Characterization of the mechanisms of two-component signal transduction involved in motility and chemotaxis of *Helicobacter pylori*

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Erklärung

Die vorliegende Arbeit wurde am Lehrstuhl für Mikrobiologie der bayerischen Julius-Maximilians-Universität Würzburg von Oktober 2001 bis April 2005 unter Betreuung von PD. Dr. Dagmar Beier angefertigt.

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt wurde. Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Würzburg, den ……

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A SUMMARY

Flagellar motility and chemotaxis are essential virulence traits required for the ability of *Helicobacter pylori* to colonize the gastric mucosa. The flagellar regulatory network and the complex chemotaxis system of *H. pylori* are fundamentally different from other bacteria, despite many similarities.

In *H. pylori* expression of the flagella is controlled by a complex regulatory cascade involving the two-component system FlgR-HP244, the sigma factors σ^{54} and σ^{28} and the anti-σ^{28} factor FlgM. Thus far, the input signal for histidine kinase HP244, which activates the transcriptional regulator FlgR, which triggers σ^{54}-dependent transcription of the flagellar class 2 genes, is not known. Based on a yeast two-hybrid screen a highly significant protein-protein interaction between the *H. pylori* protein HP137 and both the histidine kinase HP244 and the flagellar hook protein HP908 (FlgE´) has been reported recently (Rain *et al.*, 2001). So far, no function could be assigned to HP137. Interestingly, the interaction between HP137 and histidine kinase HP244 was observed in the characteristic block N sequence motif of the C-terminal ATP-binding kinase domain. In this work a potential role of HP137 in a feedback regulatory mechanism controlling the activity of histidine kinase HP244 in the flagellar regulation of *H. pylori* was investigated.
ORF *hp137* was substituted with a non-polar kanamycin resistance cassette in *H. pylori* G27. The resulting deletion mutant G27/hp137::km was non-motile on semi-solid agar plates. Moreover, quantification of the transcription of the σ^{54}-dependent flagellar genes *flaB*, *flgB*, *flgE*, and ORF *hp906* using primer extension experiments demonstrated that these genes showed enhanced transcription in the deletion mutant, while transcription of the σ^{28}-dependent flagellar gene *flaA* in the deletion mutant was unchanged as compared to the wild-type. For the complementation of G27/hp137::km two different strategies were applied resulting in non-motile mutants. Using *in vitro* phosphorylation assays only a minor inhibitory effect of HP137 on the autophosphorylation of HP244 was observed. Finally, a deletion mutant of the *Campylobacter jejuni* ortholog of *hp137*, *cj73c* was constructed. The resulting mutant showed a swarming behaviour on motility agar plates similar to the *C. jejuni* wild-type.

Although the substitution of the gene encoding HP137 by a kanamycin cassette resulted in non-motile bacteria, the failure to restore motility by the reintroduction of *hp137 in cis* into the mutant strain, and the observation that HP137 has no significant effect on the activity of histidine kinase HP244 *in vitro* indicated that HP137 is not directly involved in flagellar regulation. Therefore, it was demonstrated that HP137 does not participate in the regulation of flagellar gene expression, neither in *H. pylori* nor in the closely related bacterium *C. jejuni*.

Chemotactic signal transduction in *H. pylori* differs from the enterobacterial paradigm in several respects. In addition to a CheY response regulator protein (CheY1) *H. pylori* contains a CheY-like receiver domain (CheY2) which is C-terminally fused to the histidine kinase CheA. Furthermore, the genome of *H. pylori* encodes three CheV proteins consisting of an N-terminal CheW-like domain and a C-terminal receiver domain, while there are no orthologues of the chemotaxis genes *cheB*, *cheR*, and *cheZ*. To obtain insight into the mechanism controlling the chemotactic response of *H. pylori* the phosphotransfer reactions between the purified two-component signalling modules were investigated *in vitro*.
Using *in vitro* phosphorylation assays it was shown that both *H. pylori* histidine kinases CheAY2 and CheA´ lacking the CheY-like domain (CheY2) act as ATP-dependent autokinases. Similar to other CheA proteins CheA´ shows a kinetic of phosphorylation represented by an exponential time course, while the kinetics of phosphorylation of CheAY2

is characterized by a short exponential time course followed by the hydrolysis of CheAY2~P. Therefore, it was demonstrated that the presence of the CheY2-like receiver domain influences the stability of the phosphorylated P1 domain of the CheA part of the bifunctional protein. Furthermore, it was proven that both CheY1 and CheY2 are phosphorylated by CheAY2 and CheA´~P and that the three CheV proteins mediate the dephosphorylation of CheA´~P, although with a clearly reduced efficiency as compared to CheY1 and CheY2.
Moreover, CheA´ is capable of donating its phospho group to the CheY1 protein from *C. jejuni* and to CheY protein from *E. coli*. Retrophosphorylation experiments indicated that CheY1~P is able to transfer the phosphate group back to the HK CheAY2 and the receiver domain present in the bifunctional CheAY2 protein acts as a phosphate sink fine tuning the activity of the freely diffusible CheY1 protein, which is thought to interact with the flagellar motor.

Hence, in this work evidence of a complex phosphorelay in the chemotaxis system was obtained which has similarities to other systems with multiple CheY proteins. The role of the CheV proteins remain unclear at the moment, but they might be engaged in a further fine regulation of the phosphate flow in this complex chemotaxis system and the independent function of the two domains CheA´ and CheY2 is not sufficient for normal chemotactic signalling *in vivo*.

ZUSAMMENFASSUNG

Flagellen-basierte Motilität und Chemotaxis stellen essentielle Pathogenitätsfaktoren dar, die für die erfolgreiche Kolonisierung der Magenschleimhaut durch *H. pylori* notwendig sind. Die Mechanismen der Regulation der Flagellensynthese und das Chemotaxis-System von *H. pylori* weisen trotz einiger Ähnlichkeiten fundamentale Unterschiede zu den Systemen anderer Bakterien auf.

In *H. pylori* ist die Flagellensynthese durch eine komplex regulierte Kaskade kontrolliert, die Regulatorkomponenten wie das Zweikomponentensystem HP244/FlgR, die Sigma Faktoren σ^{54} und σ^{28} und den σ^{28}-Antagonisten FlgM enthält. Das Signal, welches über die Histidinkinase des Zweikomponentensystems HP244/FlgR die Expression der σ^{54}-abhängigen Klasse 2 Flagellengene reguliert, ist bisher noch nicht bekannt. Allerdings konnte mit HP137 ein Protein identifiziert werden, das im „yeast two-hybrid" System sowohl mit der korrespondierenden Kinase HP244 des Flagellenregulators FlgR, als auch mit der Flagellenkomponente FlgE´ interagiert (Rain *et al.*, 2001). In dieser Arbeit wurde eine mögliche Rolle von HP137 in einem Rückkopplungsmechanismus untersucht, welcher die Aktivität der Histidinkinase in der Flagellenregulation kontrollieren könnte.
ORF *hp137* wurde in *H. pylori* G27 durch eine Kanamycin-Resistenzkassette ersetzt. Die Mutante G27/hp137::km war auf Motilitätstsagar nicht mehr motil. Mit Hilfe von Primer-Extensionexperimenten konnte gezeigt werden, dass die Transkription der σ^{54}-abhängigen Gene, *flaB*, *flgB*, *flgE*, und ORF *hp906* in der Deletionsmutante deutlich aktiviert wird, die Transkriptmenge des σ^{28}- abhängigen Gens *flaA* im Vergleich zu G27 jedoch nahezu unverändert bleibt. Zwei verschiedene Strategien wurden für die Komplementation der Deletionsmutante G27/hp137::km benutzt, die jedoch nicht zum Erfolg führten. Durch *in vitro* Phosphorylierungsstudien wurde nur eine geringe Hemmung der Autophosphorylierung von HP244 durch HP137 beobachtet. Schließlich wurde das Orthologe Gen von *hp137* in *C. jejuni* durch eine Kanamycin-Resistenzkassette ersetzt. Die resultierende Mutante zeigte auf Motilitätsagar eine normale Motilität vergleichbar dem Wildtypstamm.

Obwohl die Deletion des ORF *hp137* zu einer unbeweglichen Mutante führte, legen die erfolglosen Komplementations Experimente, sowie die Beobachtung, dass HP137 *in vitro* keinen bedeutenden Effekt auf die Aktivität der Histidinkinase HP244 hat nahe, dass HP137 weder in *H. pylori* noch im nahe verwandten C. *jejuni* direkt an der Flagellenregulation beteiligt ist.

Das Chemotaxis-System von *H. pylori* unterscheidet sich vom gutuntersuchten Chemotaxis-System der Enterobakterien in einigen Aspekten. Zusätzlich zu dem CheY Response Regulator Protein (CheY1) besitzt *H. pylori* eine weitere CheY-artige Receiver-Domäne (CheY2) welche C-terminal an die Histidinkinase CheA fusioniert ist. Zusätzlich finden sich im Genom von *H. pylori* Gene, die für drei CheV Proteine kodieren die aus einer N-terminalen Domäne ähnlich CheW und einer C-terminalen Receiver Domäne bestehen, während man keine Orthologen zu den Genen *cheB*, *cheR*, and *cheZ* findet. Um einen Einblick in den Mechanismus zu erhalten, welcher die chemotaktische Reaktion von *H. pylori* kontrolliert, wurden Phosphotransferreaktionen zwischen den gereinigten Signalmodulen des Zweikomponentensystems *in vitro* untersucht.

Durch *in vitro*-Phosphorylierungsexperimente wurde eine ATP-abhängige Autophosphorylierung der bifunktionellen Histidinkinase CheAY2 und von CheA´, welches ein verkürztes Derivat von ChAY2 ohne Receiver-Domäne darstellt, nachgewiesen. CheA´ zeigt eine für an der Chemotaxis beteiligte Histidinkinasen typische

Phosphorylierungskinetik mit einer ausgeprägten exponentiellen Phase, während die Phosphorylierungskinetik von CheAY2 nur eine kurze exponentielle Phase aufweist, gefolgt von einer Phase in der die Hydrolyse von CheAY2~P überwiegt. Es wurde gezeigt, dass die Anwesenheit einer der CheY2 Domäne die Stabilität der phosphorylierten P1 Domäne im CheA Teil des bifunktionellen Proteins beeinflusst. Außerdem wurde gezeigt, dass sowohl CheY1 als auch CheY2 durch CheAY2 phosphoryliert werden und dass die drei CheV Proteine die Histidinkinase CheA´~P dephosphorylieren, wenn auch mit einer im Vergleich zu CheY1 und CheY2 geringeren Affinität. Außerdem ist CheA´ in der Lage seine Phosphatgruppen auf CheY1 aus *C. jejuni* und CheY aus *E. coli* zu übertragen. Retrophosphorylierungsexperimente weisen darauf hin, dass CheY1~P die Phosphatgruppe zurück auf die Histidinkinase CheAY2 übertragen kann und dass die CheY2-Domäne in dem bifunktionellen Protein CheAY2 als „Phosphat Sink“ agiert der den Phosphorylierungszustand und damit die Aktivität des frei diffundierbaren Proteins CheY1 reguliert, das vermutlich es mit dem Flagellenmotor interagiert. Es konnte weiterhin gezeigt werden, dass die unabhängige Funktion der beiden Domänen CheA´ und CheY2 für eine normale chemotaktische Signalgebung *in vivo* nicht ausreicht.

In dieser Arbeit wurden also Hinweise auf eine komplexe Kaskade Phosphatübertragungsreaktionen im chemotaktischen System von *H. pylori* gefunden, welches Ähnlichkeiten zu dem Syteme-Chemotaxis von *S. meliloti* aufweist an denen multiple CheY Proteine beteiligt sind. Die Rolle der CheV Proteine bleibt im Moment unklar, jedoch könnte es sein, dass sie an einer weiteren Feinregulierung der Phosphatgruppenübertragungsreaktionen in diesem komplexen chemotaktischen System beteiligt sind

B INTRODUCTION

1. General characteristics of *Helicobacter pylori*

In 1893 Bizzozero published drawings of dog stomachs infected with a gastric bacterium, which is now known as *Helicobacter heilmanni* (Bizzozero, 1893), therefore the assumption that no bacteria can colonize the highly acidic stomach lumen was abolished. Years later independent reports about the discovery of spiral organisms in the human stomach and the association of these organisms with gastric diseases were published (Freedberg & Barron, 1940; Fung *et al.*, 1979; Krientnitz, 1906; Luger & Neuberger, 1920; Steer, 1975). This human gastric bacterium was first isolated in 1982 by Warren and Marshall from a patient with a chronic active gastritis and it was first classified as *Campylobacter pylori* (Marshall & Warren, 1984). Later it was observed that the sequence of the 16S rRNA, the fatty acid content, and the flagella of *C. pylori* were different from the members of the *Campylobacter* genus, hence the new genus *Helicobacter* was defined (Goodwin *et al.*, 1989). *H. pylori* is a Gram-negative, spiral-shaped, neutrophilic, and microaerophilic bacterium that colonizes and survives in the low pH environment of the human stomach, which is the only known habitat, and is grouped together with the other members of the genus *Helicobacter*, and with the genera *Campylobacter* and *Wolinella* into the group of the ε-proteobacteria.

To date is known that half of the world's population is infected with *H. pylori* and in developing countries the rate of infected people reaches up to 90% (Feldman *et al.*, 1998). The infection is usually acquired during the childhood and can persist for the whole life. The first histological evidence of the infection is an acute gastritis, from which, if left untreated, a chronic active gastritis can develop (Blaser, 1990; Marshall & Warren, 1984; Nomura *et al.*, 1994). The chronic gastritis can persist for decades or life long and about 10-20% of the infected individuals can develop a gastric ulcer, duodenal ulcer, gastric lymphoma or gastric cancer (Ahmad *et al.*, 2003; EUROGAST, 1993; Feldman *et al.*, 1998; Nomura *et al.*, 1994; Parsonnet *et al.*, 1991; Peek & Blaser, 2002; Wotherspoon *et al.*, 1993). Both microbial (genetic diversity of *H. pylori*) and host factors (cultural, socioeconomic status, diet, environmental factors, and the genetic predisposition) contribute to different rates of infection in the world and to the development of different disease outcomes (Covacci *et al.*, 1993; Graham *et al.*, 1991; Malaty *et al.*, 1994; Weel *et al.*, 1996). The mode of transmission of *H. pylori* is unknown, but the most widely held hypotheses are that the organism is transmitted by the oral-oral route and/or the oral-faecal route (Megraud, 1995).

2. Colonization and virulence factors of *H. pylori*

The colonization of the human stomach by *H. pylori* occurs in three steps: (1) colonization of the gastric mucosa by the bacterium, (2) adaptation, and (3) persistence of the bacterium within the gastric mucous layer and pathogenesis. To assure the colonization *H. pylori* possesses several virulence factors (Fig.1). The virulence factors which have been identified include urease (Cussac *et al.*, 1992), vacuolating cytotoxin VacA (Cover *et al.*, 1994), CagA protein (**c**ytotoxin-**a**ssociated **g**en **A)**, and the *cag* pathogenicity island (Covacci *et al.*, 1993; Tummuru *et al.*, 1993), flagella (Suerbaum *et al.*, 1993), adhesins (Boren *et al.*, 1993; Ilver *et al.*, 1998; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 1999), *H. pylori* neutrophil-activating protein (HP-NAP) (Evans *et al.*, 1995; Satin *et al.*, 2000), and collagenase (Kavermann *et al.*, 2003). As in other organisms the ability to acquire iron ions also seems to contribute to the virulence of *H. pylori* (Fassbinder *et al.*, 2000; Velayudhan *et al.*, 2000)

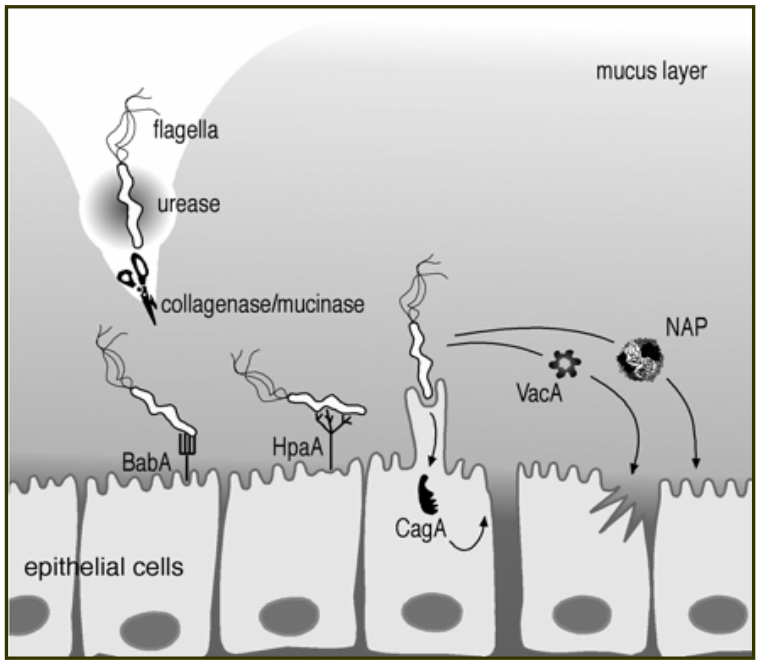

Fig.1 Summary of the main steps involved in *H. pylori* colonization, and its virulence factors. BabA and HpaA: two different adhesins; CagA: Cytotoxin associated gene A; VacA: Vacuolating cytotoxin A; NAP: neutrophil-activating protein (Covacci & Rappuoli, 2003).

2.1. Colonization of the gastric mucosa by *H. pylori*

Flagella-based chemotactic motility and urease are essential factors in the colonization of *H. pylori* (Andrutis *et al.*, 1997; Eaton & Krakowka, 1994; Eaton *et al.*, 1996; Josenhans *et al.*, 1995; Tsuda *et al.*, 1994). *H. pylori* swims into the mucus layer to contact the gastric epithelial cells. Thus the organism uses the flagella, whose motion is controlled by

chemotactic stimuli. For instance the ability of *H. pylori* to sense and move towards urea, bicarbonate ions and sodium ions is known (Mizote *et al.*, 1997).

Urea is necessary for the survival of *H. pylori* in the acidic environment of the gastric mucosa. The urea is synthesized in the liver, circulated by the blood stream at an average concentration of 5 mM (Neithercut *et al.*, 1993), and secreted into the gastric juice through capillary networks beneath the gastric epithelial surface. Consequently, a concentration gradient of urea is formed in the gastric mucus layer that could be sensed by *H. pylori*, which hydrolyzes the urea to produce ammonia. Therefore, a neutral cytoplasmic and periplasmic pH is maintained during the infection and the bacteria are protected from the attack by gastric acid. If *H. pylori* accomplishes the initial phase of the colonization, it is able to adhere to the gastric epithelium. Finally, the VacA toxin causes cytotoxic damage to the gastric epithelium and the CagA protein stimulates the activation of nuclear factors associated with the release of inflammatory mediators, such as IL-8 which attracts macrophages and neutrophils. Hence, an inflammatory reaction is produced. This reaction is the basis of all the pathologic outcomes of the infection with *H. pylori*.

2.2. Virulence factors of *H. pylori*

Urease

H. pylori produces large amounts of urease, a nickel-containing hexameric heterodimer enzyme that catalyzes the hydrolysis of urea in ammonia and carbon dioxide for neutralizing gastric acidity, thereby giving the bacteria a neutral microenvironment for their survival. A gene cluster containing seven genes encodes the two structural subunits of the enzyme, UreA and UreB, that bind Ni^{2+} ions, and the accessory proteins UreIEFGH for urea transport and nickel incorporation into the urease apoenzyme (Hu & Mobley, 1990; Labigne *et al.*, 1991; Meyer-Rosberg *et al.*, 1996; Mobley *et al.*, 1995; Scott *et al.*, 1998).

In contrast to other urease-producing bacteria, *H. pylori* releases large amounts of urease into the environment. Nevertheless, due to its pH optimum from 7.5 to 8.0 urease is active only intracellularly to generate a cytoplasmic and periplasmic neutral pH (Sachs *et al.*, 2003; Scott *et al.*, 2002; Scott *et al.*, 1998). Active secretion of urease was initially proposed (Vanet & Labigne, 1998), but most evidence now suggests that the enzyme appears on the cell surface as a consequence of cell lysis (Marcus & Scott, 2001).

The uptake of urea by *H. pylori* is achieved through the inner membrane proton-gated channel UreI that openes only at low pH. Thus, the pH-gated channel permits the access of the substrate urea to the bacterial cell in response to acidic pH. At neutral pH the urea channels

are blocked in order to avoid over-alkalinization (Rektorschek *et al.*, 2000; Weeks *et al.*, 2000).

Motility

Flagella-based motility has been demonstrated to be an essential virulence trait of *H. pylori* since non-motile mutants lacking one or both flagellins, FlaA or FlaB, are unable to colonize in the gnotobiotic piglet model (Eaton *et al.*, 1996; Josenhans *et al.*, 1995).

H. pylori possesses an unipolar bundle of two to eight flagella. Each flagellum is covered by a sheath that is an extension of the outer membrane and forms a bulb-like structure at the distal end of the flagellum. The sheath protects the flagellar filament from the stomach acid (Geis *et al.*, 1993; Suerbaum, 1995). Similar to enteric bacteria *H. pylori* flagella have three structural components (Fig.2): (1) a basal body, which is embedded in the cell wall and contains the proteins required for rotation; (2) an external helically shaped filament, which is composed of the major flagellin FlaA and the minor subtype FlaB, and which works as a propeller; and (3) a hook that serves as a joint between the basal body and the flagellar filament (Kostrzynska *et al.*, 1991; Leying *et al.*, 1992; Suerbaum *et al.*, 1993).

Most of the flagellar apparatus is localized beyond the cytoplasmic membrane. Thus, many of the flagellar proteins have to cross the membrane to reach their final destination. Components of the P and L rings are secreted by the conventional signal peptide-dependent Sec pathway. Instead the structural proteins of the filament, the hook and the rod proteins are transported by a specialized flagellum-specific pathway that possesses components with homology to type III secretion systems, which in other organism have been found to be involved also in the secretion of virulence factors (Barinaga, 1996; Covacci, 1996).

Adhesins

Adhesins are outer membrane proteins that can recognize some receptors on the host cell surface. Initially, two different types of adhesins were characterized, BabA2 with the Lewis b (Le^b) blood-group antigen as a host receptor and the outer membrane proteins AlpA and AlpB, which are adhesion-associated lipoproteins (Boren *et al.*, 1993; Ilver *et al.*, 1998; Odenbreit *et al.*, 1999). Moreover, adhesion of the HpaA protein and a lectin to sialylated glycoconjugates and to the sialic residues of laminin, respectively, was observed (Evans *et al.*, 1993; Lingwood *et al.*, 1993; Valkonen *et al.*, 1993). Recently, a new adhesin named SabA was identified, which binds to sialylated Lewis X antigens expressed during chronic inflammation (Mahdavi *et al.*, 2002).

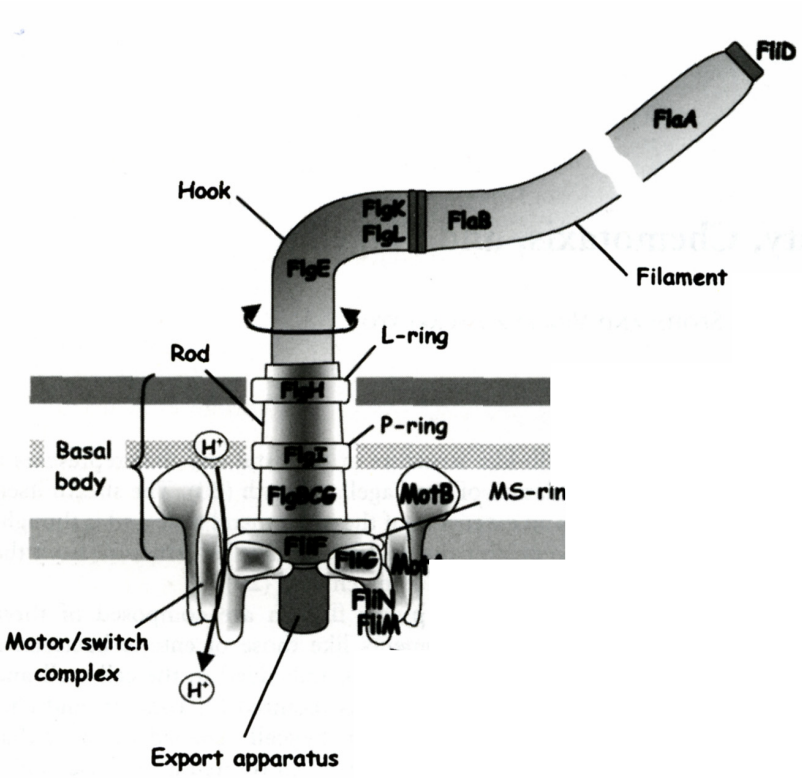

Fig.2 Schematic diagram of the *H. pylori* flagellar structural components. The protein components of the major elements of the flagellum, i.e. the external filament, the hook, the basal body, and the motor-switch complex are shown (Spohn, 2001b).

VacA

H. pylori vacuolating cytotoxin VacA is present in half of the *H. pylori* isolates and produces large cytoplasmic vacuoles in cultured cells (Cover & Blaser, 1992; Del Giudice *et al.*, 2001). The *vacA* gene encodes for a pro-toxin of approximately 140 kDa. An amino-terminal signal sequence and a carboxy-terminal fragment are proteolytically cleaved during the process of VacA secretion, which is accomplished by the autotransporter or type V secretion mechanism, and a 88 kDa mature toxin is released (Cover & Blaser, 1992; Fischer *et al.*, 2001; Schmitt & Haas, 1994). This mature toxin affects the physiological state of the stomach cells and tissues in order to favour *H. pylori* growth. For the binding of VacA to target cells, several receptors have been suggested, including the epidermal growth factor receptor (EGFR), receptor protein tyrosine phosphatases (RPTP)-α (Yahiro *et al.*, 2003), and RPTP-β (also named protein tyrosine phosphatase receptor zeta [Ptprz]) (Fujikawa *et al.*, 2003). The N-terminal region of VacA forms anion-selective chloride channels in lipid membranes (Kim *et al.*, 2004; Szabo *et al.*, 1999; Tombola *et al.*, 1999). Therefore, VacA induces the secretion of bicarbonate anions that could contribute to the buffering of the apical domain of the tissue and that could enter into the carbon cycle of *H. pylori* (Debellis *et al.*, 2001). In addition VacA could contribute to

bacterial growth also by inducing the release of urea on the apical domain. Given the importance of urea in *H. pylori* metabolism and survival, the finding that urea permeates out of the cell through the VacA channel may be a very relevant one (Tombola *et al.*, 2001). Interestingly, VacA was found to target not only ephitelial cells but also cells of the immune system and proved to have immunomodulatory activity. VacA inhibits the processing of antigenic peptides in B cells and their presentation to human $CD4^+$T-cells (Molinari *et al.*, 1998). Recently, VacA was also reported to alter T-cell function by inhibiting T-cell proliferation, suggesting a possible mechanism of how *H. pylori* might evade the adaptive immune response to establish a chronic infection (Boncristiano *et al.*, 2003; Sundrud *et al.*, 2004).

Pathogenicity island (PAI) and CagA protein

H. pylori contains a 40 kb pathogenicity island (PAI) named *cag* containing genes that encode components of a functional type IV secretion system and for the cytotoxin CagA that is associated with atrophic gastritis, peptic-ulcer disease and gastric carcinoma (Blaser & Berg, 2001; Blaser *et al.*, 1995; Parsonnet *et al.*, 1997). Based on the presence of the *cag*-PAI *H. pylori* isolates are divided into two groups: type I strains, containing the *cag*-PAI; and type II strains, lacking the *cag*-PAI. The type I strains are associated with higher grades of gastric or duodenal ulceration and are more virulent than the type II strains, which are predominantly isolated from asymptomatic carriers (Censini *et al.*, 1996; Kuipers *et al.*, 1995).

The cytotoxin CagA is translocated into gastric epithelial cells using the type IV secretion system encoded by the *cag*-PAI (Asahi *et al.*, 2000; Odenbreit *et al.*, 2000). After translocation, CagA is phosphorylated at one or several tyrosine phosphorylation motifs (EPIYA) by a Src kinase (Püls *et al.*, 2002; Selbach *et al.*, 2002; Stein *et al.*, 2000; Stein *et al.*, 2002). The phosphorylated CagA inhibits the activity of Src kinase in a negative feedback loop, which causes the dephosphorylation of a set of host cell proteins, including the actin-binding protein cortactin (Püls *et al.*, 2002; Selbach *et al.*, 2003). Phosphorylated CagA is recruited to the plasma membrane and interacts with the Src-homology 2 (SH2) domain of the protein tyrosine phosphatase SHP-2, thus stimulating its phosphatase activity (Higashi *et al.*, 2001). Under normal conditions SHP2 transmits a positive signal for the cell growth and motility, but CagA induces an abnormal proliferation and movement of gastric ephitelial cells, represented by cell scattering (cell spreading) and the “hummingbird” phenotype (cellular elongation). CagA also interacts with other signalling molecules, such as growth factor receptor-binding protein 2 (Grb2), hepatocyte growth factor (HGF)/scatter factor receptor c-

Met, and phospholipase Cγ (PLC-γ) (Churin *et al.*, 2003; Mimuro *et al.*, 2002). Furthermore, *cag*-positive strains cause the induction of the transcription factor NF-κB with consequent secretion of the proinflammatory cytokine IL-8 (Sharma *et al.*, 1998). In addition these virulent strains induce reorganization of the host cell cytoskeleton with the consequence of pedestal formation (Segal *et al.*, 1999).

Collagenase

Recently, the presence of a collagenase in *H. pylori* was reported and it was classified as a new essential virulence factor for this organism (Kavermann *et al.*, 2003), since mutants with an inactivated ORF *hp169* (encoding collagenase) could not colonize Mongolian gerbils (Kavermann *et al.*, 2003).

Collagen type I and III are important components of the extracellular matrix of the stomach epithelium. The synthesis of type I collagen is induced in regions around gastric ulcers and collagens are important for ulcer healing (Gillesen *et al.*, 1995). For *H. pylori* the degradation of collagen might be used as a source for the uptake of certain amino acids or short peptides, but so far the function of this new factor has not been determined.

3. Transcriptional regulators of *H. pylori*

The genome sequences of *H. pylori* 26695 and J99 were published in 1997 and 1999, respectively (Alm *et al.*, 1999; Tomb *et al.*, 1997). Analysis of these two *H. pylori* sequences revealed a low number of genes that encode for putative regulatory proteins. This number is approximately half of the number of regulatory genes reported for *Haemophilus influenzae* which possesses a genome size similar to *H. pylori* (Fleischmann *et al.*, 1995) and less than a quarter of the number of regulatory genes predicted for *E. coli* (Mizumo, 1997). It may reflect the adaptation of *H. pylori* to survive in the mucus layer and the absence of competition with other organisms in the same niche.

Eighteen genes are predicted to be associated with transcriptional regulation in *H. pylori*: three RNA polymerase sigma factors (Alm *et al.*, 1999; Beier *et al.*, 1998; Tomb *et al.*, 1997), three histidine kinases, five response regulators (Beier & Frank, 2000; Spohn & Scarlato, 1999b), and seven genes that encode for other regulators (Alm *et al.*, 1999; Tomb *et al.*, 1997).

The σ^{80} (RpoD) is the vegetative sigma factor of *H. pylori*. This sigma factor directs transcription during exponential growth and is involved in the transcription of the class 1 flagellar genes. The two alternative sigma factor of *H. pylori*, σ^{54} (RpoN) and σ^{28} (FliA), have

a function only in the transcriptional regulation of class 2, class 3, and the intermediate class of flagellar genes (Beier *et al.*, 1997; Beier *et al.*, 1998; Josenhans *et al.*, 2002; Josenhans & Suerbaum, 2001; Spohn & Scarlato, 1999b; Spohn & Scarlato, 2001a). In *H. pylori* homologues of either the stationary sigma factor (σ^S) or the heat-shock sigma factor (σ^{32}) have not been identified. The absence of these factors suggests that *H. pylori* possesses a different mechanism for stress response than other bacteria. Furthermore, *H. pylori* encodes the HspR repressor (HP1025) that negatively regulates the expression of the operons enconding the major chaperones of *H. pylori* (Spohn & Scarlato, 1999a), and two regulators that have been directly implicated as being involved in acid adaptation. These two regulators are the ferric uptake regulator protein Fur (HP1027) that is involved in iron-dependent regulation of transcription (Bereswill *et al.*, 1999; Bereswill *et al.*, 2000), and NikR (HP1338) which is a nickel dependent transcriptional regulates. NikR is involved in nickel-responsive induction of urease expression (Contreras *et al.*, 2003; Pflock *et al.*, 2005; van Vliet *et al.*, 2002). Additionally, other putative regulators namely *hp111*, *hp1139*, *hp1140*, and *hp1287* have been found in the genome sequences of *H. pylori* 26695. These putative regulators present homology with a transcriptional repressor (*hrcA*), which controls chaperone gene expression in *Bacillus subtilis*, a SPOJ regulator (*soj* repressor) that is a chromosome partitioning protein in *B. subtilis* and in this organism was shown to exert a direct repressive action on promoters of different sporulation genes, a biotin operon repressor *birA*, which is a repressor of the *E. coli* biotin operon, and a protein with similarity to the transcriptional activator *tenA* from *B. subtilis* (Alm *et al.*, 1999; Hommuth *et al.*, 2000; Quisel *et al.*, 1999; Tomb *et al.*, 1997).

4. Two-component systems

Two-component systems serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. Possible responses include alteration in gene expression and/or active movement towards or away from chemical or physical stimuli. In Gram-positive and Gram-negative pathogenic bacteria these sensory pathways can also control the expression of proteins important for pathogenesis (Hoch *et al.*, 1995; Parkinson & Kofoid, 1992).

The two-component systems are composed of two signalling proteins (1) a histidine protein kinase (HK), containing a conserved kinase domain, and (2) a response regulator protein (RR), containing a conserved regulatory receiver domain. In an archetypical two-component system (Fig.3) the appropriate extracellular stimulus is detected by a sensor domain of the HK, which causes ATP-dependent autophosphorylation of a specific histidine residue within

the HK transmitter domain. Then the RR catalyzes transfer of the phosphoryl group from the phospho-His in the transmitter domain to one specific aspartate residue in the regulatory domain. This phosphotransfer causes the activation of the effector domain of the RR that elicits the specific output response (Fig.3) (Stock *et al.*, 2000; West & Stock, 2001). The signalling mechanism in a two-component system involves three phosphotransfer reactions:

1-Autophosphorylation: HK-His + ATP ↔ HK-His~P + ADP

2-Phosphotransfer: HK-His~P + RR-Asp ↔ HK-His + RR-Asp~P

3-Dephosphorylation: RR-Asp~P + H_2O ↔ RR-Asp + P_i

All three reactions require divalent metal ions, with Mg^{2+} presumably being the relevant cation *in vivo*.

Most orthodox HKs, exemplified by the *E. coli* EnvZ protein, function as periplasmic membrane receptors. Nevertheless, some HKs are soluble cytoplasmic proteins that can be regulated by intracellular stimuli and/or interactions with cytoplasmic domains of other proteins. Examples of cytoplasmic HKs are represented by the chemotaxis kinase CheA (Stock, 1996) and the regulatory kinase NtrB (MacFarlane & Merrick, 1985). Moreover, elaborate hybrid kinases are found in some prokaryotic and most eukaryotic systems. These proteins contain multiple phosphodonor and phosphoacceptor sites and exhibit a complex phosphorelay. For example, *E. coli* ArcB, which functions in the anoxic redox control (Arc) system, has an architecture representative of most hybrid kinases. This protein is composed of two N-terminal transmembrane regions followed by a kinase domain, a domain similar to the regulatory domain of RRs, and finally a second His-containing region termed a His-containing phosphotransfer (HPt) domain (Stock *et al.*, 2000).

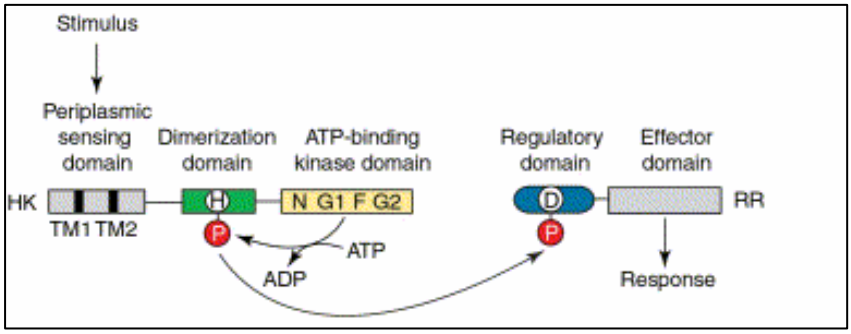

Fig.3 Scheme representing the typical phosphotransfer reaction in a two-component system. A typical two-component phosphotransfer system consists of a dimeric transmembrane sensor HK and a cytoplasmic RR. HK: histidine kinase; RR: response regulator; H: His residue; D: Asp residue; P: phosphate group; TM1: transmembrane domain 1; TM2: transmembrane domain 2 ; N, G1, F, and G2 are conserved sequence motifs located in the ATP-binding domain (West & Stock, 2001).

4.1. Two-component systems in *Helicobacter pylori*

In addition to the CheA/CheY (HP392, HP1067) two-component system (TCS) that regulates the chemotactic response (Beier *et al.*, 1997; Tomb *et al.*, 1997), there are three complete two-component systems consisting of the sensor protein (histidine kinase) and the cognate response regulator (HP165/HP166, HP244/HP703, HP1364/HP1365) and two orphan response regulators (HP1021 and HP1043) (Beier & Frank, 2000). For comparison, its close relative *Campylobacter jejuni* encodes seven TCS, as well as two orphan response regulators (Parkhill *et al.*, 2000), while *E. coli* contains about 30 TCS (Mizumo, 1997).

Two of the *H. pylori* histidine kinases (HP1364, HP165) show a sequence typical of two-component sensor proteins with a periplasmic input domain and a cytoplasmic transmitter domain. In contrast, HP244, belonging to histidine kinase subclass IIIB (Fabret *et al.*, 1999), with similarity to *E. coli* NtrB is predicted to be a cytoplasmic protein. Based on sequence similarities in their output domains the regulator proteins HP1365, HP166, and HP1043 are grouped into the OmpR subfamily of response regulators, while HP703 is an NtrC-like protein. The output domain of HP1021 does not show significant sequence similarity to other response regulator proteins, but harbours a putative DNA-binding helix-turn-helix motif close to its C-terminus (Tomb *et al.*, 1997).

To date regulated target genes have been identified only for two of the TCS HP244/HP0703 and HP165/HP166. The two-component system HP0244/HP703 (FlgR) regulates the expression of several components of the flagellar apparatus, whereas the HP165/HP166 system exhibits negative autoregulation and in response to an acidic pH acts as transcriptional activator of the urease genes and some *H. pylori* genes that encode proteins with unknown functions (Dietz *et al.*, 2002; Forsyth *et al.*, 2002; Pflock *et al.*, 2004). Moreover, on the basis of *in vitro* DNA-binding experiments, it was hypothesized that HP1043 may regulate its own expression as well as transcription of the *tlpB* gene encoding a methyl-accepting chemotaxis protein (Delany *et al.*, 2002).

5. Regulation pathways in flagellar biosynthesis

For many pathogenic bacteria, flagellum-dependent motility and chemotaxis are crucial for colonization of the host organism and establishment of a successful infection (Moens & Vanderleyden, 1996; Nakamura *et al.*, 1998). Regulatory pathways for the synthesis of flagella differ between bacterial genera. These regulatory pathways were extensively studied in organisms as *Caulobacter crescentus* (Wu & Newton, 1997), and *Salmonella typhimurium* (Aizawa, 2000; Macnab, 2003).

In *S. typhimurium* environmental signals trigger the expression of class I (early) genes, which encode the class I transcriptional master regulators FlhC and FlhD (Fig.4 A). These master regulators are required for the σ^{70}-dependent expression of class II (middle) genes that encode the flagellar basal body, hook proteins and the alternative σ-factor σ^{28}. This σ-factor controls the expression of the class III (late) flagellar genes whose transcription is initiated not until the anti-σ^{28}-factor FlgM is exported from the cell via the flagellar-specific type III secretion system when the basal body and hook structure is completed (Fig.4 A) (Macnab, 2003).

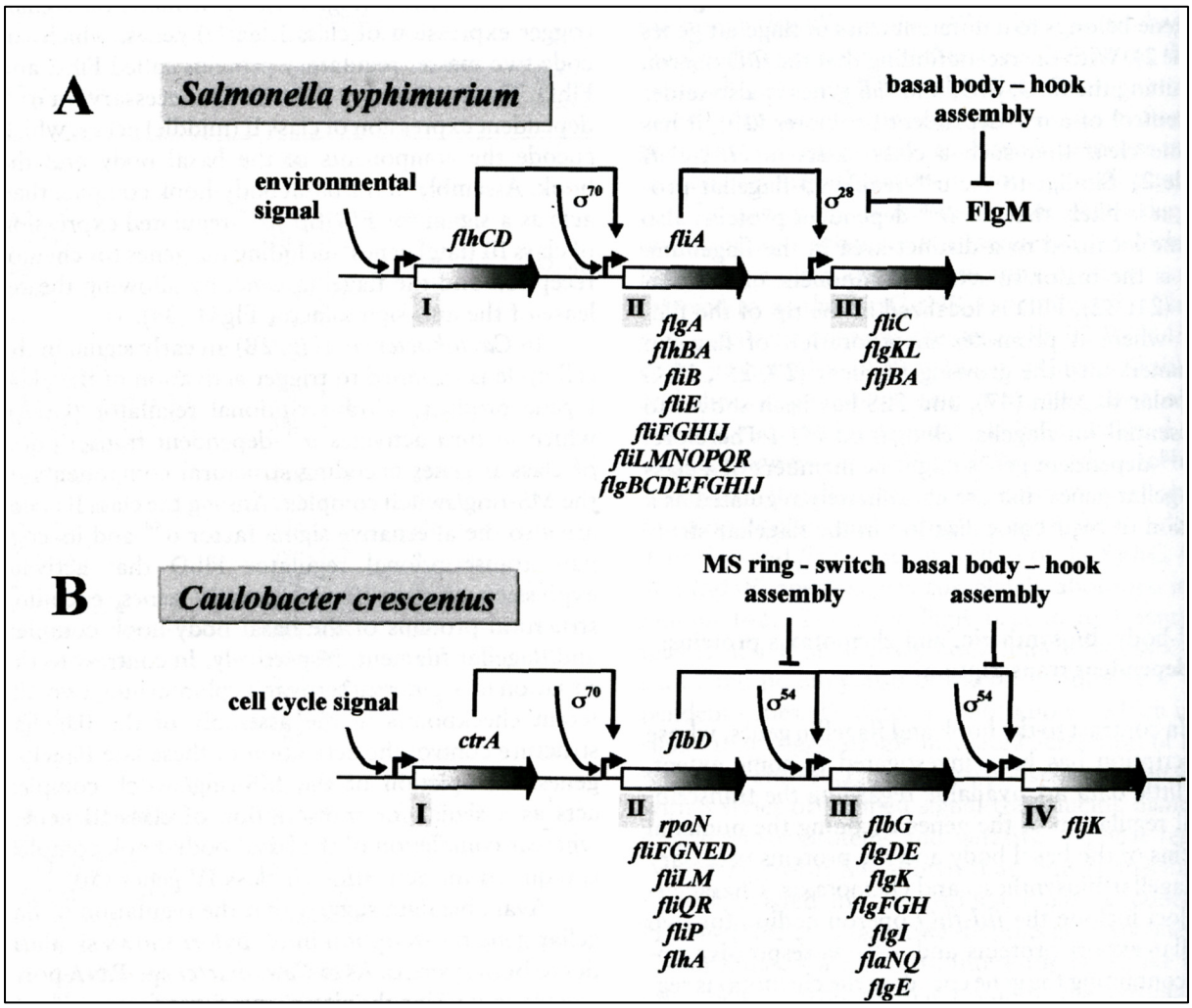

Fig.4 Comparison of flagellar regulatory hierarchies in *S. enterica* serovar Typhimurium (A) and *C. crescentus* (B). Dependence of transcription on the specified sigma factor is indicated. Classes of flagellar genes are indicated by roman numbers. Symbols: ⌐→promoter, ↘ activation , ⊥ repression (Spohn & Scarlato, 2001b).

