The role of DNA supercoiling in the coordinated regulation of gene expression in *Helicobacter pylori*

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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Würzburg, 2004

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Doktorurkunde ausgehändigt am: .....

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Hiermit erkläre ich, dass ich diese Dissertation selbstständig angefertigt und keine anderen als die von mir angegebenen Hilfsmittel und Quellen benutzt habe.

Ich erkläre außerdem, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Prüfungsverfahren vorgelegen hat.

Ich habe früher, außer den mit dem Zulassugsgesuch urkundlichen Graden, keine weiteren akademischen Grade erworben oder zu erwerben gesucht.

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Acknowledgements

I would like to express all my gratitude to Prof. Sebastian Suerbaum and P.D. Dr. Christine Josenhans for giving me the chance to do my PhD work in their lab, and for their diligent guidance and supervision in my experiments. This thesis would not be possible without their invaluable personal and professional support.

Special thanks to Prof. Jörg Hacker for providing me with the opportunity to join the “European Graduate College”, and for his kindness and support.

Many thanks also to the people in Sebastian’s lab: Eike Niehus, Christian Kraft, Allison Stack, Verena Böhm, Torsten Sterzenbach, Elena Katzowitsch, Sandra Schwarz, Patrick Olbermann, Sarah Kühne and Sae Kyung Lee for their helpful technical advice and discussions. I will never forget the pleasant time with them during these years.

I also thank my parents, my husband and my loving son for their continuous support.

The work presented in this thesis was supported by fellowships of the European Graduate College “Gene Regulation in and by Pathogenic Bacteria” of the Deutsche Forschungsgemeinschaft.

Summary

Mechanisms of global gene regulation in bacteria are not well characterized yet. Changes in global or local supercoiling of chromosomal DNA are thought to play a role in global gene silencing and gene activation. In *Helicobacter pylori*, a bacterium with few dedicated transcriptional regulators, the structure of some promoters indicates a dependency on DNA topology. For example, the promoter of the major flagellar subunit gene *flaA* (s^{28}-dependent) has a shorter spacing of 13 nucleotides (nt) in comparison to the consensus promoter (15 nt). Supercoiling changes might be a mechanism of gene-specific and global transcriptional regulation in this bacterium. The aim of this study was to elucidate, if changes in global supercoiling have an influence on global gene regulation in *H. pylori*, and on the temporal regulation of the flagellar biosynthesis pathway in this organism.

In the present work, global DNA supercoiling in *H. pylori* was visualized for the first time, by determining the supercoiling state of plasmids under different growth conditions. Using this method, we showed that cellular supercoiling was clearly growth phase-dependent in *H. pylori*. Coinciding with increased supercoiling during the growth phases, transcription of the *flaA* gene was increased, while the transcription of a second s^{28}-dependent gene with regular promoter spacing (HP0472) was reduced, supporting the hypothesis that growth phase-dependency of promoters might be mediated by changes of DNA topology. Supercoiling in *H. pylori* could be influenced in a reproducible fashion by inhibition of gyrase using novobiocin, which led to DNA relaxation and to a concomitant decrease of *flaA* transcript levels. Promoter spacer mutagenesis of the *flaA* promoter was performed. With *flaA* promoters of increased or reduced length, transcription of *flaA* was reduced, less susceptible to supercoiling changes, and, under specific conditions, inverted as compared to the wild type promoter. Transcriptional interdependence between the coupled *topA-flaB* genes and *flaA* was found by analysis of the *flaA* promoter mutants. Chromosomally linked *gyrA-flgR, and topA-flaB* genes were all dependent on supercoiling and coregulated with each other.

Comprehensive transcript profiling (DNA microarrays) of wildtype *H. pylori* with and without novobiocin treatment identified a number of genes (10% of total genes), including flagellin, virulence and housekeeping genes, which were strongly dependent on and appeared to be synchronized by supercoiling changes (transcriptional up- or downregulation). These findings indicate a tightly coupled temporal regulation of flagellar biogenesis and metabolism in *H. pylori,* dependent on global supercoiling. A specific group of genes was also regulated in *H. pylori* by overexpression of Topoisomerase I, as detected by genome-wide analysis (DNA microarray).

The DNA-bending protein HU is thought to be responsible for influencing the negative supercoiling of DNA, through its ability to wrap DNA. HU is encoded by the *hup* single gene in *H. pylori*, and constitutively expressed during the whole growth curve. An *H. pylori hup* mutant was constructed. *H. pylori* cells lacking HU protein were viable, but exhibited a severe growth defect. Our data indicate that the lack of HU dramatically changes global DNA supercoiling, indicating an important function of HU in chromosome structuring in *H. pylori*. Transcriptome analyses were performed and demonstrated that a total of 66 genes were differentially transcribed upon *hup* deletion, which include virulence genes and many other cell functions. The data indicate that HU might act as further important global regulator in *H. pylori*. Increased gene expression of heat shock proteins and a decreased transcription of the urease gene cluster may indicate a co-ordinated response of *H. pylori* to changes of environmental conditions in its specific ecological niche, mediated by HU.

After the whole genomic sequences of *H. pylori* strains 26695 and J99 were published, two ORFs (HP0116 and HP0440) were presumptively annotated as topoisomerase I orthologs. HP0116 is the functional *H. pylori* topoisomerase I (TopA). HP0440 (*topA2)* was found in only few (5 of 43) strains. Western blot analysis indicated that TopA2 is antigenically different from TopA. TopA2 is transcribed in *H. pylori*, but the protein must be functionally different from TopA, since it is lacking one functionally essential zinc finger motif, and was not able to functionally complement a TopA-deficient *E. coli*. Like *topA*, *topA2* was also transcribed in a growth phase-dependent manner. We did not find a function of TopA2 in DNA structuring or

topology, but, in the present study, we were able for the first time to establish a unique function for TopA2 in global gene regulation, by comprehensive transcriptome analysis (DNA microarray). Transcriptome analysis showed that a total of 46 genes were differentially regulated upon *topA2* deletion, which included flagellar genes and urease genes. These results suggest that TopA2 might act as a novel important regulator of both flagellar biosynthesis and urease in *H. pylori*.

Zusammenfassung

Die Mechanismen der globalen Kontrolle der Genregulation bei Bakterien sind bisher noch wenig charakterisiert. Unterschiede in der globalen oder lokalen Topologie der chromosomalen DNA spielen wahrscheinlich eine Rolle bei der globalen Kontrolle der Genexpression. Bei *Helicobacter pylori*, einem Bakterium mit wenigen funktionell definierten Transkriptionsregulatoren, spricht die Struktur einiger Promotoren dafür, daß sie durch die DNA-Topologie kontrolliert werden. Der Promotor des Hauptflagellingens *flaA*, ein Sigma-28 abhängiger Promotor, hat ein gegenüber dem Konsensuspromotor (15 Nukleotide) verkürztes Spacing von 13 Nukleotiden. Veränderungen der DNA-Superhelizität könnten ein Mechanismus der genspezifischen und globalen transkriptionellen Kontrolle in diesem Bakterium sein. Ziel dieser Untersuchungen war es zu zeigen, ob Veränderungen des globalen Supercoiling-Niveaus einen Einfluß auf die globale Genregulation von *H. pylori* haben und ob sie sich auf die zeitlich gesteuerte Regulation der Geißelbiosynthese (Beweglichkeitsorganell; essenzieller Virulenz- und Persistenzfaktor von *H. pylori*) in diesem Organismus auswirkt.

In der vorliegenden Arbeit wurde das Niveau der DNA-Superspiralisierung bei *H. pylori* erstmals durch Visualisierung des Supercoilingzustands von Plasmiden unter unterschiedlichen Wachstumsbedingungen dargestellt. Wir konnten mit dieser Methode zeigen, daß das zelluläre Supercoiling-Niveau bei *H. pylori* in Abhängigkeit von der Wachstumsphase stark variiert. In Wachstumsphasen mit erhöhtem Supercoiling war auch die Transkription des *flaA*-Gens erhöht, während die Transkription eines zweiten Sigma-28-abhängigen Gens mit normalem Promotorabstand (HP0472) reduziert war. Dieser Befund stützte die Hypothese, daß die wachstumsphasenabhängige Aktivität dieser Promotoren durch Veränderungen der DNA-Topologie bewirkt wird. Das Supercoiling-Niveau konnte reproduzierbar durch Hemmung der Gyrase mit Novobiocin beeinflusst werden. Die Gegenwart von

Novobiocin führte zur DNA-Relaxation und zu einem gleichzeitigen Absinken der Transkription von *flaA*. Es wurde eine gerichtete Mutagenese der Promotor-Spacer-Region des *flaA*-Promotors durchgeführt. Die Verlängerung oder Verkürzung des *H.pylori flaA*-Promotors führte zu einer verminderten Transkription von *flaA*, sowie zu reduzierter Empfindlichkeit der Promotoraktivität gegenüber Veränderungen des Supercoiling-Niveaus. Unter spezifischen Bedingungen war die Supercoiling-Abhängigkeit umgekehrt im Vergleich zum Wildtyppromotor. Es konnte weiterhin eine inverse transkriptionelle Abhängigkeit zwischen dem gekoppelten Genpaar *topA-flaB* und *flaA* durch Analyse der *flaA*-Promotormutanten nachgewiesen werden. Auch die chromosomal gekoppelten Gene *gyrA* und *flgR* sowie *topA* und *flaB* waren abhängig vom Supercoiling-Zustand und miteinander koreguliert. Die Analyse des Transkriptoms von *H.pylori*-Wildtypbakterien mit DNA-Microarrays mit und ohne Novobiocinbehandlung führte zur Identifizierung von zahlreichen Genen (etwa 10% des Gesamttranskriptoms), deren Expression Supercoiling-abhängig war und durch Veränderungen des Supercoilings synchronisiert verändert werden konnte. Unter diesen waren Flagellin-, andere Virulenz-, sowie Grundstoffwechsel-Gene. Diese Befunde weisen auf eine enge Verbindung zwischen der chronologischen Kontrolle der Flagellen-Biogenese und des Metabolismus bei *H. pylori*, die gemeinsam durch das Supercoiling-Niveau gesteuert werden. Eine definierte Gruppe von Genen konnte bei *H.pylori* durch Überexpression von Topoisomerase-1 reguliert werden.

Das Protein HU beeinflusst ebenfalls das Supercoiling-Niveau von DNA durch seine Fähigkeit, DNA zu biegen. HU wird bei *H. pylori* durch das Gen *hup* kodiert und ist während sämtlicher Wachstumsphasen konstitutiv exprimiert. Eine HU-defiziente Mutante wurde konstruiert. Zellen, die kein HU-Protein exprimierten, waren lebensfähig, zeigten aber einen deutlichen Wachstumsdefekt. Unsere Daten weisen daraufhin, daß der Mangel von HU sich dramatisch auf das globale DNA-Supercoiling-Niveau auswirkt, und sprechen für eine wichtige Funktion von HU bei der Kontrolle der DNA-Struktur von *H. pylori.* Mittels DNA-Microarray-Hybridisierung wurden die Transkriptome von *H. pylori*-Wildtyp und

HU-Mutante miteinander verglichen. Die Ergebnisse zeigen, daß insgesamt 66 Gene in der HU-Mutante differentiell transkribiert werden, darunter Virulenzgene und Gene für viele andere Zellfunktionen. Diese Daten deuten darauf hin, daß auch HU eine wichtige Rolle in der Kontrolle der globalen Genexpression bei *H. pylori* spielt. Die erhöhte Expression von Hitzestress-Proteinen, verbunden mit einer verminderten Transkription des Ureasegenclusters, könnte auf eine koordinierte Antwort der Bakterien auf Veränderungen der Umweltbedingungen in ihrer spezifischen ökologischen Nische hinweisen.

Nach der Publikation der Gesamtgenomsequenzen von *H.pylori* 26695 und J99 wurden 2 ORFs (HP 0116 und HP 0440) als Topoisomerase-1-Orthologe annotiert. HP 0116 ist das funktionelle *H.pylori*-Topoisomerase-1-Gen. HP 0442 (*topA2)* wurde nur in wenigen (5 aus 43) Stämmen nachgewiesen. *topA2* ist trotz seines seltenen Vorkommens kein Pseudogen und wird in *H.pylori* transkribiert. Westernblot-Analysen sprechen dafür, daß TopA2 sich antigenetisch von TopA unterscheidet. Das TopA2-Protein unterscheidet sich ebenfalls funktionell von TopA, da ihm ein funktionell essentielles Zinkfingermotiv fehlt. TopA2 konnte außerdem eine TopA-defiziente *E.coli*-Mutante nicht funktionell komplementieren. Wie bei *topA* war auch die Transkription von *topA2* von der Wachstumsphase abhängig. Eine Funktion von TopA2 bei der Kontrolle der DNA-Topologie konnte bisher nicht nachgewiesen werden, Transkriptomanalysen zeigten aber, dass TopA2 eine klare Regulationsfunktion hat, da die *topA2*-Mutante gravierende Veränderungen des Transkriptoms gegenüber dem Wildtyp aufwies. Diese Untersuchungen zeigten, daß 46 Gene in der *TopA2*-Mutante differentiell reguliert wurden, darunter Flagellengene und Ureasegene. Die Ergebnisse sprechen dafür, daß *TopA2* ein weiterer wichtiger Regulator von sowohl Flagellenbiosynthese als auch Ureasebildung bei *H.pylori* sein könnte.

Table of contents

1. Introduction

1.1. *Helicobacter pylori*, a worldwide pathogen

Helicobacter pylori is a microaerophilic, gram-negative, spiral-shaped bacterium, which was originally assigned taxonomically to the genus *Campylobacter* (Goodwin et al., 1990). In 1982, when Barry Marshall and Robin Warren first isolated this gastric pathogen by accidental extended incubation, few if any gastroenterologists would have predicted that almost 20 years later, this bacterium would have been shown to be the cause of one of the most common bacterial infections in humans and the etiologic agent of the majority of upper gastroduodenal diseases (Mitchell, 1999). Today, *H. pylori* is firmly established as a causative agent for duodenal ulcer (Graham et al., 1992), and a predisposing factor of gastric ulcer (Kuipers et al., 1995b). Furthermore, it is also considered as a risk factor for gastric adenocarcinoma (Parsonnet et al., 1993) and for B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Marshall, 1993). Accordingly, the World Health Organization has classified *H. pylori* as a type 1 carcinogen (Logan, 1994).

1.2. Epidemiology and transmission

H. pylori infection is ubiquitous and chronically infects up to 50% of the world's human population (Rocha et al., 2003; Malaty and Nyren, 2003). Although infection occurs worldwide, there are significant differences in the prevalence of infection both within and between countries (Woodward et al., 2000). In general, the overall prevalence of *H. pylori* infection in developing countries is higher than that in developed countries (Goh, 1997). This difference in infection prevalence has been attributed to the rate of acquisition of *H. pylori* in

childhood (Mitchell, 1999). Prevalence in adults ranges from 10-50% in the developed countries and up to 80-90% in developing countries (Rothenbacher and Brenner, 2003). Within each geographic region, there was also an increase in prevalence with age, with the prevalence of *H. pylori* infection in children under 10 years resident in developed countries being approximately 0 to 5% compared with 13% to 60% in children resident in developing countries (Frenck, Jr. and Clemens, 2003). In developing countries, persons of lower socioeconomic status have higher infection rates. In adulthood, the continuous risk of acquisition of *H. pylori* is approximately 1% per year. In addition, although *H. pylori* infects both males and females, in many populations, males appear to have 20-30% higher rates of infection than females (Replogle et al., 1995).

The exact source of infection has yet to be determined. A number of studies have proposed that acquisition of *H. pylori* occurs via a common environmental source (Mitchell, 1999). In particular, contaminated food, animals and water have been implicated as potential sources of infection. Laboratory experiments have found that *H. pylori* may survive up to a week in water (Fan et al., 1998). Lu et al have been able to culture the organism from an environmental water source (Lu et al., 2002).

Although the mode of transmission of *H. pylori* is not definitively known, the most widely held hypotheses are that the organism is transmitted directly from person to person by human feces (fecal-oral spread), gastric contents (gastric-oral spread), or improperly cleaned endoscopic equipment (Mitchell, 1999). Oral-oral transmission between parents and child is perhaps most important. Evidence supporting a fecal-oral spread of the organism has been reported by the Dowsett group from central America. Using PCR-based technology, investigators were commonly able to amplify *H. pylori* DNA from oral secretions (Dowsett et al., 1999). *H. pylori* has been cultured from vomitus, saliva, and diarrheal stools (Leung et al., 1999) suggesting gastric-oral routes of transmission (Covacci et al., 1999). Improperly cleaned tubes, endoscopes

or specimens in contact with the gastric mucosa from one person can inoculate *H. pylori* to other persons (Akamatsu et al., 1996). Improved disinfection of endoscopes has reduced the incidence of transmission (Katoh et al., 1993; Tytgat, 1995). In addition, the housefly, a common resident of households in developing countries has been touted as a potential mode of transmission of *H. pylori* (Frenck, Jr. and Clemens, 2003). However, whether *H. pylori* reaches the oral cavity via the gastro-oral, oral-oral, or fecal-oral route remains open for conjecture.

1.3. Pathogenesis and therapy

H. pylori causes acute and chronic inflammation in the stomach, although the magnitude of inflammation varies from strain to strain and from host to host. In the majority of infected humans, there are no clinical consequences to *H. pylori* gastritis (Covacci et al., 1999). In 20% to 30%, however, the end result of infection can be life-threatening (Covacci et al., 1999). *H. pylori* colonizes only the gastric epithelium but occasionally also areas of gastric metaplasia in the upper duodenum (Goodwin et al., 1997). There is no doubt now that *H. pylori* plays a causal role in the development of duodenal and gastric ulcer and should be eradicated in case of diagnosis of such a condition (Malfertheiner, 1997). Carriage of *H. pylori* also is strongly associated with the risk of development of atrophic gastritis (Blaser, 2000), which is a precursor lesion to gastric cancer. Thus, not surprisingly, *H. pylori* carriage also is associated with adenocarcinoma of the distal stomach (Parsonnet et al., 1993; Talley et al., 1991). Infection is associated with both the intestinal and diffuse histologic types of tumors (Nomura and Stemmermann, 1993). This association is extremely important, since gastric cancer is the second leading cause of cancer death in the world (Neugut et al., 1996).

Various other conditions have been linked to *H. pylori* infection, such as

heart disease or other extra-digestive afflictions, among them atopic diseases or sudden infant death (Gasbarrini and Franceschi, 1999), but none of these associations has been consistently demonstrated. For example, although early reports suggested that *H. pylori* infection was associated with heart disease (Mendall et al., 1994; Patel et al., 1995), later studies suggested that this association may have been confounded by other factors (Lip et al., 1996; Sandifer et al., 1996).

Treatment of *H. pylori* infection, by triple therapy with one proton-pump inhibitor and two antibiotics, has been proven to be highly effective in well-controlled clinical trials. Nevertheless, drug treatment faces various problems (Graham and Qureshi, 2000). The real efficacy of eradication treatment at the level of the general practitioner is not well known, and could be significantly lower than that reported in controlled studies. The high number of tablets to be taken daily can diminish the patient's compliance. Side effects of these treatments are not rare. The emergence of *H. pylori* strains resistant to antibiotics is seriously limiting cure rates (Megraud and Lamouliatte, 2003). In some areas for some antibiotics, resistance can approach 50%. High re-infection rates have been reported mainly from areas with high levels of transmission. Since treatment is only given to symptomatic patients, patients without symptoms would still remain at risk of developing severe complications of *H. pylori*, such as atrophic gastritis and gastric cancer (Ruggiero et al., 2003). Furthermore, antibiotics cannot be used to eradicate the infection from the whole population, especially in developing countries. It is therefore predictable that although antibiotics are a good solution for individual treatment of disease, they will not represent a definitive solution for society. Hence, vaccination – the most effective medical practice in controlling infectious diseases on a global scale – may represent the ultimate solution (Ruggiero et al., 2003; Suerbaum and Josenhans, 1999).

1.4. Morphology and microbiology

H. pylori exists in two forms: the spiral form and the coccoid form. *H. pylori* in vivo and under optimum in vitro conditions is an S-shaped bacterium with 1 to 3 turns, 2.5 x 5 µm long, and 0.5 to 1.0 µm wide, with a tuft of 5 to 7 polar sheathed flagella (Goodwin et al., 1990). Each flagellum is approximately 30 µm long and approximately 2.5 nm thick (Goodwin et al., 1990; Geis et al., 1993). Flagella exhibit a characteristic terminal bulb, which is an extension of the flagellar sheath (Goodwin et al., 1990; Geis et al., 1993). The flagellar sheath exhibits the typical bilayer structure of a membrane (Geis et al., 1993). After prolonged culture on solid or in liquid medium, coccoid forms typically predominate (Bode et al., 1993b; Nilius et al., 1993). By electron microscopy, coccoid forms appear as U-shaped bacilli with the ends of two arms joined by a membranous structure. Coccoid forms retain metabolic activity for some time; however, they cannot be cultured in vitro (Bode et al., 1993b; Nilius et al., 1993).

1.5. Genetics and diversity

H. pylori is the most genetically diverse bacterial species (Suerbaum et al., 1998b), since *H. pylori* strains isolated from different patients display a high degree of genetic variability. Comparison of the genomic sequence of two independent clinical isolates, 26695 (a *H. pylori* strain that has been isolated from an English patient with unknown medical history in the 1980s (Tomb et al., 1997)) and J99 (a *H. pylori* strain isolated from a U. S. patient with a duodenal ulcer in 1994 (Alm and Trust, 1999)) has shown that the genomes of two strains are not so diverse, since they have 1406 genes in common, however, 117 and 89 genes are specific for 26695 and J99, respectively (Suerbaum, 2000).

The infection is usually acquired in childhood, and persists for the entire life of the person (Suerbaum and Michetti, 2002). Bacteria isolated from the same patient at intervals of several years have identical DNA fingerprints, and mixed infections are uncommon (Miehlke et al., 1999). Three main sources account for the continuous evolution within the stomach of the infected person: 1) nucleotide mutations arising from replications errors on normal or damaged DNA templates (Wang et al., 1999); 2) intragenomic chromosomal rearrangement, which is frequently mediated by transposons and repetitive sequence element (Suerbaum, 2000); 3) horizontal transfer of new genes from an external donor due to natural transformation (Suerbaum et al., 1998b; Falush et al., 2001).

The family has been noted as the core unit of *H. pylori* transmission. Analysis of strains isolated from members of families with multiple *H. pylori*-infected family members have shown that these can carry almost indistinguishable strains with identical nucleotide sequences at multiple loci or very similar fingerprints (Suerbaum et al., 1998b). These observations have demonstrated that transmission of *H. pylori* is most likely to be frequent within the family or among infants within a community (Marshall et al., 1998).

1.6. Gastric *Helicobacter* species and animal models of *H. pylori* infection

The well-documented host range of gastric *Helicobacter* species extends from human and nonhuman primates to a variety of domesticated and feral land mammals (Table 1) (Solnick et al., 2001). The defining feature of gastric *Helicobacter* species is that they all express urease, and present multiple monopolar or bipolar flagella, which appears to be essential for colonization of this niche.

Table 1. Gastric *Helicobacter* taxa*

Taxon	Natural host
H. acinonychis	cheetah
H. bizzozeronii	dog
"Candidatus Helicobacter bovis"	cattle
H. felis	cat, dog
H. heilmanni	human
H. mustelae	ferret
H. pylori	human, rhesus macaque
"Candidatus Helicobacter suis"	pig
H. salomonis	dog
H. suncus	house musk shrew

*Taxonomy of the *Helicobacter* Genus (Solnick et al., 2001)

Several biochemical and genetic criteria distinguish this genus, although significant intra-genus variation has been described. Members of the genus *Helicobacter* have G+C contents ranging from 30-48% (Fox and Wang, 1997). All of these organisms are microaerobic and possess a respiratory type of metabolism (Chalk et al., 1994). Successful cultivation of *Helicobacters* requires a humid atmosphere enriched for carbon dioxide (5%-12%). The optimal growth temperature is between 37ºC and 42ºC (Solnick et al., 2001).

The need for animal models of infection became clear immediately after *H. pylori* was found to be associated with severe gastric diseases (Del Giudice et al., 2001). *Helicobacter* models were developed using animals that were either already naturally infected with closely related *Helicobacter* spp., such as ferrets (Fox et al., 1991) and nonhuman primates (Dubois et al., 1996), or with those that could be experimentally infected with *H. pylori*, including gnotobotic piglets (Eaton et al., 1991; Eaton and Krakowka, 1994) and dogs (Rossi et al., 2000). An *H. pylori* mouse model permitting the establishment of the long-term, high bacterial density *H. pylori* colonization was described (Marchetti et al.,

1995). Experimental *H. pylori* infections have also been developed in Mongolian gerbils (Hirayama et al., 1996; Honda et al., 1998; Wirth et al., 1998), guinea pigs (Shomer et al., 1998), cats (Esteves et al., 2000) and macaque monkeys (Fox et al., 1991).

Animal models are very useful in the search for antimicrobial therapies, in the analysis in vivo of potential virulence factors, in the dissection of the immune responses during infection, and in providing baseline information for the development of vaccines against *H. pylori*.

1.7. Virulence factors of *H. pylori*

Bacterial virulence factors (or virulence determinants) may be defined as molecular traits that are necessary for pathogenesis, or whose loss significantly reduces pathogenesis (Salyers, 1993). Infection with *H. pylori* results in significant morbidity and mortality. Several bacterial factors that contribute to the virulence of *H. pylori* have been identified.

1.7.1. Urease

H. pylori produces large amounts of the enzyme urease. The native urease of *H. pylori* has a molecular mass of approximately 540 kDa and is a nickel-containing hexameric molecule consisting of two subunits, UreA (30 kDa) and UreB (62 kDa), in a 1:1 molar ratio (Hu and Mobley, 1990; Dunn et al., 1991; Dunn et al., 1991). The *H. pylori* urease gene cluster contains nine genes, including the *ureA* and *ureB* structural genes, as well as regulatory genes involved in the synthesis and assembly of the holoenzyme (Mobley, 1996).

Urease is a potent stimulus of mononuclear, phagocyte activation and inflammatory cytokine production (Harris et al., 1996), and has been demonstrated as a potent virulence factor for *H. pylori*. Activity of the enzyme

is crucial for virulence, as urease is essential for colonization and persistence in the stomach (Ferrero et al., 1992; Eaton and Krakowka, 1994; Tsuda et al., 1994; Andrutis et al., 1995). Isogenic urease-negative mutants were unable to colonize gnotobiotic piglets regardless of whether the piglets had normal acid output (Eaton et al., 1991) or had been rendered achlorhydric (Eaton and Krakowka, 1994). Tsuda M. et al. demonstrated the inability of *H. pylori* urease mutants to colonise the stomachs of nude mice (Tsuda et al., 1994), and isogenic urease-negative mutants of *H. mustelae* failed to colonize ferrets (Andrutis et al., 1995).

It is generally presumed that urease activity is required for production of a neutral microenvironment for the organism within the gastric lumen (Phadnis et al., 1996). There is considerable evidence that urease is associated with the outer membrane of *H. pylori* (Hawtin et al., 1990; Dunn et al., 1990; Bode et al., 1993a). However, some urease activity also is observed within the cytoplasm (Bode et al., 1993b; Phadnis et al., 1996), suggesting a role in assimilation of organic nitrogen. The association between urease and the bacterial surface is apparently stabilized by divalent cations such as Ca^{2+} and Mg^{2+}, although other cations can inhibit the activity of the enzyme (Perez-Perez et al., 1994). A recent study has indicated that the intracellular enzyme appears to play a more important role in acid response (Scott et al., 1998).

1.7.2. Vacuolating cytotoxin A (VacA)

The vacuolating cytotoxin VacA is one of the most important virulence factors of *H. pylori*. Approximately one-half of *H. pylori* strains produce VacA in vitro (Leunk et al., 1988). Coincubation of recombinant VacA protein with gastric cell lines induces vacuole formation in the cytoplasm (Cover and Blaser, 1992), and VacA-expressing strains appear to colonize the stomach more efficiently (Salama et al., 2001). However, early studies showed that isogenic *vacA* mutants can colonize and trigger gastritis in both gnotobiotic piglets and Mongolian gerbils (Eaton et al., 1997; Wirth et al., 1998). Thus, while VacA is

believed to be a type of virulence factor, it does not appear to be absolutely required for *H. pylori* virulence.

VacA is a secreted protein toxin that is responsible for the gastric epithelial erosion observed in infected hosts(Covacci et al., 1999). VacA is expressed as a 140-kDa precursor that is processed to a functional 94-kDa toxin. Following export and exposure to acid, this toxin assembles spontaneously into oligomers. Polymorphisms in VacA account for much of the variation among clinical isolates that can be distinguished by antigenicity (Atherton et al., 1995). The association of VacA production and the occurrence of tissue damage have been demonstrated (Papini et al., 1998). It has been reported that purified VacA forms pores with a relative selectivity for anions both across planar lipid bilayers (Iwamoto et al., 1999; Tombola et al., 1999) and in Hela cell plasma membranes (Szabo et al., 1999). It was also suggested that VacA reduce the trans-epithelial electrical resistance of polarized epithelial monolayers by loosening tight junctions (Telford et al., 1994; Molinari et al., 1997; Papini et al., 1998; Pelicic et al., 1999). Tombola and colleagues report a specific function for VacA in the pathogenesis of stomach ulcers (Tombola et al., 2001). They show that *H. pylori* VacA binds preferentially to the apical plasma membrane, permeabilizing the host cell to urea (Tombola et al., 2001). VacA therefore acts as a low pH-activated transmembrane pore. It causes vacuolar degeneration of target cells by interfering with intracellular membrane fusion. The vacuoles appear to be a hybrid between lysosomal and late endosome compartments, and their generation requires the vacuolar ATPase proton pump and small GTP binding protein Rab7, which is a late endosome marker (Molinari et al., 1997; Papini et al., 1997). Recently, It has been shown that VacA causes apoptosis in the epithelial cell (Galmiche et al., 2000), and reduction of acid secretion by proapoptotic targeting of parietal cells (Boquet et al., 2003).

While all *H. pylori* strains seem to harbor the *vacA* gene, only some of them produce an active toxin. It has been shown by Atherton et al. (Atherton et al., 1995) that *vacA* has a mosaic structure with conserved and variable regions.

