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Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*

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Summary

Although successful and persistent colonization of the gastric mucosa depends on the ability to respond to changing environmental conditions and co-ordinate the expression of virulence factors during the course of infection, *Helicobacter pylori* possesses relatively few transcriptional regulators. We therefore investigated the contribution of the regulatory protein CsrA to global gene regulation in this important human pathogen. CsrA was necessary for full motility and survival of *H. pylori* under conditions of oxidative stress. Loss of *csrA* expression deregulated the oxidant-induced transcriptional responses of *napA* and *ahpC*, the acid induction of *napA*, *cagA*, *vacA*, the urease operon, and *fur*, as well as the heat shock responses of *napA*, *groESL* and *hspR*. Although the level of *napA* transcript was higher in the *csrA* mutant, its stability was similar in the wild-type and mutant strains, and less NapA protein was produced in the mutant strain. Finally, *H. pylori* strains deficient in the production of CsrA were significantly attenuated for virulence in a mouse model of infection. This work provides evidence that CsrA has a broad role in regulating the physiology of *H. pylori* in response to environmental stimuli, and may be important in facilitating adaptation to the different environments encountered during colonization of the gastric mucosa. Furthermore, CsrA appears to mediate its effects in *H. pylori* at the post-transcriptional level by influencing the processing and translation of target transcripts, with minimal effect on the stability of the target mRNAs.

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Introduction

Helicobacter pylori colonizes the stomachs of approximately half the world's population and is associated with peptic ulceration and gastric malignancy. Although a number of factors have been shown to be essential for colonization and the induction of mucosal damage (Suerbaum and Michetti, 2002), little is known of the molecular mechanisms that regulate the co-ordinated expression of genes responsible for these functions. Sequence analysis of the genomes of two *H. pylori* isolates (26695 and J99) has revealed a low abundance of regulatory networks when compared with other Gram-negative bacteria (Tomb *et al*., 1997; Alm *et al*., 1999). *H. pylori* possesses only three sigma factors RpoD (σ^{80}), RpoN (σ^{54}) and FliA (σ^{28}), and lacks homologues of the stress-response sigma factors, RpoS (σ^{38}) and RpoH (σ^{32}). There are relatively few two-component regulatory systems, with only four histidine kinases with their cognate response regulators as well as two orphan response regulators (Beier and Frank, 2000). Of the handful of other transcriptional regulators, HspR represses expression of the major chaperones of *H. pylori* (Spohn and Scarlato, 1999), and the ferric uptake regulator (Fur) functions in the regulation of iron homeostasis (Bereswill *et al*., 2000; Delany *et al*., 2001), as well as acid resistance, the nickel-responsive induction of urease expression and the regulation of the amidase and formamidase enzymes (Bijlsma *et al*., 2002; Van Vliet *et al*. 2002; 2003).

The paucity of regulatory systems may reflect the fact that *H. pylori* survives in the relatively restricted niche of the human gastric mucosa, with an apparent lack of competition from other microorganisms (Berg *et al*., 1997). However, the human stomach is far from a stable habitat, experiencing considerable fluctuations in nutrient availability, reactive oxygen species, pH and temperature, and a certain degree of adaptability would be expected to allow successful and persistent colonization. Furthermore, it is clear that *H. pylori* is able to sense and respond to diverse environmental stimuli despite the absence of

classic global regulators (Spiegelhalder *et al*., 1993; Skouloubris *et al*., 1998; Ramarao *et al*., 2000; Baker *et al*., 2001; Bijlsma *et al*., 2002). This, coupled with its ability to modulate virulence gene expression during the late log-to-stationary phase transition (Thompson *et al*., 2003) as well as during the course of infection (Blom *et al*., 2002), suggests that alternative regulatory networks are of crucial importance for survival within the gastric milieu.

Post-transcriptional regulation, through the control of mRNA stability, antisense RNA transcripts and inhibition of translation, are increasingly recognized as critical determinants of bacterial gene expression (Delihas, 1995; Regnier and Arraiano, 2000; Takayama and Kjelleberg, 2000). In *H. pylori*, there is evidence that gene expression can be regulated post-transcriptionally in response to environmental stimuli by differential mRNA processing and degradation. Transcription from the two urease promoters, P_{ureA} and P_{ureI}, is followed by pH-dependent differential mRNA decay, and this process may allow fine tuning of urease expression (Akada *et al*., 2000). Stability of the major chaperone-encoding operons of *H. pylori*, also appears to be important in defining the HspR-mediated response to sudden increases of temperature (Spohn *et al*., 2002).

The carbon storage regulator, CsrA, is a post-transcriptional regulator that was originally described as a repressor of a variety of stationary phase genes in *Escherichia coli* (Romeo *et al*., 1993; Romeo, 1998). CsrA has since been shown to function as a global regulatory protein that represses glycogen synthesis and catabolism, gluconeogenesis and biofilm formation, and activates glycolysis, motility and flagellum biosynthesis (Romeo *et al*., 1993; Romeo, 1998; Wei *et al*., 2001; Baker *et al*., 2002; Jackson *et al*., 2002). Homologues of *csrA* exhibit a broad phylogenetic distribution within the eubacterial domain and have been retained in preference to other regulatory genes by highly adapted pathogens such as *H. pylori* and *Borrelia burgdorferi* (Romeo, 1998). In *Salmonella enterica* serovar Typhimurium CsrA has been shown to inhibit invasion gene expression (Altier *et al*., 2000), and in *Erwinia carotova* and *Pseudomonas* species the *csrA* homologue, *rsmA*, represses the production of quorum-sensing-dependent secondary metabolites and factors essential for virulence (Cui *et al*., 1995; Blumer *et al*., 1999; Pessi *et al*., 2001). By controlling access to the ribosome-binding site and altering mRNA stability, CsrA is considered to function as a post-transcriptional regulator (Liu *et al*., 1995; Liu and Romeo, 1997; Romeo, 1998; Blumer *et al*., 1999; Pessi *et al*., 2001; Wei *et al*., 2001; Baker *et al*., 2002). The second well-characterized component of this novel regulatory system in *E. coli* is CsrB, a small untranslated RNA (sRNA) that forms a large ribonucleoprotein complex with approximately 18 CsrA subunits and antagonises the effects of CsrA *in vivo*. Although CsrB levels are a key determinant of CsrA activity in other bacterial species, computer-based searches have not identified a *csrB* homologue in *H. pylori*, suggesting either that this gene is absent or has diverged beyond detection. More recently, another sRNA, CsrC, which performs similar functions to CsrB, was also described in *E. coli* (Weilbacher *et al*., 2003).

The possibility that alternative regulatory networks might be of importance in controlling gene expression in *H. pylori* prompted us to examine the role of CsrA in this important human pathogen. Our results demonstrate that as well as modulating motility, CsrA has a unique role in controlling the response to environmental stress and modulating the elaboration of important virulence factors in *H. pylori*. Intriguingly, CsrA also affects the expression of important transcriptional regulators, such as Fur and HspR, implying interaction with recognized regulatory networks. We also provide the first evidence that inactivation of *csrA* significantly attenuates the virulence of a bacterial pathogen, since *H. pylori* strains unable to produce CsrA demonstrated impaired colonization of a mouse model of infection.

Results

Molecular characterization of csrA *and construction of an isogenic mutant*

We used the polymerase chain reaction to confirm the presence and distribution of the *csrA gene* in a collection of 40 clinical strains of *H. pylori* isolated from patients in different geographical locations who had undergone upper gastroduodenal endoscopy for gastric cancer, peptic ulceration and non-ulcer dyspepsia. Using oligonucleotides csrA-7 and csrA-8 (Table 1) we successfully amplified an approximately 230 bp product from each of these strains (data not shown). To determine whether the *csrA* gene was present in other species of *Helicobacter,* chromosomal DNA isolated from *H. acinonychis* (NCTC 12686)*, H. canis* (NCTC 12739)*, H. cinaedi* (NCTC 12423)*, H. fennelliae* (NCTC 11612)*, H. hepaticus* (NCTC 12886) and *H. muridarum* (NCTC 12714) was analysed by dot blot hybridization using a probe corresponding to the *csrA* nucleotide sequence. Genomic DNA isolated from *H. pylori* N6 and a C57Bl/6 mouse were used as a positive and negative control respectively. A positive signal was obtained for all the *Helicobacter* species (data not shown). These data show that the gene encoding CsrA is present in all examined strains of *H. pylori,* and is highly conserved among gastric and enterohepatic members of the *Helicobacter* genus.