In *C. crescentus* an early signal in the cell cycle is assumed to trigger activation of the class I gene product. This product is the transcriptional regulator CtrA that activates the transcription of the σ^{70}-dependent class II genes encoding structural components of the MS-ring/switch complex. Among the class II genes are also the genes encoding the σ^{54}-factor and its cognate transcriptional regulator FlbD that activate expression of class III and class IV genes,

encoding structural proteins of the basal body-hook complex and flagellar filament, respectively (Fig.4 B) (Wu & Newton, 1997).

5.1. Regulation pathways of flagellar biosynthesis in *H. pylori*

In *H. pylori* more than 40 proteins are involved in the biosynthesis and operation of the flagella and their control by the chemotaxis machinery (Alm *et al.*, 1999; Tomb *et al.*, 1997). The mechanism of flagellar gene regulation in *H. pylori* differs from the extensively studied enterobacterial paradigm (O'Toole *et al.*, 2000), but until now this mechanism is not completely understood. The current model of regulation of flagellar biosynthesis in *H. pylori* contains elements of both the *C. crescentus* and *S. typhimurium* flagellar hierarchies (Josenhans *et al.*, 2002; Spohn & Scarlato, 2001b).

The flagellar genes of *H. pylori* are divided in four classes (Fig.5) (Niehus *et al.*, 2004; Spohn & Scarlato, 1999b; Spohn & Scarlato, 2001b):

- Class 1: The first class of genes is transcribed by RNA-polymerase containing the vegetative sigma factor RpoD (σ^{80}) and contains genes that encode for components of the basal body and export apparatus, as well as for genes involved in chemotaxis and flagellar rotation.
- Class 2: Genes of the class 2 are regulated by a σ^{54}-dependent promoter. The σ^{54}-containing RNA polymerase acts together with the NtrC-like response regulator FlgR that is activated by phosphorylation via the cytoplasmic histidine kinase HP0244 (Beier & Frank, 2000; Spohn & Scarlato, 1999b). This second group contains the *flaB* gene that encodes for the minor flagellin FlaB, which is localized at the basis of the flagellar hook (Kostrzynska *et al.*, 1991). Among the class II genes are also *flgE* and its homolog *flgE´, flgK, flgL, flgB, flgC,* and *flgG*, which encode components of the flagellar hook, hook-associated proteins (HAPs), and structural proteins of the rod (Homma *et al.*, 1990a; Homma *et al.*, 1990b; O'Toole *et al.*, 1994).
- Class 3: The class 3 genes are transcribed by RNA-polymerase containing the alternative sigma factor FliA (σ^{28}) whose activity is antagonized by the anti-σ-factor FlgM. Therefore, transcription of the class III genes is regulated similarly to late flagellar gene expression in Enterobacteria (Colland *et al.*, 2001; Josenhans *et al.*, 2002). This class of genes comprises mainly *flaA* encoding the major structural component of the filament (Leying *et al.*, 1992).
- Intermediate class: The transcription of intermediate class genes is controlled by more than one promoter. This class contains genes that encode for the anti sigma factor

FlgM (anti-σ^{28}), the sigma factor FliA (σ^{28}), along with some genes encoding for structural components of the flagella such as the flagellar cap FliD that localizes at the top of the filament (Homma & Iino, 1985; Kim *et al.*, 1999), FliM and FliY, which are components of the flagellar motor switch complex, FlaG a polar flagellin (Tomb *et al.*, 1997), and FliS that has been shown to be essential for the flagellar elongation (Yokoseki *et al.*, 1995).

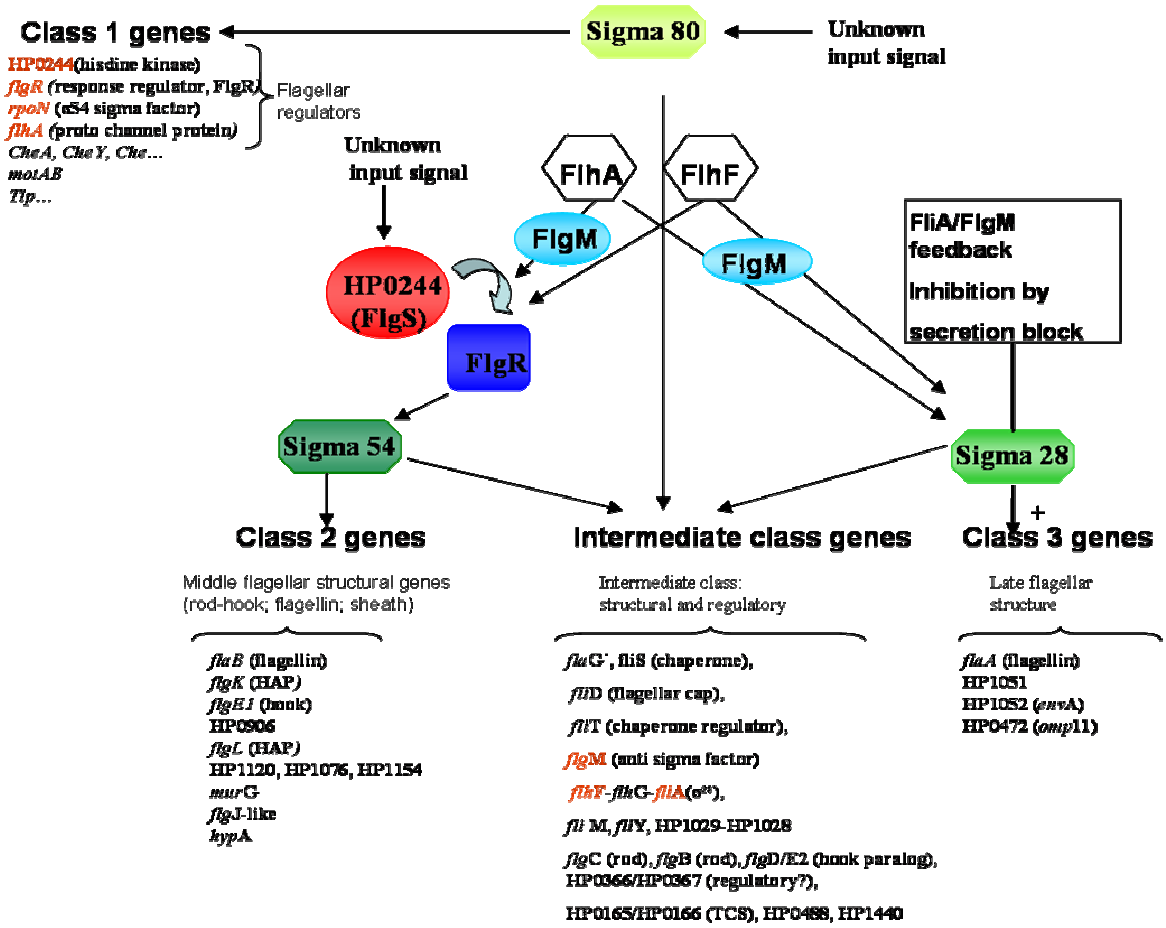

Fig.5 Current model of the regulation pathways of flagellar biosynthesis in *H. pylori*. Three different classes of flagellar genes are governed by the housekeeping sigma factor σ^{80} (class 1) and the alternative sigma factors σ^{54} (class 2) and σ^{28} (class 3). A number of intermediate genes controlled by more than one promoter are grouped in a separate category. Class 1 flagellar genes comprise, among others, most of the major regulatory genes of the flagellar system. The list of putative class 1 genes is not complete, as these regulons have not been investigated in detail. Transcription of class 2 genes is governed by RpoN, assisted by the HK HP244 and the response regulator FlgR. The class of RpoN-dependent genes could be extended by a number of novel genes indicated by HP numbers. FlhA and FlhF are both necessary for full transcription of flagellar classes 2 and 3 and the intermediate class (Niehus *et al.*, 2004).

The master regulators FlhC and FlhD of Enterobacteriaceae have not been identified in the genome sequence of *H. pylori*. Nevertheless, regulator FlgR, an RpoN (σ^{54}) activator (Spohn & Scarlato, 1999b), is an orthologue of flagellar master regulators in other bacteria as FleR and FleQ of *Pseudomonas* spp., FlbD of *Caulobacter* spp., FlrA and FlrC of *Vibrio* spp.

(Dasgupta *et al.*, 2002; Jenal, 2000; Prouty *et al.*, 2001), but functional analyses do not support a role for FlgR as a master regulator.

Furthermore, an essential membrane component of the flagellar basal body export apparatus the FlhA protein was shown to be required for expression of three flagella-associated genes, which belong to the intermediate class with both σ^{28} and σ^{54} dependent promoters (Schmitz *et al.*, 1997). Recently it was reported that FlhA of *Bacillus thuringiensis* is involved in both flagellar assembly and secretion of virulence factors (Ghelardi *et al.*, 2002). This report suggests that FlhA may connect motility/flagella with other cell functions.

Moreover, *H. pylori* contains *flhF* a putative flagellar regulatory gene, which is not found in Enterobacteriaceae, but in several other flagellated bacteria. FlhF belongs to the protein family of the signal recognition particle (SRP), which is involved in targeting protein transport across membranes (Keenan *et al.*, 2001). The *flhF* and the downstream gene (*flhG*/*fleN*) are involved in flagellar placement or regulation of flagellar numbers in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Dasgupta *et al.*, 2000; Pandza *et al.*, 2000).

In *H. pylori* the vegetative σ^{80} regulates the transcription of the class 1 genes including the gene *flgR*. The transcriptional regulator FlgR activates σ^{54}-dependent transcription of class II genes and some intermediate genes encoding σ^{28} and its antagonist (FlgM). Both σ^{28} and the anti sigma factor FlgM control the transcription of the late class III genes (Fig.5).

Thus far, it is not known which is the input signal that stimulates the histidine kinase HP244, which triggers the phosphorylation of transcriptional regulator FlgR. Interestingly, σ^{54}-dependent transcriptional activators of the NtrC type are also involved in the hierarchical order of flagellar gene expression in *C. crescentus* and *Vibrio cholerae* (Correra *et al.*, 2000; Ramakrishnan, 1990) comprising four classes of flagellar genes. Neither in *C. crescentus* nor in *V. cholerae* the environmental cues resulting in the phosphorylation of response regulators FlbD and FlrC, respectively, by their cognate histidine kinase are known. In addition to signal perception by the N-terminal input domain of a two-component sensor protein histidine kinase activity can also be controlled by the interaction of the cytoplasmic transmitter domain with a regulatory protein. The paradigm for this mode of control is the NtrB histidine kinase whose kinase and phosphatase activities are regulated by the PII protein (Pioszak *et al.*, 2000), but also other systems using a similar mechanism have been described (Garnerone *et al.*, 1999; Wang *et al.*, 1997).

6. Chemotaxis

Motile bacteria monitor changes in the chemical composition of their environment. By monitoring these changes, they migrate towards attractants (food sources) and avoid repellents (noxious chemicals). Chemotaxis is the signal transduction pathway that bacteria use to detect these chemical changes and to transmit the information from the environment to the motor of the flagella. This pathway has been studied most extensively in *E. coli* and *S. typhimurium* (Szurmant & Ordal, 2004; Wadhams, 2004), thus the chemotaxis system of these organisms is used as a model. To date is known that some organisms as *Sinorhizobium meliloti* possess a chemotaxis system that differs from the enterobaterial paradigm.

6.1. Chemotaxis in *E. coli* and *S. meliloti*

In *E. coli* the sensing of external attractants and repellents is achieved via methyl-accepting chemoreceptor proteins (MCPs), which are transmembrane proteins with a periplasmic ligand binding domain and a cytoplasmic signaling domain (Maddock & Shapiro, 1993). The transduction of the signal from the MCPs to the flagellar motor involves basically four proteins (Fig.6 A): CheA, an ATP-dependent cytoplasmic histidine kinase, CheY, a response regulator, CheW a receptor-coupling factor, and CheZ, a CheY~P specific phosphatase (Eisenbach, 1996).

The CheA and CheY proteins constitute a TCS, which differs from the archetype of a TCS because the CheY protein neither contains a DNA binding domain nor acts as a transcriptional activator (Stock, 1996). The binding of the periplasmic domain of the MCP to a ligand produces a conformational change in its cytoplasmic domain. The changed structure of the cytoplasmic domain is recognized by an associated CheA-CheW cytoplasmic complex, which binds to the MCP via CheW (Eisenbach, 1996). The histidine kinase CheA has an autokinase activity that is inhibited by the contact of the receptor with attractants and is stimulated by the contact with repellents or attractant-free receptor (Ninfa *et al.*, 1991). The stimulation of the autokinase activity initiates the phosphorylation of the response regulator CheY. Thereby, the phosphorylated CheY (CheY~P) interacts directly with FliM in the flagellar motor switch complex to cause clockwise rotation (Fig.6 A) (Sanders *et al.*, 1989b). In *E. coli* the flagellar rotatory motor turns clockwise upon interaction with CheY~P, resulting in a tumbling motion; otherwise, it turns counterclockwise, resulting in smooth swimming of the bacterial cell (Eisenbach, 1996). Finally the dephosphorylation of the response regulator CheY is controlled by the action of the CheZ phosphatase, which accelerates the decay of the unstable CheY~P (Eisenbach, 1996).

A deviation from the chemotaxis enterobactrial paradigm is represented by the soil bacterium *S. meliloti* that belongs to the α-subclass of Proteobacteria. The chemotaxis system of *S. meliloti* differs from that of *E. coli* in three characteristics: the free-swimming behaviour of *S. meliloti* is directed by variations in flagellar rotary speed, rather than by switches between clockwise and counterclockwise rotation, as in *E. coli* (Platzer *et al.*, 1997). *S. meliloti* contains two different CheY response regulators CheY1 and CheY2, but no homologue of the CheZ phosphatase (Greck *et al.*, 1995). Similar to *E. coli*, the *S. meliloti* CheA acts as ATP-dependent autokinase capable of donating its phosphate group to the two response regulators, CheY1 and CheY2 (Fig.6 B) (Sourjik & Schmitt, 1998). However, only one CheY protein (CheY2) interacts with the flagellar motor when phosphorylated, and, therefore, controls flagellar rotation directly, while the second CheY protein (CheY1) functions as a modulator of the chemotactic signalling by acting as phosphate sink which drains away the phosphoryl group from CheY2~P via retrophosphorylation of the histidine kinase CheA (Sourjik & Schmitt, 1998).

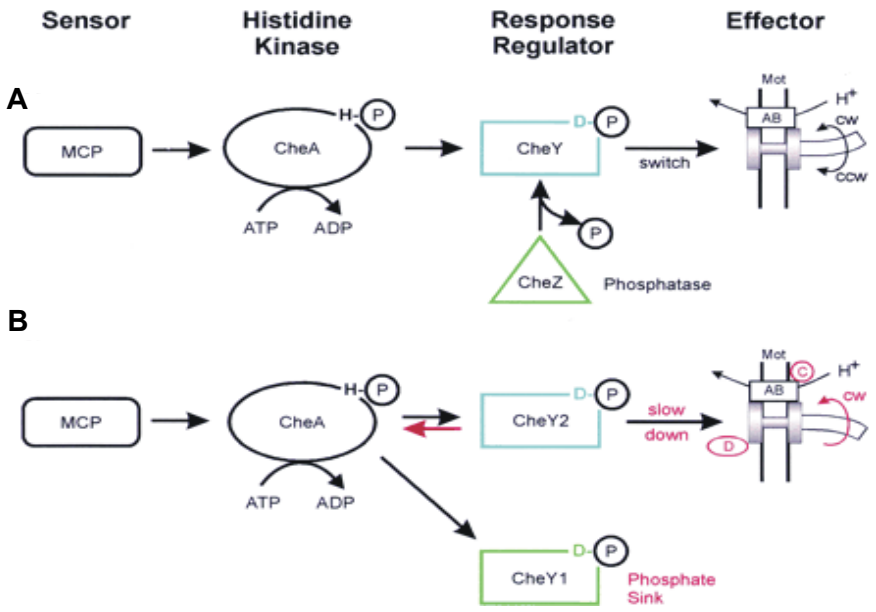

Fig.6 Scheme comparing the mechanisms of chemotactic signalling in *E. coli* (A) and *S. meliloti* (B). General components of the histidine autokinase pathway are given at the top. The corresponding receptor (MCP), chemotaxis (Che) and motor (Mot) proteins of the chemosensory transduction chain are marked within the diagrams. Histidyl (H) and aspartyl (D) phosphorylation (P), proton flow (H+) through MotA-MotB channels, and the clockwise (cw) or couterclockwise (ccw) sense of flagellar rotation are shown. Matching colours identify analogous (but differently reacting) response regulatory components in (A) and (B). Departures from the enterobacterial scheme including the motor proteins MotC and MotD are outlined in red (Schmitt, 2002).

Furthermore, this type of regulation does not appear to be unique of *S. meliloti*, because two or more CheY homologues could be found in another member of the α-subgroup of proteobacteria exemplified by *Rhodobacter sphaeroides* (Ward *et al.*, 1995) and also in the ε-subgroup of proteobacteria (Alm *et al.*, 1999; Baar *et al.*, 2003; Parkhill *et al.*, 2000; Suerbaum *et al.*, 2003; Tomb *et al.*, 1997).

6.2. Chemotaxis in *H. pylori*

As mentioned above chemotaxis is a virulence trait necessary for the colonization by *H. pylori* (Foynes *et al.*, 2000; Ottemann & Lowenthal, 2002; Terry *et al.*, 2005). During the colonization *H. pylori* has to move towards the epithelial cell surface to avoid being washed out by the mucous flow or killed by the stomach acid. It has been reported that *H. pylori* moves *in vitro* towards some amino acids (glutamine, histidine, lysine, and alanine), mucin, sodium bicarbonate, sodium chloride, and urea, whereas bile acids act as repellent (Cerda *et al.*, 2003; Mizote *et al.*, 1997; Worku *et al.*, 2004; Yoshiyama *et al.*, 1999).

Ten putative chemotaxis genes were identified in *H. pylori* by genome analysis: the histidine kinase gene *cheA* (HP392), the response regulator gen *cheY* (HP1067), *cheW*, encoding the ternary-complex-forming protein CheW (HP391), three unlinked copies of *cheV*, and genes encoding three classical MCPs (HP82, HP99, HP103), and a truncated soluble MCP orthologue (HP599) (Alm *et al.*, 1999; Tomb *et al.*, 1997).

The *cheA* gene encodes a bifunctional protein with a CheY-like domain (CheY2) at the C-terminus (Jackson *et al.*, 1995; Kelly, 1998) and, therefore, has been designated as CheAY protein (Foynes *et al.*, 2000). This histidine kinase is located in an operon together with *cheW* and *cheV3*, whereas the other two copies of *cheV* and *cheY* (CheY1) are arranged at unlinked sites in the chromosome. The *cheY* gene is part of an operon that contains stress-induced genes (Beier *et al.*, 1997). The *cheV* genes form a paralogous family and in *H. pylori* have been designated *cheV1* (HP19), *cheV2* (HP616), and *cheV3* (HP393) (Doig *et al.*, 1999). Each *cheV* gene encodes a protein, which contains an N-terminal CheW-like domain and a C-terminal CheY-like domain. CheV proteins were first identified in *Bacillus subtilis* (Fredrick & Helmann, 1994) and redundancy with CheW was reported in this organism (Rosario *et al.*, 1994). Moreover, the CheV protein in *B. subtilis* requires phosphorylation of the receiver domain for an efficient adaptation (Karatan *et al.*, 2001). Inactivation of *cheV1* in *H. pylori* reduces swarming on semi-solid agar plates while swarming was unchanged in insertion mutants of *cheV2*, *cheV3* and in a double *cheV2/cheV3* mutant (Pittman *et al.*, 2001). Therefore, the mechanisms of adaptation adopted by *H. pylori* remain unclear.

Besides a truncated soluble MCP that contains only a cytoplasmic domain, which might respond to internal physiological signals, *H. pylori* contains three genes coding for classical MCPs. These genes encode for typical transducer-like receptor proteins with a transmembrane domain and a cytoplasmic domain. This MCPs posses several conserved glutamate residues that could be methylated as part of a stimulus-adaptation system. However, there is no evidence of a potential methyltransferase (*cheR*) or methylesterase (*cheB*) (Tomb *et al.*, 1997), which could be part of a stimulus-adaptation system that acts by modulating the ability of the MCPs to induce CheA autophosphorylation through the reversible methylation of glutamic acid residues in the cytoplasmic methylation region of the MCPs. Interestingly, closely related organisms like *Helicobacter hepaticus*, *Campylobacter jejuni* and *Wolinella succinoges* encode orthologues of CheR and CheB. However, the CheB protein in these organisms does not posses a receiver domain (Baar *et al.*, 2003; Parkhill *et al.*, 2000; Suerbaum *et al.*, 2003).

Given that *H. pylori* possesses five CheY-like domains in different proteins, but that there is no ortholog of a gene that encodes a CheZ protein, it can be predicted that chemotactic signalling in *H. pylori* differs from the enterobacterial paradigm and might be similar to the chemotactic signalling pathway in *S. meliloti*.

Aims

As described above, in *H. pylori* the pathway of flagellar biosynthesis is controlled by a complex regulatory cascade. Thus far, the input signal for histidine kinase HP244, which stimulates the transcriptional regulator FlgR that controls transcription of the flagellar class 2 genes, is not known. Interestingly, the protein-protein interaction map of *H. pylori* (Rain *et al.*, 2001) indicated an interaction between the gene product of ORF *hp137* and both the histidine kinase HP244 and the flagellar hook protein HP908. The first goal of this work was the analysis of the putative function of protein HP137 in a feedback regulatory mechanism controlling the activity of histidine kinase HP244.

Analysis of the genome sequence of *H. pylori* indicated that this organism possesses a chemotaxis system which differs markedly from the well characterized enterobacterial system (Alm *et al.*, 1999; Tomb *et al.*, 1997). Therefore, in a second project the phosphotransfer reactions between the two-component signalling modules of the chemotaxis proteins were investigated in order to characterize the signal transduction processes governing the chemotactic behaviour of *H. pylori*.

C MATERIALS

1. Bacterial strains and plasmids

Tab.1 Strains and plasmids

Strain	Relevants feature(s)	Reference or Source
Helicobacter pylori		
26695	Clinical isolate	(Tomb *et al.*, 1997; Xiang, 1995)
G27	Clinical isolate	(Xiang *et al.*, 1995)
C. jejuni 4344	Clinical isolate	German Reference Laboratory for Helicobacter and Campylobacter, Freiburg
G27/hp137::km	G27 with a kanamycin resistance cassette replacing ORF *hp137*	P. Dietz and this study
G27/*cag*hp137-cm	G27/hp137::km with a fusion of the promoter region of ORF *hp139* to ORF *hp137* integrated into the *cag*-locus	This study
G27WT/*cag*hp137-cm	G27 wild-type with a fusion of the promoter region of ORF *hp139* to ORF *hp137* integrated into the *cag*-locus	This study
G27WT/hp137loc-cm	G27 wild-type with an insertion of the gene *hp137* in the wild-type locus	This study
G27/hp137loc-cm	G27/hp137::km with a reinsertion of the gene *hp137* in the wild-type locus	This study
cj4344/cj73c::km	*C. jejuni* 4344 with a kanamycin resistance cassette replacing ORF *cj73c*	This study
G27/*che*AY2::km	G27 with a kanamycin resistance cassette replacing ORF *hp392*, which encodes histidine kinase CheAY2	This study
G27/*che*A-Y2-cm	G27/cheAY2::km with two independent genes, which encode the histidine kinase domain and the CheY-like domain of CheAY2, respectively, replacing the *cheAY2* gene	This study
Escherichia coli		
DH5α	Strain used for high-efficiency transformation	Gibco
M15	Strain used for overproducing His_6-tagged proteins	QIAGEN
Plasmids		
pSL1180	Cloning vector	Amersham Biosciences
pDT2548	Plasmid containing the chloramphenicol resistance gene from *Campylobacter coli*	(Wang & Taylor, 1990)
pILL600	Plasmid containing the kanamycin resistance cassette from *Campylobacter coli*	(Labigne-Roussel *et al.*, 1988)
pQE30	Expression vector for N-terminal His_6 tag proteins	QIAGEN
pGEX-3X	GST gene fusion and expression vector	Amersham Biosciences
pRep4	Derivative of pACYC overexpressing LacI	QIAGEN
pSL-137	pSL1180 containing *Eco*RI-*Bam*HI and *Pst*I-*Sac*I fragments of 612 bp and 627 bp derived from the ORFs *hp138* and *hp136*, respectively	P. Dietz
pSL-137::km	pSL-137 containing the kanamycin resistance cassette flanked by *Eco*RI-*Bam*HI and *Pst*I-*Sac*I fragments of 612 bp and 627 bp derived from the ORFs *hp138* and *hp136*, respectively	P. Dietz
pSLhp137	pSL1180 containing a *Bam*HI-*Xba*I fragment of 1099 bp comprising a fusion of the P_{hp139}-promoter with ORF *hp137*	This study
pSL*cag*AD	pSL1180 containing *Eco*RI-*Bam*HI and *Pst*I-*Sac*I fragments of 830 bp and 862 bp derived from the *cagCD* and *cagA* genes, respectively	(Schär *et al.*, 2005)

pSL*cag*hp137	pSL*cag*AD containing a *Bam*HI-*Xba*I fragment of 1099 bp comprising a fusion of the P_{hp139}-promoter with ORF *hp137*	This study
pSL*cag*hp137-cm	pSL*cag*AD containing a *Bam*HI-*Xba*I fragment of 1099 bp comprising a fusion of the P_{hp139}-promoter with ORF *hp137* and a chloramphenicol resistance cassette	This study
pSL-137loc	pSL1180 containing a *Eco*RI-*Pst*I fragment of 1821 bp derived from ORFs *hp138*, *hp137* and *hp136* and a *Pst*I-*Sac*I fragment of 608 bp comprising the intergenic region between ORFs *hp136* and ORF *hp135* and part of ORF *hp134*	This study
pSL-137loc-cm	pSL1180 containing the chloramphenicol resistance cassette flanked by a *Eco*RI-*Pst*I fragment of 1821 bp derived from ORFs *hp138*, *hp137* and *hp136* and a *Pst*I-*Sac*I fragment of 608 bp comprising the intergenic region between ORFs *hp136* and ORF *hp135* and part of ORF *hp134*	This study
pSL-cj73::km	pSL1180 containing the kanamycin resistance cassette flanked by *Eco*RI-*Bam*HI and *PstI*-*Sac*I fragments of 722 bp and 674 bp derived from the ORFs *cj74c* and *cj73c*, respectively	This study
pQE-137	pQE30 expressing protein HP137 of *H. pylori*	This study
pQE-FlgE´	pQE30 expressing FlgE´ of *H. pylori*	This study
pSL-139-PE	pSL1180 containing a 629 bp DNA fragment from *H. pylori* 26695 comprising the promoter region and part of the coding DNA of ORF *hp139*	This study
pSL-138-PE	pSL1180 containing a 525 bp DNA fragment from *H. pylori* 26695 comprising part of the 5´ upstream region of ORF *hp138* and part of the coding DNA of ORF *hp138*	This study
pSL-137-PE	pSL1180 containing a 466 bp DNA fragment from *H. pylori* 26695 comprising part of the 5´ upstream region of ORF *hp137* and part of the coding DNA of ORF *hp137*	This study
pSL-*hp390*	pSL1180 containing a *Sac*I/*Xba*I fragment of 665 bp derived from ORF *hp390*	This study
pSL-*hp390cheV*	pSL-hp390 containing a *Nco*I-*Eco*RI fragment of 689 bp derived from ORF *hp393*	This study
pSL-*cheAY2*::km	pSL-hp390*cheV* containing the kanamycin resistance cassette flanked by *Sac*I-*Xba*I and *Nco*I-*Eco*RI fragments of 665 bp and 689 bp derived from the ORFs *hp390* and *hp393*, respectively	This study
pSL-*hp390cheVW*	pSL-hp390*cheV* containing a *Xba*I-*Bam*HI fragment of 967 bp from ORF *hp391*	This study
pSL-*hp390cheVWA*	pSL-hp390*cheVW* containing a *Eco*RI-*Bam*HI fragment of 2047 bp from ORF *hp392*	This study
pSL-*cheA´*-Y2cm	pSL-hp390*cheVWA* containing the chloramphenicol resistance cassette flanked by fragments of 665 bp and 3703 bp derived from ORFs *hp390* and ORFs *hp391*, *hp392, hp393*	This study
pTrc-*cheAY2*	pTrcHisA expressing the bifunctional CheAY2 protein of *H. pylori*	I. Delany
pTrc-*che*A´	pTrcHisA expressing a truncated derivative of CheAY2 comprising amino acids 1-676 and lacking the C-terminal CheY2 domain	I. Delany
pTrc-*che*Y2	pTrcHisA expressing the C-terminal CheY2 domain (amino acids 677-803) of the bifunctional CheAY2 protein of *H. pylori*	I. Delany
pQE-*che*Yec	pQE60 expressing the His_6-tagged response regulator CheY from *E.coli*	B. Scharf and V. Sourjik
pQE-*che*V1	pQE30 expressing the CheV1 of *H. pylori*	This study
pQE-*che*V2	pQE30 expressing the CheV2 of *H. pylori*	This study
pQE-*che*V3	pQE30 expressing the CheV3 of *H. pylori*	This study
pGEX-*che*Y1	pGEX-3X expressing CheY1 of *H. pylori*	This study
pSL-*che*Y1	pSL1180 containing a *BamH*I-*Eco*RI fragment of 373bp encoding CheY1 of *H. pylori*	This study
pGEX-*cheY1*-D53N	pGEX-3X expressing a mutated derivative of *H. pylori* CheY1 with a D53N substitution	This study
pGEX-*che*Y1cj	pGEX-3X expressing CheY1 of *C. jejuni*	This study

2. Oligonucleotides

All primers were provided by Sigma-ARK GmbH and Sigma Genosys. Oligonucleotides were dissolved in dH_2O to a final concentration of 100 pmol/µl or 50 pmol/µl.

Tab.2 Oligonucleotides used in this work

Name	Sequence (5` to 3`)[a]	Site[b]	Strand	Position[c]
M13universal	GTAAAACGACGGCCAGT			
M13reverse	CAGGAAACAGCTATGAC			
kanR-5	aattggggatccGCGAACCATTTGAGGTGATAGG	*Bam*HI		
kanR-3	atctaactgcagGTACTAAAACAATTCATCCAG	*Pst*I		
kan1	CTGCTAAGGTATATAAGCTGGT			
kan2	CCAGCTTATATACCTTAGCAG			
Cm-PstI3`	agctagctgcagACGCACTACTCTCGACAG	*Pst*I		
Cm-PstI5`	agctagctgcagATTGCGTGATATAGATTG	*Pst*I		
cagD5´	TAGATATACCGCTTCACATGTAAT		+	578118 - 578141
cagA3´	CCTTGAAAGAAAGGCAAGAAGCA		+	580501 - 580523
pQE-5	CCCGAAAAGTGCCACCTGAC			
pQE-3	TCCAGTAATGACCTAGATAG			
pGEX-5	GGGCTGGCAAGCCACGTTTGGTG			
pGEX-3	CCGGGAGCTGCATGTGTCAGAGG			
137-1	ttttatgaattcACAACCGCTCTAATATTTTGGC	*Eco*RI	-	148444-148465
137-2	ggttcgggatccGAATGGCATGCTTGGCTCTGG	*Bam*HI	+	147859-147879
137-3	gtttttctgcagCATGGGCCTCAAAAAGTCG	*Pst*I	-	147298-147316
137-4	tgataggagctcGTAAAGCGATCTTAGGGTTGTAGG	*Sac*I	+	146650-146673
137loc1	CACAGGCCCTAGAAAAGAGGGCGA		-	148507-148530
137loc2	tgatagctgcagGTAAAGCGATCTTAGGGTTGTAGG	*Pst*I	+	146650 - 146673
137loc3	tagagactgcagAGTTTAACTTTCTAACTTTCGCC	*Pst*I	-	146792-146814
137loc4	aatgacgagctcGAATGTTACAGAATGTATCGG	*Sac*I	+	146212 - 146232
P139-5	aaaaatggatccGATCACAATCCAGCGATCG	*Bam*HI	-	150573-150593
P139-3	taaatttctagaCCAAATTAAGAGTTTCAAACAC	*Xba*I	+	150129-150151
137-5´	taattgtctagaaaggagccttctATGAGTAAAGAGCTTATT TTAAAGCGC	*Xba*I	-	147889-147916
137-3´	ttccatctgcagCCTTTAGTAGAGAATGATAGCG	*Pst*I	+	147278-147299
73-1	tatagagaattcGATGGAAAGCTTTGAAGATGC	*Eco*RI	-	85384-85405
73-2	ttgaaaggatccCGATTTTACTCATAGCTTACTCC	*Bam*HI	+	84731 - 84753
73-3	atagaactgcagACTGTTTTTGGTGTTCATGG	*Pst*I	-	84118-84137
73-4	tttttcgagctcGACTTCTTCAAAGCTTACTTCC	*Sac*I	+	83415-83437
E137-5	aattttggatccAGTAAAGAGCTTATTTTAAAGC	*Bam*HI	-	147889-147913
E137-3	cattctctgcagTTAGTAGAGAATGATAGCGAC	*Pst*I	+	147281 - 147301
EFlgE´-5	agagacggatccAACGACACCTTATTAAACGC	*Bam*HI	+	959024 - 959043
EFlgE´-3	tatttcctgcagTTATTTTTTCAAGCTAATGGCTTC	*Pst*I	-	960815-960838
FlgE´-seq1	GAGGGGGAATTCCACATGGCGT		+	959270 - 959291
FlgE´-seq2	TTAGCAGGGATTCAGTAGCGAT		+	960054 - 960075
FlgE´-seq3	GCCCCTAGGCTTGCCGTCTTGGCT		-	960428-960451
RT-136	CCTTTTGAGCATGGCCTTTCG		+	146830 - 146850
RT1-136	TGATAACGCGCAATCGCATCAA		-	147035-147056
RT2-136	AAGCATGCGTTTGCCATAAGCT		+	146939 - 146960
RT-137	TAATTCAATGTCAGCTGTCCG		+	147335 - 147355
RT1-137	TTGCATGGGGTTTGTGGGATTT		-	147550-147571
RT2-137	ATGCCTTCACTCAAATTGCGCA		+	147426 - 147447
139-PE	GTTGCTGTATATGGCTGC		+	150059-150077
138-PE	GGTCGGTGATGATTTCTTCG		+	149305-149325
137-PE	GGCTCTGGCTTCTTTAATGC		+	147871-147891
139-5P	aaaaatggatccGATCACAATCCAGCGATCG	*Bam*HI	-	150573-150593
139-3P	cttcatgaattcCTGTTGTAGCTTGGCTGGC	*Eco*RI	+	149963-149984
138-5P	gataaaggatccCAAGTCAAATATGAAGATAAGG	*Bam*HI	-	149732-149754
138-3P	gtaacggaattcAAGGAGATTCTTACGATTAGC	*Eco*RI	+	149228-149249
137-5P	cagtctggatccGCGTTGCACTGAAGTATGCC	*Bam*HI	-	148232-148252

137-3P	ttcaacgaattcAAATGCTTGTATTCTTCCACC	*Eco*RI	+	147785-147806
flaA-PE	CGCATTGATATTTGTATTGACCTG		-	637314 - 637291
flgE-PE	GACACCAGACCATAAAGACC		+	922547-922566
flgB-PE	AAGACCGATAATCCAACGCC		+	1641322-1641341
flaB-PE	GCATGAGAAGTTAAAGCGGC		+	124446-124465
flgE2-PE	GGATTAATGGGAGATGGCATG		-	956484-956504
cheY1-5′	catcacggatccGAAACTACTGGTAGTAGATGA	*Bam*HI	+	1126269-1126291
cheY1-3′	tttcacgaattcTTAATCGTTTGTCCCTAAAACAACC	*Eco*RI	-	1126642-1126620
cheY1-Mut1	GTGCTTATTACAAATTGGAACATGC		+	1126413-1126437
cheY1-Mut2	GCATGTTCCAATTTGTAATAAGCAC		-	1126413-1126413
cheV1-5′	catcacggatccGCTGATAGTTTAGCGGGCATTG	*Bam*HI	+	18377-18399
cheV1-3′	tttcttggtaccTTATGCTAATTCCAAAAATTGCTTAAC	*Kpn*I	-	19321-19345
cheV2-5′	catcacggatccGTAAGAGATATTGACAAAACGAC	*Bam*HI	+	661114-661136
cheV2-3′	TttcttctgcagTTATGAAAGCGTTTTTTTAAGCATTTCATGG	*Pst*I	-	662031-662058
cheV3-5′	catcacggatccGCAGAAAAAACAGCTAACGATTTA	*Bam*HI	-	403922-403946
cheV3-3′	tttcttctgcagTTACGCATTCTTGTCTAAAATCTTAGA	*Pst*I	+	403014-403037
pTrc-seq1	TTGACAATTAATCATCCGGC			
pTrc-seq2	TTTTATCAGACCGCTTCTGC			
cheVA	tacgaagaattcCATTATCACTGAATTTAACAACG	*Eco*RI	-	403645-403668
cheVB	ttttagccatggAGATAAGAGTATTTTACAATTTTA	*Nco*I	+	402979-403003
cheAI	ttatctccatggCTAAAAAGGGGTTTTAAATGGAT	*Nco*I	-	402950-402973
cheAII	aatatcggatccTTAATAATCGCTAGGGGAATTTTTG	*Bam*HI	+	400926-400948
cheARW1	atcttaggatccTAGGAACCTAGCATGTATATTGTCTTAGCGATTGATG	*Bam*HI	-	400907-400929
cheARW2	aaaaactctagaTTGAATCAAACATTACCGCTTG	*Xba*I	+	399962-399984
cheA1	GGTAAAGTGCAATTGAGCGCG		-	401722-401742
cheA2	TCACCGTAGCACCAGCAATGC		+	401061 - 401081
cheAseq1	CTTCCCTAACACAAGCTCGCCGA		+	402103 - 402125
cheAseq2	GGGGTGATTAGCGAAAGAGACGCTG		-	401607-401631
cheAseq3	CAGCGTCTCTTTCGCTAATCACCC		+	401607 - 401630
cheAseq4	TAGTGGCTTGAAGTTGTTTGACCGC			
390-1	cttaattctagaCAAGCGGTAATGTTTGATTCAA	*Xba*I	-	339962-399984
390-2	gttactgagctcAAGAAACGTATCAGTTAGAGG	*Sac*I	+	399319-399340
cjY1-5′	catcacggatccTGAAATTGTTAGTTGTTGATGACAGTTC	*Bam*HI	-	1050660-1050632
cjY1-3′	tttcacgaattcTTACTCAGCTGCACCTTCTC	*Eco*RI	+	1051004-1051024

a. Uppercase letters indicate nucleotides derived from the genome sequences of *H. pylori* 26695 (Tomb *et al.*, 1997) or *C. jejuni* 4344. Lowercase letters indicate nucleotides introduced for cloning purposes. Underlining indicates restriction recognition sequences.

b. Restriction recognition sites.

c. Nucleotide positions in the genome sequence of *H. pylori* 26695 (Tomb *et al.*, 1997) or *C. jejuni* 4344 (Parkhill *et al.*, 2000).

3. Chemicals

All Chemicals of molecular biology research grade were obtained from the following manufactures: Amersham Biosciences, BioRad, Dibco, Genaxon, Hartman Analytic GmbH, Invitrogen, Life Technologies, MBI Fermentas, Merck, New England Biolabs, Oxoid, Perkin Elmer, Promega, QIAGEN, Roth, and Sigma.

Radioactively labelled nucleotides [γ-^{32}P] ATP (6 000 Ci/mmol) used for primer extension and *in vitro* phosphorylation and [α-^{33}P] dATP (3 000 Ci/mmol) used for sequencing, were produced by Amersham Biosciences. The radioactively labelled acetyl [^{32}P] phosphate (80 Ci/mmol) used for *in vitro* phosphorylation was obtained from Hartman Analytic GmbH.

4. Liquid medium and agar plates for *E. coli* and *H. pylori* culture

Luria-Bertani medium (LB)

10 g bacto tryptone
10 g NaCl
5 g yeast extract
volume adjusted with dH_2O to 1l

The medium was autoclaved at 121°C and 15 psi for 20 minutes and stored at 4°C.

LB agar plates

15 g agar for 1l LB medium

After autoclaving, the medium was cooled down to 60°C, appropriate antibiotics were added when required, and the medium was distributed into petri dishes and allowed to solidify at RT. The plates were stored at 4°C.

BHI medium

37 g BHI
volume adjusted with dH_2O to 900 ml

2.9 ml antibiotic supplement (see Columbia agar)
100 ml fetal calf serum (FCS)

The medium was autoclaved at 121°C and 15 psi for 20 min. After autoclaving, the medium was stored at room temperature (RT). Before use the antibiotics supplement and FCS were added into the medium. The supplemented medium was stored at 4°C.

Columbia agar plates

44 g Columbia agar (Gibco)
volume adjusted with dH_2O to 940 ml

2.9 ml antibiotic supplement
50 ml horse blood
1 ml cycloheximide (100 mg/ml stock solution in aceton, stored at 4°C)

Antibiotic supplement:

0.5 ml vancomycin (10 mg/ml stock solution in dH_2O, stored at 4°C).
1 ml trimethoprim (10 mg/ml stock solution in N,N-dimethylformamid, stored at 4°C).
0.6 ml cefsulodin (10 mg/ml stock solution in dH_2O, stored at 4°C).

0.8 ml amphotericin B (10 mg/ml in dimethylsulfoxid, stored at –20°C).

The medium was autoclaved at 121°C and 15 psi for 20 min. After autoclaving, the medium was cooled down to 60°C and antibiotic supplement, horse blood, appropriate antibiotics, and cychloheximide were added. The agar was distributed into Petri dishes and allowed to solidify at RT. The plates were stored at 4°C.

Brucella medium

28 g Brucella broth
volume adjusted with dH_2O to 900 ml

The medium was autoclaved at 121°C and 15 psi for 20 min, and then it was stored at RT.

Brucella medium with 20% glycerol

Brucella medium with 20% glycerol was used for the storage of all *H. pylori* strains at –80°C.

