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Helicobacter pylori HopQ outer membrane protein attenuates bacterial adherence to gastric epithelial cells

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Abstract

Helicobacter pylori genomes contain about 30 *hop* genes that encode outer membrane proteins. *H. pylori hopQ* alleles exhibit a high level of genetic diversity, and two families of *hopQ* alleles have been described. Type I *hopQ* alleles are found more commonly in *cag*-positive *H. pylori* strains from patients with peptic ulcer disease than in *cag*-negative strains from patients without ulcer disease. In this study, we mutated *hopQ* in four *H. pylori* strains that each contained a type I *hopQ* allele, and then analyzed interactions of the wild-type and *hopQ* mutant strains with AGS cells. In comparison to the wild-type strains, two of the *hopQ* mutant strains exhibited increased adherence to AGS cells and two *hopQ* mutants did not exhibit any detectable differences in adherence. Higher levels of tyrosine-phosphorylated CagA were detected when AGS cells were co-cultured with a hyper-adherent *hopQ* mutant strain than when co-cultured with the corresponding wild-type strain. These data indicate that in some strains of *H. pylori*, the HopQ protein can attenuate bacterial adherence to gastric epithelial cells.

Keywords

Helicobacter pylori; HopQ; adherence; CagA

INTRODUCTION

Persistent colonization of the human stomach with *H. pylori* is a risk factor for peptic ulceration and adenocarcinoma. *H. pylori* genomes contain about 30 paralogous *hop* genes, which encode outer membrane proteins (Alm *et al.*, 2000). Several Hop proteins mediate adherence of *H. pylori* to gastric epithelial cells. For example, BabA and SabA bind to fucosylated-Lewis B (Aspholm-Hurtig *et al.*, 2004; Ilver *et al.*, 1998) and sialyl-Lewis X antigens (Mahdavi *et al.*, 2002), respectively, on the surface of gastric epithelial cells. The cell-surface receptors for other *H. pylori* Hop proteins with adhesive properties [including HopH (OipA) (Dossumbekova *et al.*, 2006), HopC (AlpA), HopB (AlpB) (Odenbreit *et al.*, 1999), and HopZ (Peck *et al.*, 1999)] have not yet been identified. Several Hop proteins (e.g. HopV, HopW, HopX, and HopY) have been reported to function as porins (Alm *et al.*, 2000; Peck *et al.*, 2001).

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One outer membrane protein, HopQ (omp27), is of interest because two highly divergent families of *hopQ* alleles have been identified (Cao & Cover, 2002). Type I *hopQ* alleles are found most commonly in *H. pylori* strains that contain the *cag* pathogenicity island (PAI), which encodes CagA and a type IV secretion system involved in the translocation of CagA into gastric epithelial cells (Backert & Selbach, 2008). CagA causes numerous alterations in gastric epithelial cells, including changes in cell morphology, increased cell scattering, disruption of tight junctions, and activation of β-catenin (Backert & Selbach, 2008; Hatakeyama, 2004). Several studies have reported an association between the presence of type I *hopQ* alleles and other *H. pylori* virulence markers, including type s1 *vacA* alleles (Cao & Cover, 2002; Lehours *et al.*, 2004). Type II *hopQ* alleles are found more commonly in *cag* PAI-negative *H. pylori* strains than in *cag* PAI-positive strains (Cao & Cover, 2002). Geographical differences in the distribution of type I and type II *hopQ* alleles have been noted. For example, most *H. pylori* strains isolated in East Asia are *cag* PAI-positive and contain Type I *hopQ* alleles (Cao *et al.*, 2005). Type II alleles are commonly found in *H. pylori* strains isolated in Western countries, but are uncommon among *H. pylori* strains from East Asia (Cao *et al.*, 2005).

