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Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

Characterization of the Cag pathogenicity island in *Helicobacter pylori* from naturally infected rhesus macaques

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One sentence summary: Naturally infected rhesus monkeys carry *Helicobacter pylori* with a functional T4SS that can translocate CagA and thus use the same virulence mechanisms that are associated with disease in humans.

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ABSTRACT

Helicobacter pylori commonly infects the epithelial layer of the human stomach and in some individuals causes peptic ulcers, gastric adenocarcinoma or gastric lymphoma. *Helicobacter pylori* is a genetically diverse species, and the most important bacterial virulence factor that increases the risk of developing disease, versus asymptomatic colonization, is the cytotoxin associated gene pathogenicity island (*cag*PAI). Socially housed rhesus macaques are often naturally infected with *H. pylori* similar to that which colonizes humans, but little is known about the *cag*PAI. Here we show that *H. pylori* strains isolated from naturally infected rhesus macaques have a *cag*PAI very similar to that found in human clinical isolates, and like human isolates, it encodes a functional type IV secretion system. These results provide further support for the relevance of rhesus macaques as a valid experimental model for *H. pylori* infection in humans.

Keywords: *Helicobacter pylori*; rhesus macaques; CagA; type 4 secretion system; cag pathogenicity island; animal model

INTRODUCTION

Helicobacter pylori is a bacterium that frequently colonizes the gastric mucosa of the human stomach, infecting over 50% of the world's population. Although usually asymptomatic, ~10% of those infected with *H. pylori* will develop peptic ulcer disease and 1%–3% will progress to gastric adenocarcinoma (Wroblewski, Peek and Wilson 2010), the third most common cause of cancer death worldwide (Ferlay *et al.* 2015). The bacterial genetic locus most closely associated with clinical disease, rather than asymptomatic infection, is the *H. pylori cag* pathogenicity island (*cag*PAI). The *cag*PAI is a 40kb DNA segment that harbors 27 genes, many of which are required to produce a type IV secretion system (T4SS) that is essential for translocation of peptidoglycan and the CagA oncoprotein into host gastric epithelial cells (Odenbreit *et al.* 2000; Viala *et al.* 2004). The function of the other *cag*PAI genes is unknown.

Experimental animal models are essential to fully understand the pathogenesis of *H. pylori* infection. Most investigators use the mouse model, which is inexpensive, convenient and offers elegant host genetics, or the Mongolian gerbil, which is arguably the best disease model but has

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fewer reagents. The rhesus macaque is an alternative that has several advantages over conventional small animal models, including the anatomical and physiological similarities to humans. Moreover, unlike rodents, socially housed rhesus monkeys are naturally infected with *H. pylori* (Drazek, Dubois and Holmes 1994; Dubois *et al.* 1994). Infection is common and occurs within the first year of life (Solnick *et al.* 2003), which are features that mimic the epidemiology of *H. pylori* in humans in developing countries (Bardhan 1997) where infection is most common. All infected animals have chronic gastritis, and some develop atrophy (Dubois *et al.* 1996), which is the histologic precursor to adenocarcinoma (Correa and Piazuelo 2012).

Although *H. pylori* from rhesus macaques is genetically very similar to that from humans (Joyce *et al.* 2002), little is known about the prevalence or function of the *cag*PAI in rhesus-derived *H. pylori*. Here we demonstrate that naturally infected rhesus monkeys uniformly harbor *H. pylori* strains that have a *cag*PAI that is very similar genetically and functionally to that of human isolates. These results suggest that *H. pylori* utilizes similar virulence mechanisms to colonize rhesus and human hosts and further validate the rhesus macaque as an experimental model for *H. pylori* infection in humans.

MATERIALS AND METHODS

Animals

Male and female rhesus macaques (N = 9) aged 3 to 5 years were located at the California National Primate Research Center (CNPRC). Animals were housed in a 100 × 200 ft outdoor field cage that contained 80 to 120 male (~30%) and female (~70%) rhesus monkeys ranging in age from newborn to over 30 years. All experiments were performed in accordance with NIH guidelines, the Animal Welfare Act, and United States federal law, at the University of California, Davis, under animal use protocol #15751 approved by the UC Davis Institutional Animal Care and Use Committee.