Brucella swarm agar

28 g Brucella broth
0.3 % agar
volume adjusted with dH_2O to 900 ml

100 ml FCS
2.9 ml antibiotic supplement (see Columbia agar)
1 ml cycloheximide solution (100 mg/ml stock solution)

The medium was autoclaved at 121°C and 15 psi for 20 min. After autoclaving, the medium was cooled down to 60°C and antibiotic supplement, FCS, and cycloheximide were added. The agar was distributed into Petri dishes and allowed to solidify at RT. The plates were stored at 4°C (upright).

5. Antibiotics

Antibiotics were produced by Sigma. Stock solutions were filter sterilized and stored at -20°C.

Ampicillin: stock solution 50 mg/ml in dH_2O, working concentration 100 μg/ml for *E. coli*.

Chloramphenicol: stock solution 30 mg/ml in ethanol, working concentration 30 μg/ml for *E. coli* and 20 μg/ml for *H. pylori*.

Kanamycin: stock solution 50 mg/ml in dH_2O, working concentration 50 μg/ml for *E. coli* and 20 μg/ml for *H. pylori*.

6. Molecular weight markers

1kb DNA Ladder (Fermentas):

Fragment sizes:	9162 bp	5090 bp	1636 bp	344 bp	154 bp
	8144 bp	4072 bp	1018 bp	298 bp	134 bp
	7126 bp	3054 bp	506 bp	220 bp	75 bp
	6108 bp	2036 bp	396 bp	201 bp	

BioRad range marker for SDS-PAGE

200 kDa	myosin
116,25 kDa	β-algactosidase
97,4 kDa	phosphorylase b
66,2 kDa	bovine serum albumin
45 kDa	ovalbumin
31 kDa	carboanhydrase
21,5 kDa	trypsin inhibitor
14,4 kDa	lysozyme
6,5 kDa	aprotinin

7. Instruments

Autoclave	Webeco
Autoradiography machine	Kodak M 35 X-OMAT Processor
Cold centrifuge	Eppendorf
Spectrophotometer	Gene Quant II, Amersham
DNA sequencer	Beckman Coulter
Electrophoresis apparatus	Bio-Rad, Hartenstein
Gel dryer	BioRad 1125 B
Heating blocks	Hartenstein

Incubator with microaerophilic environment	CO_2-Auto-Zero Heraeus
Laminar flow clean bench	NUAIR
Liquid scintillation counter	Beckman coulter (LS 1800)
Macrocentrifuge	Eppendorf
Magnetic stirrer	Gerhardt
Microcentrifuge	Eppendorf
Microliter pipettes	Eppendorf
PCR machine	Bio-med, Termocycler 60
pH meter	WTW pH523, Metrohm-Herisau
Phosphorimager	Taifun, Molecular Dynamics
Refrigerators (-20°C; -80°C)	Privileg
Shaking incubator	Hartenstein
Spectrophotometer	Amersham
Speed-vac-concentrator 5301	Eppendorf
Microcentrifuge	Heraeus
UV light	Keiser
Vortexter	Hartenstein

D METHODS

1. Bacterial culture

1.1. Growth conditions for *H. pylori, C. jejuni* and *E. coli*

H. pylori and *C. jejuni* strains from frozen stocks were grown under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood, 0,2% cycloheximide and antibiotic supplement at 37°C for two or three days. After passaging on fresh plates, bacteria were cultured in a 5% CO_2-95% air atmosphere at 37°C. When required, the blood agar plates were supplemented with kanamycin or chloramphenicol at a final concentration of 20 µg/ml.
Brucella broth with 20% glycerol was used for storage of the *H. pylori* strains at -80°C.
E. coli strains were grown in LB broth. When necessary, antibiotics were added to the following final concentrations: ampicillin 100µg/ml, kanamycin 50µg/ml, and chloramphenicol 30 µg/ml.

1.2. Motility assay

To determine the swarming ability of *H. pylori* Brucella swarm agar plates were inoculated with *H. pylori* bacteria grown on blood agar plates and the plates were incubated for 4-5 days in a 5% CO_2-95% air atmosphere at 37°C.

1.3. Preparation of $CaCl_2$-competent *Escherichia coli* cells

100 ml of LB broth were inoculated with 1 ml of an overnight culture of *E. coli* DH5α or M15. For M15 cells kanamycin was added to a final concentration of 25 µg/ml to the medium. The cells were grown to an OD_{590} of 0.5, incubated for 30 min on ice and centrifuged for 10 min at 4°C and 6 000 rpm in a macrocentrifuge. After resuspension of the pellet in 1/5 volume of ice-cold 0.1 M $CaCl_2$ and incubation on ice for 15 min the suspension was centrifuged under the same conditions as described above. Finally, cells were resuspended in 1/10 volume of ice-cold 0.1 M $CaCl_2$ with 20% glycerol and aliquots of 250 µl were stored at -80°C.

1.4. Transformation

1.4.1. Transformation of $CaCl_2$-competent *Escherichia coli* cells

200 µl of competent cells (*E. coli* DH5α or M15) were mixed with 20-200 ng of plasmid-DNA or 10 µl of ligation reaction. Cells were incubated for 30 min on ice followed by incubation for 90 sec at 42°C and immediately transferred for 2 min on ice. After adding 1 ml of LB broth cells were incubated for about 60 min at 37°C in a shaking incubator for the expression of the resistance genes. Finally, the culture was centrifuged and the pellet was resuspended in 100 µl of LB and plated onto LB agar plates with the respective antibiotic(s).

1.4.2. Transformation of naturally competent *H. pylori* cells with plasmid DNA

Natural transformation of *H. pylori* was carried out on Columbia agar plates by adding 5 µg of plasmid DNA, which was resuspended in 10 µl of Brucella broth, to a spot of logarithmically growing bacteria, which was preincubated for 5 hours at 37°C. After incubation overnight at 37°C, bacteria were collected and plated on Columbia agar containing

the respective antibiotic. After 5 days of incubation single colonies were selected for further analysis. Transformants were characterized by PCR and/or Southern blot.

2. Isolation of nucleic acids

2.1. Isolation of DNA

2.1.1. Small scale preparation of chromosomal DNA from *H. pylori*

H. pylori was grown on Columbia agar plates in an atmosphere of 5% CO_2-95% air for 1 or 2 days. Bacteria from 1 plate of Columbia agar were harvested and resuspended in 1.5 ml of 1x STE buffer (100 mM NaCl; 20 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0). Then the cells were sedimented for 10 min at 14 000 rpm in a microcentrifuge. After resuspension of the pellet in 350 µl of 1x STE buffer, 4 µl of lysozyme (100 mg/ml) and 4 µl of RNase (10 mg/ml) were added to the cell suspension and the sample was incubated at 37°C for 10 min. Then 17.5 µl of 10% SDS were added and the sample was incubated at 65°C for 15 min. Finally, 5 µl of proteinase K (100 mg/ml) were added and after 2 hours of incubation at 50°C extraction of the chromosomal DNA was performed with 400 µl of phenol/chloroform/ isoamylalcohol (25:24:1). The DNA was precipitated with 2.5 volumes of absolute EtOH. Subsequently, the pellet containing the DNA was washed with 70% EtOH and dried for 10 min in a Speed-vac-concentrator (Eppendorf). Chromosomal DNA was redissolved in 20-100 µl of dH_2O.

2.1.2. Large scale preparation of chromosomal DNA from *H. pylori*

Solution 1: 50 mM Na_2EDTA
50 mM Tris-HCl (pH 8.0)

Solution 2: 400 mM Na_2EDTA
50 mM Tris-HCl (pH 8.0)
0.5 % SDS

Solution 3: 1 mM Na_2EDTA
50 mM Tris-HCl (pH 8.0)

H. pylori was grown on Columbia agar plates in an atmosphere of 5% CO_2-95% air (usually about 1 or 2 days). Bacteria from 3 to 5 plates were harvested. Bacteria were resuspended in 10 ml of PBS and centrifuged for 10 min at 5 000 rpm in a macrocentrifuge. The pellet was resuspended in 10 ml of solution 1, and frozen at -20°C for about 30 min or overnight. The sample was thawed slowly and 1 ml of lysozyme (10 mg/ml) was added into the suspension. The sample was incubated on ice for 45 min. Then 6 ml of solution 2 and 1 ml of proteinase K (20 mg/ml) were added and the sample was incubated at 50°C for 2 hours. The extraction of the DNA was performed with phenol/chloroform/isoamylalcohol (25:24:1) two times followed by an extraction with chloroform/isoamylalcohol (24:1). After each step the mixture was centrifuged for 15 min at 5 000 rpm in a macrocentrifuge. The DNA was precipitated with 0.1 volume of 5N NaCl and 2 volumes of ice-cold absolute EtOH and sedimented for 15 min at 5 000 rpm in a macrocentrifuge. The DNA was spooled using a Pasteur pipette and resuspended in 5 ml of solution 3 and 150 µl of RNAse (10 mg/ml). Then the solution was incubated for 2 hours at 37°C or overnight at 4°C. After an extraction with phenol/ chloroform/isoamylalcohol and an extraction with chloroform/isoamylalcohol as described

above, the DNA was precipitated with 2.5 volumes of ice-cold absolute EtOH and 1/10 volumes of 5M NaCl and sedimented for 15 min at 5 000 rpm in a macrocentrifuge. The pellet was washed with ice-cold 70% EtOH and after drying it was redissolved in 500 µl of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA) and stored at 4°C.

2.1.3. Mini preparation of plasmid-DNA from *E. coli*

- Solution 1: 25 mM Tris-HCl (pH 8.0)
  50 mM glucose
  10 mM Na_2EDTA
- Solution 2: 0.2 M NaOH
  1 % SDS
- Solution 3: 60 ml 5M KOAc
  11.5 ml acetic acid
  28.5 ml dH_2O
- Solution 4: 0,25 g silica
  4.0 ml Triton X-100
  47.76 g GuHCl
  adjusted with H_2O to 100 ml
- 1x TE buffer: 10 mM Tris-HCl (pH 7.5)
  1 mM Na_2EDTA

A single colony of the transformed *E. coli* cells was used to inoculate 3 ml LB medium supplemented with selective antibiotics and the culture was grown overnight at 37°C in a shaking incubator. The culture was centrifuged for one min at 14 000 rpm. The pellet was resuspended in 100 µl of solution 1 followed by the addition of 100 µl of solution 2. The resulting suspension was incubated at RT for 3 min. After adding 100 µl of solution 3 and incubation for 3 min at RT the sample was centrifugated for 5 min at RT at 14 000 rpm. The supernatant containing the DNA was transferred to a fresh Eppendorf tube and 800 µl of solution 4 were added. The sample was vortexed and centrifuged for one min at RT at 5 000 rpm. The pellet was washed with ice-cold 70% EtOH and air-dried. Finally, the purified DNA was resuspended in 30 µl of TE buffer or dH_2O.
A modification of the method described above was used in some cases. The pellet from the overnight culture was resuspended in 100 µl of solution 1. The sample was incubated for 5 min at RT, followed by the addition of 200 µl of solution 2 and then incubated for 5 min on ice. After the addition of 150 µl of solution 3 and incubation for 5 min on ice the sample was centrifuged for 10 min at 4°C and 14 000 rpm. The purification of the DNA was performed by phenol/chloroform extraction (1:1). DNA was precipitated with 2 volumes of absolute ice-cold EtOH followed by centrifugation. The resulting pellet was dried and resuspended in 50 µl of TE. The resuspended DNA was incubated with 20 µg/µl of RNase for 30 min at 37°C and stored at -20°C.

2.1.4. Maxi preparation of plasmid-DNA from *E. coli* with the Nucleobond AX-Kit from Macherey Nagel

- **S1-Buffer** 10 mM EDTA
  50 mM Tris-HCl (pH 8.0)

100 µg/ml RNase A
Storage at 4°C

- **S2-Buffer** 200 mM NaOH
  1 % SDS

- **S3-Buffer** 2.80 M KOAc (pH 5.1)

- **N2-Buffer** 900 mM KCl
  100 mM Tris
  15 % ethanol
  adjusted with H_3PO_4 to pH 6.3
  0,15 % Triton X-100

- **N3-Buffer** 1,2 M KCl
  100 mM Tris
  15 % ethanol
  adjusted with H_3PO_4 to pH 6.3

- **N5-Buffer** 1 M KCl
  100 mM Tris
  15 % ethanol
  adjusted with H_3PO_4 to pH 8.5

Using the Nucleobond®-AX-Kit the purification of DNA is performed by anion exchange chromatography. Columns and buffers are manufactured by Macherey & Nagel.
50 ml of an *E. coli* culture in LB broth were centrifuged for 10 min at 6 000 rpm. The pellet was resuspended in 4 ml of buffer S1 and 4 ml of buffer S2 were added for the disruption of bacterial cells. The mixture was incubated at RT for 5 min. 4 ml of buffer S3 were added and the suspension was mixed gently by inverting the tube 6-8 times until a homogeneous suspension was formed. This suspension was incubated on ice for 5 min and loaded onto a filter that was placed on the column. DNA binds to the silica of the column which was preequilibrated with 2 ml of buffer N2 while all debris and chromosomal DNA remain in the filter. After washing the column with 5 ml of buffer N3 elution of the plasmid DNA was performed using 2 ml of buffer N5.
Finally, the purified plasmid was precipitated with 0.7 volumes of isopropanol and centrifuged for 20 to 40 min at RT and 14 000 rpm. The pellet was washed with ice-cold 70% EtOH, dried, redissolved in dH_2O and stored at -20°C.

2.1.5. Calculation of DNA concentration

The sample was diluted 1:100 with dH_2O and the extinction coefficient was measured with a photometer (Gene Quant II, Pharmacia BIOTECH) at 260 nm and 280 nm. The concentration of the DNA was calculated using the following formula:

DNA-conc (µg/ml)= OD_{260nm} x dilution factor x 50

OD_{260}/OD_{280} indicates the purity grade of the prepared DNA. Values between 1.8 and 2 indicate a sufficient degree of purity of the DNA.

2.2. Isolation of RNA

The chemical instability of RNA molecules and the ubiquitous presence of RNAses, which do not need metal ion-cofactors and can maintain activity even after prolonged boiling or autoclaving, make the extraction of RNA more difficult than the extraction of DNA. Hence, the isolation of RNA requires some precautions. Therefore, all solutions that were used throughout the extraction were treated previously with diethylpyrocarbonate (DEPC) as follows: 1 ml of DEPC was added to 1l dH_2O and shaken thoroughly. After incubation at 37°C overnight pre-treated solutions were autoclaved.

2.2.1. Isolation of RNA from *H. pylori*

The bacteria from about a half plate of Columbia agar were harvested and resuspended in 1 ml of BHI medium. The suspension was used to inoculate 40 ml of BHI medium to an OD_{590} of about 0.1. Liquid cultures of *H. pylori* were grown at 37°C in an anaerobic jar under permanent shaking until an OD_{590} of 1. The culture was transferred to a centrifuge tube containing 125 µl of phenol and 2 ml ice-cold absolute EtOH and the sample was centrifuged for 10 min at 4°C and 6 000 rpm in a macrocentrifuge. The pellet was frozen immediately by placing the tube into liquid nitrogen and then stored at -20°C.
For the RNA extraction the pellet was resuspended in 1 ml of TRIzol reagent (monophasic solutions of phenol and guanidine isothiocyanate manufactured by Invitrogen), and incubated for 5 min at RT. Then 200 µl of chloroform were added, the sample was mixed carefully and it was incubated for 3 min at RT. After centrifugation by no more than 12 000 g for 15 min at 4°C the colorless phase was transferred to a new tube. Subsequently, 500 µl of isopropylalcohol were added and the sample was incubated for 10 min at RT. After centrifugation for 10 min under the same conditions as described above the pellet was resuspended in 1 ml of ice-cold 70% EtOH, vortexed and centrifuged at no more than 7 500 g for 5 min at 4°C. The pellet was air dried for about 20 min, and then the sample was resuspended in 50-100 µl DEPC-dH_2O and incubated for 10 min at 55-60°C. Extracted RNA was stored at -20°C.

2.2.2. Calculation of the RNA concentration

The sample was diluted 1:100 with dH_2O and the extinction coefficient was measured with a photometer (Gene Quant II, Pharmacia BIOTECH) at 260 nm and 280 nm. The RNA concentration was calculated using the following formula:

RNA-conc (µg/ml) = OD_{260nm} x dilution factor x 40

OD_{260}/OD_{280} indicates the purity grade of the prepared RNA. Values between 1.8 and 2 indicate a sufficient degree of purity of the RNA.

3. Separation of DNA and RNA by gel electrophoresis

3.1. Agarose gel electrophoresis (Sambrook *et al.*, 1989)

The agarose gel electrophoresis was employed to check the quality of purified DNA or RNA and the DNA digested by endonucleases, and DNA fragments obtained by PCR.

- 10x TBE-Puffer: 890 mM Tris-HCl
  890 mM boric acid

2 mM Na_2EDTA

1x TBE is usually used as the running buffer

- 2.5x sample buffer: 4 M urea
  50 % sucrose
  50 mM Na_2EDTA
  0,1 % bromophenol blue

Gels containing 1% and 1.5% of agarose were used to analyse DNA and RNA, respectively. Moreover, for DNA fragments smaller than 100 bp gels containing 2% agarose were used. The agarose was dissolved in 1x TBE by heating. After dissolving, the solution was cooled down to about 50°C and ethidium bromide was added to a final concentration of 0.5 µg/ml. DNA and RNA samples were electrophoresed with sample buffer (1:0.66) at 150 V and 70 V, respectively. A molecular weight marker was used as a standard (Fermentas). Nucleic acids were stained by ethidium bromide and visualized by UV light and photographed by a video printer (Keiser and Sony, respectively).

3.2. Isolation of DNA fragments from agarose gels using the QIAquick Gel Extraction Kit from QIAGEN

For the extraction of DNA-fragments with sizes ranging from 200 bp to 10 kb from agarose gels the QIAquick Gel Extraction Kit from QIAGEN was used. The DNA was electrophoresed, and then the slice of gel with the DNA fragment was excised with a scalpel and put into an Eppendorf tube. After determination of the weight of the gel slice 300 µl of QG buffer (QIAGEN) were added per 100 mg of the gel, then the mixture was incubated for 10 min at 50°C (or until the gel slice was completely dissolved). Subsequently, 100 µl of isopropanol were added per every 100 mg of gel, and then the sample was loaded onto the QIAquick column and centrifuged for 1 min. Finally, the column containing the sample was washed with 0.75 ml of PE buffer (QIAGEN), centrifugated for 1 min and the DNA was eluted with 30-50 µl of dH_2O by centrifugation for 1 min.

4. Modification of DNA

4.1. Digestion of DNA by restriction enzymes

A typical restriction reaction contains one unit of restriction endonuclease for 1 µg of DNA and the volume of the enzyme has to be no more than one tenth of the total volume of the reaction.
The concentration of the reaction buffer to be used is indicated by the manufacturer.
The digestion with two different enzymes was performed in two subsequent steps. First a digestion was performed with one of the endonucleases under appropriate conditions. Then, the reached buffer was removed with the QIAquick-spin-Column from QIAGEN and a digestion was carried out with the second enzyme under appropriate conditions.
Alternatively, a double digestion in 2x Tango buffer using two enzymes at the same time was performed. This method was used only for control samples.
The inactivation of endonucleases was performed using the QIAquick-spin-Column from QIAGEN or by the addition of 2.5x sample buffer. Restriction fragments obtained by the action of the endonucleases were analysed by agarose gel electrophoresis.

4.1.1. Isolation of DNA fragments using the QIA-quick PCR purification kit from QIAGEN

This protocol was used to separate double-stranded PCR products ranging in size from 100 bp to 10 kb from primers, nucleotides, enzymes and salts. The PCR sample was mixed with 5 volumes of PB buffer (QIAGEN). To bind DNA, the sample was loaded onto a quick spin column and centrifuged for 60 sec at 14 000 rpm. Washing was performed by adding 0.75 ml of PE (QIAGEN) buffer followed by centrifugation for 60 sec at 14 000 rpm. After discarding the flow-through, the column was centrifuged for 1 min at maximum speed. DNA fragments were eluted with 30 to 50 µl of dH_2O.

4.2. Ligation with T4 DNA ligase (Fermentas)

The T4 DNA ligase catalyzes the formation of phosphodiester bonds between adjacent 3´-OH and 5´-P ends in dsDNA in the presence of ATP. This enzyme is active at sticky and blunt ends. In the reactions with sticky ends the ratio of vector:insert is 1:3 and in the reactions with blunt ends it is 1:10. Reactions were performed for 3 hours at RT or overnight at 16°C. All ligation reactions were prepared in a final volume of 10 µl with 1 µl of 10x ligase buffer (400 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT and 5 mM ATP), 1 µl of ligase enzyme (5 U/µl), and amounts of digested DNA fragments and digested vector DNA as described above.

4.3. Dephosphorylation of DNA with calf intestinal alkaline phosphatase

Calf intestinal alkaline phosphatase (CIAP) catalyzes the dephosphorylation of 5´-ends from ssDNA, dsDNA, ssRNA, dsRNA. Therefore, this enzyme was used to remove phosphate groups from the 5´-end of a vector which was digested with a single restriction enzyme, in order to avoid the self ligation of the plasmid.
A typical dephosphorylation reaction was performed as follow:

50 µl linearized vector
3 µl CIAP (Fermentas)
10 µl 10x buffer for CIAP (Fermentas)
volume adjusted to 100 µl with dH_2O

The reaction mixture was incubated for 1 hour at 37°C and then the dephosphorylated plasmid was removed from the enzyme and the buffer using the QIAquick PCR purification Kit of QIAGEN.

5. Analysis of DNA by Southern blot and Slot blot

5.1. Southern Blot

Depurination solution:	0.25 M HCl
Denaturation solution:	1.5 M NaCl 0.5 M NaOH
Neutralizing solution:	1.5 M NaCl 0.5 M Tris HCl adjusted to pH 7.5

20x SSC buffer:	0.3 M Na_3 citrate 3 M NaCl pH 7.0
Primary wash buffer:	6 M urea 0.4 % SDS 0.5 x SSC stored at 4°C
Secondary wash buffer:	2 x SSC stored at 4°C
Hybridization buffer:	5 % blocking reagent 0.5 M NaCl volume adjusted with hybridization buffer

Blocking reagent and hybridization buffer were produced by Amersham Pharmacia Biotech.

5.1.1. Electrophoresis and processing of the gel

5-10 µg of chromosomal *H. pylori* DNA were digested in two subsequent steps with the appropriate restriction enzyme: the first incubation was carried out overnight using 100 U of the enzyme, and then another 50 U of enzyme were added and the incubation was continued for about three hours. Digested chromosomal DNA samples were electrophoresed in a 1% TBE-agarose gel at 150 V until the bromophenol blue had run two thirds of the way down the gel. After electrophoresis DNA was analysed under UV-light and photographed in the following way: a transparent ruler was placed alongside the gel parallel to the first slot in the gel containing the DNA marker, so that the distance that any band of DNA had migrated could be determined directly from the photographic image. The gel was then transferred to a glass or plastic dish and incubated with constant gentle agitation at RT in 10 gel volumes of the follow solution: (1) depurination solution for 15 min (two times), (2) denaturation solution for 20 min, (3) neutralizing solution for 20 min, and (4) 20x SSC buffer for 20 min. Between every incubation step the gel was rinsed briefly in dH_2O.

5.1.2. Transfer of DNA on a nylon membrane by capillary blotting

A piece of filter paper was placed on the bottom of an inverted dish to form a support that is longer and wider than the gel. The ends of the filter paper should drape over the edges of the dish. The support was placed into a large dish containing 20x SSC buffer. When the filter paper on the top of the support was thoroughly wet, the gel was put on this paper and the soaked Hybond-N+ nylon membrane (preincubated for 10 min with 20x SSC buffer) was placed on the top of the gel. A glass pipette was rolled across the surface of the membrane to smooth away any air bubbles. About 10 soaked and 10 dry filter papers were placed above the membrane and at the top a stack of paper towels (5-7cm) and a glass plate were put and weighed down with about 400 g weight (Fig.7).
After transfer of DNA to the membrane for 8-24 hours the membrane was marked with a pencil to distinguish the side with the transferred DNA and the transferred DNA was fixed using UV irradiation.

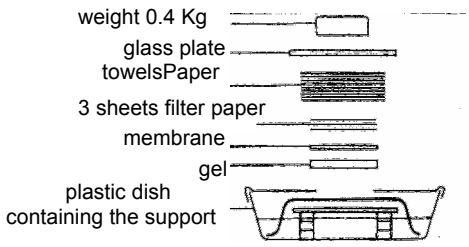

Fig.7 Schematic representation of the capillary blotting technique used for Southern blot analysis (See text for detail).

5.1.3. Labelling and hybridization of probes and signal detection

The DNA fragment to be used as a probe was diluted to a concentration of 10 ng/µl in a final volume of 10 µl and denatured for 5 min at 100°C. Immediately, the denatured sample was incubated on ice for 5 min. Then 10 µl of both labelling reagent and glutaraldehyde (ECL kit of Amersham pharmacia biotech) were added to the cooled sample. The sample was mixed and quickly centrifuged to collect the contents at the bottom of the tube. Then the sample was incubated at 37°C for 10 min. The labelled sample can be held on ice for a short period of 10-15 min.
Hybridization buffer was prepared about 2 hours before the labelling of the sample. It was prepared as follow: blocking agent, NaCl, and hybridization buffer were mixed in a beaker placed on a magnetic stirrer for about 1 hour and then the solution was pre-heated for 15-60 min at 42°C. The blot (nylon membrane with the fixed DNA) was prehybridized for 1 hour at 42°C with gentle agitation. Following pre-hybridization the labelled probe was added avoiding placing it directly on the membrane and hybridization was carried out in a shaking incubator at 42°C overnight. After hybridization the membrane was washed two times with primary wash buffer (pre-heated to 42°C) for 20 min. Subsequently, in a new container the blot was washed with secondary wash buffer at RT for 5 min. Finally, the detection of the hybridization signal was performed with a mixture of equal volumes of detection reagent 1 and detection reagent 2 (ECL kit of Amersham pharmacia biotech) with a final volume of 0.125 ml reagents mixture/cm^2 membrane. In a fresh dish containing the membrane the detection mixture was added avoiding placing it directly on the blot. After 1 min of incubation the membrane was exposed with X-ray films (Konica Minolta) for 30 sec to some hours depending on the intensity of the expected signal.

5.2. Slot Blot

5.2.1. Transfer of DNA on a nylon membrane

First, a sheet of Hybond-N+ nylon membrane and 2-3 filter papers with the same size as the slot blot apparatus were soacked with 6x SSC buffer. Soacked membrane and filter papers were placed into the slot blot apparatus and vacuum was applied. 5-10 µg of chromosomal DNA were diluted with 20x SSC to a final volume of 200-400 µl. This sample was denatured for 10 min at 100°C. Denatured DNA can be held on ice for a short period.
To transfer the DNA on the nylon membrane the denatured samples were loaded into the slots of the apparatus. After loading, the membrane was placed on a soaked filter paper with denaturation solution for 10 min and then on a filter with neutralizing solution for 5 min.
Fixation, labelling, hybridization and detection of signals were performed as described above for the Southern blot.

6. Amplification of DNA by the Polymerase chain reaction (Mullis & Faloona, 1987; Saiki *et al.*, 1988)

6.1. General PCR method

PCR is an enzymatic method for *in vitro* synthesis of multiple copies of specific sequences of DNA. The reaction mixture for PCR contained the following components:

PCR reaction mixture:

template-DNA (20 ng/µl)	1 µl
10x PCR buffer	10 µl
dNTPs (20 mM)	1 µl
oligonucleotide I (100 pmol/µl)	1 µl
oligonucleotide II (100 pmol/µl)	1 µl
$MgCl_2$ (25 mM)	4 µl
thermostable DNA polymerase	0.3 µl (5 U/µl)
dH_2O	up to 100 µl

The PCR mixture was immediately incubated in a PCR machine for amplification using a cycling program as follows:

initial denaturation:	3 min, 94°C
1-denaturation during cycling:	1 min, 94°C
2-primer annealing:	1 min, X°C (depending on the primers used)
3-primer extension:	1 min/1kb, 72°C
30 cycles (steps of 1-3)	
final extension:	3 min, 72°C

The anneling temperature is dependent on the primers used and is chosen normally 5-10°C below the Tm of the primer. The time of elongation is determined by the polymerase used and the length of the desired DNA fragments. Taq polymerase can synthesize 1 kb of DNA in 1 min. Products of the PCR were analyzed by agarose gel electrophoresis and purified using QIAquick-spin-Columns from QIAGEN.

6.2. Site-specific mutagenesis by overlapping extension

Four primers and three PCRs were used to create a site-specific mutation by overlapping extension (Ho *et al.*, 1989). One pair of primers (P5/Mut1) is used to amplify DNA that contains the mutation site together with the upstream sequence. The second pair of primers is used in a separate PCR to amplify DNA that contains the mutation site together with the dowmstream sequence (P3/Mut2). These two PCR reactions were carried out using as template a plasmid containing the gene of interest. The desired mutation(s) is located in the region of overlap and therefore in both amplified fragments. The overlapping fragments were mixed, and used as template for a third PCR using two primers that bind to the 5´ and 3´ ends of the individual fragments (P5/P3).

The sequence of the mutagenic oligonucleotide primers should share an overlap of at least 15 bp, and the mismatched base pairs within these primers should be located in the center of the oligonucleotides. The primer sequences at the 5´ and 3´ ends of the DNA fragment to be amplified can contain unique restriction endonuclease cleavage sites to aid in the subsequent cloning of the mutagenized DNA fragment. PCR reactions were performed as described above.

7. Sequencing of DNA

7.1. Automatic DNA sequencing by the CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit from Beckman Coulter

DNA sequencing was performed on a Beckman Coulter sequencer based on a method which is a variant of the dideoxynucleotide method (Sanger *et al.*, 1977).
The PCR reaction for sequencing included 15 µl of DNA template (200-1000 ng) that was denatured (when dsDNA was used) for 3 min at 96°C, 1 µl of primer (5 pmol/µl), and 4 µl of sequencing mix QSM (Quick Start Master Mix provided by Beckman Coulter in the CEQ Dye terminator cycle Sequencing Kit). The thermal cycling conditions were as follows: 96°C/20 sec; 50°C/20 sec; 60°C/4 min, for 30 cycles. The PCR product was mixed with 5 µl of stop solution (2:2:1 of 3 M NaOAc, pH 5.2; 0.1 M EDTA; and glycogen stock solution 20 mg/ml) and 60 µl of absolute EtOH. After centrifugation for 15 min at 15 000 rpm and 4°C the pellet was washed with 200 µl of 70% ice-cold EtOH and dried for about 5 min in a speedVac. Finally, the pellet was resuspended in 40 µl of SLS (Sample Loading Solution provided in the kit) and the sample was run in the Beckman Coulter Sequencer.

7.2. DNA sequencing using the ^{T7}Sequencing ^{TM} Kit (Amersham Pharmacia Biotech)

In this work the ^{T7}Sequencing ^{TM} Kit was used for the sequencing of a DNA fragment containing the promoter region of a gene whose transcription was studied in a primer extension experiment.
To denature the template 32 µl of the plasmid DNA (2 µg) were incubated with 8 µl of 2M NaOH for 10 min at RT. Subsequently, 7µl of 3M NaOAc, pH 4.8, 4 µl of dH_2O, and 120 µl of absolute EtOH were added to the sample followed by incubation overnight at -20°C. The denature sample was centrifuged for 15 min at 4°C and 15 000 rpm. Then the sedimented DNA was washed with 70% EtOH. After drying in a vacuum rotator for 10 min the pellet was resuspended in 10 µl of dH_2O. For annealing of the primer 2 µl of oligonucleotide (5 pmol/µl) and 2 µl of the annealing buffer were added to the sample followed by mixing and incubation for 5 min at 65°C. Immediately, the sample was incubated for 10 min at 37°C. Then the sample was incubated with 3 µl of labelling mix, 1.5 µl of α-^{33}P dATP (3 000 Ci/mmol) and 2 µl of diluted T7 DNA polymerase (1 µl of enzyme in 4 µl of enzyme dilution buffer) for 5 min at 37°C. In parallel four eppendorf tubes containing 2.5 µl of one of the Short-Mixes (A, C, G, T), were incubated at 37°C as well. 4.5 µl of the labelled sample-primer mixture was added to each tube containing the didesoxynucleotide mixtures and then the samples were incubated for 5 min at 37°C. Then 5 µl of the stop solution were added to each tube and the samples were incubated for 2 min at 75-80°C. Finally, the samples were electrophoresed on a 6% polyacrylamide urea gel (0.4 mm x 20 cm x 40 cm) at 1 500V.
For more information about the different solutions and the method see the handbook of ^{T7}Sequencing ^{TM} Kit (Amersham Pharmacia Biotech).

8. RNA analysis

8.1. Reverse transcription and PCR amplification (RT-PCR)

Reverse transcription is the synthesis of cDNA from RNA. In the nature there are retroviruses which are RNA viruses containing reverse transcriptase which catalyzes the conversion of the viral RNA genome into DNA in the host cell. The reverse transcriptase uses the viral RNA but also other RNA as a template. For this reason it is a key enzyme used in the laboratory.
In RT-PCR complementary DNA (cDNA) is prepared by isolating messenger RNA (mRNA) from cells and transcribing it into cDNA using the enzyme reverse transcriptase. A double stranded cDNA is obtained by a typical PCR where the single-stranded cDNA functions as a template for the thermostable DNA polymerase.
In this work the RT-PCR kit from Stratagene was used for the synthesis and amplification of DNA from prokaryotic RNA.
For the synthesis of first strand of cDNA 5-10 µg of RNA were used in a final volume of 38 µl with DEPC dH_2O. 3 µl of oligo (dt) primer (0.1 µg/µl) were added and the sample was incubated for 5 min at 65 °C followed by an incubation of 10 min at RT, thus allowing the annealing of the primer. Subsequently, other components were added in the following order: 5 µl of 10x first-strand buffer, 1 µl of RNAse block ribonuclease inhibitor (40 U/µl), 2 µl of 100 mM dNTPs and 1 µl of MMLV-RT (reverse transcriptase 50 U/µl). The sample was incubated for 1 hour at 37°C, and then for 5 min at 90°C. The amplification and synthesis of dsDNA from the synthesized cDNA was performed by PCR as described above with 5 µl of the synthesized cDNA as template.

8.2. Primer extension analysis

Primer extension is a technique that is used to determine the location of the 5´-ends of specific RNAs and to roughly quantify the amount of transcript. In this technique, a radiolabeled DNA primer, complementary to the RNA being studied is hybridized to the target RNA and extended using the enzymatic properties of reverse transcriptase. The DNA primer is designed to anneal to the sequences near the 5´-end of the target RNA and the extension reaction terminates when the reverse transcriptase reaches the extreme 5´-end of the RNA. The length of the cDNA product accurately defines the distance between the 5´-end of the radiolabelled primer and the 5´-end of the RNA (transcriptional start site), and the quantity of cDNA produced is roughly proportional to the amount of target mRNA.

8.2.1. 5`-Labelling of the oligonucleotide

For labelling of the oligonucleotide, the following components were mixed:

1 µl oligonucleotide primer (5 pmol/µl)
3 µl [γ-^{32}P]ATP (6 000 Ci/mmol)
1 µl 10x kinase buffer (Fementas)
4 µl DEPC dH_2O
1 µl T4 polynucleotide kinase (30 U/µl; Fermentas)

The reaction mixture was incubated for 30 min at 37°C, then the labelled oligonucleotide was removed from non incorporated [γ-^{32}P]ATP by the QIAquick Nucleotide Removal Kit from QIAGEN using the following protocol: labelled oligonucleotide was mixed with 10 volumes of PN buffer (QIAGEN). To bind the DNA, the sample was loaded onto the column and centrifuged for 60 sec at 6 000 rpm. Washing of the column was performed by adding 0.5 ml of PE buffer (QIAGEN) followed by centrifugation for 60 sec at 6 000 rpm. This step was repeated once. After discarding the flow-trough, the column was centrifuged for 1 min at

maximum speed. Then the elution of the oligonucleotide was performed with 30 µl of DEPC dH_2O followed by centrifugation for 1 min. Finally, 2 µl of radiolabelled primer were counted in 3 ml of scintillation fluid in a liquid scintillation counter (Beckman coulter). The labelled oligonucleotide was stored at –20°C for further applications.

8.2.2. Primer extension

6% polyacrylamide urea gel:

24 g urea
10 ml 30% polyacrylamide
5 ml 10x TBE
volume adjusted with dH_2O to 50 ml

200 µl 10% APS
45 µl Temed

30 µg of RNA were precipitated with 2 000 000 cpm of the 5´-labelled primer by addition of 1/10 volume of 3M NaOAc, pH 4.8 and 2.5 volumes of absolute ice-cold EtOH. The sample was incubated at -20°C overnight. After centrifugation of the primer-RNA mixture for 15 min at 15 000 rpm and 4°C the pellet was washed with 70% EtOH, dried and resuspended in 5 µl of DEPC dH_2O. Then 2 µl of 5x reverse transcriptase buffer (Roche) and 2 µl of dNTPs (2 mM) were added and the sample was incubated for 2 min at 100°C. After a quick centrifugation, 1 µl of AMV reverse transcritase (Roche) was added and the sample was incubated for 45 min at 45°C. After the synthesis of the cDNA the rest of RNA was removed by incubation with 1µl RNase for 10 min at RT. The reaction was stopped by addition of 5µl of stop solution. Before loading onto the 6% polyacrylamide-urea gel for electrophoresis, samples were heated to 75-80°C for 2 min. Electrophoresis was carried out at 150V for about 2 hours.
The gels were autoradiographed and when required the signals were quantified using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences) and ImageMaster TotalLab Software (Amersham Biosciences)**.**

9. Analysis of proteins

9.1. Native polyacrylamide gel electrophoresis

During native polyacrylamide gel electrophoresis proteins move in their native form. Hence, this method can be used to determine whether a protein is a monomer or a multimer and whether two proteins are associated under *in vitro* conditions.
The composition of the native polyacrylamide gradient gel is listed in Tab.3.

Tab.3 Composition of the native polyacrylamide gradient gel

Components	4% resolving gel	28% resolving gel	Stacking gel
dH_2O	8.7 ml	2.45 ml	9.1 ml
40% acryl-bisacrylamide mix	1.1 ml	7.35 ml	750 µl
3.71 M Tris-HCl (pH 8.8)	650 µl	650 µl	650 µl
sucrose	-----	2.1 g	-----
10% APS	20 µl	20 µl	20 µl
TEMED	6 µl	6 µl	6 µl

Running buffer: 25 mM Tris-HCl
1 M glycerol

Sample buffer: 50 mM Tris-HCl
20 % sucrose
0.5 % bromophenol blue

During casting of the gel, the acrylamide mixture was placed on ice. Samples were mixed with sample buffer and the electrophoresis was performed overnight at 100-150V. For protein detection the gel was stained with Coomassie blue.

9.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

In the SDS-PAGE proteins are separated according to their molecular weight. This effect is produced by the strong detergent SDS which binds to the polypeptide chains. This binding produces the SDS-protein complex with an overall negative charge and the amount of SDS that binds to the protein is almost proportional to the molecular weight of the protein. Hence, proteins move from cathode to anode in the polyacrylamide gel independent of their natural charge and natural conformation and they are separated depending on their size only. The composition of the SDS polyacrylamide gels using in this study (8x11 cm, 1 mm wide) is represented in Tab.4.

Tab.4 Composition of the SDS polyacrylamide gel

components	resolving gel (12%, 25 ml)	resolving gel (15%, 25ml)	stacking gel (5%, 10 ml)
dH_2O	8.2 ml	5.7 ml	6.8 ml
30% acryl-bisacrylamide mix	10 ml	12.5 ml	1.7 ml
1 M Tris-HCl (pH 6.8)	-----	-----	1.25 ml
1.5 M Tris-HCl (pH 8.8)	6.3 ml	6.3 ml	-----
10% SDS	250 µl	250 µl	100 µl
10% APS	250 µl	250 µl	100 µl
TEMED	10 µl	10 µl	10 µl

- 10x SDS PAGE running buffer: 30.3 g Tris-HCl
144.1 g glycerol
10 g SDS
adjust volume to 1l with dH_2O
stored at RT

- Laemmli buffer: 62.5 mM Tris-HCl (pH 8.0)
10 % glycerol
2 % SDS
5 % 2-mercaptoethanol
0.05 % bromphenol blue
stored at -20°C

After adding TEMED and APS the resolving gel was poured between two glass plates (8 x 11 cm) which are separated by a 1mm wide plastic spacebar. The gel was poured up to about 3 cm below the edge of the glass plates and the remaining space was filled with dH_2O. After the polymerisation of the resolving gel, H_2O was removed and the stacking gel was poured above the resolving gel and a teflon comb was placed into the stacking gel. Samples were mixed with Laemmli buffer and were heated to 100°C for 5 min. Finally, electrophoresis was

performed in 1x SDS-PAGE running buffer for about 60 min at 150V. The "broad-range-marker" from Biorad was used as a standard. For protein detection the gel was stained with Coomassie blue.

9.3. Staining of proteins in polyacrylamide gels with Coomassie-Blue R250

- Staining solution: 45 % methanol (could be ethanol)
  10 % acetic acid
  0.25 % Coomassie Brilliant Blue R250
  stored at RT

- Destaining solution: 45 % methanol (could be ethanol)
  10 % acetic acid
  stored at RT

Proteins in the polyacrylamide gel were stained with Coomassie blue. The gel was incubated for a minimal time of 60 min in staining solution, and was subsequently destained with distaining solution. The destained gel was kept in 10% acetic acid or dH_2O.

10. Expression and purification of proteins

10.1. Expression of recombinant 6x-His tag proteins using the QIAexpress System (QIAGEN)

Recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein of interest. The QIAexpress System is based on the selectivity and affinity of nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules which have been tagged with 6 consecutive histidine residues (His_6-tag) (Fig.8).

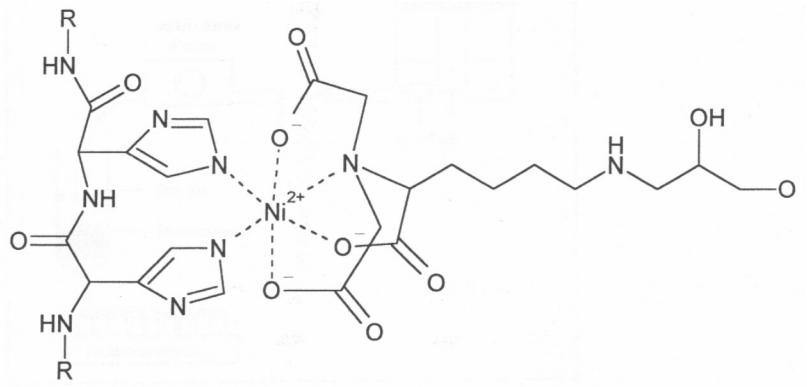

Fig.8 Interaction between neighboring residues in the 6xHis tag and the Ni-NTA matrix.