HopQ is known to be localized to the surface of *H. pylori* (Sabarth *et al.*, 2005). However, nothing is known about functional properties of HopQ. Because HopQ is an outer membrane protein, we hypothesized that it might have a role in *H. pylori* adherence to gastric epithelial cells. In addition, because type I *hopQ* alleles are found predominantly in *H. pylori* strains containing the *cag* PAI, we hypothesized that HopQ might modulate one or more *cag* PAI-associated phenotypes. In this study, we selected four *H. pylori* strains containing type I *hopQ* alleles for analysis and generated isogenic *hopQ* mutant strains. We then analyzed properties of the *hopQ* mutant strains in comparison to the parental wild-type strains.

Materials and Methods

Bacterial strains

H. pylori strains 26695 SC#7 (Busler *et al.*, 2006), J99 (Alm *et al.*, 1999), J178 and 87-29 were used in this study. Each of these strains contains a type I *hopQ* allele (Cao & Cover, 2002). We also used a 26695 SC#7 Δ*cag* PAI mutant strain, which lacks the entire *cag* PAI (Busler *et al.*, 2006). For routine growth, *H. pylori* were maintained on trypticase soy agar plates containing 5% sheep blood. Sulfite-free Brucella broth containing 5% FBS (i.e. BB-FBS) was used for growth of *H. pylori* liquid cultures, and broth cultures were grown on a rotary shaker (150 rpm). The composition of the broth culture medium was modified from the original description by the use of Bacto proteose peptone instead of peptamin (Loh *et al.*, 2007). Brucella-agar plates containing chloramphenicol 5 μg mL^{-1} were used for natural transformation experiments. All *H. pylori* cultures were grown at 37°C in room air supplemented with 5% CO_2.

Plasmid construction and mutagenesis

The *hopQ* gene was amplified from *H. pylori* strain 26695 using primers BA7676 (5’ – AACGCTAAGGCTTTATGCTTATGCTC-3’) and AND2288 (5’-TTGTAATCAAAGAAACCATAA-3’) and cloned into a pGEM-T plasmid (Promega). A GPS-LS linker-scanning system (NE Biolabs) was then used to insert a unique *Pme*I site into the *hopQ* coding sequence via a transposon. This plasmid was digested with *Pme*I, and a chloramphenicol resistance (*cat*) cassette (Wang & Taylor, 1990) was ligated into the *Pme*I site. The resultant plasmid was used to transform *H. pylori* strains 26695 SC#7, J99, J178 and 87-29 and transformants were selected on Brucella agar plates supplemented with chloramphenicol (5 μg mL^{-1}). Proper insertion of the *cat* cassette into the *H. pylori* chromosomal *hopQ* gene was confirmed by PCR analyses.

Analysis of *H. pylori* adherence to gastric epithelial cells

The human gastric adenocarcinoma cell line (AGS) was maintained in RPMI 1640 medium (GIBCO BRL) containing 10% FBS and grown at 37°C in 5% CO_2. The cells were routinely subcultured every 2–3 days. An adherence assay modified from that described (Dossumbekova *et al.*, 2006) was used to analyze *H. pylori* binding to AGS cells. Briefly, AGS cells were seeded into wells of a 24-well plate and grown overnight, prior to the addition of *H. pylori. H. pylori* were cultured in overnight in broth, and the number of *H. pylori* organisms added to cells was standardized based on measurements of optical density (OD_{600} of 1.0 = 10^9 CFU/ml). When serial dilutions of standardized *H. pylori* cultures were plated on solid media, there were no significant differences in the number of CFU/ml detected for wild-type and *hopQ* mutant strains. *H. pylori* were cocultured with AGS cells at various multiplicities of infection (MOI) for 1 h or 5 h at 37°C with gentle shaking (50 rpm) on an orbital shaker, and non-adherent bacteria were then removed by washing using PBS buffer. Bound cells were fixed with 4% paraformaldehyde for 15 min, followed by blocking in 10% normal goat serum for 1 h. After washing with PBS, the cells were incubated for 1 h with primary rabbit antiserum (1:500) generated against *H. pylori* soluble proteins (Cao *et al.*, 1998). Following 3 washes in PBS, a cy3-conjugated anti-rabbit antibody (1:500, Sigma) was then added for 1 h. Cells were washed 3 times with PBS, and then were incubated for 15 min with trypsin (0.5 mg mL^{-1}) to detach the cells, or treated with cyQUANT cell lysis buffer (Invitrogen), or 0.1% saponin in PBS to lyse the cells. The detached or lysed samples were removed, transferred to a 96-well assay plate (Corning), and fluorescence measured in a FLx800 microplate reader (BioTek).

