

Papers

Modulation of *Helicobacter pylori* induced interleukin-8 synthesis in gastric epithelial cells mediated by cag PAI encoded VirD4 homologue

Jean E Crabtree, Dangeruta Kersulyte, Shude D Li, Ivan J D Lindley, Douglas E Berg

Abstract

Background—Strains of *Helicobacter pylori* carrying the virulence associated cag pathogenicity island (PAI) induce gastric epithelial synthesis of the chemokine interleukin-8 (IL-8), a neutrophil chemo-attractant, and thereby a strong inflammatory response during chronic infection of the human gastric mucosa. Previous mutational analyses have shown that many genes in the cag PAI are needed to elicit IL-8 synthesis in gastric epithelial cells, and also that some genes are not involved.

Aim—To test the possibility that certain genes in the cag PAI also downregulate (modulate) the inflammatory response elicited by cag+ *H pylori* infection.

Methods—Cells of L5F11, a derivative of the Kato-3 gastric epithelial cell line that carries an engineered IL-8 promoter-luciferase reporter gene fusion, were co-cultured with *H pylori* strain 26695 or with an isogenic mutant in which most of the cag PAI ORF 10 gene, an *Agrobacterium* virD4 homologue, was deleted. Luciferase activity was measured to assess IL-8 gene transcription and secreted IL-8 was measured by enzyme linked immunosorbent assay to assess synthesis and release of IL-8 protein from gastric epithelial cells.

Results—Inactivation of ORF10 led to a 2.8-fold increase in IL-8 gene transcription and a 3.6-fold increase in IL-8 synthesis and secretion.

Conclusions—The results suggest that this VirD4 homologue participates in the control of inflammation that *H pylori* infection elicits by downregulating (modulating) the strong induction of IL-8 synthesis mediated by other cag encoded proteins.

(J Clin Pathol 1999;52:653-657)

Keywords: *Helicobacter pylori*; cag pathogenicity island; interleukin-8; epithelial cell; virD4

Helicobacter pylori is an extremely diverse bacterial species that chronically infects the gastric mucosa of more than half of all people

worldwide. Chronic infection is always associated with gastritis,¹ the severity of which varies widely among infected subjects, probably reflecting a complex interplay between bacterial, host, and environmental factors. In some cases (10–20% in Europe and North America) infection leads to peptic ulcer disease, and chronic infection is also an early risk factor for distal gastric cancer.^{2,3} The induction of a moderate inflammatory response by *H pylori* may provide increased nutritional factors for bacterial growth from damaged host tissues and thus contribute to the success of long term chronic infection, provided that it is insufficient to clear the bacteria or destroy the gastric epithelium on which colonisation depends.

Molecular genetic analyses of diverse *H pylori* strains have revealed there is a strong association between more severe clinical outcome (that is, peptic ulceration,⁴⁻⁶ atrophic gastritis,^{7,8} and intestinal-type gastric cancer⁹⁻¹⁰) and carriage of a 40 kb DNA segment called the cag pathogenicity island (PAI).¹¹⁻¹² Cag+ *H pylori* strains also frequently produce a vacuolating cytotoxin,¹³⁻¹⁴ and an adhesin specific for Lewis B carbohydrate structures¹⁵ that are present on the gastric epithelial surface of many subjects. In contrast, many of the strains from individuals with only chronic gastritis, at least in Europe and North America, entirely lack the cag PAI, and also are non-toxicogenic and non-adherent, even though the genes for the toxin and adhesin are not located within the cag PAI.¹²⁻¹⁵ Cag+ *H pylori* strains stimulate gastric epithelial cells to synthesise interleukin (IL)-8,¹⁶⁻¹⁸ a chemokine that attracts neutrophils to the gastric epithelium and thus contributes to the severity of the inflammatory response.

