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NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator

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The *Helicobacter pylori* protein NapA has been identified as a homologue of the *Escherichia coli* protein Dps. It is shown in this study that, like Dps, NapA is produced maximally in stationary phase cells and contributes to the ability of *H. pylori* to survive under oxidative stress conditions. Moreover, NapA co-localizes with the nuclear material, suggesting that it can interact with DNA *in vivo*. Furthermore, it is demonstrated that repression of NapA production by iron starvation was not so pronounced in a *H. pylori fur* mutant, suggesting that the ferric uptake regulator (Fur) is involved in *napA* regulation, and a potential *fur* box by which this control could be mediated is identified. This finding is consistent with the regulation of iron-binding proteins by Fur and also the modulation of Fur during oxidative stress, thus allowing NapA levels to be increased in the environmental conditions under which its ability to protect DNA from attack by toxic free radicals is most beneficial to the cell.

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INTRODUCTION

Within the unique niche in the stomach, and presumably during transmission, the important gastric pathogen *Helicobacter pylori* is exposed to oxidative stress originating from leakage of reactive oxygen species, respectively derived from the oxidative burst of invading phagocytic cells and the aerobic environment (Blaser, 1992; Marshall *et al.*, 1984; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991). Oxidative stress can also result from the formation of reactive oxygen species by the Fenton reaction, which is catalysed by excess free iron. Iron storage proteins (e.g. ferritin) mop up free intracellular iron and in so doing provide two advantages: ferritins (i) enable bacteria to store iron (an essential micronutrient) and (ii) protect against damage ensuing from oxidative stress. Dps is a structural homologue of bacterioferritin that lacks a ferroxidase centre and is implicated in the response of bacteria to oxidative stress (Grant *et al.*, 1998). Although there is no obvious DNA-binding site, the predicted structure of Dps is consistent with a model where DNA is intertwined between Dps dodecamers, thus condensing the chromosome into a compact structure and localizing its putative iron-sequestering activity to provide maximum protection to DNA.

Neutrophil-activating protein, NapA (Evans *et al.*, 1995a; Satin *et al.*, 2000), was so designated because of its ability to mediate neutrophil adhesion to endothelial cells (Yoshida *et al.*, 1993) and to bind to both mucin and neutrophil glycosphingolipids (Namavar *et al.*, 1998; Teneberg *et al.*, 1997). However, recently, NapA was shown to bind iron *in vitro* (Tonello *et al.*, 1999) and to adopt a dodecameric structure consistent with its homology to the Dps family of proteins (Grant *et al.*, 1998). Furthermore, Bijlsma *et al.* (2000) demonstrated binding of recombinant NapA to DNA in an ELISA in addition to showing that mutation of *napA* resulted in increased DNA damage in *H. pylori*.

Many proteins involved in iron acquisition and storage are regulated by the presence of iron in the environment. Dedicated regulatory pathways exist to achieve this, including the ferric uptake regulator (*fur*) (Braun, 2001; Hantke, 2001). In *H. pylori*, Fur not only performs its classical role in the regulation of iron uptake (Delany *et al.*, 2001), but also regulates iron storage (Bereswill *et al.*, 2000) and is implicated in iron-independent gene regulation (van Vliet *et al.*, 2001; Bijlsma *et al.*, 2002; Waidner *et al.*, 2002). Since NapA has been shown to be capable of interacting with iron

Abbreviations: DAPI, 4,6-diaminidino-2-phenylindole; DPP, 2,2′-dipyridyl; EDDA, ethylenediamine-*N*,*N*′-diacetic acid.

A colour version of Fig. 5 is available as supplementary material in JMM Online.

(Tonello *et al.*, 1999) and is implicated in the oxidative stress response in *H. pylori* (Olczak *et al.*, 2002), and as the presence of free radicals increases in the presence of iron, it is likely that *napA* is regulated by Fur, in order to sequester iron when abundant and thereby prevent subsequent DNA damage. Lending further weight to this notion, Fur has been shown to be regulated by oxidative stress (Zheng *et al.*, 1999).

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium or on LB agar plates at 37 °C. Where required, carbenicillin (50 μg ml^{-1}), kanamycin (100 μg ml^{-1}) or chloramphenicol (50 μg ml^{-1}) was added. *H. pylori* was grown on Oxoid blood agar no. 2, containing 7 % (v/v) horse blood, in Brucella broth containing 10 % (v/v) fetal calf serum (FCS) or in ISOSENS broth (Oxoid) supplemented with 0·2 % (w/v) cyclodextrin and 0·4 % (v/v) DENT (Oxoid) for 48 h with shaking at 37 °C in the presence of a BBL Campypakplus (Becton Dickinson) or in an atmosphere containing 86 % nitrogen, 6 % oxygen, 3 % hydrogen, 5 % carbon dioxide, 80 % humidity provided by the VAIN cabinet (MACS VA500 microaerophilic workstation, Don Whitley Scientific). Kanamycin or chloramphenicol (both at 20 μg ml^{-1}) was added to cultures of mutant strains. The *H. pylori fur* mutant 11638::*fur* and its parent, NCTC 11638 (Bereswill *et al.*, 2000; Bijlsma *et al.*, 2002), were kindly provided by Professor Dave Kelly (University of Sheffield, UK).