As another approach to analyze *H. pylori* binding to AGS cells, *H. pylori* and AGS cells were co-cultured for 5 h at 37°C with gentle shaking as described above. The wells were washed 3 times with RPMI media to remove any unbound bacteria. The cells were then incubated in PBS containing 0.1% saponin (37°C, 15 min) (Kwok *et al.*, 2002) and serial dilutions of the samples were cultured on blood agar plates. Colony forming units were enumerated 5 days after plating.

Immunoblot analysis of CagA expression

Broth cultures of *H. pylori* were grown overnight and inoculated into fresh brucella broth for 12 h before being harvested and lysed with NP-40 lysis buffer [150 mmol L^{-1} NaCl, 1% NP40, 50 mmol L^{-1} Tris-HCl (pH 8.0)] supplemented with protease inhibitors (Complete Mini; Roche Applied Science). The protein content of lysates was determined using the BCA reagent kit (Pierce). Following normalization of protein content, the proteins were separated using SDS-PAGE, and transferred to nitrocellulose membranes (Biorad). The membranes were blocked for 2 h in TBS-Tween (TTBS; Tris buffered saline containing 0.1% Tween 20) containing 3% milk, and subsequently probed with either polyclonal antiserum against CagA (1:6000 dilution, Austral Biologicals) or antiserum against *H. pylori* soluble proteins (1:10,000 dilution) (Cao *et al.*, 1998). Following a 1 h incubation, the blots were washed with TTBS, and then incubated for 1 h with a HRP-conjugated goat anti-rabbit antibody. Labeled bands were visualized on X-ray film using chemiluminescent techniques, according to the manufacturer's instructions (ECL Western Blotting Detection reagent, Amersham Bioscience). When reprobing of membranes was required, blots were incubated in Restore stripping buffer (Pierce) prior to the addition of a new primary antibody.

Analysis of CagA tyrosine phosphorylation

H. pylori were cultured in broth as described above and then co-cultured with AGS cells, using a 50:1 multiplicity of infection. Following a 5 h coculture, the cells were washed 3 times with ice-cold PBS, and then lysed with NP-40 lysis buffer containing protease inhibitors (Roche) and 2 mmol L^{-1} sodium orthovanadate. The protein concentration of

each lysate was determined (BCA reagent kit, Pierce), and all samples were normalized for protein content. To detect tyrosine phosphorylated CagA, 10 μg of each sample was subjected to SDS-PAGE and western blot analyses using a 1: 6000 dilution of a mouse monoclonal anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology). Following a 5 h incubation, the membranes were washed with TTBS and incubated for an additional 1 h with HRP-conjugated sheep anti-mouse IgG (1:10,000, Amersham Biosciences). Immunoreactive bands were then visualized using chemiluminescent techniques. Membranes were also probed with a polyclonal antibody against *H. pylori* CagA (1:6000, Santa Cruz), antiserum against *H. pylori* soluble proteins (anti-HP; 1:10,000) (Cao *et al.*, 1998), and a goat anti-actin antibody (1:10,000, Santa Cruz). Secondary antibodies included a 1:6000 dilution of HRP-conjugated goat anti-rabbit IgG (for CagA and *H. pylori* soluble proteins), and a 1:10,000 dilution of HRP-conjugated anti-goat IgG (for actin; Santa Cruz). When reprobing of membranes was required, previously bound antibodies were removed using Restore stripping buffer (Pierce).