Mutational analyses and tests with cultured gastric epithelial cell lines have shown that many of the genes in the cag PAI are needed directly to induce IL-8 synthesis in gastric epithelial cells,^{11-12,19} and also that other genes in this PAI (including the much studied cagA gene) are not needed for such IL-8 synthesis.^{11-12,20} DNA sequence analyses showed that the protein products of four cag PAI genes^{11-12,19} are homologues of four “VirB” proteins of the *Agrobacterium* Ti plasmid,

Molecular Medicine Unit, St James's University Hospital, Leeds LS9 7TF, UK
J E Crabtree
S D Li

Department of Molecular Microbiology, Washington University School of Medicine, Campus Box 8230, St Louis, Missouri 63110, USA
D Kersulyte
D E Berg

Novartis Research Institute, A-1235 Vienna, Austria
I J D Lindley

Correspondence to: Dr Crabtree.

Accepted for publication 29 April 1999

which are essential for protein and nucleoprotein (T DNA) transfer from bacterial to target cells.²¹⁻²³ They are similarly homologues of apparently corresponding Ptl proteins of *Bordetella pertussis*²⁴ and Tra proteins of conjugative bacterial plasmids²⁵ that mediate pertussis toxin and plasmid DNA transfer to human and bacterial cells, respectively. Where studied, these four VirB proteins seem to form a membrane pore on which other VirB proteins (not represented among the inferred proteins of *H pylori*) anchor for the transmission of the appropriate macromolecules to target cells.²¹ Each of the four VirB homologues in *H pylori* is needed to induce IL-8 synthesis in gastric epithelial cells^{11 12 19 26} and it is likely that they act similarly, in concert with some of the cag PAI encoded proteins with no homologues in current databases.

Also needed for T DNA transfer in the case of *Agrobacterium* is virD4, a gene that maps to a locus removed from the virB gene cluster.²¹ A virD4 homologue is also found in the cag PAI,¹² but apparently not in *B pertussis*.²¹ VirD4 is an inner membrane protein in *Agrobacterium*.^{21 27} Here we show that deletion of the virD4 homologue in the cag PAI causes enhanced IL-8 synthesis in gastric epithelial cells. This suggests that the normal role of this homologue in *H pylori* infection is to downregulate induced IL-8 synthesis and thereby to help control the intensity of the inflammatory response.

Methods

BACTERIA

The wild type *H pylori* strains used here were 26695, the strain where the genome was sequenced completely,²⁸ and NCTC 11637, the type strain of this species. Bacteria grown on blood agar base number 2 (Oxoid) with 7% fresh horse blood under microaerobic conditions at 37°C were harvested on day 3 into antibiotic-free RPMI 1640 medium (Life Technologies) supplemented with 10% heat inactivated fetal calf serum (FCS) (Sera Lab). Bacterial preparations were adjusted to 2.5×10^7 /ml and used immediately to inoculate gastric epithelial cell cultures.

CONSTRUCTION OF ISOGENIC MUTANT DERIVATIVES OF 26695 WITH INSERTIONS OR DELETIONS IN THE virD4 HOMOLOGUE ORF 10 (HP524)

The ORF10 insertion mutants, 15-1 and 16-1, were constructed in cloned ORF10 DNA by placing a camR cassette in each orientation in the EcoRI site 601 base pairs from the 3' end of the 2256 base pair gene. These constructs were then transformed into *H pylori* strain 26695, CamR was selected, and the insertion mutant structures were verified by PCR. A deletion mutant recombinant lacking 1.6 kb of internal sequence in ORF10 was constructed by PCR of cloned ORF10 containing DNA with primers facing outward from positions 341 base pairs (bp) (primer cos 36 R 7615) and 319 bp (primer cos 36 F 9198) from the 3' and 5' ends of the gene, ligation of a camR cassette in place of the deleted ORF10 sequence, and then transformation of strain 26695. The expected

transformant structure was verified by polymerase chain reaction. The camR cassette is inserted in the orientation opposite that of ORF10, but no polar effects are expected because ORF10 is the last gene in its transcription unit.¹²

STIMULATION OF EPITHELIAL CELL LINE L5F11 WITH *H PYLORI*

The reporter cell line L5F11 cell line was derived from Kato-3 gastric epithelial cells (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK) by stably transfecting them with 1200 base pairs of the IL-8 promoter driving the firefly luciferase coding region.²⁶ Initial characterisation of this IL-8 reporter assay system showed as expected a strong correlation between luciferase activity and IL-8 protein secretion following *H pylori* infection.²⁶

