harboring *HpputP*	This work
pIB6 alpA HpputP GFP	Shuttle-Vector pIB6 harboring *HpputP and GFP*	This work
pIB6 310HpputP Promotor-Region	*HpputP* Promotor-Region replacing alpA promotor in Shuttle-Vector pIB6	This work
pIB6 310HpputP promotor GFP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *GFP*	This work
pIB6 310HpputP promotor HpputP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *HpputP*	This work
pIB6 310HpputP promotor HpputP GFP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *HpputP* and *GFP*	This work
pIB6 500HpputP Promotor-Region	*HpputP* Promotor-Region replacing alpA promotor in Shuttle-Vector pIB6	This work
pIB6 500HpputP promotor GFP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *GFP*	This work
pIB6 500HpputP promotor HpputP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *HpputP*	This work
pIB6 500HpputP promotor HpputP GFP	*HpputP* Promotor-Region in Shuttle-Vector pIB6 harboring *HpputP* and *GFP*	This work

2.1.3 Oligonucleotides used in this study

Oligonucleotides were designed according to the standard parameters and ordered at Sigma-Aldrich (Germany). The primers used are listed in Table 3

Table 3. Oligonucleotides used in this study

Name	**Sequence (5’ to 3’)**	**Sequence amplified**
HpputP_s	AGGAGATCACCATGGGACATGTTG	*H. pylori putP*
HpputP_as	CTCATTTGAGCTCGAGGCATGCTTCAAGCTC	*H. pylori putP*
HpputA_s	AAAGGTAAGCATATGCAAAAAATC	*H. pylori putA*
HpputA_as	TTAATACAACTCGAGTTTTTCAGCACAG	*H. pylori putA*
D58C sP	GGAGCGAGCTGTATGAGCGGGTG	HpPutP aa substitutions
D58C asP	CCGCTCATACAGCTCGCTCCTGC	HpPutP aa substitutions
S 60C sP	AGCGATATGTGCGGGTGGCTTTTA	HpPutP aa substitutions
S60C asP	AAGCCACCCGCACATATCGCTCGC	HpPutP aa substitutions
Y143C sP	TTTATTTTTTGCATTTCTTCAGGG	HpPutP aa substitutions
Y143C asP	TGAAGAAATGCAAAAAATAAAAAA	HpPutP aa substitutions
W247C sP	GGGATTTCTTGTATGGTTATTTCT	HpPutP aa substitutions
W247C asP	AATAACCATACAAGAAATCCCAAT	HpPutP aa substitutions
E311C sP	GAAGACCCTTGTAAGATTTTCATT	HpPutP aa substitutions

E311C asP	GAAAATCTTACAAGGGTCTTCTAA	HpPutP aa substitutions
S339C sP	GCGGTGATGTGCACGGCCAGTTCG	HpPutP aa substitutions
S339C asP	ACTGGCCGTGCACATCACCGCCGC	HpPutP aa substitutions
T340C sP	GTGATGAGCTGCGCCAGTTCGCAA	HpPutP aa substitutions
T340C asP	CGAACTGGCCGTGCACATCACCGC	HpPutP aa substitutions
DelputP_A_s	CTATCATCTAAGCTTTGGATCGTGTTTTG	*H. pylori* deletion mutants
DelputP_A_as	CCACAAGTCAGGATCCGCTGAAAAATAA	*H. pylori* deletion mutants
DelputP_B_s	AACAATAGGGGATCCTAAAACAACATG	*H. pylori* deletion mutants
DelputP_B_as	GCGATTTCAGCGGCCGCACTGAGCTTTTTAC	*H. pylori* deletion mutants
DelputA_A_s	GGGTCTTCTAAGCTTAAGTCAAATTTA	*H. pylori* deletion mutants
DelputA_A_as	CCACAAGTCAGGATCCGCTGAAAAATAA	*H. pylori* deletion mutants
DelputA_B_s	TGAATCGTCGGATCCTTTTTGCATGAG	*H. pylori* deletion mutants
DelputA_B_as	TTATCTTTTGCGGCCGCTAGAGCGGTGTT	*H. pylori* deletion mutants
HpputPKP1	ATTCTTGTAATTCTAAATC	*H. pylori* deletion mutants
HpputPKP2	AAAATTACGAAATCCCGGC	*H. pylori* deletion mutants
HpputAKP3	CGCCGCTAAAATCGCGCTC	*H. pylori* deletion mutants
HpputAKP4	TGCTGAGATTGTTCTAACG	*H. pylori* deletion mutants
DelputP_A_asneu	CTTAAAGAAGGATCCAGCTTGAAGCATTAAG	*H. pylori* deletion mutants
Erm s	CATAAGTACGGATATAATACG	*rpsLErm* cassette
Erm as	CGTATTATATCCGTACTTATG	*rpsLErm* cassette
rpsL s	TTTGAAGTGATCAGCTATATC	*rpsLErm* cassette
rpsL as	ATAGCTGATCACTTCAAAGCC	*rpsLErm* cassette
Subst Primer 29	GAAATCCCAATAGTGGTCGCTTTAGGCACATCTCT	*H. pylori* deletion mutants
HpPutP pIB6compl_s	CAGGAAAGGACATATGGGACATGTTGTTTTAAG	*H. pylori* complem mutants
HpPutP pIB6compl_as	GCTTATCATGCGGCCGCTTAGTGGTGGTGG	*H. pylori* complem mutants
PutAs	GAGAAAGAAGAGGCGATTTAGTAGGGATTTCGG	*H. pylori* complem mutants
PutAas	AAGTCAATGGCTTCGCTCACTTCAGCGTCCGT	*H. pylori* complem mutants
HpPutP NdeI_s	AAGGAAAGGACATATGGGACATGTTGTTTTAAGCACCCCTATT	*H. pylori* complem mutants
HpPutP NotI_as	GCTTATCATGCGGCCGCTTAGTGGTGGTGGTGGTGGCTC	*H. pylori* complem mutants
putAF_Not_as	TTGTTAGCAGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCTTTTTCAGCACAGCATGAC	*H. pylori* complem mutants
putA_Not_as	TTGTTAGCAGCGGCCGCTCATTTTTCAGCACAGCATGAC	*H. pylori* complem mutants
gfpNde_s	GAGGAATTCCATATGGTACCAAG	*H. pylori* complem mutants
gfpNot_as	CCAAAACAGGCGGCCGCTTATTTGTATAG	*H. pylori* complem mutants
gfpXho_s	GAGGCCTCGAGCATGGTACCAAG	*H. pylori* complem mutants
gfpXho_as	AAAACAGCCGCTCGAGGCTTTGTATAGTTC	*H. pylori* complem mutants
era-F	AAGGCTAATGCGACCAGAAA	*H. pylori* constitutive gene
era-R	GGAGCCCTGGTGTGTCTAAA	*H. pylori* constitutive gene
flaA-F	CATGGGGATTATCCAAGTTG	*H. pylori* flagella genes

flaA-R	CGATACGAACCTGACCGATT	*H. pylori* flagella genes
flgE-F	GGCTAACGACCGAAATTACG	*H. pylori* flagella genes
flgE-R	TGAAATTCCCCCTCGCTTGG	*H. pylori* flagella genes
flaB-F	ACCAGAACCGACGCTAGAGA	*H. pylori* flagella genes
flaB-R	CCACATTCGCATCAAAAATG	*H. pylori* flagella genes
flgM-F	AGTGAGACCGCTCTTGATAG	*H. pylori* flagella genes
flgM-R	CCCAATAAATCCTGTGCCATT	*H. pylori* flagella genes
flhA-F	CACCATTCCTGGACTCCCTA	*H. pylori* flagella genes
flhA-R	TTAGTGAGCAACCCGTCTTT	*H. pylori* flagella genes
flhF-F	ATTAAGCCTGGAATTAATGC	*H. pylori* flagella genes
flhF-R	TTAGTGAGCAACCCGTCTTT	*H. pylori* flagella genes
rpoN-F	TGGCATTGGCGCTAAAGATG	*H. pylori* flagella genes
rpoN-R	CGCGCTTCTTCATAAAGCTCA	*H. pylori* flagella genes
fliA-F	GAATGCCCAAAGGAATTCAA	*H. pylori* flagella genes
fliA-R	AGCGAGATCGTCTTGATGGT	*H. pylori* flagella genes
ProU_s	TGCTCGCTATCCCCCAAATTTTAGT	*H. pylori* ProU
Pro U_as	AAAAATCGCCTGCCCTAAGC	*H. pylori* ProU
ProP_s	CTGTAGTTTCTGGGATTTTG	*H. pylori* ProP
ProP_as	CCTCAAATAGACAGAAATGA	*H. pylori* ProP
RTHpPutP_s	TTCAGGGCTGGTGAGTGGGGCTAA	*H. pylori* PutP
RTHpPutP_as	AATCCGTCCAGCACACCGCCTT	*H. pylori* PutP
FlgE_s2	GGCTGGTTTGTGATAGGGCC	*H. pylori* flagella genes
FlgE_as2	AATTACCGGCGCGGGTTAGA	*H. pylori* flagella genes
FlhF_s2	ATGAAATCGTTGTGGCGGTT	*H. pylori* flagella genes
FlhF_s2	GACCACATCTTCTTCATTCAATTCT	*H. pylori* flagella genes

2.1.4 Antibodies

The antibodies used in this study are listed in Table 4.

Table 4. Antibodies used in this study

Antibodies	Company
Penta His HRP-conjugate mouse antibody	Qiagen
Monoclonal Anti-Green Fluorescent Protein (GFP) antibody	Qiagen

2.1.5 Antibiotics

The antibiotics used in this study are listed in Table 5.

Table 5. Antibiotics used for selective plates and medium

Antibiotic	Abreviation	Disolved in	Final concentration in medium
Ampicillin	Amp	H_2O	100 mg/l (LB-Medium/-Plates)
Diaminopimelic acid	DAP	H_2O	14 mg/l (LB-Plates)

Kanamycin	Kan	H_2O	50 mg/l (LB-Medium/-Plates) 8 mg/l (Serumplates)
Erythromycin	Erm	EtOH	250 mg/l (*E. coli*) 10 mg/l (*H. pylori*)
Nystatin	Nys	H_2O	440 ml/l (*H. pylori*)
Streptomycin	Strep	H_2O	250 mg/l
Trimetoprim		H_2O	5 mg/ l
Vancomycin	Van	H_2O	10 mg/l

2.1.6 Culture medium

Culture media used for this study is listed in Table 6

Table 6. Culture media for *E. coli* or *H. pylori* respectively

Bacteria	Solution	Recipe
For *E. coli*	Liquid culture	Luria Bertani-Medium (Miller, 1992). 1% (w/v) bacto triptone, 0.5% yeast extract and 1% (w/v) sodium chloride. Autoclaved
	Agar plates	1.5% agar-agar was added to the LB-medium and autoclaved
	Glycerol medium	LB-medium, 20% glycerin (v/v)
For *H. pylori*	Serum plates	36 g/l GC-Agar-Base (oxoid), autoclaved, 10 ml/l vitaminmix, 80 ml/l horse serum, 10 mg/l vancomycin, 1 mg/l nystatin, 5 mg/l trimethoprim
	Vitamin mix	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 thiamin, 13 mg/l p-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-Medium	28 g/l Brucella-Broth (BD), autoclaved supplemented with 80 ml/l horse serum
	Glycerol medium	Brucella-medium, 20% glycerin, 10% FCS
	RPMI 1640 Medium	(Gibco)
	Defined Medium	RPMI 1640 medium, suplemented with 100 mg/l $Ca(NO_3)_2 \bullet 4H_2O$, 400 mg/l KC1, 100 mg/l $SO_4 .7H_2O$, 6000 mg/l NaCl, 800 mg/l Na_2HPO_4, 2000 mg/l $NaHCO_3$ 2000 mg/l Glucose, 5000 mg/l Bovine serum albumin, 5 mg/l Phenol red (optional), 2 mg/l FeSO, 50 mg/l Adenine, 3 mg/l Lipoic acid, 0.2 mg/l D-Biotin, 3 mg/l Choline chloride, 1 mg/l Folic acid, 35 mg/l my-Inositol, 1 mg/l Niacinamide, 1 mg/l p-Aminobenzoic acid, 1.25 mg/l D-Pantothenic acid, 1 mg/l Pyridoxine hydrochloride, 0.2 mg/l Riboflavin, 1 mg/l Thiamin hydrochloride, 0.005 mg/l Vitamin B12, 44.5 mg/l Alanine hydrochloride, 632 mg/l

		Arginine, 75 mg/l Asparagine, 66.5 mg/l Aspartic acid, 120 mg/l Cystine, 73.5 mg/l Glutamic acid, 300 mg/l Glutamine, 37. 5 mg/l Glycine, 110 mg/l Histidine, 262.5 mg/l Isoleucine, 262 mg/l Leucine, 362.5 mg/l Lysine, 75.5 mg/l Methionine, 165 mg/l Phenylalanine, 57.5 mg/l Proline, 52.5 mg/l Serine, 238 mg/l Threonine, 51 mg/l Tryptophan

2.1.7 Buffers and solutions

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

Buffers used for transformation of competent cells in *E. coli* are listed in Table 7

Table 7. Buffers for transformation of competent cells in *E. coli*

Buffer	Recipe
Buffer I	10mM 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

2.1.7.2 Buffers for gel electrophoresis

Buffers used for gel electrophoresis are listed in Table 8

Table 8. Buffers for gel electrophoresis

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

2.1.7.3 Buffers and solutions for membrane preparation

Buffers for membrane preparation used for this study are listed in Table 9

Table 9. Buffers and solutions for membrane preparation

Bacteria	Buffer	Recipe
For *E. coli*	Kpi Buffer	1M stock solutions of K_2HPO_4 and KH_2PO_4 were prepared and the corresponding amounts of each one were used to prepare the buffer at the desired pH 0.5L of 1M K_2HPO_4 at 174.18g mol-1 = 87.09g 0.5L of 1M KH_2PO_4 at 136.09g mol-1 = 68.045g
For *H. Pylori*	PBS	8.01 g/l NaCl, 0.20 g/l KCl ,1.78 g/l $Na_2HPO_4 \bullet 2\,H_2O$, 0.27 g/l KH_2PO_4, pH 7.4

2.1.7.4 Buffers and solutions for SDS-Polyacrylamidgel-electrophoresis

Buffers used for SDS-Polyacrylamidgel-electrophoresis are listed in Table 10

Table 10. Buffers and solutions for SDS-Polyacrylamidgel-electrophoresis

Buffer	Recipe
Spacer buffer	0.4% SDS, 0.5M Tris/HCl pH 6.76, 10% APS, 0.1% TEMED
Stacking buffer	0.4% SDS (w/v), 1.5M 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).

2.1.7.5 Buffers and solutions for Western-Blot

Buffers and solutions for Western-Blot used in this study are listed in Table 11

Table 11. Buffers and solutions for Western-Blot

Buffer	Recipe
Transfer Buffer	15.6mM Tris, 120mM 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)

2.1.7.6 Buffers and solutions for protein quantification

For protein quantification, the methods of Peterson or Bradford were used and the reagents used for each one are listed below

<u>For Peterson</u>

Solution A	CTC: 0.8N NaOH: 10% SDS

CTC: Copper tartrate/carbonate (CTC) solution

0.22% KNa-tartrate $4H_2O$
0.1% $CuSO_4$ $5H_2O$
Dissolve in 10% Na_2CO_3

Folin–Ciocalteu reagent (FCR): H_2O_{dest} (1:5)

<u>For Bradford</u>

Bradford Reagent	Serva Blue G-250 (0.07g), ethanol 96% (50ml), H_3PO_4 85% (100ml), H_2O (to 1L).

Standard curve was made from 0 to 5µg protein. 20 µl of the sample were mixed with 1 ml of the reagent, incubated for 5min at room temperature and OD was measured at 578nm

2.1.7.7 Buffers for transport measurements in proteoliposomes

Buffers used for transport measurements in proteoliposomes are listed in Table 12

Table 12. Buffers for transport measurements in proteoliposomes

Buffer	Recipe
Buffer 1	100 mM KP$_i$, 5 mM MgCl2, 2 mM ß- Mercaptoethanol
Buffer 2 (ΔµNa$^+$)	100 mM Tris/ Mes pH 7.5, 5 mM MgCl$_2$, 2 mM ß-Mercaptoethanol, 50 mM NaCl
Buffer 3 (ΔµLi$^+$)	100 mM Tris/ Mes pH 7.5 (Na$^+$- free), 5 mM MgCl$_2$, 2 mM ß-Mercaptoethanol, 50 mM LiCl
Buffer 4 (ΔµH$^+$)	100 mM Tris/ Mes pH 6.0 (Na$^+$- free), 5 mM MgCl$_2$, 2 mM ß-Mercaptoethanol
Buffer 5 (ΔµNa$^+$, pH)	100 mM Tris/ Mes pH 6.0, 5 mM MgCl$_2$, 2 mM ß-Mercaptoethanol, 50 mM NaCl, 2 mM).

2.1.7.8 Buffers and solutions for Transport measurements in *E. coli* and *H. pylori*

Buffers used for transport measurements in *E. coli* of *H. pylori* are listed in Table 13

Table 13. Buffers and solutions for Transport measurements in *E. coli and H. pylori*

Bacteria	Buffer	Recipe
E. coli	Transport Buffer	100mM Tris/Mes BufferpH 6.0
	Stop Buffer	100mM LiCl, 100mMKH$_2$PO$_4$
H. pylori	Transport Buffer	100mM Tris/Mes Buffer, 150mM KCl pH 7.0
	Stop Buffer	100mM LiCl, 100mMKH$_2$PO$_4$

2.1.8 Enzymes and Proteins used in this study

Enzymes and Proteins used in this study are listed in Table 14.

Table 14. Enzymes and Proteins used in this study

Enzyme	Company
Alkaline Phospathase (AP)-gekoppeltes Protein A Sigma DNaseI	Fermentas
Taq-Polymerase	New England Biolabs, Schwalbach
Lysozyme	Fermentas
Proteinase K	Fermentas
Restriction enzymes	New England Biolabs, Schwalbach
T4-DNA-Ligase	New England Biolabs, Schwalbach

2.1.9 Molecular markers

Molecular markers used in this study are listed in table 15

Table 15. Molecular markers

Molecular marker	Company
DNA-Gelelectrophoresis 2 log DNA Ladder	New England Biolabs
Polyacrylamid-Gelelectrophoresis PageRuler™ Prestained Protein Ladder standard	Fermentas

2.1.10 Chemicals and Reagents

Acrylamid/Methylenbisacrylamid 29:1 (30%) (Roth), NNN'N'Tetramethylendiamid (TEMED) (Merk), Tween20 (Serva), Desoxyribonukleinsäure-Triphosphat Mix (dNTPs) (Roche), Alkaline Phosphatase Calf Intestinal (CIP) (NEB), DMSO (Sigma), EDTA (Sigma), Ethidiumbromid (Sigma), Agarose (Serva), Ampicillin (Sigma), Trimethoprim (Sigma), Trypan Blue (Invitrogen), Vancomycin (Sigma), Erythromycin (Roth), Vancomycin (Sigma), Nystatin (Sigma), Trimethroprim (Sigma), Kanamycin sulfat (Roth), MES (Roth), Carbenicillin (Roth), L-hydroxyproline (Applichem), Tryptone (Roth), Agar-agar (BD Difco), Bacto yeast extract (BD), Sodium Chloride (Prolabo), Brucella-Broth Medium (Becton Dickinson), GC-Agar (Oxoid), Horse serum (PAA laboratories GmbH), Glycerin (Roth), PMSF (Sigma), Poly-L-Lysin (Sigma), IPTG (AppliChem), ATP (Roche), Bromphenolblau (ServaDAP (Sigma), *E. coli* polar lipid extract in Chloroform (acetone/ether purified, Avanti Cat.# 100600), Incidine Plus (Ecolab)

2.1.11 Commercial-Kits

Commercial kits used in this study are listed in Table16

Table 16. Commercial-Kits

Use	Kit	Company
RNA extraction	Quick-RNATM MicroPrep	ZyMo research
DNA extraction	ReliaPrep gDNA Tissues Miniprep system	Promega
Retrotranscription	RT Kit Thermo Scientific RevertAid First Strand cDNA synthesis kit	Thermo Scientific
Plasmid purification	HiYield Plasmid Mini DNA-kit	SLG®
DNA gel-extraction	HiYield PCR Clean-up/Gel extraction-kit	SLG®

2.1.12 Instruments and equipment

Biorad IQ5 Multicolor Real Time PCR detection system, nanodrop (Peqlab ND-100 Spectrophotometer), anaerobic Jar (Schuett Biotech GmbH), microflow biological safety cabinet (Nunc, InnovaTM 4330), refrigerator incubator shaker (New Brunswick Scientific), anaerobic chamber (Scholzen Microbiology systems Ag).

2.2 METHODS

2.2.1 Bacteria cultures

2.2.1.1 Cultures of *Escherichia coli*

E. coli strains were grown on Luria-Bertani agar (LB)-Plates (Miller J H, 1992) or in LB-liquid medium at 37°C. For the selection of plasmids in *E. coli*, media was supplemented with erythromycin (10mg/l), ampicillin (100 mg/l), or kanamycin (50 mg/l). For the strain collection the cell material was taken from an overnight liquid culture with glycerin (3:1) and stored at -70°C. Recombinant plasmids were transformed and maintained in *E. coli* DH5α (Bethesda Research Laboratories).

2.2.1.2 Cultures of *Helicobacter pylori*

H. pylori strains were cultured on GC agar serumplates 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% N_2. 10% CO_2. 5% 0_2) (Barwig I, 2009). When necessary for selection of *H. pylori* allelic exchange mutants, erythromycin (10mg/l) or streptomycin (10mg/l) was used in addition to the other antibiotics. For the strain collection the cells from one plate were resuspended in 1 ml brucella-medium with 20% glycerin and 10% HS and stored at -70°C.

2.2.1.3 Growth studies

Growth over time was assessed by measurement of $OD_{600.}$ Liquid cultures were grown in 15 ml volumes of medium in 100 ml conical flasks with gentle shaking (100 r.p.m.) or in 1 ml volumes in the wells of a 24-well tray (NUNC) with gentle shaking (100 r.p.m.) (Reynolds D J and Penn C W, 1994). Inocula were grown for 48h on agar plates then suspended in Brucella Broth media, and standardized to 1 x $l0^8$ cells/ml (OD_{600} of 0.2). Inoculation was carried out in a laminar-flow hood and the cultures immediately transferred to the Jar with the gas mixture and shaken at 37 °C.

For studies in defined medium, liquid cultures were grown in 1 ml volumes in the wells of a 24-well tray (NUNC) with gentle shaking (100 r.p.m.). Inocula were grown for 48h on agar plates then suspended in RPMI media, with or without addition of the mixture of amino acids, vitamins and BSA as listed in Table 6. Cells were standardized to 1 x $l0^8$ cells/ml (OD_{600} of 0.2). Inoculation was carried out in a laminar-flow hood and the cultures immediately transferred to the Jar with the gas mixture and shaken at 37 °C.

2.2.1.4 Motility assay (soft agar plates).

H. pylori cells were culture in plates for 48 h, resuspended in BB supplemented with 10% 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 microaerobically at 37°C for 5 days (Nagata K, *et al.* 2003).

2.2.1.5 Transformation of *E. coli*

Chemically competent cells; Rubidium Chloride Method.
A single colony from *E. coli* was used to inoculate 5ml of LB medium and incubated overnight at 37°C with vigorous shaking. 100µl from this overnight culture was used to prepare a new 10ml culture which was incubated 2 h at 37°C until an OD_{600} of 0.3-0.5 was reached. Cells were harvest by centrifugation 5 min at 5000rpm 4°C and resuspended in 5ml of Buffer I, washed again and respuspended in 5ml Buffer II. Cells were incubated 30 min on ice, centrifuged (5 min at 5000rpm 4°C) and finally resuspended in 1ml Buffer II.
Heatshock-Transformation of *E. coli* cells
For transformation, 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 2-3 min on ice. 800 µl LB medium was added followed by incubation for 45 min 37 °C and 750 rpm. At the end, 100 µl aliquots of the cell suspension was spread on LB selective-plates and incubated at 37°C overnight

2.2.1.6 Transformation of *H. pylori*

Natural transformation of *H. pylori* strains was performed with plasmid-DNA according to the procedure described by Haas *et al.* 1993. *H. pylori* was grown for 2-3 days on serum plates and harvested in 1mL BB containing 10% HS. The suspension was adjusted to an OD_{600} of 0.2 in a 24-well tissue culture plate (approx $3x10^7$ bacteria mL^{-1}) and 1-3µg of supercoiled plasmid or chromosomal DNA was added. The culture was incubated a minimum of 4h (5% CO_2 37°C). Cells were harvested by centrifugation at 3500rpm for 5 min and spread on serum selective plates. Afterwards plates were incubated for 4-5 days under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) at 37°C. Resulting colonies were amplified and DNA was isolated to verify the right insertion (Haas R, *et al*., 1993).

2.2.1.7 Conjugation of *H. pylori*

Natural transformation of *H. pylori* has shown lower transformation efficiency for shuttle vectors (Heuermann D and Haas R. 1998) due to DNA restriction and strain specific modification mechanisms. For the complementation of the deletion mutants with the shuttle vector *pIB6*, conjugation was used instead of transformation, as DNA restriction seems to be strongly reduced or absent during conjugal transfer.
E. coli WM3064 was transformed with the plasmid of interest by the rubidium chloride method already mentioned above. Overnight cultures of the donor (*E. coli WM3064* + plasmid+ DAP+ selective antibiotic) and recipient strains (*H. pylori ΔHpPutP, ΔHpPutA and ΔHpPutAP*) were grown. 100µl of the donor strain and 300µl of the recipient strain were spin down, washed once with medium and resuspended in 100 µl of BB. The entire volume of cells (100µl 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 degrees for about 8 hours. Afterwards, cells were washed with medium to recover the cells, centrifuged at 3500g 5 min and plated in serum-selective plates (without DAP!). Plates were incubated at 37°C under microaerophilic conditions from 3-4 days (modifyed from Webster D, 2012).

2.2.2 Biochemical methods

2.2.2.1 Western Blot

Sample preparation:

E. coli

For Western blot experiments; 300µl of overnight cultures of *E. coli* containing the desired plasmid and the selective antibiotic were inoculated in 20 ml of medium until reaching an OD_{420} nm of 1.0. The cells were then induced with IPTG (0.5M) for 2 h at 37°C. Harvested by centrifugation for 10 min at 5000rpm 4°C with KPi pH 7.5 and washed. Cells were resuspended with 3ml buffer/g of cell wet weight and sonified with 3x30s with 35% Amplitude at the Branson cell disruptor B15. The sample was centrifuged 10 min at 10,000rpm to remove the cell debris; the supernatant was recovered and ultracentrifuged for 60 min at 80,000rpm. The resulting pellet was resuspended in 50µl of PBS and stored at -20°C before use (Quick M S, *et al.*,1996).

