Functional analysis of the roles of FliQ and FlhB in flagellar expression in *Helicobacter pylori*

Susan Foynes \(^a\), Nick Dorrell \(^a\), Stephen J. Ward \(^{1.a}\), Zun W. Zhang \(^b\), Andy A. McColm \(^c\), Mike J.G. Farthing \(^b\), Brendan W. Wren \(^a,\*\)

\(^a\) Microbial Pathogenicity Research Group, Department of Medical Microbiology, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, West Smithfield, London EC1A 7BE, UK

\(^b\) Digestive Diseases Research Centre, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, London, UK

\(^c\) Systems Biology Unit, GlaxoWellcome Research and Development, Gunnels Wood Road, Stevenage, UK

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Abstract

Expression of the two *Helicobacter pylori* flagellin proteins FlaA and FlaB is required for full motility and persistent infection of the gastric mucosa. The mechanisms and regulation of the biosynthesis and export of flagella in *H. pylori* are still poorly understood. Scrutiny of the *H. pylori* 26695 genome sequence revealed homologues of FliQ and FlhB. The roles of the \(fliQ\) and \(flhB\) genes in *H. pylori* were investigated by the construction and characterisation of defined isogenic mutants. The results indicate that these genes are involved in the flagellar expression, adhesion to and colonisation of the gastric mucosa. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Helicobacter pylori*; Flagellar biosynthesis; \(fliQ\); \(flhB\); Isogenic mutant

1. Introduction

Motility is a vital adaptation for many bacterial pathogens capable of colonising mucosal surfaces. *Helicobacter pylori* is a human-specific pathogen that colonises the stomachs of at least half the world’s population, surviving largely within the gastric mucus layer without attaching to host cells [1]. Infection is associated with some of the most prevalent gastroduodenal pathologies, including chronic gastritis, peptic ulcer disease and gastric cancer [2]. *H. pylori* possesses a unipolar bundle of sheathed flagella [3,4]. *H. pylori* has been shown to be extremely motile, even under viscous conditions such as that encountered in the human stomach which are known to inhibit the motility of other bacteria [5]. *Helicobacter* flagella have distinctive features which are thought to be central to the ability of *H. pylori* to colonise the gastric mucosa [6]. The bacteria’s sheathed flagella are composed of two proteins, FlaA and FlaB, connected to the basal body by the flagellar hook protein, which is a polymer of FlgE.
Expression of both FlaA and FlaB is necessary for full motility and persistent colonisation of the gastric mucosa [8,9]. To date, only the role of FlhA (originally described as FlbA) and FliI in the regulation of the biosynthesis and export of H. pylori flagella have been investigated [10,11]. Both of these genes are homologues of the flbF/lcrD family of motility- and virulence-associated genes which are involved in the export of flagellar proteins and other virulence factors in a number of bacterial species [12]. Otherwise, the mechanisms of biosynthesis and export of flagella in H. pylori are still poorly understood.

In Escherichia coli and Salmonella typhimurium, the regulation of motility and flagellar biosynthesis has been extensively studied [13,14]. Scrutiny of the H. pylori 26695 genome sequence has identified over 40 open reading frames which are similar to known E. coli or S. typhimurium proteins that are involved in the biosynthesis and export of flagella [15]. Two of these putative genes were chosen for analysis in this study. HP0770 has homology to FlhB, which has been shown in S. typhimurium to be required for the formation of the rod structure of the basal body of the flagellar apparatus [16] and to play a role in the determination of the flagellar hook length [17]. HP1419 has homology to FliQ, which has been shown in S. typhimurium to be membrane bound, required for flagellation but not to encode any structural or regulatory component [18]. It is suggested that FliQ is likely a component of the flagellar export apparatus, which proceeds by a type III export pathway [18].

