Isogenic mutants of the *cag* pathogenicity island of *Helicobacter pylori* in the mouse model of infection: effects on colonization efficiency

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Strains of *Helicobacter pylori* that contain the *cag* pathogenicity island (*cag* PAI) are associated with increased virulence and severe clinical outcomes. To evaluate the role of the *cag* island in infection, isogenic null mutations were generated in two clinical isolates (SS1 and Iris1) with distinct genetic backgrounds. When tested for their ability to establish infection in the stomach of CD1/SPF mice, at the early phase of infection, strains in which *cagE*, ORF528, ORF527 or ORF525 were inactivated showed a reduced capacity to initiate colonization compared to the wild-type strain. Strains with a mutation in the ORF524 gene were more efficient than the other mutants, but still less efficient than the wild-type strain. Mutation in the effector protein, CagA, which is injected into host cells and tyrosine-phosphorylated, did not change the colonization efficiency. In conclusion, all *cag* genes analysed, with the exception of the effector protein, CagA, influenced the early phase of colonization in the mouse model of infection. These results suggest that the structure of the *H. pylori* secretion apparatus itself is involved in this process.

Keywords: virulence, type IV secretion system, gastric pathogen

INTRODUCTION

The micro-aerophilic, rod-shaped gastric pathogen *Helicobacter pylori* causes gastritis, peptic ulcers and increases the risk of developing gastric tumours in humans (Parsonnet *et al.*, 1991; Blaser, 1992; Mitchell, 1993; Logan, 1994; NIH consensus 1994). Genetic analysis of *H. pylori* revealed the presence of a pathogenicity island (PAI) named *cag* in the genome of a subset of strains (Censini *et al.*, 1996; Akopyants *et al.*, 1998). It has been shown that patients with overt clinical outcomes are infected with strains carrying the *cag* PAI (type I strains) and that strains that lack the *cag* PAI (type II strains) are responsible for milder clinical outcomes (Telford *et al.*, 1994; Marchetti *et al.*, 1995). *In vitro* experiments showed that the *cag* PAI plays an important role in enhancing bacterial virulence. Fol-

lowing intimate contact between the host cell and the pathogen, *cag* induces the secretion of IL-8 from eukaryotic cells and activates the nuclear transcriptional factor NF- κ B (Censini *et al.*, 1996; Segal *et al.*, 1997; Akopyants *et al.*, 1998; Glocker *et al.*, 1998). In addition, it has been shown that impairment of IL-8 secretion may affect bacterial colonization efficiency in Balb/c mice (van Doorn *et al.*, 1999). However, no causal relationship between the absence of IL-8 induction and the ability of higher colonization efficiency can be concluded since this observation seems restricted to the particular aptotype of Balb/c mice.

It has been shown that six *cag* genes encode proteins that are homologous to components of the multiprotein complexes involved in the bacterial conjugation-transport system (type IV secretion system). These include the *vir* operon of *Agrobacterium tumefaciens*, the *ptl* transporter apparatus of *Bordetella pertussis* and the *tra* system of *Escherichia coli* (Reeves *et al.*, 1994; Christie, 1997; Winans *et al.*, 1996; Zupan *et al.*, 1998; Hacker *et al.*, 1997; Weiss *et al.*, 1993; Censini *et al.*, 1996). Other VirB homologues have been identified in *Brucella suis*

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Abbreviation: PAI, pathogenicity island.

and *Legionella pneumophila*, and are important for the infection of human macrophages *in vitro* (O'Callaghan *et al.*, 1999) and for the transfer of plasmid DNA (Vogel *et al.*, 1998; Segal & Shuman, 1999), respectively. In addition to these homologies, it has been shown that *cag* is necessary for the transfer of the CagA protein into epithelial gastric cells *in vitro* (Segal *et al.*, 1999; Odenbreit *et al.*, 2000; Stein *et al.*, 2000), which further indicates that *cag* encodes a secretion apparatus that contributes to *H. pylori* virulence. It has been shown that mice can be experimentally infected by fresh clinical isolates of *H. pylori* (Karita *et al.*, 1991; Marchetti *et al.*, 1995; Lee *et al.*, 1997). This model has been extensively used because it reproduces human *H. pylori*-associated gastric disease (Marchetti *et al.*, 1995; Lee, 1998). Central to this study has been the use of this *in vivo* assay, the mouse model of infection, as a biological filter to test the importance of *cag* in the initial step of gastric colonization. We used two recipient type I *H. pylori* strains to construct defined isogenic mutants missing components of the secretion apparatus, i.e. *cagE* (homologous to *virB4*), ORF528 (homologous to *virB9*), ORF527 (homologous to *virB10*), ORF525 (homologous to *virB11*), ORF524 (homologous to *virD4*) and the secreted CagA protein. Each gene was deleted individually and the mutants were analysed for their ability to colonize the stomach of mice during the early stages of colonization.