In order to determine whether the *H. pylori* CsrA protein was functional in *E. coli*, the *H. pylori csrA* gene was expressed in an *E. coli* strain (TR1-5) that lacks the native

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′-3′)
Oligonucleotides used for mutagenesis	
csrA-1	ccatcgatAAGAGCTTGCAAAAACCGCTTACGCC
csrA-2	ggaattcTTATCATCAATGACAATCCC
csrA-3	gcggatccAACCAAAAAGCTTCTGTGTGCG
csrA-4	aaaactgcagAATGACTTCGCCATAAGAAGTGGCG
csrA-5	aaactgcagATGCTCATACTCAGCCGCAAAG
csrA-6	ggaattcAGGCTTAATGACCTTTTTGATG
napA-1	TGCGATCGTGTTGTTTATG
napA-2	ATGAGCTTCTAGCATCCAA
napA-3	ggcagatctAGAATTTCTTTAAAGAT
napA-4	ggcagatctGAATTTAAAGAGCTCTC
Oligonucleotides used to synthesize probes	
csrA-7	ATGCTCATACTCAGCCGCAAAG
csrA-8	AGGCTTAATGACCTTTTTGATG
HP1443-1	GCATGTGTTTGGTCAAAGAC
HP1443-2	GCTTGGTTAGTGAGTAAAGC
HP1444-1	GCTTTTAGGCTCTGAAGTGAAGGC
HP1444-2	GACTTTGTCTTTTGTCGTGCAATC
flaA-1	AACAAAGAATTCCAAGTAGGGGC
flaA-2	AACGCTCGCATAGGCTTTAACGCC
flaB-1	AAACTTTAGAAAGCCGAAGAGCGC
flaB-2	TTTCAATCTTATAATCATTCACGCC
napA-5	AAATTCTAAAACATTTGCAAGCG
napA-6	AACTTGGCTAATTGATCATCCGC
ahpC-1	ATGTTAGTTACAAAACTTGCCCC
ahpC-2	AAGCTTAATGGAATTTTCTTTGAG
katA-1	ACGAGATCTCAAACCAATTTGCC
katA-2	TACTTCTTAGCATCTTCTTCTGGC
sodB-1	TAGCGTTTGATTTCCACCATGGG
sodB-2	ATAGCTAAGAGAAACAAAATGCC
ggt-1	ATCACTAAAGAAGATTTAGCC
ggt-2	GTTTGATTTGAGAGCTTGGCG
cagA-1	GATAACAGGCAAGCTTTTGAGGG
cagA-2	CCATGAATTTTTGATCCGTTCGG
vacA-1	GCTGGGATTGGGGGAATGCCG
vacA-2	GCTCTTGTTGCAGCGCTATAG
ureA-1	CCGGATGATGTGATGGATGGCG
ureA-2	CCTTACCGCTGTCCCGCTCGC
ureI-1	GGACTTGTATTGTTATATGTTGGG
ureI-2	GGATAAAGAGCAACCAAGCAGGG
fur-1	ATGAAAAGATTAGAAACTTTG
fur-2	ACATTCACTCTCTTGGCATTC
groESL-1	GGAGAAAGGGTCTTAGTAG
groESL-2	GTGATCATGACAGCAAGC
hspR-1	CGTCGTGGCTAAAATCTTAGGC
hspR-2	GTTTGTGCAGAGCGTCTTGC

Underlining indicates *Bam*HI (GGATCC), *Bgl*II (AGATCT), *Cla*I (ATCGAT), *Eco*RI (GAATTC) and *Pst*I (CTGCAG). Lower case letters in the primer sequences indicate nucleotides that were added at the 5′ end to create a restriction site.

csrA gene and which accumulates glycogen (Romeo *et al*., 1993). Expression of *H. pylori* CsrA was confirmed by SDS–PAGE (data not shown) and the presence of glycogen in bacterial colonies was demonstrated by exposure to sublimated iodine within a sealed container. Expression of *H. pylori* CsrA in strain TR1-5 did not prevent the accumulation of glycogen, indicating that was unable to functionally complement this *csrA* defective *E. coli* mutant (Fig. 1). In contrast, expression of *P. aeruginosa* RsmA on plasmid pME3849 (Pessi *et al*., 2001) was able to inhibit glycogen accumulation in this *E. coli* strain (Fig. 1).

Isogenic mutants of *H. pylori csrA* were generated from a plasmid construct (pJEN1) carrying a central 112 bp deletion of the *H. pylori csrA* gene coupled with insertion of a non-polar cassette composed of the *aphA-3* kanamycin resistance gene (Trieu-Cout *et al*., 1985) lacking its promoter and terminator regions (Ménard *et al*., 1993). *H. pylori csrA* mutants in strains N6 and SS1 were obtained by allelic exchange after natural transformation with the recombinant plasmid. The genotype of the constructed mutants was verified by PCR using primers flanking *csrA* and Southern hybridization (data not shown). Northern blot hybridization using a probe corresponding to the *csrA* DNA sequence detected a single transcript of approximately 900 bp in the wild-type strain that was not produced in the *csrA* mutant (data not shown). Production of mRNA transcripts corresponding to the downstream genes, HP1443 and HP1444, was demonstrated in the *csrA* mutant by similar analysis using probes specific to these genes (data not shown). This confirmed that there was no polar disruption of gene expression downstream of *csrA*. The kanamycin-resistant *csrA* mutants grew normally *in vitro* and had similar growth rates to the parent strains under microaerobic conditions.

A functional csrA *gene is essential for full motility in* H. pylori

Because a functional *csrA* gene is essential for motility and flagellum biosynthesis in *E. coli* (Wei *et al*., 2001), we

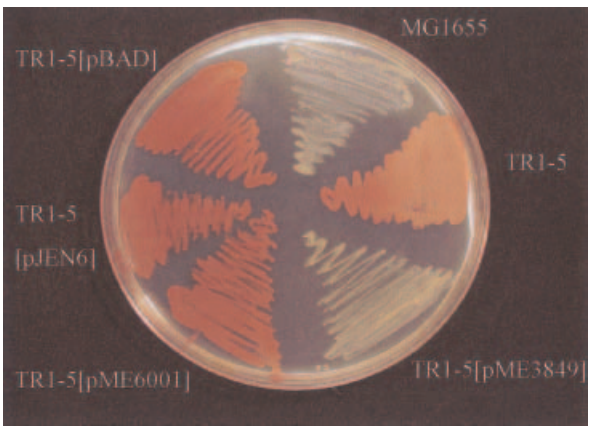

Fig. 1. Lack of complementation of a *csrA*-deficient mutant of *Escherichia coli* (strain TR1-5) with *Helicobacter pylori* CsrA. Cultures were streaked onto LB medium supplemented with 1% L-arabinose and incubated at 37°C before staining with iodine. MG1655, wild-type *E. coli* strain; TR1-5, *E. coli csrA* mutant; TR1-5[pME3849], *E. coli csrA* mutant carrying *P. aeruginosa rsmA* gene on pME6001 cloning vector; TR1-5[pME6001], *E. coli csrA* mutant carrying pME6001 cloning vector; TR1-5[pJEN6], *E. coli csrA* mutant carrying *H. pylori csrA* gene on pBAD cloning vector; TR1-5[pBAD], *E. coli csrA* mutant carrying pBAD cloning vector.

wished to examine whether inactivation of *csrA* had any effect on *H. pylori* motility. Growth on semisolid agar revealed that the swimming zone of the *H. pylori csrA* mutant was reduced by approximately 50% compared with the parental, wild-type strain (Fig. 2A). Similar results were observed in both *H. pylori* genetic backgrounds. The morphology of the *csrA*-mutant bacteria, as determined by electron microscopy, was similar to the wild-type strain, with a unipolar bundle of four or five flagella (data not shown). Although elevated levels of *flaA* and *flaB* mRNA transcript were detected in the *csrA* mutant (Figs 2B.C), immunoblotting of cell extracts developed with monoclonal antibody F2B9 (which reacts with both FlaA (53.3. kDa) and FlaB (53.9 kDa) (Jenks *et al*., 1997) indicated that the major flagellin proteins were present at similar levels in the mutant and wild-type strain (Fig. 2D).

Analysis of susceptibility to oxidative stress

H. pylori has adapted to survive within an environment which is bathed in oxygen-derived free radicals generated from the bacterium's own metabolism and the inflammatory defences of the host. The ability of *H. pylori* to neutralize reactive oxygen species confers resistance against phagocytic killing (Ramarao *et al*., 2000) and has recently been shown to be essential for long-term survival in the murine gastric mucosa (Harris *et al*., 2003). To determine whether the *csrA* mutant was more susceptible to oxygen-dependent cell death, we measured the ability of cells to survive periods of oxygen exposure. After a 6-h period of exposure, the population of the wild-type cells decreased by approximately 10-fold [from 10^7 to 10^6 colony-forming units (cfu) ml^{-1}]. Although the *csrA* mutant demonstrated slightly decreased survival under these conditions, this did not reach statistical significance (data not shown). To investigate whether mutation of *csrA* had an effect on *H. pylori* viability under conditions of oxidative stress, the susceptibility of the wild-type and *csrA* mutant strains to oxidative stress inducers was measured with a disk inhibition assay. Hydrogen peroxide or methyl viologen were added to filter paper discs applied to plates streaked for confluent growth and susceptibilities were measured as zones of inhibition around the discs. The *csrA* mutant was more sensitive to hydrogen peroxide and methyl viologen, with significantly greater zones of inhibition compared with the wild-type strain ($P < 0.01$; Fig. 3A) and similar results were observed in both genetic backgrounds. Susceptibility to oxidative stress was also tested by measuring the ability of cells to survive when challenged with 50 μM methyl viologen. In this experiment, the survival of the *csrA* mutant was compared with that of a *napA* mutant as well as the parent strain. Both the *csrA* and *napA* mutants survived significantly less well after 4 h of exposure to methyl viologen ($P < 0.05$; Fig. 3B).

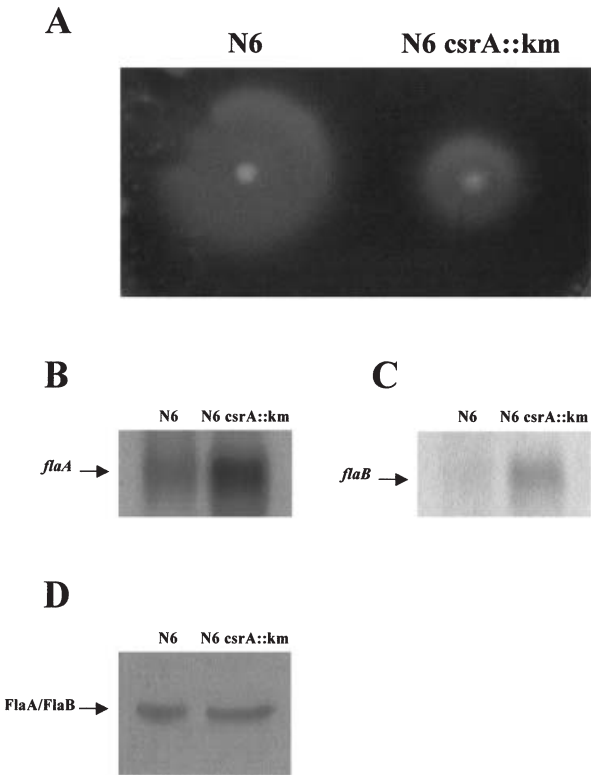

Fig. 2. Motility and expression of the major flagellins of wild-type and *csrA* mutant strains. Motility was assessed after 5 days growth on semisolid BHI agar supplemented with 0.2% cyclodextrin and 0.35% agar (A). For Northern blot analysis of the transcription of *flaA* (B) and *flaB* (C), RNAs were prepared after 18 h liquid cultures and hybridized with DIG-labelled PCR fragments corresponding to *flaA* and *flaB*. For Western blot analysis of the expression of the major flagellins of wild-type and *csrA* mutant strains (D), whole cell extracts were prepared from 18 h liquid cultures and membranes were reacted with antiserum F2B9.

