Analyse der Topologie und Funktion der c-Untereinheit der F_1F_0-ATPase von *Helicobacter pylori*

Dissertation

Zur Erlangung des akademischen Grades

des Doktors der Naturwissenschaften (Dr. rer. nat.)

an der Universität Konstanz, Fachbereich für Biologie

Vorgelegt von Tessa Schmidt-Petri

Tag der mündlichen Prüfung: 22. Oktober 2003
Referent: Prof. Dr. K. P. Schäfer
Referent: Prof. Dr. W. Welte

Analysis of the topology and function of the c subunit of the *Helicobacter pylori* F_1F_0-ATPase

Dissertation

For the degree of
Dr. rer. nat.

Submitted to the examination board
of the
Department of Biology
University of Konstanz

by
Tessa Schmidt-Petri

Konstanz, May 2003

Danksagung

Die vorliegende Doktorarbeit wurde in den Abteilungen „Functional Genomics and Proteomics" und „Biotechnology" bei ALTANA Pharma in Konstanz angefertigt.

Herrn Prof. Dr. Schäfer danke ich für die Bereitstellung des Themas und des Arbeitsplatzes. Die hervorragenden Arbeitsbedingungen ermöglichten spannende Forschung! Sein Enthusiasmus und die Unterstützung in allen Belangen sorgten für eine anregende Arbeitsatmosphäre.

Für die Betreuung dieser Arbeit über *Helicobacter pylori* möchte ich auch Herrn Dr. Melchers danken, der aus der Nähe und der Ferne immer wieder hilfreiche Anregungen gegeben hat. Außerdem vielen Dank für das Korrekturlesen, das Internet macht´s möglich!

Allen Mitgliedern der Abteilungen FG und BT möchte ich für das tolle Laborklima danken, ohne das sicherlich einige Momente viel härter gewesen wären. Ihre bereitwillige Hilfe zu jeder Zeit und ihre wertvollen Tipps haben immer wieder zum Fortkommen dieser Arbeit beigetragen. Marion Eisenhauer, Waltraud Burckhardt-Boer und Sabine Drewitz konnten mir die proteinbiochemischen Methoden näher bringen.

Ganz besonders muß das DNA Labor hervorgehoben werden. Anita Buhmann und Marina Mollenhauer möchte ich für die Einführung in die Arbeiten mit *H. pylori* danken. Ob Hp wohl heute gut drauf ist? Zusammen mit Michael Herrmann, Gisela Schüßler und Tanja Weller haben wir viel Spaß im Labor gehabt!

Herzlichster Dank gilt Kay für alle Unterstützung.

Dr. Anne Busch, Dr. Sandra Glockzin und Dr. Tina Kartmann danke ich sehr für immer neue Motivation und das rosa Glücksschwein. Tja, die Lerngruppe hat (fast) ausgelernt!

Außerdem möchte ich ganz herzlich meiner Familie danken, die mir diese Ausbildung ermöglicht und mich während des ganzen Studiums unterstützt hat! Dem Philosophen danke ich für das Korrekturlesen dieser „unverständlichen" Arbeit.

Hiermit versichere ich, die vorliegende Arbeit ohne unerlaubte Hilfsmittel verfasst zu haben.

Konstanz, Mai 2003

Table of contents

Abbreviations

aa	Amino acid
amp	ampicillin
BHI	Brain-Heart-Infusion Medium
bp	Base pair
cam	chloramphenicol
cat	chloramphenicol-acetyl-transferase
cat_{GC}	chloramphenicol-acetyl-transferase with promoter
DNA	Desoxyribonucleic Acid
FCS	Fetal calf serum
Fig.	Figure
gDNA	genomic DNA
h	hour
HS	Horse serum
kDa	kilodalton
LB	Luria Broth medium
min	minute
mV	Millivolt
MW	Molecular
OD	optical densitiy
ORF	open reading frame
PAGE	polyacrylamid gel electrophoresis
PAI	pathogenicity island
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P*flaA*	*flaA* promoter
pI	isoelectric point
rpm	rounds per minute
RT	room temperature
SDS	Sodiumdodecylsulfate
Tricin	N-Tris-(hydroxymethyl)-methylglycin
V	Volt
wt	wild type

1 Introduction

Helicobacter pylori is a spiral-shaped bacterium that lives in the acidic human stomach and can cause severe diseases. Spiral microorganisms were already detected in the animal stomach by the end of the 19th century (Bottcher, 1874) and described in dogs and cats (Rappin, 1881; Bizzozero, 1893). A detailed description about the flagellar structure and motility in the animal host, the handling in the laboratory and even infection of mice with, as now known, *Helicobacter felis* was provided by Salomon (1896). By this time, the so-called "Spirocheta" were also described in the human stomach (Jaworski, 1889, Pel, 1899) and a role in gastric disease was suggested. But the dogma "No acid, no ulcer" (Schwarz, 1910) and the general opinion that the acidic environment of the stomach with an average pH of 1.5 permitted no bacterial life, dominated the scientific discussion. In 1938, Doenges systematically examined more than 200 victims of accidents and found spiral organisms in over 40% (Doenges, 1938). Although some investigators believed in the association of spiral bacteria and pathological phenomena in the human stomach (Krienitz, 1906; Konjetzny, 1928; Doenges, 1939; Freedberg, 1940), their opinion was contradicted in the 1950s (Ivy, 1950; Palmer, 1954) when bacteria in the stomach were thought to result from contamination during the operation due to unclean tools. Only in 1975, Steer proposed a possible etiopathological role of the bacteria in gastritis and gastric ulceration.

Warren and Marshall "rediscovered" *H. pylori* in 1983, when they showed that *H. pylori* infected patients developed gastritis and ulcers or, in some cases, eventually even cancer. They were able to fulfil Koch's postulates (1884), which demand isolation and cultivation of a pure culture of organisms from the diseased organ and the proof of induction of disease after reinfection with this culture.

Marshall himself ingested a bacterial culture and consequently developed gastritis thereafter and *H. pylori* were reisolated from a biopsy taken 10 days after infection (Warren and Marshall, 1983, Marshall and Warren, 1984).

This link resulted in an increasing interest in research on *H. pylori* and 16512 papers have been published since 1987 as referenced in PubMed (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed).

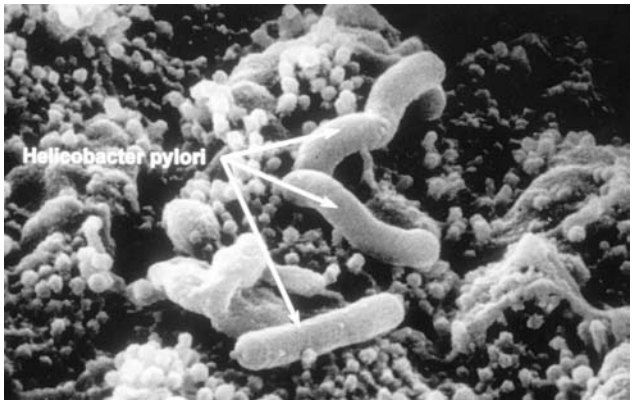

Figure 1.1: *H. pylori* are spiral shaped bacteria of up to 4µm length with 4 to 6 unipolar flagellae. They are found on the gastric epithelium of the human antral mucosa (figure provided by G. Bode, University of Ulm).

1.1 Gastroduodenal disease

H. pylori infection occurs throughout the world but there are significant differences between countries with different socioeconomic status. These differences arise due to differing childhood infection rates. The prevalence in developed countries varies between 0 and 5% in contrast to developing countries where 13 to 60% of the children under 10 years are infected. Thereafter, infection rates increase annually between 0.5 and 2%. The route of infection is unclear and environmental reservoirs have been proposed but no significant source of contamination could be identified. Direct transmission from person-to-person seems to be the most likely way of transmitting the disease (reviewed in Mitchell, 2001).

Several diseases have been associated with *H. pylori* infection. The association with peptic ulcer disease, chronic gastritis, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma has been well documented (Eslick et al., 1999). However, the role in gastropathy associated with non-steroidal anti-inflammatory drugs (NSAiDs), gastroesophageal reflux disease (GERD) and both uninvestigated and non-ulcer dyspepsia, dyspepsia being “a pain or discomfort centred in the upper abdomen”, remain controversial. The benefit of eradication and long-term symptom relief in non-ulcer dyspepsia is not obvious (Moayyedi *et al*. 2000, Laine *et al*., 2001). GERD seems to be less common in *H. pylori* infected individuals but eradication of the bacterium does not negatively influence the course of reflux disease (Moayyedi *et al*., 2001).

Recommendations for the treatment of *H. pylori* infection are given in the Maastricht 2-2000 Consensus Report (Malfertheiner *et al*., 2002).

The association between *H. pylori* infection and gastric cancer has been documented in several studies and meta-analyses concluded that *H. pylori* infection increases the risk for gastric adenocarcinoma (Huang *et al*., 1998; Danesh, 1999; Eslick *et al*., 1999). An animal model using Mongolian gerbils is also available and is contributing to understand the course of disease (Watanabe *et al*., 1998).

Although these studies give evidence for an association between *H. pylori* infection and gastric disease, they also show that the disease outcome is influenced by environmental, bacterial and host co-factors.

The course and development of disease is shown in Figure 1.2.

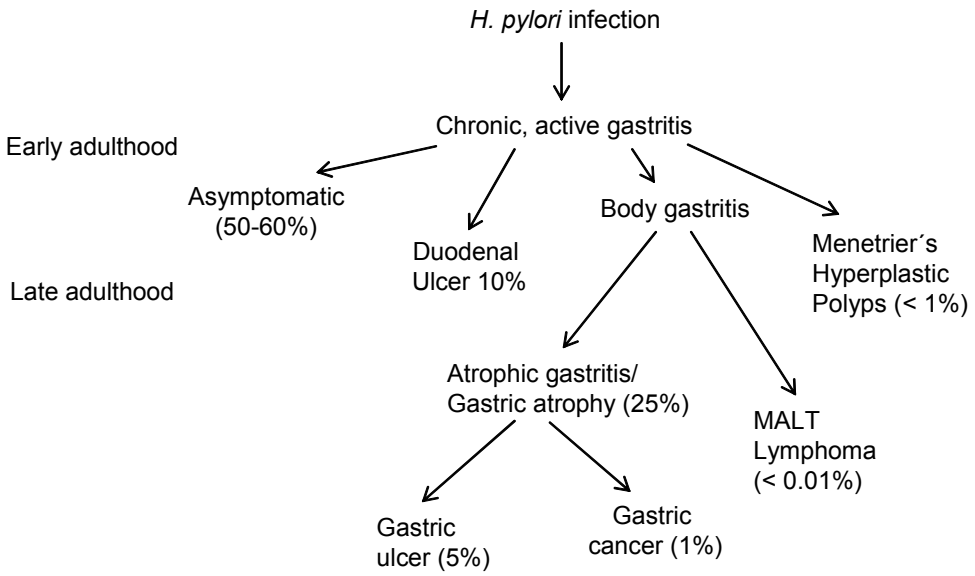

Figure 1.2: The natural history of *H. pylori* infection in the USA. (adapted from Houghton *et al*., 2002). The disease develops over several years and can have different outcomes. (MALT: mucosa associated lymphoid tissue)

Various tests are available for the diagnosis of *H. pylori* infection, invasive and non-invasive methods. Non-invasive tests include the urea breath test, serologic tests and stool antigen assays. Endoscopic biopsy is indicated in patients with alarming symptoms, such as anaemia, gastrointestinal bleeding or weight loss, as well as in older patients (> 50 years) (Suerbaum and Michetti, 2002). The biopsy is analysed by a urease test and, in rare occasions, antibiotic sensitivity testing is performed.

An *H. pylori* specific therapy is not available yet. The standard regimen is a triple therapy that includes a proton pump inhibitor and two antibiotics. The proton pump inhibitor improves the effectiveness of the antibiotics, both by optimising the impact of the antibiotics and by destroying *H. pylori's* acidic ecological niche. At rare occasions quadruple therapies are used to clear persistent infections or as a second line treatment (for a review see Suerbaum and Michetti, 2002).

Furthermore, the development of an *H. pylori* vaccine is also recognised as an important factor for disease control. A better understanding of the manipulation of the host immune system and of immune evasion strategies of *H. pylori* are needed (see Banerjee and Michetti, 2001 for a review).

1.2 Properties of *H. pylori*

H. pylori is a gram-negative bacterium of 2.5-4µm length and 0.5-1.0µm width. Its appearance varies from spiral-shaped to a rod-like form. Coccoids are also observed in older cultures or under unfavourable culture conditions and might be responsible for the persistence of *H. pylori*. They still show reduced metabolism (Bode *et al*., 1992, 1993) but recultivation has not been reported. Kusters *et al*. (1997) suggest that coccoids appear as a form of cell death. The conversion to the coccoid form is not influenced by the inhibition of protein or RNA synthesis, which indicates a passive process. Moreover the coccoids lack a membrane potential. Infection of mice with non-cultivable coccoids has been reported (Cellini *et al*., 1994; Aleljung *et al*., 1996) and viable forms could be reisolated. However, it remains controversial if the cultures were completely free from spiral-shaped bacteria. Additionally, very high concentrated bacterial cultures were used which could have influenced the outcome of disease.

H. pylori is a microaerophilic bacterium that needs a complex medium with additional growth supplements. These supplements include inactivated horse serum or blood derivatives for growth *in vitro* in liquid or solid media. *In vivo*, *H. pylori* is an exclusively human pathogen and lives on the gastric mucosa in the antral region of the human stomach.

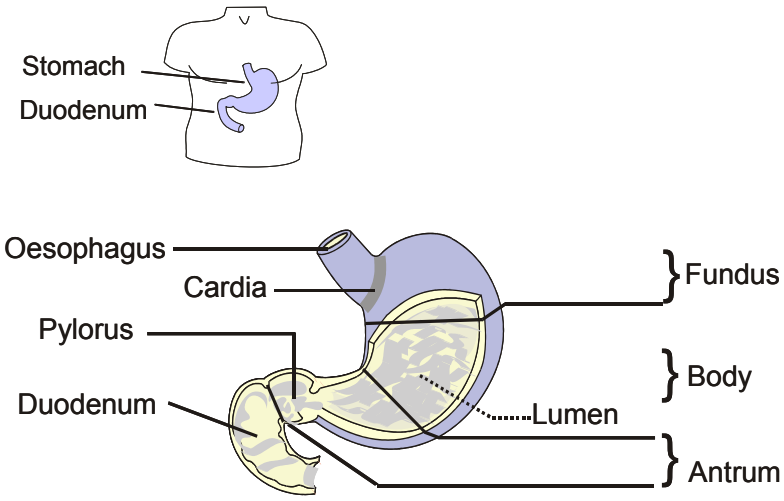

Figure 1.3: The four regions of the human stomach are cardia, fundus, body and antrum. *H. pylori* is mostly found in the antrum, other regions are only colonised under conditions of low acid secretion.

The human stomach is divided into four regions: cardia, fundus, body and antrum. *H. pylori* is predominantly found in the antral region of the stomach. Acid secretion occurs in the fundic region of the stomach in the fundic glands. These glands contain a specialised cell type, the parietal cells that express the gastric H^+, K^+- ATPase for acid secretion.

H. pylori adheres to gastric epithelial cells in the antral region of the stomach where acid secretion is not observed. The more acidic fundic or body region is only colonized under conditions of low acid secretion as a result of pathological or medical alterations.

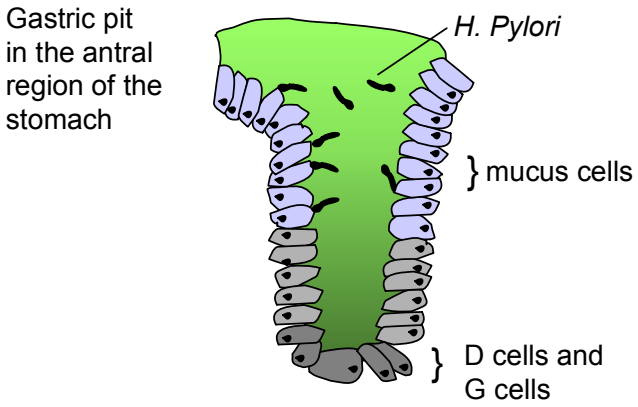

Figure 1.4: *H. pylori* are mostly found in the antral region of the stomach. They adhere to mucus-secreting cells in the upper part of the gastric pit or live in the mucus layer. In this region of the stomach, acid-secreting parietal cells are lacking. The D-cells possess a pH sensing receptor and somatostatin is released as a response to increasing acidity. Somatostatin inhibits secretion of gastrin from G endocrine cells. Gastrin stimulates acid-secretion by parietal cells, which are found in the body and the fundic region of the stomach. Gastrin secretion by G-cells is stimulated by the presence of aromatic amino acids at a pH > 3 and inhibited at a pH < 3.

Many factors allow *H. pylori* to inhabit the hostile environment of the human stomach, creating its own ecological niche (Haas, 1995). The passage through the acidic gastric lumen has to be overcome to reach the more neutral mucus layer and the underlying gastric epithelial cells. It has been shown that motility is essential for infection in various animal models (Eaton *et al*., 1989, 1992, 1996), as is the potent urease (Andrutis *et al*., 1995; Eaton and Krakowka, 1994; Tsuda *et al*., 1994). Other factors such as adherence to the gastric epithelium, the production of cytotoxins and the effective evasion of the host immune system influence the pathogenicity of *H. pylori*.

1.3 Virulence factors

Several bacterial factors, so-called virulence factors, influence the pathogenesis of *H. pylori* and the host-pathogen interaction.

Three categories of virulence factors are classified depending on the phase of infection: factors for *colonisation* (transit through lumen, entry into the mucus), factors important for *persistence* (immune system cannot eliminate the bacterium) and factors that induce tissue damage and *chronic infection* (for instance toxins). After infection, the bacterium has to overcome gastric acidity and colonizes the gastric epithelium of the human stomach. Once in the niche in the gastric mucus layer, *H. pylori* displays pathogenic features and generally persists for the host's lifetime (Blaser, 1993).

1.3.1 Motility

At first, motility as part of a chemotactic movement is essential for *H. pylori* infection and non-motile *H. pylori* are unable to infect gnotobiotic piglets (Eaton *et al*., 1992, 1996, Ottemann *et al*., 2002) and the mucosae of mice (Kim *et al*., 1999). Motility is mediated by 1 to 8 unipolar flagella (O´Toole and Clyne, 2001). The flagella consist of the structural components of the filament, the hook and the flagellar basal body (O´Toole *et al*., 1994). Analysis of two *H. pylori* genomes identified 50 proteins that are involved in the regulation of flagellar assembly (Tomb *et al*. 1997, Alm *et al*., 1999), yet not all of them have been analysed in detail. FlaA and FlaB of the central filament have been extensively studied (Leying *et al*., 1992, Haas *et al*., 1993, Suerbaum *et al*., 1993). Both genes, *flaA* and *flaB*, are essential for full motility (Josenhans *et al*., 1995). The filament is surrounded by a membranous sheath, which is an extension of the outer

membrane and might prevent degradation of the filament in the acid environment (Geis *et al*., 1993). Motility together with the spiral shape and the secretion of mucus hydrolysing enzymes allows *H. pylori* to enter the mucus layer.

The pH gradient between the lumen (pH 1-2) and the mucus layer (pH 6-7) probably directs the bacterium to the epithelial cells (Haas *et al*., 1995). Chemotaxis towards various compounds has been observed, including the amino acids glutamine, histidine, lysine and alanine (Worku *et al*., 1997), mucin (Turner *et al*., 1997), urea, sodium bicarbonate and sodium chloride (Mizote *et al*., 1997). A concentration gradient of urea, bicarbonate and sodium ions exists in the mucous layer of epithelial cells and may direct the bacteria. Chemotactic movement was enhanced and dependent on urease in a viscous environment (Nakamura *et al*., 1998, Yoshiyama *et al*., 1999). The rotation of the flagellar motor was shown to depend on a proton motive force (Nakamura *et al*., 1998).

1.3.2 Adherence

Once in the protective mucus layer, adherence and interaction with the host play an important role. The bacterial outer membrane consists of lipid A of the lipidpolysaccharides and phospholipids. The lipidpolysaccharides (LPS) are important for the identification of bacterial species, as well as their interaction with their environment. Therefore, LPS have an important role in bacterial pathogenesis: they possess various endotoxic properties and can transfer lethal toxicity but they are also highly variable which might contribute to virulence and immune evasion.

H. pylori expresses several genes that mediate adherence to gastric epithelial cells. Adherence might be essential for the persistence of *H. pylori* in the stomach as clearance of the bacteria is impeded and higher densities of bacteria are achieved. Adherence renders the bacteria more resistant to antibiotics than non-adherent bacteria (Mégraud *et al*., 1991). However, investigation of the role of single proteins in adhesion is difficult because other adhesion proteins may mask the effect of a specific knockout. Additionally, the variety of human cell lines and *H. pylori* strains complicates the comparison of the different studies. Various groups of *H. pylori* adhesins have been identified. They bind to different cell receptors on the gastric epithelium such as Lewis blood group antigens, laminin, type IV collagen, plasminogen and mucin (Haas, 1995; Logan, 1996).

BabA (blood group antigen-binding adhesin) is found on the outer membrane of *H. pylori* and binds to Lewis^b-blood group antigen on epithelial cells (Boren *et al*., 1993; Ilver *et al*., 1998). This might explain an increased risk of gastric cancer in persons with blood group 0. Strains expressing BabA colonize in higher densities and provoke an IL-8 response and higher granulocytic infiltration, which might lead to increased mucosal damage (Rad *et al*., 2002). A novel adhesin, SabA, has been identified with its receptor, the sialyl-Lewis x glycosphingolipid (Mahdavi *et al*., 2002). This receptor appears to be induced with increasing inflammation and SabA binding might therefore support chronic infection.

Immune evasion seems to be another factor in chronic infection. The O-antigen of *H. pylori* LPS seems to promote infection by molecular mimickry of Lewis antigens (a, b, X and Y), normally expressed on gastric epithelium (Appelmelk *et al*., 1997).

1.3.3 Toxins

H. pylori does secrete proteins, which induce a host cell response. The different strains can be classified by their variable expression of these secreted proteins.

VacA is a secreted exotoxin of 95kD. The toxin inserts itself into the epithelial cell membrane and forms a hexameric anion-selective, voltage-dependent channel. Bicarbonate and organic anions are released through this channel, possibly providing *H. pylori* with nutrients (Szabo *et al*., 1999). VacA can also insert into the mitochondrial membrane, where it causes release of cytochrome c and induces apoptosis (Galmiche *et al*., 2000). The vacuolating VacA induces vacuoles *in vitro* in HeLa cells and in primary gastric epthelial cells (Covacci *et al*., 1993; Cover *et al*., 1993, Phadnis *et al.*, 1994). It can also induce apoptosis in AGS gastric cell line (Kuck *et al*., 2001). Different alleles of the vacA gene exist: three different signal sequences s1a, s1b, s2 and two different middle sequences, m1 and m2, are known (Atherton *et al*., 1995). They influence vacA production and also the disease outcome although VacA is not essential for colonization.

CagA (cytotoxin associated antigen) is expressed by type I strains ($CagA^+/VacA^+$) in contrast to type II strains ($CagA^-/VacA^-$). Type I strains are associated with the development of duodenal ulcer, atrophic gastritis and adenocarcinoma (Cover *et al*., 1990; Cover *et al*., 1995; Kuipers *et al*., 1995; Blaser *et al*., 1995). The type IV secretion system delivers CagA into the mammalian cytosol. The genes for the type IV

secretion system are encoded on the 40 kb cag pathogenicity island (PAI), together with other factors. CagA contains various phopshorylation motifs, despite its amino acid diversity, and is phosphorylated at a tyrosine residue by an unidentified host cell kinase (Evans *et al*., 2001; Backert, *et al*., 2001; Puls, *et al*., 2002; Stein, *et al*., 2002). The activation of NFκB and AP-1 transcription factors and deregulation of the cellular phosphatase SHP-2 by CagA may induce abnormal cellular growth and might promote gastric cancer (Ferber, 2001). Additionally, IL-8 secretion of gastric epithelial cells can be observed in response to secretion of CagA. IL-8 is a chemoattractant for neutrophils and IL-8 secretion results in an inflammatory response.

Many factors contribute to the establishment of a chronic infection by *Helicobacter pylori*, as summarized in Figure 1.5 below:

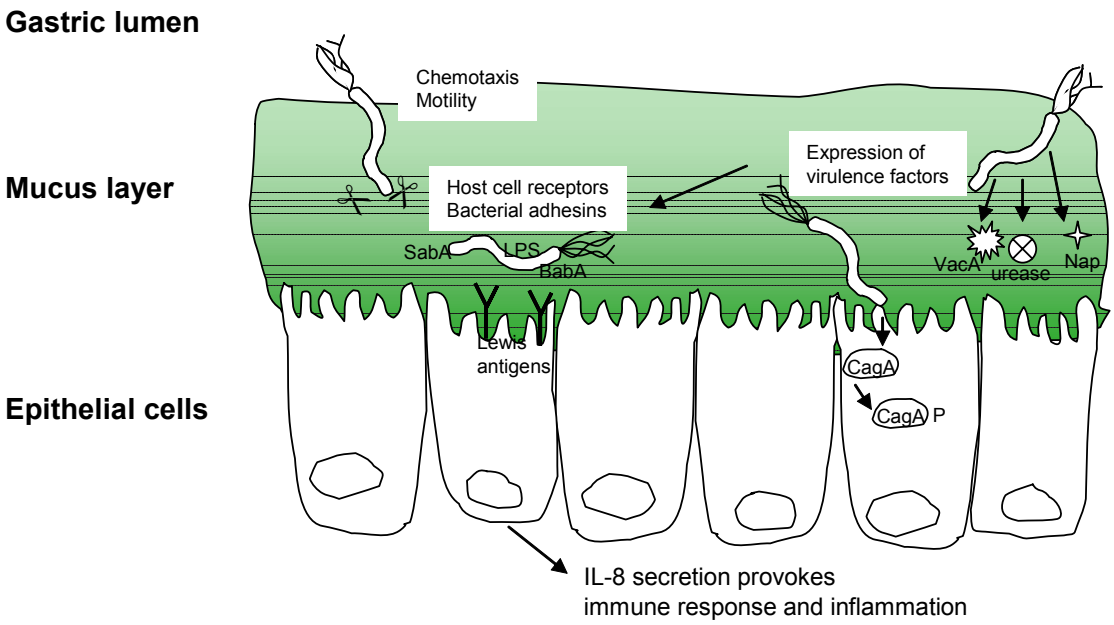

Figure 1.5: A schematic description of *H. pylori* infection. The bacteria reach the gastric mucus due to chemotactic orientation. The spiral shape and several flagella enable *H. pylori* to move through the mucus, which is solubilized by mucinase and collagenase. Urease is essential for the passage of the gastric lumen and survival. *H. pylori* adhere to epithelial cells. Identified adhesins are BabA, SabA and HpaA. Virulence factors are released into the cell, CagA is phosphorylated and modifies signalling pathways. Other secreted molecules are VacA and NAP. The secretion of virulence factors induces IL-8 response from the epithelial cells, provoking an immune response and further cell damage.

1.4 Genomics and proteomics

The first genome sequence for *H. pylori* was published in 1997 (Tomb *et al*., 1997). The chromosome of strain 26695 has 1.67 Mbp and 1590 predicted open reading frames.

The sequence of a second, unrelated strain J99 became available in 1999 and has been compared to the sequence of strain 26695 (Alm *et al*., 1999). The comparison of the two genomes was the first comparison of this kind and revealed species-specific characteristics as well as strain specific differences.

The overall structure of the two genomes is similar with only ten variant sequence arrangements. Eight of these show characteristics of insertion elements in at least one of the two strains. Between 6% and 7% of the genes are strain specific and these genes appear at various places, disrupting the overall gene order. Almost 60% of the genes were attributed a predicted function, 24% were conserved in other bacterial strains. 17% of the genes were *H. pylori* specific, even when the genome was compared to the very closely related *C. jejuni* genome (Parkhill *et al*., 2000).

The *H. pylori* specific genes might provide unique drug targets but further investigation of their function *in vitro* and *in vivo* is necessary.

More than 70% of the predicted proteins have an isoelectric point greater than 7, compared to ~40% in *E. coli* and *H. influenzae*, and basic amino acids, arginine and lysine, occur twice as frequently. These characteristics might be reflecting the ability of *H. pylori* for acid adaptation.

The paucity of transcriptional regulators in *H. pylori* (Tomb *et al*., 1997) probably indicates adaptation to its very specific niche. This phenomenon has been described in other bacteria and a correlation between number of transcriptional regulators and adaptation to a special niche has been observed: 9.6% of the genome of *P. aeruginosa* encodes transcriptional regulators or two-component systems (Stover *et al*., 2000). In *E. coli* it is only 5.8% and in the highly adapted *M. tuberculosis* it is 3%. In *H. pylori,* only 1.1% of all genes regulate gene expression (Tomb *et al*., 1997, Stover *et al*., 2000) and the low percentage emphasizes the high specialization. The creation and analysis of a genome-scale metabolic model for *H. pylori* 26695 also describes a limited metabolism (Schilling *et al*., 2002). A high degree of adaptation to the human host in a nearly competitor-free environment might account for these limitations.

The high variation at the nucleotide level can explain the overestimation of genetic variability, previously predicted by pulsed-field gel electrophoresis (PFGE) (Jiang *et al*., 1996). Average nucleotide identity is 94% whereas protein similarity is 95.4% for genes and their proteins with predicted function. This observation is also valid for the *atpE* gene where 22 base pairs were exchanged in the different strains, only resulting in two amino acid exchanges.

With the completion of the two genome sequences, the development and use of microarrays allows the comparison of different *H. pylori* strains grown in various environmental conditions. 15 *Helicobacter* strains were compared with microarrays that represent 98.6% of 26695 and J99 (Salama *et al*., 2000). The minimal functional core comprises 1281 genes that are common to all tested strains and represent genes with metabolic, biosynthetic, cellular and regulatory functions. 362 open reading frames (ORF) were even strain-specific and absent from one or more strains. This fact might be responsible for adaptation of *H. pylori* to its specific host. Most of these genes were found in two regions with high plasticity: the plasticity zone (PZ) and the pathogenicity island, which also vary in their GC content. Comparing two clinical isolates with different disease outcomes in the gerbil model revealed that the less proinflammatory strain had a large deletion of the cag PAI (Israel *et al*., 2000). The PAI is relevant for disease outcome but does probably not represent a lineage. PAI containing strains are not more related to each other than to PAI lacking strains.

Microarrays are also used for the comparison of messenger RNA (mRNA) expression levels in different environmental conditions to detect important genes. *H. pylori* colonizes over years the acidic human stomach and genes responsible for this unique ability might provide new drug targets. Ang *et al*. (2001) performed microarray experiments to investigate acid response. They grew bacteria on agar plates of pH 7.2 and 5.5 for 48 hours. The expression profile using a macroarray with 1534 predicted ORFs of strain 26695 (96%) identified 80 acid-upregulated ORFs. 16 ORFs were already known to be involved in acid response but 43 functionally annotated ORFs were previously not assumed to be involved in acid response. This might reflect the complexity of acid response in *H. pylori* but can also represent an experimental artefact due to elongated growth on agar plates.

The variation of mRNA levels is only an indication for differential protein expression. More accurate results are obtained by two-dimensional gel electrophoresis (2-DE), which is used to identify proteins expressed under varying conditions. Comparison of 2-D gel images can identify differentially expressed proteins that are identified by mass spectroscopy. 2-D gel electrophoresis allows the separation of up to 10000 protein species in one run (Klose and Kobalz, 1995) and is therefore sufficient for proteome analysis of *H. pylori* with 1495 (J99) or 1590 (strain 26695) predicted open reading frames. Strain specificities were identified. Three strains, 26695, J99 and SS1, were compared by 2-D gel electrophoresis (Jungblut *et al*., 2000).

Separation conditions were from pI 4-10, MW 5-150kDa. The number of detected proteins varied between all three strains: About 1863 protein spots were detected in 26695, 1448 in SS1 and 1622 in J99 indicating again high strain variability. However, single amino acid exchanges already result in a clearly detectable shift in the 2-D gel. Subsequently, the 2-D gels were used to identify antigens in combination with antisera from infected and non-infected patients.

Unfortunately, the analysis of membrane proteins by proteomic analyses is difficult and membrane proteins are often underrepresented (Santoni *et al*., 2000). They have very important functions *in vivo*, for instance as receptors. A different method was used by Santoni *et al*. (2000) to disrupt the membrane with high pH and using proteinase K to generate short peptides for identification. The percentage of identified membrane proteins seems to correspond to the predictions of the genome analyses of 20-30% of all open reading frames. Also, posttranslational modifications can be detected with this method.

Rain *et al*. (2001) constructed a protein-protein interaction map for *H. pylori* using a modified yeast-two-hybrid screen. 261 bait plasmids were constructed. A highly complex library of prey plasmids with encoded polypeptides was generated. Interactions were grouped according to selected interacting domains that were identified comparing common sequences shared by a group of prey fragments. The relevance of interactions was evaluated with a reliability score and 1200 interactions were identified, which represents 47% of the proteome.

The relevance of these investigations for the *in vivo* situation is sometimes questioned because very diverse results have been obtained (Covacci and Rappuoli, 2003). *In vivo*

essential genes were recently identified with the signature tagged mutagenesis method (Kavermann *et al*., 2003). Among known genes that are essential for colonisation, new genes were identified such as collagenase, also proposing unknown mechanisms for gastric colonization.

1.5 Acid resistance

H. pylori is able to survive in the acidic human stomach. At least during primary colonization it has to overcome pH values between 1 and 2, the diurnal median pH in the gastric lumen being 1.4 (Teyssen *et al*., 1995). Following the passage through the acidic gastric lumen, *H. pylori* reaches the gastric surface where the pH is assumed to be close to neutral. Still, this is a matter of debate. It has been shown that the pH at the gastric surface is equivalent to the luminal pH when it decreases to pH 2 (Schade *et al*., 1994). *H. pylori* has to penetrate the gastric mucosa for colonization of the gastric epithelium but the role of the mucosa for protection or infection is unclear.