Stress tests. *H. pylori* strains were resuspended to an OD_{600} of 0·35 in PBS containing 50 mM H_2O_2, 50 mM H_2O_2/6·25 μM ethylenediamine-*N*,*N*′-diacetic acid (EDDA), 50 mM H_2O_2/200 μM 2,2′-dipyridyl (DPP) or 50 μM paraquat. Incubation was performed at 37 °C in the VAIN cabinet and duplicate samples were removed for analysis at 0, 0·5, 1, 2 and 4 h. Dilutions between 10^{-1} and 10^{-5} were performed in PBS containing 2000 U catalase and aliquots of 8 μl were removed, serially diluted and plated onto blood-agar plates for enumeration. The number of viable cells present before imposition of stress-inducing conditions was taken as 100 % survival and subsequent survival values were calculated relative to this.

Analysis of iron-regulated proteins. *H. pylori* strains were subjected to iron stress as described by Bereswill *et al.* (2000). *E. coli* strains were grown overnight in LB containing either 6·25 μM ferric sulphate, 0·227 mM EDDA or 0·2 mM DPP and were subjected to fractionation to isolate outer membranes, which were analysed by SDS-PAGE.

Fractionation of *H. pylori* outer membranes. Pelleted cells from 1 ml culture were resuspended in 0·1 ml ice-cold 10 mM Tris/HCl (pH 7·4)/20 % (w/v) sucrose containing 0·1 mg lysozyme ml^{-1}. An equal volume of ice-cold 10 mM Tris/HCl (pH 7·4)/1 mM EDTA was then added and the mixture was incubated for 30 min on ice with gentle shaking. Magnesium sulphate was added to a final concentration of 0·2 M and spheroplasts were harvested by centrifugation at 3000 ***g*** for 3 min. Following resuspension in 75 μl 20 mM Tris/HCl (pH 7·4)/0·7 % (w/v) sodium lauryl sarcosine and incubation at room temperature for 25 min, outer membranes were pelleted by centrifugation at 3000 ***g*** for 30 min, washed in 20 mM Tris/HCl (pH 7·4) and resuspended in the same buffer. Outer-membrane proteins were precipitated with 10 % (v/v) trichloroacetic acid for 60 min on ice prior to SDS-PAGE analysis.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed as described in Hardie *et al.* (1996), except that PBS with 0·5 % (v/v) Tween 20 (PBST) replaced TBST. The primary antibodies anti-NapA (Evans *et al.*, 1995b), kindly provided by Doyle Evans (VA Medical Center, Houston, TX, USA), and our anti-NapA antiserum (see below) were respectively used at dilutions of 1 : 10 000 and 1 : 1000. Western blots were developed using enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions. Whole-cell extracts were prepared by collecting cells by centrifugation for 3 min in a microfuge and resuspending in SDS-PAGE loading buffer. Samples were sonicated for 10 s and boiled for 5 min before being adjusted to equal total protein content according to the OD_{600} of the harvested bacterial culture, and were then subjected to SDS-PAGE.

DNA manipulation. DNA was manipulated by standard methods (Sambrook *et al.*, 1989). Restriction enzymes (Promega) were used according to the supplier's instructions. For isolation of plasmid DNA from *E. coli*, the Qiagen Mini and Midi kits were used. Genomic DNA was extracted from *H. pylori* according to Atherton (1997). Standard methods were used for preparation of competent cells and for electroporation of plasmids into *E. coli* (Sambrook *et al.*, 1989). Southern blots were performed using DIG-labelled probes (prepared according to the manufacturer's instructions; Boehringer Mannheim) following PCR amplification from *H. pylori* strain N6 and pNap2 (see below) using primer pairs nap1 (5′-TGCGATCGTGTTGTTTATG)/nap3 (5′-ATGAGCTTCTAGCATCCAA) and Kan374 (5′-ATAGAAGAAACCCAGGACAATAACC)/KanR778 (5′-ATAGAAGTTCCACATCATAGGTGG), respectively.

Mutant strain construction. Following PCR amplification (5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C, 5 min at 72 °C) of genomic DNA from *H. pylori* strain 26695 with primers nap1 and nap3, the majority of open reading frame HP0243 (Tomb *et al.*, 1997) was cloned into the pTAg vector using the LigATor kit (Novagen) according to the manufacturer's instructions. The cloned fragment was then recloned into the plasmid vector pUC19 (Amersham Pharmacia Biotech) using the restriction enzymes *Pst*I and *Xba*I (creating pNap1). A 25 bp deletion and unique *Bgl*II site were then engineered into *napA* by inverse PCR mutagenesis (IPCRM) using primers nap6 (5′-GGCAGATCTAGAATTTCTTTAAAGAT) and nap7 (5′-GGCAGATCTGAATTTAAAGAGCTCTC) as described previously (Wren *et al.*, 1994). A *Bam*HI fragment containing a 1·4 kb kanamycin-resistance cassette (*aph-3*) (Trieu-Cuot *et al.*, 1985) was cloned into the unique *Bgl*II site generated by IPCRM, creating pNap2.

