Identification of Novel *Helicobacter pylori* Adhesins that Bind the Host Cell Receptor Decay-Accelerating Factor (DAF)

Emily M. Alsentzer, Jennifer M. Noto, W. Hayes McDonald, and Richard M. Peek, Jr.

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BRIEF. *H. pylori* adherence is critical for gastric cancer development, and this study has identified novel adhesins responsible for DAF-mediated adherence.

ABSTRACT. Gastric cancer is the second leading cause of cancer-related deaths worldwide, and infection with *Helicobacter pylori* is the strongest known risk factor for this malignancy. Over half of the world's population is infected with *H. pylori*, but only a fraction of those infected ever develop gastric cancer. Adherence of *H. pylori* to the stomach is critical for cancer development, and the human protein Decay-accelerating factor (DAF) is important for *H. pylori* binding. The goal of this study was to identify the *H. pylori* proteins that specifically bind to DAF. Six candidate proteins were identified, and two proteins, FlaA and HopQ, were confirmed to contribute to DAF binding, providing new insights into the pathogenesis of *H. pylori*-mediated gastric cancer. Identification of novel factors required for *H. pylori* colonization may lead to discovery of novel targets for antimicrobial therapy or vaccine development.

INTRODUCTION.

Gastric cancer is the second leading cause of cancer-related death worldwide, accounting for 700,000 deaths each year and a 5-year survival rate of <15% in the U.S. [1]. Infection with *Helicobacter pylori*, a bacterium that colonizes the stomach of more than half of the world's population, is the strongest known risk factor for the development of gastric cancer [2]. Almost all individuals infected with *H. pylori* develop chronic gastritis, inflammation of the stomach lining. Once infected by *H. pylori* the stomach undergoes a series of changes from chronic gastritis to gastric cancer. Interestingly, although greater than 50% of the world's population is infected with *H. pylori*, only a fraction of infected individuals ever develop gastric cancer. Such observations show the importance of identifying mechanisms on a cellular level that are involved in the interactions between *H. pylori* and their hosts as a means to better understand the diseases induced by *H. pylori*.

It is widely established that binding of *H. pylor*i to the stomach is important for the development of gastric inflammation. A number of human proteins have been identified that aid in *H. pylori* binding to the stomach. Decay-accelerating factor (DAF), a human host cell receptor that protects host cells from lysis or cell death , is one such receptor that assists *H. pylori* adherence and is involved in *H. pylori*-induced gastric inflammation [3]. A number of pathogens capable of causing chronic infections bind to DAF, such as *E. coli*, coxsackieviruses, enteroviruses, and echoviruses [4]. Increased expression of DAF has also been observed in breast [5] and lung cancer cells [6], which suggests that DAF is involved in the development of cancer.

Previous studies have shown that more DAF is expressed within human stomachs infected with *H. pylori* compared to uninfected stomachs [7]. *H. pylori* bind cells that express DAF more readily than cells that lack DAF, suggesting that DAF is important in *H. pylori* binding. DAF-deficient mice infected with *H. pylori* develop significantly less severe inflammation compared with infected wild-type mice that express DAF. This suggests that *H. pylori* binding to DAF aids in *H. pylori*-induced stomach inflammation (Fig. S1) [8]. However, the specific bacterial proteins involved in the interaction between DAF and *H. pylori* have not been identified. Due to the importance of *H. pylori* adherence in the development of gastric cancer, understanding the interaction between *H. pylori* and DAF is critical in comprehending how *H. pylori* binds to receptors in the stomach. By discovering the mechanisms by which *H. pylori* binds to the stomach, these pathways can later be targeted for treatment and prevention of this disease. Thus, the aim of this study is to determine how *H. pylori* utilizes DAF as a receptor.

MATERIALS AND METHODS.

Bacterial Strains and Protein Preparation.

H. pylori strains J166 and 7.13 and *flaA*$^{-}$ and *hopQ*$^{-}$ isogenic mutants were grown in *Brucella* broth with 10% fetal bovine serum (FBS) for 18 hours at 37°C and 5% CO_2. To lyse *H. pylori*, samples were subjected to French press-mediated lysis. To harvest membrane fractions, samples were subjected to ultracentrifugation. Total protein levels were determined by the bicinchroninic acid (BCA) assay.

Immobilized Metal Affinity Chromatography.

