Growth phase-dependent and differential transcriptional control of flagellar genes in *Helicobacter pylori*

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***Helicobacter pylori* possesses two different flagellin genes, *flaA* and *flaB*, which are unlinked on the chromosome and transcribed from σ^{28} and σ^{54} promoters, respectively. Both flagellins are hypothesized to be present in varying amounts in the flagellum, to adapt the physical properties of the flagellar filament to different environmental conditions. The influence of growth phase and environmental conditions on the transcriptional regulation of both flagellin genes has not been investigated so far. Using three different reporter genes as well as Northern blot analyses and RT-PCR, it was determined that both flagellin genes are transcribed in a growth phase-dependent fashion. Growth phase dependency was also found for the flagellar basal body export apparatus gene *flhA* which is involved in the transcriptional regulation of both flagellin genes. Peak transcription of *flaB* and *flhA* occurred earlier during the growth phase than that of *flaA*, possibly consistent with a hook-proximal localization of the minor flagellin FlaB. Of the reporter gene systems, luciferase fusions reflected best the dynamic regulation patterns of *H. pylori* flagellin genes. Growth phase *in vitro* had the strongest influence on transcriptional control of *H. pylori* *flaA* and *flaB*, while differences in supplements to a rich culture medium had only a modest modulatory effect on flagellin gene transcription.**

Keywords: gene regulation, transcription, flagellin, reporter genes, flagellar assembly

INTRODUCTION

The eubacterial genus *Helicobacter* comprises the gastric *Helicobacter* species that infect the stomach mucosa of many different kinds of mammals. All of these have developed specific adaptation mechanisms to permit persistent life in the inhospitable environment of the stomach. *Helicobacter pylori*, the causative agent of chronic gastritis and peptic ulcers in humans, like other *Helicobacter* species depends on its strong motility to move through the viscous mucus gel lining the epithelium of the human stomach, to establish chronic infection of the human gastric mucosa and to persist in the gastric mucus (Hazell *et al.*, 1986; Worku *et al.*, 1999). The flagella of gastric *Helicobacter* sp. (*H. pylori*, *H. mustelae*, *H. felis*) possess complex flagellar filaments composed of two flagellin subunits, FlaA and FlaB.

Flagellar filaments contain FlaA and FlaB subunits in a stoichiometric ratio of approximately ten to one (Kostrzynska *et al.*, 1991; Suerbaum *et al.*, 1993; Josenhans *et al.*, 1995a, 1999). Mutants lacking the major flagellin FlaA show strongly impaired motility *in vitro* and possess only truncated flagella, whereas *flaB* mutants have moderately diminished motility and apparently normal flagella. In the gnotobiotic piglet animal model and the *H. mustelae*-ferret model, isogenic mutants lacking either the FlaB or the FlaA subunits were both severely impaired in their ability to colonize (Andrutis *et al.*, 1997; Eaton *et al.*, 1996). Those results demonstrated the importance of both flagellins *in vivo*.

The *H. pylori* *flaA* and *flaB* genes are unlinked on the chromosome and their transcription is regulated by two different promoters (Leying *et al.*, 1992; Suerbaum *et al.*, 1993). Expression of *flaA* is controlled by a σ^{28} -dependent promoter, a class of promoters responsible for the transcriptional activation of late flagellar genes in many flagellated bacteria (class 3; Aizawa, 2000; Josenhans *et al.*, 2002). *flaB* and several other flagellar

Abbreviations: CAT, chloramphenicol acetyl transferase; GFP, green fluorescent protein; RLU, relative luminescence unit.

genes of *H. pylori* are preceded by a σ^{54} promoter and have been found to be under the transcriptional control of FlgR, a transcriptional activator of σ^{54} -dependent genes (Spohn & Scarlato, 1999, 2001). Dependency of a number of flagellar genes on the 'environmentally regulated' σ^{54} factor has also been observed in *Caulobacter crescentus*, *Vibrio cholerae* and *Campylobacter* species, (Anderson *et al.*, 1995; Jagannathan *et al.*, 2001; Prouty *et al.*, 2001). However, the evolutionary, environmental and functional basis for the importance of σ^{54} promoters in flagellar regulation in all these bacterial species is not well established.

The genetic background and global regulation of flagellar motility and signal transduction in *Helicobacter* species have not been thoroughly investigated yet. The complete genome sequences of two *H. pylori* strains have confirmed that both strains possess most motility genes known from other bacteria, but that these are much less frequently organized in operons than in most other bacteria (Alm *et al.*, 1999; Tomb *et al.*, 1997). Based on homology searches, some genes known from other bacteria to be important in motility regulation have not been identified in *H. pylori* (Josenhans & Suerbaum, 2001). Notably, *H. pylori* lacks homologues of genes encoding the flagellar master regulators FlhD and FlhC. The mechanisms of motility regulation in *H. pylori* are thus significantly different from those in other bacteria, most likely due to specific adaptation to their ecological niche. We hypothesized that *H. pylori* motility, and in particular the expression level of *H. pylori* FlaB, can be regulated in response to specific environmental signals to change the mechanical properties of the flagellar filament.