A mutant in *napA* was created by natural transformation and allelic exchange mutagenesis as follows. *H. pylori* strain N6 (Ferrero *et al.*, 1992) was subcultured twice for 24 h on blood-agar plates and the cells were then harvested into 1 ml sterile cold 5 % (w/v) sucrose/15 % (v/v) glycerol. Cells were then washed three times in this buffer and finally resuspended in 30 μl of the same buffer. Plasmid DNA (pNap2) was then added to the cells and electroporation was carried out at 2·4 V, 200 Ω. Ice-cold ISOSENS broth was then added and cells were incubated on a blood-agar plate for 24 h at 37 °C in the VAIN cabinet. Mutants that had undergone allelic exchange were then selected on plates containing 100 μg kanamycin ml^{-1} and denoted as *H. pylori* strain N6::*napA*.

Overproduction of rNapA in *E. coli*. For high expression, the *napA* open reading frame was amplified by PCR from *H. pylori* strain N6 (5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C, 5 min at 72 °C) with primers *napA*F*Nde* (5′-CCCATCCATATGAAAACATTTGAAATTCTAAAACAT) and *napA*R *Bam*HI (5′-ATGGCAGGATCCTTAAGCCAAATGGGC) and cloned into pET3a (Novagen), creating pCC2. Clones were confirmed by restriction digestion and sequencing. Automated non-radioactive sequencing reactions were carried out using the BigDye terminator cycle-sequencing kit in conjunction with a 373A automated sequencer (Perkin Elmer Applied Biosystems).

Antibody generation. To supplement the limited supply of anti-NapA (Evans *et al.*, 1995b), a rabbit polyclonal anti-NapA antiserum was

generated as follows. *H. pylori* N6 cells were resuspended in water and incubated for 20 min at room temperature. After centrifugation for 15 min at 17 000 ***g***, the pellet was again incubated in water for 20 min and then centrifuged for 15 min at 25 000 ***g***. The proteins in the resulting supernatant were separated through 15 % SDS-PAGE. NapA was excised from the gel and electroeluted into 50 mM ammonium bicarbonate/0·1 % (w/v) SDS. Following confirmation of the identity of the purified protein by N-terminal sequencing, rabbit polyclonal antibodies were raised according to a protocol based on Harlow & Lane (1988). New Zealand White rabbits were immunized subcutaneously biweekly with between 50 and 400 μg antigen, mixed 50 : 50 with Freund's complete adjuvant on the first immunization and subsequently with Freund's incomplete adjuvant. A test bleed was carried out following the third immunization. A fourth immunization was carried out before finally obtaining complete bleeds from the rabbits. Adsorption of contaminating anti-*E. coli* antibodies was carried out by incubation at 37 °C for 60 min of 50 μl serum with 1 ml of lysates of *E. coli* DH5α(pBluescript) and *E. coli* BL21(DE3)(pLysS,pET3a) prepared in PBSA.

Immunofluorescence. *E. coli* strains BL21(DE3)(pLysS,pCC2) and BL21(DE3)(pLysS) were grown in LB containing carbenicillin and chloramphenicol for 3 h. IPTG (1 mM final concentration) was then added and the incubation continued for a further 8 h. One millilitre of culture was added to 10 ml of 80 % (v/v) methanol, mixed very gently and left overnight at 4 °C. Following centrifugation at 2700 r.p.m. for 5 min at 4 °C, cells were resuspended in 1 ml of 80 % (v/v) methanol. An aliquot (10 μl) of this cell suspension was air-dried onto a polylysine-coated slide for 20 min and then covered with 50 μl of 25 mM Tris/HCl (pH 8)/50 mM glucose/10 mM EDTA containing 2 mg lysozyme ml^{-1} and incubated at room temperature for 5 min. The slides were then covered sequentially in 4 ml of 99 % (v/v) methanol for 1 min and 4 ml acetone for 1 min before air-drying. Fifty microlitres PBST containing 2 % (w/v) BSA was then applied and covered with a cover slip. Following a 15 min incubation at room temperature, 50 μl PBST/BSA containing a 1 : 200 dilution of our anti-NapA antiserum was applied and the slide was incubated for 1 h in a moist chamber. Following three washes in PBST, 50 μl PBST/BSA containing a 1 : 500 dilution of FITC-conjugated protein A (Sigma) was applied and the slide was incubated for 1 h in a moist chamber. Following three washes in 5 ml PBST, 10 μl 10 μg 4,6-diaminidino-2-phenylindole (DAPI) ml^{-1} was added and the slide was incubated for 1 min before removal and air-drying. Samples were analysed by phase-contrast and fluorescence microscopy.

RESULTS

NapA is produced maximally in stationary phase *H. pylori*

NapA has been purified from *H. pylori* (Evans *et al.*, 1995b) and also following production in the heterologous host *Bacillus subtilis* (Tonello *et al.*, 1999) and subjected to a range of investigations *in vitro* to assess its predicted ability to interact with iron and DNA.

To determine the temporal production of NapA, *H. pylori* strain N6 was grown until late stationary phase and whole-cell samples were removed at selected time-points for Western blot analysis following adjustment for total cell number (Fig. 1). NapA production occurred throughout the growth of *H. pylori*, but was enriched in stationary phase, consistent with its proposed functions in iron storage and DNA protection.