L5F11 cells were routinely maintained in RPMI 1640 medium supplemented with 10% FCS, 40 mg/ml gentamicin, 400 mg/ml geneticin (Life Technologies), and 2 mM glutamine (Life Technologies). Before infection with *H pylori*, the cells were resuspended in antibiotic-free medium and cultured in 96-well plates (Corning Costar) at a density of 5×10^5 /ml. Cells were cocultured with *H pylori* 26695 and the isogenic mutants 15-1, 16-1, 28-1, 28-2, and 28-3 at a bacteria to L5F11 cell ratio of 50:1 in quadruplicate for 10 hours at 37°C in a 95% air/5% CO₂ humidified incubator. An isogenic total cag PAI deletion mutant derivative of 26695¹² and an unrelated clinical isolate that is naturally cag- (G50) served as negative controls. The cag+ strains 26695 and NCTC 11637 served as positive controls. These negative and positive bacterial controls were included in each assay, and tumour necrosis factor α (TNF α) (5 ng/ml; R&D System) was used as an additional positive control. After 10 hours of culture, supernatants were harvested for IL-8 protein assay, and luciferase activity was determined in cell lysates.

LUCIFERASE ASSAY

Luciferase activity was assayed as previously described.²⁶ Briefly, L5F11 cells were washed twice using cold phosphate buffer saline (pH 7.4) and lysates were prepared using the cell culture lysis reagent (Promega). Luciferase concentrations in lysates were measured using a Luciferase assay kit (Promega). Luciferase activity was measured in a scintillation counter (Canberra Packard) and the levels of the enzyme were expressed as counts per minute (cpm). A blank control, cell lysis buffer control, and uninfected L5F11 cell control were included in each assay.

IL-8 ELISA

IL-8 protein was assayed in duplicate by enzyme linked immunosorbent assay (ELISA) as described previously^{16 17} using a murine monoclonal antibody to IL-8 (Novartis) and phosphatase conjugated goat anti-IL-8 antibody (Novartis). Concentrations of IL-8 were determined from a standard curve of recombinant IL-8 (Novartis) and expressed in ng/ml.

Table 1 Interleukin 8 transcription induced by wild type and *cag* ORF isogenic mutant *H pylori* strains in L5F11 gastric epithelial cells

Strain	Allele	n	Luciferase (cpm × 10 ⁶)
26695	ORF10-WT	10	0.67 (0.08)
15-1	ORF10-insertion	8	0.65 (0.09)
16-1	ORF10-insertion	8	0.57 (0.09)
NCTC 11637	Unrelated <i>cag</i> +	10	2.22 (0.26)
8-1	Total <i>cag</i> deletion	10	0.12 (0.03)*
G50	Natural <i>cag</i> -	10	0.15 (0.02)*
TNF α		10	2.82 (0.25)

Comparison of the ability of *H pylori* isogenic mutant strains with insertions in parent strain 26695 and other negative and positive control strains to induce luciferase activity in L5F11. Luciferase activity is expressed as mean (SEM) after subtraction of background cell control values. The cytokine TNF α can induce interleukin 8 synthesis independent of *H pylori*.

* $p < 0.001$ v wild type 26695 strain.

cpm, counts per minute; TNF α , tumour necrosis factor α ; WT, wild type.

STATISTICAL ANALYSIS

Data are expressed as mean (SEM). The statistical significance of observed differences between the isogenic mutants and parent strain 26695 were analysed by Student's *t* test. A probability (*p*) value of < 0.05 was considered statistically significant.

Results

The ability of strain 26695 derivatives with simple insertion mutations in the 3' end of ORF10 to induce luciferase activity as a measure of IL-8 gene transcription in L5F11 cells was tested first (table 1). Consistent with our previous studies with the L5F11 cell line, *H pylori* strain 26695 induced a 3.3-fold significantly lower ($p < 0.001$) level of IL-8 gene transcription than the type strain NCTC 11637.²⁶ However, strain 26695 still induced some fourfold more IL-8 transcription than did its isogenic derivative lacking the entire *cag* PAI (8-1) or the wild type naturally *cag* PAI negative strain G50. The levels of IL-8 gene transcription induced in L5F11 cells by the mutant strains 15-1 and 16-1, with simple insertions at the 3' of ORF 10, were similar to that of the 26695 wild type (table 1). This result suggested that the VirD4 homologue, unlike VirB homologues studied earlier,^{11 12 19 26} might not be important for *H pylori* induced IL-8 synthesis, or that these insertions, which are near the 3' end of the gene, did not eliminate VirD4 function.

