Helicobacter pylori type IV secretion, host cell signalling and vaccine development

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Abstract. ***Helicobacter pylori*** **is a bacterial pathogen specialised to colonise and persist the gastric mucosa and to cause severe gastroduodenal disease. A major disease-associated bacterial component is a type IV secretion system (TFSS) encoded by the cytotoxin-associated genes pathogenicity island (*cag*PAI). Among the multiple responses in *H. pylori*-infected epithelial cells, the induction of proinflammatory cytokines and chemokines, cell spreading and motility associated with the "hummingbird" phenotype appear strictly dependent on the functional transporter complex in the *cag*PAI. *H. pylori* is also capable of occasionally entering epithelial cells and manipulates the host immune system for immune evasion. Attached bacteria actively translocate the CagA protein into epithelial cells by a TFSS-dependent process and translocated CagA undergoes tyrosine phosphorylation in the carboxy terminal EPIYA sequence repeat motif (Y-972) by kinases of the Src family. Furthermore, we have identified a novel TFSS in *H. pylori* involved in horizontal DNA-transfer. Host cell signalling events and cellular phenotypes provoked by the *cag*PAI, the investigation of mechanisms related to gastric cancer as well as the development of a *Salmonella* based live recombinant vaccine are in the focus of additional departmental activities.**

Key words: CagA, interleukin-8, molecular pathogenesis, pathogenicity island, Salmonella, type IV secretion, tyrosine phosphorylation, virulence

Introduction

H. pylori is a highly successful bacterial pathogen that colonises the stomach of about half of the human population world-wide. Infection with this bacterium has been recognised as the major cause of chronic gastric inflammation that can further progress to a variety of diseases such as peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma.[1-3] Pathogenicity of *H. pylori* relies on its capacity to adapt to a hostile environment and to escape from the host response. Resistance to acidity, motility, adhesion, molecular mimicry, resistance to phagocytosis, expression of virulence factors and induction of an inflammatory response are important strategies developed by this pathogen to colonise persistently and damage gastric tissue. On the other hand, host factors such as interleukin (IL)-1β polymorphism are also known to increase the risk of gastric disease.[4]

Although *H. pylori* has generally been considered an extracellular pathogen, it has been shown that it is capable of occasionally entering epithelial cells *via* a zipper-like mechanism as known from *Yersinia*, *Neisseria* or *Listeria*.[5] *In vitro*, attached *H. pylori* induce several host responses in epithelial cells such as cellular vacuolation, actin rearrangements associated with the so-called "hummingbird" phenotype and nuclear responses resulting in the secretion of a large set of inflammatory molecules such as IL-1, IL-6, tumor necrosis factor α (TNF-α), IL-8, Gro-α, RANTES, ENA-78, MCP-1 and Mip-1 α.[6,7] This scenario leads to a dense infiltration of different monocytic cells causing acute inflammation of the gastric mucosa. However, despite a strong inflammatory response bacteria survive in the stomach for years or even decades without being cleared by the immune system of the host.

To date a number of virulence factors have been identified including the vacuolating cytotoxin (VacA), the neutrophil-activating protein (NapA) and the *cag*-PAI, a locus of about 40 kb containing up to 31 genes.[1-3] Genetic and functional studies provided evidence that *cag*PAI encodes a specialised TFSS for the delivery of virulence factors such as the immunodominant 120–150 kDa CagA and probably also other proteins.[1,3,7] This type of TFSS is evolutionary related to conjugative plasmid transfer systems.[8] Six of the

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*H. pylori cag*PAI genes are homologous to the well-known virulence genes *virB4*, *virB7*, *virB8*, *virB9*, *virB10*, *virB11* and *virD4* from TFSS's of *Agrobacterium tumefaciens*, *Bordetella pertussis* or *Legionella pneumophila*.[1] It is now well established that *H. pylori* actively injects CagA into target cells in a *cag*PAI-dependent manner,[9–13] where CagA becomes tyrosine-phosphorylated ($CagA^{P\text{-}Tyr}$) and interacts physically with the tyrosine phosphatase Shp-2.[14] $CagA^{P\text{-}Tyr}$-mediated signalling finally results in the development of the "hummingbird" phenotype.[10,15] This phenotype is characterised by spreading and elongation of the infected host cell, the presence of lamellipodia (thin actin sheets at the edge of the cell), and filapodia (spikes containing a tight bundle of actin filaments) but particular signalling leading to these events is not yet understood.[10]

A complex interplay between host and bacterial factors is of importance for the clinical outcome of infection. Our laboratory has started several research projects to investigate global mechanisms underlying *H. pylori* infection and to develop a vaccine. The major scientific focus is on host cell signalling and cellular phenotypes associated with a functional *cag*PAI, the activation of target genes, horizontal gene transfer and the investigation of putative pathways leading to the development of gastric cancer.

