Neutrophil-activating protein (HP-NAP) versus ferritin (Pfr): comparison of synthesis in *Helicobacter pylori*

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Abstract

We recently reported that the neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is capable of binding iron in vitro. To more fully understand the relationship between iron and HP-NAP the synthesis of HP-NAP was compared to that of Pfr, another iron-binding protein of *H. pylori*. Synthesis of HP-NAP and Pfr in growing cultures of *H. pylori* was analysed under iron depletion and iron, copper, nickel and zinc overload. The synthesis of HP-NAP and Pfr in *H. pylori* was also analysed under conditions of varying pH and oxidative stress. In addition, recombinant HP-NAP and Pfr were produced in *Escherichia coli* to assess the contribution of the two proteins to increased survival of *E. coli* under heavy metal overload. Our data reveal that both HP-NAP and Pfr accumulate in the stationary phase of growth. HP-NAP synthesis is not regulated by iron depletion or overload or by the presence of copper, nickel or zinc in liquid medium and it does not confer resistance to these metals when produced in *E. coli*. Except for an increase in the synthesis of Pfr at pH 5.7 neither the pH or oxidative stress conditions investigated had an affect on the synthesis of either protein. An increase in Pfr synthesis was observed under iron overload and a decrease was observed under conditions of copper, nickel and zinc overload confirming previous reports. Recombinant Pfr, as well as conferring resistance to iron and copper as previously reported, also conferred resistance to zinc overload when produced in *E. coli*. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Neutrophil-activating protein; HP-NAP; Pfr; Ferritin; Stress; *Helicobacter pylori*

1. Introduction

*Helicobacter pylori*, a Gram-negative bacterium which colonises the gastric mucosa of humans is now recognised as the major aetiological agent of chronic gastritis and peptic ulcers and has been linked with the development of gastric carcinoma (for review see [1]). *H. pylori*-induced gastritis is typically associated with infiltration of the infected stomach mucosa by neutrophils and there is a good correlation between this neutrophil infiltration and mucosal damage (for review see [2]). A protein capable of promoting neutrophil adhesion to endothelial cells in water extracts of *H. pylori* has been identified [3]. This 17-kDa protein, termed HP-NAP for neutrophil-activating protein, is also capable of inducing the production of reactive oxidative intermediates [4,5]. HP-NAP is a major antigen in the human immune response to *H. pylori* infection and is a strong candidate as a part of a multicomponent recombinant anti-*H. pylori* vaccine [5,6]. We recently reported a computer modelling, spectroscopic and electron microscopic study of HP-NAP and revealed that, while it is structurally similar to the *Escherichia coli* DNA-binding protein Dps it was also capable of binding up to 500 atoms of iron in vitro [7,8]. The evidence therefore pointed to a possible function for HP-NAP in iron binding in *H. pylori*. Indeed Evans et al. [9] originally identified HP-NAP as a bacterioferritin based on the nucleotide sequence of the *napA* gene. The gene has several A+T-rich regions of dyad symmetry immediately upstream of its start codon which could function as Fur-regulated promoters for iron regulation. In addition the observed stability [7] to thermal denaturation and denaturing agents of HP-NAP is also a characteristic of many bacterioferritins [10].
To date a number of groups have identified and characterised mechanisms by which \textit{H. pylori} acquires and then utilises iron \cite{11,12}. Bereswill et al. \cite{13} reported the characterisation of the \textit{pfr} gene which encodes a \textit{H. pylori} ferritin originally described by Frazier et al. \cite{14} and very recently showed that the synthesis of the protein is regulated by the ferric uptake regulator homolog (Fur) in \textit{H. pylori} \cite{15} and that this Fur-mediated \textit{pfr} expression is repressed by iron depletion and by nickel, copper, manganese and zinc overload \cite{15}.