Bacteria in general survive by maintaining a relatively constant proton motive force (PMF) across their cytoplasmic membrane. For *H. pylori*, the PMF amounts to ~ 200 mV between pH 4.0 and 8.2 and growth is observed between pH 6.0 and 8.0. This behaviour classifies them as neutralophiles. Neutralophiles are able to grow at neutral pH and survive in the range of pH 4 to 8.5 (Padan *et al*., 1981). Additionally, *H. pylori* has developed special mechanisms for survival in the acidic environment of the stomach and can therefore be classified as an acid-tolerant neutralophile. In contrast to acidophiles, which thrive at pH values between 1 and 4, *H. pylori* is only able to survive in pH 1 for several hours in the presence of urea (Stingl *et al*., 2001).

Acid resistance is primarily mediated by the enzyme urease, which constitutes up to 10% of total cell protein (Bauerfeind *et al*., 1997). The hydrolysis of urea produces NH_3, resulting in local pH elevation by protonation, either of the cytoplasm or, after diffusion, the periplasm. This issue is still controversially discussed (Stingl *et al*., 2001; Sachs *et al*., 2002). About 1.7-3.4mM urea are present in the human stomach (Mobley and Foxall, 1994).

The constitutive production of urease was recognized early as a significant factor for bacterial survival in acidic pH and in the stomach (Marshall *et al*., 1990) and the generation of various mutants demonstrated that urease is essential for colonisation in

several animal models (Eaton and Krakowka, 1994; Andrutis *et al*., 1995; Tsuda *et al*., 1994).

The sequence of the urease gene cluster revealed an operon structure with the genes *ureABIEFGH* in this order (Clayton *et al*., 1990; Labigne *et al*., 1991; Cussac *et al*., 1992) and similar operons are found in other organisms that express an active urease (Mobley *et al*., 1995).

The urease gene cluster and a model for the assembly of active urease are displayed in Figure 1.6 below.

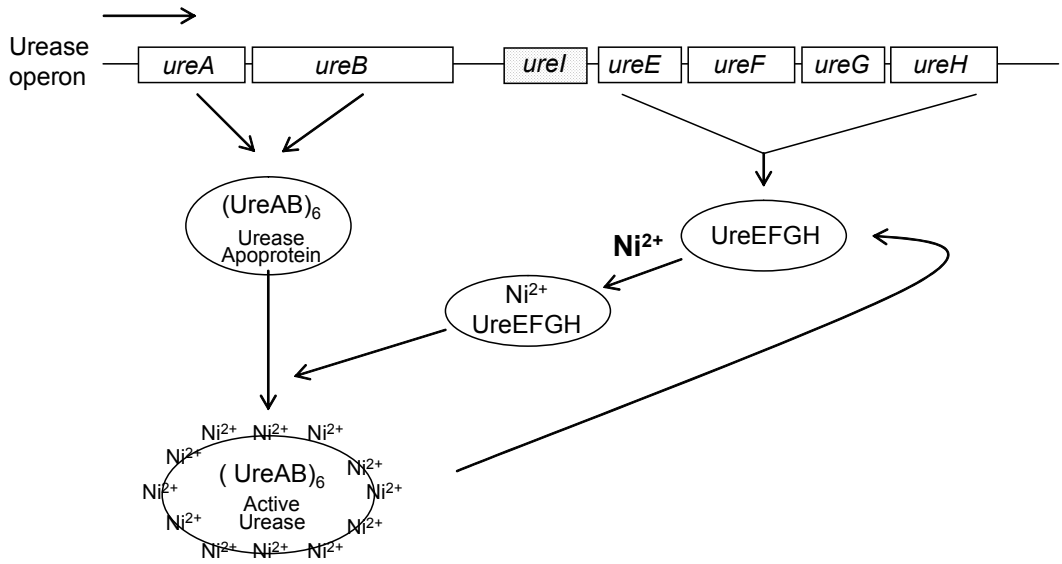

Figure 1.6: The urease gene cluster and the formation of active urease. A hexamer of UreAB dimers constitutes the urease apoprotein. The incorporation of twelve Ni^{2+} ions by the accessory proteins UreEFGH mediates activity. Ni^{2+} is taken up by specialized transport proteins such as NixA and stored in the cell, bound to different proteins such as Hpn or HspA. UreI forms an inner membrane urea channel and regulates urease activity by regulating urea uptake into the cells (Weeks *et al*., 2000). (Modified from Mobley, 1996).

Urease is a hexadimer of the two structural subunits UreA and UreB and requires Ni^{2+} ions in its active centre for catalytic activity. The accessory proteins UreE, F, G and H are responsible for the incorporation of Ni^{2+} ions into the apoenzyme and Ni^{2+} is essential for urease activity and animal colonization (Nolan *et al*., 2002). The availability of the urease cofactor Ni^{2+} in response to pH constitutes a regulatory mechanism (van Vliet *et al*., 2001 and 2002) and Hpn has been described as a Ni^{2+} storage protein (Gilbert *et al*., 1995).

Urease has been proposed to be located in the periplasm, generating an acid neutralizing cloud of ammonia around the bacterium (Hazell, 1990). The mechanism of the proposed

urease export is unclear. It is suggested that urease export results from bacterial lysis, termed altruistic autolysis (Dunn and Phadnis, 1998). In contrast, several arguments substantiate a cytoplasmic localization of urease (Scott *et al*., 1998). Free urease is acid unstable and completely inactivated at a pH < 4.5 (Scott *et al*., 1998). Inactivation at pH values below 4.5 would render the enzyme useless when it is most needed. Moreover, the pH optimum of free urease is between pH 7.5 and 8.5. However, maximal urease activity in intact organisms is obtained at pH 5.5 and maintained down to pH 2 (Scott *et al*., 1998; Rektorschek *et al*., 2000). The $K_{m,app}$ decreases from ~200mM at pH 7 to ~1mM at pH 5.5 (Scott *et al*., 1998; Weeks *et al*., 2000). This trend can be explained by the pH dependent availability of urea to urease as mediated by UreI (Weeks *et al*., 2000). The pH-regulated urea channel UreI is a membrane protein with six transmembrane domains. Extensive mutagenic analysis of the periplasmic and cytoplasmic domains has identified the sites of pH regulation (Weeks and Sachs, 2001). Protonatable residues in the second periplasmic loop and the C terminus are responsible for acid activation of UreI. Especially histidine 123, 131 and 193, aspartic acid 129 and 140, glutamic acid 138 and a positive charge at position 132 are required to maintain urea transport. The pK of histidine is 6.04, which is close to half-maximal activity of urease in *H. pylori*. The pK of the dicarboxylic amino acids aspartic acid and glutamic acid is about 4.

Some membranous residues are probably also involved in conformational changes because replacement of all UreI periplasmic loops in *S. salivarius* by the *H. pylori* sequences did not mediate the same acid activation pattern (Weeks and Sachs, 2001). UreI has only been found in urease operons of some organisms. In *Helicobacter* species, UreI is found in gastric species but absent in non-gastric species (Scott *et al*., 2000). This fact substantiates a specialized role in acid resistance. UreI is required both for luminal transit and persistence of *H. pylori* G1.1 in the gastric mucosa of the gerbil model (Mollenhauer-Rektorschek *et al*., 2002).

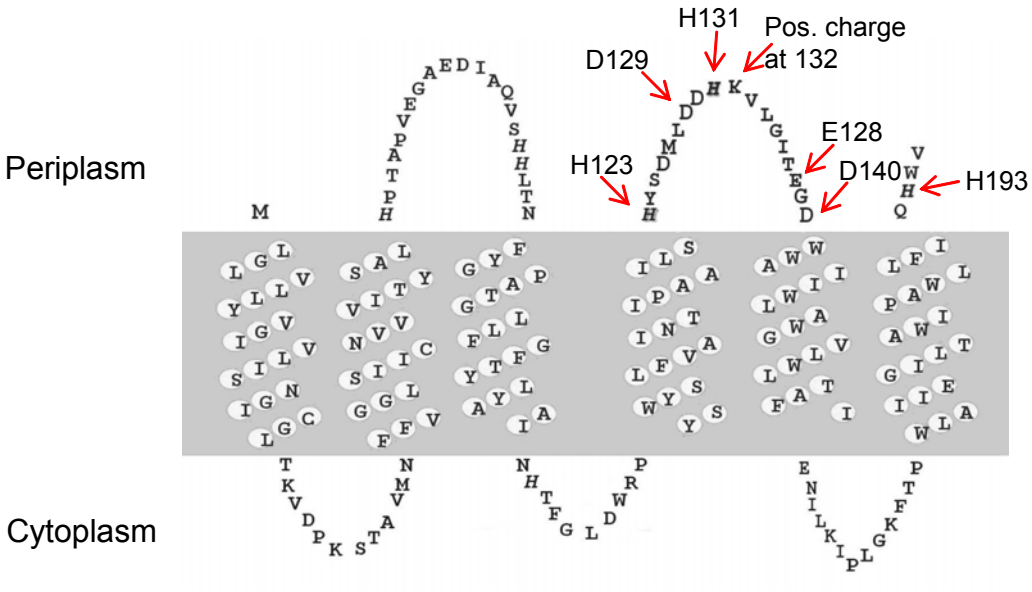

Figure 1.7: The structure of the UreI protein. Protonatable residues in the second periplasmic loop are responsible for acid dependent urea uptake.

The tight regulation of urease activity is essential for the survival of *H. pylori* in the varying acidic environment. Urease activity is lethal at higher external pH and restricts survival to an external pH of 8 (Clyne *et al*., 1995).

Moreover, urease-independent mechanisms for acid survival also exist. Bijlsma *et al*. (1998) generated urease-positive, acid-sensitive mutants. Among others, mutations in *atpF´*, the gene encoding the b´ subunit, has been identified to be involved in survival at low pH but not for acid shock (Bijlsma *et al*., 2000). This mutant contains a duplication in *atpF´* and the preceding gene, which probably results in polar effects on the whole *atp* operon. *atpF* encodes a subunit of the F_1F_0-ATPase, a multisubunit enzyme that uses a pH gradient for the generation of ATP.

The role of the F_1F_0-ATPase for acid survival of *H. pylori* is unclear.

1.6 The F_1F_0-ATPase of *H. pylori*

In addition to the mechanisms of acid resistance mentioned in the previous chapter, the F_1F_0-ATPase of *H. pylori* could have a unique function in acid survival for the organism. The F_1F_0-ATPase is a multisubunit enzyme in the cytoplasmic membrane that has been studied extensively in *E. coli* (Senior *et al*., 2002). It consists of the F_0 and F_1 subunit, F_0 being the transmembrane channel and F_1 the catalytic domain.

The F_1F_0 proton-translocating ATPase, also called ATP synthase, plays a central role in the maintenance of the proton motive force (PMF). In most organisms, it uses the H^+ electrochemical gradient to produce ATP but it can also catalyse the reverse reaction, the generation of a PMF at the expense of ATP.

As a gram-negative bacterium, *H. pylori* possesses two membranes. In the outer membrane, water-filled porins allow diffusion of hydrophilic substances with a molecular weight of approximately 6 kDa. The cytoplasmic membrane encloses the cytoplasm creating the periplasmic space between outer and inner membrane. The phospholipid bilayer of the cytoplasmic membrane is impermeable to most compounds. Various proteins tightly regulate the flow of nutrients and metabolic products, thereby allowing cytoplasmic homeostasis in a changing environment. Moreover, it has been observed that many membrane proteins in *H. pylori* have a higher isoelectric point (pI) compared to *E. coli* (Tomb *et al*., 1997) which is achieved by insertion of positive charges.

The PMF consists of an electrical potential, owing to the separation of charge, and a chemical gradient of H^+, sometimes Na^+. The external pH differs from the internal pH of 7.4 and 7.8. The relationship between these two forces is expressed in the equation $PMF = \Delta\mu_{H+}/F = \Delta\psi - 2.3RT\Delta pH/F$. $\Delta\psi$ is the electrical transmembrane potential in millivolts (R: gas constant, T: absolute temperature, F: Faraday's constant). The maintenance of the PMF within a narrow range is vital for the cells and an increasing pH gradient diminishes the transmembrane potential. *H. pylori* lives in the variable environment of the human stomach. The acidity varies from below pH 1 to as high as pH 6 just after a meal within a few hours and the availability of nutrients depends on the food intake of the host. Thus, additional mechanisms must have evolved to survive in this wide pH range.

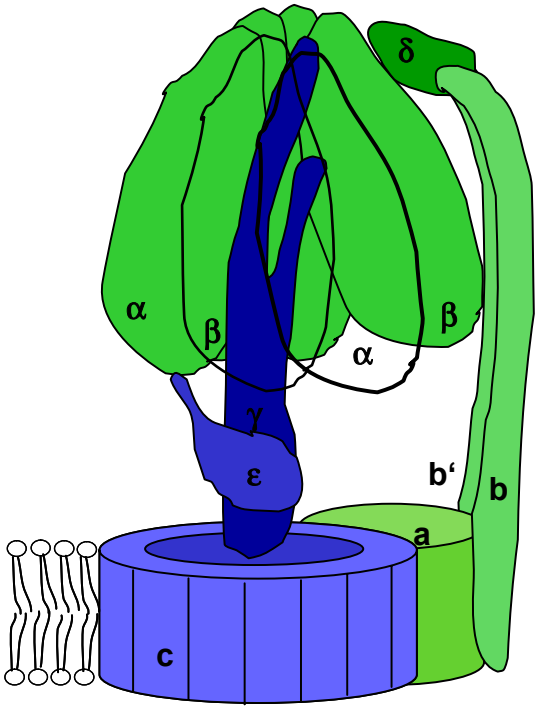

Figure 1.8: A structural model of the F_1F_0-ATPase. The overall architecture of the F-type ATPases from various organisms is similar but the exact mechanism of ion translocation is still controversially discussed. Elements of the rotor are shown in blue whereas the stator part is coloured in green.

The F_1F_0-ATPase can be divided into several subunits. The F_0 sector has three subunits a, b and c with the stochiometry $a_1b_2c_{10}$ (Foster and Fillingame, 1982) but the number of c subunits is still a matter of debate. The exact number might vary in different species and is dependent on metabolic conditions (Tomashek and Brusilow, 2000). In yeast, 10 subunits were identified by x-ray (Stock *et al*., 1999). Crosslinking and genetic studies suggest 10 c subunits for the *E. coli* enzyme (Fillingame *et al*., 2000) whereas 11 and 14 subunits have been identified for *P. modestum* and chloroplast F_0 by atomic force microscopy (Muller *et al*., 2001).

F_1 is organized as a $\alpha_3\beta_3\gamma\delta\varepsilon$ oligomer in the bacterial cytoplasm. The structure of the F_1 complex of the bovine F_1F_0 ATPase was first clarified by x-ray crystallography (Abrahams *et al*., 1994). The γ subunit extends through the αβ trimer, comprising the rotor stalk together with ε (Nakamoto *et al*., 1999; Stock *et al*., 1999; Capaldi *et al*., 2000). b_2 is anchored in the membrane and in contact with subunit a (Dmitriev *et al*., 1999; McLachlin *et al*., 2000; Jiang and Fillingame, 1998). It forms the stator stalk together with δ, which interacts with the α subunit (Wilkens *et al*., 2000; Ogilvie *et al*., 1997).

Especially the c subunit has been studied extensively because it plays a crucial role in H^+ translocation (Deckers-Hebestreit *et al*., 1996). In *E. coli*, the c subunit forms a helical hairpin with two hydrophobic domains of more than 20 amino acid residues, with C- and N-terminus located in the periplasm. This conformation has been verified by several independent experiments (Deckers-Hebestreit *et al*., 1986, 1987; Lötscher *et al*., 1984;). The hydrophilic loop points towards the cytoplasm and it has been proposed that it binds F_0 to F_1 (Girvin *et al*., 1989). Subunits a and c together form the proton translocation channel but all F_0 subunits are necessary for ion translocation (Schneider and Altendof, 1987). Two different theories try to explain the mechanism of H^+ or Na^+ translocation (Junge *et al*., 1997; Dimroth *et al*., 1999).

The F_1F_0-ATPase is involved in H^+ metabolism. The β subunit has been shown to be essential in *H. pylori* (McGowan *et al*., 1997). In *H. pylori*, the proton translocating F_0 subunit exhibits features that might add to acid resistance. The pI of the c subunit is 8.9 as compared to *E. coli* subunit c with a pI of 6.3. Most strikingly, the gene *atpE*, which encodes the c subunit, is elongated compared to most other organisms. Therefore, the *H. pylori* c subunit exhibits a third hydrophobic domain as is demonstrated in this work. This domain probably adds an additional structural feature to the c subunit in *H. pylori* and its function is investigated in this work.

1.7 Objective

H. pylori inhabits a special niche in the varying acidic environment of the human stomach. Several mechanisms have evolved that confer resistance to high acidity. The regulation of membrane permeability is of high importance. An H^+ gated urea channel regulates the availability of urea and therefore cytoplasmic urease activity. The c subunit of the F_1F_0 ATPase forms part of the inner membrane channel for H^+ translocation. Several differences were noted when the amino acid sequence was compared to that of *E. coli*. The *H. pylori* genomic sequence of the F_1F_0-ATPase c subunit predicts a possible additional stretch of hydrophobic amino acids except for few protonatable residues. However, expression of a c subunit with three instead of two transmembrane segments has never been shown. Hence, it was analysed whether this sequence can be expressed, and when expressed, whether it spans the membrane. Function and role of this special segment and the entire *H. pylori atpE* sequence were

examined by truncation and deletion experiments and sequence replacement methods to gain insight as to the role of the *atpE* gene in *H. pylori*. Investigation of the structure and function of this stretch should show a potential role in acid tolerance.

2 Materials and methods

2.1 Materials

2.1.1 Laboratory equipment

Instruments were purchased from different companies: Abimed, Bachhofer, Beckmann, Bender + Hobein, Biometra, BioRad, Braun-Melsungen, Eppendorf, Heraeus, Hermle, Hoefer IKA Labortechnik, Julabo Labortechnik GmbH, Kontron, Merck, Mettler-Toledo, Millipore, Perkin-Elmer, Pharmacia Scientific Instruments, Sauter, Sigma, Vakubrand.

2.1.2 Chemicals

Roth, Sigma, Serva and Merck supplied chemicals. Radio chemicals were ordered from Amersham-Pharmacia.

2.1.3 Buffers and Solutions

1x BSS:	138 mM NaCl, 5mM KCl, 0.81mM Na_2HPO_4, 0.11 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$
BSSgg:	BSS supplemented with 10 mM Glucose, 1 mM Glutamine.
DNA loading buffer:	20% Ficoll 400, 100 mM EDTA, 0.25% Xylen-Cynol, 0.25% Bromphenolblue, 0.25% OrangeG
1x PBS (Phosphate buffered saline):	140 mM NaCl, pH 7.25; 6.5mM Na_2HPO_4; 2.5mM KCl, 1.5 mM KH_2PO_4
1x TBE:	100 mM Tris base, 100 mM boric acid, 2.5 mM EDTA
1x TBS (Tris buffered saline):	20 mM Tris-HCl, pH 7.5; 150 mM NaCl
2x Tricine Sample buffer:	8% SDS, 24% glycerol, 10 mM Tris pH 6.8, 0.02% Serva Blue G, add 4% β-mercaptoethanol prior to use

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Monoclonal antibody against alkaline phosphatase (Caltag, South San Francisco)

Polyclonal antibody against *E. coli* c subunit of the F_1F_0 ATPase (R α *c* IgG8, 9.9.87, kindly provided by Prof. Altendorf, Osnabrück)

Polyclonal antibody against *H. pylori* c subunit of the F_1F_0 ATPase, made against the peptide AHDGGMGGMDMIKSY, corresponding to amino acid residues 17 to 31 of the *H. pylori* c subunit (Neosystems)

2.1.4.2 Secondary antibodies

Peroxidase conjugated AffiniPure Goat Anti Mouse IgG (H+L)

Peroxidase conjugated AffiniPure Goat Anti Rabbit IgG (H+L)

(Jackson Immunoresearch Laboratories, provided by Dianova)

2.1.5 Kits

Dneasy Tissue Kit	Qiagen
Expand High Fidelity PCR System	Roche
Genomic DNA preparation tips	Qiagen
Lumi Light Plus Western Blotting Substrate	Roche
Qiagen plasmid isolation kits	Qiagen
QIAquick PCR purification/gel extraction/nucleotide removal Kit	Qiagen
Quik Change Site directed mutagenesis Kit	Stratagene
TNT Quick Coupled Transcription and Translation System	Promega
Wizard Plasmid Isolation Kit	Promega

2.1.6 Enzymes

The enzymes used were purchased from Roche and New England Biolabs. Taq DNA Polymerase was purchased from Gibco BRL.

2.1.7 Media

M9 (minimal medium): 33.7 mM Na_2HPO_4, 22 mM KH_2HPO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 0,002% (w/v) Thiamine, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.04% (w/v) Glucose

Luria-Bertani broth and agar was purchased from Gibco BRL.

SOC medium was provided by Invitrogen.

Brain-Heart-Infusion broth BHI (Difco): 36g/l brain-heart-infusion, 0.25% yeast extract, 10% foetal calf serum (FCS, PAA Laboratories GmbH) added after sterilisation.

BHI-Agar (Difco) was prepared following the manufacturer's instruction. 10% horse serum (Eurobio) was added before pouring the agar plates.

GC Agar base was provided by Remel, for supplementation 10% horse serum was added.

2.1.8 Antibiotics

Ampicillin: 50 mg/ml in H_2O (aliquots stored at –20°C)

Kanamycin: 50mg/ml (Roche) (stored at 4°C)

Chloramphenicol: 30 mg/ml in 70% Ethanol (stored at –20°C)

The amounts of antibiotics used for cultivation of *E. coli* and *H. pylori* in liquid culture and on agar plates are listed below.

	E. coli	*H. pylori*
Ampicillin	50-100 µg/ml	-
Kanamycin	30 µg/ml	8 µg/ml
Chloramphenicol	30 µg/ml	6-8 µg/ml

Table 2.1: The amount of antibiotics for agar plates and liquid culture medium for *E. coli* and *H. pylori*.

2.1.9 Bacterial strains and plasmids

2.1.9.1 Bacterial strains

The *E. coli* strains used in this work are listed in the Table below:

Strain	Genotype	Source, reference
Top 10	F$^-$ *mcrA* Δ (*mrr-hsd*RMS-*mcrBC*) Δ80*lacZ*ΔM15 ΔlacX74 *deoR recA*1 *araD*139 Δ (ara-leu)7697 *galU galK rpsL*(StrR) *endA*1 *nupG*	Invitrogen
LMG 194	F- delta(lacIPOZY)X74 *galE galK* thi *rpsL* Δ*phoA* ara714	Dr. M. Ehrmann, University of Cardiff Guzman LM *et al*., 1995

Table 2.2: *E. coli* strains used in this work.

The *H. pylori* strains used in this study are listed in the Table below:

Strain	Description	Source, reference
69A	wild type	Prof. R. Haas (Max-von-Pettenkofer Institut, Munich, Germany)
26695	wild type	Prof. Krakowka; Tomb *et al*., 1997
888-0	wild type	Prof. R. Haas (Max-von-Pettenkofer Institut, Munich, Germany)
G1.1	wild type, infectious in mongolian gerbil	Prof. Wirth, Kantonsspital Zurich, Switzerland

Table 2.3: *H. pylori* strains.

2.1.9.2 Vectors and plasmids

Name	Description	Reference
pBSIIKS-	pBluescript II KS-, expression vector for *E. coli*	Stratagene
pRK2013	Ori_{colE1}, RK2-transfer genes, $kann^R$	Figurski *et al*., 1979
pHel3	Expression vector for *H. pylori*	Heuermann and Haas, 1998

Table 2.4: Plasmids that were used to construct the different mutants.

Name	Description	Basic vector	Reference
M16.1	Knockout of *H. pylori atpE* by homologous recombination	pBSIIKS-	This work
M59.33	Expression of *E. coli atpE* in *H. pylori*	pHel3	This work
M64.3	Knockout of *H. pylori atpE* by homologous recombination	pBSIIKS-	This work
M64.14	Knockout of *H. pylori atpE* by homologous recombination	pBSIIKS-	This work
M75.28	*E. coli atpE* with H. pylori N-terminus for homologous recombination in H. pylori	pBSIIKS-	This work
M84.35	Bascic vector for generation of histidine mutant	pBSIIKS-	This work
M85.28	Expression of *H. pylori atpE* in H. pylori	pHel3	This work
M85.42	Expression of truncated *H. pylori atpE* in *H. pylori*	pHel3	This work
M89.25	Histidine mutant for insertion in *H. pylori* by homologous recombination	pBSIIKS-	This work

Table 2.5: Listing of plasmids that were used in this work.

Listing of all the plasmids that were used for the topology analysis.

Name	Description	Basic vector	Reference
pBADphoA	Expression of alkaline phosphatase with araBAD promoter	pBAD22	Melchers *et al*., 1999
phoA1	Expression of fusion protein of alkaline phosphatase with first transmembrane domain of *H. pylori atpE*	pBADphoA	This work
phoA2	Expression of fusion protein of alkaline phosphatase with first and second transmembrane domain of *H. pylori atpE*	pBADphoA	This work
phoA3	Expression of fusion protein of alkaline phosphatase with *H. pylori atpE*	pBADphoA	This work

Table 2.6: Plasmids that were used for the *in vivo* topology analysis.

Name	Description	Basic vector	Reference
M0	Expression of part of α and β subunit of the gastric H+/K+ ATPase for use in topology analysis	pGEM7zf+	Bamberg and Sachs, 1994
M1	Expression of part of α and β subunit of the gastric H+/K+ ATPase with one transmembrane domain for use in topology analysis	pGEM7zf+	Bamberg and Sachs, 1994
H1	Expression of the first transmembrane domain in M0 and M1	M0 and M1	This work
H2	Expression of the second transmembrane domain in M0 and M1	M0 and M1	This work
H2*	Expression of the second transmembrane domain in M0 and M1	M0 and M1	This work
H3	Expression of the third transmembrane domain in M0 and M1	M0 and M1	This work
H1-H2	Expression of the first and second transmembrane domain in M0 and M1	M0 and M1	This work
H1-H2*	Expression of the first and second transmembrane domain in M0 and M1	M0 and M1	This work
H1-H3	Expression of the first to third transmembrane domain in M0 and M1	M0 and M1	This work
H2-H3	Expression of the second and third transmembrane domain in M0 and M1	M0 and M1	This work

Table 2.7: Plasmids that were used for the *in vitro* topology analysis

2.1.10 Oligonucleotides

3´-atpE-as	5´- tgg cat tta ttt gga ata aaa ct -3´
5´-atpE-s	5´- ctt gct tgt att ttt gaa agt ag -3´
atpEa-BHI	5´- ccg gat ccc gca ccc gat aaa att gta g –3´
atpEas-BHI	5´- cgg gat ccc tac gcg aca gcg aac atc –3´
atpEbN-EcatpE	5´- gat cca tat tca ggt ttt cca tcc cac cca ttc cgc cat c –3´
atpEb-popacat	5´- ggc gga tta aca aaa acc gga gca ctc cgt ttc aaa aat tag –3´
atpEc-popacat	5´- tgg cag ggc ggg gcg taa ggg ttt tgt tgg gct aaa tc –5´
atpEd-SacI	5´- tcg gag ctc taa aac gct ctc tct ttt aat c –3´
cats	5´- atg gag aaa aaa atc act gga t –3´
EcatpE-RBS	5´- ttt ata aca agg agt tac aac aat gga aaa cct gaa tat gg –3´
EcatpEs	5´- atg gaa aac ctg aat atg gat c –3´
flaA-RBS	5´- tgt tgt aac tcc ttg tta taa a -3´
H1 as	5´- cca cca agc ttt cat gag cga aag caa cgc cc -3´
H1 forw	5´- acg gag aga tct Aat gaa att ttt agc g -3´
H2 forw	5´- tga tag atc ttt att cta tct tag gag cga tga tc -3´
H2*as	5´- gga tta agc ttc tcg ctg tgc ctg taa tg -3´
H2as	5´- cct gta agc tta tgg tcg ctg cgg ccg c -3´
H3 forw	5´- tgc tag atc tta tgt ttg tcg cca tgg cga tg -3´
H3as	5´- cct taa agc ttc tta aga atg ggt tac tat a -3´
His-ggg-as	5´- cat tcc gcc atc ccc agc gaa agc gac -3´
His-ggg-s	5´- cat tcc gcc atc ccc agc gaa agc gac -3´
HpatpEas-BHI	5´- cgg gat cct taa ctt aag aat ggg tta cta ta –3´
HpatpE-cat	5´- cca gtg att ttt ttc tcc att taa ctt aag aat ggg tta cta -3´
HpatpEphoAas1	5´- ccg ggt acc gat tta atc ata tcc atc cc -3´
HpatpEphoAas2	5´- ccg ggt acc tta ccg ccc act cct gg -3´
HpatpEphoAas3	5´- ccg ggt acc gat aag aat ggg tta cta taa ata g -3´
HpatpEphoAs	5´- cgg ggt acc taa att ttt agc gtt att ttt tct gg -3´
HpatpEs-BHI	5´- ccg gat cca tga aat ttt tag cgt tat ttt ttc tg –3´
PflaA-ClaI	5´- cca tcg ata aag ccc ttt aaa att tca aac –3´
PflaA-HpatpE	5´- ttt ata aca agg agt tac aac aat gaa att ttt agc gtt att ttt tct g –3´
PflaA-HpatpEk	5´- ttt ata aca agg agt tac aac aat gga tat gat taa atc tta ttc -3´
popacatas	5´- tta cgc ccc gcc ctg cca –3´
popacat-EcatpE	5´- ggc gga tta aca aaa acc gga cta cgc gac agc gaa cat c –3´
popacats	5´- tcc ggt ttt tgt taa tcc gcc –3´

Table 2.8: Listing of all oligonucleotides used in this work.

2.2 Methods

2.2.1 Cultivation of *E. coli*

E. coli was grown under aerobic conditions at 37°C on LB agar plates or in LB broth containing the appropriate antibiotics. Liquid cultures were incubated on a shaker at 220rpm.

Glycerol stocks were prepared by mixing liquid cultures 1:1 with 40% glycerol (v/v) and kept at -80°C for long-term storage.

2.2.2 Cultivation of *H. pylori*

H. pylori were grown under microaerophilic conditions using AnaerocultC gas packs in anaerobic jars (Merck) at 37°C. Glycerol stocks were prepared by using 40% glycerol (v/v) in a 1:1 mixture with liquid cultures in BHI/10% FCS and stored at -80°C.

H. pylori were streaked out from the glycerol stock on BHI/10% HS plates with or without antibiotics and left to grow for 48-72 hours. Bacteria were then either restreaked on agar plates or resuspended in BHI/10% FCS broth with swabs (Falcon) and cultured until the desired OD_{580} was reached. Liquid cultures were incubated in cell culture flasks in anaerobic jars with shaking at 120 rpm.

The viability was surveyed by microscopic analysis.

2.2.3 Transformation of *E. coli*

Competent LMG 194 cells were prepared as follows: cells from an overnight culture were diluted and cultured until OD_{580} 0.7-0.9 was reached. 30 ml were centrifuged (10 min at 6000 rpm) and the pellet was resuspended in 1.5ml LB. 1.5ml 2x TSS was added (20% PEG 8000 w/v, 100 mM $MgCl_2$, 10% DMSO freshly added). 200 µl aliquots were frozen at -80°C. For transformation 10-20 µl ligation reaction or 1 µl of prepared plasmid DNA were added and cells were incubated on ice for 10 min. After addition of 1 ml SOC cells were grown at 37°C at 220 rpm for 60 min and the whole reaction was plated on selective agar plates.

2.2.4 Transformation of *H. pylori*

Most *H. pylori* strains are naturally competent (Haas *et al*., 1993). Bacteria from an overnight culture were resuspended in BHI/10%FCS and the OD_{580} was determined.

The transformation was performed in a 24 well plate where each well contained 1 ml BHI/10% FCS. The bacteria were inoculated at OD_{580} 0.1 and left to grow under microaerophilic conditions with slight shaking in an anaerobic jar. After 5-6 hours 5 µl of DNA (50 ng/µl) was added and the bacteria were left to grow overnight under the same conditions. The transformation reaction was centrifuged (14000 rpm, 2 min) and 950 µl of the supernatant were discarded. The pellet was resuspended in the remaining liquid and plated on BHI/10% HS plates with the appropriate antibiotics. Colonies were usually detected 3-4 days after successful transformation.

2.2.5 Conjugation of *H. pylori*

The recipient (*Helicobacter pylori* wild type) was streaked out on BHI/10% HS and left to grow for two days. After two days *H. pylori* was replated on a new BHI/10% HS plate. The *E. coli* donor and the mobilisator (HB101 [pRK 2013]) were also plated on antibiotic selection LB plates. After overnight culture some of the *E. colis* were replated on new LB antibiotic plates to obtain *E. coli* in the logarithmic growing phase after 2-3 hours.

For the conjugation all bacteria were resuspended in BHI (without FCS) and the OD at 580 nm was determined. The ratio of donor, mobilisator and recipient was 1:1:10. The suitable amount of *H. pylori* recipient for one reaction corresponds to 20 µl culture with OD_{580} 50. Accordingly 20 µl culture with OD_{580} 5 for donor and mobilisator were used. The mix was centrifuged for 2 min at full speed and nearly all of the supernatant was removed. The bacteria were resuspended in the remaining supernatant (10-20 µl) and put on 0.2 µm cellulose nitrate filter (Sartorius), which lied on BHI /10% HS plates. The filters were incubated at 37°C, 10% CO_2 for exactly 2 hours. The bacteria from the filter were resuspended with 1ml BHI medium (without FCS) in 50ml tubes (Falcon). The suspension was transferred to an Eppendorf cup and centrifuged for 2 min at full speed. The pellet was resuspended in about 50 µl and the bacteria were plated on selection plates: GC Agar/10% HS/Colistin/antibiotic (Colistin=Polymyxin E, Sigma). After incubation under microaerobic conditions for 4-5 days, bacterial colonies were propagated for further analysis.

