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Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in *Salmonella* Typhimurium and is relieved by nitric oxide

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Oxidative and nitrosative stresses including nitric oxide (NO), superoxide ($O_2^{-\cdot}$) and peroxynitrite play key roles in determining the outcome of bacterial infections. In order to survive within the host and allow proliferation within immune cells such as macrophages, *Salmonella* isolates have a number of inducible proteins that are able to detoxify these highly reactive species, notably the anoxically functioning NO reductase NorVW, and the aerobically functioning flavohaemoglobin, Hmp, which catalyses the reaction between oxygen and NO to produce relatively inert nitrate. However, in the absence of NO but in the presence of reducing substrates and oxygen, $O_2^{-\cdot}$ is generated from Hmp-mediated electron transfer to bound oxygen and may form a variety of further oxidative species. Hence, Hmp expression is under tight negative regulation by the transcription factor NsrR, abolition of which causes an increase in the production of Hmp. In a previous study, this increase in Hmp levels conferred resistance to the nitrosating agent *S*-nitrosoglutathione but, perhaps surprisingly, the organism became more sensitive to killing by macrophages. Here, we report that an *nsrR* mutant that constitutively overexpresses Hmp is also hypersensitive to peroxynitrite *in vitro*. This sensitivity is alleviated by deletion of the *hmp* gene or pre-incubation of growing bacteria with NO-releasing agents. We hypothesize that Hmp-expressing cells, in the absence of NO, generate reactive oxygen species, the toxicity of which is exacerbated by peroxynitrite *in vitro* and in macrophages. RT-PCR confirmed that peroxynitrite causes oxidative stress and upregulation of *katG* and *ahpC*, whilst *hmp* and *norV* expression are affected very little. The *katG* gene upregulated by peroxynitrite encodes a catalase peroxidase enzyme with well-established roles in detoxifying peroxides. Here, we report that KatG is also able to enhance the breakdown of peroxynitrite, suggesting that the protective role of this enzyme may be wider than previously thought. These data suggest that spatial and temporal fluctuations in the levels of NO and reactive oxygen species will have important consequences for bacterial survival in the macrophage.

INTRODUCTION

In parallel with the development of biological nitric oxide (NO) chemistry, peroxynitrite has become a focus for intense research. Peroxynitrite is formed in cells and tissues from the reaction of NO and superoxide ($O_2^{-\cdot}$) at near diffusion-controlled rates (Huie & Padmaja, 1993). $O_2^{-\cdot}$ is a product of the immune system but also a by-product of incomplete oxygen respiration and, under normal physiological conditions, is removed by the potent activities of superoxide dismutases (SODs) (Beckman & Koppenol, 1996; Fridovich, 1995; Miao & St Clair, 2009). However, when NO concentrations reach approximately 10 μM near macrophages, the reaction of $O_2^{-\cdot}$ with NO becomes competitive with the SOD-catalysed reaction and peroxynitrite is formed, with a half-life of as little as 10 ms *in vivo* (Ferrer-Sueta & Radi, 2009).

The peroxynitrite anion ($ONOO^-$) and its conjugate acid (ONOOH) are powerful nitrating and oxidizing agents, resulting in protein and lipid nitration and extensive damage to critical macromolecules. Previous studies have shown that peroxynitrite, generated within a macrophage from NO synthase-derived NO and $O_2^{-\cdot}$ generated in the respiratory burst, is able to react with a variety of targets including invading pathogens both inside and within the vicinity of the phagocyte (Alvarez *et al.*, 2004). Transcriptomic analysis of *Escherichia coli* exposed to peroxynitrite revealed a multitude of targets of this reactive species,

Abbreviations: H_2O_2, hydrogen peroxide; NO, nitric oxide; $O_2^{-\cdot}$, superoxide; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase.

including damage to lipids, iron–sulfur clusters and thiols. Cellular responses included protective mechanisms against reactive oxygen species and defence against nitration and nitrosylation (McLean *et al.*, 2010b). Other studies have demonstrated that oxidative stress response enzymes such as the alkylhydroperoxide reductase AhpC (Bryk *et al.*, 2000), catalase (Gebicka & Didik, 2009; Sahoo *et al.*, 2009) and the catalase-hydroperoxidase KatG (McLean *et al.*, 2010a; Wengenack *et al.*, 1999) are able to enhance the breakdown of peroxynitrite.