Thus, the two genes chosen for this post-genomic analysis included one with likely a function in the flagellar assembly and another with likely a role in export. To characterise the role of the putative FliQ and FlhB proteins in the production of flagellar, control of motility and subsequent pathogenesis of H. pylori, isogenic fliQ and flhB mutants were constructed and characterised in vitro and in vivo.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in BHI broth supplemented with 10% v/v foetal calf serum or on Helicobacter selective agar (DENT), consisting of Blood Agar Base number 2 supplemented with 7% v/v lysed defibrinated horse blood and Dent’s selective supplement, in a micro-aerobic atmosphere at 37°C. E. coli strains were routinely grown in Luria-Bertani (LB) broth or on LB agar.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. pylori</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26695</td>
<td>Virulent wild-type strain</td>
<td>[15]</td>
</tr>
<tr>
<td>SS1</td>
<td>Virulent wild-type strain</td>
<td>[29]</td>
</tr>
<tr>
<td>SF3</td>
<td>Kn’ H. pylori SS1 fliQ mutant</td>
<td>This study</td>
</tr>
<tr>
<td>SF5</td>
<td>Kn’ H. pylori SS1 flhB mutant</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td>Cloning strain</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL2-Blue MRF’</td>
<td></td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap’</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pJM130</td>
<td>Kn’; source of Kn’ BamHI cassette</td>
<td>[30]</td>
</tr>
<tr>
<td>pSF3</td>
<td>pUC19 plus 0.33-kb PCR gene fragment of H. pylori flQ</td>
<td>This study</td>
</tr>
<tr>
<td>pSF3-TI</td>
<td>pSF3 with 4-bp deletion in flQ</td>
<td>This study</td>
</tr>
<tr>
<td>pSF3-TIK</td>
<td>pSF3-TI plus Kn’</td>
<td>This study</td>
</tr>
<tr>
<td>pSF5</td>
<td>pUC19 plus 1.05-kb PCR gene fragment of H. pylori flhB</td>
<td>This study</td>
</tr>
<tr>
<td>pSF5-TI</td>
<td>pSF5 with 10-bp deletion in flhB</td>
<td>This study</td>
</tr>
<tr>
<td>pSF5-TIK</td>
<td>pSF5-TI plus Kn’</td>
<td>This study</td>
</tr>
</tbody>
</table>

Abbreviations: Ap’, ampicillin resistant; Kn’, kanamycin resistant.

Table 1
Bacterial strains and plasmids used in this study
The antibiotics used for selection purposes were ampicillin (100 µg ml⁻¹) and kanamycin (20 µg ml⁻¹ for *H. pylori* and 50 µg ml⁻¹ for *E. coli*).

2.2. Construction of *H. pylori* *fiIQ* and *flhB* mutants

The putative *H. pylori* *fiIQ* and *flhB* genes were amplified by PCR using *H. pylori* 26695 chromosomal DNA as a template. The oligonucleotide primers used for PCRs are summarised in Table 2. The primers SF3F and SF3R were designed from the HP1419 sequence to amplify a 328-bp gene fragment. The primers SF5F and SF5R were designed from the HP0770 sequence to amplify a 1050-bp gene fragment. The amplified PCR products were cloned into pUC18 to produce pSF3 and pSF5, respectively. pSF3 and pSF5 were sequenced by the dideoxynucleotide chain termination method with a PRISM sequencing kit (Applied Biosystems, Warrington, UK). The data were compared with the HP1419 and HP0770 nucleotide sequences on the TIGR web site (http://www.tigr.org).

4-bp and 10-bp deletions plus unique *Bgl*II sites were introduced into the cloned *fiIQ* and *flhB* gene fragments in pSF3 and pSF5, respectively, by inverse PCR mutagenesis using the primer pairs SF3 TIF and SF3 TIR and SF5 TIF and SF5 TIR to form pSF3-TI and pSF5-TI, as described previously [19,20]. A 1.4-kb *Bam*HI restriction fragment of plasmid pJMK30 containing a gene encoding resistance to kanamycin (*aph3'-III*) [21] was cloned into the unique *Bgl*II site of pSF3-TI and pSF5-TI to form pSF3-TIK and pSF5-TIK. These constructs were introduced into the *H. pylori* SS1 wild-type strain by electroporation [22]. Kanamycin resistant colonies were selected after 4–5 days growth.

2.3. *In vitro* characterisation of mutants

The loss of functional flagella in both mutants was confirmed using the standard 0.3% stab agar motility test as described previously [8,11].

Overnight cultures of SS1, SF3 and SF5 were analysed by electron microscopy for the presence of flagellar organelle structures. 0.5 ml of each culture was mixed with 0.5 ml 2% (v/v) glutaraldehyde solution, incubated at room temperature for 10 min, then the bacteria were collected by centrifugation at 4000 rpm for 2 min. The bacterial pellet was resuspended in 150 µl H₂O. The whole bacterial cells were negatively stained with 1.5% w/v potassium phoshphotungstate (pH 6.4) and examined by transmission electron microscopy.