2.2.6 PCR

2.2.6.1 Real Time TaqMan PCR

Real Time TaqMan PCR (Applied Biosystems) was used to distinguish between *H. pylori* wild type and the histidine mutants where histidine 18 in the *atpE* gene was exchanged against glycine.

TaqMan probes have a reporter dye (fluorophore) at their 5´ end and a quencher dye (quencher) at the 3´ end. The intact probe is only weakly fluorescent when excited by a light source because the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes. This process is called Fluorescent Resonance Energy Transfer (FRET).

The TaqMan probe is highly specific and anneals between the upstream and downstream primer in PCR. During the PCR reaction the AmpliTaq Gold DNA Polymerase (TaqMan PCR Universal Master Mix, Applied Biosystems) with 5´-3´ exonuclease activity cleaves the fluorophore from the probe. Since the fluorophore is now no longer in close proximity to the quencher, fluorescence can be detected and the amount is in direct proportion to the amount of target DNA accumulation during the PCR cycles. No cleavage is observed when the TaqMan probe is not bound specifically to its target DNA because the weakly bound probe will rather be displaced by the polymerase before being cleaved.

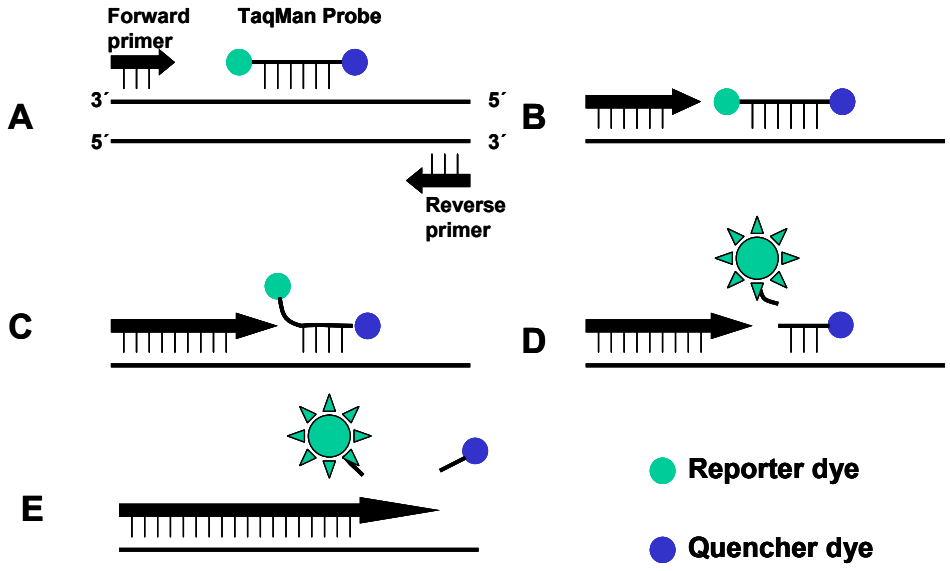

Figure 2.1: The strategy of TaqMan PCR. The reporter dye is illustrated in green, the quencher dye in blue. When the target sequence is amplified (B, C), the probe is cleaved and the reporter starts to fluorescence (D). The fluorescent signal is detected by the Perkin Elmer Abi Prism 7700 Sequence Detector.

2.2.6.2 Standard PCR

Standard PCR reaction was carried out in 100 µl volume using the Robocycler Gradient 40 from Stratagene. The following reagents were mixed: Expand High Fidelity PCR System Polymerase (2.6 Units) with the supplied buffer 2, 100 ng template DNA, 30 pmol of each primer, 10 mM dNTPs each. The cycling protocol was started with a denaturation step for 1 min at 94°C followed by 30 cycles with 1 min at 94°C denaturation, 1 min 48-60°C primer annealing and 1 min at 72°C polymerization, followed by a final polymerization step at 72°C for 10 min. 40 mer primers with 20 base pairs overlap were generated for crossover PCR fragments.

PCR fragments were analysed on 0.8-1.5% agarose gels prepared with 1x TBE. The gels were stained with ethidium bromide (0.5 µg/ml in H_2O, stock 10 mg/ml), destained in water and analysed with the Gene Genius Bio Imaging System from Syngene. For further use PCR fragments were purified using the Qiaquick PCR Purification kit (Qiagen) with additional washing with 35% guanidine HCl. Removal of all primers is necessary to avoid unspecific crossover PCR products.

The purified PCR fragments were used for the crossover PCR. The crossover PCR reaction was carried out using the Perkin Elmer Cetus DNA Thermal Cycler Version 2.1 with the following protocol: denaturation for 4 min at 94°C, then 30 cycles with 1 min 94°C, 1 min 56°C, 2.5 min 72°C and a final elongation step with 10 min at 72°C. Up to 4 different templates were annealed in one reaction; 1 µl of each template was used. The 100 µl reaction volume contained 2.6 units Expand Polymerase and buffer 2 with $MgCl_2$, 30 pmol of each primer, 10 mM of each dNTP. The PCR products were analysed as described above. Gel extraction was used if various products were generated.

2.2.6.3 Crossover PCR

Overlap extension by PCR (Horton *et al*., 1989, Ho *et al*., 1989) was adapted for *H. pylori* and used to generate the different constructs for mutagenesis.

The desired fragments of a construct are amplified in separate PCR reactions. The purified fragments are used in the next PCR reaction with the external primers to produce a crossover PCR product without any additional sequences.

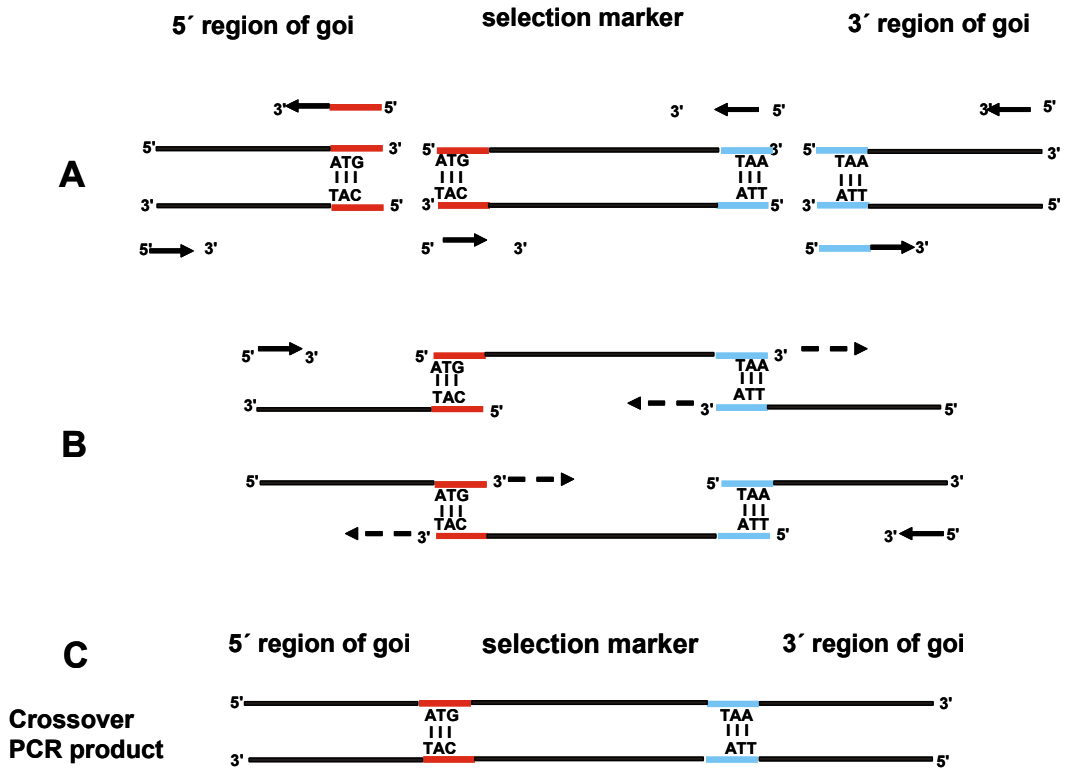

Figure 2. 2: Crossover PCR was used to generate constructs for knockout mutagenesis of various genes by homologous recombination. In separate PCR reactions the 5´ and 3´ regions from the gene of interest and the selection marker were amplified (A). The 5´ and 3´ regions were amplified with primers that show an overlap with the selection marker of about 20 base pairs. In the crossover PCR reaction the products of the previous reactions are mixed as template and the crossover PCR product is amplified with the external primers (B). The complementary ends also anneal and serve as primers so that the generated crossover PCR product does not contain any additional restriction sites (C). goi: gene of interest.

2.2.7 Cloning procedures

PCR fragments and plasmids were digested with the appropriate restriction endonucleases using 10 U/µg DNA following the supplier's manual. After purification or gel extraction (QIAquick Gel extraction Kit) ligation of DNA fragments was performed using T4 DNA ligase (New England Biolabs).

E. coli Top 10 (Invitrogen) were used for plasmid transformation according to instructions.

Plasmid isolation from 2 ml of an overnight culture was performed using Qiaprep Spin Kit and sequences were verified by restriction endonuclease analysis, gel analysis and sequencing (GATC Biotech).

Larger amounts of plasmid DNA were prepared with the Qiagen Plasmid Maxi Kit.

2.2.8 Generation of mutants for topology analysis

2.2.8.1 The *in vitro* topology analysis with the M0/M1 vectors

The primers used for the in vitro topology analysis are listed in detail with restriction enzyme sites base pairs that are needed for in frame cloning.

H1forw	5´acg gag aga tct Aat gaa att ttt agc g3´
H2 forw	5´tga tag atc tTt att cta tct tag gag cga tga tc3´
H3 forw	5´tgc tag atc tTa tgt ttg tcg cca tgg cga tg3´

Table 2.9: The sense primers for amplification of the different membrane domains are shown. The BglII site is underlined. The capital letter indicates an additional base pair, which is necessary to fuse the insert in frame into the fusion protein.

H1 as	5´cca cca aGC Ttt cat gag cga aag caa cgc cc3´
H2 as	5´cct gta aGC Tta tgg tcg ctg cgg ccg c3´
H2* as	5´gga tta aGC Ttc tcg ctg tgc ctg taa tg3´
H3 as	5´cct taa aGC Ttc tta aga atg ggt tac tat a3´

Table 2.10: The antisense primers for constructing the different inserts are listed. The capital letters indicate the amino acid serine. The following t is necessary for the HindIII site (underlined) but can change the last amino acid of the inserted sequence as shown below.

2.2.8.2 *In vivo* topology analysis with *phoA*-fusionproteins

HpatpEphoAs	5´cgg ggt acc taa att ttt agc gtt att ttt tct gg3´
HpatpEphoAas1	5´ccg ggt acc gat tta atc ata tcc atc cc3´
HpatpEphoAas2	5´ccg ggt acc tta ccg ccc act cct gg3´
HpatpEphoAas3	5´ccg ggt acc gat aag aat ggg tta cta taa ata g3´

Table 2.11: The primers for the phoA topology analysis.

The primers for cloning the *phoA* constructs are listed. The capital letter in the sense primer indicates an additional base pair before the *H. pylori atpE* sequence begins. The capital letters in the antisense primer indicate the last triplet of the cloned *atpE* sequence. The C is necessary for the *Kpn*I site (underlined). Therefore, it may be necessary to change the sequence of the last triplet.

2.2.9 Generation of mutants

2.2.9.1 Construction of the *atpE* deletion mutant

The crossover PCR method was used for the generation of the construct for knockout of *H. pylori atpE* at genomic level by homologous recombination.

The 5´ region, the 3´ region and the antibiotic resistance gene chloramphenicol-acetyl-transferase (cat_{GC}) were amplified in separate PCR reactions. The purified PCR products were used together as templates in the crossover PCR with external primers. All reactions were carried out at 56°C annealing temperature. The resulting PCR product was purified by gel extraction and ligated in the vector pBSIIKS- (Stratagene). The resulting vector M16.1 with cat_{GC} was verified by sequence analysis (GATC Biotech AG).

Description	PCR	Template	Sense primer	Antisense primer
cat_{GC}	PCR 97	AsTnMax5	popacats	popacatas
5´ Hp atpE	PCR 106	gDNA 26695	atpEa-BHI	atpEb-popacat
3´ Hp atpE	PCR 107	gDNA 26695	atpEc-popacat	atpEd-SacI
Crossover PCR	PCR 109.1	PCR 97, 106, 107	atpEa-BHI	atpEd-SacI

Table 2.12: Listing of the PCR fragments for generation of the *atpE* deletion mutants.

The plasmid M16.1 was used for homologous recombination in *H. pylori* and selection took place on BHI/10%HS/cam6 agar plates.

2.2.9.2 Generation of a truncated variant of *H. pylori atpE*

A variant was generated for expression of a shortened protein under control of the *flaA* promoter. The *flaA* promoter fragment was amplified at 48°C. Amplification of the truncated *atpE* and the crossover PCR were carried out at 56°C.

Description	PCR	Template	Sense primer	Antisense primer
PflaA	PCR 215	gDNA 26695	PflaA-ClaI	flaA-RBS
Hp atpE k	PCR 296	gDNA 26695	PflaA-HpatpEk	HpatpEas-BHI
Crossover PflaA-HpatpEk	PCR 303	PCR 215, 296	PflaA-ClaI	HpatpEas-BHI

Table 2.13: Description of the reactions for construction of a truncated variant of *H. pylori atpE* in *H. pylori*.

The crossover PCR product was ligated into the vector pHel3 for expression in *H. pylori*, pHel3 being a shuttle vector for *H. pylori* (Heuermann and Haas, 1998). The resulting vector was called M85.42.

Additionally, a control plasmid with *H. pylori atpE* was generated with crossover PCR for expression of the wild type sequence. The *flaA* promoter was cloned in front of the gene to enable efficient gene expression.

Description	PCR	Template	Sense primer	Antisense primer
PflaA	PCR 215	gDNA 26695	PflaA-ClaI	flaA-RBS
Hp atpE	PCR 295	gDNA 26695	PflaA-HpatpE	HpatpEas-BHI
Crossover PflaA-HpatpE	PCR 302	PCR 215, 295	PflaA-ClaI	HpatpEas-BHI

Table 2.14: Description of the reactions for construction of *H. pylori atpE* for expression in *H. pylori*.

The crossover PCR product was ligated into the vector pHel3, yielding the vector M85.28, which was verified by sequencing (GATC Biotech).

Selection took place on kanamycin plates. Knockout of the atpE wild type sequence should then take place with the plasmids M64.3 and M64.14.

2.2.9.3 Expression of *E. coli atpE* in *H. pylori*

The *flaA* promoter region was amplified in a standard PCR reaction using the sense primer PflaA-ClaI and the antisense primer flaA-RBS as with 48°C annealing temperature from *H. pylori* strain 26695 genomic DNA. *E. coli atpE* was amplified from genomic DNA of MM294 using EcatpE-RBS and atpEas-BHI as primers at 52°C. The purified PCR products were annealed in a third PCR reaction using PflaA-ClaI and atpEas-BHI as primers at 52°C. The resulting crossover PCR product of 350 base pairs was purified with the Qiagen PCR Purification Kit.

Description	PCR	Template	Sense primer	Antisense primer
PflaA	PCR 215	gDNA 26695	PflaA-ClaI	flaA-RBS
E. coli atpE	PCR 214	gDNA MM 294	EcatpE-RBS	atpEas-BHI
Crossover	PCR 216	PCR 214, 215	PflaA-ClaI	atpEas-BHI

Table 2.15: Generation of the construct for expression of *E. coli atpE* in *H. pylori*.

The crossover PCR product was ligated into the vector pHel 3 leading to the vector M59.33 which was verified by sequencing (GATC Biotech).

2.2.9.4 *E. coli atpE* with *H. pylori* N-terminus

The *H. pylori* N-terminus was fused to *E. coli atpE* to assess the possible function of a fusionprotein. Four fragments, containing the *H. pylori* N-terminus, *E. coli atpE*, the cat_{GC} gene for selection and the H. *pylori* 3´ region, were annealed in the crossover PCR at 54°C.

Description	PCR	template	Sense primer	Antisense primer	$T_{anneal.}$
5´ Hp atpE with N-terminus	PCR 248.1	gDNA 26695	atpEa-BHI	atpEbN-EcatpE	48°C
Ec atpE	PCR 229.4	gDNA MM294	EcatpEs	popacat-EcatpE	52°C
cat_{GC}	PCR 278.1	Vector AsTnMax 5	popacats	popacatas	58°C
3´ Hp atpE	PCR 237	PCR 177 (gDNA 26695)	atpEc-popacat	atpEd-SacI	56°C
Crossover HpNterm-EcatpE	PCR 285	PCR 248.1, 229.4, 278.1, 237	atpEa-BHI	atpEd-SacI	54°C

Table 2.16: Description of the construction of a construct for introduction of *E. coli atpE* with the *H. pylori* N-terminus by homologous recombination.

The crossover PCR product was ligated into pBS II KS- via BamHI/ SacI restriction sites. The resulting vector M75.28 was verified by sequencing (GATC Biotech) and was used for homologous recombination in *H. pylori*.

2.2.9.5 Generation of histidine mutants

The histidine mutants were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions.

Briefly, a plasmid was constructed for insertion of the *atpE* gene into the genome of *H. pylori* by homologous recombination and selection with chloramphenicol.

description	PCR	Template	sense primer	antisense primer	$T_{anneal.}$
atpE gene with overlap to cat	PCR 300.1	26695K1#I gDNA	HpatpEs-BHI	HpatpE-cat	56°C
cat	PCR 197	AsTnMax5	cats	popacatas	52°C
3´ HP *atpE*	PCR 177	26695K1#I gDNA	atpEc-popacat	atpEd-SacI	56°C
Crossover PCR	PCR 304	PCR 300.1, 197, 177	HpatpEs-BHI	atpEd-Sac	56°C

Table 2.17: The templates and primers for construction of the wild type plasmid M84.35 are shown.

The plasmid M84.35 contains PCR 304 as insert, in which the wild type *atpE* sequence is followed by the chloramphenicol-acetyl-transferase gene for selection and the 3´ region after the *atpE* gene to accomplish homologous recombination.
Two primers, his-ggg-s and his-ggg-as, were designed for use with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The base pairs cat, which code for histidine were replaced by ggg. This triplet has the highest codon usage for glycine in *H. pylori*. The plasmid was amplified with these primers by PCR. The methylated wild type plasmid was digested with DpnI. The mutated plasmid was transformed into *E. coli* Top10 for propagation and reisolated.
The resulting mutated plasmid M89.25 was used for homologous recombination in three different *H. pylori* strains: 69A, 26695 and G1.1.
Transformants were plated on BHI/10%HS/cam6 agar plates and colonies were detected after four days in strain 69A. Eight clones, His1 to His8, were propagated.
Integration and presence of the mutated sequence was verified by TaqMan PCR.

2.2.10 Isolation of genomic DNA

Bacteria from one agar plate were resuspended in PBS and pelleted by centrifugation. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen) or Qiagen Genomic Tips following the manufacturer's instructions for gram-negative bacteria.

2.2.11 SDS PAGE

The following protocol was used for preparation of polyacrylamide gels (Laemmli, 1970).
For Laemmli gels:
Running buffer: 25 mM Tris, 192 mM Glycine, 0.1 % SDS (w/v).
4x stacking gel buffer: 0.5 M Tris-HCl, pH 6.8.
4x separating gel buffer: 1.5 M Tris-HCl, pH 8.8.

Tricine gels and tricine gradient gels were purchased from Invitrogen. The anode buffer consisted of 0.2 M Tris-HCl, pH 8.9. Cathode buffer was 0.1 M Tris, 0.1 M Tricine, 0.1% SDS (w/v). The Seeblue marker (Invitrogen) was used as a protein standard.
Minigels were run at 100V.

	Stacking gel (1 gel) 4%	Stacking gel (2 gels) 4%	Separating gel (2 gels) 7.5%	Separating gel (1 gel) 10%	Separating gel (2 gels) 10%	Separating gel (1 gel) 12.5%	Separating gel (2 gels) 12.5%
H_20	1.5 ml	3 ml	4.95 ml	1.4 ml	2.79 ml	1 ml	1.95 ml
Stacking gel buffer (4x)	0.625 ml	1.25 ml	-	-	-	-	-
Separating gel Buffer (4x)	-	-	2.5 ml	1.25 ml	2.5 ml	1.25 ml	2.5 ml
Acrylamid/Bis (30%/ 0.8%)	0.34 µl	0.67 ml	2.5 ml	1.7 ml	3.33 ml	2.09 ml	4.17 ml
SDS	50 µl	0.1 ml	0.1 ml	50 µl	0.1 ml	50 µl	0.1 ml
glycerol	-	-	1.2 ml	0.6 ml	1.2 ml	0.6 ml	1.2 ml
TEMED	1.9 µl	3.8 µl	7.5 µl	3.8 µl	7.5 µl	3.8 µl	7.5 µl
APS	19 µl	37.5 µl	50 µl	37.5 µl	75 µl	37.5 µl	75 µl

Table 2.18: Composition of various SDS gels

2.2.12 Western blotting

Western blotting was performed using a PVDF membrane (Millipore). After incubation in methanol the membrane was soaked in blotting buffer: 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol, 0.1% SDS (v/v). Protein transfer was performed for 45min at 950mA (TE Series Transphor Electrophoresis Unit by Hoefer). The Blot was incubated for at least 1 hour at RT in blocking solution (5% BSA in 1x TBS). The first antibody was diluted in 0.5% BSA in 1x TBS and the blot was incubated at 4°C overnight with slight shaking. After four consecutive washes with 1x TBS, 0.5 % Tween 20 for 15 min each, the membrane was incubated with the second antibody in 1x TBS, 0.5% Tween 20 for two hours at RT. After four consecutive washes with 1x TBS, 0.5% Tween 20 for 15min each the detection was performed using the Lumi Light Western Blotting Substrate from Roche. The signal was recorded using the LAS-1000 from Fuji film.

2.2.13 *In vitro* transcription and translation

The *in vitro* transcription and translation was performed using the TNT Quick Coupled Transcription/Translation System from Promega with Canine Pancreatic Microsomal Membranes, equally purchased from Promega.

For 19 reactions, 500 µl of lysate were mixed with 25 µl of Redivue L-S^{35}-methionine (1000 Ci/mmol at 10 mCi/ml, Amersham pharmacia). Each reaction was then prepared

separately: 4 µl plasmid DNA (0.2 µg/µl) were mixed with 21 µl of the lysate by pipetting. 16µl were transferred to a new cup and 1 µl of microsomal membranes was added (+ reaction). This reaction was mixed by vortexing and left on ice until all reactions were prepared. Transcription and translation took place at 30°C for 90 min. The samples were prepared by addition of 2x Tricine sample buffer including 10% β-mercaptoethanol (5 µl to the – reaction, 17 µl to the + reaction). Denaturation took place at 37°C for 30 min.

5 µl of the – reaction and 15 µl of the + reaction were loaded on a 12% Tricine gel (Invitrogen). After completion, the proteins were fixed in the gel by incubation in fixing solution (50% methanol, 10% glacial acetic acid, 40% H_2O) for 30 min. The gel was dried using a gel dryer (Hoefer) and exposed to an imaging plate for auto radiography for 1 to 4 days. The imaging plate was scanned with a Fuji film Bas-2500 Reader.

2.2.14 Alkaline phosphatase assay

Bacteria from an overnight culture in LB with the appropriate antibiotic were diluted 1:20 and grown for 2-3 hours with or without 100 µM arabinose. 0.2 ml cells and 0.8 ml 10 mM Tris HCl pH 8, 150 mM NaCl were mixed. The bacteria were washed 2x at RT in this buffer. 750 µl were used to determine OD_{600} and 100 µl cells were added to 0.9 ml 1M Tris HCl pH8, 0.1 mM $ZnCl_2$ for the assay. After addition of 25 µl 0.1% SDS and 25 µl $CHCl_3$ the reaction was mixed by vortexing and incubated for 5 min at RT. The addition of 0,1 ml 0,4% pNPP (4-Nitrophenylphosphat) in 1 M Tris HCl pH 8 started the phosphatase reaction at 37°C (or 28°C). After colour developed the reaction was stopped by adding 120 µl 2.5M K_2HPO_4 and centrifuged for 5 min to remove cells. The OD at 420 nm was determined and AP units were calculated using the following formula:

$$\text{AP activity (Units)} = \frac{OD_{420}}{\text{Time} \times OD_{600} \times \text{ml of cells}} \times 1000$$

One unit of AP activity corresponds to 1 µmole of pNPP hydrolysis per minute at 37°C (28°C).

2.2.15 Examination of the histidine mutants

The physiological effect of the exchange of histidine 18 against glycine was investigated with a cytosensor microphysiometer perfusion system (Molecular Devices).

The design and use of the Cytosensor is described in detail (Hafeman *et al*., 1988, McConnell *et al*., 1992, Owicki and Parce, 1990 and 1992). Although mostly used to investigate mammalian cell metabolism (Baxter *et al*., 1992, Parce *et al*., 1989), it was already used to measure *H. pylori* metabolism and urease activity (Rektorschek *et al*., 1998, 2000).

The cells are retained in microflow chambers in diffusive contact with a silicon chip that functions as a very sensitive light-addressable potentiometric pH sensor (LAPS). The insulating layer of the surface of the silicon chip binds protons and this creates a pH dependent electrical current. The primary sensor output is in voltage (mV) and the metabolic rates during pump off are recorded in μV/s.

Perfusion is controlled by a pump with a cycle time of one minute. Each cycle is divided into 40 seconds perfusion (pump on) and a stop for 16 seconds (pump off), with two seconds interruption in between. Valves enable media changes from two different reservoirs during the experiment. The computer connected with the microphysiometer monitors and displays the pH changes in two configurations termed raw and rate data.

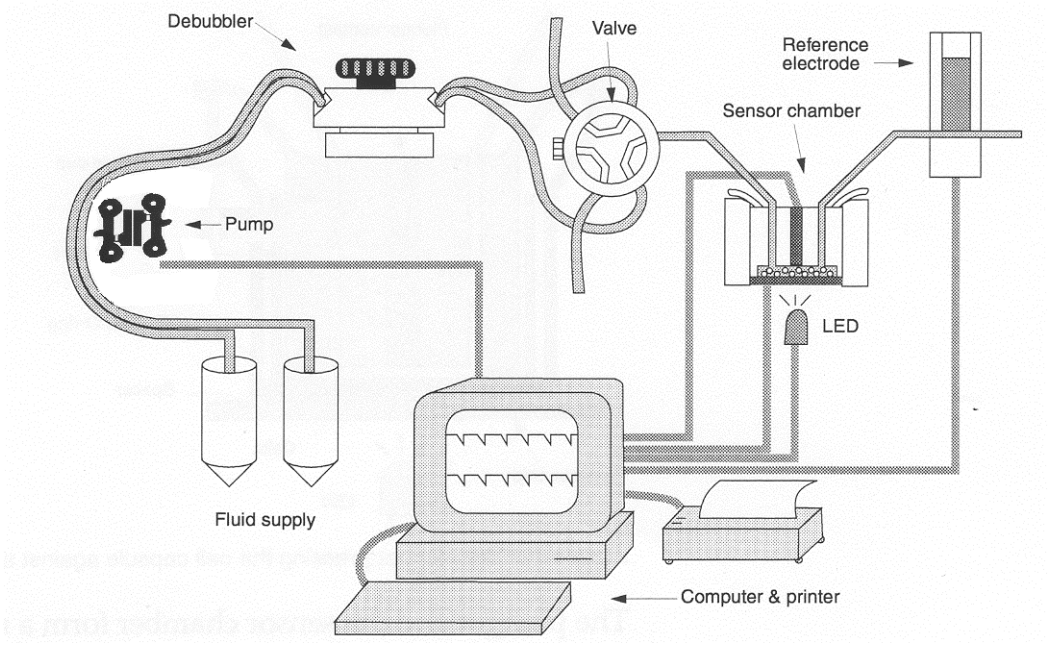

Figure 2.3: The Cytosensor. There are eight sensor chambers in our system.

Raw data are collected every second during the entire experiment and are displayed in millivolts. The rates of acidification or alkalinization are calculated every minute from

the slope measurements recorded during the pump off period. Rate data are recorded and displayed in µV/s and are proportional to the change of the H^+ concentration in the perfusion medium: 1 µV/s approximates 1 x 10^{-3} pH units per minute. Net changes of pH in the microflow chamber are obtained by comparing the pH measured in the chamber to the pH of the perfusate.

Microphysiometer experiments were run with *H. pylori* widltype and histidine mutants immobilized in the chambers by using agarose cell entrapment medium (Molecular Devices). Approximately 1 to 5 x 10^5 bacterial cells (9 µl of 1:4 agarose: *H. pylori* (OD_{580} = 2) were trapped between two micro porous membranes and loaded into each of the sensor chambers. The sensor chambers were perfused with balanced salt solution (BSSgg) with various pH, adjusted with HCl or NaOH. BSSgg is a weakly buffered medium and does not allow growth of *H. pylori* during incubation in the sensor chambers of the microphysiometer. Therefore effects on pH regulation are not influenced by bacterial growth.

Bacteria were challenged with a gradual reduction of medium pH, beginning at pH 7.4 then from pH 5.5 to pH 2.5 in 0.5 pH units with and without 2.5 mM urea for 30 min each. The recovery from acid exposure was measured in BSSgg pH 7.4.

3 Results

In this study, the c subunit of the *H. pylori* F_1F_0-ATPase was examined. A unique operon structure was shown, and the topology of the *H. pylori* c subunit was solved.
The special features of the *H. pylori* c subunit were compared to that of other organisms and their possible functions discussed.

3.1 Organization of *atp* operons

The genes of the F_1F_0-ATPase are arranged as an operon-like structure. In contrast to the other organisms examined, two genes from the *H. pylori atp* operon are located separately from the other *atp* genes (Tomb *et al*., 1997, Alm *et al*., 1999).
The different subunits in *E. coli* and *H. pylori* with their corresponding gene names and the annotation of the open reading frame are compared in Table 3.1 below.

	subunits	gene	ORF in *E.coli*	ORF in *H.pylori*
F_1 subunit	α	*atpA*	b3734	HP1134
	β	*atpD*	b3732	HP1132
	γ	*atpG*	b3733	HP1133
	δ	*atpH*	b3735	HP1135
	ε	*atpC*	b3731	HP1131
F_0 subunit	a	*atpB*	b3738	HP0828
	b	*atpF*	b3736	HP1136
	b´	*atpF´*	-	HP1137
	c	*atpE*	b3737	HP1212

Table 3.1: Annotation of the different subunits of F_1F_0 ATPase and the corresponding gene names. The numbers of the open reading frames of *E. coli* K-12 and *H. pylori* 26695 are listed to illustrate the differences between the *H. pylori atp* operon and most other bacterial *atp* operons represented by the *E. coli* operon (see below) (orf: open reading frame).

The *atp* genes encoding the subunits of the F_1F_0-ATPase are highly conserved among eubacterial species (Deckers-Hebestreit and Altendorf, 1996; Dimroth, 1997; Weber and Senior, 1997). The *atp* operon has been described in a variety of species. The most detailed analysis is available for *E. coli* (Harold and Maloney, 1996). The *atp* operon has also been described for a variety of other bacteria such as aerobic and anaerobic gram-positive bacteria (e. g. *Bacillus*, *Streptococcus*) and the photosynthetic bacterium

Rhodobacter capsulatus (Borghese *et al*., 1998), and in Cyanobacteria. At least 100 sequenced genomes facilitate the analysis (refer to www.ncbi.nlm.nih.gov/PubMed).

Two complete genomes of *H. pylori* are available for the comparison of the *H. pylori atp* operon with other species: strain 26695 (Tomb *et al*., 1997) and J99 (Hancock *et al*., 1998; Alm *et al*., 1999).

In *E. coli,* the *atp* (formerly *unc*) operon consists of nine genes in the following order: *atpIBEFHAGDC*. The protein product of *atpI* has no known function for the ATP synthase and is dispensable. The *E. coli atp* operon is transcribed as a polycistronic mRNA which suggests additional regulation by mRNA secondary structures (Harold and Maloney, 1996). *atpBEF* encode the subunits a, b and c which form the F_0 subunit. *atpHAGDC* encode the five subunits that form the catalytic F_1 part: α_3, β_3, γ, δ, ε. This structure of the operon is relatively conserved among bacteria. Only the order of the genes encoding F_0 varies in some organisms, e.g. *Streptococcus pneumoniae* (*atpEBFHAGDC*, Martin-Galiano *et al*., 2001) and *Lactococcus lactis* (*atpEBFHAGDC*, Koebmann *et al*., 2000). In contrast to the composition of the *atp* operons described above, which are similar despite of changes in the gene order, *H. pylori* contains a modified operon where *atpE* and *atpB* are found separated from the other genes. This particularity is also found in *Campylobacter jejuni*, which is closely related to *H. pylori*. In Figure 3.1 below, the operons of different bacteria are compared and aligned using the "Phylosopher" program (Genedata).