Salmonella Typhimurium is a leading cause of gastroenteritis in humans and is characterized by diarrhoea and vomiting. In order to survive within the host, this enteropathogen possesses a multitude of defences against the host immune response which allow proliferation within immune cells such as macrophages (Hébrard *et al.*, 2009; Prost *et al.*, 2007). Among these defences is the two-domain flavohaemoglobin, Hmp, which possesses a globin domain containing a *b*-type haem and a flavin-containing reductase domain (Poole & Hughes, 2000). Aerobically, Hmp catalyses the reaction between oxygen and NO to produce relatively inert nitrate (Gardner *et al.*, 1998; Hausladen *et al.*, 1998). In the absence of NO, $O_2^{-\cdot}$ is formed, which reacts with a variety of compounds leading to the formation of other oxidative stresses such as hydrogen peroxide (H_2O_2) (Membrillo-Hernández *et al.*, 1996; Orii *et al.*, 1992; Wu *et al.*, 2004). Anaerobically, NO reacts with the haem of Hmp to yield a nitrosyl anion with subsequent formation of nitrous oxide (Hausladen *et al.*, 1998; Kim *et al.*, 1999; Liu *et al.*, 2000).

The transcriptional regulation of Hmp expression is complex (Spiro, 2007). In *E. coli* and *Salmonella*, five transcription factors have been implicated: *hmp* transcription is activated by MetR (Membrillo-Hernández *et al.*, 1998) and RamA (Hernández-Urzúa *et al.*, 2007) and is repressed by NsrR (Bodenmiller & Spiro, 2006), FNR (Cruz-Ramos *et al.*, 2002; Poole *et al.*, 1996) and Fur (D'Autreaux *et al.*, 2002; Hernández-Urzúa *et al.*, 2007). Abolition of one of these transcription factors, NsrR, causes a large increase in the production of Hmp in *Salmonella* (Gilberthorpe *et al.*, 2007). In a previous study, this increase in Hmp levels was reported to confer resistance to the nitrosating agent *S*-nitrosoglutathione and also hypersensitivity to killing by macrophages (Gilberthorpe *et al.*, 2007). This was attributed to the ability of Hmp to generate $O_2^{-\cdot}$ in the absence of NO (Membrillo-Hernández *et al.*, 1996; Orii *et al.*, 1992; Wu *et al.*, 2004).

During the course of investigating the role of Hmp in response to the presence of peroxynitrite, we made the surprising observation that NO protects *Salmonella* cells that constitutively express Hmp. Here, we report evidence for a fine balance between the presence of NO and oxidative species in determining the outcome of bacterial exposure to peroxynitrite.

METHODS

***Salmonella* growth conditions.** The strains used were wild-type *Salmonella enterica* serovar Typhimurium ATCC 14028s and the isogenic mutants shown in Table 1. Bacteria were cultured in defined minimal media with glycerol as the sole carbon source, as described previously (Flatley *et al.*, 2005) at 37 °C and with orbital shaking at 200 r.p.m. Cells were grown with ampicillin (150 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) as required in 20 ml defined medium in 250 ml flasks fitted with side arms for measurement of optical density with a Klett–Summerson photoelectric colorimeter (Klett Manufacturing) fitted with a no. 66 red filter. Anaerobic cultures were grown in Klett-compatible 8.5 ml screw cap vials filled completely with defined minimal media supplemented with fumarate as a terminal electron acceptor (50 mM). Cultures were incubated on roll bars at 37 °C.