Gene transcription under oxidative stress conditions

Because inactivation of *csrA* had rendered *H. pylori* more susceptible to oxidative stress, we wished to determine whether CsrA was involved in regulating the expression of genes that protect the bacterial cell against reactive oxygen species. For this purpose, wild-type and *csrA* mutant bacterial cell suspensions were exposed for 30 min to 0 and 50 μM methyl viologen and the transcription of neutrophil activating protein (*napA*), alkyl hydroperoxide reductase (*ahpC*), catalase (*katA*) and superoxide dismutase (*sodB*) was analysed by Northern blotting. As a negative control, Northern blotting was also performed with a probe specific for gamma-glutamyltranspeptidase (ggt), an enzyme not involved in oxidative stress protection. No difference in the survival of the wild-type and mutant bacterial cells was observed after a period of exposure to methyl viologen that was considerably shorter than used in the viability experiments

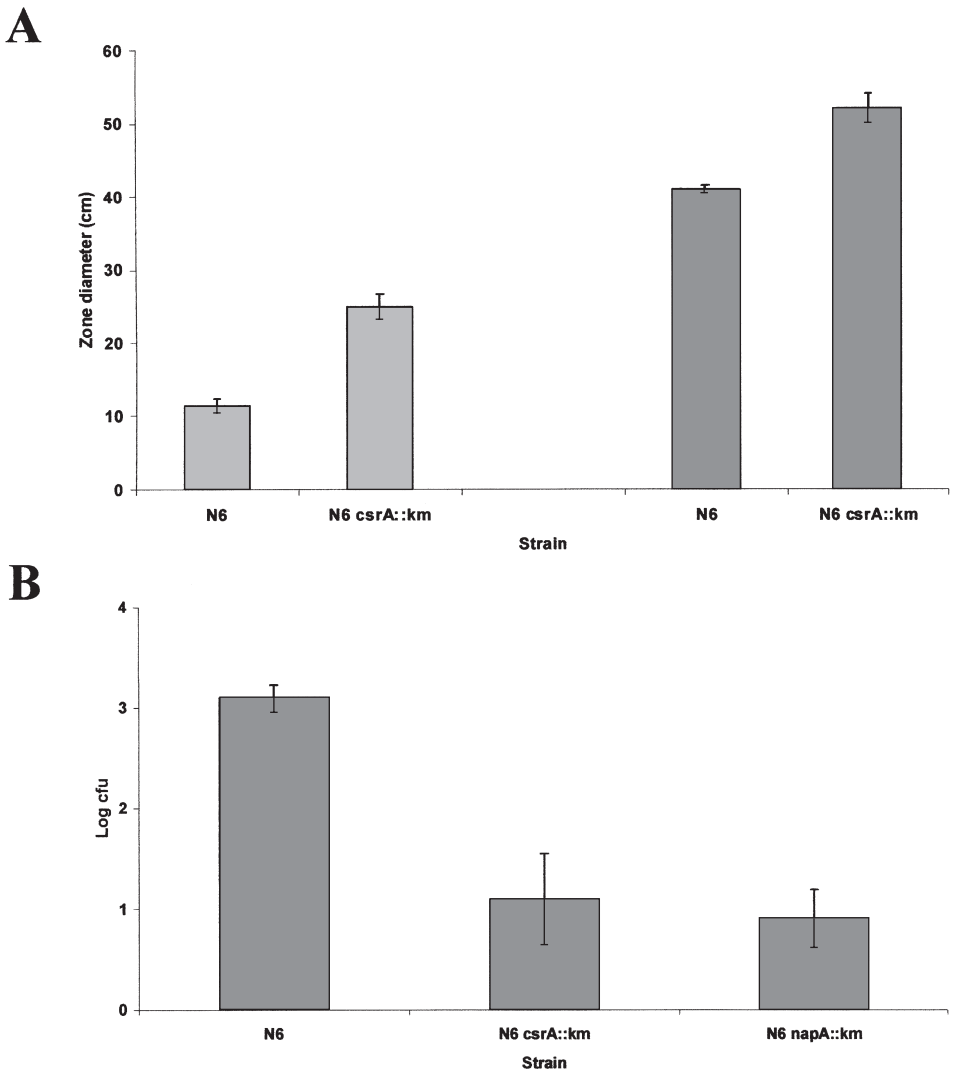

Fig. 3. Susceptibility of wild-type, *csrA* and *napA* mutant strains to oxidative stress. For the disc inhibition assay (A), hydrogen peroxide or methyl viologen were added to filter paper discs applied to plates streaked for confluent growth and susceptibilities were measured as zones of inhibition around the discs. To determine bacterial survival (B), cells were incubated in the presence of 50 μM methyl viologen and enumerated after 4 h. Hydrogen peroxide in light grey, methyl viologen in dark grey.

(30 min as compared with 4 h). The *katA*, *sodB* and *napA* genes were transcribed as monocistronic units (of approximately 2200 and 1150 and 600 bp respectively), and the transcription of these genes in the wild-type strain was induced after exposure to methyl viologen (*napA* shown in Fig. 4A, *katA* and *sodB*, data not shown). Two transcripts, of approximately 700 bp and 1900 bp, were detected with the *ahpC*-specific probe gene (Fig. 4B), consistent with *ahpC* being co-transcribed from its promoter with the downstream gene, HP1564, a 813 bp gene that encodes an outer membrane protein. The genes that lie immediately upstream and downstream of this putative operon are both transcribed in the opposite direction. In all experiments performed ($n = 10$), the level of *napA* mRNA in untreated cells was higher in the *csrA* mutant than the wild-type strain (Fig. 4A). No significant change in the level of either *napA* or *ahpC* mRNA transcripts was observed in the *csrA* mutant after exposure to methyl viologen. The transcriptional responses of *katA* and *sodB* in the *csrA* mutant on exposure to methyl viologen were not significantly different to those observed in the wild-type strain (data not shown). There was no significant difference between the levels of *ggt* transcript in the wild-type or mutant, and no alteration in level was observed in either strain after exposure to methyl viologen (Fig. 4C).

To determine whether differences in expression were manifested at the protein level, wild-type and *csrA* mutant bacterial cell suspensions were exposed for 30 min to increasing concentrations of methyl viologen, and con-