METHODS

***H. pylori* strains and culture conditions.** *H. pylori* strain Iris1 was recovered from human gastric biopsies obtained from the Medical Pathology Department, Siena Hospital, Siena, Italy. The strain was analysed for the presence of *cag* PAI by PCR analysis and tested for its ability to infect mice. The primers used to analyse the structure of the *cag* PAI are listed in Table 1. PCR amplification was performed under standard conditions. The *vacA* gene was analysed by sequencing the mid-region (m1 and m2) as described by Atherton *et al.* (1995) and Pagliaccia *et al.* (1998). *H. pylori* strain SS1 is an Australian type I strain obtained by Dr A. Lee (University of New South Wales, Australia) and capable of colonizing the gastric mucosa of mice at high density (Lee *et al.*, 1997). These strains were used to obtain isogenic mutants for infection experiments. *H. pylori* CCUG 17874 was obtained from the University of Gotheberg and used as a representative laboratory strain that is unable to colonize mice. Table 2 shows the phenotypic and genotypic characteristics of these three strains. Bacteria were grown on Columbia agar plates supplemented with 10% horse blood plus vancomycin (10 µg ml⁻¹), cefsulodin (5 µg ml⁻¹), polymyxin B (2.5 U ml⁻¹), cyclohexamide (50 µg ml⁻¹), trimethoprim (5 µg ml⁻¹), amphotericin B (8 µg ml⁻¹), β-cyclodextrin (0.2%) and bacitracin (200 µg ml⁻¹) using the Anaerojar system with the Campygen atmosphere-generating system (Oxoid). For kanamycin-resistant bacteria, plates were supplemented with kanamycin (20 µg ml⁻¹).

Animal colonization. Six 1-week-old CD1/SPF (Specific-Pathogen-Free) male mice were purchased from Charles River and kept in a 12 h light/dark schedule in an air-conditioned animal facility. Water and food was provided *ad libitum*. Mice were fasted for 24 h immediately before inoculation and being sacrificed. Each mouse received 250 µl sodium bicarbonate

oro-gastrically to neutralize gastric acid, according to the protocol described previously (Marchetti *et al.*, 1995). Half an hour later, 1 × 10⁹ c.f.u. bacteria in 150 µl sterile PBS was administered to each mouse through the same route. As a control, a similar group of mice was inoculated with PBS alone. Mice were sacrificed by cervical dislocation at pre-determined times and colonization was assessed. Animals were inoculated and cared for according to institutional guidelines.

Assessment of *H. pylori* colonization. The stomach of sacrificed mice was removed and opened through the lesser curvature. The number of viable infecting bacteria was estimated by making serial dilutions of the homogenized tissue in sterile PBS and plating on blood agar plates supplemented with bacitracin alone or supplemented with specific selective antibiotics and incubated for 3–5 days at 37 °C in a micro-aerophilic jar. Growing bacteria were identified by Gram-staining and morphology. The remaining material was immediately frozen in liquid nitrogen and stored at –80 °C until required.

PCR on gastric samples. The frozen samples were defrosted and DNA was extracted as described by Clayton *et al.* (1991); 5 µl of the recovered material was used for PCR with D008/R008 *cagA*-specific primers (Xiang *et al.*, 1995; Table 1). Gene amplification was carried out in a Perkin-Elmer thermal cycler for 35 cycles. Each cycle consisted of a denaturation step at 94 °C for 5 min, a primer annealing step at 60 °C for 30 s and an extension step at 72 °C for 30 s.