To isolate *H. pylori* proteins that bind DAF, immobilized metal affinity chromatography was used. Full-length, recombinant DAF (rDAF) with a 6X Histidine tag was utilized as bait. Strains J166 and 7.13 were incubated with rDAF for 1 hour at 4°C. To form Nickel-nitrilotracetic acid (Ni-NTA)-DAF complexes, rDAF, J166, 7.13, J166/rDAF, or 7.13/rDAF mixtures were incubated with the Ni-NTA matrix. Samples were then washed and complexes were eluted using a magnetic separator. DAF-binding *H. pylori* proteins were eluted and stored for subsequent analyses.

Silver Staining and Far Western Blot Analysis.

Eluted *H. pylori* DAF-binding protein complexes were separated by SDS-PAGE and visualized by mass spectrometry compatible silver staining. To validate these results, eluted proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with rDAF, which was detected using an anti-DAF antibody. Far Western blotting differs from traditional Western blotting in that membranes are probed with a protein then antibodies as opposed to antibodies alone. Proteins were separated by SDS-PAGE, and bands that appeared in the lanes with *H. pylori* proteins and DAF, but not in lanes with *H. pylori* proteins alone were extracted and subjected to liquid chromatography-mass spectrometry.

Chinese Hamster Ovary Cell Culture Model.

To determine whether the inactivation of certain H. pylori genes abolished DAF binding, adherence assays were performed using a DAF-expressing or deficient Chinese Hamster Ovary (CHO) cell culture model. Wild-type and isogenic *H. pylori* mutants were co-cultured with DAF+ or DAF- CHO cells to quantify *H. pylori* adherence. CHO cells expressing full-length DAF or vector alone were seeded in tissue culture dishes at 3 X 10^6 cells/dish. Wild-type and isogenic mutant *H. pylori* were co-cultured with DAF+ or DAF- CHO cells at a multiplicity of infection (MOI) of 10:1 or 100:1. *H. pylori*:CHO cell co-cultures were washed to remove non-adherent bacteria, and adherent bacteria were enumerated by quantitative culture.

Supplemental Information.

Please refer to the supplemental information for methods pertaining to the generation of *H. pylori* mutants and liquid chromatography-mass spectrometry.

RESULTS.

Visualization of DAF Binding Proteins.

Metal chromatography and SDS-PAGE analysis identified three bands with an approximate molecular weight of 50-75 kDa that were identified in both the lanes containing *H. pylori* strain J166 and DAF as well as *H. pylori* strain 7.13 and DAF, but no such bands were present in lanes containing J166 or 7.13 alone, suggesting that these proteins bind specifically to DAF (Figure 1A).

To account for potential nonspecific binding, and the fact that the molecular weight of DAF overlapped with the molecular weights of the bands of interest, a DAF far Western was performed to confirm the DAF-binding ability of the bands of interest between 50 and 75 kDa. A doublet was visualized in the J166 membrane fraction, corresponding in molecular weight to those visualized via SDS-PAGE (Figure 1B).

DAF Binding Proteins of Interest.

Tandem mass spectrometry identified 33 *H. pylori* proteins present in the excised bands from the lanes containing 7.13 and DAF (Table S1). The *H. pylori* proteins FlaA, HopQ, AlpA, HofC, FlaB, and AlpB were selected for subsequent analyses using the Comprehensive Microbial Resource database based on their abundance, molecular weight (50-75 kDa), and their biological roles in adherence and motility (Table 1). HopQ, AlpA, HofC, and AlpB were of particular interest due to their roles as outer membrane proteins and adhesins; however due to their prevalence, subsequent analyses were performed on FlaA and HopQ.

Expression of HopQ and FlaA Increases Cellular Binding of H. pylori in a DAF-dependent Manner.

To determine whether the candidate DAF-binding proteins mediate *H. pylori* adherence to host cells, CHO cells transfected with human DAF cDNA or vector alone were co-cultured with the *H. pylori* strain 7.13. Binding was assessed by quantitative culture. Wild-type *H. pylori* strain 7.13 bound to CHO cells expressing DAF at higher levels than CHO cells not expressing DAF, confirming previous studies that show DAF increases *H. pylori* adherence *in vitro* (Figure 2) [8]. Both *hopQ*- and *flaA*- mutant strains showed considerably less binding to CHO cells expressing DAF than wild-type *H. pylori*, suggesting that FlaA and HopQ mediate *H. pylori* adherence to DAF. However, there was no significant difference in *H. pylori* binding to CHO cells that do not express DAF, suggesting that FlaA and HopQ mediate adherence in a DAF-dependent manner.