Peroxynitrite and NO donor preparations. Peroxynitrite was purchased from Calbiochem and tetramethylammonium peroxynitrite was purchased from Alexis Biochemicals; stock solutions were diluted in 0.1 M NaOH for addition to cultures. Peroxynitrite concentrations were determined by measurement of absorbance (*A*) at 302 nm using the molar absorption coefficient of $\varepsilon_{302}=1670\ M^{-1}\ cm^{-1}$.

NOC-5 and NOC-7 were purchased from Alexis Biochemicals and were solubilized in 0.1 M NaOH to yield 10 mM stocks. NOC solutions were added to cultures directly at a 1 : 1000 dilution. Controls showed that small additions of NaOH only did not affect culture growth.

RNA isolation and real-time PCR. Cells were harvested directly into RNA Protect (Qiagen) to stabilize RNA, which was purified using an RNeasy Mini kit (Qiagen) as recommended by the suppliers. The integrity of the RNA was confirmed by electrophoresis on a 1.25 % agarose gel in 1 × MOPS. The concentration and purity of the RNA was measured at 260 and 280 nm on a Beckman DU 650 spectrophotometer

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source/reference
***S.* Typhimurium**		
ATCC 14028s	Wild-type	Crawford & Goldberg (1998)
ATCC 14028s *hmp*	*hmp*::*kan*^{r}	Crawford & Goldberg (1998)
ATCC 14028s *nsrR*	*nsrR*::*cat*	Gilberthorpe *et al.* (2007)
ATCC 14028s *nsrR hmp*	*nsrR*::*cat hmp*::*kan*^{r}	Gilberthorpe *et al.* (2007)
XF1000: ATCC 14028s *katG*	*katG*::pBR10Δ*trfA*, Pen^r Amp^r	Buchmeier *et al.* (1995)
Plasmids		
pBR322	Amp^r Kan^r	Laboratory stock
pBR322-*hmp*	Amp^r Kan^r	Laboratory stock

(Sambrook and Russell, 2001). RT-PCR analysis of isolated RNA was carried out as described previously (Flatley *et al.*, 2005) and the relative levels of expression of genes of interest were calculated by comparison to a constitutively transcribed control gene (*carA*).

SDS-PAGE and Western blot analysis. Cell cultures were harvested by centrifugation, washed in 100 mM phosphate buffer (pH 7.4) plus protease inhibitors, and lysed by sonication. Clarified supernatant (10 μg) was run on 12 % SDS-PAGE gels. Relative Hmp protein levels were determined by transfer to nitrocellulose membranes and subsequent incubation with polyclonal antibodies raised to purified *E. coli* Hmp, followed by washing and incubation with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma). Membranes were exposed to X-ray film followed by reaction with ECL reagents as described previously (Gilberthorpe *et al.*, 2007).

Purification and peroxynitritase activity assays of KatG. Pure KatG enzyme was obtained as described previously (McLean *et al.*, 2010a). Briefly, His_6-tagged enzyme was purified by immobilized metal affinity chromatography using a Talon (Clontech) column and elution with imidazole. Pure enzyme was subsequently desalted on a PD-10 desalting column. KatG concentrations were determined using the molar absorption coefficient ε_{280}=143 350 M^{-1} cm^{-1} (Wilkins *et al.*, 1999).

Peroxynitrite decomposition was monitored using an Applied Photophysics stopped flow spectophotometer. Peroxynitrite solutions in 0.01 M NaOH were mixed in a 1 : 1 ratio with KatG buffered in 100 mM phosphate, pH 7.4, and 100 μM diethylenetriaminepentaacetic acid. The breakdown of peroxynitrite was monitored by a decrease in absorbance at 302 nm; the molar absorption coefficient of ε_{302}=1670 M^{-1} cm^{-1} (Hughes & Nicklin, 1968) was used to calculate substrate concentration.