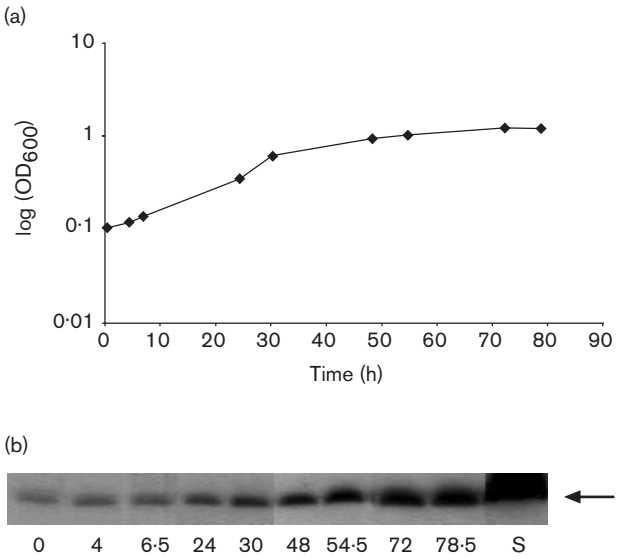

Fig. 1. NapA production increases during *H. pylori* growth. *H. pylori* strain N6 was grown in ISOSENS broth. Samples were removed at the times indicated for measurement of the OD_{600} (a) and to prepare whole-cell extracts. (b) Following separation of samples equalized for total protein concentration by 15 % SDS-PAGE, NapA (arrow) was detected by Western blot using the specific antibody raised in this study and is shown next to a recombinant NapA preparation (S) for orientation.

Mutation of *napA* decreases the survival of *H. pylori* in the presence of oxidative stress

In order to assess the function of NapA *in vivo*, a mutant of *napA* was created in *H. pylori* strain N6 (Ferrero *et al.*, 1992) as described in Methods and confirmed by PCR, Southern blot and Western blot analysis (data not shown, see Fig. 4b). Mutation of *napA* did not result in a severe growth impediment, as evidenced by the similar OD_{600} reached after growth for 48 h in Brucella broth supplemented with FCS and either 500 μM NaCl or 100 or 500 μM iron chloride (OD_{600} = 1·04, 1·46, 1·36, respectively), compared with the parent strain *H. pylori* N6 (OD_{600} = 1·08, 1·1, 1·2, respectively; see Fig. 4 for description of methods employed).

Although mutation of *napA* was well tolerated when *H. pylori* is grown in nutrient-rich conditions, the survival of the mutant was reduced in adverse conditions. The wild-type *H. pylori* strain N6 and the *napA* mutant N6 : : *napA* were exposed to hydrogen peroxide in broth culture and viable counts were taken over the subsequent 4 h (see Methods). Fig. 2 shows that the wild-type strain survived significantly better under these conditions. Similar results were observed when the wild-type and mutant strains were challenged in broth cultures with 50 μM paraquat, which generates superoxide ions rather than hydroxyl free radicals (Fig. 2). One of the major effectors of DNA damage (leading ultimately to cell death) in oxidative conditions is free intracellular ferrous iron. The homology of NapA to the iron-storage protein

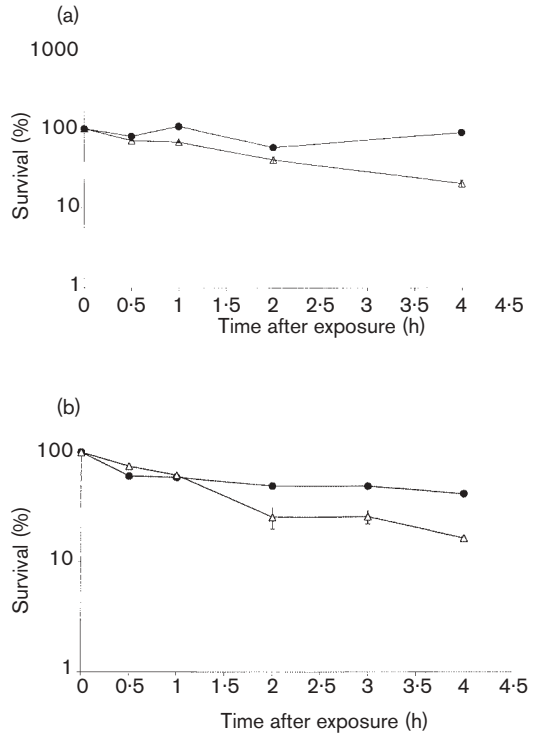

Fig. 2. A *napA* mutant survives less well than wild-type *H. pylori* upon exposure to oxidative stress. *H. pylori* strains N6 (●, wild-type) and N6 : : *napA* (△, *napA* mutant) were subjected to oxidative stress induced by the presence of 50 mM hydrogen peroxide (a) or 50 μM paraquat (b). At the times indicated, samples were removed and survival was assessed by calculating the number of c.f.u. present relative to those present initially (see Methods). The graph shows the $\log_{10}$ percentage survival plotted against the time of exposure to hydrogen peroxide or paraquat. Data shown are representative of four independent experiments and the standard errors of the mean c.f.u. are indicated by the error bars.

bacterioferritin led us to hypothesize that, in the absence of iron (generated by iron chelators), the lack of NapA would not be as detrimental. However, addition of the iron chelators DPP and EDDA to the growth medium simultaneously with hydrogen peroxide did not improve the viability of the *H. pylori napA* mutant reproducibly (data not shown).