In the present work, we constructed and characterized reporter gene fusions of the *H. pylori* *flaA* and *flaB* promoters and of the flagellar basal body gene *flhA* of *H. pylori* in order to study flagellin gene regulation *in vitro* and *in vivo*. Fusions with promoterless *cat* [encoding chloramphenicol acetyl transferase (CAT)], *gfp* [encoding green fluorescent protein (GFP)] and *luxAB* (encoding luciferase) genes were utilized for this purpose. The *luxAB* operon is described for the first time as a transcriptional reporter in *H. pylori*. Expression and activity levels of the reporter proteins in the *H. pylori* fusion mutants were determined during the growth phases in liquid culture and under various growth conditions *in vitro*. We found that transcription of the regulatory gene *flhA* as well as of both flagellin genes was profoundly influenced by the growth phase but only slightly modulated by the different *in vitro* growth conditions used. The results support a model of a predominantly growth phase-dependent differential regulation of both σ^{28} - and σ^{54} -controlled *H. pylori* flagellin genes.

METHODS

Bacterial strains and growth conditions. *H. pylori* N6 wild-type strain (Cussac *et al.*, 1992) was chosen for the allelic exchange mutagenesis because of its high transformation

efficiency. *Escherichia coli* strains MC1061 and DH5 α were used for the DNA cloning experiments. For the transposon mutagenesis of *H. pylori* *flaB* in *E. coli*, we used *E. coli* strains S17-1[pTCA, pILL638], *E. coli* lambda *cre* Rif and *E. coli* DH1 (Labigne-Roussel *et al.*, 1988).

H. pylori strains were cultured on blood agar plates (Columbia agar base II, Oxoid), supplemented with 10% sheep blood and the following antibiotics: vancomycin (10 mg l⁻¹), polymyxin B (2500 U l⁻¹), trimethoprim (5 mg l⁻¹) and amphotericin B (4 mg l⁻¹). Plates were incubated for 48 h at 37 °C under microaerobic conditions. *flaA-cat* mutants were propagated on plates with the addition of chloramphenicol (10 mg l⁻¹). For selection of *flaB-cat* mutants, kanamycin at 100 mg l⁻¹ was added to the plates.

For the CAT assays, *H. pylori* cells harvested in mid-exponential phase were inoculated (about 5 × 10⁸ cells per 100 ml, corresponding to an initial OD₆₀₀ of approx. 0.04–0.06) into BHI broth supplemented with 10% fetal calf serum and the above-mentioned antibiotics, but without chloramphenicol or kanamycin. They were incubated in a rotary shaker (110 r.p.m.) under microaerobic conditions for several days. Growth was monitored at each harvest by measuring the OD₆₀₀ of the cultures. Special care was taken to standardize the conditions of inoculation and growth.

E. coli strains were propagated in Luria broth or on Luria–Bertani plates supplemented with antibiotics as required: kanamycin (100 mg l⁻¹), chloramphenicol (20 mg l⁻¹), rifampicin (100 mg l⁻¹), spectinomycin (100 mg l⁻¹, or for transposon mutagenesis, 500 mg l⁻¹), ampicillin (100 mg l⁻¹) and tetracycline (10 mg l⁻¹).

DNA methods. DNA purification and cloning procedures were performed as described elsewhere (Sambrook *et al.*, 1989). Large-scale plasmid purifications were performed using Qiagen column purification protocols. DNA fragments were extracted from agarose gels with the QiaEX DNA purification kit (Qiagen). DNA restriction and modification enzymes were obtained from Invitrogen Life Technologies or Roche Biochemicals and were used according to the manufacturers' protocols. Plasmids used in this study are listed in Table 1.

Construction of *flaA*-, *flaB*- and *flhA-cat* fusions in *H. pylori*

***flaA-cat* fusion.** For construction of *flaA-cat* reporter gene strains, a plasmid (pSUS24) was used that contains the *H. pylori* *flaA* gene with a *cat* cassette inserted into its unique *Bam*HI restriction site (Suerbaum *et al.*, 1993). A chromosomal transcriptional fusion of the *flaA* promoter with *cat* was generated by electroporation of *H. pylori* N6 with pSUS24 and selection of chloramphenicol-resistant allelic exchange mutants. Electroporation was performed as described by Ferrero *et al.* (1992).

***flaB-cat* fusion.** The σ^{54} promoter of *flaB* *in vitro* allows for a relatively low transcriptional activity in *H. pylori*. Therefore, we used a transposon mutagenesis approach to insert a promoterless *cat* cassette into *flaB*. A miniTn3-Km-*cat* transposon, which contains a kanamycin resistance marker (*aphA*-3) with a strong promoter in addition to the promoterless *cat* cassette, was used to perform a transposon mutagenesis of *flaB* (pSUS19), using a protocol described by Labigne-Roussel *et al.* (1988). *E. coli* cells containing transposon-mutated plasmids were selected on kanamycin-containing plates and characterized by restriction mapping and PCR. One plasmid, pSUS19::9, had a transposon insertion within the first third of the *H. pylori* *flaB* gene that created a transcriptional fusion of the *flaB* promoter and the *cat* cassette. pSUS19::9 was