Analysis of IL-8 secretion

To monitor IL-8 secretion by AGS cells, supernatants from coculture experiments were clarified by centrifugation at 12,000 g. IL-8 levels in the supernatants were subsequently determined using an IL-8 cytometric bead assay (BD Biosciences), as per the manufacturer's instructions. The samples were analyzed using a BD LSR II flow cytometer and a high throughput system. IL-8 standards provided with the IL-8 cytometric bead assay were used for determination of IL-8 concentrations. Data analysis was performed using FCAP Array Software v.1.0.1 (BD Bioscience, San Diego, CA).

RESULTS AND DISCUSSION

To study functional properties of HopQ, we first generated two independent *hopQ* mutants (hopQ6 and hopQ9) derived from *H. pylori* strain 26695 SC#7, as described in the Materials and Methods. To test the hypothesis that HopQ might have a role in the adherence of *H. pylori* to epithelial cells, we cultured wild-type *H. pylori* strain 26695 and its isogenic *hopQ* mutants with AGS cells, and the levels of *H. pylori* adherence were monitored as described in the Materials and Methods, using a polyclonal rabbit antiserum against *H. pylori* followed by a cy3-labeled anti-rabbit antibody. These studies revealed that the *hopQ* mutant strains bound to AGS cells to a significantly greater extent than did the wild-type strain at each of the tested MOI's (Figure 1A, B). In addition, as shown in Figure 1C, the number of viable *hopQ* mutant bacteria recovered from these co-culture experiments was significantly higher than the number of wild-type bacteria recovered. We also analyzed the adherence properties of three other wild-type *H. pylori* strains (J99, J178, and 87-29) and the corresponding *hopQ* mutants. Similar to strain 26695, each of these strains contains a type I *hopQ* allele (Cao & Cover, 2002). As shown in Figure 1, a *hopQ* mutant derived from strain J178 exhibited significantly increased adherence to AGS cells compared to wild-type J178. In contrast, *hopQ* mutagenesis did not alter the binding of *H. pylori* strains J99 and 87-29 to AGS cells (Figure 1D). These data provide evidence that the HopQ outer membrane protein modulates *H. pylori* adherence in a strain-specific manner. Strain-specific variations have previously been detected with several other *H. pylori* phenotypes (Baldwin *et al.*, 2007; Lee *et al.*, 2006; Pflock *et al.*, 2007; Tan *et al.*, 2005).

Because type I *hopQ* alleles are found predominantly in *H. pylori* strains containing the *cag* PAI, we hypothesized that HopQ might modulate one or more *cag* PAI-associated phenotypes. As a first step, we tested whether a wild-type *H. pylori* strain and a *hopQ* mutant strain differed in the expression of CagA. For these studies we analyzed *H. pylori* strain 26695 SC#7, which is known to translocate CagA into gastric epithelial cells (Busler *et al.*, 2006), and an isogenic *hopQ* mutant. *H. pylori* strains were cultured in broth, and

CagA expression was assessed by immunoblotting. As shown in Figure 2A, wild-type *H. pylori* strain 26695 and an isogenic *hopQ* mutant did not differ in CagA expression.

To determine whether expression of HopQ has any effect on CagA translocation into gastric epithelial cells, wild-type strain 26695 and an isogenic *hopQ* mutant strain were co-cultured with AGS cells, and the levels of tyrosine phosphorylated CagA were then analyzed as described in the Methods. As shown in Figure 2B, a higher level of tyrosine-phosphorylated CagA was detected in AGS cells co-cultured with the *hopQ* mutant than in AGS cells co-cultured with the wild-type strain. Similarly, a higher level of total CagA was detected in samples containing the *hopQ* mutant strain than in samples containing the wild-type strain. When the blots were reprobed with anti-actin antibody, the levels of actin were similar, indicating that there was minimal variation in the number of AGS cells present within each sample. When the blots were reprobed with an antiserum against soluble *H. pylori* proteins, immunoreactivity was stronger in samples containing the *hopQ* mutants than in samples containing the wild-type strain. This result is consistent with the data in Figure 1, showing that *hopQ* mutants derived from strain 26695 exhibit increased adherence to AGS cells compared to wild-type *H. pylori*. Increased bacterial adherence presumably facilitates increased translocation of CagA into host cells.