A logical progression from our previous studies was therefore to identify any other similarities between HP-NAP and Pfr. This was done by first comparing the synthesis of HP-NAP and Pfr by \textit{H. pylori} in liquid medium and then by determining whether this synthesis was affected by the addition of iron and other metals to the medium. The ability of recombinant HP-NAP and Pfr in conferring metal resistance when overexpressed in \textit{E. coli} was also compared. During the inflammation process iron catalyses the generation of reactive oxygen intermediates from superoxide anions produced by neutrophils and monocytes \cite{16}. The sequestration of iron by bacterial ferritins is believed, in some cases, to protect against such oxidative stress \cite{17}. By using the radical mediator methyl viologen (paraquat) \cite{18} the effect of oxidative stress on oxidative stress \cite{17}. By using the radical mediator methyl viologen (paraquat) \cite{18} the effect of oxidative stress on oxidative stress \cite{17}. By using the radical mediator methyl viologen (paraquat) \cite{18} the effect of oxidative stress on oxidative stress \cite{17}. By using the radical mediator methyl viologen (paraquat) \cite{18} the effect of oxidative stress on oxidative stress \cite{17}. By using the radical mediator methyl viologen (paraquat) \cite{18} the effect of oxidative stress on oxidant mediators such as paraquat \cite{18}.

2. Materials and methods

2.1. Bacterial strains and growth conditions

\textit{H. pylori} strain CCUG 17874 was used in this study and was grown under microaerophilic conditions for 3 days at 37°C on \textit{Campylobacter} agar base (Oxoid) containing 7% lysed horse blood and supplemented with cefsulodin (6 mg 1\(^{-1}\)), amphotericin (5 mg 1\(^{-1}\)), vancomycin (10 mg 1\(^{-1}\)). For analyses in liquid culture, \textit{H. pylori} strain CCUG 17874 was grown in \textit{Brucella} broth (Difco) supplemented with 0.4% (w/v) \(\beta\)-cyclodextrin and the above antibiotics at 200 rpm in an orbital shaker at 37°C. \textit{E. coli} strain XL-1 blue (supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F\({}^\text{proAB}\) lacIq OADC15 Tn10 (Tet\(^{r}\)) (Stratagene) was grown in LB medium supplemented with ampicillin (50 mg 1\(^{-1}\)) where required. Metal overload was established by the addition of salts of ferrous chloride, zinc chloride, nickel chloride and copper sulfate. The growth medium was deprived of iron by the addition of the iron chelator desferrioxamine B (Sigma). Oxidative stress was generated with the radical mediator methyl viologen (Sigma). The pH of \textit{Brucella} broth (pH 6.8) was adjusted by adding 2 N HCl and was controlled with a pH electrode.

2.2. Cloning of \textit{napA} and \textit{pfr}

The \textit{napA} (encoding HP-NAP \cite{9}) and \textit{pfr} genes \cite{14} were amplified by PCR from \textit{H. pylori} strain CCUG 17874 using primers N1 and N2 for \textit{napA} and P1 and P2 for \textit{pfr} as indicated in Table 1. The primers were designed such that the putative promoter region of each gene was also amplified. The PCR was carried out using standard methods on genomic DNA template prepared by the CTAB–NaCl method \cite{19}. The thermal cycling parameters were: 1 cycle of 94°C (5 min) followed by 30 cycles of 94°C (1 min), 55°C (1 min), 72°C (2 min), and with a final elongation step at 72°C (10 min). The \textit{napA}– and \textit{pfr}–amplified fragments were then cloned into pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen) resulting in plasmids pNAP and pPFR respectively. Both plasmids were sequenced to confirm the presence and correct insertion of \textit{napA} and \textit{pfr}.