3-Nitrotyrosine assays. 3-Nitrotyrosine formation in the presence of peroxynitrite was assayed as described previously (Gebicka & Didik, 2009). The 3-nitrotyrosine concentration was determined spectrophotometrically using the molar absorption coefficient ε_{430}=4400 M^{-1} cm^{-1} (Crow, 1999).

RESULTS

An *nsrR* knockout is hypersensitive to peroxynitrite stress

Given the well documented evidence that Hmp plays a key role in NO detoxification (Poole *et al.*, 1996; Poole & Hughes, 2000; Pullan *et al.*, 2007; Stevanin *et al.*, 2002) and the proposal that, aerobically, this reaction may involve an Hmp-bound peroxynitrite intermediate (Poole & Hughes, 2000), we wanted to determine whether this flavohaemoglobin could play a role in the detoxification of peroxynitrite. We therefore investigated the effect of altered levels of Hmp and NsrR, the key regulator of Hmp, on the response of cells to peroxynitrite. Cultures of the wild-type and *hmp*, *nsrR* and *hmp–nsrR* mutant strains (Table 1) were grown in defined minimal medium in batch culture and treated with a bolus addition of 50 μM peroxynitrite. Culture density has been shown to play a critical part in determining the outcome of a peroxynitrite stress, so cultures were always used when they had reached a turbidity of 50 Klett units (McLean *et al.*, 2010b). Although 3-morpholinosydnonimine (SIN-1) has been used in several studies, including in bacteria (Acosta *et al.*, 2003; Brunelli *et al.*, 1995; Makino *et al.*, 2005), to generate peroxynitrite (by simultaneous generation of NO and $O_2^{-\cdot}$), a recent study highlights the formation of cytotoxic products other than peroxynitrite generated following SIN-1 decomposition in culture media (Konishi *et al.*, 2009). We therefore chose to employ commercial preparations of peroxynitrite as used in our detailed transcriptomic analyses of chemostat-grown *E. coli* cells (McLean *et al.*, 2010b). Cell growth was monitored for several hours both before and after the addition of stress. Peroxynitrite caused a small lag in the growth of wild-type cells and deletion of the *hmp* gene caused a more significant lag in growth (Fig. 1a and b). Although peroxynitrite preparations are not expected to contain NO, which could be detoxified by Hmp in wild-type cells, commercial peroxynitrite is frequently contaminated with nitrite, which could explain this result.

Deletion of *nsrR*, which encodes a negatively acting transcription factor for *hmp* regulation (Bodenmiller & Spiro, 2006; Rodionov *et al.*, 2005), had a much more dramatic effect upon the culture, with a complete arrest in growth (Fig. 1c). NsrR regulates expression of not only *hmp* but also several other genes in a small regulon. To test the hypothesis that Hmp overexpression, and not that of other NsrR-regulated gene products (such as *ytfE*, *hcp–hcr*, *tehA*, *tehB* and *ygbA*) is responsible for hypersensitivity to peroxynitrite, we determined the effect of peroxynitrite on a double mutant (*hmp* and *nsrR*). Growth of this strain was unaffected by the addition of peroxynitrite (Fig. 1d) demonstrating that altering the regulation of other genes under the control of this transcription factor does not cause the sensitivity seen in the *nsrR* single knockout (Fig. 1c). However, alteration in the regulation of these other genes could be beneficial to the cell in its resistance to peroxynitrite as this mutant does not show the mild lag in growth seen in the wild-type culture. A wild-type phenotype was demonstrated on complementation of the *nsrR* mutant with an *nsrR*-expressing plasmid (Fig. 1e). This provides convincing evidence that elevated Hmp levels are solely responsible for the peroxynitrite-elicited inhibition of growth seen in Fig. 1(c).