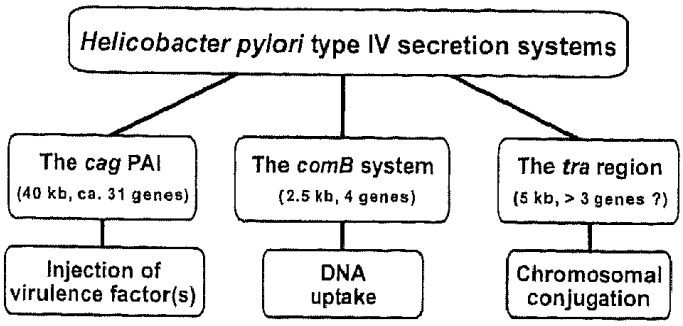

Fig. 1. Schematic presentation of the three type IV secretion systems (TFSS's) identified in *Helicobacter pylori*. (I) Components of the *cag*PAI were first discovered by the groups of Antonello Covacci (Chiron, Siena, Italy), Doug E. Berg (Washington University, St. Louis, USA) and Martin Blaser (New York University, N.Y., USA). This TFSS is important for CagA translocation, modulation of host cell signalling and nuclear responses. (II) Individual gene products encoded by the *comB* locus also show significant homology to VirB proteins of *Agrobacterium*, and a novel mechanism for natural DNA transformation using basic components of a TFSS was proposed by the group of Rainer Haas (Ludwig-Maximilians University, Munich, Germany). (III) In addition to the *cag*PAI and the *com*B systems, our group has identified components of a third TFSS in a different part of the *H. pylori* chromosome, called the *tra* region. We have shown that this gene cluster encodes *virD2* and *traG* homologs, essential for conjugative transfer of chromosomal DNA between *H. pylori* isolates (20; S. Backert *et al.*, manuscript submitted).

Identification of a Novel Conjugative DNA Transfer System in *H. pylori*

H. pylori is one of the most diverse bacterial species exhibiting extreme genetic variability. The origin of this diversity has been attributed to several mechanisms, including an elevated mutation rate, impaired DNA repair, frequent DNA transfer and recombination events.[16] Recent investigations indicated a novel mechanism for *comB*-dependent transformation using a second TFSS for the uptake of naked DNA.[17,18] Kuipers and co-workers[19] described the DNase-insensitive exchange of genetic information among *H. pylori* strains but mechanisms of DNA transfer *in vivo* were unknown. We sought to determine whether mechanisms of DNA exchange other than transformation exist in this pathogen. Besides the *cag*PAI and the *comB* locus, we have now identified a third TFSS in *H. pylori* for the horizontal transfer of chromosomal DNA *via* a conjugative mechanism (S. Backert, V. Brinkmann and T.F. Meyer, unpublished data). Our current knowledge about these three TFSS's is summarised in Fig. 1.

To establish a test system to monitor DNA transfer in *H. pylori*, we produced a set of strains carrying specific chomosomal resistance markers. Mating experiments combined with electron microscopy indicated the presence of pilus-like structures of about 12 nm in diameter and 1–3 μm in length that were reminiscent of conjugative pili or the agrobacterial T-DNA pili, suggesting that conjugative DNA transfer might occur in *H. pylori*. DNA transfer by plasmids or bacteriophages (transduction) was excluded in our system. Instead, we found that both a DNaseI-sensitive mechanism (transformation) and a DNase-resistant/*comB*-independent mechanism (conjugation) involving tight cell contact contributed to the horizontal exchange of genetic information between *H. pylori* isolates.

The conjugative DNA transfer was analysed in detail and was found to be independent of the *cag*PAI. Instead, we discovered three novel conjugative transfer proteins (two *virD2* genes, called relaxases, and a *traG* coupling factor) in the *tra* locus a few hundred kb downstream of the *cag*PAI which share homology to agrobacterial T-DNA transfer proteins.[20] The DNA transfer mechanism in *H. pylori* was further characterised by mutagenesis of these genes and confirmed by genetic complementation using shuttle vector constructs. In addition to the exchange of the chromosomal resistance markers, we detected few other genomic rearrangements in the transconjugands as identified by random amplified polymorphic DNA (RAPD) PCR and by hybridisation of DNA to a whole-genome microarray chip carrying all genes (>1,600) of the en-

tirely sequenced *H. pylori* strains 26695 and J99. In conclusion, we identified a third TFSS in *H. pylori* and described a novel conjugative mechanism for chromosomal DNA transfer in this pathogen.