Table 1. Plasmids used in this study

Plasmid	Brief description	Resistance*	Source
pILL638	Donor plasmid of the MiniTn3-Km- <i>cat</i> minitransposon, derived from MiniTn3-Km (pILL553), containing a promoterless <i>cat</i> gene and a kanamycin resistance cassette (<i>aphA</i> 3'-III)	km ^r	A. Labigne
pHL319-2-4	<i>H. pylori flaA</i> gene	ap ^r	Leying <i>et al.</i> (1992)
pSUS19	<i>H. pylori flaB</i> gene	spc ^r	Suerbaum <i>et al.</i> (1993)
pSUS24	<i>H. pylori flaA-cat</i> fusion	ap ^r , cm ^r	Josenhans <i>et al.</i> (1995a)
pSUS19::9	<i>H. pylori flaB-cat</i> -Km	cm ^r , km ^r	This study
pCM4	Plasmid containing promoterless <i>cat</i> gene	ap ^r	Amersham/Pharmacia Biotech
pSUS59	<i>H. pylori flhA</i> gene in pILL570 [from pUC derivative pSUS40 (Schmitz <i>et al.</i> , 1997)]	ap ^r	This study
pSUS59::1	<i>H. pylori flhA-cat</i> transposon fusion	ap ^r , cm ^r , km ^r	This study
pSUS132	<i>H. pylori flhA</i> fused to a promoterless <i>cat</i> cassette	ap ^r , cm ^r	This study
pSUS78	<i>H. pylori flaB-gfp</i> transcriptional fusion	km ^r	Josenhans <i>et al.</i> (1998)
pSUS128	<i>H. pylori flaA-gfp</i> transcriptional fusion	km ^r	Josenhans <i>et al.</i> (1998)
pSUS1609	<i>H. pylori flaB-luxAB</i> transcriptional fusion	km ^r	This study
pSUS1601	<i>H. pylori flaA-luxAB</i> transcriptional fusion	km ^r	This study

* Resistance to ampicillin (ap^r), chloramphenicol (cm^r), kanamycin (km^r), spectinomycin (spc^r).

electroporated into *H. pylori* N6 to generate the transcriptional *H. pylori flaB-cat* fusion mutant.

***flhA-cat* fusion.** The *H. pylori flhA-cat* fusion was generated by MiniTn3-Km-*cat* shuttle transposon mutagenesis of plasmid pSUS59, a pILL570 derivative that contains a PCR-amplified part (1.6 kb) of the *flhA* gene. One transposon clone carrying the transposon insertion within the first third of the *flhA* coding sequence (pSUS59::1) was isolated from *E. coli* and introduced by electroporation into *H. pylori* N6. In addition, a plasmid pSUS132, which contained a direct insertion of the promoterless *cat* cassette into *flhA* in pSUS59, was also used to generate allelic exchange mutants.

The *H. pylori flaA-cat*, *flaB-cat* and *flhA-cat* mutants were characterized by PCR, Western blotting, transmission electron microscopy and motility testing. In addition, the promoter regions of all mutants were resequenced to exclude selection of promoter mutants during the process of allelic exchange mutagenesis. The mutants were shown to exhibit the same phenotypic characteristics as previously described for *H. pylori flaA*, *flaB* and *flhA* mutants constructed by the insertion of a kanamycin resistance cassette (Suerbaum *et al.*, 1993; Josenhans *et al.*, 1995a; Schmitz *et al.*, 1997).

CAT assays. The expression of CAT by the *H. pylori cat* fusion mutants was determined with the Roche CAT-ELISA detection kit. Samples of 5 ml were harvested from *H. pylori* liquid cultures at different time points, washed once in 0.9% NaCl and the bacteria pelleted by centrifugation. The pellets were resuspended in 2 ml of 0.9% NaCl and lysed by sonication. Cell debris was separated by centrifugation and discarded. The protein concentration of the supernatant was determined with the bichinchoninic acid (BCA) protein assay. Finally, the samples were diluted to a protein concentration of 0.005 µg µl⁻¹ (*flaA-cat*), 0.01 µg µl⁻¹ (*flaB-cat*) and 0.1 µg µl⁻¹ (*flhA-cat*), respectively. 0.01 µg protein of the *flaA-cat* samples per well, 0.07 µg protein of the *flaB-cat* samples per well and 1–5 µg of the *flhA-cat* samples were used in the Roche microtitre-plate CAT assay which was performed as described

by the manufacturer. All assays were performed in triplicate. For quantitative standardization of CAT expression, the Roche purified CAT standard was used.

***gfp* reporter gene fusions of *H. pylori flaA* and *flaB* genes.** The reporter gene fusions with the red-shifted and enhanced *gfp* variant *gfpmut2* (Cormack *et al.*, 1996) were constructed as described previously (Josenhans *et al.*, 1998). The phenotypes were checked by the same methods as for the *cat* fusion mutants and were shown to be similar (see above, data not shown).

Determination of GFP fluorescence. Bulk measurement of GFP was performed in a Bio-Rad fluorometer equipped with a filter system for red-shifted GFP (excitation wavelength 490 nm, emission 515 nm, narrow band pass filter). Samples were diluted to the same protein concentration of 0.1 µg µl⁻¹ in PBS and measured as samples of 2 ml for 10 s to get a stable reading.