To examine these possibilities strain 26695 with a deletion that removed most of ORF10 was constructed. Coculture of L5F11 gastric epithelial cells with ORF10 deletion mutant recombinants 28-1, 28-2, and 28-3 (each probably identical to the others) resulted in a significant 2.8-fold increase ($p < 0.01$, 28-1; $p < 0.05$, 28-2 and 28-3) in IL-8 gene transcription compared with the parental wild type strain 26695 (fig 1). This stimulation contrasted markedly with the significant ($p < 0.001$) reduction in IL-8 gene transcription observed with the total *cag* PAI deletion strain 8-1 (fig 1) and with many other *cag* PAI insertion or deletion mutations.^{26 29}

To test that the increase in IL-8 transcription was associated with increased IL-8 protein secretion, culture supernatants were assayed

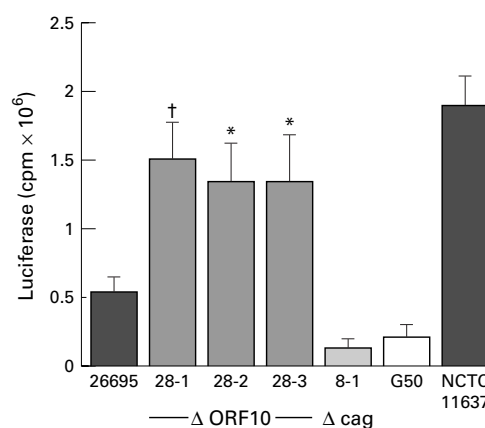


Figure 1 Comparison of the ability of *H pylori* isogenic mutant strains with deletions in ORF10 (28-1, 28-2, and 28-3) and the total *cag* PAI deletion mutant (8-1) and the parent strain 26695 and other positive and negative controls to induce luciferase activity in the L5F11 gastric epithelial cell line. G50 is a wild type *cag* PAI negative strain and NCTC 11637 is the *cag* PAI positive type strain. Values are means after subtraction of background cell control luciferase activity for nine independent experiments; error bars = SEM.

* $p < 0.05$; † $p < 0.01$ v 26695 parent strain.

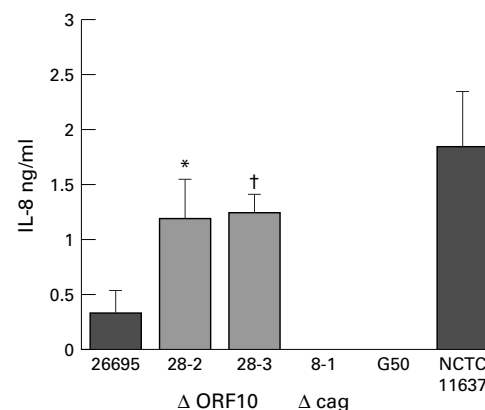


Figure 2 Comparison of the ability of *H pylori* isogenic mutant strains with deletions of ORF10 (28-2, 28-3) and the total *cag* PAI deletion mutant (8-1) and the parent strain 26695 to induce IL-8 secretion over 10 hours in the L5F11 gastric epithelial cell line. G50 is a wild type *cag* PAI negative strain and NCTC 11637 is the *cag* PAI positive type strain. Values are means of four independent experiments; error bars = SEM.

* $p < 0.05$; † $p < 0.01$ v 26695 parent strain.

for IL-8 by ELISA in a subset of experiments. No secretion of IL-8 was observed from L5F11 cultured in the absence of *H pylori*, or after culture with 26695 lacking the entire *cag* PAI (8-1), or with the wild type *cag* PAI negative *H pylori* strain G50 (fig 2). Secretion of IL-8 induced by NCTC 11637 was greater than that induced by strain 26695. The ORF 10 deletion mutants 26695 derivatives 28-2 and 28-3 induced significantly greater secretion of IL-8 (3.6-fold, $p < 0.05$, 28-2; $p < 0.01$, 28-3) than their isogenic parental strain 26695 (fig 2).