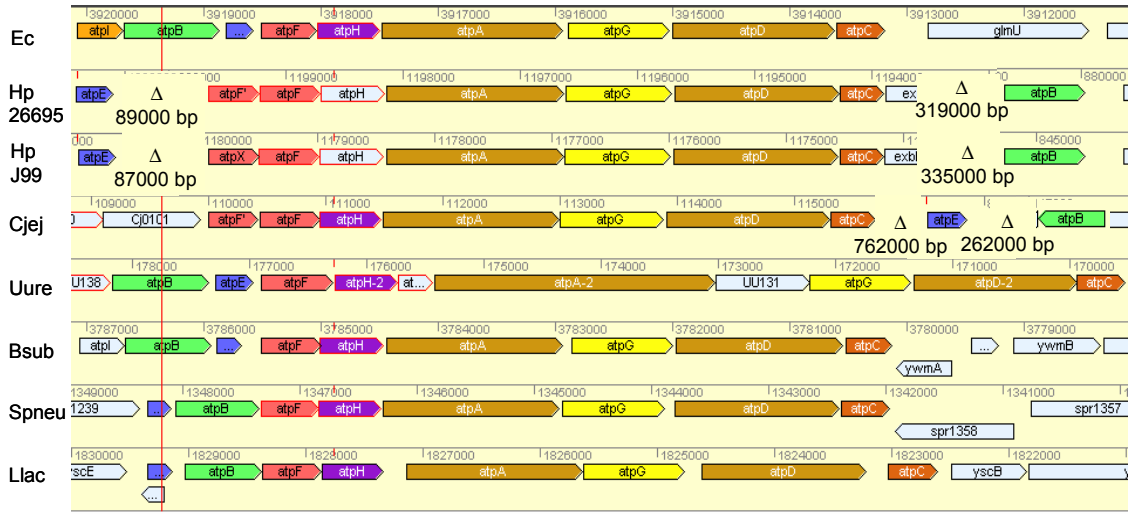

Figure 3.1: *atp* operons of different bacteria. The abbreviations and the Genbank entries of the different bacteria are described as follows: Ec: *E. coli* K12 (NC_000913), 26695: *H. pylori* 26695 (AE000511), J99: *H. pylori* J99 (AE001439), Cj: *C. jejuni* (NC_002163), Uure: *U. urealyticum* (NC_002162), Bsub: *B. subtilis*, Spneu: *S. pneumoniae* R6 (AE005672), Llac: *L. lactis* subsp. *Lactis IL* 1403 (AE005176).

3.2 Alignment of *atpE* DNA sequences from different *H. pylori* strains

The *atpE* genes and the surrounding region of the three *H. pylori* strains (69A, 888, G1.1) used in this study were sequenced from genomic DNA. These sequences were compared to the published sequences of two other *H. pylori* strains, 26695 and J99 (Tomb *et al*., 1997, Hancock *et al*., 1998; Alm *et al*., 1999).

The alignment of the *H. pylori* strains G1.1, 69A, 888, 26695 and J99 is shown in Figure 3.2 below, the open reading frame of the *atpE* gene is shown in red.

```
  G1.1    AAATCGCTTTGGATTTGGCTTAAAATTTTAAAAAGCGTTTTTTAAAAGAGATTTTAAGCT
   69A    AAATCGCTTTGGATTTGGCTTAAAATTTTAAAAAGCGTTTTTTAAAAGCGTTTTTAAGCT
   888    ACAACCCTATAAAAA---CTTACT-TTTTAAAAAGCGTTTTTTAAAAGCGTTTTTAAGCT
 26695    AAATCGCTTTAGATTTGGCTTAAAATTTTAAAAAGCGTTTTTTAAAAGCGTTTTTAAGCT
   J99    AAATCGCTTTGGATTTGGCTTAAAATTTTAAAAAGCGTTTTTTAAAAGAGATTTTAAGCT

  G1.1    AGTTTGTATTAAAATAAATTCTTGCTTGTATTTTTGAAAGTAGAGGAACGATTCGAAATG
   69A    AGTTTGTATTAAAATAAATTCTTGCTTGTATTTTTGAAAGTAGAGGAGCGATTCGAAATG
   888    AGTTTGTATTAAAATAAGTTCTTGCTTGTATTTTTGAAAGTAGAGGAACGATTCGAAATG
 26695    AGTTTGTATTAAAATAAATTCTTGCTTGTATTTTTGAAAGTAGAGGAACGATTCGAAATG
   J99    AGTTTATATTAAAATAAGTCCTTGCTTGTATTTTTGAAAGTAGAGGAACGATTCGAAATG
                                                                1

  G1.1    AAAATCAAGCGCAGAAACATTTGAAAACGGAGTGCGAAAATGAAATTTTTAGCGTTATTT
   69A    AAAATCAAGCCCA---ATTTTTGAAA-CGGAGTGCGAAAATGAAATTTTTAGCGTTATTT
   888    AAAATCAAGCCCA---ATTTTTAAAA-CGGAGTGCGAAAATGAAATTTTTAGCGTTATTT
 26695    AAAATCAAGCCTA---ATTTTTGAAA-CGGAGTGCGAAAATGAAATTTTTAGCGTTATTT
   J99    AAAATCAAGCCCA---ATTTTTGAAA-CGGAGTGCGAAAATGAAATTTTTAGCGTTATTT
            22                                                      81

  G1.1    TTTCTGGCTTTAGCGGGCGTTGCTTTCGCTCATGATGGTGGAATGGAGGGGATGGATATG
   69A    TTTCTGGCTTTAGCGGGCGTTGCTTTCGCTCATGATGGTGGAATGGGTGGGATGGATATG
   888    TTTCTGGCTTTAGCGGGCGTTGCTTTCGCTCATGATGGCGGAATGGGTGGGATGGATATG
 26695    TTTCTGGCTTTAGTGGGCGTCGCTTTCGCTCATGATGGCGGAATGGGTGGGATGGATATG
   J99    TTTCTGGCTTTAGCGGGCGTTGCTTTCGCTCATGATGGTGGCATGGGTGGGATGGATATG
            82                                                     141

  G1.1    ATTAAATCTTATTCTATCTTAGGAGCGATGATCGGTCTAGGGATTGCTGCTTTTGGTGGG
   69A    ATTAAATCTTATTCTATCTTAGGGGCGATGATCGGTTTAGGGATTGCCGCTTTTGGTGGG
   888    ATTAAATCTTATTCTATCTTAGGGGCGATGATCGGTCTAGGGATTGCCGCTTTTGGTGGG
 26695    ATTAAATCTTATTCTATCTTAGGGGCGATGATCGGTCTAGGGATTGCCGCTTTTGGTGGG
   J99    ATTAAATCCTATTCTATCTTAGGGGCGATGATCGGTCTAGGGATCGCCGCTTTTGGTGGG
            142                                                    201

  G1.1    GCAATCGGCATGGGGAATGCGGCCGCAGCGACCATTACAGGCACAGCGAGGAATCCAGGA
   69A    GCGATCGGCATGGGGAATGCGGCCGCAGCGACCATTACAGGCACAGCGAGAAACCCAGGA
   888    GCGATCGGCATGGGGAATGCGGCCGCAGCGACCATTACAGGCACAGCGAGAAACCCAGGA
 26695    GCGATCGGCATGGGGAATGCGGCTGCAGCGACCATTACAGGCACAGCGAGAAACCCAGGA
   J99    GCGATCGGTATGGGGAATGCGGCCGCAGCGACCATTACAGGCACAGCGAGAAACCCAGGA
            202                                                    261

  G1.1    GTGGGTGGTAAATTGCTCACCACTATGTTTGTCGCCATGGCGATGATTGAAGCGCAAGTG
   69A    GTGGGCGGTAAATTGCTCACCACCATGTTCGTGGCCATGGCGATGATTGAAGCGCAAGTG
   888    GTGGGCGGTAAATTGCTCACAACCATGTTCGTGGCCATGGCGATGATTGAAGCGCAAGTG
 26695    GTGGGCGGTAAATTGCTCACTACCATGTTTGTGGCCATGGCGATGATTGAAGCGCAAGTG
   J99    GTGGGCGGTAAATTGCTCACAACCATGTTCGTGGCCATGGCGATGATTGAAGCGCAAGTG
            262                                                    318

  G1.1    ATTTATACTCTAGTGTTTGCTATTATCGCTATTTATAGTAACCCATTCTTAAGTTAAGGG
   69A    ATCTATACTCTAGTGTTTGCTATTATCGCTATTTATAGTAACCCATTCTTAAGTTAAGGG
   888    ATTTATACTCTAGTGTTTGCTATTATCGCTATTTATAGTAACCCATTCTTAAGTTAAGGG
 26695    ATTTATACTCTAGTGTTTGCTATTATCGCTATTTATAGTAACCCATTCTTAAGTTAAGGG
   J99    ATTTATACTCTAGTGTTTGCTATTATCGCTATTTATAGTAACCCATTCTTAAGTTAAGGG
```

```
  G1.1    TTT--TGATGGGCTAAATTATTGCTTAAAAAGCGTAATTTGGCTATAATATTTGCTT---
   69A    TTTTTGATGGGCTAAATTATTGCTTAAAAAGCATGATTTAGCTATAATATCTGTTT---
   888    TTT--TGTTGGGCTAAATCATTGCTTAAAAAGCGTGATTTAGCTATAATATTCGTTTATT
 26695    TTT--TGTTGGGCTAAATCATTGCTTAAAAAGCGTGATTTAGCTATAATATTTGTTTATT
   J99    TTTT-TGATGGGCTAAATTATTGCTTAAAAAGCATGATTTAGCTATAGTATTTGTTT---

  G1.1   -TAAATTTTTAGCACTGGTGGTGGAATTGGTAGACACGCCATCTTGAGGGGGTGGTGGGA
   69A   -TAAATTTTTAGCACTGGTGGTGGAATTGGTAGACACGCCATCTTGAGGGGGTGGTGGGA
   888   TTAAATTTTTAGCACTGGTGGTGGAATTGGTAGACACGCCATCTTGAGGGGGTGGTGGGA
 26695   TTAAATTTTTAGCACTGGTGGTGGAATTGGTAGACACGCCATCTTGAGGGGGTGGTGGGA
   J99   -TAAATTTTTAGCACTGGTGGTGGAATTGGTAGACACGCCATCTTGAGGGGGTGGTGGGA
```

Figure 3.2: Alignment of *atpE* from different *H. pylori* strains with the surrounding region. The open reading frame is shown in red, the surrounding region in blue.

Twenty-two changes in base pair composition were detected, which result in only two amino acid exchanges. Alanine at position 12 is exchanged against valine in strain 26695, both are amino acids with aliphatic side chains. More interesting is the exchange of glycine at position 23 against glutamic acid in strain G1.1. Glutamic acid has an acidic side chain in contrast to glycine, which only has a hydrogen atom.

All other changes in base pairs result only in different codon usage but no patterns for codon usage between strains could be recognized.

A potential Shine-Dalgarno sequence for ribosome binding, ggag, is present, varying slightly from the core sequence ggagg known for *E. coli* (Schurr *et al*., 1993), which is also valid for *H. pylori* (Ma *et al*., 2002). The Shine-Dalgarno sequence is the same in all *H. pylori* strains analysed in this study. The spacing to the atg start codon is 7 bases, whereas in *E. coli*, optimal spacing ranges from 8 to 10 bases (Ringquist *et al*., 1992; Chen *et al*., 1994).

3.3 Homology among c subunits of different bacteria

Protons enter the cell through a channel between subunit a and a multimer of 10-14 c subunits, driven by the proton motive force, thereby generating ATP. This reaction is reversible and H^+ ions can be pumped out of the cytoplasm by ATP-hydrolysis. *atpE* encodes the c subunit of F_1F_0-ATPase that forms part of the inner membrane proton channel.

The alignment of various protein sequences reveals a striking feature of *H. pylori atpE* encoded peptide (Figure 3.3). *H. pylori*, *C. jejuni* and *U. urealyticum* exhibit an elongated N-terminus compared to the other organisms.

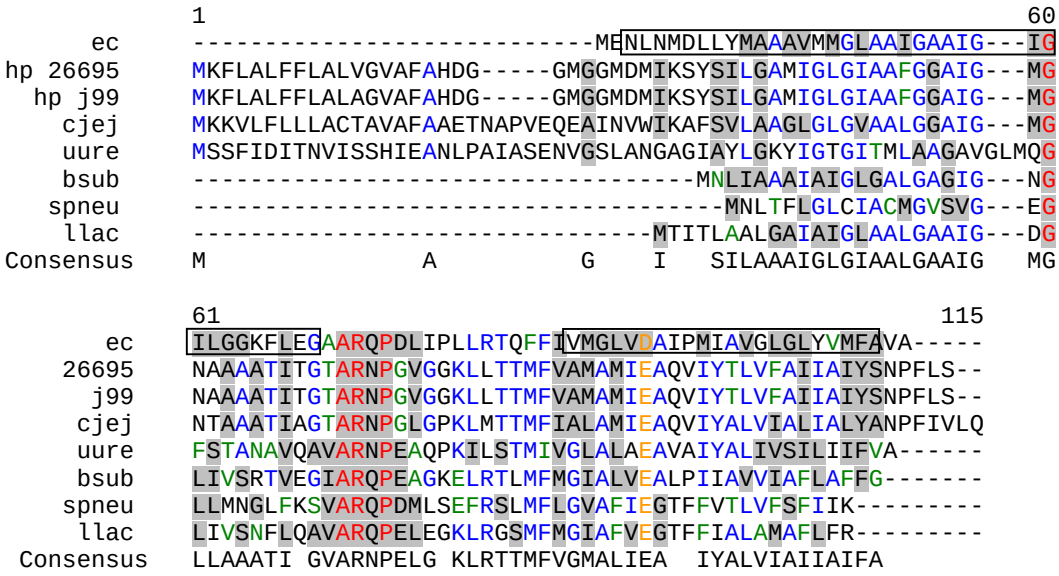

Figure 3.3: Alignment of *atpE* from different bacterial organisms. Only *H. pylori,* the closely related *C. jejuni* and *U. urealyticum* possess an N-terminal elongation compared to the other organisms. Ec: *E. coli* K12 (NC_000913), hp 26695: *H. pylori* 26695 (AE000511), hp J99: *H. pylori* J99 (AE001439), cjej: *C. jejuni* (NC_002163), uure: *U. urealyticum* (NC_002162), bsub: *B. subtilis*, spneu: *S. pneumoniae* R6 (AE005672), llac: *L. lactis subsp. Lactis* IL 1403 (AE005176). The boxes mark the transmembrane domains in *E. coli atpE*. Strictly conserved residues are shown in red. Aspartatic acid at position 61 (*E. coli* nummeration) is shown in orange. It corresponds to glutamic acid in all other organisms.

and is essential for proton translocation (Deckers-Hebestreit and Altendorf, 1996). The two hydrophobic transmembrane domains probably span residue 3-38 and 50-77 in *E. coli* (Dmitriev *et al*., 1999). The most variable region is the N-terminus.

H. pylori atpE contains an N-terminal elongation compared to most other organisms. A closely related sequence is found in *C. jejuni*. *U. urealyticum* also contains a varied N-terminus. *U. urealyticum* is found in the normal human genital tract and also possesses a potent urease (Pollack, 2001). *atp* genes for other urease positive organisms have not been sequenced (e. g. *Proteus mirabilis*, *Streptococcus salivarius*, *Yersinia spp*.). All sequences have a conserved alanine residue at position 17. Two charged residues, histidine and aspartatic acid, followed by alanine at position 17 in *H. pylori*. A histidine residue and a glutamic acid residue are detected at position 14 and 16 of *U. urealyticum* sequence. Only one glutamic acid is found at position 19 is found in *C. jejuni*.

In *H. pylori*, the N-terminal stretch is mostly hydrophobic. Signal sequence analysis (signalP at www.cbs.dtu.dk, Nielsen *et al*., 1997) predicts a function as a signal sequence for the N-terminal stretch.

The result of the signal sequence analysis of *H. pylori atpE* performed with SignalP is shown in Figure 3.4 below:

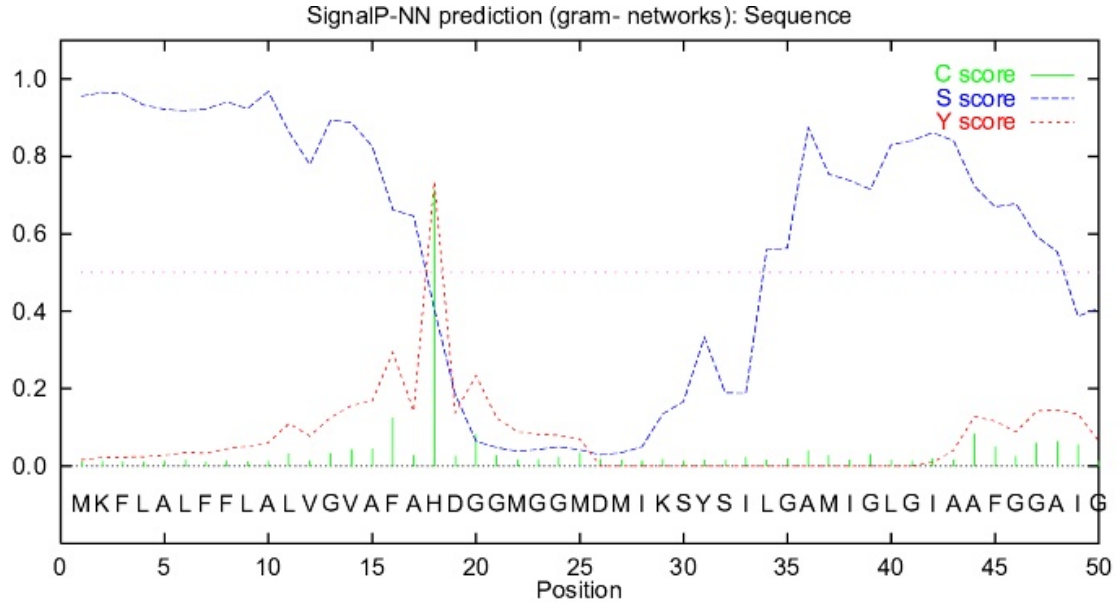

Figure 3.4: Results from the signalP analysis for the N-terminal sequence of *H. pylori*. The C-score, termed "raw cleavage site score" is high at position +1 immediately after the cleavage site and low at all other positions. The S-score, the "signal peptide score", discriminates signal peptides from non-signal-peptide positions. Its value is high at all positions before the cleavage site and low at 30 positions before the cleavage site and in the N-terminus of non-secretory proteins. The Y-score, the "combined cleavage site score", combines C- and S-score by combining the height of the C-score with the slope of the S-score.

For *H. pylori atpE* the peak of the Y-score (green bar) suggests a signal sequence with a cleavage site between amino acid residues 17 and 18: AFA ✂ HD.

SignalP analysis for the *U. urealyticum* sequence does not predict a signal sequence (Figure 3.5 below).

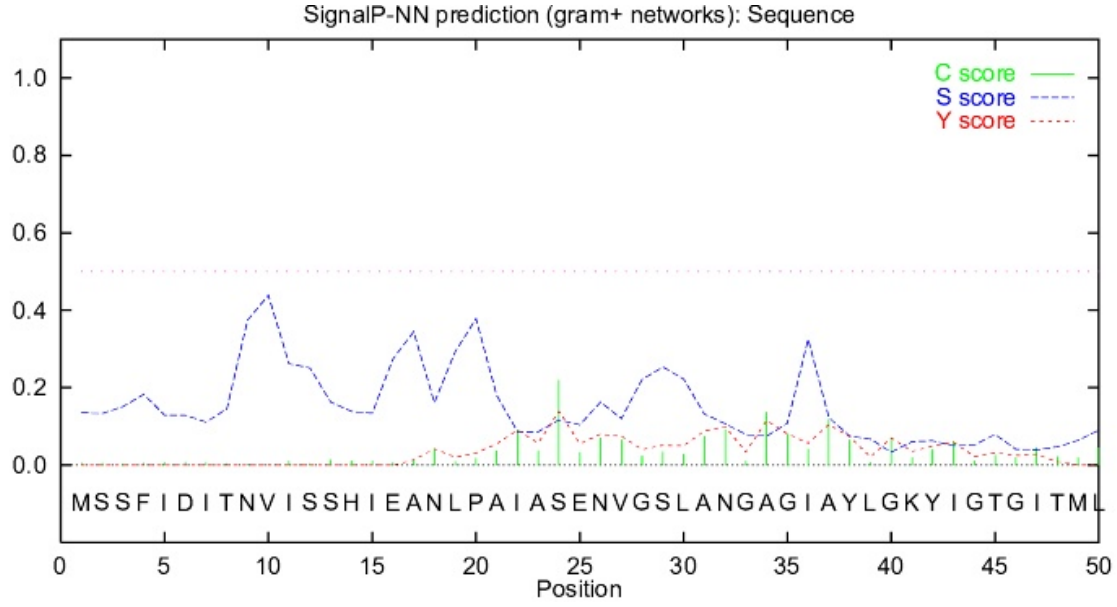

Figure 3.5: The signalP analysis for the *U. urealyticum* sequence.

On the other hand, the GGMGG sequence also indicates a turn in the protein structure, which could indicate a third membrane domain. Histidine 18 could possibly be located in the periplasmic loop and function as an H^+ dependent gate of the channel. Aspartatic acid 19 could also be involved.

The charged amino acids might also play a role in a pH sensing mechanism that is unique for *H. pylori* and enables it to survive in the acidic human stomach.In order to elucidate these peculiar characteristics of the *H. pylori* c subunit, the topology was studied in detail in the next chapter 3.4.

3.4 Topological analysis of the c subunit of *H. pylori* F_1F_0-ATPase

In order to solve the question whether the additional N-terminal sequence of *H. pylori* c subunit serves as a signal sequence or a third transmembrane segment, the nature of the gene product was analyzed by various means. A computational analysis based on several structural transmembrane prediction programmes was followed by *in vitro* and *in vivo* topology analysis.

3.4.1 Computational analysis and predictions

Various prediction programmes are available to investigate the possible tertiary structure of a peptide. Hydrophobic regions can be identified if each amino acid is assigned a score reflecting its relative hydrophobicity based on a number of physical characteristics: e.g. solubility and the free energy of transfer through a water-vapour phase transition. Several algorithms are available that display the hydrophobic index plotted against the residue number, including: Kyte and Doolittle, 1982, Rao and Argos, 1986, Eisenberg *et al*., 1984 and Klein *et al*., 1985. The results from a Kyte and Doolittle analysis are shown in Figure 3.6 below but the results obtained with the other algorithms were similar.

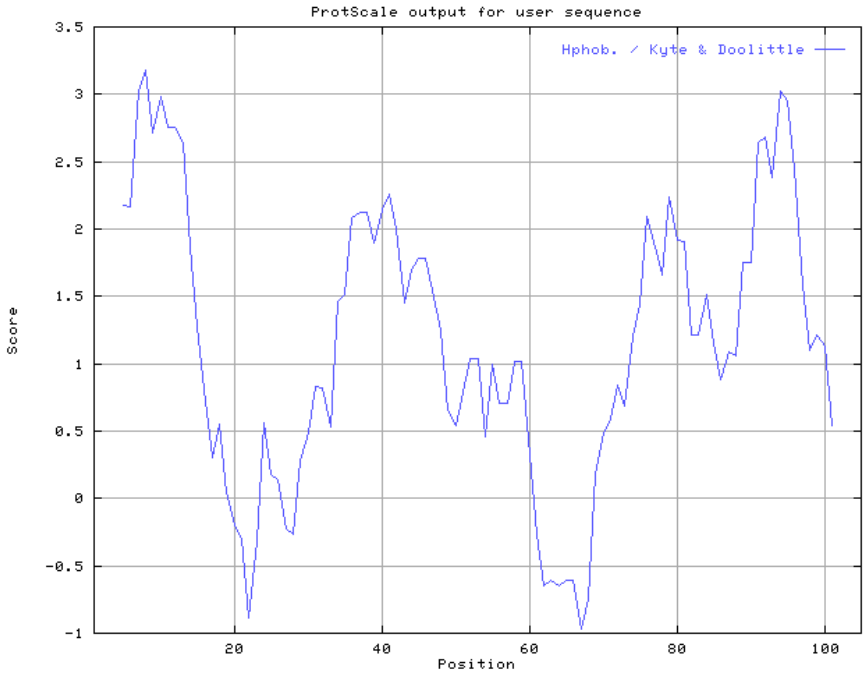

Figure 3.6: Result of a Kyte-Doolittle hydropathy determination using Vector NTI (Biomax). The hydropathy score (y-axis) is plotted against the amino acid position (x-axis). A higher, positive value indicates a higher hydrophobicity whereas amino acid residues with a more negative score are more hydrophilic. Three peaks with positive score are detected and indicate the presence of three hydrophobic, possibly membranous, domains.

Three peaks with a positive score indicating three hydrophobic domains are identified with the Kyte and Doolittle algorithm.

However, hydrophobicity analysis can give indications about transmembrane domains but they can also be embedded in globular domains. More complex programmes, mostly based on neural networks, attempt to predict tertiary structures such as transmembrane domains.

A summary of the results of different programs for transmembrane domain prediction is given in Table 3.2 below. All programs were challenged with the amino acid sequence of the c subunit from *H. pylori* strains 26695 and G1.1.

***H. pylori* strain**	**Pre-diction method**	**Location of possible membrane domains and their orientation (i, o)**			**Source of the prediction**	**reference**
		TM 1: i→o	TM 2: o→i	TM 3: i→o		
Strain 26695	TM Pred	3-21	33-49	85-105	www.ch.embnet.org	K. Hofmann & W. Stoffel (1993)
	TM HMM	3-25	35-57	77-99	www.cbs.dtu.dk	
	Hmmtop	6-28	33-52	73-97	www.enzim.hu	Tusnády and I. Simon (2001)
	Sosui	2-24	36-58	75-97	http://sosui.proteome.bio.tuat.ac.jp/	
	Memsat 2	7-24 (o→i)	31-51 (i→o)	76-99 (o→i)	http://bioinf.cs.ucl.ac.uk/psipred/	Jones DT. (1999)
	phoA	30	71	105		This work
	IVTT	1-19	31-59/ 31-64	76-105		This work
Strain G1.1	TM Pred	1-18	33-49	85-105	www.ch.embnet.org	K. Hofmann & W. Stoffel (1993)
	TM HMM	3-20	35-57	77-99	www.cbs.dtu.dk	
	Hmmtop		27-51	72-96	www.enzim.hu	Tusnády and I. Simon (2001)
	Sosui	2-24	34-56	75-97	http://sosui.proteome.bio.tuat.ac.jp/	
	Memsat 2	7-25 (o→i)	33-51 (i→o)	77-100 (o→i)	http://bioinf.cs.ucl.ac.uk/psipred/	Jones DT. (1999)

Table 3.2: Comparison of the predictions of transmembrane domains of various prediction programmes. TM 1 to 3 indicates the predicted transmembrane domains. The orientation is marked by i for inside and o for outside, the arrow marking the direction of the membrane spanning sequence. IVTT is the *in vitro* transcription and translation method used for topology analysis in this work. phoA designates the alkaline phosphatase experiments also described in this work.

Although the predictions of the different programmes for the transmembrane domains of the *H. pylori* c subunit correspond fairly well to one another, there are some important differences. For *H. pylori* 26695, all programs predict three transmembrane domains with the same orientation. For *H. pylori* G1.1, where two amino acids are exchanged (see Figure 3.2), Hmmtop only predicts two membrane domains. The

orientation of the predicted transmembrane domains is reversed when using Memsat 2. These findings illustrate the necessity to verify the predictions by experimental means although it seems most likely that three transmembrane domains are present.

3.4.2 *In vitro* topology analysis of the c subunit

The topology of putative membrane segments was analysed with help of the M0 and M1 vectors (Bamberg and Sachs, 1994). These vectors were initially used to investigate the membrane topology of the α subunit of the H^+, K^+-ATPase (Bamberg and Sachs, 1992, 1994) but have also been successfully applied to determine the topology of integral membrane proteins of *H. pylori* (Melchers *et al*., 1996, Bayle *et al*., 1998). This method enables the user to distinguish between signal anchor sequences and stop transfer sequences. This system was used to investigate the nature of the c subunit of *H. pylori* that is encoded by the *atpE* gene.

The basic vector is pGEM7zf$^+$ (Promega) and the fusion proteins are expressed in a reticulocyte lysate with ^{35}S methionine under control of the T7 promoter. The inserts consist of the 101 (M0) or 139 (M1) N-terminal amino acids of the α subunit of the H^+, K^+-ATPase (Bamberg *et al*., 1992). This sequence is followed by a linker sequence of four amino acids and the 177 C-terminal amino acids of the β subunit (Reuben *et al*., 1990), which contains five N-linked glycosylation sites. The linker sequence can be replaced by the region of interest.

The putative transmembrane segments of the c subunit of the *H. pylori* F_1F_0-ATPase were amplified by PCR and ligated into the *Bgl*II and *Hin*dIII sites of the expression vectors M0 and M1.

The M0 vector is used to identify signal anchor sequences. A fusion protein with signal anchor activity will be translocated across the microsomal membrane and the β subunit can therefore be glycosylated. Glycosylation results in a shift of the molecular weight of the protein when separated by SDS polyacrylamide gel electrophoresis and subsequently visualized by autoradiography.

The M1 vector contains the first transmembrane domain of the gastric H^+, K^+-ATPase that displays signal anchor activity. Therefore, the β subunit is translocated into the lumen of the microsomes and subsequently glycosylated. Presence of an additional stop transfer sequence inhibits glycosylation.

All constructs were synthesized by PCR (refer to materials and methods) and ligated into the M0 and M1 vector. Inserts were verified by sequencing (GATC Biotech).

The different constructs that were used for the *in vitro* topology analysis are shown in Figure 3.7 below.

Construct	Amino acid sequence
H1	M1-H18 (D19→E)
H2	Y31-I59
H2*	Y31-R64
H3	M76-L104 (S105→R)
H1-2	M1-I59
H1-2*	M1-R64
H1-H3	M1-L104 (S105→R)

Table 3.3: The three putative transmembrane segments (H1-H3).H2* is a longer sequence for the identification of the second transmembrane domain. All constructs were inserted in the M0 and the M1 vector and their ability for membrane insertion was tested by *in vitro* transcription/translation with and without microsomal membranes.

The synthesized proteins were separated by SDS-PAGE and visualized by autoradiography. The results of the *in vitro* transcription and translation (IVTT) experiments are shown in the Figures below.

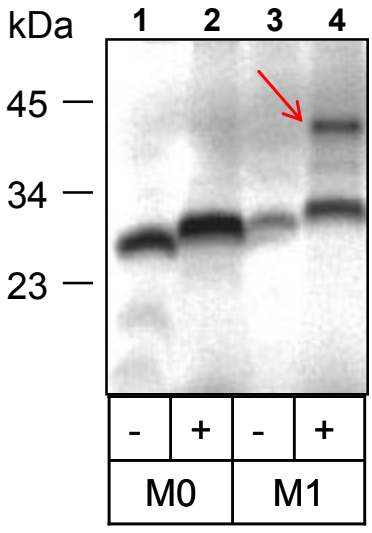

Figure 3.7: The M0 and M1 vector translation products before insertion of the potential transmembrane domains H1 to H3. The M1 translation product is glycosylated in the presence of membranes as can be detected by a band shift. The molecular weight in kD is indicated on the left. – and + indicate the absence or presence of microsomal membranes.

The experiments with the M0 and M1 vector without insertion of sequences of the *H. pylori* c subunit are shown in Figure 3.7. No glycosylation is visible when the M0

vector is translated in the presence of microsomal membranes. In contrast, the M1 vector translation product is glycosylated when microsomal membranes are present. The glycosylation can be seen by the increase in the molecular weight of the fusion protein, which contains the first membrane domain of the α subunit of the gastric H^+, K^+-ATPase. Induced by the signal anchor activity of this membrane domain, the β subunit is translocated across the microsomal membrane. The transfer efficiency was about 50% since not all proteins were translocated.

The results from IVTT of the four inserts H1, H2, H2* and H3 in the M0 vector are shown in Figure 3.8 below:

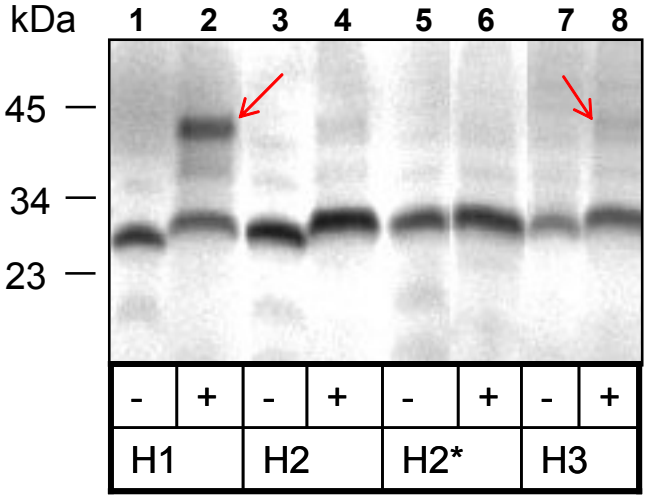

Figure 3.8: All putative transmembrane domains were cloned into the M0 vector to investigate their signal anchor activity. A strong signal is detected for H1, whereas no signal can be seen with the second potential transmembrane domain. A very weak signal might be detected for H3. – and + indicate the absence or presence of microsomal membranes during IVTT.

The potential transmembrane domain H1, which constitutes the first putative transmembrane domain of the c subunit, shows strong signal anchor activity in M0 when incubated with microsomal membranes. The transfer efficiency is similar compared to the M1 vector translation product (Figure 3.8, lane 1 and 2).

Two sequences with different length were chosen to analyze the properties of the second putative membrane domain designated H2. No glycosylation was observed when M0/H2 and M0/H2* were translated in the presence of microsomal membranes (Figure 3.8, lane 3 to 6). Therefore, H2 (amino acids 31-59 of *H. pylori atpE*) and H2* (amino acids 31-64) showed no signal anchor activity.

To examine the signal anchor properties of the third putative transmembrane domain H3, amino acids methionine at position 76 to leucine at position 104 were inserted into

the M0 vector. When M0/H3 was translated in the presence of membranes a very weak signal was detectable at the size of the glycosylated fusion protein compared to the reaction without microsomal membranes. Hence, the signal anchor activity of the isolated H3 domain is low in contrast to H1 (Figure 3.8, lane 7 and 8).

Subsequently, all four constructs were inserted in the M1 vector for investigation of their stop transfer activity.