TFSS-dependent Translocation of the CagA Protein, Tyrosine Phosphorylation and Cellular Effects in Gastric Epithelial Cells

The TFSS encoded by the *cag*PAI is responsible for changes within the host cell such as reorganisation of the actin cytoskeleton and the activation of host signalling pathways leading to the expression of cytokines and chemokines or even oncogenes. Systematic mutagenesis has revealed that many genes throughout the whole *cag*PAI are essential for both CagA translocation and the induction of proinflammatory responses in gastric epithelial cells and phagocytes.[21–23]

We and other laboratories have demonstrated that the translocated CagA protein becomes phosphorylated on tyrosine residues in so-called EPIYA repeat motifs.[15,24] Our *in vitro* and *in vivo* studies showed that CagA is strongly phosphorylated on Y-972 by kinases of the Src family.[25] Moreover, phosphorylation of Y-972 is essential for the initiation of host cytoskeletal rearrangements associated with the "hummingbird" phenotype in AGS gastric epithelial cells.[15] This phenotype is characterised by spreading and elongation of the host cell as described above as well as the loss of cell contacts and enhanced motility.[10,15] Time-lapse, live-cell imaging illustrated the cellular dynamics during four hours of *H. pylori* infection (Fig. 2). We further demonstrated that transient expression of full-length CagA and phosphorylation in the EPIYA repeat motifs is essential but not sufficient for the hummingbird phenotype suggesting the involvement of yet unknown bacterial factors.[15] In addition, full-length CagA ($p135^{CagA}$) can be processed into a amino-terminal $p100^{CagA}$ and a carboxy-terminal $p35^{CagA}$ fragment, however, the role of these products is not known.[26–28]

Recently, the protein tyrosine phosphatase (PTPase) Shp-2 was shown to bind specifically to transiently expressed $CagA^{P\text{-}Tyr}$ *via* its src homology 2 (SH2) domain followed by the activation of the Shp-2 PTPase activity.[14] Interestingly, previous studies reported that translocation and tyrosine phosphorylation of CagA are temporally correlated with dephosphorylation of yet unidentified host proteins p80 and p120.[11,12,29] These events might be important for the induction of actin cytoskeletal rearrangements and the hummingbird phenotype. A hypothetical model for CagA-induced host cell signalling is shown in Fig. 3.

We further produced a series of truncated CagA constructs expressed from a shuttle vector in *H. pylori* followed by investigation of the phenotypical outcome

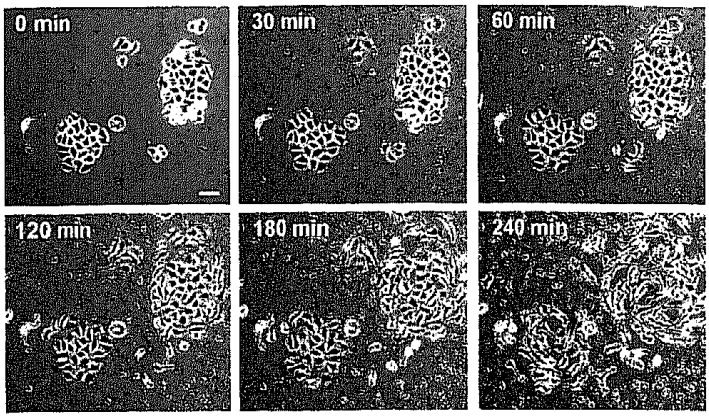

Fig. 2. Live-cell imaging of AGS cells infected with wild-type *H. pylori*. One particular field of the coverslip was photographed in a time-course over four hours of infection. The pictures clearly indicate two specific host cell responses. First, the cells lose contacts to neighbouring cells and start to migrate 30–60 min after bacterial attachment. Second, after 3–4 hours 65–80% of the infected AGS cells form long needle-like protrusions (25–70 μm in length), which exceed the size of uninfected control cells up to four times, known as the hummingbird phenotype.[10] Whereas enhanced cellular motility is a *cag*PAI-dependent but CagA-independent process, expression of the hummingbird phenotype is strictly dependent on CagA translocation and phosphorylation on Y-972.[15] Bar represents 40 μm.