The assay for adherence of *H. pylori* to AGS cells was performed as described previously [23]. A FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to measure bacteria adhering to AGS cells, gated to include single cells and to exclude cell debris and unbound bacteria.

2.4. *In vivo* characterisation of mutants

Female outbred mice (HSD/ICR strain, Harlan, Bicester, UK) of approximately 20 g body weight (4–6 weeks old) were challenged orally on successive days with SS1, SF3 or SF5 [24]. Prior to the challenge, all strains were pre-treated with acidified 5 mM urea (pH 2) in order to boost the urease activity and thus optimise the colonisation potential [24]. At 2 and 8 weeks post-infection, 10 mice from each group were killed by CO₂ inhalation, the stomachs removed and opened along the greater curvature and analysed for colonisation [24]. Also after 8 weeks, the mice were exsanguinated and the individual serum samples stored at −20°C.

*H. pylori* SS1 cells were harvested from DENT agar plates, washed twice with PBS and lysed with three 30 s bursts of ultrasound (Ultrasonic Processor, Jencons Scientific, Leighton Buzzard, UK) with 30 s cooling periods on ice between each burst. The insoluble material was removed (13000 rpm for 20 min) and the soluble material was used to coat

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**Table 2**

<table>
<thead>
<tr>
<th>Name</th>
<th>PCR method</th>
<th>Strand</th>
<th>Sequence (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>SF3F</td>
<td>PCR</td>
<td>+</td>
<td>AACTGCAGATTTAGAAGAAATCTT</td>
</tr>
<tr>
<td>SF3R</td>
<td>PCR</td>
<td>−</td>
<td>AAAAGCTTTAATGGTTTCTAGCT</td>
</tr>
<tr>
<td>SF5F</td>
<td>PCR</td>
<td>+</td>
<td>AAGAAGAAAACCGAAC</td>
</tr>
<tr>
<td>SF5R</td>
<td>PCR</td>
<td>−</td>
<td>AAGAAGAAAACCGAAC</td>
</tr>
<tr>
<td>SF3 TIF</td>
<td>IPCRM</td>
<td>+</td>
<td>GAGATCTTTCAAGCCACCTCA</td>
</tr>
<tr>
<td>SF3 TIR</td>
<td>IPCRM</td>
<td>−</td>
<td>GAGATCTTTCAAGCCACCTCA</td>
</tr>
<tr>
<td>SF5 TIF</td>
<td>IPCRM</td>
<td>+</td>
<td>GAGATCTTTCAAGCCACCTCA</td>
</tr>
<tr>
<td>SF5 TIR</td>
<td>IPCRM</td>
<td>−</td>
<td>GAGATCTTTCAAGCCACCTCA</td>
</tr>
</tbody>
</table>

Underlined nucleotides represent *Bgl*II (SF3 TIF, SF3 TIR, SF5 TIF and SF5 TIR) restriction endonuclease sites.
wells of an EIA/RIA 96 well plate (Corning Costar, High Wycombe, UK) for 18 h at 4°C (1 μg well⁻¹ in 0.1 M NaHCO₃, pH 9.5). The antibody levels within individual serum samples were determined as described previously [25].

3. Results

3.1. Construction of \textit{H. pylori} \textit{fliQ} and \textit{flhB} mutants

\textit{H. pylori} \textit{fliQ} and \textit{flhB} mutants were constructed by allelic replacement as summarised previously [11]. Successful double recombination was checked by PCR for the mutants SF3 and SF5 using the primer pairs SF3F and SF3R and SF5F and SF5R, respectively, and confirmed by Southern blotting (data not shown). No differences in the colony morphology or growth rates were observed when grown on DENT plates or in BHI broth, respectively, between SF3, SF5 and the wild-type strain SS1.

3.2. In vitro characterisation of mutants

After stabbing into semi-solid media, both mutants produced small, compact colonies (indicating a lack of motility) in contrast to the diffuse spreading colonies seen with the wild-type strain SS1 (data not shown).

The mutants SF3 (Fig. 1B) and SF5 (Fig. 1C) were analysed by transmission electron microscopy using negative staining and compared with SS1 (Fig. 1A). No flagella were detected in either mutant. In a previously constructed \textit{H. pylori} \textit{flhA} mutant (initially described as \textit{flhA}), short structures of about 30–100 nm in length were observed that were suggested to represent flagellar hooks [10]. No such structures were observed in either the \textit{fliQ} or \textit{flhB} mutants.