Endoscopy with gastric biopsy

Endoscopy with gastric biopsy and subsequent *H. pylori* isolation were performed essentially as described previously (Solnick *et al.* 2003). Homogenized gastric biopsies were inoculated onto brucella agar containing 5% newborn calf serum (NCS; Invitrogen, Carlsbad, CA) and TVPA antibiotics (trimethoprim, 5 mg/l; vancomycin, 10 mg/l; polymyxin B, 2.5 IU/l, amphotericin B, 2.5 mg/l; Sigma-Aldrich, St. Louis, MO, USA), and incubated for up to 8 days. A total of 10 single colonies confirmed as *H. pylori* were isolated from each monkey.

Bacterial culture

Helicobacter pylori was cultured on brucella agar (BBL, Becton Dickinson, Sparks, MD, USA) supplemented with 5% heat-inactivated NCS and TVPA antibiotics. All *H. pylori* cultures were grown at 37°C under microaerophilic conditions generated by a fixed 5% O_2 concentration (Anoxomat, Advanced Instruments, Norwood, MA, USA).

Helicobacter pylori DNA fingerprinting

Genomic DNA from each single colony was prepared from plate-grown bacteria using a Qiagen DNA extraction kit (DNeasy® Blood & Tissue Kit, QIAGEN Sciences, MD, USA) or phenol-chloroform DNA extraction. Repetitive extragenic palindromic PCR (REP-PCR) was performed as previously described (Solnick *et al.* 2003) using the degenerate oligonucleotide primers REP1R-DT and REP2-DT (Table 1). DNA fragments were separated by electrophoresis in a 1.5% agarose gel for 16 h at 18 V and visualized by ethidium bromide staining.

DNA sequence analysis

The entire *cag*PAI from one rhesus *H. pylori* isolate from monkey 1 was sequenced by PCR amplification of 10 overlapping DNA segments with flanking primers (Table 1) and the Expand Long Template PCR system (Roche, Indianapolis, IN, USA). Purified PCR products were sequenced directly by the Sanger method and assembled using Sequencher analysis software (GeneCodes Corporation, Ann Arbor, MI, USA). The resulting open reading frames were compared to sequenced human *H. pylori* isolates J99 (Alm *et al.* 1999) and 26695 (Tomb *et al.* 1997) using the Vector NTI software package (Life technologies, Foster City, CA, USA). To confirm correct assembly of the *cagY* 5′ repeats, the *cagY* PCR products were electrophoresed on 0.4% agarose gels for 16 h at 18 V and the total *cagY* size was estimated. In addition, genes essential for IL-8 induction were amplified from two isolates from a second monkey (monkey 5) using flanking primers (Table 1). Sequencing and analysis were performed as above. All DNA sequences were deposited in GenBank under accession numbers KX683298 (for entire *cag*PAI) and KX807112-807127 (IL-8 essential *cag*PAI genes).

IL-8 ELISA

IL-8 was measured as described previously (Barrozo *et al.* 2013). AGS gastric adenocarcinoma cells (ATCC, Manassas, VA) were co-cultured with *H. pylori* at an MOI of 1:100 for 18–24 h. Supernatants were harvested and diluted 1:8 prior to IL-8 assay by ELISA (Invitrogen, Camarillo, CA) performed according to the manufacturer's protocol. *Helicobacter pylori* J166 WT and its isogenic *cagY* deletion mutant (Barrozo *et al.* 2013) were included as positive and negative controls, which typically induce around 3000 pg/ml and 200 pg/ml IL-8, respectively.

cagA PCR, CagA expression and translocation

cagA was amplified using primers D008 and R008 (Table 1) in PCR reactions as previously described (Figura *et al.* 1998). Expression and phosphorylation of CagA were detected by immunoblot as previously described (Barrozo *et al.* 2013). Briefly, lysates from *H. pylori* co-cultured with AGS gastric carcinoma cells were electrophoresed in a 7.5% polyacrylamide gel (BioRad, Hercules, CA), transferred to a PVDF membrane (Millipore, Billerica, MA), and incubated separately with IgG antibodies to CagA (Austral Biological, San Ramon, CA) and to phosphotyrosine (PY99, Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected by incubation with horseradish peroxidase-conjugated anti-IgG, visualized using ECL reagents (Thermo Scientific, Rockford, IL, USA) and exposure to X-ray film. Films were imaged with GelDoc XR+ imaging system (BioRad) using the white light conversion screen and the silver stain application. The Band Analysis tools of ImageLab software version 5.2.1 (BioRad) were used to select the bands in the blots and determine their relative density using results for J166 WT as reference.