by transmission and scanning electron microscopy. Morphological appearance of both the pathogen and the cells as well as features of colonisation and attachment were evaluated. During infection, bacterial cell membranes progressively enlarged and often formed (i) unique protrusion structures (50–200 nm in length) connecting the bacteria with the host cell and (ii) outer membrane vesicles containing VacA were increasingly secreted from *H. pylori*, coinciding with increased cellular damage. Immunogold transmission electron microscopy demonstrated that components of the TFSS in the *cag*PAI (e.g. Cag3, Cag7 or Cag13) are typically clustered in the bacterial membranes with associated CagA signals. $CagA^{P\text{-}Tyr}$ was detected in the host cell membrane directly underneath the attached bacteria and co-localises with proteins from the actin-cytoskeleton (S. Backert, V. Brinkmann; unpublished data).

Our findings support the view that *H. pylori* actively translocates virulence determinants, including CagA, that could change the host protein tyrosine phosphorylation status, which is involved in the induction of host cell scattering and rearrangements of the actin cytoskeleton. The function of CagA in the host and interacting partners are currently under investigation. Collectively, our data indicate that *H. pylori* exhibits several TFSS-dependent host responses, which may play an essential role in the development of certain gastric diseases.

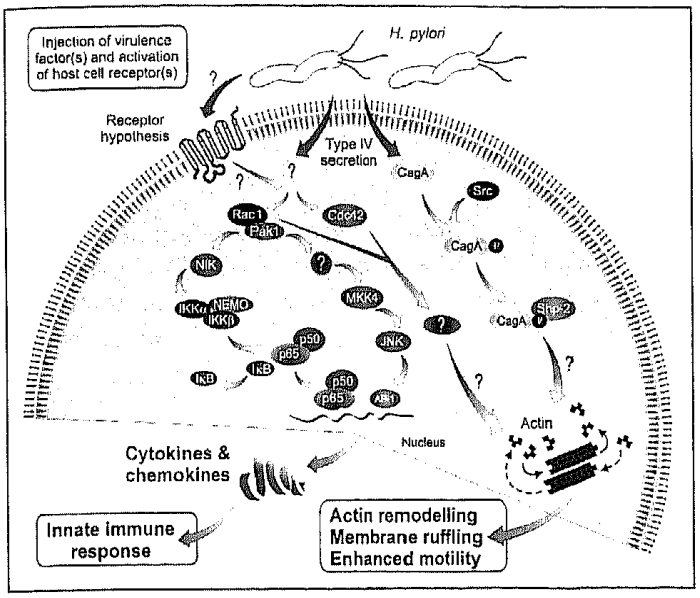

Fig. 3. Model for type IV secretion (TFSS)-dependent induction of host cell signalling by bacterial protein injection or activation of contact-dependent eukaryotic receptor(s) on the cell surface. The activation of CagA proceeds through tyrosine phosphorylation by members of the Src kinase family. Binding of phosphorylated CagA to Shp-2 and possibly other factors induces actin-cytoskeletal rearrangements leading to the hummingbird phenotype. Multiple other CagA-independent but TFSS-dependent host responses include the activation of small GTPases (Rac1 and Cdc42) which is possibly involved in membrane ruffling, enhanced cell motility and nuclear responses.[36] For example, the activation of transcription factors NF-κB[30–33] and AP-1[34,35] are major early responses and Rac1 phosphorylation contributes to this signalling.[36] Nuclear translocation of NF-κB involves IKKα and IKKβ activation as well as IκBα phosphorylation and degradation. Upstream of the IKK complex are two kinases, called NIK and PAK1.[37] During *H. pylori* infection, PAK1 has also been considered to control AP-1 activation *via* a MAPK kinase 4 (MKK4) and JNK pathway. Both, NF-κB and AP-1 contribute considerably to the transcription and subsequent release of cytokines and chemokines (like IL-8). This signalling is important for initiating the innate immune response during infection with *H. pylori*.