H. pylori

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 liquid medium supplemented with 10% HS and Kanamycin (8µg/ml) if necessary. Cells were spin down at 3500 rpm 4°C and a new fresh culture was inoculated from the pre-culture.

Cells were harvested, resuspended in a mixture of PBS, complete and disrupted by sonification at 3x30s with 35% Amplitude at the Branson cell disruptor B15. The sample was centrifuged 10 min at 10,000rpm to remove the cell debris; the supernatant was recovered and ultracentrifuged for 60 min at 80,000rpm. The resulting pellet was resuspended in 50µl of PBS and stored at -20°C before use (modifyed from Quick M S, *et al.*,1996).

2.2.2.2 Protein quantification

Determination of protein was performed according to a modified Lowry method (Peterson, 1977) for total membrane protein, according to Bradford (1976) for detergent- solubilized protein, and by the Amido Black method (Schaffner and Weissmann, 1973) for protein in proteoliposomes.

2.2.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein separation was performed by SDS-PAGE according to Lämmli (1970). Proteins were separate 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, 2-mercaptoethanol (beta-mercaptoethanol/BME), a reducing agent was added, which further denatures the proteins by reducing disulfide 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) Glycerin, 0,005 % (w/v) Bromphenolblue 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.* (1989). The acrylamide concentration in the gel was 12% (Table 17).

For *E. coli* cells 0,35mg/ml were loaded and for *H. pylori* samples 50 µg of protein was applied to the gels.

Table 17. SDS-Gel preparation used in this study

Reagent	Spacergel	Stackingel
Acrylamid (%)	4,9	12,5
5 x SpacerBuffer **(0,4% (w/v) SDS, 1,5 M Tris/HCl, pH8,8)**	-	6 ml
5 x Stackingbuffer **(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 (Fermentas) was used as protein standard. And the electrophoresis was performed at 200 V for 1 h in SDS 1x buffer

2.2.2.4 Detection of proteins in polyacrylamide-gels

After the electrophoresis, proteins in polyacrilamide gels can be stained to be visualized. Detection can be performed using the Comassie solution (Marshall and Williams, 1992). Therefore, SDS gels were incubated with the Coomassie solution for 20 min with gentle agitation. Afterwards, gels were destained in destaining solution. The solution was several times replenished until background of the gel was fully destained. A the end, the destained gel was dryed and stored

2.2.2.5 Immunological analysis for proteins with a His-tag

For Western blot experiments, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by the method of Towbin *et al,* 1979.
The blots were first incubated with blocking Buffer for 1 h to avoid unspecific binding. Washed 10 min with TBS buffer and then incubated either with a Penta His HRP-conjugate mouse antibody raised against a synthetic His-tag at the *HpputP* C-terminus or with an antibody raised against GFP fused to the C- terminal part of *HpputP* in the *H. pylori* complementation plasmids. Both antibodies were used in a dilution of 1:20,000. Finally the blot was washed twice with TBS-TT buffer and bound antibodies were visualized with an Uptima-uptilight HRP blot substrate

2.2.2.6 Protein Purification

For the purification of *H. pylori* PutP preparation of inverted membrane vesicles was performed first. For this, *E. coli WG170* harboring plasmid *pRHpputP6H* was grown aerobically in LB medium containing 100 mg/mL ampicillin at 37 °C, and expression was initiated by addition of 0.3 mM isopropyl thio-ß-Dgalactoside (IPTG) at the middle of the exponential growth phase. After further growth for 3 h, cells were harvested by centrifugation, washed with 100 mM KPi, pH 7.5/2 mM ß-mercaptoethanol, and resuspended in the same buffer to give 0.4 g of cells (wet weight)/mL. Inverted membrane vesicles were prepared by passage of the cell suspension through a high-pressure cell disruptor followed by low-speed centrifugation at 12000g for 30 min at 4 °C to remove unbroken cells. Membranes were collected by centrifugation at 230000 xg for 90 min at 4 °C, washed with 25 mM Tris pH 7.5, 300 mM NaCl, 10% Glycerol, 2 mM ß-Mercaptoethanol, resuspended in the same buffer, and stored at -80 °C (Jung H *et al.*, 1998b).

For solubilization of HpPutP, inverted membrane vesicles were diluted into 50 mM KPi, pH 8.0, to yield a protein concentration of 10 mg/mL. The membrane suspension was supplemented with 2 mM ß -mercaptoethanol and 10% glycerol. n-Dodecyl-ß-D-pyranosid (DDM) was added stepwise to yield a final concentration of 1.5% (w/v) while stirring on ice. After additional stirring for 30 min, the sample was centrifuged at 230, 000g for 20 min

The protein purification was performed in an Äkta Purifier (GE Healthcare) instrument, in which a cartridge pre-filled with Ni-NTA Superflow was used (Quiagen). Finally the column was equilibrated with washing buffer (25 mM Tris pH7.5, 300 mM NaCl, 2 mM ß-Mercaptoethanol, 0,04% DDM, 0,01 M Imidazol pH8.0) and loaded with the solubilized protein. The protein was finally eluted by using an elution gradient with the washing buffer up to 200 mM Imidazol.

2.2.2.7 Functional reconstitution of HpPutP

First, lipids were prepared; therefore an appropriate amount of lipids was transfered into a large beaker or flask (e.g., 15 ml chloroform containing 300 mg lipids). Chloroform was removed under a stream of argon to obtain a thin layer of dry lipids. The lipids were resuspended in argon saturated buffer PL [100 mM KPi pH 7.5/ 2 mM β-Mercaptoethanol/ 1.5 % octylglucoside (w/v) (This OG concentration corresponds to the onset of lipid solubilisation) (e.g., add 15 ml buffer PL to 300 mg of lipids to obtain 20 mg lipids/ml). Aliquots of the lipids were frozen and stored in liquid nitrogen

The permease used was purified by Ni-NTA chromatography in 0.04 % dodecylmaltoside (w/v).

The pretreatment of biobeads was performed as previously described (According to Holloway P.W. 1973). Work was done at room temperature. 200 ml methanol was added to 30 g Bio-Beads SM-2 (BioRad) and stirred for 15 min. Beads were collected on sintered glass funnel and washed with further 500 ml Methanol. Immediately after the beads were washed with 1000 ml water (The beads are not allowed to dry). The moist beads were washed slowly with 2000 ml water using a chromatography column. Beads were stored under water until required and finally washed with 100 mM KPi pH 7.5 before use.

Finally, for the protein reconstitution, lipids were thawed and extruded through 400nm filter, lipids were adjusted to a final concentration of 5mg/ml with 100mM Kpi pH 7.5 (room temperature) and destabilized with 0.12% Triton X-100. Afterwards biobeads were added. Lipids were mixed with purified permease at the desired lipid to protein ratio [e.g., 150 to 1 (w/w)] and incubated at room temperature under gentle agitation for 15 min. Biobeads were added [beads to detergent ratios: 5 mg beads (wet weight) to

1 mg Triton X-100; 10 mg beads (wet weight) to 1 mg DM] (e.g., add 260 mg biobeads to a solution containing 50 mg Triton X-100 and 1 mg DM)

This mixture was incubated at room temperature under gentle agitation for 1 h. Afterwards the same amount of biobeads was added again and incubation continued at room temperature under gentle agitation for further 1 h.

More biobeads (double amount as above) were added for the third time and then incubated at 4°C under gentle agitation overnight. Biobeads were removed by filtration on glass silk or by pipetting carefully using a narrow pipette tip. The proteoliposome suspension was dialyzed two times against 2 l of 100 mM KPi pH 7.5/ 2mM β-Mercaptoethanol at 4°C. Proteoliposomes were concentrated by centrifugation and stored in liquid nitrogen (Jung H *et al.*, 1998b).

2.2.2.8 Determination of Na^+

Na^+ concentrations in buffers used for transport assays were determined with a Varian AA240 atomic absorption spectrometer.

2.2.2.9 Transport experiments

2.2.2.9.1 ^{14}C L-proline-uptake of HpPutP in proteoliposomes

An aliquot of the proteoliposomes was resuspended in Buffer 1 (100 mM KP_i, 5 mM MgCl2, 2 mM ß-Mercaptoethanol) and extruded through a 400nm polycarbonat membrane filter.

The extruded proteoliposomes were sedimented at 180, 000 g for 30 min at 20°C and resuspended in the minimum amount of buffer 1. The ^{14}C L-proline uptake in proteoliposomes was performed under different conditions Buffer 1 (100 mM KP_i, 5 mM MgCl2, 2 mM ß- Mercaptoethanol, negative control); Buffer 2 ($\Delta\mu Na^+$): 100 mM Tris/ Mes pH 7.5, 5 mM $MgCl_2$, 2 mM ß- Mercaptoethanol, 50 mM NaCl; Buffer 3 ($\Delta\mu Li^+$): 100 mM Tris/ Mes pH 7.5 (Na^+- free), 5 mM $MgCl_2$, 2 mM ß- Mercaptoethanol, 50 mM LiCl; Buffer 4 ($\Delta\mu H^+$): 100 mM Tris/ Mes pH 6.0 (Na^+- free), 5 mM $MgCl_2$, 2 mM ß-Mercaptoethanol; Buffer 5 ($\Delta\mu Na^+$, pH): 100 mM Tris/ Mes pH 6.0, 5 mM $MgCl_2$, 2 mM ß-Mercaptoethanol, 50 mM NaCl, 2 mM).

For the measurements, 400µl of the buffers together with 10 µM ^{14}C- L- proline (26Ci/ mol) were incubated at 25°C for 5 min. Immediately before starting the reaction, 1µl of proteoliposomes and Valinomycin with an end-concentration of 0.2µM were added. The reaction was stopped after different time intervals by adding twice 3ml of cold calcium lithium stop buffer. Proteoliposomes were filtered in cellulose-ester-filters (0.22 µM, Millipore) which were transferred into scintillation vials with 4.5 ml of scintillation liquid (EcoLite; MP Biomedicals). Radioactivity of bacteria retained on the filters was quantified using a ß-Counter.

2.2.2.9.2 ^{14}C L-proline-uptake of HpPutP in *E. coli*

Active transport was measured in *E. coli WG17*0 (Δ*putPA)* harboring derivatives of plasmids *pTHpputP6H* or encoding *HpputP* (*HPP12_0049*) with given nucleotide replacements. The cells were grown aerobically in Luria-Bertani (LB) medium containing 100 µg/mL ampicillin at 37 °C. Overnight cultures were diluted 25-fold and were allowed to grow up to an optical density at 420 nm (OD_{420}) of 1.0, followed by induction with 0.5 mM IPTG for 2 hours. Cells were harvested by centrifugation at 13,200 xg for 10 min and washed up to 2 times with 100 mM Tris/Mes, pH 6.0 at 4 °C. For transport assays, cells were resuspended in the same buffer and adjusted to a total protein concentration of 0.35 mg/mL. Transport of 10 μM ^{14}C- L-proline (26 Ci/mol) was assayed under standard test conditions in the presence of 20 mM D-lactate (Na^+ salt) and 50 mM NaCl. Transport assays were determined at various time points (0, 0.17, 0.5, 1, 2, 5, 10, 30 min) using the rapid filtration method as described by Jung *et al.* 1998. Initial rates of transport were calculated from the initial linear portion of the time course. Standard deviations were determined from at least three independent experiments.

2.2.2.9.3 Proline kinetic of HpPutP in *E. coli*

E. coli WG170 Cells, with the *pTHpputP6H* plasmid were cultured and prepared as for the ^{14}C L-proline uptake of HpPutP in *E.coli.* 200μl of the cell suspension was preincubated with 20 mM D-lactat at 25°C for 5 min. The reaction was started with the addition of 50 mM NaCl and different concentrations of ^{14}C-L-proline. The transport measurements were carried out with 9 different ^{14}C-L-proline concentrations from 0.12 μM to 100 μM. For each ^{14}C-L-proline concentrations two time points mere measured, 0 and 10 sec. The reaction was stopped as previously described. Finally, for each reaction of different concentrations of ^{14}C-L-proline the velocity of the reaction v (nmol/mg*min) was calculated based on the difference of the two measurements.

2.2.2.9.4 ^{14}C L-proline uptake by HpPutP in *H. pylori*

The procedure was adapted from that used by Leduc D, *et al.* in 2010. *H. pylori* parental strain and the isogenic knockout single or double mutants were amplified on plates for 2 days, grown in 25ml liquid medium supplemented with 10% HS and the corresponding antibiotic if necessary for 24 h. Afterwards, cells were spin down at 3500 rpm 4°C and a new fresh 50ml culture was inoculated from the pre-culture. After 12h this late log cells were collected by centrifugation and washed with 100 mM Tris-morpholineethanesulfonic acid (MES) buffer, pH 7.0, with 150mM KCl. Cells were adjusted to an OD_{600} of 0.8 and from the prepared suspension, 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 1mM 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.

2.2.2.9.5 Proline kinetics of HpPutP in *H. pylori*

Cell cultures were grown, harvested and prepared as those for the ^{14}C L proline-uptake of HpPutP in *H. pylori* as mentioned above. 200μl of the cell suspension was preincubated at 37°C until use. The reaction was started with the addition of 50 mM NaCl and different concentrations of ^{14}C-L-proline. The transport measurements were carried out with 9 different ^{14}C-L-proline concentrations from 0.12 μM to 100 μM. For each ^{14}C-L-proline concentrations two time points were measured, 0 and 10 sec. The samples were incubated for 1 min and immediately filtrated on Durapore 0.45-µm membrane filters (Millipore) that were abundantly washed with 3 ml of cold Stop-Buffer. The reaction was stopped as previously described. Finally, for each reaction of different concentrations of ^{14}C-L-proline the velocity of the reaction v (nmol/mg*min) was calculated based on the difference of the two measurements.

2.2.2.9.6 Sodium kinetic of HpPutP in *H pylori*

Cell cultures were grown, as those for the ^{14}C L-proline uptake of HpPutP in *H. pylori* as mentioned above. Cells were washed for 5 times with Na^+-free 100 mM Tris-MES buffer, pH 7.0, with 150mM KCl and the cell suspension was adjusted to an OD_{600}nm of 8.0. Aliquots of 200µl of the cell suspension were kept at 37°C until use. Before starting the transport reaction 10 µM ^{14}C-L-proline and different concentrations of NaCl (5 µM- 250 mM) were added. The reaction time point was 1 min and for the 0 time point just one Na^+-concentration was measured. The velocity of the reaction v (nmol/mg*min) was calculated based on the difference of the measurements at 1 min minus the 0 time point measurement.

For the determination of the $K_{0.5}$ [Na^+], the measurement of the Na^+ concentration in the cell suspension was considered in the calculations; therefore, it was determined with an atomic absorption spectrophotometer

All kinetic analysis in this work, either for sodium or ^{14}C L-proline uptake were based on the Michaelis-Menten Kinetics

Equation of Michaelis-Menten:

$$\mathbf{v = V_{max} \times [S] / K_m + [S]}$$

v = velocity;

[S] = Substrate concentration;

K_m = Michaelis-constant;

V_{max} = maximum rate

Lineweaver-Burke:

$$\mathbf{1/v = 1/[S] \times K_m/V_{max} + 1/V_{max}}$$

Eadie-Hofstee:

$$\mathbf{v = V_{max} - (K_m \times V) / [S]}$$

2.2.2.10 ATP measurements

To determine the ATP levels in *H. pylori* cells, the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) was used. Briefly, samples were collected from liquid cultures in logarithmic phase, adjusted to an OD_{600} of 1 in an opaque-welled multiwell plate in

culture medium (e.g., 100µl 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 100µl of reagent to 100µl 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 10pMof ATP).

2.2.3 Molecular Methods

2.2.3.1 Isolation of Plasmid-DNA of *E. coli*

Plasmid DNA of *E. coli* was extracted using the HiYield Plasmid Mini DNA-kit (SLG), according to the manufacturer's instructions

2.2.3.2 Extraction of genomic DNA from *H. pylori*

Genomic DNA from 2-day-old *H. pylori* plate cultures 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).

2.2.3.3 Isolation of RNA *H. pylori*

RNA was extracted from *H. pylori* strain P12 or from the *ΔHpputP, ΔHpputA* and *ΔHpputAP* mutants from cells grown in liquid culture at the late-log phase using the Quick-RNA™ MiniPrep (Zymo Research) according to the manufacturer's instructions. RNA prepared was analyzed by gel electrophoresis for no sign of degradation and quantified by NanoDrop ND-100 (Peqlab)

2.2.3.4 Polymerase-chain reaction

PCRs were performed in Mastercycler personal thermal cycler (Eppendorff). 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 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- 60°C (depending on the calculated melting temperatures of the primers) for 30-45sec, and extended at 72°C for 1 to 3 min. A total of 35 cycles was performed.

2.2.3.5 DNA-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 100V for 40 min. To make DNA or RNA bands visible for agarose gel electrophoresis ethidium bromide (EtBr) was used (1 mg/l). It fluoresces under UV light when intercalated into the major groove of DNA (or

RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20 ng DNA becomes distinctly visible

2.2.3.6 DNA restriction by restriction enzymes

Restriction of the desired PCR fragments, were done according to the manufacturer's instructions. Restriction was performed for 2 h at 37°C

2.2.3.7 Ligation

Ligation was performed at 16°C for 2h or overnight. For cohesive (sticky) ends, 1 µl of T4 DNA Ligase in a 20µl reaction was used. To avoid self-ligation of the restricted vector and decrease the vector background in the cloning, 1µl Alkaline Phosphatase, Calf Intestinal (CIP) (NEB) was added to remove the 5´ phosphate groups from DNA, deoxyribonucleoside triphosphates.

2.2.3.8 Site directed mutagenesis

Desired nucleotide substitutions in the *HpputP* were generated by two-step PCR with *Phusion*-DNA polymerase using plasmid *pTHpputP6H* as a template and synthetic mutagenic oligonucleotides. Altered sequences were cloned into plasmid *pTHpputP6H*. Resulting plasmid DNA was verified by sequencing using an ABI 3730 device.

2.2.3.9 Construction of *H. pylori* mutants

The construction of the *H pylori* mutants was based on the contraselection procedure established by Dailidiene *et al.* in 2006. Briefly, chromosomal inactivation of *hpPutP* (encoding HpPutP), *hpPutA* (encoding HpPutA), and a double mutant for both *hpPutP/hpPutA* (encoding HpPutP and HpPutA) genes was performed in *H. pylori* strain P12. Deletions were introduced by allelic exchange using a suicide plasmid pBluescript 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* and used as suicide plasmids in *H. pylori*. *H. pylori* mutants were obtained by natural transformation with these suicide plasmids as previously described (Haas R. *et al.,* 1993). The double mutant was constructed in one step, due to the contraselection property of the rpsLerm cassette. Correct chromosomal insertion of the cassette and correct allelic exchange were verified by PCR using primers *HpputPKP1, HpputPKP2, HpputPKP3* and *HpputPKP4* (Table 3).

2.2.3.10 Construction of *H. pylori* complemented mutants

For the construction of the complementation mutants, *HpputP* was amplified with primers *HpputP pIB6compl_s* and *HpputP pIB6compl_as* (Table 3) and cloned into the *NotI* and *NdeI* sites of *pIB6* to obtain plasmid *pIB6 alpA HpputP*. This plasmid was used for the complementation of the Δ*HpputP* and Δ*HpputAP* mutants by conjugation. For the quantification and verification of the expression of HpPutP in *H. pylori* we used *GFP* as a reporter gene fused to: (1) The c-terminus of *HpputP* to give the plasmid *pIB6 alpA HpPutP GFP* (primers *gfpXho_s* and *gfpXho_as*), or (2) to the empty vector to give place to the plasmid *pIB6alpAGFP* (primers *gfpNde_s* and *gfpNot_as*). This last plasmid was used as a control of expression of *pIB6* in *H. pylori*

Furthermore 2 different sizes (310 or 500bp upstream of *HpputP*) of the putative *HpputP* promoter region were cloned into the plasmid *pIB6* replacing the *alpA* promoter (plasmids *pIB6 310 GFP* and *pIB6 500 GFP*). Also in this case *HpputP* was cloned behind these promoters (plasmids *pIB6 310 HpputP* and *pIB6 500 HputP*) and also *GFP* was fused to the c-terminus of *HpputP* to give *pIB6 310 HpputP GFP* and *pIB6 500 HpputP GFP* plasmids.

2.2.3.11 DNAseI 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 1µl of 50mM EDTA was added and the reaction was incubated for 10 min at 65°C

2.2.3.12 Retrotranscription

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

2.2.3.13 Real-time PCR

Real-time PCR primers for seven flagellar-associated genes and a housekeeping gene (*era HPP12_0523*) were evaluated. Primer sequences were taken from Ryan, K A *et al.* 2005 and modified according to our strain. qRT-PCR was performed using a BioRad IQ5 Multicolor instrument (BioRad). Each 12.5 µl reaction contained 400 nM of each primer and 12.5 µl of IQ SYBR Green Supermix (from BioRad). 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.

Era was used as reference gene. We refer to the calibrator as the normal wild-type strain and sample to the knockout strain. qRT-PCRs were repeated on three different sets of cultures collected on separate days.

2.2.4 Microscopy Methods

2.2.4.1 Fluorescent microscopy

Samples were taken from liquid cultures in the logarithmic phase and bacterial suspensions were fixed to the slides with poly-L-lysine to allow adhesion. A stock solution of poly-L-lysine (1mg/ml, in bidestilated water filtrated) was diluted 1:20 with bidestilated water to get the working solution (storage at -20°C). A slide was covered with 200 and 500µl of the working solution and incubated for 2h at 37°C (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 20min, washed 2-times with PBS to remove unfixed cells. Fluorescence pictures were obtained using a fluorescent Leica DM-IRE2 microscope.

2.2.4.2 Scanning electron microscopy (SEM) observation and image analysis

For scanning electron microscopy (SEM), drops of the sample were placed either onto a glass slide, covered with a cover slip and rapidly frozen with liquid nitrogen. The cover slip was removed with a razor blade and the glass slide was immediately fixed with 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.0), postfixed with 1% osmium tetroxide in fixative buffer, dehydrated in a graded series of acetone solutions and critical-point dried after transfer to liquid CO_2. Specimens were mounted on stubs, coated with 3 nm platinum using a magnetron sputter coater, and examined with a Zeiss Auriga scanning electron microscope operated at 1-2 kV.

2.2.5 HpPutP homology model

Homology model was generated by using HHpred (Soding J, 2005) using its maximum accuracy (MAC) alignment algorithm (Soding J *et al.,*2005). The backbone coordinates for the core of the homology model were built based on the crystal structure of the inward-facing galactose/glucose vSGLT transporter from *V. parahaemolyticus* (PDB: 3DH4) as template (Faham S, *et al*, 2008). UCSF Chimera was used to generate figures with details of the model (Pettersen E F *et al*., 2004).

3. RESULTS

3.1 Biochemical characterization of HpPutP

3.1.1 Construction of plasmids for heterologous expression of *HpputP* in *E. coli*

For characterization of the putative proline transporter of *H. pylori*, the genes *HpputP* and *HpputA* from strain P12 were cloned and heterologously expressed in *E. coli*. The cloning steps were performed using NEB technology according to the manufacturer's instructions. Briefly, the genes coding for the selected proteins (HpPutP and HpPutA) were amplified by PCR and flanked with *NcoI* and *XhoI* specific recombination sites. All the genes were also extended with a sequence coding for a His-tag at the C- terminal end of the constructs. The PCR products were purified and cloned into different destination vectors allowing expression from the promoters P_{lac} and P_{T7} (*pTHpputP6H*) or P_{trc} (*pRHpputP10H*). For the subsequent experiments, plasmids were transformed into the *E. coli* strain *WG170.*

3.1.2 Purification and functional reconstitution of HpPutP

3.1.2.1 Purification of HpPutP

HpPutP was purified according to a protocol originally established for EcPutP (Jung H. *et al.* 1998). Inverted membrane vesicles were prepared from *E. coli* expressing *HpputP* from plasmid *pRHpputP6H* and n-Dodecyl-ß-D-maltopyranoside (DDM) was used for the subsequent solubilization and purification of HpPutP by Ni^{2+}-NTA affinity chromatography (see section 2.2.2.6 for details). The purified protein was analyzed by SDS PAGE (Figure 7), according to which HpPutP falls within the range of 38 kDa. The molecular mass determined from the amino acid sequence (496aa) is 54.04 kDa, suggesting that molecular weight on SDS-PAGE underestimates, by about 16 kDa, the true molecular weight of the protein. The SDS-gel also revealed a band of aproximately 78kDa that could correspond to the dimeric state of HpPutP. Membrane transporter proteins exist in a complex dynamic equilibrium between various oligomeric states that include monomers, dimers, dimer of dimers and higher order oligomers. These higher order oligomerization states have been proposed as important factors that modulate the function (Veenhoff L M, *et al.* 2002). The amount of purified protein from 1 L culture was 0.37 mg and purity was between 90-95%.

3.1.2.2 Functional reconstitution of HpPutP

For further characterization of HpPutP and to test for the energetic requirements of L-proline transport, purified HpPutP was reconstituted into proteoliposomes. Liposomes were prepared from *E. coli* polar lipid extracts as described before for EcPutP (Jung H *et al.*, 1998). Destabilization of liposomes was achieved with Triton X-100, before addition of the protein, and the detergent was removed by stepwise addition of polystyrene beads (Figure 8).

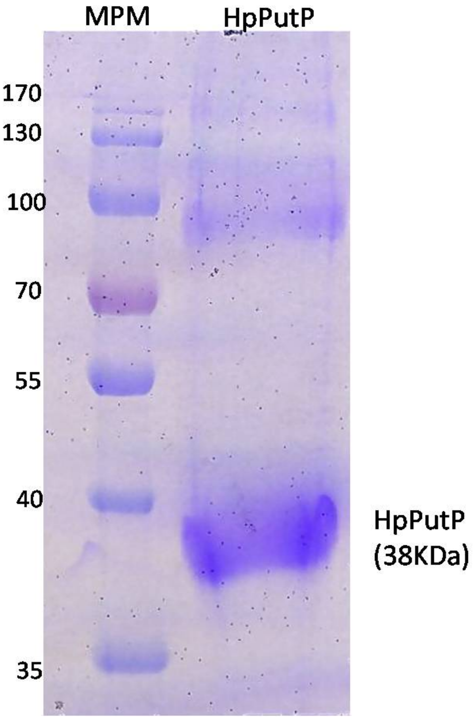


Figure 7. Purification of HpPutP heterologously expressed in *E. coli*. *E. coli* cells WG170 were transformed with the overexpression plasmid pT*HpputP6H*. After solubilization with 1.5% (w/v) *n*-dodecyl-β-D-maltoside and purification via Ni^{2+}-NTA affinity chromatography, protein was resolved using 10% SDS-PAGE and detected by Coomassie staining. HpPutP run at a M_r of around 38 kDa .