10.1.1. Overexpression of the protein

The pQE-30 vector was used for the cloning of the tagged genes. This plasmid contains the T5 promoter which is recognized by the *E .coli* RNA polymerase, a *lac* operator sequence that increases Lac repressor binding and ensures repression of the T5 promoter, the β-lactamase gene (*bla*), which confers resistance to ampicillin, and a His_6-tag 5′ of the multiple cloning site in the vector, so that the 6xHis-tag is located at the N-terminus of the tagged protein. For

the expression of the His_6-tag proteins the strain *E. coli* M15 was used. This strain contains the low-copy number repressor plasmid pREP4, that confers kanamycin resistence and expresses the Lac repressor protein encoded by the *lac* I gene.
The expression vector was designed for direct cloning of PCR products. For expression and purification of the tagged protein 500 ml of LB broth were inoculated with 20 ml of an overnight culture (M15 cells with pQE-plasmid) in the presence of ampicillin and kanamycin at final concentrations of 100 and 25 µg/ml, respectively. The cells were grown at 37°C for about 2 hours until an OD_{600} of 0.5 was reached. The high-level expression of the His-tag protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) and the incubation was continued for 2 to 3 hours at 37°C. After centrifugation at 10 000 rpm for 20 min at 4°C the pellet was resuspended in 10 ml of French Press buffer. After centrifugation for 10 min at 4°C and 6 000 rpm the pellet was stored at -20°C for further purification of the His_6-tag protein. For the expression of some proteins the appropriate time of induction, the temperature of incubation and the concentration of IPTG were defined experimentally.

10.1.2. Determination of target protein solubility

The interaction between the Ni-NTA matrix and the His_6-tag of the recombinat protein does not depend on the tertiary structure, therefore purification of the target protein can be performed either under native or under denaturing conditions. To set up the best purification strategy, it is important to determine whether the protein is soluble in the cytoplasm or located in cytoplasmic inclusion bodies.
10 ml of LB broth were inoculated with 0.2 ml of an overnight culture and grown to an OD_{590} of 0.5, then 1 ml of the sample was removed immediately before the induction (noninduced control). The cells were sedimented and then resuspended in 100 µl of SDS-PAGE sample buffer. The sample was boiled and frozen until use. The remaining culture was used for the induction of protein expression by addition of 1 mM IPTG and further incubation for about 3 hours at 37°C. At the end of the incubation 1 ml of the cell culture was centrifuged and mixed with 100 µl of SDS-PAGE sample buffer (induced control) and the rest of the cells were sedimented by centrifugation and resuspended in 1 ml of BugBuster (mixture of non-ionic detergents that is capable of the gentle disruption of the cell wall without denaturing soluble proteins, produced by Novagen) per gram of pellet and 1 µl of the endonuclease benzonase (Novagen) per ml of detergent. The suspension was incubated for 20 min at RT, centrifuged and pellet and supernatant were separated. All the samples were analyzed in an SDS polyacrylamide gel, which was stained with Coomassie blue. When the tagged protein is found in the pellet the protein is located in inclusion bodies in the cytoplasm and the purification of the protein was performed under denaturing conditions. When the tagged protein is in the supernatant the protein is in a soluble form in the cytoplasm and the purification was performed under native conditions.

10.1.3. Lysis of bacterial cells using a French-Press

French-Press (FP) buffer:

50 mM	Tris-HCl (pH 7.5)
50 mM	KCl
1 mM	DTT
1 mM	PMSF

The activity of some proteins can be affected by temperature, therefore the entire purification was performed at 4°C in a cold room and the solutions and the French Press apparatus were held also at 4°C. The pellet containing the overexpressed protein was resuspended in 10 ml of

ice-cold FP buffer and cells were lysed by pressure in a French Press cell by applying about 1000 bar. Then the lysed cells were centrifuged for 30 min at 15 000 rpm and 4°C, resulting in the separation of soluble proteins (in the supernatant) from cell debris and the non soluble proteins (in the pellet). Moreover, proteases are ubiquitous, hence protease inhibitors (protease inhibitor cocktail tablets from Roche and PMSF) were added after the lysis and were used throughout the procedure.

10.1.4. Purification of 6xHis-tagged proteins expressed in *E. coli* under native or denaturing conditions

Ni-NTA-Agarose is the matrix of the column that is used in the metal affinity chromatography for the purification of His_6-tag proteins. The imidazole rings of the histidine residues of the His_6-tag protein bind to the nickel ions and disrupt the binding of dispersed histidine residues in nontagged background proteins.

10.1.4.1 Preparation of the Ni-NTA-Agarose column

For 1l of culture 5 ml of Ni-NTA agarose suspension were used. The resin was loaded onto the column (procured by BioRad) and washed twice with 20 ml of dH_2O and twice with 20 ml of the FP buffer. After setting of the agarose, the column was fitted to a peristaltic pump (Fig.9).

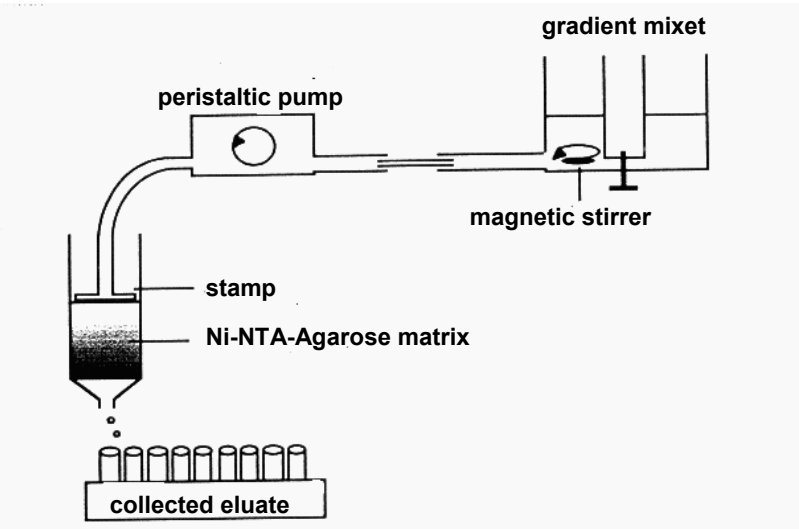

Fig.9 Experimental setup for the purification of proteins by affinity chromatography

10.1.4.2 Purification under native conditions

Wash buffer:

50 mM	Tris-HCl (pH 7.5)
50 mM	KCl
10 %	glycerol
1 mM	DTT
1 mM	PMSF
20 mM	imidazol
5 mM	$MgCl_2$

Elution buffer:

50 mM	Tris-HCl (pH 7.5)
50 mM	KCl
10 %	glycerol
1 mM	DTT
1 mM	PMSF
250 mM	imidazol
5 mM	$MgCl_2$

Dialysis buffer:

50 mM	Tris-HCl (pH 7.5)
50 mM	KCl
20 %	glycerol
1 mM	DTT
1 mM	PMSF
5 mM	$MgCl_2$

Soluble proteins (after treatment with BugBuster or French Press in the supernatant) were extracted under native conditions. After the cell lysis in the French Press the supernatant (about 30 ml) was loaded onto the column at a flow rate of 0.5ml/min, then the column was washed with wash buffer at a flow rate of 1ml/min for 1 hour and the protein was eluted with a gradient ranging from 0 mM to 250 mM imidazol. The eluate was collected in a final volume of 60 ml in aliquots of 1.5 ml. Every second fraction was analysed on a SDS polyacrylamide gel, which was stained with Coomassie blue and the fractions containing protein were pooled together for dialysis. Dialysis was performed in 2 l of dialysis buffer in the cold room for about 24 hours with one change of buffer. Finally, the protein concentration was determined using the Bio-Rad protein assay and the sample was divided into small aliquots and stored at -80°C.

10.1.4.3 Purification under denaturing conditions

Denaturation buffer:

50 mM	Tris-HCl (pH 7.5)
50 mM	KCl
10 %	glycerol
6 M	GuHCl
1 mM	DTT
1 mM	PMSF
5 mM	$MgCl_2$

The proteins forming inclusions bodies upon overexpression were extracted under denaturing conditions. After the lysis using the French Press and centrifugation of the cell suspension the pellet was resuspended in 20 ml of cold FP buffer with 1% Triton X-100 and incubated on ice with stirring for 30 min. Then the sample was centrifuged for 30 min at 15 000 rpm and 4°C. After a second incubation with PF buffer with 1% Triton X-100 and centrifugation under identical conditions, the pellet was resuspended in 30 ml of cold denaturation buffer and incubated for 30 min on ice with stirring. Finally, the suspension was centrifuged for 20 min at 15 000 rpm and 4°C and the supernatant containing denatured proteins was loaded onto the column at a flow rate of 0.5ml/min, then the column was washed with denaturation buffer containing GuHCl at a flow rate of 1ml/min for 1 hour. After renaturation of the protein using

a gradient of GuHCl ranging from 6 M to 0 M with a flow rate of 1ml/min for 1 hour the column was washed with wash buffer with a flow rate of 1ml/min for 1 hour as well. Finally, elution, dialysis and storage of the purified protein were performed as described above for the purification of proteins under native conditions.
A schemematic representation of the expression and purification procedure for His_6-tagged proteins is represented in Fig.10.

10.2. Expression of recombinant GST-tagged proteins and purification by affinity chromatography

In this system a GST (glutahione-S-transferase) tagged protein is constructed by recombinant DNA techniques. The GST-fusion protein is purified by affinity chromatography with Glutathione Sepharose 4B that is bound by the enzyme GST. The binding between GST and sepharose is possible only when the enzyme is active, hence the purification can be performed only under native conditions.

10.2.1. Overexpression of recombinant GST-tagged protein

The pGEX-3X vector was used for the cloning of the tagged genes. This plasmid contains a *lac* operator sequence encoding for the Lac repressor protein that increases Lac repressor binding, the β-lactamase gene (*bla*), which confers resistance to ampicillin, and a *gst*-gene that encodes for the glutahione-S-transferase at the 5´ of the multiple cloning site in the vector pGEX-3X (the GST is located at the N-terminus of the tagged protein). The high-level expression of the recombinant protein encoded by a gene in the pGEX-3X vector is induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the Lac repressor protein and inactivates it.
The expression vector was designed for direct cloning of PCR products. The propagation of the plasmid with the tagged gene was performed in the *E. coli* strain DH5α. 500 ml of LB broth were inoculated with 20 ml of an overnight culture (DH5α cells with pGEX-plasmid) in the presence of ampicillin at a final concentration of 100 µg/ml. The cells were grown at 37°C for about 2 hours until an OD_{600} of 0.5 was reached. The high-level expression of the GST-fusion protein was induced by the addition of 0.1 to 1 mM IPTG and the incubation was continued for 2 to 3 hours at 30°C. After centrifugation at 10 000 rpm for 20 min at 4°C the pellet was resuspended in 10 ml of French Press buffer, and centrifuged for 10 min at 6 000 rpm and 4°C. The pellet was stored at -20°C for further purification of the GST-fusion protein. For the expression of some proteins the appropriate time of induction, the temperature of incubation and the concentration of IPTG were defined experimentally.

10.2.2. Purification of GST-tagged proteins expressed in *E. coli*

10.2.2.1. Preparation of the Glutathione Sepharose 4B column

For 1l of culture 5 ml of Glutathione Sepharose 4B were used. The sepharose was loaded onto the column (BioRad) and washed 3 times with 20 ml of PBS buffer. After setting of the sepharose, the column was fitted to a peristaltic pump (Fig.9).

10.2.2.2. Purification of the GST-targged protein

10x PBS:

80 g	NaCl
2 g	KCl

26.8 g Na_2HPO_4-7 H_2O
2.4 g KH_2PO_4
adjusted with HCl to pH 7.4

Elution buffer:

50 mM Tris-HCl (pH 8.0)
10 mM reduced glutathione

Dyalisis buffer:

50 mM Tris-HCl (pH 7.5)
50 mM KCl
20 % glycerol
1 mM DTT
1 mM PMSF

After lysis using the French Press the supernatant (about 30 ml) was diluted with one volume of 1x PBS buffer containing 1% Triton, and then the sample was loaded onto the column at a flow rate of 1ml/min, then the column was washed with 1x PBS buffer at a flow rate of 1ml/min for 1 hour. Then the protein was eluted with 50 ml of elution buffer. The eluate was collected in aliquots of 1.5 ml. Every second fraction was analysed on a SDS polyacrylamide gel which was stained with Coomassie blue and the fractions containing protein were pooled together for dialysis. Dialysis was performed in 2 l of dialysis buffer in the cold room for about 24 hours with one change of buffer. Finally, the protein concentration was determined using the Bio-Rad protein assay and the sample was divided into small aliquots and stored at -80°C. A schemematic representation of the expression and purification of GST-tagged proteins is illustrated in Fig.10.

10.3. Quantification of proteins using the Bio-Rad Protein Assay

The Bio-Rad Protein Assay was used to quantify the purified proteins. This dye-binding assay is based on the observation that the maximum absorbance for a solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.
Protein concentrations were determined using BSA as a standard. 1, 2, 4, 6, 8, 10, and 12 µl of BSA (1µg/µl) were added to different tubes containing 800 µl of dH_2O. Subsequently, 2 and 5 µl of purified protein were added to the same volume of dH_2O. Then 200 µl of the dye reagent were added to the tubes containing the standard (BSA) and samples (purified protein). After vortexing, all standard and sample reactions were measured at a wavelength of 595 nm against a reagent blank (only dH_2O and dye reagent) in a spectrophotometer (Pharmacia). Finally, a standard curve was drawn with the values of the BSA and the protein concentration of the test samples.

11. *In vitro* phosporylation assays

11.1. Analysis of the phosphorylation of histidine kinase proteins and phosphotransfer to response regulators

Histidine kinase proteins catalyze their autophosphorylation on a conserved His residue. The autophosphorylation of the histidine kinase was observed *in vitro* by the addition of radiolabelled ATP ([γ ^{32}P] ATP).

All phosphorylation reactions were performed in the following buffer:

5x phosphorylation buffer:

250 mM	Tris-HCl (pH 7.5)
250 mM	KCl
50 mM	$MgCl_2$

Components of the reaction are listed below:

ATP mix:

ATP (10mM, Amersham Biosciences)	10 µl
[γ-^{32}P]ATP (Amersham Biosciences: (6 000 Ci/mmol)	25 µl
dH_2O	adjust to 40 µl

Phosphorylation reaction:

5x phosphorylation buffer	5 µl
ATP mix	5 µl
response regulator	x µM
histidine kinase	x µM
dH_2O	adjust to 25 µl

The reaction was incubated for 5-15 min at RT or 30°C depending on the histidine kinase (HK). To an aliquot of 10 µl of the reaction were added 7 µl of Laemmli buffer containing 50 mM Na_2EDTA. Autophosphorylation of HK proteins, as well as transphosphorylation of RR proteins in the presence of a HK under multiple turnover conditions was initiated by the addition of 50 nM [γ-^{32}P]ATP (Amersham Biosciences, 6 000Ci/mmol). To study the kinetics of phosphorylation of the HKs the phosphorylation reactions were carried out in a final volume of 100 µl and samples (10 µl) were taken at different time points. Chase experiments were performed in the presence of 10 mM of unlabelled ATP. Concentration of proteins and the reaction times are indicated in the results part. Samples were electrophoresed for about 60 min at 160 V in a 12 or 15% SDS polyacrylamide gel. The radiolabelled bands were detected using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences) and quantified using ImageMaster TotalLab Software (Amersham Biosciences).

11.2. Analysis of the phosphorylation of the response regulator by acetyl phosphate

Several response regulators are phosphorylated by small molecules that function as phosphor-donors. One of these molecules is acetyl phosphate which can be used to phosphorylate some RR in the absence of the cognate HK (Lukat *et al.*, 1992; McClearly, 1996).

Phosphorylation reaction:

5x phophorylation buffer	5 µl
acetyl [^{32}P] phosphate (80 Ci/mmol) (Hartman Analytic GmbH)	1 µl
response regulator	x µM
dH_2O	adjust to 25 µl

The appropriate concentration of the response regulators was determined experimentally. This concentration depends on the efficiency of the phosphorylation reaction. The reaction

mixtures were incubated for 10 min at RT, and the reaction was stopped by the addition of 7 µl of Laemmli buffer containing 50 mM Na_2EDTA. To determine the dephosphorylation rate of a response regulator the reaction was carried out in a final volume of 200 µl. The response regulator was phosphorylated in the presence of acetyl [^{32}P] phosphate (80 Ci/mmol) for 10 min and then reaction was chased by adding unlabelled acetyl phosphate at a final concentration of 10 mM. At different time points samples were removed and analyzed. Retrophosphorylation experiments were performed in a final volume of 50 µl in the presence of acetyl [^{32}P] phosphate (80 Ci/mmol). Analysis of samples and signal quantification were performed as described above.

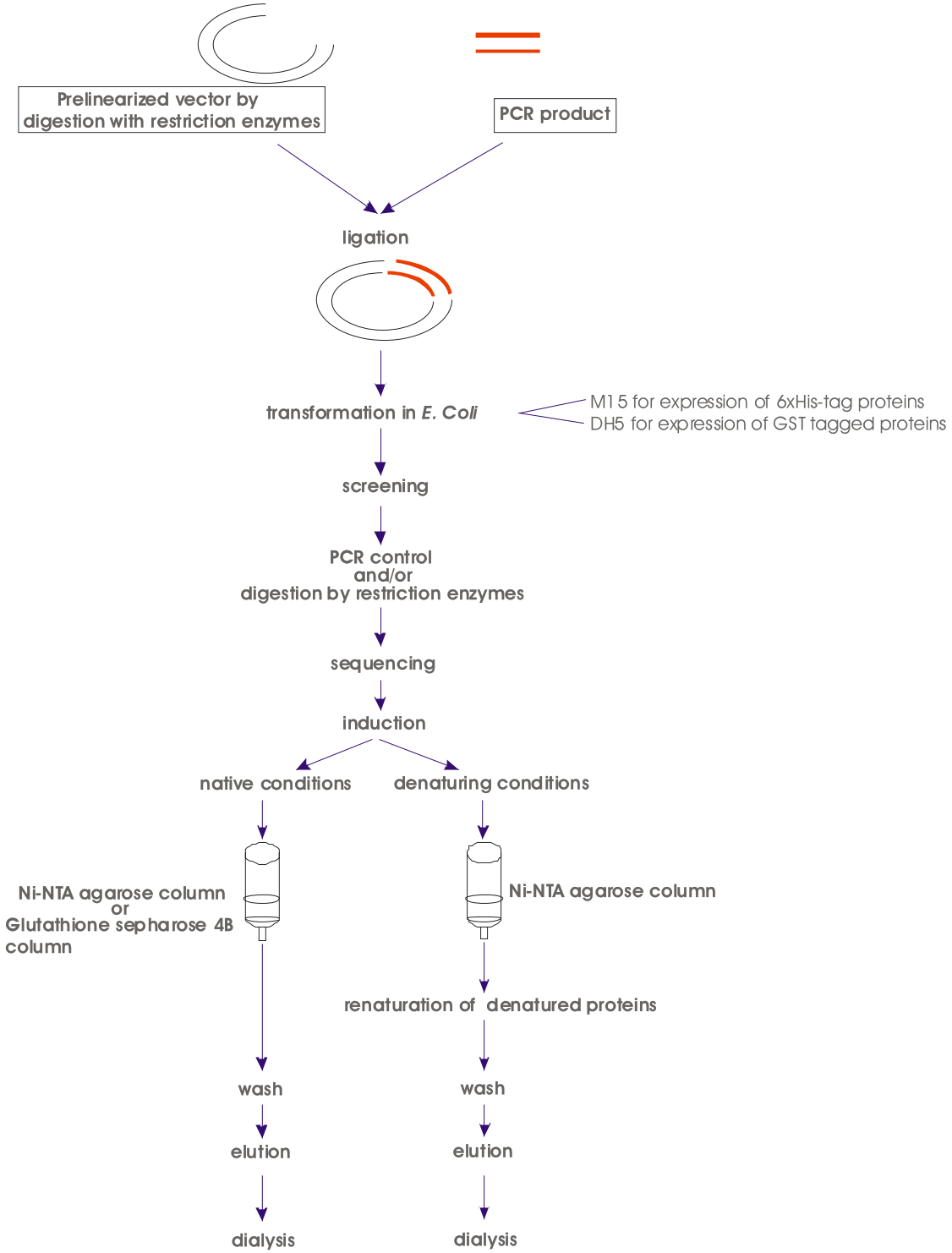

Fig.10 Strategy for the expression and purification of a 6xHis-tagged and a GST-tagged protein.

E RESULTS

1. ORF *hp137* and its putative function in the flagellar regulation of *H. pylori*

As described in Fig.5 in *H. pylori* the expression of flagella is controlled by a complex regulatory cascade involving the two component system FlgR-HP244, the sigma factors σ^{80}, σ^{54}, and σ^{28} and the anti-σ^{28} factor FlgM. Thus far, the input signal for histidine kinase HP244, which activates the transcriptional regulator FlgR, is not known. Five years ago Rain and coworkers published the first prokaryotic protein-protein interaction map, which is based on the genome sequence of *H. pylori* (Rain *et al.*, 2001). The strategy used for the creation of this map is a variation of the two-hybrid assay (Fromont-Racine *et al.*, 1997). Protein-protein interaction maps are built on experimental data that ideally yield a heuristic value for each connection. The map was established in three steps. First, positive prey fragments were clustered into families of overlapping fragments. The common sequences shared by these fragments were referred to as the selected interacting domain (SID). Second, SIDs that did not code for part of *H. pylori* ORFs were discarded. Third, for every remaining SID, a PIM biological score (PBS) was computed. The PBS is based on a statistical model of the competition for bait-binding between fragments. It was computed like a classical expected value (E value), and ranges from 0 (specific interaction) to 1 (probable artefact). For practical use the scores were divided into four categories, from A (score very close to 0) to D (close to 1). A fifth category, E, was added to distinguish interactions involving only highly connected prey domains (SIDs found as prey with frequency greater than a fixed threshold). These are more probably two-hybrid artefacts (Rain *et al.*, 2001).

Interestingly, the protein-protein interaction map of *H. pylori* (htt://pim.hybrigenics.com) indicates a significant interaction between the protein HP137 that possesses an unknown function and both the histidine kinase HP244 and the flagellar hook protein HP908 (FlgE´) (PBS of $1e^{-33}$ with HP908 and $1e^{-46}$ with HP244, and both category A). The selected interacting domain (SID) mapped for the interaction of HP244 and HP137 comprises amino acids 270-304 of HP244 including the characteristic block N sequence motif of the C-terminal ATP-binding kinase domain (Parkinson & Kofoid, 1992). On the basis of the observed interaction between HK HP244 and HP137 and the fact that HP244 is a cytoplasmic HK whose input signal is not known it was hypothesized in this work that the protein HP137 might be involved in a feedback regulatory mechanism controlling the activity of histidine kinase HP244.

1.1. Construction of *H. pylori* G27/hp137::km

To investigate the putative function of ORF *hp137* in the regulation of flagellar gene expression the *hp137* gene was substituted by a non-polar kanamycin resistance cassette. This substitution was performed in *H. pylori* G27 by homologous recombination using suicide plasmid pSL-137::km (Fig.11).

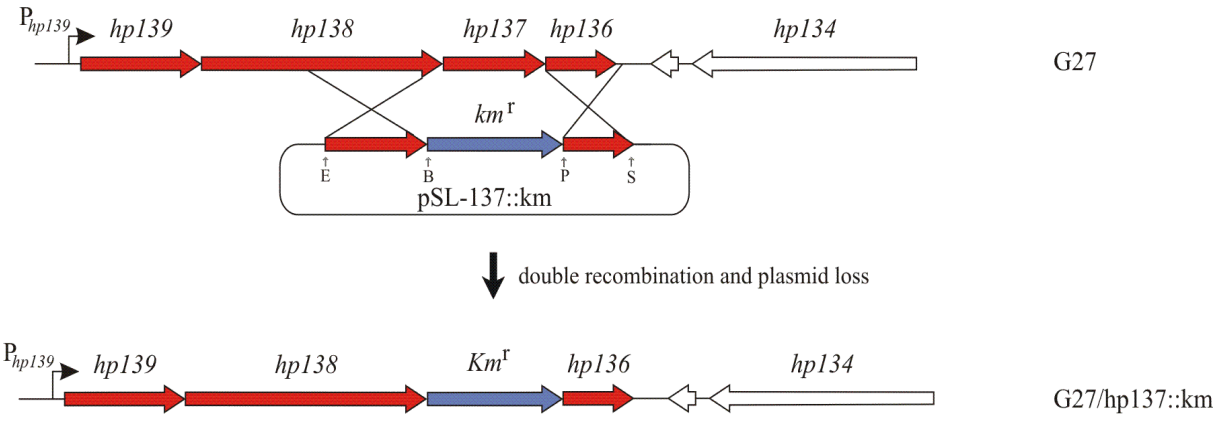

Fig.11 Strategy for the construction of *H. pylori* strain G27/hp137::km by allelic exchange mutagenesis. Non-coding DNA is represented as a black bar, ORFs are indicated as arrows. Red and white arrows represent ORFs of the *hp139-hp136* operon and adjacent downstream ORFs, respectively. The antibiotic resistance gene is represented as blue arrow. The P_{hp139}-promoter is marked by an arrowhead. *km*^{r}: kanamycin resistance gene; E: *Eco*RI; B: *Bam*HI; P: *Pst*I; S: *Sac*I.

To construct the suicide plasmid pSL-137::km fragments of 612 bp encoding amino acids (aa) 298-482 of ORF *hp138* and 627 bp encoding ORF *hp136* were amplified with primer pairs 137-1/137-2, and 137-3/137-4, respectively. The resulting fragments were digested with restriction enzymes *Eco*RI-*Bam*HI, and *Pst*I-*Sac*I, respectively. Chromosomal DNA of *H. pylori* G27 was used as the template DNA in the PCR reactions. Both PCR products were ligated into pSL1180 vector DNA resulting in plasmid pSL-137. Subsequently, a non-polar kanamycin resistance cassette from *C. coli* (Labigne-Roussel *et al.*, 1988) was digested with *Bam*HI-*Pst*I and was inserted between these fragments resulting in pSL-137::km. This plasmid was obtained from Patricia Dietz (P. Dietz, PhD thesis). The non-polar kanamycin resistance cassette was used to ensure the transcription of the gene *hp136*, which is located downstream of gene *hp137*, and both ORFs *hp137* and *hp136* are part of a putative operon together with ORFs *hp139* and *hp138*.

H. pylori G27 was transformed by natural transformation with the plasmid pSL-137::km, in order to substitute ORF *hp137* by the kanamycin resistance cassette generating *H. pylori* G27/hp137::km. Mutants were controlled by PCR with primer pair 137-1/137-4. As expected the obtained PCR products had a length of 1704 bp and 2639 bp for the *H. pylori* G27 wild-

type and the deletion mutant containing the kanamycin cassette, respectively (data not shown). Moreover, Southern blot analysis was performed with chromosomal DNA of the mutant G27/hp137::km and the G27 wild-type (negative control). *H. pylori* DNA was hybridized with a DNA fragment of 1400 bp containing the kanamycin resistance cassette. A hybridization signal was detected only in the case of the chromosomal DNA of G27/hp137::km which contains the kanamycin cassette (Fig.16 A).

To analyse the motility of the deletion mutants 16 characterized colonies of *H. pylori* G27/hp137::km from two independent transformation experiments were tested on motility agar plates. Eight mutants obtained in the first transformation experiment were analysed by P. Dietz. 16 analyzed clones proved to be non-motile on semi-solid agar plates (Fig.12). As shown in Fig.12 the *H. pylori* G27 parent strain showed chemotactic swarming, which is apparent by the formation of characteristic concentric rings originating from the inoculation site. The mutant bacteria, which are deficient in motility, remained close to the site of inoculation. Furthermore, electron microscopy revealed that the mutant strain G27/hp137::km did not produce flagella (data not shown). These results suggested that deletion of ORF *hp137* causes a defect in flagella biosynthesis.

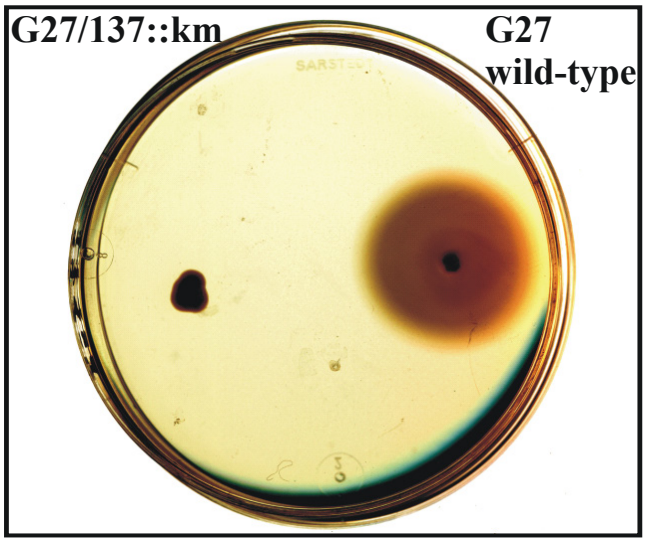

Fig.12 Analysis of the motility of the *H. pylori* wild-type strain G27 and the deletion mutant G27/hp137::km. The swarming ability on semi-solid agar plates was tested. The two strains were stabbed into the agar and the plates were incubated for 5 days at 37°C under microaerophilic conditions.

1.2. Analysis of *flaA*, *flaB*, *flgB*, *flgE* and *flgE´* transcription in the mutant G27/hp137::km using primer extension

To analyse whether the absence of motility on semi-solid agar and the lack of flagella biosynthesis observed in the electron microscopy was due to an alteration of σ^{54}-dependent transcription of flagellar genes in strain G27/hp137::km, semi-quantitative primer extension

experiments were performed on equal amounts of RNA (30 µg) isolated from the *H. pylori* G27 wild-type and the mutant G27/hp137::km with oligonucleotides specific for the genes *flaA*, *flaB*, *flgB*, *flgE* and ORF *hp906*. Transcription of the *flaA* gene is controlled by σ^{28} (Leying *et al.*, 1992), while for the genes *flaB*, *flgB*, *flgE* and ORF *hp906* transcription has been demonstrated previously to be under positive control of the NtrC-like response regulator FlgR and σ^{54} (Spohn & Scarlato, 1999b). The specific oligonucleotides *flaA*-PE, *flaB*-PE, *flgB*-PE, *flgE*-PE, and *flgE2*-PE were used in the primer extension experiments. RNA samples were isolated from liquid cultures of *H. pylori* grown to the late logarithmic phase (OD_{590}=1.0).

As shown in Fig.13 transcription of *flaB*, *flgB*, *flgE* and *hp906* was increased in *H. pylori* G27/hp137::km, while transcription of *flaA* was not significantly altered as compared to the wild-type strain. This result suggested a role of HP137 in the control of the activity of the two-component system HP244/FlgR.

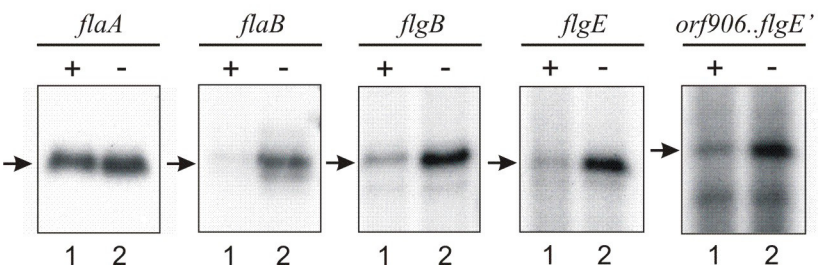

Fig.13 Transcription of σ^{28}- and σ^{54}- dependent flagellar genes in *H. pylori* G27 and G27/hp137::km. Primer extension experiments with oligonucleotides specific for *flaA*, *flaB*, *flgB*, *flgE* and orf*906* were performed with equal amounts of RNA (30 µg) extracted from the *H. pylori* wild-type strain G27 (lanes 1/+) and G27/hp137::km (lanes 2/-). Arrows on the left indicate the specific transcripts.

1.3. Complementation of G27/hp137::km by chromosomal reintegration of ORF *hp137*

To test whether the non-motile phenotype, lack of flagella, and alteration in the transcription of some flagellar genes in G27/hp137::km was indeed the consequence of the deletion of ORF *hp137*, complementation experiments were performed. To be able to express ORF *hp137* under control of its own promoter in the complemented strains mapping of the promoter of ORF *hp137* was first performed.

1.3.1. Mapping of the promoter of ORF *hp137* using primer extension

The genome sequence of *H. pylori* 26695 suggests that ORF *hp137* is part of an operon comprising ORF *hp139* encoding a FeS oxidoreductase, ORF *hp138* encoding a conserved

protein with a ferredoxin-like domain and ORF *hp136* encoding a protein with a domain commonly found in alkyl hydroperoxide reductases (Fig.14 A) (Tomb *et al.*, 1997).

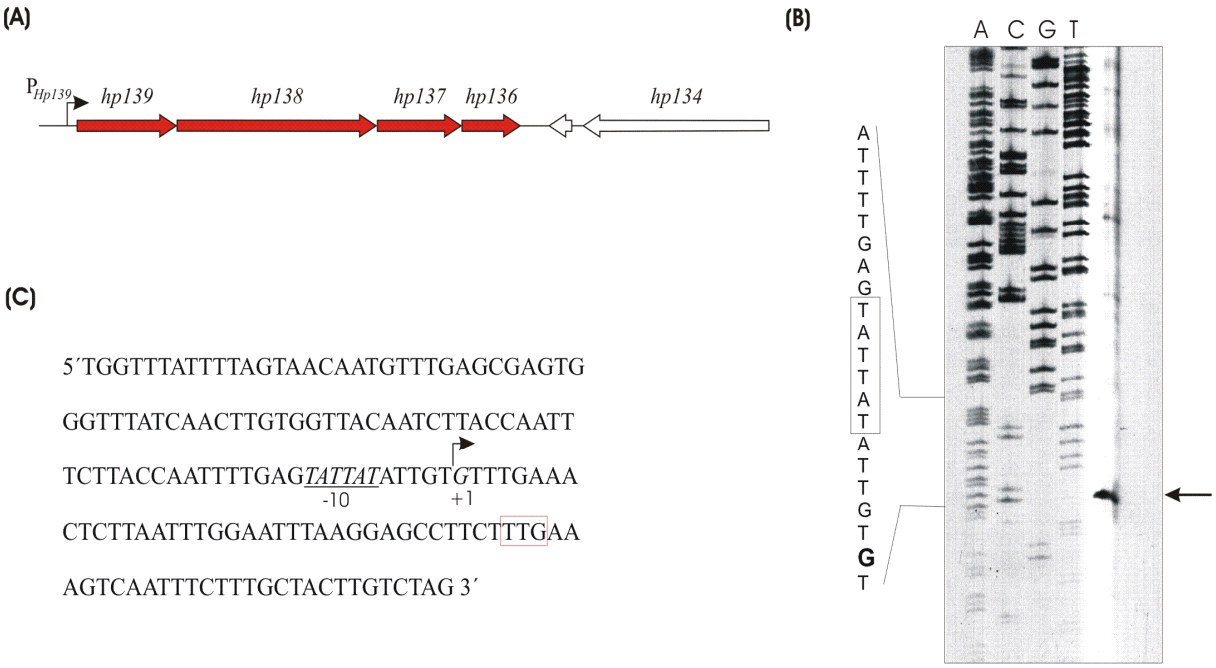

Fig.14 Identification of the promoter of the *hp139-hp137* operon. (A) Schematic representation of the genomic organization of ORF *hp137*. Red and white arrows represent the ORFs of the *hp139-hp137* operon and adjacent downstream ORFs, respectively. The arrowhead indicates the position of the P_{hp139}-promoter of the operon. (B) Determination of the transcriptional start site of ORF *hp139* by primer extension analysis. Total RNA extracted from *H. pylori* 26695 was hybridized to the radiolabelled oligonucleotide 139-PE. The transcriptional start site is indicated by an arrow. A part of the promoter sequence is shown on the left. The -10 promoter element is boxed. The sequencing reaction (lanes A, C, G, and T) was performed by using oligonucleotide 139-PE and plasmid pSL-139-PE as the template. (C) Schematic representation of the promoter region of hp139-hp136. The transcriptional start site is indicated by an arrow, and the -10 promoter element is underlined. The start codon is boxed.

To determine the transcriptional start site of gene *hp137* primer extension experiments were performed with oligonucleotides specific for the 5' ends of ORFs *hp139*, *hp138*, and *hp137*. Plasmids containing the upstream region of ORFs *hp139*, *hp138*, and *hp137*, which were used as templates in sequencing reactions used as a size standard in the PE experiments were obtained as follows: PCR reactions with chromosomal DNA from *H. pylori* 26695 as a template were performed with primer pairs 139-5P/139-3P, 138-5P/138-3P, and 137-5P/137-3P. The resulting PCR fragments (sizes 629 bp, 525 bp, and 466 bp), respectively, were digested with the restriction enzymes *Bam*HI-*Eco*RI and were cloned into pSL1180 vector DNA yielding plasmids pSL-139-PE, pSL-138-PE, and pSL-137-PE.

The primer extension reactions were carried out with primers 139-PE, 138-PE, and 137-PE, which are specific for the 5′ ends of the ORFs *hp139*, *hp138*, and *hp137*, respectively. PE experiments were performed with 30 µg of total RNA from *H. pylori* 26695. The RNA was

isolated from a liquid culture of *H. pylori* grown to the late logarithmic phase (OD_{590}=1.0). An extension product was obtained only when cDNA synthesis was performed with the oligonucleotide specific for *hp139*. Thus the transcriptional start site of the operon was defined to position -38 with respect to the translational start site of ORF *hp139* corresponding to a TATTAT -10 promoter element (Fig.14 B and C).

1.3. 2. Reintroduction of ORF *hp137* in *H. pylori* G27/hp137::km

As described in the introduction section only *H. pylori* type I but not type II strains contain the *cag*-locus (Censini *et al.*, 1996), and consequently this locus is not necessary for the colonization and to survive *in vivo* and *in vitro*. Hence, the *cag*-locus was chosen for the chromosomal integration of gene *hp137* into *H. pylori* G27/hp137::km. Plasmid pSL*cag*hp137-cm containing a fusion of the P_{hp139} promoter to ORF *hp137* was used to reintroduce ORF *hp137* in *H. pylori* G27/hp137::km. To construct the plasmid pSL*cag*hp137-cm fragments of 465 bp containing the P_{hp139} promoter and 635 bp encoding ORF *hp137* were amplified with primer pairs P139-5/P139-3, and 137-5´/137-3´, and digested with restriction enzymes *Bam*HI-*Xba*I, and *Xba*I-*Pst*I, respectively. Chromosomal DNA of *H. pylori* G27 was used as template DNA in the PCR reactions. Both PCR products were ligated into pSL1180 vector DNA, which was digested with *Bam*HI-*Pst*I resulting in plasmid pSLhp137. A fragment of 1100 bp containing both the promoter region and ORF *hp137* was excised from pSLhp137 with the restriction enzymes *Bam*HI-*Pst*I and inserted into pSL*cag*AD, which contains 830 bp and 862 bp *Eco*RI-*Bam*HI, and *Pst*I-*Sac*I fragments derived from the *cag*CD and *cag*A genes, respectively (Schär *et al.*, 2005) yielding plasmid pSL*cag*hp137. Finally, a chloramphenicol resistance cassette was digested with *Pst*I and inserted behind *hp137* into pSL*cag*hp137 vector DNA cleaved with *Pst*I yielding plasmid pSL*cag*hp137-cm. This plasmid was controlled by PCR with the primer pairs listed in Tab.5.

Tab.5 Primer pairs used for the PCR characterization of plasmid pSL*cag*hp137-cm and *H. pylori* strain G27/*cag*hp137-cm. The length of the expected PCR fragments, which were obtained in the respective reactions is indicated.

Primer pair	Fragment size
139-5 and cagA3´	2765 bp
137-5 and cagA3´	2300 bp
cm5´ and cm3´	805 bp

H. pylori G27/hp137::km was transformed by natural transformation with the plasmid pSL*cag*hp137-cm yielding *H. pylori* G27/*cag*hp137-cm. By homologous recombination the

ORF *hp137* was integrated into the *cag* pathogenicity island (*cag*-locus) of deletion mutant *H. pylori* G27/hp137::km (Fig.15 A). The resulting transformants were controlled by PCR with the primer pairs listed in Tab.5.

Several colonies of the complemented strain G27/*cag*hp137-cm were analysed on semi-solid agar plates and turned out to be non-motile (data not shown). Therefore, as control the parent strain G27 was transformed with plasmid pSL*cag*hp137-cm. Surprisingly, as the strain G27/*cag*hp137-cm the resulting transformants named G27WT/*cag*hp137-cm were non motile on semi-solid agar plates (data not shown). Therefore, for unknown reasons the *cag*-locus proved to be not suitable as integration site in complementation experiments addressing motility functions.

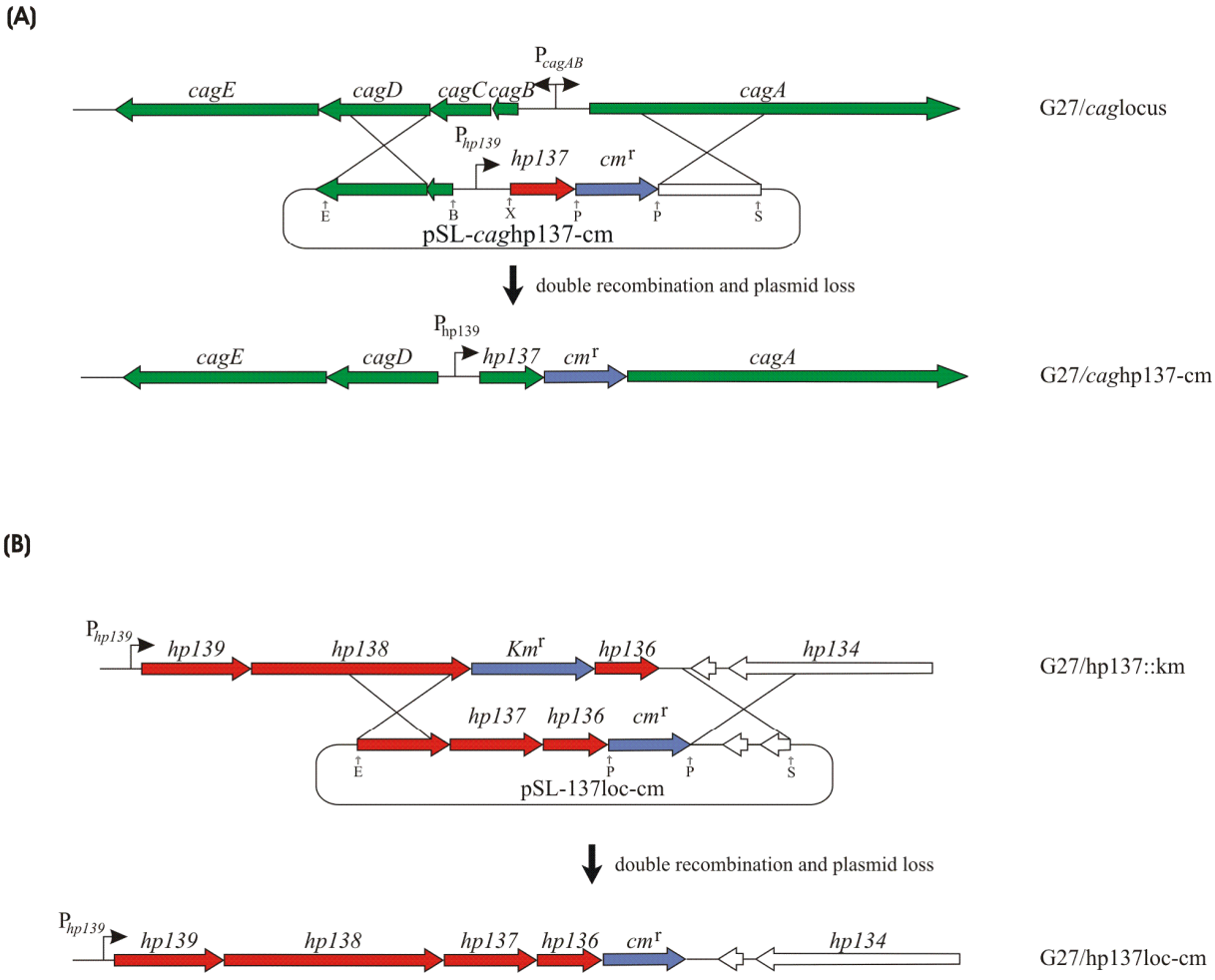

Fig.15 Schematic representation of the strategy applied for the generation of *H. pylori* strains G27/*cag*hp137-cm (A) and G27/hp137loc-cm (B) by allelic exchange mutagenesis. Non-coding DNA is represented as a black bar, ORFs are indicated as arrows. Red, green, and white arrows represent ORFs of the *hp139-hp136* operon, *cag*-locus and adjacent upstream ORFs, respectively. Antibiotic resistance genes are represented as blue arrows. The P_{hp139}- and P_{cagAB}-promoters are represented by an arrowhead. *cm*^{r}: chloramphenicol resistance gene; *km*^{r}: kanamycin resistance gene; E: *Eco*RI; B: *Bam*HI; P: *Pst*I; S: *Sac*I; X: *Xba*I.