Table 1. Primers used for PCR.

Amplification	Name	Sequence (5′ to 3′)[a]	26695 region
REP-PCR	Rep2-DT	NCG NCT TAT CNG GCC TAC	NA
	Rep1R-DT	III NCG NCG NCA TCN GGC	
cagA PCR detection	D008	ATA ATG CTA AAT TAG ACA ACT TGA GCG A	581152-581449
	R008	TTA GAA TAA TCA ACA AAC ATC ACG CCA T	
cag1-4	HP0519:548L21	CGC TCA AAC CTG AAA GAT CAA	546585-550329
	cag5:2135U20	TGG GGA AAG TGC CTA ATC AA	
cag5-6 (including virB11)	cag4:381U28	CAA CAA AAG CGT GTA TCA ATT AGT AGA A	549969-554299
	cag7:5691U27	CAA ATC TGT GGT AGA TGA AAT TAT CAA	
cag7	cag6:116L24	CTT GCG GAT CGT TGC TAT CTT TTA	553930-560178
	cag8:1395U28	ATG GTA TAG AGT TAA TGA AAT TGC AGA A	
cag8-10	cag7:12L21	TTC TTC ATT CAT GTC TTA ACG	559978-563935
	cag10:61U25	GCC CTT GAT AGA TTG GCT AAA CTC A	
cag11-14	cag10:627L24	AAG CCA AAA GGA TTG ATG ATA AGA	563346-567505
	cag14: 18U21	ACG CAT TAG AGA TCC GAA CAA	
cag15-18	cag14:357L25	CTA GAG TCT TAC TTG AGA GAC ACT C	567142-571474
	cag18:110U22	CCA ACC AAC AAG TGC TCA AAA A	
cag19-22	cag18:512L25	GTC TGT GAA GCA GTG ATT AAG GAA G	571048-575001
	cag22:153U27	CCT TAC CGC TCT TTA TGA TTT TTC TAA	
cag23-25	cag22:681L23	CGC TCA TAT CAA TCT GAA TCC AA	574451-579074
	cag25:14U25	CAA GAA TCA CTG ACA GCT ACA AGA A	
Intergenic space between *cag25* and *cag26* (*cagA*)	cag25:271L20	ATA CCG CCT GCC ACC GCT AA	578798-580381
	cagA:437L25	GGG GGT TGT ATG ATA TTT TCC ATA A	
cag26 (*cagA*)	cagA:223U26	AAT AAA GCG ATC AAA AAT TCT ACC AA	580143-[b]
	Rhesus pai reverse	GCT AAA TGC TTT CCA TCC ACA AAC	
cagY PCR-RFLP	HP0527R	CCG TTC ATG TTC CAT ACA TCT TTG	554810-560058
	HP0528F	CTA TGG TGA ATT GGA GCG TGT G	

[a]For degenerate REP-PCR primers, I = inosine; N = any nucleotide
[b]Rhesus pai reverse primer does not match 26695; NA = not applicable.

cagY PCR-RFLP

cagY genotyping was performed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as described previously (Barrozo *et al.* 2013), using primers HP0527R and HP0528F (Table 1).

Statistical analysis

Data were analyzed with GraphPad Prism 7.0 using one-way ANOVA with Dunnett's post hoc test and Pearson correlation as indicated. A *P*-value <0.05 was considered statistically significant.

RESULTS

Naturally infected rhesus macaques are colonized with clonally diverse strains of *H. pylori*

Rhesus monkeys at the CNPRC are housed in one-half acre outdoor field cages that contain a breeding colony of males and females. Animals are sometimes removed for experimentation, but new animals are typically not introduced other than by birth because this disrupts the social structure and sometimes leads to aggression. Since *H. pylori* strains within each field cage are more closely related than across different field cages (Solnick *et al.* 2003), we obtained gastric biopsies from 9 monkeys housed in 9 different cages to better represent the diversity of *H. pylori* at the CNPRC. All monkeys were culture positive for *H. pylori*. To assess clonality of the 9 isolates, we performed REP-PCR on a single isolate from each monkey. As expected, the strains were more similar to one another than is typical among human isolates (Go *et al.* 1995), but subtle differences were apparent by REP-PCR analysis (Fig. 1).