We also investigated the possibility that a *hopQ* mutation might alter the ability of *H. pylori* to stimulate IL-8 secretion by AGS cells. Wild-type *H. pylori* strain 26695 and an isogenic *hopQ* mutant strain each induced expression of IL-8 (Figure 3), whereas a *cag* PAI mutant strain did not (data not shown). There was no significant difference in the levels of IL-8 secretion stimulated by the wild-type strain and the *hopQ* mutant strain (Figure 3). The data shown in Figure 3 suggest that under the conditions of this assay, a sufficient number of adherent wild-type or mutant bacteria were present at each of the tested MOI's to induce maximal levels of IL-8 induction, and therefore, despite a difference in the adherence of wild-type and *hopQ* mutant strains, no differences in the IL-8-inducing capacity of wild-type bacteria versus mutant bacteria were detected.

In conclusion, the results of this study indicate that the presence of HopQ modulates the adherence properties of *H. pylori* in a strain-specific manner. All of the strains tested in the current study contained type I *hopQ* alleles. Further studies will be required to determine whether type II HopQ proteins exhibit anti-adhesive properties, or whether this phenomenon is limited to type I HopQ proteins. The molecular basis for the observed increased adherence of 26695 and J178 *hopQ* mutant strains compared to the respective wild-type strains is not yet understood. One possibility is that the expression of HopQ on the bacterial cell surface may sterically block the adhesive properties of another bacterial adhesin. Another possibility is that HopQ is localized within a complex of molecules in the *H. pylori* outer membrane, and the presence or absence of HopQ may influence the adherence properties of other molecules in the complex. Interestingly, both *H. pylori* strains J99 and 87-29 are known to bind to express the BabA adhesin and bind Lewis B antigens, whereas strains 26695 and J178 are BabA negative and unable to bind to Lewis B (Hennig *et al*., 2004; Hennig *et al*., 2006). Based on this observation, it seems possible that the anti-adhesive properties of HopQ might be most readily detectable in *H. pylori* strains that lack adhesins such as BabA. As shown in Figure 2, higher levels of tyrosine-phosphorylated CagA were detected when AGS cells were co-cultured with a hyper-adherent *hopQ* mutant strain than when co-cultured with the corresponding wild-type strain. Therefore, by attenuating *H. pylori* adhesion to gastric epithelial cells, HopQ may downregulate *H. pylori*-induced gastric epithelial cell alterations.

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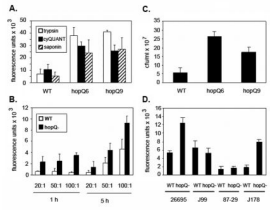

Figure 1. Adherence of wild-type *H. pylori* and *hopQ* mutant strains to AGS gastric epithelial cells