For that reason an alternative strategy for the complementation of G27/hp137::km by the reintegration of ORF *hp137* into the *hp139-hp136* locus was carried out. Suicide plasmid

pSL-137loc-cm was constructed and used for the transformation of the deletion mutant G27/hp137::km. To construct the plasmid pSL-137loc-cm fragments of 1821 bp encoding aa 298-482 of ORF *hp138*, ORF *hp137* and ORF *hp136* and 608 bp comprising the intergenic region between ORFs *hp136*, ORF *hp135* and part of ORF *hp134* (encoding aa 400-450) were amplified with primer pairs 137-1/137loc2, and 137loc3/137loc-4. The resulting fragments were digested with restriction enzymes *Eco*RI-*Pst*I, and *Pst*I-*Sac*I, respectively. Chromosomal DNA of *H. pylori* G27 was used as the template DNA in the PCR reactions. Both PCR products were ligated into pSL1180 vector DNA, which was digested with *Eco*RI-*Sac*I yielding pSL-137loc. This plasmid was controlled by PCR with primer pairs 137-1/137loc2, and 137loc3/137loc-4 and sequenced with primers M13universe and M13reverse to ensure proper PCR amplification. Subsequently, a chloramphenicol resistance cassette of *C. coli* (Wang & Taylor, 1990) was inserted between these fragments yielding pSL-137loc-cm. Plasmid pSL-137loc-cm was controlled by PCR with the primer pairs listed in Tab.6. *H. pylori* G27/hp137::km was transformed by natural transformation with the plasmid pSL-137loc-cm in order to replace the kanamycin cassette by ORF *hp137* via homologous recombination and restore the operon comprising the ORFs *hp139-hp136* (Fig.15 B). Chromosomal DNA of two chloramphenicol-resistant, kanamycin-sensitive transformants (G27/hp137loc-cm1 and G27/hp137loc-cm2) was analysed by PCR with the primer pairs listed in Tab.6. DNA of strains G27/hp137::km and G27 was used as a control.

Tab.6 Primer pairs used for the PCR characterization of plasmid plasmid pSL-137loc-cm and *H. pylori* strains G27/hp137loc-cm1 and G27/hp137loc-cm2. The length of the expected PCR fragments, which were obtained in the respective reactions is indicated.

Primer pair	Fragment size	G27 wild-type	G27/hp137::km
137-5 and 137-3	635 bp	635 bp	> 1400 bp
137loc1 and 137loc2	1860 bp	1860 bp	2625 bp
cm5´ and cm3´	805 bp	_____	_____
kanR3 and kanR5	_____	_____	1400 bp

Moreover, Southern blot analysis was performed with chromosomal DNA of G27/hp137loc-cm1, G27/hp137loc-cm2, G27/hp137::km (positive control), and the wild-type strain G27 (negative control). The DNA samples of the *H. pylori* strains under study were hybridized with a DNA fragment of 1400 bp containing the kanamycin resistance cassette. A hybridization signal was detected only in the case of the chromosomal DNA of G27/hp137::km which contains the kanamycin cassette (Fig.16 A). Hence, it was shown that

the complemented transformants G27/hp137loc-cm1, G27/hp137loc-cm2 did not contain the kanamycin cassette, and as expected the complemented transformants were unable grow on Columbia agar plates containing kanamycin.

Transformants G27/hp137loc-cm1 and G27/hp137loc-cm2 were analysed on semi-solid agar plates for their swarming behaviour and similar to strain G27/*cag*hp137-cm the mutants turned out to be non-motile, while transformation of the wild-type strain G27 with plasmid pSL-137loc-cm yielded chloramphenicol-resistant transformants with normal motility (data not shown). Tab.7 summarizes the results of the motility assays performed on semi-solid agar for all *H. pylori* strains tested.

Tab.7 Analysis of the motility on semi-solid agar of the *H. pylori* strains under study.

Strain	motility
G27 wild-type	+
G27/hp137::km	_
G27WT/*cag*hp137-cm	_
G27/*cag*hp137-cm	_
G27WT/hp137loc-cm	+
G27/hp137loc-cm1	_
G27/hp137loc-cm2	_

Moreover, RT-PCR experiments were performed with RNA isolated from the transformants with the restored *hp139-hp136* operon to analyze whether ORFs *hp137* and *hp136* are expressed in the complemented strains. RNA from G27/hp137::km and G27 was used as control. RNA samples were isolated from liquid cultures of *H. pylori* grown to the late logarithmic phase (OD_{590}=1.0). 5-10 µg of each RNA sample were used for the synthesis of the cDNA with random hexanucleotide primers that bind to the mRNA at a variety of complementary sites and lead to partial length (short) cDNA´s (Invitrogen). Subsequently, synthesized cDNAs were used as template for a PCR reaction yielding dsDNA. A PCR product of 145 bp was observed for G27/hp137loc-cm1, G27/hp137loc-cm2, and the G27 wild-type when the primer pair RT1-137/RT2-137 specific for ORF *hp137* was used. A PCR fragment of 117 bp was observed for G27/hp137loc-cm1 and G27/hp137loc-cm2, G27/hp137::km, and the G27 wild-type when the primer pair RT1-136/RT2-136 specific for ORF *hp136* was used (Fig.16 B). This result showed that ORF *hp137* is transcribed in the complemented transformants G27/hp137loc-cm1 and G27/hp137loc-cm2. Furthermore, ORF *hp136* is transcribed in these transformants but also in G27/*cag*hp137-cm, where its transcription is driven by the promoter directing transcription of the upstream km^r gene.

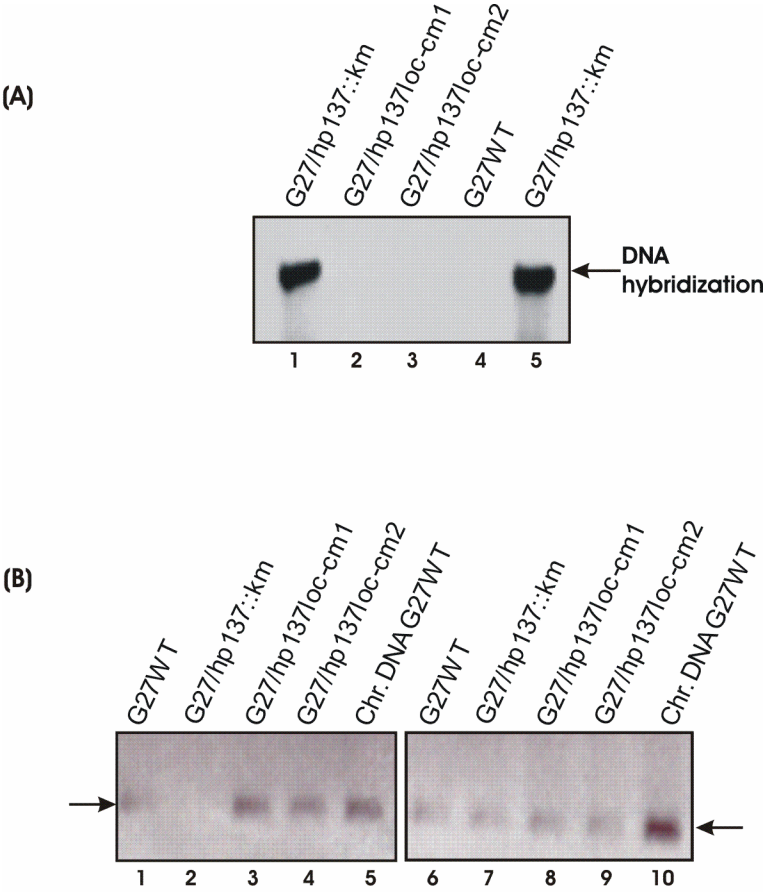

Fig.16 Characterization of the mutant G27/hp137loc-cm. (A) Southern blot performed with chromosomal DNA from strains *H. pylori* G27/hp137::km (lane 1 and 5), G27/hp137loc-cm1 and 2 (lane 2, 3), and G27 wild-type (lane 4). DNA was hybridized with a DNA probe that contains the kanamycin resistance gene (B) RT-PCR performed on RNA extracted from *H. pylori* wild-type G27 (lane 1, 6), G27/hp137::km (lane 2, 7), and G27/hp137loc-cm1 and 2 (lane 3, 4 and 8, 9). Synthesized cDNAs were amplified with specific primers for ORFs *hp137* (lanes 1-5) and *hp136* (lanes 6-10). Chromosomal DNA of the *H. pylori* G27 wild-type was used as a positive control (lane 5, 10). Arrows on the left and on the right indicate PCR fragments derived from the specific cDNA of the *hp137* and *hp136* transcript, respectively.

To analyze whether the enhanced transcription of genes controlled by the NtrC-like response regulator FlgR was still observed in the complemented strain *H. pylori* G27/hp137loc-cm1 and G27/hp137loc-cm2 semi-quantitative primer extension was performed on equal amounts of RNA (30 µg) isolated from *H. pylori* G27, G27/hp137::km, and the complemented strains G27/hp137loc-cm1 and G27/hp137loc-cm2 with the oligonucleotide *flaB*-PE, which is specific for the *flaB* gene. RNA samples were isolated from liquid cultures of the respective *H. pylori* strains grown to the late logarithmic phase (OD_{590}=1.0). Interestingly, transcription of *flaB* in *H. pylori* G27/hp137loc-cm1 and G27/hp137loc-cm2 containing the restored *hp139-hp136* operon was similar to the parent strain G27/hp137::km (Fig.17).

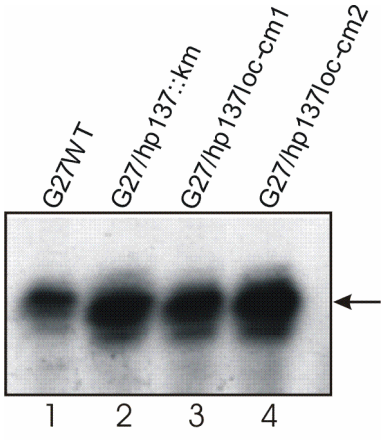

Fig.17 Transcription of the *flaB* gene in *H. pylori* G27, G27/hp137::km and derivatives of G27/hp137::km containing a reconstituted *hp139-hp136* operon (G27/*cag*hp137-cm1 and G27/*cag*hp137-cm2). Primer extension experiments with a *flaB*-specific oligonucleotides were performed with equal amounts of RNA (30 µg) extracted from the *H. pylori* wild-type strain G27 (lane 1), strain G27/hp137::km (lane 2) and the mutants G27/hp137loc-cm1 (lane 3) and G27/hp137loc-cm2 (lane 4). The arrow on the right indicates the *flaB*-specific transcript.

1.4. Analysis of the influence of a putative protein-protein interaction between HP137 and HP244 on autophosphorylation and phosphotransfer to the response regulator FlgR

As mentioned, a highly significant protein-protein interaction between HP137 and both histidine kinase HP244 and the flagellar hook protein FlgE' was observed in the yeast two-hybrid system (Rain *et al.*, 2001). The selected interacting domain (SID) mapped for the interaction of HP244 and HP137 comprises amino acids 270-304 of HP244 (Rain *et al.*, 2001) including the characteristic block N sequence motif of the C-terminal ATP-binding kinase domain (Parkinson & Kofoid, 1992). To investigate the effect of these putative protein-protein interactions on histidine kinase activity and the phosphotransfer reaction to the response regulator FlgR *in vitro* phosphorylation assays were performed.

1.4.1. Expression and purification of fusion proteins HP137 and FlgE´ with an N-terminal His_6-tag

Both proteins HP137 and the flagellar hook protein homolog FlgE' (HP908) were overexpressed and purified as N-terminal His_6-fusions. Plasmid pQE-137 overexpressing the His_6-tagged protein HP137 (23 kDa) was obtained by ligating a 638 bp *Bam*HI-*Pst*I fragment amplified with primer pair E137-5 and E137-3 from chromosomal DNA of *H. pylori* G27 into pQE30 vector DNA. This plasmid was controlled by PCR with the primer pair E137-5/E137-3 and by sequencing with primers pQE-5 and pQE-3. To construct plasmid pQE-FlgE' overexpressing His_6-tagged FlgE' (66 kDa) a 1822 bp *Bam*HI-*Pst*I fragment amplified with primer pair EFlgE'-5 and EFlgE'-3 from chromosomal DNA of *H. pylori* G27 was ligated into pQE30 vector DNA. This plasmid was controlled by PCR with the primer pair EFlgE'-5 /EFlgE'-3 and by sequencing with primers pQE-5, pQE-3, FlgE´-seq1, FlgE´-seq2, and FlgE´-seq3.

Overexpression of both His_6-fusion proteins HP137 and FlgE´ was carried out in *E. coli* M15 cells. In several attempts to overexpress the proteins under conditions with modified concentrations of inducer and different incubation temperatures and incubation times both proteins were always found to be located in cytoplasmic inclusion bodies. Therefore, purification of the proteins His_6-HP137 and His_6-FlgE´ was performed under denaturing conditions as described in the methods section. Purification of protein HP137 was performed repeatedly, but due to a low solubility the protein precipitated during dialysis, although various dialysis buffers with different pH or salts concentrations were used. Therefore, the purified protein could be obtained in a soluble form only at very low concentrations (Fig.18 lane 5).

The response regulator FlgR (42.5 kDa) was fused to an N-terminal His_6-tag, whereas the transmitter domains of histidine kinases HP244 (52 kDa) and HP165 (53 kDa) which served as a control were fused to glutathione-S-transferase. Plasmids pQE-703, pGEX-244, and pGEX-165 were used for the overexpression of the above mentioned three proteins in *E. coli* cells (Beier & Frank, 2000). Purified proteins were provided by Dagmar Beier. All the purified proteins were analysed by SDS-PAGE and the gels were stained with Coomassie blue (Fig.18 A).

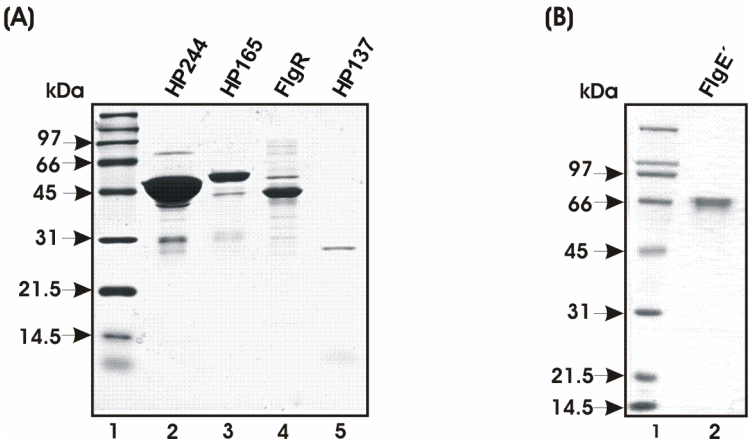

Fig.18 Analysis of the purified fusion proteins by SDS polyacrylamide gel electrophoresis on 15% SDS polyacrylamide gels. The following proteins were loaded (A) Histidine kinase HP244 (52 kDa, lane 2), histidine kinase HP165 (53 kDa, lane 3), response regulator HP703 (42.5 kDa, lane 4), protein HP137 (23 kDa, lane 5). (B) 12% SDS polyacrylamide gel containing the flagellar protein FlgE´ (66 kDa, lane 2). Protein marker (BioRad) was used as a size standard in the lane 1 of both gels A and B.

1.4.2. Analysis of the protein-protein interaction between HP137 and HP244 using native polyacrylamide gel electrophoresis

In a native polyacrylamide gel proteins are electrophoresed in their native form. Hence, this method can be used to compare the electrophoretic mobility of one protein alone and in

combination with other proteins. Differences in the electrophoretic mobility can indicate an association between two proteins *in vitro*. To analyse the putative interaction between HP137 and histidine kinase HP244 native gel electrophoresis was performed.

Three proteins with known molecular weight were used as protein standard: BSA (bovine serum albumin, 66.2 kDa), CEA (chicken egg albumin, 45 kDa), and α-Lact (α-Lactalbumin bovine milk, 14.2 kDa). Moreover, a protein marker (BioRad) was electrophoresed parallel to the three standard proteins and the samples under study (data not shown).

The purified recombinant proteins HP137 and HP244 were electrophoresed alone and after mixing then in equimolar amounts (1 µM: 1 µM) in a reaction with a final volume of 100 µl. Under these conditions no differences in the electrophoretic mobility of HP244 in the absence and in the presence of HP137 were observed (data not shown). In contrast, a slight retardation in the electrophoretic mobility was detected when HP137 was present in a fourfold molar excess to the HK (1 µM HP244: 4 µM HP137) (Fig.19 lane 3). Interestingly, protein HP137 was never observed in the gel, while HP244 was detected in the absence of the other protein (Fig.19 lane 1, 2, 4, and 5). Moreover, as a control HP137 was electrophoresed after mixing it with the *H. pylori* histidine kinase HP165 under the same conditions (1 µM HP165: 4 µM HP137) resulting in no alteration of the electrophoretic mobility of HP165 in the presence of HP137 (Fig.19 lanes 6, 7, 8). Histidine kinase HP165 was used as control because this protein does not possess a function in the flagellar biosynthesis and does not show an interaction with HP137 in the yeast two-hybrid system (Rain *et al.*, 2001). The difference in the electrophoretic mobility of HP244 in the presence of HP137 suggested an interaction between these two proteins and confirmed the results of the genome-wide two-hybrid approach.

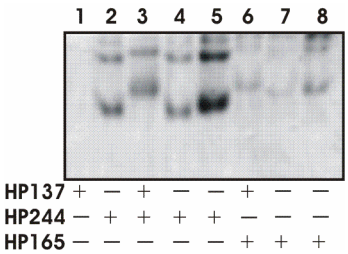

Fig.19 Analysis of the interaction between HP137 and HK HP244 using native polyacrylamide gel electrophoresis. The interaction between HP244 and HP137 was analyzed on native polyacrylamide gels with a gradient of polyacrylamide ranging from 4 to 28%. Purified proteins were used in the following amounts: Lane 1: 2 µM HP137; Lane 2: 1 µM HP244; Lane 3: 1 µM HP244, 4 µM HP137; Lane 4: 1 µM HP244; Lane 5: HP244 (represents total amount of protein in line 3); Lane 6: 1 µM HP165, 4 µM HP137; Lane 7: 1 µM HP165; Lane 8: HP165 (represents total amount of protein in lane 6).

1.4.3. *In vitro* phosphorylation assays

To investigate the effect of the interaction between HP244 and HP137 on the autophosphorylation of HP244 and the phosphotransfer to the response regulator FlgR *in vitro*

phosphorylation assays under multiple turnover conditions were performed. 1 µM of HK HP244 was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) and 1 and 4 µM of protein HP137 for 5 min at RT. After incubation 10 µl of the sample were taken and the reaction was stopped by the addition of sample buffer. Samples were electrophoresed on a 12% SDS polyacrylamide gel. Signals were detected using a Typhoon 9200 Variable Mode Imager. When histidine kinase HP244 and protein HP137 were incubated in equimolar concentrations (1 µM HP244: 1 µM HP137) no effect of HP137 on the autophosphorylation of HP244 was observed. However, when HP137 was present in a fourfold excess (1 µM HP244: 4 µM HP137) autophosphorylation of HP244 was slightly decreased, while autophosphorylation of the *H. pylori* histidine kinase HP165 was not affected by the presence of HP137 (Fig.20 A). To determine whether HP137 interferes with the phosphotransfer from HK HP244 to RR FlgR 1 µM of HP244 and 2 µM of FlgR were incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) and in the presence and absence of 4 µM HP137 under the same conditions as described above. As shown in the Fig.20 A transfer of the phosphate group to the RR FlgR was not affected by the presence of HP137 in the reaction mixture.

Furthermore, in similar reactions HK HP244 was combined in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) with either FlgR, HP137, and FlgE´, or HP137 and FlgE´, or FlgE´ only (Fig.20 lane 6, 7, and 8). As mentioned above FlgE´ (HP908) is a flagellar hook protein and as HP244, shows a highly significant protein-protein interaction with the putative protein HP137 in the yeast two-hybrid system (Rain *et al.*, 2001). Assuming that there exists an interaction between these proteins *in vivo*, FlgE´ might be involved in the hypothesized regulatory mechanism controlling the activity of HP244. In the reactions containing FlgE´ dephosphorylation of HK HP244 was detected, while the phosphotransfer to the RR FlgR was not affected. To determine whether FlgE´ interferes directly with the autophosphorylation of HP244, autophosphorylation of HK HP165 was carried out in the presence of FlgE´ as a control. Interestingly, similar to HP244 histidine kinase HP165 was dephosphorylated in the presence of FlgE´ (Fig.20 A). The same effect was observed with different preparations of FlgE´. Therefore, it was hypothesized that the protein preparations of FlgE´ were contaminated with a phosphatase. The purified recombinant FlgE´ was subjected to a second round of purification on Ni-NTA agarose which increased the ratio of His_6-tagged protein to contaminating proteins in the preparation. When this protein preparation was used in the phosphorylation assay with HP244, dephosphorylation of HP244~P was less pronounced, argueing in favour of the presence of a contaminating phosphatase. Addition of HP137 to a reaction containing both HP244 and FlgE' did not change the phosphorylation properties of

HP244 exhibited in the presence of FlgE' alone, nor was the phosphotransfer to FlgR affected by the presence of both FlgE' and HP137 (Fig.20 A).

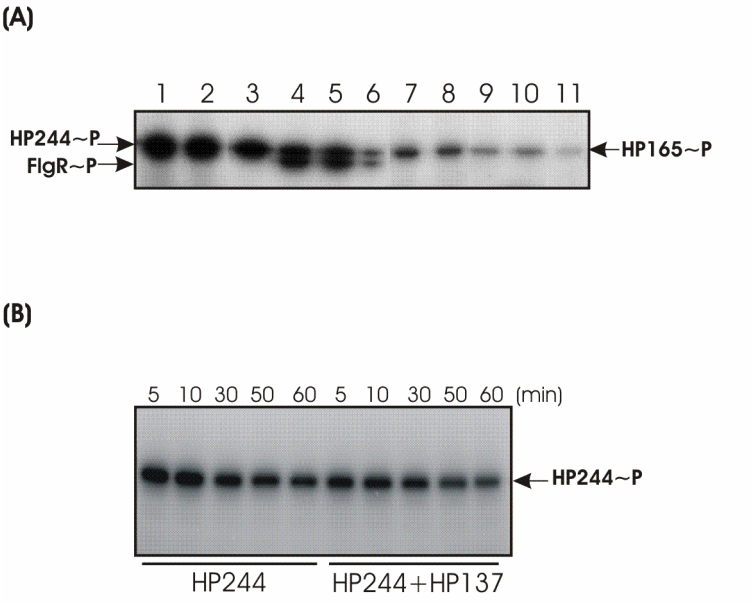

Fig.20 Autophosphorylation and phosphotransfer reactions of histidine kinase HP244 in the presence of HP137. (A) HP244 was incubated for 5 min with 50 nM [γ^{32}P]ATP and various combinations of the proteins FlgR, HP137 and FlgE' as indicated. Lane 1: 1 μM HP244; lane 2: 1 μM HP244, 1 μM HP137; lane 3: 1 μM HP244; 4 μM HP137; lane 4: 1 μM HP244, 2 μM FlgR; lane 5: 1 μM HP244, 2 μM FlgR, 4 μM HP137; lane 6: 1 μM HP244, 2 μM FlgR, 4 μM HP137, 1 μM FlgE'; lane 7: 1 μM HP244, 1 μM FlgE'; lane 8: 1 μM HP244, 1 μM FlgE', 4 μM HP137. Histidine kinase HP165 of *H. pylori* was used as a control. Lane 9: 1 μM HP165; lane 10: 1 μM HP165, 4 μM HP137; lane 11: 1 μM HP165, 1 μM FlgE'. (B) Autoradiogram of the phosphorylation of HK HP244 in the presence of HP137. Time course experiments were performed with HP244 (1 μM) and HP137(4 μM). Samples were removed at indicated time points and analysed by SDS PAGE (15% gel). Signals were detected using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences).

Moreover autophosphorylation of histidine kinase HP244 in the presence of HP137 was examined in a time course experiment of 60 min. Both proteins HP244 (1 μM) and HP137 (4 μM) were incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol). Subsequently, samples (10 μl) were taken at defined time points (5, 10, 15, 30, and 60 min) and electrophoresed on a 12% SDS polyacrylamide gel. Signals were detected using a Typhoon 9200 Variable Mode Imager. Autophosphorylation of HP244 was not significantly affected by HP137 during the 60 min of incubation (Fig.20 B).

1.5. Construction of *Campylobacter jejuni* 4344/cj73c::km

C. jejuni, a close relative of *H. pylori*, harbours orthologs of ORFs *hp139-hp137* which are also arranged in an operon (*cj75c-cj73c*) (Parkhill *et al.*, 2000), while the ortholog of the gene

hp136 is encoded at an unlinked locus. The *C. jejuni* orthologues of HP244 and FlgR show a high degree of similarity to the two-component proteins of *H. pylori* (Cj793c-HP244: 42% identity, 65% similarity; Cj1024c-FlgR: 55% identity, 70% similarity) and there is also an ortholog of the *flgE'* gene (*cj43*). It was assumed that if protein-protein interaction was a mode of regulating kinase activity of HP244 in *H. pylori* a similar mechanism should be working in the related flagellar regulatory system of *C. jejuni*.

To investigate the putative function of ORF *cj73c* in the regulation of flagellar gene expression the gene *cj73c* was substituted by a kanamycin resistance cassette. This substitution was performed in *C. jejuni* 4344 by homologous recombination using suicide plasmid pSL-cj73::km.

To construct the suicide plasmid pSL-cj73::km fragments of 722 bp encoding aa 244-479 of ORF *cj74c* and of 674 bp comprising the 5'-end of ORF *cj73c*, the intergenic region between ORFs *cj73c* and *cj72c* and ORF *cj72c* were amplified with primer pairs 73-1/73-2, and 73-3/ 73-4. The resulting fragments were digested with restriction enzymes *Eco*RI-*Bam*HI, and *Pst*I-*Sac*I, respectively. Chromosomal DNA of *C. jejuni* 4344 was used as the template DNA in the PCR reactions. Both PCR products were cloned into pSL1180 vector DNA. Subsequently, a kanamycin resistance cassette from *C. coli* (Labigne-Roussel *et al.*, 1988) was inserted between these fragments resulting in pSL-cj73::km. This plasmid was controlled by PCR with primer pairs 73-1/kan2, and 73-4/kan1. As expected the obtained PCR products were 1422 bp and 1374 bp in length, respectively (data not shown). Primers kan1 and kan2 were designed in a way that they anneal to different strands in the middle of the kanamycin resistance gene.

C. jejuni 4344 was transformed by natural transformation with the plasmid pSL-cj73::km. By homologous recombination the ORF *cj73* was substituted by the kanamycin resistance cassette generating *C. jejuni* 4344/cj73c::km. Mutants were controlled by PCR with primer pairs 73-1/kan2, and 73-4/kan1. As expected, the obtained PCR products had a length of 1422 bp and 1374 bp, respectively. No PCR product was obtained with chromosomal DNA of *C. jejuni* 4344 as template, which was used as a negative control (data not shown).

To analyze the motility of *C. jejuni* 4344/cj73c::km transformants were stabbed into semi-solid agar plates and the bacteria were incubated for 5 days. Interestingly, in contrast to *H. pylori*, substitution of ORF *cj73c* with a kanamycin resistance cassette in *C. jejuni* 4344 resulted in a strain whose swarming behaviour on motility agar plates was indistinguishable from the wild-type (data not shown).

2. Characterization of the two-component signalling modules of the chemotaxis system of *H. pylori*

2.1. Phosphotransfer between two-component signalling modules of the chemotaxis system of *H. pylori*

Analysis of the genome sequence of *H. pylori* indicates that this organism possesses a chemotaxis system which differs markedly from the well characterized enterobacterial system (Alm *et al.*, 1999; Tomb *et al.*, 1997). As can be deduced from the genome sequence (Fig.21) *H. pylori* lacks a *cheZ* gene encoding the CheZ phosphatase but contains two *cheY* genes one of which is fused to the *cheA* gene forming the *cheAY2* gene encoding the unorthodox histidine kinase protein CheAY2. In addition, *H. pylori* encodes three CheV proteins, which are composed of a N-terminal CheW-like domain and a C-terminal receiver domain. Moreover, this organism possesses three classical MCPs, and a truncated soluble MCP orthologue (Alm *et al.*, 1999; Tomb *et al.*, 1997). Different from *E. coli* but similar to *S. meliloti H. pylori* contains more than one response regulator CheY, but no homolog of the CheZ phosphatase.

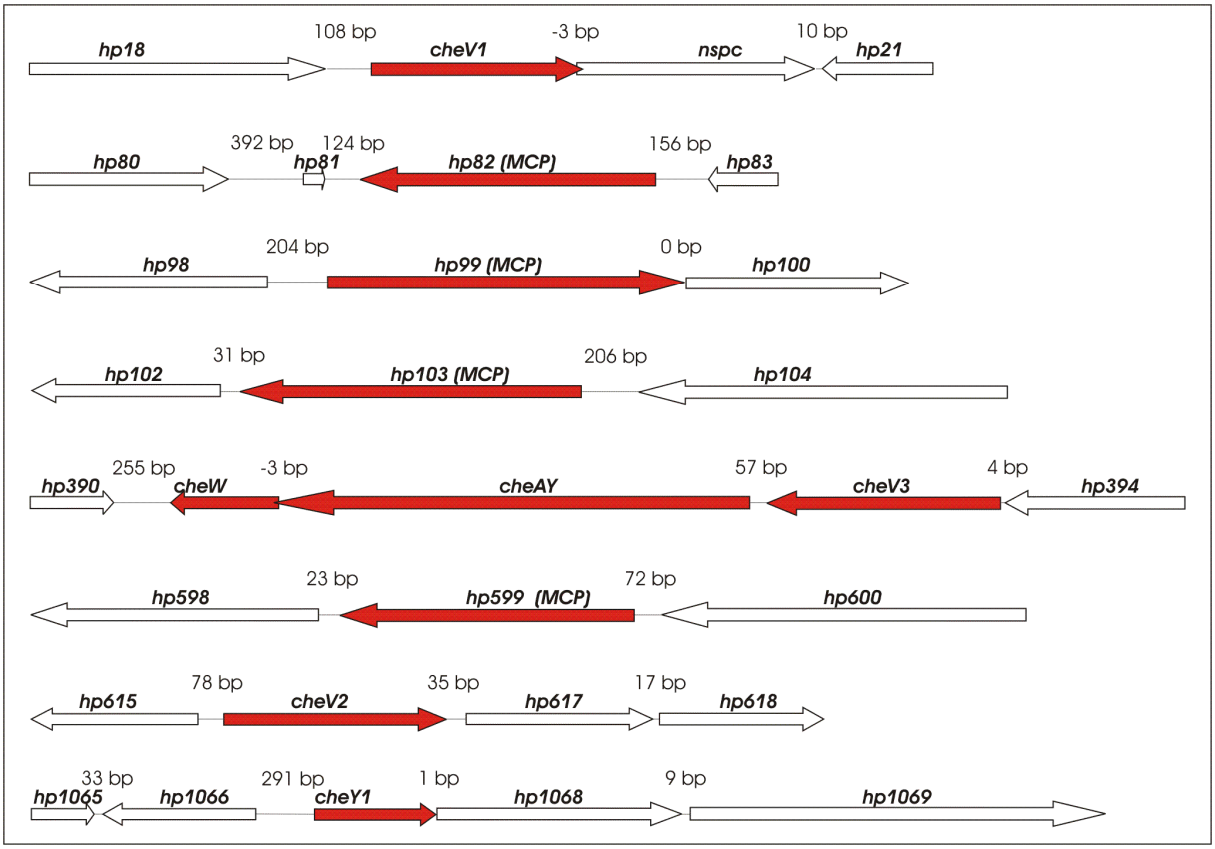

Fig.21 Genome organization of the *H. pylori* chemotaxis genes. ORFs are indicated by arrows and non-coding DNAs are represented by black bars. Red and white arrows represent ORFs that encode chemotaxis proteins and up- and downstream ORFs, respectively. The figure is not drown to scale.

In *S. meliloti* two CheY-like response regulators are phosphorylated by the histidine kinase CheA, however, only one response regulator (CheY2) interacts with the flagellar motor controlling the flagellar rotation directly, while the other CheY (CheY1) functions as a modulator of the chemotactic signalling by acting as phosphate sink which drains away the phosphoryl group from CheY2~P via retrophosphorylation of CheA (Sourjik & Schmitt, 1998).

As mentioned above *H. pylori* possesses a CheY protein (CheY1) and four proteins with a CheY-like domain, the CheAY2 (CheY2), and the three CheV proteins (Alm *et al.*, 1999; Tomb *et al.*, 1997). All *H. pylori* CheY-like proteins possess the highly conserved residues Asp12, Asp13, Asp57, and Lys109 that form the active site of the *E. coli* CheY protein (Fig.22). Hence, it is likely that all the *H. pylori* CheY-like domains are phosphorylated by the histidine kinase CheA.

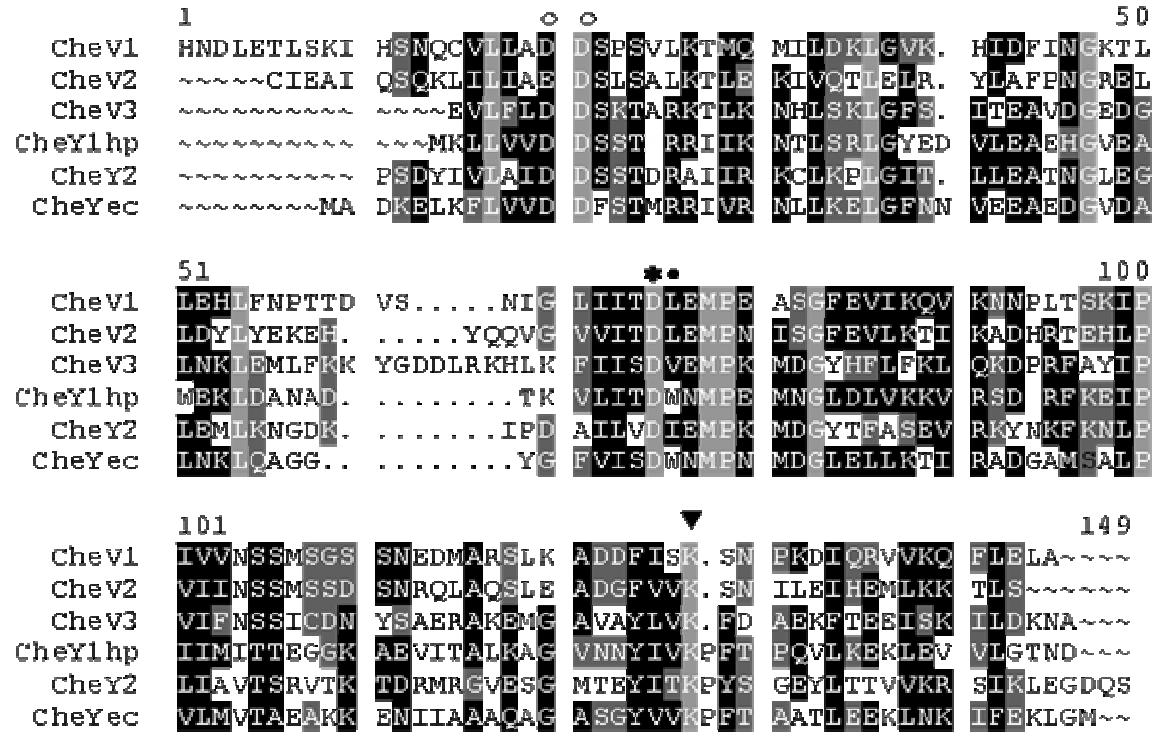

Fig.22 Alignment of the receiver domains of the three *H. pylori* CheV paralogues (HP19/CheV1, HP616/CheV2, and HP393/CheV3), the *H. pylori* CheY1 protein (CheY1hp), the CheY domain of the CheAY2 protein (CheY2), and the *E. coli* CheY (CheYec) is shown. The similarity between the homologous proteins is highlighted by different shading. Light grey: all amino acids of a column are identical; black: more than half of the amino acids of a column are identical or belong to a strong similarity group; dark grey: more than half of the amino acids of a column belong to a weak similarity group or the amino acid marked in dark grey could be grouped into a weak similarity group with every amino acid of the same column belonging to a strong similarity group marked in light grey. Key *E. coli* CheY active-site residues D12, D13 (circle), D57 (the site of phosphorylation; asterisk) and K109 (filled circle) are conserved in all of the *H. pylori* CheY domains. The triangle indicates the position of the tryptophane residue in the *E. coli* CheY which reports conformational changes upon phosphorylation by fluorescence quenching (Lukat, 1992). Although this residue is conserved in *H. pylori* CheY, it is replaced by aliphatic hydrophobic amino acids in all of the CheV proteins.

Furthermore, one or more of these proteins might interact with the flagellar motor or might control the phosphorylation reactions by acting as phosphate sink. To determine whether the *H. pylori* histidine kinase CheA is capable of donating its phosphate group to the different chemotactic receiver domains and to investigate the phosphotransfer reactions between the two-component signalling modules of the chemotaxis proteins *in vitro* phosphorylation assays were performed.

2.1.1. Construction of plasmids expressing chemotaxis proteins fused to His_6 or GST

To investigate the signal transduction processes between the two-component signalling modules of the chemotaxis system of *H. pylori* plasmids for the overexpression of the two-component chemotaxis proteins were constructed.

The bifunctional histidine kinase CheAY2 and two derivatives of CheAY2, CheA´ lacking the CheY-like domain and CheY2 comprising only the CheY-like domain (CheY2) were overexpressed and purified as N-terminal His_6 proteins. Plasmids pTrc-*cheAY2* overexpressing the His_6-tagged protein HP392/CheAY2 (89 kDa), pTrc-*cheA´* overexpressing the His_6-tagged histidine kinase domain CheA´ (74 kDa), and pTrc-*cheY2* overexpressing only the His_6-tagged CheY-like domain protein CheY2 (16 kDa) were provided by Isabel Delany (Molecular Immunology Unit, Chiron Vaccines, Siena, Italy). DNA fragments encoding CheAY2, CheA´, and CheY2 used for the construction of the plasmids were amplified from chromosomal DNA of *H. pylori* G27. Plasmids pTrc-*cheA´*, pTrc-cheAY2, and pTrc-*cheY2* were controlled by sequencing with primers pTrc-seq1 and pTrc-seq2 for proper PCR amplification.

Protein CheY1 (39 kDa) of *H. pylori* and the ortholog of CheY1 in *C. jejuni* (CheY1cj/39 kDa) were overexpressed as GST-fusion proteins. The *che*Y1 genes of *H. pylori* and *C. jejuni* were amplified from chromosomal DNA of *H. pylori* G27 and *C. jejuni* 4344 with the primer pairs cheY1-5´/cheY1-3´, and cjY1-5´/cjY1-3´, respectively, yielding PCR products of 370 bp and 390 bp, respectively. The PCR fragments were cloned into pGEX-3X vector DNA after restriction with *Bam*HI-*Eco*RI generating plasmids pGEX-*cheY1* and pGEX-*cheY1cj* encoding for CheY1 of *H. pylori* and CheY1 of *C. jejuni*. These two plasmids were controlled by sequencing with primers pGEX5´ and pGEX3´. Moreover, the *Bam*HI-*Eco*RI fragment encoding *H. pylori* CheY1 was also used for the construction of pGEX*cheY1-D53N,* which encodes the *H. pylori* response regulator CheY1 with a substitution of the putative phosphorylation site (D53) by asparagine. For this purpose the cheY1 PCR fragment was digested with restriction enzymes *Bam*HI-*Eco*RI and was ligated into pSL1180 vector DNA

yielding plasmid pSL-*cheY*1. pSL-*cheY*1 was used as a template for site-specific mutagenesis by overlapping extension creating the PCR product *cheY1-D53N* containing the substitution of the aspartate 53 by asparagine. Finally, this PCR product was digested with restriction enzymes *Bam*HI-*Eco*RI and ligated into pGEX-3X vector DNA yielding plasmid pGEX*cheY1-D53N*, which overexpresses the protein CheY1-D53N. Plasmid pGEX*cheY1-D53N* was controlled by sequencing with primer pGEX5´ and pGEX3´.

Plasmid pQE-*cheYec* overexpressing the C-terminal His_6-tagged protein CheY of *E. coli* (13 kDa) was constructed by Victor Sourjik and provided by Birgit Scharf (Institute of Biochemistry, Genetics and Microbiology, University of Regensburg, Germany). This plasmid was controlled by sequencing with primer pQE-5 and pQE-3.

The three *H. pylori* CheV proteins HP19/CheV1 (36 kDa), HP616/CheV2 (35 kDa), and HP393/CheV3 (35 kDa) were overexpressed as His_6 proteins. The *cheV1*, *cheV2*, and *cheV3* genes were amplified from chromosomal DNA of *H. pylori* G27 with primer pair cheV1-5´/cheV1-3´, cheV2-5´/cheV2-3´, and cheV3-5´/cheV3-3´, respectively. The PCR fragments with a size of 960 bp, 930 bp, and 940 bp were digested with restriction enzymes *Bam*HI-*Kpn*I, *Bam*HI-*Pst*I, and *Bam*HI-*Pst*I, respectively, and ligated into pQE30 vector DNA yielding plasmid pQE-*cheV*1, pQE-*cheV*2, and pQE-*cheV*3. These plasmids were controlled by sequencing with primers pGEX5´ and pGEX3´.

In Fig.23 A a schematic representation of the *H. pylori* chemotaxis proteins under study is shown.

