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Research Article

RESEARCH ARTICLE

Outer inflammatory protein a (OipA) of *Helicobacter pylori* is regulated by host cell contact and mediates CagA translocation and interleukin-8 response only in the presence of a functional *cag* pathogenicity island type IV secretion system

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One sentence summary: This study demonstrates that expression of OipA of *Helicobacter pylori* is necessary for translocation of the oncoprotein CagA into gastric cells *in vitro*.
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ABSTRACT

OipA is a phase-variable virulence factor of *Helicobacter pylori*. Mutations in *oipA* to turn the gene phase on in a *cag* pathogenicity island (PAI)-negative strain of *H. pylori* (J68) or phase off in a *cag* PAI-positive strain (26695) demonstrated that phase on *oipA* alleles in both strains had both increased *oipA* mRNA and human gastric adenocarcinoma (AGS) cell adherence compared to isogenic *oipA* phase off mutants. An *oipA* phase off mutant of *H. pylori* 26695 demonstrated decreased IL-8 secretion by AGS cells and failure to translocate the *cag* PAI effector CagA. Increased attachment by OipA expressing *cag* PAI-negative *H. pylori* J68 failed to alter secreted IL-8 levels. Thus, OipA is necessary but not sufficient for the induction of IL-8; however, it is necessary for translocation of the oncoprotein CagA. Perhaps the nearly invariant phase on status of *oipA* alleles among *cag* PAI-positive *H. pylori* isolates relates to the role of this outer membrane protein in effective translocation of CagA. *oipA* mRNA comparisons between AGS cell-adherent and non-adherent *H. pylori* 26695 revealed significantly greater levels in the adherent cells. This may allow *H. pylori* to adapt to conditions of host cell contact by altering expression of this virulence factor.

Keywords: phase variation; adhesin; CagA translocation; interleukin-8

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INTRODUCTION

Helicobacter pylori resides in the human stomach and is associated with chronic gastritis, gastric ulcers, duodenal ulcers and gastric cancer. The global prevalence of *H. pylori* infection is ∼50% of the population. Infection with strains of *H. pylori* containing the crucial virulence locus, the *cag* pathogenicity island (*cag* PAI), and thus the oncoprotein cytotoxin-associated antigen A (CagA) is strongly associated with more severe clinical outcomes (Hatakeyama 2004).

The *cag* PAI of *H. pylori* is a 40-kb chromosomal region, identifiable as a horizontally acquired DNA element by its lower GC content (Terry *et al.* 2005). The *cag* PAI encodes a type IV secretion system (T4SS), and its presence in the *H. pylori* genome is positively associated with the presence of highly active alleles of the secreted vacuolating cytotoxin (VacA) (Tegtmeyer, Wessler and Backert 2011). VacA enhances colonization and pathogenesis in host cells by several experimentally documented activities (Foegeding *et al.* 2016). There are >30 open reading frames in the *cag* PAI with *cagA* located at one end of the island. CagA is translocated into host epithelial cells via the T4SS upon attachment (Noto and Peek 2012). Such secretion systems involve conjugative structures to transport proteins through a complex channel structure directly through the membrane into the cytoplasm of the host cell (Backert and Tegtmeyer 2017). Once inside the cell, CagA is frequently tyrosine phosphorylated at EPIYA domains by a host kinase, and proceeds to interact with systems leading to host cell junction damage, cytoskeletal changes and cell proliferation although the non-phosphorylated form of CagA has specific cellular effects as well (Hatakeyama 2008; Backert, Tegtmeyer and Fischer 2015).

In a separate sequence of events, non-translocated proteins of the T4SS encoded by the *cag* PAI are involved in inducing proinflammatory cytokine production by host cells, including interleukin 8 (IL-8) (Fischer *et al.* 2002). Secretion of IL-8 recruits an innate immune system response, resulting in inflammation of the gastric mucosa. The *cag* PAI is found disproportionately in *H. pylori* isolates from patients with chronic active gastritis, peptic ulcer disease and gastric cancer, indicating that it is an important virulence factor (Cover 2016). Thus, *cag* PAI-positive strains are innately more virulent, while *cag* PAI-negative strains lacking this PAI are much less virulent.

Outer membrane inflammatory protein A, or OipA, is an outer membrane protein unique to *H. pylori*. Approximately 4% of the *H. pylori* genome encodes a large set of outer membrane proteins, many with unique functions including adhesion and pH regulation (Oleastro and Ménard 2013). OipA, encoded by the gene *oipA* (previously *hopH—HP0638*), was originally named for its role in inducing inflammation in the host, as evidenced by high mucosal IL-8 levels; however, this finding is controversial as results from various studies indicate differing effects of OipA on inflammation (Dossumbekova *et al.* 2006; Odenbreit *et al.* 2009; Matsuo, Kido and Yamaoka 2017). Additionally, the particular means by which OipA might induce inflammation are unclear as the effects can often be attributed to *cag* PAI-mediated pathways.

In a more widely agreed upon manner, OipA has been shown to play a role in adherence to host cells and thus host colonization (Dossumbekova *et al.* 2006). OipA is a member of the Hop (Helicobacter Outer Proteins) outer membrane family of proteins, several of which promote binding to the gastric epithelium (Oleastro and Ménard 2013). The gastric epithelial cell receptor for OipA is unknown, but has been hypothesized to be in the integrin family (Posselt, Backert and Wessler 2013).

OipA expression is regulated by slipped-strand mispairing (SSM) within a hypermutable CT dinucleotide repeat motif located in the 5′ region of the gene (Miftahussurur and Yamaoka 2015). SSM is a mutagenic process that occurs during DNA replication of repetitive sequences (Torres-Cruz and van der Woude 2003). The poly CT repeat tract in *oipA* is just one of numerous occurrences of this repeat *in H. pylori*. Addition/deletion of CT repeats in *oipA* results in a frame shift that drives phase variation in protein expression. While there is great variation in the number of CTs in the dinucleotide repeat of *oipA* between *H. pylori* strains (Zhang *et al.* 2014), SSM within *oipA* during *in vitro* growth appears to be less frequent than quantified in *sabA*, another outer membrane protein gene possessing a 5' poly CT tract than during similar *in vitro* growth (Yamaoka *et al.* 2006; Goodwin *et al.* 2008).

In a study of 410 *H. pylori* patient isolates, this CT sequence ranged from 3 to 12 repeats, with short repeats predominating in East Asia, where gastric cancer caused by *H. pylori* is much more prevalent compared to strains from Western countries (Yamaoka *et al.* 2002). Studies show that a functional 'phase on' status of *oipA* is associated with increased risk for peptic ulcer disease and gastric cancer. The strong correlation of functional OipA and the virulence of the bacterium has made OipA a candidate for potential vaccines against *H. pylori* (Chen *et al.* 2012). Additionally, functional OipA is associated with high *H. pylori* density in infected stomachs and severe neutrophil infiltration (Liu *et al.* 2013). However, the positive association of *oipA* phase on status with the presence of the *cag* PAI and *vacA* s1m1 alleles makes the role of OipA with these disease states difficult to dissect.