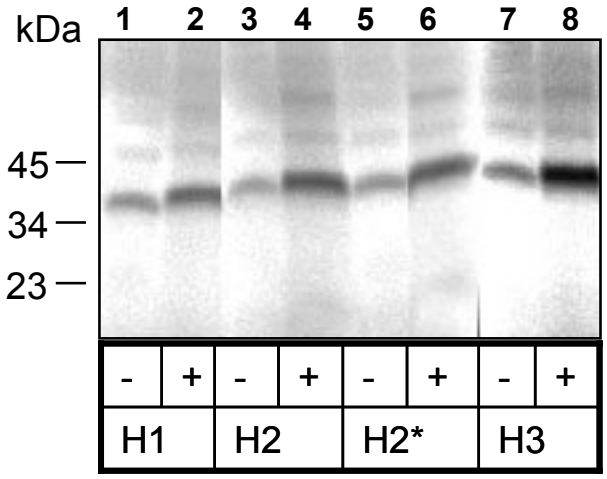

Figure 3.9: All putative transmembrane domains were also inserted into the M1 vector to investigate their stop transfer activity. No glycosylation of any of the constructs can be detected. – and + indicate the absence or presence of microsomal membranes during IVTT. (the stripes were assembled in Adobe Photoshop to obtain this order.)

When H1 was inserted into the M1 vector and translated in the presence of microsomal membranes glycosylation was inhibited (Figure 3.9, lane 1 and 2). Therefore M1-H18 acts as stop transfer sequence in combination with the first membrane spanning sequence of the gastric H^+, K^+-ATPase.

Glycosylation was also completely inhibited when M1/H2 and H2* were translated (Figure 3.9, lane 3 to 6). This indicates a stop transfer activity for the second putative transmembrane segment.

M1/H3 also shows stop transfer activity. No glycosylation could be observed when this construct was translated in the presence of membranes as shown in Figure 3.9, lane 7 and 8. Therefore, no stop transfer activity could be detected for any of the single potential transmembrane domains.

The context of different transmembrane domains can be important for the proper insertion of a protein into the membrane. Consequently, combinations of

transmembrane segments were also inserted into M0 and M1 to elucidate the ability for membrane insertion.

In Figure 3.10 and Figure 3.11 below the results from IVTT of combinations of potential transmembrane domains in the M0 and the M1 vector are shown.

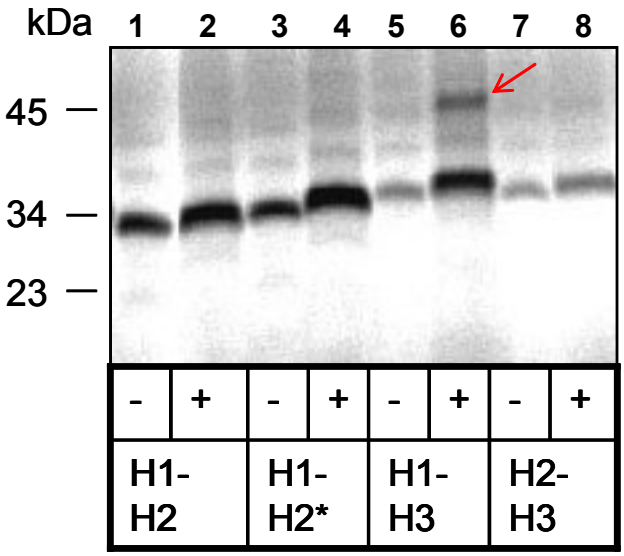

Figure 3.10: Different combinations of the putative transmembrane domains were inserted into the M0 vector. – and + indicate the absence or presence of microsomal membranes during IVTT. Glycosylation is only observed when all three transmembrane domains are expressed together (lane 6, arrow marked).

When M0/H1-H2 or M0/H1-H2* were translated in the presence of membranes no glycosylation was observed (lane 1-4). This result is presented in Figure 3.10 and indicates that H1 has a signal anchor activity as shown Figure 3.8. The sequences H2/H2* act as strong stop transfer signals in context of the H1 signal anchor sequence of *H. pylori atpE*. H2 exhibits stop transfer activity in this context.

Glycosylation was observed when M0/H1-H3 was translated in the presence of membranes (Figure 3.10). This shows that H3 exhibits signal anchor activity when expressed with the complete amino acids sequence of the *H. pylori* c subunit.

H2-H3 was also inserted into the M0 vector. No glycosylation was observed when this construct was translated in the presence of membranes (lane 7). Obviously, H2 and H3 can act as a pair of membrane spanning domains when expressed together in the M0 vector as seen in lane 7 and 8 of Figure 3.10.

The combined transmembrane domains were subsequently inserted in the M1 vector.

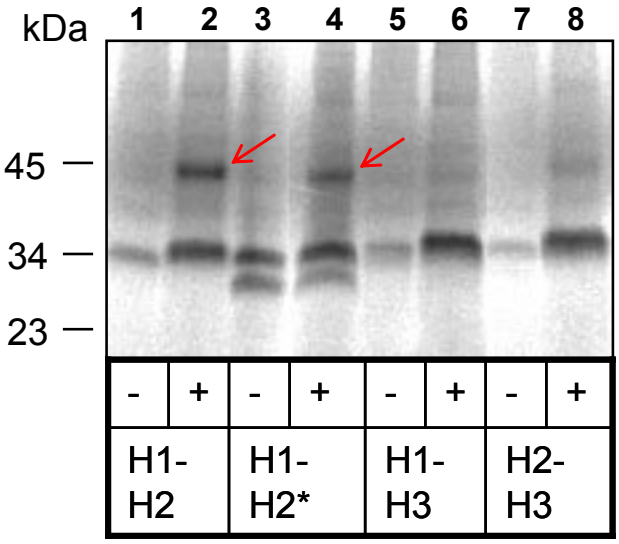

Figure 3.11: The different combinations of membrane domains were also inserted into the M1 vector. – and + indicate the absence and presence of microsomal membranes during IVTT. Glycosylation can be observed when the 1st and 2nd transmembrane domains are expressed together.

When H1-H2 and H1-H2* were inserted into M1, glycosylation could be observed (Figure 3.11). In this context, H1 showed stop transfer activity in combination with the M1 membrane domain and hence, signal anchor activity is forced on H2. The same result is found with H2*. Additionally, a lower molecular weight translation protein product can be observed with H1-H2*. This indicates that the longer sequence might provoke a premature stop of translation.

No glycosylation can be seen when H1-H3 are inserted into M1, which might possibly be due to the even number of membrane segments (Figure 3.11).

A weak transfer across the microsomal membrane followed by glycosylation can be observed when H2-H3 is inserted into the M1 vector. In this combination H2 shows stop transfer activity and H3 acts as a signal anchor. The weak transfer might be explained by the weak signal anchor activity of H3 (Figure 3.8).

The *in vitro* topology analysis of the *H. pylori* c subunit shows that three transmembrane domains are present in this organism. Expecially the first N-terminal transmembrane domain exhibits strong signal anchor activity. The N-terminus is found in the cytoplasm. Due to the uneven number of transmembrane domains, the C-terminus is in the periplasm. This is in contrast to *E. coli*, where two transmembrane domains are found (Deckers-Hebestreit and Altendorf, 1996) and N- and C-terminus are found in the periplasm.

3.4.3 *In vivo* topology analysis of *H. pylori* c subunit

In vivo topology analysis of *H. pylori atpE* encoded c subunit was performed in *E. coli* using fusion proteins with alkaline phosphatase. Alkaline phosphatase must be translocated to the periplasm for correct folding of disulfide bonds. Hybrid proteins of *H. pylori* c subunit and alkaline phosphatase were expressed in *E. coli*. The three different constructs contained the potential first transmembrand domain (phoA1), the first and second transmembrane domains (phoA2) and first to third transmembrane domains (phoA3). Fusion joints were chosen close to the potential end of periplasmic and cytoplasmic domain, as determined by the M0/M1 experiments, to ensure that topological signals are present. pBAD*phoA* (Melchers *et al*., 1999) was used as a vector in which signal sequenceless *phoA* was cloned into pBAD22 (Guzmann L.M. *et al*., 1995). Three constructs phoA1, phoA2 and phoA3 ligated into pBAD*phoA* where they are expressed und control of the inducible araBAD promoter. Constructs were verified by sequencing (GATC Biotech).

Construct	Amino acid sequence
phoA1	K2-S30
phoA2	K2-K 71
phoA3	K2-S105

Table 3.4: The constructs, which were inserted into pBADphoA, are listed with the corresponding amino acid sequence as derived from *H. pylori* strain 26695.

The expression of the different constructs after induction with arabinose was analysed. The expression levels of the fusion proteins are shown by Western blotting in Figure 3.12 B.

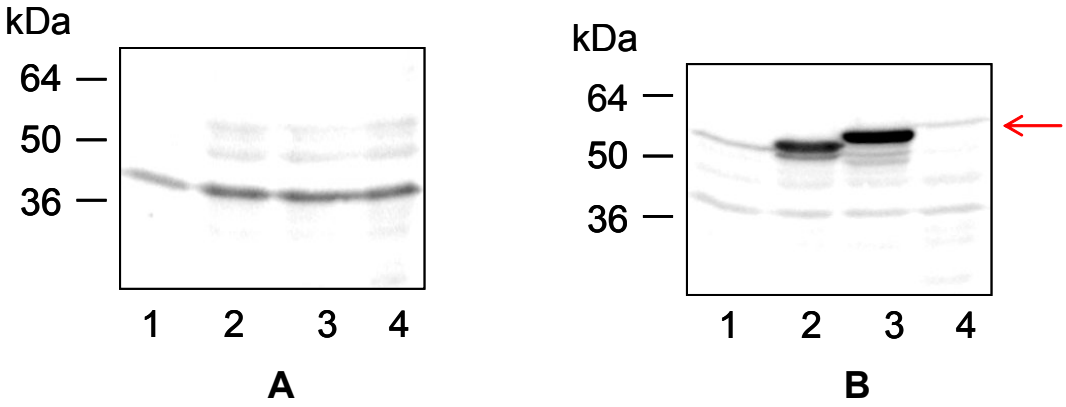

Figure 3.12: Bacterial lysate of *E. coli* LMG 194 harbouring the different expression plasmids was separated on a 10% Laemmli gel. The same samples are analysed in figures A and B. In both figures in lane 1, bacterial lysate of LMG 194 with pBADphoA was separated. In lane 2 to 4, LMG 194 phoA1, phoA2 and phoA3 are shown. No fusion protein can be detected in figure A. In figure B, the protein expression was induced by addition of 100µM arabinose whereas no arabinose was added to bacteria analysed in figure A. After western blotting on a PVDF membrane, alkaline phosphatase was detected by a monoclonal antibody.

The araBAD promoter is a tightly regulated promoter (Lee *et al*., 1981). This fact is also demonstrated in Figure 3.12 A. No alkaline phosphatase protein can be detected without induction with arabinose. In Figure 3.12 B, 100µM arabinose is added to the LB medium. Bacteria were grown for 3 hours in average and were lysed in sample buffer. Subsequently, equal amounts were analysed by SDS PAGE and Western blotting. The wild type protein can be detected at about 50 kDa, which is the upper band in Figure 3.12 B, lane 1. The following constructs (lane 2 to 3) have a slightly higher molecular weight with 55, 59 and 60 kDa. The increasing size corresponds to the longer parts of the *H. pylori atpE* gene that are cloned in front of the *phoA* gene. In several experiments, the *E. coli* LMG 194 harbouring phoA3 (lane 4) grew slower than the other strains. This was the case for the overnight culture as well as the newly inoculated cultures. The protein expression level of the phoA3 construct was also low compared to the other constructs, especially phoA1 and phoA2. Despite the variation of expression levels in the different strains the enzyme activity showed remarkable differences. The enzyme activities were compared to those of the uninduced cultures. The results from the alkaline phosphatase assay are described in the Table 3.5 below.

	- Arabinose (Units)	+ Arabinose (100µM) (Units)
phoA	1,31 (+/- 0,078)	2,21 (+/- 1,75)
phoA1	12,80 (+/- 1,08)	818,21 (+/- 416, 59)
phoA2	1,13 (+/- 0,25)	34,90 (+/- 9,64)
phoA3	9,79 (+/- 0,87)	194,55 (+/- 34,31)

Table 3.5: The alkaline phosphatase activity in units was measured as described in materials and methods. Three separate cultures were analysed and the mean activity with the standard deviation of one representative experiment is shown.

Three different constructs, phoA1, 2 and 3, were used to investigate the topology of the c subunit of the *H. pylori* F_1F_0-ATPase. The empty pBADphoA vector was used as a control.

In the first row of Table 3.5, the activity of alkaline phosphatase in pBAD*phoA* was noted. Protein synthesis was induced with arabinose (see Figure 3.12 B, lane 1) but did not yield an active protein. Despite protein synthesis there was no measurable enzyme activity. This result confirms that signal sequenceless *phoA* is not translocated across the bacterial inner membrane and therefore not folded into its active conformation.

PhoA1 in contrast showed high protein expression (Figure 3.12 B, lane 2) and alkaline phosphatase activity (Table 3.5, second row). In this construct, the first putative transmembrane domain of the *H. pylori* c subunit with the putative periplasmic loop was cloned in front of the alkaline phosphatase. The activity indicates that the protein is translocated into the periplasm and folded into an active enzyme. Therefore, the orientation of this sequence is from the inside to the outside of the membrane.

Low enzyme activity can be observed when expression of phoA2 is induced by addition of arabinose (Table 3.5, third row). The first two putative transmembrane domains might insert into the membrane as a pair and the alkaline phosphatase remains in the cytoplasm where it cannot be folded properly. Despite high protein expression (see Figure 3.12 B, lane 3), the activity was very low compared to phoA1.

The whole c subunit followed by alkaline phosphatase is expressed in the construct phoA3. High activity can be observed which indicates that alkaline phosphatase is translocated across the membrane (Table 3.5, fourth row). The enzyme activity must be related to the amount of protein expressed in strain LMG 194 phoA3. Although there is about 20x less protein than in phoA 2 (compare lane 3 and 4 in Figure 3.12 B), the

enzyme activity is about six-fold higher than in phoA2. PhoA3 therefore yields an active alkaline phosphatase protein, indicating that the C-terminus of the c subunit is translocated to the periplasm.

The results of the *in vivo* topology analysis confirm the results from the *in vitro* topology analysis and show that the *H. pylori* c subunit has three transmembrane domains.

3.5 Generation of mutants of the c subunit

The c subunit of the *H. pylori* F_1F_0-ATPase exhibits several unique features compared to most other bacterial c subunits. Thus, the generation of mutants was attempted to elucidate the function. In short, the various constructs of the *H. pylori* c subunit are summarized in Table 3.6 below.

Insert	Vector	Description
Δ*atpE*::cat_{GC} (M16.1)	pBSIIKS-	Chapter 3.5.1
PflaA_Hp*atpE* (M85.28)	pHel3	Chapter 3.5.2
PflaA_Hp*atpE*k (M85.42)	pHel3	Chapter 3.5.2
Ec *atpE* (M59.33)	pHel3	Chapter 3.5.3
Hp Nterm_Ec*atpE* (M75.28)	pHel3	Chapter 3.5.3
Histidine 18 to glycine mutant (M89.25)	pBSIIKS-	Chapter 3.5.4

Table 3.6: Listing of the different mutants of the *H. pylori* c subunit. A detailed description is provided in the text below.

The deletion of *atpE* in *H. pylori* was attempted by homologous recombination and replacement with an antibiotic resistance gene. These experiments are described in chapter 3.5.1. Alternatively, the complementation of *atpE* with a subsequent deletion was done with *H. pylori atpE* and a truncated variant of *H. pylori atpE* and is described in chapter 3.5.2. The replacement of *H. pylori atpE* with *E. coli atpE* is described in chapter 3.5.3. *E. coli atpE* was also elongated with the *H. pylori* N-terminal stretch as demonstrated in chapter 3.5.3.

In summary, replacement of *H. pylori atpE* proved impossible (see the chapter 3.5.1 below), so the replacement by several mutant structures was performed.

Finally, one amino acid residue, histidine 18, was replaced by glycine in the *H. pylori* N-terminal stretch, these experiments are described in chapter 3.5.4.

3.5.1 Deletion of *atpE*

Homologous recombination is an indispensable tool for the mutational analysis of genes and their function in bacteria. The knowledge of two genomic sequences for *H. pylori* (Tomb *et al*., 1997, Hancock *et al*., 1998; Alm *et al*., 1999) and its accessibility for genetic manipulation facilitate the analysis of gene function.

The constructs for knockout mutagenesis of *atpE* were constructed by crossover PCR (chapter 2.2.6.3). Recombinants were analysed by PCR. The number of colonies resulting from one transformation is shown in Table 3.7 below:

	Strain 69A	Strain 888	Strain 26695	Strain G1.1
M16.1	2	2	0	43
K+ (pY 198)	> 6000	1376	476	548

Table 3.7: The different H. pylori strains used for transformation are mentioned in the first row. M16.1 is the plasmid used for knockout of *atpE*, pY 198 was used as control plasmid and knocks out *urel* by homologous recombination. Some potential knockout mutants were received, but very few colonies were detected compared to the control reactions. Transformation efficiency varied depending on the transformed strain, and one representative outcome is shown. The experiments were repeated at least three times.

Only few colonies were detected after transformation with the plasmid M16.1 compared to the transformation with the control plasmid (knockout of *ureI*, Rektorschek *et al*., 1998). This indicates that the gene *atpE* is essential for *H. pylori*. Some of the potential knockout clones were picked and propagated for further analysis.

The analysis of genomic DNA of nine clones by PCR is shown in Figure 3.13 below:

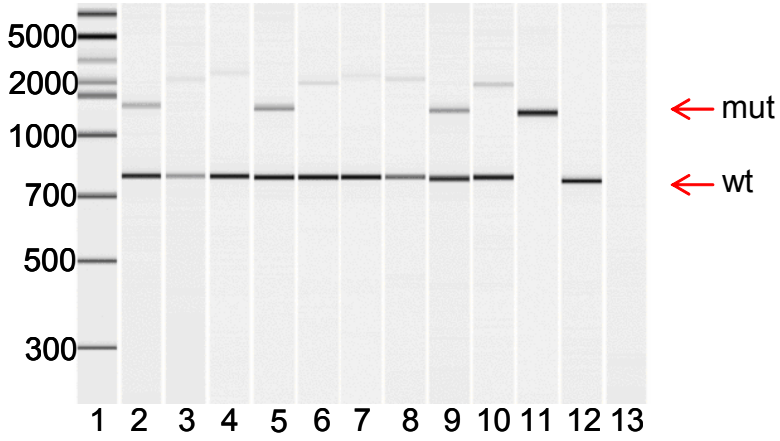

Figure 3.13: PCR of potential *atpE* knockout mutants. In all clones analysed, the wild type sequence was still detectable (band at 800kb). The marker is shown in lane 1. In lane 2-8, potential knockouts in strain G1.1 were analysed. In lane 9 and 10, potential knockouts in strain 888 were analysed. The construct M16.1 was analysed for comparison, displayed in lane 11. The wild type genomic DNA is analysed in lane 12. Lane 13 shows the no template control. The PCR products were analysed with the Bioanalyzer from Agilent.

The fragment of roughly 800 base pairs contains the *H. pylori atpE* wild type sequence with the surrounding regions. The knockout construct is 1756 base pairs in length.
Mutant PCR products were detected in three potential knockouts (lane 2, 5, 9, control lane with mutant plasmid lane 11) but the wild type sequence was also detected in all clones displayed (lane 1 to 9, control lane 12). Despite several attempts, it was not possible to perform a knockout of *atpE* in different *H. pylori* strains. Therefore, *atpE* was identified as an essential gene for *H. pylori*.

3.5.2 Expression of a truncated variant of *H. pylori atpE*

The creation of knockout mutants for *H. pylori atpE* was unsuccessful which substantiates an essential role for *atpE* in *H. pylori*.
A truncated variant of *H. pylori atpE* was created to investigate a possible function of the N-terminal stretch.
H. pylori atpE and the truncated variant were cloned into the shuttle vector pHel 3 with a kanamycin resistance marker, which allows expression of *atpE* genes. The genomic knockout was then performed by homologous recombination.
The first 26 amino acid residues were omitted from the *H. pylori atpE* gene. The resulting vectors expressing the full length *atpE* (M85.28) and the truncated variant (M85.42), were used for conjugation in two different *H. pylori* strains: 69A and G1.1. The results of a representative conjugation after selection on kanamycin plates are shown in Table 3.1 below.

	M85.28 Full length	M85.42 truncated	Control pHel2
Strain 69A	12	7	204
Strain G1.1	0	2	112

Table 3.8: The number of colonies for a conjugation of M85.28 and M85.42 in *H. pylori* strain 69A and G1.1 is shown. The empty vector pHel 2 was used as control.

Subsequently, the knockout was performed using the constructs for homologous recombination that contained the chloramphenicol-acetyl-transferase without (M64.3) and with promoter (M64.14). The plasmids were introduced into *H. pylori* by transformation.
Few transformants were received and stored in glycerol stocks after propagation. When the clones were replated for analysis, only strain 69A with the truncated variant of *H.*

pylori atpE and subsequent knockout of *atpE* with cat and cat_{GC} would grow. Six clones were analysed by genomic DNA preparation.

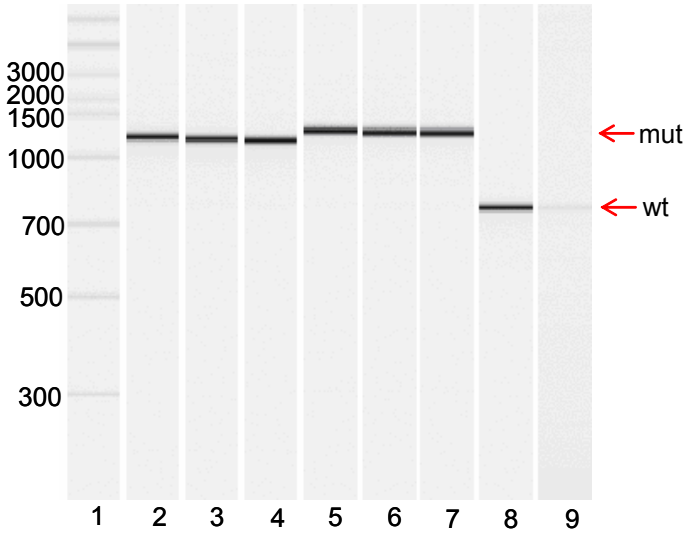

Figure 3.14: Analysis of the potential mutants expressing a truncated variant of *H. pylori atpE* under control of the *flaA* promoter from the plasmid pHel 3 (69A M85.42 M64.3 K1-3 and 69A M85.42 M64.14 K1-3, lane 2-7) compared to the wild type sequence of strain 69A (lane 8). The mutants in lane 2-4, the cat gene without promoter is present, in lane 5-7, the cat gene with promoter is present. The size difference of 400 base pairs to the wild type sequence in lane 8 indicates that the knockout has taken place.

The size difference of the wild type sequence of *H. pylori atpE* and the knockout mutants amounts to 400 bases. This difference was detectable in the mutants analysed. The size difference of 100 base pairs of the constructs amplified in lanes 2-4 and 5-7 results from the presence of the chloramphenicol-acetyl-transferase without and with promoter. Substitution of the *H. pylori* wild type gene *atpE* with a truncated variant without the N-terminal stretch is possible. Surprisingly, the mutants expressing the wild type *atpE* sequence (M85.28) would not grow after freezing.

3.5.3 Expression of *E. coli atpE* in *H. pylori*

The insert for expression of *E. coli atpE* in *H. pylori* was constructed by crossover PCR. The *H. pylori flaA* promoter region and the *E. coli atpE* gene were amplified in two separate reactions and ligated in a third PCR reaction to yield *E. coli atpE* for expression in *H. pylori*.

The vector M59.33 was introduced into *H. pylori* by conjugation, resulting in 21 colonies in strain 69A of which four were further propagated. All three colonies resulting from conjugation in strain G1.1 were propagated.

Protein expression of *E. coli atpE* in *H. pylori* is shown in the Western blot in the Figure 3.15 below.

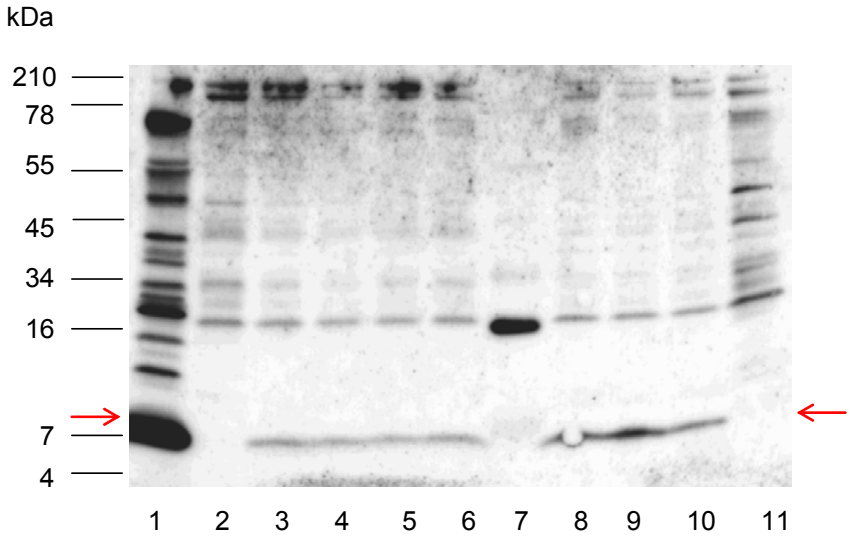

Figure 3.15: Western blot demonstrating the expression of *E. coli atpE* in *H. pylori* strains 69A and G1.1, using the polyclonal anti *E. coli* c subunit antibody (kindly provided by Prof. Altendorf). Lane 7 is the marker lane. Bacterial lysate of *E. coli* wild type HB 101 is analysed in lane 1 as control: the band above 7 kDa (8.2 kDa) is the *E. coli* c subunit, in lane 2 69A wild type with the corresponding mutants 69A M59.33 K1-K4 in lane 3 to 6. G1.1 wild type is analysed in lane 11 with the corresponding mutants G1.1 M59.33 K1-K3 in lane 8-10. No *E. coli* c subunit was detected in 69A and G1.1 wild type in contrast to the mutants expressing the *E. coli atpE*.

The expression of the *E. coli* c subunit in *H. pylori* was clearly demonstrated with a specific antibody for the *E. coli* c subunit. The antibody did not recognize a protein of the corresponding size in the *H. pylori* wild type lysates (Figure 3.15, lane 2 and 11). *E. coli atpE* was detected in the bacterial lysate of the mutants (band above 7kD in lane 3-6 and 8-10.

Subsequently, knockout of *H. pylori atpE* in the strains expressing *E. coli atpE* was attempted. These strains were transformed with the constructs M64.3 and M64.13, which allow replacement of *H. pylori atpE* with the chloramphenicol-acetyl-transferase, by homologous recombination. Genomic DNA was isolated and examined by PCR. The expected PCR product of the wild type sequence has roughly 800 base pairs whereas the mutated sequence with cat or cat_{GC} has roughly 1200 base pairs and 1300 base pairs respectively. The PCR products were analysed with the Bioanalyzer (Agilent), the results are displayed in Figure 3.16 below:

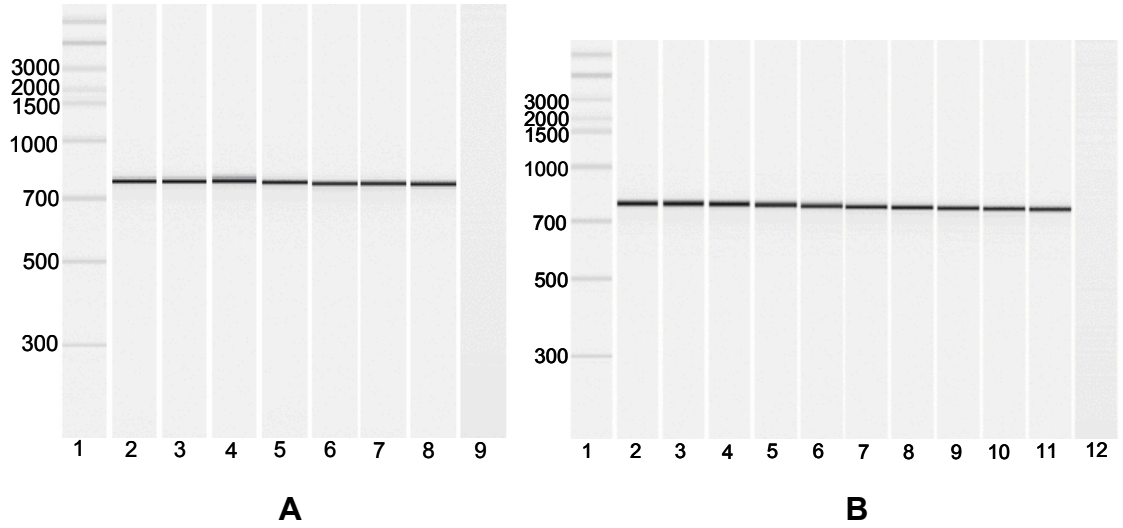

Figure 3.16: Strain 69A harbouring the plasmid M59.33, from which *E. coli atpE* is expressed. Subsequently, the knockout of *H. pylori atpE* was attempted on genome level with the constructs M64.3 and M64.14 that should replace *H. pylori atpE* by a chloramphenicol-resistance gene without and with promoter. In figure A, the plasmid M64.3 was used for knockout. Genomic DNA was prepared from the resistant colonies and analysed with the primers 5´atpEs and 3´atpEas. The wild type genomic DNA was used as a control (lane 8) and no size difference was detected. Therefore knockout has not taken place. The same observation can be made in figure B, where the plasmid M64.14 was used for knockout, the control with the wild type DNA being in lane 11. Equally, no size difference was observed.

The same strategy was performed with strain G1.1. Resistant colonies were amplified and genomic DNA was prepared. The results of the PCR analysis are shown in Figure 3.17.

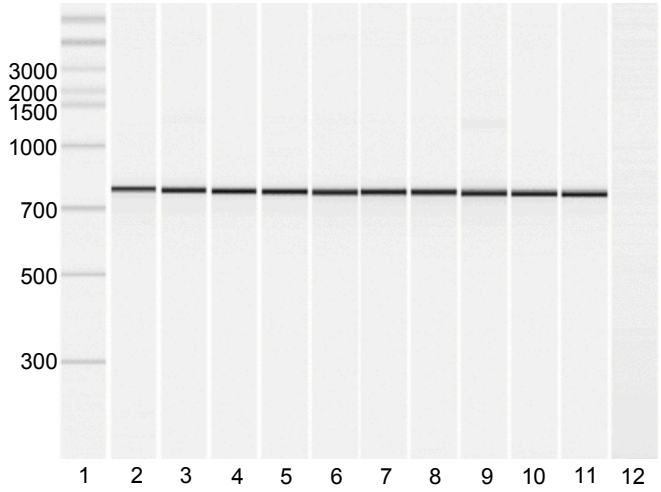

Figure 3.17: Analysis of the PCR products, testing genomic DNA for the potential knockout of *atpE* and replacement by cat_{GC}. The control with G1.1 wild type genomic DNA is shown in lane 11. Again, no size difference of the potential mutants and the wild type was detected. In lane 12, the no template control is shown.

The mutated sequence, which is around 400 base pairs longer in size than the wild type sequence, was not detectable in any of the clones analysed (Figure 3.16 and Figure 3.17).

Despite the expression of *E. coli atpE*, as shown by Western blot in Figure 3.15, the knockout of *H. pylori atpE* was not possible.

To test whether the N-terminal sequence of *H. pylori atpE* was responsible for this effect, *E. coli atpE* with the additional N-terminal sequence from *H. pylori atpE* was constructed for homologous recombination (M75.28). The first 26 amino acid residues were selected: MKFLALFFLALVGVAFAHDGGMGGD.

Twelve clones in strain G1.1 were received after transformation of plasmid M75.28 with efficient controls. From eight glycerol stocks, five clones were recovered and genomic DNA was prepared. The DNA was analysed by PCR.

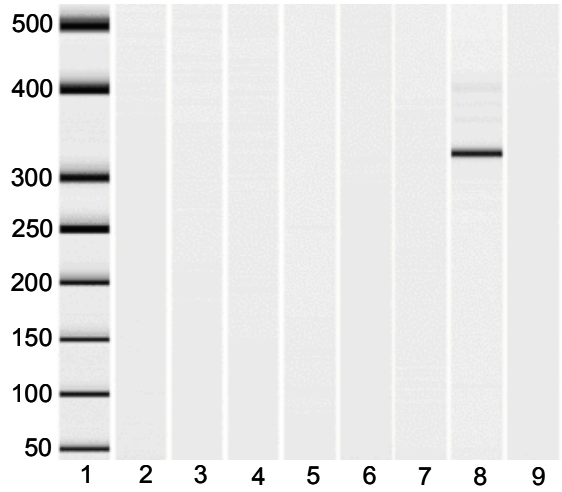

Figure 3.18: Analysis of the potential mutants expressing *E. coli atpE* with the N-terminus from *H. pylori atpE*. Mutants were analysed by PCR with the primers atpEs und popacat-Ec atpE. In lane 8, the plasmid M75.28 was used as a template showing that the primers can be used together to generate a PCR product of about 350 base pairs.

No mutants were detected. The exchange of the wild type *H. pylori atpE* sequence against *E. coli atpE* with the *H. pylori* N-terminus by homologous recombination was not possible.

3.5.4 Generation of the histidine mutants

Charged amino acid residues are susceptible to modification under varying pH conditions. Histidines were shown to be involved in pH dependent gating of the urea channel UreI. Thus, the histidine residue in the periplasmic loop of the c subunit was was exchanged against glycine to identify a possible role in acid resistance.

The plasmid M84.35 contains the wild type *atpE* sequence followed by the chloramphenicol-acetyl-transferase gene for selection and the 3´ region after the *atpE* gene to accomplish homologous recombination.

The base pairs CAT, which code for histidine were replaced by GGG. This triplet has the highest codon usage for glycine in *H. pylori*. The resulting mutated plasmid M89.25 was used for homologous recombination in three different *H. pylori* strains: 69A, 26695 and G1.1.