cagPAI DNA sequence of a rhesus *Helicobacter pylori* strain is highly related to that from strains found in human infection

All *H. pylori* strains isolated from rhesus monkeys were positive by PCR for the *cagA* gene (Fig. 2A), which was used as a marker for the presence of the *cag*PAI. We next sequenced the entire *cag*PAI of the isolate from monkey 1 and compared the results with the *cag*PAI from human isolates J99 and 26695, whose complete genomes are sequenced. The rhesus *H. pylori* strain carried the same *cag*PAI gene content as the human strains and had a high sequence similarity, typically $>93\%$ (Table 2). The most notable exceptions were *cag7* (*cagY*) and *cag26* (*cagA*), which are also highly variable among human isolates and known to be under strong diversifying selection (Olbermann *et al.* 2010). *cag2* from rhesus *H. pylori* was also very dissimilar from human isolates, which is not surprising since *cag2* is sometimes absent among human strains and may be a pseudogene (Azuma *et al.* 2004). These results indicate that the *cag*PAI gene sequences of *H. pylori* derived from rhesus monkeys are closely related to those of human isolates, with the exception of genes that are already known to be variable among human isolates.

Helicobacter pylori isolated from natural infected rhesus macaques has a functional T4SS

After confirming that the monkey isolates were uniformly positive for the *cag*PAI, with sequence similarity to human isolates,

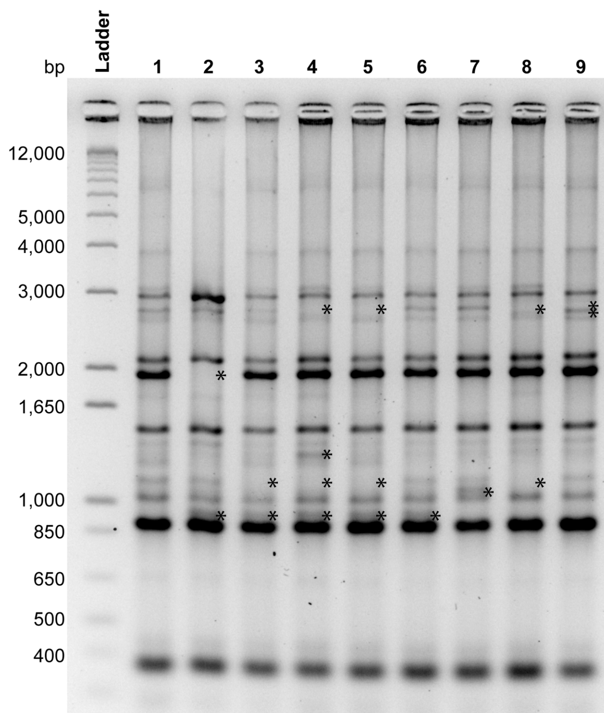

Figure 1. REP-PCR DNA fingerprints of a single *H. pylori* isolate from each monkey (N = 9). Although related, each strain is unique, with one or more bands (*) that distinguish it from every other. Stars indicate bands that are either absent or present in each isolate compared to the isolate from monkey 1.