Identification of putative *fur* boxes within the *napA* promoter

Despite its small size, the *H. pylori* genome encodes a number of systems involved in iron uptake and storage, including the regulatory protein Fur (Tomb *et al.*, 1997). We observed that there are several A+T-rich regions of dyad symmetry immediately upstream of the start codon of *napA* that could function as a *fur* box. We used the consensus *H. pylori fur* box sequence proposed recently by Delany *et al.* (2001) to identify putative *fur* boxes centred between 20 and 180 bp upstream of genes in *H. pylori* strain J99 (Alm *et al.*, 1999) involved in iron storage and detoxification using the analysis tools available at http://rsat.ulb.ac.be/rsat/. Searches of the 200 bp upstream of *napA* with this consensus (NtaTNaN_5ttTT aatNAaAATNataAaANatT) identified two regions (Fig. 3a), each with 8/10 of the absolutely conserved bases (indicated in upper case) plus 11 of the pyrimidine bases (indicated in lower case). The two missing conserved bases were replaced by a pyrimidine. One of the putative *fur* boxes was centred at position −167 and the other at −111 from the ATG. The spacing from the mapped transcription start site (Olczak *et al.*, 2002) is also indicated in Fig. 3(a), and parallel searches revealed the *fur* boxes located experimentally by Delany *et al.* (2001) upstream of *frpB1* (Fig. 3b).

NapA is regulated by *fur*

Since putative *fur* boxes are located upstream of *napA* (see above), we investigated whether the production of NapA (like that of the *H. pylori* bacterioferritin, Pfr; Bereswill *et al.*, 1998) was regulated by iron via Fur. The *H. pylori fur* mutant 11638 : : *fur* and its parent, NCTC 11638 (Bereswill *et al.*, 2000; Bijlsma *et al.*, 2002), were grown overnight in Brucella broth containing 10 % FCS and supplemented with either 100 μM $FeCl_3$, 500 μM $FeCl_3$, 40 μM of the iron chelator desferal or 500 μM NaCl to exclude any effect of osmotic stress. To assess the effect of NapA upon survival under these conditions, *H. pylori* strains N6 and N6 : : *napA* were analysed similarly. Whole-cell extracts containing matched total protein contents were prepared and SDS-PAGE revealed modulation of the production of a 19 kDa protein, presumed to be Pfr by analogy with the data reported by Bereswill *et al.* (2000), in response to iron availability (Fig. 4a). As reported previously, when the growth medium was supplemented with desferal, the level of Pfr was higher in a *fur* mutant than in the parent strain. Also in accordance with this previous study, the OD of cultures grown in the presence of desferal was considerably reduced (OD_{600} = 0·3, 0·3, 0·2 and 0·1 for *H. pylori* strains N6, N6 : : *napA*, NCTC 11638 and 11638 : : *fur*, respectively, compared with 1·1–1·4 in the absence of desferal). Although the levels of many proteins appeared to stay the same or to fall in the presence of desferal, one in particular, at 66 kDa, displayed a marked increase under these conditions in both strains. Western blots demonstrated that NapA production in *H. pylori* strains N6 and NCTC 11638 is regulated by iron in a manner similar to that demonstrated previously for Pfr. In *H. pylori* strain 11638 : : *fur*, NapA levels were at least twofold higher under all growth conditions and increased most significantly (approximately fivefold) in the presence of desferal (Bereswill *et al.*, 2000; Fig. 4b). NapA is absent from N6 : : *napA* (Fig. 4b).

Since NapA is predicted by homology to bacterioferritin to bind iron, although it does not possess an active ferroxidase centre, we hypothesized that its production might influence the expression of other iron-regulated proteins. However, no effect was observed on the expression of iron-regulated proteins such as the visible 19 kDa (presumed Pfr) protein when the *H. pylori napA* mutant was compared with its parent strain (data not shown). Similarly, high-level produc-