Discussion

Previous mutational studies have implicated the four *Agrobacterium* VirB homologues as well as several other *cag* PAI encoded proteins in the induction of IL-8 synthesis in gastric epithelial cells,^{11 12 26} and thereby the strong inflammatory response in host tissues. Inflam-

mation might be beneficial to *H pylori* if, for example, the bacteria feed on exudate from inflamed tissue.³⁰ However, strong unchecked inflammation could be deleterious to the bacteria, for example by direct action on them or eventual destruction of gastric epithelial tissue upon which *H pylori* persistence depends. We suggest that these opposing forces could have selected for the development of a sophisticated system in *H pylori* for modulation of the inflammatory response. The results reported here show that inactivation of ORF 10, the cag PAI gene in strain 26695 that encodes an *Agrobacterium* VirD4 homologue, results in increased induction of IL-8 synthesis in L5F11 gastric epithelial cells. This implicates the VirD4 homologue in the downregulation of the inflammatory response. Also illustrated in our data are differences in the innate abilities of different wild type cag+ strains to elicit IL-8 synthesis, confirming earlier studies.¹⁶⁻¹⁸ The NCTC 11637 type strain induced severalfold more IL-8 synthesis than did 26695 (the strain chosen for these studies because its genome has been completely sequenced²⁸).

The four *Agrobacterium* VirB proteins with homologues encoded in the cag PAI form part of a multiprotein pore through which other specific proteins or nucleic acids may pass in the bacterial membrane during delivery to target cells.²¹ The VirD4 protein is needed for such transfer, and is associated with the inner membrane of *Agrobacterium*, whereas the related bordetella system (which delivers pertussis toxin to target human cells) may function without such a VirD4 homologue.²¹ Adapting these models to *H pylori*, conceivably the VirD4 homologue achieves a regulatory role by modification of the potency or availability of the VirB homologue secretory machine or other critical membrane components. Alternatively the VirD4 homologue might stimulate production of anti-inflammatory agents such as the cytokine IL-10 in host cells. Clearly other models are also tenable, and the specific macromolecules from *H pylori* that stimulate IL-8 transcription in epithelial cells also remain to be identified.

Recent studies using rhesus monkeys as a human-like infection model have indicated diversity among individual hosts in susceptibility to particular *H pylori* strains, and a corresponding diversity among *H pylori* strains in their ability to colonise a given host either transiently or persistently.³¹⁻³² Similar transient infection has been documented occasionally in humans, and might result from clearance by inflammatory and immune responses.³³⁻³⁶ The finding of a patient with a mixture of cag+ and cag- *H pylori* strains, in whom cag- recombinant strains were most abundant,³⁷ suggests that strains that are less potent in turning on inflammatory responses might be selected in some persons. The host inflammatory cytokine response to *H pylori* may also differ markedly in intensity.³⁸ Anti-inflammatory cytokines such as IL-10 are likely to have important downregulatory functions in the gastric mucosa,³⁹ and gastric mRNA expression of IL-10 is preferentially stimulated by cag+

H pylori strains.⁴⁰ We would suggest that such human genetic and physiological diversity would select for a corresponding diversity in *H pylori* strains: cag- versus cag+ at one level, and then different signalling inductive capacities among those strains that are cag+. The level of inflammation elicited by a given cag+ strain in a given individual would be shaped in part by the balance of activities favouring induction of IL-8 synthesis (for example, those of the VirB homologue complex) compared with those that counteract it (for example, VirD4 and collaborating proteins).

CONCLUSIONS

Our studies have suggested that the product of ORF10, the virD4 homologue in the cag PAI of *H pylori*, helps modulate induction of IL-8 synthesis in gastric epithelial cells during *H pylori* infection. We propose that this balance between positive (VirB homologue based) and negative (VirD4 homologue based) factors helps to control this induction process, that it contributes importantly to the remarkable chronicity of established *H pylori* infection, and that it is a critical determinant of whether *H pylori* infection will or will not lead to the development of overt disease.

These studies were supported by the British Digestive Foundation, the European Commission (contract number IC18CT950024), Yorkshire Cancer Research, and the US National Institutes of Health (A138166, DK48029).

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