Colonies were detected after four days in strain 69A. Eight clones, His1 to His8, were propagated and the mutation was analysed by TaqMan PCR. Two different reporter dyes were used for the probes. A vic-labeled probe was designed to identify the mutants whereas a fam-labeled probe bound to the wild type sequence.

In Figure 3.19 and Figure 3.20 below the results of the TaqMan PCR with the vic- and fam-labeled probe for His1 to His8 and the controls are shown. The ΔRn value on the y-axis visualizes the fluorescent signal and is calculated as the difference of the normalised reporter signal less the reporter signal before the PCR. The number of cycles is indicated on the x-axis. The Ct value is the number of cycles where the cycle threshold is first exceeded. The cycle threshold is defined as 10 times the standard deviation of the background fluorescence, e.g. by the plastic of the microtiter plate or the optical devices, measured between cycle 3 and 15.

In Figure 3.19, the results with the vic-labeled probe are displayed. The first triplicate of curves shows the amplification of the mutant sequence in the plasmid M89.25. The Ct value of 18.77 signifies that a significant signal is detected after 18 cycles. The low Ct value indicates that the template is a purified plasmid. In the next set of six curves the mutation in the 69A His1 and His5 mutant is amplified. The Ct values of about 21 indicate that a few more cycles are needed for amplification. The third set of curves shows that 69A His3, 4, 7 and 8 also contain the mutation. No amplification was detected when the template was 69A wild type genomic DNA or the potential mutant 69A His2 and 7 were tested. Six out of eight potential mutants were positive for the mutation and negative when assayed with the wild type probe (Figure 3.20). Therefore, His1, 3, 4, 5, 7 and 8 are real mutants.

The analysis of the potential histidine mutants is shown in Figure 3.19.

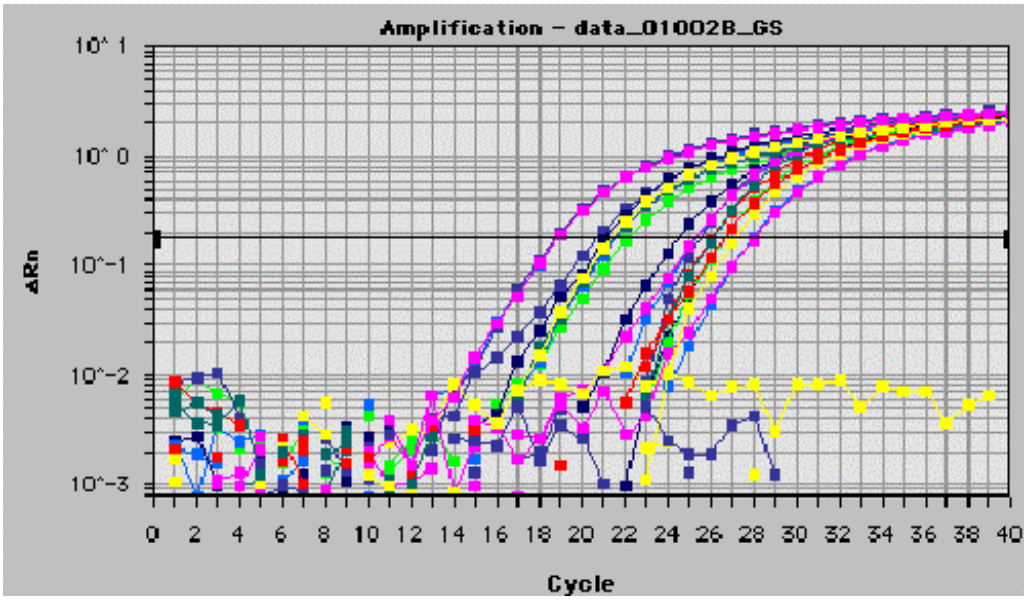

Figure 3.19: TaqMan analysis of the histidine mutants with the vic-labeled probe. The vic-labeled probe detects the mutated *atpE* sequence. The mutation was detected in M89.25, His1, 3, 4, 5, 7 and 8.

In Figure 3.20, the results from the analysis with the fam-labeled probe are shown, which detects the wild type sequence.

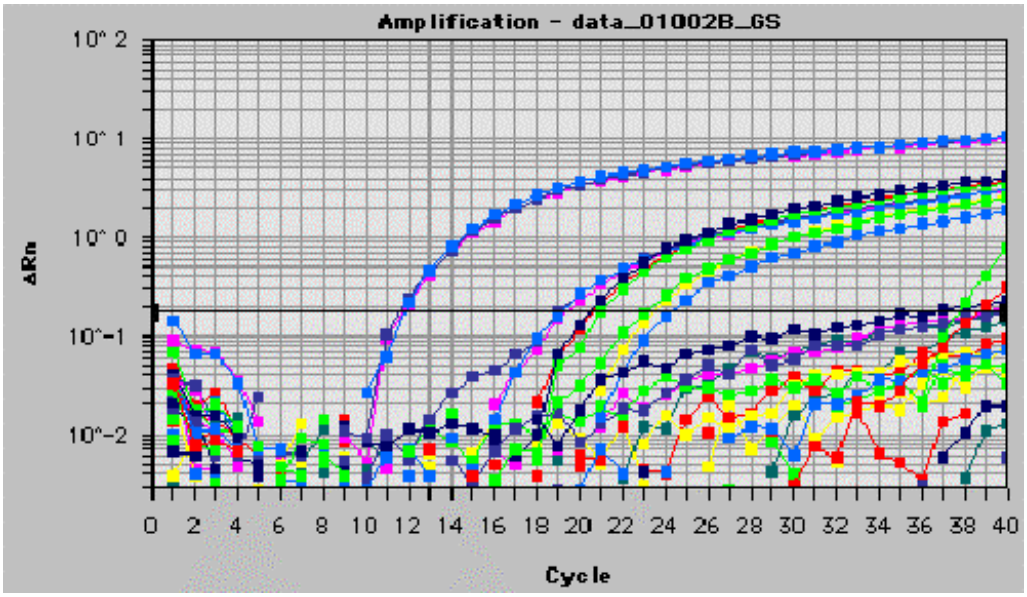

Figure 3.20: Result of the TaqMan PCR identifying the histidine mutants. The number of PCR cycles is shown on the x-axis. On the y axis the fluorescent signal is applied as the ΔRn value. Amplification of the wild type DNA was obtained when using wild type genomic DNA (first set of curves) and the mutant plasmid M84.35 (last set of curves). His2 and His6 also contained the wild type sequence (third and second set of curves).

In Figure 3.20, a fluorescent signal with the fam-labeled probe was detected in genomic DNA, the plasmid M84.35, His2 and His6

In the table below, the Ct values with the vic- and fam-labeled probes and all samples are summarized:

sample	Ct value (vic-labeled probe)	Ct value (fam-labeled probe)
No template	40	40
M84.35	40	12.1 +/- 0.05
M89.25	18.77 +/- 0.026	40
gDNA wt	40	23.99 +/- 0.53
His1	21.57 +/- 0.37	40
His2	40	20.95 +/- 0.26
His3	26.55 +/- 0.04	40
His4	27.62 +/- 0.49	40
His5	21.0 +/- 0.36	40
His6	40	19.64 +/- 0.16
His7	25.3 +/- 0.09	40
His8	25.48 +/- 0.93	40

Table 3.9: Summary of the Ct values for analysis of the potential histidine mutants. In the first column the results with the vic-labeled probe, which detects the mutant sequence, are shown. The mutation was detected in M89.25, His1, 3, 4, 5, 7, and 8. In the second column, the results with the fam-labeled probe are listed, and the wild type sequence was detected in M84.35, genomic wild type DNA (gDNA) and His2 and 6.

Expression of the mutated c subunit was verified by Western blotting. The polyclonal antibody anti-HP c subunit I was generated against the *H. pylori* N-terminal sequence, amino acids alanine 17 to tyrosine 31 (Neosystems).

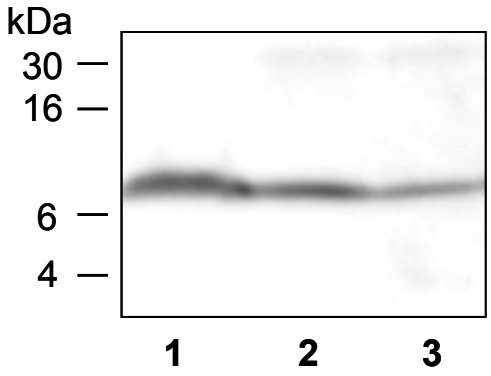

Figure 3.21: In lane 1 to 3, bacterial lysates of *E. coli* HB 101, *H. pylori* 69A wild type and 69A His1 were separated on a 10-20% Tricine gel (Novex). The c subunit was detected with a custom made polyclonal antibody against the *H. pylori* c subunit (Neosystems). The molecular weight of the *E. coli* c subunit is 8.2 kDa; the molecular weight of the *H. pylori* c subunit is 10.7 kDa.

The two clones 69A His1 and His3 as detected in lane 2 and 3 express the mutated c subunit. Unfortunately, the polyclonal antibody also recognizes the *E. coli* c subunit in lane 1. It was not possible to generate a species-specific antibody against the unique N-

terminus because N-terminal sequence was not usable as immunizing peptide due to its hydrophobicity.

3.5.5 Analysis of the histidine mutants

His mutants were analysed in a cytosensor (Molecular Devices) to detect differences in pH regulation. The pH of the surrounding medium of bacteria, which are trapped in microflow chambers can be measured with a highly sensitive pH sensor. Urease activity produces NH_3 and CO_2 with alkalinization of the medium, therefore monitoring medium pH enables assessment of urease activity. If the exchange of histidine against glycine alters the H^+-influx in the cells, urease activity might also be altered.

The cytosensor experiments were carried out with strain 69A wild type and the mutant 69A His1. The cells were incubated in BSSgg, a weakly buffered salt medium, with gradually decreasing pH, starting from pH 7.4, then down from pH 5.5 to pH 2.5 with 2.5 mM urea. Each incubation lasted for 30 minutes before decreasing in 0.5 pH units. This experiment is called shift down (Rektorschek *et al*., 1998) and lasts for 3.5 hours. The viability of the cells was measured after the experiment but no difference between the wild type bacteria and the His1 mutants could be detected.

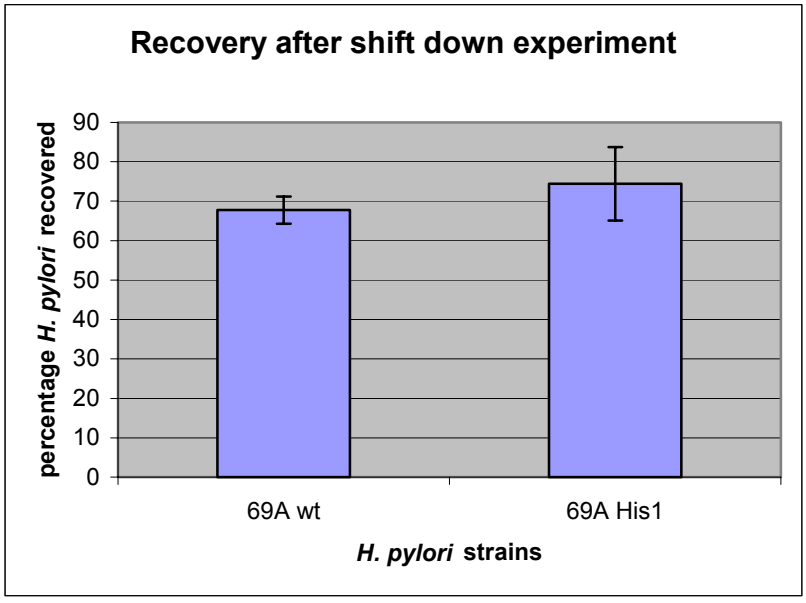

Figure 3.22: The recovery after the shift down experiment was measured. No significant difference between the wild type bacteria and the His1 mutants was detected.

Both strains, the 69A wild type and the His1 mutant, show a recovery rate of approximately 70% after the shift down assay.

The results from the shift down experiment are shown in Figure 3.23 below. The pH of the flow through medium is indexed on the x-axis. The pH of the perfusate is shown on the y-axis. The characteristics of strain 69A wild type (brown curve) and 69A His1 (blue curve) during pH challenge from pH 7.4 down to pH 2.5 with 2.5mM urea are shown.

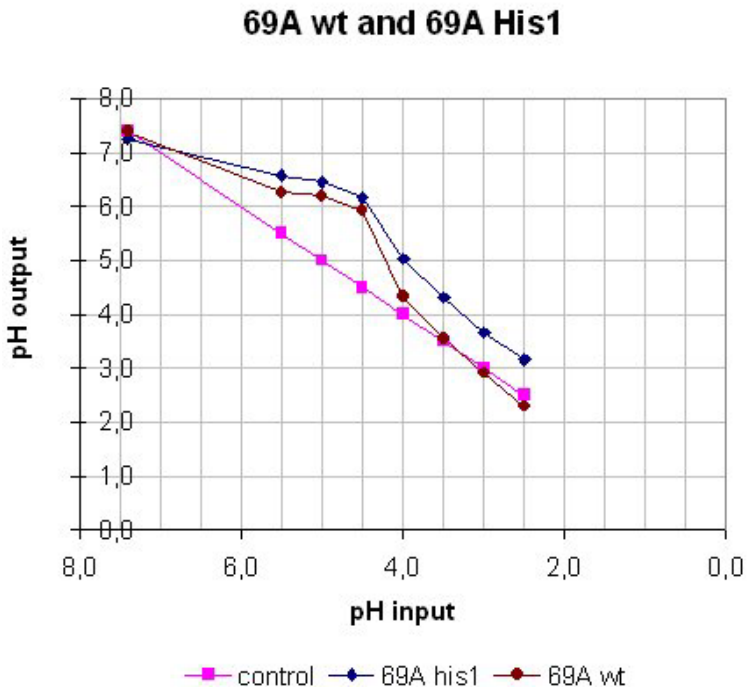

Figure 3.23: Results of the Cytosensor experiments (shift down). Each value represents the mean of eight experiments.

The brown curve shows the behaviour of strain 69A wild type in the shift down experiment with 2.5 mM urea. The bacteria incubated in BSSgg without urea (pink curve) are unable to elevate the chamber pH. When urea is present, the potent urease stabilizes the chamber pH at pH 6.2 until the perfusion pH reaches 4.5. Below pH 4.5 the urease is not able to elevate the medium pH significantly and the pH remains at the input value as is also the case for the control (pink curve). In contrast, the 69A His1 mutant (blue curve) is able to keep the pH above the input value, even below the input pH of 4.5. The medium pH value can be elevated one pH unit above the input value.

4 Discussion

The importance of *H. pylori* for the development of gastroduodenal disease was rediscovered in 1983 (Warren and Marshall, 1983) and led to an enormous increase of research on *H. pylori*. The aim is today to understand the mechanisms that allow *H. pylori* to survive and persist in the inhospitable environment of the acidic human stomach where the luminal pH can drop as low as 1. *H. pylori* does not only have survive the transit through the stomach but is also able to colonize the gastric mucosa.

Several virulence factors have already been identified that influence the severity of the infection. These include motility, lipopolysaccharides, adhesins, the pathogenicity island and mechanisms for acid survival. Acid survival is most importantly influenced by the enzyme urease, which amounts to 5-10% of the total protein (Bauernfeind *et al*., 1997). Urease-deficient mutants are not able to colonize the gastric mucosa in various animal models (Mobley, 1996; Eaton and Krakowka, 1994; Eaton *et al*., 1991). Hydrolysis of urea to carbon dioxide and ammonia buffers acidic pH. Ammonia immediately reacts to ammonium in an acidic environment, therefore neutralizing H^+ ions, but whether the buffered compartment is the cytoplasm or the periplasm is still being discussed controversially (Stingl *et al*., 2001; Sachs *et al*., 2002).

The F_1F_0-ATP synthase is also involved in H^+-metabolism but its role in pathogenicity and acid survival in *H. pylori* remains to be clarified. The multisubunit enzyme plays a central role in free energy metabolism in all living cells and has been found in the cytoplasmic membrane of bacteria, as well as in the thylakoid membrane of chloroplasts and the inner membrane of mitochondria (Senior, 1988). Depending on physiological conditions, ATP is synthesized when H^+ enters the cell through the F_0 proton channel of the F_1F_0-ATPase along an electrochemical H^+ gradient. On the other hand, H^+ can be exported at the expense of ATP. At first sight, the universality of the enzyme might seem to preclude the possibility to be an interesting drug target. However, the *H. pylori* F_1F_0-ATPase exhibits some unique features compared to most other organisms.

The overall structure is similar in all F_1F_0-ATPases: F_1 is the catalytic entity composed of the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ (Walker, 1998). F_0 is the membrane-spanning domain consisting of subunits a, b and c with a stochiometry of 1:2:10-14 (Fillingame *et al*.,

2000). F_0 functions as a proton channel whereas F_1 exhibits ATPase activity when separated by a mild detergent. The stochiometry of the c subunit remains controversial and might be species-specific (Stock *et al*., 1999, Seelert *et al*., 2000) or an experimental artifact (Pedersen *et al*., 2000).

In organisms with a respiratory chain, such as *E. coli* and *B. subtilis*, the primary function of the enzyme is the synthesis of ATP driven by the proton gradient across the cytoplasmic membrane. The proton gradient results from respiration in the presence of an electron acceptor. In contrast, in organisms lacking a respiratory chain, or in the absence of electron acceptors, the enzyme hydrolyses ATP. The hydrolysis of ATP supplies the energy to pump protons out of the cell thereby leading to a transmembrane proton gradient and regulating cytoplasmic pH homeostasis.

The F_1F_0-ATPase constitutes a rotary motor and subunits are also classified according to their location in the rotor or stator part. The rotor comprises 10-14 c subunits that are bound to subunits γ and ε. Subunits γ and ε transmit the rotary energy to $\alpha_3\beta_3$ where ATP is synthesized in three steps. The stalk consists of subunits ab_2 and δ. The actual H^+ transport occurs between the fixed a subunit and the rotating complex of the several c subunits.

In this study, the gene a*tpE*, which encodes the c subunit of the F_1F_0-ATPase, was chosen for analysis because it is part of the proton-conducting channel. The proton channel lies between subunit a and c and the regulation of proton conductance might play a role in cytoplasmic pH regulation, beside a role in ATP synthesis, functions in cytoplasmic pH homeostasis. Since the pH even of the periplasmic pH can fall to acidic values, the function of the *H. pylori* enzyme must have been adapted to the varying acidity of the microorganism´s environment. Also, specific structural features were identified that distinguish the *H. pylori* c subunit from other c subunits.

4.1 Analysis of the gene *atpE* and its protein - the c subunit

The F_1F_0-ATPase is a very conserved enzyme among species (Deckers-Hebestreit and Altendorf, 1996; Dimroth, 1997; Weber and Senior, 1997). Along with sequencing efforts, many ATPase-operons have been identified. Two *H. pylori* genomes have been sequenced and are available for comparison (Tomb *et al*., 1997; Hancock *et al*., 1998; Alm *et al*., 1999). Generally, the genes for the eight subunits are present in the

following order: *atpIBEFHAGDC*. The function of *atpI* is still unclear. The structure of the operon is conserved among many bacteria although the order of genes in the genome for the F_0 subunit (*atpBEF*) is sometimes changed to *atpEBF*, e.g. in *Streptococcus pneumoniae* (Martin-Galiano *et al*., 2001) or *Lactococcus lactis* (Koebmann *et al*., 2000).

The *H. pylori* operon deviates from this well-known structure. The genes for the F_0 subunit are arranged in a different way (Figure 3.1). First of all, there are two homologous genes coding for the b subunit, *atpF* and *atpF´*. Two b subunits form part of the stalk that connects the F_0 and F_1 subunits. A duplication of the gene, e. g. for adaptation of gene expression, is conceivable. Knockout mutagenesis has shown that neither *atpF* nor *atpF´* can be eliminated (T. Schmidt-Petri, unpublished results). This result indicates that both subunits might have distinct functions that cannot be replaced by the other gene. Such a diverged and duplicated form of the b subunit is also found in plants and photosynthetic bacteria (Kelly *et al*., 2001).

Secondly, the genes *atpB* and *atpE*, which encode the subunits a and c, do not form part of the *atp* operon in *H. pylori*. *atpE* is found 89kb upstream and *atpB* is found 319kb downstream of the operon in *H. pylori* 26695 (as detailed in Figure 3.1). Subunit a (encoded by *atpB*) and at least 10 copies of subunit c (encoded by *atpE*) form the inner membrane proton channel in *E. coli* (Deckers-Hebestreit and Altendorf, 1996).

H. pylori, also shown to be a neutralophilic bacterium (Meyer-Rosberg *et al*., 1996), cannot only survive but also thrives in the acidic environment of the human stomach. This might require adaptations in perhaps structure and regulation of the *H. pylori* F_1F_0-ATPase, especially the F_0 part. Subunits a and c are primarily involved in proton translocation and proton influx must probably be limited or at least regulated under highly acidic conditions. The regulation of the *atpB* and *atpE* gene separately from the operon can be a requirement for rapid and independent expression and hence enzyme activity and function.

The *atpE* DNA sequence of different *H. pylori* strains, 69A, 888 and G1.1, was determined and compared to the two publicly available sequences of strain 26695 and J99 (Tomb *et al*., 1997; Hancock *et al*., 1998; Alm *et al*., 1999). Strains 69A, 888 and G1.1 are frequently used in our laboratory for various analyses. Strain G1.1 is used in the gerbil animal model, whereas strain 69A and 888 are not able to infect the gerbil.

The aim was to identify strain specific differences in the *atpE* sequence (chapter 3.2) and two amino acid exchanges were identified when all strains were compared (Figure 3.2). In contrast to strain 26695, the other strains examined carry a valine at position 12 instead of an alanine. Both amino acid residues carry aliphatic side chains so that this exchange is not likely to result in variation of function when the pH varies. The second exchange was found in strain G1.1 when compared to strain 26695 and the other strains. Glycine at position 23 was exchanged against glutamic acid in strain G1.1. Glutamic acid has an acidic side chain with a typical pK of 4.07. This amino acid exchange is more interesting because the acidic side chain is susceptible to modification and might influence the mode of action of the c subunit under varying pH conditions. This speculation is substantiated by the observation that especially strain G1.1 shows infectivity in the gerbil model. Glutamic acid at position 23 could possibly play a role in this model. The results from the topology analysis show that residue 23 is part of the periplasmic loop between transmembrane domains 1 and 2 (see Figure 4.2), where regulation of enzyme activity might take place. Protonation of glutamic acid at low pH could result in conformational changes.

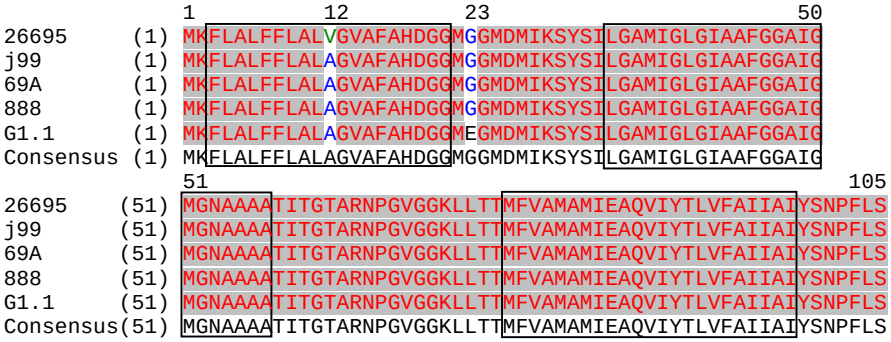

Figure 4.1: Comparison of the amino acid sequences of the c subunits of the different *H. pylori* strains. The boxes designate the first, second and third transmembrane domain. Glutamic acid in strain G1.1 is found in the periplasmic loop.

All other base pair exchanges only resulted in different codon usage but no patterns for codon usage between strains were recognized. A high degree of variability in DNA sequence was detected in *H. pylori* (Jiang *et al*., 1996). Every patient seems to have “his own” strain and genetic variability might also influence the outcome of disease.

The twelve base pairs upstream of the atg start codon of *atpE*, including the potential Shine-Dalgarno sequence, are identical in all strains. The regulation of the *atpE* gene therefore seems to be identical in all strains.

A potential Shine-Dalgarno sequence for ribosome binding, ggag, is present, varying slightly from the core sequence, ggagg, known for *E. coli* (Schurr *et al*., 1993). This core sequence is also valid for *H. pylori* (Ma *et al*., 2002). The spacing to the atg start codon amounts to seven base pairs compared to *E. coli* where optimal spacing ranges from eight to ten base pairs (Ringquist *et al*., 1992; Chen *et al*., 1994). Length and nucleotide composition of the spacer influence the activity of the ribosome binding site.
Correlations between the Shine-Dalgarno sequence and expected expression levels have been investigated recently by comparing 30 prokaryotic genomes (Ma *et al*., 2002) and showed that a strong Shine-Dalgarno sequence correlates with high gene expression.
The comparison of the amino acid sequence of the c subunits of several bacteria revealed that the *H. pylori*, *C. jejuni* and *U. ureaplasma* c subunits possess an elongated N-terminus of about 25 amino acids compared to the other bacteria (Figure 3.3). In all three organisms, there is a conserved alanine at position 17. In all *H. pylori* strains known so far, alanine 17 is followed by the two charged residues histidine and aspartatic acid (chapter 3.3). Charged residues are susceptible to modification in a varying pH environment. The typical pK of histidine is 6.04 and that of glutamic acid is 4.07 (Dawson *et al*., 1986). A histidine is also found in *U. urealyticum* at position 14.
No such residue is found in the N-terminus of *C. jejuni* and, in contrast to *H. pylori* and *U. urealyticum*, *C. jejuni* does not possess a urease or nickel transport system (Parkhill *et al*., 2000). The protonatable residues could therfore be involved in acid toleranc in urease containing organisms.
A helix-breaking motif with glycine, GGMGG, is found at positions 20 to 24 indicating the presence of an additional transmembrane domain.
In *H. pylori*, the N-terminal stretch is mostly hydrophobic except for the residues mentioned above. Analysis with SignalP suggested that it might be a signal sequence (signalP at www.cbs.dtu.dk, Nielsen *et al*., 1997, Figure 3.4) although the hydrophobicity can also indicate a transmembrane domain. The topology analysis confirms that the N-terminal stretch is a third transmembrane domain.

4.2 Topology of the c subunit

The topology of the c subunit was scrutinized because the computational analysis indicated either a signal sequence (Figure 3.4) or a third transmembrane domain (Table 3.2) for the additional N-terminal region of the *H. pylori* c subunit compared to the other bacteria (Figure 3.3). The first approach to identify transmembrane regions was the computational analysis of the amino acid sequence. The availability of various algorithms for the prediction of hydrophobic and transmembrane domains allows the comparison and interpretation of the different predictions as shown in Table 3.2. The predictions have been ameliorated significantly in the last years by incorporating data from crystallization studies but the different results from the analyses emphasize the necessity for experimental data (Table 3.2).

The structural analysis of membrane proteins is difficult because reconstitution into a native conformation is nearly impossible and the use of membrane extracts and solvents hinder the analysis. Several other methods are available for the experimental topology analysis of proteins and two different methods were chosen to investigate the presence and the orientation of the transmembrane domains of the *H. pylori* c subunit: the *in vitro* topology analysis with microsomal membranes and the generation of fusion proteins with alkaline phosphatase.

Three hydrophobic domains were identified with the Kyte and Doolittle algorithm (Figure 3.6). Hydrophobicity is only one indication for transmembrane domains because hydrophobic domains can also be embedded inside a hydrophilic region of a protein. The membrane topology is determined by information in the membrane integrating hydrophobic domain and the flanking sequence (Wickner and Lodish, 1985). Sequence analysis of several proteins revealed that positive charges influence the orientation of the membrane domain (von Heijne and Gavel, 1988). Positive charges before a hydrophobic segment direct the N-terminus of this segment to remain in the cytoplasm. The N-terminus is found in the periplasm when the hydrophobic domain is followed by a positive amino acid residue. This finding was also substantiated by the observation that arginine and lysine residues are found more often in cytoplasmic loops than in periplasmic loops (von Heijne, 1986).

Specific programmes for the prediction of transmembrane domains are also available and some were used to elucidate the transmembrane domains of *H. pylori atpE* (Table

3.2). They do not only rely on hydrophobic scores but take other parameters into account: the flanking region of the hydrophobic domain and information about other proteins with related domains.

In contrast to *E. coli* and most other bacteria, where two transmembrane domains for the c subunit are present (Deckers-Hebestreit and Altendorf, 1996), three transmembrane domains are predicted in the *H. pylori atpE* sequence of strain 26695 and G1.1 with only one exception (see Table 3.2).

The various DNA fragments of the *H. pylori atpE* gene for the topology analysis were chosen according to the predictions and the "positive inside" rule (von Heijne, 1986).

The *in vitro* topology analysis with the M0/M1 vector system has already been used for the analysis of membrane topology for a *H. pylori* protein, the P-type ATPase 439 and CopA (Melchers *et al*., 1996, Bayle *et al*., 1998). Originally used for the analysis of the gastric H^+/K^+-ATPase (Bamberg and Sachs, 1994) and the mammalian Ca^{2+}-ATPase (Bayle *et al*., 1995), the system proved valid also for *H. pylori* membrane proteins. Membrane insertion is detected by glycosylation of the c-terminal region in the presence of microsomal membranes.

Four DNA fragments of *H. pylori atpE* containing the potential transmembrane segments, H1, H2, an elongated version of the second transmembrane domain H2* or H3, were cloned into the M0 and M1 vector. In an *in vitro* transcription and translation system with and without microsomal membranes, signal anchor and stop transfer activity of membrane domains can be investigated. It is also important to test the potential membrane domains together in the system because the context also carries information for folding and membrane insertion of the whole protein. H1 showed strong signal-anchor activity (Figure 3.8). Signal-anchor activity describes an internal signal for membrane insertion and membrane insertion and translocation of the C-terminal region was observed for H1. No stop transfer activity was detected for H1. H2 and H2* alone did neither show signal anchor activity nor stop transfer activity. Information of the other domains is probably necessary for correct membrane insertion of the second transmembrane domain.

Only very weak signal anchor activity could be detected for H3 (Figure 3.8). When individual domains were inserted into the M1 vector, which already contains a signal anchor domain, no stop transfer activity of any of the inserts could be detected.

Subsequently, the whole c subunit with domains H1 to H3 was expressed in the M0 vector and translocation of the c-terminus into the microsomes could be detected (Figure 3.10). The C-terminus of *H. pylori* c subunit is translocated into the periplasm and therefore the N-terminus is located in the cytoplasm. These experiments give evidence that all three potential domains are real transmembrane domains. The context of all domains is necessary to identify H2 as a stop transfer domain and H3 as a signal anchor domain.

In an additional approach, fusion proteins of the *H. pylori* c subunit at the N-terminus followed by alkaline phosphatase were used to test the potential membrane domains in an *in vivo* system in *E. coli*. Enzymatic activity of alkaline phosphatase is only detected when the enzyme is located in the periplasm where its disulfide bonds can be correctly formed (Derman and Beckwith, 1991).

Three constructs were generated for the *in vivo* topology analysis: the first potential transmembrane domain, the first and the second transmembrane domain, and the whole amino acid sequence of the *H. pylori* c subunit.

High enzyme activity was detected when the first potential transmembrane domain was expressed as part of the fusion protein (Table 3.5). Therefore, the alkaline phosphatase was properly folded in the periplasm, and the N-terminus of the c subunit remained in the cytoplasm. No activity was detected when the first two domains were expressed. When the whole c subunit was expressed as a fusion protein with alkaline phosphatase, enzyme activity was detected although the amount of protein was low (Figure 3.12). The C-terminus of the c subunit is transported to the periplasm and alkaline phosphatase is then folded into its active conformation. Therefore, the N-terminus is found in the cytoplasm.

Again, these experiments prove that the *H. pylori* c subunit contains three transmembrane domains with the N-terminus in the cytoplasm and the C-terminus in the periplasm. Both systems, the *in vitro* transcription and translation system as well as the *in vivo* analysis of fusion proteins with alkaline phosphatase are valid to examine the existence and orientation of potential transmembrane domains of bacterial proteins. This has also been illustrated by the topology analysis of the CadA-ATPase (Melchers *et al*., 1996) and CopA (Bayle *et al*., 1998). The topology of the *H. pylori* c subunit is illustrated in Figure 4.2:

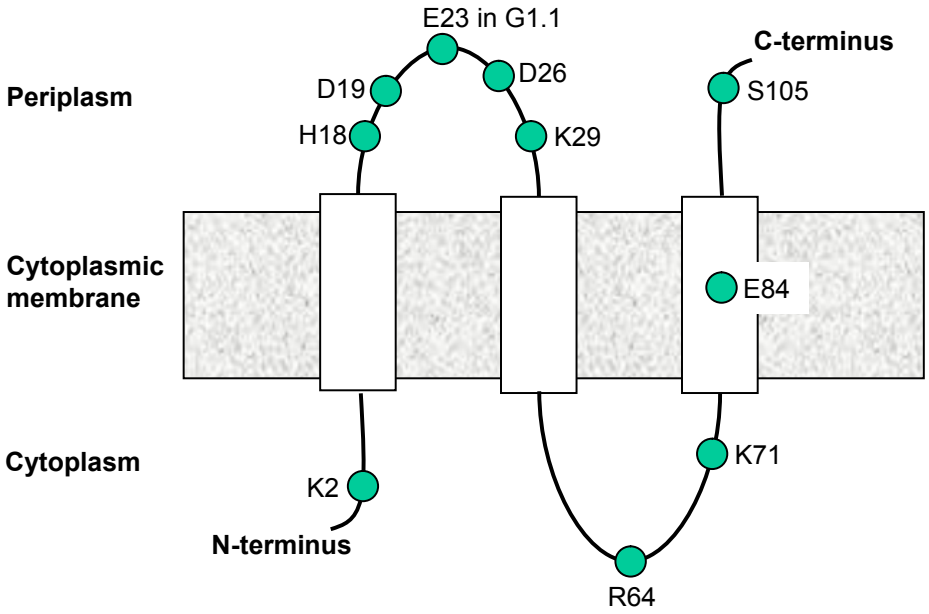

Figure 4.2: A model for the c subunit of *H. pylori* as derived from the experiments described in this work. Loaded residues and their likely position in the c subunit model are shown.