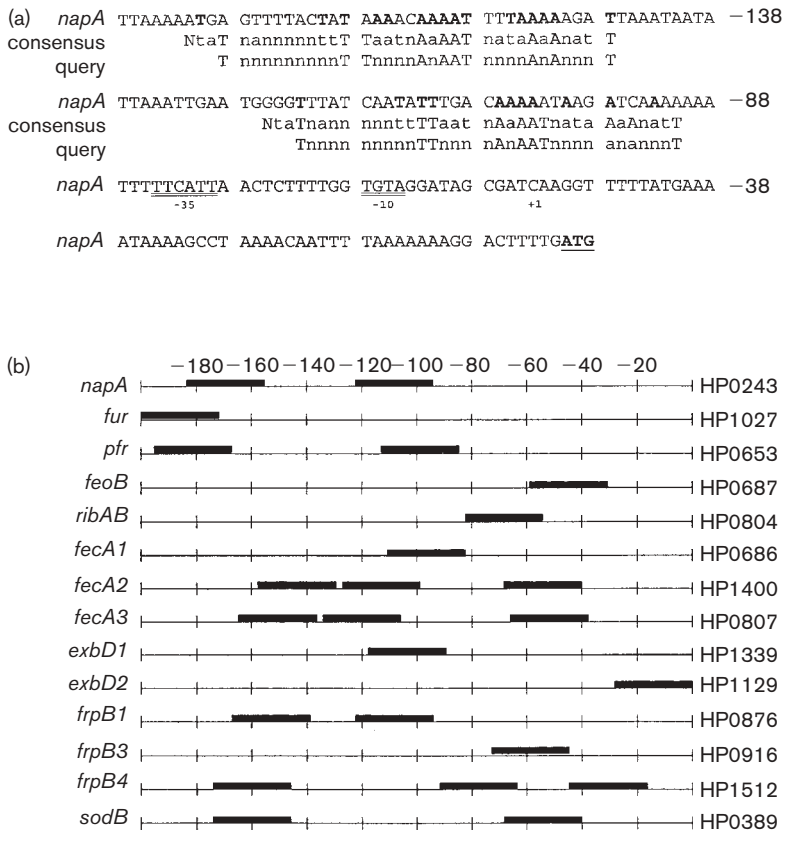

Fig. 3. Proposed *fur* box upstream of *H. pylori napA*. (a) The DNA sequence immediately upstream of the start codon of *napA* (bold and underlined), with the A+T-rich dyad symmetrical regions that could function as a *fur* box in bold and the −10 and −35 regions proposed by Olczak *et al.* (2002) double-underlined, is aligned with the consensus *H. pylori fur* box (Delany *et al.*, 2001) and the sequence used as the query to locate the *napA fur* box. (b) Alignment of the upstream regions of several genes known to be regulated by *fur*. The likely *fur* boxes are indicated as solid boxes and the TIGR ORF identifiers are listed on the right (Tomb *et al.*, 1997). The apparent discrepancy in the position of *fur* boxes between these data and those presented in Delany *et al.* (2001) stems from indicating the spacing relative to the ATG and the transcriptional start site, respectively.

tion of soluble recombinant NapA in *E. coli* did not induce the expression of iron-regulated proteins, despite the possibility that this would lower intracellular iron concentrations (data not shown).

NapA co-localizes with cellular DNA *in vivo*

The use of fluorescent microscopy has allowed us to visualize the co-localization of rNapA with DNA directly within the living *E. coli* cell and also demonstrates that, in so doing, the DNA is concentrated to one part of the cell. *E. coli* BL21(DE3)(pLysS) harbouring either pCC2 (encoding *napA*) or the empty control plasmid vector (pET3a) was grown for 8 h and the production of rNapA by the former was verified by SDS-PAGE (Fig. 5) and Western blot analysis (data not shown). When cells were lysed in a French pressure cell at 12 000 p.s.i. and insoluble material was pelleted by centrifugation at 10 000 ***g*** for 10 min, more than 90 % of the rNapA detected was present in soluble cell fractions and, thus, the high-level production was not leading to the formation of inclusion bodies (data not shown). DNA was stained with DAPI and the cells were probed with anti-NapA antisera and FITC-conjugated protein A. Microscopy was then performed to visualize the cells by phase-contrast, plus the distribution of DAPI and FITC. Fig. 5 shows that, in BL21(DE3)(pLysS,pCC2), the DAPI (Fig. 5b) and FITC (Fig. 5c) co-localized to a region of visually increased density seen in phase-contrast images (Fig. 5a). In contrast, with strain BL21(DE3)(pLysS,pET3a), no areas of increased density were seen by phase-contrast (Fig. 5d), the DNA appeared to be generally spread throughout the cell, except for occasional polar concentration in some cells, and the FITC staining was at background levels. Similar results were obtained on three separate occasions, suggesting that the overproduced rNapA protein co-localizes with DNA and may indeed induce compartmentalization of the DNA within the cell. The observed compartmentalization of the DNA was not due to rNapA simultaneously forming insoluble inclusion bodies. The two strains grew comparably, as judged by OD_{600} measurements taken throughout growth (data not shown). Unfortunately, technical problems prevented us from observing this effect in *H. pylori*.

DISCUSSION

Our observations that the antioxidant, iron-binding protein NapA can protect cells against oxidative stress-induced lethality and is produced maximally during stationary phase are in accordance with recent data presented by Dundon *et al.* (2001) and Olczak *et al.* (2002). The stationary phase induction is likely to be mediated through the sigma factor homologous to RpoS, and these observations extend the noted sequence similarity of NapA to Dps and bacterioferritin to one of likely functional conservation. It has been shown that production of *E. coli* Dps is induced under conditions of starvation (Almiron *et al.*, 2000) and, during stationary phase, nutrients become scarce. In addition, cell death is likely to result in increased levels of iron and free radicals, which will induce oxidative stress via catalysis of the Fenton