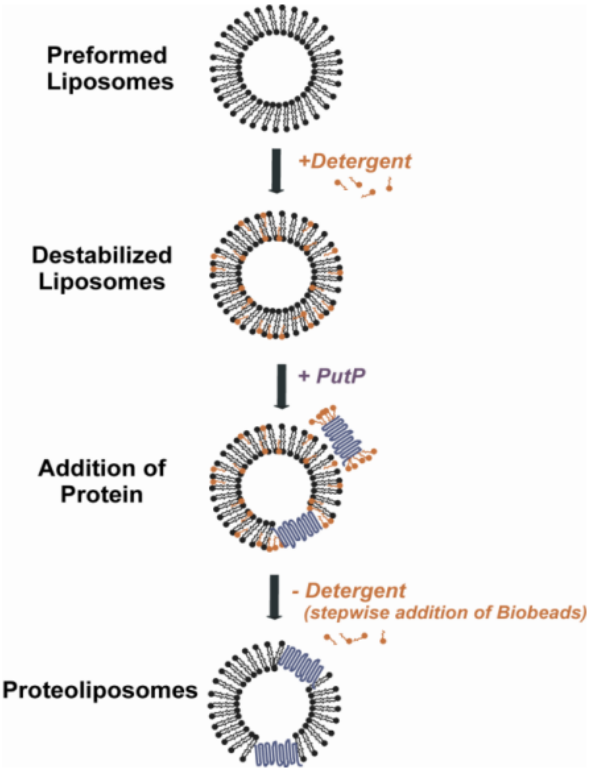


Figure 8. Process of protein reconstitution in proteoliposomes. The purified HpPutP was reconstituted into preformed, detergent-destabilized liposomes. Detergent was removed by adsorption to polystyrene beads in three steps at room temperature.

3.1.3 Ion dependency of L-proline uptake via HpPutP in proteoliposomes

Proline uptake was meassured in *E. coli* lipids. Ion and pH dependency were assessed with the help of the different buffers containing the different coupling ions. The cation-coupled symport activity was assessed by measuring ^{14}C-L-proline accumulation in proteoliposomes loaded with 100 mM KPi, pH 7.5. A membrane potential ($\Delta\Psi$) and desired ion gradients were imposed across the proteoliposome membrane by creating an outward-directed K^+ diffusion gradient in the presence of valinomycin and changing the ionic composition or pH of the dilution buffer. Results demostrated that the imposition of an electrochemical Na^+ or Li^+ gradient ($\Delta\mu_{Na+}$ or $\Delta\mu_{Li+}$) lead to accumulation of ^{14}C-L-proline in the proteoliposomes whereas the absence of a driving force did not give significant transport activity. In addition, analysis of ($\Delta\mu_{Na+}$) Na^+-driven transport at pH 6.0 did not reveal a significant difference in transport activity compared to meassurements at neutral pH (Figure 9).

Therefore, determination of Na^+-coupled (Na^+ motive force (smf)-driven) transport in proteoliposomes confirmed that proline can be transported coupled to Na^+, can be substituted by Li^+, but not by H^+.

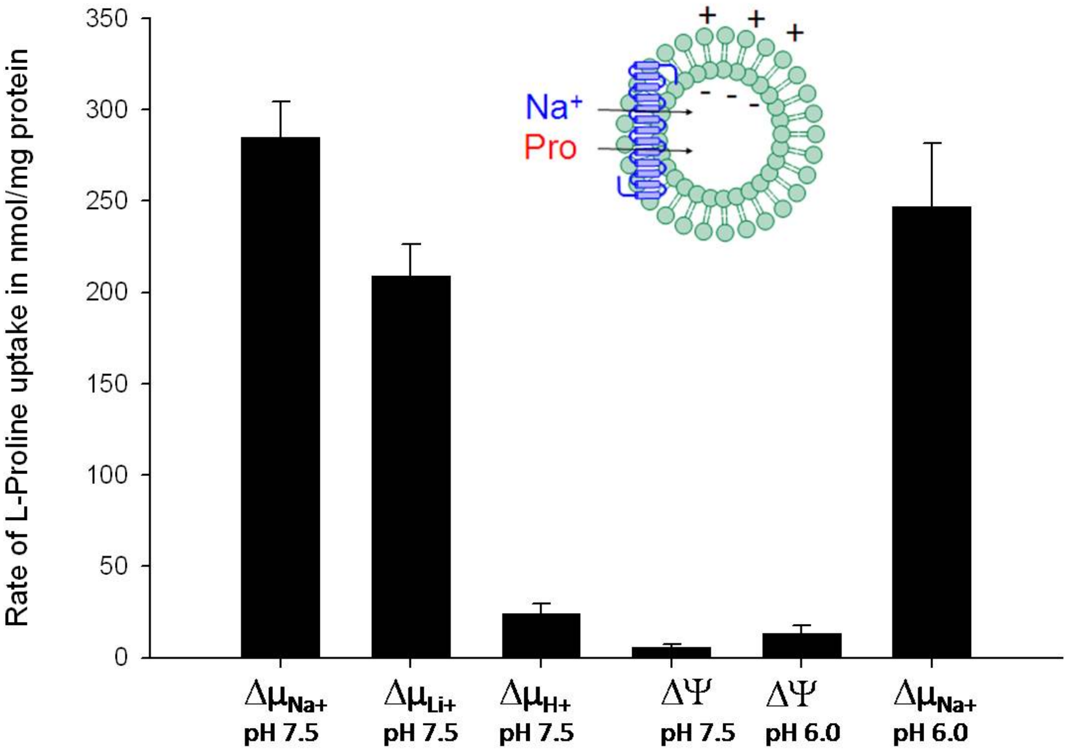
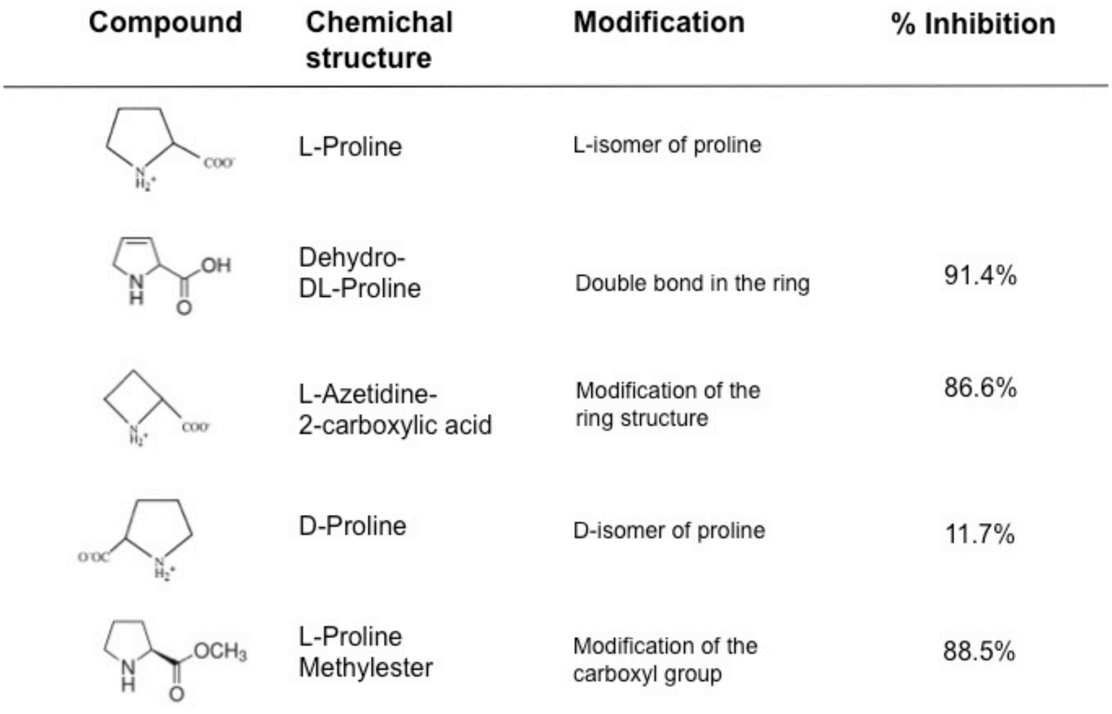


Figure 9. Energy requirements of L-proline uptake into proteoliposomes. Purified HpPutP was reconstituted in *E. coli* lipids and ion and pH dependency were assessed. For the reaction, 400µl of the buffers together with 10 µM ^{14}C- L- proline (26Ci/ mol) were incubated at 25°C for 5 min. immediately before starting the reaction, 1µl of proteoliposomes and valinomycin with an end-concentration of 0.2µM were added. The reaction was stopped after different time intervals by adding twice 3ml of cold calcium lithium stop buffer. The plot shows from left to right transport in the presence of: 50 mM NaCl and neutral pH ($\Delta\mu_{Na+}$ pH7.5); with 50 mM LiCl ($\Delta\mu_{Li+}$ pH7.5); in the absence of a coupling ion ($\Delta\mu_{H+}$); with no further additions ($\Delta\Psi$, interior negative); $\Delta\Psi$ with pH 6.0; and with 50 mM NaCl with slightly acid pH ($\Delta\mu_{Na+}$ pH 6.0)

3.1.4 Inhibition of proline transport by proline analogues in proteoliposomes

To further analyze the specificity of HpPutP, we tested several proline analogues with structures similar to L-proline (Table 18).

Table 18. Structures of proline analogues and effect on HpPutP activity



Compound	Chemichal structure	Modification	% Inhibition
	L-Proline	L-isomer of proline	
	Dehydro-DL-Proline	Double bond in the ring	91.4%
	L-Azetidine-2-carboxylic acid	Modification of the ring structure	86.6%
	D-Proline	D-isomer of proline	11.7%
	L-Proline Methylester	Modification of the carboxyl group	88.5%

Using the conditions optimized for L-proline transport by HpPutP, we determined the percentage of inhibition for each analogue in reconstituted proteoliposomes. The inhibition of the putative proline permease by proline analogues was determined by assaying the inhibition of ^{14}C-L-proline uptake by the unlabelled analogues. Our results showed that with all the different concentrations tested (10-100μM of the competing analogue), there were three strong inhibitors of proline transport: Dehydro-DL-proline, an analogue with a double bond in the ring structure, showed 91.4% inhibition; L-azetidine-2-carboxylic acid, an analogue with a different ring structure, showed 86.6% inhibition; and L-proline-methylesther, an analogue with alteration in the carboxyl group, showed 88.5% inhibition. D-proline showed almost no inhibition 11.7% of proline transport (Figure 10).

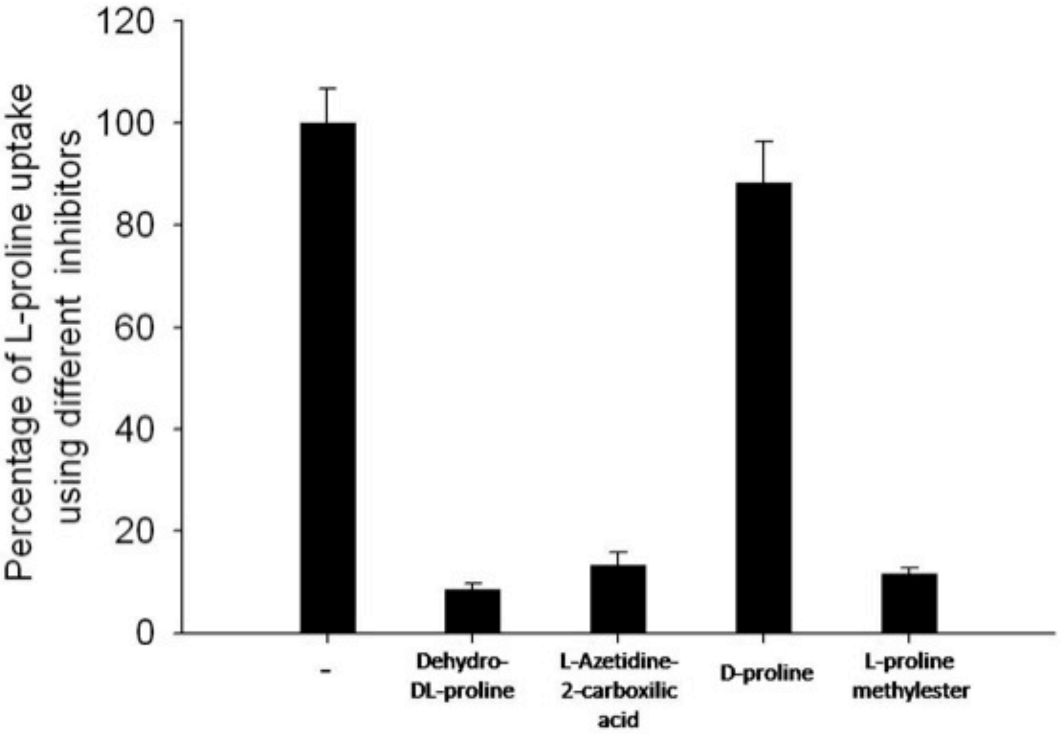


Figure 10. Inhibition studies of L-proline uptake in proteoliposomes. Transport was measured as L-proline uptake into proteoliposomes. The plot shows transport of 10μM ^{14}C L-proline in the presence of 100μM of the competing analogue. Bars represent from left to right transport in the presence of: 50 mM NaCl and neutral pH ($\Delta\mu_{Na+}$ pH 7.5) positive control; the same conditions as before adding the different inhibitors: Dehydro-DL-proline; L-Azetidine-2-carboxylic acid; D-proline; and L-proline Methylester.

Our results for the specificity of HpPutP provide insight into the structural requirements for substrate recognition. The structures of the analogues that are recognized or ignored by HpPutP suggest the following. (a) Only the L-isomer is recognized, indicating that the proline permease is stereospecific; (b) a 5-member ring structure preferentially interacts with the substrate binding pocket but a smaller 4-member ring molecule also fits into the substrate binding pocket of proline; and (c) modifications of the carboxyl group indicate that the charged carboxyl group is not essential.

3.1.5 Proline uptake kinetics in HpPutP expressed in *E. coli*

For further characterization of HpPutP in *E. coli,* the analysis of proline uptake was performed in whole cells. The plasmid *pTHpputP6H* was transformed in *E. coli WG170* (*putP* deletion mutant) for transport assays. Cells were resuspended and adjusted to a total protein concentration of 0.35 mg/ml. Transport of different ^{14}C L-proline concentrations (26 Ci/mol) was assayed under standard test conditions. The amount of substrate transported was determined by calculating the difference between two different time points (0 sec, 10 sec) for each L-proline concentration.

Reaction rate v [nmol/mg*min] values for the kinetics were calculated using SigmaPlot. In the Michaelis Menten graphic the reaction rate v is in function of the substrate concentration, in this case the proline concentration.

When assayed over a range of proline concentrations, this system showed Michaelis-Menten kinetics with a K_m of 1.3±0.5 µM and a V_{max} of 30.8±1.7 nmol/mg*min (Figure 11).

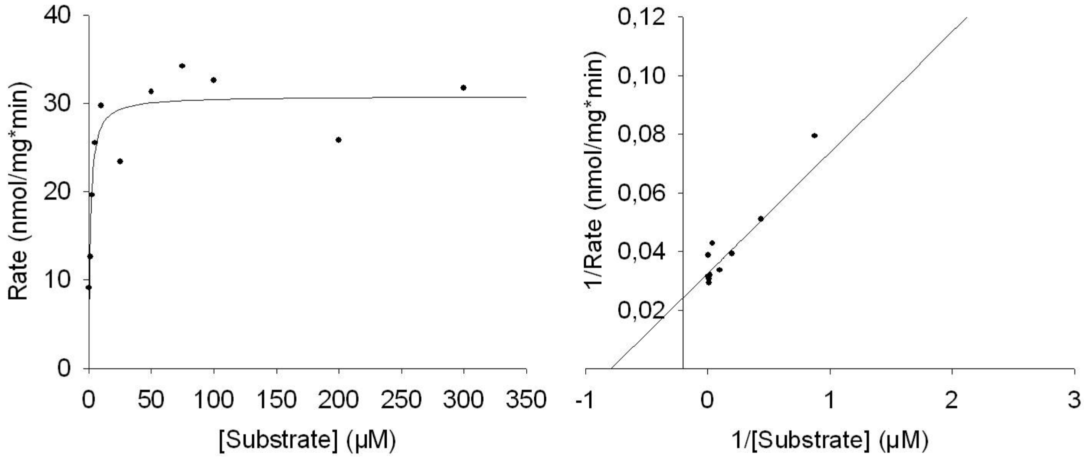


Figure 11. Kinetics of ^{14}C-L-proline uptake into *E. coli WG170* containing the plasmid *pTHpputP6H*. The reaction rate (nmol/mg protein) is plotted according to (**A**) Michaelis- Menten and (**B**) Lineweaver- Burk. The L-proline concentration was varied between 0.12 µM und 300 µM. According to Michaelis - Menten calculations V_{max} corresponds to 30.8±1.7 nmol/mg*min, and K_m to 1.3±0.5 µM.

From theses assay we conclude that heterologous expression of HpPutP was able to complement an *E. coli putP* deletion mutant, therefore HpPutP could be involved in the proline uptake in this bacterium

3.1.6 Functional important amino acids of HpPutP

Sequence alignment of HpPutP and EcPutP with CLUSTALO (1.1.0) revealed 50% identical amino acids (suppl. Figure 1). On the other hand, HpPutA and EcPutA share only 20% identical amino acids (suppl. Figure 2), and the amino acids found to be particularly important for EcPutP function are conserved in HpPutP. Previous studies and biochemical characterization identified various residues of EcPutP directly involved in Na^+ and/or L-proline binding and translocation (Olkhova E, et al. 2011; Hilger D *et al.,* 2008; Raba M, *et al.,* 2008; Hilger D *et al.,* 2009; Pirch T, *et al.,* 2002; Quick M and H Jung, 1997; Quick M *et al.,* 1996).

In order to test whether these residues are also of functional significance in HpPutP, these amino acids were replaced and transport activity was evaluated. Amino acids corresponding to D55, S57, Y140, W277, E311, S340, T341 of EcPutp (in *H. Pylori*; D58, S60, Y143, W280, E310, S399, T340) were substituted with Cys. Active transport was measured in *E. coli WG170* containing plasmid-encoding *HpputP* (*HPP12_0049*) with given nucleotide replacements.

Desired nucleotide substitutions in *HpputP* were generated by PCR with Phusion-DNA polymerase using plasmid *pTHpputP6H* as a template and synthetic mutagenic oligonucleotides (Table 3). Altered sequences were cloned into plasmid *pT7-5/HpputP*. Resulting plasmid DNA was verified by sequencing using an ABI 3730 device. Transport measurements showed that most of the substitution caused a significant inhibition of transport except for the mutant W280C (Figure 12).

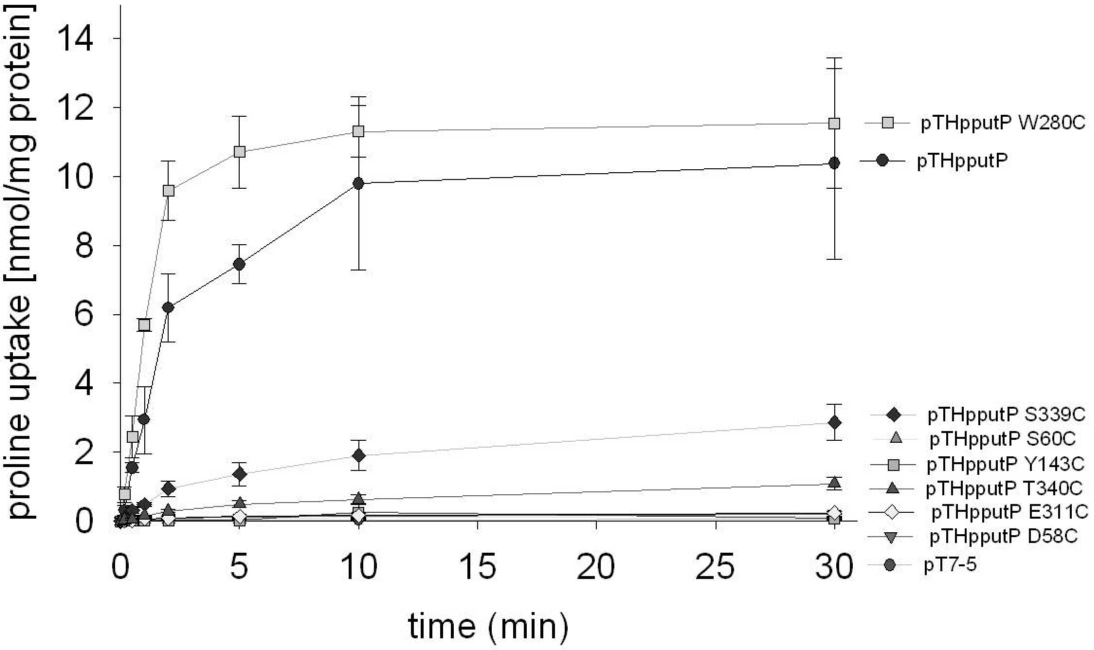


Figure 12. Influence of amino acid replacements in HpPutP on L-proline transport in *E. coli WG170* ($PutP^-A^-$). Cells were cultured as described in section 2.2.2.9.2. Time courses of proline uptake into intact cells of *E. coli* WG170 were assayed in the presence of 10 µM L-^{14}C-proline (26 Ci/mol), 50 mM NaCl, and 20mM D-lactate (Na-salt) as the electron donor at 25 °C under aerobic conditions. Transport assays were determinated at various time points (0, 0.17, 0.5, 1, 2, 5, 10, 30 min) using the rapid filtration method. Initial rates of transport were calculated from the initial linear portion of the time course. Standard deviations were determined from at least three independent experiments.

The initial rates and maximum accumulation of the mutants were highly reduced except for HpPutP containing the W280C substitution, which showed a higher activity compared to the wild type (Figure 13).

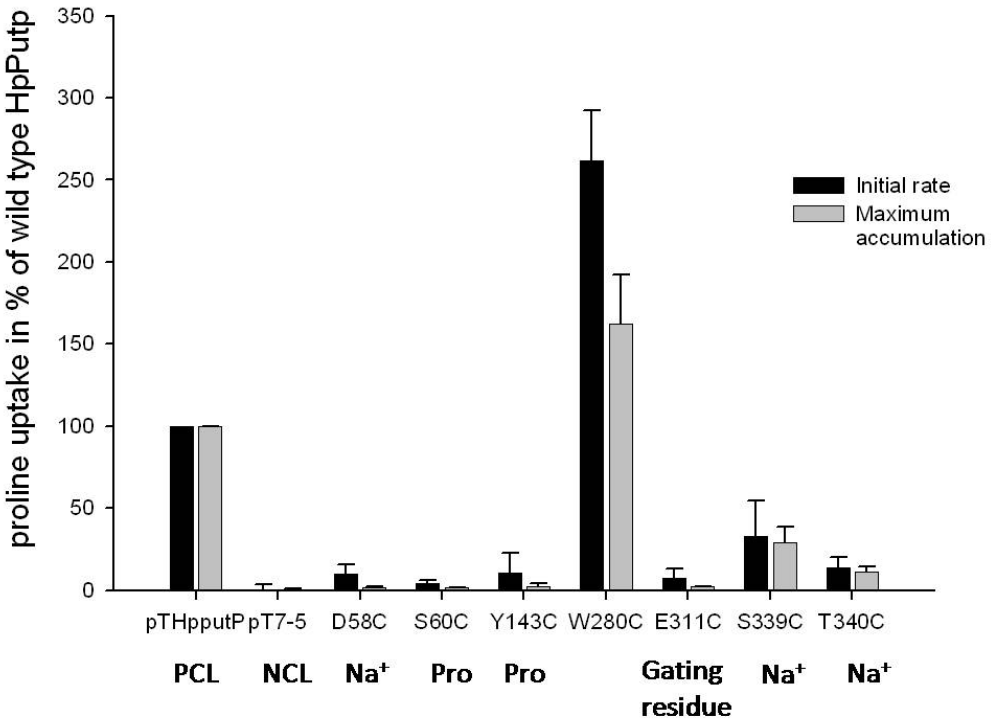


Figure 13. Initial rate and maximal accumulation of L-proline uptake in *E. coli WG170* (*$PutP^-A^-$*) encoding *HpputP* with given nucleotide replacements. Cells were cultured as described in section 2.2.2.9.2. Transport of 10 μM L-^{14}C-proline (26 Ci/mol) was assayed under standard test conditions in the presence of 20 mM D-lactate (Na$^+$ salt) and 50 mM NaCl. Transport assays were determinated using the rapid filtration method. Initial rates of transport were calculated from the initial linear portion of the time course. Standard deviations were determined from at least three independent experiments. PCL (positive control), NCL (negative control). Na$^+$ refers to the residues in charge for Na$^+$ recognition. Pro refers to the residues in charge for proline recognition.

A Western blot analysis revealed that these amino acid replacements resulted in amounts of transporter molecules in the membrane similar as the observed for HpPutP in the case of the D58C, W280C, E311C, S339C and T340C mutants. This indicates that diferences in proline uptake cannot be attributed to different amounts of transporter molecules in the membrane. Mutant S60C did not show activity even though the protein content was higher compared to the wild type. For the Y143C mutant, the lack of activity may be reduced in part due to the lower amounts of protein (Figure 14).