OipA and the *cag* PAI are two important *H. pylori* virulence factors, and there is great interest in the relationship they have to one another. Ando *et al.* (2002) demonstrated that while the vast majority (>96%) of *cag* PAI-positive isolates contained *oipA* phase on alleles, none of the *cag* PAI-negative isolates in the study possessed phase on *oipA*, yet the gene was uniformly present and highly conserved. While both OipA and the *cag* PAI are thought to be involved in the secretion of cytokines such as IL-8 by host epithelial cells, most likely via a mechanism involving transcription factor NF-κB, how they work together (or if they work together) is largely unknown (Matsuo, Kido and Yamaoka 2017). This study aims in part to determine the relationship between *oipA* and the *cag* PAI, and to shed light on why *oipA* is so highly conserved in *cag* PAI-negative strains in which the protein is apparently seldom, if ever, expressed. We additionally sought to determine what pressure maintains *oipA* as a phase on allele in the presence of the *cag* PAI. Here, we show that OipA is necessary, but not sufficient for IL-8 secretion *in vitro*; however, it is necessary for translocation of CagA from *H. pylori* 26695 to human gastric adenocarcinoma (AGS) cells. We also demonstrate that *oipA* transcript levels are higher in cells in contact with gastric epithelial cells *in vitro*.

MATERIALS AND METHODS

Helicobacter pylori culture

Helicobacter pylori strains were cultured on tryptic soy agar II with 5% sheep's blood (BBL) for 24–48 h at 37°C in an ambient air/5% CO_2 atmosphere. Liquid cultures of *H. pylori* were grown in Sulfite-Free Brucella Broth (SFBB) supplemented with 1X cholesterol (Gibco Life Technologies), 20 μg vancomycin/mL, shaking at 150 rpm.

Table 1. Plasmids used for creation of *H. pylori* mutants.

Name	Description
p*rdxA*	TOPO TA cloning vector pCR4 containing a1556 bp amplicon of the *H. pylori* strain 26695 *rdxA* gene
pΔ*rdxA*	p*rdxA* with a 390-bp deletion in the *rdxA* gene
pOipA (26695 and J68)	TOPO TA cloning vector pCR4 containing a 2300-bp amplicon including the entire *oipA* gene, as well as the untranslated regions both upstream and downstream
pOipA.*Bam*HI (26695 and J68)	pOipA containing a *Bam*HI site in the cloned allele of *oipA*
pMM672	*Helicobacter pylori* 26695 plasmid in which the coding region of *rdxA* is deleted (Loh *et al.* 2011)
pOipA::CAT-*rdxA* (26 695 and J68)	pOipA.*Bam*HI with CAT-*rdxA* cassette cloned into the *Bam*HI restriction site
p26695.*oipA*OFF	pOipA 26695 with CT repeat tract consisting of 5 CT repeats
pJ68.*oipA*ON	pOipA J68 with CT repeat tract consisting of 11 CT repeats
pJ68*oipA*ON.FLAG	pJ68.*oipA*ON containing a FLAG tag
pJ68*oipA*OFF.FLAG	pOipA J68 containing a FLAG tag
pICB:CAT	Plasmid containing the *cag*PAI, including *cagE* with CAT inserted (Harvey *et al.* 2014)

Table 2. *Helicobacter pylori* mutant strains.

Name	Description
26695 wild type	Mtz^S and Cm^S, contains *oipA* with a CT repeat tract consisting of 6 CT repeats (phase on)
J68 wild type	Mtz^S and Cm^S, contains *oipA* with a CT repeat tract consisting of 10 CT repeats (phase off)
26695 *rdxA*$^-$ & J68 *rdxA*$^-$	26695 and J68 WT containing a 390-bp deletion in the *rdxA* gene. Mtz^R and Cm^S
26695 *oipA*$^-$ and J68 *oipA*$^-$	26695 *rdxA*$^-$ and J68 *rdxA*$^-$ containing the *oipA*::CAT-*rdxA* cassette. Mtz^S and Cm^R
26695 *oipA* phase Off	26695 *rdxA*$^-$ containing mutant *oipA* with an altered CT repeat tract consisting of 5 CT repeats (phase off). Mtz^R and Cm^S
J68 *oipA* phase on	J68 *rdxA*$^-$ containing mutant *oipA* with an altered CT repeat tract consisting of 11 CT repeats (phase on). Mtz^R and Cm^S
J68 *oipA* phase on-FLAG	J68 *rdxA*$^-$/*oipA* phase on::FLAG. Mtz^R and Cm^S
J68 *oipA* phase off-FLAG	J68 *rdxA*$^-$/*oipA* phase off::FLAG. Mtz^R and Cm^S
26695 *cagE*$^-$	26695 *rdxA*$^-$ containing *cagE*::CAT. Mtz^R and Cm^R
26695 *oipA* phase off-*cagE*$^-$	26695 *rdxA*$^-$/*oipA* phase off containing *cagE*::CAT. Mtz^R and Cm^R

Nucleotide sequence accession

The nucleotide sequence determined for *oipA* of *H. pylori* strain J68 has been deposited in GenBank and has the accession number BankIt2035716 Helicobacter MF576477.

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) was employed to determine natural variation in the *oipA* CT dinucleotide repeat tract of wild-type *H. pylori strains* grown *in vitro* as described in Goodwin *et al.* (2008) and Harvey *et al.* (2014). AFLP primers were designed to amplify ~270 bp beginning upstream of *oipA* and including the CT dinucleotide repeat tract from both strains J68 and 26695 (Table S1, Supporting Information). Separate primers were designed to amplify a 294-bp control region of *oipA* from a non-repeat bearing region as a control. In each case, reverse primers contained fluorescent VIC tags on the 5′ end (Applied Biosystems, Foster City, CA, USA)

rdxA mutant of *cag* PAI-negative *Helicobacter pylori* strain J68 for counterselection of *oipA* mutants

oipA mutant strains of *H. pylori* were generated using an antibiotic counterselection method designed at Vanderbilt University Medical Center (Loh *et al.* 2011) to create markerless mutations in the *oipA* locus of *H. pylori*. We used a metronidazole-resistant (Mtz^R) strain of the *cag* PAI-positive strain of *H. pylori* 26695 (a gift of Drs Mark McClain and Timothy Cover) designated 26695 *rdxA*$^-$. We created an analogous mutant of the *cag* PAI-negative strain J68 by deleting an internal portion of *rdxA*, thereby generating an *H. pylori* J68 Mtz^R mutant. This was accomplished by amplifying a 1556-bp amplicon containing the full-length *rdxA* gene (HP0954) using the HP0955 Fwd and HP0953 Rev primers (Table S1). The amplicon was cloned into pCR4 (Invitrogen) according to the manufacturer's protocol. The resultant plasmid p*rdxA* (Table 1) was modified by inverse PCR to delete a 390-bp portion of *rdxA* using the inverse PCR *rdxA* Fwd and Rev primers (Table S1) with 5′ phosphorylation to aid in ligation. The amplicon was purified using PCR Purification kit (IBI) and digested with *Dpn*I restriction endonuclease to destroy the p*rdxA* template, and then ligated using T4 DNA ligase (Quick Ligation Kit—New England Biolabs) and transformed into *Escherichia coli* DH5α. The confirmed plasmid construct was designated pΔ*rdxA* (Table 1) and used in natural transformation of the *cag* PAI-negative, vacA s2/m2 strain, J68 (a gift from Dr Richard Peek Jr., Vanderbilt University Medical Center). Mtz^R colonies capable of growing on 5 μg metronidazole/mL SFBB 10% Newborn Calf Serum plates were selected, and the mutation was confirmed by PCR and DNA sequencing and the strain named J68 *rdxA*$^-$ (Table 2).