Overexpression of Hmp causes hypersensitivity to peroxynitrite

To test directly the hypothesis that *hmp* gene overexpression causes hypersensitivity to peroxynitrite, wild-type cells were transformed with an *hmp*-expressing plasmid (pBR322-hmp^+) or empty plasmid as a negative control and exposed to 50 μM peroxynitrite at 50 Klett units. Wild-type cells and cells containing the empty vector showed a mild lag in growth rate after peroxynitrite treatment; however, the growth of cells containing the pBR322-*hmp* overexpression plasmid was much slower after exposure (Fig. 2a). In order to confirm the elevated Hmp protein levels, Western blot analysis against anti-

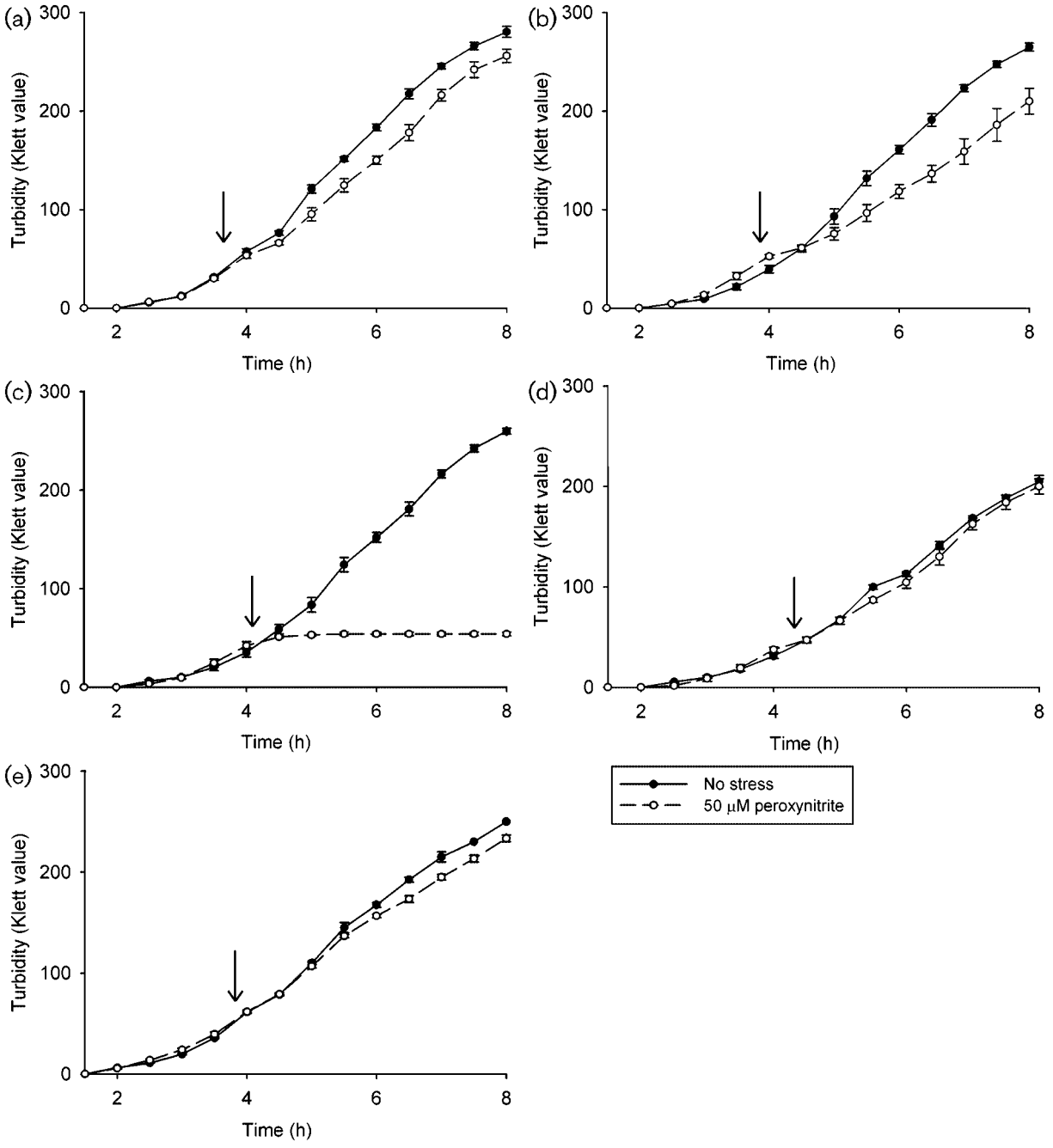

Fig. 1. The effect of peroxynitrite on wild-type and mutant *S.* Typhimurium growth. Cells were grown in defined minimal medium and growth was monitored every 30 min using a Klett meter. At a turbidity of 50 Klett units, cultures were injected, where appropriate (arrow), with 50 μM peroxynitrite (final concentration). Growth of the following strains was monitored: (a) wild-type, (b) *hmp*, (c) *nsrR*, (d) *hmp–nsrR* and (e) *nsrR* complemented with the pBR322-*hmp* vector. $n \geqslant 5$; values shown are mean ± SEM.