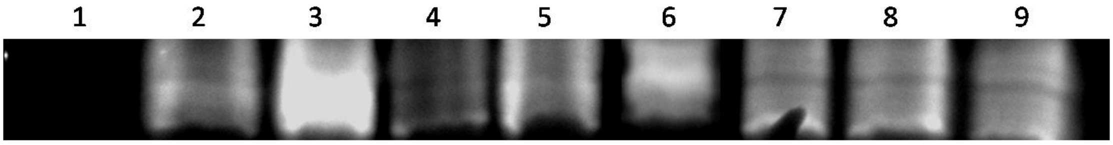


Figure 14. Western blot analysis of HpPutP with desired amino acid replacements expressed in *E. coli*. Cells were cultured as described in section 2.2.2.9.2. Twenty-five micrograms of total membrane protein of each mutant was separated by 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and probed with mouse anti-His-tag monoclonal antibody linked to horseradish peroxidase. Detection was performed according to the enhanced chemiluminescence method. Lanes correspond to: **1** pT7-5 without *putP* served as negative control, **2** D58C, **3** S60C, **4** Y143C, **5** W280C, **6** E311C, **7** S339C, **8** T340C, **9** plasmid pTHpPutP (Wt) served as positive control.

3.1.7 Homology model of HpPutP

We used protein modeling to predict the structure of HpPutP from its sequence to get more insights into the structure/function relationship of the transporter. This is a good alternative when high resolution structures are not available as in the case of EcPutP, HpPutP or any other L-proline transporting integral membrane protein.

Insight into the fold of a member of the SSS family was gained by the crystallization of the Na^+-dependent galactose symporter vSGLT of *Vibrio parahaemolyticus* (Faham S, *et al.* 2008). Based on this and the homology model of EcPutP (Olkhova E, *et al*. 2011) the homology model we designed of HpPutP was based on the crystal structure of vSGLT. According to our results and comparison of the generated HpPutP model with the recently published homology model of EcPutP, most of the residues analyzed either for Na^+ or proline binding overlap between HpPutP and EcPutP (Figure 15).

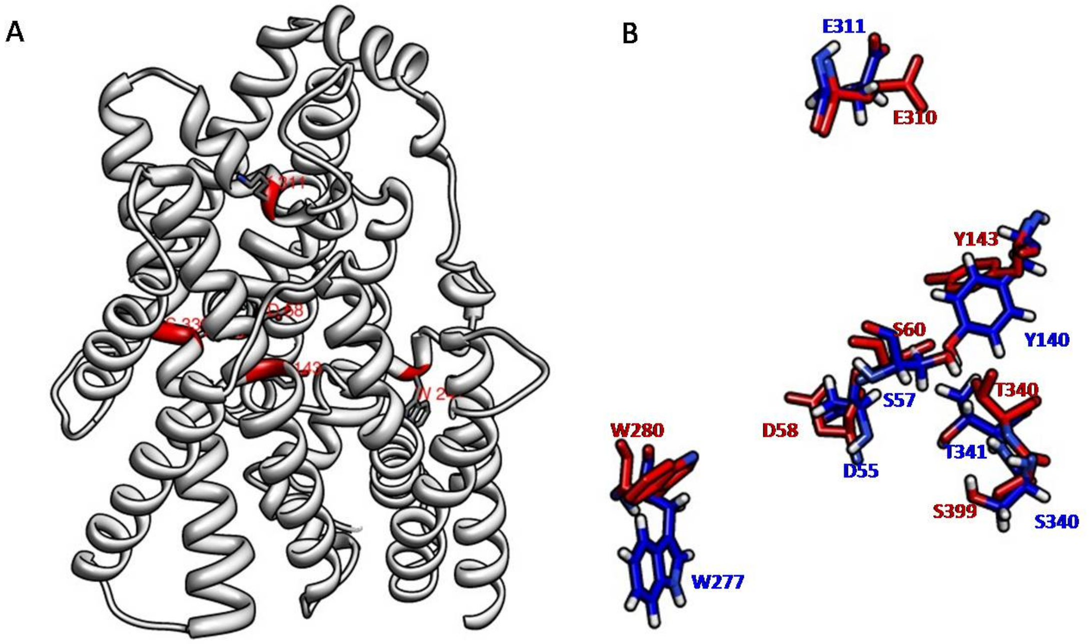


Figure 15. Tertiary structure model of HpPutP generated based on the crystal structure of vSGLT. A) Functional important residues in HpPutP are highlited in red. B) Overlay of important residues from HpPutP in red and EcPutP in blue. The figure was created with the program UCSF Chimera (Pettersen *et al.*, 2004).

3.2 Physiological relevance of HpPutP

3.2.1 Deletion of *HpputP, HpputA* and *HpputP/HpputA* of *H.pylori* strain P12 using a contraselection strategy

Due to the fact that handling of *H. pylori* needs special working conditions like containment level 2 facilities, equipment, and operational practices for work involving infectious or potentially infectious materials, the initial characterization of HpPutP was through heterologous expression in *E. coli*.

It is known that expression of genes in heterologous organisms has allowed the isolation of many important genes (e.g. for nutrient uptake and transport) and has contributed a lot to the functional analysis of the gene products (Frommer W B, and O Ninnemann, 1995). However characterization and confirmation of the function of HpPutP in *H. pylori* was needed, consequently, first, culture conditions were standardized for the subsequent construction of the deletion mutants.

The generation of the *H. pylori* mutants was based on the Dailidiene D, *et al.,* 2006 procedure. This consists of a two-gene cassette (1.5 Kb) containing the *Campilobacter jejuni rpsL* gene (dominant streptomycin susceptibility) upstream of an erythromycin resistance gene (Figure 16), flanked by *H. pylori* sequences that normally flank the *HpputP*, *HpputA* or both *HpputP/HpputA* genes. The suicide plasmid *pBluescript* II SK^+ ($pBSK^+$) was used for cloning procedures in *E. coli* and transformed in *H. pylori P12*. *H. pylori* mutants were obtained by natural transformation according to Haas R, *et al.,* 1993.

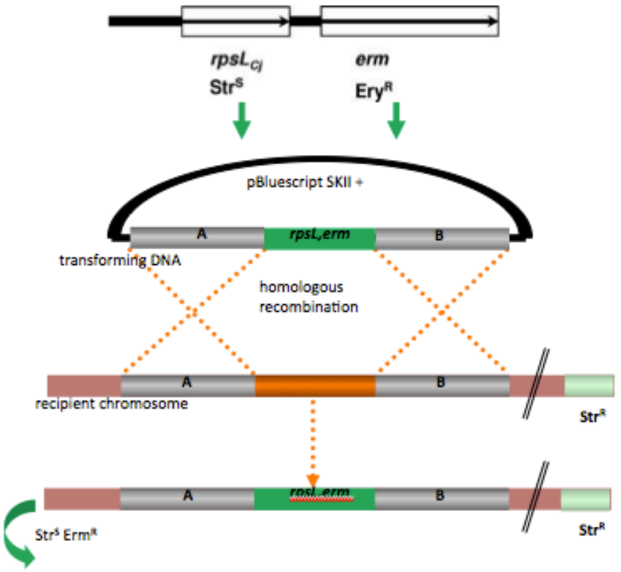


Figure 16. Contraselection strategy for the generation of the *H. pylori* deletion mutants. A two-gene cassette (*rpsLerm*) confering erythromycin resistance and stepromycin susceptibility was inserted in the middle of the flanking regions of the selected gene and cloned into the suicide vector *pBluescript* SK^+. Upon transformation in *H. pylori* the transforming DNA was transferred to the recipient chromosome by homologous recombination in regions of sequence homology. This results in the replacement of the original sequence for the *rpsLerm* cassette and a change in the bacteria phenotype from Erm^r to Erm^s (Dailidiene D, *et al.,* 2006).

PCR tests showed that the Erm^r Str^s transformants tested contained the expected *rpsLerm* cassette in the right place of the full-length gene (Figure 17). Colonies were streaked to streptomycin/erythromycin-containing agar to confirm the desired phenotype and finally DNA sequence confirmed the deletion in the right genome position.

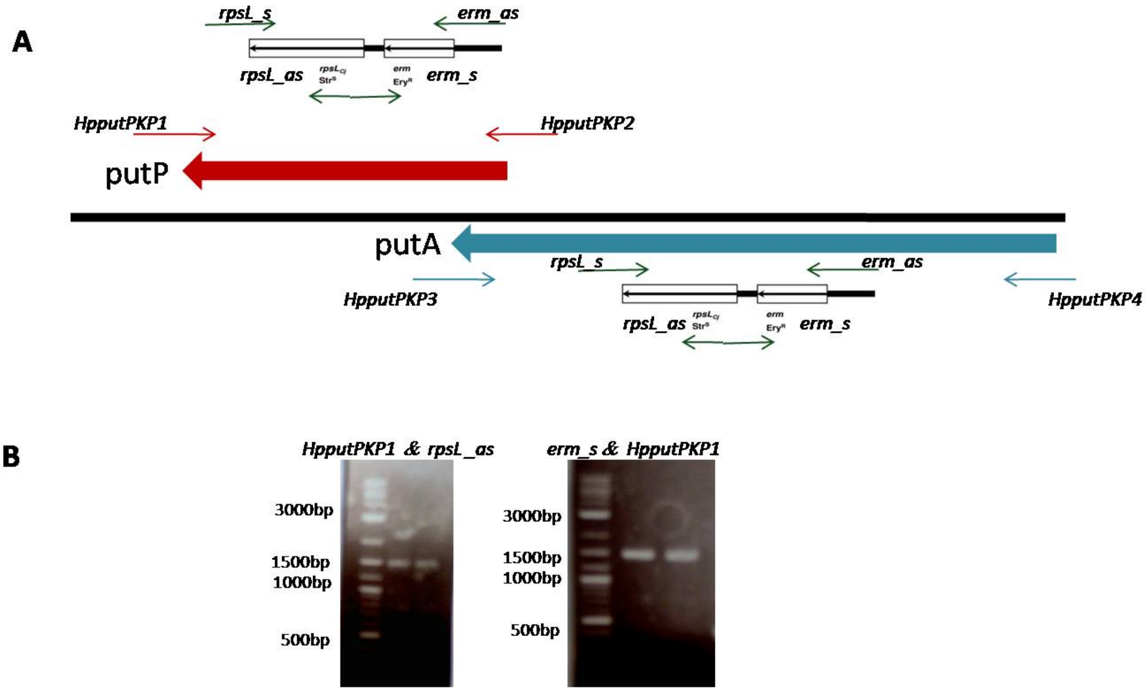


Figure 17. PCR tests performed to confirm the *Erm*^{r} *Str*^{s} transformants in *H. pylori.* (A) To confirm the insertion of the two-gene cassette (*rpsLerm*) in the middle of the flanking regions of the selected gene (*HpputP, HpputA* or in between the two genes), PCR tests from genomic DNA were performed (using primers: *rpsL_s* & *erm_as* or *erm_s* & *rpsL_as*). PCR products containing full-length of *HpputP* (primers *HpputPKP1 & HpputPKP2*), *HpputA* (primers *HpputPKP3 & HpputPKP4*) and *HpPutAP* (primers *HpputPKP1 & HpputPKP4*) were also confirmed. Furthermore the right insertion of the cassete was analyzed with PCR (using primers *HpputPKP1 & rpsL_as* and *HpputPKP2 & erm_s*) for the *HpputP* gene, (*HpputPKP3 & rpsL_as* and *HpputPKP4 & erm_s*) for the *HpputA* gene, and finally (*HpputPKP1 & rpsL_as* and *HpputPKP4 & erm_s*) for the double deletion mutant. In **(B)** PCR products containing half-length of the *HpputP* gene are shown, confirming the right insertion of the *rpsLerm* cassette in the Δ*HpputP* mutant. The Δ*HpputA* and double deletion mutant were confirmed using the same strategy.

3.2.2 Phenotypic characterization of Δ*HpputP*, Δ*HpputA* or Δ*HpputP/HpputA* mutants

3.2.2.1 Growth curves

Culture of *H. pylori* is the most accurate way of assessing viability and for analyzing differences between strains (Saito N *et al.,* 2003). For the phenotypic characterization of the *H. pylori* deletion mutants, growth was evaluated. Growth over time was assessed by measurement of OD$_{600}$ nm using 1 ml samples in 24 well plates. The Δ*HpputP*, Δ*HpputA* or Δ*HpputP/HpputA* mutants had growth rates comparable to the growth rate of the parental strain under standard conditions (Figure 18).

Furthermore, growth curves using increasing L-proline concentrations in the culture medium did not reveal significant differences of the mutants compared to the wild type (Figure 19).

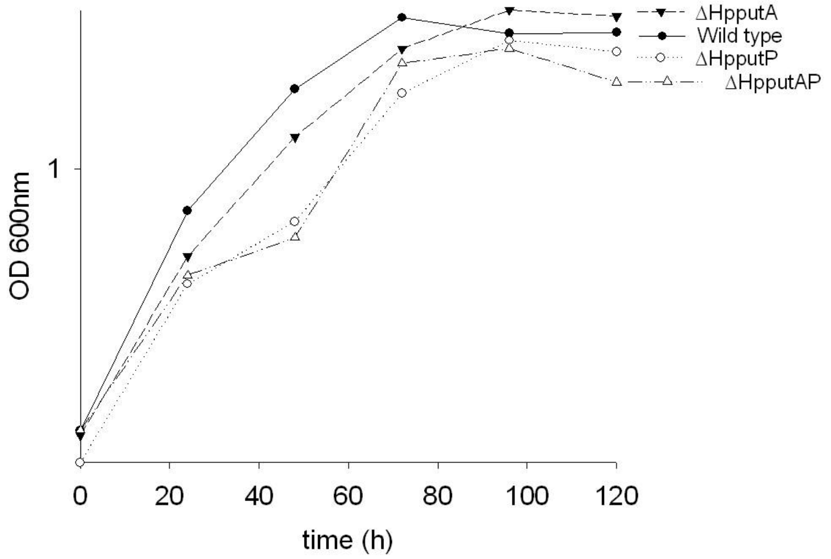


Figure 18 Growth curves of the deletion mutants *H. pylori* Δ*HpputP*, Δ*HpputA* or Δ*HpputP/HpputA* compared to the Wt in complete medium. Inocula was recovered from 48h agar plates and resuspended in Brucella Broth medum (BB). Cells were adjusted to 1 x 10^8 cells/ml (OD_{600} of 0.2) in 24-well plates with gentle shaking (100 r.p.m.). OD measuremens were performed over 5 days of culture.

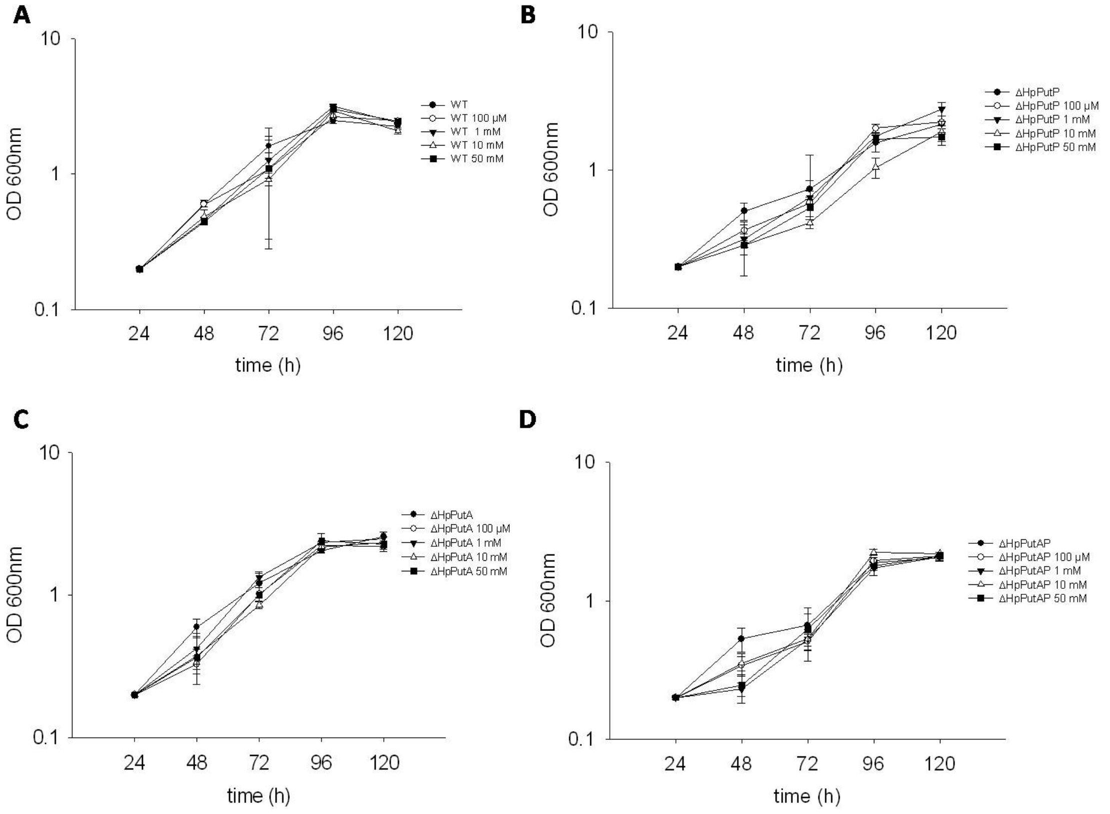


Figure 19. Growth curves of *ΔHpputP, ΔHpputA, and ΔHpputA/P* compared to *H pylori P12* exposed to different L-proline concentrations in the growth media. Inocula were grown for 48h on agar plates then resuspended in Brucella Broth media, and standardized to 1 x 10^8 cells/ml (OD_{600} of 0.2). Different L-proline concentrations were applied to the medium (0μM, 100μM, 1mM, 10mM and 50mM) for each strain and OD was recorded every 24h. A) Wild type; B) *ΔHpputP*; C) *ΔHpputA* and D)*ΔHpputAP.*

In addition, we tried to reproduce the defined medium described by Reynolds D J and C W Penn in 1994. These media was reported to support the growth of different strains in the presence of amino acids and BSA. A medium in which all components are chemically defined would facilitate metabolic studies of *H. pylori*. However our results showed half of the growth than the one obtained with the complex medium and OD values decreased dramatically after 72h (Figure 20).

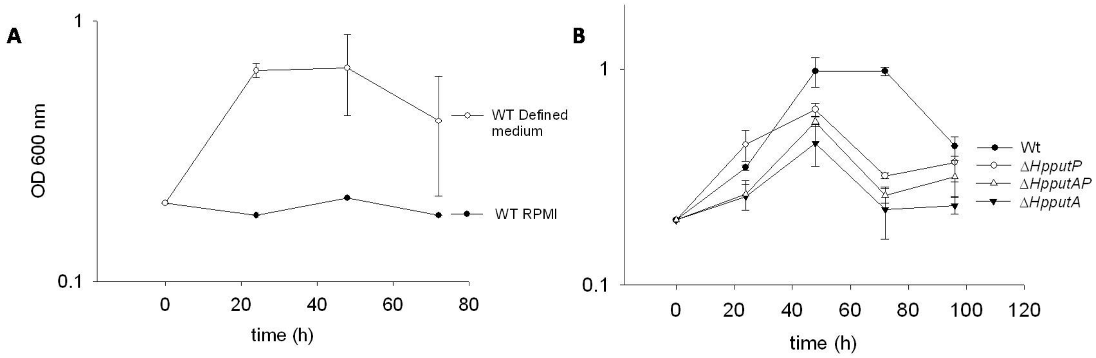


Figure 20. Growth patterns of *H. pylori* in a defined medium. Inocula were grown for 48h on agar plates then resuspended in RPMI media. Cells were standardized to 1 x l0^8 cells/ml (OD$_{600}$ of 0.2). RPMI medium was suplemented with a mixture containing amino acids, vitamins and BSA. OD 600nm was recorded every 24h. A) Wild type growth with RPMI or RPMI suplemented with the mixture previously described (defined medium); B) Growh of the *ΔHpputP, ΔHpputA*, and *ΔHpputAP* mutants compared to the wild type in defined medium.

3.2.2.2 Electron microscopy observation of flagella of *H. pylori* wild type and deletion mutants

In 2008 Nakajima and collaborators showed that a *putA* disrupted mutant, Δ*HpputA*, did not show respiratory activity using L-proline, no motility in response to amino acids and no swarming activity. As a result of these deficiencies, the mutant lost its ability to colonize the stomach of nude mice. These findings indicate that *putA* may play an important role *in H. pylori* colonization on the gastric mucus layer. This is similar to the data reported by Kavermann H*, et al.,* in 2003 regarding the loss of infectivity by the putative proline transporter in *H. pylori*, HpPutP.

Since the motility is affected in the mutant, morphology changes could be involved in the infectivity capacity of the mutant. Consequently, electron microscopy was proposed as a good evaluation method. To investigate the impact of *HpputP* deletion on the morphology of *H. pylori* cells, bacteria were cultured and harvested as for transport experiments. Cells were collected and fixed and samples were prepared and examined by Prof. Dr. Gerhard Wanner.

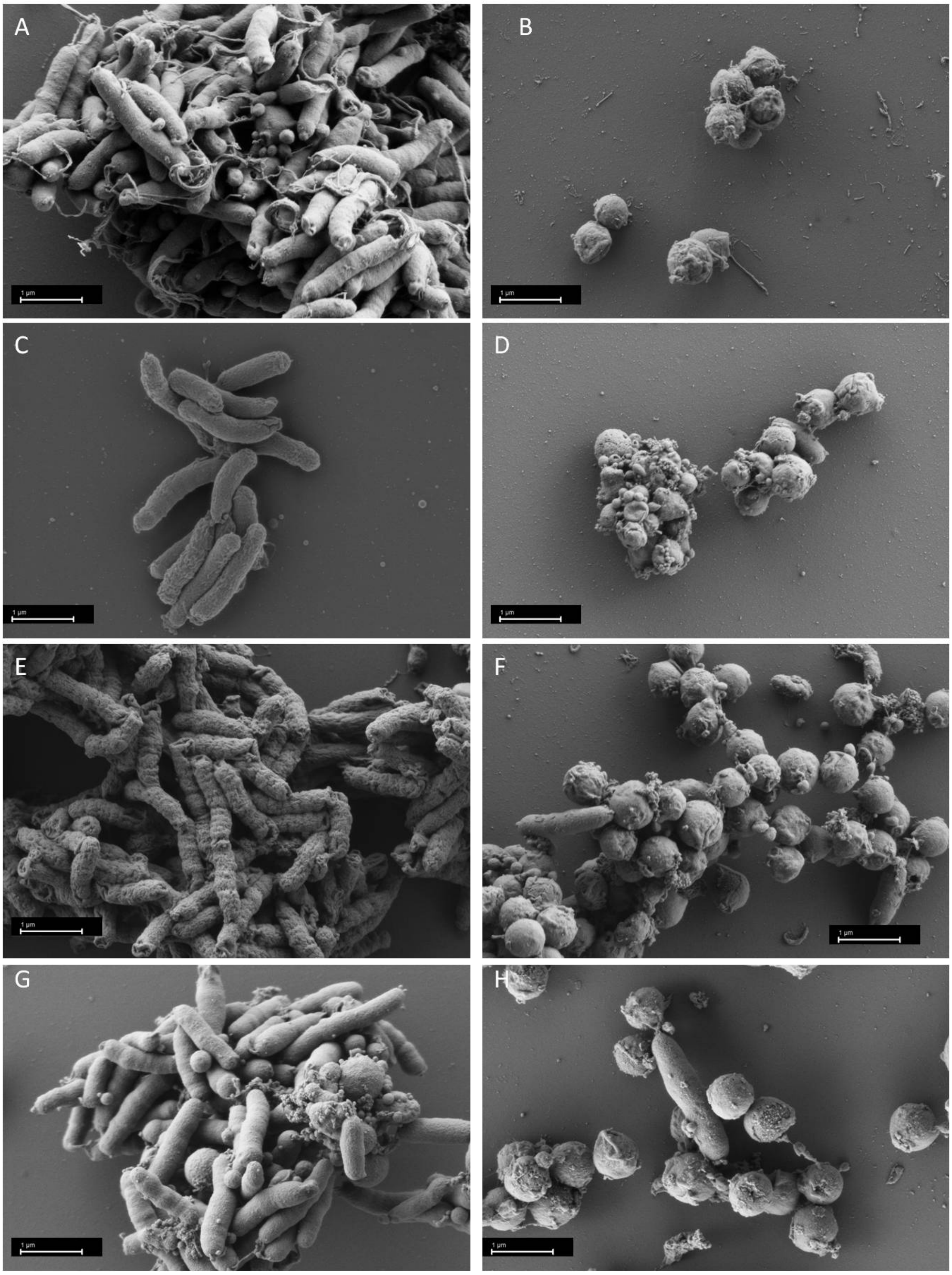


Figure 21. Electron microscopy pictures of *H. pylori* wild type and deletion mutants. Scaning electron microscopy of (A) bacillary form of *H. pylori* P12 (Wt) at day 2 in logaritmic phase; (B) coccoid form of the Wt at day 4 in stationary phase; (C) bacillary form of *H. pylori* P12 Δ*HpputP* at day 2 in logaritmic phase;(D) coccoid form of the Δ*HpputP* at day 4 in stationary phase ;(E) bacillary form of *H. pylori* P12 Δ*HpputA* at day 2 in logaritmic phase; (F) coccoid form of the Δ*HpputA* at day 4 in stationary phase ; (G) bacillary form of *H. pylori* P12 Δ*HpputAP* at day 2 in logaritmic phase; (H) coccoid form of the Δ*HpputAP* at day 4 in stationary phase (electron microscopy by Prof. Dr. Gerhard Wanner).

Scanning electron microscopy (SEM) analysis revealed a clear difference in the morphology of the mutants compared to the wild type (Figure 21). The wild type showed a bundle of polar flagella at the logarithmic phase as expected (Figure 21A). On the contrary, at the same growth phase the three mutants lost their flagella (Figure 21C, 21E, 21G). Since the motility of *H. pylori* depend on the flagella, this result could explain the loss of infectivity when these genes are absent in *H. pylori*. Furthermore, we confirmed the coccoid conversion from all the strains at the stationary phase (day 4) (Figure 21B, 21D, 21F, 21H) as described by Benaissa M, *et al.* in 1996.

The deletion mutants were further characterized by motility assay and they showed no motility compared to the wild type, confirming the previous observations of the electron microscopic images (Figure 22).