A) AGS cells were co-cultured with *H. pylori* wild-type strain 26695 (WT) or two isogenic *hopQ* mutant strains (hopQ6 and hopQ9) (MOI 50:1). After coculture for 1 h at 37°C, cells were fixed with paraformaldehyde and blocked with 10% goat serum. Cells were then incubated with a polyclonal antibody against *H. pylori* soluble proteins (1:500), followed by a cy3-labeled anti-rabbit antibody (1:500 dilution). The samples were treated with either 0.5 mg mL^{-1} trypsin, cyQUANT lysis buffer, or 0.1% saponin, and fluorescence was quantified using a FLx800 microplate reader (BioTek). B) *H. pylori* strain 26695 (WT) and an isogenic hopQ mutant strain were cocultured with AGS cells at MOI's of 20:1, 50:1 and 100:1, and then the co-cultures were incubated with 0.5 mg mL^{-1} trypsin and analyzed as in Figure 1A. C) Following coculture of wild-type strain 26695 or *hopQ* mutant strains with AGS cells for 5h at 37° C, the cells were treated with PBS containing saponin (Kwok *et al.*, 2002). Serial dilutions of samples were then plated on blood agar plates and colony forming units were enumerated 5 days after plating. D) *H. pylori* strains 26695, J99, 87-29, J178 and isogenic *hopQ* mutant strains were cocultured with AGS cells for 1 h. Fluorescent-based adherence assays were then performed as described above. For each of the panels, the results represent mean and standard deviation based on triplicate samples from a single experiment. All experiments were performed three times with similar results. Statistical significance was analyzed using Student's t-test.

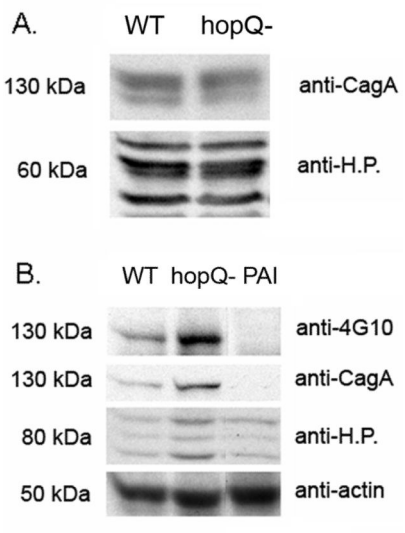

Figure 2. Analysis of CagA expression and CagA tyrosine phosphorylation

A) Immunoblot analysis of CagA expression in *H. pylori* strain 26695 (WT) and an isogenic *hopQ* mutant strain (*hopQ*-). *H. pylori* cells were cultured in brucella broth, and then lysed with NP-40 lysis buffer. Standardized quantities of *H. pylori* lysates (5 μg protein) were analyzed by immunoblotting. Membranes were immunoblotted sequentially with an anti-CagA antibody followed by an antiserum to soluble *H. pylori* proteins (anti-H.P.), as described in the Methods. B) Comparison of CagA translocation into AGS cells by *H. pylori* strain 26695 (WT), an isogenic *hopQ* mutant (hopQ-), or a Δ*cag* PAI mutant strain (PAI). *H. pylori* cells were cultured in brucella broth and then were added to AGS cells (50:1 MOI) for 5 h. Samples were then washed with PBS, lysed with NP-40 lysis buffer, and standardized quantities of lysates (10 μg protein) were analyzed by immunoblotting. Membranes were sequentially probed with an anti-phosphotyrosine antibody (anti-4G10), anti-CagA, anti-actin, and antiserum to *H. pylori* proteins (anti-H.P.). The experiments were performed three times with similar results.

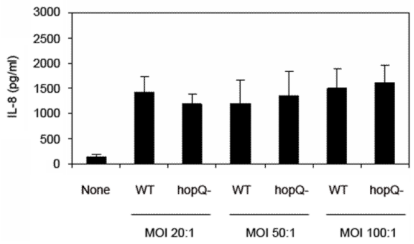

Figure 3. Analysis of IL-8 induction

H. pylori wild-type strain 26695 (WT) and a *hopQ* mutant strain (hopQ-) were cultured in brucella broth and then added to AGS cells at MOI's of 20:1, 50:1 and 100:1. Control AGS cells (labeled "none") were maintained in medium alone. Following a 5 h incubation, supernatants were removed and the IL-8 content was quantified as described in the Materials and Methods. The results represent mean and standard deviation based on triplicate samples from a single experiment. No significant differences were detected when comparing the effects of wild-type versus *hopQ* mutant strains (Student's t-test). This experiment was performed three times with similar results.