Cloning of *rdxA* complement/chloramphenicol-resistant plasmids

Subsequent to the creation of these Mtz^R *H. pylori* clones, a chloramphenicol (Cm) resistance gene (CAT or chloramphenicol acetyl transferase) together with an intact version of *rdxA* was

inserted within the *oipA* gene of *H. pylori* strains 26695 *rdxA*$^-$ and J68 *rdxA*$^-$. To accomplish this, the entire *oipA* gene from both strains 26695 and J68, including regions both upstream and downstream, was amplified using the *oipA* universal Fwd and Rev primers designed based on a consensus sequence built from several *H. pylori* sequenced genomes (Table S1). The resultant ~2300 bp amplicons were cloned into pCR4 (Invitrogen), and the plasmids were designated pOipA 26695 and pOipA J68 (Table 1). GeneArt Site-Directed Mutagenesis System (Invitrogen) was utilized according to the manufacturer's protocol to insert a *Bam*HI site into the cloned allele of *oipA* using the *oipA Bam*HI Fwd and Rev mutagenic oligos (Table S1). Resulting plasmids, pOipA.*Bam*HI 26695 and pOipA.*Bam*HI J68 (Table 1), were digested with *Bam*HI for cloning of the selectable markers CAT and *rdxA*. This two-gene cassette was purified as a *Bam*HI fragment from pMM674 (Loh *et al*. 2011), a gift of Dr Mark McClain and Dr Timothy Cover of Vanderbilt University Medical Center, and cloned into pOipA.*Bam*HI, resulting in pOipA::CAT-*rdxA* 26695 and pOipA::CAT-*rdxA* J68 (Table 1). These plasmids were naturally transformed into *H. pylori* 26695 *rdxA*$^-$ or *H. pylori* J68 *rdxA*$^-$ with selection using 10 μg chloramphenicol/mL. Resultant strains were confirmed via PCR of the *oipA* locus and sequencing, and designated 26695 *oipA*$^-$ and J68 *oipA*$^-$. These serve as both intermediates in the isolation of markerless mutants and *oipA* null mutants, and are each Mtz^S and Cm^R.

Cloning of *oipA* mutant plasmids

oipA mutagenic oligos were designed specific to each strain to alter the number of CT dinucleotide repeats in the 5′ region of *oipA* in order to turn the gene phase off in the *cag* PAI-positive *H. pylori* strain 26695, and phase on in the *cag* PAI-negative *H. pylori* strain J68. This was accomplished by deleting one CT in strain 26695 to go from 6 CT repeats to 5, resulting in a frame shift in *oipA* from phase on to phase off and deleting one CT in the J68 *oipA* allele from 9 to 8 dinucleotide repeats (*oipA* phase off to phase on). Mutagenesis was performed using the 26695.*oipA*OFF and J68.*oipA*ON mutagenic primers (Table S1), respectively, and clones were screened via sequencing. Mutant bearing plasmids were named p26695.*oipA*OFF and pJ68.*oipA*ON (Table 1).

Confirmed plasmids were used in natural transformations and allelic exchange with the appropriate *H. pylori oipA*$^-$ strains to replace the *oipA*::CAT-*rdxA* locus on the recipient chromosome. Such allelic replacement mutants were selected for with SFBB plates containing 5 μg metronidazole/mL. These strains, 26695 *oipA* phase off and J68 *oipA* phase on (Table 2), are Mtz^R/Cm^S, and possess a mutated *oipA* gene with no antibiotic resistance genes left behind in the locus.

FLAG epitope-tagged *oipA* mutants

To determine OipA protein expression in the mutant *H pylori* strains via western blotting, we designed mutagenic primers containing the epitope tag FLAG (YKDDDKD) to insert this seven amino acid epitope encoding sequence into the gene *oipA* between codons 161 and 162 (Table S1). These primers were used in site-directed mutagenesis with pJ68.*oipA*ON and pJ68.*oipA* (containing the wild-type phase off *oipA* allele). Successful plasmids were confirmed via sequencing and named with the appropriate strain and phase of *oipA*, e.g. pJ68*oipA*OFF.FLAG (Table 1). Plasmids were naturally transformed into the appropriate *H. pylori* strain *oipA*$^-$, selected for Mtz^R Cm^S phenotype and confirmed by PCR and sequencing. These strains, J68 *oipA* phase on-FLAG and J68 *oipA* phase off-FLAG (Table 2), were used in western blotting using ANTI-FLAG antibodies (Sigma-Aldrich).

Western blotting

Mutant and control strains of *H. pylori* were grown 24–36 h on blood agar plates and 0.4 OD_{600} units of cells were denatured and reduced using Lamelli buffer (Bio-Rad) with 2-mercaptoethanol, and total proteins were separated by SDS-PAGE. Proteins were transferred from the gel to nitrocellulose blotting membranes (Bio-Rad). OipA expression was detected using monoclonal anti-FLAG M2 antibody (Sigma-Aldrich), followed by goat anti-mouse IgG-Peroxidase. Chemiluminescence was accomplished using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized with X-ray film.

cagE null mutants

To render the *cag* PAI T4SS non-functional in the *H. pylori* strain 26695, we created *cagE* null mutants in both *oipA* phase on and phase off mutants. This was accomplished using pICB::CAT (Tummuru, Sharma and Blaser 1995), a plasmid possessing a chloramphenicol resistance gene in the gene *cag*E, previously named *picB*. This plasmid was naturally transformed into both *H. pylori* 26695 *rdxA*$^-$ with *oipA* unaltered and thus phase on, and *H. pylori* 26695 *oipA* phase off. Clones with the mutation were selected for using SFBB plates with 10 μg Cm/mL, and the CAT insertion within *cagE* was confirmed via PCR and DNA sequencing. Strains were designated *H. pylori* 26695 *cagE*$^-$ and *H. pylori* 26695 *oipA* off-*cagE*$^-$ (Table 2).

RNA extraction, cDNA synthesis and real-time quantitative PCR

Helicobacter pylori cells were inoculated in SFBB/10 μg vancomycin/mL containing 1X Cholesterol (Gibco/BRL) at ~0.2 OD_{600} and incubated shaking at 150 rpm for 12–24 h in a 5% CO_2/ambient air mixture. A total of $\times 10^8$ cells were collected during exponential phase of growth (OD_{600} between 0.7 and 1.2) and suspended in 1 mL of RNAzol RT (Molecular Research Center, Inc.). Total RNA was extracted from each pellet according to the manufacturer's protocol. RNA samples were then used in cDNA synthesis. One microgram of purified RNA was combined with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad), and cDNA was synthesized using the manufacturer's cDNA synthesis protocol. cDNA was used for RT-qPCR.