TFSS-dependent Activation of Rho GTPases (Rac1 and Cdc42) during *H. pylori* Infection

Attachment of *H. pylori* to gastric epithelial cells leads to a variety of nuclear and cytoskeletal responses in the host cells.[3] The activation of nuclear factor-kappaB (NF-κB)[30–33] and activator protein 1 (AP-1)[34,35] represent major early responses (Fig. 3). In this signalling scenario, we have demonstrated that Rho GTPases Rac1 and Cdc42 become activated during infection of gastric epithelial cells with wild-type *H. pylori*.[36] In contrast, no activation of Rho GTPases was observed when *H. pylori* mutant strains (Δ*virB7* and Δ*cag*PAI) were used that lack a functional TFSS encoded by the *cag*PAI. Moreover, we provided evidence that Rac1 and Cdc42 were recruited to the sites of bacterial attachment and are probably involved in the regulation of local and overall cytoskeleton rearrangement in host cells and motogenic responses characterised by enhanced motility of the infected cells (Fig. 2).

We further demonstrated that *H. pylori*-induced activation of Rac1 and Cdc42 led to the activation of p21-activated kinase 1 (PAK1) mediating nuclear responses, whereas the mutant strain Δ*cag*PAI had no effect on PAK1 activity.[36] In addition, we have shown that activated PAK1 binds and activates NF-κB inducing kinase (NIK) to trigger NF-κB activation.[37] Activation of PAK1 might also be involved in activation of the c-Jun N-terminal kinase (JNK) pathway to stimulate AP-1.[34] A model for this signalling is included in Fig. 3. Activation of Rac1, Cdc42 and PAK1 represented a very early event in colonisation of AGS cells by *H. pylori* carrying a functional *cag*PAI. Interestingly, the *H. pylori* Δ*cagA* mutant strain also induced Rho GTPase activation suggesting that the translocated CagA protein does not play a role in this process. This supports the view that Rho GTPase activation is triggered either by (i) translocation of a yet unknown bacterial effector molecule or by (ii) activation of host cell surface receptor(s) induced by binding of the TFSS itself (Fig. 3). For example, epidermal growth factor receptor (EGFR) is phosphorylated in a *cag*PAI-dependent manner but the molecular mechanism of this activation is not known.[38] However, the activation of EGFR, Rho GTPases and motogenic responses provide new insights how *H. pylori* infection could be involved in gastric tumour progression.

TFSS-independent B-Raf/Rap1 Signalling Induces the Histidine Decarboxylase (HDC) Promoter during *H. pylori* Infection

HDC is the key enzyme for gastric histamine synthesis, and enhanced HDC expression is critically involved in the pathogenesis of several gastric disorders, including gastroduodenal ulcer disease as described above. The molecular mechanism underlying the activation of the HDC promoter in *H. pylori*-infected gastric epithelial cells did not require a functional TFSS encoded by the *cag*PAI.[39,40] The obtained data are summarised in Fig. 4. In particular, *H. pylori* infection of gastric epithelial cells activated a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK1/ERK1) signalling cascade through accumulation of the second messenger cyclic AMP (cAMP). cAMP binds to specific guanine nucleotide exchange factors (GEF's) such as EPAC (exchange protein activated by cAMP), which directly activates Rap1. Indeed, this resulted in the stimulation of Rap1 and B-Raf, but not Ras/c-Raf-1, leading to potent transactivation of the human HDC promoter. *H. pylori*-triggered elevation of