Two regions have sequence differences important enough to define variants (alleles), i.e., the *s (s1/s2)* region encoding a signal peptide and the *m (m1/m2)* region encoding an intermediary region (Atherton et al., 1995; Van Doorn et al., 1999). The amount of cytotoxic activity produced is maximum with the *s1m1* allele, followed by the *s1m2* allele, while no cytotoxic activity is found when *s2m2* is present (Forsyth et al., 1998). The worldwide distribution of these alleles is not homogeneous (Ito et al., 1997).

1.7.3. Adhesins

Although the majority of *H. pylori* organisms in infected patients are free living in the mucus layer (Lee, 1994), a proportion appears to adhere to the epithelial cells of the gastric tissue (Hessey et al., 1990; Lee et al., 1993). Several different *H. pylori* adhesins that mediate binding to gastric epithelial cells have been characterized, including the blood-group antigen binding adhesins (BabA) targeting Lewis b epitopes (Boren et al., 1993), sLex-binding adhesin (SabA) (Mahdavi et al., 2002) and several other outer membrane proteins (AlpA, AlpB, HopZ, etc.) (Odenbreit et al., 1999; Peck et al., 1999; Yamaoka et al., 2000). Among them, The BabA is intensively investigated because patients with blood group 0 run a greater risk of developing gastric ulcer (Clarke and Thomson, 2002). BabA adhesin was shown to be localized on the outer membranes of bacterial cells, and mediates attachment to the difucosylated Lewis^b blood group antigen (Ilver et al., 1998). In addition, sulfate and lipid-binding adhesin (Lingwood et al., 1993; Kamisago et al., 1996) and laminin-binding adhesin (Trust et al., 1991) have been reported, but these adhesions have yet to be fully characterized.

The *H. pylori* Hop family of outer membrane protein (Alm et al., 2000) contains many potential adhesins. Odenbreit et al. reported that two additional outer membrane proteins, named AlpA and AlpB, both function in specific adherence of *H. pylori*. Isogenic mutants of *alpA* and of *alpB*, respectively, showed greatly reduced adherence to surface epithelial cells on human gastric

tissue sections (Odenbreit et al., 1999). Peck et al. (Peck et al., 1999) showed that isogenic mutants of the gene encoding the outer membrane protein HopZ have greatly reduced adherence to human gastric epithelial cells; expression of *hopZ* appears to be regulated by slipped-strand mispairing within a CT dinucleotide repeat motif in the coding region of the signal sequence. Namavar et al. (Namavar et al., 1998) identified a 16kDa surface protein that adhered to oligosaccharide ligands such as sulfated Lewis^a and present on mucin glycoproteins. Recently, Mahdavi et al. (Mahdavi et al., 2002) identified the sLex-binding adhesin SabA, which also belongs to this family.

Considering the adhesins previously described and the large family of lipoproteins and OMPs identified (Tomb et al., 1997), it appears that *H. pylori* can use several redundant adherence mechanisms for successful attachment to epithelial cells.

1.7.4. Heat shock protein

The chaperones of the human gastric pathogen *H. pylori* have provoked particular scientific interest because of their possible implication in the pathogenesis of this organism. The sequence of the gene encoding the HspB protein of *H. pylori* is highly conserved compared with those of heat shock proteins of other bacteria and humans (Macchia et al., 1993). *hspB* is part of a bicistronic operon (*hspA*-*hspB*), which has been cloned and sequenced (Suerbaum et al., 1994). The *hspA* gene, which is located upstream of *hspB*, codes for the *H. pylori* homolog of the GroES heat shock protein. The *H. pylori hspA* gene is unique in that it contains a nickel-binding site at its C-terminus (Suerbaum et al., 1994). Expression of the *hspA* and *hspB* heat shock proteins together with the *H. pylori* urease increases the activity of urease in functional complementation experiments (Suerbaum et al., 1994). Thus, *hspA* may play a role in the integration of nickel into the functional urease molecule (Suerbaum et al., 1994).

The urease enzyme and the GroESL chaperone machinery both can be

found on the extracellular surface of *H. pylori* cells, where they are thought to be closely associated (Kansau and Labigne, 1996; Vanet and Labigne, 1998).

1.7.5. Flagella and motility

The motility of *H. pylori* is considered a virulence factor, more correctly a colonization factor, since it has been demonstrated repeatedly that non-motile strains are unable to colonise or survive in the host. The flagella of *H. pylori* have been extensively studied, and convincing evidence demonstrated that the flagella play key a role in the colonization of the human gastric mucosa.

Normally, *H. pylori* possesses two to six polar, sheathed flagella, whose filaments are a copolymer of the flagellin subunits FlaA (Leying et al., 1992) and FlaB (Suerbaum et al., 1993). FlaA is the predominant subtype and FlaB is the minor subtype, localized close to the base of the flagellum (Kostrzynska et al., 1991). Both flagellins have a similar molecular mass, 53 kDa for FlaA and 54 kDa for FlaB. The two flagellins display considerable amino acid homology (58% identity), and the respective genes are unlinked on the chromosome (Suerbaum et al., 1993). The experimental results obtained by Josenhans et al. (Josenhans et al., 1995; Niehus et al., 2002) suggest that relative composition of the flagellar filaments may vary and adapt to environmental conditions.

The motility of *H. pylori* is essential for colonization. It allows the bacteria to spread through the viscous mucus covering the epithelial cells of the gastric mucosa (Hazell et al., 1986). Strains defective in the expression of the gene for the major and minor flagellar subunits, FlaA and FlaB, respectively, were compared with the wild-type strain for colonization and persistence in the well-established gnotobiotic piglet mode (Eaton et al., 1996). The wild type strain colonized all piglets and persisted for two, four and ten days after inoculation. In contrast, strains lacking either flagellin and thus with impaired motility, were recovered after two and four days, but not ten days after inoculation. The double mutant was isolated after two days but not thereafter. The data indicate the importance of motility in the colonization by *H. pylori* of a

new host. The studies of Haas et al. (Haas et al., 1993) and Josenhans et al. (Josenhans et al., 1995) have also confirmed the role of the flagella and resulting motility in *H. pylori* pathogenicity. Complementary results were obtained in a broadly similar examination of the importance of motility in the closely related *H. mustelae* (Andrutis et al., 1997) and *H. felis* (Josenhans et al., 1999).

Like other bacteria, *H. pylori* and the closely related *H. mustelae*, the gastric pathogen of ferrets, possess a hook, which is essential for biosynthesis of the flagellar filament (O'Toole et al., 1994). Mutants with mutations in the *flgE* gene, which encodes the flagellar hook protein, are nonmotile and aflagellate but continue to synthesize flagellins (O'Toole et al., 1994). Mutants with mutations in the *flbA* (*flhA*) gene, which is involved in the regulation of motility, are nonmotile and fail to express either flagellin or the hook protein (Suerbaum, 1995; Schmitz et al., 1997). Mutants in *fliD*, which encodes a hook associated protein, produce truncated flagella and are severely impaired in motility and their ability to colonize the gastric mucosa of mice (Kim et al., 1999)

Some genes required for flagellum assembly have similarities to those involved in type III secretion systems (Aizawa, 2001). The proteins forming the P and L rings have able to cleave signal peptides and are exported through the *sec*-dependent pathway, whereas the external flagellar components are secreted through a specialized pathway encoded by flagellar genes. The flagellar export pathway is assembled from the gene *fliH, fliI, fliP, flhA, flhB, fliQ, fliR* and others (Aizawa, 2001). Worku et al (Worku et al., 1999) confirmed that *H. pylori* demonstrates chemotaxis, which mediated, in particular, through the amino acids glutamine, histidine, lysine and alanine. Similarly, the studies of Yoshiyama et al. (Yoshiyama et al., 1998) and Nakamura et al. (Nakamura et al., 1998) lead to the conclusion that movement of *H. pylori* in the viscous mucus layer is enhanced by chemotactic activity toward urea and bicarbonate.

Attempts have been made to correlate the degree of *H. pylori* motility with cytokine response levels and severity of disease. For example, a correlation

has been described between motility levels and IL-8 induction (Watanabe et al., 1997). It has also been reported that there is a significantly lower degree of motility in strains isolated from remnant gastritis, as distinct from chronic gastritis, peptic ulceration or gastric cancer (Kurihara et al., 1998). This may indicate that the type and stage of gastric disease could dictate the selective pressure for maintenance of high levels of motility.

1.7.6. Cytotoxin-associated gene A (CagA) and the *cag* pathogenicity island (PAI)

Many strains of *H. pylori* produce an immunodominant protein called CagA (Covacci et al., 1993; Tummuru et al., 1993). The *cagA* gene that encodes CagA is localised at one end of *cag* pathogenicity island (*cag* PAI), an approximate 40 kb DNA segment that has been assumed to have been acquired by *H. pylori* during its evolution (Censini et al., 1996; Akopyants et al., 1998). The *cag* PAI contains 31 genes, which are thought to encode components of an *H. pylori*-specific type IV secretion system, through which bacterial proteins are translocated from the inside to the outside of the bacterial cells and into host cells.

Early studies have indicated that *cag*-positive *H. pylori* strains are associated with higher grades of gastric inflammation, and they are thus considered to be more virulent than the *cag*-negative strains (Kuipers et al., 1995a; Crabtree et al., 1991). Consistently, many epidemiological studies have shown a positive relationship between *cag*-positive *H. pylori* and atrophic gastritis, peptic ulcer disease, and gastric cancer (Blaser et al., 1995; Parsonnet et al., 1997; Rugge et al., 1999; Rudi et al., 1997; Torres et al., 1998; Shimoyama et al., 1998; Nomura et al., 2002).

Although CagA is regarded as an important *H. pylori* virulence determinant, the presence or absence of CagA is not sufficient for the prediction of a clinical outcome associated with *H. pylori* infection. The genetic diversity of the infecting *H. pylori* strains could also play an important role in the development

of disease. Indeed, CagA is characterized by its amino acid sequence polymorphism, and the molecular mass of CagA protein varies from 120 to 145 kDa, primarily depending on the duplication of various numbers of a 34-amino acid sequence located at the C–terminal region of the protein (Covacci et al., 1993; Yamaoka et al., 1998; Yamaoka et al., 1999; Segal et al., 1999; Asahi et al., 2000; Stein et al., 2000; Odenbreit et al., 2000). This structural diversity among different CagA proteins raises the possibility that the degree of virulence of CagA may vary from one strain to the other, depending on the sequence variation. Recently, Amieva et al. (Amieva et al., 2003) demonstrate that CagA appears to target *H. pylori* to host cell intercellular junctions and to disrupt junction-mediated function.

In *H. pylori*, the *cag* PAI induces epithelial cells to secrete interleukin-8, a mediator of inflammation, by activating nuclear factor kappa B complexes (Crabtree et al., 1999).

Both a functional *cagA* gene and a functional type IV injection system were found to be important for virulence of *H. pylori*.

1.7.7. Other virulence factors

There are other pathogenic factors of *H. pylori* that have been proposed to be directly or indirectly responsible for mucosal damage (Megraud, 1996).

LPS (Lipopolysaccharide): *H. pylori* LPS disrupts the gastric mucus coat by interfering with the interaction between mucin and its mucosal receptor. However, the outstanding feature of the *H. pylori* LPS is its low proinflammatory activity (Piotrowski et al., 1995). The phenomenon is mediated by its unique lipid A structure and affects binding to CD14 (Kirkland et al., 1997).

NAP: the neutrophil activating protein is one molecule of *H. pylori* that acts directly on the surrounding tissues. NAP is an oligomeric protein made of 10 to 12 copies of a 17kD polypeptide with homology to iron-binding proteins. This protein has the capacity to activate neutrophils and may be in involved in the

recruitment of these cells to the site of inflammation (Evans, Jr. et al., 1995; Montecucco and de Bernard, 2003).

It is clear today that no single factor can be considered responsible for a specific outcome of *H. pylori* infection. We can hypothesise that a combination of factors increases the risk of developing severe sequelae.

1.8. DNA supercoiling

1.8.1. Concept of DNA supercoiling

DNA supercoiling is a phenomenon occurring in covalently closed, circular duplex DNA molecules when the number of turns differs from the numbers found in DNA molecules of the same length but containing an end that can rotate (Drlica, 1992). Supercoiling creates strain in closed DNA molecules, and a deficiency of duplex turns generates negative supercoiling. Negative supercoiling makes DNA more flexible, facilitating loop formation, wrapping of DNA around proteins, and the formation of left-handed Z-DNA (Drlica, 1992).

1.8.2. DNA topoisomerases

DNA topoisomerases are the enzymes that maintain the optimum topological state of DNA in the cell (Zechiedrich et al., 2000). The basic role for topoisomerases is to separate the two strands of the helix either temporarily, as in transcription or recombination, or permanently, as in replication. During DNA replication, the two strands of the DNA must be completely unlinked by topoisomerases, and transcription itself generates positive supercoiling ahead of and negative supercoiling behind the translocating RNA polymerase that are rapidly resolved by DNA gyrase and DNA topoisomerase I, respectively (Wu et al., 1988; Wang, 1998). In addition, transcription from some promoters in bacteria require a minimal level of negative supercoiling, but too much supercoiling will be a sign of disaster. Also in the cells, completely replicated

chromosomes must be resolved by topoisomerases before partitioning and cell division can occur (Dasgupta et al., 2000; Weitao et al., 2000; Nordstrom and Dasgupta, 2001). These examples illustrate that the topological state of DNA itself must be adjusted to optimize DNA function.

1.8.2.1. Classification of topoisomerases

DNA cleavage by all topoisomerases is accompanied by the formation of a transient phosphodiester bond between a tyrosine residue in the protein and one of the ends of the broken strand. DNA topology can be modified during the lifetime of the covalent intermediate, and the enzyme is released as the DNA is religated. These enzymes that cleave only one strand of the DNA are defined as type I, and are further classified as either type IA subfamily members if the protein link is to a 5'-phosphate, or type IB subfamily members if the protein is attached to a 3'-phosphate. Topoisomerases that cleave both strands to generate a straggered double-strand break are grouped in the type II subfamily of topoisomerases (Wang, 1996).

1.8.2.2. Cellular role of topoisomerases

There are four known topoisomerases in the Gram-negative bacterium *Escherichia coli*. These enzymes consist of the two type IA enzymes, DNA topoisomerase I and III, and the two type IIA enzymes, DNA gyrase and DNA topoisomerase IV (Deibler et al., 2001). Although some overlap of function has been shown genetically, each of the DNA topoisomerases appears optimized to carry out its own particular set of topological manipulations. DNA gyrase is the only known topoisomerase able to generate negative supercoiling by hydrolyzing ATP, and is responsible for global generation of negative supercoiling in the bacterial chromosome (Snoep et al., 2002). Such global supercoiling in combination with the activity of the *E. coli* Muk proteins is essential for chromosome condensation leading to proper chromosome

partitioning at cell division (Holmes and Cozzarelli, 2000; Sawitzke and Austin, 2000). Topoisomerase IV also relaxes negative supercoiling in the cell (Zechiedrich et al., 2000), implicating topoisomerase IV along with topoisomerase I (Tse-Dinh, 1998) as activities that prevent excessive negative supercoiling by DNA gyrase. Together, topoisomerase I and IV along with DNA gyrase set the steady-state level of negative supercoiling that is required for the initiation of replication and for transcription from at least some promoters (Zechiedrich et al., 2000).

An analysis of the genome sequences of 17 mesophilic eubacterial organisms for which a complete annotated sequence is available reveals that several bacteria (*Haemophilus influenzae, Bacillus subtilis,* and *Xylella fastidiosa)* have the same topoisomerase complement as *E. coli*. It seems likely that these enzymes share the same relative distribution of functions discussed above. However, given the apparent functional redundancy of topoisomerases III and IV mentioned above, it is not surprising to find a number of mesophilic eubacteria that appear to lack a homologue of topoisomerase III but still possess the other three topoisomerases. This group includes *Borrelia burgdorferi, Chlamydophila pneumoniae, Chlamydia trachomatis, Mycoplasma pneumoniae, Mycoplasma genitalium, Neisseria meningitidis, Rickettsia prowazekii, Synechocystis PCC6803,* and *Ureaplasma urealyticum*. Apparently in these cases topoisomerase IV is solely responsible for unlinking percatenates as well as daughter molecule catenates (see review, (Wang, 1996).

Based on the current level of genome annotation, a number of mesophiles (*Helicobacter pylori, Campylobacter jejuni, Deinococcus radiodurans, Mycobacterium tuberculosis,* and *Treponema pallidum*) appear to possess only two topoisomerase genes: one is homologous to the gene encoding *E. coli* DNA gyrase and a second to the *E. coli* topoisomerase I *topA* gene (Champoux, 2001). This configuration probably represents the minimal topoisomerase makeup for a bacteria cell. In these bacteria, the DNA gyrase

as the sole type II enzyme would negatively supercoil the DNA, relax positive supercoils associated with transcription and replication, and decatenate replicated DNA. In turn, topoisomerase I would function to relax negative supercoils associated with transcription and to prevent excessive negative supercoiling by DNA gyrase.

A reverse gyrase was found in the two hyperthermophilic bacteria, *Aquifex aeolicus* and *Thermotoga maritima*. This enzyme uses the energy from ATP hydrolysis to introduce positive supercoils into the chromosomal DNA. The exact role of reverse gyrase in *Hyperthermophiles* remains uncertain, but it seems likely that positive supercoiling of DNA functions to counteract the helix unwinding and strand separation effects of growth at high temperature (Forterre, 1996).

Topoisomerase V, originally identified in the hyperthermophile *Methanopyrus kandleri* (Slesarev et al., 1993; Slesarev et al., 1994), currently remains the only known example of a type IB topoisomerase in bacteria.

1.8.3. Gene regulation by DNA supercoiling

Available information concerning the nature of the DNA in the bacterial nucleoid strongly suggests that DNA supercoiling could play a key role in controlling gene expression. There are several ways in which transcription initiation can be either activated or repressed by the superhelical state of the DNA template (Gellert, 1981; Pruss and Drlica, 1989; Wang, 1996). If the rate-limiting step of the initiation reaction is polymerase binding, the effect of negative DNA supercoiling on the helical twist of the promoter can alter the alignment of RNA polymerase recognition elements on the face of the DNA helix (Opel and Hatfield, 2001). Depending on their spacing, this can either promote or inhibit RNA polymerase binding (Wang and Syvanen, 1992; Wang, 1997). The ability of negative superhelicity to destabilize local regions of the DNA duplex can also affect open complex formation and activate transcription initiation in cases where it is the rate-limiting step (Borowiec and Gralla, 1987;

Ehrlich et al., 1985; Amouyal and Buc, 1987).

The problem of unambiguously identifying DNA supercoiling as a regulatory motif is illustrated by the case of the leucine biosynthetic operon from *S. typhimurium* (Tan et al., 1994; Mojica and Higgins, 1996). A promoter-like mutation, *leu-500*, in this operon has played an important role in the history of research into the biology of DNA supercoiling in bacteria. Strains carrying this lesion are leucine auxotrophs due to a failure to transcribe *leu* from the *leu-500* promoter (Gemmill et al., 1984). It was subsequently found that a suppressor of *leu-500, supX,* is an allele of *topA* (Trucksis et al., 1981; Pruss and Drlica, 1985), and strains harbouring *supX* have increased levels of negative supercoiling in their DNA (Pruss and Drlica, 1985; Richardson et al., 1988). The *leu-500* mutation is an AT to GC transition in the *leu* promoter (Gemmill et al., 1984), suggesting that *supX* suppresses *leu-500* by increasing negative supercoiling at the promoter, thereby meeting the requirement for additional free energy of supercoiling for strand separation imposed by AT to GC transition. Treatment with DNA gyrase-inhibitors both reduces intracellular DNA supercoiling levels and eliminates suppression of *leu-500* by *topA* mutations, supporting the model of *leu-500* sensitivity to changes in DNA supercoiling (Pruss and Drlica, 1985). Later studies demonstrate the *leu-500* promoter to be completely dependent on adjacent transcription for activation (Chen et al., 1992; Tan et al., 1994; Wu et al., 1995; Mojica and Higgins, 1996; Spirito and Bossi, 1996; Chen et al., 1998). In particular, the promoter was active when positioned near the gene for membrane-anchored, tetracycline resistance determinant or in the region between divergently oriented promoters (Chen et al., 1992; Chen et al., 1998). These studies have shown that the *leu*-500 promoter can sense changes of negative supercoils resulting from the transcription of adjacent sequences. The *E. coli tyrT* promoter, which controls the minor tyrosine tRNA gene, was found to be exquisitely sensitive to mutations and to drugs that alter the level of DNA supercoiling (Free and Dorman, 1994; Bowater et al., 1994). The response of the *tyrT* promoter to

supercoiling is qualitatively similar to that of a known supercoiling-sensitive tRNA gene promoter, *hisR* (Figueroa et al., 1991; Figueroa-Bossi et al., 1998). Specifically, these studies also demostrate that treatments which increase in vivo DNA supercoiling levels enhance transcription of these tRNA genes. Significantly, the promoters of the *topA, gyrA* and *gyrB* genes are themselves supercoiling sensitive, with increases in negative supercoiling activating *topA* and decreases favoring expression of *gyrA* and *gyrB* (Menzel and Gellert, 1987; Tse-Dinh and Beran, 1988).

1.8.4. Histone like protein

Histone-like proteins have been shown to be involved in DNA supercoiling. The FIS, IHF, H-NS and HU protein were found in many bacteria, and play a role to turn on or turn off gene by bending the DNA to a certain angle. Also relevant may be the observation that cells do not tolerate the combined absence of HU, IHF, and H-NS, although cells are viable when only one of the three proteins is present. Perhaps at least one of the three bending proteins is needed for chromosome compaction (Drlica, 1992).

HU (heat unstable): HU was initially classified as a histone-like protein on the basis of its amino acid composition and its ability to wrap DNA into nucleosome-like particles *in vitro.* HU is a protein with long flexible arms that reach around DNA and force it to make a U-turn. HU does not recognize a specific nucleotide sequence, and was thought to provide a general bending activity. Several examples have been found in which HU serves as an architectural protein, assisting in the formation of site-specific DNA protein complexes that carry out site-specific recombination (Li and Waters, 1998; Kamashev and Rouviere-Yaniv, 2000). HU also provides the DNA bending needed for certain repressors to bring distant regions of DNA together in loops that block initiation of transcription (Lewis et al., 1999; Semsey et al., 2002).

IHF (integration host factor): IHF is a small heterodimeric bending protein

that is closely related to HU. IHF recognizes specific nucleotide sequence, and can cause DNA to turn 180 degree on itself. Many examples have been found in which IHF helps form a DNA loop between promoters and transcription activators far upstream from promoters (Giladi et al., 1998; Zhong et al., 2001; Gerstel et al., 2003), thereby facilitating initiation of transcription. IHF also participates as an architectural protein during the formation of site-specific DNA-protein complexes (Snyder et al., 1989; Segall and Nash, 1996). The best known of these is the intasome generated by bacteriophage lambda during integration into the bacterial chromosome (Snyder et al., 1989).

FIS (factor for inversion stimulation): FIS functions as a homodimer, regulates gene expression by binding to and bending gene promoters containing the degenerate consensus sequence (Pan et al., 1996). Because of its ability to alter DNA topology (Thompson et al., 1987; Gille et al., 1991), FIS is regarded as a sensor and global regulator (Xu and Johnson, 1995a; Xu and Johnson, 1995b), which serves as auxiliary element that is able to integrate environmental signals in order to adapt the expression of stress-inducible genes to the conditions in the surrounding medium (Hengge-Aronis, 1999). FIS acts as an upstream activator of transcription (Ross et al., 1990; Gonzalez-Gil et al., 1996). It is also a repressor of transcription and negatively autoregulates the promoter responsible for transcription of its own gene, *fis* (Walker et al., 1999). Importantly, *fis* has been shown to regulate levels of DNA supercoiling by repressing the *gyrA* and *gyrB* genes coding for DNA gyrase and activating *topA* gene coding for topoisomerase I in *E. coli* (Schneider et al., 1999; Weinstein-Fischer et al., 2000).

H-NS (histone-like nucleoid structuring): H-NS is unlike the other two, it does not actively bend DNA. Instead, it binds to DNA that is already bent. If an appropriate bend is near the promoter of a gene, H-NS will bind and act as mild repressor. H-NS is regarded as a global repressor. Genetic work using whole-genome methods has shown that H-NS plays a negative role in controlling expression of many unrelated bacterial genes, with up to 5% of the

genes in *E. coli* responding to its presence or absence (Hommais et al., 2001; Schroder and Wagner, 2002). H-NS action appears to be a general way to keep the expression of many genes down-regulated until their products are needed.

1.8.5. DNA supercoiling is altered by the environment

DNA supercoiling levels should ideally be environmentally responsive, in order to play a useful regulatory function in vivo. Many examples demonstrate that this is the case. Genetic work provided evidence that an ability to increase supercoiling of DNA was necessary for anaerobic growth of the facultative anaerobe *S. typhimurium* (Yamamoto and Droffner, 1985; Hsieh et al., 1991a; Marshall et al., 2000). Experiments showed a higher level of negative supercoiling of reporter plasmid DNA isolated from bacteria grown anaerobically, with gyrase activity being required for this elevation (Dorman et al., 1988). Increase in growth medium osmolarity has been shown to change reporter plasmid supercoiling in both *E. coli* and *S. typhimurium* (Higgins et al., 1988). Significantly, the deletion effects of the *topA*::Tn10 insertion mutation in *E. coli* were found to be offset by a reduction in growth medium osmolarity (Dorman et al., 1989) and increased sensitivity to oxidative challenges (Tse-Dinh, 2000). Changes in growth temperature also alter plasmid supercoiling as well, although the direction of change depends on the species of bacterium (Goldstein and Drlica, 1984; Dorman et al., 1990; Ogata et al., 1994; Camacho-Carranza et al., 1995; Mizushima et al., 1997). In fact, analyses of reporter plasmid supercoiling have shown the level of DNA supercoiling in vivo to be in a state of constant flux (Dorman et al., 1988; Parekh et al., 1996; Conter, 2003), providing the cell with a highly pleiotropic physiological barometer for environmental interpretation and response. This mechanism would provide a means of dealing with multiple environmental influences on the coordinated regulation genes contributing to completely different aspects of metabolism and survival (Weinstein-Fischer et al., 2000;

Conter, 2003); evidence from studies of sensitive promoters in enteric bacteria suggests that fluctuating levels of DNA supercoiling provides an overlap between responses to different environmental stimuli (Bhriain et al., 1989).

1.8.6. DNA supercoiling and pathogenesis

Bacterial infection processes may be described as adaptations by the pathogen to a series of microenvironments in the host. Every environmental factor offers the bacterium information concerning its situation in relation to the host. The pathogen can use these cues to ensure expression of features that are important for their survival (Soutourina et al., 2001).

Most virulence genes or presumptive virulence genes possess an element of environmental control in their regulation. This should make them excellent candidates for control by the constantly fluctuating levels of DNA supercoiling found in bacterial cells (Dorman et al., 1990). The OmpC and OmpF porins contribute to virulence in *S. typhimurium*, and the genes that code for them posses supercoiling-sensitive promoters (Tsui et al., 1988; Graeme-Cook et al., 1989; Dorman et al., 1989). In *C. jejuni,* DNA supercoiling is also involved in the regulation of major outer membrane protein porin expression (Dedieu et al., 2002). The chromosomally encoded invasion genes of *S. typhimurium* are unambiguous virulence factors, and their expression is sensitive to changes in DNA supercoiling (Galan and Curtiss, III, 1990; Leclerc et al., 1998; Morris et al., 2003). In this case, the environmental signal normally involved in their control is high osmolarity, a stress characteristic of the intestinal lumen. *Salmonella* invasiveness has also been shown to be dependent on growth phase and anaerobiosis (Lee and Falkow, 1990), both of which influence intracellular DNA supercoiling levels (Dorman et al., 1988; Bhriain et al., 1989; O'Byrne et al., 1992). Transcription of the invasion genes encoded by the virulence plasmid of *Shigella flexneri* is temperature regulated (Maurelli and Sansonetti, 1988; Bhriain and Dorman, 1993), and a role for DNA topology in this regulation has been described (Dorman et al., 1990; Falconi et al., 1998).

DNA topology has been identified as a regulatory factor in the transcriptional control of the *alg* genes (responsible for alginate production) of *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients (DeVault et al., 1991). These genes are activated by osmolarity, and their products contribute to the biosynthesis of alginate in the cystic lung. Interestingly, these genes belong to a regulon governed transcriptionally by a two-component signal transduction system closely related to the OmpR-EnvZ system, that regulates the OmpC and OmpF porins in enteric bacteria (Berry et al., 1989; Deretic et al., 1989; Bang et al., 2002). In addition to these control elements, the *alg* regulon is dependent on a histone like protein (AlgR3) for optimal control (Kato et al., 1990). These proteins contribute to the regulation of several DNA supercoiling-sensitive genes (e.g. *tyrT* and *rrnB* (Nilsson et al., 1990)), and they have been described as affecting the expression of a number of virulence factors, including adhesins. For example, phenotype of a loss of motility has been associated with a mutation in *hns* (Soutourina et al., 1999). The HN-S protein plays a role in the thermal control of transcription of the *pap* operon (Goransson et al., 1990; White-Ziegler et al., 1998), and also for optimal transcription of the *esp* operon in *E. coli* (Beltrametti et al., 1999). IHF is absolutely required for the stimulation of both FimB- and FimE-mediated site-specific DNA inversion, that controls phase variation of type 1 fimbriae expression (Blomfield et al., 1997), and also mediates repression of flagella in *E. coli* (Yona-Nadler et al., 2003). It also appears that the osmZ/virR locus, identified as affecting invasion gene expression in *S. flexneri*, includes the gene for H-NS and IHF (Dorman et al., 1990; Tobe et al., 1993; Porter and Dorman, 1997). Significantly, Fis is also emerging as an important regulator of virulence genes in bacterial pathogens. In addition to promoting transcription of invasion genes in *S. flexneri* (Falconi et al., 2001), Fis contributes to virulence gene regulation in *S. typhimurium* (Wilson et al., 2001) and enteropathogenic *E. coli* (Goldberg et al., 2001), and to biofilm formation by enteroaggregative *E. coli* (Sheikh et al., 2001).