Hmp antibodies was carried out on cultures grown to 50 Klett units and exposed, where appropriate, to 50 μM peroxynitrite. In both the presence and absence of peroxynitrite, the *nsrR* mutant and the strain carrying the hmp^+ plasmid had highly elevated Hmp levels compared with the wild-type (Fig. 2b). Fig. 2(b) also shows qualitatively that the *nsrR* mutant expresses higher levels of Hmp than does the pBR322-*hmp* containing wild-type, suggesting a reason for the knockout mutant's complete arrest of growth while cells containing the Hmp-expressing plasmid are able to grow slowly. Fig. 2(b) demonstrates that peroxynitrite has no effect on Hmp synthesis in wild-

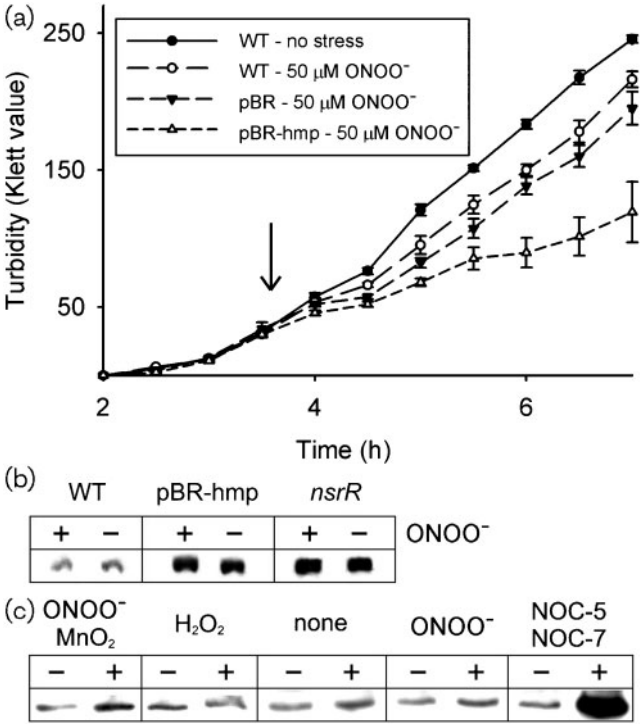

Fig. 2. Expression of Hmp is linked to growth rate under conditions of peroxynitrite stress. (a) Cells were grown in defined minimal medium to a turbidity of 50 Klett units before addition of 50 μM peroxynitrite where appropriate (arrow). Growth was monitored every 30 min; $n \geqslant 5$, values shown are mean ± SEM. (b) Western blots with anti-Hmp antibody of wild-type (WT), wild-type carrying the pBR322-*hmp* vector (pBR-hmp) and *nsrR* cells immediately prior to (−) and 1 h after (+) exposure to 50 μM peroxynitrite. (c) Western blots of wild-type cells raised against anti-Hmp antibody. Cells were grown without stress (none) or in the presence of 50 μM MnO_2-treated peroxynitrite, 400 μM H_2O_2, 50 μM peroxynitrite and 10 μM NOC-5/NOC-7 for 1 h prior to harvesting. Samples were taken immediately prior to (−) or 1 h after (+) the stress.