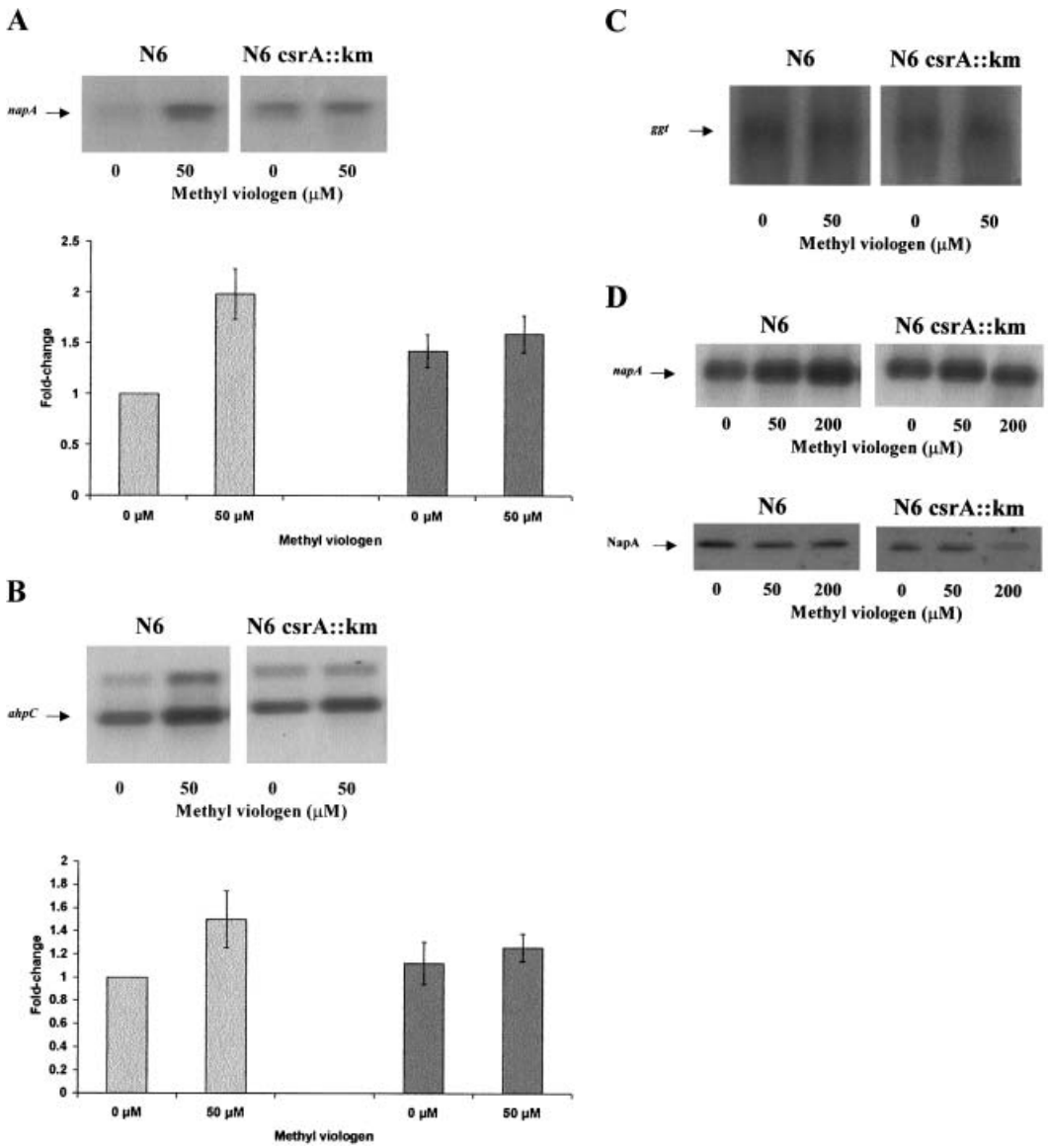

Fig. 4. Gene expression under oxidative stress conditions. Northern blot analysis of the transcription of *napA* (A), *ahpC* (B) and *ggt* (C) and concomitant Northern and Western blot analysis of the expression of *napA* (D) of wild-type and *csrA* mutant strains under conditions of oxidative stress. RNAs and whole cell extracts were prepared after 30 min exposure to increasing concentrations of methyl viologen. Membranes were reacted with DIG-labelled PCR fragments corresponding to *napA* (A and D), *ahpC* (B), *ggt* (C) or anti-NapA serum (D). Standardized peak densities were obtained by densitometry using Quantity One Software (Bio-Rad).

comitant samples were subjected to either Northern blotting using a *napA*-specific probe or Western blotting using specific antisera raised against NapA (Evans *et al.*, 1995). In contrast to the data from the Northern analysis, the amount of NapA protein detected was consistently lower in the *csrA* mutant than the parent strain (Fig. 4D). While the level of NapA protein remained relatively constant in the wild-type strain, exposure of the *csrA* mutant to methyl viologen resulted in the loss of NapA protein to almost undetectable levels (Fig. 4D). To determine whether the synthesis of other proteins under these conditions were also affected in the *csrA* mutant, we examined the expression of AhpC by Western blot analysis using specific antisera to this proteins (O'Toole *et al.*, 1991). No significant difference between the levels of AhpC was observed between the wild-type and *csrA* mutant in steady-state cells or after exposure to methyl viologen (data not shown).

Gene transcription under acidic conditions

Survival of *H. pylori* in the human stomach is also dependent on the ability to adapt gene expression to exposure

to gastric acidity. In order to investigate whether loss of CsrA function affected the expression of genes under acidic conditions, Northern blot analysis using probes for *napA*, cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin (*vacA*), the urease operon (*ureA* and *ureI*), *fur* and *ggt* was therefore performed on bacterial cell suspensions exposed for 30 min to pH 7.0 and 4.0. The survival of wild-type and mutant bacterial cells was similar after exposure to these conditions. Figure 4 shows that in the wild-type strain, exposure to pH 4, as compared with pH 7, resulted in a 1.5- to 2.0-fold increase in the level of mRNA of *napA*, *cagA*, *vacA* and *fur*, as well as the transcript corresponding to *ureABIE´* (3.7 kb). At pH 7.0 the level of mRNA of *napA*, *cagA*, *vacA* and *ureABIE´* was higher in the *csrA* mutant than wild-type strain, and incubation of the *csrA* mutant at pH 4 resulted in minimal change or a reduction in the level of these transcripts (Fig. 5A–D). In contrast, incubation under acidic conditions resulted in a greater induction of *fur* in the *csrA* mutant than the wild-type strain (Fig. 5E). There was no induction of *ggt* at pH 4.0, and the transcript level was unaffected by inactivation of *csrA* (data not shown).

The expression of the NapA and UreA/B in the samples was also examined by immunoblotting with specific antisera to these proteins (Ferrero *et al*., 1994; Evans *et al*., 1995). Again, less NapA protein was detected in the *csrA* mutant than the parent strain at pH 7.0 (Fig. 5A). Exposure to pH 4.0 resulted in a reduction in NapA to virtually undetectable levels in both wild-type and mutant strains (Fig. 5A). Although both strains exhibited similar expression of the UreA and UreB urease subunits (data not shown), quantitative determination of urease activity using an assay based on the Bertholet reaction (Cussac *et al*., 1992) revealed that the urease activity of the *csrA* mutant (9.62 ± 0.21 μmol urea min^{-1} mg^{-1} protein) was higher than the wild-type strain (8.46 ± 0.24 μmol urea min^{-1} mg^{-1} protein; $P < 0.05$).

Gene transcription under heat stress conditions

Because *H. pylori* will experience fluctuations of temperature during ingestion of food by the host, we also determined whether CsrA is involved in the response to sudden temperature increase. To do this, wild-type and *csrA* mutant bacterial cell suspensions were incubated for 10 min at 37°C or 40°C and the transcription of *napA*, *groESL (hspA/B*), *hspR* and *ggt* were analysed by Northern blotting. Exposure to these conditions had no effect on the viability of the wild-type or mutant strain. The amount of *napA*, *groESL* and *hspR* was consistently higher in the *csrA* mutant than the parent strain at 37°C (Fig. 6). Temperature upshift resulted in an increase in the amount of each transcript in the wild-type strain, a response that was attenuated in the *csrA* mutant (Fig. 6A–C). There was no induction of *ggt* at 40°C, and the transcript level was unaffected by inactivation of *csrA (*data not shown). We conclude that *napA*, as well as *hspR* and *groESL*, exhibits a typical heat shock response as temperature increases to 40°C and that this response is impaired in the *csrA* mutant.

The expression of the NapA, GroES and GroEL in these samples was also examined by Western blotting with specific antisera to these proteins (Suerbaum *et al*., 1994; Evans *et al*., 1995). Less NapA was detected in the *csrA* mutant than the parent strain at 37°C and 40°C (Fig. 6A). In contrast, GroES and GroEL were expressed at higher levels in the *csrA* mutant at both temperatures (Fig. 6B). Exposure to increasing temperature did not significantly alter the level of expression any of these proteins in either the wild-type or mutant strains.

Effects of CsrA on mRNA stability

In *E. coli*, CsrA-mediated regulation occurs through binding mRNA and altering transcript stability (Liu *et al*., 1995; Wei *et al*., 2001; Baker *et al*., 2002). To determine whether the increase in levels of the *napA* transcript in the *csrA* mutant was explained by a decrease in the rate of its degradation (i.e. by increased mRNA stability), we examined its decay after the addition of rifampicin to liquid cultures of wild-type and *csrA* mutant strains. In three independent experiments, inactivation of *csrA* had no significant effect on the stability of the *napA* transcript, which had a half-life of approximately 36 min in both the wild-type and mutant strains (Fig. 7). Further experiments were also performed to examine the stability of *napA* mRNA in wild-type and mutant strains at pH 4.0 and in the presence of 20 μM methyl viologen, and also *groESL*. The rate of decay of the *napA* transcript under these conditions and also of *groESL* was again similar in the wild-type and *csrA* mutant (data not shown).

Estimation of DNA damage

Bacterial strains deficient in oxidative-stress response proteins are more prone to DNA damage caused by reactive oxygen species. Our observation that NapA, which has high similarity to the bacterial DNA-protecting protein Dps, was under the control of CsrA led us to compare the susceptibility to oxidant-induced DNA fragmentation of *napA* and *csrA* mutants to the wild-type strain. *H. pylori* strains were exposed to hydrogen peroxide or methyl viologen and DNA fragmentation was assessed by alkaline (denaturing) gel analysis. The control cells show no DNA migration from the blocks, indicating a negligible amount of DNA fragmentation (Fig. 8A). Treatment for 30 min with 100 mM hydrogen peroxide or 100 μM methyl viologen caused DNA fragmentation, with the *napA* mutant show-