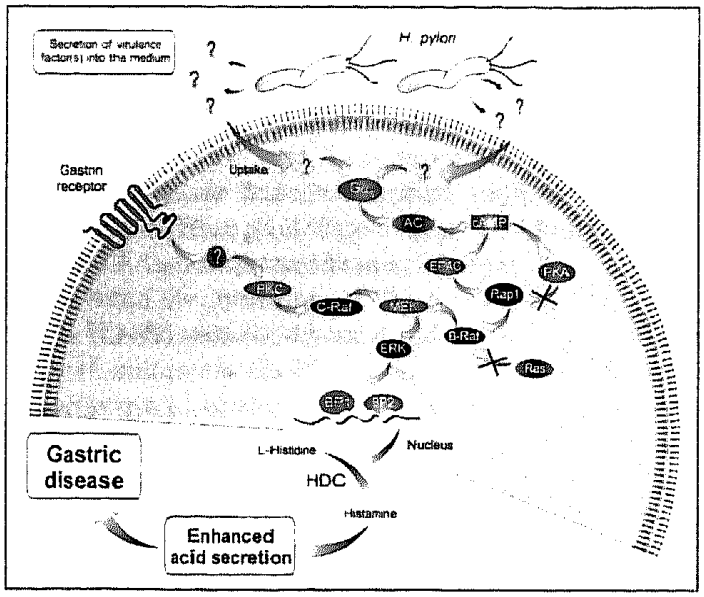

Fig. 4. Schematic presentation of *H. pylori*-induced signalling leading to the activation of the histidine decarboxylase (HDC) promotor in gastric epithelial cells. Enhanced transcription of the HDC gene without *H. pylori* infection was shown to proceed by binding of gastrin to the cholecystokinin/gastrin receptor involving protein kinase C (PKC), c-Raf-1 and MAP kinase (MEK/ERK) signalling. During infection with *H. pylori*, the HDC promotor is activated by a different pathway. This signalling is triggered by a unknown low molecular weight component from the bacteria which is secreted into the culture medium and is independent of the TFSS in the *cag*PAI. The signalling cascade includes cAMP accumulation induced by $G\alpha_S$ followed by the activation of ERK1 and MEK1. Activation of the MAP kinases is mediated by the small GTPase Rap1 *via* B-Raf but not c-Raf-1. The differential activation of MAP kinases by cag^+ and cag^- *H. pylori* was also described.[41] The presented model was adapted from Wessler and co-workers.[40]

adenylate cyclase activity was directed by $G\alpha_S$-subunit of heterotrimeric G protein. The mechanism how *H. pylori* induces $G\alpha_S$ (e.g. receptor activation) remained unknown.

Stimulation of this signalling cascade was triggered independently of bacterial host cell contact by a small molecular weight component(s) (<1 kDa) released by *H. pylori*. These studies demonstrated for the first time that the $G\alpha_S \rightarrow$ cAMP $\rightarrow$ Rap1 $\rightarrow$ B-Raf $\rightarrow$ MEK1 $\rightarrow$ ERK1 pathway is critical for *H. pylori*-dependent gene regulation, which can be induced *via* a bioactive component(s).[40] This bacterial factor is secreted into the culture medium which proceeds in a *cag*PAI-independent manner. Opposing data were obtained from Meyer-ter-Vehn *et al.*[35] and Keates *et al.*[38,41] indicating that ERK activation is *cag*PAI-dependent. These results might be due to strain-specific differences. However, the data described above may further elucidate the molecular mechanisms underlying interaction of *H. pylori* with gastric epithelial cells and could help to define potential molecular targets for therapeutic interventions in the context of *H. pylori*-related gastric diseases.

H. pylori Infection and the Role of Cyclooxygenase-2 (COX-2) in the Development of Gastric Cancer

The pathogenic influence of *H. pylori* on the gastric mucosa is multifactorial. Epidemiological factors as well as host factors and bacterial virulence factors contribute to the overall pathogenic action of this bacterium. The world health organization WHO has classified *H. pylori* as a class I carcinogen, however, the molecular mechanisms underlying the carcinogenic effect of this pathogen remained largely unknown. Current studies indicate that increased COX-2 gene expression is involved in the carcinogenic cascade triggered by *H. pylori* in the stomach.[2,3,42]

COX-2 is the inducible key enzyme for the generation of prostaglandins and these products are involved in the regulation of cellular proliferation, apoptosis, angiogenesis and adhesion processes.[42–44] *H. pylori* infection leads to increased expression of COX-2 gene in the gastric mucosa, whereas eradication of the bacterium reverts this effect. This observation indicates a possible link between *H. pylori* infection and COX-2 expression in the stomach. The aim of our laboratory is to elucidate the role of *H. pylori*-induced COX-2 expression within the pathogenic sequence leading to gastritis and possibly to intestinal metaplasia and gastric cancer.

Investigation of this phenomenon in the mice animal model system by a variety of molecular approaches including cDNA microarray and immunobiological methods is currently in progress (summarised in Fig. 5). Special emphasis lies on the identification of *H. pylori* target genes regulated through a COX-2-dependent mechanism as well as the bacterial virulence factor(s) that affect COX-2 expression itself. In the latter case, a number of low molecular weight fractions of *H. pylori* secretions have been identified by high pressure liquid chromatography (HPLC), which have specific stimulatory activity on the expression of the COX-2 gene and the process of identification of the component(s) is underway using electrospray ionisation mass spectrometry (ESI/MS).