2.1.2. Expression and purification of fusion proteins

The His_6 tagged proteins CheAY2, CheA´, CheY2, and the three CheV proteins from *H. pylori*, and *E. coli* CheY were overexpressed in *E. coli* M15 cells. After overexpression under various conditions CheA´, CheY2, and the three CheV proteins were found in cytoplasmic inclusion bodies. Therefore, purification of these proteins was performed under denaturing conditions. In contrast the bifunctional CheAY2 protein was found soluble in the cytoplasm and was purified under native conditions. Purified proteins were analysed on SDS gels stained with Coomassie blue (Fig.23 B). The proteins CheA´, CheAY2, CheV1, and CheV3 were repeatedly obtained in low concentrations, and therefore the respective protein solutions were concentrated with Aquacid II (a sodium salt of carboxymethylcellulose) for about 3 hours at 4°C (cold room).

The GST-fusion proteins CheY1, CheY1-D53N, and *C. jejuni* CheY1 were overexpressed in *E. coli* DH5α. When overexpression was performed for 2 hours at 30°C in the presence of

0.05 mM IPTG the proteins CheY1 and CheY1-D53N were found soluble in the cytoplasm. *C. jejuni* CheY1 was found soluble in the cytoplasm when protein expression was induced with 0.1 mM IPTG for 3 hours at 30°C. Purified proteins were analysed by SDS-PAGE and the gels were stained with Coomassie blue (Fig.23 B).

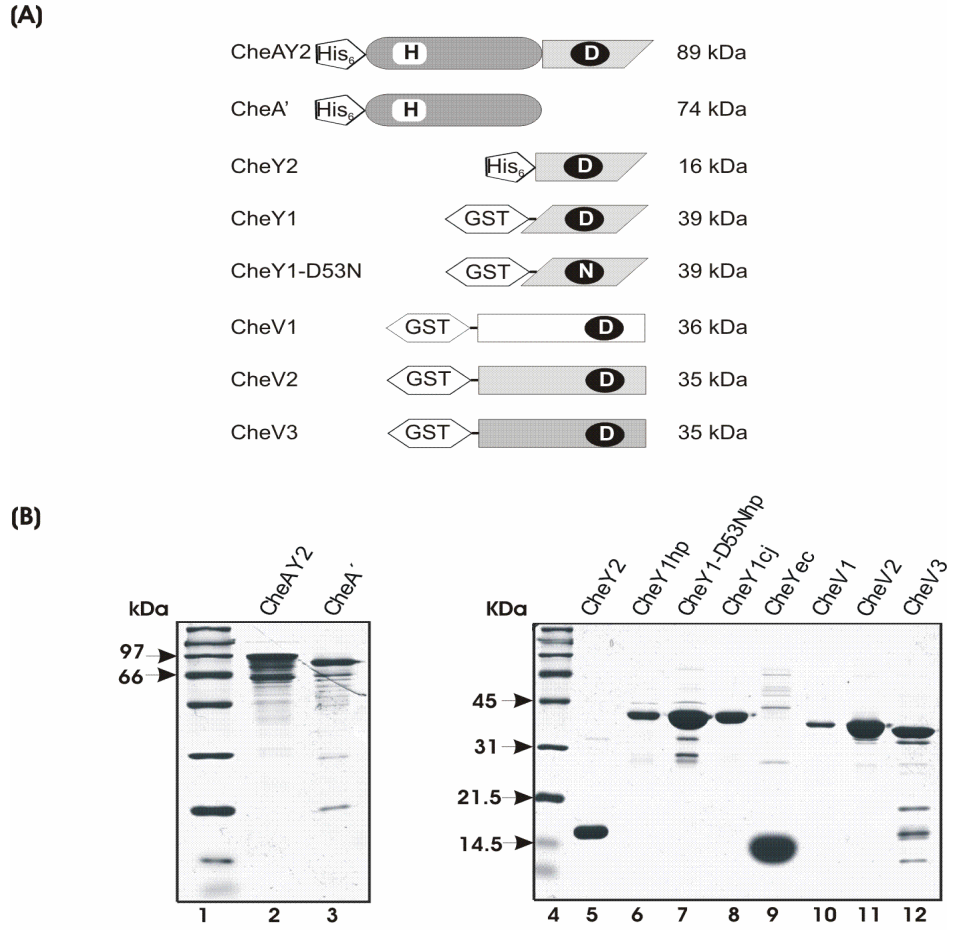

Fig.23 Schematic representation of the chemotaxis two-component proteins of *H. pylori*. (A) Representation of the *H. pylori* chemotaxis two-component proteins used in this study. The phosphorylated histidine residue in the P1 domain of the histidine kinase (H) and the phosphate-accepting aspartic acid residues in the response regulator domains (D) are highlighted. The molecular weights of the proteins are given on the right. (B) Analysis of the purified fusion proteins by SDS polyacrylamide gel electrophoresis on 15% SDS polyacrylamide gels. The following proteins were loaded: *H. pylori* chemotaxis histidine kinases CheAY2 (89 kDa. lane 2), truncated histidine kinase CheA´ (74 kDa, lane 3), *H. pylori* CheY2 (16 kDa, lane 5), *H. pylori* CheY1hp (39 kDa, lane 6), CheY1-D53Nhp (39 kDa, lane 7), *C. jejuni* CheY1cj (39 kDa, lane 8), *E. coli* CheYec (13 kDa, lane 9), and *H. pylori* CheV1 (36 kDa, lane 10), CheV2 (35 kDa, lane 11), and CheV3 (35 kDa, lane 12). Protein marker (BioRad) was used in both gels (lanes 1 and 4). The gels were stained with Coomassie-blue.

2.1.3. Kinetics of *H. pylori* CheAY2 and CheA´ autophosphorylation

2.1.3.1. Analysis of the autokinase activity of CheAY2 and CheA´

As mentioned above the bifunctional histidine kinase CheAY2 and two derivatives of CheAY2, CheA´ and CheY2 were overexpressed and purified as N-terminal His_6 proteins. To determine whether histidine kinase CheAY2 and histidine kinase CheA´ show ATP-dependent autophosphorylation *in vitro*, phosphorylation assays were performed. Different

concentration (0.2 µM, 0.5 µM, 2 µM, 3 µM, and 5 µM) of histidine kinases CheAY2 and CheA´ were incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) in independent reactions. After 10 min of incubation at RT reactions were stopped by the addition of sample buffer and the mixtures were electrophoresed on a 12% SDS polyacrylamide gel and the signals were detected using a Typhoon 9200 Variable Mode Imager. Autophosphorylation of both histidine kinases, CheAY2 and CheA´, was detected (data not shown). Moreover, the reactions were incubated at different temperatures (on ice, RT, 20°C, 30°C). It was established that optimal autophosphorylation of both chemotaxis histidine kinases is obtained at a protein concentration of 2 µM at a reaction temperature of 30°C (data not shown).

2.1.3.2. Kinetics of autophosphorylation of CheAY2 and CheA´

To analyze the kinetics of the autophosphorylation of the *H. pylori* histidine kinases CheAY2 and CheA´ phosphorylation experiments were performed in a time course of 60 min. 2 µM of CheAY2 was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) at 30°C in a final volumen of 100 µl. At defined time points (2, 4, 6, 8, 10, 15, 20, 25, 30, and 60 min) samples (10 µl) were removed and the reaction was terminated by the addition of sample buffer containing 50 mM Na_2EDTA. The samples were electrophoresed on a 15% SDS polyacrylamide gel and quantification of the signals was performed using Image Master TotalLab Software. Then the signal intensity of the strongest signal on the gel was arbitrarily set to 100%. Subsequently, the signal intensities of the other bands in the gel were calculated with respect to the most intense signal. The experiment was performed more than three times and each data point in the graphic shown in Fig.24 A represents the average of three independent experiments. The standard deviation was calculated using Microsoft Excel. As shown in the Fig.24 A the maximal signal in the kinetics of the phosphorylation of histidine kinase CheAY2 was detected after 8 min of incubation. Interestingly after this time of incubation the dephosphorylation of CheAY2 prevails in the reaction (Fig.24 A).

As described for CheAY2, 2 µM of CheA´ was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) at 30°C in a final volume of 100 µl. At the indicated time points samples (10 µl) were removed and the reaction was terminated by the addition of sample buffer containing 50 mM Na_2EDTA. The samples were electrophoresed on a 15% SDS polyacrylamide gel. Quantification of the signals was performed essentially as described above. The experiment was performed more than three times and each data point in the graphic represents the average of three independent experiments (Fig.24 A). As shown in the Fig.24 A autophosphorylation of CheA´ follows a simple exponential time course reaching

the maximum level of phosphorylation after 15 min of incubation. This signal remains constant over a period of 60 min of incubation (Fig.24 A). Therefore, the phosphorylation kinetics suggested that the CheY-like domain of CheAY2 (CheY2) accelerates the dephosphorylation of the P1 domain of CheAY2 containing the highly conserved histidine residue.

2.1.4. Dephosphorylation rates of *H. pylori* CheAY2~P and CheA´~P

To determine the dephosphorylation rates of CheAY2~P and CheA´~P chase experiments were performed. 2 µM CheAY2 was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) in a reaction with a final volume of 100 µl. After 10 min of incubation a sample (10 µl) was taken and then the reaction was chased by adding unlabelled ATP to a final concentration of 10 mM. Then samples (10 µl) were removed at defined time points (15, 30, 45, 60 sec, 2.5, 5, 10, 15, and 30 min) and electrophoresed on a 15% SDS polyacrylamide gel. Quantification of signals was performed as described in 2.1.3.2. The signal intensity of the sample taken immediately before the chase with unlabelled ATP was arbitrarily set as 100% and this time point is referred to as t_0. Subsequently, the signal intensities of the other bands in the gel were calculated with respect to the 100% (t_0). The experiment was performed more than three times and each data point in the graphic shown in Fig.24 B represents the average of three independent experiments. The standard deviation was calculated using Microsoft Excel. Moreover, the half-life of the phosporylated proteins was calculated using computer generated least square fits of the data to each curve (Fig.24 B). As observed in the Fig.24 B CheAY2~P showed a half-life of 3 min and hydrolysis of CheAY2~P was almost complete after 15 min.

As in the dephosphorylation experiment with CheAY2, 2 µM of CheA´ was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) in a reaction with a final volume of 100 µl. After 15 min of incubation a sample (10 µl) was taken and then the reaction was chased by adding unlabelled ATP to a final concentration of 10 mM. Then samples (10 µl) were removed at the indicated time points and electrophoresed on a 15% SDS polyacrylamide gel. Quantification of signals was performed as described in 2.1.3.2. The experiment was performed more than three times and each data point in the graphic represents the average of three independent experiments (Fig.24 B). In contrast to the complete dephosphorylation of CheAY2 after 15 min of chase, half the amount of CheA´~P was still detectable at this time point. After 30 min of incubation 40% of the initial signal of CheA´~P (t_0) were detected and after 90 min of incubation 35% of the initial phosphorylation signal were still detectable

(Fig.24 B and C). Therefore, the chase experiments corroborate the conclusion that the presence of CheY2 in the bifunctional CheAY2 protein significantly decreases the half-life of the phosphorylated histidine kinase.

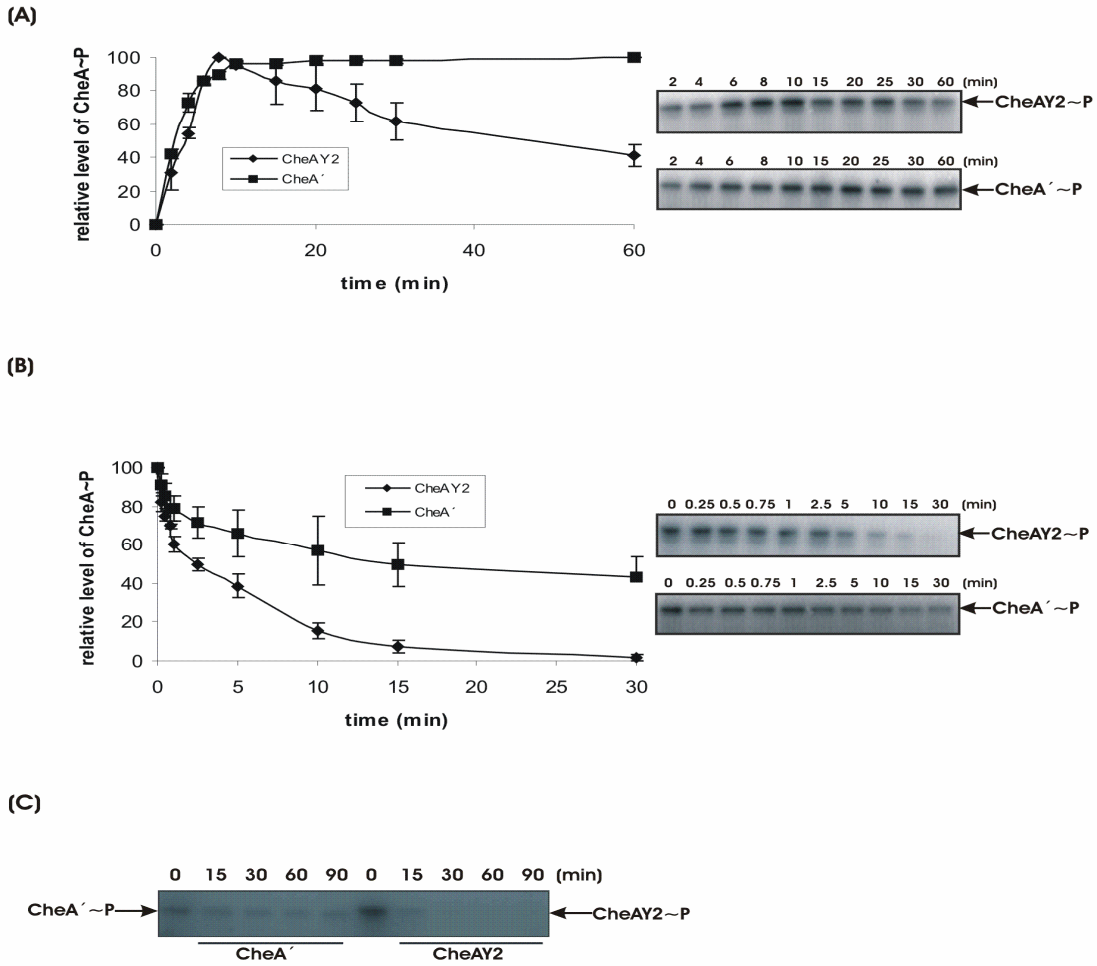

Fig.24 Kinetics of the ATP-dependent autophosphorylation of CheAY2 and CheA´ and dephosphorylation rates of histidine kinase CheAY2~P and CheA´~P. (A) Time course of CheAY2 and CheA´ autophosphorylation. 2 µM of the purified proteins CheAY2 and CheA´ were incubated with 50 nM [γ-^{32}P]ATP and at indicated time points the reactions were stopped by the addition of sample buffer containing 50 mM Na_2EDTA. On the right autoradiograms of representative 15% SDS-polyacrylamide gels are shown. Quantification of signals was performed using Image Master TotalLab Sofware. (B) Time course of the dephosphorylation of CheAY2~P and CheA´~P. 2 µM of the purified proteins CheAY2 and CheA´ were phosphorylated with 50 nM [γ-^{32}P]ATP for 10 and 15 min, respectively. Then the reactions were chased by the addition of unlabelled ATP to a final concentration of 10 mM and at indicated time points reactions were stopped by the addition of sample buffer containing 50 mM Na_2EDTA. On the right autoradiograms of a representative 15% SDS-polyacrylamide gel are shown. Quantification of signals was performed using Image Master TotalLab Sofware. Data points in (A) and (B) represent the means of three independent experiments. (C) Autoradiogram of the dephosphorylation of CheAY2~P and CheA´~P within a 90 min time-course.

2.1.5. Phosphotransfer reactions between chemotaxis proteins

2.1.5.1. Phosphotransfer reactions between chemotaxis histidine kinases from H. pylori and the response regulators CheY1 and CheY2

To analyse the phosphorylation of both histidine kinases CheAY2 and CheA´ in the presence of the response regulators CheY1 and CheY2 *in vitro* phosphorylation assays under multiple turnover conditions were performed. 2 µM of CheAY2 was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) and 4 µM of CheY1 or 4 µM of CheY2 at 30°C for 10 min. Then 10 µl of the sample were removed and the reaction was stopped with sample buffer. Samples were electrophoresed on a 15% SDS polyacrylamide gel. Detection of signals was performed as described in 2.1.3.2. Dephosphorylation of CheAY2 was observed in the presence of both response regulators CheY1 and CheY2, however, only CheY2 was phosphorylated stably enough so that CheY2~P could be detected after 10 min of incubation in the presence of CheAY2 (Fig.25 A, lanes 1, 2, and 3). In a similar reaction 2 µM of CheA´ was incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) and 4 µM of CheY1 or 4 µM of CheY2 at 30°C for 15 min. Dephosphorylation of CheA´~P in the presence of the proteins CheY1 and CheY2 indicated transfer of the phosphoryl group to the respective receiver domain. Similar as in the presence of CheAY2, only CheY2 was phosphorylated stably enough to be detected on the gel after 15 min of incubation (Fig.25 A, lanes 4, 5, 6). As control CheY1 and CheY2 were incubated in the presence of [γ-^{32}P]ATP only and as expected no phosphorylation could be detected (data not shown).

Furthermore, phosphorylation of histidine kinase CheA´ was analyzed in the presence of two different concentrations of the proteins CheY1 and CheY2. 2 µM CheA´ and 1 µM or 4 µM of CheY1 or CheY2 were incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) under multiple turnover conditions for 15 min. As described for the other experiments samples (10 µl) were taken and electrophoresed on a 15% SDS polyacrylamide gel. Total dephosphorylation of CheA´ occurred when the response regulators were added in both half or twofold of the amount of the histidine kinase (Fig.25C, lanes 2-3 and 10-11). Moreover, transfer of the phosphate group of the CheA´ protein to the aspartate 53 of the protein CheY1 was confirmed by a phosphorylation reaction containing CheA´ and CheY1-D53N, which is a derivative of CheY1 with a D53N substitution (Fig.25 C, lanes 4 and 5). In this case no dephosphorylation of CheA´ was observed.

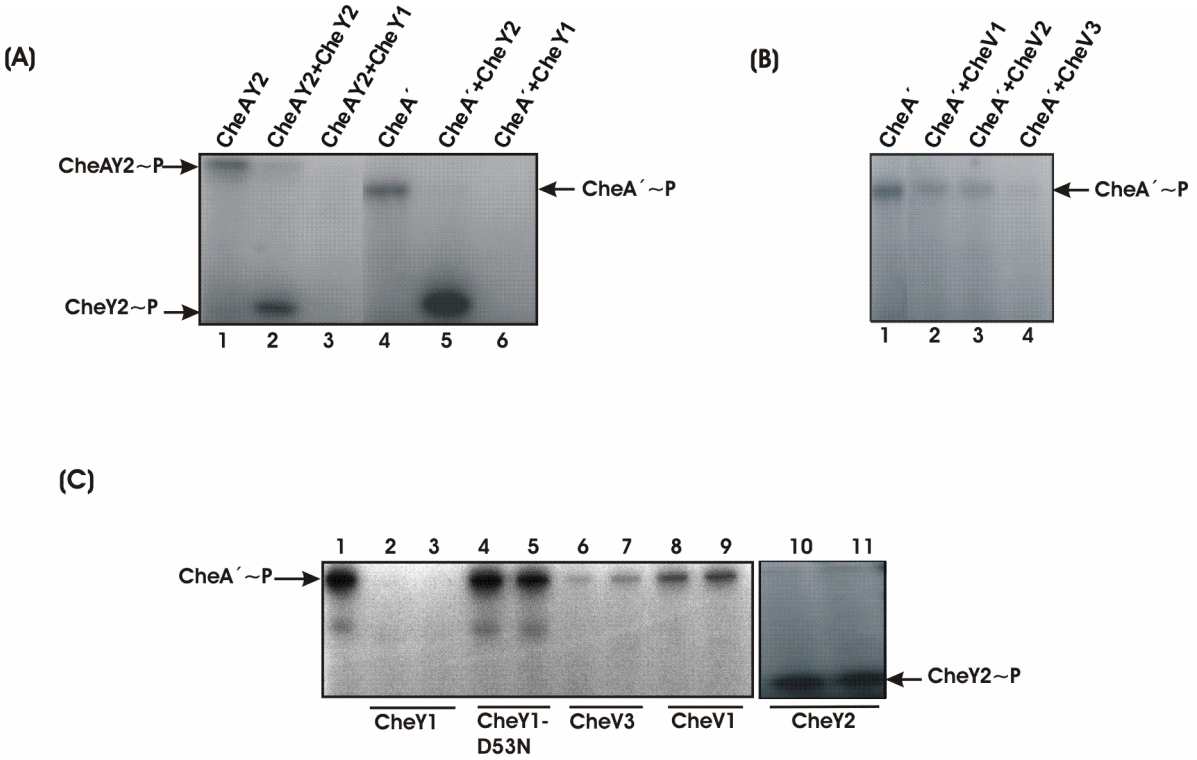

Fig.25 Analysis of the phosphoryl group transfer between the CheA histidine kinase and the *H. pylori* CheY-like proteins under multiple turnover conditions. (A) Autophosphorylation of 2µM CheAY2 (1) or 2µM CheA´ (4) and phosphotransfer to 4µM CheY2 (2, 5) and CheY1 (3, 6). (B) Autophosphorylation of CheA´ (1) and phosphotransfer to 4µM CheV1 (2), CheV2 (3) and CheV3 (4). (C) Autophosphorylation of CheA´ (1) in the presence of 1µM and 4µM of CheY1 (2, 3), CheY1-D53N (4, 5), CheV3 (6, 7), CheV1 (8, 9), and CheY2 (10, 11).

To analyse the dephosphorylation of CheA´~P in the presence of CheY1 or CheY2 within 60 sec time course experiments were performed. 2 µM of CheA´ was phosphorylated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol). After 15 min of incubation 10 µl of the reaction was removed and the reaction was stopped by the addition of sample buffer (t_0). Subsequently, 4 µM of CheY1, CheY1-D53N, or CheY2 were added in independent reactions. At defined time points (15, 30, 45, and 60 sec) samples (10 µl) were removed and the reaction was stopped by the addition of sample buffer. As shown in Fig. 26 total dephosphorylation of CheA´ in the presence of CheY1 and CheY2 was observed when the first sample was analysed after 15 sec of incubation, while in the presence the CheY1-D53N the radiolabelled band of CheA´~P did not change its intensity within 60 sec and was still detectable after 20 min of incubation (Fig.26 and data not shown).

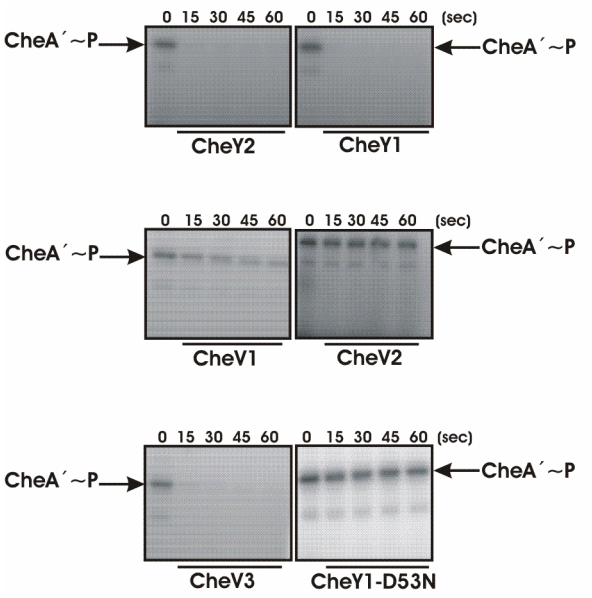

Fig.26 Time course of dephosphorylation of CheA´~P in the presence of the *H. pylori* CheY and CheV proteins. 2 µM CheA´ was incubated with 50 nM [γ-^{32}P]ATP for 15 min (t_0), and after the addition of 4 µM of the CheY-like proteins (CheY1, CheY1-D53N, CheY2, CheV1, CheV2, and CheV3) reactions were stopped at the indicated time points. Detection of signals was performed using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences).

A similar experiment investigating the phosphorylation of CheA´ in the presence of non *H. pylori* CheY proteins was performed. 2 µM of CheA´ was phosphorylated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol). After 15 min of incubation 10 µl of the reaction were removed and the reaction was stopped with the addition of sample buffer (t_0). Subsequently, 4 µM of the ortholog of *H. pylori* CheY1 from *C. jejuni* (CheY1cj) or *E. coli* CheY (CheYec) were added. At defined time points (15, 30, 45, and 60 sec) samples (10 µl) were removed and the reactions were stopped by the addition of sample buffer. Dephosphorylation of *H. pylori* CheA´~P was observed when CheA´ was incubated in the presence of CheY1cj and CheYec indicating a phosphotransfer from the chemotaxis histidine kinase CheA´ from *H. pylori* to the heterologous response regulators. Similar as in the reaction with *H. pylori* CheY1, CheY1~P from *C. jejuni* and CheY~P from *E. coli* were not detected after an incubation time of 15 sec or more (data not shown). However, when samples were taken after 5 and 10 sec of incubation in time course experiments with CheA´~P phosphorylated CheY1hp, CheY1cj, and CheYec could be detected. CheA´~P was completely dephosphorylated when the first sample was analysed after 5 sec of incubation. These data clearly demonstrated the phosphotransfer from the *H. pylori* CheA´ histidine kinase to CheY1 from *H. pylori* and the orthologous proteins of *C. jejuni* and *E. coli* (Fig.27 A).

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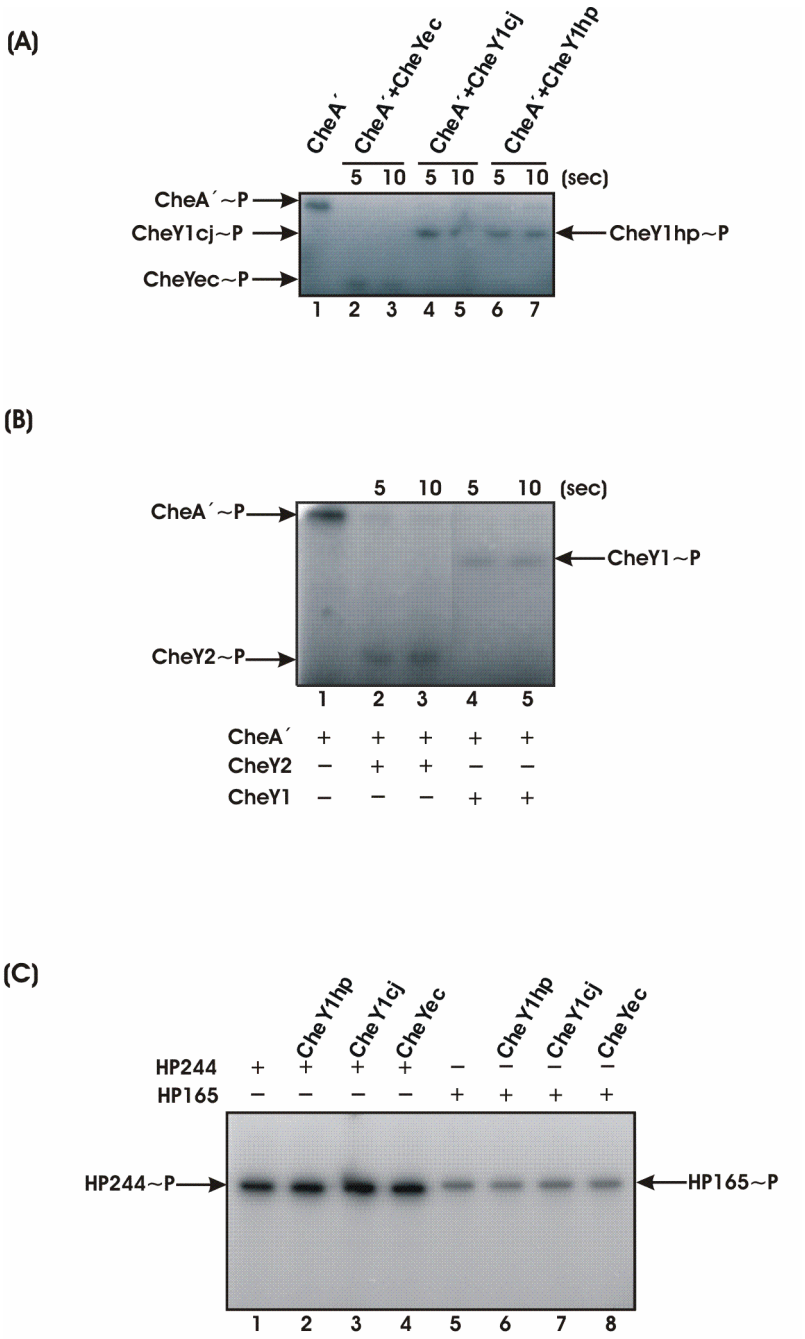

Fig. 27 Analysis of the phosphoryl group transfer between the CheA´ histidine kinase and the chemotaxis CheY-orthologs under multiple turnover conditions. (A) 2 µM CheA´ was phosphorylated with 50 nM [γ-^{32}P]ATP for 15 min (1), then 4 µM *E. coli* CheYec (2, 3), *C. jejuni* CheY1 (4, 5), or *H. pylori* CheY1hp (6, 7) were added and the reactions were stopped after 5 (2, 4, 6) and 10 sec (3, 5, 7) of incubation at RT. (B) 2 µM CheA´ was phosphorylated with 50 nM [γ-^{32}P]ATP for 15 min (1), then 4 µM CheY2 (2, 3) or CheY1 (4, 5) were added and the reactions were stopped after 5 (2, 4) and 10 sec (3, 5) of incubation on ice. (C) As a control autophosphorylation of HKs HP244 and HP165 (1 and 5, respectively) was carried out in the presence of *H. pylori* CheY1hp (2, 6), *C. jejuni* CheY1cj (3, 7), and *E. coli* CheYec (4, 8).

In a similar time-course experiment CheA´~P was completely dephosphorylated in the presence of CheY1 after 5 sec of incubation on ice, whereas in presence of CheY2 20% of the initial signal of CheA´~P was still detected after 10 sec of incubation on ice (Fig.27 B). This result indicated that CheY1 has a higher affinity for CheA´ than CheY2. As a control

phosphorylation assays with two *H. pylori* histidine kinases, HP244 and HP165, with no function in chemotaxis were performed in the presence of the CheY proteins CheY1hp, CheY1cj, CheYec, and CheY2 (Fig.27 C and data not shown). In these control experiments no dephosphorylation of both HKs HP244 and HP165 was observed.

2.1.5.2. Phosphotransfer reactions between the histidine kinase CheA´ and the proteins CheV1-CheV3

To investigate the phosphorylation of histidine kinase CheA´ in the presence of CheV1-CheV3, 2 µM CheA´ was phosphorylated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) and 4 µM of one of the three paralogous CheV proteins under multiple turnover conditions. The reactions were incubated for 15 min at 30°C. As described above samples (10 µl) were taken and the reaction was stopped with sample buffer. All samples were electrophoresed on a 15% SDS polyacrylamide. Detection of signals was performed as described above. Dephosphorylation of CheA´ was observed in the presence of all three CheV proteins (Fig.25 B). Interestingly, CheA´ exhibited a moderate dephosphorylation in the presence of CheV1 and CheV2, whereas in the reaction with CheV3 the kinase CheA´ was almost completely dephosphorylated. Similar to the analysis of the CheY proteins, a CheV~P signal could not be detected for CheV1, CheV2, and CheV3 after incubation for 15 min. Furthermore, phosphorylation of histidine kinase CheA´ was analyzed in the presence of two different concentrations of the proteins CheV1, CheV2, or CheV3. 2 µM CheA´ and 1 µM or 4 µM of each CheV protein were incubated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) under multiple turnover conditions for 15 min in independent reactions. As described for the other experiments samples (10 µl) were taken and electrophoresed on a 15% SDS polyacrylamide gel. Dephosphorylation of CheA´ occurred when the response regulator was present in half or twofold of the amount of the histidine kinase and with comparable efficiencies as in the experiment shown in Fig.25 B (Fig.25 C lanes 6-9 and data not shown).
Time course experiments were performed to analyse the dephosphorylation of CheA´ in the presence of the three CheV proteins in a time course of 60 sec. 2 µM of CheA´ was phosphorylated in the presence of 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol). After 15 min of incubation 10 µl of the reaction mixture were removed and the reaction was stopped with the addition of sample buffer (t_0). Subsequently, 4 µM of CheV1, CheV2, or CheV3 were added. At defined time points (15, 30, 45, and 60 sec) samples (10 µl) were removed and the reactions were stopped by the addition of sample buffer. As shown in Fig. 26 total dephosphorylation of CheA´ was observed within 15 sec only in the presence of CheV3,

while in the presence of CheV1 and CheV2 radiolabelled bands were detected after 60 sec of incubation exhibiting signal intensity of 45% and 60% with respect to the signal of CheA´~P at t_0. In a second time course experiments samples were removed after 5 and 10 sec of incubation on ice or at RT. CheA´~P was slightly dephosphorylated in presence of the CheV3 after 10 sec of incubation. In contrast to the CheY proteins, CheV~P was not detected in case of any CheV protein after 5 and 10 sec of incubation (data not shown). As a control phosphorylation of the two *H. pylori* histidine kinases HP244 and HP165 with no function in chemotaxis was performed in the presence of the CheV proteins. In this control no dephosphorylation of both HKs HP244 and HP165 was observed (data not shown). In addition, the three CheV proteins were not phosphorylated in presence of [γ-^{32}P]ATP alone (data not shown).

2.1.6. CheA´-dependent phosphorylation of CheY2 in the presence of other chemotaxis response regulator proteins

To determine whether phosphorylation of CheY2 is affected in the presence of the other *H. pylori* chemotaxis response regulators, CheA´ dependent phosphorylation of CheY2 in the presence of CheY1 and CheV1-CheV3 was analysed.

4 µM CheY2 was incubated in the presence of 2 µM CheA´ and 50 nM [γ-^{32}P]ATP (6 000 Ci/mmol) for 15 min. Then a sample (10 µl) was removed and the reaction was stopped by the addition of sample buffer (Fig.28 A lane 2). In parallel reactions CheA´-dependent phosphorylation of CheY2 was analysed under multiple turnover conditions in the presence of either 1 µM or 4 µM of *H. pylori* CheY1, CheY1-D53N, and CheV1-CheV3. As shown in Fig. 28 A CheY2~P was not detected in the presence of *H. pylori* CheY1 in a reaction containing both proteins CheY1 and CheY2 in equimolar concentrations, while CheY2~P was detected when CheY2 was present in a fourfold excess relative to CheY1, but the signal was clearly reduced as compared to the control reaction containing only CheY2. In contrast, CheY2~P exhibited a stable phosphorylation in the presence of CheY1-D53N and CheV1-CheV3 and the signal intensity was not altered as compared to the control reaction. Interestingly, in a similar experiment CheY2~P was not detected in the present of 4 µM of *C. jejuni* CheY1 and *E. coli* CheY (Fig.28 B). These results suggested that CheY1 from *H. pylori* and its ortholog from *C. jejuni* and *E. coli* strongly prevail over CheY2 in competition for the phosphoryl group donor CheA´~P.

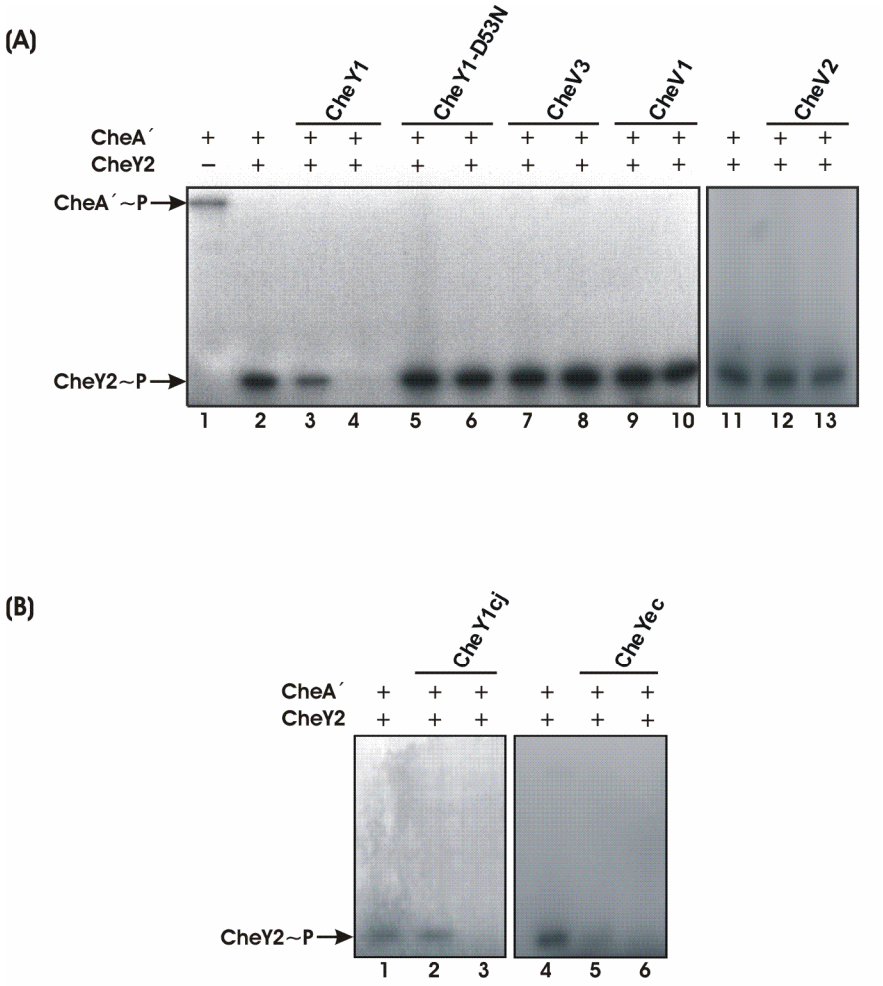

Fig.28 CheA´-dependent phosphorylation of CheY2 in the presence of other chemotaxis response regulators. (A) CheA´-dependent phosphorylation of CheY2 in the presence of other *H. pylori* chemotaxis response regulators. Reactions mixtures contained 2 µM CheA´ (1), 2 µM CheA´ and 4 µM CheY2 (2, 11), and 2 µM CheA´, 4 µM CheY2 and 1 µM or 4 µM of CheY1 (3, 4), CheY1-D53N (5, 6), CheV3 (7, 8), CheV1 (9, 10), and CheV2 (12, 13). (B) CheA´-dependent phosphorylation of CheY2 in the presence of CheY1 from *C. jejuni* (CheY1cj) and CheY from *E. coli* (CheYec). Reactions mixtures contained 2 µM CheA´, 4 µM CheY2 (1, 4) and 1 µM or 4 µM of CheY1cj (2, 3), CheYec (5, 6).

To further characterize the CheA´ dependent phosphorylation of CheY2 in the presence of other chemotaxis response regulator proteins time course experiments were performed as follows: 2 µM CheA´ and 2 µM CheY2 were incubated in the presence of 50 nM [γ-32P]ATP at 30°C in a reaction with a final volume of 100 µl. After 15 min of incubation a sample (10 µl) was taken and 2 µM of the respective chemotaxis proteins (CheY1, CheY1-D53N, CheV1-CheV3, CheYcj, and CheYec) were added to the reaction mixture. Then samples (10 µl) were removed at defined time points (15, 30, 45, 60 sec, 2.5, 5, 10, 15, and 30 min) and were electrophoresed on a 15% SDS polyacrylamide gel. Quantification of signals was performed as described in 2.1.3.2. The signal intensity of the sample taken immediately before adding the respective response regulator protein was arbitrarily set as 100% (t_0). Subsequently, the signal intensities of the other bands in the gel were calculated with respect

to the 100% (t_0). The experiments were performed more than three times and each data point in the graphics shown in Fig.29 represents the average of three independent experiments. The standard deviation was calculated using program Microsoft Excel. When necessary the half-life of CheY2~P was calculated using computer generated least square fits of the data to each curve. When CheY2 was incubated only in the presence of CheA´, CheY2~P could be detected after 30 min of incubation with a signal intensity of 45% with respect to the initial signal at t_0. In contrast, when CheY1 was added CheY2~P hydrolyzed with a half-life of 3 min (Fig.29 A). As expected the dephosphorylation rate of CheY2~P was not altered in the presence of CheY1-D53N (Fig.29 A).

To determine whether the effect on CheY2~P in the presence of CheY1 is a consequence of a competition of both response regulators CheY1 and CheY2 for the phosphate group of the histidine kinase CheA´, a chase experiment with unlabelled ATP was carried out. 2 µM of CheA´ and 2 µM of CheY2 were incubated in the presence of 50 nM [γ-^{32}P]ATP at 30°C in a reaction with a final volume of 100 µl. After 15 min of incubation a sample (10 µl, t_0) was taken and then the reaction was chased by adding unlabelled ATP to a final concentration of 10 mM. Interestingly, in this experiment a similar kinetics for the dephosphorylation of CheY2 was observed as in CheA´-dependent phosphorylation of CheY2 in the presence of CheY1. The half-life for CheY2~P was calculated to be 3.3 min. In a similar experiment the simultaneous addition of CheY1 and unlabelled ATP had no significant effect on the half-life of CheY2~P (Fig.29 C). The difference in the kinectics of dephosphorylation of CheY2~P observed in the presence and in the absence of an excess of unlabelled ATP indicated that in the unchased reaction phosphorylation of CheA´ and phosphotransfer to CheY2 continuously occurred in the investigated time period resulting in the presence of a higher relative amount of CheY2~P observed after 30 min of reaction.

In another experiment 2 µM CheA´ and 2 µM CheY2 were incubated in the presence of 50 nM [γ-^{32}P]ATP at 30°C in a reaction with a final volume of 100 µl. After 15 min of incubation 2 µM of *C. jejuni* CheY1 or *E. coli* CheY were added. Then samples (10 µl) were removed at defined time points (15, 30, 45, 60 sec, 2.5, 5, 10, 15, and 30 min) and were electrophoresed on a 15% SDS polyacrylamide gel. The experiment was performed more than three times and each data point in the graphic shown in Fig.29 D represents the average of three independent experiments. In the presence of CheY1cj and CheYec CheY2~P showed a kinetics of dephosphorylation similar as in the reaction in the presence of *H. pylori* CheY1 (Fig.29 D). The half-life for CheY2~P was calculated to be 3.3 min and 2 min in the presence of CheY1cj and CheYec, respectively.