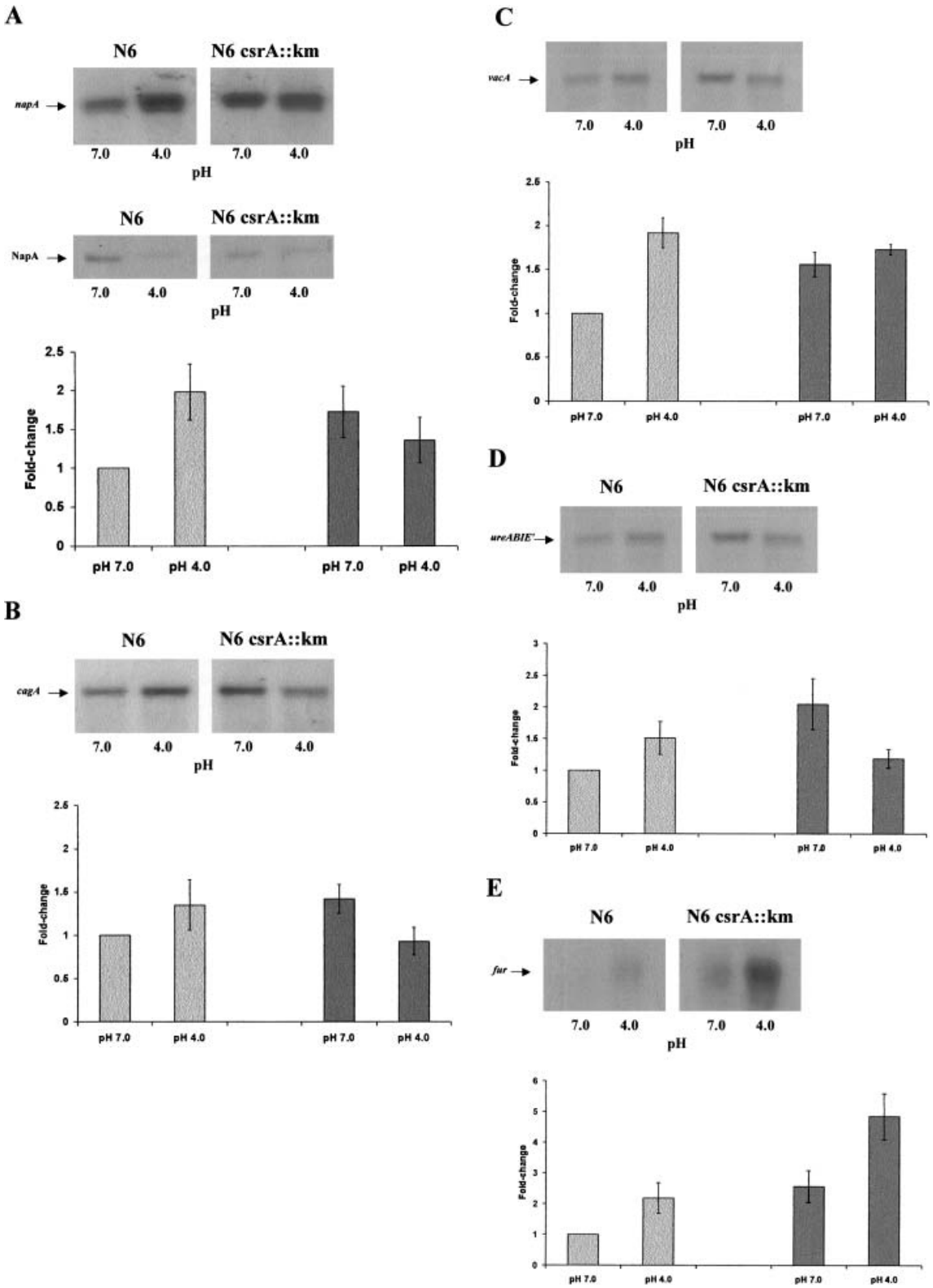

Fig. 5. Gene expression under acid stress conditions. Concomitant Northern and Western blot analysis of the expression of *napA* (A), and Northern blot analysis of the transcription of *cagA* (B), *vacA* (C), *ureABIE′* (D) and *fur* (E) of wild-type and *csrA* mutant strains under conditions of acid stress. RNAs and whole cell extracts were prepared after 30 min exposure to pH 7.0 or pH 4.0. Membranes were reacted with DIG-labelled PCR fragments corresponding to *napA* (A), *cagA* (B), *vacA* (C), *ureI* (D) or *fur* (E), or anti-NapA serum (A). Standardized peak densities were obtained by densitometry using Quantity One Software (Bio-Rad).

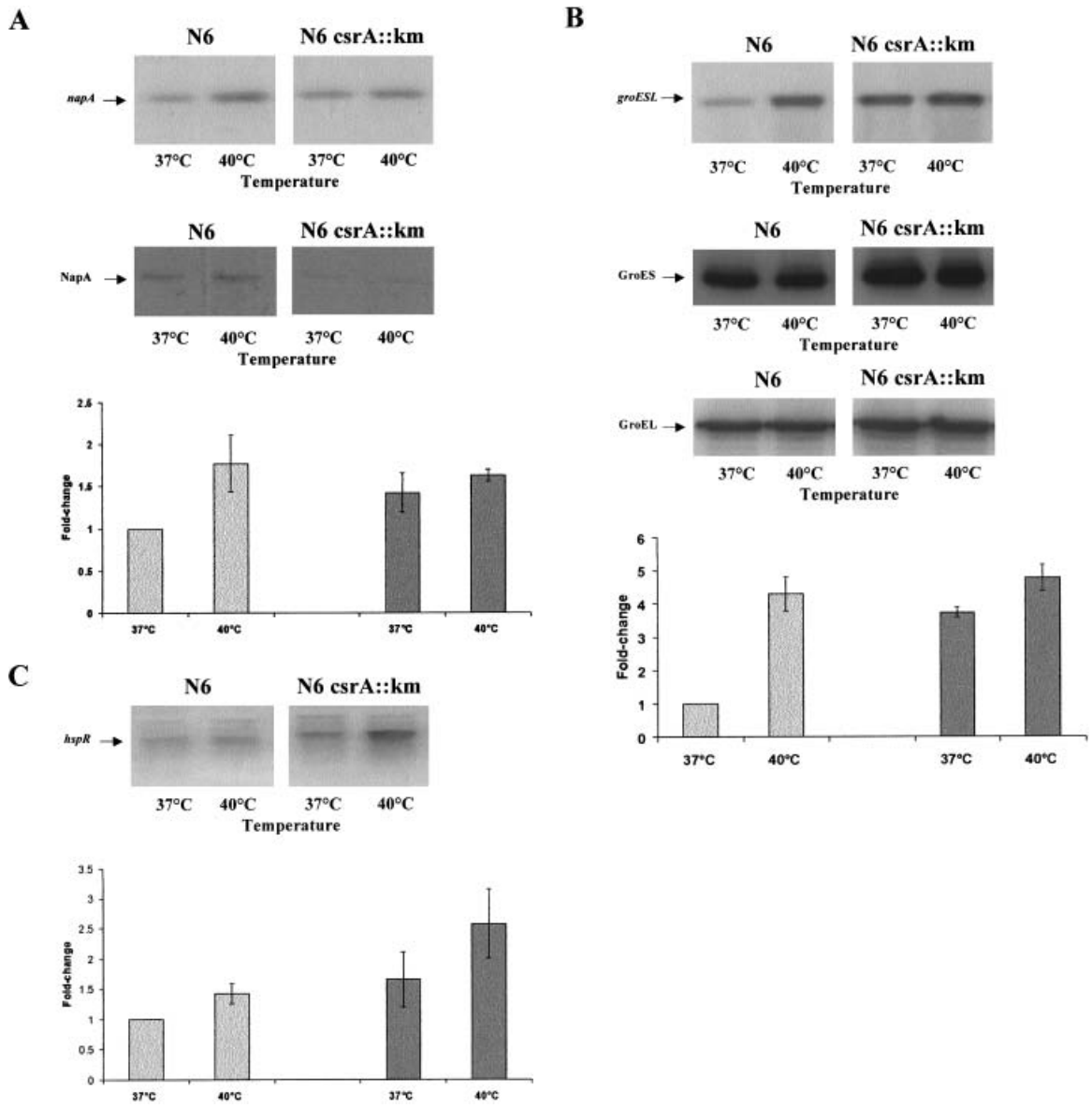

Fig. 6. Gene expression under heat stress conditions. Concomitant Northern and Western blot analysis of the transcription of *napA* (A) and *groESL* (B) and Northern blot analysis of the transcription of *hspR* (C) of wild-type and *csrA* mutant strains under conditions of heat stress. RNAs and whole cell extracts were prepared after 30 min exposure to 37°C or 40°C. Membranes were reacted with DIG-labelled PCR fragments corresponding to *napA* (A), *groESL* (B) or *hspR* (C), or anti-NapA (A), GroES or GroEL antiserum (B). Standardized peak densities were obtained by densitometry using Quantity One Software (Bio-Rad).

ing the greatest increase in length of DNA distribution (Fig. 8B and C). The *csrA* mutant showed DNA distributions with relative lengths that were intermediate to those of the *napA* mutant and the parent strain.

Mouse colonization by the csrA *mutant*

To assess the role of CsrA in colonization, we examined the potential of wild-type and mutant bacteria to infect the SS1 *H. pylori* mouse model. Six experiments were conducted in which two independently constructed *csrA* mutants, as well as the parental SS1 strain, were used to orogastrically inoculate mice (10^7–10^8 cfu per mouse). Colonization by the respective SS1 and SS1-derivative mutants was assessed by quantitative culture of the stomachs (Jenks *et al*., 1999). Previous work has demonstrated that insertion of the *aphA3* gene, which confers kanamycin resistance in the mutant strain, does not affect fitness for colonization of the murine stomach by *H. pylori* strain SS1 (Salama *et al*., 2001; Loughlin *et al*., 2003). When each strain was inoculated separately, both *H. pylori* SS1 and the *csrA* mutants were able to colonize

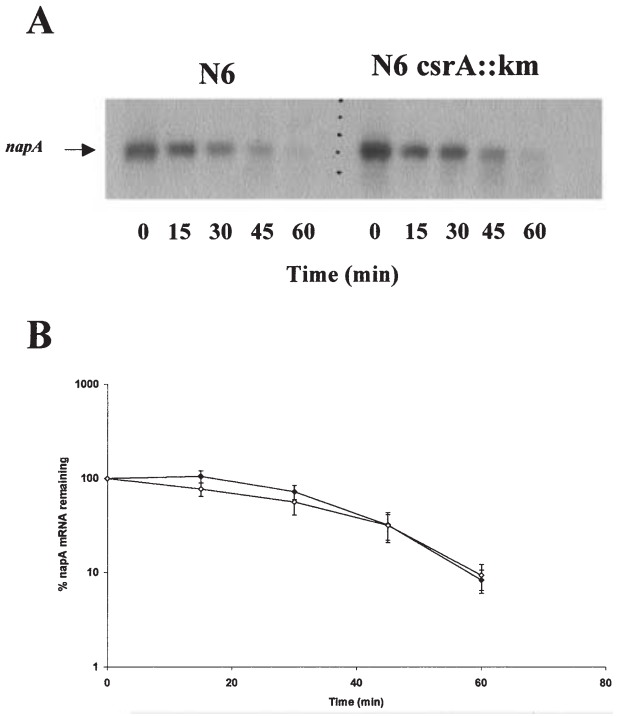

Fig. 7. Analysis of *napA* transcript stability in wild-type and *csrA* mutant strains. Cultures of wild-type and *csrA* mutant strains were treated with rifampicin and harvested at several time points thereafter. Total RNA was isolated and hybridized with DIG-labelled PCR fragments corresponding to *napA* (A). The bands obtained were quantified by densitometry (B) and the values for each strain normalized relative to the amount of transcript at the time of addition of rifampicin ($T = 0$ min). ◆: *H. pylori* N6; ◇: *H. pylori* N6csrA::km.