2.3. SDS–PAGE and Western immunoblotting

Protein samples were resuspended in loading buffer \cite{19} and separated by SDS–PAGE using standard methods \cite{19}. Following SDS–PAGE the resolved proteins were...
transferred electrophoretically onto a nitrocellulose membrane (Protran, Schleicher and Schuell). The membrane was incubated at 4°C overnight in blocking reagent (5% dried skimmed milk in PBS) and then incubated with primary antibody in blocking reagent for 60 min at room temperature. The membrane was washed three times in PBS-Tween (0.15% Tween-20) for 10 min. It was then incubated with secondary antibody (1/2000 goat anti-rabbit IgG (Calbiochem)) diluted in blocking reagent for 1 h at room temperature. The membrane was washed as above and then developed using the SuperSignal 0 chemiluminescent substrate (Pierce).

3. Results

3.1. Analysis of HP-NAP and Pfr synthesis in H. pylori grown in liquid medium

To compare the synthesis of HP-NAP and Pfr under normal in vitro growing conditions H. pylori strain CCUG 17874 was grown in Brucella broth liquid medium at 200 rpm in an orbital shaker at 37°C for 120 h. Bacteria taken at specific timepoints were pelleted at 16,000×g. Each bacterial pellet was resuspended in 30 μl of PBS, freeze-thawed (liquid N2/37°C) three times and centrifuged for 10 min at 16,000×g. The total protein concentrations of the supernatants following freeze-thawing were calculated using the Bradford assay and bovine serum albumin as standard. Aliquots (15 μg) were subjected to SDS-PAGE and blotted onto nitrocellulose membrane as described in Section 2. The membrane was subsequently incubated simultaneously with anti-HP-NAP and AK128 antiserum. The results of the Western blotting revealed that both HP-NAP and Pfr were similarly synthesised during exponential growth and then accumulated in stationary phase (Fig. 1).

3.2. Effect of metals on HP-NAP and Pfr synthesis in liquid culture

H. pylori strain CCUG 17874 was grown in liquid medium for 48 h as described above. Ferrous chloride, nickel chloride, zinc chloride, copper sulfate were added to final concentrations of 1 mM, 750 μM, 500 μM, and 500 μM respectively. These were the highest concentrations of each metal that did not inhibit bacterial growth. Desferrioxamine B was added to a final concentration of 25 μM according to Bereswill et al. [13]. Sodium chloride and sodium sulfate were also added in control experiments to final concentrations of 1 mM and 500 μM respectively to exclude the effect of osmotic stress on the synthesis of HP-NAP and Pfr. At specific timepoints, aliquots were taken from each growth condition, were processed as described.
above and were immunoblotted simultaneously with anti-HP-NAP and AK198 antiserum. The results reported in Fig. 2a show that Pfr synthesis was increased on iron overload, confirming previous findings by Bereswill et al. [13,15]. The 19-kDa band corresponding to Pfr was more apparent in the sample taken from the culture with added ferrous chloride than in culture with no added iron or desferrioxamine B. In fact desferrioxamine B did not repress Pfr synthesis as previously reported [13] (see Section 4). The presence of nickel and copper significantly repressed the synthesis of Pfr while zinc repressed synthesis for at least 24 h with levels of Pfr synthesis beginning to return to normal at 48 h (Fig. 2b). No difference in HP-NAP synthesis was observed under any of the growth conditions analysed and no change in the synthesis of either protein was observed in the presence of sodium chloride or sodium sulfate (data not shown).