All strains were analysed for the ability to adhere to cultured human AGS cells. SF5 adhered to AGS cells at a level comparable to SS1, whilst SF3 showed a reduced level of adherence. The SF3 adherence was measured as 62.8 ± 13.2% and SF5 as 96.8 ± 3.7%, compared to 92.8 ± 2.6% seen with SS1.

3.3. In vivo characterisation of mutants

Three groups of 20 mice were infected with 1 ml volumes of overnight cultures of SS1, SF3 and SF5, respectively, on two successive days. The number of bacteria administered to each mouse was between $5 \times 10^7$ and $1 \times 10^8$ cfu ml⁻¹. All mice inoculated...
with SS1 were colonised at 2 and 8 weeks post-infection. However, mice inoculated with SF3 or SF5 showed no colonisation by either mutant at either 2 or 8 weeks post-infection.

Serum isolated from individual mice 8 weeks after oral inoculation were analysed for the presence of anti-

*H. pylori* antibodies by ELISA. Seven out of the 10 mice inoculated with SF3 generated a significant anti-

*H. pylori* serum response. Similarly, six of the mice challenged with SF5 seroconverted. In comparison, nine out of the 10 mice challenged with the SS1 wild-type strain seroconverted (Fig. 2). The mean antibody titres induced by both SF3 and SF5 were not significantly different (*P* > 0.05) to the antibody level seen with wild-type-challenged mice as measured by the Student’s *t*-test.

4. Discussion

Motility has been shown to be a key factor in the ability of *H. pylori* to colonise the gastric mucosa [9]. Scrutiny of the *H. pylori* 26695 genome sequence has identified by homology at least 40 proteins that may be involved in the regulation, secretion and assembly of flagella [15]. We have constructed mutants in two of these putative genes, *fliQ* and *flhB*, which have been shown to play a role in the flagella expression in *E. coli* and *S. typhimurium*. Subsequent characterisation of these isogenic mutants demonstrated that they were non-motile, aflagellate and were unable to colonise the *H. pylori* mouse model. These results suggest the involvement of FliQ and FlhB in the assembly and export of flagella in *H. pylori*.

The *fliQ* mutant has a reduced ability to adhere to AGS cells, in contrast to the *flhB* mutant which showed a level of adherence similar to that of the wild-type strain. In *S. typhimurium*, it is suggested that FliQ is involved in a type III export pathway [18]. The reduced levels of adherence of the *fliQ* mutant may be due to the inability to export a protein with a role in adherence. This hypothesis is supported by the wild-type levels of adherence observed with the *flhB* mutant, where the proposed type III export pathway involving FliQ would be functional. The aflagellate nature itself of these *H. pylori* mutants does not appear to affect the ability to adhere to epithelial cells directly, suggesting that adhesion to and colonisation of the gastric mucosa are separate stages in the *H. pylori* infection.

Neither mutant showed evidence of colonisation of the mouse gastric mucosa at either 2 or 8 weeks post-infection. However, both mutants elicited a host immune response, which was indistinguishable from that induced by the wild-type strain. In the gnotobiotic piglet model, it was shown that even though non-motile *H. pylori* mutants can cause only a very transient colonisation, these mutants could still elicit a humoral immune response [9]. More recently, similar results have also been observed in the mouse model with a *flaA* mutant of SS1 [26]. Considered together, these results suggest that some of the aflagellate bacteria must adhere to gastric mucin allowing persistence for several days and stimulating an immune response, before leaving the stomach due to their inability to penetrate and colonise the mucus layer. Surprisingly, the *fliQ* mutant appears to elicit a stronger host immune response, despite the mutant’s reduced ability to adhere to AGS cells in vitro. This only serves to underline the complexity of *H. pylori*-associated host-bacterial interactions [27].

This study demonstrates the application of genomic analysis to the study of *H. pylori* [28]. Bioinformatic analysis suggests a function by virtue of homology. These hypotheses can then be tested by a mutational analysis through the construction of defined isogenic mutants. We have confirmed the hypothesis that FliQ and FlhB are involved in flag-
cellular expression in *H. pylori*, but also have shown that FliQ has a role in adhesion to gastric epithelial cells.

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**References**