1.8.7. DNA topology and global gene regulation

To enable an organism to be both metabolically efficient and rapidly adaptive, mechanisms must exist to coordinate its global patterns of gene expression to its growth and environmental conditions (Schroder and Wagner, 2002). Many examples demonstrate that DNA supercoiling plays a functional role in cellular adaptation and survival under various suboptimal growth conditions and during growth state transitions. Well-characterized examples include gene expression changes that occur in stationary phase (Kusano et al., 1996), osmotic stress (Higgins et al., 1988) and aerobic to anaerobic growth transitions (Dorman et al., 1988). Since changes in environmental parameters such as osmolarity and anaerobiosis result in a DNA supercoiling change of reporter plasmid (Higgins et al., 1988; Dorman et al., 1989), the environmental change must become manifest in a modulation of DNA topoisomerase activity. Increasing or decreasing DNA supercoiling levels may also activate certain promoters and can have consequences for other topological perturbations of DNA (e.g., looping or bending) that affect transcription (Koo and Crothers, 1988; Friedman, 1988). Physiological control of the genes coding for histone like protein (Nilsson et al., 1990), and the possible covalent modification of some of these proteins in response to physiological changes (Drlica and Rouviere-Yaniv, 1987), will affect protein-directed changes in DNA topology. Steck et al. (Steck et al., 1993) have used O'Farrell two-dimensional gels to show that topoisomerase mutations that cause *in vivo* changes in DNA supercoiling affect the levels of expression of more than one-third of cellular proteins. These results demonstrate that changes in DNA supercoiling levels affect the expression of many genes. Such a global regulatory network has equal value to the bacterium both as a commensal and as a pathogen.

1.9. Observation and aim of the work

H. pylori possesses only a topoisomerase I gene, and genes encoding a and ß subunits of gyrase (topoisomerase II), which are unlinked on the chromosome (Tomb et al., 1997; Alm and Trust, 1999). Other active topoisomerase-like genes could not be identified in the complete genome sequences, which seems to indicate the existence of a relatively simple system to maintain the cellular DNA supercoiling state in *H. pylori* (Suerbaum et al., 1998a). In addition, *H. pylori* possesses relatively few genes encoding regulatory factors in the published whole genome sequences (Tomb et al., 1997; Alm and Trust, 1999). Its bacterial genome is relatively small, and there are only three sigma factors (s^{80}, s^{54}, and s^{28}) and one anti-sigma factor, FlgM (Colland et al., 2001; Josenhans et al., 2002b). Only four genes encoding sensor kinases of two component signal transduction systems have been identified so far, as well as six corresponding response regulators (Beier and Frank, 2000; McDaniel et al., 2001), in comparison to more than 30 two component systems for *e. g. Pseudomonas aeruginosa* and *Bacillus subtilis* (Stover et al., 2000; Kunst et al., 1997). In addition, it is not entirely clear, how the formation of new flagellar filaments, which is most intimately coupled with cell division in *H. pylori,* insofar that a set of new, unipolar filaments is completed exactly at the moment of partition of the two daughter cells, can be so tightly regulated, since flagellar master regulators FlhC and FlhD are lacking in *H. pylori* (Tomb et al., 1997; Alm and Trust, 1999). These findings indicate that the mechanisms of flagellar gene regulation in *H. pylori* are significantly different from other bacteria, which may be most due to its special ecological niche.

DNA topology plays a role in the genetic processes that involve DNA, like DNA replication, transcription and recombination. DNA topology was found to be involved in the virulence regulation in other pathogens, such as the plasmid-encoded virulence genes in *S. flexneri* (Dorman et al., 1990; Tobe et

al., 1993; Porter and Dorman, 1997; Falconi et al., 2001) and type I fimbriae in *E. coli* (Blomfield et al., 1997), but no such role has been assigned with certainty in *H. pylori*. The following genetic hints indicate supercoiling-dependency of the *H. pylori* flagellar regulatory network: The *flaA* s^{28} promoter was characterized and found to be unusual since its spacing was shortened to 13 nucleotides instead of the s^{28} consensus of 15 nucleotides (Leying et al., 1992; Suerbaum et al., 1998a). This was a first hint that this promoter might be supercoiling-dependent. Genes involved in flagellar biosynthesis are closely coupled on the *H. pylori* genomes with genes implicated in DNA topology: The *topA* gene is located directly upstream of the *flaB* gene (Suerbaum et al., 1998a), and these two genes are transcribed in divergent orientation by overlapping promoters. The *gyrA* gene is co-transcribed in the same operon with the *flgR* gene, which is the NtrC-like transcription activator of s^{54} regulated flagellar genes (Spohn and Scarlato, 1999b).

These findings suggested that transcriptional regulation of *flaA* and possibly other flagellar genes could be directly or indirectly regulated by DNA supercoiling, considering the pleiotropic nature of DNA supercoiling function, in response to special environmental signals. The aim of this study was to determine the role of global DNA supercoiling changes on the flagellar regulatory hierarchy of *H. pylori* and on its global regulatory network.

2. Material and Methods

2.1. Material

2.1.1. Bacterial strains

Strain	Description	Reference/source
E.coli		
DH5a	for the DNA cloning experiments	(Hanahan, 1983)
MC1061	for the DNA cloning experiments	
H.pylori		
N6	Wild type strain, for allelic exchange mutagenesis and for the transformation with shuttle plasmids	
N6(*flgR*::km)	Km^R, N6 derivative in which 515 bp of *flgR* have been substituted by a km^R cassette	This study
N6(*hup*::km)	Km^R, N6 derivative in which 85 bp of *hup* have been substituted by a km^R cassette	This study
N6(*topA2*::km)	Km^R, N6 derivative in which *topA2* has been substituted by a km^R cassette	(Brauer-Steppkes, 1999)
N6(11N)	N6 derivative in which *flaA* promoter region between –10 and –35 was change to 11bp	(Brauer-Steppkes, 1999)
N6(12N)	N6 derivative in which *flaA* promoter region between –10 and –35 was change to 12bp	(Brauer-Steppkes, 1999)
N6(14N)	N6 derivative in which *flaA* promoter region between –10 and –35 was change to 13bp	(Brauer-Steppkes, 1999)
N6(15N)	N6 derivative in which *flaA* promoter region between –10 and –35 was change to 14bp	(Brauer-Steppkes, 1999)
N6(pSUS316)	Cm^R, N6 obtained by transformation with plasmid pSUS316	This study
N6(pSUS1812)	Cm^R, N6 obtained by transformation with plasmid pSUS1812	This study

Table 2: Bacterial strains used in this study

2.1.2. Plasmid

Vector/ plasmid	Description	Reference/ source
vectors		
pILL570	Cloning vector, *Spec*^{R}	
pUC18	Cloning vector, *Amp*^{R}	
pHel2	*Cm*^{R}, E.coli/H.pylori-shuttle-vector	(Heuermann and Haas, 1998)
pHel3	*km*^{R}, E.coli/H.pylori-shuttle-vector	(Heuermann and Haas, 1998)
plasmid		
pILL600	*Km*^{R}, Plasmid containing the kanamycin resistance cassette (*aphA*3'-III) from *Campylobacter coli*	(Labigne-Roussel et al., 1988)
pSUS1802	*Spec*^{R}, pILL570 derivative with a *Bam*HI insert encoding the *flgR* gene	This study
pSUS1807	*Km*^{R}, *Spec*^{R}, pSUS1802 derivative containing the kanamycin resistance cassette (*aphA*3'-III) of pILL600 inserted at the *Eco*RI site of *flgR*	This study
pSUS316	*Cm*^{R}, plasmid containing *H.pylori topA* uncluding its own promoter	(Suerbaum et al., 1998a)
pSUS1812	*Cm*^{R}, plasmid derivative of pSUS316	This study
pSUS1810	*Spec*^{R}, pILL570 derivative with a *Bgl* II /*Bam*HI insert encoding the *hup* gene	This study
pSUS1814	*Km*^{R}, *Spec*^{R}, pSUS1810 derivative containing the kanamycin resistance cassette (*aphA*3'-III) of pILL600 inserted at the *Eco*RI site of *hup*	This study

Table 3: plasmid used in this study

2.1.3. Oligonucleotides for cloning and sequencing

Primer	Sequence(5' to 3')	stran	site[a]	position
OLHPTopA-IA1	AGGCGTTCCATAAGCACCTTATTATCGTAGAATC	+		238-272
OLHPTopA-IA2	ATAAGGTGCTTATGGAACGCCTTTAATGGAATG	-		227-260
OLHPFlgR-1	ATA**GGATCC**CACTACCACAAGCATTCTTG	+	*Bam*HI	upstr. of *flgR*
OLHPFlgR-2	TAT **GGATCC**AATCATCATTAAGTGGTTAGC	-	*Bam*HI	downstr.of *flgR*
OLHPFlgR-3	CTGCGAAA**GAATTC**AGAGAAG	+	*Eco*RI	875-854
OLHPFlgR-4	TAT **GAATTC**TTGGTGCGATAGATGGATTC	-	*Eco*RI	338-318

OLHPFlgR-5	TATCACGCATTATGATCATAG	+		Upstr.of *flgR*
OLHphup-4	ATA**GGATCC**CCTTATGAAGAGATCATAGC	+	*Bam*H	Upstr.of *hup*
OLHphup-5	ATA**GGATCC**CTAATCTTATAAGGCTAGTGG	_	*Bam*HI	Downstr. of *hup*
OLHphup-6	ATA**GAATTC**AAGTAAAGGCACTGATCGC	+	*Eco*R I	91-73
OLHphup-7	ATA**GAATTC**AAGAAGGTAAAGTGCCAGG	_	*Eco*R I	176-194

Table 4: Oligonucleotides for cloning and sequencing used in this study

a) Bold and underlined nucleotides are restriction recognition sites

2.1.4. Oligonucleotides for RT-PCR

Gene No.	Gene Name	Primer	Sequence (5'to 3')	Tm	Extension time(sec.)	cycles	Reference /source
HP0010 HP0011	*GroEL-GroES*	HP0010-RT1 HP0011-RT1	TCTGTCTTTGACATCATGGC AGGCATTGTGGGCTCAGG	53	90	16	This study
HP0071	*ureA*	HP0071-RT1 HP0071-RT2	GAT CCT AAA AGC ACT GCG TGG TCA TCA AGC ATA TCG C	50	60	16	This study
HP0072	*ureB*	HP0072-RT1 HP0072-RT2	ATG AGC CAA TCC AAC AAC C TAG CTG CCA TAG TGT CTT CC	53	60	20	This study
HP0073	*ureE*	HP0073-RT1 HP0073-RT2	GTT AGA CAA GTT GAT GCT CC TTT TAC ATA GTT GTC ATC GC	50	60	19	This study
HP0099	*tlpA*	HP0099-RT1 HP0099-RT2	AATGAGAACGCTTCA AGAGGT TCT TTCAAATCAGGGCTTGC	55	60	25	(Niehus et al., 2004)
HP0100	*hyp*	HP0100-RT3 HP0100-RT4	GCT GCT CAG TGG ATA ACC CAG AAT GCA GCA ATT CAA GG	51	60	25	(Niehus et al., 2004)
HP0115	*flaB*	HPFlaBn1 HPFlaBn2	ATCGCTGATAGCTTA AGGAGTC GGATCTTGTCCA ATTGCGTGC	57	90	22	(Niehus et al., 2002)

HP0116	*topA*	HPTopA-9 HPTopA-10	CCTTATAGCTGATGAGTTGC ATTACTAGTCACCTTATTATCGTAG AATCC	52	60	27	This study
HP0166	*ompR*	HP0166-RT1 HP0166-RT2	ACA GAG ATG ATG TAT TGC GG AAA GCA CTA GAT TAT GGG GC	53	60	19	This study
HP0232		HP232-RT1 HP232-RT2	ACCCACATTATAGTGCGCG TAGTAGGCCTACTAGAGCC	53	60	25	(Niehus et al., 2004)
HP0393	*cheV*	HP393-RT1 HP393-RT2	AAGTGAGATAGAACTCGTGG TCCACAGCTTCAGTGATGC	53	60	25	(Niehus et al., 2004)
HP0440	*topA*	HPtopA2-4 HPtopA2-5	ACCCTGATAGAGAGGGCTATGG CAGCTTGTGAGTTTTTGCCAGC	61	90	30	This study
HP0472	*omp11*	HP0472-2 HP0472-5	ATCAGCAAGGTCGCTATGG GAATAGTAGCGGATAAGCCC	53	60	20	(Niehus et al., 2004)
HP0501	*gyrB*	HPGyrB-1 HPGyrB-2	CATGGTGTATGAAGTCGTGG AAGTGCGCGTTTCTTCATCC	55	60	26	This study
HP0525	*virB11*	HP0525-RT1 HP0525-RT2	CCTCTAAGGCATGCTACTG TTCTTCACTGCTCCCTGC	51	60	27	This study
HP0528	*cag8*	HP0528-RT1 HP0528-RT2	TTGGCTGTTTCTGTCTTGG CATGTCCTCTAGTCGTTCC	51	60	27	This study
HP0529	*cag9*	HP0529-RT1 HP0529-RT2	ACATCTATTGGTGCATCAGG AGATTGACTACACCATATGC	51	60	27	This study
HP0532	*cag12*	CagT-1Rfor. CagT-1Rrev.	ATGAAACTGAGAGCAAGTGTTTTA ATCGGTG TCACTTACCACTGAGCAAACTTCT GATTTGA	59	60	20	This study
HP0541	*cag20*	HP0541-RT1 HP0541-RT2	TCATCTCAAGCACAAGTGG GTGGTCATAGGATTAGTGC	51	60	25	This study
HP0543	*cag22*	CagF1 CagF2	ACTGAACAAAACCTTACCGC GGAGTCCATGATCAAATGCG	53	60	19	This study
HP0544	*cag23*	HP0544-RT1 HP0544-RT2	AGGACAAACTAGCTTTCACC AGTATCAAAGTGTGTCCTGC	53	60	20	This study

HP0547	*cag26*	CagAN1554 CagAN1555	AAGGAGAAACAATG(AG)CTAACGAA CTGCAAAAG(AC)TTGTTTGGCAGA	58	60	16	This study
HP0601	*flaA*	OLHPFlaA-4 OLHPFlaA-9	ATTGATGCTCTTAGCGTC CAAGCGTTATTGTCTGGTC	49	60	16	(Niehus et al., 2002)
HP0679	*wbpB*	HP0679-RT1 HP0679-RT2	ACCTTGTCTTTA ACGCTTCC ATATTCATGATAGCGTGGGG	53	60	23	This study
HP0701	*gyrA*	HPGyrA-1 HPGyrA-2	GCTTATTCCATGAGCGTGATC CGTCTATGATTTCATCCATCC	53	60	22	This study
HP0703	*flgR*	HP0703_RT1 HPflgR-4	ATGCGTAAAAGCCTGGAGC TAT**GAATTC**TTGGTGCGA TAG ATG GATTC	55	60	23	This study
HP0751	*flaG2*	HP0751-1 HP0751-2	TTCCCACATCTCACACAAGC TCACCTTATCCCCATTAGCG	55	60	23	(Niehus et al., 2002)
HP0752	*fliD*	HP0752-1 HP0752-2	TTACAGGCGATGCGTTGAGT AATCGCTTTCTTGGGTGGTG	55	60	23	(Niehus et al., 2002)
HP0753	*fliS*	HP0753-1 HP0753-2	TACCAGCATAACCGAGTGAG CTCTAGCCACATTCA ACACC	55	60	23	(Niehus et al., 2002)
HP0770	*flhB*	HP0770-RT3 HP0770-RT4	AGAGCTGTTTAACCAACTGG CTTCTTGCTGTTTGTATTCG	51	60	25	(Niehus et al., 2002)
HP0835	*hup*	HPhup-2 HPhup-3	AGCGGAATTTATTGATTTGG CTTCAACTTTTTGTTTAAGGG	51	60	30	This study
HP0869	*hypA*	HP0869-RT1 HP0869-RT2	AATAACATTCTTGCTGTGGC CGTTTCTTCTTTAATCGCTC	51	30	21	(Niehus et al., 2002)
HP0869 – HP0870	*HypA-flgE1*	HP0869-RT1 HP0870-RT1	AATAACATTCTTGCTGTGGC CTAGTA ATGTGGATTTGAGC	51	60	25	(Niehus et al., 2002)
HP0870	*flgE1*	HPflgE1 HPflgE2	CTCAAGTCA AACTCATCG CTTGCCGTCTAA ATA AGC	47	90	30	(Niehus et al., 2002)

HP1051	*Hyp.*	HP1051-1 HP1051-2	AAAATGTAAAACGGCGTGTTC AAAAGGCTTTCGCTTAATCC	50	60	21	(Niehus et al., 2004)
HP1052	*envA*	HP1052-1 HP1052-2	AAGAAGTGTATTTGCCCATC ATTAAAGAACTAGACGCTCC	50	60	21	(Niehus et al., 2002)
HP1076	*Hyp.*	HP1076-1 HP1076-2	GGCGATGTTGAAACAAGCGA TTGGCATGAAGGCTTTAGGG	55	60	23	(Niehus et al., 2002)
HP1119	*flgK*	HP1119-1 HP1119-2	CAACATTACCCGCACTATCG GACATTGATGAAGCGAGCCT	55	60	23	(Niehus et al., 2002)
HP1120	*hyp.*	HP1120-1 HP1120-2	TAATCGCAAGTGTCATGGGG AAGCTCGCTAAACACACCGA	55	60	23	(Niehus et al., 2002)
HP1152	*ffh*	HP1152-RT1 HP1152-RT2	ACGATGTGCATCATAAAGTGG CACAAACACGTCTAAATCAGG	55	60	25	(Niehus et al., 2002)
HP1154	*Hyp.*	HP1154-RT1 HP1154-RT2	GAGCA ATTAACGAAGTGCG TAAATAGCCTCTATGCCTTGC	55	60	23	(Niehus et al., 2002)
HP1154- HP1155	*Hyp-* *murG*	HP1154-RT1 HP1155-RT2	GAGCATATTAACGAAGTGCG TTGTTTGGTGAGTTTGGGAG	53	60	25	(Niehus et al., 2002)
HP1233	*flgJ*	HP1233-RT1 HP1233-RT2	TCCACCAGAAAATCTCCCC TGTAAGCGTTTTGGTAGCG	51	60	23	(Niehus et al., 2002)
	flhA	HPflaC21 HPflaC22	TA**GGATCC**AAATTCGGCTTGGATT TGAG TA**GGTACC**CTTAAAAGTTAATATG GATCG	49	60	33	(Niehus et al., 2002)
	HP16s rDNA	HP16s I HP16s II	GCTAAGAGATCAGCCTATGTCC TGGCAATCAGCGTCAGGTAAT G	55	60	15	This study

Table 5:Oligonucleotides for RT-PCR used in this study

2.1.5. Bacterial growth conditions

H. pylori strains were grown on blood agar plates (Columbia agar base 2, Oxoid, Wesel, Germany), containing 10% horse blood, vancomycin (10 mgl^{-1}),

polymyxin B (2500 Ul^{-1}), trimethoprim (5 mgl^{-1}) and amphotericin B (4 mgl^{-1}). Plates were incubated at 37°C under microaerobic conditions for 48 hours. Selective antibiotics (kanamycin, 100 mgl^{-1}; chloramphenicol 20 mgl^{-1}) were added to the media as required. For the assays of plasmid DNA supercoiling, and RNA isolation of different growth phases, *H. pylori* cells were grown in liquid culture as follows: Liquid culture were done in brain-heart infusion (BHI) broth (Oxoid) supplemented with 10% heat-inactivated horse serum and the above-mentioned antibiotics as required. The medium was inoculated with the bacteria that had been harvested from plates at a concentration of approximately 1 x 10^9 cells per 100 ml, to an initial OD_{600} of 0.06. They were incubated in a rotary shaker (125 rpm) under microaerobic conditions for up to three days. Growth was monitored at each harvest by measurement of the OD_{600} of the cultures. Different concentrations of the gyrase inhibitor novobiocin were added to the liquid culture at a standardized time point, in order to reduce negative supercoiling of the DNA.

E. coli strains were grown in Luria broth or on Luria-Bertani (LB) plates, containing kanamycin (100 mgl^{-1}), chloramphenicol (20 mgl^{-1}), spectinomycin (100 mgl^{-1}), tetracycline (10 mgl^{-1}) or ampicillin (50 mgl^{-1}) as required.

2.1.6. Antisera

A rabbit antiserum against *H. pylori* topoisomerase I was from the lab collection (unpublished).

2.1.7. Reagents and enzymes

All the reagents used in the experiments were purchased from Sigma, Roth, Merck, Bio-Rad, Amersham, Boehringer, Difco, Fluka, Life Technologies and Qiagen. Restriction and DNA-modification enzymes were purchased by Boehringer, Roche, Life Technologies, Invitrogen, New England Biolabs and Pharmacia.

2.2. Methods

2.2.1. Preparation of *E. coli* competent cells and transformation

Competent *E. coli* cells were prepared by the method of Hanahan (Hanahan, 1983).

2.2.2. Transformation of *E. coli* (Sambrook et al., 1989)

Two hundred microliters of competent *E. coli* cells were mixed with 100-300 ng of plasmid DNA or the respective amount of a ligation reaction and incubated for 30 min on ice. The mixture was then heat shocked for 2 min at 42ºC and after that, reincubated on ice for 2 min. Thereupon 500 µl SOC was added and samples were incubated at 37ºC for 1 h for the expression of antibiotic resistance genes. One hundred microliter of the suspension were then plated on a selective agar plate. Then the plate was incubated overnight at 37ºC for growth of bacterial colonies.

2.2.3. Natural transformation of *H. pylori*

H. pylori cells from a 24 h-cultured plate were scraped off with a cotton swab and spread on a BHI agar plate in a diameter of 8-10 mm. After continued incubation for 2 hours, 10 µl of DNA (1-5 µg) in TE buffer was spotted directly onto the inoculated area (bacterial lawn). After an overnight incubation at 37°C under microaerobic conditions, the bacteria were collected, and streaked onto BHI plates that contained selective antibiotics. The plates were read after growing the transformants for 5-7 days. Single colonies were then selected for further analysis.

2.2.4. Small and medium scale isolation of plasmid DNA from *E. coli* and *H. pylori* (Sambrook et al., 1989)

For small scale preparation of plasmid DNA, 5 ml of LB medium containing the

proper antibiotic supplement were inoculated with a single bacterial colony and incubated overnight in a shaker at 37ºC. On the next day, 3ml of the bacterial culture were centrifuged, the supernatant was discarded and the pellet was resuspended in 100 µl of ice cold solution I (25 mM Tris-HCl pH=7.8; 50 mM Glucose; 1 mM EDTA). After 5 min incubation at room temperature, 200 µl of solution II (0.2 N NaOH; 1% SDS) are added, the sample was mixed carefully and incubated for 5 min on ice. Cellular proteins and chromosomal DNA were then precipitated by addition of 150 µl of solution III (3M sodium acetate pH=4.8) and a further 5 min incubation on ice. Cell debris was removed by centrifugation at 13000rpm for 10min, and the supernatant was first extracted with a mixture of phenol/chloroform and then precipitated by addition of 1 ml ethanol. After 2 min incubation at room temperature the precipitated plasmid DNA was centrifuged at 13000 rpm for 30 min, dried under vacuum for 10 min and resuspended in 30 µl TE (10 mM Tris-HCl pH=8.0; 1 mM sodium EDTA).

For midi scale plasmid preparations, 25 ml (with high copy plasmids) LB medium supplemented with the proper antibiotic were inoculated with a single bacterial colony and incubated over night at 37ºC in a shaker. Bacteria were then pelleted by centrifugation at 6000 rpm at 4ºC for 10 min, and plasmid DNA was purified with the QIAgen® midi-column plasmid purification kit (Qiagen Inc.) according to the manufacturer's instructions.

Plasmids from *H. pylori* were prepared with QIAgen® mini plasmid kit (Qiagen Inc.) for the determination of plasmid supercoiling.

2.2.5. Preparation of chromosomal DNA from *H. pylori*

For small scale isolation of *H. pylori* chromosomal DNA, cells from 24-48 hours grown plates were resuspended in 200 µl 0.9% NaCl, then cells were pelleted by centrifugation at 13000 rpm at 4ºC for 1 min, and genomic DNA was purified with QIAamp® DNA mini kit (Qiagen Inc.) following the manufacturer's instructions.

2.2.6. Isolation of DNA fragment from agarose gels

DNA fragments or PCR amplification products from agarose gels were extracted from agarose gels and purified with the QIAquick® gel Extraction kit (Qiagen Inc.) following the manufacturer's instructions.

2.2.7. Cloning of DNA fragments

Cloning experiments were performed according to standard protocol (Sambrook et al., 1989). Typically, 100 ng of the linearized vector DNA were mixed with 200-300 ng of DNA fragment of 20 µl of 1 x T4 ligase buffer and 2 units of T4 DNA ligase were added. After an overnight incubation at 4°C, the sample was used for transformation of appropriate *E. coli* competent cells.

2.2.8. PCR amplification and DNA sequence determination

PCR amplification was performed according to standard procedures. PCR reactions were performed in a Biometra thermal cycler. In each reaction, 5 pmol of target DNA, 10 pmol of each primer, and 10 mM of deoxynucleotides were used. The PCR reactions were denatured at 94ºC for 1min, annealed at temperatures between 49 to 61ºC (depending on the calculated melting temperature of the primers) for 1-2 min and extended at 72ºC for 2 min. 25-30 cycles were performed. PCR products were purified with QIAquick® PCR Purification kit (Qiagen Inc.). DNA sequence determination of PCR products was done using a fluorescent dye terminator kit (Applied Biosystem) and subsequent separation in an Applied Biosystem model 377 sequencing apparatus (Suerbaum et al., 1998a). The plasmids and oligonucleotides that were used are list in table 1 and 2, or indicated in the methods.

2.2.9. Preparation of Digoxygenin (Dig)-labeled probes

Digoxygenin-labelled probes were generated by PCR with specific primes. Each reaction mixture contained about 100 ng of plasmid or chromosomal DNA, 1x PCR buffer (supplied 10x by the manufacturer), 10 mM of dATP,

dGTP, dCTP, 7 mM of dTTP, 3.5 mM DIG-11-dUTP and 50 nM of each specific oligonucletide. One microliter of Taq polymerase was then added and PCR was performed according to the standard protocol.

2.2.10. Protein determination

Protein was determined according to Bradford (Bradford, 1976) with bovine serum albumin as the standard.

2.2.11. SDS-PAGE and Western blotting

For Western blot analysis, equal amount of protein were loaded onto the gel. Gel electrophoresis was in 10% SDS-PAGE (Laemmli, 1970). Transfer of proteins to nitrocellulose membranes was done in a semidry blotting device using Towbin buffer. Immunoblotting was done according to standard procedures as follows: The membrane was washed with TBST for 10 min, then blocked with 5% Skim Milk (SM) in TBST for 1h, then incubated with primary antibody in 5% SM in TBST at 4°C overnight. On the following day, the membrane was first washed with TBST for 3 times, each for 15 min, then incubated with secondary antibody for 1h at room temperature, followed by 3 washings with TBST, each for 15 min. Then the blot was covered with chemiluminescent substrate, incubated for 5 min at room temperature, and chemiluminescence detected by hyperfilmMP (Amersham) for 20 s and 5 min.

2.2.12. Whole RNA preparation from *H. pylori*

RNA was isolated from liquid cultures of *H. pylori*. Bacteria were harvested, and spun down for 30 s at 13000 rpm at 4°C in an Eppendorf centrifuge. Bacterial pellets were quick frozen in liquid nitrogen and stored at –80°C until the RNA preparation was carried out. Before RNA isolation, bacteria (approximately $12x10^8$) were resuspended in 850ul of the cell lysis buffer of the Qiagen RNeasy kit (10 $\mu l ml^{-1}$ of ß-mercaptoethanol), mixed with glass beads in 2 ml FastRNA tube-Blue (BIO 101) and lysed for 45 s at speed of 6.5 units in a

bead beater FP120 shaker (Savant). Further preparation was done according to the standard Qiagen RNeasy column kit. After column elution, RNA was treated with RNase-free DNase I (Roche) and, in case of use for microarray hybridizations and RT-PCR, again purified with the Qiagen RNeasy column kit. RNA preparations were quality-controlled by a PCR reaction using DNA–based primers (OLHPFlaA-4 and OLHPFlaA-PV4, upstream of the *flaA* gene.) in a non-transcribed region. RNA was further quality-checked on the agarose gels, and RNA amounts were measured by a spectrophotometer.

2.2.13. Northern blotting

Whole RNA preparations (1 to 4 µg) were used to perform a denaturing agarose gel electrophoresis (0.24 M formaldehyde) at 100 volts for 2 hours. Afterwards, the gel was treated with 50 mM NaOH and 0.1M Tris-HCl (pH=8) for 5 minutes each and was vacuum-blotted on to positively charged nylon membrane (Roche). Dig-labelled probes were generated by PCR with primes specific for *flaA* (OLHPFlaA4 and OLHPFlaA9) and 16S rDNA (OL16S1 and OL16S2), with incorporation of DIG-labelled dUTP nucleotides during the reaction. They were adjusted to 200 $ngml^{-1}$ of hybridization buffer. Following high stringency washes, the Dig-labeled probes were detected with the CSPD chemiluminescence detection system (Roche) and Hyperfilm® MP film (Amersham) for 1 and 5 minutes.

2.2.14. Hybridization of microarray

Hybridization of microarray was essentially as described by Josenhans et al. (Josenhans et al., 2002a). Briefly, thirty microgram of DNase I-treated RNA were reverse transcribed using random primers and Superscript II reverse transcriptase (Invitrogen), and the cDNA was concomitantly labeled using the dyes Cy3 and Cy5, according to the design of the respective experiment. The RNA was removed after reverse transcription by alkaline hydrolysis. Oligonucleotide slides (MWG Biotech AG) were pretreated by blocking with

4xSSC, 0.5% SDS, 1% BSA for 2 hours. PCR slides were pretreated with QMT blocking solution (Quantifoil Micro Tools GmbH). After blocking, the slides were washed five times in bi-distilled water and dried. The hybridization mixture containing the Cy-labeled cDNA in hybridization buffer (50% Formamide, 6x SSC, 0.5% SDS, 50 mM $NaPO_4$, pH=8.0, 5x Denhardts' solution) was denatured for 5 min and co-incubated with slides for 16 hours at 42°C (PCR slides at 50°C). Washing was carried out in three subsequent steps with increased washing stringency: 2x SSC, 0.1% SDS, followed by 1x SSC, 0.1% SDS, and 0.5x SSC for 10 min at room temperature. Afterwards, slides were spun dry and scanned at wavelengths using a GMS418 Array Scanner (MWG Biotech AG) at six different fixed intensities.