The relative quantity of *oipA* mRNA was determined as in Acio-Pizzarello *et al*. (2017) using a TaqMan Gene Expression assay (Life Technologies) performed on the Applied Biosystems StepOne apparatus. The assays were performed in technical triplicate for each gene and each strain according to the manufacturer's protocol using custom TaqMan Custom Gene Expression assays (Thermo Fisher), including the *oipA*.Taq, *ftsZ*.Taq and *gyrB*.Taq probes (Table S1). *ftsZ* was used as the normalizer. Relative expression of genes among the various mutants was calculated using the $2^{\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) and processed using the DataAssist software (Applied Biosystems).

AGS cell culture

AGS cells were a gift from Timothy Cover of Vanderbilt University Medical Center. Cells were grown in RPMI 1640 medium (Life Technologies), supplemented with 10 mM HEPES 10% NCS

and penicillin/streptomycin (Gibco/BRL). Cultures were grown at 37°C in an ambient air/5% CO_2 atmosphere in either six-well tissue culture treated plates (CytoOne) or T-75 tissue culture flasks (Thermo Fisher).

Adhesion assay and IL-8 ELISA

A total of 2.5 × 10^5 AGS cells were seeded in each well of a six-well plate for 24 h. Cells were washed three times with antibiotic-free RPMI 1640. *Helicobacter pylori*, suspended in this same medium, was introduced to AGS cells at a multiplicity of infection of 100:1 and co-cultured for 5 h. At the completion of adhesion assays, medium was collected for subsequent IL-8 ELISAs. Following three washes, AGS cells were lysed using 1 mL of PBS/0.1% saponin for 15 min with shaking at 50 rpm. Lysates were serially diluted and spotted onto blood agar plates, and titers were calculated at 5 days. Media from *H. pylori*-infected AGS cells were centrifuged at 3300 × *g*, and supernatant was assayed for IL-8 concentration. A 96-well plate (BioLegend) was coated with IL-8 capture antibody (BioLegend) overnight at 37°C. ELISA was performed according to the manufacturer's suggested protocol. Plates were read at 450 and 570 nm using a Bio-Rad iMark Microplate Reader.

CagA translocation assay

AGS cells were seeded in six-well plates as described above and incubated until ∼80% confluence. *Helicobacter pylori* strains to be assayed for CagA translocation into AGS cells were added at an MOI of 100 and infection allowed to proceed for 14 h. Non-adherent *H. pylori* cells were removed by aspiration and AGS monolayers were washed three times with PBS and then lysed using RIPA Buffer (25 mM Tris-HCl [7.5], 150 mM NaCl, 5mM EDTA, 1% Triton X-100, 0.1% SDS) with 2 mM Na_3VO_4 and Protease Inhibitor Cocktail (Thermo). Twenty micrograms of soluble protein was resolved on 7% TGX polyacrylamide gels (Bio-Rad) and western blotted to nitrocellulose. Translocated and tyrosine phosphorylated CagA was visualized using a rabbit monoclonal antibody to phospho-Tyrosine (Abcam), while total CagA was detected in identical blots, run simultaneously with identical protein loads, using a mouse monoclonal anti-CagA (Sigma-Aldrich). Detection utilized horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse IgG (Abnova) and SuperSignal West Pico Chemiluminescent Substrate (Thermo). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected as a protein loading control using a mouse monoclonal anti-GAPDH (Sigma Aldrich).

Statistical analysis

A Welch's unpaired *t*-test of unequal variance was used to determine statistically significant differences in gene transcription, adhesion and immunoassay studies. These statistics were calculated with α-level = 0.05.

RESULTS

Phase variation in the CT repeat tract of *oipA* is rare *in vitro*

Outer membrane inflammatory protein (OipA) is regulated via SSM in a CT dinucleotide repeat tract in the 5′ end of the gene and results in phase on status in most *cag* PAI-positive strains of *Helicobacter pylori*, and phase off in *cag* PAI-negative strains, nearly without exception (Ando *et al.* 2002). Typically, longer repeat sequences are more likely to vary in length during SSM that occurs during replication (Harvey *et al.* 2014). In order to quantify the natural variation in this CT repeat length when strains of *H. pylori* are grown *in vitro*, we employed AFLP analysis.

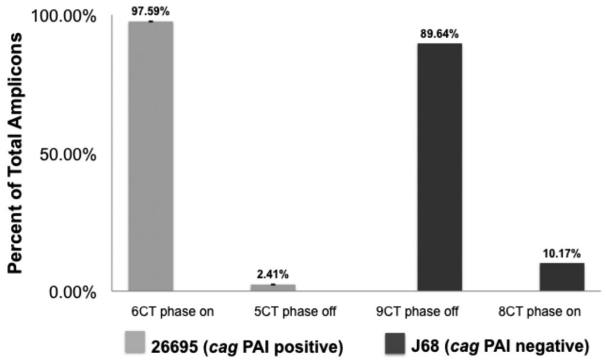

Figure 1. AFLP analyses demonstrates that phase variation in *oipA in vitro* is infrequent. Fluorescently labeled amplicons containing the poly CT repeat tract of *oipA* in both *cag* positive (26695) and *cag* PAI negative (J68) strains of *H. pylori* demonstrate relatively minor levels of variation in length, indicating that the expression phase status of *oipA* is quite stable when *H. pylori* is grown *in vitro*. Bars represent the mean ± standard deviation calculated using three biological replicates. Amplification of a similar sized amplicon from a non-repeat bearing region of *oipA*, as a PCR control, revealed no fragment polymorphisms (data not shown).

Our results suggest that despite the ability of SSM in the CT tract to generate variability, there was little variation in the number of CT repeats present within *in vitro* grown wild-type *H. pylori* strains 26695 and J68 (Fig. 1). In the *cag* PAI-positive strain 26695, >97% of the amplicons generated from the poly CT region contained six CTs, consistent with a phase on *oipA* allele. In the *cag* PAI-negative *H. pylori* strain J68, while the majority of the amplicons generated from this same region possess nine CTs, indicating a phase off *oipA* allele, there were slightly more variants (∼10% amplicons with eight CTs, suggesting a phase on *oipA* allele) among the amplicons (Fig. 1). Overall, this apparent paucity of *in vitro* variation in the poly CT tract suggests that the relationship between the presence/absence of the *cag* PAI and the expression phase of *oipA* tends to be maintained, despite the repetitive sequence in the 5′ region of *oipA*.

Experimental alterations to the CT tract result in changes in *oipA* expression

To quantify the effect of CT tract changes on *oipA* expression, RT-qPCR was used to quantify transcript levels, as phase status is known to affect mRNA levels (Acio-Pizzarello *et al.* 2017). The deletion of an internal portion of *rdxA* in order to create a metronidazole-resistant control within wild type, phase on *oipA* in strain 26695 (Loh *et al.* 2011) did not produce a significant difference in *oipA* transcript levels compared to the wild-type *H. pylori* strain, indicating that it is a reliable control (Fig. 2). Altering the number of CTs in *oipA* in strain 26695 such that the gene is phase off decreases the transcript levels of the gene by more than 80% compared to the isogenic phase on control. Additionally, the relative quantity of *oipA* transcript when the gene is phase off is not significantly different from the *oipA*$^-$ null mutant (Fig. 2).