The proton flow is between the a subunit and the rotary motor of several c subunits. The third transmembrane domain containing glutamic acid at position 84 is probably oriented towards the a subunit because it has been shown that the equivalent aspartic acid at position 61 in *E. coli* is involved in proton translocation.

4.3 Analysis of mutants of the c subunit

Various mutants were generated to elucidate the function of the *H. pylori* c subunit, encoded by the *atpE* gene.

The arrangement of the different subunits in the F_1F_0-Type ATPase has been shown in various organisms by different methods (Abrahams *et al*., 1994, Stock *et al*., 1999). The proton channel lies at the interface between several c subunits arranged in a circle and subunit a (Vik *et al*., 2000). It is still unclear how many c subunits are involved and the quantity might be species-specific. The c subunits form a rotating multimer whereas the a subunit is embedded in the membrane with five or six transmembrane domains (Fillingame *et al*., 2000, Deckers-Hebestreit *et al*., 2000). Hydrophilic channels subunit a at the interface between subunit a and c allow the passage of protons or other cations from the periplasm to the cytoplasm. The mechanism of cation translocation is still

controversially discussed and two concepts for proton translocation exist: the one-channel theory (Dimroth *et al*., 1999) and the two-channel theory (Junge *et al*., 1997).

The one-channel theory predicts that a proton enters the interface between subunits a and c and is then bound to aspartatic acid at position 61 at the c subunit. Aspartic acid controls the flow of cations through the channel. The c subunit rotates by a small degree at the a-c interface due to interactions of several charged residues and the proton is released to the cytoplasm.

The two-channel theory predicts two half-channels in subunit a, one connected to the periplasm, the other open to the cytoplasm. The proton enters from the periplasm and the neutralised charge distribution induces a shift towards the membrane. The c subunit complex has to fulfil a rotation of nearly 360° before the proton is released into the cytoplasm. In *H. pylori*, the subunit a exhibits 4 to 5 transmembrane domains as shown by computational analysis (Tmpred, data not shown). The c subunit exhibits three transmembrane domains in contrast to the *E. coli* structure. Several residues shown to be involved in proton translocation in subunit a are also conserved in *H. pylori*: serine 206, arginine 210 asparagine 214, aspartic acid 219.

```
Ec subunit a   (1) MASENMTPQDYIGHHLNNLQLDLRTFSLVDPQNPPATFWTINIDSMFFSV
Hp subunit a   (1) --------------------MEHRVFTIANFFSSNHDFIT------GFFV
Consensus      (1)                     LD R FSI         F T       F V

Ec subunit a  (51) VLGLLFLVLFRSVAKKATSGVPGKFQTAIELVIGFVNGSVKDMYHGK-SK
Hp subunit a  (25) VLTAVLMFLISLGASRKMQMVPMGLQNVYESIISAILSVAKDIIGEELAR
Consensus     (51) VL  L L L    A K    VP   Q   E II  I    KDI     AK

Ec subunit a  100) LIAPLALTIFVWVFLMNLMDLLPIDLLPYIAEHVLGLPALRVVPSADVNV
Hp subunit a  (75) KYFPLAGTIALYVFFSNMIGIIP----------GF--ES----PTASWSF
Consensus    (101)    PLA TI LWVF  NLI IIP              A    PSA

Ec subunit a (150) TLSMALGVFILILFYSIKMKGIGGFTKELTLQPFNHWAFIPVNLILEGVS
Hp subunit a (109) TLVLALIVFFYYHFEGIRVQG---FFKYFAHFAGPVKWLAPFMFPIEIIS
Consensus    (151) TL LAL VF    F  IKM G   F K            P    IE IS

Ec subunit a (200) LLSKPVSLGLRLFGNMYAGELIFILIAGLLPWWSQWILNVPWAIFHILII
Hp subunit a (156) HFSRIVSLSFRLFGNIKGDDMFLLIMLLLVPW----AVPVAPFMVLFFMG
Consensus    (201)   SK VSL  RLFGNI A DL  III  LLPW     L V   I    I

Ec subunit a (250) TLQAFIFMVLTIVYLSMASEEH----
Hp subunit a (202) ILQAFVFMILTYVYLAGAVLTDEGH-
Consensus    (251)  LQAFIFMILT VYLA A
```

Figure 4.3: Comparison of the a subunits of *E. coli* and *H. pylori*. Some amino acid residues involved in proton translocation are marked with a box.

The similar organisation of subunit a does probably not influence the mechanism of proton translocation. On the other hand, an extra periplasmic loop of subunit c results from existence of a third transmembrane domain at the N-terminus. The periplasmic loop and the adjacent region contain several charged residues that could influence the

regulation of the *H. pylori* proton channel under varying conditions (Figure 3.3, Figure 4.2). Several mutants were generated to elucidate the function of the c subunit.

4.3.1 Knockout of *H. pylori atpE*

Knockout mutagenesis is an essential tool for the analysis of genes *in vitro* as well as *in vivo*. A gene can be essential *in vitro*, which complicates further analysis because loss-of-function analysis is not possible. On the other hand, if the gene is non-essential *in vitro*, manipulation is easily possible and it can still be important for infection in the animal model.

The knockout of *H. pylori atpE* was performed by homologous recombination in strains 69A, 888, 26695 and G1.1. A genetic construct for knockout mutagensis was created by crossover PCR with which the wild type sequence should be replaced by an antibiotic resistance cassette.

Very few antibiotic resistant colonies were detected in all strains (Table 3.7) and the analysed bacteria still contained the *atpE* wild type gene (Figure 3.13).

It was not possible to delete the *atpE* gene under standard conditions (chapter 3.5.1) although we have used this method successfully several times in our laboratory for the knockout of other genes in *H. pylori*. For example, UreI has been deleted successfully *in vitro* and was shown to be essential *in vivo* (Rektorschek *et al*., 2000 and 2003).

The gene *atpE* was classified as an essential gene for *H. pylori* under normal growth conditions on BHI-agar plates. Normal growth conditions were chosen because essentiality should be tested under standard conditions.

For *E. coli*, several mutants defective in *atp* genes have been described (Harold and Maloney, 1996). Defective mutants cannot be grown in succinate medium but can still grow aerobically when ATP is supplied by glycolysis and energy supplied by the citric acid cycle. However, they show lower growth yields (Fillingame, 1990).

The F_1F_0-ATPase is crucial for sufficient ATP energy supply in all living cells. The c subunit is essential for proton translocation therefore a knockout is impossible although it might be possible to isolate mutants with partially defective subunits. No other protein can substitute for this essential part of the enzyme and no other enzyme can substitute for the F_1F_0-ATPase.

It has also been shown that *atpD*, coding for the β subunits, was essential in *H. pylori* (McGowan *et al*., 1997).

4.3.2 Truncated *atpE*

The comparison of the peptide sequence of c subunits from several organisms revealed that the *H. pylori* c subunit has an elongated N-terminus compared to most other organisms (Figure 3.3). A similar elongation is found in the closely related *C. jejuni* and a different one in *U. urealyticum*. Therefore, it was tempting to try whether the F_1F_0-ATPase with a N-terminal truncated c subunit could be generated and could retain function. The first 26 amino acids were omitted to yield a truncated peptide that naturally starts with methionine.

An expression plasmid was constructed that contained the truncated *atpE* gene with the constitutive *flaA* promoter from *H. pylori*. The knockout of the genomic *atpE* copy was performed with constructs for homologous recombination.

After several attempts, the knockout on the genomic level was successful in strain 69A when tested with PCR (Table 3.8, Figure 3.14).

Apparently, the truncated c subunit expressed from the plasmid was able to retain a functioning F_1F_0-ATPase under standard growth conditions. The N-terminus and part of the periplasmic loop are not essential but a function for survival under acidic conditions cannot be ruled out. Further experiments are necessary to elucidate the role of the N-terminus under varying pH. Moreover, it would be very interesting to see whether these mutants are infectious in the gerbil animal model. Unfortunately, mutants were only received in strain 69A whereas mutants in strain G1.1, which is infectious in the animal model, could not be regrown after storage indicating instability of the mutants.

4.3.3 Expression of *E. coli atpE* in *H. pylori*

E. coli atpE was expressed from plasmid M59.33 in *H. pylori* strain 69A and G1.1 under control of the *flaA* promoter. The expression was shown on a Western blot with a polyclonal antibody against the *E. coli* c subunit (Figure 3.15). The knockout at the genomic level of the *atpE* wild type sequence was performed subsequently by homologous recombination.

No knockout of the *atpE* wild type sequence could be shown at the genomic level by PCR (Figure 3.16).

Despite the expression of the *E. coli atpE* gene, as shown by Western blot (Figure 3.15), it was not possible to delete the corresponding *atpE* gene in *H. pylori* expressing the *E. coli atpE* gene. Hence, the *E. coli atpE* gene cannot replace the function of the *H. pylori*

atpE gene. The amino acid sequences of *E. coli* and *H. pylori* share the general structure with the hydrophobic domains and few very conserved amino acids. The motif AR(Q/N)P is found in all analysed organisms. This motif is part of the cytoplasmic loop and is probably involved in binding of F_0 to F_1 (Birkenhäger *et al*., 1999). Strikingly, an asparagine (Q) is found in *H. pylori*, *C. jejuni* and *U. urealyticum* whereas glutamine (N) is found in the other organisms (Figure 3.3). This motif is found in the cytoplasmic loop between the two conserved transmembrane segments. Aspartic acid at position 61 is essential for function in *E. coli*, which can be blocked by binding of DCCD to aspartic acid at position 61. In all other organisms, glutamic acid is found at this position. This might be one reason why *E. coli atpE* cannot substitute for *H. pylori atpE*.

4.3.4 *E. coli atpE* with *H. pylori* N-terminus

The unique N-terminus could be important for function of the c subunit in *H. pylori*, although in strain 69A few mutants with a truncated *atpE* were received (chapter 3.5.2). Therefore, a mutant was constructed that contained the *E. coli atpE* sequence preceded by the *H. pylori* N-terminal sequence. The first 26 amino acids of the *H. pylori* subunit c were selected including the GGMGG helix-breaking sequence. The sequences of the *H. pylori* N-terminus and *E. coli atpE* were annealed by crossover PCR so that no additional restriction enzyme sequences were necessary to fuse the two sequences. A fragment for homologous recombination was constructed and inserted into a suicide vector.

The construct could not be amplified from genomic DNA of the potential *H. pylori* mutants when analysed by PCR (Figure 3.18) after transformation.

The little number of mutants in strain G1.1 only already indicated that the exchange with this construct was not easy to achieve. Only five clones from eight glycerol stocks were recovered, which is unusual, and they did not contain the desired fragment (Figure 3.18).

It is not the N-terminus, which is essential for the assembly of the F_1F_0-ATPase but the last two transmembrane domains. This conclusion can also be drawn from the experiments with the truncated *atpE* (chapter 3.5.2). However, it must be noted that only few mutants with the truncated *atpE* gene in strain 69A were recovered. The fusion protein with *E. coli atpE* cannot assemble in the right manner although *E. coli atpE* is

expressed and recognized by a specific antibody (Figure 3.15). The second and third transmembrane domain of *H. pylori atpE* must possess features important for protein assembly that are not existant in *E. coli*. The N-terminal region is probably only involved in acid resistance.

4.4 Histidine 18 – its role in acid survival

Histidine amino acid residues in the urea channel UreI were shown to play an essential role in the pH regulation of this channel (Weeks and Sachs, 2001). A histidine residue is found in the *H. pylori* c subunit at position 18. The topology analysis revealed that the N-terminus of the c subunit is located in the cytoplasm (chapter 3.4, model in Figure 4.2). Histidine 18 will therefore be part of the periplasmic loop or at least lie at the border of membrane and periplasm (Figure 3.3). Histidine has an average pK of 6.04. At this pH, half maximal urea uptake in *Xenopus* oocytes and half maximal intrabacterial urease activity is observed. The median diurnal pH in the human stomach is 1.4 (Teyssen *et al*., 1995) but might be significantly higher at the mucosal surface (Coskun *et al*., 2001; Schade *et al*., 1994), so that a histidine could have an influence in regulating F_1F_0-ATPase activity. At pH values below the pK, the histidine residue should be predominantly protonated and the degree of protonation might influence the working mechanism of the c subunit in *H. pylori*. The behaviour of the wild type and the His-mutant was analysed in so-called shift-down experiments where the pH was attenuated in 0.5 pH units from pH 7.2 to 2.5 (chapter 3.5.5). Each incubation lasted for 30 minutes and the behaviour of the bacteria was monitored with a cytosensor. The cytosensor allows measurement of the rate at which the bacteria acidify or alkalinize the weakly buffered medium. The wild type bacteria and the His1 mutants were able to elevate the pH to about pH 6.2 of the perfusion chamber as the pH was decreased from pH 6 to pH 4.5. This has also been shown previously (Rektorschek *et al*., 1998). Below pH 4.5, the wild type and the His1 mutant show a different behaviour. Whereas the His1-mutant is still able to elevate the outer pH about one unit above the input pH, the wild type bacteria do not significantly elevate the chamber pH above the input pH (Figure 3.23). This indicates that the His1 mutant, where histidine 18 is exchanged against glycine perhaps has an on control of periplasmic pH. The regulation of the c subunit is changed when histidine 18 is exchanged against glycine. Glycine has only a

hydrogen atom as its side chain, whereas histidine possesses an imidazole ring as side chain.

The exchange of these amino acids might provoke a conformational change in the periplasmic region that probably also influences all transmembrane domains.

The F_1F_0-ATPase could have the following role in *H. pylori*: at close to neutral pH, the F_1F_0-ATPase functions as ATP-Synthase. At lower periplasmic pH, also resulting in lower cytoplasmic pH due to H^+ leakage, it regulates cytoplasmic pH by extrusion of protons. In the His1-mutant, the proton extrusion is perhaps partly impaired which results in lower cytoplasmic pH. Urease has maximal activity at pH 5 in sodium citrate buffer (Stingl *et al*., 2001) and a high urease activity results in buffering of the cytoplasm and also the periplasm by extrusion of NH_3. McGowan *et al*. (1997) found that *H. pylori* survival at pH 4 and 5 was not inhibited when the F_1F_0-ATPase was blocked with DCCD whereas survival was decreased at pH values close to neutral, pH 6 and 7. DCCD had no effect on survival of *E. coli* at any of these pH values, therefore substantiating the finding that *atp* mutants have been generated in *E. coli*. In contrast, this was not possible for *H. pylori* (this work, McGowan *et al*., 1997). Apparently, *H. pylori* is dependent on the F_1F_0-ATPase at pH values close to neutral.

The mutants are viable at low pH as are the wild type bacteria because the recovery rates are the same for the 69A wild type bacteria and the His1-mutants after the whole experiment (Figure 3.22).

On the other hand, the binding of H^+ to the histidine residue could also regulate the speed of rotation of the rotor of the different c subunits. The more H^+ are bound, depending on pH, the faster the motor and more ATP is generated. The incoming H^+ react with NH_3 that is produced by the highly active urease therefore neutralizing acidity in the cytoplasm and periplasm. In the mutant, more H^+ enter the cells therefore inducing a higher urease acitivity with higher buffering capacity. Probably at very low pH values, the urease will become inactive as the cytoplasm acidifies too much.

ATP is the energy currency in the cell but cannot be used as a storage for energy. ATP is needed for biosynthesis of metabolites, transport processes and motility, chemotaxis. Therefore, *H. pylori* might need more ATP in an acidic environment to sense the acidity and to move away to a more favourable niche.

4.5 Conclusion

The aim of this work was to analyse the topology and function of the c subunit of the *H. pylori* F_1F_0-ATPase.

It has been shown that the *H. pylori* c subunit possesses three transmembrane domains in contrast to most other organisms where only two transmembrane domains are present. Essentiality of the *atpE* gene in *H. pylori* was shown by knockout mutagenesis, emphasizing the importance of this subunit for the functioning of the entire enzyme.

The *E. coli atpE* gene cannot substitute for the *H. pylori atpE* although it was possible to express the *E. coli* protein in *H. pylori*. This might be a structural problem because only two transmembrane segments are present in *E. coli*. The expression of a fusion protein of *E. coli atpE* with the *H. pylori* N-terminus instead of *H. pylori atpE* was equally not possible, probably due to variations in protein folding, which influence the association of the several c subunits that form the c subunit rotor.

Expression of the truncated *atpE* gene in *H. pylori* was only observed in few mutants and only in *H. pylori* strain 69A. Therefore, the strain specific genetic background might have influenced this outcome and the N-terminus is nevertheless likely to be essential for *H. pylori* in the acidic human stomach.

Protonatable residues in the periplasmic loop, especially a histidine residue at position 18, were identified in the *H. pylori* c subunit and might have a function in survival at low pH values.

These unique features of the *H. pylori* c subunit emphasize that further analysis of this interesting target would help to understand its role for acid survival of *H. pylori*.

4.6 Outlook

The *atpE* gene of *H. pylori* has been shown to be an interesting target with unique features. Having shown that three transmembrane domains are present in the *H. pylori*. c subunit, further analysis is necessary to understand the differences between the mutants of the *atpE* gene generated in this work.

The role of histidine 18 in acid survival has to be further evaluated. Especially the survival and energetics at pH 1 in acid precipitated medium as shown by Stingl *et al*. (2001) could help to understand its influence at very low pH. ATP measurements under different pH conditions could help to understand further the role of the F_1F_0-ATPase in

acid survival. No effect was seen with DCCD, a specific inhibitor of the *E. coli* F_1F_0-ATPase in our cytosensor experiments (M. Mollenhauer, personal communication), therefore specific inhibition of the *H. pylori* F_1F_0-ATPase was not possible and has to be investigated by other means.

Moreover, the truncated mutant has to be investigated for its behaviour under varying pH conditions. Although mutants in (only) strain 69A were received under standard conditions, an essential function in acid survival cannot be ruled out.

The *H. pylori* coexpressing the *E. coli atpE* probably assemble hybrid c subunit rotors which might result in varied behaviour under acidic conditions.

5 Summary

Helicobacter pylori is recognized today as a human pathogen responsible for several disorders in the human stomach and intestine. The diseases range from chronic gastritis and ulcer to MALT-Lymphoma and a risk factor for gastric cancer.

It is important to understand the mechanisms that allow survival in the acidic human stomach, as well as to identify specific proteins involved herein for the discovery of new targets and the development of selective drugs against *H. pylori*.

In this study, the c subunit of the F_1F_0-ATPase from *H. pylori* has been analysed. The F_1F_0-ATPase or ATP-synthase uses an electrochemical H^+ gradient across the cytoplasmic membrane for the generation of ATP. On the other hand, protons can be pumped out of the cytoplasm during hydrolysis of ATP. Thus, the F_1F_0-ATPase is involved in H^+ metabolism. The c subunit of this multimeric enzyme is directly involved in proton translocation across the membrane and thus might play a role in acid survival. Several characteristics were identified that distinguish the *H. pylori* F_1F_0-ATPase from most other organisms.

The genetic organization of the *atp* genes is found to be an operon in most organisms. In *H. pylori,* the genes for the subunits a and c that form the proton-conducting channel are found separated from the remaining operon. Therefore, the varied regulation of gene expression might influence the mechanism of proton translocation.

Here it is shown for the first time that the *H. pylori* c subunit contains three membrane spanning segments instead of the usual two segments found in most other organisms.

This has been demonstrated with an *in vitro* transcription and translation system with microsomal membranes and in an *in vivo* system, where fusion proteins with potential transmembrane sequences of *H. pylori atpE* with alkaline phosphatase were expressed in *E. coli*. Both systems could show that an additional transmembrane domain is located in the N-terminal region of the *H. pylori* c subunit and that the N-terminus is located in the cytoplasm. The additional N-terminal region creates unique structural features and a periplasmic loop is found between the first and second transmembrane domain. This periplasmic loop has several protonatable amino acid residues that might be involved in regulation of the *H. pylori* c subunit in an environment with varying pH: H18, D19,

D26, K29 and Y31. It was also shown that strain G1.1, which is infectious in the gerbil animal model, exhibits an additional glutamic acid at position 23.

Knockout of the *atpE* gene, which encodes the c subunit, was impossible. Very few viable clones were detected. For instance, only two potential mutants were received in strain 69A compared to the control with more than 6000 mutants obtained. These potential mutants still contained the wild type *atpE* sequence when genomic DNA was analysed by PCR. Thus, the *atpE* gene is essential for *H. pylori* under standard growth conditions.

The construction of mutants revealed that the *E. coli* c subunit cannot replace the *H. pylori* c subunit. Even when the *H. pylori* N-terminus was added to the *E. coli* sequence, it was not possible to exchange the *H. pylori atpE* gene against the *E. coli* variant. This indicates a crucial role of the conserved second and third transmembrane segment, corresponding to the complete *E. coli* c subunit, for protein assembly and function.

On the other hand, it has also been shown that a mutant with a truncated version of the *H. pylori atpE* gene is viable. Nevertheless, an essential role of the additional N-terminal segment cannot be excluded because the truncation was only possible in *H. pylori* strain 69A. Additionally, a specialized role of the N-terminal segment in survival in an acidic environment is conceivable.

The involvement of the histidine residue at position 18 in the periplasmic loop of the *H. pylori* c subunit under varying pH conditions was examined. Histidine was exchanged for glycine and the mutants exhibited a different behaviour in the acidic environment, indicating that the exchange of histidine affects control of cytoplasmic and periplasmic pH.

Further analysis is necessary to elucidate the function of the *H. pylori* c subunit. The amino acids in the periplasmic loops have to be examined for their influence on the survival in an acidic environment. One candidate for adaptation to the acidic environment appears to be histidine at position 18 in the periplasmic loop of the *H. pylori* c subunit.

Moreover, the characteristics of the truncated mutants have to be investigated.

The c subunit is a promising target due to its unique structure and the involvement in proton translocation. Proton metabolism has to be differentially regulated in *H. pylori* in order to enable the pathogen to inhabit the human stomach, its ecological niche.

6 Zusammenfassung

Das Bakterium *Helicobacter pylori* ist heute als ein humanes Pathogen anerkannt, das unterschiedlichste Krankheitsbilder in Magen und Darm verursacht. Die verursachten Beschwerden reichen von entzündlicher Gastritis und Geschwüren hin zu MALT-Lymphomen. Außerdem stellt eine *H. pylori* Infektion einen Risikofaktor für die Entwicklung von Magenkrebs dar.

Die Forschung an *H. pylori* möchte vor allem klären, wie dieser Keim im sauren Magen überlebt. Die Identifizierung der Mechanismen, die ein Überleben im sauren Milieu ermöglichen, können zur Entdeckung neuer Angriffspunkte für Medikamente beitragen.

In dieser Arbeit wurde die c-Untereinheit der F_1F_0-ATPase von H. pylori untersucht. Die F_1F_0-ATPase, auch ATP-Synthase genannt, nutzt den elektrochemischen Protonengradienten über der inneren bakteriellen Membran, um ATP zu bilden. Unter veränderten Bedingungen kann diese Reaktion auch umgekehrt werden, so daß Protonen unter ATP-Verbrauch aus der Zelle hinaus gepumpt werden. Die F_1F_0-ATPase ist also am Protonenhaushalt der Zelle beteiligt. Die c-Untereinheit bildet einen Teil des Kanals durch die innere Membran und ist direkt am Protonenfluß beteiligt. Einige Besonderheiten unterscheiden die c-Untereinheit von *H. pylori* von den c-Untereinheiten der meisten anderen Organismen.

In den meisten Organismen sind die *atp* Gene in einem Operon angeordnert. Bei *H. pylori* sind die Gene, die für die Untereinheiten a und c kodieren, nicht in diesem Operon enthalten. Die Untereinheiten a und c bilden den Protonenkanal und diese veränderte Anordnung deutet auf veränderte Regulation dieser Gene in *H. pylori* hin.

Zudem wurde in dieser Arbeit erstmals gezeigt, daß die *H. pylori* c-Untereinheit drei Transmembrandomänen besitzt. Damit unterscheidet sie sich deutlich von den c-Untereinheiten der meisten anderen Organismen, für die eine helicale Haarnadel-Struktur mit zwei Transmembrandomänen beschrieben wird. Diese Struktur wurde sowohl mit einem *In vitro* System als auch mit einem *In vivo* System nachgewiesen. Im ersten System wurde die Translokation über mikrosomale Membranen analysiert, während bei der zweiten Methode Fusionsproteine von potentiellen Membrandomänen mit alkalischer Phosphatase in *E. coli* exprimiert wurden. Beide Systeme haben gezeigt,

das bei der *H. pylori* c-Untereinheit der N-Terminus im Cytoplasma und der C-Terminus im Periplasma liegt. Durch den zusätzlichen N-Terminus entsteht eine periplasmatische Domäne, die mehrere protonierbare Aminosäurereste enthält und daher an der Regulation der c-Untereinheit beteiligt sein könnte: H18, D19, D27, K29 und Y31. Außerdem konnte gezeigt werden, daß im *H. pylori* Stamm G1.1, der im Tiermodell infektiös ist, einen zusätzliche Glutaminsäure an Position 23 vorhanden ist.

Das Ausschalten des Gens *atpE,* welches für die c-Untereinheit kodiert, war nicht möglich. Während bei der Kontrolle mehr als 6000 Mutanten erhalten wurden, waren es beim Versuch mit *atpE* nur 2 bis 43, je nach Stamm. Allerdings stellte sich bei der PCR Analyse heraus, daß in der genomischen DNA immer noch die Wildtyp-Sequenz vorhanden war. Das Gen *atpE* wurde folglich als essentiell unter Standardbedingungen klassifiziert.

Weiterhin wurde versucht, das *H. pylori atpE* Gen mit der Sequenz des *E. coli atpE* Gens auszutauschen. Obwohl die Expression des *E. coli* Gens nachgewiesen werden konnte, war dieser Austausch nicht möglich. Selbst wenn der N-Terminus der *H. pylori* Sequenz an die *E. coli* Sequenz fusioniert wurde, konnten die Gene nicht ausgetauscht werden. Das deutet darauf hin, daß die zweite und dritte Membrandomäne für die Funktionalität der c-Untereinheit notwendig sind. Allerdings konnten einige wenige *H. pylori* Mutanten in einem Stamm generiert werden, die eine verkürzte Form des *atpE* Gens exprimieren. Trotzdem schliessen diese Versuche nicht aus, daß der N-Terminus, vor allem unter sauren Bedingungen, notwendig für das Überleben von *H. pylori* ist.

Die Funktion des Histidin-Rests in der periplasmatischen Domäne wurde anhand von Mutanten untersucht, in denen Histidin gegen Glycin ausgetauscht wurde. Diese Mutanten zeigen ein verändertes Verhalten im sauren Milieu, was auf eine regulatorische Funktion hinweist.

Nachdem die Topologie der c-Untereinheit bestimmt ist, sind weiterführende Versuche notwendig, um die exakte Funktion der *H. pylori* c-Untereinheit aufzuklären. Dazu müssen auch die anderen protonierbaren Aminosäuren in der periplasmatischen Domäne untersucht werden, ebenso wie die Mutanten mit dem verkürzten *atpE*.

Die c-Untereinheit besitzt mehrere spezielle Eigenschaften und ist daher ein interessantes Protein, welches eine wichtige Rolle im Protonenhaushalt von *H. pylori* haben könnte.

7 References

Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 A resolution of F_1-ATPase from bovine heart mitochondria. Nature 370: 621-628

Aleljung, P., Nilsson, H.O., Wang, X., Nyberg, P. Morner, T., Warsame, I. and Wadstrom, T. (1996) Gastrointestinal colonisation of BALB/cA mice by Helicobacter pylori monitored by heparin magnetic separation. FEMS Immunol. Med. Microbiol. 13 (4): 303-309

Alm, A. R., Ling, L. S. L., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonges, B. L., Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelsen, M., Millis, D. M., Ives, C., Gibson, R., Merberg, D., Vovis, G. F. and Trust, T. J. (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature 397: 176-180

Andrutis, K.A., Fox, J.G., Schauer, D.B., Marini, R.P., Murphy, J.C., Yan, L. and Solnick, J.V. (1995) Inability of an isogenic urease-negative mutant strain of *Helicobacter mustelae* to colonize the ferret stomach. Infect. Immun. 63: 3722-3725

Ang, S., Lee, C.U., Peck, K., Sindici, M., Matrubutham, U., Gleeson, M.A. and Wang, J.T. (2001) Acid-induced gene expression in *Helicobacter pylori*: study in genomic scale by microarray. Infect. Immun. 69: 1679-1686

Appelmelk, B.J., Negrini, R., Moran, A.P. and Kuipers, E.J. (1997) Molecular mimicry between *Helicobacter pylori* and the host. Trends Microbiol. 5: 70-73

Atherton J.C., Cao P., Peek R.M. Jr, Tummuru M.K., Blaser M.J., Cover T.L. (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. J. Biol. Chem. 270 (30):17771-17777

Backert, D., Moese, S., Selbach, M., Brinkmann, V. and Meyer, T. F. (2001) Phosphorylation of tyrosine 972 of the *Helicobacter pylori* CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells. Mol. Microbiol. 42: 631-644

Bamberg, K. and Sachs, G. (1994) Topological Analysis of H^+, K^+-ATPase using *in vitro* translation. J. Biol. Chem. 269 (24): 16909-16919

Bamberg, K., Mercier, F., Reuben, M. A., Kobayashi, Y., Munson, K. B. and Sachs, G. (1992) cDNA cloning and membrane topology of the rabbit gastric H^+/K^+-ATPase alpha-subunit. Biochim. Biophys. Acta 1131: 69-77

Banerjee, S. and Michetti, P. (2001) Development of a *Helicobacter pylori* Vaccine. From: *Helicobacter pylori*: Molecular and Cellular Biology edited by Achtmann, M. and Suerbaum, S.