Construction of *H. pylori* isogenic mutants. Isogenic SS1 and Iris1 *cagA* mutants were obtained by natural transformation with the clone A plasmid obtained by Antonello Covacci (Chiron Vaccines) and described by Xiang *et al.* (1995). The other *cag* mutations were constructed by inserting a non-polar kanamycin resistance determinant, *aphA-3'* of *Campylobacter coli*, recovered using the *Pst*I restriction site from a plasmid generously donated by Antonello Covacci (Chiron Vaccines). Primers (Table 3) were constructed by use of an Applied Biosystems synthesizer and the automated phosphoramidite coupling method. For each gene we used PCR to amplify two fragments containing *Kpn*I/*Pst*I and *Pst*I/*Not*I restriction sites at the ends. These fragments were cloned into the *Kpn*I/*Not*I site of pBluescript SK+ (Stratagene). The kanamycin cassette was cloned into the *Pst*I site of the gene. The final constructs were transformed into competent *E. coli* DH10B (Gibco BRL). Each recombinant plasmid (Table 2) was sequenced with an ABI Prism Dye Terminator Sequencing Kit (Applied Biosystems). The purified DNA was used to mutagenize the wild-type locus by allelic exchange with the recipient SS1 and Iris1 strains. The isogenic mutants were selected by plating on selective media. DNA was extracted from three colonies for each mutant and stored at –80 °C. The extracted DNA was used for PCR analysis with primers listed in Table 4 to confirm the recombination event. A single recombinant clone for each mutant was then used for each infection experiment.

Production of fusion proteins and polyclonal antibodies. We used the *H. pylori* genome sequence to construct the fusion proteins for ORF528, ORF527, ORF525 and ORF524 (Tomb *et al.*, 1997). Each of the fusion proteins was constructed by use of the QIA-expression system (Qiagen). Briefly, the hydrophilic region of each ORF [according to the Kyte–Doolittle analysis (Kyte & Doolittle, 1982)] was amplified by PCR with specific primers (Table 5). The amplified fragments were cloned into the pQE30 expression vector containing a

Table 1. Primers used to study the *cag* PAI structure

Oligonucleotide	Corresponding ORF	Strand	Sequence (5'–3')
D008	<i>cagA</i>	+	ATAATGCTAAATTAGACAACCTTGAGCGA
R008	<i>cagA</i>	–	TTAGAATAATCAACAAACATCACGCCAT
E1	<i>cagE</i>	–	TCAAGCCTATGAACCTGTTTT
E2	<i>cagE</i>	+	GTCTTCTCTGTTTGGATTTAAG
B1	ORF525	–	AACCATTTGATGTGAGAGACAG
B2	ORF525	+	GTTTCATCATTAACCTGTAACAG
E64T	Middle zone (intervening sequence)	+	GATAACAGCGCGATAATCTTTCAT
HR6	Middle zone (intervening sequence)	–	GTTTGATCAACGAGGCAATAGATT
9KPFa	ORF528	–	CTCGGTAAAAAGT
9PNRb	ORF528	+	AAAATTGCTCTTAGTTT
10KPFa	ORF527	–	AACAGGAATGCGAG
10PNRb	ORF527	+	CGCCACTATATCCTCTAGA
4KPFa	ORF524	–	CGGAAGACTTT
4PNRb	ORF524	+	TTCGCTTGAACCCACAG