Similarly, there was no significant difference in *oipA* mRNA levels between the *H. pylori cag* PAI-negative J68 *rdxA*$^-$ control and the wild-type strain J68 (*oipA* phase off), and the J68 *oipA*$^-$ null mutant (Fig. 2). However, when the J68 *oipA* CT dinucleotide

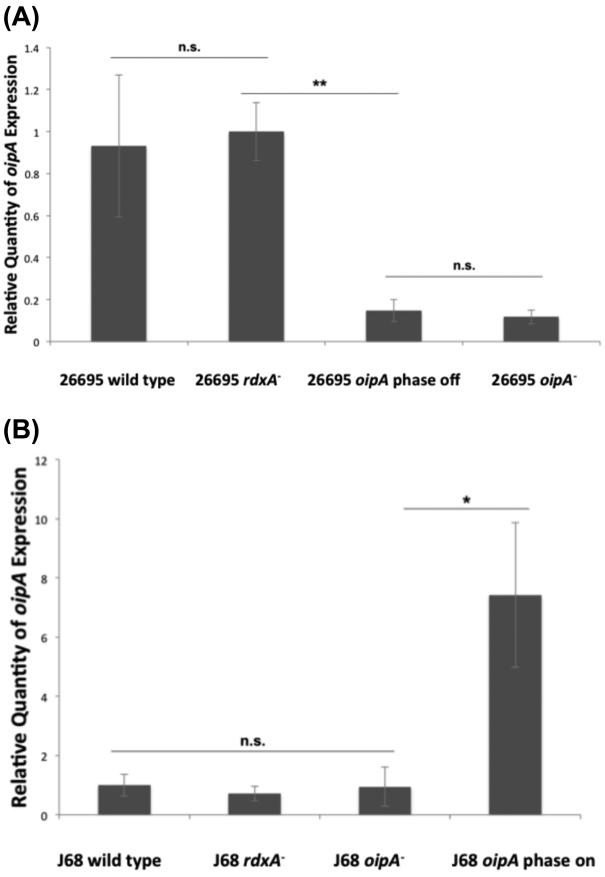

Figure 2. Phase off status of *oipA* in both *cag* PAI-positive and negative strains of *H. pylori* causes a significant decrease in the relative quantity of transcripts expressed compared to phase on status. RT-qPCR was used to determine the relative expression of *oipA* in the *cag* PAI-positive *H. pylori* 26695 wild-type and the isogenic *rdxA*$^-$ control strain, possessing *oipA* phase on, as well as to 26695 *oipA* phase off and *oipA* null mutants **(A)**. Additionally, wild-type *cag* PAI-negative *H. pylori* strain J68 and isogenic *rdxA*$^-$ control, as well as to the J68 *oipA* phase on and J68 *oipA* null mutants **(B)**. The data shown here are representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance with 26695 *rdxA*$^-$ (*oipA* phase on) as the control ($^{**} = P \leq 0.01$, $^{*} = P \leq 0.05$, n.s. $= P > 0.05$).

repeat tract was mutated such that *oipA* was in frame and thus phase on, there was a significant increase in *oipA* transcript levels, further indicating that *oipA* mRNA levels are tied to its expression phase status.

Phase on *oipA* confers an increased host adherence phenotype

To demonstrate the increased *oipA* mRNA levels documented in the phase on *H. pylori* J68 mutant resulted in OipA protein expression, a 21-nucleotide sequence encoding the FLAG epitope was inserted into the *oipA* allele of *H. pylori* strain J68, and a monoclonal antibody to this epitope was used to detect OipA protein. In this *cag* PAI-negative and naturally *oipA* phase off *H. pylori* strain, when *oipA* was experimentally turned phase on, there was a novel ~34 kDa protein detected (Fig. 3), indicating a successful switch from phase off to on results in the novel expression of OipA.

We next investigated whether the demonstrated decrease in *oipA* transcript level in the *cag* PAI-positive strain 26695 (Fig. 2)

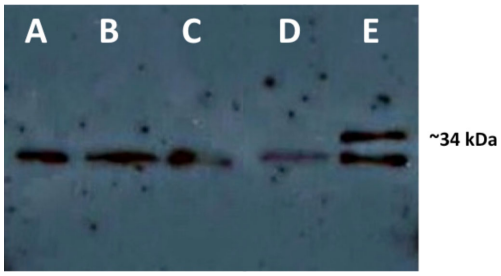

Figure 3. Phase on status of *oipA* in the *cag* PAI-negative *H. pylori* strain J68 results in expression of the protein OipA. An anti-FLAG western blot was performed using 0.4 OD_{600} units of **(A)** wild-type *H. pylori* J68 *oipA* phase off control, **(B)**: *H. pylori* J68 *oipA* phase on mutant, **(C)**: *H. pylori* J68 *oipA* phase off FLAG, **(D)**: *H. pylori* J68 *oipA* phase on FLAG. This revealed the ~34 kDa FLAG epitope tagged OipA only when *oipA* is phase on. An ~30 kDa cross-reactive protein in *H. pylori* J68 appeared in all strains.

and OipA expression in the *cag* PAI-negative, *oipA* phase on mutant strain J68 would alter *H. pylori* adherence to gastric epithelial cells. When *oipA* was turned phase off in strain 26695, *H. pylori* attachment levels decreased by ~80% (Fig. 4). *oipA* phase was also strongly correlated to *H. pylori* attachment capability in *H. pylori* strain J68, as attachment levels more than doubled when *oipA* was turned phase on. These results indicate that OipA has the ability to mediate attachment to host cells independent of the *cag* PAI and demonstrate that the *cag* PAI-negative strain J68 *oipA* allele is functional when phase on.

OipA effects on IL-8 production and CagA translocation

Due to the differences in attachment ability observed between *H. pylori* strains with *oipA* phase on and phase off, and the theorized involvement of OipA in the host inflammatory response, we hypothesized that the altered attachment phenotype might be associated with altered epithelial cell IL-8 production. To quantify alterations in IL-8 concentration, AGS cell attachment assay culture medium was used in ELISAs. AGS cells infected with the *cag* PAI-positive strain 26695 with *oipA* experimentally mutated to phase off produced ~80% less IL-8 than those infected by isogenic *H. pylori* with *oipA* phase on (Fig. 5). This is nearly the same reduction in IL-8 secretion from AGS cells infected with a *cagE* null mutant that lacks a functional *cag* PAI T4SS. There was no significant difference in the amount of IL-8 produced by AGS cells infected with *H. pylori* strain 26695 possessing an *oipA* null mutation relative to the isogenic *oipA* phase off mutant, and both of these IL-8 concentrations were comparable to the amount of IL-8 produced by uninfected AGS cells (Fig. 5).

In contrast, in *H. pylori* strain J68 there was no significant difference in IL-8 production by AGS cells, whether infected with *oipA* phase on, *oipA* phase off, *oipA* null mutant strains or left uninfected (Fig. 5). This stark contrast between *cag* PAI-positive and *cag* PAI-negative *H. pylori* strains indicates that while OipA has the ability to mediate IL-8 production in the presence of the T4SS encoded by the *cag* PAI, OipA cannot itself alone mediate the host inflammatory response in the absence of the *cag* PAI. Thus, OipA appears necessary but not sufficient for inducing IL-8 secretion by AGS cells.