mice and infections resulted in similar bacterial loads (10^5–10^6 cfu g^{-1}) at various time points up to 20 weeks post-inoculation. In a co-infection experiment, in which mice were inoculated with a 50:50 mixture of the strains, both wild-type and mutant bacteria were recovered from mice sacrificed 2 weeks post-inoculation. However, the *csrA* mutant was out competed by the SS1 parental strain [CI = 0.074 (range 0.024–0.200, results from five mice)]. This defect was further amplified in bacteria recovered from the stomachs of mice sacrificed 4 weeks after infection [CI = 0.009 (range 0.002–0.030, results from five mice)]. In a parallel experiment, we checked the *in vitro* growth of a similar mixed culture at 0 and 48 h. At both time points, the culture consisted of approximately 50% wild-type and 50% mutant bacteria (data not shown). Thus the *csrA* mutation seems to confer a specific disadvantage for colonization rather than growth. Finally, the ID_{50} of the wild-type and mutant strains was determined by infecting mice with 10-fold serially diluted bacteria and determining the number of animals colonized at each dose. The Reed–Muench calculation was used to determine the number of bacteria required to obtain colonization of 50% of the animals (Reed and Muench, 1938). Table 2 shows that while the ID_{50} for the wild-type SS1 strain was 1.4×10^4 bacteria, the ID_{50} of the *csrA* mutant was approximately 10-fold higher with a value of 1.4×10^5.

Discussion

Although genome analysis has revealed that *H. pylori* has

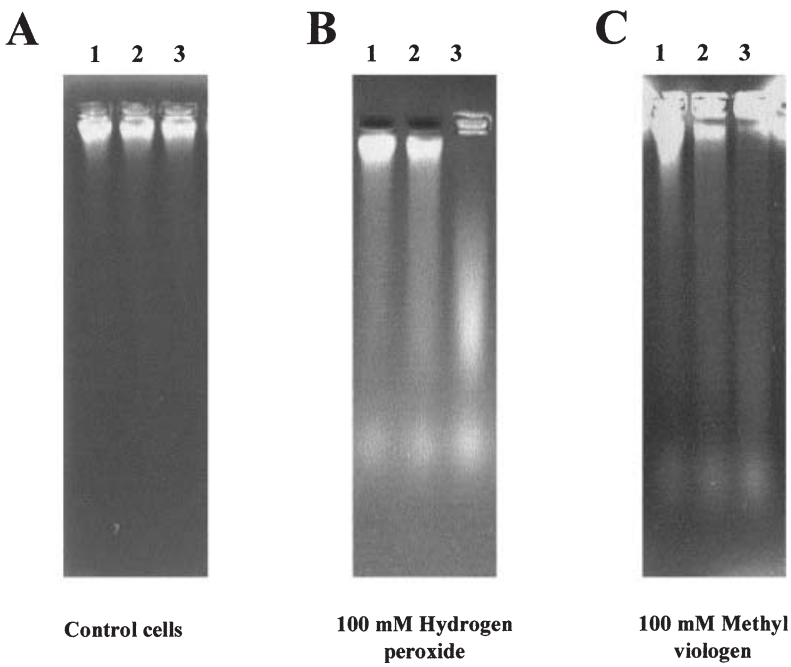

Fig. 8. Oxidative stress-induced DNA fragmentation of wild-type (lane 1), *csrA* mutant (lane 2) and *napA* mutant (lane 3). Exponential phase bacterial cells were exposed for 30 min to 0 mM (A) or 100 mM (B) hydrogen peroxide, or 100 μM (C) methyl viologen. Cells were lysed in agarose plugs that were inserted in the wells of a denaturing alkaline agarose gel and electrophoresed to determine the extent of genomic DNA fragmentation

Table 2. ID_{50} determination for wild-type and *csrA* mutant strains in mice.

Dilution	Number of mice		% of total mice infected
	Infected	Uninfected	
Wild type			
2.5×10^8	5	0	100
2.5×10^7	5	0	100
2.5×10^6	5	0	100
2.5×10^5	5	0	100
2.5×10^4	3	2	60
2.5×10^3	1	4	20
csrA mutant			
2.5×10^8	5	0	100
2.5×10^7	5	0	100
2.5×10^6	5	0	100
2.5×10^5	3	2	60
2.5×10^4	1	4	20
2.5×10^3	0	5	0

Mice were inoculated with wild-type SS1 bacteria or the *csrA* mutant at the indicated dilutions. Reed–Muench ID_{50} calculations were 1.4×10^4 cfu ml^{-1} for wild type and 1.4×10^5 cfu ml^{-1} for the *csrA* mutant.

relatively few regulatory networks, our knowledge of the molecular mechanisms that control its response to environmental challenges and mediate the expression of virulence genes remains rudimentary. Post-transcriptional regulatory mechanisms are critical determinants of gene expression in many bacterial species, and there is evidence that such systems may be important in facilitating the response of *H. pylori* to diverse environmental stimuli (Akada *et al*., 2000; Spohn *et al*., 2002). The series of experiments described in the present study indicate that the global post-transcriptional regulator CsrA has a broad role in *H. pylori* physiology, regulating motility, mediating the responses to environmental stresses, and controlling the elaboration of virulence factors. Furthermore, our finding that a mutant defective for CsrA production is attenuated for colonization of a mouse model of infection suggests an important role for this regulator in establishing and maintaining infection of the gastric mucosa.

While many regulatory genes were lost during the evolution of highly adapted pathogens such as *H. pylori*, *csrA* homologues appear to have been retained (Romeo, 1998). Our finding that the gene encoding CsrA was ubiquitous in clinical isolates of *H. pylori* and was conserved among gastric and enterohepatic members of the *Helicobacter* genus, supports the hypothesis that CsrA is a critical determinant of gene expression in this species. Standard BLAST analysis has failed to identify CsrB and CsrC homologues in eubacterial families beyond the *Enterobacteriaceae* (Romeo, 1998; Weilbacher *et al*., 2003), possibly because function of these sRNA molecules requires limited sequence conservation (Wassarman *et al*., 1999). Despite performing a detailed *in silico* search, including algorithms that identify conservation of adjacent genes, the presence of palindromes and quasi-palindromes, as well as a hidden Markov model that identifies conserved motifs upstream of functional genes (Petersen *et al*., 2003), we were unsuccessful in identifying non-coding RNAs that might be associated with the function of CsrA in *H. pylori*. If these molecules do exist in *H. pylori*, their identification is likely to require experimental approaches similar to those used in *E. coli* (Liu *et al*., 1997; Weilbacher *et al*., 2003).

In *E. coli*, CsrA stimulates expression of the *flhDC* master regulon, and hence positively regulates motility and flagellum biosynthesis (Wei *et al*., 2001). Although the *H. pylori* motility regulation cascade lacks *flhDC*, we observed that the *csrA* mutant had reduced motility and produced elevated levels of *flaA* and *flaB* mRNA transcript as compared with the wild-type strain. Despite this, the *csrA* mutation appeared to have a minimal effect on either flagellin levels or flagellar morphology, suggesting that CsrA either affects the ability to use flagella (similar to a Mot-deficient phenotype) or mediates its effect at a relatively late stage in the motility regulatory hierarchy. Previous work has shown that the ratio of the major flagellins in the flagellum of *H. pylori* varies in response to changes in environmental and physiological conditions (Suerbaum, 1995). It would be interesting to determine whether CsrA is part of the adaptive response pathway that controls expression of these flagellins in response to changing environmental stimuli.

H. pylori produces a number of detoxifying enzymes that protect against the effects of oxygen-derived free radicals and have a key role in its ability to survive in the gastric mucosa. This study provides formal transcriptional analysis of the antioxidant enzyme-encoding genes of *H. pylori*, and our results demonstrate that these are upregulated in response to oxidative stress conditions, despite the fact that this organism lacks both the RpoS sigma factor and the oxidatively activated transcriptional regulators OxyR and SoxRS. The ability to sense and respond to these stress conditions may be mediated either by an alternative, unrecognized transcriptional regulator, or represent an additional, compensatory function of those regulators that are present. In contrast to a previous study, which reported the mono-cistronic transcription of *ahpC* (Lundstrom and Bolin, 2000), our analysis indicated co-transcription with the downstream open reading frame (ORF) (HP1564), which encodes an outer membrane protein. Although this discrepancy may reflect interstrain differences in the transcriptional organization of these genes, the higher molecular weight transcript was initially faint and only became clearly visible after exposure to methyl viologen, raising the possibility that this transcript might have been overlooked in the earlier study.

The *H. pylori* neutrophil-activating protein (NapA) was

originally described as a promoter of neutrophil adhesion to endothelial cells (Yoshida *et al*., 1993) and has significant homology to bacteriferritins and the *E. coli* DNA-binding protein from starved cells, Dps (Evans *et al*., 1995). The latter is a non-specific DNA-binding protein that is induced by starvation and exposure to oxidative and osmotic stresses (Almiron *et al*., 1992), and which protects DNA from oxidative damage by sequestering iron that might otherwise generate free radicals through the Fenton reaction (Martinez and Kolter, 1997; Grant *et al*., 1998). Other Dps homologues also bind DNA and have been shown to protect against oxidative and acid stress (Almiron *et al*., 1992; Chen *et al*., 1995; Valdivia and Falkow, 1996; Choi *et al*., 2000). In this study, we showed that the transcription of *napA* was increased under conditions of oxidative, as well as acid and heat stress, and that this response was impaired in the *csrA* mutant. Induction of *napA* by diverse environmental stimuli suggests that *H. pylori* NapA is involved in protecting *H. pylori* against various stress conditions, and is consistent with recent evidence that NapA production is increased in *ahpC* mutants (Olczak *et al*., 2002) and that this protein is essential for growth of *H. pylori* at low pH (Bijlsma *et al*., 2000). Also consistent with this is our observation of significantly more oxidant-induced genomic DNA damage in a *napA* mutant than either the *csrA* mutant or the wild-type strain. The intermediate degree of DNA fragmentation seen in the *csrA* mutant appears to correlate with the reduced levels of NapA expressed in this strain under these conditions. These data are likely, in part, to explain the increased susceptibility of the *csrA* mutant to oxidative-stress conditions, and provide evidence that NapA has a DNA-protective role in *H. pylori*.