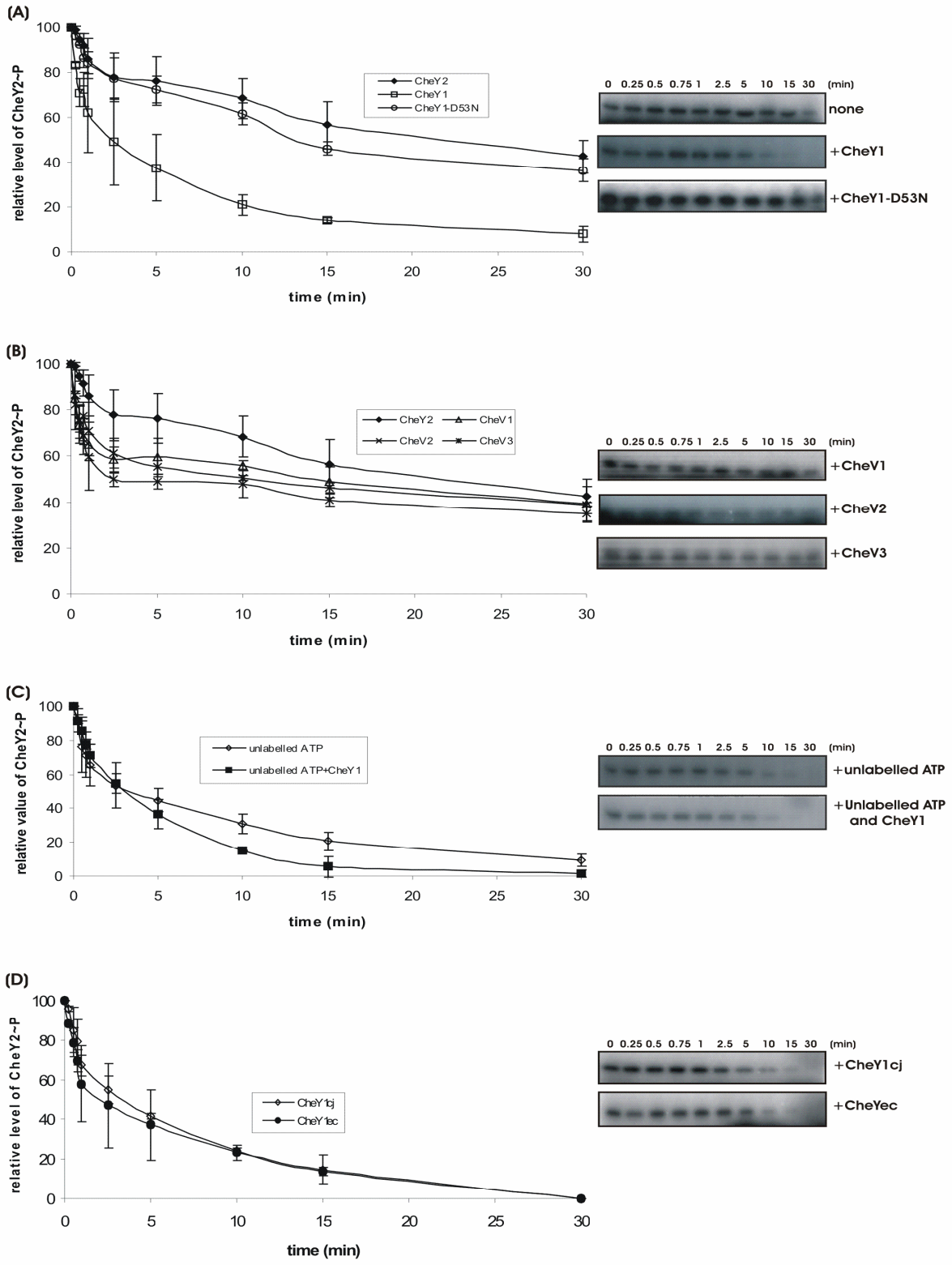

Fig. 29 Kinetics of dephosphorylation of CheY2~P in the presence of other chemotaxis response regulators. Kinetics of dephosphorylation of CheY2~P in the presence of CheY1 and CheY1-D53N (A); CheV1, CheV2, and CheV3 (B); an excess of unlabelled ATP and CheY1 (C); CheY from *C. jejuni* (CheY1cj) and CheY form *E. coli* (CheYec) (D). On the right autoradiograms of a representative 15% SDS-polyacrylamide gel are shown. Quantification of signals was performed using Image Master TotalLab Sofware (Amersham Biosciences). Data points represent the means of three independent experiments.

Furthermore, in a similar time course experiment 2 µM CheA´ and 2 µM CheY2 were incubated in the presence of 50 nM [γ-^{32}P]ATP and the *H. pylori* CheV proteins in independent reactions. Dephosphorylation of CheY2~P was slightly enhanced in the presence of the three CheV proteins but only in the first 15 min of the reaction. After 30 min of incubation CheY2~P exhibited a signal intensity of about 40% of the initial signal at t_0 (Fig.29 B).

The data from the time course experiments clearly suggested that CheY1 interacts more efficiently with CheA´~P than CheY2 possibly through a higher affinity for the histidine kinase. This is also supported by the observation that CheY1 caused the complete dephosphorylation of CheA´~P within 10 sec at 0°C (Fig.27 B), while CheA´~P remained detectable in the presence of CheY2.

2.1.7. Phosphorylation of the chemotaxis response regulators with acetyl phosphate

Several response regulators are phosphorylated *in vitro* by small molecules that function as phospho-donors. One of these molecules is acetyl phosphate which is the major secondary source of phosphoryl groups. Assuming that the chemotaxis system of *H. pylori* is similar to the chemotaxis system of *S. meliloti*, *in vitro* phosphorylation assays were performed to analyze phosphorylation of response regulators by acetyl phosphate and a possible retrophosphorylation of *H. pylori* CheA by the response regulators.

2.1.7.1. Phosphorylation of CheY1, CheY2, and CheV1-CheV3 by acetyl phosphate

To determine the optimal conditions for the *in vitro* phosphorylation of the *H. pylori* chemotaxis response regulators CheY1, CheY2, CheV1, CheV2, and CheV3 different amounts of proteins were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) for different times of incubation (1, 5, 10, and 30 min) and at different temperatures (on ice, RT, and 30°C) in independent reactions for each protein. The reactions, which were carried out in a final volume of 25 µl, were stopped with sample buffer containing 50 mM of Na_2EDTA and electrophoresed on a 15% SDS polyacrylamide gel. Detection of radiolabelled bands was performer using a Typhoon 9200 Variable Mode Imager (data not shown). Phosphorylation of CheY1, CheY2, and CheV2 was detected when the proteins were present in a concentration of 7 µM, 24 µM, and 20 µM, respectively, and when the incubation was performed for 10 min at RT (Fig.30). Interestingly, a labelled band of CheY1~P was not detected when the reaction was stopped with sample buffer without Na_2EDTA, while CheV2~P was detected when sample buffer without Na_2EDTA was used. As shown in Fig.30

CheV1 and CheV3 were not phosphorylated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) under the same conditions. However, due to the low concentrations of the proteins solutions obtained after the protein purification CheV1 and CheV3 were present in the reaction mixture only in a concentration of 4 µM and 6 µM, respectively.

Interestingly, when 7 µM of *C. jejuni* CheY1 was phosphorylated under the same conditions as described above, a strong signal of CheY1~P could be detected demonstrating that CheY1cj behaved similarly as *H. pylori* CheY1 (Fig.32 lane 1).

As a control CheY1-D53N was incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) for 10 min at RT. As expected CheY1-D53N~P was not detected (data not shown).

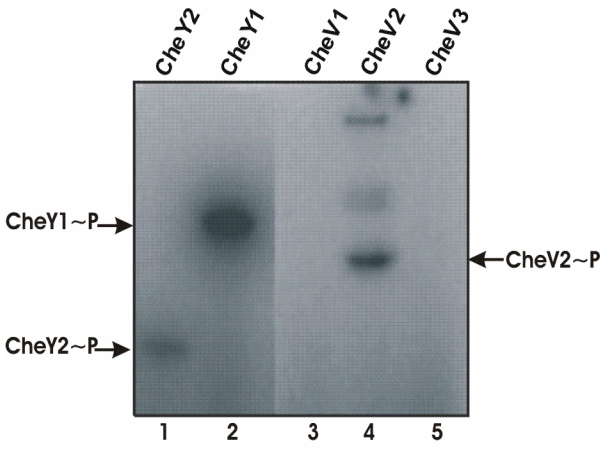

Fig.30 Phosphorylation of CheY1, CheY2 and CheV proteins by acetyl phosphate. CheY2 (24 µM, lane 1), CheY1 (7 µM, lane 2), CheV1 (4 µM, lane 3), CheV2 (20 µM, lane 4), and CheV3 (6 µM, lane 5), respectively, were incubated in the presence of acetyl 5 µCi [^{32}P] phosphate for 10 min, and samples were analysed in a SDS 15% polyacrylamide gel.

2.1.7.2. Dephosphorylation rates of CheY1~P and CheY2~P

To determine the dephosphorylation rates of CheY1~P and CheY2~P chase experiments were performed. 7µM CheY1 was incubated in the presence of acetyl [^{32}P] phosphate (80 Ci/mmol) in a reaction with a final volume of 200 µl. After 10 min of incubation at RT a sample (20 µl) was taken and then the reaction was chased by adding unlabelled acetyl phosphate to a final concentration of 10 mM. Then samples (20 µl) were removed at defined time points (15, 30, 45, 60 sec, 2.5, 5, 10, 15, and 30 min) and electrophoresed on a 15% SDS polyacrylamide gel. Quantification of signals was performed as described above. The intensity of the signal of the sample taken before the chase with unlabelled acetyl phosphate was arbitrarily set as 100% (t_0) and the signal intensities of the other bands in the gel were calculated with respect to the sample taken at t_0. The experiment was performed more than three times and each data point in the graphic shown in Fig.31 represents the average of three independent experiments. The standard deviation was calculated using program Microsoft

Excel. The half-life of CheY1~P was calculated using computer generated least square fits of the data point in the curve (Fig.31). As shown in Fig.31 dephosphorylation of CheY1~P after the addition of the unlabelled acetyl phosphate was fast and the half-life of CheY1~P was determined to be 20 sec. As described for CheY1, a time course experiment analysing the dephosphorylation rate of CheY2~P was performed. For this purpose 50 µM of CheY2 was incubated in the presence of acetyl [^{32}P] phosphate (80 Ci/mmol) in a reaction with a final volume of 200 µl under the conditions mentioned above. The experiment was performed more than three times and each data point in the graphic shown in Fig.31 represents the average of three independent experiments. In contrast to CheY1~P, CheY2~P proved to be very stable under these conditions. After 30 min of chase 55% of the signal intensity of CheY2~P present at t_0 could still be detected (Fig.31).

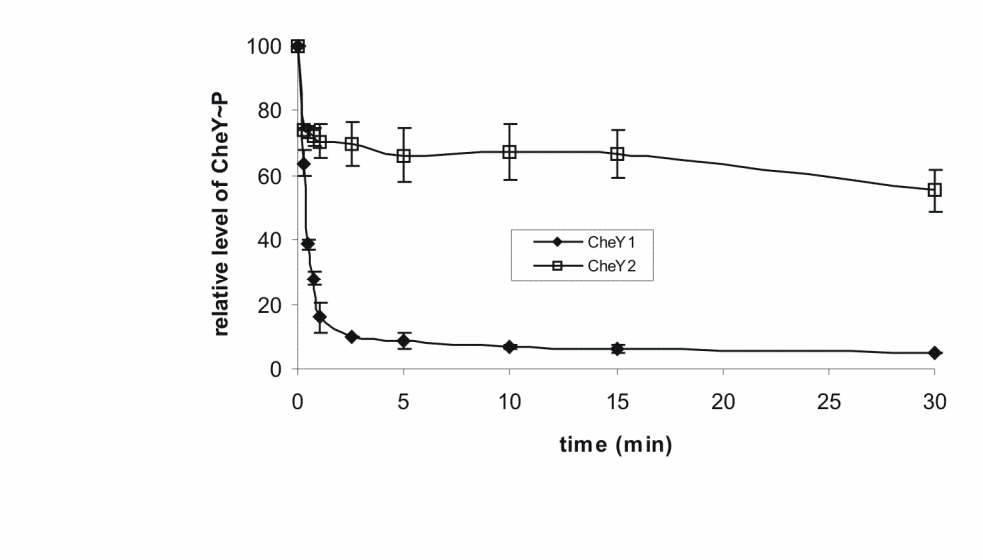

Fig.31 Time course of the dephosphorylation of CheY1~P and CheY2~P. 7 µM CheY1 and 50 µM CheY2 were incubated in the presence of 5 µCi [^{32}P] acetyl phosphate for 10 min. Then the reactions were chased by the addition of unlabelled acetyl phosphate to a final concentration of 10 mM. At indicated time points the reactions were stopped and the samples were analysed as described above.

2.1.7.3. Phosphorylation of CheY1 and CheY2 by acetyl phosphate in the presence of CheA´

To investigate the possibility of reverse phosphorylation from CheY1~P or CheY2~P to the *H. pylori* chemotaxis histidine kinase *in vitro* phosphorylation reactions were carried out. 7µM CheY1 were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) and in the presence or absence of 3.5 µM CheA´ or 8 µM CheAY2 for 10 min at RT. After the incubation the reactions were stopped by the addition of sample buffer and were electrophoresed on a 15% SDS polyacrylamide gel. Dephosphorylation of CheY1~P was observed in the presence of both histidine kinases CheAY2 and CheA´ (Fig.32 A and data not shown). These results suggested that the phosphate group of *H. pylori* CheY1~P can be

transferred to the histidine kinase. Moreover, as shown in Fig.32 A lane 2 a radiolabelled band of CheAY2~P was detected in the presence of CheY1~P. However, in the described experiments phosphorylated CheA´ could not be detected directly. As control 13 µM of CheAY2 was incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) (Fig.32 A lane 5) and no phosphorylation of CheAY2 could be detected under these conditions.

When 50 µM of CheY2 were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) and in the presence of 3.5 µM CheA´ or 8 µM CheAY2 for 10 min at RT, no dephosphorylation of CheY2~P was observed as compared to the control reactions without the histidine kinase proteins and no phosphorylation of CheA´ or CheAY2 could be detected. (data not shown and Fig.32 A).

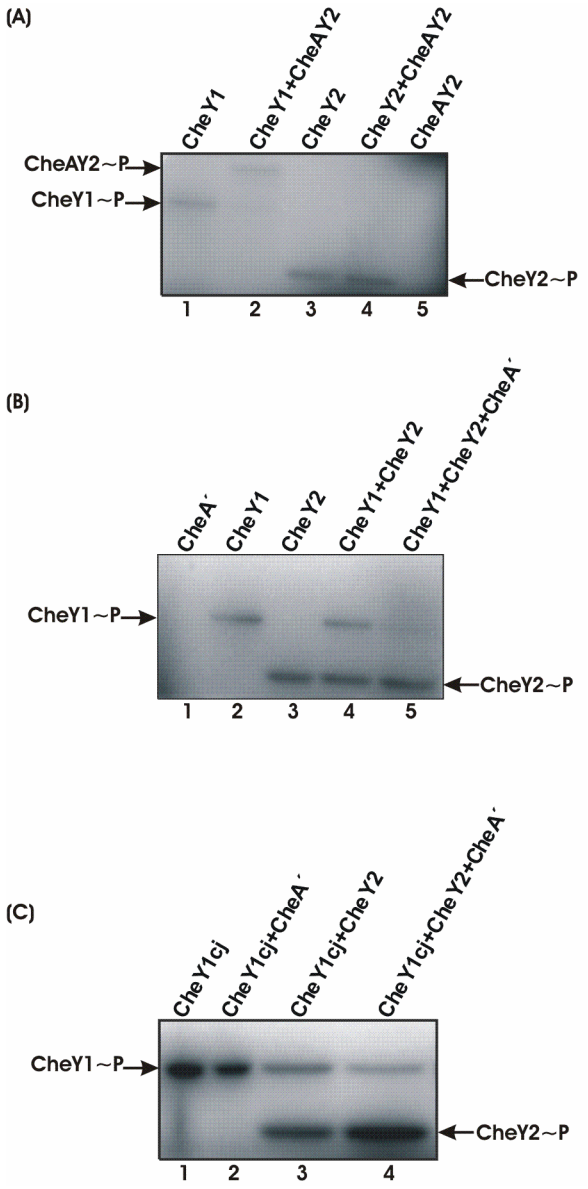

Fig.32 Phosphorylation of CheY1 and CheY2 by acetyl phosphate in the presence of the histidine kinases CheAY2 and CheA´, respectively. (A) Retrophosphorylation of CheAY2 by CheY1~P. 7 µM CheY1 or 50 µM CheY2 were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate in the absence (lane 1, 3) or presence of 8 µM CheAY2 (lane 2, 4), respectively. As a control 13 µM CheAY2 was incubated with acetyl [^{32}P] phosphate

(lane 5). (B) Phosphotrasnfer from CheY1~P to CheY2 via CheA´. 7 µM CheY1 (lane 2), 50 µM CheY2 (lane 3), and a mixture of either 7 µM CheY1 and 50 µM CheY2 (lane 4) or 7 µM CheY1, 50 µM CheY2 and 3.5 µM CheA´ (lane 5) were incubated with 5 µCi acetyl [^{32}P] phosphate. As additional control CheA´ was also incubated with 5 µCi acetyl [^{32}P] phosphate (lane 1). (C) Phosphotransfer from CheY1~P of *C. jejuni* to CheY2 via CheA´. 7 µM CheY1cj (lane 1) was incubated with acetyl [^{32}P] phosphate in the presence of 3.5 µM CheA´ (lane 2), 50 µM CheY2 (lane 3), and both 50 µM CheY2 and 3.5 µM CheA´ (lane 4), respectively, for 10 min. The reactions were stopped by the addition of sample buffer containing 50 mM Na_2EDTA and the reaction mixtures were analysed by electrophoresis on a SDS-15% polyacrylamide gel.

From the observation that a radiolabelled band of CheAY2~P was detected only in the presence of CheY1 but not in the presence of CheY2, it can be concluded that retrotransfer of the phosphate group to the histidine kinase occurs efficiently in case of CheY1~P but not in case of CheY2~P. In a similar reaction 50 µM of CheY2 and 7 µM of CheY1 were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) and in the presence of 3.5 µM CheA´ or 8 µM CheAY2 for 10 min at RT. Interestingly, an enhanced phosphorylation signal of CheY2~P was observed in the presence of both proteins CheY1 and CheA´, as well as in the presence of CheY1 and CheAY2 (Fig.32 A and B). As a control both response regulators CheY1 (7 µM) and CheY2 (50 µM) were incubated together in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) under the same conditions, but in the absence of histidine kinase protein dephosphorylation of neither CheY1 nor CheY2 was observed.
Furthermore, 7 µM of CheY1 from *C. jejuni* was incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) in the presence or absence of 3.5 µM CheA´ for 10 min at RT. Similar to the *H. pylori* CheY1 CheY1cj was phosphorylated by acetyl phosphate and dephosphorylation of CheY1cj~P was observed in the presence of the *H. pylori* histidine kinase CheA´. Moreover 7 µM of CheY1 from *C. jejuni* was incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol) and both 3.5 µM CheA´ and 50 µM CheY2. As observed with CheY1 from *H. pylori*, a dephosphorylation of CheY1cj~P and an enhancement in the signal of CheY2~P was detected in this reaction (Fig.32 C). As control both response regulators CheY1cj (7 µM) and CheY2 (50 µM) were incubated together in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol). As expected, dephosphorylation of neither CheY1cj nor CheY2 was observed (Fig.32 C lane 3). Again as a control CheAY2 and CheA´ were incubated in the presence of 5 µCi acetyl [^{32}P] phosphate (80 Ci/mmol). In these reactions no radiolabelled bands were detected (Fig.32 A lane 5 and B lane 1).

A summary of the model of the phosphotransfer reactions between the chemotaxis proteins of *H. pylori* as deduced from the experiments reported in this work is shown in Fig.33.

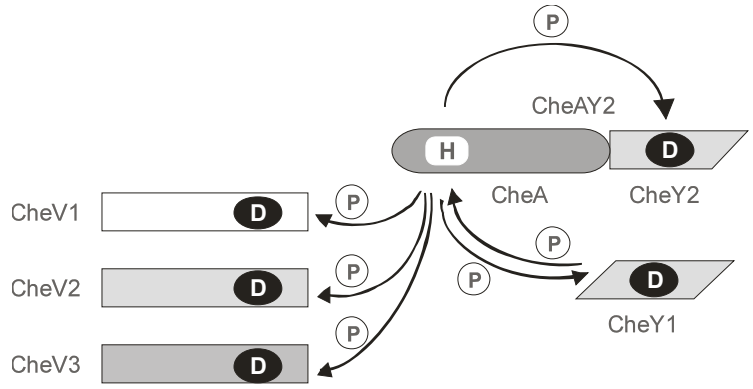

Fig.33 Scheme of the phosphotransfer reactions between the CheA histidine kinase and the chemotaxis response regulator proteins CheY1, CheY2, and CheV1-CheV3. Arrows indicate the direction of phosphorylation.

2.2. Construction of *H. pylori* G27/cheAY2::km and G27/cheA-Y2-cm

To determine whether normal chemotactic signalling can occur *in vivo* when the histidine kinase domain and the CheY-like domain of CheAY2 are expressed as two separate proteins the mutant *H. pylori* G27/cheA-Y2cm was constructed. In this mutant ORF *hp392*/*cheAY2* was substituted by two separates genes, which encode for the CheA´ histidne kinase domain and for the CheY-like domain of the CheAY2 protein, respectively.

First the gene *che*AY2 was substituted by a kanamycin cassette in the motile *H. pylori* G27 wild-type strain by homologous recombination using suicide plasmid pSL-*cheAY2*::km (Fig.34). To construct suicide plasmid pSL-*cheAY2*::km fragments of 665 bp encoding ORF *hp390* and of 689 bp encoding amino acids (aa) 92-312 of ORF *hp393* (*cheV3*) were amplified with primer pairs 390-1/390-2, and cheVA/cheVB, and the fragments were digested with restriction enzymes *Xba*I-*Sac*I, and *Eco*RI-*Nco*I, respectively. Chromosomal DNA of *H. pylori* 26695 was used as the template DNA in the PCR reactions. First the PCR fragment of 665 bp was ligated into pSL1180, which was digested with restriction enzymes *Xba*I-*Sac*I resulting in pSL-*hp390*. After the PCR control with primer pair 390-1/390-2, and sequencing with primers M13universal and M13reverse, pSL-*hp390* was digested with *Eco*RI-*Nco*I and the second fragment of 689 bp was inserted yielding pSL-*hp390cheV*. This plasmid was controlled by PCR with primer pairs 390-1/390-2, cheVA/cheVB, and 390-1/cheVB and sequencing with the primers M13universal and M13reverse. Subsequently, a kanamycin resistance cassette from *C. coli* (Labigne-Roussel *et al.*, 1988) was inserted into pSL-*hp390cheV* between the two fragments yielding pSL-*cheAY2*::km. Plasmid pSL-*cheAY2*::km was controlled by PCR with the primers combinations listed in Tab.8

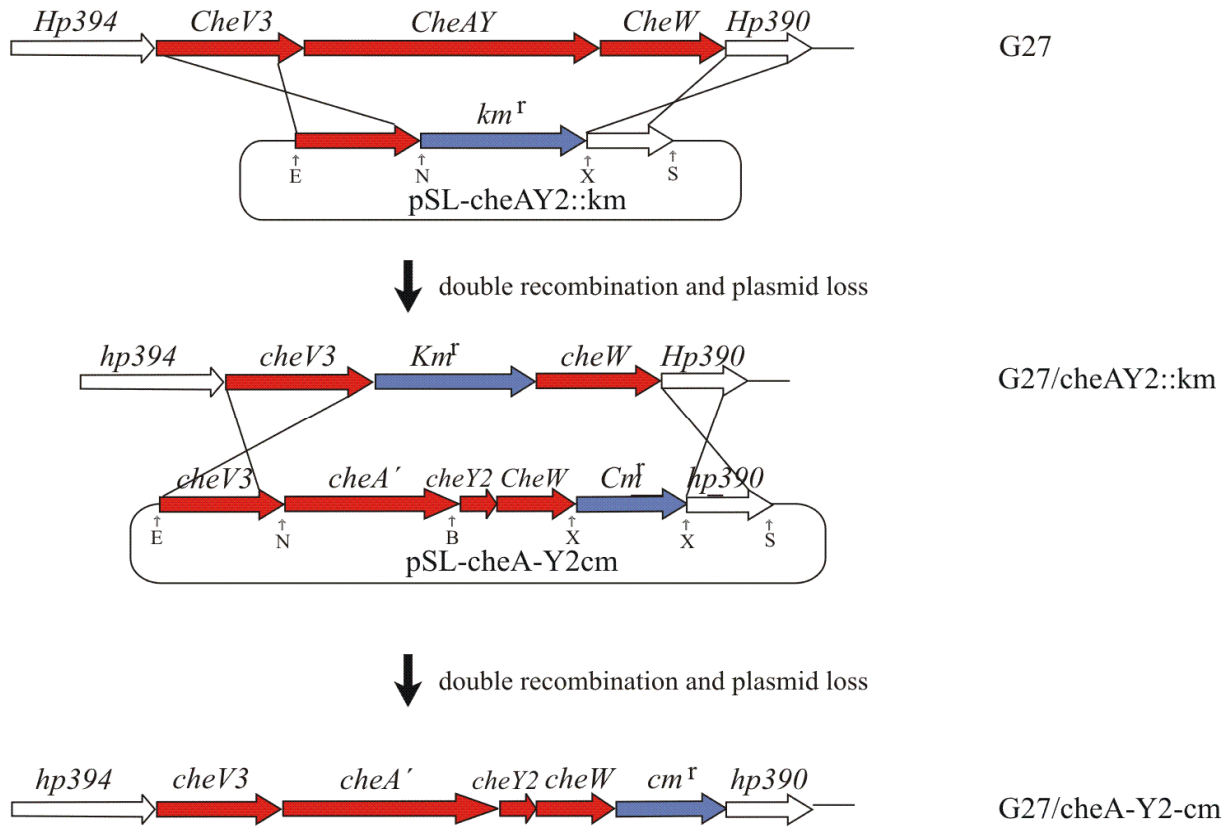

Fig.34 Strategy for the construction of *H. pylori* strains G27/cheAY2::km and G27/cheA-Y2-cm by allelic exchange mutagenesis. Non-coding DNA is represented as a black bar, ORFs are indicated as arrows. Red and white arrows represent genes encoding chemotaxis proteins and adjacent ORFs in the *H. pylori* genome, respectively. Antibiotic resistance genes are illustrated as blue arrows. *cm*^{r}: chloramphenicol resistance gene; *km*^{r}: kanamycin resistance gene; E: *Eco*RI; B: *Bam*HI; S: *Sac*I N: *Nco*I; X: *Xba*I. (See text for details).

The *H. pylori* G27 wild-type strain was transformed by natural transformation with the plasmid pSL-*cheAY2*::km in order to replace the ORF *hp392* encoding the bifunctional protein CheAY2 by the kanamycin resistance cassette generating *H. pylori* G27/*che*AY2::km. Deletion mutants were controlled by PCR with the primer pairs listed in Tab.8 and in all cases the expected PCR products were obtained.

Tab.8 Primer pairs used for the PCR characterization of plasmid pSL-*cheAY2*::km and *H. pylori* strain G27/cheAY2::km. The length of the expected PCR fragments, which were obtained in the respective reactions is indicated.

Primer pair	Fragment size	G27 wild-type
cheVA and cheVB	689 bp	689 bp
390-1 and 390-2	665 bp	665 bp
kanR5´ and kanR3´	1400 bp	_____
CheVA and 390-2	2754 bp	4368 bp
CheVA and kanR3´	2089 bp	_____
390-2 and kan1	1390 bp	_____

Moreover, slot blot analysis was performed with chromosomal DNA of G27/*che*AY2::km and G27. The DNA samples of both *H. pylori* strains were hybridized with two different DNA probes. The first probe of 687 bp contained part of the sequence of the histidine kinase domain and the second probe of 967 bp contained the sequence of *cheW* and *cheY2* encoding the CheY-like domain of the histidine kinase CheAY2. The two DNA fragments were amplified by PCR with primer pair cheA1/cheA2, and cheARW1/cheARW2 using chromosomal DNA of *H. pylori* 26695 as template. A hybridization signal was detected for both DNA probes only in the case of the chromosomal DNA of the wild-type strain G27 confirming the absence of the *cheAY2* gene in the *H. pylori* G27/*che*AY2::km, which possesses resistance to kanamycin (Fig. 35 A). To analyse the motility of the mutant, G27/*che*AY2::km was stabbed into semi-solid agar plates and the plates were incubated for 5 days at 37°C. The G27 wild-type was analysed in parallel as a positive control. As expected G27/*che*AY2::km proved to be non-motile (Fig.35 B).

To introduce two separate genes encoding the histidine kinase domain CheA´ and the receiver domain CheY2, respectively, *H. pylori* G27/*che*AY2::km was transformed with plasmid pSL-*cheA´-Y2*cm (Fig.34). To construct plasmid pSL-*cheA´-Y2*cm fragments of 2047 bp encoding aa 1-676 of the histidine kinase CheAY2 and of 967 bp encoding aa 677-881 of CheAY2 and CheW were amplified with primer pairs cheAI/cheAII and cheARW-1/cheARW-2. Primer cheARW-1 contains a Shine-Dalgarno sequence (AGGA) in the appropriate distance to the synthetic start codon of ORF *cheY2* to ensure protein synthesis. Chromosomal DNA of *H. pylori* 26695 was used as the template DNA in the PCR reactions. The DNA fragments were digested with restriction enzymes *Bam*HI-*Nco*I, and *Bam*HI-*Xba*I, respectively. First the PCR fragment of 967 bp was ligated into pSL-*hp390cheV*, which was digested with *Bam*HI-*Xba*I resulting in pSL-*hp390cheVW*. After PCR control with primer pairs cheARW-1/cheARW-2, cheVA/cheARW2, and 390-2/cheARW1, which yielded PCR products of the expected sizes (967 bp, 1656 bp, and 1632 bp) sequencing using primers cheARW1 and cheARW2 was performed. Then plasmid pSL-*hp390cheVW* was digested with *Bam*HI-*Nco*I and the second fragment of 2047 bp was inserted yielding pSL-*hp390cheVWA*. This plasmid was controlled by PCR with primer pairs cheAI/cheAII, cheARW1/cheARW2, 390-1/390-2, and cheVA/cheVB, and was sequenced using primers cheAI, cheAII, cheAseq1, cheAseq2, cheAseq3, and cheAseq4. Subsequently, a chloramphenicol resistance cassette of *C. coli* (Wang & Taylor, 1990) was inserted into pSL-hp390*cheVWA* between the *cheW* gene and the fragment derived from ORF *hp390* yielding pSL-*cheA´-Y2*cm (Fig.34). Plasmid pSL-*cheA´-Y2*cm was controlled by PCR with the primer pairs listed in Tab.9 and was sequenced

with the primers M13universal, M13reverse, cheVB, CheAI, cheAII, cheARW1, cheARW2, 390-1, 390-2, cheAseq1, cheAseq2, cheAseq3, cheAseq4, cheAseq5, and cheAseq6. By this way the correct insertion of the different fragments and proper PCR amplification of the cloned DNA-fragments were ensured.

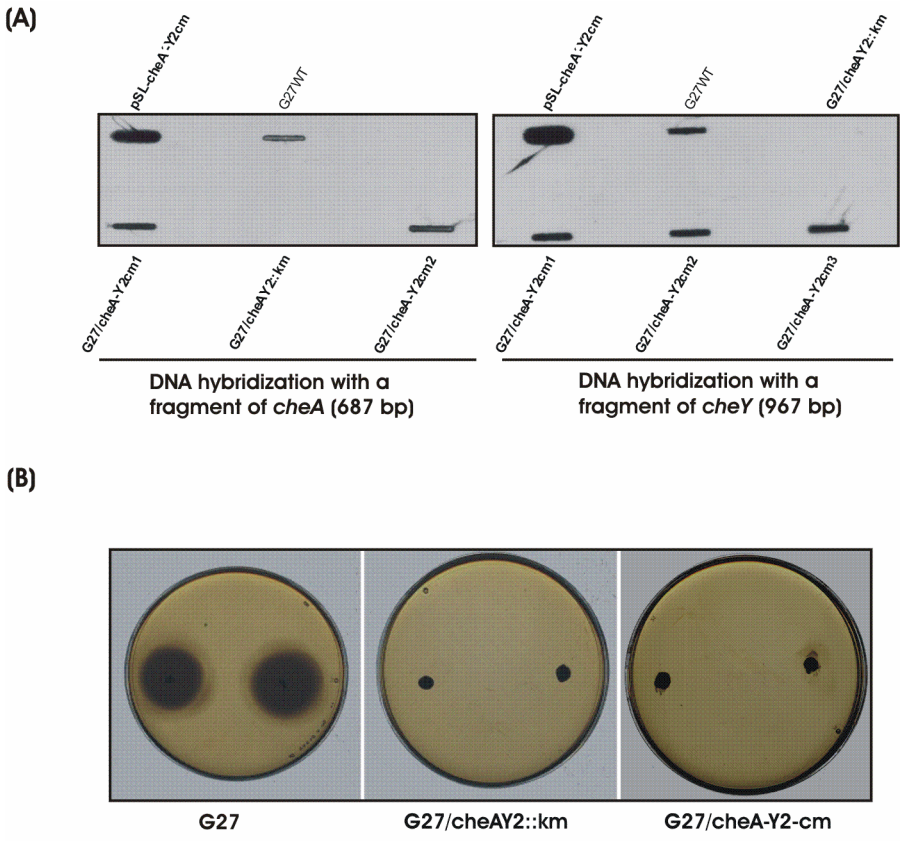

Fig.35 Characterization of the mutants G27/cheA-Y2-cm. (A) Southern blot performed with chromosomal DNA from the *H. pylori* wild-type strain G27, G27/cheAY2::km, and G27/cheA-Y2-cm. Template DNA was hybridized with two different DNA probes. The first probe of 687 bp contained part of the sequence of the histidine kinase domain and the second probe of 967 bp contained part of the sequence enconding the CheY-like domain of CheAY2. (B) Analysis of the motility of *H. pylori* strains G27 wild-type, G27/cheAY::km, and G27/cheA-Y2-cm by testing the swarming ability on semi-solid agar. The strains were incubated for 5 days at 37°C under microaerophilic conditions.

H. pylori G27/*che*AY2::km was transformed by natural transformation with the plasmid pSL-*cheA´-Y2*cm to yield strain G27/cheA-Y2-cm. In this strain the kanamycin resistance cassette present in G27/*che*AY2::km was substituted by the two independent genes encoding the histidine kinase domain CheA´ and the receiver domain CheY2, respectively. Mutants were controlled by PCR with the primer pairs listed in Tab.9 and in all cases the expected PCR products were obtained.

Tab.9 Primer pairs used for the PCR characterization of plasmid pSL-*cheA´-Y2*-cm and *H. pylori* strain G27/cheA-Y2cm. The length of the expected PCR fragments, which were obtained in the respective reactions, is indicated.

Primer pair	Fragment size	G27 wild-type
390-1 and 390-2	689 bp	689 bp
cheVA and cheVB	665 bp	665 bp
cheARW1 and cheARW2	967 bp	967 bp
cheAI and cheAII	2047 bp	2047 bp
390-2 and cm5´	1470 bp	_____
390-2 and cm3´	_____	_____
cheARW2 and cheAseqX	1661 bp	1661 bp
cheVA and cheAseq X	1099 bp	1099 bp

Moreover, slot blot analysis was performed with chromosomal DNA of the mutants G27/cheA-Y2-cm, G27/*che*AY2::km, and the G27 wild-type. The DNA samples of the three *H. pylori* strains were hybridized with the DNA probes described above. A hybridization signal was detected for both DNA probes in the case of the chromosomal DNA of strains G27/cheA-Y2-cm and wild-type G27 confirming the presence of the genes encoding CheA´ and CheY2 in the *H. pylori* strain G27/cheA-Y2-cm (Fig. 35 A).

To analyze the motility of the mutant, G27/cheA-Y2cm was stabbed into semi-solid agar plates and the plates were incubated for 5 days at 37°C. Strains G27 and G27/*che*AY2::km were analysed in parallel as a control. Mutant G27/cheA-Y2-cm proved to be non-motile in this assay (Fig.35 B).

F DISCUSSION

In various studies it has been established that motility and chemotaxis are essential virulence traits in *H. pylori*. The flagellar regulatory network and the complex chemotaxis system in *H. pylori* are fundamentally different from other bacteria, despite many similarities. In this study the putative regulatory function of protein HP137 in the biosynthesis of the flagella and the mechanism of two component signalling in the chemotactic response of *H. pylori* were analyzed.

1. Putative function of HP137 in the flagellar biosynthesis of *H. pylori*

As mentioned in the introduction section the expression of flagella in *H. pylori* is controlled by a complex regulatory cascade involving the two component system FlgR-HP244, the sigma factors σ^{80}, σ^{54}, and σ^{28} and the anti-σ^{28} factor FlgM. Thus far, the input signal for histidine kinase HP244, which activates the transcriptional regulator FlgR, is not known. Based on a yeast two-hybrid screen a highly significant protein-protein interaction between the *H. pylori* protein HP137 and both the histidine kinase HP244 and the flagellar hook protein HP908 (FlgE´) has been reported recently (Rain *et al*., 2001). So far, no function could be assigned to HP137. Interestingly, the interaction between HP137 and histidine kinase HP244 was observed in the characteristic block N sequence motif of the C-terminal ATP-binding kinase domain suggesting an inhibitory effect of HP137 binding due to interference with ATP binding. In this work the following hypothesis was investigated: the transcriptional regulator FlgR activates σ^{54}-dependent transcription of class II genes and some intermediate genes encoding σ^{28} and its antagonist (FlgM). Both σ^{28} and the anti sigma factor FlgM control the transcription of the late class III genes. It was assumed that in the presence of an excess of cytoplasmic FlgE´ which might accumulate in the cell after complete assembly of the flagellar basal body and hook structure, FlgE´ could interact with the HP137 protein and then interaction of the protein complex with the histidine kinase HP244 could result in an inhibitory effect on the autophosphorylation of the histidine kinase HP244 and a feedback control. Therefore, transcription of the class 2 flagellar genes would be stopped when FlgE´ is in excess.

It is well known that the activity of two-component histidine kinases can be controlled by the interaction with regulatory proteins. One such example is the *E. coli* histidine kinase NtrB, which together with the cognate RR NtrC regulates nitrogen assimilation by controlling genes like *glnA*, which encodes the glutamine synthetase (GS) and other nitrogen-regulated genes (Kamberov *et al*., 1994; Pioszak *et al*., 2000). The kinase and phosphatase activities of NtrB

are regulated by the PII protein. In *E. coli* the PII protein regulates not only the activity of the histidine kinase NtrB but also the activity of the enzyme adenylyltransferase (ATase) involved in the covalent modification of the GS producing a non active adenylylated form of GS (Atkinson & Ninfa, 1999; van Heeswijk *et al*., 1996). The regulation of ATase and the nitrogen regulation of gene transcription by NtrBC utilize a common sensory and signal transduction system consisting of the uridylyltransferase/uridylyl-removing enzyme (UTase/UR) and the PII protein (Magasanik & Neidhardt, 1987; Rhee *et al*., 1985). Under nitrogen-limiting conditions PII is uridylylated and under conditions of nitrogen excess PII is unmodified (Magasanik & Neidhardt, 1987; Rhee *et al*., 1985). PII and PII-UMP then communicate conditions of nitrogen sufficiency or limitation, respectively, to the PII receptors ATase and NtrB. Both PII and PII-UMP interact with the bifunctional ATase activating ATase adenylylation and deadenylylation activities, respectively (Reitzer & Magasanik, 1985; Rhee *et al*., 1985). In contrast, PII but not PII-UMP, interacts with NtrB (Atkinson *et al*., 1994). In the presence of PII-UMP, NtrC is phosphorylated by NtrB and transcription of the *glnA* is induced while in the presence of PII interaction of PII with NtrB activates the phosphatase activity of NtrB towards NtrC~P and consequently NtrC is converted to its inactive state. These regulatory mechanisms enable the cell to rapidly adjust GS activity in response to changes in the availability of the preferred nitrogen source, ammonia. When nitrogen is in excess, cell contain little GS and the enzyme is mostly adenylylated; under condition of nitrogen limitation, the intracellular concentration of GS is 7 to 10-fold higher and the enzyme is mostly unadenylylated (Magasanik & Neidhardt, 1987; Rhee *et al*., 1985; Rhee *et al*., 1989).

Similar mechanisms have been described in the phosphorelay signal transduction system which functions in the initiation of sporulation in *B. subtilis* (Wang *et al*., 1997). In this system the reponse regulator SpoOF receives a phosphate group from two different kinases, KinA and KinB. However, SpoOF is not a transcription factor but only an intermediate in the ultimate activation of the transcription factor SpoOA (Burbulys *et al*., 1991; Hoch, 1993). The regulation of the activity of one of the histidine kinases, KinA, is important for the initiation of transcription at the onset of sporulation. Its kinase activity is regulates by the protein KipI, which is an inhibitor of the autophosphorylation of the KinA by ATP. The KipI protein targets the carboxy-terminal catalytic domain of KinA where it interferes with the ATP-dependent reactions of the kinase (Wang *et al*., 1997).

In this work a potential role of HP137 in a feedback regulatory mechanism controlling the activity of histidine kinase HP244 in the flagellar regulation of *H. pylori* was investigated.

To obtain a first hint whether ORF *hp137* might be involved in the regulation of flagellar gene expression this ORF was substituted with a non-polar kanamycin resistance cassette in *H. pylori* G27. The resulting deletion mutant G27/hp137::km was non-motile on semi-solid agar plates (Fig.12). Furthermore, electron microscopy revealed that the mutant strain did not produce flagella (data not shown). Moreover, quantification of the transcription of *flaB*, *flgB*, *flgE*, and ORF *hp906* which are σ^{54}-dependent flagellar genes using primer extension experiments demonstrated that these genes showed enhanced transcription in the deletion mutant, while transcription of the σ^{28}-dependent flagellar gene *flaA* in the deletion mutant was unchanged as compared to the wild-type (Fig.13). These results suggested that deletion of ORF *hp137* causes an abnormal synthesis of the flagella proteins as a consequence of an unregulated activity of the histidine kinase with a continuous autophosphorylation of this protein and a high concentration of FlgR~P.

To prove that the non-motile phenotype of *H. pylori* G27/hp137::km was due to the deletion of ORF *hp137* complementation experiments were performed. As suggested by the analysis of the genome sequence of *H. pylori* 26695 (Tomb *et al.*, 1997) and as confirmed in this work (Fig.14) transcription of ORFs *hp139-hp136* is directed from a single promoter. The transcriptional start site of the operon *hp139-hp136* was identified at the position -38 with respect to the transcriptional start site of ORF *hp139* corresponding to a TATTAT -10 promoter element using primer extension analysis (Fig.14 B). To reintroduce *hp137* under control of its own promoter into G27/hp137::km the *cag*-locus was chosen (Fig.15 A). As described previously only *H. pylori* type I strains contain the *cag*-locus (Censini *et al.*, 1996), demonstrating that this locus is not necessary for the colonization and to survive *in vivo* and *in vitro*. Analysis of the complemented mutant G27/*cag*hp137-cm containing a fusion of the promoter region of the *hp139-hp136* operon to a copy of the *hp137* gene in the *cag*-locus turned out to be non-motile (data not shown). Furthermore, the analysis of the transcription of *flaB*, which is controlled by the NtrC-like response regulator FlgR, showed that there was no difference in the amount of *flaB* transcript (data not shown), as compared to the parent strain. This result suggested three explanations: (1) although *hp137* is transcribed under control of its natural promoter HP137 is not produced in an appropriate amount; (2) deletion of the *hp137* gene has a polar effect on the transcription of the *hp136* gene, which is the last gene of the *hp139-hp136* operon, and *hp136* might be required for flagellar synthesis; or (3) the insertion of any sequence in the *cag*-locus causes a non-motile phenotype. The last hypothesis was supported by the observation that a derivative of *H. pylori* G27 obtained by transformation

with the same plasmid used for the complementation of G27/hp137::km was non-motile (data not shown). A similar observation was reported by Figura *et al*. who showed that the disruption of the *cag*A gene strongly reduced the expression of both flagellin A and flagellin B (FlaA and FlaB) causing a non-motile phenotype (Figura *et al.*, 2004). However, Marchetti *et al*., showed that *H. pylori* mutants with the insertion of a kanamycin resistance cassette within the *cagA* gene are able to colonize mice with the same efficiency as the wild-type strain (Marchetti & Rappuoli, 2002). It is widely accepted that flagellar based motility is an essential colonization factor, and, therefore these obviously contradictory results remain unexplained.