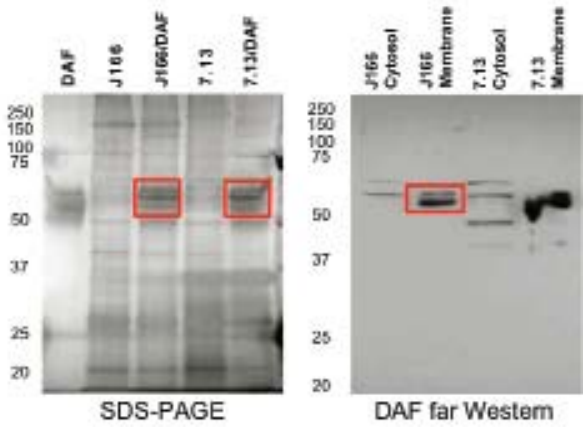

Figure 1. Visualization of DAF-binding proteins via metal affinity chromatography and DAF far Western blotting. A) SDS-PAGE with mass spectrometry compatible silver staining showing DAF, *H. pylori* membrane proteins, and both DAF and membrane proteins. The molecular weight of DAF is similar to the molecular weights of the DAF-binding proteins. Red boxes represent regions excised for mass spectrometry analysis B) Far Western analysis of *H. pylori* lysates probed with rDAF and followed by an anti-DAF antibody. Red box designates corresponding bands in the far Western blot.

Table 1. H. pylori proteins identified via mass spectrometry listed according to percent abundance. DAF-binding proteins were filtered according to percentage of total spectra, correlation of molecular weight with predicted molecular weight of DAF-binding proteins, and cellular role, which was determined via the Comprehensive Microbial Resource database (J.Craig Venter Institute, www.cmr.jcvi.org).

Protein Description	Molecular Weight	Cellular Role
FlaA	53 kDa	Chemotaxis & motility
HopQ	69 kDa	Outer membrane protein
AlpA	56 kDa	Porin and adhesin
HofC	59 kDa	Outer membrane protein
FlaB	58 kDa	Chemotaxis & motility
AlpB	57 kDa	Porin

DISCUSSION.

Colonization of humans by pathogenic bacteria is common, but gastric cancer occurs only in a fraction of infected persons. Increased DAF expression has been recently linked to increased adherence of *H. pylori* to the gastric mucosa and augmented *H. pylori*-induced inflammation *in vivo*, implicating its role in *H. pylori* pathogenesis [8]. This study expands upon this recent discovery by identifying six novel potential DAF binding *H. pylori* proteins, which may function in DAF-mediated adherence to gastric epithelial cells. These studies will ultimately aid in identification of novel antimicrobial therapy targets.

Of the six candidate *H. pylori* proteins identified, it is worth noting the possibility that more than one protein may contribute to DAF binding as demonstrated by the reduction in binding of both *hopQ*- and *flaA*- mutants to DAF-expressing CHO cells. The hallmark of the gastric inflammatory response to *H. pylori* is its capacity to persist for decades, and one strategy *H. pylori* employs to accomplish this involves varying the antigenic repertoire of surface-exposed proteins [9].

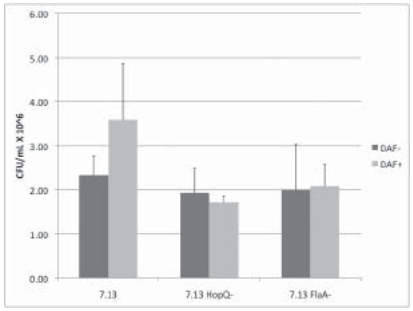

Figure 2. *H. pylori hopQ*- and *flaA*- mutants bind to CHO cells expressing DAF at lower levels than wild-type *H. pylori.* CHO cells transfected with DAF or vector alone were incubated with *H. pylori* strain 7.13, and bacterial adherence was assessed using quantitative culture. Error bars represent standard error. (n=2)

By having the capacity to bind more than one *H. pylori* adhesin, DAF may also contribute to *H. pylori*'s persistence in the gastric mucosa, as DAF binding is not as easily abolished by the deletion of HopQ and FlaA. Following affinity purification of potential DAF-binding proteins, three bands were identified via SDS-PAGE that correspond to proteins capable of binding DAF (Figure 1A). Although the most intense of these bands is likely DAF due to its correspondence in molecular weight to the lane with DAF alone, the other two bands have the potential to be DAF-binding proteins, and this possibility was confirmed by the presence of a doublet in the DAF far Western (Figure 1B). HopQ and FlaA have been shown to be involved in DAF binding (Figure 2); however, other proteins with similar molecular weights may exist in these bands.