2.2.15. Bioinformatics

The microarrays were analysed using IMAGENE 5.5 and GENESIGHT LITE® software (BioDiscovery) for spot location, array alignment and background subtraction. Cy3 and Cy5 intensities were adjusted for local background and normalized to the median intensity over all the spots in the respective channel in which it was scanned. The Cy3/Cy5 ratio of all the spots was calculated. Microsoft EXCEL was used for statistical analysis of the IMAGENE output files. For each treated and untreated strain, the arithmetical means of the mean signal ratio for each spot was calculated for all three experiments. A change of transcription was considered significant when the average mean signal ratio was smaller than 0.5 or greater than 2. All genes with low signal intensity (less than twofold of the background signal) were excluded from the evaluation. To account for biological variability in the experiment, competitive microarray hybridization was also done using RNAs from two independently grown cultures of the stains. All genes in the biological variation experiment remained within a cut-off range between 0.5 and 2.

2.2.16. Measurement of DNA supercoiling

Topoisomer distributions of reporter plasmid were determined by electrophoresis in 0.5% agarose gels containing 1.5 $\mu g ml^{-1}$, 10 $\mu g ml^{-1}$, and 25 $\mu g ml^{-1}$ chloroquine (dissolved in 1% acetic acid), respectively. The electrophoresis buffer was TBE (90mM Tris (pH=8.3), 90mM borate, 10mM EDTA) containing the same concentration of chloroquine as in the gel, and gels were run at 5 $V cm^{-1}$ for 18 hours at 4°C. After electrophoresis, the gels were first soaked in 1 mM $MgCl_2$ for 2 h, followed by 1 h in water, and then stained with ethidium bromide for 1 h.

2.2.17. Novobiocin MIC test

The minimal inhibitory concentration (MIC) of novobiocin was determined by the agar dilution test method, using blood agar plates and a cell suspension calibrated at 0.5 McFarland. Twofold differences in concentrations were used, and ranged from 0.5 $mg l^{-1}$ to 32 $mg l^{-1}$. Plates were inoculated with a suspension of plate-grown bacteria in phosphate-buffered saline (PBS), pH=7.2, with a turbidity equal to that of a McFarland 0.5 standard. 10 µl of this suspension was applied to the plates. Plates were read after 72 hours of incubation at 37°C under microaerobic conditions. Results were recorded as growth or no growth at each concentration. The MIC was defined as the lowest concentration of antimicrobial agent at which no visible growth was observed.

2.2.18. RT-PCR

First, 1.5 µg of DNase I-treated RNA were reverse transcribed using random hexamer primers and Superscript II reverse transcriptase (Invitrogen) at 42°C for 2 hours, followed by RNase H digestion, then the cDNA was amplified in different PCR reactions on 2.5 µl cDNA samples with specific primers (Table 3). The reactions were performed in a 25 µl reaction volume with the following PCR conditions: 94°C for 5 minutes, followed by 15-30 cycles of 94°C for 1 minute, 49-57°C (depending on the calculated melting temperature of the

primers) for 1 minute followed by 72°C for 1 minute. RT-PCR products were visualized on 0.8% agarose gels.

2.2.19. Real time quantitative PCR on the LightCycler®

PCR was performed with the LC FastStart DNA Master SYBR Green I® (Roche Molecular Biochemicals) in a standard PCR reaction according to the manufacturer's protocol using specific primers as required. The reactions were performed in a 20 µl reaction volume with the following PCR conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds followed by 72°C for 40 seconds. A series of 10 fold DNA dilutions (PCR product 10^1-10^6 pg/ul) as standards were prepared and included in each amplification run to give the standard curve. Fresh dilutions were prepared for each assay. For measuring the reproducibility of the assay, all samples were run in duplicates.

3. Results

3.1. Establishment of conditions of supercoiling assays in *H. pylori* using a shuttle plasmid

In order to study the role of DNA supercoiling in the coordinated gene regulation of *H. pylori*, our first aim was to establish a reproducible system to visualize changes of DNA supercoiling in *H. pylori*. Since analysis of DNA supercoiling in *H. pylori* had not been achieved previously, our first approach was to develop a method to determine DNA supercoiling in *H. pylori*, based on the visualization of reporter plasmid supercoiling states, as was previously described for *E. coli* (Kato and Furuno, 1992).

3.1.1. Determination of methods for extracting plasmid DNA

Topoisomer analysis of reporter plasmid supercoiling was used to determine the effects of intracellular growth on DNA topology. We used the *E. coli* /*H. pylori* shuttle plasmid pHel3 as a reporter plasmid for analysis of DNA supercoiling (Heuermann and Haas, 1998). *H. pylori* wild type strain N6 transformed with this plasmid was grown in liquid culture, and harvested at mid-log phase, then plasmid DNA were isolated. Since plasmid pHel3 preparations from the *H. pylori* yielded low amounts of DNA, three different methods were initially tested, which are the Wizard® Plus Miniprep DNA purification isolation kit (Promega), the Tip20 plasmid isolation kit (Qiagen), and the alkaline lysis method as described in the methods part. The same amount of plasmid DNA from different methods was analyzed on 0.8% agarose gels that contained chloroquine at a concentration of 7.5 µg/ml. Fig 1 shows that the plasmid DNA extracted with the Qiagen kit was the best method for getting clear multiple topoisomers of the plasmid from *H. pylori*.

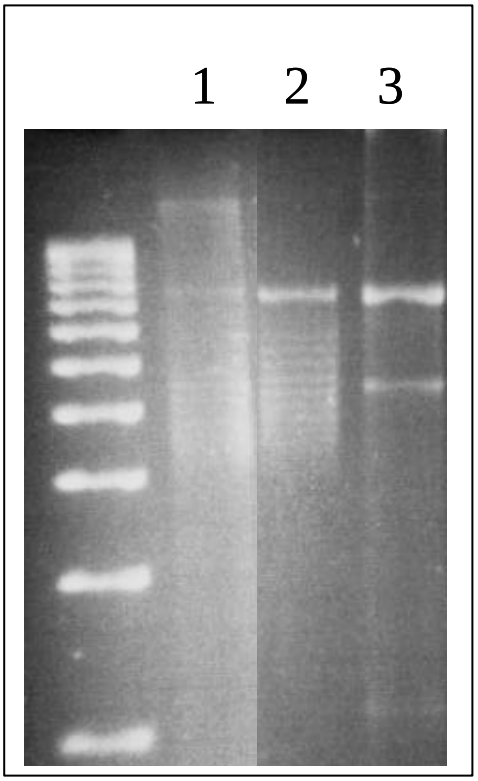

Fig 1. Reporter plasmid DNA in *H. pylori* was extracted with three different methods, and checked by agarose gel electrophoresis in the presence of 7.5 µg/ml of chloroquine. Lane 1) plasmid DNA was extracted with Wizard® Plus Minipreps DNA purification isolation kit; Lane 2) plasmid DNA was extracted with Tip20 plasmid isolation kit (Qiagen); 3) plasmid DNA was extracted using the alkaline lysis method.

3.1.2. Determination of running buffer

To obtain maximum resolution of the pHel3 plasmid DNA, 0.5% agarose gels were used. Three different buffers were used for analyzing plasmid DNA supercoiling in *E. coli*, which are TAE (40 mM Tris-acetate, pH=8.0, 1 mM EDTA), TBE (45 mM Tris-borate, pH=8.3, 1 mM EDTA) and TPE (50 mM Tris-phosphate, pH=7.2, 1 mM EDTA). Among them, TAE is the most commonly used buffer, but its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis (Sambrook et al., 1989). We chose TBE and TPE since they both have significantly higher buffer capacities. The plasmid DNA was isolated from *H. pylori*, and equal amounts of DNA were loaded onto chloroquine gels (7.5 µg/ml of chloroquine) that were prepared with TBE and TPE buffer alternatively. The electrophoresis buffers

were TBE or TPE, respectively, containing the same concentration of chloroquine as the gels, and gels were run at 5 Vcm^{-1} for 18 hours at 4°C. Fig 2 shows that TBE buffer was the best buffer for resolving multiple topoisomers of shuttle plasmid pHel3, in comparison to TPE buffer.

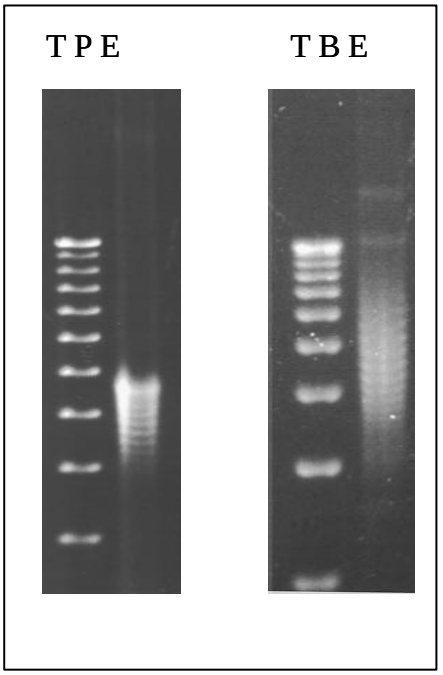

Fig 2. Reporter plasmid DNA was extracted from *H. pylori* with the Oiagen kit, and analyzed by agarose gel electrophoresis in the presence of 7.5 µg/ml of chloroquine in TBE or TPE buffers. 1) TPE buffer; 2) TBE buffer.

3.1.3. Determination of concentration of chloroquine gel

Chloroquine is an intercalating agent that unwinds DNA, allowing analysis of the distribution of DNA topoisomers in agarose gels (Shure et al., 1977). Chloroquine gels are the standard technique for determination of the relative degree of plasmid DNA supercoiling in *E. coli*. *H. pylori* N6 carrying the plasmid pHel3 was grown in liquid culture, harvested at mid-log phase, and the plasmid DNA was isolated as described. The same amount of plasmid DNA was analyzed on agarose gels that contained different concentrations of chloroquine. In separate experiments, the reporter plasmid topoisomer distribution was examined at chloroquine concentrations of 1.5, 10 and 25 µg/ml. Consistent with findings from *E. coli*, in the presence of 1.5 µg/ml of

chloroquine, the more supercoiled DNA migrated faster than the relaxed forms of DNA. In the presence of 10 µg/ml and 25 µg/ml chloroquine in the gel, the more relaxed forms of DNA migrated faster than the highly supercoiled forms. Our data showed that 10 µg/ml of chloroquine was the best concentration for resolving the multiple topoisomers of plasmid pHel3. This concentration was chosen for the subsequent experiments.

3.2. Establishment of two different systems for modifying the DNA supercoiling in *H. pylori*

The next aim of this study was to develop methods to achieve reproducible and detectable changes of DNA supercoiling in *H. pylori*, in order to observe the effects on the transcription of flagellin gene *flaA* and possibly other genes. Since topoisomerase I and gyrase are the two major enzymes which maintain the optimum DNA supercoiling in bacteria, two different approaches were chosen to change DNA supercoiling: a) inhibition of *H. pylori* gyrase activity by the addition of novobiocin, which is a inhibitor of gyrase B subunit; b) the overexpression of topoisomerase I in *H. pylori*, which should both lead to a relaxation of DNA supercoiling.

3.2.1. Decrease of DNA supercoiling by inhibition of *H. pylori* gyrase activity

The use of inhibitors of gyrase to inhibit enzyme activity was described to be useful for changing DNA supercoiling in other bacteria. Novobiocin is a DNA gyrase inhibitor but indirectly influences the optimal supercoiling in the cell (Maxwell, 1997), its binding decreases the affinity of *gyrB* for the ATP nucleotide, which is required for DNA breakage and strand passage, so the DNA will keep stay intact and maintain lower supercoiling states.

3.2.1.1. Determination of the minimum inhibitory concentration (MIC) of novobiocin

Critical for the use of inhibitory substances, such as novobiocin, in order to affect exclusively the DNA supercoiling state of the bacterium and not vital cell functions, is to determine the minimum inhibitory concentration (MIC). To elucidate the MIC in *H. pylori*, an agar dilution test was used to determine the MIC of novobiocin as described in the methods. The tested concentrations were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/ml. The results demonstrated that the MIC of novobiocin in *H. pylori* is 16 µg/ml. It seems therefore that *H. pylori* is sensitive to novobiocin.

3.2.1.2. Determination of the growth rate with different concentrations of novobiocin in *H. pylori* liquid culture

In order to determine the right time course of novobiocin addition treatment for *H. pylori*, we tested growth rates of the bacteria at the concentrations from 0.001 to 16 µg/ml at different incubation times of 6,12 and 24 hours. *H. pylori* N6 was grown in liquid culture in a big flask (one liter Fernbach flask) with shaking, until an OD_{600} of 0.6 was reached, then the culture was aliquoted into 20 ml, and each culture was supplemented with subinhibitory concentrations (0.001, 0.25, 0.5, 1, 2, 4, 8 and 16 µg/ml) of novobiocin. At 6, 12 and 24 hours after subculturing, the OD_{600} of all cultures were measured. After 6 h, the growth at all novobiocin concentrations tested, was indistinguishable. After 12 h and 24 h, even at the lowest concentration, a severe reduction of growth was observed, which indicated that the response to the inhibitor remains constant over the time measured.

3.2.1.3. Measurement of DNA supercoiling with different concentrations of novobiocin in *H. pylori* liquid culture

To further test the effect of inhibition of gyrase on the changes of DNA

supercoiling, *H. pylori* N6 carrying shuttle-plasmid pHel3 was grown in liquid culture and kanamycin for optimal maintenance of the plasmid. The cultures were incubated at 37°C under microaerobic conditions. In early log phase, the culture was aliquoted into a series of small volumes, and each suspension was inoculated with different subinhibitory concentrations of novobiocin as above (0.001, 0.25, 0.5, 1, 2, 4, 8 and 16 µg/ml). The cells were incubated for an additional 6 and 12 hours at 37°C before the plasmid DNA was extracted. The effect of increasing novobiocin concentrations on plasmid DNA supercoiling was analyzed by agarose gel electrophoresis in the presence of 10 µg/ml chloroquine. In this gel system, the more relaxed topoisomers migrate faster than the more highly supercoiled plasmids.

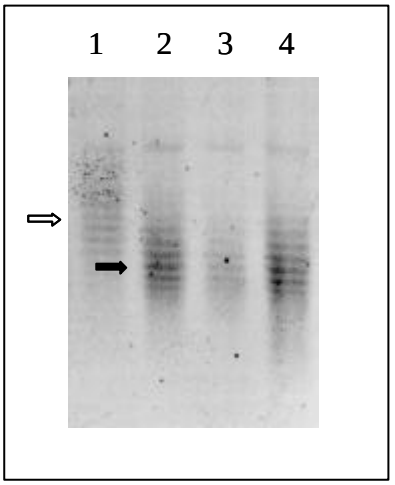

Fig 3. Sensitivity of negative supercoiling of plasmid pHel3 DNA to the gyrase inhibitor novobiocin. *H. pylori* N6 strains carrying pHel3 were grown to early log phase, at which time novobiocin 0.5 µg/ml (lane 2), 2 µg/ml (lane 3) and 8 µg/ml (lane 4) was added. Lane 1 shows a control without novobiocin treatment. Extracted plasmid DNA samples were analyzed by electrophoresis in agarose gels in the presence of 10 µg/ml chloroquine. White arrow indicates the supercoiling peak in the control sample, black arrow shows the shift of the peak to a more relaxed form in the treated samples.

The changes of supercoiling in the treated versus the untreated samples were visible at all concentrations after 12 hours, except samples treated with 0.001, 0.25, 0.5 and 2 µg/ml at 6 hours. Fig 3 shows the preparation of plasmid DNA from the samples treated with 0.5, 2 and 8 µg/ml novobiocin. A change of DNA

supercoiling was observed after novobiocin treatment. Plasmid DNA from the culture in the presence of novobiocin migrated faster than that from the control in the absence of novobiocin. This indicates relaxation of plasmid DNA supercoiling can be controlled in *H. pylori* using novobiocin treatment. This series of experiments demonstrated that addition of the gyrase inhibitor novobiocin reproducibly decreases the negative supercoiling in *H. pylori*.

3.2.1.4. *flaA* transcription is sensitive to changes of DNA supercoiling

After a reproducible system for changing DNA supercoiling in *H. pylori* was successfully established, we sought to determine the influence exerted by DNA supercoiling changes on the transcription of the major flagellin gene *flaA.* As has been shown previously, the short spacing of 13 bp in the *flaA* promoter is characteristic of all *H. pylori* strains analyzed, and a similarly short spacing was also found in the *flaA* genes of other *Helicobacter* species, like *H. felis* and *H. mustelae* (Suerbaum et al., 1998a). This was a first hint this promoter could be supercoiling-dependent. The wild type strain N6 was grown at 37°C in microaerobic condition as described in the methods, until an OD_{600} of 0.6 was reached. Then the culture was aliquoted into several equal volumes, and novobiocin was added at subinhibitory concentrations of 0, 4, 8, 16 µg/ml, and the cells were harvested after an additional incubation period of 6h. At this time point, novobiocin did not alter the growth rate of the bacteria, but induced a detectable decrease in plasmid DNA supercoiling. Total mRNA was extracted, and Northern blots of these RNA preparations with probes of DIG-*flaA* and DIG-16S rDNA were performed. Fig 4 shows that *flaA* transcription decreased with increasing novobiocin concentration when compared to a control sample. The *flaA* transcription was slightly decreased at 4 µg/ml, and severely reduced at 8 µg/ml and 16 µg/ml. By this method, it could clearly be shown that the transcription of the *H. pylori flaA* gene is exquisitely sensitive to changes of DNA supercoiling. This finding suggests that promoter spacing renders the *flaA* transcription sensitive to the changes of DNA supercoiling. Final assay

conditions for further studies were adjusted to harvesting at 6 hours of incubation, and to a novobiocin concentration of 8 µg/ml.

In contrast, transcription of the minor flagellin gene *flaB* showed no change under these conditions, which is different from previous findings obtained by Spohn et al. (Spohn and Scarlato, 1999b), probably because different concentration of novobiocin and different conditions were used *in vitro*.

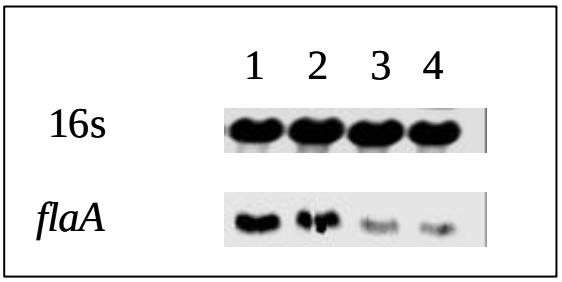

Fig 4. *flaA* expression by *H. pylori* treated with different concentration of novobiocin. Northern Blots of RNA samples under indicated conditions were prepared.1) control for 6 hours; 2) samples treated with 4 ug/ml novobiocin for 6 h; 3) samples treated with 8 ug/ml novobiocin for 6 h; 4) samples treated with 16 ug/ml novobiocin for 6 h.

3.2.1.5. Different promoter spacer mutants lead to different *flaA* transcription under conditions of changed DNA supercoiling

In order to further characterize the role of the *flaA* promoter in supercoiling-dependent changes of *flaA* transcription, the *flaA* s^{28} promoter, which has unusually short promoter spacer 13 bp between –10 and –35 region instead of the s^{28} consensus of 15 nucleotides (Leying et al., 1992; Suerbaum et al., 1998a), was chosen for site-directed mutagenesis. These strains were constructed and partially characterized by Tanja Brauer-Steppkes in the course of her Ph.D. studies (Brauer-Steppkes, 1999) and further characterized in this work. The strategy employed was to change the length of the *H. pylori flaA* promoter spacing region (Fig. 5) by introducing or deleting one or more nucleotides in this stretch of DNA sequence. The suicide plasmids containing mutated spacer sequences were then used in a two-step mutagenesis strategy via a *km-sacB* insertion in *flaA* to introduce the mutated spacer segments into

the genome of *H. pylori* as unmarked mutations. By this strategy, spacer segments of 11, 12, 14, and 15 nucleotides were obtained in the *H. pylori flaA* promoter.

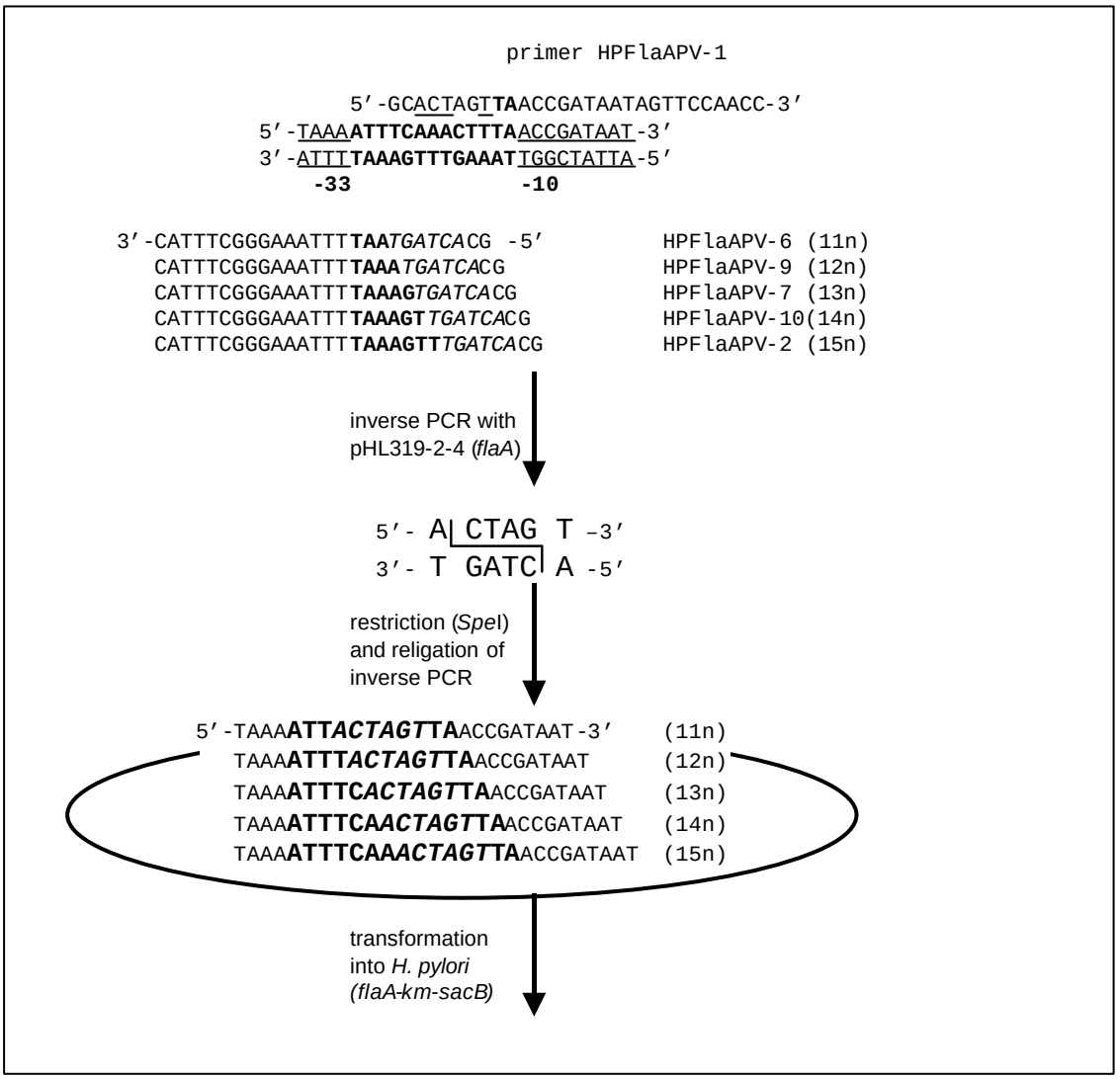

Fig 5. The strategy employed was to change the length of the *H. pylori flaA* promoter spacing region by introducing or deleting one or more nucleotides in this stretch of DNA sequence (Brauer-Steppkes, 1999)

Western blotting and immunodetection had shown (Brauer-Steppkes, 1999) that the expression of the FlaA protein was significantly reduced in the 12n and 14n strains (both show one nucleotide difference in comparison to the original promoter spacing). The 11n and 15n strains (two nucleotides difference in length compared to the original promoter, 15n corresponds to consensus s^{28}

promoters) expressed almost no FlaA. The morphology of the promoter mutant strains as assessed by transmission electron microscopy (Performed by C. Josenhans) was significantly different and differed as well from the wild type strain (Brauer-Steppkes, 1999). Strains that differed from the wild type *flaA* promoter by 1 nucleotide in length (12n, 14n) displayed less flagella than the wildtype strain, which were, in addition, mostly of uneven lengths. Bipolar arrangement of flagella was more frequently observed than in the wildtype strain. In addition, more than 20% of the 12n and 14n spacing mutant bacteria did not produce flagella, whereas wild type bacteria always produced flagella. P_{flaA} 11n bacteria assembled no (majority of cells) or only truncated flagellar filaments, which were almost exclusively composed of the minor flagellin FlaB. P_{flaA} 15n mutant bacteria produced no flagella (majority of cells) or one single long flagellum, which contained FlaA in addition to FlaB. These results had revealed that the promoter spacing is crucially involved in the protein expression of FlaA and in flagellar biogenesis.

To address directly the possible susceptibility of the *flaA* promoter activity to supercoiling and a role of the promoter structure in supercoiling-dependent transcriptional regulation, the following approach was taken in this work: 11n, 12n, 14n, 15n mutants and wild type were grown in liquid culture. When the culture reached an OD_{600} of 0.6, each culture was aliquoted into two equal amounts, and one was incubated with novobiocin at the concentration of 8 µg/ml, the other one was left untreated. After additional 6 h of incubation, the cells were harvested and RNA was extracted for semi-quantitative RT-RCR. In the absence of novobiocin, *flaA* transcript levels of the 14n mutant were reduced significantly in comparison to the wild type strain. In 11n, 12n and 15n spacing strains, no *flaA* transcript could be detected at this time point (Fig. 6A). In the presence of novobiocin, the *flaA* transcription increased almost twofold in 14n mutant (reconfirmed by Northern blotting (Fig. 6B)), while it decreased in the wildtype strain compared to the untreated sample (Fig. 6A). In 11n, 12n, and 15n mutants, no or very low amounts of *flaA* transcript could be detected.

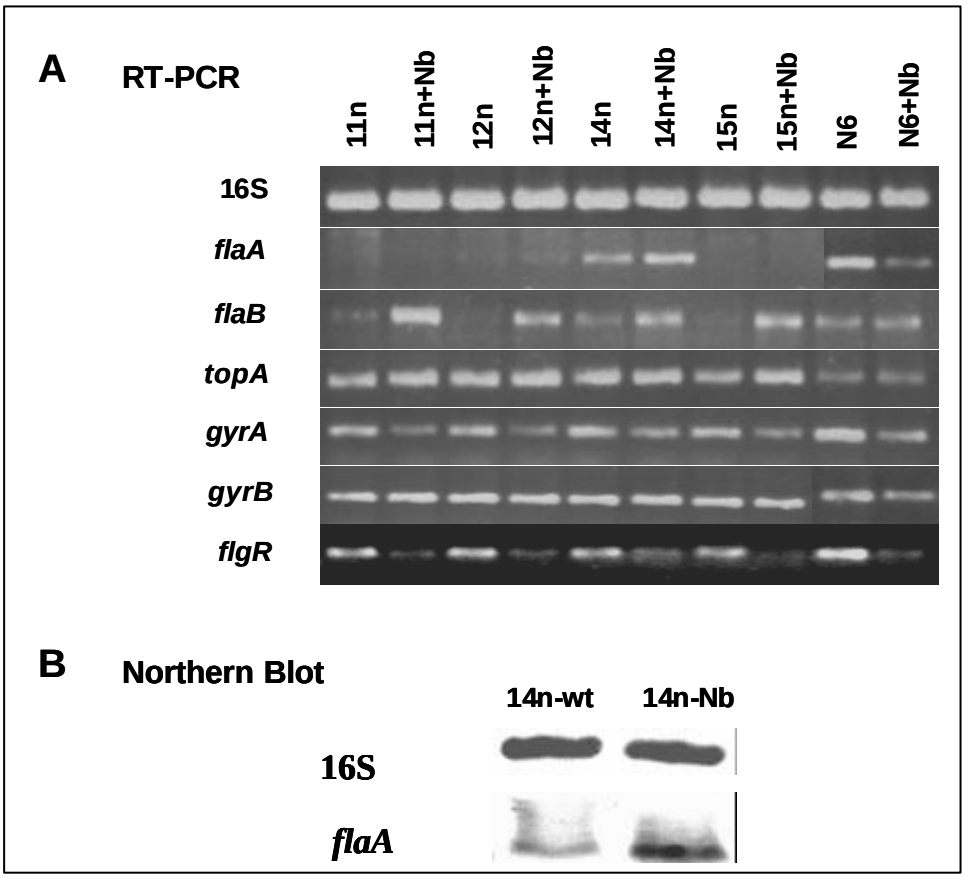

Fig 6. Different *flaA* promoter spacer mutants react differently to changes of DNA supercoiling. Promoter mutant strains 11n, 12n, 14n, 15n and wild type strain N6 were grown in liquid culture. 8 µg/ml novobiocin was added when the culture reached an OD_{600} of 0.6. After 6 additional hours, the cells were harvested and total RNA were prepared. A) Semi-quantitative RT-PCR done from RNA preparations from 11n, 12n, 14n, 15n and wildtype N6 strains in the absence /presence of novobiocin (+Nb), with primers specific for *flaA, flaB, topA, gyrA, gyrB, flgR* and 16s rDNA, are shown as indicated above the lanes; B) Northern Blots of whole RNA preparations of 14n strain in the absence /presence of novobiocin (+Nb) were hybridized with DIG-labelled probes specific for *H. pylori flaA* and 16s rDNA, are shown as indicated above the lanes.

These findings indicate that changes of promoter spacing reduce transcription and alter the reaction of the promoter to DNA supercoiling, and that the 13n spacing is optimal for *flaA* transcription in *H. pylori*. Transcript levels of *flaB* and *topA* did not change in the wild type strain in the presence of novobiocin as compared to the untreated samples. *gyrA* and *gyrB* transcript

levels were decreased in the wild type strain in the presence of novobiocin, indicating feedback regulation under conditions of gyrase inhibition. Strikingly, however, in the promoter spacer mutants, an increase of *flaB* and *topA* transcript levels was observed in the presence of novobiocin. *gyrA* transcript levels were reduced, but *gyrB* transcripts did not change by novobiocin in the *flaA* promoter mutants, as in the wild type strain.