Given the microenvironment in which they exert their effect, it is not surprising that the expression of several *H. pylori* virulence factors is regulated in response to pH. In this study, we have shown that CsrA has a critical role not only in controlling transcript levels of these genes, but also in mediating the acid-induced changes in their expression. While the wild-type strain demonstrated a typical induction of *cagA* at low pH (Karita *et al*., 1996), this response was reversed in the *csrA* mutant, with a higher level of *cagA* mRNA at pH 7.0, and a reduction in transcript levels at pH 4.0. Acid activation is also important for the biological activity of VacA, increasing secretion of activated toxin, promoting dissociation into monomers and facilitating the formation of transmembrane pores which permeabilize the gastric epithelium to urea (De Bernard *et al*., 1995; Molinari *et al*., 1998; Tombola *et al*., 1999, 2001; Jungblut *et al*., 2000). Here we provide evidence that transcription of the *vacA* gene is induced under acidic conditions and that this pH-dependent response requires the presence of CsrA. This result differs from a recent array-based study that found that the expression of *vacA* was repressed by exposure to acid and which, in contrast to previous publications, also demonstrated acid-induced repression of *cagA* (Merrell *et al*., 2003). These discrepancies may be due to the use of different media, but are more likely to reflect differences in gene expression that are intricately linked to the strain being examined (Merrell *et al*., 2003). The acid induction of the *ureABIE′* transcript, which is known to be regulated post-transcriptionally by mRNA decay in response to environmental pH (Akada *et al*., 2000), was also absent in the *csrA* mutant. Although expression of the UreA and UreB subunits appeared similar, higher urease activity was observed in the *csrA* mutant as compared with the wild-type strain, providing evidence of an additional level of regulation of this important enzyme. While *H. pylori fur* mutants are unaffected in their ability to survive acid shock, their growth is severely impaired at acid pH, and transcription of *fur* is repressed after 24 h growth under acidic conditions (Bijlsma *et al*. 2002). We found that exposure to pH 4.0 for 30 min resulted in increased transcription of *fur*, which suggests that Fur is involved in both the acute response and long-term adaptation to low pH. The observation that there are temporal variations in the expression of this regulatory gene at low pH, and that the acid induction of *fur* was more marked in the *csrA* mutant, suggests that the response to acid exposure is complex and likely to be co-ordinated through the interaction of a number of regulatory mechanisms.

The operons encoding the major chaperones of *H. pylori* exhibit a typical heat shock response that is followed by an adaptation phase specific for each promoter, the onset of which is determined by the half-life of respective RNAs (Spohn *et al*., 2002). Transcriptional responses to different stimuli may therefore be time dependent, with changes in mRNA stability allowing adaptation to different environmental conditions. We showed that temperature upshift resulted in an increase in the amount of *groESL* and *hspR* mRNA in the *csrA* mutant and wild type, and that the amount of each transcript was higher in the *csrA* mutant than the parent strain at all temperatures studied. It is possible that these results could be explained by direct binding and destabilization of these transcripts by *H. pylori* CsrA. However, it was recently demonstrated that puromycin induces transcription from these promoters, implying that environmental stresses are intracellularly sensed through the accumulation of non-native proteins (Spohn *et al*., 2002). Preliminary evidence suggests that increased degradation of proteins occurs in the *H. pylori csrA* mutant, particularly under conditions of stress, and this could provide an alternative explanation for the increased transcription of these genes in the absence of CsrA.

In *E. coli*, CsrA regulates gene expression by a post-transcriptional mechanism that involves binding to the untranslated leader of target transcript, which either

blocks translation and causes rapid degradation of the transcript (Baker *et al*., 2002) or results in direct activation of protein synthesis (Wei *et al*., 2001). It is thought that the changes in mRNA stability are secondary to the effect of CsrA on translation, and that the rate of transcript degradation is also influenced by RNA secondary structure and endolytic ribonuclease activity (Liu *et al*., 1995). The series of experiments described in this study suggest that in *H. pylori*, CsrA also mediates its action through affecting the translation and processing of transcripts. Although elevated levels of the *napA* transcript were observed in the *csrA* mutant, this was associated with a marked reduction in the production of NapA protein, and this paradoxical effect was exaggerated under conditions of environmental stress. This is consistent with CsrA activating translation of the *napA* transcript, reminiscent of its function in the regulation of *flhDC* in *E. coli* (Wei *et al*., 2001). However, the observation that *H. pylori* CsrA is unable to complement an *E. coli csrA* mutant suggests that it mediates its effect on translation through an alternative mechanism. Furthermore, we were unable to demonstrate any significant difference in the half-life of the *napA* transcript in the *csrA* mutant as compared with the wild-type strain at any of the conditions tested, which suggests that CsrA mediates its effects on protein synthesis without affecting transcript stability. It is notable that the half-life of *napA* (36 min) and *H. pylori* transcripts in general (Akada *et al*., 2000; Spohn *et al*., 2002) are much longer than in *E. coli* [e.g. 3.5 min for *flhDC* (Wei *et al*., 2001)]. While this makes it difficult to detect relatively subtle changes in transcript stability that could still result in changes in total transcript levels, it may also reflect specific features of RNA secondary structure in *H. pylori* that provide a protective barrier to ribonuclease degradation. In other bacteria, CsrA blocks ribosome binding and the absence of translating ribosomes is thought to facilitate a series of endonucleolytic cleavages (Liu *et al*., 1995). If *H. pylori* CsrA is able to inhibit or stimulate translation of target transcripts without affecting ribosomal binding, this would provide an alternative explanation for the lack of any major effect on RNA turnover.

Our *in vivo* experiments provide the first evidence that inactivation of *csrA* significantly attenuates the virulence of a bacterial pathogen. Although mutations in the *csrA* gene of *H. pylori* strain SS1 resulted in bacteria that were able to colonize the murine gastric mucosa, the ID_{50} of the *csrA* mutant was 1 log unit higher than the wild-type strain, which is likely, in part, to be reflect the reduced motility of this strain (Eaton *et al*., 1992). A more detailed analysis using co-infection experiments revealed that *csrA* mutants were out competed in the mouse stomach in the presence of the wild-type strain, and the degree exclusion of the *csrA* mutant increased with time. This strongly suggests that CsrA is crucial for the ongoing survival of *H. pylori* in the face of exposure to environmental and host-derived stresses. This observation was made despite the fact that *H. pylori* SS1 induces relatively mild inflammatory changes in the murine gastric epithelium (Crabtree *et al*., 2002), and we plan to determine whether a more profound colonization defect is seen in other animal models of infection where the host response is more pronounced. Taken together, our results provide evidence that the post-transcriptional regulator CsrA is a key regulatory element in *H. pylori*, which may be particularly important in adaptation to the different environments encountered during colonization of the gastric mucosa. Our finding that inactivation of *csrA* affects the expression of important regulators such as Fur and HspR, suggests that gene regulation in this important gastric pathogen is more complex than previously thought.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli strains MC1061 (Casadaban and Cohen, 1980) and MG1655 (Jensen, 1993) were used as hosts for plasmid cloning experiments, and were grown at 37°C on solid or liquid Luria–Bertani medium containing spectinomycin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), ampicillin (75 μg ml^{-1}), chloramphanicol (12.5 μg ml^{-1}) or L-arabinose (1%) as required.

Strains *H. pylori* N6 and SS1 were used in the experiments (Ferrero *et al*., 1992; Lee *et al*., 1997). A total of 40 *H. pylori* strains from the United States, South America, Asia and Europe were used to study the distribution of *csrA* in clinical isolates of *H. pylori*. *H. pylori* strains were routinely cultured under microaerobic conditions (86% nitrogen, 6% oxygen, 3% hydrogen and 5% carbon dioxide) at 37°C on a blood agar (BA) base 2 (Oxoid) plates supplemented with 10% horse blood and an antibiotics/fungicide mix consisting of: vancomycin (10 μg ml^{-1}), polymyxin (2.5 IU l^{-1}), trimethoprim (5 μg ml^{-1}) and amphotericin B (4 μg ml^{-1}). Liquid cultures of *H. pylori* were grown under identical conditions in brain–heart infusion (BHI) broth containing 0.2% cyclodextrin and the antibiotics/fungicide mix. Transformation of *H. pylori* was carried out as described previously (Jenks *et al*., 2001) with selection on kanamycin (25 μg ml^{-1}) or chloramphanicol (20 μg ml^{-1}).

DNA techniques

DNA manipulations were carried out using standard techniques (Sambrook *et al*., 1989). Mini or midi Qiagen columns and a QiaAmp DNA extraction kit (Qiagen) were used for plasmid and rapid chromosomal DNA preparations respectively. PCR was carried out according to the manufacturer's instructions using *Taq* polymerase (Promega).