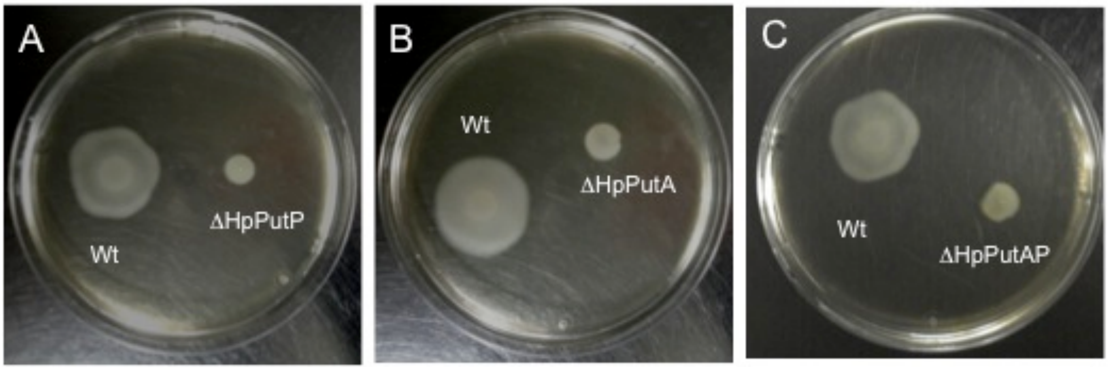


Figure 22. Assesment of motility in *H. pylori* wild type and deletion mutants. *H. pylori* cells were cultured in plates for 48 h, resuspended in BB supplemented with 10% horse serum and adjusted to bacterial concentrations of 1x 10^8 cells per ml (OD_{600} of 0.2). Bacterial cells were stabbed with toothpicks into plates containing brucella broth with 0.3% agar and incubated microaerobically at 37°C for 5 days. (A) Wild-type strain and Δ*HpputP;* (B) Wild-type strain and Δ*HpputA;* (C) Wild-type strain and Δ*HpputAP*.

3.2.3 Complementation of the mutants Δ*HpputP*, Δ*HpputA* and Δ*HpputP/HpputA* mutants

In order to test whether the deletion of Δ*HpputP,* Δ*HpputA* and Δ*HpputP/HpputA* is responsible of the loss of the flagella in the deletion mutants, complementation was necessary. *E. coli-H. pylori* shuttle vectors are available and can replicate autonomously in *H. pylori* (Heuermann and Haas, 1998). Prof. Dr. Rainer Haas (MvP, LMU Munich) kindly provided us a shuttle vector (*pIB6*) (Figure 23) which allows the cloning of *H. pylori* genes in *E. coli* without expressing them in this heterologous system. This is due to the harboring of a constitutive *H. pylori*-specific promoter (*alpA*-Promoter) which is not active in *E. coli* (Barwig I, 2009).

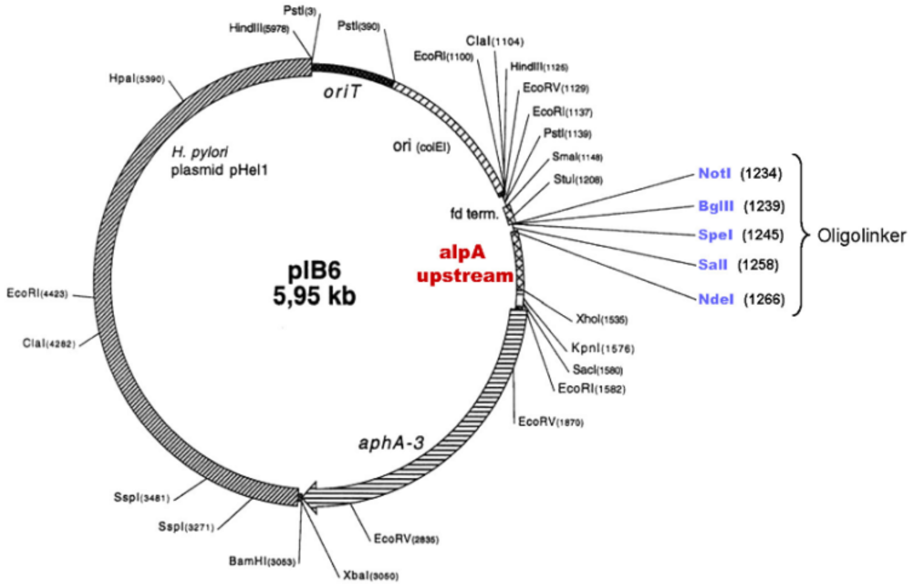


Figure 23. Shuttle-Vector *pIB6.* The Shuttle- Vector *pIB6* was constructed based on the plasmid *pHel3*. It contains a portion of a cryptic *H. pylori*-Plasmids, a kanamycin resistence, a *ori*T for conjugation, a *ori*(colEI) as replication origin in *E. coli*, the upstream region and the promoter of the *alpA* and *alpB genes from H. pylori* and a oligolinker with the restriction sites for the enzymes *NdeI*, *SalI*, *SpeI*, *BglII* and *NotI* (Barwig I, 2009).

HpPutP-GFP fusions were generated in the shuttle vector *pIB6* to monitor and visualize the expression of this membrane protein. It is known that protein tags such as luciferase, *β*-galactosidase (*β*-gal) and green fluorescent protein (GFP) can be detected through their innate activity. GFP has the advantage that it is stable for months, can be visualized easily via standard confocal or fluorescent microscopy and can be detected in living cells (Lippincot-Schwartz J, 2003).
We used this stable fusion protein to detect HpPutP in *H. pylori* by observing the fluorescence. Different plasmids were generated using either a constitutive promoter (*alpA*) or the putative native *HpputP* promoter to evaluate complementation (Figure 24). Plasmids with the different promoters harboring *HpputP* but without *GFP* were constructed in case that the fusion of *GFP* would interfere with the right localization of the protein.

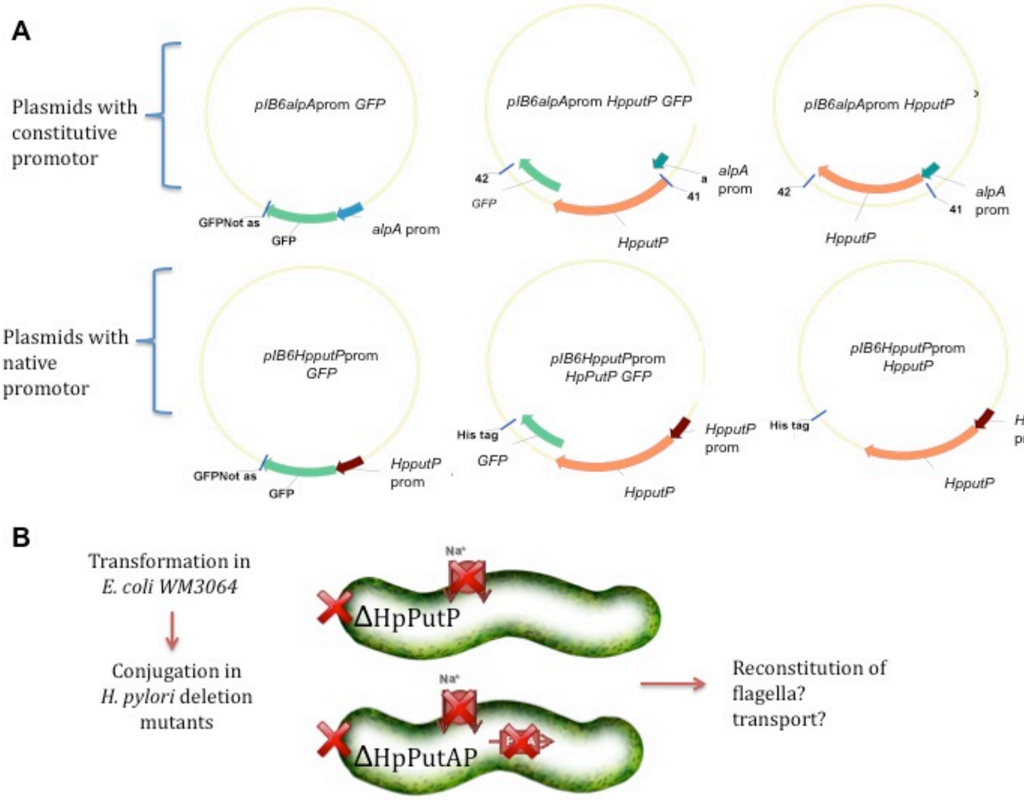


Figure 24. Cloning strategy for evaluation of the function of the expression system and expression of *HpputP* in the deletion mutants. (A) The Shuttle-Vector *pIB6* was used to construct the complementation plasmids for *H. pylori* deletion mutants. *GFP* was fused directly behind of the constitutive *alpA* promoter or to the putative native promoter of HpputP to evaluate the expression in the system (control). Expression of GFP fused to the C-terminus of HpputP was also evaluated when fused to the two different promoter regions. And finally, *HpputP* was also fused to both promoters without the reporter gene. **(B)** Plasmids were individually transformed in *E. coli WM3064* and afterwards introduced to *H. pylori* deletion mutants *ΔHpputP* and *ΔHpputAP* by conjugation. Colonies were striped in plates containing the resistance antibiotic (Kanamycin) for selection.

Representative samples of the recovered colonies after conjugation are shown in Figure 25. Fluorescent microscopy pictures showed GFP expression in the mutants harboring the plasmids containing GFP and no GFP expression was detected in the wild type where no plasmid was introduced (Figures 25A-C). The expression of GFP and therefore of HpPutP in the deletion mutants containing the HpPutP-GFP fusion was comparable when the constitutive (Figures 25D-F) or the native promoter (Figures 25G-I) were present, indicating no additional requirement for the function of the native promoter in our culture conditions.

The subcellular localization of GFP in the deletion mutants containing the plasmid *pIB6* with *GFP* immediately downstream of the constitutive or native promoter (*pIB6 alpA GFP, pIB6 HpputP*prom *GFP*) was in the cytoplasm (Figure 26A). For the constructs containing either the constitutive or native promoter with the HpPutP-GFP fusion (*pIB6 alpA HpputP GFP, pIB6 HpputP*prom *HpputP GFP*), GFP was localized at the membrane (Figure 26B).

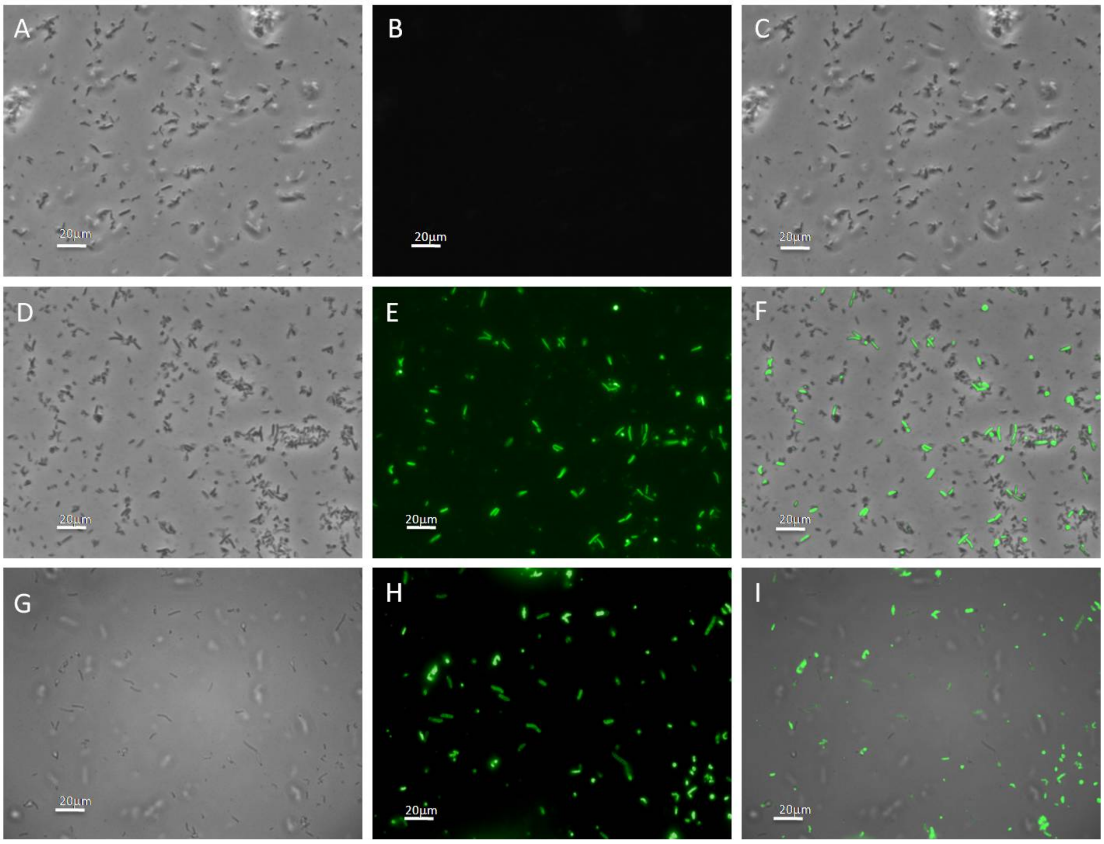


Figure 25. Fluorescent microscopy of the wild type and complemented mutants. *H pylori P12 in* **(A)** phase contrast; **(B)** GFP; **(C)** overlay. The deletion mutant, *ΔHpputP*, harboring *pIB6 alpA GFP* (positive control of the expression system in *H. pylori*) in **(D)** phase contrast; **(E)** GFP; **(F)** overlay. Deletion mutant, *ΔHpputP*, complemented with *pIB6 alpA HpputP GFP* in **(G)** phase contrast; **(H)** GFP; **(I)** overlay.

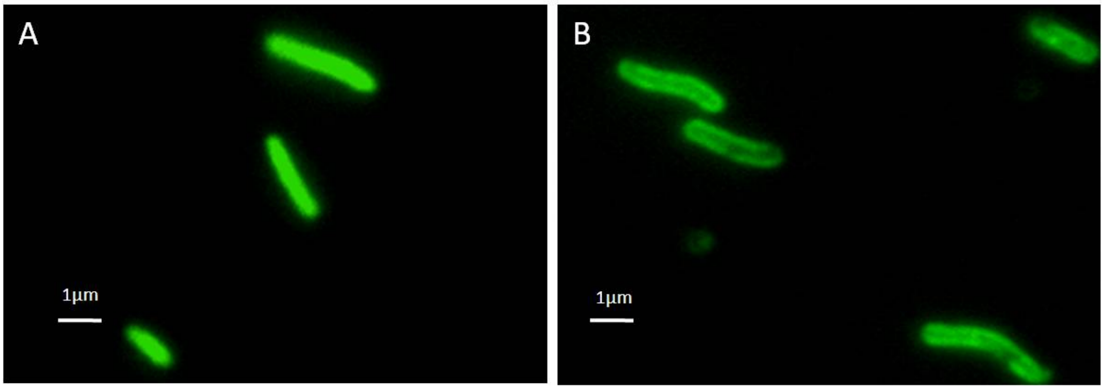


Figure 26. Fluorescent microscopy of *H. pylori ΔHpputP* deletion mutant harboring a shuttle vector containing GFP or HpPutP-GFP fusion. Samples were taken from liquid cultures in the logarithmic phase and fixed as described in section 2.2.4.1. **A)** Cytoplasmic localization of GFP in the deletion mutant *ΔHpputP*, harboring *pIB6 alpA GFP*; **B)** Membrane localization of the HpPutP-GFP fusion in the deletion mutant, *ΔHpputP*, harboring *pIB6 alpA HpputP GFP.*

In addition, the expression of HpPutP in the *H. pylori* complemented mutants was evaluated by Western blot analysis where the expected size of recombinant protein was confirmed (Figure 27). HpPutP has a molecular weight of 54,036 kDa and GFP of 27 kDa, therefore the HpPutp-GFP fusion protein has a predicted molecular weight of 81 kDa. In the Western blot analyses, HpPutp-GFP runs with about 65 kDa, 16 kDa below the theoretical size. This is again due to the typical faster migration of membrane proteins observed in SDS-Gels. Nevertheless, the size of 65 kDa observed for the fusion protein is in full agreement with the experimental molecular weight of the transporter (38 kDa) alone plus the weight of GFP (27 kDa).

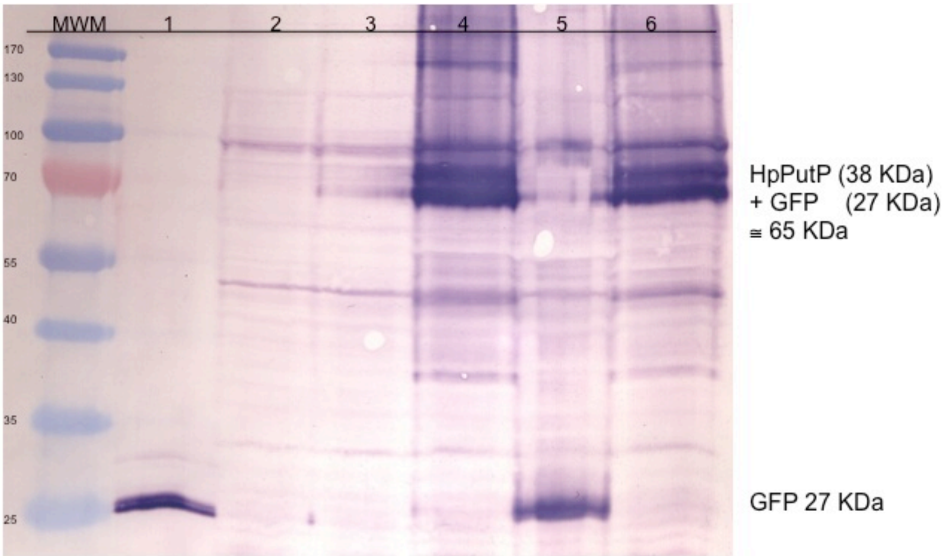


Figure 27. Western blot analysis of HpPutP in *H. pylori*. *H. pylori* samples were prepared from liquid cultures in the log phase. Cells were disrupted by sonification and membranes were loaded on a SDS-PAGE gel. Polyacrylamid gel was transferred to a nitrocellulose membrane and then incubated with anti-GFP antibody and finally developed by Alkaline phosphatase. MM: molecular weight marker, Lane 1: Pure extract of GFP which is a 27 kDa protein (positive control), Lane 2: wild type (negative control), Lane 3: Double deletion mutant (negative control), Lane 4: *ΔHpputP* mutant complemented with *pIB6 HpputP-GFP*, Lane 5: Deletion Mutant expressing GFP, Lane 6: *ΔHpputAP* mutant complemented with *pIB6 HpputP-GFP.*

3.2.4 Proline transport in *H. pylori*

Transport of L-proline by HpPutP in *E. coli* was demonstrated above, however L-proline uptake in *H. pylori* needed to be tested. Therefore transport measurements were performed as previously described for *E. coli* but with the respective adjustments to the protocol as mentioned in material and methods in section 2.2.2.9.4. Analysis of the time course of proline uptake revealed active transport activity in *H. pylori* (Wt) cells compared to the deletion mutants (*ΔHpputP* and *ΔHpputAP*) where transport was not detected (Figure 28). The complemented mutants were also assessed in order to corroborate that the proline uptake was due to the loss of the *HpputP* gene. Deletion mutants harboring the plasmid *pIB6 alpA HpputP* were tested and results indicate that deletion mutant cells harboring the plasmid were able to transport the amino acid almost like the wild type (Figure 28), while those devoid of it did not show any activity.

The increase of proline uptake at the beginning of the reaction is linear up to 3 minutes and the maximum accumulation of the measured points was after 10 minutes. Initial rates of transport were taken from the initial linear portion of the time course (1 min). Most of the initial rate values were around 1.2 nmol/mg*min and 9 nmol/mg for the maximum accumulation. The complemented mutant showed a maximum accumulation of 80% compared to the wild type (Figure 29).

Our results demonstrated that HpPutP is the proline transporter, allowing proline-specific incorporation into the cells. Thus, we could prove that HpPutP, which was previously found to be essential for colonization in the Mongolian gerbil model (Kavermann H, 2003), is the solely proline transporter in *H. pylori*. under the condition tested.

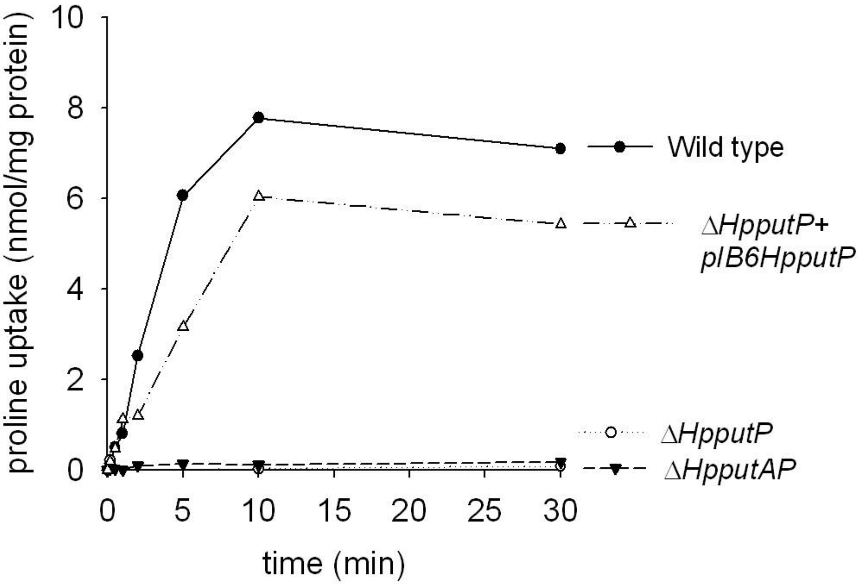


Figure 28. Time course of active proline uptake in *H. pylori* cells. Measurements were performed as described in section 2.2.2.9.4. The transport data corresponds to 200 µl of a cell suspension which was afterwards incubated at 37°C shortly before use. Reactions were performed with simultaneus addition of 50 mM NaCl and 10 µM ^{14}C-L-proline (26 µCi/µmol) and stopped after different time intervals(0 sec, 10 sec, 30 sec, 1 min, 2 min, 5 min, 10 min, 30 min) with a total of 6 ml potassium-lithium-Buffer. From the filters used, the accumulated proline in the cells was determined in a ß- Counter.

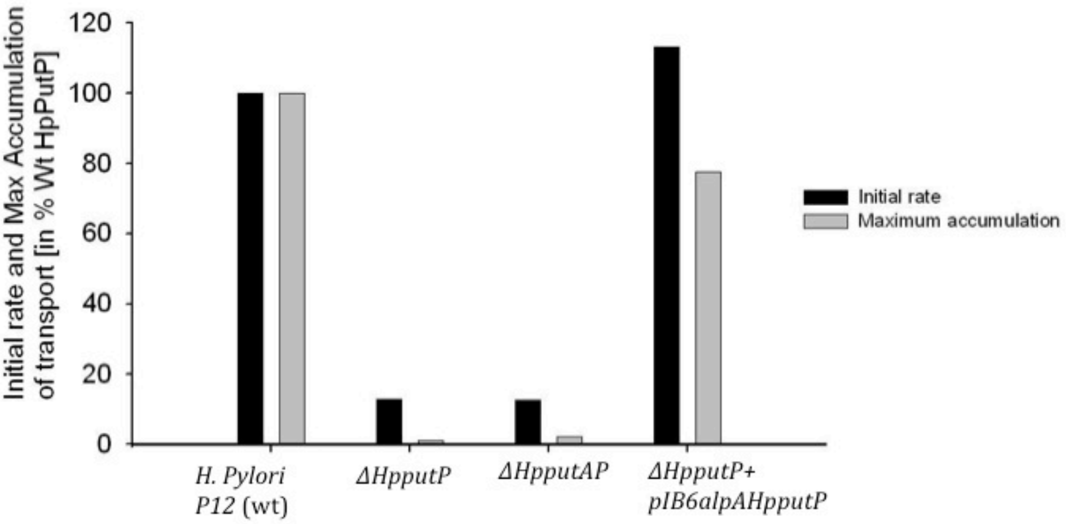


Figure 29. Initial rate and Maximum accumulation of proline uptake in *H. pylori* cells. Measurements were performed with *H. pylori* cells as described in section 2.2.2.9.4. The transport data corresponds to 200 µl of a cell suspension which was afterwards incubated at 37°C shortly before use. Reactions were performed with simultaneus addition of 50 mM NaCl and 10 µM [U-14C]-L-proline (26 µCi/µmol) and stopped after different time intervals(0 sec, 10 sec,

30 sec, 1 min, 2 min, 5 min, 10 min, 30 min) with a total of 6 ml potassium- lithium- Buffer. From the filters used, the accumulated proline in the cells was determined in a ß- Counter.

3.2.5 Michaelis-Menten kinetics of proline into *H. pylori*

Kinetic analysis of HpPutP in *H. pylori* revealed K_m values of 19.4±6.9 µM and a V_{max} of 2.6±0.26 nmol/mg*min (Figure 30). These results seem to correspond to a low affinity binding. The proline concentrations tested were in the range of 0.12 µM to 250 µM. Furthermore the proline kinetics showed a biphasic behavior that could represent two binding sites in HpPutP.

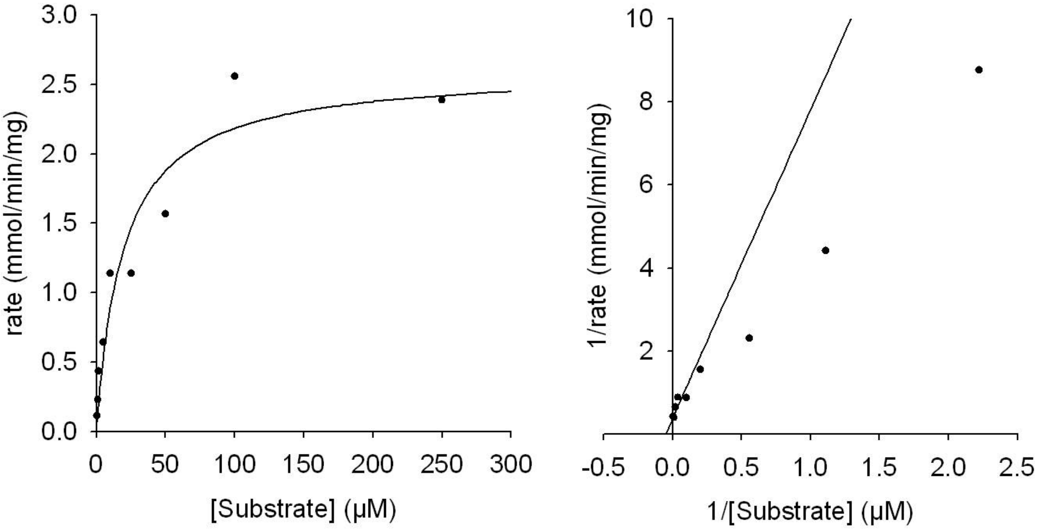


Figure 30. Kinetics of ^{14}C-L-proline uptake into *H. pylori P12*. The reaction rate v (nmol/mg min) is plotted (**A**) as Michaelis- Menten, (**B**) Lineweaver- Burk versus the L-proline-concentration, which lay between 0,12 µM and 250 µM. According to Michaelis - Menten calculations V_{max} corresponds to 2.6±0.26 nmol/mg*min, and a K_m of 19.4±6.9 µM. Maximum accumulation 2.3 µM

The cation-coupled symport activity of HpPutP in *H. pylori* was also investigated. Here, kinetics of Na^+-dependent L-proline transport in *H. pylori* were analyzed. The results showed Michaelis-Menten kinetics with a K_m of 1.0±0.5 mM and a V_{max} of 1.8 nmol/mg*min, (Figure 31).