Because the *cag* PAI-positive *H. pylori* strain 26695 was greatly altered in the ability to induce the secretion of IL-8 by AGS cells depending on the expression phase of OipA, we next asked how *oipA* phase variation affected the translocation of the *cag* PAI effector protein CagA into AGS cells. We visualized translocated,

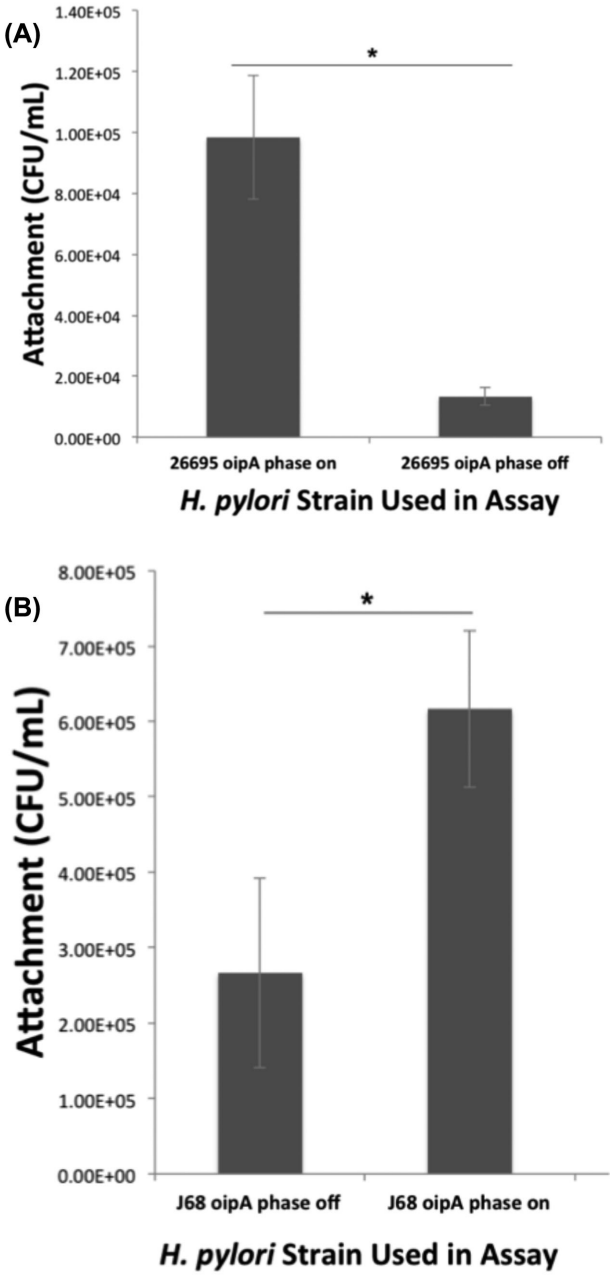

Figure 4. *Helicobacter pylori* exhibits increased adherence ability when *oipA* is phase on in both *cag* PAI-positive or negative strains. Attachment assays were performed to determine whether *oipA* phase was important in the ability of *H. pylori* to adhere to AGS cells, particularly in the *cag* PAI-negative strain, J68, in which OipA is not expressed. Panel **A** shows the *cag* PAI-positive strain 26695, with *oipA* in alternate expression phases and panel **B** shows *H. pylori* J68 with *oipA* in each expression phase. The data shown here are representative of results obtained in independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance ($^* = P \leq 0.05$).

tyrosine phosphorylated CagA in western blots of 14-h *H. pylori*-infected AGS cells (Fig. 6). While *oipA* phase on *H. pylori* strain 26695 was capable of translocating CagA into the AGS cells, the isogenic *oipA* phase off strain could not. This indicates a role for OipA in the translocation of this oncoprotein from *H. pylori* to AGS cells *in vitro*.

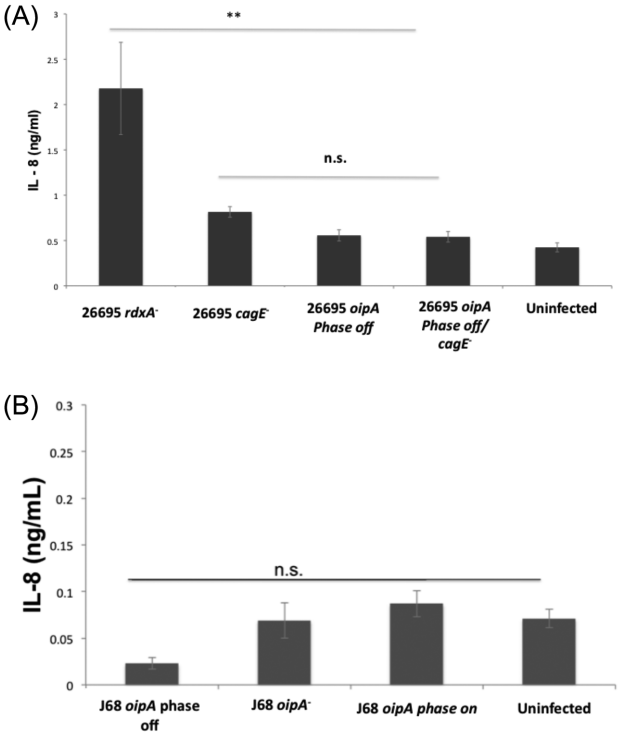

Figure 5. Phase on status of *oipA* in *H. pylori* mediates gastric epithelial cell IL-8 production only in the presence of the *cag* PAI. Cell medium from AGS cells infected with *H. pylori* 26695 *oipA* phase on (26695 *rdxA*$^-$), *oipA* phase on/*cagE* null mutant (26695 *cagE*$^-$), *oipA* phase off (26695 *oipA* phase off), *oipA* phase off/*cagE* null mutant (26695 *oipA* phase off/*cagE*$^-$) as well as from uninfected AGS cells was collected after a 5-h infection and used in ELISA to quantify AGS cell IL-8 production. This is a representative of three independent experiments **(A)**. The same assay was performed for the *cag* PAI-negative *H. pylori* strain J68 using *oipA* phase off (J68 *rdxA*$^-$), *oipA* null mutant (J68 *oipA*$^-$) and *oipA* phase on (J68 *oipA* phase on). Medium form uninfected cells after a 5-h incubation is also shown. The data shown here are representative of the results obtained in three independent experiments, each conducted in technical triplicate **(B)**. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance ($^{**} = P \leq 0.01$, n.s. $= P > 0.05$).

oipA mRNA transcript levels are increased in AGS cell-adherent *Helicobacter pylori* compared to non-adherent

We next asked if there was a difference in the amount of *oipA* mRNA transcripts in *H. pylori* strain 26695 cells attached to AGS cells versus those that were also incubated with host cells, but remained unattached. In order to determine this, attachment assays were performed as before; however, after the 5-h incubation, infected AGS cell culture supernatants were collected and the non-adherent *H. pylori* population was harvested, as were adherent *H. pylori* after saponin-mediated lysis of infected AGS cells. RT-qPCR was used to quantify relative *oipA* transcript levels in both populations of *H. pylori*. Due to the variation in *H. pylori* cell numbers in these two populations and subsequently mRNA and cDNA amounts compared to traditional mRNA extraction preparations, a housekeeping gene was used in RT-qPCR experiments. *gyrB* was employed as a comparison to *oipA*.