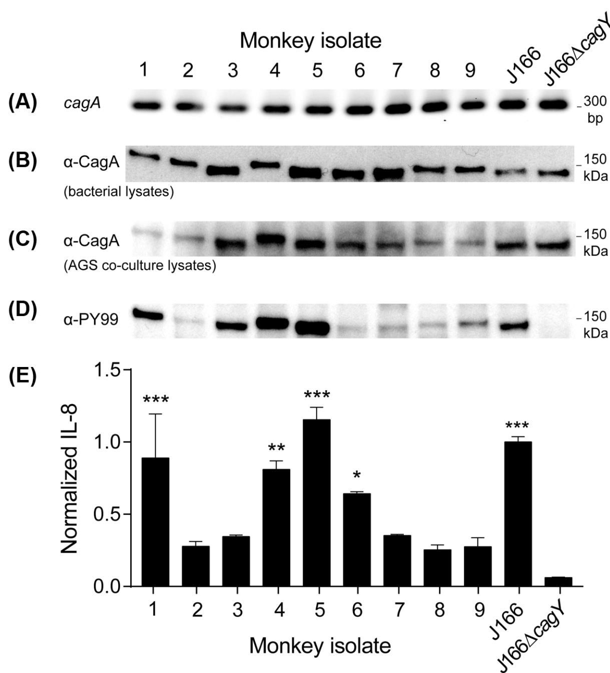

Figure 2. Functional characterization of the *cag*PAI in *H. pylori* isolates from naturally infected rhesus macaques. All strains contained *cagA* (**A**) and expressed CagA in bacterial culture (**B**) and during co-culture with gastric epithelial cells (**C**). Each *cag*PAI encoded a functional T4SS as indicated by CagA phosphorylation (**D**) and IL-8 induction (**E**). Values of IL-8 induction are mean ± SEM from three biological replicates normalized to the results for *H. pylori* strain J166. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to *H. pylori* J166 *cagY* deletion mutant (ANOVA with Dunnett's test).

we assessed if the *cag*PAI expressed CagA and encoded a functional T4SS. Immunoblot of agar plate-cultured bacteria, as well as of *H. pylori* co-cultured with human AGS cells, demonstrated that all rhesus isolates expressed CagA (Fig. 2B and C). To determine if rhesus-derived *H. pylori cag*PAI encodes a functional T4SS, we performed co-culture experiments with isolates from each monkey, and compared the results to WT J166 and its isogenic *cagY* deletion mutant as positive and negative controls, respectively (Barrozo *et al.* 2013). In co-culture with AGS cells, *H. pylori* induced IL-8 and CagA was tyrosine phosphorylated, suggesting that the rhesus-derived *H. pylori* contains a *cag*PAI that encodes a functional T4SS (Fig. 2D, E). Similar to what is found in *cag*PAI positive clinical isolates from humans (Olbermann *et al.* 2010), *H. pylori* strains from individual animals showed marked variability in IL-8 induction, which was strongly correlated with total CagA ($r = 0.685$, $P = 0.042$) and phosphorylated CagA ($r = 0.85$, $P = 0.004$, Pearson correlation) detected in co-culture with AGS cells. Since T4SS function is modulated by recombination in *cagY* (Barrozo *et al.* 2013), we next performed *cagY* PCR-RFLP on the isolates to determine if T4SS function was associated with differences in *cagY*. Digestion of amplified *cagY* with DdeI and BfuCI showed four distinct RFLP fingerprints among the nine isolates (Fig. 3), suggesting that *cagY* can undergo recombination during transmission and natural colonization of monkeys similar to what is seen in human infection. However, no consistent pattern was found between T4SS function and *cagY* RFLP. Together, these results demonstrate that all rhesus *H. pylori* isolates expressed a *cag*PAI that encoded a functional T4SS.

Heterogeneity of T4SS function among *Helicobacter pylori* colonies from within individual rhesus macaques

We previously showed that individual *Helicobacter pylori* isolates from experimentally infected rhesus monkeys sometimes displayed marked variability in T4SS function, which in some cases was related to recombination in *cagY* (Barrozo *et al.* 2013). To better understand T4SS function within a population of *H. pylori* in naturally infected monkeys, we isolated 10 colonies from each monkey and examined their capacity to induce IL-8. Most isolates from within an individual animal showed similar IL-8 induction (Fig. 4A). Similar to what we have observed before in experimentally infected monkeys (Barrozo *et al.* 2013), strains with relatively high levels of IL-8 induction (e.g. monkeys 1 and 5) showed greater variability. This might be measurement error, resulting from variability in host or bacterial cell density, or pipetting error, but could also reflect differences in T4SS function among isolates within an individual animal. To address this, we performed biological replicate assays on the colonies from monkey 5, which demonstrated that one colony (open circle) consistently showed greater induction of IL-8 (Fig. 4B). However, *cagY* PCR-RFLP and DNA sequence analysis of 16 *cag*PAI genes implicated in T4SS function (*cag3-4*, virB11, *cag7-12*, *cag16*, *cag18-20*, *cag23*, *cag25-26*) showed no differences between the isolate inducing high IL-8 (open circle) and one of the others (open square) (data not shown). These results suggest that there are sometimes reproducible differences in T4SS function among strains within an animal, but that they are not necessarily attributable to sequence differences in *cag*PAI genes.

DISCUSSION

Socially housed rhesus monkeys are naturally infected with *Helicobacter pylori* (Drazek, Dubois and Holmes 1994; Dubois *et al.*

Table 2. Percent identity of rhesus *cag*PAI genes compared to human strains 26695 and J99.