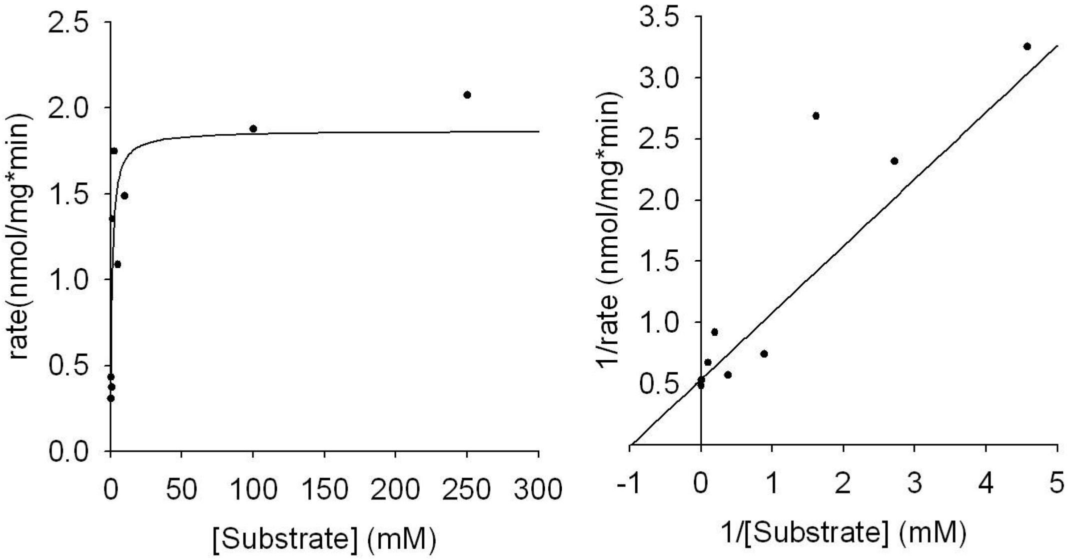


Figure 31. Na+ dependence of proline uptake into *H. pylori P12.* The reaction rate v (nmol/mg*min) is plotted (**A**) as Michaelis- Menten, and (**B**) as Lineweaver- Burk versus the Na+ concentration, which lay between 5 µM und 250 mM. According to Michaelis - Menten calculations v_{max} corresponds to 1.8 nmol/mg*min and a $K_{0,5}$ of 1±0.5 mM.

3.2.6 Electron microscopy observation of flagella of *H. pylori* wild type and complemented mutants

Fluorescent microscopy localization of HpPutP, western blot analysis and transport measurements demonstrated the successful complementation of the deletion mutants. Therefore, evaluation of the complemented mutants by electron microscopy was necessary in order to elucidate if deletion of *HpputP* is directly responsible for the loss of the flagella or if this could be the result of an indirect effect. Samples were prepared as those used for transport measurements and drops of the sample were placed onto a glass slide and fixed as described in material and methods. Samples were further analyzed by Prof. Dr. Gerhard Wanner, with a Zeiss Auriga scanning electron microscope.

The wild type is shown in figure 32A with the flagella bundle and figure 32B also shows the wild type but with a small flagella. These cells were recovered from the same culture at the same time point, however remains the possibility that not all of them were synchronized and were in a different growth phase.

On the other hand the deletion mutant Δ*HpputP* showed no flagella (Figure 32C) and the complemented mutant Δ*HpputP* + *pIB6 alpA HpputP* shows small structures with the polar localization for the flagella but not with the typical morphology of the late log phase (Figure 32D).

Therefore if the complemented mutant needs more time to reach the late log phase or the energy status is impaired, our results suggest that the loss of flagella could be due the depletion of proline to the bacteria. By this means further characterization must be performed in order to further confirm these results.

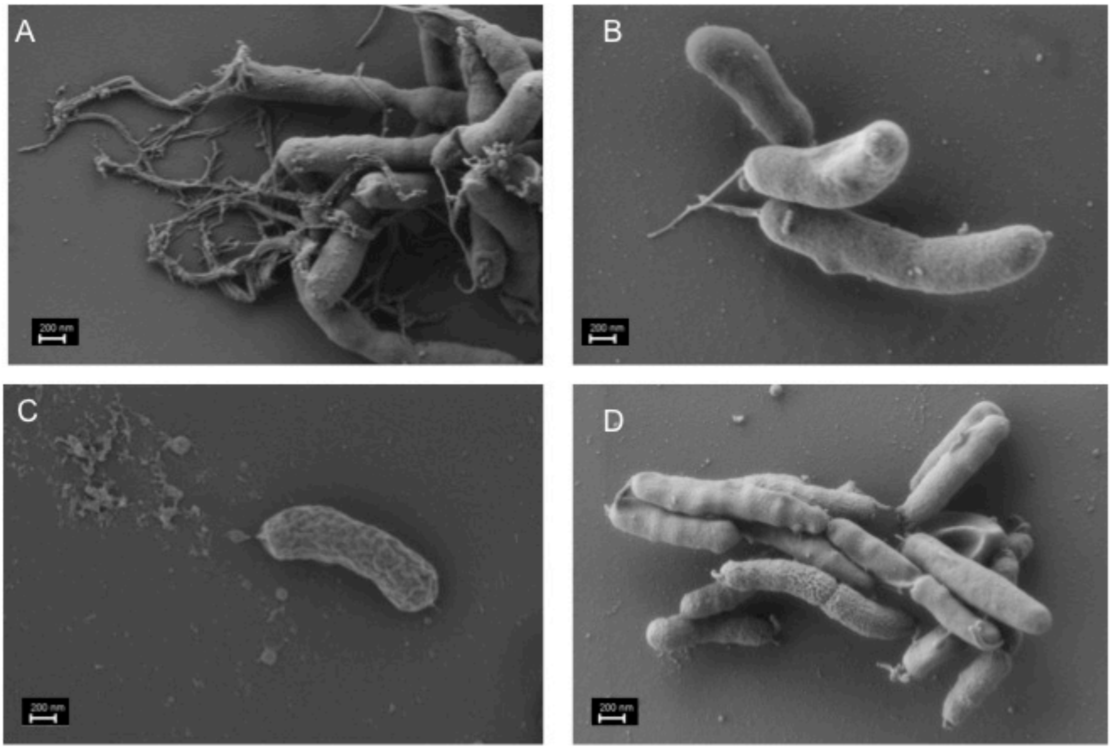


Figure 32. Scanning electron microscopy of *H. pylori* wild type and complemented mutant. (A) Bacillary form of *H. pylori* P12 (Wt) at day 2 in logarithmic phase; (B) bacillary form of *H. pylori* P12 (Wt) at day 2 in logarithmic phase; (C) bacillary form of *H. pylori* P12 Δ*HpputP* at day 2 in logarithmic phase; (D) bacillary form of *H. pylori* P12 Δ*HpputP* + pIB6 *HpputP* at day 2 in logarithmic phase.

The complemented mutant was also characterized by the soft agar assay and results showed that motility was reestablished (Figure 33). Therefore probably the complemented mutant needs more time to reach the late log phase and the flagella assembly. However, as the typical flagella bundle was not present, further electron microscopy analysis at later growth time points should be assessed for the complemented mutant in order to confirm this hypothesis.

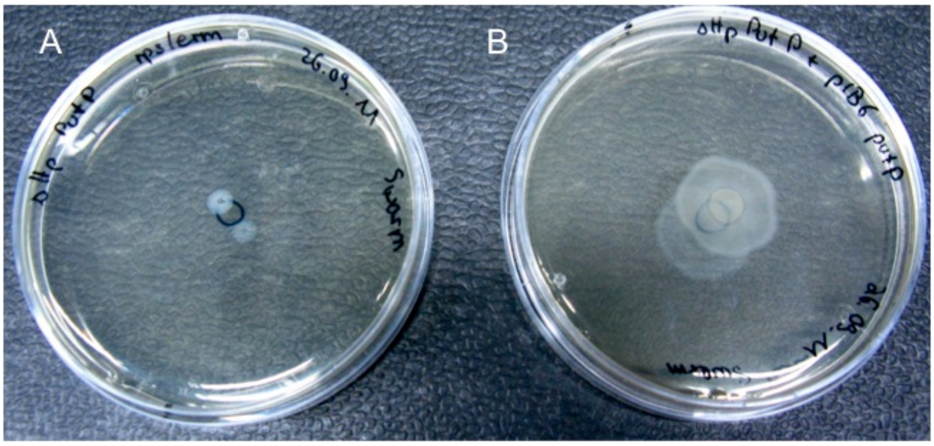


Figure 33. Assessment of motility in *H. pylori* wild type and complemented mutant. *H. pylori* cells were cultured in plates for 48 h, resuspended in BB supplemented with 10% horse serum and adjusted to bacterial concentrations of 1x 10^8 cells per ml (OD_{600} of 0.2). Bacterial cells were stabbed with toothpicks into plates containing brucella broth with 0.3% agar and incubated microaerobically at 37°C for 5 days. (A) Deletion mutant Δ*HpputP;* (B) Deletion mutant Δ*HpputP* harboring *pIB6 alpA HpputP* (complemented mutant).

3.2.7 L-proline effects on the energy status in *H. pylori*

Some *H. pylori* flagellar genes have evolved to respond to changes in DNA supercoiling in response to: temperature, osmolarity, pH and cellular energy level (ATP/ADP ratio) or entry into stationary phase.

It is known that the assembling of the flagella involves a high-energy cost and this could explain the lack of flagella in the deletion mutants if L- proline is the main energy source for the bacteria. For this, ATP levels were measured in the mutants compared to the wild type. Results showed a highly reduced amount of ATP levels in the *ΔHpputP, ΔHpputA*, and *ΔHpputAP* mutants compared to the wild type (Figure 34).

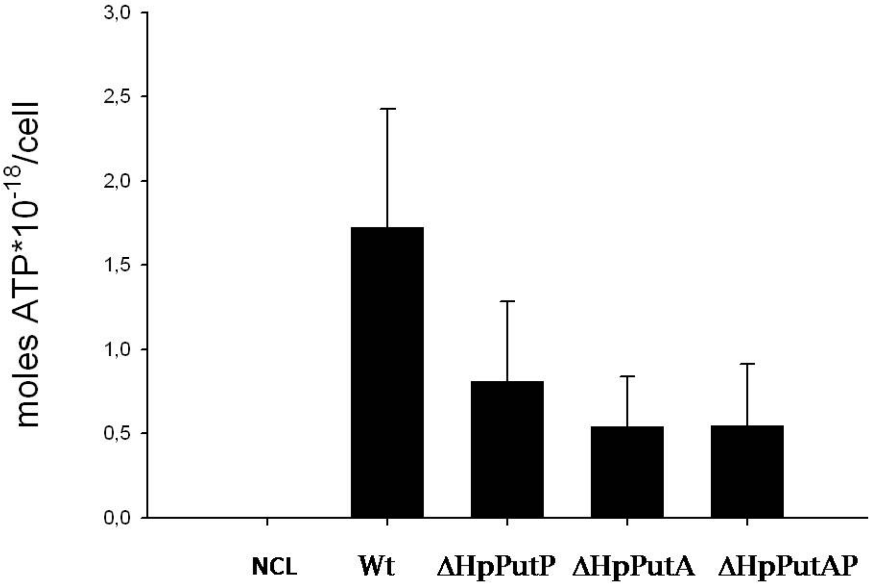


Figure 34. Intracellular ATP levels in *H. pylori P12* wild type and deletion mutants*.* ATP levels in *H. pylori* cells were measured with the BacTiter-Glo™ Microbial Cell Viability Assay (Promega). Samples were collected from liquid cultures in logarithmic phase, adjusted to an OD_{600} of 1 in an opaque-multiwell plate in culture medium (100µl 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. The content was mixed, 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 (10pM to 1µM of ATP). NCL (negative control of medium).

On the other hand, the complemented mutant was also measured showing significantly higher ATP levels than the wild type (Figure 35).

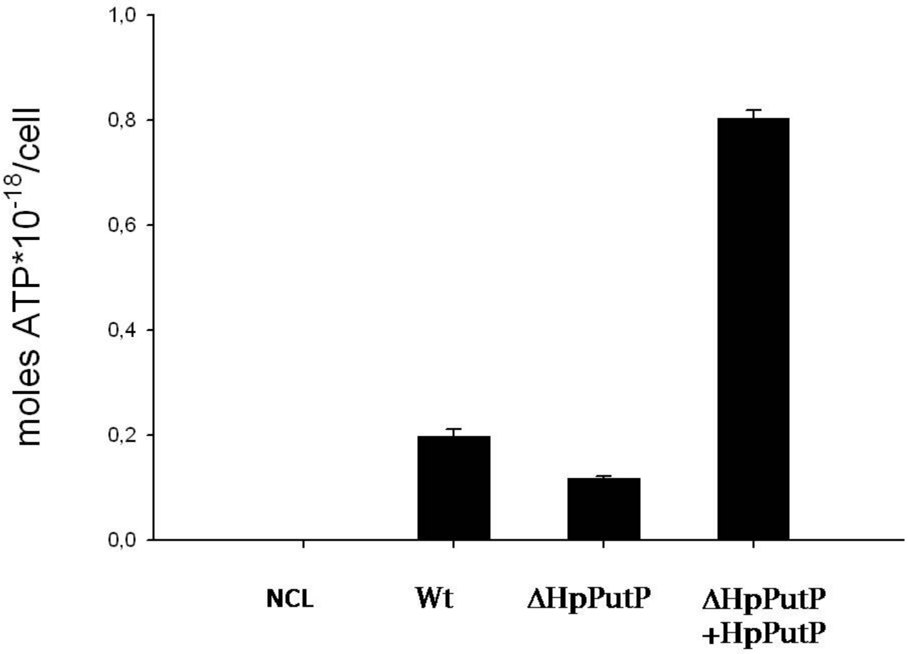


Figure 35. ATP levels in *H. pylori P12* (Wt), Δ*HpPutP* and complemented mutant (Δ*HpPutP+pIB6 HpPutP*). ATP levels in *H. pylori* cells were measured with the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) as described before. NCL (Negative control of medium)

3.2.8 Transcription analysis of flagellum-related genes in *H. pylori* deletion mutants

The current model for the regulation of flagellar biosynthesis in *H. pylori* involves four classes of genes. The expression of class 1 early genes is under the control of an unknown signal and involves important regulators such as flgR, which is an activator of the sigma factor σ^{54} (Brahmachary P, *et al.* 2004) and flhA (Schmitz A, *et al.* 1997). Activation of σ^{54} in turn initiates transcription of class 2 middle genes, a group that includes structural proteins such as FlaB and FlgE. Following completion of middle gene expression, the FliK homolog HP0906 triggers a switch in expression to class 3 late genes under the control of FliA (σ^{28}) (Ryan K A, *et al.* 2005). Furthermore, there exists an intermediate class of regulatory and structural genes that contain both σ^{54} and σ^{28} regulatory elements (Niehus E, *et al.* 2004). Many elements of this complex pathway are yet to be resolved.

To further investigate the basis for the lack of flagella by the deletion mutants, we examined the transcription levels of a number of important genes in each of the four classes described above using real-time reverse transcriptase PCR.
As mentioned previously, the mutants showed similar growth characteristics to the wild-type strain. This is important to remark due to the fact that previous studies showed that *H. pylori* motility is affected by bacterial morphology and phase of growth (Worku M L et al., 1999).

Our results showed that *era* expression was unchanged between the mutants and the wild type (Figure 36). Transcription of the class I genes *rpoN* (σ^{54}) and *flhA* showed a slight variation compared to the wild type. By contrast, class 2 genes *flaB* and *flgE* was slightly increased in the mutants. *flaB* expression was increased approximately twofold and *flgE* fivefold compared to that of the wild type. Expression of the class 3 gene *flaA* was severely decreased compared to the wild type (Figure 36). Reduced expression of this important structural flagellar protein alone would certainly account for the lack of flagella observed on the mutant. Expression of intermediate class genes *flgM* and *flhF* were slightly downregulated compared to the wild type.

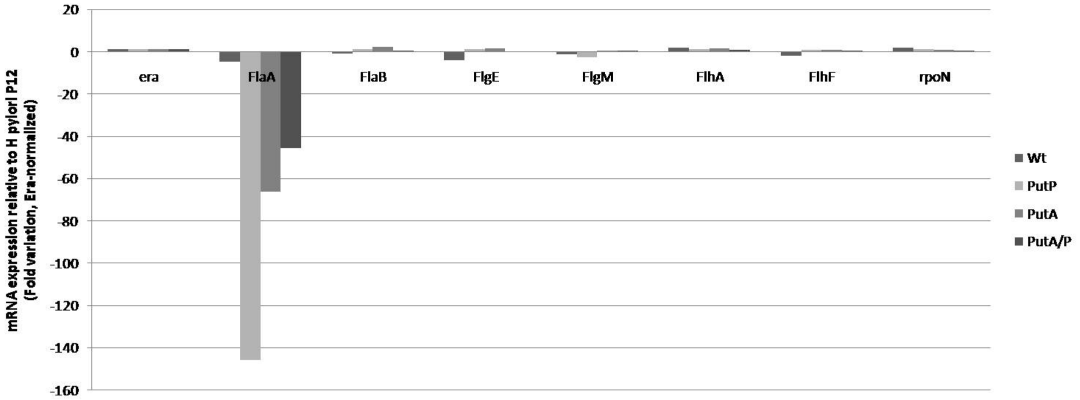


Figure 36. Flagella gene expression of *H pylori P12 ΔHpputP, ΔHpputA*, and *ΔHpputAP* mutants compared to wild type Fold expression compared to wild-type was calculated using *era* as reference gene as described in Section 2.2.3.13. Expression values are representative of three independent biological experiments.

It is clear that *flaA* downregulation is responsible for the loss of the flagella in the deletion mutants when proline is not available, probably due to lower energy levels in the cells. However, a deeper characterization of this effect should still be addressed.

4. DISCUSSION

It is estimated that *H. pylori* is responsible for 5.5% of all human cancer cases, or approximately 592,000 gastric cancer cases per year (Parkin D M, 2006). The ability of *H. pylori* to live in the stomach acidic environment makes its physiology unique, and much research has focused on understanding the factors that enable this bacterium to survive in such environment.

Besides the urease enzyme and genes involved in motility of *H. pylori*, only few further genes have been shown to be essential for colonization in animal models (Eaton K A, *et al.* 1999). Kavermann H, *et al.* in 2003 used the signature tagged mutagenesis (STM) method and performed infection studies in the Mongolian gerbil (*Meriones unguiculatus*), a well-established *H. pylori* animal model, for the identification of *H. pylori* genes essential for colonization. This model appears to be most suitable for screening as it has several advantages such as a low gastric pH as in humans, the induction of typical gastritis, gastric ulceration, and carcinoma upon infection with *H. pylori,* similar to the situation in humans (Ikeno T H, *et al.* 1999).

Among the genes essential for colonization was the gene *hpp12_49* encoding a putative proline transporter (HpPutP) (Kavermann H, *et al.* in 2003). The finding suggests that *H. pylori* relies on the exogenous uptake of this amino acid from the stomach mucosa. Furthermore, Nagata and coworkers demonstrated in 2003 that i) consumption of L-serine and L-proline as respiratory substrates stimulates rates of oxygen consumption; ii) *H. pylori* cells contain high levels of L-proline; and iii) gastric juice from patients with *H. pylori* showed a significantly higher level of L-proline than those from uninfected subjects. Taken together, these results suggest that *H. pylori* cells utilize L-proline as a major energy source in their habitat, the mucous layer.

4.1 Biochemical characterization of HpPutP

The L-proline transporter in *H. pylori* has not been experimentally characterized yet and at the moment it remains annotated as a predicted symporter based on sequence alignments with PutP of *E. coli.* EcPutP is the best-characterized bacterial L-proline transporter of the Na^+/substrate symporter family (SSSF, TC 2.A.21) (Jung H *et al.* 2012; Olkhova E *et al.* 2011; Reizer J, *et al.* 1994; Turk E and Wright E M *et al.* 1997). It catalyzes the symport of Na^+ and L-proline with a stoichiometry of 1:1 (Yamato I and Y Anraku, 1993). EcPutP shares 50% identical amino acids with the putative proline transporter of *H. pylori.* Therefore we started the characterization of HpPutP in order to experimentally test whether it is the responsible for proline translocation in this bacterium.

For practical reasons *E. coli* was first used as a heterologous expression system to study the mechanism of proline uptake by HpPutP. The *HpputP* gene was cloned and sequenced and the gene product was solubilized, purified and reconstituted in proteoliposomes. This was performed because it is known that the proper incorporation of a purified membrane protein into closed lipid vesicles, to produce proteoliposomes, allows the investigation of transport and/or catalytic properties of any membrane protein without interference by other membrane components (Rigaud J L *et al*. 2003).

Therefore transport activity of radioactive labeled L-proline was evaluated first in proteoliposomes, and as proteins of this family utilize a SMF to drive uphill transport of substrates, ion dependency was also assessed. The results showed that proline transport in HpPutP seems to depend on an electrochemical Na^+ gradient. Na^+ can be substituted by lithium (Li^+) but not by protons (H^+). This is in agreement with data reported for *E. coli* where H^+-driven L-proline uptake by EcPutP could not be demonstrated (Jung H *et al.,* 1998a; Jung H *et al.* 1998b; Yamato I and Y Anraku, 1993; Chen C C *et al.* 1985; Cairney J *et al,* 1984).

Inhibition studies in proteoliposomes revealed that HpPutP is stereospecific recognizing only the L- but not the D-isomer of proline. This could have a physiological reason and fits to the high content of L-proline in infected samples of human gastric juice (Nagata, *et al.* 2003). Furthermore proline analogs already known to inhibit proline transport by EcPutP strongly inhibit also HpPutP-catalyzed transport. This indicates again a strong functional similarity between both proteins. The analogs tested were dehydro-DL-proline (double bond in the ring structure), L-azetidine-2-carboxylic acid (smaller ring) and L-proline-methylesther (alteration in the carboxyl group), showing 91.4%, 86.6% and 88.50% inhibition, respectively.

The next step was the analysis of proline uptake into *E. coli* cells heterologously expressing *HpputP*, which also showed complementation of the transport activity. The kinetic parameters of proline uptake and Na^+ dependency were evaluated (K_m of 1,3 μM) and these were in the same order of magnitude as those for EcPutP (kd(Pro) and K_m(Pro) of 2 μM) (Wood J M and D Zadworny, 1979; Yamato I and Y Anraku, 1993; Jung H *et al.* 1998). Therefore, PutP of *H. pylori* seems to be a high affinity L-proline transporter under these conditions. Furthermore, a Na^+ concentration required for half-maximum stimulation of L-proline uptake ($k_{0.5(Na+)}$) of 17±0.9 μM suggests a high affinity also for Na^+.

One of the current major challenges in the investigation of transporters is the identification of regions involved in the binding and translocation of Na^+ and substrate. Labeling experiments, random and site-directed mutagenesis have been employed to identify functionally important sites in PutP of *E. coli,* where substitution of Asp-55, Ser-340 and Thr-341 dramatically reduces the apparent affinity for Na^+ and Li^+ (Hilger D, *et al*., 2008; Pirch T, *et al.* 2002). On the other hand, Ser-57, Tyr-140, and Trp-244, are involved in proline binding (Pirch T, *et al.* 2002). All these residues are conserved in HpPutP, thus, we systematically studied the effect of individual amino acid substitutions on the corresponding HpPutP residues to identify the functionally important sites in HpPutP. As mentioned in the results section, proline uptake was severely affected upon replacement of the corresponding residues in HpPutP. Initial rates and steady-state level of proline accumulation were also reduced compared to HpPutP wild type. This leads to the assumption that EcPutP and HpPutP share the same important residues for proline and sodium binding and probably a very similar protein structure.

During evolution, the protein structure is more stable and changes much slower than the associated sequence, so that similar sequences adopt practically identical structures and distantly related sequences still fold into similar structures. This relationship was first identified by Chothia and Lesk in 1986 and later quantified by Sander and Schneider in 1991. In 1999, Rost could derive a precise limit for this rule. As long as the length of two sequences and the percentage of identical residues fall in the region marked as “safe,” (>40%) the two sequences are practically guaranteed to adopt a similar structure. Therefore, as EcPutP and HpPutP show 50 % identical amino acids, is highly probable that both share the same structure. To date there is no high resolution structure of PutP or any other L-proline transporting integral membrane protein available. But crystallization of the Na^+-dependent galactose symporter vSGLT of *Vibrio parahaemolyticus* (a member of the SSS family) gave the first hints (Faham S *et al*. 2008). A homology model of *E. coli* PutP was generated based on the crystal structure of vSGLT (Olkhova E *et al.* 2011). We also designed a homology model of HpPutP based on vSGLT (Figure 15). This model shows a high similarity to the EcPutP homology model including the sites of proline and Na^+ binding. Therefore, this observations support our previous results of transport when amino acid replacements were assessed.

All the data recovered up to this point were obtained from heterologous expression systems, which are based on the assumption that the basic principles of protein expression and function are similar in all organisms (Frommer W B and O Ninnemann, 1995). Nevertheless, important differences exist between cells in terms of the presence and composition of cell walls, and the presence of specialized organelles. The *H. pylori* outer membrane composition is unique in its protein content and lipopolysaccharide (LPS) structure (Aspinall G O *et al.* 1996). The bacterium's LPS consists predominantly of a variety of the tetra-acylated lipid A, which is known to exhibit 1000-fold reduced bioactivity as compared to *E. coli* LPS (Moran AP, *et al.* 1997). The peptidoglycan of *H. pylori* also differs substantially from that of *E. coli* (Costa K, *et al.* 1999).