type cells, suggesting a lack of significant NO levels in peroxynitrite preparations.

As a control, Hmp levels were investigated when wild-type cultures were exposed to a variety of stresses including peroxynitrite, H_2O_2 and NO-generating compounds (Fig. 2c). Hmp expression levels were not significantly increased in response to peroxynitrite or H_2O_2. However, when the NO-releasing compounds NOC-5 and NOC-7 were present, expression of the flavohaemoglobin was significantly increased indicating that of these reactive species, only NO causes upregulation.

Oxygen is necessary for peroxynitrite toxicity in the *nsrR* mutant

Previous work has shown that peroxynitrite is less toxic under anaerobic conditions compared with in aerobic environments due to a reduction in nitrosylation and nitration reactions as well as a decrease in the formation of hydroxyl radicals (Kikugawa *et al.*, 2004). To investigate the toxicity of peroxynitrite under anaerobic conditions, the peroxynitrite hypersensitive *nsrR* mutant was grown anaerobically and stressed with 100 μM peroxynitrite, twice the concentration used in the aerobic growth experiments and showed no effect (Fig. 3a). Thus, the presence of oxygen is key to the Hmp-exacerbated toxicity of peroxynitrite.

Commercially available preparations of peroxynitrite may contain H_2O_2 as an unused substrate from synthesis (Saha *et al.*, 1998). This contaminant has the potential to influence growth and physiological data and we have