In a novel approach to identify genes regulated by COX-2 in the host, infected mice were treated for 19 weeks with NS398, an experimental drug that specifically inhibits COX-2 activity. Gene expression was studied in the gastric mucosa of these treated mice using a mouse cDNA microarray. This study resulted in the identification of several groups of genes that are regulated in a COX-2-dependent manner. These investigations could give new insights into the mechanisms responsible for the pathogenic action of *H. pylori* in the

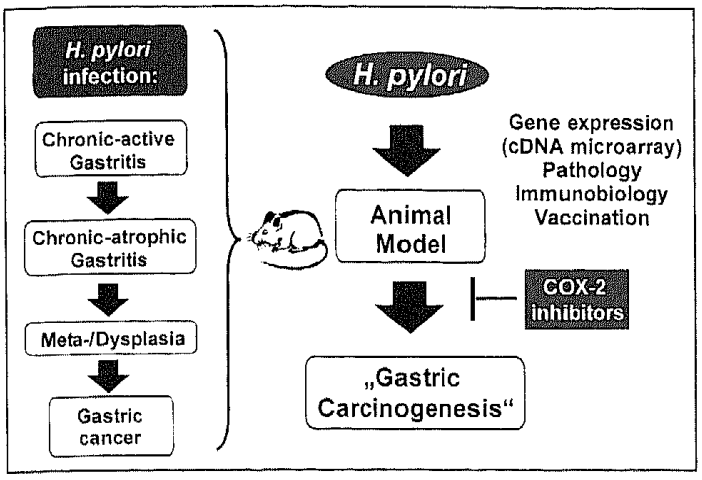

Fig. 5. Schematic presentation of developmental stages in *H. pylori*-induced gastric carcinogenesis. A striking feature of *H. pylori* infection is that it may take years or decades to lead to a symptomatic disease in patients. Since the mice and Mongolian gerbil models have been reported to be colonised by *H. pylori*, our laboratory uses these animal models to study by a broad variety of molecular biological approaches the development of *H. pylori*-induced diseases, the role of COX-2 and the relationship between *H. pylori* infection and malignancy.

stomach and may lead to the development of new therapeutic strategies.

Development of a Vaccine Against *H. pylori* Based on Recombinant *Salmonella ssp.*

H. pylori is now recognised as the most widespread pathogen that can persist for life in the stomach of humans. To combat this infection, vaccination would be a very cost effective way.[45] The aim of our laboratory in this project is to develop a safe, effective human vaccine against *H. pylori* for prophylactic and therapeutic applications based on live recombinant *Salmonella spp.*[46–49] This research program is structured into four principle projects: (i) The development of safe and effective *Salmonella* carrier strains; (ii) identification of new *H. pylori* antigens as candidate vaccine molecules or diagnostic tools; (iii) elucidating the mechanism of protection in the mouse model for selection or improvement of carrier strains; and (iv) combining the knowledge gained in the pre-clinical models to construct carrier strains based on the attenuated, *Salmonella enterica* Serovar Typhi Ty21a for clinical testing in human volunteers.

Two strategies are followed to optimise potential carrier strains. Using a genome wide insertional mutagenesis approach, a large panel of attenuated mutants have been identified by *in vitro* screening. Optimisation of the expression system driving the production of the vaccine antigen in the recombinant *Salmonella* for T cell induction is complementary to the carrier identification.[50,51] The protocol for successful screening of mutants for attenuated phenotypes directly *in vivo* has been established. According to the ovalbumin system, promoters activated upon *Salmonella* invasion are useful to optimise expression of antigens for T cell induction by these bacterial carriers.[47] Protection against *H. pylori* is afforded by $CD4^+$ cells and optimising antigen expression in *Salmonella* for T helper cell activation should improve vaccine efficacy.[52] Thus, *S. typhimurium* strains expressing Urease A and B subunits under the control of a number of *in vivo* induced promoters were constructed and used to vaccinate mice against a challenge infection. The results of these experiments suggest that extrapolation from the ovalbumin model antigen system is a valid way to improve vaccine efficacy.

Vaccination induced protection is mediated by $CD4^+$ T cells, but the mechanism of protection remains obscure. Data from our laboratory have shown that this is also the case for *Salmonella* immunised hosts. Three to eight hours after infection with *H. pylori*, a significantly lower number of bacteria were recovered from immunised mice as compared to naive mice. However, *H. pylori* became established in all mice and similar numbers of bacteria were detected in immunised and naive mice at days one and three after infection. A significant reduction of bacterial colonisation was again observed five days after infection in immunised mice, and mice remained protected for at least one year. The early reduction was not observed in mice immunised with *H. pylori* extract combined with Cholera toxin and studies with antibody deficient mice showed that the very early reduction was not due to antibody production. Fluorescence-activated cell sorting (FACS) analyses of lymphocytes infiltrating the mucosa and immunohistochemical analyses revealed that infection with *H. pylori* stimulated a rapid influx of inflammatory cells into the mucosa with a marked change in the cellular composition over time, which was dominated by $CD8^+$ T cells at the beginning in *Salmonella* treated mice and became strongly $CD4^+$ T cell-biased in protected mice over time.