Bauerfeind, P., Garner, R., Dunn, B.E. and Mobley, H.L.T. (1997) Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. Gut 40: 25-30

Baxter, G. T., Miler, D. L., Kuo, R. C., Wada, H. G. and Owicki, J. C. (1992). PKCe is involved in GM-CSF signal transduction: evidence from microphysiometer and antisense oligonucleotide experiments. Biochemistry 31: 10950-10954

Bayle, D., Weeks, D. and Sachs G. (1995) The membrane topology of the rat sarcoplasmic and endoplasmic reticulum calcium ATPases by *in vitro* translation scanning. J Biol Chem 270 (43):25678-84

Bayle, D., Wangler, S., Weitzenegger, T., Steinhilber, W., Volz, J., Przybylski, M., Schäfer, K.P., Sachs, G. and Melchers, K. (1998) Properties of the P-type ATPases encoded by the copAP operons of *Helicobacter pylori* and *Helicobacter felis*. J. Bacteriol. 180 (2): 317-329

Bijlsma, J.J., Gerrits, M.M., Imamdi, R., vandenbroucke-Grauls, C.M. and Kusters, J.G. (1998) Urease-positive, acid-sensitive mutants of *Helicobacter pylori*: urease-independent acid resistance involved in growth at low pH. FEMS Microbiol. Lett. 167: 309-313

Bijlsma, J.J., Lie, A.L., Nootenboom, I.C., Vandenbrouke-Grauls, C.M. and Kusters, J.G. (2000) Identification of loci essential for the growth of *Helicobacter pylori* under acidic conditions. J. Infect. Dis 182: 1566-1569

Birkenhäger, R., Greie, J.-C., Altendorf, K. and Deckers-Hebestreit, G. (1999) F_0 complex of the *Escherichia coli* ATP synthase. Eur. J. Biochem. 264: 385-396

Bizzozero, G. (1893) Über die schlauchförmigen Drüsen des Magendarmkanals und die Beziehungen ihres Epithels zu dem Oberflächenepithel der Schleimhaus. Arch. Für Mikrosk. Anat. 42: 82-152

Blaser, M. J., Perez-Perez, G. I., Keanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., stemmermann, G. N. and Nomura, A. (1995). Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adocarcinoma of the stomach. Cancer Res. 55: 2111-2115

Blaser, M.J. (1993) *Helicobacter pylori*: microbiology of a "slow" bacterial infection. Trends Microbiol. 1: 255-260

Boas, I. (1907) Diseases of the stomach. Davis, Philadelphia

Bode, G., Mauch, F. and Malfertheiner, P. (1993). The coccoid forms of *Helicobacter pylori*. Criteria for their viability. Epidemiol. Infect. Immun. 111:483-490

Bode, G., Mauch, F., Ditschuneit, H. and Malfertheiner, P. (1992). Mikrobiologische Aspekte von *Helicobacter pylori*. Z. Gastroenterol. 30 (2): 4-8

Boren, F., Flak, P., Roth, K. A., Larson, G., Nilius, M. and Ditschuneit, H. (1993). Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science 262: 1892-1895

Borghese, R., Turina, P., Lambertini, L. and Melandri, B. A. (1998). The *atpIBEXF* operon coding for the F_0 sector of the ATP synthase from the purple nonsulfur photosynthetic bacterium *Rhodobacter capsulatus*. Arch Microbiol. 170: 385-388

Bottcher, G. (1874) Dorpater Med. Z. 5: 184

Capaldi, R.A., Schulenberg, B., Murray, J. and Aggeler, R. (2000) Cross-linking and electron microscopy studies of the structure and functioning of the *Escherichia coli* ATP synthase. J. Exp. Biol. 203: 29-33

Cellini, L., Allocati, N., Angelucci, D., Iezzi, T., Di Campli, E., Marzio, L., Dainelli, B.. (1994) Coccoid *Helicobacter pylori* not culturable *in vitro* reverts in mice. Microbiol Immunol. 38 (11): 843-50

Chen, H., Bjerknes, M., Kumar, R. and Jay, E. (1994). Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. Nucleic Acids Res. 22: 4953-4957

Clayton, C.L., Pallen, M.J., Kleanthous, H., Wren, B.W. and Tabaychali, S. (1990) Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleid Acids Res. 18: 362

Clyne, M., Labigne, A. and Drumm, B. (1995) *Helicobacter pylori* requires an acidic environment to survive in the presence of urea. Infect. Immun. 63: 1669-1673

Coskun, T. chu, S. and Montrose, M.H. (2001) Intragastric pH regulates conversion from net acid to net alkaline secretion by the rat stomach. Am. J. Physiol. 281: G870-G877

Covacci, A., Censini, S., Bugnoli, M., Petracca, R. Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z. and Figura, N. (1993) Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA 90 (12): 5791-5795

Covacci, A. und Rappuoli, R. (2003) *Helicobacter pylori*: After the Genomes, Back to Biology. J. Exp. Med. 197 (7): 807-811

Cover, T. L., Dooley, C. P. and Blaser, M. J. (1990). Characterization of a human serologic response to proteins in Helicobacter pylori broth culture supernatants with vacuolation cytotoxin activity. Infect. Immun. 58: 603-610

Cover, T. L., Cao, L. P,, Lind, C. D., tham, K. T. and Blaser, M. J. (1993) Correlation between vacuolation cytotoxin production by *Helicobacter pylori* isolates *in vitro* and *in vivo*. Infect. Immun. 61:5008-5012

Cover, T. L., Glupczynski, Y. an Lage, A. P. (1995). Serologic detection of infection with cagA$^+$ *Helicobacter pylori* strains. J. Clin. Microbiol. 33: 1496-1500

Cussac, V., Ferrero, R.L. and Labigne, A. (1992) Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J. Bacteriol. 174: 2466-2473

Danesh, J. (1999) *Helicobacter pylori* infection and gastric cancer: systematic review of the epidemiological studies. Aliment. Pharm. Therap. 13: 851-856

Davies, B.J., de Vries, N., Rijpkema, S.G., van Vliet, A.H.M. and Penn, C.W. (2002) Transcriptional and mutational analysis of the *Helicobacter pylori* urease promoter. FEMS Microbiol. Lett. 213: 27-32

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) Data for Biochemical Research (3rd ed.) pp. 1-31, Oxford Science Publications

Deckers-Hebestreit, G. and Altendorf, K. (1986) Accessibility of F_0 subunits from *Escherichia coli* ATP synthase. A study with subunit specific antisera. Eur. J. Biochem. 161: 225-231

Deckers-Hebestreit, G., Schmid, R. Kilts, H.-H. and Altendorf, K. (1987) F_0 portion of *Eschrichia coli* ATP synthase: Orientation of subunit c in the membrane. Biochemistry 26: 5486-5492

Deckers-Hebestreit, G. and Altendorf, K. (1996)The F_0F_1-type ATP synthases of bacteria: structure and function of the F_0 complex. Annu. Rev. Microbiol. 50: 791-824

Deckers-Hebestreit, G., Greie, J., Stalz, W. and Altendorf, K.The ATP synthase of *Escherichia coli*: structure and function of F_0 subunits. (2000) Biochim Biophys Acta 1458 (2-3): 364-73

Derman, A.I., Beckwith, J. (1991) *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. J. Bacteriol. 173: 7719-7722

Dimroth, P., Wang, H., Grabe, M. and Oster, G. (1999) Energy transduction in the sodium F-ATPase of *Propionigenium modestum*. Proc. Natl. Acad. Sci. USA 96: 4924-4929

Dmitriev, OY., Jones, P.C. and Fillingame, R.H. (1999) Structure of the subunit c oligomer in the F_1F_0 ATP synthase: model derived from solution structure of the monomer and cross-linking in the native enzyme. Proc. Natl. Acad. Sci. USA 96: 7785-779

Dmitriev, O., Jones, P.C., Jiang, W. and Fillingame, R.H. (1999) Structure of the membrane domain of subunit b of the *Escherichia coli* F_0F_1 ATP synthase. J. Biol. Chem. 274: 15598-15604

Doenges, J.L. (1938) Spirochetes in gastric glands of macaus rhesus and human without definite history of related disease. Proc. Soc. Exp. Biol. Med. 38: 536-538

Dunn, B.E. and Phadnis, S.H. (1998) Structure, Function and Loacalization of *Helicobacter pylori* Urease. Yale J. Biol. Med. 71: 63-73

Eaton, K.A., Morgan, D.R. and Krakowka, S. (1989) *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Imm. 57: 1119-1125

Eaton, K. A., Morgan, D. R. and Krakowa, S. (1992).Motility as a factor in the colonization of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. 37, 123-127

Eaton, K.A. and Krakowka, S. (1994) Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect. Immun. 62: 3604-3607

Eaton, K. A., Suerbaum, S., Josenhans, C. and Krakowka, S. (1996). Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. Infect. Immun. 64: 2445-2448

Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R. (1984). Analysis of Membrane and Surface Protein Sequences with the Hydrophobic Moment Plot. J. Mol. Biol. 179: 125-142

Eslick, G.D., Lim, L.L.-Y., Byles, J.E., Xia, H.H.-X. and Talley, N.J. (1999) Association of *Helicobacter pylori* infection with gastric carcinoma: a meta-analysis. Am. J. Gastroenterol. 94: 2373-2379

Evans, D. G., Karjalainen, T. K., Evans, D. J. jr., Graham, D. Y. and Lee, C. H. (1993). Cloning, nucleotide sequence and expression of a gene encoding an adhesin subunit protein of *Helicobacter pylori*. J. Bacteriol. 175: 674-683

Evans, D. J. J. and Evans, D. G. (2001). *Helicobacter pylori* CagA analysis of sequence diversity in relation to phosphorylation motifs and implications for the role of CagA as a virulence factor. Helicobacter 6: 187-198

Ferber, D.(2001) Carcinogenic bacteria. Cracking gut bugs´ cell-skewing streategy. Science 294: 2269

Figurski, D.H., Meyer, R.J. and Helinski, D.R. (1979) Suppression of ColE1 replication properties by the Inc P-1 plasmid RK2 in hybrid plasmids constructed *in vitro*. J. Mol. Biol. 133 (3): 295-318

Fillingame, R.H. (1990) Molecular mechanics of ATP synthesis by F_1F_0-type H^+ transporting ATP synthases in The Bacteria, Vol. XII, ed. Krulwich, T.A., Academic Press

Fillingame, R.H. *et al*. (2000) The oligomeric subunit c rotor of the F_0 sector of ATP synthase. Unresolved questions in our understanding of function. J. Bioenerg. Biomembr. 32: 433-440

Fillingame, R.H., Jiang, W., Dimitriev, O.Y. and Jones, P.C. (2000) Structural interpretations of F(0) rotary function in the Escherichia coli F_1F_0 ATP synthase. Biochim. Biophys. Acta 1458: 387-403

Foster, D.L. and Fillingame, R.H. (1982) Stoichiometry of subunits in the H^+-ATPase complex of *Escherichia coli*. J. Biol. Chem. 257: 2009-2015

Freedberg, A.S. and Barron, L.E. (1940) The presence of Spirochetes of the human. Am. J. Dig. Dis. 38: 443-445

Galmiche, A., Rassow, J., Doye, A. *et al*., (2000) The N-terminal 34kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome c release. EMBO J. 19: 6361-6370

Geis, G., Suerbaum, S., Forsthoff, B., Leying, H. and Opferkuch, W. (1993). Ultrastructure and biochemical studies of the flagellar sheath of *H. pylori*. J. Med. Microbiol. 38: 371-377

Gilbert, J.V., Ramakrishna, J., Sundermann Jr, F.W., Wright, A. and Plaut, A.G. (1995) Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*. Infect immun. 63: 2682-2688

Girvin, M.E., Hermolin, J., Pottorf, R. and Fillingame, R.H. (1989) Organization of the F_0 sector of *Escherichia coli* H+-ATPase: the polar loop region of subunit c extends from the cytoplasmic face of the membrane Biochemistry 28 (10): 4340-4343

Guzman LM *et al*. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. 177: 4121-4130,

Haas, R., Meyer, T. F. and van Putten, J. P. M. (1993). Aflagellated mutants of *Helicobacter pylori* generated by genetic transdormation of naturally competent strains using transoposon shuttle mutagenesis. Mol. Microbiol. 8 (4): 753-760

Haas, R. (1995) Mikrobiologie, Pathogenität und milekulare mechanismen der Vrulenz von *Helicobacter pylori*. Biospektrum 3: 35-40

Hafeman, D. G., Parce, J. W. and McConnell, H. M. (1988). Light-addressable potentiometric sensor for biochemical systems. Science 240, 1182-1185

Hancock, R. E. W., Alm, R., Bina, J. and Trust, T. (1998). *Helicobacter pylori*: A surprisingly conserved bacterium. Nat. Biotech. 16: 216-217

Harold and Maloney (1996) Energy transduction by ion currents, in *Escherichia coli* and *Salmonella typhimurium*, Cellular and molecular biology, ed. by Neidhart, F.C., ASM Press, Washington D.C., USA

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77 (1): 51-59

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68

Houghton, J., Fox, J.G. and Wang, T.C. (2002) *Helicobacter pylori* and other causes of gastric ulceration: Gastric cancer: Laboratory bench to clinic. J. Gastroen. Hepatol. 17: 495-502

Huang, J.-Q., Sridhar, S. Chen, Y. and Hunt, R. (1998) Meta-analysis of the relationship between *Helicobacter pylori* seropositivity and gastrric cancer. Gastroenterology 114: 1169-1179

IARC Monographs (1994) Volume 61: page 177 (ISBN 9 2832 1262 2)

Ilver, D., Arnqvist, A., Ägren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L. and Bóren, T. (1998). *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 279: 373-377

Israel, D.A., Salama, N., Arnold, C.N., Moss, S.T., Ando, T., Wirth, H.P., Tham, K.T., Camorlinga, M., Blaser, M.J., Falkow, S. and Peek, R.M. Jr. (2000) *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. J. Clin. Invest. 107: 611-620

Ivy, A.C., Grossman, M.I. and Bachrach, W.H. (1950) Peptic Ulcer. Philadelphia: Blackiston Co., p. 395-396

Jaworski, W. (1889) Podrecznik Chorob zoladka. Wydawnictwa Dziel Lakarskich Polskich. 32

Jiang, Q., Hiratsuka, K. and Taylor, D.E. (1996) Variability of gene order in different *Helicobacter pylori* strains contributes to genome diversity .Mol. Microbiol. 20: 833-842

Jiang, W. and Fillingame, R.H. (1998) Interacting helical faces of subunits a and c in the F_1F_0 ATP synthase of *Escherichia coli* defined by disulfide cross-linking. Proc. Natl. Acad. Sci. USA 95: 6607-6612

Jones, D.T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292: 195-202.

Jones, A.C., Logan, R.P.H., Foynes, S., Caockayne, A., Wren, B.W. and Penn, C.W. (1997) A flagellar sheath protein of *Helicobacter pylori* is identical to HpaA, a putative N-acetyl-neuraminyllactose-binding hemagglutinin, but is not an adhesin for AGS cell. J. Bacteriol. 179: 5643-5647

Josenhans, C., Labigne, A. and Suerbaum, S. (1995) Comparative ultrastuructural and functional studies of *Helicobacter pylori* and *Helicobacter mustelae* flagellin mutants: both flagellin subunits, FlaA and FlaB, are necessary for full motility in *Helicobacter* species. J. Bacteriol. 177:3010-3020

Jungblut, PR., Bumann, D., Haas, G., Zimny-Arndt, U., Holland, P., Lamer, S., Siejak, F., Aebischer, A. and Meyer, T.F. (2000) Comparative proteome analysis of *Helicobacter pylori*. Mol. Microbiol. 36 (3): 710-725

Junge, W., Lill, H. and Engelbrecht, S. (1997) ATP synthase: an electrochemical transducer with rotatory mechanics. Trends Biochem. Sci 22: 420-423

Hofmann, K. and Stoffel, W. (1993) TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler 374: 166

Kavermann H, Burns BP, Angermuller K, Odenbreit S, Fischer W, Melchers K, Haas R. (2003) Identification and characterization of Helicobacter pylori genes essential for gastric colonization. J. Exp. Med.197 (7): 813-22

Kim, J.S., Chang, J.H., Chung, S.I. and Yum, J.S. (1999) Molecular cloning and characterization of the *Helicobacter pylori fliD* gene, an essential factor in flagellar structure and motility. J. Bacteriol. 181: 6969-6976

Klein, P., Kanehisa, M. and Delisa, C. (1985) The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta 815: 468-476

Klose, J. and Kobalz, U. (1995) Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis 16: 1034-1059

Koch, R. (1884) Die Aetiologie der Tuberkulose. Mittheilungen aus dem Kaiserlichen Gesundheitsamte, Vol. 2, pages 1-88

Koebmann, B. J., Nilsson, D., Kuipers, O. P. and Jensen, P. R. (2000). The Membrane-Bound H^+-ATPase Complex is essential for Growth of *Lactococcus lactis*. J. of Bacteriology 182 (17): 4738-4743

Konjetzny, G.E. (1928) Die Entzündungen des Magens. In: Handbuch Pathologie, Anatomie und Histologie IV. 2: 768-1116

Krienitz, U. (1906) Über das Auftreten von Spirochaeten verschiedener Form im Mageninhalt bei Carcinoma vetriculi. Dtsch. Med. Wschr. 32: 872

Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P. H., Stremmel, W. and Rudi, J. (2002) Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis int the human gastric epithelial cell line AGS. Infect. Immun. 69 (8): 5080-5087

Kuipers, E. J., Perez-Perez, G. I. Meuwissen, S. G. M. and Blaser, H. J. (1995). *Helicobacter pylori* an atrophic gastritis: importance of the cagA status. J. Natl. Cancer Instut. 87: 1777-1780

Kusters. J. G. (1997). Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. Infect. Immun. 65 (9): 3672-3679

Kyte, J. and Doolittle, R.F. (1982). A Simple Method for displaying the hydropathic character of a Protein. J. Mol. Biol. 157: 105-132

Labigne; A., Cussac, V. and Courcoux, P. (1991) Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. 173: 1920-1931

Laemmli, U. K. (1970). Cleavage of Structural Proteins during Assembliy of the Head of Bacteriophage T4. Nature 227: 680-685

Laine, L., Schoenfield, P. and Fennerty, M.B. (2001) Therapy for *Helicobacter pylori* in patients with non-ulcer dyspepsia: a meta-analysis of randomized controlled trials. Ann. Intern. Med 134: 361-369

Lee N.L., Gielow W.O. and Wallace R.G. (1981) Mechanism of araC autoregulation and the domains of two overlapping promoters, Pc and PBAD, in the L-arabinose regulatory region of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 78 (2): 752-6

Leying, H., Suerbaum, S., Geis, G. and Haas, R. (1992) Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol. Microbiol. 6 (19): 2863-2874

Lotscher, H.R., deJong, C. and Capaldi, R.A. (1984) Inhibition of the adenosinetriphosphatase activity of Escherichia coli F1 by the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide is due to modification of several carboxyls in the beta subunit. Biochemistry 23 (18): 4134-40

Logan, R. P. H. (1996). Adherence of *Helicobacter pylori*. Aliment Pharma. Therap. 10 (1): 3-15

Logan, R.P.H. (1994) *Helicobacter pylori* and gastric cancer. Lancet 344: 1078-1079

Ma, J., Campbell, A. and Karlin, S. (2002). Correltaions between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. J. Bacteriol. 184 (20): 5733-5745

Mahdavi, J., Sondén, B., Hurtig, M., Olfat, F.O., Forsberg, L., Roche, N., Angström, J., Larsson, T., Teneberg, S., Karlsson, K.A., Altraja, S., Wadström, T., Kersulyte, D. Berg, D.E., Dubois, A., Petersson, C., Magnusson, K.E., Norberg, T., Lindh, F., Lundskog, B.B., Arnqvist, A., Hammarström, L. and Borén, T. (2002) *Helicobacter pylori* SabA Adhesin in Persistent Infection and Chronic Inflammation. Science 297: 573-578

Malfertheiner, P., Megraud, F., O´Morain, C. *et al*. (2002) Current concepts in the management of *Helicobacter pylori* infection: the Maastricht 2-2000 Consensus Report. Aliment. Pharm. Therap. 16: 167-180

Marshall, B. J. and Warren, J.R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1 (8390): 1311-1315

Marshall, B.J., Barrett, L.J., Prakash, C., McCallum, R.W. and Guerrant, R.L. (1990) Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. Gastroenterology 99: 697-702

Martin-Galiano, A. J., Ferrandiz, M. J. and de la Campa, A. G. (2001). The promoter of the operon encoding the F_0F_1 ATPase of Streptococcus pneumoniae is inducible by pH. Mol. Microbiol 41 (6): 1327-1338

McConnell, H. M., Owicki, J. C., Parce, J. W., Miller, D. L., Baxter G. T., Wada, H. G. and Pichford, S. (1992) The Cytosensor microphysiometer: biological applications of silicon technology. Science 257: 1906-1912

McGowan, C.C., Cover, T.L. and Blaser, M.J. (1997) Analysis of the F_1F_0-ATPase from *Helicobacter pylori*. Infect. Immun. 65 (7): 2640-2647

McLachlin, D.T., Coveny, A.M., Clark, S.M. and Dunn, S.D. (2000) Cross-linking and electron microscopy studies of the structure and functioning of the *Escherichia coli* ATP synthase. J. Biol. Chem. 275 (23): 17571-17577

Megraud, F., Trimoulet, P., Lamouliatte, H. and Boyanova L. (1991) Bactericidal effect of amoxicillin on *Helicobacter pylori* in an *in vitro* model using epithelial cells. Antimicrob. Agents Chemother. 35: 869-872

Melchers, K., Weitzenegger, T., Buhmann, A., Steinhilber, W., Sachs, G. and Schäfer, K.P. (1996) Cloning and membrane topology of a P type ATPase from *Helicobacter pylori*. J. Biol. Chem. 271 (1): 446-457

Meyer-Rosberg, K., Scott, D.R., Rex, D., Melchers, K. and Sachs, G. (1996) The effect of environmental pH on the proton motive force of Helicobacter pylori. Gastroenterology 111 (4): 886-900

Mitchell, H.M. (2001) Epidemiology of Infection in: *Helicobacter pylori*: Physiology and Genetics, edited by Mobley, H.L.T., Mendz, G.L. and Hazell, S.L. ASM Press, Washington, D.C., USA

Mizote, T., Yoshiyama, H. and Nakazawa, T. (1997). Urease-independent chemotactic response of *Helicobacter pylori* to urea, urease inhibitors, and sodium bicarbonate. Infect. Immun. 65: 1519-1521

Moayyedi, P., Bardhan, C., Young, L., Dixon, M.F. Brown, L. and Axon, A.T. (2001) *Helicobacter pylori* eradication does not exacerbate reflux symptoms in gastroesophageal reflux disease. Gastroenterology 121: 1120-1126

Moayyedi, P., Soo, S., deeks, J. *et al*. (2000) Systematic review and economic evaluation of *Helicobacter pylori* eradication treatment for non-ulcer dyspepsia. Brit. Med. J. 321: 659-664

Mobley, H.L., Island, M.D. and Hausinger, R.P. (1995) Molecular biology of microbial ureases. Microbiol. Rev. 59 (3): 451-480

Mobley, H.L.T and Roxall, P.A. (1994) *H. pylori* urease. In *Helicobacter pylori*: Basic mechanisms to clinical cure. Hunt, R.H. and Tytgat, G.N.J. (eds). Boston: Kluwer Academic Publishers, pp. 41-58

Mollenhauer-Rektorschek, M., Hanauer, G., Sachs, G. and Melchers, K. (2002) Expression of UreI is required for intragastric transit and colonization of gerbil gastric mucosa by Helicobacter pylori. Res. Microbiol. 153 (10): 659-66

Muller, D.J., Dencher, N.A., Meier, T., Dimroth, P., Suda, K., Stahlberg, H., Engel, A., Seelert, H. and Matthey, U. (2001) ATP synthase: constrained stoichiometry of the transmembrane rotor. FEBS Lett. 504: 19-22

Nakamoto, R.K., Kechum, C.J. and Al-Shawi, M.K. (1999) Rotational coupling in the F_0F_1 ATP synthase. Annu. Rev. Biophys. Biomol. Struct. 28: 205-234

Nakamura, H., Yoshiyama, H. takeuchi, H., Mizote, T., Okita, K., and Nakazawa, T. (1998) Urease plays an important role in the chemotactic motility of *Helicobacter pylori* in viscous environment. Infect. Immun. 66: 4832-4837

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997). Identification of prokaryotic and eukarotic signal peptides and prediction of theri cleavage sites. Protein Eng. 10: 1-6

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1-6

Nolan, K.J., McGee, D.J., Mitchell, H.M., Kolesnikow, T., Harro, J.M., O´Rourke, J., Wilson, J.E., Danon, S.J., Moss, N.D., Mobley, H.L. and Lee, A. (2002) *In vivo* behaviour of a *Helicobacter pylori* SS1 nixA mutant with reduced urease activity. Infect. Immun. 70 (2): 685-691

O´Toole, P. W., Logan, S. M., Kostrzynska, M., Wadstrom, T. and Trust, T. J. (1994). Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. Mol. Microbiol. 14 (4): 691-703

O´Toole, P.W. and Clyne, M. (2001) Cell Envelope in: *Helicobacter pylori*: Physiology and Genetics by Mobley, H.L.T., Mendz, G.L. and Hazell, S.L. (editors). ASM Press, Washington, USA

Ogilvie, I., Aggeler, R. and Capaldi, R.A. (1997) Cross-linking of the delta subunit to one of the three alpha subunits has no effect on functioning, as expected if delta is a part of the stator that links the F1 and F0 parts of the *Escherichia coli* ATP synthase. J. Biol. Chem. 272: 16652-16656

Ottemann, K. M. and Lowenthal, A. C. (2002). *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect. Immun. 70: 1984-1990

Owicki, J. C. and Parce, J. W. (1990). Bioassays with a microphysiometer. Nature 344: 271-272

Owicki, J.C. and Parce, J. W. (1992). Biosensors based on the energy metabolism of living cells, the physical chemistry and cell biology of extracellular acicification. Biosens. Bioelectron 7: 255-272

Padan, E., Zilberstein, D. and Schuldner, S. (1981) pH Homeostasis in bacteria. Biochim. Biophys. Acta 650: 151-166

Palmer, E.D. (1954) Investigation of the gastric mucosa spirochetes of the human. Gastroenterology 27: 218-220

Parce, J. W., Owicki, J. C., Kercso, K. M., Sigal, G. B., Wada, H. G., Muir, V. C., Bousse, L. J., Ross, K. L., Sikic B. I. and McConnell, H. M. (1989). Detection of cell-affecting agents with a silicon biosensor. Science 246: 243-247

Parkhill, J., Wren, B.W., Mungall, K., Ketley, C., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Witehead, S. and Barrell, B.G. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403: 665-668

Pel, P.K. (1899) De ziekten van de Maag met het oog op de behoeften der geneeskundige praktijk geschetst. Ed. by Bohn., Harlem

Phadnis, S. H., Ilver, D., Janzon, L., Normark, S. and Westblom, T. U. (1994). Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. Infect. Immun. 62: 1557-1565

Pollack, J.D. (2001) *Ureaplasma urealyticum*: an opportunity for combinatorial genomics. Trends Microbiol. 9 (4): 169-175

Puls, J., Fischer, W. and Haas, R. (2002). Activation of *Helicobacter pylori* CagA by tyrosine phosphorylation is essential for dephosphorylation of host cell proteins in gastric epithelial cells. Mol. Microbiol. 43: 961-969

Rad, R., gerhard, M., Lang, R. (2002). The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J. Immunol. 168: 3033-3041

Rain, J.-C., Selig, L., De Reuse, H., Battaglia, V., Reverdy, C., Simon, S., Lenzen, G., Petel, F., Wojcik, J., Schächter, V., Chemama, Y., Labigne, A. and Legrain, P. (2001) The protein-protein interaction map of *Helicobacter pylori*. Nature 409: 211-215

Rao, M.J.K. and Argos, P. (1986). A comformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta 869: 197-214

Rappin (1881) Contribution à l´étude des bactéries da la bouche a l´état normal et dans la fièvre typhoide. PhD thesis. Collége de France, Nantes

Rektorschek, M., Buhmann, A., Weeks, D., Schwan, D., Bensch, K. W., Eskandari, S., Scott, D., Sachs, G. and Melchers, K., (2000). Acid resistance of *Helicobacter pylori* depends on the UreI membrane protein and an inner membrane proton barrier. Molecular Microbiology 36 (1): 141-152

Rektorschek, M., Weeks, D., Sachs, G. and Melchers, K. (1998) Influenc of pH on Metabolism and Urease Activity of *Helicobacter pylori*. Gastroenterology 115: 628-641

Reuben, M. A., Lasater, L. S., and Sachs, G. (1990). Characterization of a beta subunit of the gastric H+/K(+)-transporting ATPase. Proc. Natl. Acad. Sci. USA 87: 6767-6771

Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G. D. and Gold, L. (1992). Translation initiation in *Escherichia coli*: sequences within the ribosome-binding site. Mol. Microbiol. 6: 1219-1229

Sachs, G., Scott, D., Weeks, D. and Melchers, K. (2002) The compartment buffered by the urease of *Helicobacter pylori*: cytoplasm or periplasm? Trends Microbiol. 10 (5): 217-218

Salama, N., Guillemin, K., McDaniel, T.K., Sherlock, G., Tompkins, L. and Falkow, S. (2000) A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. Proc. Natl. Acad. Sci. USA 97 (26): 14668-14673

Salomon, H. 1896, Über das Spirillium des Säugetiermagens und sein Verhalten zu den Belegzellen. Zentralblatt für Bakteriol. Parasitenkd. Infektionskr. 19: 434-442

Santoni, V., Molloy, M and Rabilloud, T. (2000) Membrane proteins and proteomics: un amour impossible? Electrophoresis 21: 1054-1070

Schade, C. *et al*. (1994) Hydrogen ion concentration in the mucus layer on tooop of acid-stumulated and –inhibited rat gastric mucosa. Gastroenterology 107: 180-188

Schade, C., Flemstrom, G. and Holm, L. (1994) Hydrogen ion concentration in the mucus layer on top of acid-stimulated and –inhibited rat gastric mucosa. Gastroenterologiea 107: 180-188

Schilling, C.H., Covert, M.W., Famili, I., Church, G.M., Edwards, J.S. and Palsson, B.O. (2002) Genome-Scale metabolic model of *Helicobacter pylori* 26695. J. Bacteriol. 184 (16): 4582-4593

Schneider, W. and Altendorf, K. (1987) Bacterial adenosine 5'-triphosphate synthase (F_1F_0): purification and reconstitution of F_0 complexes and biochemical and functional characterization of their subunits. Mirobiol. Rev. 51: 477-497

Schurr, T., Nadir, E. and Margalit., H. (1993). Identification and characterization of *E. coli* ribosomal binding sites by free energy computation. Nucleic Acids Res. 21: 4019-4023

Schwartz, K. (1910) Über penetrierende Magen-und Jejunal-Geschwüre. Beitr. Klin. Chir. 67: 96-128

Scott, D.r., Marcus, W.A., Weeks, D.L., Lee, A., Melchers, K. and Sachs, G. (2000) Exptression of the *Helicobacter pylori* ureI Gene is required for Acidic pH activation of cytoplasmic urease. Infect. Imm. 68 (2): 470-477

Scott, D.R., Weeks, D., Hong, C. Postius, S. Melchers, K. and Sachs, G. (1998) The role of internal urease in acid resistance of *Helicobacter pylori*. Gastroenterologia 114: 58-70

Seelert, H., Poetsch, A., Rohlfs, M. and Dencher, N.A. (2000) Dye-ligand chromatographic purification of intact multisubunit membrane protein complexes: application to the chloroplast H^+-F_0F_1-ATP synthase. Biochem J 346 (1): 41-44

Senior, A.E. (1988) ATP synthesis by oxidative phosphorylation. Physiol. Rev. 68 (1): 177-231

Senior AE, Nadanaciva S, Weber J.The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase. Biochim Biophys Acta 2002 Feb 15;1553(3):188-211

Smith, D.G., Russell W.C., Ingledew W.J. and Thirkell, D. (1993) Hydrolysis of urea by *Ureaplasma urealyticum* generates a transmembrane potential with resultant ATP synthesis. J. Bacteriol. 175 (11): 3253-3258

Steer, H.W. and Colin-Jones, D.G. (1975) Mucosal changes in gastric ulceration and their response to carbenoxolone sodium. Gut 16: 590-597

Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W. J. and Covacci, A. (2002). C-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. Mol. Microbiol. 43: 971-980

Stingl, K., Uhlemann, E.M., Deckers-Hebestreit, G., Schmid, R., Bakker, E.P. and Altendorf, K. (2001) Prolonged Survival and cytoplasmic pH homeostasis of *Helicobacter pylori* at pH 1. Infect. Immun. 69: 1178-1180

Stingl, K., Altendorf, K. and Bakker, E.P. (2001) Acid survival of *Helicobacter pylori*: how does urease activity trigger cytoplasmic pH homeostasis? Trends Microbiol. 10 (2): 70-74

Stock, D., Leslie, A.G.W. and Walker, J.E. (1999) Molecular architecture of the rotary motor in ATP synthase. Science 286: 1700-1705

Stover, C., Pham, X., Erwin, A., Mizoguchi, S., Warrener, P. Hickey, M., Brinkman, F., Hufnagle, W., Kowalik, D., Lagrou, M. *et al*., (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406: 959-964

Suerbaum, S. and Michetti, P. (2002) *Helicobacter pylori* infection. New Eng. J. Med. 347 (15): 1175-1186

Suerbaum, S., Josenhans, C. and Labigne, A. (1993). Cloning and genetic characterization of the Heicobacter pylori and *Helicobacter* mustelae flaB flagellin genes and construction of *H. pylori flaA*- and *flaB*-negative mutants by elctropopration-mediated allelic exchange. J. Bacteriol. 175 (11): 3278-3288

Szabo, I., Brutsche, S., Tombola, F. *et al*. (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. EMBO J. 18: 5517-5527

Teyssen, S., Chari, S.T., Scheid, J. and Singer, M.V. (1995) Effect of repeated boluses of intravenous omeprazole and primed infusions of ranitidine on 24-hour intragastric pH in healthy human subjects. Digest Dis. Sci. 40: 247-255

Tomashek, J.J. and Brusilow, W.S.A. (2000) Stochiometry of energy coupling by proton-translocating ATPases: a history of variability. J. Bioenerg. Biomembr. 32: 493-500

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G. *et al*. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature 388: 539-547

Tsuda, M. Karita, M. Morshed, M.G., Okita, K. and Nakazawa, T. (1994) A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect. Immun. 62: 3586-3589

Turner, G.A., Logan, R.P.H., Chinnery, R., Cockayne, A., Hawkey, C.J. and Boriello, S.P. (1997). Trefoil peptides are unique chemotaxins for *Helicobacter pylori* . Gastroenterology 112: A1107 (abstract)

Tusnády and I. Simon (2001) The HMMTOP transmembrane topology prediction server. Bioinformatics 17: 849-850

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N. and Schlemper, R.J. (2001) *Helicobacter pylori* infection and the development of gastric cancer. New Eng. J. Med. 345: 784-789

Van Vliet, A.H.M., Kuipers, E.J, Waidner, B., Davies, B.J., de Vries, N., Penn, C.W., Vandenbroucke-Grauls, C.M.J.E., Kist, M., Bereswill, S. and Kusters, J.G. (2001) Nickel-responsive induction of urease expression in *Helicobacter pylori* is mediated at the transcriptional level. Infect. Immun. 69: 4891-4897

Van Vliet, A.H.M., Poppelaars, S.W., Davies, B.J., Stoof, J., Bereswill, S., Kist, M., Penn, C.W., Kuipers, E.J. and Kusters, J.G. (2002) NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. Infec. Immun. 70: 2846-2852

Vik, S.B., Long, J.C., Wada, T. and Zhang, D.A. (2000) Model for the structure of subunit a of the *Escherichia coli* ATP synthase and its role in proton translocation. Biochim Biophys Acta: 1458 (2-3): 457-66

Von Heijne, G. (1986) The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology. EMBO J. 5: 3021-3027

Von Heijne, G. and Gavel, Y. (1988) Topogenic signals in integral membrane proteins. Eur. J. Biochem., 174: 671-678

Walker, J.E. (1998) ATP synthesis by rotary catalysis. Angew. Chem. Int. Ed. 37: 2309-2319

Warren, J.R. and Marshall, B.J. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 4: 1273-1275

Watanabe, T., Tada, M., Nagai, H., Sasaki, S., Nakao, M. (1998) *H. pylori* infection induces gastric cancer in *Mongolian gerbils*. Gastroenterology 115: 642-648

Weber, J. und Senior, A.E. (1997) Catalytic mechanism of F1-ATPase. *Biochim. Biophys. Acta* 1319: 19-58

Weeks, D. and Sachs, G. (2001) Sites of pH regulation of the urea channel of *Helicobacter pylori*. Mol. Microbiol. 40 (6): 1249-1259

Weeks, D.L., Eskandari, S., Scott, D.R. and Sachs, G. (2000) A H^+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Science 287: 482-485

Wickner, W.T. and Lodish, H.F. (1985) Multiple mechanisms of protein insertion into and across membranes. Science: 230 (4724): 400-407

Wilkens, S., Zhou, J., Nakayama, R., Dunn, S.D. and Capaldi, J. (2000) Localization of the delta subunit in the *Escherichia coli* F_1F_0-ATPsynthase by immuno electron microscopy: the delta subunit binds on top of the F_1. Mol. Biol. 295: 387-391

Worku, M., Sidebotham, R.L., Wren, B.W. and Karim, Q.N. (1997) Chemotaxis of *H. pylori* in presence of human plasma. Gut 41: A25 (abstract)

Yoshiyama, H., Nakamura, H., Komoto M., Okita K. and Nakazawa T. (1999) Chemotaxis and motility of *Helicobacter pylori* in a viscous environment. J. Gastroenterol. 34: 18-23