Construction of *luxAB* fusions with the *H. pylori flaA* and *flaB* promoters. *flaA*- and *flaB*-promoter fusions with the *luxAB* operon from *Vibrio harveyi* were constructed by inverse PCR from the plasmids pSUS81 (*flaA*) and pSUS128 (*flaB*) which contain the *fla-gfp* fusions together with a kanamycin cassette. Primers HPF_{flaA}NotI1/HPF_{flaB}NotI1 and HpgfpNotI2 were used to delete the *gfp* gene from these constructs by inverse PCR and subsequent religation. The deleted *gfp* fragment was then replaced with the *luxAB* operon (*V. harveyi*) that had been amplified from MiniTn5*luxAB* (De Lorenzo *et al.*, 1990; Heuner *et al.*, 1999) using primers containing NotI sites (LuxABNotI1 and LuxABNotI2). The resulting plasmids pSUS1609 and pSUS1601 were used to generate allelic exchange mutants in *H. pylori* by natural transformation. The geno- and phenotypes were determined by the same methods as for the *cat* and *gfp* fusion mutants and found to be the same (data not shown).

Measurement of luciferase activity. A Hamamatsu photon counting device (Hamamatsu MTP reader) was used for bulk

measurements of luciferase activity in microtitre plates. Preparation of the bacterial cultures for measurement was as follows. *H. pylori* bacteria containing the luciferase gene fusions were grown in liquid medium and harvested at different time points throughout the growth phase. The OD₆₀₀ was measured at each point and adjusted to 0.1 (approx. 3×10^7 bacteria ml⁻¹) for each sample. Then 10 µl (approx. 3×10^5 bacteria) bacterial suspension were gently mixed with 30 µl reaction buffer (50 mM sodium phosphate buffer, pH=7, 50 mM β-mercaptoethanol, 2% w/v BSA), to which the substrate, n-decyl aldehyde, diluted 1:2000 from the stock solution (Sigma), had been added directly prior to the measurement. Samples were pipetted into a black 96-well microtitre plate and measured in the photon counter using the MTP reader CAA software (Hamamatsu Photonics). Counts were taken 5 and 10 min after starting the reaction. The counting interval was 2 s. In general, at 10 min after addition of the substrate, the counts were stable and remained stable for at least another 10 min. All measurements were performed in triplicate on two independent samples and each experiment was performed at least three times on different days. Luciferase activity in the graphs is expressed in relative luminescence units (RLU).

RNA preparation, Northern blotting and RT-PCR. RNA was prepared from *H. pylori* bacteria harvested from liquid cultures in different growth phases using the Qiagen RNeasy kit with slight modifications as described elsewhere (Josenhans *et al.*, 2002). Northern blots were performed on DNase I-treated whole RNA samples, which were separated on denaturing formamide agarose gels. Probes for Northern blots were generated and DIG-labelled as previously described (Josenhans *et al.*, 2002). Semiquantitative RT-PCRs were performed on 2 µg DNase I-treated RNA samples from different time points. Reverse transcription was performed using a random hexamer primer mix and Superscript IITM reverse transcriptase (Invitrogen) at 42 °C for 1 h. After RNase H digestion, the cDNA was amplified in different PCR reactions on 2.5 µl cDNA sample with primers specific for *flhA*, *flaA* and *flaB* (primer sequences available on request).

RESULTS AND DISCUSSION

Construction and evaluation of transcriptional reporter fusions of *H. pylori* flagellar genes

Systematic studies about temporal gene regulation in *H. pylori* are scarce. Few studies describe environmentally and growth phase-regulated expression of virulence-related genes in *H. pylori* (Karita *et al.*, 1996; Spohn *et al.*, 1997; van Vliet *et al.*, 2001; Allan *et al.*, 2001; Delany *et al.*, 2001; Joyce *et al.*, 2001). Reporter genes that have been used so far in *Helicobacter* are *xylE*, *cat*, *gfp* and *lacZ* (Josenhans *et al.*, 1995b, 1998; Karita *et al.*, 1996; van Vliet *et al.*, 2001; de Vries *et al.*, 2001). Recently, a *lacZ* reporter transposon strategy has been devised to identify stress-regulated genes in *H. pylori* (de Vries *et al.*, 2001). There is so far no published experimental evidence that permits a direct comparison of the applicability and performance of different reporter gene systems in *Helicobacter* species. In the present study, we compared three different reporter systems, *cat*, *gfp* and *luxAB*, to evaluate the transcriptional activities of the *H. pylori* *flaA* and *flaB* flagellin gene promoters, and of the early flagellar basal body gene *flhA* promoter (previously *flbA*; Schmitz *et al.*, 1997). The phenotypes of the fusion

mutants with all three reporter genes described below were found to be non-polar and similar to the phenotypes of the isogenic *flaA*, *flaB* and *flhA* mutants that were created previously by allelic exchange with a kanamycin cassette-disrupted copy of the genes (Suerbaum *et al.*, 1993; Josenhans *et al.*, 1995a; Schmitz *et al.*, 1997; data not shown). The two flagellin genes have a distinct regulation via their σ^{28} and σ^{54} promoters, and their protein expression levels are known to differ greatly (Kostrzynska *et al.*, 1991; Suerbaum *et al.*, 1993). Initially, fusions of the major flagellin gene *flaA*, which is transcribed very efficiently by means of its σ^{28} promoter, were constructed by inserting a promoterless *cat* cassette into the *flaA* coding sequence. The *cat* cassette was chosen to serve the two purposes of providing i) a selective marker for recombinant *H. pylori* *flaA*Δ*cat* strains and ii) a reporter gene, whose expression under the control of the *flaA* promoter can be assayed. For the weakly transcribed *flaB* and *flhA* genes, a shuttle transposon mutagenesis approach was used in addition to a simple insertion mutagenesis (Josenhans *et al.*, 1998). For constructing transposon insertions in *E. coli*, a *km-cat*Tn3 derivative, which contains a *km*-cassette with a strong promoter and an additional promoterless *cat*-cassette, was utilized, which creates a transcriptional fusion of the *cat* gene to the gene it is inserted into. Transposon insertions in *flaB* and *flhA* were integrated into the *H. pylori* chromosome by allelic exchange using the mutagenized suicide plasmids. *flaA-cat* as well as *flaB-cat* and *flhA-cat* reporter gene fusions led to detectable chloramphenicol resistance in the respective *H. pylori* mutants.