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Characteristics	Reference or source
<i>H. pylori</i>			
CCUG 17874	Type I; <i>cag</i> ⁺ <i>vacA</i> ⁺ (m1)	Laboratory strain, unable to infect mice	Gotheberg
SS1	Type I; <i>cag</i> ⁺ ORF7 [–] <i>vacA</i> ⁺ (m2)	Competent for natural transformation, able to infect mice	Lee <i>et al.</i> (1997); Salama <i>et al.</i> (2000)
Iris1	Type I; <i>cag</i> ⁺ <i>vacA</i> ⁺ (m2)	Fresh isolate, competent for natural transformation, able to infect mice	This study
<i>E. coli</i>			
DH10B	F [–] <i>mrcAΔ</i> <i>φ80d lacZΔM15 ΔlacX74 deoR(ara leu)7697 recA1 endA1 araD139 galU galKd rpsL nupG rpsL λ[–]</i>		Gibco BRL
M15(pREP4)	Nal ^s Str ^s Rif ^s Thi [–] Lac [–] Ara ⁺ Kan ^r Gal ⁺ Mtl [–] F [–] RecA ⁺ Uvr ⁺ Lon ⁺		Qiagen
Plasmids			
pBluescript SK + p9K9	pUC19-derived; ColE1 <i>ori lacZ</i> Amp ^r ; cloning vector pBluescript SK + containing the ORF528 gene with the aphA-3' Kan ^r cassette inserted		Stratagene This study
p10K10	pBluescript SK + containing the ORF527 gene with the aphA-3' Kan ^r cassette inserted		This study
p11K11	pBluescript SK + containing the ORF525 gene with the aphA-3' Kan ^r cassette inserted		This study
p4K4	pBluescript SK + containing the ORF524 gene with the aphA-3' Kan ^r cassette inserted		This study
pQE30	ColE1 <i>ori bla</i> Amp ^r <i>lacZ</i> 6×His tag; cloning vector		Qiagen
pQE9K9	pQE30 containing the hydrophilic region of ORF528		This study
pQE10K10	pQE30 containing the hydrophilic region of ORF527		This study
pQE11K11	pQE30 containing the hydrophilic region of ORF525		This study
pQE4K4	pQE30 containing the hydrophilic region of ORF524		This study

Table 3. Oligonucleotide primers used for PCR, cloning and sequencing

Lower case letters in the primers sequences indicate cleavage recognition sequence. Underlining indicates sites for *KpnI* (GGTACC), *PstI* (CTGCAG) and *NotI* (GCGGCCGC) that were added at the 5' end to create restriction sites.

Oligonucleotide	Corresponding ORF	Strand	Sequence (5'–3')
EK	<i>cagE</i>	–	ccggGGTACCAATGGGCTTGTATTATACAAAT
EPS	<i>cagE</i>	+	tgcaCTGCAGACAAACACCTCTCTCTCTT
EP	<i>cagE</i>	–	tgcaCTGCAGAGCTAATTATAGAGCAAGAG
EN	<i>cagE</i>	+	ccttGCGGCCGCTAAGTGGATGTCATTAGCCAT
9KPF	ORF528	–	ccggGGTACCTCGGTAAAAAGT
9KPR	ORF528	+	tgcaCTGCAGCGCTTGGTAGTAATTGTA
9PNF	ORF528	–	tgcaCTGCAGCCTGAAAAACGCTCTAAACATATT
9PNR	ORF528	+	ccttGCGGCCGCAAAATTGCTCTTAGTTTCTGC
10KPF	ORF527	–	ccggGGTACCAACAGGAATGCGAG
10KPR	ORF527	+	tgcaCTGCAGTCGTCAATGGGATTACCATTTTCT
10PNF	ORF527	–	tgcaCTGCAGTAAAAAGAAAGAAAGAAAAACAAG
10PNR	ORF527	+	ccttGCGGCCGCGCCACTATATCCTCTAGAAGA
11KPF	ORF525	–	ccggGGTACCGTTTCAATG
11KPR	ORF525	+	tgcaCTGCAGCAACCTTGTT
11PNF	ORF525	–	tgcaCTGCAGGGCGAATGGCAACCATTGATGTG
11PNR	ORF525	+	ccttGCGGCCGCGGACAATCATATCAAATCAAAT
4KPF	ORF524	–	ccggGGTACCGGAAGACTTT
4KPR	ORF524	+	tgcaCTGCAGACATGAGATC
4PNF	ORF524	–	tgcaCTGCAGGGACTAAAAAGGGGCTTGAGTTTG
4PNR	ORF524	+	ccttGCGGCCGCTTCGCTTGAACCCACAGGCACT