Our results indicate that strain 26695 *oipA* phase on *H. pylori* cells that were attached to AGS cells after the 5-h infection had seven times the amount of *oipA* transcript compared to non-adherent cells, while housekeeping gene *gyrB* transcript levels did not vary (Fig. 7). Independent experiments using the *H. pylori*

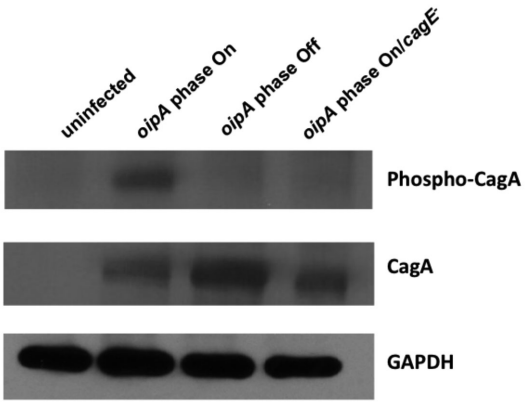

Figure 6. OipA is Involved in CagA Translocation from *H. pylori* 26695 into AGS cells. AGS cells were infected 14 h with *H. pylori* 26695 *rdxA*⁻ (*oipA* phase on), *H. pylori* 26695/*rdxA*⁻/*oipA* phase off, *H. pylori* 26695/*rdxA*⁻/*oipA* phase on/*cagE*⁻ or left uninfected. Adherent *H. pylori* and AGS cells were subjected to western blotting using either anti phosphotyrosine to visualize translocated CagA in its tyrosine phosphorylated form, anti-CagA, or anti glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. CagA protein is translocated and tyrosine phosphorylated only in the presence of phase on *oipA* allele and a functional T4SS. Changing the expression phase of *oipA* to phase off or ablating the *cagE* gene of the T4SS prevented the translocation of this effector protein. These results are representative of the results obtained in two independent experiments.

26695 *oipA* phase off mutant also showed significantly increased *oipA* mRNA levels, relative to the non-adherent population. Genomic DNA preparations from adherent and non-adherent *H. pylori* cells from identical experiments were isolated and used in AFLP analyses described above. There was no detectable length polymorphism in the amplicons generated from these two populations (data not shown), indicating that neither was the non-adherent population of *H. pylori* was enriched for naturally occurring phase off *oipA* allele bearing cells nor were the adherent bacteria enriched for cells bearing phase on *oipA* alleles.

DISCUSSION

OipA and the *cag* PAI are two well-documented *Helicobacter pylori* virulence factors. The *cag* PAI encodes a T4SS that induces the production of proinflammatory cytokines by host gastric epithelial cells (Khatoon *et al.* 2017). OipA acts as an adhesin; however, the role of OipA in eliciting a proinflammatory response, such as IL-8 secretion, is a subject of controversy. Some studies have claimed that while OipA is an adhesin, it has no effect on IL-8 secretion by host cells (Fischer *et al.* 2002; Odenbreit *et al.* 2002; Dossumbekova *et al.* 2006). Studies have shown that the majority of *cag* PAI-positive strains of *H. pylori* possess an *oipA* allele that is phase on while *cag* PAI-negative strains contain a phase off, yet highly conserved, allele of *oipA* (Ando *et al.* 2002; Matsuo, Kido and Yamaoka 2017). Despite this and the importance of both virulence factors in *H. pylori* virulence, the relationship between OipA and the *cag* PAI is somewhat enigmatic.

Despite the ability of *oipA* to undergo phase variation (Miftahussurur and Yamaoka 2015), our AFLP results suggest that there is little variation in the CT tract length in populations of *H. pylori* grown *in vitro*. This indicates that, while possible, phase variation at this locus may be rare. Rates of mutation in this poly CT tract length may vary between strains as Zhang *et al.* (2014) demonstrated that >75% of *oipA* alleles of western *H. pylori* isolates have relatively long poly CT tract lengths, most between 6 and 10 CT repeats. *Helicobacter pylori* isolates of Asian origin

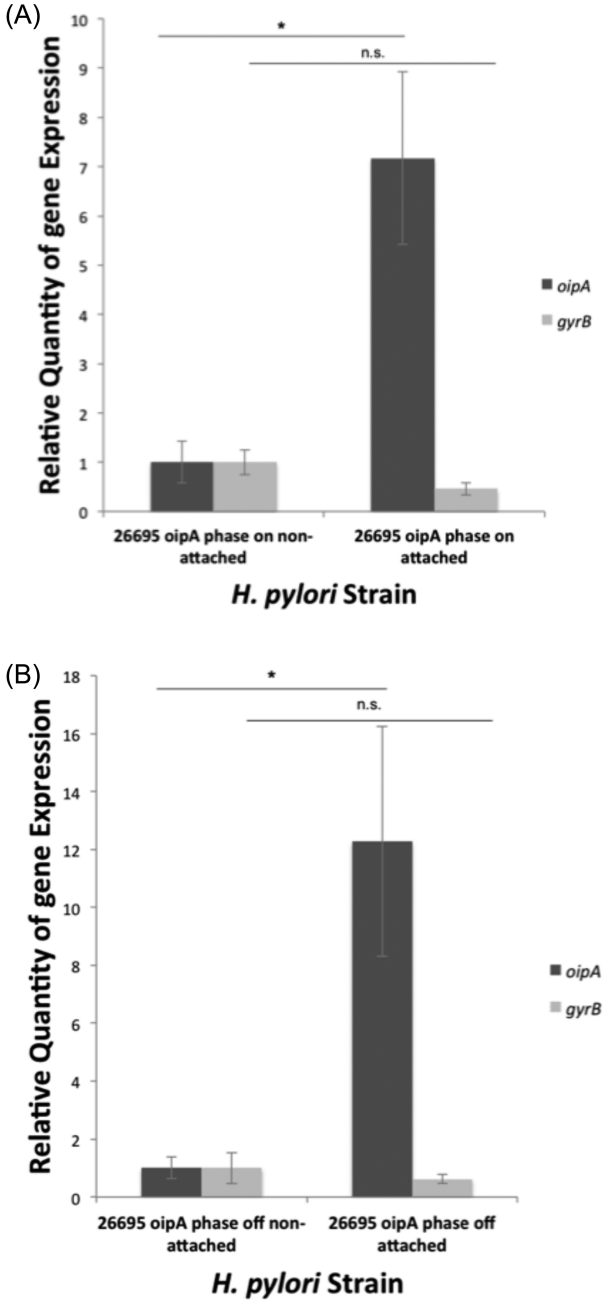

Figure 7. *Helicobacter pylori* attached to AGS cells possess higher levels of *oipA* transcript. Attachment assays were performed using 26695 *oipA* phase on (**A**) and 26695 *oipA* phase off (**B**). For each infection, *H. pylori* in the supernatant was collected separately from the bacteria attached to the AGS monolayer. RNA was extracted, and cDNA synthesized was used in RT-qPCR to determine relative quantities of *oipA* transcripts in attached cells compared to non-attached. The data shown here are representative of the results obtained in independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired t-test of unequal variance (* = $P \leq 0.05$, n.s. = $P > 0.05$).