3.2.2. Decrease of DNA supercoiling by overexpression of TopA

3.2.2.1. Determination of TopA protein in N6(pSUS316) and N6(pSUS1812)

To establish a second experimental system of changing DNA supercoiling, in order to confirm independently the results obtained by external addition of a gyrase inhibitor, a second approach was chosen: *H. pylori* N6 was transformed with a plasmid which contains the *H. pylori* topoisomerase I gene *topA* including its own promoter pSUS316 (Suerbaum et al., 1998a). As a control plasmid for unaltered supercoiling, a derivative of pSUS316 of the same size, but with a frame shift in the immediate 5' coding region of the *topA* gene, pSUS1812, created by site-directed mutagenesis by using primers HPTopA-IA1 and HPTopA-IA2, was likewise introduced into the wild type strain N6. To determine the amount of the TopA proteins present in the wild-type strain N6, TopA overexpression strain N6(pSUS316), and strain N6(pSUS1812), the three strains were grown in liquid culture and harvested at an OD_{600} of 1.2.

A Western blot analysis was performed with polyclonal antibodies against the TopA protein. Five micrograms of protein from each strain were loaded onto the gels. Proteins were separated by electrophoresis through 10% SDS-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (0.45-um pore size). Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody (Jackson ImmunoResearch). As shown in Fig 7, due to a gene copy effect, the level of TopA in strain

pSUS316 was approximately fivefold higher than in the wild-type strain and in the control strain pSUS1812.

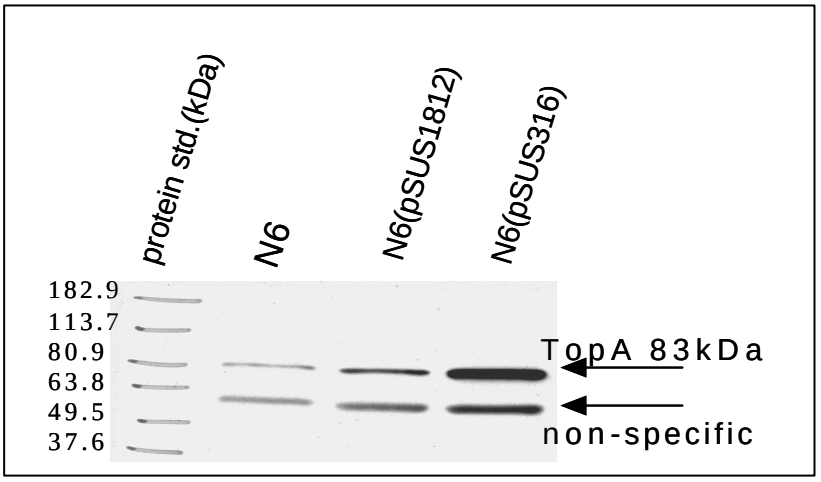

Fig 7. Determination of TopA protein in N6, N6(pSUS1812) and N6(pSUS316) by Western blot with anti-*topA* polyclonal antibody. 5 µg protein samples were separated by electrophoresis through 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Similar results were obtained in two independent experiments. 1) wild-type N6 2) N6(pSUS1812) 3) N6(pSUS316). The BenchMark™ Protein ladder was purchased from Invitrogen.

3.2.2.2. DNA supercoiling of pSUS316 and pSUS1812

In an attempt to further explore the effect of overexpression of TopA on the regulation of DNA supercoiling, plasmid DNA was extracted from N6(pSUS316) and N6(pSUS1812) strains and plasmid superhelicity was analyzed by agarose gel electrophoresis in the presence of 10 µg/ml chloroquine. Comparison of DNA supercoiling states of the two plasmids from both cultures showed (Fig8A) that plasmid pSUS316 which can overexpress TopA (Fig 7) caused a significant decrease of DNA supercoiling, in comparison to the plasmid pSUS1812, which has just the normal TopA expression from the chromosomal *topA* copy. This result demonstrated that overexpression of TopA decreased the DNA supercoiling in *H. pylori*.

Finally, both experimental methods indicated that a decrease of negative supercoiling can be achieved by both gyrase inhibition and TopA overexpression.

3.2.2.3. *FlaA* transcription after changes of DNA supercoiling by overexpression of TopA

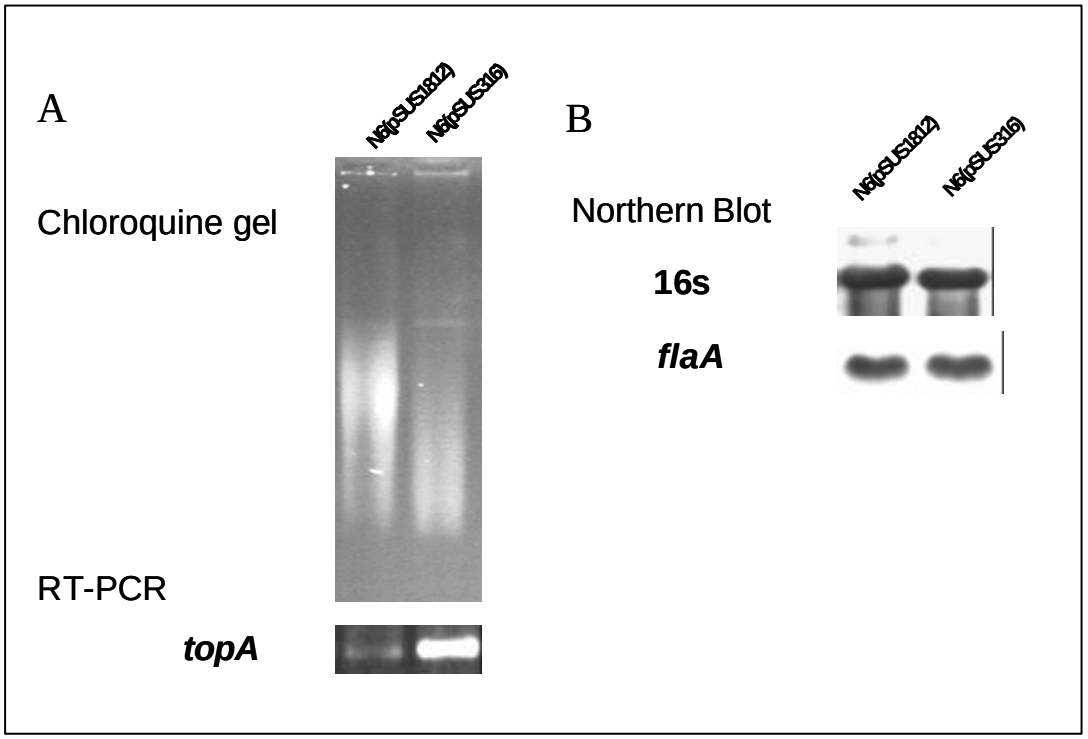

Fig 8. Response of *flaA* transcription on changes of DNA supercoiling by overexpression of topoisomerase I. N6(pSUS1812) and N6(pSUS316) strains were grown in liquid culture and harvested when an OD_{600} of 1.2 was reached. The plasmid DNA was isolated and total RNA was prepared. A) isolated plasmid DNA was checked on a 10 µg/ml chloroquine gel.1) pSUS1812; RT-PCR was done with primers specific for *topA.* 2) pSUS316; B) Northern Blot of whole RNA preparations hybridized with DIG-labeled probes specific for *H. pylori flaA* and 16s rRNA.1) N6(pSUS1812); 2) N6(pSUS316)

In order to check if two different experimental systems have the same effect on *flaA* transcription, associated with changing the DNA supercoiling, we grew *H. pylori* strains that carried the plasmid pSUS316 or pSUS1812 at the same conditions, and the cells were harvested at the same OD_{600} as above. The total

mRNA was extracted and *flaA* transcripts were checked by Northern blot. Unfortunately, the Northern blot result did not show the same results as obtained by the novobiocin experiments (Fig. 8B), since the *flaA* transcription did not change after the changes of DNA supercoiling. One possible explanation for this result may be that overexpression of TopA does not change DNA supercoiling sufficiently to cause a detectable change of *flaA* transcription (see discussion).

3.2.2.4. *flaA* expression upon the overexpression of TopA

To determine the influence of overexpression of TopA on *flaA* transcription in the *flaA* promoter spacer mutants, all *flaA* promoter spacing mutants (11n, 12n, 14n, and 15n) and the wild type strain N6 were also transformed with the test plasmids pHel2 (negative control) or pSUS316 (overexpression of TopA) and analyzed for *flaA* transcript. All the strains were grown in liquid culture, and harvested when an OD_{600} of 0.6, 1.5 or 2.5 was reached. Total RNA was extracted for Northern blot analysis. The wildtype strain showed no changes in *flaA* transcript amount upon overexpression of TopA at three time points, probably because DNA gyrase and topoisomerase I affected gene transcription in a different manner, and constitutive overexpression of TopA could not be achieved, indicating that this system may not be an optimal one for analysis of sensitivity of the *flaA* promoter to supercoiling.

3.2.3. Transcriptome analysis of global gene expression on the changes of DNA supercoiling

We next addressed the question of whether the two different systems used to change DNA supercoiling, would induce different responses at the level of global transcription in *H. pylori*. Many of the proteins involved in virulence are expressed at particular conditions resembling the host environment. Since DNA topology, which, by maintaining of the balance between topoisomerase I and gyrase, was found to be subject to changes in *H. pylori*, could most likely

function as a more global regulator of transcriptional activity in the cell, we sought to determine those global influences by whole genome microarray technology. Glass slide oligonucleotide microarrays spotted to a high density with a set of oligonucleotides corresponding to almost all open reading frames of the whole genomes of *H. pylori* 26695 and J99, and our custom produced PCR product arrays (representing 96.1% of the unique ORFs found in both 26695 and J99 *H. pylori* strains (Niehus et al., 2004)) were used for this purpose by a competitive hybridization methodology (Josenhans et al., 2002a). Two assay conditions, corresponding to the ones previously utilized to deduce supercoiling -dependency of *flaA*, were used again for the microarray experiments: 1) novobiocin-treated bacteria were compared with bacteria grown in standard liquid media; 2) TopA-overexpressing *H. pylori* N6(pSUS316) were compared with the control strain N6(pSUS1812).

3.2.3.1. Effect of DNA gyrase inhibitor novobiocin on the gene expression pattern

The wild type strain N6 was grown in liquid culture to an OD_{600} of 0.6, then the culture was aliquoted into two equal volumes, and one was incubated with novobiocin (8 µg/ml), and the other was used as control in the absence of novobiocin. After both cultures continued to grow for 6h, the cells were harvested. Four independent experiments were conducted on separate days, and total RNA was prepared from all the samples and used for transcriptome analysis. For each experiment, cDNA synthesized from RNA from the treated sample was used as reference and was labeled with Cy3 while cDNA synthesized from RNA from untreated sample was labeled with Cy5. Each Cy3 and Cy5 pair was hybridized to an oligonucleotide microarray (MWG). Ratios of each Cy5 and Cy3 fluorophore for each spot were obtained by scanning with an MWG418 scanner. Data for each experiment were evaluated statistically (see methods), and genes showing altered patterns of expression in both slides are list in Table 6.

Table 6. Genes showing major expression changes after novobiocin treatment

(ratio: untreated vs. treated)

1)Up-regulated gene					
		MWG slides(n=2)		PCR slides(n=3)	
TIGR No.	putative gene product	mean	SD.	mean	SD.
HP1432*	histidine and glutamine-rich protein	0.11	0.01	0.29	0.13
HP0011*	*groES* co-chaperone	0.17	0.04	0.2	0.07
HP0966	conserved hypothetical protein	0.18	0.02	0.72	0.08
HP0472*	*omp11* outer membrane protein	0.21	0.04	0.32	0.07
HP1322	hypothetical protein	0.24	0.03	0.45	0.17
HP1527	hypothetical protein	0.27	0.06	0.33	0.04
HP1431	*ksgA* 16S rRNA (adenosine-N6,N6-)-dimethyltransferase	0.29	0.05	0.34	0.1
HP0783	hypothetical protein	0.29	0.00	0.48	0.07
HP1564	outer membrane protein	0.30	0.01	0.35	0.1
HP1536	hypothetical protein	0.31	0.07	not present	
HP1153	*valS* valyl-tRNA synthetase	0.33	0.02	0.57	0.07
HP0901	hypothetical protein	0.34	0.10	0.9	0.03
HP1233*	hypothetical protein	0.34	0.07	0.64	0.16
HP0473	*modA* molybdenum ABC transporter	0.36	0.07	0.46	0.22
HP0010*	*groEL* chaperone and heat shock protein	0.37	0.04	0.23	0.05
HP0081	hypothetical protein	0.37	0.02	0.73	0.11
HP1415	*miaA* tRNA delta(2)-isopentenylpyrophosphate transferase	0.39	0.02	1.1	0.1
HP1238	*aimE* aliphatic amidase	0.39	0.01	0.49	0.09
HP0317	*omp9* outer membrane protein	0.41	0.02	1.25	0.2
HP0876	*frpB* iron-regulated outer membrane protein	0.41	0.07	0.87	0.1
HP1537	hypothetical protein	0.41	0.00	0.86	0.06
HP1065	hypothetical protein	0.41	0.04	0.77	0.07
HP0811	hypothetical protein	0.41	0.03	0.72	0.04
HP0602	endonuclease III	0.41	0.09	0.87	0.03
HP1254	*bioC* biotin synthesis protein	0.42	0.06	0.49	0.19
HP1204	*rpL33* ribosomal protein L33	0.42	0.08	0.63	0.03
HP0015	hypothetical protein	0.42	0.03	0.6	0.12
HP0012	*dnaG* DNA primase	0.42	0.02	0.42	0.11
HP1427*	*hpn* histidine-rich, metal binding polypeptide	0.43	0.02	0.46	0.05
HP0444	hypothetical protein	0.43	0.06	1.05	0.21
HP1051*	*envA* operon (sigma 28 regulated)	0.43	0.04	0.5	0.05
HP1324	hypothetical protein	0.44	0.01	0.62	0.08
HP0963	hypothetical protein	0.44	0.13	0.66	0.03
HP1526	*lexA* exodeoxyribonuclease	0.44	0.02	0.42	0.08
HP1343	putative Inner membrane protein	0.44	0.06	not present	
HP0174	hypothetical protein	0.44	0.09	0.97	0.09

HP1554	*rps2* ribosomal protein S2	0.44	0.02	0.38	0.13
HP0118	hypothetical protein	0.45	0.04	0.7	0.03
HP1203	*nusG* transcription termination factor NusG	0.46	0.08	0.41	0.12
HP0475	*modD* molybdenum ABC transporter, ATP-binding protein	0.46	0.00	0.86	0.12
HP1152*	*ffh* signal recognition particle protein	0.46	0.16	0.79	0.02
HP0366	spore coat polysaccharide biosynthesis protein C	0.46	0.01	0.62	0.11
HP0899	*hypC* hydrogenase expression/formation protein	0.47	0.02	0.81	0.17
HP1323	*rnhB* ribonuclease HII	0.47	0.22	0.5	0.14
HP0975	*gatC* Glu-tRNA(Gln) amidotransferase, subunit C	0.47	0.06	0.69	0.10
HP1074	hypothetical protein	0.47	0.01	0.92	0.06
HP1060	hypothetical protein	0.47	0.06	0.38	0.10
HP1240	conserved hypothetical protein	0.47	0.17	1.01	0.02
HP0377	thiol:disulfide interchange protein (dsbC), putative	0.47	0.06	0.46	0.11
HP0902	hypothetical protein	0.47	0.23	0.72	0.05
HP0878	hypothetical protein	0.48	0.05	1.11	0.15
HP0514	*rpl9* ribosomal protein L9	0.48	0.03	0.45	0.11
HP0051	DDEM cytosine specific DNA methyltransferase	0.49	0.05	1.2	0.11
HP0536	*cag15* cag pathogenicity island protein	0.49	0.13	0.62	0.06
HP0089	*pfs* pfs protein	0.49	0.04	0.52	0.07
HP0172	*moeA* molybdopterin biosynthesis protein	0.50	0.09	0.85	0.07
HP0359	hypothetical protein	0.50	0.15	0.95	0.03
JHP1462	putative	0.50	0.02	0.94	0.03
HP1211	hypothetical protein	0.50	0.01	1.11	0.24
HP1052	*envA* UDP-3-0-acyl N-acetylglcosamine deacetylase	0.50	0.04	0.66	0.10

2) Downregulated genes

		MWG slides (n=2)		PCR slides(n=3)	
TIGR No.	putative gene product	mean	SD.	mean	SD.
HP0643	*gltX* glutamyl-tRNA synthetase	2.00	0.20	1.10	0.90
HP0679*	*wbpB* lipopolysaccharide biosynthesis protein	2.01	0.18	1.10	0.91
HP0506	conserved hypothetical s ecreted protein	2.01	0.24	1.13	0.89
HP0397	*serA* phosphoglycerate dehydrogenase	2.03	0.12	1.07	0.95
HP1185	conserved hypothetical integral membrane protein	2.05	0.23	1.14	0.91
HP0541*	*cag20* cag pathogenicity island protein	2.05	0.18	1.12	0.94
HP0947	hypothetical protein	2.07	0.28	1.18	0.89
HP0785	conserved hypothetical secreted protein	2.07	0.20	1.13	0.94
HP0166*	ompR response regulator	2.08	0.54	1.31	0.77
HP1459	conserved hypothetical protein	2.10	0.18	1.14	0.96
HP0100*	conserved hypothetical	2.13	0.16	1.14	0.98
HP0644	conserved hypothetical integral membrane protein	2.16	0.21	1.19	0.97
HP1293	*rpoA* DNA-directed RNA polymerase, alpha subunit	2.18	0.15	1.16	1.01
HP0649	*aspA* aspartate ammonia-lyase	2.18	0.52	1.35	0.83

HP0125	*rpl35* ribosomal protein L35	2.18	0.38	1.28	0.90
HP0178	spore coat polysaccharide biosynthesis protein E	2.19	0.05	1.12	1.07
HP0424	hypothetical protein	2.20	0.43	1.31	0.88
HP1463	hypothetical protein	2.20	0.02	1.11	1.09
HP0822	*metL* homoserine dehydrogenase	2.20	0.65	1.42	0.78
HP0575	conserved hypothetical membrane protein	2.20	0.20	1.20	1.00
HP0607	*acrB* acriflavine resistance protein	2.23	0.40	1.31	0.92
HP0165	hypothetical protein	2.23	0.20	1.22	1.02
HP1397	hypothetical protein	2.24	0.59	1.41	0.83
HP1012	*pqqE* protease	2.24	0.03	1.13	1.10
HP1297	*rpl36* ribosomal protein L36	2.24	0.19	1.22	1.02
HP1014	*hdhA* 7-alpha-hydroxysteroid dehydrogenase	2.25	0.36	1.31	0.94
HP0426	hypothetical protein	2.26	0.54	1.40	0.86
HP0382	YJR117W zinc-metallo protease	2.27	0.42	1.35	0.92
HP0526	*cag6* cag pathogenicity island protein	2.27	1.07	1.67	0.60
HP0792	sigma-54 interacting protein	2.27	0.84	1.56	0.71
HP0716	conserved hypothetical protein	2.27	0.14	1.21	1.07
HP0756	hypothetical protein	2.29	0.49	1.39	0.90
HP1081	hypothetical protein	2.30	0.00	1.15	1.15
HP0916	*frpB* iron-regulated outer membrane protein	2.32	0.09	1.20	1.12
HP0944	conserved hypothetical protein	2.33	0.55	1.44	0.89
HP0308	hypothetical protein	2.35	0.40	1.37	0.98
HP0950	*accD* acetyl-CoA carboxylase beta subunit	2.36	0.58	1.47	0.89
HP0529*	*cag9* cag pathogenicity island protein	2.37	0.27	1.32	1.05
HP0126	*rpl20* ribosomal protein L20	2.38	0.48	1.43	0.95
HP1186	carbonic anhydrase	2.38	0.13	1.26	1.13
HP0150	hypothetical protein	2.41	0.48	1.44	0.96
HP0701	*gyrA* DNA gyrase, sub A	2.42	0.68	1.55	0.87
HP0890	conserved hypothetical protein	2.45	0.64	1.55	0.91
HP1454	hypothetical protein	2.47	0.16	1.31	1.15
HP0642	NAD(P)H-flavin oxidoreductase	2.50	0.34	1.42	1.08
HP0227	*omp5* outer membrane protein	2.52	0.68	1.60	0.92
HP0231	hypothetical protein	2.58	0.20	1.39	1.19
HP0561	*fabG* 3-ketoacyl-acyl carrier protein reductase	2.64	0.07	1.35	1.29
HP1361	*comE3* competence locus E	2.69	0.47	1.58	1.11
HP0287	hypothetical protein	2.71	0.21	1.46	1.25
HP0056	delta-1-pyrroline-5-carboxylate dehydrogenase	2.72	0.03	1.37	1.34
HP1466	conserved hypothetical integral membrane protein	2.73	0.30	1.51	1.22
HP0393*	*cheV* chemotaxis protein	2.74	0.02	1.38	1.36
HP1350	protease	2.75	0.09	1.42	1.33
HP0232*	secreted protein involved in flagellar motility	2.76	0.42	1.59	1.17
HP0791	*cadA* cadmium-transporting ATPase, P-type	2.79	0.36	1.57	1.22

HP0388	conserved hypothetical protein	2.80	0.60	1.70	1.10
HP0543*	*cag22* cag pathogenicity island protein	2.81	0.02	1.42	1.40
HP0570	*pepA* aminopeptidase a/i	2.84	0.18	1.51	1.33
HP0164	signal-transducing protein, histidine kinase	2.93	1.04	1.98	0.94
HP0560	hypothetical protein	3.00	0.18	1.59	1.41
HP0156	hypothetical protein	3.01	0.72	1.86	1.14
HP0574	*lacA* galactosidase acetyltransferase	3.08	0.56	1.82	1.26
HP1075	conserved hypothetical protein	3.15	0.41	1.78	1.37
HP0524	*cag5* cag pathogenicity island protein	3.16	1.42	2.29	0.87
HP0790	*prrB* anti-codon nuclease masking agent	3.16	0.36	1.76	1.40
HP0153	*recA* recombinase	3.20	0.40	1.80	1.40
HP0770*	*flhB* flagellar biosynthetic protein	3.23	0.14	1.69	1.55
HP0425	hypothetical protein	3.30	1.25	2.27	1.03
HP0002	*ribE* riboflavin synthase beta chain	3.35	0.03	1.69	1.66
HP0680	*nrdA* ribonucleoside-diphosphate reductase 1 alpha subunit	3.35	0.34	1.85	1.50
HP1294	*rps4* ribosomal protein S4	3.48	1.18	2.33	1.15
HP0006	*panC* pantoate-beta-alanine ligase	3.53	0.43	1.98	1.55
HP1123	*slyD* peptidyl-prolyl cis-trans isomerase, FKBP-type rotamase	3.62	0.12	1.87	1.75
HP0534	*cag13* cag pathogenicity island protein	3.63	1.06	2.35	1.28
HP0305	hypothetical protein	3.66	0.12	1.89	1.77
HP0001	hypothetical protein	3.68	1.57	2.62	1.06
HP0545	*cag24* cag pathogenicity island protein	3.73	0.18	1.95	1.78
HP0645	*slt* soluble lytic murein transglycosylase	3.73	1.17	2.45	1.28
HP0544*	*cag23* cag pathogenicity island protein	3.75	0.55	2.15	1.60
HP1192	secreted protein involved in flagellar motility	3.81	0.11	1.96	1.85
HP0234	conserved hypothetical integral membrane protein	4.13	0.20	2.17	1.97
HP0558	*fabF* beta ketoacyl-acyl carrier protein synthase II	4.60	0.11	2.35	2.25
HP1168	*cstA* carbon starvation protein	4.87	0.91	2.89	1.98
HP0133	*sdaC* serine transporter	4.90	3.23	4.06	0.84
HP0183	*glyA* serine hydroxymethyltransferase	5.13	3.36	4.24	0.89
HP0185	hypothetical protein	5.51	0.54	3.02	2.49
HP1019	*htrA* serine protease	5.77	1.11	3.44	2.33
HP1020	conserved hypothetical protein	6.06	3.25	4.65	1.41
HP0306	*hemL* glutamate-1-semialdehyde 2,1-aminomutase	6.59	1.50	4.04	2.55
HP0184	hypothetical protein	6.78	0.65	3.72	3.07
HP0824	*trxA* thioredoxin	6.79	0.30	3.54	3.25

*Confirmed by RT-PCR

It is evident that the expression level of many genes did not vary significantly after treatment of novobiocin (90% of spots varied by <2 fold). This suggests

that a large number of genes are not affected by DNA supercoiling under these conditions.

3.2.3.2. Classification of DNA supercoiling regulated genes

A total of 178 genes displayed altered transcription upon changes of DNA supercoiling (Table 6 (The complete microarray results are given in the Supplementary material)). Seventy of those showed up-regulation, and 103 showed down-regulation at this time point. We grouped these genes into functional classes as defined by “The Institute for Genomic Research”. This analysis revealed that a number of different biological functions were affected by DNA supercoiling.

1) Up-regulated genes

The first functional group induced by the change of DNA supercoiling, included some flagellar genes. Among these were σ^{28}-regulated flagellar gene, HP0472, whose promoter has a 15 nucleotides consensus spacing, but apparently a different transcriptional pattern from *flaA*. HP1233 is σ^{54}-regulated gene, of the non-flagellar genes. Heat shock genes (HP0010, HP0011), encoding stress-related proteins HspA and HspB. HP0536, which is a *cag* island gene. HP1427, which is a histidine-rich Hpn protein, was thought to play a major role in nickel storage (Kansau and Labigne, 1996). HP1052 is an orthologue of *envA* of *E.coli* encoding UDP-3-O-acyl N-acetylglucosamine deacetylase. This enzyme catalyzes the second step of lipid A biosynthesis and is a check point of regulation for LPS biosynthesis (Sorensen et al., 1996). HP1051 and HP1052 are co-transcribed.

2) Down-regulated genes

A second set of genes which showed a very distinct pattern of regulation under conditions of reduced DNA supercoiling, included a number of genes in the *cag* pathogenicity island (Censini et al., 1996; Fischer et al., 2001), encoding

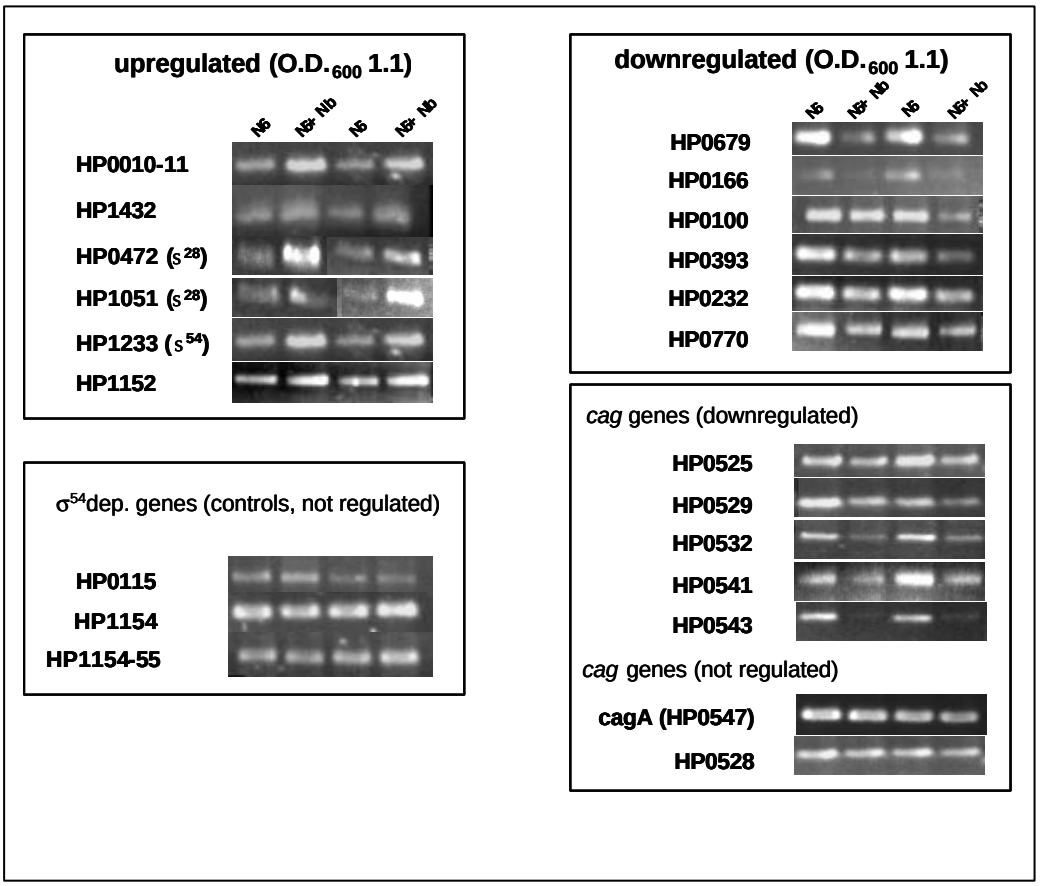

Fig 9. Confirmation of transcriptional changes of selected DNA supercoiling regulated genes by semi-quantitative RT-PCR. PCRs with two independent cDNA preparations derived from *H. pylori* N6 wildtype strain in the absence (wt)/presence of novobiocin (*Nb*) are shown as indicated above the lanes. Gene-specific products are indicated by ORF number and gene name. The σ^{54}-dependent genes *flaB* and *HP1154-1155* genes do not show any transcriptional changes under the conditions of changed DNA supercoiling. All other genes are upregulated or downregulated under the same conditions in concordance with the microarray data (Table 6).

structural components of the *cag* type IV secretion system. Not all genes in the *cag* island were affected to the same extent, e. g. transcription of the *cagA* gene, encoding for the translocated substrate CagA, was not affected by changes of supercoiling. HP0100 and HP0393 are chemotasis genes. HP0679 (*wbpB*), an enzyme which is involved in lipopolysaccharide biosynthesis (Tomb et al., 1997), and a two-component system composed of histidine kinase HP0165 and response regulator HP0166, which belongs to the OmpR family of two-component regulators (Beier and Frank, 2000), were also

down-regulated by the relaxation of DNA supercoiling. Two-component systems enable bacteria to regulate cellular functions in response to changing environmental conditions.