Table 4. Oligonucleotide primers designed to check for *cag* inactivation

Oligonucleotide	Corresponding ORF	Strand	Sequence (5'–3')
AcloneF	<i>cagA</i>	+	ATCCTCTCGTTTCCTTAGAGA
AcloneR	<i>cagA</i>	–	CACAGGCAATTATGATGAAGT
EPCRf	<i>cagE</i>	–	ACATATTAGCACCGTAGAGTC
EPCRr	<i>cagE</i>	+	TGTTTCATCATCAGCCTGCTGCT
9FTEST	ORF528	–	AGTGAATGTGCCATCATCAA
9RTEST	ORF528	+	CAATATGTGCATAATGCATT
10FTEST	ORF527	–	GGCCTGTTTGACAGGGCTTGT
10RTEST	ORF527	+	AAATATCAGTGAAATCGCTCTTA
11FTEST	ORF525	–	GGCTTTCCTGTCTCTCACAT
11RTEST	ORF525	+	ACTGAGATTTGTTACAATGG
4FTEST	ORF524	–	TTTGACAAACTCAAGCCCCT
4PCRr	ORF524	+	TAGCAATCAAGCGCGAAATCT

6× His affinity tag and electroporated into the K12 derivative *E. coli* M15(pREP4) (Table 2) (Villarejo & Zabin, 1974). IPTG was added to induce the expression of the recombinant proteins and the fusion protein was purified by binding the affinity tag to Ni-NTA resin. Polyclonal antibodies were obtained from rabbits immunized with each of the purified recombinant proteins (50 µg per dose + MF59, four doses), according to standard protocols, and used for Western blot analysis. Wild-type and mutant *H. pylori* strains were grown on blood agar plates, recovered in PBS and boiled in the presence of SDS-PAGE loading buffer. The bacterial lysates

were then used for Western blot analysis. Antisera (diluted 1:5000) and a peroxidase-labelled goat anti-rabbit IgG (H + L) (Gibco BRL) were used for SDS-PAGE immunoblot analysis. The immunoblot was developed by the ECL Western blotting detection system (Amersham).

Bacterial transformation. All the *H. pylori* mutants were obtained following the transformation protocol described by Copass *et al.* (1997). Briefly, bacteria were grown on blood agar plates for 48 h and then streaked onto fresh non-selective plates and incubated for 6 h. DNA (1 µg) was then mixed with the growing bacteria. Plates were incubated for a further 16 h

Table 5. Oligonucleotide primers designed to obtain fusion proteins

Underlined sequences indicate sites for *Kpn*I (GGTACC) and *Pst*I (CTGCAG). Lower case letters indicate the cleavage recognition sequence.

Oligonucleotide	Corresponding ORF	Strand	Position on the reference sequence*	Sequence (5'–3')
9BPF	ORF528	–	17713	cgg <u>GGTACCT</u> CGGTAAAAAGTAATCTC
9BPR	ORF528	+	16795	tgca <u>CTGCAGC</u> GCTTGGTAGTAATTGTA
10BPF	ORF527	–	12826	cgg <u>GGTACCA</u> AGTGTTTGGATGTTTGAGCGA
10BPR	ORF527	+	12050	tgca <u>CTGCAGAC</u> GTCATGGGATTACCATTTTCA
11BPF	ORF525	–	10515	cgg <u>GGTACCG</u> TTTCAATG
11BPR	ORF525	+	10352	tgca <u>CTGCAGCA</u> ACCTTGTT
4BPF	ORF524	+	8392	cgg <u>GGTACCG</u> GAAGACTTT
4BPR	ORF524	–	7276	tgca <u>CTGCAGAC</u> ATGAATC

* The position refers to the reference sequence from Akopyants *et al.* (1998).

in a controlled micro-aerophilic atmosphere (8% O₂/10% CO₂). Bacteria were transferred onto selective plates and grown in an anaerobic jar. DNA was extracted from the single colonies that appeared after 3–6 days and analysed.

RESULTS

Status of *cag* PAI of the *H. pylori* strains used in infection experiments

We analysed *H. pylori* strain Iris1 for the structure of the *cag* PAI by PCR analysis, looking for the presence of *cagA*, *cagE*, ORF527, ORF528, ORF524 and ORF525 (Table 1). These genes were chosen because they are well distributed over the *cag* PAI. The presence of an interrupted or uninterrupted *cag* region was investigated by PCR using primers directed to the region flanking IS605. If IS605 was present, we would expect a band of 2100 bp; in the case of fused *cagI* and *cagII* regions, the resulting band would be 600 bp. Finally, if the intervening sequence divided the two *cag* regions, the PCR could not be completed because of the size of the fragment to be amplified. Strain Iris1 turned out to be a type I strain (*cag*⁺) with an uninterrupted *cag* region. Strain SS1, the other strain used in infection experiments, has been shown to have a *cag* PAI lacking ORF 7 (*cag*⁺ ORF7[–]) (Salama *et al.*, 2000; Table 2). From the functional point of view, SS1 has been shown to be unable to induce an IL-8 response (van Doorn *et al.*, 1999).