PutP is an integral protein of the cytoplasmic membrane of *E. coli* (Pirch, T *et al.,* 2002). In *E. coli*, the plasma membrane consists predominantly of phosphatidylethanolamine, which constitutes 80% of total lipid (Cooper GM. (2000). On the contrary the *H. pylori* total lipid content (by weight) consists of 6% neutral lipids, 20.6% glycolipids, and 73.4% phospholipids (Hirai Y, *et al.* 1995). The major phospholids are phosphatidylethanolamine, cardiolipin, and phosphatidylglycerol. Phosphatidylserine was detected as a minor phospholipid. Furthermore, *H. pylori* performs the unique action of using non-esterified colesterol and glucosylated cholesterols as membrane lipid composition. cholesterol glucosides are very rare in animals and bacteria (Haque M, *et al.* 1996). The role of cholesterol glucosylation in *H. pylori* was first identified by Wunder *et al*. in 2006. They demonstrated that *H. pylori* cells glucosylate the non- esterified cholesterol extracted from the lipid raft of gastric epithelial cells to evade the host immune responses. The glucosylation of the non-esterified cholesterol absorbed into the cell membrane allows the bacterium to colonize the gastric mucosa tissues of hosts for long periods by conferring resistance against the phagocytosis of macrophages and regulating the activation of antigen-specific T cells (Wunder C *et al*. in 2006). Because of these differences in membrane composition, L-proline uptake needed to be tested in *H. pylori* cells .

To evaluate proline kinetics in *H. pylori* (wt) we conducted several experiments at multiple substrate concentrations and measured initial velocity at each substrate concentration. Results showed a K_m of 19.4±6.9 μM in *H. pylori* that compared to the values obtained from the heterologous system (K_m of 1.3±6.9 μM) seems to indicate a lower affinity for proline. Assuming a stable pH, temperature, and redox state, the K_m is constant, however, a possible explanation for this discordance is the different membrane environment between *H. pylori* and *E. coli*. Furthermore meassurements were performed with the cells exposed to high oxygen concentrations that can be detrimental for *H. pylori.*

We had evidence that the accumulation of proline is achieved in the presence of sodium, therefore measurements of sodium kinetics were performed and showed a $K_{0.5Na+}$ of 1.0±0.5mM. Sodium concentrations in the sample supernatants were between 0.07 to 0.12 mM and attempts to reduce them after several washing steps lead to inhibition of transport. According to our results, *H. pylori* seems to respond to Na+ concentrations in the milimolar range, which differs from the values obtained previously in the heterologous system ($K_{0,5}$ of 17±0.9 μM) (Bracher S and H Jung, unpublished data). The different membrane environment could be a reason. But this could also be an adaptation from the bacteria to the gastric environment since sodium concentrations in the gastric juice are in the range of 30.2 to 69.8 miliequivalents (mEq) in healthy individuals or higher in individuals with chronic gastritis and peptic gastric ulcer (Meeroff J C *et al.,* 1973).

In addition, salt resistance can induce changes in expression of outer membrane proteins, such as the transporters ProP, ProU, BetT, and BetU in *E. coli* (Wood J M 2007). In pathogenic bacteria, osmotic stress can also serve as a signal that controls expression of virulence factors. For example, a recent study showed that elevated salt concentrations result in alterations in expression of the virulence factor CagA in *H. pylori* strain 26695 (Loh J T *et al.* 2007). Furthermore recent reports suggest that there is a strong epidemiological link between high salt intake and *H. pylori*-induced disease (Tsugane S and S Sasazuki 2007). Thus, the bacterium could sense fluctuations in salt concentration due to dietary intake and alters its growth and gene expression accordingly.

4.2 Physiological relevance of HpPutP

The precise role of *putP* in *H. pylori* metabolism and virulence is not known. HpPutP may be required to utilize L-proline as a nutrient ensuring, for example, the supply of energy for transmembrane transport processes and bacterial cell motility. And it cannot be excluded that the PutP-catalyzed accumulation of L-proline contributes also to the resistance of *H. pylori* against osmotic or oxidative stress. Therefore to evaluate the physiological relevance of HpPutP, deletion mutants of *HpputP, HpputA* and *HpputAP* were constructed. To obtain the right mutation several trials had to be performed, maybe because *H. pylori* stands out as the bacterial species with the highest recombination (Perez-Losada M, *et al.* 2006) and mutation rates (Bjorkholm B, *et al.* 2001). Furthermore just few colonies were obtained after each transformation. Previous studies showed that the yields of the replacements among selected Str^r colonies varied from ~17% to ~90%, depending on the gene targeted and the strain background (Dailidiene D, *et al.* 2006). This may mean that deletion of genes of the *put* operon in *H. pylori* is highly deleterious for the viability of these bacteria. However further growth studies did not support this hypothesis.

After confirming the correct deletion of the selected genes, initial characterization of the mutants was evaluated through growth as it is the best way to evaluate cell viability and differences between strains (Saito N, *et al.,* 2003). Results showed no difference between mutants compared to the wild type. Supplementing bacterial cultures with L-proline did not affect growth in any strain. We must take into account that we used a complex medium that most probably contains high amounts of L-proline. In addition L-serine and D-alanine can also be used as alternative energy sources as they also function as respiratory substrates (Nagata K, *et al.,* 2003). Furthermore *in vitro* studies already showed that proline is not an essential amino acid for *H. pylori* (Nedenskov P, 1994).

In order to better evaluate the effect of the deletion genes on growth, we tried to reproduce the defined medium for *H. pylori* established by Reynolds and Penn in 1994. This is a medium in which all the constituents are defined, allowing each of them to be selectively omitted or further components added. However our results showed that growth in the defined medium was reduced 50% compared to complex medium and after 72 h OD values decreased dramatically. Probably the differences we observed to those previously reported are strain dependent as the strains previously tested were different including also clinical isolates. We used Bovine serum albumin (BSA) as the main substitute for the serum and blood factors required normally for the culture of *H. pylori.* It has been reported that BSA is sufficient to support and maintain a population of *H. pylori* cells (Goodwin C S *et al*., 1989; Marshall B J and C S Goodwin. 1987) by reducing the toxic effects of fatty acids, which inhibit the growth of *H. pylori* (Hazell S L *et al.,* 1990). However serum may contain other growth-stimulatory factors required by our strain.

For characterization of the mutants, colony forming units (CFU) measurements were also assessed but data are not presented in this work, as the results did not reflect the growth of colonies from single cells because of the clumps that the bacteria formed. Reports that *H. pylori* cultures in liquid media develop clumping of up to 50 to 100 cells have been described (Shahamat M, *et al.* 1991)

Previous phenotypic characterization by Nakajima K *et al.*, in 2008 of a *putA* mutant showed that the mutant did not show motility and full-length sheathed flagella were rarely observed. The mutant was also incapable to colonize the stomach of mice. Kavermann H, *et al*., in 2003 also observed the loss of infectivity when the *HpputP* gene was depleted. Therefore the loss of infectivity in the HpPutP mutant could also be due to effects on the flagella assembly. To corroborate this hypothesis, we used analysis by scanning electron microscopy and the results revealed that the deletion mutants harvested at the log phase (48h) did not show any flagella structure, while in the wild type the flagella bundle was perfectly visible. These data were confirmed by the lack of swarming activity in the deletion mutants. Furthermore, conversion to the coccoid shape was observed in all strains at the stationary phase (96h). This morphological transition from spiral to coccoid has been appointed as a manifestation of cell adaptation to non-optimum environments as the bacterium moves into a viable but non-culturable state (Azevedo N F, *et al.* 1997). Our results suggest that the *HpputP* and *HpputA* genes are probably necessary for flagella assembly. This is another important evidence that proline uptake and metabolism are determinants required for colonization and morphological changes in *H.pylori* as already described before by Kavermann H *et al.* 2003 for HpPutP and Nakajima K *et al.* 2008 for HpPutA respectively.

Motility is an important virulence factor, as no colonization in the stomach is observed in mice infected with a strain with disrupted flagella (Eton K A, 1999). Therefore the previously reported failure of Δ*HpputP* and Δ*HpputA* mutants to cause colonization may be due to the loss of flagella and therefore motility. Loss of motility was also confirmed for the Δ*HpputP,* Δ*HpputA* and Δ*HpputAP* mutants by the soft agar plates assay.

To restore the phenotypic effects in the *H. pylori* mutants, and furthermore to use the complementation system in transport experiments, the shuttle vector pIB6 was used. Based on this, different vectors for native/constitutive-promoter driven expression were created using GFP as a reporter to evaluate the expression of the system by fluorescent microscopy.

We searched for differences between expression of the protein under regulation of the putative native promoter sequence of HpPutP (to identify possible differences in gene expression in response to proline) or the constitutive promoter *alpA* (Barwig I 2009). Plasmids derived from *pIB6* were introduced into *H. pylori* by conjugation to overcome the *H. pylori* strain-specific restriction barriers that frequently lead to restriction of plasmid DNA derived from *E. coli* or heterologous *H. pylori* strains. This phenomenon was repeatedly observed in our case, showing complete resistance to transformation when using shuttle vectors as previously reported (Tsuda M *et al*., 1993; Wang G *et al.*, 1993; Heuermann D and Haas R, 1998).

No evident difference in expression between the two promoters was observed when GFP was fused directly behind the native or constitutive promoter region, showing GFP localization in the cytoplasm. When the translational HpPutP-GFP fusion was expressed with either of the two promoters, the protein was localized in the membrane, which is consistent with the expected localization of HpPutP as a membrane protein. As the *HpputP* expression in the putative native promoter did not need an additional stimulus for expression, if L-proline is the triggering stimulus for expression, this still needs to be determined. It is important to remark that *H. pylori* was cultured in a high complex medium containing proline, therefore whether proline concentrations trigger the expression of *HpputP* is not known and as mentioned before, trials to reproduce a defined medium for *H. pylori* culture were not successful. We can not discard the possibility that the expression of *HpputP* could be constitutive as indicates the missing DNA binding domain of HpPutA. We also confirmed by western blot the expression of HpPutP-GFP in samples from *H. pylori* membranes.

Once *HpputP* expression was confirmed by a second methodology, we focused upon further investigating the proline uptake in *H. pylori* cells. Analysis of L-proline uptake revealed active transport activity in *H. pylori* (Wt) cells, while in the deletion mutants (*ΔHpputP* and *ΔHpputAP*) transport was not detected. The complemented mutants (Δ*HpputP* and *ΔHpputAP* harboring the plasmid *pIB6 alpA HpputP*) were able to reestablish the function to up to 80% of maximum accumulation in respect to the wild type. This means that HpPutP is the proline permease in *H. pylori.* Thus, we could prove that HpPutP, which was previously found to be essential for colonization in the Mongolian gerbil model (Kavermann H, *et al.* 2003), is the sole proline transporter in *H. pylori* under the conditions tested. Under other conditions ProP and ProWX (potential proline transporters) could also play a role.

Another important question is: why does the lack of proline in the *ΔHpputP, ΔHpputAP* and *ΔHpputAP* mutants lead to the loss of the flagella? Scanning electron microscopy pictures of the complemented mutants were performed to address if reestablishment of the expression of *HpputP* could restore the flagella. Interestingly the pictures showed the presence of flagella-like structures and therefore partial complementation. Similar pictures were observed in the wild type at earlier time points. This could mean three things: a) that the complemented mutant was somehow delayed in assembling the flagella compared to the wild type; b) that side effects from the deletion play a role in the flagella assembly or c) that the copy number of the gene (in this case the plasmid) could lead to an overexpression which could alter the biogenesis of the flagella.

The soft agar assay showed that motility was reestablished. Therefore probably the complemented mutant has a delayed flagella assembly compared to the wild type. However, to confirm this hypothesis, further electron microscopy analysis at later growth time points should be assessed for the complemented mutant. Our results directly link the lack of proline to loss of flagella and therefore loss of infectivity. Motility by means of flagella is very expensive for cellular economy in terms of the number of genes and the energy required for flagellar biosynthesis and functioning (Macnab RM, 1996). In *H. pylori,* ATP production seems to be coupled to the respiratory activity with D- and L-alanine, L-proline, L-serine and pyruvate as respiratory substrates (Nagata K *et al.,* 2003). Besides ATP production, membrane integrity, and mRNA transcription have all been used as indicators of viability (Adams B I *et al.,* 2003; Conway T and G K Schoolnik, 2003; Gribbon L T and Barer, 1995; Nilsson H O *et al.,* 2002; Sheridan G E C *et al.,* 1998). In this regard, we decided to measure the energy status (ATP levels) of the deletion mutants compared to the wild type. Our results showed that the mutants displayed a lower energy status compared to the wild type and this means that proline uptake and metabolism are important for energy production in the cells. This is confirmed by the fact that the ATP levels in the complemented mutant were restored.

In humans collagen has the potential role of storage reservoir for amino acids, in particular proline (Guszczyn T and K Sobolewski, 2004). For *H. pylori* this means that in the gastric mucosa there is a great reservoir of proline stored as collagen that can be used as energy source. *H. pylori* apparently relies on the exogenous uptake of amino acids and precursors in the stomach mucosa, as shown previously for growth of *H. pylori* in vitro (Stark R M *et al*. 1997). Such an amino acid exchange between bacteria and host is frequently observed in primary and secondary symbionts of plants and animals. The amino acid dependence might be in support of the view that *H. pylori* originated as a commensal of humans. *H. pylori* colonization shows the markers for a relatively benign co-existence with its host (high prevalence, chronicity of carriage and marginal or no effects on host reproductive capacity), which may include all of the entire spectrum of interactions from parasitism, through commensalism, to symbiosis (Blaser M J, 1997).

According to our results, the energy status of the mutants might be in part involved in the failure of the flagella assembly but might not be the only reason. It remains the possibility that the lack of flagella on the surface of the mutants could be due to an alteration in the expression of flagella genes, a deficiency of protein production, or a failure to export these components to the cell surface.

The expression of some flagellar genes was analyzed in order to elucidate if deletion of *HpputP* affects the flagella regulation. Strikingly, the expression levels of the major flagellin gene, *flaA* were severely downregulated. Although flagellar motility is essential for the colonization of the stomach by *H. pylori,* little is known about the regulation of flagellar biosynthesis in this organism. The flagellar regulon is not contained in operons in this organism, which further confounds the apparent lack of regulation (Tomb, J F, 1997). Flagellar biosynthesis is a hierarchical process that is subject to temporal and growth phase regulation involving the sequential activation of approximately 40 genes. Furthermore, a master regulator of flagellar biosynthesis (such as the enterobacterial *flhCD* genes) has not been found in *H. pylori* (Spohn and Scarlato, 2001). It has been proposed that some *H. pylori* flagellar genes have evolved to respond to changes in DNA supercoiling. It was demonstrated that a decrease in negative supercoiling induced by the gyrase inhibitor noboviocin coincided with lowered transcription of the late flagellin gene *flaA* (Fang Yea, et al. 2007). Nobiocin is the only aminocoumarin which has been licensed for the treatment of human infections, and its efficacy has been confirmed in several clinical trials (Raad I *et al.* 1995; Raad I *et al.*1998). This drug inhibits the ATP hydrolysis required for ATP-dependent DNA supercoiling (Maxwell A and D M Lawson 2003). DNA topology changes are known to occur in response to temperature, anaerobiosis, osmolarity, pH, and cellular energy level (ATP/ADP ratio) (Drlica, 1992; Higgins et al., 1988; Hsieh et al., 1991) and entry into stationary phase (Balke and Gralla, 1987; Bang et al., 2002; Camacho-Carranza et al., 1995). Therefore the lowered levels of ATP in the mutants could be the reason for a change in the supercoiling status of the DNA in the mutants that lead to the downregulation of *flaA* and loss of the flagella.

In conclusion, low energy levels due to the lack of proline could lead to DNA relaxation, downregulation of *flaA* and finally loss of the flagella in the deletion mutants (Figure 37). While restoring proline uptake, energy levels are compensated and the flagella can be assembled. Therefore, in *H. pylori* the control of transcription by supercoiling could also be intricately coupled with cellular energy levels (ATP/ADP ratio) (Hsieh et al., 1991; van Workum et al., 1996; Travers A and G Muskhelishvili, 2005b), that could determine the effective superhelicity for individual genes or regulons in the chromosomal DNA (Travers A et al., 2001; Travers A and G Muskhelishvili, 2005).

The results of this thesis showed complementation of the HpPutP function. However, due to the lack of full length flagella in the SEM pictures we may not discard the possibility of partial complementation for the flagella assembly which may be explained by pleiotropic effects of supercoil relaxation. Previous reports show that several genes involved in virulence, such as *cag* pathogenicity island genes, are supercoil sensitive, reacting to relaxation with decreased transcription. Another observation connecting flagellar genes and supercoiling concerns the proximity of topoisomerase and flagellar genes on the *H. pylori* chromosome: topA is immediately adjacent to *flaB*, the minor flagella subunit (Spohn and Scarlato, 1999; Niehus et al., 2004). It has been proposed that feedback mechanisms exists by which the expression level of *flaA* influences the expression of other genes involved in flagellar motility (*flaB*) and supercoiling (*topA*) (Fang Yea, et al. 2007). Furthermore, supercoiling in *H. pylori*, appears to be less tightly controlled compared to other bacteria and fluctuates as the culture passes through the growth phases. Therefore a response to supercoiling could occur at many levels (Hatfield G W and C J Benham, 2002; Travers A and G Muskhelishvili, 2005b) and this could also be happening in our system.

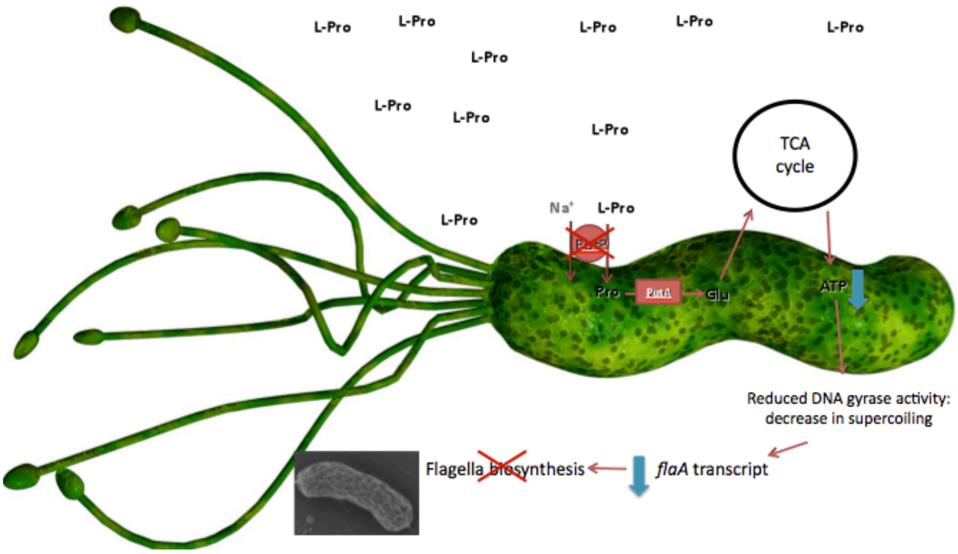


Figure 37. L-proline significance in *H pylori P12*. Results showed that HpPutP is the L-proline transporter in *H. pylori*. Lack of proline transport in *H. pylori P12* impairs the oxidation of proline by HpPutA, which in turn affects the energy status of the cells as documented by reduced amounts of ATP in the cell. As a consequence, the ATP-dependent gyrase activity may be reduced. Previously, it was already shown that a inhibition of gyrase activity leads to downregulation of *flaA* expression and finally loss of the flagella in the deletion mutants (Fang Yea, *et al*. 2007). Complementation of HpPutP transport activity in the deletion mutants restores energy levels and partial flagella structures.

4.3 OUTLOOK

HpPutP is the proline permease in *H. pylori* and transport activity depends on a sodium gradient. However for a deeper characterization of the transporter, several experiments still need to be performed. For example:

Calculation of the inhibition constant *Ki* for the inhibitors tested should be performed. Using the conditions optimized for proline transport by HpPutP, we could determine the *Ki* of the available proline analogs and determine the relative affinity for each analog.

The turnover number for HpPutP (the number of substrate molecules transported per second per transport molecule when the protein is saturated with substrate (Nelson D I and M M Cox, 2005; Becker W M *et al.,* 2006)) should be determined.

In addition, it is important to unravel whether HpPutP has two binding sites.

Scanning electron microscopy of the complemented mutant at later time points (growth phase) should be performed.

DNA superhelicity changes in *H. pylori* (mutants Δ*HpputP* and Δ*HpputAP* compared to the wild type) should be evaluated based on the experiments performed by Fang Yea, et al. in 2007 in order to test if DNA topology changes due to the energy status of each particular strain have implications on the flagellar gene regulation.

It is also important to analyze the impact of HpPutP on the functionality of other virulence factors of *H.pylori* like the CagA-system

Finally, the significance of other putative proline transporters (ProP, ProWX) for *H. pylori* physiology and stress resistance should be evaluated.

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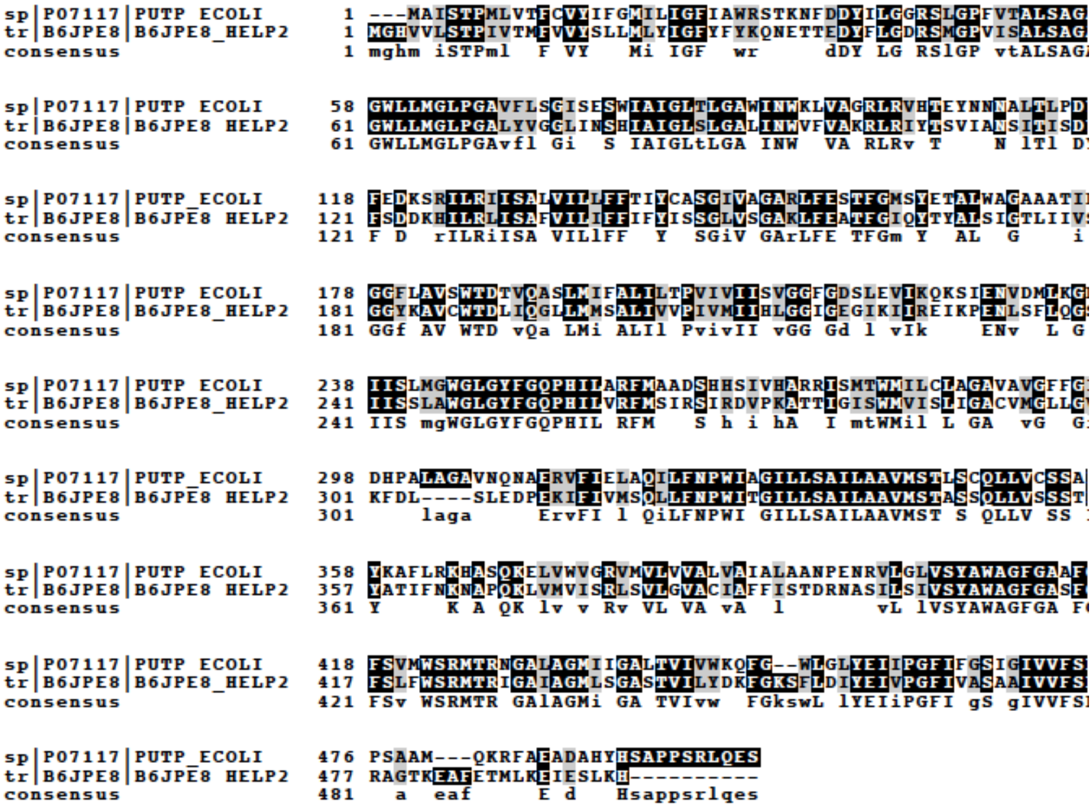
6. SUPPLEMENTARY MATERIAL

6.1 Sequence alignment of EcPutP and HpPutP

Sequence alignment of HpPutP and EcPutP with CLUSTAL O (1.1.0) revealed 50% identical amino acids (suppl. Figure 1). Furthermore, the amino acids found to be particularly important for EcPutP function are conserved in HpPutP.



```
sp|P07117|PUTP_ECOLI      1 ---MAISTPMLVTFCVYIFGMILIGFIAWRSTKNFDDYILGGRSLGPFVTALSAG
tr|B6JPE8|B6JPE8_HELP2    1 MGHVVLSTPIVTMFVVYSLLMLYIGFYFYKQNETTEDYFLGDRSMGPVISALSAG
consensus                 1 mghm iSTPml  F VY   Mi IGF  wr     dDY LG RSlGP vtALSAG

sp|P07117|PUTP_ECOLI     58 GWLLMGLPGAVFLSGISESWIAIGLTLGAWINWKLVAGRLRVHTEYNNNALTLPD
tr|B6JPE8|B6JPE8_HELP2   61 GWLLMGLPGALYVGGLINSHIAIGLSLGALINWVFVAKRLRIYTSVIANSITISD
consensus                61 GWLLMGLPGAvfl Gi  S IAIGLtLGA INW  VA RLRv T    N lTl D

sp|P07117|PUTP_ECOLI    118 FEDKSRILRIISALVILLFFTIYCASGIVAGARLFESTFGMSYETALWAGAAATI
tr|B6JPE8|B6JPE8_HELP2  121 FSDDKHILRLISAFVILIFFIFYISSGLVSGAKLFEATFGIQYTYALSIGTLIIV
consensus               121 F D  rILRiISA VILlFF  Y  SGiV GArLFE TFGm Y  AL  G   i

sp|P07117|PUTP_ECOLI    178 GGFLAVSWTDTVQASLMIFALILTPVIVIISVGGFGDSLEVIKQKSIENVDMLKG
tr|B6JPE8|B6JPE8_HELP2  181 GGYKAVCWTDLIQGLLMMSALIVVPIVMIIHLGGIGEGIKIIREIKPENLSFLQG
consensus               181 GGf AV WTD vQa LMi ALIl PvivII vGG Gd l vIk    ENv  L G

sp|P07117|PUTP_ECOLI    238 IISLMGWGLGYFGQPHILARFMAADSHHSIVHARRISMTWMILCLAGAVAVGFFG
tr|B6JPE8|B6JPE8_HELP2  241 IISSLAWGLGYFGQPHILVRFMSIRSIRDVPKATTIGISWMVISLIGACVMGLLG
consensus               241 IIS mgWGLGYFGQPHIL RFM   S h i hA  I mtWMil L GA  vG  G

sp|P07117|PUTP_ECOLI    298 DHPALAGAVNQNAERVFIELAQILFNPWIAGILLSAILAAVMSTLSCQLLVCSSA
tr|B6JPE8|B6JPE8_HELP2  301 KFDL----SLEDPEKIFIVMSQLLFNPWITGILLSAILAAVMSTASSQLLVSSST
consensus               301     laga     ErvFI l QiLFNPWI GILLSAILAAVMST S QLLV SS

sp|P07117|PUTP_ECOLI    358 YKAFLRKHASQKELVWVGRVMVLVVALVAIALAANPENRVLGLVSYAWAGFGAAF
tr|B6JPE8|B6JPE8_HELP2  357 YATIFNKNAPQKLVMVISRLSVLGVACIAFFISTDRNASILSIVSYAWAGFGASF
consensus               361 Y     K A QK lv v Rv VL VA vA  l       vL lVSYAWAGFGA F

sp|P07117|PUTP_ECOLI    418 FSVMWSRMTRNGALAGMIIGALTVIVWKQFG--WLGLYEIIPGFIFGSIGIVVFS
tr|B6JPE8|B6JPE8_HELP2  417 FSLFWSRMTRIGAIAGMLSGASTVILYDKFGKSFLDIYEIVPGFIVASAAIVVFS
consensus               421 FSv WSRMTR GAlAGMi GA TVIvw  FGkswL lYEIiPGFI gS gIVVFS

sp|P07117|PUTP_ECOLI    476 PSAAM---QKRFAEADAHYHSAPPSRLQES
tr|B6JPE8|B6JPE8_HELP2  477 RAGTKEAFETMLKEIESLKH----------
consensus               481   a  eaf     E d   Hsappsrlqes
```

Suppl Figure 1. Sequence alignment of EcPutP and HpPutP. The alignment was generated using ClustalO (1.1.0) with sequences (FASTA) obtained from a blast search (www.ncbi.nlm.nih.gov/BLAST) against PutP of *E. coli* K12 (swiss-prot entry P07117) and PutP of H. pylori P12 (entry HPP12_0049). Alignment was optically improved by the Boxshade 3.21 program (www.ch.embnet.org), highlighting conserved residues in black (50% identical to consensus).