As a second reporter gene fusion system, mainly for subsequent use in visualization of gene regulation *in vivo* and in cell culture models, *flaA* and *flaB* fusions with a promoterless *gfp* gene (*gfp*mut2; Cormack *et al.*, 1996) were generated as previously described (Josenhans *et al.*, 1998). Finally, since the results of the experiments with *fla-gfp* and *fla-cat* reporter gene assays were ambiguous in some aspects (see below), we constructed *flaA* and *flaB* transcriptional fusions with the *luxAB* operon of *V. harveyi*, which has been used as a highly sensitive reporter system in different bacterial species and eukaryotes, and is a good reporter for use *in vivo* and in infected cultures of eukaryotic cells (Baldwin *et al.*, 1984; Kirchner *et al.*, 1989; Heuner *et al.*, 1999). The sensitivities of both CAT expression as measured by ELISA and luciferase activity measured in a photon-counting device were high. The maximal values of CAT expression driven by the *flaA* promoter were 10000 pg soluble protein µg⁻¹ in comparison to a background in negative controls of 50–100 pg µg⁻¹ (Fig. 1a). For luciferase, the maximal activity was approximately 50000–60000 RLU for *flaA* (per 3×10^5 bacteria) in comparison to ± 0 units for the background in negative controls, which means that it was possible to detect the luminescence emission of five to ten bacteria (Fig. 1b). A lower sensitivity was found for *gfp*, where highest *flaA* transcriptional activities were only 60-fold higher (3000 units of fluorescence per 200 µg soluble protein) than the

Fig. 1. Growth phase-dependent activity of *H. pylori* *flaA* and *flaB* flagellin gene promoters, measured in three different reporter gene systems. Depicted are growth curves (solid lines) and reporter gene measurements (dotted lines) for all three systems used. Solid triangles represent points during the growth curves for *flaA*, solid squares for *flaB*. Open triangles depict the reporter measurements for *flaA*, open squares measurements for *flaB*. The growth phases were followed for at least 100 h (4 days) for the *cat* and *gfp* reporters (a, b). For the *luxAB* reporter genes (c), reporter activities decreased to and stayed near zero after 41 h (*flaB*) and 60 h (*flaA*) of growth, therefore the growth curve was stopped at 60 h. All three parts show the mean values and standard deviations from three independently performed experiments. The last two values of the *flaA-gfp* reporter measurements (b) are the results of one experiment, so no error bars are shown. The left y-axes show reporter measurements. Note that the scales of reporter measurements (left y-axes) have been chosen differently for *flaA* and *flaB*, since the changes between *flaB* measurements during the growth phases could be assessed more easily. The right y-axes show measurement of growth (as OD₆₀₀; an OD₆₀₀ of 1:0 corresponds to approx. 3×10^8 c.f.u. live bacteria in exponential growth phase) and are drawn to logarithmic scale in each panel. Time of growth (h) is depicted on the x-axis. (a) Combined growth curves and determination of CAT expression [$\mu\text{g CAT } (\mu\text{g protein})^{-1}$] of *H. pylori* *flaA*- and *flaB*-*cat* fusion mutants. Note that expression of the flagellin subunits is regulated roughly in parallel. (b) Growth curves and relative fluorescence measurements of the transcriptional *flaA-gfp* and *flaB-gfp* fusion mutants during the growth phases (measured in relative fluorescence units per $\mu\text{g protein}$). Note the differential transcription of *flaA* and *flaB*. (c) Growth curves and luminescence measurements of the transcriptional *flaA-luxAB* and *flaB-luxAB* fusions (measured in RLU per 3×10^5 bacteria).

background (50 units per 200 $\mu\text{g protein}$; Fig. 1c). The *gfp* system was also compromised by high auto-fluorescence of the *H. pylori* strains, especially in later

phases of growth (Josenhans *et al.*, 1998). In comparison to the systems used in this study, for the *xylE* reporter gene, enzymic activities of approximately 1000 mU (mg

Fig. 2. Ratios of *flaA* to *flaB* promoter activities for all three reporter gene systems. Note that the *flaA/flaB* ratio is lower in the early to mid-exponential phases (up to approx. 20 h growth, OD_{600} of about 0.5) in all systems and increases from mid-exponential phase on (OD_{600} of approx. 1). Growth curves for the *lux* reporter fusions were only followed for 60 h (see Fig. 1).

protein)⁻¹ were reported in the literature when it was used as a reporter gene for the *H. pylori vacA* and *ureA* promoters, while the background in negative control measurements was near zero (Karita *et al.*, 1996). Stability of XylE activity was reported to be high. Recently, *lacZ* was first used as a reporter gene in *H. pylori*, to measure transcriptional activity of the urease operon (van Vliet *et al.*, 2001). However, it has to be noted that even for one of the most active promoters in *H. pylori* (*ureA*) this system yielded only very low

reporter gene activities, which may be limiting for *in vivo* gene expression measurements, or for the study of weak promoters.