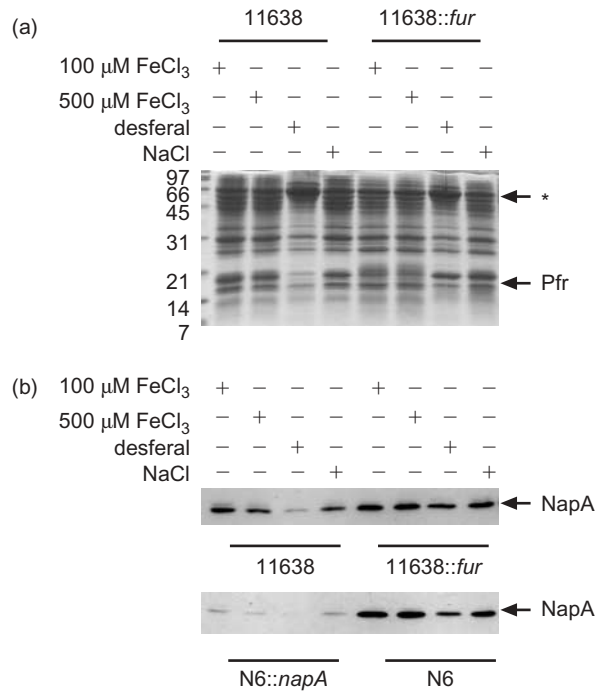

Fig. 4. NapA is controlled by *fur* in *H. pylori*. *H. pylori* strains 11638 : : *fur* (*fur* mutant), NCTC 11638 (parent), N6 : : *napA* (*napA* mutant) and N6 (parent) were grown in iron-rich conditions (100 or 500 μM $FeCl_3$), iron-depleted conditions (40 μM desferal) or 500 μM NaCl for 16 h. Cultures were equalized according to OD_{600} and cells were harvested and resuspended in SDS-PAGE sample buffer. Whole-cell protein profiles were analysed following Coomassie staining of 15 % SDS-PAGE gel (a) and NapA levels were assessed by Western blotting with the antiserum raised in this study (b). Molecular masses of markers are shown in kDa and the positions of ferritin (Pfr) and NapA are indicated by arrows, whilst a 66 kDa protein induced in iron-starved conditions is indicated by the asterisk.

reaction. Indeed, higher levels of intracellular iron have been observed in stationary phase cells (Abdul-Tehrani *et al.*, 1999). Furthermore, increased production of enzymes involved in the oxidative stress response (catalase, superoxide dismutase, AhpC) has been reported and linked to the regulator Fur (Dubrac & Touati, 2002; Hantke, 2001). Moreover, direct regulation of Fur by the oxidative-stress response has been observed (Zheng *et al.*, 1999), an understandable link as, under oxygen-rich conditions, iron is a source of dangerous radicals.

The presence of multiple *fur* boxes within the *napA* promoter is not unusual (Fig. 3) and has also been noted experimentally (Delany *et al.*, 2001). It is possible that one of the *fur* boxes upstream of *napA* serves as a high-affinity, iron-regulated gene regulator, whilst the other binds Fur with a low affinity and is not influenced by iron availability, in a manner similar to that described for *frpB1* by Delany *et al.* (2001). Indeed, Fur has been shown to respond to environmental signals other than iron concentration. For instance, Fur has been implicated in regulation of *H. pylori* genes in response to a range of conditions unrelated to iron availability, such as the modulation of *H. pylori* urease in response to nickel (van Vliet *et al.*, 2001), differential regulation of *pfr* in the presence of a range of different metals (Bereswill *et al.*, 2000) and *H. pylori* acid resistance (Bijlsma *et al.*, 2002). The sequence variation within the proposed *fur* boxes is not unusual, since Fur appears to have a flexible mode of DNA interaction, providing it with the ability to behave both as a very specific repressor and as a more general regulator (de Lorenzo *et al.*, 1988; Escolar *et al.*, 1999). Experimental confirmation is required to identify these sequences as true *fur* boxes and to evaluate their relative contribution to recognition of the *napA* promoter by Fur.

Although it would be predicted that the Pfr ferritin of *H. pylori* is regulated by iron availability via *fur*, there is disagreement in the literature. Bereswill *et al.* (2000) demonstrated, using the chelator desferal, that Pfr was repressed by the removal of iron, whereas Dundon *et al.* (2001) reported that they could not show this repression. Since Dundon *et al.* (2001) were also unable to demonstrate an effect of desferal on NapA, they concluded that *fur* was not involved in *napA* regulation. The inconsistency in these results may reflect different growth conditions, since Bereswill *et al.* (2000) grew their *H. pylori* cultures in broth supplemented with FCS, whilst Dundon *et al.* (2001) appeared to supplement *H. pylori* broth cultures with cyclodextrin. Cyclodextrin affects the iron-chelating properties of desferal, negating its effect upon cells and leading to an absence of expected iron-regulation (A. H. M. van Vliet, personal communication). We were able to repeat the results of Bereswill *et al.* (2000), indicating iron-dependent regulation of Pfr, identify putative *fur* boxes upstream of the *pfr* and *napA* genes (Figs 3b and 4) and, in parallel, demonstrate repression of *napA* by desferal. This would suggest that *fur* does regulate *napA*, and this was confirmed by our demonstration that the effect of desferal upon NapA production is less evident in a *fur* mutant of *H. pylori*. There appears to be approximately fivefold more NapA in the presence of desferal when an *H. pylori* strain deficient in *fur* is analysed. This difference is not a reflection of growth-phase-dependent NapA production noted earlier, since the optical densities of the cultures analysed were comparable. Moreover, since the level of NapA is elevated under all the growth conditions analysed in the *H. pylori fur* mutant compared with its parent, it can be concluded that Fur influences the production of NapA. However, since the *H. pylori fur* mutant retained the ability to modulate the level of NapA in response to the availability of iron, additional regulatory influences must be at work, e.g. aconitases (Hantke, 2001).