Significant variation exists between the potential DAF-binding proteins of *H. pylori* strains 7.13 and J166. These two strains show considerable variation in the number and location of bands in the far Western analysis, particularly in the cytosolic fraction (Fig. 1B). This suggests that perhaps the number and identity of DAF-binding proteins varies depending on the *H. pylori* strain. In this study, the region of the gel containing strain 7.13 *H. pylori* proteins was subjected to mass spectrometry. In the future, the corresponding region of the gel containing strain J166 proteins could also be excised, and the proteins identified could be compared to those of 7.13 to determine whether variation of DAF-binding proteins exists among *H. pylori* strains. This would be particularly interesting as it may shed light onto why certain *H. pylori* strains are more virulent than others.

Extensive research has been conducted on FlaA and FlaB as cell motility proteins, but the roles of FlaA and FlaB in mediating adherence have also been investigated. It has been speculated that the flagellar sheath of *H. pylori* plays a role in promoting adherence to the gastric mucosa, and flagellin has also been proposed as an adhesin in the binding of the bacteria *Campylobacter jejuni* to cultured cells [10]. However, a study conducted by Clyne *et al.* demonstrated that *flaA*$^-$ and *flaB*$^-$ mutants showed no reduction in adherence to primary human or ferret gastric epithelial cells compared to wild-type parental strains [11]. Similarly, *hopQ* has been shown to attenuate rather than mediate *H. pylori* adherence to gastric epithelial cells [12]. *H. pylori* strain 26696 *hopQ* mutants exhibited either increased adherence to human gastric adenocarcinoma (AGS) cells or undetectable differences in adherence. This study provides a different insight into the roles of HopQ and FlaA in mediating adherence of *H. pylori*. In contrast to these studies, our studies have shown that FlaA and HopQ may contribute to DAF-mediated adherence to CHO cells. Differences in the function of FlaA and HopQ in mediating bacterial adherence may largely depend on host cell specificity and/or different *in vitro* cell culture systems. However, these previous studies support our results in that they indicate that FlaA and HopQ are not independently responsible for adherence of *H. pylori* to the gastric mucosa. Because a mutation of only one protein did not completely abolish adherence, these proteins could act in a larger complex or in concert with one another to mediate *H. pylori* adherence.

Two other proteins identified as potential DAF-binding proteins are AlpA and AlpB. These adhesins have been shown to bind host laminin and influence gastric inflammation in gerbils [13]. Abrogation of *H. pylori* AlpA and AlpB has been shown to reduce binding of *H. pylori* to laminin, and the expression of plasmid-borne AlpA or AlpB has been shown to confer laminin-binding ability to *Escherichia coli*. Thus, AlpA, AlpB, and HofC are additional candidate proteins that may mediate DAF binding, and the investigation of the adherence of AlpA, AlpB, and HofC mutants will extend these current studies well into the future.

Studies using a CHO cell culture model have demonstrated that HopQ and FlaA are likely involved in DAF-mediated adherence, suggesting that these may also mediate *H. pylori* adherence to the gastric mucosa. In the future, these results can be confirmed via immunofluorescence microscopy and DAF far Westerns of the mutants. *In vivo* studies with these mutants should be conducted in the future using a murine model of gastric cancer, in which mice overexpress gastrin making them susceptible to gastric carcinogenesis. It has been previously determined that *flaA*$^-$ isogenic mutants cannot colonize the gastric mucosa and therefore cannot be used in this *in vivo* model. However, wild-type INS-GAS and DAF deficient INS-GAS mice have been infected with wild-type *H. pylori* strain 7.13 and the *hopQ*- isogenic mutant to assess the role of DAF and HopQ on *H. pylori*-mediated gastric carcinogenesis. The level of inflammation in these mice will be assessed, which may provide additional insights into the relationship between the expression of HopQ and the induction of inflammation and injury in the gastric mucosa.

DAF may potentially mediate *H. pylori*-induced inflammation in more ways than simply mediating the adherence of *H. pylori* to the gastric mucosa. Additional avenues of research will involve investigating the role DAF plays in mediating cytoskeletal rearrangements and disrupting junctional complexes, techniques that could contribute to increased inflammation in gastric mucosa (Figure S2). The role of DAF in the pathogenesis of gastric cancer remains largely undefined, and further research is necessary to determine how DAF mediates inflammation, which can be a precursor to gastric cancer.

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SUPPORTING INFORMATION.

Supplemental Methods

Figure S1. Preliminary DAF adherence data

Table S1. Candidate *H. pylori* binding proteins

Figure S2. Model of DAF-mediated epithelial cell adherence

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Emily M. Alsentzer is a student at Hume-Fogg Academic Magnet High School and enrolled in the School for Science and Math at Vanderbilt.