Growth phase-dependent transcriptional regulation of *H. pylori* flagellar genes *flaA*, *flaB* and *flhA*

One of our major aims was to determine, if transcription of the flagellar genes in *H. pylori* is dependent on the growth phases in liquid culture, and if flagellar genes

Fig. 3. Northern blots and semiquantitative RT-PCRs of RNA samples prepared from different time points during the growth phases of the *H. pylori* N6 wild-type strain in liquid culture. The results of this experiment are representative for two independently performed experiments. Shown in the upper panels are results for Northern blots with *flaA* and 16S rRNA (control for equal amounts of loaded RNA) probes. Below as indicated, semiquantitative RT-PCRs done from RNA preparations of the same growth curve with primers specific for *flaA*, *flaB* and *flhA* (control of RNA amounts with 16S rRNA-specific primers, not shown). RT-PCRs for *flaA*, *flaB* and *flhA* were allowed to proceed for 20, 25 and 33 cycles, respectively.

belonging to different regulons (σ^{54} -dependent, σ^{28} -dependent) are differentially transcribed during growth in liquid culture *in vitro*.

In late exponential phase, *flaA* promoter activity was eight- to 12-fold higher than the activity of the *flaB* promoter, as measured by determination of CAT expression of *H. pylori* *flaA-cat* and *flaB-cat* mutants and by fluorescence measurement of the GFP reporter protein (Fig. 2). Using the *luxAB* reporter gene fusions, the ratio of *flaA*/*flaB* reporter activities was even higher in late exponential phase, up to more than 100-fold.

All three reporter gene systems showed that the expression of both flagellin genes varied strongly in different growth phases (Fig. 1). In addition, in all three systems the ratio of *flaA* to *flaB* transcriptional activities varied significantly during the growth phases (Fig. 2), from 3:1 (*flaA* vs *flaB*) at the beginning of the growth phase to more than 10:1 (*flaA* vs *flaB*) in later growth phase.

The growth phase-dependent regulation of flagellin genes was reflected slightly differently in the three different reporter gene systems. In the CAT system, where the total amount of CAT protein was measured, reporter activities of both flagellin genes increased exponentially during the mid- to late exponential phases (OD₆₀₀ between approx. 0.5 and 2.0). Reporter levels from both flagellin gene promoters peaked almost in parallel in late exponential phase (OD₆₀₀ of approx. 2.0) and remained at a high level for up to 120 h, probably because of accumulation and high stability of the CAT reporter protein (Fig. 1a). Sometimes a second peak of transcriptional activity was visible after approx. 4 days (96 h) of culture. This growth phase dependency corresponds to findings by Alm and colleagues for *Campylobacter coli* (Alm *et al.*, 1993), where *flaB* σ^{54} promoter-driven CAT production was maximal in mid- to late exponential phase.

In the GFP reporter system, where fluorescence of correctly folded, active GFP is measured, the differential

transcription of both flagellins was most clearly visible. *flaB* transcription peaked in early exponential phase, then declined again gradually, whereas *flaA* transcription peaked only in late exponential phase (Fig. 1b). Luciferase activities (measured as substrate processing; Fig. 1c), of *flaB* and *flaA* transcriptional fusions peaked closely together in the early to mid-exponential phase, with *flaB* slightly preceding *flaA* transcriptional activity. In the luciferase system, both *flaB* and *flaA* transcription rapidly decreased to basal levels in late exponential growth phase, in contrast to the results in the CAT system, where the amounts of reporter proteins appeared to rise almost continuously during the growth cycle, an effect that was likely to be due to the well-known high stability of the CAT reporter protein.

The results obtained with the three reporter gene systems were compared with Northern blots and semi-quantitative RT-PCRs of RNA isolated from the *H. pylori* N6 wild-type strain. These direct experiments on the mRNA level gave slightly different results, but overall confirmed that significant and differential growth phase-dependent changes occur for *flaA* and *flaB* transcription (Fig. 3). Of the three reporter gene systems, only luciferase reflected the transient and dynamic changes visible on the mRNA level at different time points throughout the growth phases, and was the only system that showed a transcriptional downregulation of *flaA* at the onset of stationary phase, similar to the Northern blot results. The highest peak of *flaA* mRNA concentration in the Northern blots and RT-PCRs was in late exponential phase (OD₆₀₀ approx. 2.5), later than the highest reporter gene activity of the *flaA-lux* fusion. This might indicate that additional mechanisms modulate the outcome of transcriptional regulation in the reporter systems, rather than just a temporal increase in transcription. *flaB* transcription during the growth phases as measured by semiquantitative RT-PCR and Northern blot (not shown) was significantly different from *flaA* (Fig. 3). It peaked in early to mid-exponential phase, when *flaA* transcription was still very low. In contrast, no *flaB* mRNA was detectable at the later time points during late exponential phase, when *flaA* transcription was highest. However, for the early time points during growth, transcription of *flaA* and *flaB* occurred in parallel.