6.2 Sequence alignment of EcPutA and HpPutA

On the other hand HpPutA and EcPutA share only 20% identical amino acids (suppl. Figure 2).

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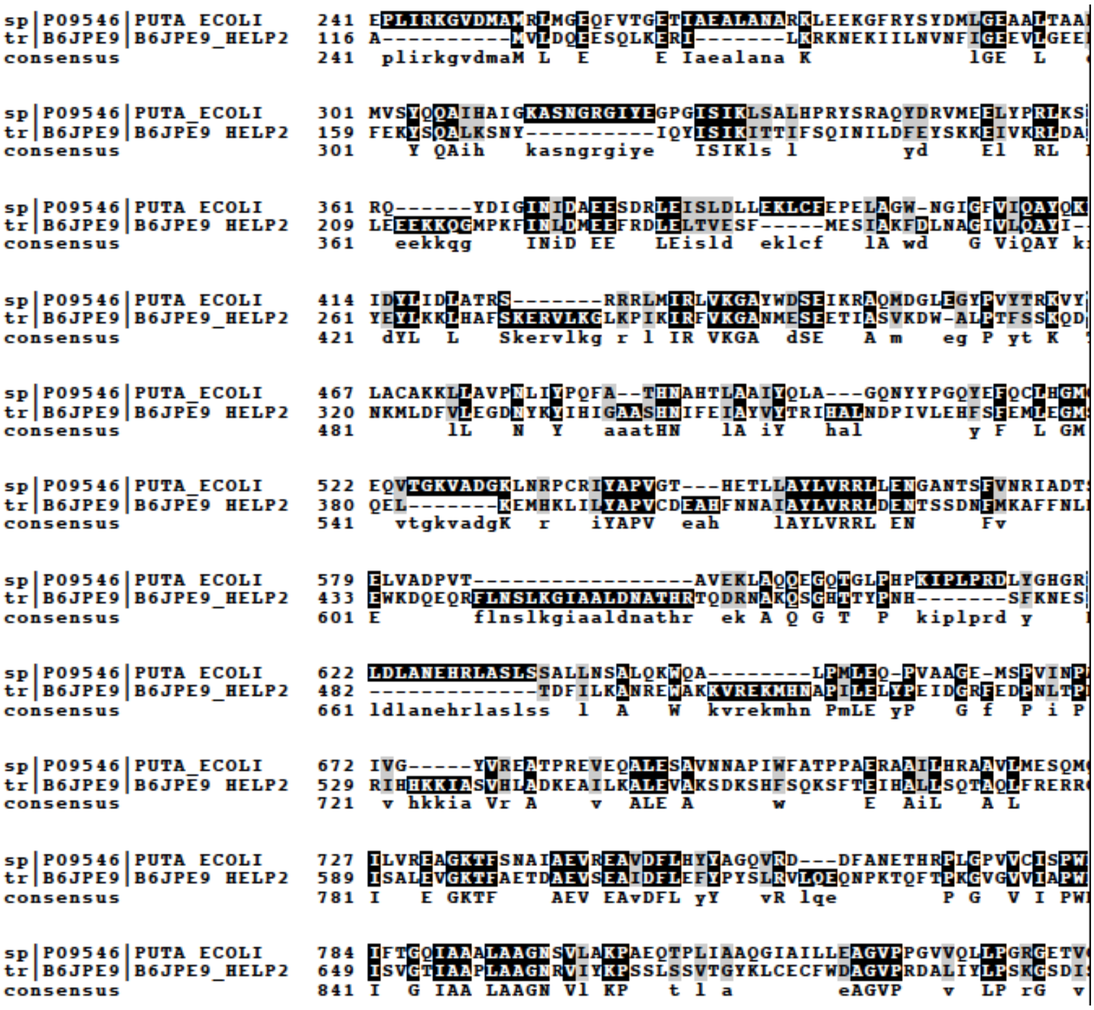
sp|P09546|PUTA_ECOLI      1 MGTTTMGVKLDDATRERIKSAATRIDRTPHWLIKQAIFSYLEQLENSDTLPELPA
tr|B6JPE9|B6JPE9_HELP2    1 -------------------------------------------------------
consensus                 1 mgtttmgvklddatreriksaatridrtphwlikqaifsyleqlensdtlpelpa

sp|P09546|PUTA_ECOLI     61 ANESDEAPTPAEEPHQPFLDFAEQILPQSVSRAAITAAYRRPETEAVSMLLEQAR
tr|B6JPE9|B6JPE9_HELP2    1 -------------------------------------------------------
consensus                61 anesdeaptpaeephqpfldfaeqilpqsvsraaitaayrrpeteavsmlleqar

sp|P09546|PUTA_ECOLI    121 AEQAHKLAYQLADKLRNQKNASGRAGMVQGLLQEFSLSSQEGVALMCLAEALLRI
tr|B6JPE9|B6JPE9_HELP2    5 IDDSLELAKKLQDSISNHLSEQE--KAFHSKMQKLLNNPENKVMLIELMDRSFRC
consensus               121  e    LA  L D l N      ra      lQ         V Lm L e   R

sp|P09546|PUTA_ECOLI    181 RDALIRDKISNGNWQSHIGRSPSLFVNAATWGLLFTGKLVSTHNEASLSRSLNRI
tr|B6JPE9|B6JPE9_HELP2   63 RFEMIEHVLDK--YKSRE--IFSPFEKLLLMGFLSFGKMLPDMSVP---FFVNKI
consensus               181 R  lI   i  gnw Sh gr  S F      G L  GKlv      sls  lNrI
```



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sp|P09546|PUTA ECOLI     241 EPLIRKGVDMAMRLMGEQFVTGETIAEALANARKLEEKGFRYSYDMLGEAALTAA
tr|B6JPE9|B6JPE9_HELP2   116 A----------MVLDQEESQLKERI-------LKRKNEKIILNVNFIGEEVLGEE
consensus                241  plirkgvdmaM L  E     E Iaealana K            lGE  L

sp|P09546|PUTA_ECOLI     301 MVSYQQAIHAIGKASNGRGIYEGPGISIKLSALHPRYSRAQYDRVMEELYPRLKS
tr|B6JPE9|B6JPE9 HELP2   159 FEKYSQALKSNY----------IQYISIKITTIFSQINILDFEYSKKEIVKRLDA
consensus                301    Y QAih   kasngrgiye   ISIKls l        yd    El  RL

sp|P09546|PUTA ECOLI     361 RQ------YDIGINIDAEESDRLEISLDLLEKLCFEPELAGW-NGIGFVIQAYQK
tr|B6JPE9|B6JPE9 HELP2   209 LEEEKKQGMPKFINLDMEEFRDLELTVESF-----MESIAKFDLNAGIVLQAYI-
consensus                361   eekkqg    INiD EE   LEisld  eklcf   lA wd   G ViQAY k

sp|P09546|PUTA ECOLI     414 IDYLIDLATRS-------RRRLMIRLVKGAYWDSEIKRAQMDGLEGYPVYTRKVY
tr|B6JPE9|B6JPE9_HELP2   261 YEYLKKLHAFSKERVLKGLKPIKIRFVKGANMESEETIASVKDW-ALPTFSSKQD
consensus                421  dYL  L   Skervlkg r l IR VKGA  dSE   A m   eg P yt K

sp|P09546|PUTA_ECOLI     467 LACAKKLLAVPNLIYPQFA--THNAHTLAAIYQLA---GQNYYPGQYEFQCLHGM
tr|B6JPE9|B6JPE9 HELP2   320 NKMLDFVLEGDNYKYIHIGAASHNIFEIAYVYTRIHALNDPIVLEHFSFEMLEGM
consensus                481       lL   N  Y   aaatHN   lA iY   hal        y F  L GM

sp|P09546|PUTA_ECOLI     522 EQVTGKVADGKLNRPCRIYAPVGT---HETLLAYLVRRLLENGANTSFVNRIADT
tr|B6JPE9|B6JPE9 HELP2   380 QEL-------KEMHKLILYAPVCDEAHFNNAIAYLVRRLDENTSSDNFMKAFFNL
consensus                541   vtgkvadgK  r   iYAPV  eah    lAYLVRRL EN     Fv

sp|P09546|PUTA ECOLI     579 ELVADPVT-----------------AVEKLAQQEGQTGLPHPKIPLPRDLYGHGR
tr|B6JPE9|B6JPE9_HELP2   433 EWKDQEQRFLNSLKGIAALDNATHRTQDRNAKQSGHTTYPNH-------SFKNES
consensus                601 E       flnslkgiaaldnathr  ek A Q G T  P  kiplprd y

sp|P09546|PUTA ECOLI     622 LDLANEHRLASLSSALLNSALQKWQA--------LPMLEQ-PVAAGE-MSPVINP
tr|B6JPE9|B6JPE9_HELP2   482 -------------TDFILKANREWAKKVREKMHNAPILELYPEIDGRFEDPNLTP
consensus                661 ldlanehrlaslss  l  A   W  kvrekmhn PmLE yP   G f   P i P

sp|P09546|PUTA_ECOLI     672 IVG-----YVREATPREVEQALESAVNNAPIWFATPPAERAAILHRAAVLMESQM
tr|B6JPE9|B6JPE9 HELP2   529 RIHHKKIASVHLADKEAILKALEVAKSDKSHFSQKSFTEIHALLSQTAQLFRERR
consensus                721  v hkkia Vr A    v  ALE A      w      E  AiL   A L

sp|P09546|PUTA ECOLI     727 ILVREAGKTFSNAIAEVREAVDFLHYYAGQVRD---DFANETHRPLGPVVCISPW
tr|B6JPE9|B6JPE9 HELP2   589 ISALEVGKTFAETDAEVSEAIDFLEFYPYSLRVLQEQNPKTQFTPKGVGVVIAPW
consensus                781 I   E GKTF    AEV EAvDFL yY   vR lqe        P G  V I PW

sp|P09546|PUTA ECOLI     784 IFTGQIAAALAAGNSVLAKPAEQTPLIAAQGIAILLEAGVPPGVVQLLPGRGETV
tr|B6JPE9|B6JPE9_HELP2   649 ISVGTIAAPLAAGNRVIYKPSSLSSVTGYKLCECFWDAGVPRDALIYLPSKGSDI
consensus                841 I  G IAA LAAGN Vl KP   t l a        eAGVP   v  LP rG  v
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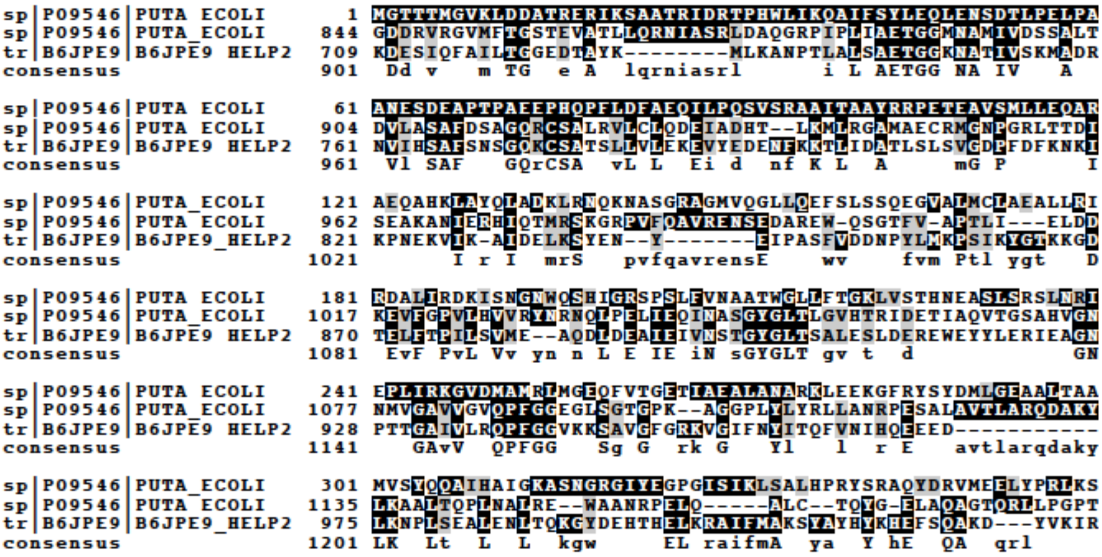

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sp|P09546|PUTA ECOLI       1 MGTTTMGVKLDDATRERIKSAATRIDRTPHWLIKQAIFSYLEQLENSDTLPELPA
sp|P09546|PUTA_ECOLI     844 GDDRVRGVMFTGSTEVATLLQRNIASRLDAQGRPIPLIAETGGMNAMIVDSSALT
tr|B6JPE9|B6JPE9 HELP2   709 KDESIQFAILTGGEDTAYK--------MLKANPTLALSAETGGKNATIVSKMADR
consensus                901  Dd v   m TG  e A  lqrniasrl      i L AETGG NA IV   A

sp|P09546|PUTA ECOLI      61 ANESDEAPTPAEEPHQPFLDFAEQILPQSVSRAAITAAYRRPETEAVSMLLEQAR
sp|P09546|PUTA ECOLI     904 DVLASAFDSAGQRCSALRVLCLQDEIADHT--LKMLRGAMAECRMGNPGRLTTDI
tr|B6JPE9|B6JPE9 HELP2   761 NVIHSAFSNSGQKCSATSLLVLEKEVYEDENFKKTLIDATLSLSVGDPFDFKNKI
consensus                961  Vl SAF   GQrCSA  vL L  Ei d  nf K L  A     mG P      I

sp|P09546|PUTA_ECOLI     121 AEQAHKLAYQLADKLRNQKNASGRAGMVQGLLQEFSLSSQEGVALMCLAEALLRI
sp|P09546|PUTA ECOLI     962 SEAKANIERHIQTMRSKGRPVFQAVRENSEDAREW-QSGTFV-APTLI---ELDD
tr|B6JPE9|B6JPE9_HELP2   821 KPNEKVIK-AIDELKSYEN--Y-------EIPASFVDDNPYLMKPSIKYGTKKGD
consensus               1021       I r I  mrS   pvfqavrensE    wv    fvm Ptl ygt   D

sp|P09546|PUTA ECOLI     181 RDALIRDKISNGNWQSHIGRSPSLFVNAATWGLLFTGKLVSTHNEASLSRSLNRI
sp|P09546|PUTA ECOLI    1017 KEVFGPVLHVVRYNRNQLPELIEQINASGYGLTLGVHTRIDETIAQVTGSAHVGN
tr|B6JPE9|B6JPE9 HELP2   870 TELFTPILSVME--AQDLDEAIEIVNSTGYGLTSALESLDEREWEYYLERIEAGN
consensus               1081  EvF PvL Vv yn n L E IE iN sGYGLT gv t  d            GN

sp|P09546|PUTA ECOLI     241 EPLIRKGVDMAMRLMGEQFVTGETIAEALANARKLEEKGFRYSYDMLGEAALTAA
sp|P09546|PUTA_ECOLI    1077 NMVGAVVGVQPFGGEGLSGTGPK--AGGPLYLYRLLANRPESALAVTLARQDAKY
tr|B6JPE9|B6JPE9 HELP2   928 PTTGAIVLRQPFGGVKKSAVGFGRKVGIFNYITQFVNIHQEEED-----------
consensus               1141    GAvV  QPFGG   Sg G  rk G   Yl   l  r E   avtlarqdaky

sp|P09546|PUTA_ECOLI     301 MVSYQQAIHAIGKASNGRGIYEGPGISIKLSALHPRYSRAQYDRVMEELYPRLKS
sp|P09546|PUTA ECOLI    1135 LKAALTQPLNALRE--WAANRPELQ-----ALC--TQYG-ELAQAGTQRLLPGPT
tr|B6JPE9|B6JPE9_HELP2   975 LKNPLSEALENLTQKGYDEHTHELKRAIFMAKSYAYHYKHEFSQAKD---YVKIR
consensus               1201 LK  Lt  L  L  kgw     EL raifmA  ya  Y hE  QA  qrl
```

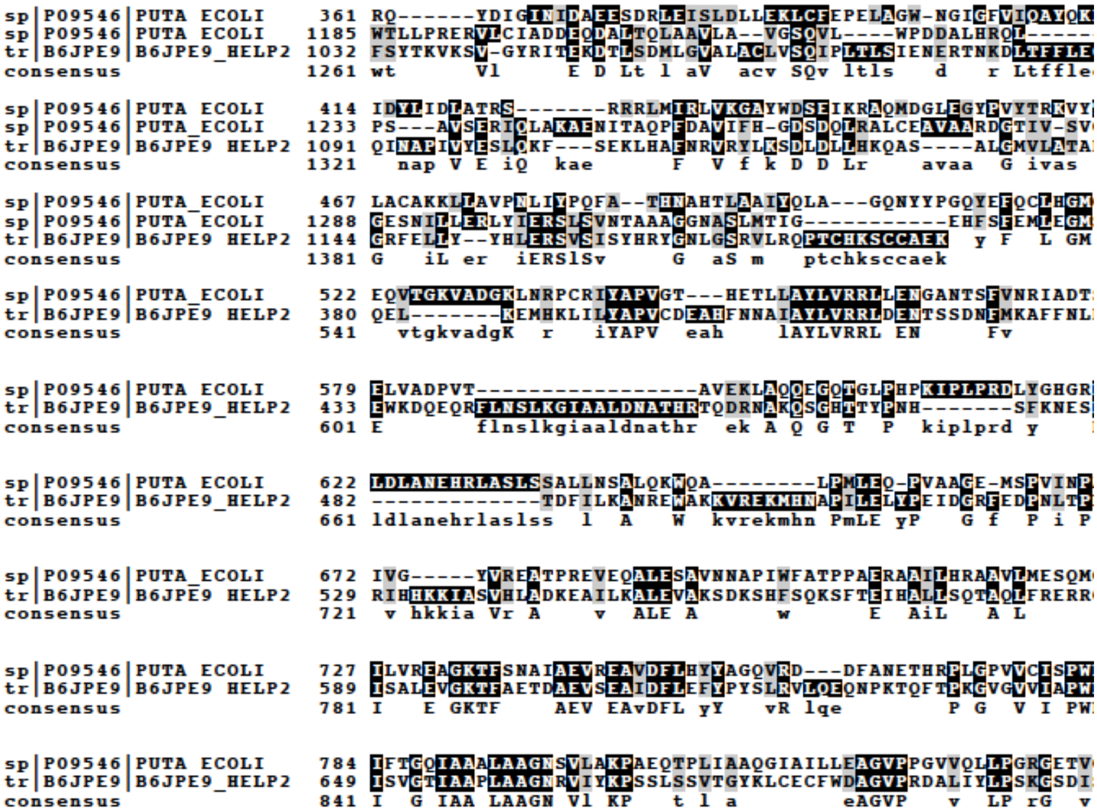

```
sp|P09546|PUTA ECOLI     361 RQ------YDIGINIDAEESDRLEISLDLLEKLCFEPELAGW-NGIGFVIQAYQK
sp|P09546|PUTA ECOLI    1185 WTLLPRERVLCIADDEQDALTQLAAVLA--VGSQVL----WPDDALHRQL-----
tr|B6JPE9|B6JPE9_HELP2  1032 FSYTKVKSV-GYRITEKDTLSDMLGVALACLVSQIPLTLSIENERTNKDLTFFLE
consensus               1261 wt      Vl     E D Lt l aV  acv SQv ltls   d   r Ltffle

sp|P09546|PUTA ECOLI     414 IDYLIDLATRS-------RRRLMIRLVKGAYWDSEIKRAQMDGLEGYPVYTRKVY
sp|P09546|PUTA_ECOLI    1233 PS---AVSERIQLAKAENITAQPFDAVIFH-GDSDQLRALCEAVAARDGTIV-SV
tr|B6JPE9|B6JPE9 HELP2  1091 QINAPIVYESLQKF---SEKLHAFNRVRYLKSDLDLLHKQAS----ALGMVLATA
consensus               1321   nap V E iQ  kae      F  V f k D D Lr    avaa  G ivas

sp|P09546|PUTA_ECOLI     467 LACAKKLLAVPNLIYPQFA--THNAHTLAAIYQLA---GQNYYPGQYEFQCLHGM
sp|P09546|PUTA ECOLI    1288 GESNILLERLYIERSLSVNTAAAGGNASLMTIG-----------EHFSFEMLEGM
tr|B6JPE9|B6JPE9 HELP2  1144 GRFELLY--YHLERSVSISYHRYGNLGSRVLRQPTCHKSCCAEK  y F  L GM
consensus               1381 G   iL er  iERSlSv     G  aS m   ptchksccaek

sp|P09546|PUTA_ECOLI     522 EQVTGKVADGKLNRPCRIYAPVGT---HETLLAYLVRRLLENGANTSFVNRIADT
tr|B6JPE9|B6JPE9 HELP2   380 QEL-------KEMHKLILYAPVCDEAHFNNAIAYLVRRLDENTSSDNFMKAFFNL
consensus                541   vtgkvadgK  r   iYAPV  eah    lAYLVRRL EN     Fv

sp|P09546|PUTA ECOLI     579 ELVADPVT-----------------AVEKLAQQEGQTGLPHPKIPLPRDLYGHGR
tr|B6JPE9|B6JPE9_HELP2   433 EWKDQEQRFLNSLKGIAALDNATHRTQDRNAKQSGHTTYPNH-------SFKNES
consensus                601 E       flnslkgiaaldnathr  ek A Q G T  P  kiplprd y

sp|P09546|PUTA ECOLI     622 LDLANEHRLASLSSALLNSALQKWQA--------LPMLEQ-PVAAGE-MSPVINP
tr|B6JPE9|B6JPE9_HELP2   482 -------------TDFILKANREWAKKVREKMHNAPILELYPEIDGRFEDPNLTP
consensus                661 ldlanehrlaslss  l  A   W  kvrekmhn PmLE yP   G f  P i P

sp|P09546|PUTA_ECOLI     672 IVG-----YVREATPREVEQALESAVNNAPIWFATPPAERAAILHRAAVLMESQM
tr|B6JPE9|B6JPE9 HELP2   529 RIHHKKIASVHLADKEAILKALEVAKSDKSHFSQKSFTEIHALLSQTAQLFRERR
consensus                721  v hkkia Vr A    v  ALE A      w      E  AiL   A L

sp|P09546|PUTA ECOLI     727 ILVREAGKTFSNAIAEVREAVDFLHYYAGQVRD---DFANETHRPLGPVVCISPW
tr|B6JPE9|B6JPE9 HELP2   589 ISALEVGKTFAETDAEVSEAIDFLEFYPYSLRVLQEQNPKTQFTPKGVGVVIAPW
consensus                781 I   E GKTF    AEV EAvDFL yY   vR lqe        P G  V I PW

sp|P09546|PUTA ECOLI     784 IFTGQIAAALAAGNSVLAKPAEQTPLIAAQGIAILLEAGVPPGVVQLLPGRGETV
tr|B6JPE9|B6JPE9_HELP2   649 ISVGTIAAPLAAGNRVIYKPSSLSSVTGYKLCECFWDAGVPRDALIYLPSKGSDI
consensus                841 I  G IAA LAAGN Vl KP   t l a        eAGVP   v  LP rG  v
```

Suppl Figure 2. Sequence alignment of EcPutA and HpPutA. The alignment was generated using ClustalO (1.1.0) with sequences (FASTA) obtained from a blast search (www.ncbi.nlm.nih.gov/BLAST) against PutA of *E. coli K12* (swiss-prot entry P09546) and PutA of *H. pylori P12* (entry HPP12_0050). Alignment was optically improved by the Boxshade 3.21 program (www.ch.embnet.org), highlighting conserved residues in black (20% identical to consensus).

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2006-2008 MSc in Immunology. National School of Biological Sciences, National Polytechnic Institute. Mexico City, Mexico. Thesis: Effect of *Mycobacteruim tuberculosis* Beijing genotype in the response of T lymphocytes. Overall average grade **9.13/10**.

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2008 **Poster:** "Induction of IFN-γ^+ and IL-10^+ double producer cells in peripheral blood mononuclear cells stimulated with *Mycobacterium tuberculosis*". XVIII National Congress of Immunology. Mexican Society of Immunology A C. April 20-24, Huatulco, Oaxaca.

2008 **Poster:** "Effect of the *Mycobacterium tuberculosis* components in Phagocytic cells". XXXVI National Congress of Microbiology. Morelia Michoacan, Mexico.

2007 **Poster**: Over-expression of HBD-2 and TNF-α by leukocytes treated with dialyzable leukocyte extracts.Assistance to the 13th International Congress of Immunology from August 21-25. Rio de Janeiro, Brazil.

2006 **Poster:** "Dialyzable leukocyte extracts induce the over-expression of beta-defensins in peripheral blood mononuclear cells". XVII National Congress of Immunology from May 2-5. Mexican Society of Immunology A C. Chihuahua, Chihuahua.