Construction of *H. pylori* isogenic mutants

Mutations within the *cagA*, ORF528, ORF527, ORF525 and ORF524 genes were constructed in two type I *H. pylori* strains (Iris1 and SS1). After natural transformation, using the appropriate plasmid, each clone was selected by plating on kanamycin blood agar plates. PCR was used to verify that the gene had been inactivated by insertion of the kanamycin cassette using primers mapping on chromosomal DNA just up- and

Fig. 1. PCR analysis of the SS1 parent and transformants. Each pair of primers maps just up- and downstream of the cloned kanamycin cassette of each specific gene. For each isogenic mutant we amplified a band of 1400 bp corresponding to the size of the kanamycin cassette, indicating that it had been correctly inserted.

downstream of the kanamycin insertion site in each gene (Table 4). As shown in Fig. 1, each mutant had a PCR product of 1.4 kb, indicating that the gene had been inactivated by the insertion of the kanamycin cassette.

Fig. 2. Immunoblots of ORF527 (a), ORF528 (b), ORF525 (c) and ORF524 (d) proteins in cell extracts of the Iris1 wild-type and the corresponding isogenic mutants. Arrows indicate the band corresponding to the protein recognized by specific polyclonal antibodies.

The recombinant colony, one from each mutant, was then chosen and used for colonization experiments.

Phenotypic characterization of *H. pylori* *cag* gene mutants

To test whether the *cagA*, *cagE*, ORF528, ORF527, ORF525 and ORF524 mutants had lost the ability to express the antigen encoded by the corresponding gene, we used immunoblot analysis of the whole-cell extracts. In each case, the corresponding antigen was present in the parental strain, but absent from the isogenic mutant. Fig. 2 shows representative immunoblot assays obtained for Iris1. The same results were obtained for SS1 (data not shown). In the case of ORF527, the specific antibodies recognized, as predicted, a 189 kDa band and an additional 36 kDa band. These bands were absent from the Δ ORF527 mutant (Fig. 2a). In the Δ ORF527 mutant (Fig. 2a) a lower band was seen. Although we do not have an explanation for this band, we believe it is due to the extreme development of the cross-reacting band of similar molecular mass in the wild-type strain. Antibodies raised against the fusion protein for ORF528 identified a 56 kDa protein. This corresponds to the predicted size of the VirB9 protein, which is expressed by the wild-type strain, but not by the Δ ORF528 mutant (Fig. 2b). Anti-ORF525 antibodies

recognized a 32 kDa protein in the wild-type, but not in the mutant (Fig. 2c). Finally, polyclonal antisera obtained by immunizing rabbits with a fusion protein constructed for ORF524 recognized a 90 kDa band in the wild-type bacterial lysate, but not in the isogenic mutant strain (Fig. 2d). To test whether the mutations affected the growth capacity of the organisms, the mutants were cultured in blood agar plates and in liquid medium. The growth capacity of the mutants did not differ when compared to the corresponding wild-type strains (not shown).