An alternative complementation strategy was used based on the reintroduction of the gene *hp137* into the operon *hp139-hp136* (Fig.15 B). Similar to the mutant G27/*cag*hp137-cm strain G27/hp137loc-cm showed not only the same level of *flaB* transcription as the parent strain G27/hp137::km (Fig.17), but was also non-motile on semi-solid agar plates (data not shown). Using RT-PCR it was confirmed that *hp136* is transcribed in *H. pylori* G27/hp137::km, G27/*cag*hp137-cm, and G27/hp137loc-cm and that *hp137* is also transcribed in the complemented strains (data not shown and Fig.16 B). As a control *H. pylori* G27 was transformed with the plasmid reintegrating *hp137* into the *hp139-hp136* operon. As expected this strain was motile on semi-solid agar plates (data not shown). From the observation that both complementation strategies did not result in a motile phenotype it can be concluded that deletion of *hp137* is not responsible for the loss of motility in strain G27/hp137::km. Therefore it seems likely that substitution of this ORF for an unknown reason favours the occurrence of an unknown secondary site mutation affecting motility.

As mentioned above interaction between HP137 and histidine kinase HP244 was observed using a yeast two-hybrid screen (Rain *et al*., 2001). This interaction was mapped to the characteristic block N sequence motif of the C-terminal ATP-binding kinase domain (Parkinson & Kofoid, 1992). *In vitro* phosphorylation assays were performed to determine the effect of the putative protein-protein interaction on histidine kinase activity. Only a slight decrease in the autophosphorylation of histidine kinase HP244 was detected when HP137 was present in the reaction mixture in a fourfold excess with respect to HP244 (Fig.20 A), while no effect was seen when both proteins were present in equimolar concentrations. This result indicated a slight inhibitory interaction between the proteins and is consistent with the observation that under native conditions a slight difference in the electrophoretic mobility of HP244 was detected when the protein was combined with HP137 in the same molar ratio (Fig.19 lane 3). The presence of HP137 had no effect on the autophosphorylation efficiency

of HP165 indicating that the protein interaction between HP244 and HP137 is specific. Phosphotransfer from HP244~P to the response regulator FlgR was not affected in the presence of protein HP137 (Fig.20 A). The minor effect of HP137 on the autophosphorylation efficiency of HP244 was not affected in the presence of FlgE´, which as well as HK HP244 showed an interaction in the yeast two-hybrid screen with HP137 (Fig.20 A) arguing against a role of HP137 and FlgE´ in feedback inhibition of histidine kinase activity. Due to the small effect observed the *in vitro* data argue against a regulatory role of an interaction between HP137 and HP244 for histidine kinase activity, unless the recombinant HP137 lost its biological function as a consequence of improper refolding during the purification process. Furthermore, the observation of Niehus *et al*. that the putative operon containing ORF *hp906* and the flagellar genes *flgD* and *flgE'*, possesses not only a σ^{54}-dependent promoter, but also an additional σ^{80}-dependent promoter (Niehus *et al.*, 2002) argues against a role of FlgE´ in a feedback regulation of the activity of HP244.

As in *H. pylori*, in the other species, in which part of the flagellar biosynthesis machinery is under the control of a two-component system and σ^{54}, like *V. cholera*, *C. crescentus*, and *C. jejuni*, the signals that triggers the autophosphorylation of the sensor proteins of these systems have not yet been identified (Beier & Frank, 2000; Jacobs *et al.*, 2003; Wosten *et al.*, 2004). Given that *C. jejuni* is a close relative of *H. pylori* and orthologs of all regulatory and structural components of the flagellar apparatus are present in both organisms it is likely that both organisms posses a similar mechanism of flagellar regulation. Therefore, to investigate the putative role of HP137, a deletion mutant of the *C. jejuni* ortholog of *hp137*, *cj73c*, was constructed using *C. jejuni* 4344 as the parent strain yielding *C. jejuni* 4344/cj73c::km. This mutant presented a swarming behaviour on motility agar plates similar to the *C. jejuni* wild-type. This result proved that ORF *cj73c* does not posses a function in the flagellar regulation in *C. jejuni* and also argues against a possible role of *hp137* in flagellar regulation in *H. pylori*.

The original observation of an interaction between HP137 and both proteins HP244 and FlgE´ using the yeast two-hybrid system may therefore not be of relevance *in vivo*. Since HP137 is part of an operon encoding three other proteins possibly involved in the catalysis of redox reactions, HP137 may participate in the function of these enzymes. In fact, HP137 belongs to a family of highly related proteins of unknown function, all containing the domain DUF162 (InterPro database). Conforming to the organization of *hp137* in an operon with genes encoding enzymes involved in redox reactions, the HP137 ortholog MTH140 of *Methanobacterium thermoautotrophicum* contains similarities to ferredoxin-like proteins.

2. Two-component signalling in *H. pylori* chemotaxis

The chemotaxis system of *H. pylori* contains multiple signal transduction proteins which interact in a complex way. From the genome sequence it can be predicted that chemotactic signalling in *H. pylori* differs from the enterobacterial paradigm (Alm *et al.*, 1999; Tomb *et al.*, 1997) and might be similar to other organisms that lack the CheZ phosphatase and contain more than one CheY protein like *S. meliloti*, *R. sphaeroides*, *Caulobacter crescentus*, and *Agrobacterium tumefaciens* (Armitage & Schmitt, 1997). The domain architecture of the chemotaxis histidine kinase CheA is different from most HKs. *E. coli* CheA, contains five domains, designed P1-P5. The N-terminal P1 domain contains the autophosphorylation site (histidine 48). Adjacent to P1 is the P2 domain that contains a binding site for the proteins CheY and CheB. P3 is the dimerization domain, and P4 is the kinase domain. The P5 domain mediates the interactions of CheA with the chemotaxis receptors and a coupling protein CheW (Bilwes *et al.*, 1999; Hess *et al.*, 1988). The *H. pylori* chemotaxis histidine kinase CheAY2 is a bifunctional protein containing not only the five domains present in *E. coli* CheA, but also a C-terminal CheY-like receiver domain. Such bifunctional CheAY proteins are also present in the other members of the ε-proteobacteria (*H. hepaticus, C. jejuni, Wolinella succinogenes*) whose genomes have been sequenced so far (Baar *et al.*, 2003; Parkhill *et al.*, 2000; Suerbaum *et al.*, 2003), as well as in the Gram-negative soil bacterium *Myxococcus xanthus* (Acuna *et al.*, 1995). In addition to the bifunctional histidine kinase CheAY2 *H. pylori* contains other nine proteins (CheW, CheY1, CheV1-CheV3, and four MCPs) that are homologous to chemotaxis proteins with a characterized function in the chemotaxis systems of other organisms such as *E. coli*, *B. subtilis*, and *S. meliloti*. In this work the *in vitro* phosphotransfer between five key components (CheAY2, CheY1, CheV1, CheV2, and CheV3) of the *H. pylori* chemotaxis signal transduction pathway was analyzed. The full length bifunctional histidine kinase CheAY2 was expressed and purified, as well as two derivatives of CheAY2, one lacking the C-terminal CheY2 domain, the other comprising the separate CheY2 receiver domain (Fig.23 A and B lane 2, 3, 5).

Using *in vitro* phosphorylation assays it was shown that both histidin kinases CheAY2 and CheA´ lacking the CheY-like domain (CheY2) act as ATP-dependent autokinases. Similar to the CheA proteins of *E. coli* and *S. meliloti* (Sourjik & Schmitt, 1998; Tawa & Stewart, 1994) *H. pylori* CheA´ shows a kinetics of phosphorylation represented by an exponential time course reaching the maximum level of phosphorylation after 15 min and the signal remaining stable over 60 min. In contrast, the kinetics of phosphorylation of CheAY2 is characterized by

an exponential increase in signal intensity up to 8 min of incubation, and after this time the hydrolysis of CheAY2~P predominates in the reaction (Fig.24 A). Furthermore, in a chase experiment in the presence of unlabelled ATP CheAY2~P has a half-life of 3 min, while after 15 min half of the initial amount of CheA´~P was still detectable and about 35 % of the initial amount of CheA´~P were detected after 90 min of incubation (Fig.24 B and C). Therefore, it was demonstrated that the presence of the CheY2-like receiver domain influences the stability of the phosphorylated P1 domain of the CheA part of the bifunctional protein. This modulation of the half-life of the phosphorylated P1 domain is probably due to an intramolecular phosphoryl group transfer from the histidine residue in the P1 domain to the phosphate-accepting aspartic acid residue in the C-terminal CheY2 domain and the subsequent rapid hydrolysis of CheY2~P. In accordance with this conclusion, rapid dephosphorylation of the P1 domain was also observed when the separated CheY2 domain was added to the phosphorylated CheA´ histidine kinase (Fig.26). A similar effect of the receiver domain on the autophosphorylation kinetics of an unorthodox histidine kinase has been reported for the redox sensor ArcB of *E. coli* which is composed of a kinase domain, a receiver module and a C-terminal Hpt-domain (Georgellis *et al.*, 1997). Since in chase experiments the phosphorylated CheY2 protein exhibited a higher stability in the absence of CheA´ than in its presence it is conceivable that the interaction with the CheA´ histidine kinase stimulates the hydrolysis of CheY2~P. This effect might be even more pronounced in the bifunctional CheAY2 protein.

As shown in Fig.22 all *H. pylori* CheY-like proteins possess the highly conserved residues of the *E. coli* CheY protein (Asp12, Asp13, Asp56, and Lys109) that form the active site for catalyzing the phosphotransfer reaction and, therefore, should be able to accept a phosphoryl group from a cognate histidine kinase (Sanders *et al.*, 1989b; Volz, 1993). Using *in vitro* phosphorylation assays it was determined that the *H. pylori* histidine kinases CheAY2 and CheA´ are dephosphorylated in the presence of both response regulators CheY1 and CheY2 (Fig.25 A). While CheY2~P was detected after 15 minutes of incubation with the kinases under multiple turnover conditions, CheY1 was not detectable under the same conditions. Nevertheless, using time course experiments the phosphorylation of CheY1 was demonstrated to occur, since after 5 and 10 seconds upon the addition of CheY1 to CheA´~P phosphorylated CheY1 could be detected (Fig.27 A and B). Therefore, it was confirmed that as in *S. meliloti* (Sourjik & Schmitt, 1998) CheA~P is dephosphorylated in the presence of both response regulators CheY1 and CheY2 and that this dephosphorylation is due to the transfer of the phosphate group to the response regulators. This last conclusion was further

substantiated by the observation that CheA´~P is not dephosphorylated in the presence of the protein CheY1-D53N, which contains a substitution of the aspartate 53, which corresponds to the phosphate accepting aspartic acid residue in *E. coli* CheY, by asparagine (Fig.25 C). On ice dephosphorylation of CheA´ occurred within 5 sec in the presence of CheY1, while in the presence of CheY2 after 10 sec 20% of the initial amount of CheA´~P were still detectable (Fig.27 B). This observation suggested that CheY1 exhibits a higher affinity to CheA´ than CheY2. For the intrinsic dephosphorylation of CheY1~P a half-life of 20 sec (Fig.31) was determined which is in the same range as the half-life of CheY~P from *E. coli* (Lukat *et al.*, 1991) but approximately twice as long as the half-life of the phosphorylated CheY1 and CheY2 proteins from *S. meliloti* (Sourjik & Schmitt, 1998). The half-life of CheY2~P in the absence of CheA´ could not be determined, because in chase experiments CheY2 presented a stable phosphorylation until 30 min of incubation. 55% of the initial signal intensity were detected at that time (Fig.31). These results suggested that differences in the kinetics of phosphorylation and dephosphorylation of CheY1 and CheY2 are the key of distinctive features of the two response regulators.

Dephosphorylation of CheA´ was observed not only in the presence of *H. pylori* CheY1 but also in the presence of its ortholog in *C. jejuni* (CheY1cj), which shows a similarity of 82 % with *H. pylori* CheY1. Furthermore, CheA´~P was able to transfer the phosphoryl group to the CheY protein of *E. coli*, which possesses a similarity of 49,6 % with *H. pylori* CheY1 (Fig.27 A).

Phosphorylation of CheY of *E. coli* by CheA´ was somewhat surprising because the P2 domain, which in *E. coli* has been demonstrated to be involved in the binding of CheY (Welch *et al.*, 1998) exhibits the lowest degree of conservation between the CheA orthologues of *H. pylori*, *C. jejuni* and *E. coli*. Nevertheless, it is known that a derivative of *E. coli* CheA lacking the P2 domain can phosphorylate the cognate CheY protein, however with a 25-fold slower rate of phosphorylation than the wild-type CheA (Stewart *et al.*, 2000). Furthermore, CheB, which shares only three of the 13 residues of *E. coli* CheY necessary for the interaction with the P2 domain of *E. coli* CheA, interacts efficiently with the chemotaxis histidine kinase (McEvoy *et al.*, 1998; Welch *et al.*, 1998). Therefore, McEvoy *et al*. concluded that there is no molecular “code” of amino acid residues that is used by CheA to recognize the response regulators (McEvoy *et al.*, 1998). Interestingly, as observed with CheY1 from *H. pylori*, phosphorylated *E. coli* CheY1 did not accumulate under our experimental conditions, while efficient phosphorylation of *E. coli* CheY by its cognate *E. coli* histidine kinase CheA under steady state conditions has been reported (Bourret *et al.*, 1990). The same result was obtained

with CheY1cj~P from *C. jejuni* (data not shown), and similar as in case of CheY1hp both CheYec~P and CheY1cj~P were detected only in a time course reaction of CheA´~P dephosphorylation at incubation times of 5 and 10 sec (Fig.27 A). These results together with the fact that the CheY2~P protein exhibited a higher stability in the absence of CheA´ than in its presence suggest that the CheA´ domain of the bifunctional protein CheAY2 might possess a function as phosphatase of CheY~P. Many HKs possess a phosphatase activity, enabling them to dephosphorylate their cognate RRs, for example EnvZ and NtrB in *E. coli* (Kamberov *et al.*, 1994; Zhu *et al.*, 2000). However, such a function has not been identified for a CheA chemotaxis histidine kinase so far. Another explanation for the fact that CheY1~P could not be detected might be efficient retro-transfer of the phosphate group from CheY~P to the histidine kinase (see below).

In vivo experiments previously indicated that interspecies interactions between chemotaxis proteins are possible. For example, it was shown that swarming in a *cheW* mutant of *E. coli* was partially restored by the complementation with *cheW1* and *cheW3* of *Rhodobacter sphaeroides*, and chemotaxis in wild-type *E. coli* cells was impaired by the overexpression of the *cheW* genes of *R. sphaeroides* (Hamblin *et al.*, 1997; Shah *et al.*, 2000). Furthermore, it was possible to partially complement *cheA*, *cheY*, and *cheZ* mutants of *E. coli* with the *cheA2* and the four *cheY* genes of *R. sphaeroides*, which is an organism containing multiple copies of the *che* genes but lacks a CheZ phosphatase (Shah *et al.*, 2000). These latter experiments in particular suggested phosphotransfer reactions between chemotaxis proteins originating from different bacterial genera. Therefore, it is likely that interactions between certain components of the chemotaxis systems of *H. pylori*, *C. jejuni* and *E. coli* may also occur *in vivo*. However, *H. pylori che*W was apparently unable to complement an *E. coli che*W mutant (Pittman *et al.*, 2001). Pittman *et al*. explained this result by an insufficient functional similarity, or an inappropriate level of expression of the *H. pylori* chemotaxis protein, since an overexpression of CheW in the *E. coli* wild-type caused the inhibition of swarming and induced a smooth-swimming phenotype similar to the overexpression of the homologous *E. coli cheW* (Pittman *et al.*, 2001; Sanders, 1989a). Therefore, overexpression of the *H. pylori* chemotaxis proteins in wild-type *E. coli* cells and in isogenic chemotaxis mutants might deepen our understanding of the function of some components of the chemotaxis pathway in *H. pylori*. For example, an interesting experiment could be the complementation of *E. coli cheY* or *cheZ* mutants with *cheY1* or *cheY2* of *H. pylori*.

As mentioned above in contrast to CheY1 of *H. pylori* and *C. jejuni*, and to CheY of *E. coli* CheY2 was stably phosphorylated by CheA´ under multiple turnover conditions.

Interestingly, the half-life of CheY2~P decreased markedly in time course experiments in the presence of CheA´ and of the other three investigated CheY proteins (CheY1hp, CheY1cj, and CheYec) as compared to the presence of CheA´ alone and was similar to the half-life of CheY2~P determined in chase experiments in the presence of CheA´ and an excess of unlabelled ATP. These results suggested that CheY1 of *H. pylori* and *C. jejuni* and CheY of *E. coli* have a clearly higher affinity for the phosphate group of CheA´~P than CheY2 and that their affinity for CheA´~P is probably similar. Therefore, it is conceivable that CheY1 of *H. pylori* and *C. jejuni* and CheY of *E. coli* possess a similar function *in vivo* in the different organisms. However, this hypothesis has to be verified by additional experiments. In particular, investigating the putative protein-protein interaction between the CheY proteins of *H. pylori* and the protein of the flagellar motor will help to clarify the function of these proteins. Furthermore, it is possible that phosphorylation of CheY2 is favoured by the intracellular phosphotransfer reaction in the bifunctional CheAY2 protein.

Signalling events in a chemotaxis system have to occur relatively rapid, enabling a cell to respond to a chemotactic stimulus within 50-100 milliseconds (Khan *et al.*, 1993; Sourjik & Berg, 2002). For example, CheY~P from *E. coli* presents an intrinsic half-life of 20 sec. This time is decreased in the presence of the CheZ phosphatase to 200 milliseconds. This reduction is essential for chemotaxis (Segall *et al.,* 1982). The half –life of CheY1~P was determined to be similar to the half-life of *E. coli* CheY, while CheY2~P proved to be extremely stable in the absence of CheA´. Therefore, it is likely that *H. pylori* possesses mechanisms to accelerate the dephosphorylation of the CheY protein, which interacts with the flagellar motor. However, in *H. pylori* no homologues of proteins with a known CheY~P specific phosphatase activity like CheZ, CheC and CheX are present. CheC was identified in *B. subtilis* and works in concert with another chemotaxis protein CheD (Szurmant & Ordal, 2004). So far only the function of CheX from *Thermatoga maritima* was analysed in some detail although orthologues of the CheX gene are currently found in more than twenty sequenced bacterial genomes (Park *et al.*, 2004). CheC and CheX share a sequence signature which is present also in the flagellar switch protein FliY from both organisms and consequently FliY was also demonstrated to act as a phosphatase on CheY~P (Szurmant *et al.*, 2004). *H. pylori* contains an ortholog of FliY, however, the sequence similarity to the regions conserved between FliY, CheC, and CheX from *B. subtilis* and *T. maritima* is rather low. Therefore, the observation that *H. pylori* contains a CheY1 protein and the bifunctional CheAY2 histidine kinase suggested a similar mechanism of termination of chemotactic signalling as was observed for the first time in the chemotaxis system of *S. meliloti*. In this system a CheY protein acts as a

phosphate sink which drains away the phosphate group from the other CheY protein via retrophosphorylation of the CheA kinase. To investigate this possibility, the phosphotransfer from CheY1~P and CheY2~P phosphorylated with acetyl phosphate to the CheA´ and CheAY2 proteins was analyzed. Phosphorylation of both CheY1 and CheY2 was observed in the presence of acetyl phosphate. Interestingly, for the detection of CheY2~P CheY2 had to be present in a concentration of 24 μM, while a concentration of 7 μM was sufficient for the detection of phosphorylated CheY1, indicating a higher efficiency for the phosphoryl group transfer from acetyl phosphate to CheY1 as compared to CheY2. Upon incubation of the CheY proteins with acetyl phosphate and CheAY2, phosphorylation of CheAY2 was observed in the presence of CheY1 but not in the presence of CheY2 indicating efficient retro-transfer of the phosphoryl group from CheY1~P to CheAY2 and suggesting a role of CheY2 as a phosphate sink involved in signal termination (Fig.32 A). Direct phosphorylation of the CheY2 domain of CheAY2 by acetyl phosphate can be ruled out since CheAY2~P was not detected when it was incubated in identical concentrations in the presence of acetyl phosphate only. Phosphorylation of CheA´ could not be observed under the same conditions. However, when both CheY1 and CheY2 were incubated with CheA´ in the presence of acetyl phosphate a decrease in the phosphorylation of CheY1 and an increase in the phosphorylation of CheY2 was observed, indicating retrophosphorylation of CheA´ by CheY1~P with subsequent phosphorylation of CheY2. In this three component reaction where CheY1 is present in its phosphorylated form while the P1 domain of CheA´ is initially unphosphorylated the phosphotransfer from CheA´~P is clearly biased towards CheY2, despite of the higher affinity of CheY1 for CheA´. From the fact that in the retrophosphorylation experiments CheAY2 but not CheA´ could be detected in the retrophosphorylated form, we conclude that the phosphorylation signal generated by CheAY2 derives mainly from the phosphorylated C-terminal CheY2 domain and not from the P1 domain. In a similar reaction containing CheY1 from *C. jejuni*, CheA´ and CheY2 dephosphorylation of CheY1cj and an enhancement in the phosphorylation of CheY2 was observed confirming the result obtained with the *H. pylori* chemotaxis proteins and stressing the functional similarity between the chemotaxis system of *H. pylori* and *C. jejuni*.

As was observed with the CheY proteins of *S. meliloti* (Sourjik & Schmitt, 1998) CheY1 and CheY2 of *H. pylori* did not exhibit phosphatase activity towards each other (Fig.32 B lane 4). Taken together the described results suggest a role of the reverse phosphotransfer from CheY1~P to CheAY2 in the regulation of chemotactic signalling in *H. pylori*. In a similar mechanism as observed in *S. meliloti* CheY2 may act as a phosphate sink, while according to

the data presented here CheY1 would interact with the flagellar motor. Since the phosphorylated form of the separated CheY2 domain presented an extended half-life it is possible that *H. pylori* contains a phosphatase specific for the phosphorylated phosphate sink domain (CheY2~P). Recent results have demonstrated that *S. meliloti* contains a CheX protein with a phosphatase activity towards the CheY protein acting as the phosphate sink (Birgit Scharf, personal communication). However, the half-life of CheY2~P might be also very much different in the context of the bifunctional CheAY2 protein. Retrophosphorylation of a HK has also been implicated in other two-component systems, which do not function in chemotaxis. For example the phosphotransfer from the OmpR response regulator (aspartyl phosphate) to its cognate histidine kinase EnvZ (phosphoramidate) has been observed *in vitro* in the *E. coli* osmotic sensory system (Dutta & Inouye, 1996), where it appears to play a physiological role in dephosphorylating phospho-OmpR.

Following the *S. meliloti* model, the CheY1 protein of *H. pylori* which is freely diffusible in the cell would interact with the flagellar motor. It was shown that the MCPs together with CheW and CheA form higher order clusters which are localized at the cell pole in *E. coli* and *R. sphaeroides* (Maddock & Shapiro, 1993; Martin *et al.*, 2003; Sourjik & Berg, 2000; Wadhams *et al.*, 2000). In swarmer cells of *C. crescentus* the chemoreceptor McpA has been demonstrated to be localized to the flagellated cell pole (Alley *et al.*, 1992). Assuming a similar localization of the chemoreceptor complex in the monotrichously flagellated bacterium *H. pylori*, direct interaction of CheAY2 with the flagellar motor is conceivable but seems little likely due to the complex stoichiometrie of the MCP-CheW-CheA complex which is a prerequisite for proper signal amplification (Levit *et al.*, 2002; Shimizu *et al.*, 2000). Interestingly, an alignment of the CheY proteins of *E. coli*, *S meliloti*, *H. pylori* and *C. jejuni* revealed two positions which might discriminate the CheY proteins interacting with the flagellar motor (CheY-*Ec*; CheY2-*Sm*; CheY1-*Hp*; CheY1-*Cj*) from those acting as a "phosphate sink" (CheY1-*Sm*; CheY2-*Hp*; CheY2-*Cj*): At the position corresponding to A74 in CheY of *E. coli* there is a lysine in the phosphate sink proteins and the position corresponding to I95 of *E. coli* CheY (V in CheY1-*Hp* and CheY2-*Sm*) is substituted by lysine or arginine in the "phosphate sink" proteins (Fig.36). However, this positions are probably not involved in the interaction with the FliM protein (McEvoy *et al.*, 1999; Shukla *et al.*, 1998).

Computerized tracking of the movement of the *H. pylori* strain N6 revealed a swimming pattern consisting of short straight runs with frequent changes of direction, whereas mutants of *H. pylori* N6 with inactivated histidine kinase CheAY2 moved in long straight runs

(Foynes *et al.*, 2000) suggesting that phosphorylation of CheAY2 induces tumbling. Furthermore, in these assays a mutant with an insertional inactivation of *cheY1* exhibited excessive tumbling. This observation is not consistent with the putative interaction of CheY1 with the flagellar motor proposed by our data, but would rather suggest a role of CheY1 in the termination of the chemotactic signalling. For the moment this apparent contradiction remains unexplained.

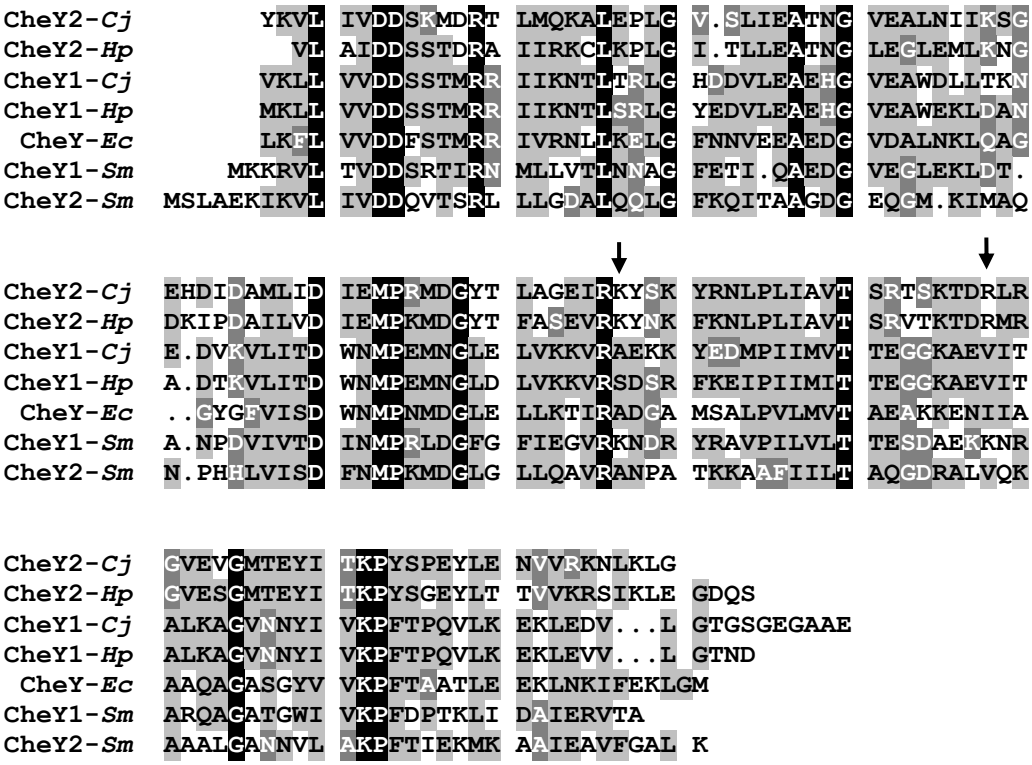

Fig.36 Alignment of the amino acid sequences of the CheY proteins of *H. pylori* (Hp), *C. jejuni* (Cj), *E. coli* (Ec), and *S. meliloti* (Sm). Gaps introduced to maximize the alignments are indicated by dots. The similarity between the homologous proteins is highlighted by different shading. Black: all amino acids of a column are identical; light grey: more than half of the amino acids of a column are identical or belong to a strong similarity group; dark grey: more than half of the amino acids of a column belong to a weak similarity group or the amino acid marked in dark grey could be grouped into a weak similarity group with every amino acid of the same column belonging to a strong similarity group marked in light grey. Positions which are different in the CheY proteins interacting with the flagellar motor and acting as phosphate sink, respectively, are marked by arrows.

So far it is unclear what is the outcome of the interaction of CheY~P with the flagellar motor of *H. pylori*. In *E. coli* the free-swimming behaviour is directed by switches between clockwise and counterclockwise rotation, rather than by variations in flagellar rotatory speed, as in *S. meliloti* (Eisenbach *et al.*, 1996; Platzer *et al.*, 1997). Examination of the effects of overproduction of chemotaxis proteins such as CheY, CheZ, and CheW has greatly contributed to the understanding of the function of these proteins (Clegg, 1984; Kuo, 1987; Russo, 1983; Sanders *et al.*, 1989a). For example in *E. coli* an increase in clockwise flagellar rotation was observed upon overexpression of CheY, while in strains lacking CheY, incessant

counterclockwise rotation was observed (Parkinson & Houts, 1982). In fact, whereas other counterclockwise processing mutants can reverse direction upon repellent addition, a *cheY* mutant cannot (Parkinson & Houts, 1982). These facts suggested in 1982 that the CheY protein generates clockwise rotation. Analysis of the behavioural consequences of overproduction of chemotaxis proteins together with the analysis of free-swimming cells can help in the complete understanding of the functions of the components of the chemotaxis signalling pathway of *H. pylori*.

One of the unusual features of the *H. pylori* chemotaxis system is the presence of the three paralogues of CheV. CheV proteins are present only in some Gram-positive and Gram-negative bacteria but not in Archaea. The chemotaxis protein CheV consisting of an N-terminal CheW-like domain and a C-terminal receiver domain was first identified in *B. subtilis* (Fredrick & Helmann, 1994), but is present in many bacteria including the enterobacterium *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, and two organisms closely related to *H. pylori*, *C. jejuni* and *W. succinogenes*, which contain one and five putative *cheV* genes, respectively (Baar *et al.*, 2003; Parkhill *et al.*, 2000; Pittman *et al.*, 2001). The presence of the N-terminal CheW domain in all CheV proteins may suggest a function in the early stages of signal transduction, from the receptor to CheA. In *B. subtilis* a partial redundancy of CheV and CheW in coupling the MCPs to CheA was reported (Rosario *et al.*, 1994) and it was shown that phosphorylated CheV is involved in the adaptation to attractants (Karatan *et al.*, 2001). However, the actual function of CheV in chemotaxis has not been elucidated. The three *H. pylori* CheVs show a sequence identity of less than 30%. HP393 (CheV3) is most closely related to the CheV protein of *C. jejuni*, whose gene as *cheV3* is also part of a *cheV-cheAY-cheW* operon. HP019 (CheV1) and HP616 (CheV2) are more similar to each other than either is to HP393 (CheV3). As mentioned, the highly conserved residues that form the active site of *E. coli* CheY are present in all three *H. pylori* CheV proteins or correspond at least to the more general receiver consensus sequence (Fig.22). These include D57, the site of phosphorylation (Sanders *et al.*, 1989b), D12 and D13 (D12 is replaced by a glutamate in CheV2), which are involved in the co-ordination of a Mg^{2+} ion that is essential for the phosphotransfer reactions (Volz, 1993), and K109, involved in the phosphorylation-induced conformational change (Volz, 1993). The position corresponding to T87 in *E. coli* CheY is occupied by an amino acid containing a hydroxyl group (threonine or serine) in virtually all known response regulators (Volz, 1993). In the CheY domains of the three CheV´s, the residue corresponding to T87 is serine. Therefore, it can be assumed that the CheY domains of each of the CheV proteins of *H. pylori*

could accept phosphoryl groups. However, only CheV1, was shown to be required for normal chemotactic motility in swarming assays on motility agar plates (Pittman *et al.*, 2001). Moreover, it was shown that the CheV proteins can not functionally substitute for CheW, since a *cheW* mutant of *H. pylori* was non-motile (Pittman *et al.*, 2001). In this work it was shown that CheA´ is dephosphorylated in the presence of all CheV proteins indicating the transfer of the phosphoryl group to the C-terminal receiver domain of the CheV proteins. Therefore, it is likely that as in *B. subtilis* the function of the CheV proteins is connected to their ability to get phosphorylated (Karatan *et al.*, 2001). CheV3 whose gene is encoded upstream of *cheAY2* showed the highest affinity for CheA´. However, a signal of the phosphorylated CheV proteins was not observed in the presence of CheA´ suggesting that these proteins have a higher dephosphorylation rate than the proteins CheY1 and CheY2, or the histidine kinase CheA´ possesses a phosphatase activity for CheV~P, or a retrophosphorylation mechanism with the phosphotransfer from CheV~P to histidine kinase CheA´ is operating (Fig.25 B, C and 26). Moreover, similar to the phosphotransfer reaction between CheA~P and CheV of *B. subtilis* (Karatan *et al.*, 2001) in *H. pylori* this reaction in case of CheV1 and CheV2 shows a slow phosphate flow (Fig.26) compared to the phosphotransfer reaction between *E. coli* CheA~P and CheY (Stewart, 1997). As mentioned above in time course experiments CheY2~P showed a decreased half-life in the presence of CheA´ and CheY1 from *H. pylori, C jejuni,* and *E. coli*, which was shown to be due to the predominance of CheY1 in the competition for the phosphate group of CheA´~P. In the *in vitro* phosphorylation experiments neither of the CheV proteins was able to interfere with the phosphorylation of CheY2 (Fig.28 A and 29 B). Consequently any interference of the CheV proteins with the phosphorylation of CheY1 which has a higher affinity for CheA´ than CheY2 can be excluded. Due to the low affinity of the CheV proteins to the CheA histidine kinase a role of the CheV proteins as a phosphate sink in terminating the chemotactic signalling seems rather unlikely. In accordance with the observation that binding of acetyl phosphate to CheV2 was detected by fluorescence spectroscopy (Pittman *et al.*, 2001) we could directly show the phosphorylation of CheV2 in the presence of acetyl [^{32}P] phosphate (Fig.30). However, high protein concentrations were required in order to detect CheV2~P which is in agreement with the low affinity of CheV2 for acetyl phosphate observed in the spectroscopic analysis (Pittman *et al.*, 2001). Interestingly, phosphorylation signals from proteins of higher molecular weight than the monomeric CheV2 could also be detected which might suggest phosphorylation-induced oligomerization of CheV2 since contaminating proteins of similar sizes could not be detected in significant amounts when the purified

CheV2 was analysed by SDS-PAGE and Coomassie staining (Fig.30 and data not shown). The intrinsic phosphatase activity of CheV2 seems to be rather low, since CheV2~P remained completely stable for 5 minutes in chase experiments (data not shown). Phosphorylation of CheV1 and CheV3 by acetyl phosphate could not be detected. It can not be ruled out that non detection of CheV1~P and CheV3~P was a consequence of the rather low amount of protein which could be analysed due to the low solubility of these proteins.

A schematic representation of the phosphotransfer reactions between the CheA histidine kinase and the chemotaxis response regulators established in this study is shown in the Fig.33. Using *in vitro* analysis it was shown that CheA´ lacking the CheY-like domain acts as ATP-dependent autokinase and that the CheY-like domain (CheY2) of the bifunctional protein CheAY2 was phosphorylated by its cognate histidine kinase CheA´, demonstrating that both domains can function independently *in vitro*. To test whether the independent expression of these domains as separate proteins can restore chemotactic behaviour *in vivo* the mutant *H. pylori* G27/cheA-Y2-cm was generated, which contains a substitution of the *cheAY2* gene by two separate genes encoding for CheA´ (only histidine kinase domain) and CheY2 (only receiver domain) (Fig.34). *H. pylori* G27/cheA-Y2-cm was non-motile on semi-solid agar plates (Fig.35 B). This result suggested that the independent function of the two domains CheA´ and CheY2 is not sufficient for normal chemotactic signalling *in vivo* indicating differences in the characteristics of the CheY2 domain when present in the CheAY2 protein and as a separate protein, respectively. However, synthesis of both proteins has to be proven in the *H. pylori* G27/cheA-Y2-cm mutant before any final conclusion can be drawn. Moreover, the obtained result has to be controlled by a complementation experiment restoring the cheAY2 gene, in the parent strain G27/cheA-Y2-cm.

In conclusion the phosphate flow in the chemotactic response system of *H. pylori* illustrated in the Fig.33 was dissected. It was proven that CheA is the cognate histidine kinase of the five CheY-like proteins in *H. pylori*. Moreover, CheA´ is capable of donating its phosphoryl group to the CheY1 protein from *C. jejuni* and the CheY protein from *E. coli*. Retrophosphorylation experiments indicated that the receiver domain present in the bifunctional CheAY2 protein acts as a phosphate sink fine tuning the activity of the freely diffusible CheY1 protein which is thought to interact with the flagellar motor. Hence, there is evidence of a complex phosphorelay *in vitro* which has similarities to other chemotaxis systems with multiple CheY proteins. The role of the CheV proteins remain unclear at the moment, but they may be engaged in a further fine regulation of the phosphate flow in this complex chemotaxis system.

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H Appendix

1. Abbreviations and chemical symbols

A	adenine
°C	Grad Celsius
µ	micro-
aa	amino acid
abs.	absolut
$AcPO_4$	acetyl phosphate
ADP	adenosine diphosphate
Amp	ampicillim
APS	ammonium persulfate
Asp	aspartate
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
$CaCl_2$	calcium chloride
cag	Cytotoxin associated gene
cagA	Cytotoxin associated gene A
CagA	Cytotoxin associated protein
C. coli	*Campylobacter coli*
C. crescentus	*Caulobacter crescentus*
cDNA	complementary DNA
CheY1cj	CheY1 from *C. jejuni*
CheYec	CheY from *E. coli*
CheY1hp	CheY1 from *H. pylori*
Ci	curie
CIAP	Calf intestinal alkaline phosphatase
C. jejuni	*Campylobacter jejuni*
Cm	chloramphenicol
cm	centimeter

CO_2	carbon dioxide
cpm	counts per minute
C-Terminal	carboxyl group terminal
CTP	cytosine triphosphate
d	deoxy
dATP	2´-desoxyadenosin-5´-triphosphat
dCTP	2´-desoxycitidin-5´-triphosphat
dH_2O	distilled water
DEPC	diethylpyrocarbonate
dGTP	2´-desoxyguanosin-5´-triphosphat
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dNTPs	deoxyribonucleoside triphosphates
dsDNA	double strand of deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetraacetate
et al.	et alteri (and others)
EtOH	ethanol
FCS	fetal calf serum
Fig	figure
g	gram
G	guanine
GS	glutamine synthetase
GST	glutahione S-transferase
GTP	guanosine triphosphate
GuHCl	guanidine clorid
h	hour
HCl	hydrochloric acid
HK	histidine kinase
H_2O	water
H. pylori	*Helicobacter pylori*
His	histidine
IAA	isoamyl alcohol

IPTG	isopropyl-β-D-thiogalactoside
Kb	kilobase pairs
Km	kanamycin
KCl	potassium chloride
KH_2PO_4	potassium phosphate, monobasic; potassium dihydrogenphosphate
K_2HPO_4	potassium phosphate, dibasic; potassium hydrogenphosphate
l	liter
LB	Luria Bertani
M	molar
MCP	methyl-accepting chemoreceptor protein
mg	milli gram
MgCl2	magnesium chloride
min	minute(s)
ml	milli liter
mM	milli molar
mRNA	messenger RNA
MW	molecular weight
Na-EDTA	disodium ethylenediaminetetraacetate
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NAP	neutrophil-activating protein
ng	nano gram
nmol	nano mole
N-Terminal	group terminal
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	primer extension
pmol	pico mole
PMSF	phenyl-methyl-sulfonyl-fluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute

RR	response regulator
RT	room temperature
RT-PCR	reverse Transcription-PCR
sec	second (s)
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel
S. meliloti	*Sinorizobium meliloti*
spp.	species
T	thymine
Tab.	Table
TBE	tris-borate-EDTA
TCS	two-component system
TEMED	N,N,N´,N´-tetramethylethylenediamine
t_0	time zero
Tris	Tris(hydroxymethyl)-aminomethane
U	uracil
µg	micro gram
µl	micro liter
µM	micro molar
UV	ultraviolet
V	volt
VacA	vacuolating cytotoxin A
WT	wild-type

2. Lebenslauf

Persönliche Angaben

Name:	María Antonieta Jiménez Pearson
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Schulbildung

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1993-1999:	Universidad de Costa Rica, San José, Costa Rica Abschluss (Licenciatura) in Mikrobiologie und klinischer Chemie
Januar 1999-Juni 1999:	Praktikum im klinischen Labor des Krankenhauses „Hospital San Juan de Dios San José, Costa Rica“

Berufstätigkeit:

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Fortbildung

Oktober 2000-März 2001:	Intensiver Deutschkurs am Goethe Institut in Mannheim Deutsche Sprachprüfung für den Hochschulzugang ausländischer Studienbewerber (DSH) zur Aufnahme eines Fachstudiums (durchgeführt durch das Goethe Institut und die Universität Mannheim).

April 2001-September 2001: Forschungsprojekt zur Erlangung eines Diplom-Äquivalents an der Julius-Maximilians Universität Würzburg; am Lehrstuhl für Mikrobiologie von Prof. Dr. W. Goebel. Betreuer der Forschungsprojekt: PD. Dr. D. Beier. Thema: "Untersuchungen zur Charakterisierung der regulatorischen Gene *tenA* und *nikR* aus *Helicobacter pylori* ".

Promotionsstudium

Ab Oktober 2001: Doktorarbeit an der Julius-Maximilians Universität Würzburg; am Lehrstuhl für Mikrobiologie von Prof. Dr. W. Goebel. Betreuer der Doktorarbeit: PD. Dr. D. Beier. Thema: "Characterization of the mechanisms of two-component signal transduction involved in motility and chemotaxis of *Helicobacter pylori* ".

Publikationsliste

María-Antonieta Jiménez-Pearson, Patricia Dietz and Dagmar Beier. (2005). Protein-protein interaction of HP137 with histidine kinase HP244 does not contribute to flagellar regulation in *Helicobacter pylori*. Microbiol. Res. 160: 299-305

María-Antonieta Jiménez-Pearson, Isabel Delany, Vicenzo Scarlato and Dagmar Beier.(2005). Phosphate flow in the chemotactic response system of *Helicobacter pylori*. Microbiology. Accepted.

Posterpräsentationen

María-Antonieta Jiménez-Pearson and Dagmar Beier: Phosphotransfer between two-component signalling modules of the chemotaxis system of *Helicobacter pylori*. Workshop: Proteome analysis of bacterial pathogens, Würzburg, Germany (July, 2004)

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María-Antonieta Jiménez-Pearson, Isabel Delany, Vicenzo Scarlato and Dagmar Beier: Phosphate flow in the chemotactic response system of *Helicobacter pylori*. International Workshop "Multidisciplanary approaches in studies of microbial pathogens". Umea, Sweden (June, 2005)