Under conditions of excess iron, the *fur* mutant displayed a smear of proteins at the expected mobility for Pfr (Fig. 4). This is likely to be due to (i) the ability of ferritin to assume a number of different conformational forms depending on the extent of iron loading due to oxidation (Welch *et al.*, 2001), particularly since such isoforms have been described for *H. pylori* Pfr by Waidner *et al.* (2002), and (ii) the susceptibility of *H. pylori* Pfr to post-translational modification (Waidner

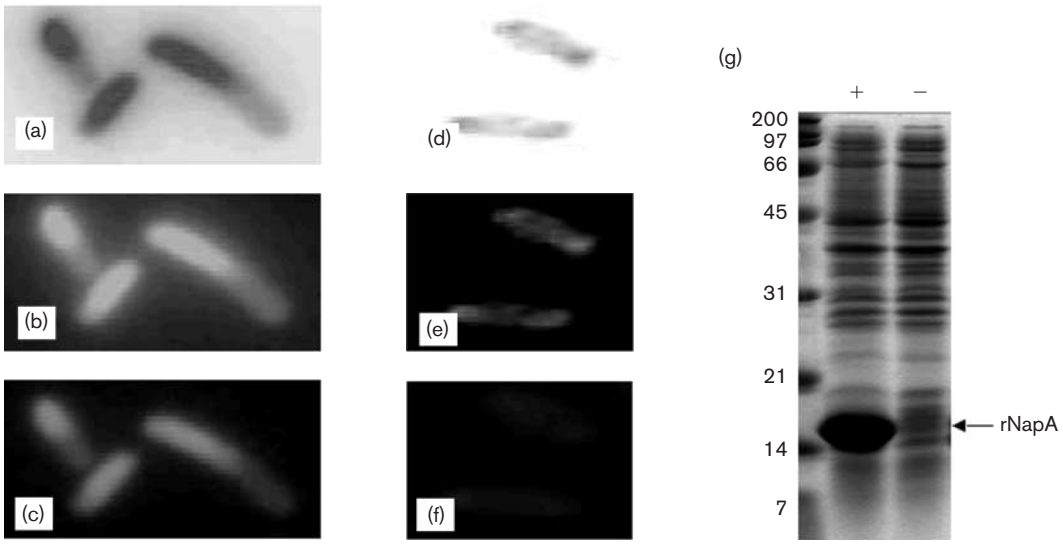

Fig. 5. NapA co-localizes with cellular DNA. *E. coli* BL21(DE3) harbouring either pCC2 (bearing *napA*) [a–c, g (lane +)] or the empty plasmid vector pET3a [d–f, g (lane −)] were grown for 8 h in LB broth containing 1 mM IPTG at 30 °C with shaking and the production of rNapA by the former was verified by analysis of whole-cell extracts subjected to Coomassie-stained SDS-PAGE (g) and Western blot analysis (data not shown). Cells were stained with DAPI and probed with FITC-conjugated anti-NapA antiserum according to Methods. Microscopy was then performed to visualize the cells by phase-contrast (a, d) and DAPI under UV (b, e) and FITC with a blue filter (c, f). Representative images from three separate experiments are shown. In (e), molecular masses of markers are shown in kDa and the position of rNapA is indicated by the arrow. A colour version of the figure is available as supplementary material in JMM Online.

et al., 2002). In agreement with the former suggestion, the dispersed nature of the 19 kDa protein was less evident when cultures were supplemented with NaCl to control for osmotic effects on protein production and thus contained lower iron concentrations. It is not entirely surprising that overproduction (recombinant NapA in *E. coli*) or absence (*H. pylori napA* mutant) of a single iron-binding protein did not result in alteration of the levels of proteins shown to be regulated by iron, since the total iron pool would neither increase nor be depleted thanks to the many systems present to deal with iron, ensuring the provision of iron in an accessible form and avoiding its potentially toxic effects. The presence of multiple iron-recycling pathways and their careful regulation result in compensatory modulation of these interconnected systems, allowing cells to balance iron storage and removal to maintain optimal growth (Abdul-Tehrani *et al.*, 1999).

For the first time, co-localization of NapA with cellular DNA was demonstrated *in vivo*, as has been shown for the other members of the Dps family of proteins (Azam *et al.*, 2000). Previous attempts to show this *in vitro* by DNA mobility assays and ELISA have met with varying success (Tonello *et al.*, 1999; Bijlsma *et al.*, 2000). Because *in vitro* analysis of DNA interactions requires the maintenance of protein function during overproduction and purification, failure to observe binding is not conclusive proof of its absence. Therefore, we chose to investigate whether NapA interacts with DNA *in vivo*. Indirect evidence exists that suggests that NapA interacts with DNA, resulting in protection against DNA damage (Bijlsma *et al.*, 2000); we were able to support this observation by co-localizing NapA with DNA through fluorescence microscopy using specific antisera. The association between NapA and DNA appeared to cause a condensation of the DNA within one part of the cell, which was not the result of NapA forming insoluble inclusion bodies. This condensation may be due to the high level of rNapA in the cells (approx. 80 % of total protein), however, as Dps was shown previously to be evenly distributed within *E. coli* cells (Azam *et al.*, 2000). This observation is consistent with DNA wrapping around a hexameric array of NapA in a pattern similar to that proposed for Dps (Grant *et al.*, 1998).

In summary, we have shown that NapA production is affected by Fur and, consistent with its homology to Dps, it is produced maximally in stationary phase and co-localizes with DNA, causing it to accumulate in one area of the bacterial cell. This is likely to protect the DNA from damage by free radicals, a suggestion that is borne out by the loss of viability of an *H. pylori napA* mutant when exposed to oxidative stress. It will be interesting to investigate whether the protective role of NapA facilitates the pathogenicity of *H. pylori*.

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