3.3. Expression of HP-NAP and Pfr in E. coli strain XL-1 blue

To compare the effect of overexpression of HP-NAP on metal resistance in E. coli to that of Pfr both the napA and the pfr genes from H. pylori strain CCUG 17874 were cloned into pCR2.1.TOPO® and transformed in E. coli strain XL1-Blue. The expression of recombinant HP-NAP and Pfr in E. coli was confirmed by Western blot using anti-HP-NAP and AK198 antiserum revealing a higher level of synthesis of HP-NAP than Pfr (Fig. 3). The E. coli recombinants and the parental XL1-blue strain were grown in triplicate in LB medium supplemented with 5.5 mM ferrous chloride, 4.5 mM copper sulfate, 2.5 mM zinc chloride, and 3 mM nickel chloride respectively. For ferrous chloride, copper sulfate and zinc chloride the concentrations used were the lowest at which growth inhibition of at least one of the strains was observed, whereas for nickel chloride the concentration used was the highest at which growth of the strains was not inhibited. The growth of each strain was monitored over an 8-h period and as it can be seen from Fig. 4a, both the recombinants and parental E. coli strain grew well under normal growth conditions. However, in the presence of a high concentration of iron, copper and zinc significant growth inhibition of the parental strain and the recombinant expressing HP-NAP was observed (Fig. 4b–d) while the Pfr-producing recombinants grew much better under these heavy metal overload conditions. No difference in growth between the different recombinants and the parental strain was observed when grown at high concentrations of nickel (data not shown).
3.4. Comparison of HP-NAP and Pfr synthesis under environmental stress conditions

The effect of both acid and oxidative stress on the production of both HP-NAP and Pfr was investigated. Strain CCUG 17874 was grown for 24 h under oxidative stress conditions established by the addition of 5 \( \mu \text{M} \), 10 \( \mu \text{M} \), and 20 \( \mu \text{M} \) of methyl viologen respectively (Fig. 5a) and was also grown for 24 h in liquid medium whose initial pH of 6.8 was adjusted to values of 5.7 and 4.7 respectively (Fig. 5b). As can be seen from the graphs in Fig. 5a a significant decrease in growth of \( H. pylori \) was observed at concentrations of 10 \( \mu \text{M} \) and 20 \( \mu \text{M} \) methyl viologen and at pH 5.7. No growth of \( H. pylori \) was observed at pH 4.7. Except for an increase in Pfr synthesis at pH 5.7 no effect on either HP-NAP or Pfr synthesis was observed under any of the growth conditions investigated (Fig. 5a,b).

4. Discussion

Our previous studies on HP-NAP in vitro revealed that
the protein is structurally similar to Dps [7]. Although HP-NAP does not possess the DNA-binding function of Dps it was shown to bind up to 500 atoms of iron per monomer and this pointed to a possible function of HP-NAP in iron binding/sequestration in *H. pylori*. Data presented here show that HP-NAP synthesis is not regulated by the presence or absence of iron or by the presence of other heavy metals in the growth medium. In contrast Pfr synthesis increased on iron overload and decreased on nickel and copper overload in agreement with recent data from Bereswill et al. [13,15] indicating that Pfr probably plays more than just an iron binding/sequestration role in *H. pylori*. Indeed Bereswill et al. [15] have already clearly shown that Pfr is a fur-regulated ferritin whose synthesis can be regulated by the presence of a variety of metal ions including iron. However, not all our data on Pfr synthesis were in complete agreement with Bereswill et al. [13,15]. Firstly, we did not observe the reported decrease in Pfr synthesis on the addition of desferrioxamine B and the repression of Pfr synthesis by zinc was not maintained over the 48-h period investigated. One explanation for these differences is that different strains of *H. pylori* were used in the two studies. *H. pylori* strain NCTC 11638 was used by Bereswill et al. [13,15] while in this study strain CCUG 17874 (identical to strain NCTC 11637) was used. It is possible that the regulation of Pfr synthesis varies between these two strains and that the zinc in the growth medium was titrated out by unidentified zinc-binding proteins and/or enzymes that require zinc as a co-factor thereby reducing the repressive effect of zinc on Pfr synthesis.