Virulence of the isogenic mutants *in vivo*

To test the ability of each *cag* mutant to colonize the stomach of CD1/SPF mice, the six mutants and the corresponding parental strains were used to inoculate 10 mice for each bacterial strain in two independent experiments. The laboratory strain CCUG 17874, that is unable to establish infection (Marchetti *et al.*, 1995), was used as negative control. The SS1 and Iris1 wild-type strains and relative mutant strains had a similar number of *in vitro* passages before being used for infection experiments to avoid the possibility that repetitive laboratory passages, in particular for the mutant strains, influenced their ability to colonize. Infection was assessed 10 days after the last bacterial inoculum by quantifying the number of bacteria in the stomach of each mouse after homogenization and plating out serial dilutions. As expected, none of the control mice inoculated with the laboratory strain CCUG 17874 were infected. Comparison of the two wild-type strains revealed that the colonization efficiency of SS1 was higher than that of Iris1 (Fig. 3). Infection with Iris1 resulted in approximately 8.9×10^4 c.f.u. (g wet wt gastric mucosa)⁻¹, whereas infection with SS1 resulted in three times more c.f.u. [2.5×10^5 c.f.u. (g wet wt gastric mucosa)⁻¹]. In all the experiments between 90 and 100% of the mice inoculated with the wild-type strains or with the *cagA* mutants were infected. Remarkably, only a mean of 20–40% of the mice inoculated with the other *cag* mutants were infected (data not shown). The degree of colonization in mice that had been infected with the *cagA* mutant was not significantly lower than in the corresponding parental strains. In marked contrast, ablation of the *cagE* (*virB4*), ORF527 (*virB10*) and ORF528 (*virB9*) genes significantly reduced the number of colonies recovered from infected mice in both strains (Fig. 3). When the Iris1 Δ *cagE* mutant was used a 10³-fold reduction in infection was observed. Following bacterial inoculation with SS1 Δ *cagE*, we recovered a mean of 90 c.f.u. (g wet wt gastric mucosa)⁻¹ and no colonies were recovered from most of the inoculated mice (83%). The level of infection following inoculation with the Iris1 Δ ORF528 (*virB9*) mutant strain was about 1000-fold lower than with the corresponding wild-type. The colonization rate of SS1 Δ ORF528 was approximately 1300-fold lower [1.9×10^2 vs 2.5×10^5 c.f.u. (g wet wt gastric mucosa)⁻¹] than that of the corresponding wild-type. When ORF527 (*virB10*) was mutated the number of colonies recovered

Fig. 3. Colonization phenotype of the *H. pylori* wild-type strains and isogenic mutants. Groups of mice were infected and then killed 10 days after infection. Half of the stomach from each mouse was homogenized and dilutions plated on blood agar plates. The values shown represent the c.f.u. recovered. Results were obtained with SS1 (a) and Iris1 (b) and the corresponding isogenic mutants, respectively. Each value is the mean $\pm$ SD of all mice that received the same bacterial inoculum in two independent experiments.

was approximately 1000-fold lower for the SS1 mutant and 300-fold lower for the Iris1 mutant than for the corresponding wild-type strains. The colonization efficiency of the SS1 Δ ORF525 mutant was approximately 200-fold lower than that of the corresponding parental strain. The colonization efficiency of SS1 Δ ORF524 (*virD4*) was 40 times lower than in the wild-type and that of the Iris1 mutant was about 20 times lower.

Assessment of colonization by PCR

The infection experiments revealed that the number of mice infected with the *cag* mutant strains, except for *cagA*, was always lower than the number of mice infected with the wild-type strains. Thus, we decided to use a more sensitive system to reveal the presence of very small numbers of infecting organisms. Chromosomal DNA was extracted from the gastric tissue of infected

Fig. 4. Assessment of colonization by PCR. PCR was performed on DNA extracted from the homogenized tissue of half of the stomach of infected mice (see Methods). Specific primers for *cagA* were used (D008/R008). (a) PCR on mice infected with the *H. pylori* CCUG 17874. (b) Representative PCR on mice inoculated with SS1 wild-type and SS1 Δ *cagE*; in this case the amplification was used for Southern blotting with a *cagA* probe (c). SS1 DNA was used as positive control (C+).

animals (see Methods) and PCR was performed with primers specific for *cagA* (D008/R008) (Xiang *et al.*, 1995; Table 1). Fig. 4 shows the results obtained after analysing the stomachs of two representative mice infected with SS1 and positive for colony recovery and the isogenic mutant SS1 Δ *cagE* in which we could not recover any colonies on plates. Mice inoculated with the laboratory strain CCUG 17874 were used as control because this strain is unable to infect mice. From the

gastric tissue of half a mouse stomach we could always recover approximately $100 \mu\text{g}$ DNA ml^{-1} and 100 ng was used for PCR amplification. As expected, we did not get any PCR product from mice that received strain CCUG 17874, confirming that this laboratory strain cannot infect mice (Fig. 4a). When we analysed mice infected with SS1 and the ΔcagE mutant, we found that all the mice sacrificed 10 days after infection gave a positive signal (Fig. 4b) and that the band was stronger for mice inoculated with wild-type SS1 than for those that received the ΔcagE mutant. We also performed Southern blot hybridization using *cagA* as probe to confirm that the PCR reaction was specific (Fig. 4c). This indicated that in these mice the infection was strongly reduced, but not abolished, and that the low number of infecting organisms could be detected only by this more sensitive system.