Plasmid construction

To construct an *H. pylori csrA* deletion, oligonucleotides

(csrA-1 and csrA-2; Table 1) were used to amplify a 524 bp DNA fragment (fragment 1) containing the 5′ region of the *csrA* gene flanked by *Cla*I and *Eco*RI restriction sites. A second pair of primers (csrA-3 and csrA-4; Table 1) were used to generate a 554 bp DNA fragment (fragment 2) containing the 3′-region of the *csrA* gene flanked by *Bam*HI and *Pst*I restriction sites. After PCR amplification, fragment 1 was restricted with *Cla*I and *Eco*RI and cloned into the plasmid vector pILL570 (Labigne *et al*., 1991) cut with the same enzymes. The non-polar kanamycin cassette liberated from pUC18K2 (Ménard *et al*., 1993) by restriction with *Eco*RI and *Bam*HI was inserted into the intermediate recombinant plasmid (containing fragment 1) that had been linearized with the same enzymes. Finally, the resultant plasmid (containing fragment 1 and the non-polar cassette) was linearized with *Bam*HI and *Pst*I, and the *Bam*HI- and *Pst*I-restricted fragment 2 was inserted. The resulting construct, pJEN1, carries a 112 bp deletion of the *csrA* gene replaced with the kanamycin cassette. Plasmid pJEN6 contained the *H. pylori csrA* gene that had been amplified using primers csrA-5 and csrA-6 and cloned into the pBAD-TOPO® expression vector (Invitrogen) according to the manufacturer's instructions.

Plasmid pJEN2 was constructed by inverse PCR mutagenesis, as previously described (Jenks *et al*., 1997). Briefly, the *napA* gene was amplified using primers napA-1 and napA-2 (Table 1) and cloned into the pTAG vector (Novagen), before subcloning via *Pst*I and *Xba*I sites into pUC19 (Yannisch-Perron *et al*., 1985). A 25 bp deletion and unique *Bgl*II site were then engineered into *napA* by inverse PCR using primers napA-3 and napA-4 (Table 1). A *Bam*HI fragment containing the *aphA-3* kanamycin resistance gene (Trieu-Cout *et al*., 1985) was then cloned into the *Bgl*II site to generate pJEN2. Resultant recombinant plasmids were introduced into *H. pylori* by natural transformation, and allelic replacement of the intact chromosomal gene by the mutated gene was confirmed by PCR and Southern hybridization.

Motility assay and electron microscopy

The motility plate assay was initiated by stabbing a colony from a 24 h culture into semisolid agar (BHI supplemented with 0.2% cyclodextrin and 0.35% agar). The plates were incubated under microaerobic conditions at 37°C for up to 5 days. For electron microscopy, bacteria that had been grown overnight in BHI broth supplemented with 10% fetal bovine serum (Gibco BRL) were harvested by centrifugation at 500 *g* for 5 min, resuspended in BHI broth and stained on copper EM grids coated with formvar and carbon, using 1% phosphotungstate (pH = 7.0). EM grids were viewed in a Joel 1010 transmission electron microscope at an acceleration voltage of 80 kV.

Oxidative stress sensitivity assays

The toxicity of oxygen on wild-type and mutant strains of *H. pylori* was assessed as described by Seyler *et al*. (2001). Bacterial cells grown for 48 h on BA plates were suspended in BHI broth containing 0.2% cyclodextrin to an optical density at 600 nm (OD_{600}) of 0.1. Ten millilitres of the suspension was incubated at 37°C under conditions of 85% nitrogen, 10% hydrogen and 5% carbon dioxide, or normal atmospheric conditions. Samples were removed at appropriate intervals, serially diluted and plated directly on BA plates for enumeration. Results were obtained from three independent determinations

Oxidative stress resistance was determined by disc inhibition assay and survival curves. Sterile 6-mm-diameter paper discs were applied to BA plates which had been streaked for confluent growth. Samples (10 μl) of 1.9% methyl viologen or 37% hydrochloric acid were applied to the discs and the zone of inhibition of growth was measured after incubating the plates for 48 h at 37°C. To determine survival of bacterial cells, methyl viologen (final concentration of 50 μM) was added to cell suspensions (*c*. 10^7 cfu ml^{-1}) in 5 ml of BHI supplemented with 0.2% cyclodextrin and incubated under microaerobic conditions at 37°C with shaking (100 r.p.m). Samples were removed at appropriate intervals, serially diluted and plated directly BA plates for enumeration. For all experiments, results were obtained from three independent determinations and were analysed using the *t*-test. A *P*-value of ≤ 0.05 was considered significant.

RNA preparation

H. pylori was grown in 50 ml of BHI broth containing 0.2% cyclodextrin at 37°C to mid-log phase. The bacterial cell suspensions were then diluted with fresh, pre-warmed media to an OD_{600} of 0.1 before exposure to various stresses. For RNA stability studies, liquid cultures were treated with rifampicin to inhibit the initiation of transcription (Schlessinger *et al*., 1977) and sampled at 15 min intervals. Extraction of RNA was performed immediately using RNeasy spin columns (Qiagen) or TRIzol Reagent (Invitrogen) according to the manufacturers' instructions. The RNA concentration was determined spectrophotometrically at 260 nm using a GeneQuant *pro* RNA/DNA calculator (Pharmacia-Biotech). Results are representative of data obtained from up a minimum of two independent experiments.

Hybridization

For Southern and Northern blot hybridization, DNA fragments and RNA were blotted onto nylon membranes (Boehringer Mannheim) by capillary transfer in 20× SSC. The efficiency of transfer of RNA was verified by methylene blue staining. Hybridizations were performed using a digoxigenin (DIG) labelling and detection system according to the manufacturer's instructions (Roche). DIG-labelled DNA probes were synthesized by random incorporation of Dig-11-dUTP by PCR using primers specific to the corresponding gene (Table 1), and hybridizations were performed in DIG EasyHyb (Roche). For Southern blots, hybridizations were performed at 37°C and the filters were washed twice with 2× SSC and 0.1% SDS at room temperature and then with 0.1× SSC and 0.1% SDS at 68°C. For Northern blots, hybridizations were performed at 42°C, the filters were washed twice with 2× SSC and 0.1% SDS at room temperature, and then with 0.1× SSC and 0.1% SDS at 50°C. Membranes were developed using the CDP-Star chemiluminescent detection system (Roche) according to the manufacturer's instructions. Standardized

peak densities were determined using RNA Quantity 1 software (Bio-Rad).

SDS–PAGE and immunoblot analysis

Protein concentrations were estimated using a commercial version of the Bradford assay (Sigma Chemicals). Solubilized protein preparations were analysed on slab gels comprising a 5% acrylamide stacking gel and 12–15% resolving gel according to the procedure of Laemmli (1970). Electrophoresis was performed at 200 V using a mini-gel apparatus (Bio-Rad). Proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane at 0.8 mA cm^{-2} using a Mini Trans-Blot transfer cell (Bio-Rad). The efficiency of transfer of proteins was verified by Ponceau red staining. After blocking with 5% milk powder in PBS and Tween 1%, membranes were reacted at 4°C overnight with antisera diluted in 5% milk powder in PBS-0.2% Tween, and washed in PBS-0.2% Tween. Immunoreactants were detected with anti-rabbit peroxidase-linked immunoglobulin (Amersham) diluted 1:10 000 and reaction products were visualized on autoradiographic film by chemiluminescence using the ECL Western blotting detection system (Amersham).

Alkaline gel DNA analysis

Twenty-four hour mid-exponential wild-type and *csrA* mutant broth cultures were diluted to an OD_{600} of 0.1 in fresh, prewarmed BHI containing 0.2% cyclodextrin and then hydrogen peroxide or methyl viologen were added to a final concentration of 50 and 100 mM, and 100 μM respectively. After 30 min of incubation in a microaerobic incubator at 37°C with shaking (100 r.p.m), cells were harvested by centrifugation and analysed for DNA fragmentation by alkaline gel electrophoresis as described elsewhere (Zirkle and Krieg, 1996). Briefly, plugs consisting of 10 μl of cells and 50 μl of 1% low melting point agarose (Roche) were incubated at 55°C for 1 h and then at room temperature overnight in a lysing solution consisting of 0.25 mM EDTA, 0.5% sodium lauryl sarcosine and 0.5 mg l^{-1} proteinase K (Sigma). The agarose plugs were then placed into the preformed wells of a 0.8% agarose gel prepared under alkaline conditions (50 mM NaOH, 1 mM EDTA) and the gel was subjected to electrophoresis for 4 h at 30 mV. The gel was then neutralized for 1 h in 30 mM NaCl/50 mM Tris-HCL (pH 6.0), stained for 30 min with ethidium bromide (0.5 μg ml^{-1}), destained in distilled water, and visualized under UV light.

Animal colonization

Six- to 8-week-old specific pathogen-free CD1 mice (Charles River) were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water *ad libitum*. All animal experimentation was performed in accordance with Home Office license 40/2340 and institutional guidelines. Aliquots of 100 μl, containing 10^7–10^8 *H. pylori* strains in BHI supplemented with 0.2% cyclodextrin, were administered orogastrically to mice as described elsewhere (Jenks *et al*., 1999). For all experiments, inoculating suspensions of SS1 and two independently constructed *csrA* mutants were prepared from identical, low subculture stocks (between 8 and 12 *in vitro* passages). Mice were killed at various time points up to 20 weeks after inoculation, and colonization with *H. pylori* was assessed by quantitative culture and serology as described previously (Jenks *et al*., 1999). For competitive infections, serial dilutions of the stomach homogenate were plated onto BA plates to determine the numbers of both wild-type and mutant bacteria or onto plates supplemented with 25 μg of kanamycin ml^{-1} to detect *csrA* mutant bacteria only. The results were expressed as a competitive index defined as the output ratio of *csrA* mutant to wild type divided by the input ratio of *csrA* mutant to wild type (Freter *et al*., 1981). To determine the 50% infective dose (ID_{50}), mice were infected with serial dilutions of bacteria as described above. The inoculum was serially diluted and plated to determine the actual bacterial dose. The mice were sacrificed and the stomachs were cultured as above to determine the number of animals infected at each dose. The Reed–Muench calculation was used to determine the ID_{50} (Reed and Muench, 1938).

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