Growth of the recombinant *E. coli* expressing Pfr under high iron, copper and zinc conditions was observed due to the metal resistance conferred on *E. coli* by Pfr. The iron resistance has previously been shown by Bereswill et al. [13] to be due to the sequestration of iron by Pfr and the subsequent formation of cytoplasmic iron aggregates. It is possible that a similar mechan isim is involved in resistance to copper and zinc suggesting that Pfr is also capable of binding these two metals and sequestering them. Bereswill et al. [13] showed that a Pfr-deficient mutant of *H. pylori* was more susceptible to killing by copper ions compared to the parental strain suggesting that Pfr plays a role in copper resistance in *H. pylori* which is in agreement with the present data using the *E. coli* system. Therefore, our data suggest that Pfr also confers resistance to zinc overload in *H. pylori*. This could be confirmed by testing the susceptibility of a Pfr-deficient mutant to zinc overload. Binding and sequestration of nickel by Pfr does not appear to occur. Even though the expression level of HP-NAP in the *E. coli* recombinant was higher than that of Pfr the data presented clearly indicate that HP-NAP does not have a metal binding/sequestration function in the *E. coli* recombinant.

Normal growth of *H. pylori* was analysed over a 120-h period and it was observed that HP-NAP and Pfr accumulated in the stationary phase of bacterial growth. The data on the accumulation of Pfr at stationary phase are in good agreement with similar data on the ferritin (FtnA) of *E. coli*. FtnA is also known to accumulate during stationary phase due to a 10-fold increase in *ftnA* expression [20]. The reason why HP-NAP accumulates at stationary phase is unclear. It is possible that the protein protects the bacterium against deleterious environmental effects. Expression of Dps in *E. coli* is induced under conditions of bacterial starvation [21]. However, *Brucella* broth is a rich medium so the accumulation of HP-NAP would appear not to be a result of bacterial starvation.

At pH 5.7 a change in Pfr synthesis was observed. *H. pylori* colonises the gastric mucus layer, where the pH gradient can range from pH 2 on the luminal side to nearly pH 7 at the epithelia surface. The bacterium has been shown to possess genes that are induced or repressed on acid stress [22] most likely facilitating *H. pylori* colonisation of the acidic gastric environment. It is also possible that at lower pH an increase in the synthesis of Pfr is required by *H. pylori* to maintain iron homeostasis within the bacterium. However the fact that Pfr synthesis is unchanged at pH 4.7 leads us to think that the increased synthesis of Pfr at pH 5.7 does not reflect in vivo events. Clearly, the effect of pH on Pfr synthesis requires further investigation.

Neither the synthesis of HP-NAP or Pfr was altered under the oxidative stress induced by paraquat. Again, this result confirms the studies on Pfr by Bereswill et al. [13] who showed that under conditions of oxidative stress the growth of a *pfr*-deficient mutant did not significantly differ from the parental strain. Due to the structural similarities between HP-NAP and Dps-like proteins which are believed to protect nucleotides from oxidative damage [21] and the fact that some ferritins have been shown to protect against oxidative stress, it was thought that the synthesis of HP-NAP might be increased under conditions of oxidative stress. This however does not seem to be the case for HP-NAP.

Comparison of the *napA* nucleotide sequence from 20 *H. pylori* strains from different geographical regions have revealed that the gene and therefore the protein is highly conserved (our unpublished observations). This would indicate that HP-NAP most likely plays a defined and important role in *H. pylori* growth and/or survival. What this function is remains to be elucidated. It would appear from our present data that the iron-binding capability of HP-NAP is an in vitro characteristic that is not evident in conditions that are closer to the in vivo situation.

It is of note that the neutrophil-activating activity in vitro of iron-saturated HP-NAP and iron-depleted apo-HP-NAP are identical suggesting that iron is not a requirement for the neutrophil-activating activity of HP-NAP [7] although this does not exclude a possible role for iron in other, as yet unknown functions, of the protein. One could hypothesise that the original function of HP-
NAP was as an iron-binding protein but that this function has now been superseded by the more important task of activating neutrophils during the course of *H. pylori* infection. By activating neutrophils and inducing a moderate inflammatory reaction leading to alteration of the epithelial tight junctions and basal membranes, HP-NAP possibly promotes the release of nutrients from the mucosa to support the growth of *H. pylori* [23].

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References