Gene	Start	Stop	Length	Direction	26695 Designation	% Identity	J99 Designation	% Identity
cag1	1	348	348	+	HP0520	96.0	JHP0469	96.3
cag2	379	1122	744	+	HP0521	17.1	JHP0470	48.7
cag3	1115	2560	1446	+	HP0522	97.2	JHP0471	94.4
cag4	2570	3079	510	+	HP0523	95.7	JHP0472	94.4
cag5	3554	5800	2247	−	HP0523	96.8	JHP0472	95.8
virB11	5809	6801	993	−	HP0525	97.7	JHP0474	96.8
cag6	6816	7405	590	−	HP0526	97.3	JHP0475	97.8
cag7	7543	12235	4693	−	HP0527	78.5	JHP0476	80.5
cag8	12274	13842	1569	−	HP0528	97.9	JHP0477	96.4
cag9	13895	15502	1608	−	HP0529	97.6	JHP0478	97.7
cag10	15507	16265	759	−	HP0530	97.2	JHP0479	96.8
cag11	16645	17295	651	+	HP0531	94.8	JHP0480	94.7
cag12	17308	18150	843	+	HP0532	98.1	JHP0481	97.2
cag13	18350	18919	570	−	HP0534	96.3	JHP0482	97.3
cag14	19390	19770	381	−	HP0535	96.9	JHP0483	97.9
cag15	20514	20858	345	−	HP0536	93.9	JHP0484	94.2
cag16	21258	22388	1131	+	HP0537	98.9	JHP0485	96.6
cag17	22403	23323	921	+	HP0538	97.6	JHP0486	97.4
cag18	23405	24118	714	−	HP0539	97.2	JHP0487	97.5
cag19	24115	25260	1146	−	HP0540	96.2	JHP0488	96.9
cag20	25271	26383	1113	−	HP0541	97.6	JHP0489	97.4
cag21	26400	26828	429	−	HP0542	97.9	JHP0490	97.4
cag22	26883	27689	807	−	HP0543	96.4	JHP0491	96.4
cag23	27691	30642	2952	−	HP0544	97.6	JHP0492	96.7
cag24	30651	31274	624	−	HP0545	96.0	JHP0493	95.4
cag25	31276	31623	348	−	HP0546	95.7	JHP0494	96.6
cagA	34355	37996	3642	+	HP0547	90.0	JHP0495	87.7

1994, 1996; Solnick *et al.* 2003), and some develop the associated diseases that affect humans, including peptic ulcer, gastric adenocarcinoma and MALT lymphoma (Kimbrough 1966; Parker, Gilmore and Dubois 1981; Solnick, Eaton and Peek 2016). Therefore, rhesus monkeys have been used as a model host to study *H. pylori* pathogenesis, treatment and vaccine development (Solnick, Eaton and Peek 2016). In humans, expression of CagA is the major determinant for the outcome of infection, but it is unknown if the same is true for macaques. In fact, all *Helicobacter* species that infect animals other than humans lack the *cag*PAI (Vermoote *et al.* 2011; Smet *et al.* 2013), and it might thus be possible that *cag*PAI-negative *H. pylori* are favored in naturally infected monkeys. Here we studied the prevalence and function of the *cag*PAI in naturally infected monkeys and showed that rhesus-derived *H. pylori* uniformly expresses CagA and a functional T4SS with high gene sequence similarity to human strains. The similarity between rhesus- and human-derived *H. pylori* in *cag*PAI, and in whole genome DNA microarrays (Joyce *et al.* 2002), suggests that rhesus monkeys in captivity may have acquired the infection from humans. Transmission of helicobacters has indeed been shown to occur between humans and other species (Haesebrouck *et al.* 2009; Sabry, Abdel-Moein and Seleem 2016).