The *flhA* promoter activity, which was only measured in the CAT reporter assay and by semiquantitative RT-PCR, because of the low activity of the promoter, was also strongly dependent on the growth phase, with a first peak of transcription slightly preceding peak activities of the *flaA* and *flaB* promoters in the CAT reporter gene assays (Fig. 4). The FlhA protein is one candidate master regulator in *H. pylori* for growth phase-dependent coordinated transcription of flagellin genes (Schmitz *et al.*, 1997). In a *H. pylori flhA* mutant, transcription of *flaA*, *flaB* and *flgE1* (encoding the major hook protein) genes as well as expression of the respective proteins was greatly reduced, dependent on the growth phase (Schmitz *et al.*, 1997). FlhA has been characterized as being one of the early flagellar proteins and part of the

Fig. 4. *flhA* promoter activity during the growth phases as measured by CAT expression of the *flhA-cat* reporter gene mutant. For better comparison, *flaB*-CAT expression is included in the graph. Solid lines/solid squares represent the growth curve. Dotted lines represent the reporter measurements (open triangles: *flaB*; open circles: *flhA*). The left y-axis shows reporter measurements, the right y-axis the OD₆₀₀ (determination of growth). The x-axis shows time of growth (h). The peak of reporter protein expression of the *flhA* mutant precedes the peak activity in the *flaB* mutant. *flhA* promoter activity is significantly lower than that of *flaB* (one-twentieth), therefore values of *flhA* measurements were multiplied by 10 to use the same scale on the left y-axis.

flagellar basal body export apparatus in *Salmonella* (Minamino *et al.*, 1994; Minamino & Macnab, 2000; Kihara *et al.*, 2001). A promoter consensus sequence could not be identified upstream of *flhA*, except for a TAAA – 10 box, similar to those of ‘housekeeping’ σ^{80} promoters of *H. pylori* (Beier *et al.*, 1998; Vanet *et al.*, 2000). In the present study, the *flhA* promoter was characterized to be weakly active, with only about one-twentieth of the activity of the *flaB* promoter in the *cat* reporter system (i.e. 200-fold lower transcription levels than the *flaA* gene). Using semiquantitative RT-PCR of *flhA* transcription, we could confirm the growth phase dependency of this gene, surprisingly showing a biphasic peak distribution, one in mid-exponential phase (roughly in parallel with *flaB* transcription), one in the early stationary phase (Fig. 3, bottom panel). However, even with an extended number of polymerization cycles, only very weak signals were obtained. The biphasic distribution of *flhA* transcriptional activity was also observed in all experiments using the CAT reporter (data not shown).

Influence of environmental factors on regulation of flagellar genes

The influence of different medium supplements and environmental conditions on the regulation of the two flagellin genes *flaA* and *flaB* was studied using the *cat* reporter gene system under the same growth conditions as above (liquid culture in rich, non-defined medium supplemented with serum, see Methods). It would have been preferable to perform such experiments in defined media. However, such media will not support growth of *H. pylori* to high densities and at reproducible growth

rates, which is why the results concerning the effect of medium supplements have to be interpreted with some caution.

In an evaluation phase, we determined the influence of different medium supplements and incubation temperatures on motility in plate motility assays (Josenhans *et al.*, 1995a). Addition of mucin, which has been reported to be a chemotactic stimulus for *H. pylori* (Foyne *et al.*, 2000), and of a vitamin (thiamin) to the rich and complex growth medium used in this study did not lead to changes in motility in a soft agar motility assay. Therefore, these parameters were not tested in reporter gene assays. Temperature changes from the optimal *H. pylori* growth temperature of 37 °C caused a drastically reduced growth rate of the bacteria, which also led to exclusion of this parameter from further testing.

We evaluated the influence of the following parameters on *flaA* and *flaB* transcription using the *cat* reporter: addition of metal ions (1 mM FeSO₄, 10 mM MgSO₄), urea (8.5 mM, approx. two to three times the concentration present in human serum), glutamine (5 mM), different medium viscosities (13 cP, 75 cP, 1000 cP, created by the addition of different percentages of methylcellulose to the medium). Two assays were performed for each condition. Addition of FeSO₄ and MgSO₄ led to slightly better growth but not to a significant change in *flaA-cat* or *flaB-cat* transcription. Increased viscosities or addition of glutamine, a chemo-attractant for a number of bacteria, had no significant effect on growth rate or CAT expression. Addition of 8.5 mM urea increased *flaB* transcription about twofold in all growth phases, while *flaA* transcription was unaffected. The urea supplement did not alter growth rates of both reporter strains. The medium pH constantly remained at approximately 7.5 under the conditions used.

Another crucial condition in the lifestyle of *H. pylori* in the human stomach mucus is likely to be pH. Low pH values are not easily stabilized in *H. pylori* *in vitro* culture in the presence of urea, because of medium alkalization by urea metabolism of the bacterium. Therefore, in our hands, regular *H. pylori* liquid media such as Brucella or BHI broth supplemented with bovine or horse serum were not useful to maintain a constantly low pH throughout growth phase regulation experiments. In a special buffered pH-stable culture system (serum-free/urea-free Brucella medium, adjusted to pH values between 4 and 7.5 in steps of 0.5, using 15 mM sodium phosphate buffer, and supplemented with 0.1 % cyclodextrin), we attempted to measure pH dependence of flagellin gene expression during the growth phases. The *flaA*-fusion mutants did not grow well nor reproducibly in this medium. The *flaB* mutants grew, but only reproducibly at pH values between 6 and 7.5. There was no difference in *flaB*-dependent CAT expression within this pH range. Recently, Allen & Griffiths (2001) described pH-dependent activities of the *Campylobacter jejuni* *flaA* promoter, measured by a luciferase reporter system. In their system, *flaA* transcriptional activity was