In previous studies, we demonstrated that *flaA* transcript was decreased after relaxation of DNA supercoiling by Northern Blot and RT-PCR. A significant decrease of *flaA* was not observed in the microarray analysis. Therefore, we reinvestigated the *flaA* gene expression by quantitative real–time reverse transcription–PCR as described in the methods. The real time PCR result indicated a fourfold decrease in the *flaA* transcripts in the treated sample (the ratio of *flaA* transcript amount in the absence/presence (4.653E+04/1.026E+04) of novobiocin was 4.5), confirming the previous Northern blot and RT-PCR results.

We did not detect a repression of *flaB* in our studies, either using the microarray or RT-PCR (Fig 9).

3.2.4. **Effect of overexpression of TopA on the gene expression pattern**

Table 7.Genes showing major expression changes after overexpressing TopA

(ratio: HPN6(pSUS1812) vs HPN6(pSUS316))

1) Upregulated genes			
		PCR slides(n=2)	
TIGR No.	Putative gene product	mean	SD
HP1427*	*hpn* histidine-rich, metal binding polypeptide	0.44	0.05
HP0117	conserved hypothetical protein	0.46	0.03
HP0318	conserved hypothetical protein	0.47	0.04
HP0011*	*groES*,co-chaperone	0.47	0.18
HP0111	hypothetical protein	0.48	0.19
HP0010*	*groEL* chaperone and heat shock protein	0.48	0.07
HP0110	*grpE* co-chaperone and heat shock protein	0.49	0.08
HP1588	conserved hypothetical protein	0.49	0.06
2) Downregulated genes			
HP1247	hypothetical protein	2.15	1.20

*Confirmed by RT-PCR

H. pylori N6(pSUS316) and N6(pSUS1812) were both grown in liquid culture to an OD_{600} of 1.2, and total RNA was extracted from the cultures and used for transcriptome analysis. Two independent experiments were conducted on separate days. For each experiment, cDNA synthesized from N6(pSUS1812) RNA was used as reference and was labeled with Cy3, while cDNA synthesized from N6(pSUS316)RNA was labeled with Cy5. Each Cy3 and Cy5 pair was hybridized to PCR product arrays (see above). Data for each experiment were evaluated statistically (methods), and genes showing altered patterns of expression in both slides are listed in Table 7 (The complete microarray results are given in the Supplementary material).

3.2.4.1. Classification of DNA supercoiling regulated genes

The transcriptome comparison between the TopA overexpressing strain N6(pSUS316) and the control strain N6(pSUS1812) showed only few genes whose expression was changed. Eight genes showed up-regulation at this time point, and only one gene down-regulated under these conditions was detected in this microarray analysis. This condition did obviously entail less severe consequences for the cellular equilibrium. The response to TopA overexpression was clearly different when compared with the novobiocin-induced response. There are three genes, the heat shock genes HP0010 and HP0011, and HP1427, which overlapped with supercoiling-dependent genes identified by novobiocin treatment. The possible reason for this will be discussed below.

Again, as *flaA* did not appear regulated under these conditions, we used real time PCR to check *flaA* transcription. The result indicated that transcript levels of *flaA* were substantially less decreased than after novobiocin treatment (the ratio of control sample /overexpression ($7.898x10^4/5.802x10^4$) of TopA sample is 1.36).

3.3. DNA supercoiling in *H. pylori* is growth phase dependent

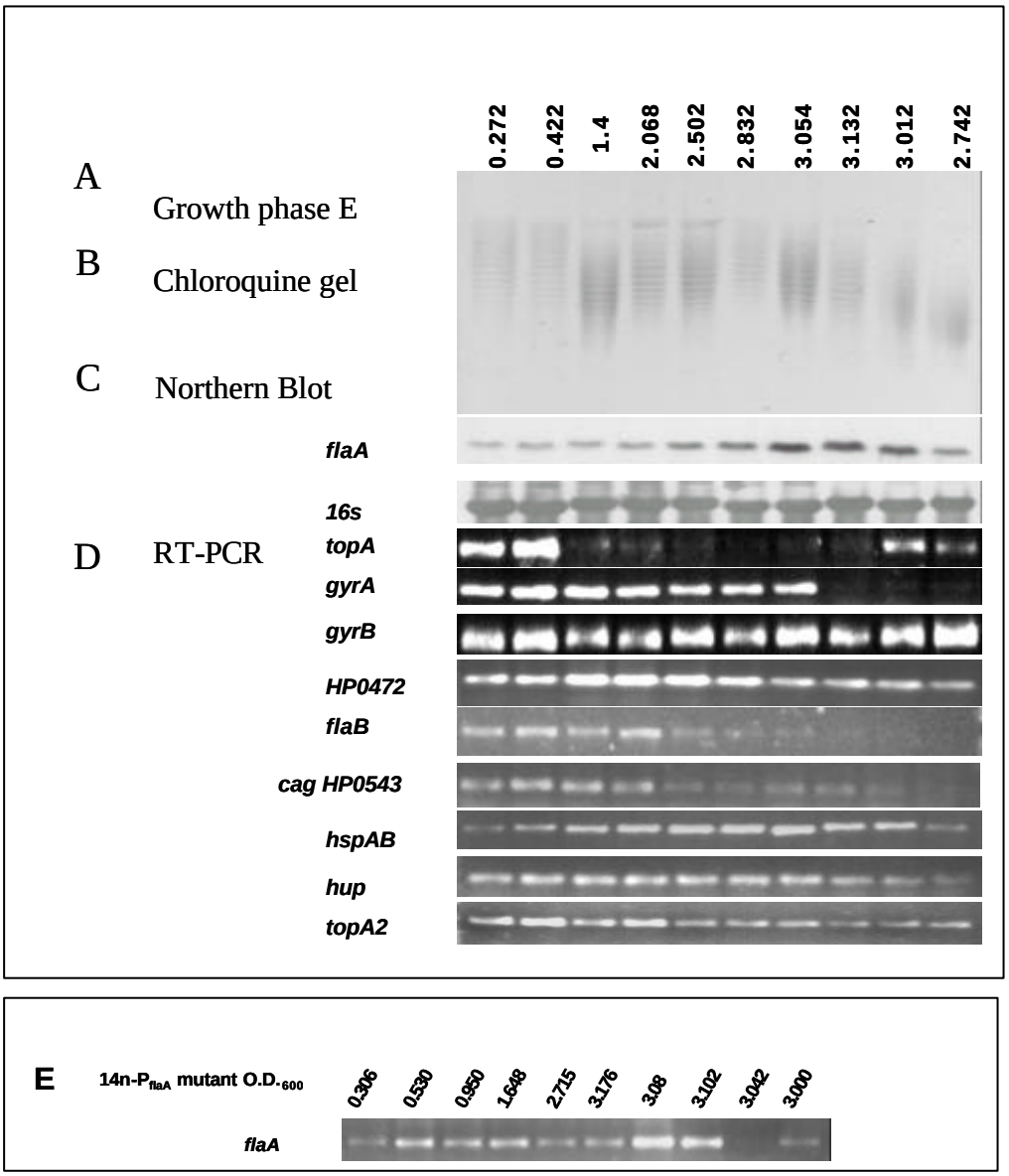

Fig. 10. *flaA* transcription and DNA supercoiling are growth phase dependent. A) Growth profile of *H. pylori* transformed strain N6(pHel3). Growth was monitored by measuring absorbance at OD_{600}. Cells were harvested at the time points indicated by arabic numbers, and used for DNA and mRNA analysis. B) Topoisomer analysis of plasmid DNA with bacterial native superhelicity. Samples were analyzed by electrophoresis in chloroquine agarose gels. The arrow indicates the time point of highest superhelicity. C) Northern blots of whole RNA preparations were hybridized with DIG-labelled probes specific for *H. pylori flaA* and 16S rDNA. D) semiquantitative RT-PCRs done from RNA preparations of the same growth curve with primers specific for *flaA, topA, gyrA, gyrB, topA2, hup, flaB, HspAB*, HP0543 and HP0472 were allowed to proceed for 16,30,22, 23,30,16, 22, 20, 19 and 30 cycles respectively. E) *flaA* transcription of 14n promoter mutant.

In order to achieve a better understanding of the regulation of DNA supercoiling during the growth phases, the level of supercoiling and expression of the *flaA* gene were determined throughout the growth curve. The first approach used was to determine, if global supercoiling changes can be observed throughout the growth phases of a native limited *H. pylori* culture in the absence of gyrase inhibitors. The N6 strain carrying shuttle-plasmid pHel3 was grown in liquid culture with shaking. OD_{600} was used to monitor cell growth. Fig. 10A shows the growth profile. Plasmid DNA was isolated at various growth stages as indicated in Fig 11B. Superhelicity of the plasmid DNA was examined by agarose gel electrophoresis in the presence of 10 µg/ml chloroquine (Fig. 10B). Increase of plasmid superhelicity was observed while the cells were growing exponentially, the highest superhelicity reached when the cells were in late exponential phase. Partial relaxation of plasmid DNA occurred when the culture reached stationary phase. The plasmid DNA became more relaxed after 72h than 64h. Clearly, the DNA supercoiling was growth phase-dependent.

Total RNA was extracted from the same time points during the growth curve, and prepared for Northern blot and semi-quantitative RT-PCR. Fig. 10C shows that the flagellin gene *flaA* was also transcribed in a growth phase-dependent manner (Niehus et al., 2002). *flaA* transcription increased during growth, a peak of *flaA* transcript level was observed at later log phase. The highest *flaA* transcription was correlated with the highest superhelicity during the growth phase. Thus, a link between growth phase, DNA superhelicity and *flaA* transcripts was suggested. To test if growth phase and possibly the associated DNA supercoiling can regulate different s^{28} promoters, we tested the transcription of another s^{28}-dependent gene, HP0472. The s^{28} promoter of this gene has a regular spacing of 15 nucleotides as compared with the 13 nucleotides of the *flaA* promoter. The result was that the transcriptional profile of HP0472 was completely different from that of *flaA* during the same growth

curves, the peak of HP0472 transcription was in the mid log phase, and was also correlated with lower DNA superhelicity. These data imply a potential role of DNA supercoiling for flagellin gene regulation and regulation of other σ^{28}-dependent genes throughout the growth phases in *H. pylori*. We also tested the *flaA* transcription of the 14n promoter mutant during the growth curve, and found a similar profile as in wild type, with a second transcription peak in early log phase (Fig. 10E). *FlaA* transcription in the 14n promoter mutant was also growth phase-dependent. The local configuration in the mutant might be slightly changed, make it slightly less sensitive to the DNA supercoiling. Our present hypothesis that growth phase regulation of late flagellar biosynthesis (Filament elongation by exclusive *FlaA* synthesis), which is necessary for the coordinated elongation of the flagellar filament in a certain phase of the growth cycle, is dependent on supercoiling will be further discussed below. We also included three additional genes, a functionally essential gene of the virulence-associated *H. pylori cag* island (HP0543) and the *hspAB* operon, whose transcription was shown by global transcriptome analysis to be supercoiling-dependent (Fig 9), in our growth phase transcript profiles. These genes showed quite distinct transcript profiles during the growth phases (Fig. 11D). The transcript profile of HP0543, although this gene is not σ^{54}-dependent (Niehus et al., 2004), was similar to *flaB*.

3.4. Regulation of *H. pylori* topoisomerase I and gyrase during the growth phase, and under conditions of changed supercoiling

At this stage of our analyses, the crucial question concerning the possible role of supercoiling changes *in vivo* remained to be addressed. As mentioned in the introduction, genome analysis of *H. pylori* strains indicate a highly simplified

system of DNA supercoiling controlled by only two enzymes, topoisomerase I and gyrase in this organism. The expression of the two enzymes and the balance between their activities had not been investigated previously, so that nothing could be presumed about a possible role of their respective activities in regulation. In *E. coli*, the main topoisomerases have been shown to be expressed in a constitutive manner (McEachern and Fisher, 1989). *topA* transcription in *E. coli* is under the control of four promoters, of which P1 is induced in response to heat shock (Qi et al., 1996). In contrast to *E. coli*, the *H. pylori topA* gene had previously been shown to have only one promoter (Suerbaum et al., 1998a). It had also been implied by several unsuccessful approaches at mutagenizing the gene that *topA* is essential for growth in *H. pylori*, which indicated that no compensating enzymatic activities exist. To look at the balance of genes that could influence supercoiling in *H. pylori* under in vitro growth conditions, we determined the transcript levels of *topA* gene and gyrase genes during different growth phases in broth culture. To verify topoisomerase I and gyrase transcription, semi-quantitative RT-PCR were performed with specific primers for *topA, gyrA*, and *gyrB* during the same growth curve. Fig10D shows that *topA, gyrA* and *gyrB* genes were differentially transcribed. *topA* was transcribed more highly in the earlier growth phases. In contrast, transcript levels of *gyrA* were almost constant during the exponential phase, and suddenly decreased when entering the stationary phase. *gyrB* showed almost no changes of transcript during the whole growth phase. Therefore, it can be assumed that global DNA supercoiling in *H. pylori* is subject to changes during the growth phases, and those changes might be dependent predominantly on changes of Topoisomerase I activity.

3.5. Characterization of TopA2

After the whole genomic sequences of the *H.pylori* strains 26695 and J99 were

published (Tomb et al., 1997), two ORFs (HP0116 and HP0440) were presumptively annotated as topoisomerase I. HP0116 consists of 2208 nucleotides, encoding a polypeptide of 736 amino acids and a predicted molecular mass of 83.2 kDa. HP0440 consists of 2031 nucleotides, encoding a protein of 677 amino acids and a predicted molecular mass of 77.6 kDa. Suerbaum et al. (Suerbaum et al., 1998a) demonstrated that H0116 functions as the *H. pylori* topoisomerase I. The function of HP0440, encoded by a gene in a variable segment of the *H. pylori* ("plasticity zone"), whose amino acid sequence is 30.5% identical to *H. pylori* TopA, and 23.7% identical to *E. coli* TopA, has not been intensively studied (Suerbaum et al., 1998a). We named HP0440 *topA2.* Five of 43 *H. pylori* strains tested had *topA2* (Brauer-Steppkes, 1999). Successful inactivation of *topA2* had previously shown that this gene is not essential for growth in *H. pylori*. Similarity between the *topA* and *topA2* genes of *H. pylori* raised the question whether TopA2 can have a similar function as TopA.

3.5.1. Growth phase dependency of *topA2*

To better understand the function of TopA2, the first approach of our study was to determine the profile of *topA2* expression during the growth curve. RT-PCR was performed with specific primers for *topA2* (Fig 10D). Fig 10D showed that *topA2* was also highly transcribed in the early exponential phase, and decreased during the growth phase, until the stationary phase was reached. Clearly, *topA2* was also transcribed in a growth phase-dependent manner, but the transcription pattern was not indentical with *topA* transcription.

3.5.2. Detection of the TopA2 protein in *H. pylori*

To determine the amount of TopA2 protein present in the wild-type starin N6 and *topA2* mutant, a Western blot analyses was performed with polyclonal antiserum against the TopA protein. Wild type strain N6 and the *topA2* mutant were grown in liquid culture, up to an OD_{600} of 1.2, and the cells were

harvested. The same amounts of protein of both strains were separated by electrophoresis through 10% SDS-polyaclamide gels and electrophoretically transferred to a nitrocellulose membrane. Horseradish peroxidase conjugated anti-rabbit IgG was used as secondary antibody. As shown in Fig 11, the antiserum reacted with a 83 kDa protein band which is TopA protein as confirmed before, but there was no second band corresponding to TopA2 in either strain. These results showed that TopA2 was not recognized by TopA antibody, suggesting that either TopA2 might not be expressed in *H. pylori* or that TopA2 is antigenically distinct from TopA. We then tested whether topA2 deletion would affect the global DNA supercoiling (Fig. 12 and below).

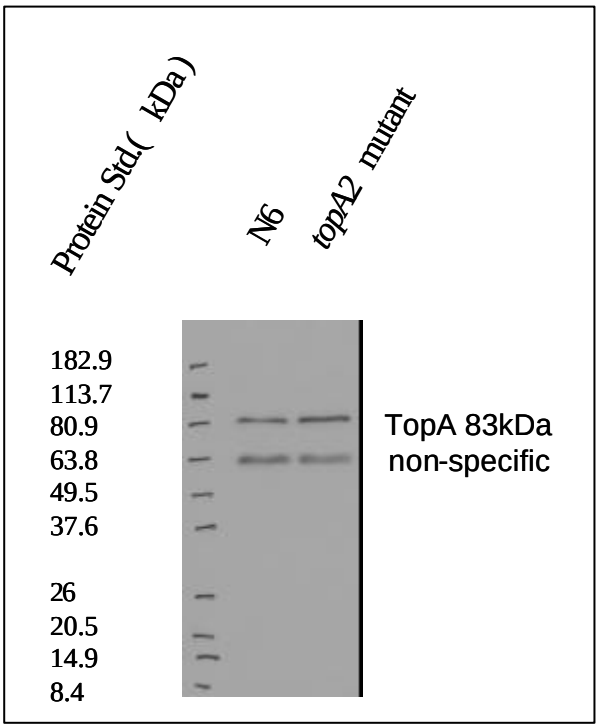

Fig 11 Determination of TopA protein in N6 and *topA2* mutant A) Western Immunoblot analyses of extracts from wild-type strain N6 and *topA2* mutant with anti-*topA* polyclonal antibody. 5µg protein samples were separated by electrophoresis through 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Similar results were obtained in two independent experiments. 1) wild-type N6 2) TopA2 mutant. The BenchMark™ Protein ladder was purchased from Invitrogen.

3.5.3. Determination of DNA supercoiling

In order to test whether TopA2 contributes to the changes of global DNA

supercoiling, the following approach was utilized. The shuttle plasmid pHel2 was transformed into the *topA2* mutant. The pHel2 carrying wild type strain and *topA2* mutant were grown in liquid culture, and the cells were harvested at an OD_{600} of 1.2. The growth rates of the two strains were indistinguishable. The same amount of plasmid DNA was isolated and visualized in the presence of 10 µg/ml chloroquine in the agarose gel. Fig 12 showed no detectable differences in the level of superhelicity when the topoisomers of the plasmid were isolated from both wildtype and *topA2* mutant. We can conclude that TopA2 does not contribute to global DNA supercoiling *in vivo*, although it might still regulate local topology or be more active under different conditions.

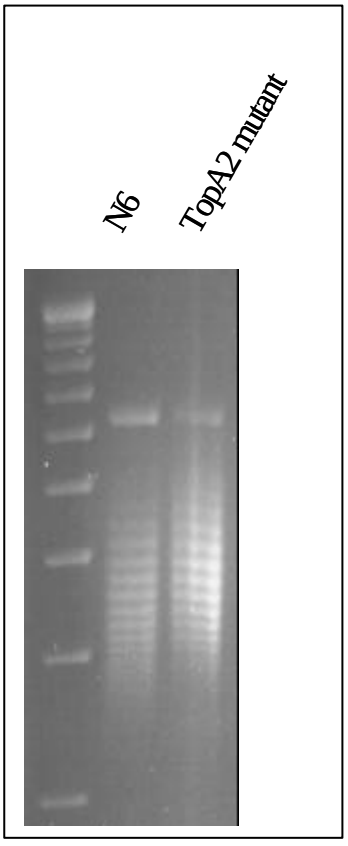

Fig 12. Determination of DNA supercoiling in *topA2* mutant: N6 strain and *topA2* mutant carrying the shuttle plasmid pHel2 were grown in liquid culture and harvested when an OD_{600} of 1.2 was reached. The plasmid DNA were isolated and checked on the 10 µg/ml chloroquine gel. 1) plasmid DNA from N6 2) plasmid DNA from *topA2* mutant

3.5.4. **Transcriptome analysis of the *topA2* mutant**

In order to better understand the role of TopA2 *in vivo*, a whole genome microarray analysis was performed. Wild type N6 strain and *topA2* mutant were both grown in liquid culture. Cells were rapidly harvested when culture reached an OD_{600} of 1.2, and total RNA was prepared and used for transcriptome analysis. Two independent experiments were conducted on separate days. For each experiment, cDNA synthesized from N6 RNA was used as reference and was labeled Cy3, while cDNA synthesized from *topA2* mutant RNA was labeled with Cy5. Each Cy3 and Cy5 pair was hybridized to an oligonucleotide array (MWG) and 2 PCR product arrays (3 slides in total). Data for each experiment were evaluated statistically, and genes showing altered patterns of expression in both slides are listed in Table 8 (The complete microarray results are given in the Supplementary material). Transcription of some genes was confirmed by semi-quantitative RT-PCR (Fig. 13 and Table 8).

Table 8. Genes showing major expression changes after loss of *topA2*

(ratio: wildtype (N6) vs. *topA2* mutant)

Up-regulated genes				
		MWG (n=1)	PCR slides (n=2)	
GeneName	Gene Description	ratio	mean	SD
HP1155*	transferase, peptidoglycan synthesis	0.25	0.63	0.39
HP0561	3-ketoacyl-acyl carrier protein reductase	0.28	0.47	0.13
HP0870*	*flgE* flagellar hook	0.28	0.66	0.33
HP0472*	*omp11* outer membrane protein	0.32	0.63	0.25
HP1119	*flgK* flagellar hook-associated protein 1 (HAP1)	0.32	0.71	0.00
HP0562	ribosomal protein S21	0.32	0.62	0.15
HP1076*	hypothetical protein (flagella associated)	0.33	0.62	0.44
HP0114	hypothetical protein	0.35	0.53	0.25
HP1154*	hypothetical protein	0.35	0.46	0.00
HP1233*	hypothetical protein (flagella associated)	0.36	0.36	0.00
HP0366	spore coat polysaccharide biosynthesis protein C	0.36	0.43	0.00
HP0073*	*ureA* urease, alpha subunit	0.37	0.39	0.05
HP1305	ribosomal protein S8	0.38	0.61	0.15

HP1298	translation initiation factor EF-1	0.41	0.65	0.01
HP1317	ribosomal protein L23	0.41	0.56	0.19
HP1120*	hypothetical protein (possibly flagella associated)	0.42	0.81	0.37
HP1306	ribosomal protein S14	0.42	0.70	0.11
HP0367	hypothetical protein	0.43	0.76	0.35
HP1302	ribosomal protein S5	0.44	0.52	0.19
HP0752*	*fliD* flagellar hook-associated protein 2 (flagellar cap)	0.44	0.60	0.00
HP1245	*ssb* single-strand DNA-binding protein	0.45	0.72	0.06
HP0560	hypothetical protein	0.45	0.82	0.28
HP1307	ribosomal protein L5	0.46	0.58	0.21
HP0071*	*ureI* urease accessory protein	0.46	0.65	0.08
HP0375	hypothetical protein	0.47	1.10	0.09
HP1430	conserved hypothetical ATP-binding protein	0.48	0.79	0.02
HP1497	peptidyl-tRNA hydrolase	0.49	0.60	0.07
HP0185	hypothetical protein	0.49	0.68	0.03
HP1428	conserved hypothetical protein	0.50	0.60	0.11
HP0084	ribosomal protein L13	0.51	0.60	0.00
HP1246	ribosomal protein S6	0.51	0.74	0.01
HP1429	*kpsF* polysialic acid capsule expression protein	0.51	0.71	0.08
HP0869	hydrogenase expression/formation protein	0.52	0.58	0.16
HP0072*	*ureB* urease beta subunit (urea amidohydrolase)	0.55	0.50	0.13
HP0751*	polar flagella-associated	0.58	0.66	0.22
HP0753*	flagellar protein (*fliS*)	0.59	0.49	0.00
Down-regulated genes				
HP0724	anaerobic C4-dicarboxylate transport protein	2.02	drop out	drop out
HP0909	hypothetical protein	2.04	0.99	0.00
HP0106	*metB* cystathionine gamma-synthase	2.05	0.96	0.36
HP0944	conserved hypothetical protein	2.08	1.85	0.64
HP1427	*hpn* histidine-rich, metal binding polypeptide	2.16	1.57	0.55
HP0243*	neutrophil activating protein (napA)	2.16	1.97	0.18
HP0013	hypothetical protein	2.49	1.14	0.25
HP1432	histidine and glutamine-rich protein	2.69	1.71	1.12
HP1074	hypothetical protein	2.94	drop out	drop out
HP0610	toxin-like outer membrane protein	3.86	1.73	0.00

*Confirmed by RT-PCR

3.5.4.1. Classification of *topA2*-regulated genes

A total of 46 genes displayed significantly altered transcription upon *topA2* deletion. The majority of these (36 genes) were induced, while 10 genes

showed a decrease in transcript levels. The data show that a *topA2* mutation has a different effect on *H. pylori* gene expression than *topA overexpression*. Table 8 shows the up-regulated and down-regulated genes. Fig. 13 shows confirmation of results by RT-PCR.

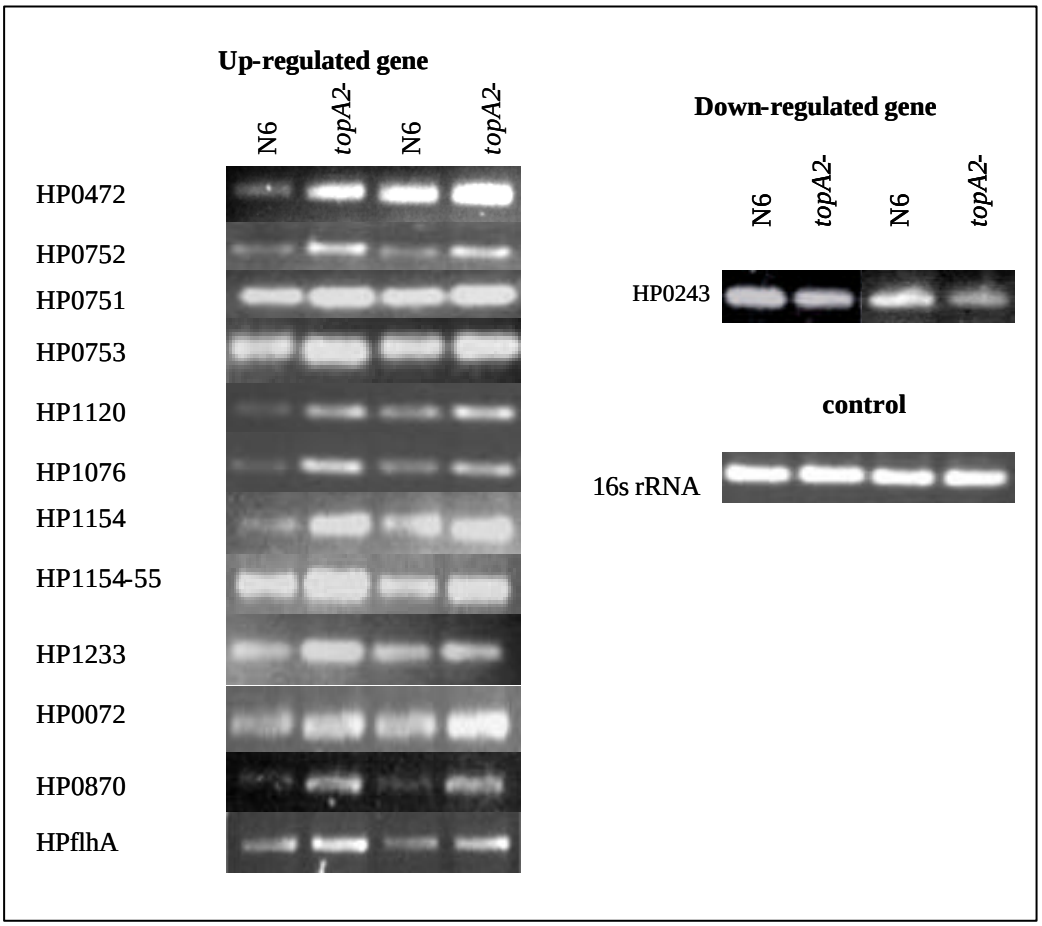

Fig.13 Confirmation of transcriptional changes of selected TopA2-dependent genes by semi-quantitative RT PCR. PCRs with two independent cDNA preparations (two biological experiments) derived from *H. pylori* N6 wildtype strain (N6) and *topA2* mutant (*topA2*-) are shown as indicated above the lanes. Gene-specific products are indicated by ORF number and gene name. The 16S rRNA control does not show any transcriptional changes in the *topA2* mutant compared to the wildtype strain. All other genes are upregulated or downregulated in the *topA2* strain in concordance with the microarray data (Table 9).

We grouped these genes into functional classes as defined by "The Institute for Genomic Research", which revealed that a number of different biological

functions are affected by the loss of *topA2*. These include a large number of genes predicted to encode hypothetical or conserved proteins of unknown function, chemotaxis and motility genes, proteins involved in metabolism and protein syntheses, components of the cell envelope, and factor involved in transport and binding.

1) Up-regulated genes

One of the most striking findings of our microarray analysis was the altered expression of a large number of genes that are components of the flagellar apparatus (table 9). These genes included HP0472, which is a s^{28}-regulated flagellar gene; HP0870, which is the s^{54}-dependent flagellar hook gene *flgE*; HP1119 and HP0572, which are flagellar hook-associated and cap proteins FlgK and FliD, respectively; HP0751 and HP0752, which encode polar flagellar component FlaG and the flagellin chaperone FliS, respectively. Other upregulated genes included HP1076, HP1233 and HP1154, which encode proteins of unknown function, presumably involved in flagellar function (Niehus et al., 2004). HP1155 (*murG*), which encodes a glycosyltransferase active in peptidoglycan biosynthesis, and HP0869 (*hypA*) which encodes a nickel-binding protein involved in hydrogenase and urease maturation (Mehta et al., 2003). Niehus et al. (Niehus et al., 2004) demonstrated that all of these genes were also downregulated in *H. pylori flhA* mutants. However, a significant increase of *flhA* was not observed in the microarray analysis. Therefore, we reinvestigated the *flhA* gene transcription by semi-quantitative PCR. The RT-PCR result indicated a two-fold increase in the *flhA* transcripts in the *topA2* mutant. *flhA* transcript could not be measured by microarray analysis since its transcription levels are very low (Niehus et al., 2002). In conclusion, these results suggested that TopA2 indirectly affects transcription of some class 2, class 3 and intermediate class flagellar genes mediated by negative influence on *flhA*. Taken together, TopA2 might be another global

transcriptional regulator that is involved in flagellar regulation under specific conditions.

Another group included on the list of upregulated genes are part of the urease operon, which are *ureA*, *ureB* and *ureI*. Activity of the enzyme is crucial for virulence, as urease is essential for colonization and persistence of *H. pylori* in the human stomach.