Helicobacter pylori infection in developing countries is common, is acquired very early in life and is almost uniformly positive for the *cag*PAI—all features that are similar to *H. pylori* infection in rhesus monkeys. As the *H. pylori* prevalence declines in developed countries, so too does the prevalence of the *cag*PAI (Perez-Perez *et al.* 2002). The reasons for this are unknown, but it might indicate a role for the *cag*PAI in transmission of *H. pylori* between hosts. This is supported by the observation that transmission in mice has been found only with defective acid secretion (Bjorkholm *et al.* 2004), which is promoted by *cag*PAI gene products that downregulate parietal cell H,K-ATPase expression (Saha *et al.* 2010). Alternatively, features of the *H. pylori* microbial ecology in developing countries—and among socially housed macaques—might favor the *cag*PAI. Since the *cag*PAI appears to be important for acquisition of iron from the gastric epithelium (Tan *et al.* 2011; Noto *et al.* 2013, 2014), one possibility is that relative iron deficiency selects for *cag*PAI-positive strains. Another possibility is that the inflammatory response induced by the *cag*PAI, including expression of defensins and other antimicrobial peptides (Hornsby *et al.* 2008; Bauer *et al.* 2012, 2013), protects the host against other enteric infections, which are common in both developing countries and among socially housed rhesus monkeys. These similarities between *H. pylori* in macaques and in humans where infection is most common suggest that the rhesus model can be used to better understand the role of the *cag*PAI in the ecology of *H. pylori*.

Another similarity between *H. pylori* infection in humans and in naturally infected rhesus monkeys is that the *cag*PAI in rhesus *H. pylori* encodes a functional T4SS that translocates CagA and induces IL-8. Yet in experimental infection of rhesus monkeys, T4SS function is frequently lost by recombination in *cagY*, much like what happens uniformly during infection of mice (Barrozo *et al.* 2013). The different outcome of experimental challenge versus natural infection in monkeys may result from the larger inoculum or perhaps from introduction of *H. pylori* directly into the stomach. Moreover, since natural *H. pylori* infection typically occurs in young macaques (and children), T4SS function may be preserved because they are relatively immunotolerant. This is consistent with results in infant mice, where *cag*PAI function is relatively preserved (Arnold *et al.* 2010), and it may also explain the observation that the prevalence of the *cag*PAI is lower

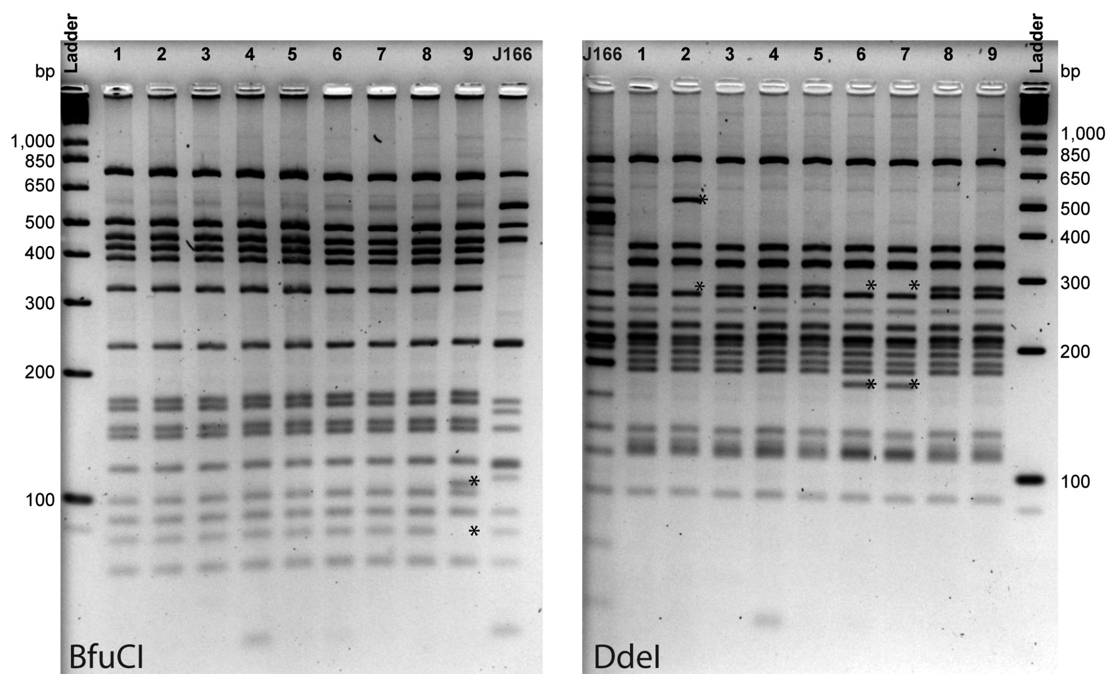

Figure 3. *cagY* PCR-RFLP of a single *H. pylori* isolate from each monkey. *cagY* was PCR amplified, digested with BfuCI or DdeI and separated by agarose gel electrophoresis. Four unique patterns were identified, with variant bands present and absent (*) compared to the most common pattern found in isolates from monkeys 1,3,4,5 and 8.