moderately higher (about twice) at acidic compared to neutral pH. In a recently published paper dealing with the influence of decreased pH (5.5) on the complete transcriptome of *H. pylori* using macroarrays, *flaA* was not among the genes under low-pH control, but other flagella-related genes like *flaB* showed increased transcriptional activities at an acidic pH of 5.5 compared to pH 7.2 (Allan *et al.*, 2001). Since pH changes are clearly one of the key parameters in the natural *H. pylori* ecological niche, further experiments will have to be performed to determine the influence of pH on flagellin promoter activities in this organism.

Conclusions

In this work we determined the influence of growth phase and several environmental parameters on the transcriptional regulation of flagellar genes in *H. pylori* by means of reporter gene analyses and direct determination of mRNA levels. This is also the first systematic comparison of three different reporter genes performed for *H. pylori*, applied to three different gene promoters of very different activities. The advantages and limitations of the three reporter systems, compared to each other, and with methods of direct mRNA detection, were elucidated. The environmental stimuli tested in this work appeared to play a minor role in transcription of *H. pylori* *flaA* and *flaB* genes, while leaving growth-phase-related regulation intact. It cannot be excluded that environmental conditions exist *in vivo* that would lead to more profound changes of differential flagellin gene transcription. Presumably, growth phase regulation by a factor of more than 100-fold is one important mechanism superimposed over other stimuli as far as regulation of structural flagellar components is concerned. In further studies of *H. pylori* gene regulation, especially for microarray experiments, great care should be taken to take the influence of growth phase and growth rate into account, since the differences of transcription at different time points were vast.

In *Cau. crescentus*, *E. coli* and *Salmonella typhimurium*, growth phase-dependent or, where synchronization of bacterial cells was possible, growth cycle-dependent flagellar gene expression has been demonstrated. This tightly controlled chain of events has been termed temporal expression of motility-associated genes and is closely linked to other events during the cell cycle (e.g. cell division). Systematic investigations of the temporal regulation of flagellar genes in other bacteria have been done by reporter gene analyses as well as by proteome and transcriptome approaches (Grunenfelder *et al.*, 2001; Kalir *et al.*, 2001; Laub *et al.*, 2000). In *H. pylori*, *flaB* showed a transcription peak prior to *flaA* during the growth curve, possibly consistent with the ultra-structural finding that FlaB is a hook proximal flagellin subunit (Kostrzynska *et al.*, 1991), which is incorporated into the growing filament before FlaA. Methods to synchronize *H. pylori* cells are not available, and thus a direct measurement of growth cycle influences is not possible. However, we believe that some extrapolations from the growth phases upon growth cycle effects are

possible, especially at early and late time points. *flhA*, a gene encoding an early (putative class 1) flagellar protein, which is part of the flagellar type III secretion apparatus in the bacterial membrane, showed its transcriptional peak in parallel or slightly prior to *flaB* and significantly prior to *flaA* during the growth phases. Dependence of transcription of late flagellar proteins on FlhA, as has been observed previously (Schmitz *et al.*, 1997), might be one of the mechanisms of temporally regulated flagellar biosynthesis in *H. pylori*. In *V. cholerae* and *Cau. crescentus*, two bacterial species of the *Proteobacteria*, which, like *H. pylori*, express more than one flagellin, but do not possess a flagellar master operon *flhCD*, a large part of the flagellar biosynthesis programme appears to be under the control of σ^{54} , independently of environmental conditions (Prouty *et al.*, 2001; Grunenfelter *et al.*, 2001). The same seems to be true for *H. pylori*. Bacteria have apparently developed at least two separate pathways during evolution to control the ordered transcription of early and late flagellar genes. One pathway, exemplified by the *Salmonella* paradigm, is characterized by the superimposed regulation by the master regulators FlhCD, the other is characterized by the dependency of many flagellar genes on σ^{54} and further unknown master regulators (one of them probably FlhA), such as in *Vibrio*, *Cau. crescentus*, *Helicobacter* and *Campylobacter*. Our own preliminary results of comprehensive transcript analyses (E. Niehus, S. Suerbaum and C. Josenhans, unpublished data) indicate that σ^{54} -dependent genes (such as *flaB* and the hook subunit gene *flgE*), in addition to being transcribed prior to σ^{28} -dependent genes (such as *flaA*) during the growth phases, are subject to a more sophisticated order of transcription.

In this study, we have laid the groundwork for understanding growth phase-related regulation of flagellar genes in *H. pylori*, which will continue to be of use to validate candidate genes for regulation studies *in vivo*. These cornerstones are also a prerequisite for further work on flagellar gene regulation and the global regulatory networks in this fastidious slow-growing bacterium. It would be preferable to directly measure cell cycle-dependent events in *H. pylori*, using cell cycle-synchronized bacteria, which has not been established so far for any slow-growing bacterium. Further investigations to clarify the temporal regulation and the regulatory network governing *Helicobacter* motility and flagellar biosynthesis during the growth cycle *in vitro* and *in vivo* are presently under way in our laboratory.

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