DISCUSSION

Since their discovery, PAIs have generated great interest and they have been described in an increasing number of pathogens (Springer & Goebel, 1980; Hacker *et al.*, 1997). In 1996, a pathogenicity island was identified in the human gastric pathogen *H. pylori* (Censini *et al.*, 1996). On the basis of the presence or absence of the *cag* PAI, *H. pylori* strains are now grouped in two large families: type I and type II, respectively (Censini *et al.*, 1996; Akyopants *et al.*, 1998). The presence of *cag* PAI in the bacterial genome is associated with more severe clinical outcomes (Telford *et al.*, 1994; Marchetti *et al.*, 1995). Moreover, its product is homologous to components of the multiprotein complexes found in *A. tumefaciens* and *B. pertussis* (type IV secretion system), and it is necessary for the delivery of the CagA protein into epithelial gastric cells *in vitro* (Segal *et al.*, 1999; Odenbreit *et al.*, 2000; Stein *et al.*, 2000).

We selected two strains: Iris1, isolated by us from a clinical sample, and SS1, well described in literature. Both of them are *cag*⁺ as demonstrated by genetic analysis with SSI lacking ORF7. These two *H. pylori* strains were used in our experimental infections and the colonization phenotype of the *cagA*, *cagE*, ORF528, ORF527, ORF525 and ORF524 mutants has been assessed in the mouse model of infection. The results show that these genes can influence bacterial colonization in our mouse model of infection. The mutants did not show significant differences in growth under laboratory conditions, suggesting that the observed phenotype is strictly *in vivo*-dependent. The colonization phenotype was assessed during the early stages of infection, 10 days after inoculation. This time was chosen according to the results obtained for non-motile flagellin mutant strains, showing that a time shorter than 10 days could give false results for the bacterial colonization phenotype (Eaton *et al.*, 1996; Kim *et al.*, 1999). The colonization of the *cag* mutants normalized following a longer infection period (data not shown). This is consistent with the results obtained by Eaton *et al.*

(2001) and Ogura *et al.* (2000) and suggests that the reduced colonization density observed for the *cagE*, ORF528, ORF527, ORF525 and ORF524 mutants could be related to the function(s) that these components may play in the initial step of the colonization process. However, the mechanism responsible for the observed phenotype is not known. We showed that the inactivation of these *cag* genes did not abolish infection but that it was sufficient to diminish their ability to establish infection. The inactivation of the *cag* genes may disrupt the *cag* system, thus reducing bacterial virulence. As a consequence, bacteria need more time before they recover their normal colonization efficiency. However, not all of the *cag* genes are involved in the observed phenotype, and mutations of *cagA*, encoding the secreted protein CagA, did not significantly affect the bacterial density. This observation is consistent with previous findings that demonstrated that *cagA* mutants are able to infect animal models with the same efficiency as the wild-type strain (Wirth *et al.*, 1998).

Considering the short time course of infection, we could not expect any histopathological change in the gastric tissue of mice infected with the wild-type or with the mutant strains. Other studies have shown that gastritis induced by wild-type strains is similar to that induced by *H. pylori cagA* mutants in the first weeks of infection (Ghiara & Marchetti, 1998). Two *H. pylori* strains were used in parallel in the infection experiments. Mutants for the same genes in all the wild-type strains showed a comparable colonization phenotype. This suggests that the *in vivo cag*-dependent phenotype is independent of the bacterial genetic background.

In conclusion, we have shown that the presence of *cag*, at least for some of the *cag* genes, increases the ability of type I strains to colonize the stomach of mice. The mechanisms involved and the pathological traits associated with these *cag* genes *in vivo* remain unknown.

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