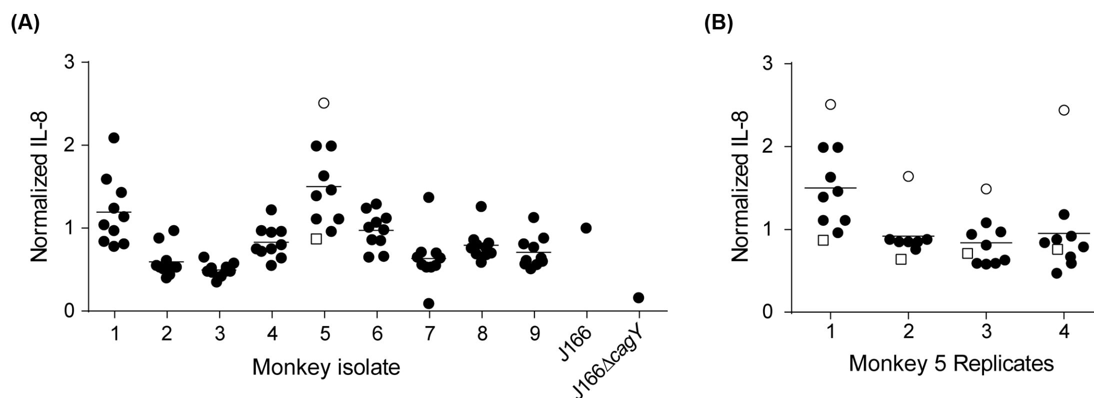

Figure 4. Heterogeneity of IL-8 induction among multiple individual *H. pylori* colonies isolated from each monkey. (**A**) Multiple isolates from within most individual monkeys showed similar induction of IL-8, though in some cases (e.g. monkeys 1 and 5) there was considerable variability. (**B**) Biological replicates of strains recovered from monkey 5 showed that the isolate with the highest IL-8 (open circle) was consistently higher than all others. The *cag*PAI sequence of this isolate was later compared to one of the isolates that induced low IL-8 (open square). IL-8 values are normalized to the results for *H. pylori* strain J166.

in countries where infection is acquired at an older age (Perez-Perez *et al.* 2002).

Although T4SS function among isolates from a single individual is little studied, we found that some monkeys were colonized with strains that appeared clonal by REP-PCR, but that differed reproducibly in T4SS function (Fig. 4). Since we previously demonstrated *cag*Y-dependent modulation of T4SS function, we used PCR-RFLP to determine if *cag*Y genotype was related to T4SS function. Although *cag*Y was variable among strains from different monkeys (Fig. 3), it did not correlate with T4SS function, and no differences were found among isolates from monkey 5 that differed in their induction of IL-8 (Fig. 4). Moreover, the high and low IL-8 inducing strains from monkey 5 did not differ at other *cag*PAI genes that are associated with T4SS function, suggesting that *cag*PAI genes whose function is unknown, or genes outside the *cag*PAI, can regulate T4SS function. Several examples of non-*cag*PAI gene regulation of T4SS function have been described, including the *babA* adhesin (Ishijima *et al.* 2011), the ferric uptake regulator (Pich *et al.* 2012) and a predicted glycosyltransferase that is induced by epithelial cell contact and upregulates expression of *cagA* (Bhattacharya *et al.* 2016).

In summary, we have shown that *H. pylori* strains isolated from naturally infected, socially housed monkeys have a functional T4SS that translocates CagA, induces IL-8 and is highly related to human *cag*PAI. These results suggest that *H. pylori* can use the same virulence mechanisms in monkeys as in humans, and support the relevance of the rhesus monkey as a model for *H. pylori* infection in humans.

AUTHOR CONTRIBUTIONS

ECS, SLD, HDE, LMH and JVS: conceived and designed the experiment. ECS, SLD, HDE and LMH: performed experiments. ECS, SLD, HDE, LMH and JVS: analyzed data. ECS, SLD and JVS: wrote the paper.

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Conflict of interest. None declared.

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