have much shorter CT repeat tract lengths, most having condensed CT repeat lengths of 3 (Ando *et al.* 2002). These Asian *H. pylori* isolates are invariable *oipA* phase on. Our results showing that alterations in CT tract length are rare for *oipA* grown *in vitro* are in contrast to phase variation in other outer membrane proteins possessing poly CT tracts (Solnick *et al.* 2004; Styer *et al.* 2010), including sialic acid binding adhesin (SabA)

(Harvey *et al.* 2014). *sabA* alleles vary in lab-cultured populations such that there are three distinct subpopulations with varying CT tract lengths within a population of *H. pylori* strain 26695 with 60% possessing six CT repeats, 10% with seven repeats and 30% with eight (Goodwin *et al.* 2008). This same study demonstrated similar phase variation in *H. pylori* strain J99 as well. This inconsistent phase variation between *oipA* and *sabA* may suggest that factors other than the repetitive nature of CT repeat sequences might impact SSM and thus phase variation. While Asian isolates of *H. pylori* have utilized a condensation of the CT repeat tract to minimize *oipA* phase variation and thus maintain *oipA* phase on status, western isolates, with longer poly CT tracts, yet reduced phase variation, may have utilized an alternate evolutionary strategy to limit phase variation. Perhaps epigenetic influences on this locus may impact phase variation of *oipA*.

The vast majority of bacterial genome sequences encode protein or structural RNA. Additionally, when bacterial species transition to permanent host interactions, they undergo genomic reduction (Moran 2002). This adds intrigue to the conundrum that phase off alleles of *oipA* are nearly always found in *cag* PAI-negative strains of *H. pylori* (Ando *et al.* 2002), yet these strains have not lost the gene for this protein that is apparently unexpressed. We speculate that phase on and thus expressed alleles of *oipA* must serve an important function in these *H. pylori* strains of reduced virulence. *In silico* correction of *oipA* revealed that the protein produced when the CT tract is altered to generate a phase on allele in the *cag* PAI-negative strain used in this study, J68, it shares 97% amino acid identity with the protein produced by *cag* PAI-positive strain 26695 (data not shown). Based on this finding, we hypothesized that turning *oipA* phase on in *cag* PAI-negative strains of *H. pylori* would lead to the production of a full-length protein, and an increased ability of these cells to adhere to AGS cells and to elicit proinflammatory cytokine (IL-8) production. Our creation of a phase on allele of *oipA* in *H. pylori* strain J68 resulted in novel protein expression and increased attachment to AGS cells *in vitro*. However, this OipA expression in *cag* PAI-negative isolate J68 is not sufficient to elicit IL-8 secretion from AGS cells. Phase on *oipA* confers an increase in host adherence ability in both *cag* PAI-positive and negative strains. These findings show that OipA has the ability to mediate host attachment independent of the *cag* PAI. We hypothesize that the expression of OipA must be important under as yet undetermined circumstances during host infection by these reduced virulence strains, otherwise we would predict evidence of decay and perhaps deletion of the gene, rather than persistent conservation in the absence of apparent expression.

IL-8 induction experiments demonstrated that phase off status of *oipA* in the *cag* PAI-positive strain 26695 correlates not only with a decrease in host attachment, but also with decreased host production of this proinflammatory cytokine. It is striking that in the absence of OipA expression, there is a significant decrease in IL-8 production even in the presence of a functional *cag* PAI-encoded T4SS. This indicates that the *cag* PAI cannot mediate IL-8 induction independent of OipA. This finding is also supported by the fact that OipA expression does not elicit significant IL-8 secretion by AGS cells when infected by the *cag* PAI-negative strain J68 with *oipA* experimentally turned phase on. We hypothesize that OipA works in conjunction with the *cag* PAI T4SS to induce the host inflammatory response, and that both *H. pylori* surface structures must be present to stimulate host proinflammatory cytokine production.

Studies by Odenbreit *et al.* (2002) and Dossumbekova *et al.* (2006) concluded that IL-8 induction and CagA translocation are independent of OipA. In contrast, in the current study, we demonstrate that both IL-8 induction and CagA translocation are dependent on the expression of OipA. This discrepancy may be due to the use of different *H. pylori* strains or differing cell lines used in infections. Additionally, a counterselection method for introduction of markerless mutations initially described in a study by Loh *et al.* in 2011 (Ando *et al.* 2002) was employed in our study. The different nature of mutations in *oipA* may help explain the differing results between these studies. While our study demonstrates the necessity of OipA for IL-8 induction, this outer membrane protein alone cannot stimulate this proinflammatory event. The novel experimental ability to express OipA in a *cag* PAI-negative strain in the absence of any *cag* PAI proteins or VacA s1/m1 may allow better investigation of the role of this outer membrane protein in altered host cell responses. OipA joins BabA and HopQ (Ishijima *et al.* 2011; Javaheri *et al.* 2016; Koniger *et al.* 2016) as *H. pylori* outer membrane proteins encoded outside the *cag* PAI to facilitate the translocation of CagA into host cells. We believe that part of the explanation for the nearly uniform possession of phase on *oipA* alleles among *cag* PAI-positive *H. pylori* isolates lies in the association between OipA and the translocation of this oncoprotein. Selective pressure to maintain the expression of accessory proteins, such as OipA, in isolates of *H. pylori* bearing the *cag* PAI may explain why these more virulent isolates usually express OipA while the *cag* PAI-negative isolates would not experience this same pressure.

In vitro infection of AGS cells by *H. pylori* strain 26695 and subsequent RT-qPCR experiments revealed a significant increase in the quantity of *oipA* mRNA transcripts in the *H. pylori* cells adhered to AGS cells, compared to the non-adherent population in the same experiments. We initially proposed two alternative hypotheses regarding these results. First, we hypothesized that, despite the relative rarity of phase variation in *oipA in vitro*, there may be selection within the population of *H. pylori* used in the infection that allows cells expressing OipA to better adhere to host cells. However, our AFLP analyses on the poly CT region of *oipA* in both the adherent and non-adherent populations of *H. pylori* strain 26695 revealed neither enrichment for phase on *oipA* alleles in adherent *H. pylori* nor selection for phase off *oipA* alleles in the non-adherent population.

An alternative hypothesis is that contact with AGS cells may trigger increased transcription of *oipA* or increased stability of *oipA* mRNA as a response to the presence of the gastric epithelium. This hypothesis is supported by data showing the same increase in *oipA* transcript levels occurs in attached cells compared to non-attached, even when *oipA* is experimentally rendered phase off in 26695. The ability to regulate *oipA* expression may be adaptive in colonizing the stomach in the face of mucus shedding and epithelial cell turnover (Oleastro and Ménard 2013). Other investigators have demonstrated *H. pylori* gene expression alterations in response to gastric epithelial cell contact (Joyce *et al.* 2001; van Amsterdam *et al.* 2003; Silva *et al.* 2017). Affected genes include genes of the *cag* PAI, the vacuolating cytotoxin and genes within the plasticity zone. The ability to sense and respond to host cell contact (Johnson, Gaddy and Cover 2012) is almost certainly an important part of the exquisite adaptation of *H. pylori* to the host microcosm that contributes to the decades long infection that is a hallmark of *H. pylori* pathogenesis.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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Conflict of Interest. None declared.

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