The very early transient reduction of colonisation in immunised mice is probably the result of innate, but inducible mechanisms to a large degree induced by the *Salmonella* carrier, and that the second, persistent reduction is mediated by a T cell response. The cellular infiltrate over time shifts to resemble that of intestinal lymphocyte populations. This work forms the basis for further studies and defined the optimal time windows for analysing the onset of the protective immune response (A. Walduck, B. Lucas and A. Aebischer, unpublished data).

Our lab now aims to improve vaccine delivery based on our pre-clinical data indicating that recombinant

Salmonella expressing vaccine antigens only *in vivo* during infection have dramatically improved protective properties. *S. enterica* Serovar Typhi Ty21a (Thyphoral®) have been engineered for *in vivo* induced expression of *H. pylori* Urease A and B subunits. These strains will now be tested for their safety as well as immunigenicity in human volunteers non-pretreated with the carrier. Volunteers will subsequently be challenged with a *cag*PAI-negative *H. pylori* strain to evaluate the potential protective effect of the vaccine.

Identification of New Vaccine Candidate Antigens from *H. pylori*

In the past two years, our laboratory has continued the characterisation of the *H. pylori* proteome using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization (MALDI) mass peptide fingerprinting with a special focus on surface-exposed proteins that could be involved in the direct contact with the host and may mediate important pathogen-host interactions.[53,54] In particular, new techniques have been developed to obtain secreted proteins with minimal contamination by material from spontaneously lysed bacteria, and to identify surface proteins using selective biotinylation and affinity purification.[54] Using these techniques, 23 secreted and 18 surface-exposed proteins were identified, many of which had escaped theoretical predictions for surface-associated proteins. Interestingly, there was a large overlap between secreted and surface-associated proteins suggesting that many secreted proteins are re-adsorbed by *H. pylori*.

In addition, *H. pylori* antigens recognised by sera from patients with different *H. pylori* related pathologies were characterised by immuno-proteome analyses using 2-DE and MALDI-MS.[55] A complement of 310 proteins was recognised and 32 most frequently recognised proteins were identified. Nine of these proteins were newly discovered and 23 confirmed from other studies. The data were incorporated in a 2DE-database available on the internet (http://www.mpiib-berlin.mpg.de/2D-PAGE/). This immuno-proteome is currently extended by adding the data from a similar analysis of more than 60 patient sera that include a large cohort of gastritis, peptic ulcer and gastric cancer patients. To compare these data with *in vitro* induced host responses, *H. pylori* isolated from these patients were tested in the AGS cell model system for their ability to express *cag*PAI-specific phenotypes such as IL-8 secretion, CagA translocation and phosphorylation as well as induction of the hummingbird phenotype. This study revealed a high percentage (>65%) of the isolates expressing full *cag*PAI functions between all groups of patients listed above.

In another project, serum antibody responses in mice infected with *H. pylori* were compared to data for the infected human patients. Despite a large individual variation, the general pattern of recognised antigens was rather similar for mice and humans suggesting similar *H. pylori in vivo* gene expression patterns, and comparable antigen exposition to the host immune system. The murine *Helicobacter* model thus appears to be appropriate for pre-clinical screening of antigens.

Concluding Remarks

The molecular crosstalk between the bacterium and the host is highly complex and determines the clinical outcome of infection. *In vivo* and *in vitro* studies on the interaction between *H. pylori* and host target cells provide powerful tools for identification of both virulence factors from the bacterium and the function of various eukaryotic signalling networks as well as risk factors. To clinically control *H. pylori* it is important to further continue vaccination studies and to reveal and better understand basic mechanisms underlying the infection. For the efficient analysis of host signalling pathways, widely automated techniques in the field of genomics, transcriptomics and proteomics are of growing importance. In future it will be also very interesting to study in detail the mechanisms of horizontal DNA transfer including the transfer of virulence genes. Finally, the availability of animal models, such as gerbils or mice with targeted gene deletions, coupled with unique experimental approaches should result in a more detailed understanding of the complex alliance between the pathogen and host.

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