2) Down-regulated gene

The list of down-regulated gene includes components of the cell envelope, such as HP0610, encoding a vacuolating-like toxin outer membrane protein, which belongs to the Hop family (Alm et al., 2000). Another downregulated gene was *napA*. NapA, the neutrophil activating protein, is a putative virulence factor that acts directly on the surrounding tissues. This protein has the capacity to activate neutrophils and may be in involved in the recruitment of these cells in response to tissue inflammation (Evans, Jr. et al., 1995). NapA also has high homology to Dps, a DNA binding protein in *E. coli* (Cooksley et al., 2003).

3.6. HU-histone like protein

3.6.1. **Growth phase dependency**

After the whole genomic sequences of the *H. pylori* strains of HP26695 and J99 were published, only one histone-like protein gene could be identified in *H. pylori* which is *hup*. In *E. coli*, HU is encoded by two subunit genes, *hupA* and *hupB* (Claret and Rouviere-Yaniv, 1996), and the levels of the mRNAs varied during the growth cycle (Claret and Rouviere-Yaniv, 1997). To study the transcriptional regulation of the *hup* gene and its influence on gene regulation, the first step was to examine whether *hup* transcription is growth phase-dependent. RT-PCR was performed at different time points during the

growth phase with specific primers for *hup*. Fig 10D showed that *hup* transcripts are constitutively and highly expressed during the whole growth phase, indicating that HU is an abundant DNA-binding protein.

3.6.2. Construction of the *H. pylori hup* mutant

In order to better understand the role of HU in *H. pylori*, the first step was the construction of a HU mutant. The procedure of the construction was as follows: A genomic fragment of *H. pylori* 26695, encompassing ORFs 0834-0836 (1370bp), was amplified by polymerase chain reaction (PCR) using primers OLHPhup4 and OLHPhup5, and cloned into plasmid pILL570 (*Bgl*II–*Bam*HI). The resulting recombinant plasmid pSUS1810 was further amplified in an inverse PCR reaction using oligonucleotides OLHPhup6 and OLHPhup7. Thereby, a 85-bp deletion in the *hup* gene was created, which was closed by inserting the kanamycin resistance cassette (*aphA*3'-III) prepared from pILL600 using *Eco*RI sites. The resulting suicide plasmid pSUS1814, containing the *km*-cassette in the same transcriptional orientation as the *hup* gene, was introduced into *H. pylori* by natural transformation. Recombinant clones in *H. pylori* were selected on kanamycin-containing plates and isolated. Four clones out of each experiment were genetically characterized by PCR with primers OLHPhup4 and OLHPhup5, as well as oligonucleotides binding to the *aphA3'*-III gene. All clones proved to carry the genomic disruption of the *hup* gene as a result of a double-crossover event.

3.6.3. Growth properties of the *hup* mutant

The *H. pylori* HU mutant has a growth defect that is similar as in *E. coli* (Huisman et al., 1989). Figure 15 shows the growth profile of wild type strain and *hup* mutant. The doubling time of the *hup* mutant was significantly longer than that of the wild type strain under normal growth conditions. The *hup* mutant was 15h later than wild type strain to reach the stationary phase. It was notable that the mutant exhibited increased sensitivity to chloramphenicol. This

special sensitivity to chloramphenicol was observed during the transformation of the shuttle plasmid pHel2. During growth on agar plates, the wild type strain N6 carrying pHel2 formed colonies in the presence of 20 µg/ml chloramphenicol, while the HU mutant carrying pHel2 failed to form colonies at this concentration. It formed only small colonies on the plates that contained 10 µg/ml chloramphenicol. MIC determination of chloramphenicol in *hup* mutant was 16 µg/ml. Possible reasons for this increased sensitivity of the *hup* mutant to chloramphenicol will be discussed below.

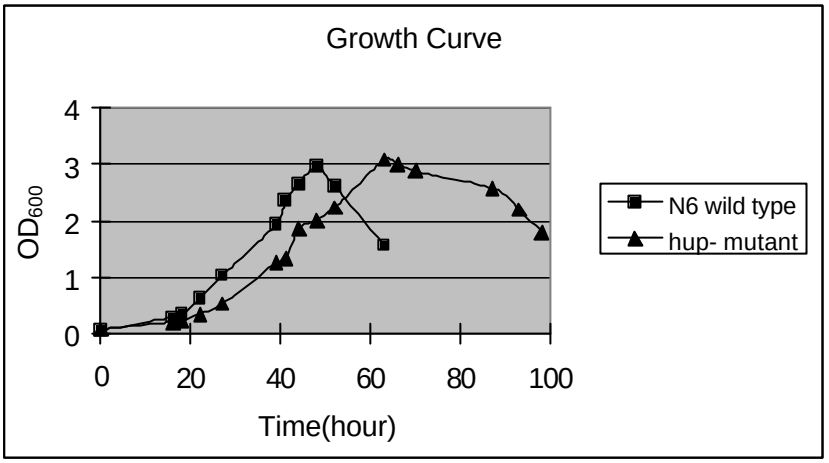

Fig 14 Growth profile of *H. pylori* transformed strain N6 and *hup* mutant. Growth was monitored by measuring absorbance at 600nm

3.6.4. Effect of *hup* mutation on plasmid supercoiling

The availability of a mutant in the *hup* gene encoding HU allowed us to examine whether the absence of HU affects DNA supercoiling. The shuttle plasmid pHel2 was transformed into *hup* mutant. pHel2 carrying Wild type strain N6 and *hup* mutant were grown in liquid culture up to an OD_{600} of 1.2. The cells were harvested and plasmid DNA was isolated. The same amount of DNA was analyzed in regular agarose gels and in the presence of 10 µg/ml of chloroquine in the gel. The plasmid DNA isolated from the *hup* mutant appeared as a smear in the agarose gel with one strong band of high supercoiling, which might be due to change of supercoiling of the DNA,

indicating that HU was needed for chromosome compaction, and/or protection of DNA from high gyrase activities. Fig 15 shows that the loss of HU changes supercoiling of extracted DNA, indicating that HU was a factor to change DNA supercoiling.

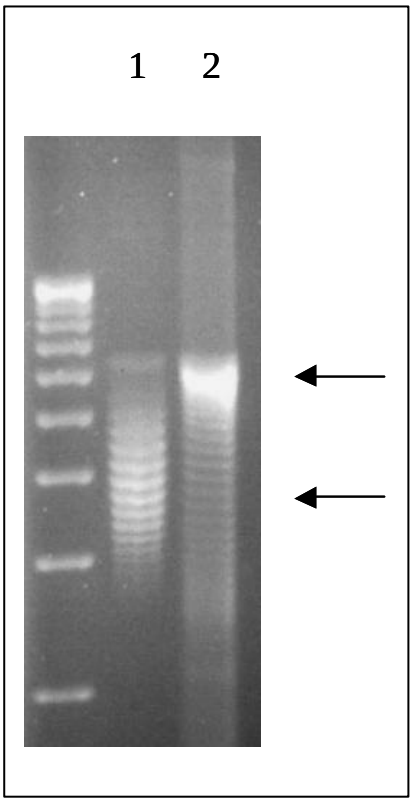

Fig. 15. Determination of DNA supercoiling in the *hup* mutant: N6 strain and *hup* mutant carrying the shuttle plasmid pHel2 were grown in liquid culture and harvested, when an OD_{600} of 1.2 was reached. The plasmid DNA was isolated and checked on 10 µg/ml chloroquine agarose gel. 1) plasmid DNA from N6. 2) plasmid DNA from *hup* mutant

3.6.5. Transcriptome analysis of *hup* mutant

A genome microarray analysis was performed to assess whether *hup* is a global regulator of gene expression in *H. pylori*. *H. pylori* wild type N6 and *hup* mutant were both grown in liquid culture and harvested at the same time point as above. Total RNA was prepared from the cultures and used for transcriptome analysis. Two independent experiments were conducted on separate days. For each experiment, cDNA synthesized from N6 RNA was used as a reference and was labeled with Cy3, while cDNA synthesized from from *hup* mutant RNA was labeled with Cy5. Each Cy3 and Cy5 pair was

hybridized to PCR product arrays (see above). Data for each experiment were evaluated statistically, and genes showing altered patterns of expression in both slides are listed in Table 9 (The complete microarray results are given in the Supplementary material). Various transcripts were confirmed by RT-PCR (Fig. 16).

Table 9. Genes showing major expression changes upon loss of *hup*

(ratio: wildtype (N6) vs. *hup* mutant)

		PCR slides (n=2)	
		mean	SD
Up-regulated genes			
HP0010*	chaperone and heat shock protein	0.27	0.05
HP0686	iron(III) dicitrate transport protein	0.28	0.04
HP0110	co-chaperone and heat shock protein	0.30	0.06
HP0111	hypothetical protein	0.31	0.03
HP0011*	co-chaperone	0.42	0.10
HP1401	conserved hypothetical protein	0.43	0.03
HP0294	aliphatic amidase	0.45	0.16
HP0109	chaperone and heat shock protein 70	0.46	0.10
HP0057	hypothetical protein	0.47	0.05
HP0769	molybdopterin-guanine dinucleotide biosynthesis protein A	0.49	0.05
HP0229	outer membrane protein	0.50	0.08
HP0725	omp17 outer membrane protein (Hop family)	0.50	0.06
Down-regulated genes			
HP0067	*ure H*, urease accessory protein	2.00	0.01
HP0827	ss-DNA binding protein 12RNP2 precursor	2.02	0.07
HP0126	ribosomal protein L20	2.02	0.00
HP0069	*ureF*, urease accessory protein	2.04	0.14
HP1275	*algC*,phosphomannomutase	2.04	0.13
HP0588	ferrodoxin-like protein	2.04	0.25
HP0835	histone-like DNA-binding protein HU	2.07	0.12
HP1269	NADH-ubiquinone oxidoreductase, NQO10 subunit	2.08	0.06
HP0549	glutamate racemase	2.14	0.11
HP0608	hypothetical protein	2.15	0.38
HP0388	conserved hypothetical protein	2.16	0.32
HP0124	translation initiation factor IF-3	2.17	0.21
HP0071*	*ureI*, urease accessory protein	2.17	0.15
HP0658	Glu-tRNA(Gln) amidotransferase, subunit B	2.18	0.03

HP1199	ribosomal protein L7/L12	2.20	0.50
HP0300	dipeptide ABC transporter, permease protein	2.23	0.23
HP1200	ribosomal protein L10	2.23	0.59
HP1186	carbonic anhydrase	2.24	0.08
HP1175	conserved hypothetical integral membrane protein	2.26	0.29
HP1272	NADH-ubiquinone oxidoreductase, NQO13 subunit	2.26	0.03
HP0591	ferredoxin oxidoreductase, gamma subunit	2.26	0.28
HP0499	phospholipase A1 precursor	2.26	0.10
HP1514	transcription termination factor NusA	2.28	0.16
HP0077	peptide chain release factor RF-1	2.31	0.33
HP1160	conserved hypothetical protein	2.33	0.06
HP0590	ferredoxin oxidoreductase, beta subunit	2.34	0.17
HP0302	dipeptide ABC transporter, ATP-binding protein	2.36	0.27
HP0942	D-alanine glycine permease	2.41	0.23
HP0498(db)	sodium- and chloride-dependent transporter	2.46	0.10
HP0692	3-oxoadipate coA-transferase subunit B	2.48	0.01
HP0070	*ureE*,urease accessory protein	2.52	0.08
HP0140	L-lactate permease	2.54	0.38
HP1432	histidine and glutamine-rich protein	2.64	0.24
HP0875	catalase	2.67	0.06
HP0691	3-oxoadipate coA-transferase subunit A	2.71	0.23
HP1192	secreted protein involved in flagellar motility	2.73	0.52
HP0696	N-methylhydantoinase	2.78	0.11
HP0487	hypothetical protein	2.97	0.17
HP0695	hydantoin utilization protein A	3.02	0.33
HP0825	thioredoxin reductase	3.29	0.11
HP0824	thioredoxin	3.35	0.41
HP0141	L-lactate permease	3.37	0.51
HP1564	outer membrane protein	3.49	0.09
HP0874	KapA protein	4.15	0.16
HP0697	hypothetical protein	4.49	0.21

*Confirmed by RT-PCR

3.6.5.1. Classification of *hup*-regulated gene:

We identified a total of 58 genes whose transcription was significantly changed in the *hup* mutant versus the wildtype strain. 12 genes were induced, while 46 genes showed a decrease in transcript levels. Overall, almost four times as many genes were down-regulated as opposed to up-regulated. We sorted these genes into functional classes as defined by “The Institute for Genomic

Research", which revealed that a number of different biological functions are affected by the loss of the *hup* gene. These include a large number of genes predicted to encode hypothetical or conserved proteins of unknown function, proteins involved in metabolism, translation, transport and binding, components of the cell envelope.

3.6.5.2. Gene transcripts up-regulated by *hup*

One group of genes organised in two operons significantly upregulated in *hup* mutant are stress-related genes, which included *dnaK, grpE*, and *hrcA* (*dnaK* operon), and *groEL* and *groES* (*groESL* operon). Another group of genes are the putative iron uptake gene, *fecA* (HP0686), which is an iron (III) dicitrate transport protein, was highly induced in the *hup* mutant. This protein may contribute to virulence, since *H. pylori* has an extensive ability to scavenge iron (Barabino, 2002). Interestingly, another gene, *amiE* (HP0294), encoding an aliphatic amidase, was also induced. As a group, bacterial amidases catalyze the hydrolysis of short-chain aliphatic amides to ammonia and the corresponding organic acid and play a valuable role in nitrogen metabolism (Skouloubris et al., 2001).

3.6.5.3. Gene down-regulated by *hup*

hup downregulated genes were associated with diverse functions. Many amino acid biosynthesis enzymes and amino acid transporters were detected as downregulated. In line with this finding, the transcription of genes coding for ribosomal proteins and for enzymes indirectly linked to amino acid biosynthesis was also decreased, which might suggest that loss of HU leads to a decrease in protein biosynthetic activity (one possible explanation for the observedgrowth retardation).

Urease is virulence factor that identified in *H. pylori*. Urease synthesis is directed by a seven-gene cluster, of which *ureAB* encode the structural

components of the enzyme and *ureIEFGH* encode accessory genes (Labigne et al., 1991; Cussac et al., 1992). Included on the list of downregulated genes are four genes that are part of the urease operon. The reduced level of urease gene transcripts in the *hup* mutant strain suggests that *hup* positively affects the transcription of urease cluster genes.

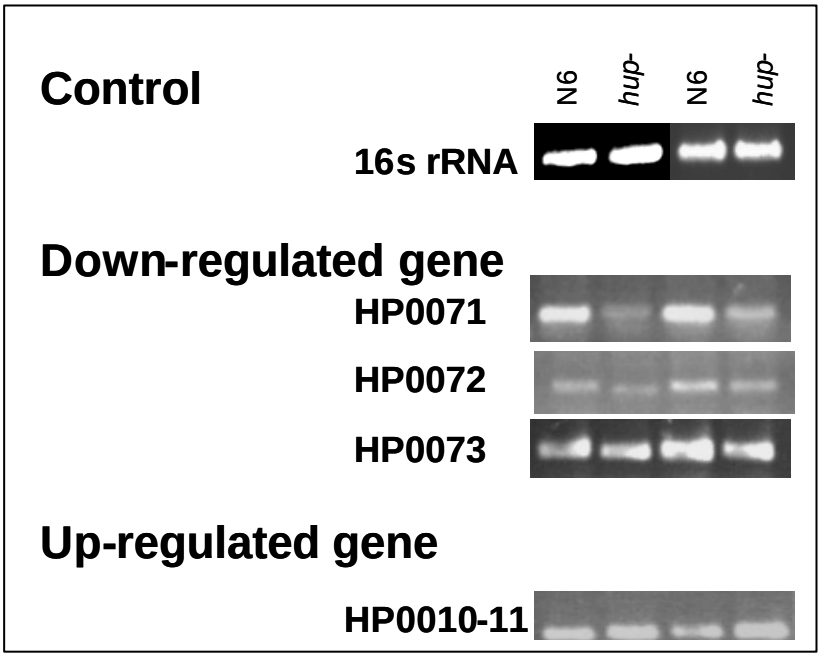

Fig 16. Confirmation of transcriptional changes of selected Hup-dependent genes by semi-quantitative RT-PCR. PCRs with two independent cDNA preparations (two biological experiments) derived from *H. pylori* N6 wildtype strain (N6) and *hup* mutant (*hup*) are shown as indicated above the lanes. Gene-specific products are indicated by ORF number and gene name. The 16S rRNA control does not show any transcriptional changes in the *hup* mutant compared to the wildtype strain. All other genes are upregulated or downregulated in the *hup* strain in concordance with the microarray data (Table 9).

3.6.5.3. Common effect

Since HU was a factor to change DNA supercoiling, it was of interest to know whether the inactivation of *hup* or novobiocin treatment affected the same genes. Table 10 shows that only a small set of genes are common to both sets of regulated genes. Among them, two genes are upregulated, four genes are downregulated, and two genes have opposite response in two different systems.

Table 10. Effects on gene transcriptions common between novobiocin treatment and *hup* mutant

TIGR No.	Putative product	Novobiocin treatment		*hup* mutant
		MWG slides	PCR slides	PCR slides
		mean of ratio (n=2)	mean of ratio (n=3)	mean of ratio (n=2)
HP0010	chaperone and heat shock protein ?	0.37	0.23	0.27
HP0011	co-chaperone ?	0.17	0.2	0.42
HP0126	ribosomal protein L20 ?	2.38	1.23	2.02
HP0388	conserved hypothetical protein ?	2.8	2	2.16
HP1186	carbonic anhydrase ?	2.38	1.73	2.24
HP1192	secreted protein involved in flagellar motility ?	3.81	4.1	2.64
HP1432	histidine and glutamine-rich protein	0.11 ?	0.29	2.73 ?
HP1564	outer membrane protein	0.3 ?	0.35	3.49 ?

*?: up-regulation?: downregulation

3.7. The s^{54} activator FlgR is coregulated with *gyrA by* DNA supercoiling

As mentioned above, there is genomic linkage between *flgR,* an NtrC-like RpoN activator (Spohn and Scarlato, 1999b), and *gyrA* in *H. pylori.* Co-regulation of these two proteins by supercoiling might provide a further level of control in the s^{54} flagellar regulon (Niehus et al., 2004). We were able to confirm *gyrA* and *flgR* coregulation (probably by cotranscription) by DNA supercoiling. *gyrA* and *flgR* transcript levels were reduced by more than half under the conditions of decreased supercoiling (Fig. 6).

3.7.1. Definition of the *flgR* regulon I: Construction of the *H. pylori flgR* mutant by allelic exchange

To gain insight into the transcriptional regulation by FlgR, which is controlled by supercoiling/gyrase activity, the first step was to construct a *flgR* mutant. The procedure of the construction of *flgR* mutant was as described in the following:

A genomic fragment of *H. pylori* 26695, encompassing ORFs 0702–0704 (1505bp), was amplified by polymerase chain reaction (PCR) using primers OLHPFlgR1 and OLHPFlgR2, and cloned into plasmid pILL570 (*Bam*HI–*Bam*HI). The resulting recombinant plasmid pSUS1802 was further amplified in an inverse PCR reaction using oligonucleotides OLHPFlgR3 and OLHPFlgR4. Thereby, a 515-bp deletion in the *flgR* gene was created, which was closed by inserting the kanamycin resistance cassette (*aphA*3'-III) prepared from pILL600 using *Eco*RI sites. The resulting suicide plasmid pSUS1807, containing the *km*-cassette in the same transcriptional orientation as the *flgR* gene, was introduced into *H. pylori* by natural transformation. Recombinant clones in *H. pylori* were selected on kanamycin-containing plates and isolated. Four clones out of each experiment were genetically characterized by PCR with primers OLHPFlgR1 and OLHPFlgR2, as well as oligonucleotides binding to the *aphA3'* gene. All clones proved to carry the genomic disruption of the *flgR* gene as a result of a double-crossover event.

3.7.2. Definition of the *flgR* regulon II: Transcriptome analyses of *flgR* mutant

To define the set of FlgR controlled target genes, we performed a genome-wide transcript profiling using the isogenic *flgR* mutant of the *H. pylori* strain N6.

H. pylori N6 and *flgR* mutant were both grown in liquid culture to an OD_{600} of 0.35, and total RNA was prepared from the cultures and used for transcriptome analysis. Two independent experiments were conducted on separate days. For each experiment, cDNA synthesized from N6 RNA was utilized as reference and was labeled with Cy3, while cDNA synthesized from *flgR* mutant RNA was labeled with Cy5. Each Cy3 and Cy5 pair was hybridized to custom-produced PCR product arrays (see above). Data for each experiment were evaluated statistically, and genes showing altered patterns of expression in both slides are listed in Table 11 (The complete microarray results are given in the

Supplementary material). There were 10 genes were downregulated in the *flgR* mutant. Among them, flagellar genes HP0115, HP1159 and HP1119-HP1120 were in agreement with previous findings (Spohn and Scarlato, 1999b). In addition, five new genes were detected as being dependent on FlgR. Four of the novel genes (HP1154, HP0367, HP1034 and HP1029) encode proteins of unknown function, HP1155 (*murG*) encodes a glycosyltransferase active in peptidoglycan biosynthesis, which has a housekeeping function in all cell wall-possessing bacteria (Niehus et al., 2004). In comparison to the transcript profile of *rpoN* mutants, two differences were observed: A gene cluster containing the *flhF/flhG* and *fliA* gene (HP1035 to HP1028), and the gene HP0367 appeared negatively regulated in *flgR* but not in *rpoN* mutants (Niehus et al., 2004). Under conditions of decreased supercoiling, the whole set of FlgR controlled flagellar genes may also be downregulated (see discussion).

Table 11. Genes showing major expression changes upon loss of FlgR

(ratio: wildtype (N6) vs. *flgR* mutant)

Downregulated genes			
		PCR-slides (n=2)	
TIGR No.	putative gene product	mean	SD.
flaB	*flaB* flagellin B	9.03	1.42
HP0295	flagellin B homolog	5.78	0.68
HP1120	hypothetical protein	3.55	1.17
HP1155	*murG* transferase, peptidoglycan synthesis	3.50	0.22
HP1154	hypothetical protein	3.02	0.17
HP0367	hypothetical protein	2.69	0.32
HP0115	*flaB* flagellin B	2.64	0.41
HP1034	*ylxH* ATP-binding protein	2.37	0.33
HP1559	*flgB* flagellar basal-body rod protein	2.33	0.1
HP1029	hypothetical protein	2.32	0.13
HP1119	*flgK* flagellar hook-associated protein 1 (HAP1)	2.09	0.1

4. Discussion

4.1. DNA supercoiling is a global regulator in *H. pylori*

4.1.1. **Changes of DNA supercoiling affect *flaA* transcription**

H. pylori typically possesses a polar bundle of two to six sheathed flagella that are essential for colonization in animal models of infection (Haas et al., 1993; Eaton et al., 1996; Foynes et al., 1999; Kim et al., 1999; Ottemann et al., 1999; Clyne et al., 2000). It is believed that motility plays an important role in that it is necessary to guide the bacterium from the extremely acidic stomach lumen to the more basic mucus layer of the gastric epithelium which is the preferred colonization site of *H. pylori* (Suerbaum, 1995; Schreiber et al., 1999; Schreiber et al., 2004). Two flagellin genes, *flaA* and *flaB*, are regulated by σ^{28} and σ^{54}, respectively, and are necessary for full motility (Josenhans et al., 1995). The existence of two distinct flagellin subunits, present in different amounts and under the regulation of different sigma factors, is characteristic of *Helicobacter* species (Josenhans et al., 1995; Josenhans et al., 1999). It has been suggested that *H. pylori* has the ability to alter the relative level of FlaA and FlaB in response to different environmental stimuli (Suerbaum, 1995). By doing so, the bacterium might adapt to microenvironments by alteration of the filament structure and flagellar flexibility.

In this work, we demonstrate that changes of DNA supercoiling in *H. pylori* affect gene regulation of the highly expressed late flagellar gene *flaA*. It was reported that the transcription of *flaA* is σ^{28}-dependent and repressed by the anti-sigma factor FlgM (Josenhans et al., 2002a). However, it was already clear that there is a further level of superimposed regulation that has not been elucidated so far. The *flaA* promoter has an unusually short spacer sequence, only 13 bp between the -10 and -35 regions, rather than the σ^{28} consensus of

15 nucleotides (Leying et al., 1992; Suerbaum et al., 1998a), and it has been suggested that such short spacing can render promoters sensitive to changes of DNA supercoiling (Wang and Syvanen, 1992). DNA supercoiling is modulated by the antagonistic actions of topoisomerase I and DNA gyrase. We have modulated supercoiling artificially using novobiocin to change supercoiling levels. Novobiocin is a DNA gyrase inhibitor that indirectly influences the supercoiling in the cell by inhibiting the activity of the gyrase B subunit (Maxwell, 1997). We used novobiocin to reproducibly and detectably alter cellular supercoiling in *H. pylori*. Subsequently, we found that inhibition of DNA gyrase with novobiocin causes a substantial reduction of *flaA* transcription. In addition, the peak of supercoiling (higher negative supercoiling) observed in late log phase bacterial cultures was accompanied by a significant increase in *flaA* transcript. These results indicate that the normal expression of the *flaA* locus is critically dependent upon the promoter structure and DNA supercoiling. The role of the promoter region was further investigated by the analysis of site-directed mutants in the spacer that have elongated or shortened spacing in comparison to the wild type strain. When the spacing of the *flaA* promoter was changed in the chromosomal copy of the *flaA* gene, less efficient *flaA* transcription, and decreased dependency of *flaA* transcription on DNA supercoiling were observed. Under specific conditions, the response of a mutated (14n) *flaA* promoter to supercoiling was inverted in comparison to the 13n wildtype promoter. This promoter structure might mediate a growth cycle control of the *flaA* gene. Various hypotheses have been put forward to account for the supercoiling sensitivity of promoters such as that of *flaA*. A consideration is that the poor fit to the consensus sequence of such promoters may make them more sensitive to alterations in DNA topology than promoters with a sequence closer to the consensus (Borowiec and Gralla, 1987). Many promoters, like the *E. coli tyrT* promoter, despite being some of the strongest in the bacterial cell, show poor homology to the consensus –10 and –35 sequences for s^{70} promoters, and also tend to display sub-optimal spacing

between the two regions. This promoter structure has been proposed to be important for growth-rate control and stringent regulation (Lamond, 1985; Travers et al., 1986).

Other flagellar genes tested, which are very likely needed at earlier stages of flagellar assembly, were regulated completely differently from *flaA* during the growth phases even though some of them also possess s^{28} promoters in *H. pylori*. As far as they could be identified and characterized, those show a regular consensus promoter spacing of 15 nucleotides and have much lower transcriptional activities (Josenhans et al., 2002a; Niehus et al., 2004). For instance, HP0472 encoding an outer membrane protein (Hop family), which has a s^{28} promoter with a 15n spacing, is the only gene except *flaA* which is exclusively dependent on FliA (Josenhans et al., 2002a), but its growth phase-dependency and response to novobiocin treatment are very different from those of *flaA*. Transcription of both these genes is growth phase-dependent as well as supercoiling dependent. These results are consistent with a necessity for the bacterium to specifically and more stringently control the highly expressed *flaA* gene, and provide an exclusive mechanism to switch on *flaA* transcription when it is needed during the growth phase and under specific environmental conditions. This stringent transcriptional control should also ensure the coordinated expression of the flagellar filament subunits for filament elongation at the exact time point in the growth cycle, after flagellar basal bodies and hook structures have been fully assembled. The two different s^{28} promoters seem to have adapted to different requirements of gene expression of the respective genes, with respect to transcription levels and temporal regulation. Such a differential regulation of two genes during the growth cycle might be related to changes in cellular energy and global supercoiling. However, this hypothesis cannot be experimentally tested at present, since no method is available to synchronize *H. pylori* cells.

It is well established that changes in the level of DNA supercoiling influence

the activity of many promoters (Gomez-Eichelmann, 1981; Wang, 1996), and that environmental variations alter this level (Balke and Gralla, 1987; Dorman et al., 1988; Bhriain et al., 1989; Ogata et al., 1994; Camacho-Carranza et al., 1995). In general, stationary-phase cells exhibit a number of morphological and physiological changes in order to survive starvation, and these changes include resistance to several harmful conditions, condensation of the nucleoid, increased protein degradation, and a general decrease in transcription and translation (Kolter et al., 1993). Many authors have described a DNA plasmid relaxation when cells enter into stationary phase (Balke and Gralla, 1987; Dorman et al., 1988; Reyes-Dominguez et al., 2003), but the role of DNA topoisomerases during the cellular response to these conditions is not yet completely understood. The nutritional up-shift has been shown to cause a very rapid increase of DNA supercoiling followed by relaxation (Balke and Gralla, 1987). The initial effect is due to an increase in the ATP/ADP ratio required for the activation of DNA gyrase (Hsieh et al., 1991b; Drlica, 1992; Jensen et al., 1995; van Workum et al., 1996). We suggest that growth-rate regulation of *flaA* and HP0472 expression implies a primary role for DNA topology in gene regulation. Modulation of DNA supercoiling during the growth cycle and by environmental conditions may provide a fine-tuned regulation mechanism of gene transcription, which is superimposed to and cooperates with regulation by transcription factors.

A genomic linkage between *flgR* and *gyrA* indicates that *flgR* is cotranscribed with *gyrA,* and both genes are coregulated by a single promoter. This suggests that changes in the expression of genes involved in regulation of DNA supercoiling might concomitantly work to change the expression of a subset of flagellar genes. Our data showed that, in addition, transcript levels of *flgR* were decreased under conditions of decreased supercoiling, similar to *gyrA*. This may imply that a coordinated regulation of both genes takes place in response to the same environmental stresses. The *topA* gene is located directly upstream of the *flaB* gene, and these two genes are transcribed in

divergent orientation by overlapping promoters (Suerbaum et al., 1998a), which suggests mutual interference of promoter activities. Spohn et al. reported that expression of *flaB* was also sensitive to changes of DNA supercoiling (Spohn and Scarlato, 1999b) under high concentrations of novobiocin. Unfortunately, in our studies we did not detect regulation of *flaB* by supercoiling in the wild type strain. The reason for this is unclear, but probably reflects differences of *in vitro* conditions, or the different concentrations of novobiocin that have been used by us compared to the work by Spohn and colleagues (Spohn and Scarlato, 1999b). However, our present findings of concomitant *flaB* and *topA* upregulation in all *flaA* promoter mutants with low *flaA* transcriptional activities also suggest that there is a distinct connection between transcriptional activities of the *flaA* promoter and the activities of the overlapping promoters of *flaB* and *topA*, respectively, such that inverse regulation between *flaA* and *flaB* can occur. The exact mechanism of this inverse regulation will be investigated in the future, but is most likely related to the specific requirements for flagellar biosynthesis during the growth cycle linked to chromosomal replication.

To establish another experimental system of changing DNA supercoiling to confirm independently the results obtained by the external addition of gyrase inhibitor, a second approach was chosen: *H. pylori* topoisomerase I was overexpressed (Suerbaum et al., 1998a; plasmid pSUS316). Under two different experimental conditions, which both led to changes of DNA supercoiling, inhibition of gyrase and overexpression of topoisomerase I, different influences of DNA supercoiling on *flaA* transcription emerged. Increased DNA relaxation by gyrase inhibition led to a significant decrease of *flaA* transcript, while the overexpression of *TopA* had no effect on *flaA* transcription. This difference might be due to the different mechanisms of function of DNA gyrase and topoisomerase I that might affect gene transcription in a different manner. Alternatively overexpression of TopA might not change DNA supercoiling in the *flaA* promoter region sufficiently to cause a

detectable change of *flaA* transcription. It also has to be kept in mind that inhibition of gyrase (novobiocin) is a strong negative manipulation which cannot be counteracted by the bacterial regulation mechanisms, while positive overexpression of topoisomerase using its own promoter, can be modulated by the bacteria.

4.1.2. Regulation of *H. pylori* topoisomerase I and gyrase during the growth phase, and under conditions of changed supercoiling

TopA and gyrase appear to be the master enzymes dedicated to maintaining DNA topology in *H. pylori*, as in other bacteria. Questions about the possible mechanisms of supercoiling changes in *H. pylori*, which could be responsible for a growth cycle-dependent or environmental regulation, remain, although a partial explanation might be offered in this study by the differential regulation of topoisomerase I (*topA*) and gyrase genes throughout the growth phases. In *E. coli*, constitutive transcription of *topA* throughout the growth phases was reported (Qi et al., 1996), since TopA expression in *E. coli* is under the control of four promoters (Tse-Dinh and Beran, 1988), which have balanced transcriptional activities during the growth phase. In contrast to *E. coli*, we observed changes of the *topA* transcriptional activity during the growth phases, most likely dependent on the activities of just one single *topA* promoter in *H. pylori* as previously described (Suerbaum et al., 1998a). The highest amounts of *topA* transcript were found in early log phase. Together with the finding that gyrase seems to be almost constitutively expressed throughout the growth phases in *H. pylori*, this indicates that TopA activity might be responsible for the observed changes of DNA supercoiling during exponential growth. Thus, TopA activity levels could work as a global regulator for a subset of susceptible genes, among them flagellar genes, cell division and virulence genes, under certain growth conditions and corresponding energy levels in *H. pylori*. In *E. coli*, DNA supercoiling homeostasis is maintained by balancing the expression of topoisomerase I and gyrase (Snoep et al., 2002), but this does not exclude

different overall levels of supercoiling under certain growth conditions (Reyes-Dominguez et al., 2003). In further studies, environmental conditions and cellular energy levels, involved in supercoiling-driven gene regulation, will have to be tested in *H. pylori.* In other bacteria, heat shock, changes in osmolarity, pH, temperature, or oxygen availability are all conditions which can lead to differential changes in supercoiling and gene expression, possibly by influencing cellular energy levels and the activity of different topoisomerase enzymes (Dorman, 1996). These might also influence global gene expression patterns, but this has only been tested sporadically so far. In *H. pylori*, changes during the growth phases, which correlate with changes in the metabolic activities as well as, to a limited extent, with changes in the bacterial cell cycle, led to changes of DNA supercoiling and changes in transcriptional activities throughout exponential growth *in vitro*. The hypotheses that the balance of gyrase and topoisomerase I activities is responsible for the changes of DNA supercoiling during the growth phases will have to be investigated in more detail in future work.

4.1.3. Influence of DNA supercoiling on global gene regulation

A recent genome-wide study in *E. coli* reported that changes in osmolarity/ supercoiling led to a switch in the global gene expression program (Cheung et al., 2003). Among other cell functions (such as energy metabolism and nutrient transport), motility and flagellar biosynthesis were affected by these changes. In *H. pylori*, changes during the growth phases, which correspond to changes in the composition of the growth medium, the energy levels of the cell, and to changes in DNA supercoiling, were previously correlated to changes in transcriptional activities of some flagellar genes (Niehus et al., 2002). Transcriptome analysis was employed in this study to find out, if changes of supercoiling were related to a global gene regulation programme. The majority of *H. pylori* genes showed no change of transcriptional activities under the conditions of decreased supercoiling *in vitro*. However, 178 genes were

affected under the same conditions. Among those were flagellar genes as well as genes involved in metabolism and virulence.

1. *cag* pathogenicity island (PAI)

Most virulence genes or presumptive virulence genes possess an element of environmental control in their regulation. This would make them prime candidates for control by the environmental changes of DNA supercoiling suggested for *H. pylori* by the current results. Microarray analysis for the role of supercoiling in global gene regulation in *H. pylori* revealed that some genes affected by supercoiling changed similarly to *flaA*, such as genes in the *cag* gene cluster. The *cag* pathogenicity island encodes a novel type IV secretion apparatus present in some strains of *H. pylori* (Censini et al., 1996; Akopyants et al., 1998), and it is well accepted that *H. pylori* strains carrying the PAI are more likely to be associated with serious manifestations of the *H. pylori* infection (Xiang et al., 1995). In the present study, the transcript levels of the majority of the genes in the *cag* PAI were decreased upon relaxation of DNA, including HP0524-HP0526, HP0529 and HP0541-HP0545. All of these genes have already been shown to be necessary for the function of the type IV secretion apparatus in protein translocation and cellular activation (Fischer et al., 2001). Not all genes in the *cag* PAI were affected to the same extent, for example, transcription of the *cagA* gene, encoding for the translocated substrate CagA (Hatakeyama, 2003), was not affected by changes of DNA supercoiling under our experimental conditions.

2. Flagellar synthesis

One of our aims was to analyze the expression of flagellar genes in response to DNA supercoiling. We demonstrated by single gene analysis that *flaA* transcripts were decreased after relaxation of DNA supercoiling.

Other supercoiling-dependent flagellar genes, which were induced by the decrease of negative DNA supercoiling, include a σ^{54}-regulated gene, HP1233

(possibly involved in the biosynthesis of the flagellar sheath), and three σ^{28}-regulated class 3 flagellar genes (Niehus et al., 2004), which are HP0472 and HP1051-HP1052. HP0472 (Hop family), whose promoter has a 15 nucleotides consensus spacing, has apparently a very different transcriptional pattern from *flaA* (see above). Chemotaxis genes HP0100 (neighbor to a chemotaxis sensor gene) and HP0393 (*cheV*-ortholog) were also decreased under conditions of decreased negative supercoiling, indicating a coregulation with *flaA* and filament elongation under low energy conditions. Although *flgR* was decreased under the same conditions, not the whole set of FlgR controlled genes was downregulated, suggesting a still more complex mechanism of regulation of flagellar genes.

In addition to *flaA*, we found some unusual promoter structures in weakly active genes of the flagellar and chemotaxis pathways (For example, the *motAB* promoter has a σ^{28} like promoter with a short 13n spacing). In the present studies, we were not able to detect any influence of supercoiling changes on these genes. From our and other studies, it is quite clear that not all transcriptional changes, especially driven by weak promoters, can be detected by microarray analyses, since the sensitivity is far lower than that of direct methods, such as Northern blots or primer extension. The possible activity of these unusual promoters will have to be further investigated.

3. heat-shock response

The stress response in bacteria is essential for effective adaptation to changes in the environment, as well as to the changes in the physiological state of the bacterial culture itself. Exposure of bacteria to environmental stresses such as high temperature or high osmolarity generally leads to the transient induction of heat-shock proteins, which protect the cell from damage caused by the formation of unproperly folded polypeptides (Neidhardt, 1987; Lindquist and Craig, 1988). It was known that plasmid DNA in exponentially growing *E. coli* immediately relaxes after heat shock and that relaxed DNA quickly supercoils

again after a short period of time, even when heat shock conditions are not interrupted (Ogata et al., 1994; Mizushima et al., 1997). Many heat shock proteins play an essential role in cellular metabolism under all growth conditions, assisting the folding, assembly and translocation of cellular proteins (Craig, 1993; Georgopoulos and McFarland, 1993; Hartl et al., 1994), therefore, the heat-shock response is a widespread phenomenon found in all living cells. The heat shock proteins of the human gastric pathogen *H. pylori* have been studied in some detail, because of their potential involvement in specific virulence mechanisms (Macchia et al., 1993; Suerbaum et al., 1994; Huesca et al., 1996). Microarray analyses show that the *groES-groEL* operon is induced by changes of DNA supercoiling, but the *hrcA-grpE-dnaK* operon shows no change. Spohn et al. demonstrated that both the *groESL* operon and the *dnaK* operon are transcribed by the housekeeping sigma factor s^{80} and negatively regulated by the transcriptional repressor HspR (Spohn and Scarlato, 1999b). They also showed transcription of the *groESL* operon was induced by osmotic shock, while transcription of the *dnaK* operon was not affected. These results taken together, we propose that regulation of the *groESL* operon in response to changes of environmental conditions is modulated by the topology of DNA, but not regulation of the DnaK operon.

4. Regulatory function (two-component systems)

Two-component systems are important prokaryotic signal transduction systems that allow regulation of cellular functions in response to changes of environmental conditions (Beier and Frank, 2000). Each of these systems is composed of a sensor protein and a cognate response regulator, which frequently works by acting as a transcriptional activator. The whole genome sequence of *H. pylori* 26696 shows only four open reading frames (ORFs) with homology to two-component histidine kinases, and six genes encoding response regulators (Tomb et al., 1997). One sensor-response regulator pair CheA-CheY has been identified as the *H. pylori* two component system

regulating chemotaxis (Tomb et al., 1997; Beier and Frank, 2000; Foynes et al., 2000). Histidine kinase HP0244 and the NtrC-like response regulator HP0703 (see below), have been demonstrated to positively regulate the transcription of genes encoding components of the flagellar basal body and hook and minor flagellin *flaB* (Spohn and Scarlato, 1999b; Beier and Frank, 2000; Niehus et al., 2004). Only one two-component system, consisting of HP0165-HP0166, showed decreased expression upon changes of DNA supercoiling in our studies. HP0166 (Dietz et al., 2002) is a response regulator that is similar to the OmpR regulator, which in *E. coli* regulates genes in response to osmolarity, pH and membrane-damaging agents. HP0166 was demonstrated to be an essential gene (Beier and Frank, 2000; Dietz et al., 2002); (Forsyth et al., 2002). Recently HP0166 was shown to be autoregulatory and to regulate expression of a number of genes of unknown function (Dietz et al., 2002). It has been suggested that acid stress induces *ompR* transcription in *S. typhimurium* by altering local DNA topology, not by changing the phosphorylation status of OmpR (Bang et al., 2002). In analogy to *E. coli* OmpR, recent genetic evidence suggests that HP165 stimulates gene regulation in response to acidification of the environment (Pflock et al., 2004).

Regulation of gene expression allows bacteria to better adapt to environmental changes. By turning on and off the transcription of a set of genes, bacteria can save energy and acquire nutrients in each environment. An organism like the gastric pathogen *H. pylori* undoubtedly encounters many environmental stresses within the human stomach, and must deal with drastic changes of osmolarity, pH and temperature. Sequencing of the *H. pylori* genome revealed a remarkably low number of transcriptional regulators, compared with other pathogens, which has been interpreted as an adaptation of this pathogen to its unique ecological niche (Tomb et al., 1997; Beier and Frank, 2000). Since the level of DNA supercoiling varies with cellular energy charge (Drlica, 1992), it can change rapidly in response to wide variety of altered nutritional and environmental conditions. The change of DNA

supercoiling appears to be a global alteration in *H. pylori*, affecting the entire chromosome and the expression levels of multiple operons whose promoters appeared sensitive to DNA supercoiling. Similar results have also been obtained for *E. coli* (Cheung et al., 2003). Thus, it appears that gene expression patterns are stringently regulated according to the nutritional requirements of the cell and environmental conditions, mediated by global supercoiling changes. Our analyses of the effect of changed DNA superhelicity on transcription using gyrase inhibitor confirms that DNA supercoiling adds a level of complexity to the previously known mechanisms of transcriptional regulation and regulons, and acts as a global superimposed regulatory mechanism in *H. pylori*, which interlinks several cellular functions. In recent work, a global switch of *H. pylori* gene expression was observed during the growth phases (Thompson et al., 2003). This switch might be due to the same global mechanism of supercoiling changes. We propose that global levels of gene expression from specific promoters are coupled to the growth, nutritional states, and environmental conditions through the regulation of transcription activity by mechanisms that are sensitive to DNA supercoiling. Future experiments to characterize the role of some of the identified factors in response to changes of DNA supercoiling should provide valuable information concerning the mechanisms used by *H. pylori* during its colonization within the gastric environment of the human host. Since DNA supercoiling is affected by environmental factors, genes that are sensitive to DNA supercoiling might constitute an important part of the ability of *H. pylori* to survive in its human host and cause disease. The study of some of these factors may facilitate elucidation of novel targets for vaccine development and antimicrobial therapy.

The sensitivity of gene expression to DNA supercoiling may partially explain, why supercoiling levels in *H. pylori* are tightly controlled. For some genes, the basal level of supercoiling sensitivity may have evolved to facilitate an adaptive response to changes in environment that elicit changes in supercoiling (Pruss and Drlica, 1989). Which genetic characteristics mediate supercoiling

sensitivity? Among the more interesting factors that vary from promoter to promoter is the twist angle between the –10 and –35 regions. This angle, which depends also on the number of nucleotides separating the two boxes, is sensitive to supercoiling and can either increase or decrease promoter activity (Brahms et al., 1985; Borowiec and Gralla, 1987; Aoyama and Takanami, 1988). Protein-dependent gene activation and repression that act through supercoiling-sensitive DNA binding and bending of DNA could be another factor imposed on the basal sensitivity. DNA supercoiling is also known to drive transitions to a wide variety of alternative secondary structures (Hatfield and Benham, 2002), including local denaturation (Kowalski et al., 1988), transitions to Z-form (Benham, 1987) and to H-form (Htun and Dahlberg, 1989), and cruciform extrusion (Lilley, 1986). The formation of alternative DNA structures can play regulatory roles, either by forming or modifying a regulatory binding site, or by altering the level of unconstrained supercoiling in the balance of the domain (Benham, 1996). The twin supercoiled-domain model (Liu and Wang, 1987) proposes that a local domain having a high level of DNA supercoiling can be generated between, and influences the activities of divergently transcribed promoters. Mojica et al. demonstrated that localized domains of increased negative DNA supercoiling are generated upstream of an actively transcribed promoter (Mojica and Higgins, 1996). They showed DNA supercoiling-mediated transcriptional coupling between the divergently oriented *tetA* and mutant *leu*-500 promoters. In addition, sensitivity can also arise indirectly from other supercoiling-sensitive genes whose products affect transcription. Thus assigning a cause for supercoiling sensitivity in vivo is not straightforward.

In the TopA overexpressing strain, in contrast to the control sample containing mutated inactive TopA plasmid, a much lower number of genes displayed altered expression upon the changes of DNA supercoiling by overexpression TopA. The response to TopA-overexpression showed some clear differences when compared with the novobiocin-induced response. As

described earlier, a key step of difference in the DNA supercoiling reactions between gyrase and topoisomerase I is that the DNA gyrase mediates ATP-dependent double-stranded cleavage and resealing of DNA, whereas topoisomerase I mediates single strand cleavage and resealing of DNA without additional input of energy. This functional difference may result in different effects on DNA supercoiling.

4.2. HU acts as another important global regulator in *H. pylori*

Histone-like proteins were thought to be involved in DNA supercoiling and play a role in gene regulation by bending the DNA to a certain angle. HU is thought to be responsible for maintaining the negative supercoiling of DNA (Broyles and Pettijohn, 1986; Drlica and Rouviere-Yaniv, 1987), through its ability to wrap DNA. It is likely that the interaction of DNA-binding proteins with DNA may be temperature-sensitive, and their release from DNA at high temperature may cause DNA relaxation catalyzed by DNA topoisomerases (Ogata et al., 1994), thus leading to a transient heat shock response.

HU is encoded by a single gene *hup* in *H. pylori*, and constitutively expressed during the whole growth phase, indicating that HU is an abundant DNA-binding protein. In *H. pylori*, although cells lacking HU protein are viable, they exhibit a growth defect that is similar as in *E. coli*. The doubling time of *hup* mutants was significantly longer than that of the wild type strain under normal growth conditions. This may be due to an influence on a number of HU-dependent cellular processes that have been reported and include, among others, DNA replication, DNA recombination, DNA transposition, and DNA repair (Bramhill and Kornberg, 1988; Dri et al., 1992; Lavoie and Chaconas, 1993; Boubrik and Rouviere-Yaniv, 1995). We also found a negative influence of HU in *H. pylori* on protein biosynthesis, which might also be involved in the observed growth retardation. The HU mutant exhibited increased sensitivity to

chloramphenicol, which may be due to the effect that altered plasmid supercoiling might influence the expression of the plasmid-borne chloramphenicol resistance gene. Our data indicate that HU plays an important role in the control of DNA supercoiling, possibly by its capacity to stabilize higher order nucleoprotein complexes through its interaction with supercoiled double-stranded DNA, similar to the interaction in *E. coli* (Drlica and Rouviere-Yaniv, 1987). It is known that, by serving as an indicator for physiological conditions, any changes in HU level will modulate the topology of DNA by constraint. More compellingly, HU may play multiple roles in determining the architecture of prokaryotic chromatin (Broyles and Pettijohn, 1986; Drlica and Rouviere-Yaniv, 1987; Dorman and Deighan, 2003; Rimsky, 2004).

Transcriptome analysis has shown that a total of 66 genes were differentially regulated upon *hup* deletion. Our data indicate that HU not only changes the global DNA supercoiling, but at the same time causes induction or repression of the expression of many genes. The response to inactivation of *hup* was clearly different when compared with the novobiocin-induced response. Only a small set of genes were common to both sets of regulated genes (table 10). This difference in regulation might be dependent on HU and gyrase ultilizing different mechanisms to affect gene transcription and to alter DNA structure.

In the *hup* mutant, all known stress-related genes were found to be significantly upregulated in this study, which included both the *dnaK* operon (*dnaK, grpE*, and *hrcA*), and the *groESL* operon (*groEL* and *groES*), while only *groESL* was dependent on a net change of supercoiling. Expression of both *the groESL* and the *dnaK* operons is repressed by the HspR repressor protein (Spohn and Scarlato, 1999b). Therefore, one plausible explanation for this finding is that HU may be involved in the regulation of the DNA binding affinity of HspR. The heat shock proteins of the human gastric pathogen *H. pylori* have provoked particular scientific interest because of their possible implication in the pathogenesis of this organism (Homuth et al., 2000).

Urease is a virulence factor of *H. pylori*. Urease synthesis is directed by a seven-gene cluster, of which *ureAB* encode the structural components of the enzyme and *ureIEFGH* encode accessory genes (Labigne et al., 1991; Cussac et al., 1992). Included on the list of downregulated genes are four genes that are part of the urease operon, which are *ureE, ureF, UreH* and *ureI*. It has been shown recently that UreI acts as an inner membrane proton-gated, urea - specific channel (Weeks et al., 2000). The UreI pore opens as the pH of the medium drops below 6.5 and as the cytoplasmatically localized urease gains access to its urea substrate. As urease activity neutralizes the cytoplasm and the local environment, the pore closes and urea transport stops, thus providing a regulated level of intracellular enzyme activity (Weeks et al., 2000). The reduced level of urease gene transcripts in the *hup* mutant strain, indicates that *hup* can use changes of environmental conditions (such as pH) as a signal to affect the transcription of urease cluster gene, via a mechanism requiring structural alteration of the promoter DNA upon protein binding (Gosink et al., 1993).

We hypothesize that HU might act as another important global regulator in *H. pylori*. The microarray data show increased gene expression of heat shock proteins and a decreased transcription of the urease gene cluster. This finding indicates a co-ordinated response of *H. pylori* to changes of environmental conditions in its specific ecological niche. There are two variables that might affect HU-dependent promoter activity: the superhelical density of DNA and the occupation of HU binding sites (Semsey et al., 2002). Such a combination of direct and indirect effects would allow for fine tuning of gene expression in response to changes in environmental conditions. The experimental confirmation of a definitive role of HU in the expression of genes involved in many cell functions in various bacteria has triggered a considerable interest in the possible mechanisms involved. The direct DNA - binding effect of HU may contribute to the observed difference in supercoiling and gene expression between the wild type and *hup* mutant. Although HU typically binds to DNA

nonspecifically, *E. coli* HU binds to the H1 sequence with high affinity and specificity, forming an intasome (Segall et al., 1994). Comparable HU binding specificity has also been observed in higher order nucleoprotein structures, such as the *gal* repressor complex (Coombs and Cann, 1996) and the Mu transpososome (Lavoie and Chaconas, 1993; Lavoie and Chaconas, 1996). HU binding to nicked (Castaing et al., 1995), cruciform (Pontiggia et al., 1993; Bonnefoy et al., 1994) and negatively supercoiled DNA exhibits similar binding specificity. HU binding to DNA causes a conformational change in DNA, on the other hand, and conformational changes can in turn affect HU binding affinities. We further suggest that HU might also stimulate reduced translation of genes by modifying RNA secondary structure to facilitate or inhibit ribosome binding.

To our best knowledge, the present work describes for the first time in a genome-wide study that HU is involved in global gene regulation in bacteria. Our data show that HU not only contributes to virulence gene regulation, but also to many other cell functions. Future experiments to characterize in more detail the function of HU in the regulation of various genes identified in this study will provide valuable information concerning the mechanisms used by *H. pylori* during colonization within the gastric environment of the human host.

4.3. TopA2 acts as a global regulator of flagella and urease in *H. pylori*

After the whole genomic sequences of *H. pylori* strains 26695 and J99 were published (Tomb et al., 1997), two ORFs (HP0116 and HP0440) were presumptively identified as topoisomerase I orthologues in the sequence annotation. Suerbaum et al. demonstrated that HP0116 is the functional *H. pylori* topoisomerase I (Suerbaum et al., 1998a). HP0440 (*topA2*), a gene of the variable plasticity zone (Suerbaum S, 1998), was found in only few (5 of 43)

strains (Brauer-Steppkes, 1999), which indicated that its acquisition might be a consequence of evolution of *H. pylori*. Horizontal transfer of new genes (Suerbaum et al., 1998a; Miehlke et al., 1999; Suerbaum, 2000) has been demonstrated to contribute to evolution. Western blot analysis indicated that TopA2 might be antigenically different from TopA. In comparison to *topA*, whose sequence contains four zinc finger motifs (Suerbaum et al., 1998a), there is one zinc finger motif missing in the TopA2 sequence, which might help us to understand the functional difference between these two topoisomerases.

In this study, we found that *topA2* was also transcribed in a growth phase-dependent manner, but differently from *topA* transcription. It was also transcribed highly in the early exponential phase, similarly to *topA*, but decreased continuously while the cells were growing, until the stationary phase was reached. We conclude that Topoisomerase I plays a more important role than TopA2 in controlling and maintaining DNA supercoiling in *H. pylori*. This conclusion is based on the following: 1) Topoisomerase I/*topA* is an essential protein/gene, and, in contrast, we demonstrated that *topA2* was not essential for growth in *H. pylori*. The growth rate of a *topA2* mutant showed no detectable difference in comparison to the wild type strain, indicating that this activity is not essential for cell growth. 2) In addition, the finding that TopA2 failed to complement a defect in an *E. coli topA* mutant (Suerbaum et al., 1998a) also supports this conclusion. 3) Topoisomerase I relaxes negative DNA supercoils, but in contrast, an assay of reporter plasmid supercoiling suggests that TopA2 does not contribute to global DNA supercoiling *in vivo*. Since reporter plasmids might not necessarily reflect slight local changes in chromosomal supercoiling *in vivo*, it cannot be excluded that it might still regulate local DNA topology, and changes of local superhelicity might also affect expression of relevant genes located in those regions for physiological need (Opel et al., 2001). However, despite a sofar elusive role of TopA2 in DNA topology, we were able to establish a function for TopA2 in gene regulation by comprehensive transcriptome analysis in this study.

H. pylori possesses a small set of obvious regulatory genes, among them three RNA polymerase sigma factors (σ^{80}, σ^{54}, σ^{28}; Tomb et al., 1997; Beier et al., 1998; Alm and Trust, 1999), which are all involved in flagellar biosynthesis (Beier et al., 1997; Spohn and Scarlato, 1999b; Spohn and Scarlato, 1999a; Josenhans et al., 2000; Josenhans et al., 2002a). No flagellar master regulator similar to FlhCD in the *Enterobacteriaceae* has been found in the *H. pylori* genome. The results of microarray analyses show that *topA2* has different effects on the gene expression in *H. pylori* as compared to *topA*. One of the most striking findings of our microarray analysis was the increased expression of a large number of genes that encode components of the flagellar apparatus (Table 9). These genes belong to class 2, class 3 and intermediate class flagellar genes (Niehus et al., 2004). *H. pylori* FlgR, an NtrC-like RpoN activator (Spohn and Scarlato, 1999b), is an ortholog of flagellar master regulators in other bacteria (FleR, FleQ of *Pseudomonas* spp., CtrA and FlbD of *Caulobacter*, FlrA and 22 FlrC of *Vibrio* spp.; (Jenal, 2000; Prouty et al., 2001); (Dasgupta et al., 2002), but our microarray analysis of the *flgR* mutant does not support a role of FlgR as a flagellar master regulator. Niehus et al. (Niehus et al., 2004) recently demonstrated that all of the genes summarized here were also downregulated in *H. pylori flhA* mutants, whose gene product works as a global flagellar regulator in *H. pylori*. We found that *flhA* transcript was increased twofold in the *topA2* mutant. We propose that TopA2 indirectly affects transcription of some flagellar class 2, class 3 and intermediate class genes mediated by *flhA*. Taken together, TopA2 works as a global transcriptional regulator involved in flagellar regulation and coordination of regulation of flagellar and non-flagellar genes. Its mode of activation, which might be coupled to a DNA binding function, will have to be determined in future studies. Understanding the regulatory pathway of flagellar synthesis will provide insights into the pathogenesis of *H. pylori* as well as an enhanced understanding of underlying features in the control of gene expression in this organism.

Another group included in the list of upregulated genes in the *topA2* mutant are part of the urease operon, which are *ureA*, *ureB* and *ureI*. UreI (gated urea channel) was found down-regulated in the *hup* mutant, which indicates that *topA2* and *hup* may play opposite roles in the regulation of this gene.

The list of down-regulated genes includes components of the cell envelope, like HP0610, a vacuolating- toxin like outer membrane protein, which belongs to the Hop family (Alm et al., 2000). The Hop subfamily contains the largest number of OMPs in *H. pylori*, including several proteins that are predicted to function as porins (Doig et al., 1995; Exner et al., 1995) or adhesins (Odenbreit et al., 1999). Little is known about the selectivity or function of most of the Hop proteins. It has been suggested that changes in expression of Hop protein, specifically those acting as porins, would presumably affect the permeability of the outer membrane and play a previously unappreciated role in iron-uptake and/or storage (Alm et al., 2000).

These results suggest that TopA2 may contribute to global virulence gene regulation. However, several questions remain regarding the function and physiological role of *H. pylori* TopA2. Although TopA2 did not affect the global supercoiling, it is possible that specific DNA structures are particularly sensitive to TopA2 activity, or might even be activated/repressed directly by binding TopA2. Further experiments will have to be performed to determine the precise function of *topA2* in the life style of *H. pylori* and the mechanisms of regulation by TopA2. Also the question, why TopA2 is highly strain specific, and if *topA2*-like genes with similar function exist in other strains will have to be investigated in the future.

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6. Abbreviations

Amp	ampicillin
BHI	brain-heart infusion
bp	base pair
cagA	Cytotoxin-associated gene A
cm	centimeter
Cm	chloramphenicol
Da	Dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetate
FIS	factor for inversion stimulation
g	gram
h	hour
H-NS	histone-like nucleoid structuring
HU	heat unstable
IHF	integration host factor
k	kilo
Km	kanamycin
l	liter
LB	Luria-Bertani
LPS	Lipopolysaccharide
m	mili
µ	micro
MALT	mucosa-associated lymphoid tissue
min	minute
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
nt	nucleotides
OD	optical density
PAI	pathogenicity island
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNase	ribonuclease
RNA	ribonucleic acid
rpm	rounds per minute
SDS	sodium dodecyl sulfate
sec	second
Spec	spectinomycin
U	Unit
V	Volt
VacA	Vacuolating cytotoxin A

7. Curriculum Vitae

Name:	Fang Ye
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1998.7--2000.8	MSc. (In Ecology and Environmental Quality) Dept. of Biochemistry, Tel Aviv University, Israel Master Thesis: The antioxidant system of the obligate anaerobe, green sulfur photosynthenic bacterium *Chlorobium limicola*
2000.9--2004	Experimental work for Ph.D. (Microbiology) Inst. of Hygiene and Microbiology (Supervisor: Prof. Dr. Sebastian Suerbaum) University of Wuerzburg, Germany Ph.D. Thesis: The role of DNA supercoiling in the coordinated regulation of gene expression in *Helicobacter pylori* Member of the European Graduate College "Gene Regulation in and by Pathogenic Bacteria"

8. List of publications

Fang Ye, Tanja Brauer, Christine Josenhans, Eike Niehus and Sebastian Suerbaum. Global gene regulation in *Helicobacter pylori* is dependent on changes in DNA supercoiling (submitted)

Eike Niehus, Helga Gressmann, **Fang Ye**, Ralph Schlapbach, Michaela Dehio, Christoph Dehio, Allison Stack, Thomas F. Meyer, Sebastian Suerbaum and Christine Josenhans. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori.* (Molecular Microbiology, May 2004 OnlineEarly edition)

Eike Niehus, **Fang Ye**, Sebastian Suerbaum and Christine Josenhans. (2002). Growth phase-dependent and differential transcriptional control of flagellar genes in *Helicobacter pylori.* Microbiology, 148, 3827-3837