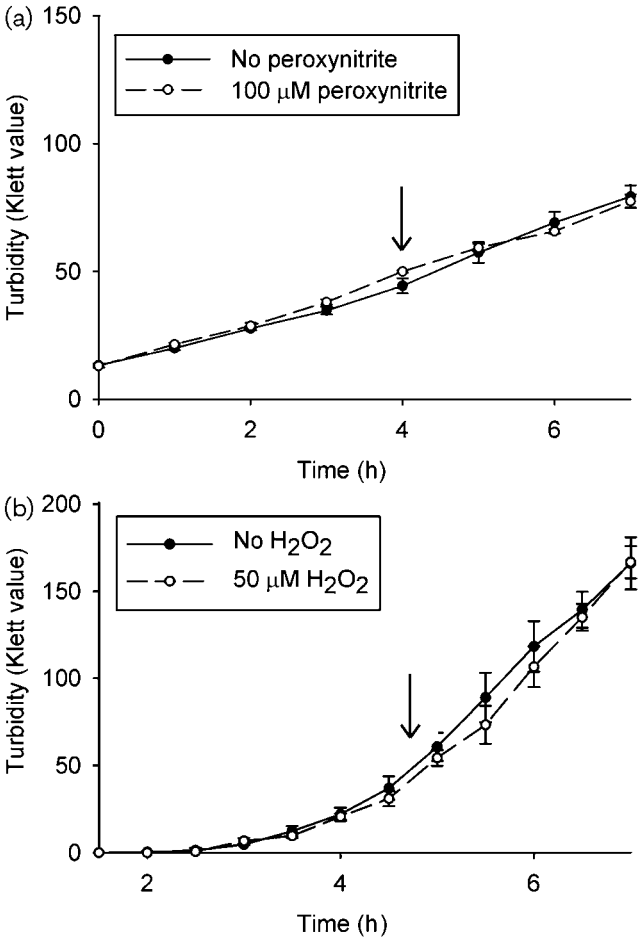

Fig. 3. Growth of the *nsrR* mutant is unperturbed by H_2O_2 and the toxicity of peroxynitrite is eliminated under anaerobic conditions. (a) *nsrR* mutants were grown in defined minimal medium supplemented with 50 mM fumarate to act as the terminal electron acceptor in the absence of oxygen. Cell growth was monitored every hour and at a turbidity of 50 Klett units; 100 μM peroxynitrite was added where appropriate (arrow). $n=5$; values shown are mean ± SEM. (b) The *nsrR* mutant was grown in defined minimal media to a turbidity of 50 Klett units before addition of 50 μM H_2O_2 where necessary (arrow). $n \geqslant 3$; values shown are mean ± SEM.

included appropriate controls in previous transcriptomic studies (McLean *et al.*, 2010b). However, addition of 50 μM H_2O_2 to cultures as the stressor had no significant impact upon the growth of the *nsrR* strain (Fig. 3b).

NO rescues an *nsrR* mutant from peroxynitrite hypersensitivity

Under aerobic conditions and in the absence of NO, Hmp forms the highly reactive species $O_2^{-\cdot}$ via the reduction of molecular oxygen (Membrillo-Hernández *et al.*, 1996; Orii *et al.*, 1992; Wu *et al.*, 2004); however, in the presence of NO, the relatively inert nitrate ion is formed in a denitrosylase or dioxygenase reaction (Gardner *et al.*, 2000; Hausladen *et al.*, 2001; Poole & Hughes, 2000). As Hmp levels are elevated in an *nsrR* mutant (Fig. 3b) we investigated whether this mutant's hypersensitivity towards peroxynitrite could be reduced by the presence of NO, effectively reducing $O_2^{-\cdot}$ levels by the production of nitrate.

Growth experiments were undertaken in the presence and absence of the NO donors NOC-5 and NOC-7. NOC-7 has a half life at pH 7.0 and 37 °C of 5 min and so provided an initial burst of NO to the cells, whereas NOC-5 has a half life of 25 min under the same conditions and gave a slower sustained release of NO (Cruz-Ramos *et al.*, 2002). A mixture (10 μM final concentration of each NOC compound) was added to the cultures as required; this concentration of NOC-5 and NOC-7 did not cause any significant growth inhibition in the *nsrR* mutant (data not shown). The NO donors were delivered to cultures approximately 5 min before the injection of 50 μM peroxynitrite, a concentration shown to completely inhibit growth of *Salmonella* under these conditions (Fig. 1). Remarkably, NO offers significant protection from peroxynitrite in this Hmp-overexpressing strain (Fig. 4). This suggests that diversion of the electron flux to form nitrate anion in the presence of NO alleviates oxidative stress.

Real-time PCR of oxidative and nitrosative stress response genes upon exposure to peroxynitrite

Peroxynitrite has been described as both an oxidative and nitrosative stress (Ferrer-Sueta & Radi, 2009) so, in order to ascertain how *Salmonella* was responding to this stress, RT-PCR analysis of both nitrosative (*hmp* and *norV*) and oxidative (*sodA*, *katG* and *ahpC*) stress response genes was undertaken (Fig. 5a). Wild-type *S.* Typhimurium cultures were grown to a turbidity of 50 Klett units in defined minimal medium and treated with 50 μM peroxynitrite. Samples were removed into RNAprotect (Qiagen) immediately prior to and 5 min after exposure for analysis by RT-PCR. The nitrosative stress response genes were upregulated to a small degree and the *hmp* result is consistent with the lack of expression of Hmp protein (Fig. 2c); however, the oxidative stress response gene upregulation, specifically that of *katG* and *ahpC*, was much more pronounced. KatG has been shown to enhance the breakdown of peroxynitrite in *Mycobacterium tuberculosis* (Wengenack *et al.*, 1999) as has AhpC in *S.* Typhimurium (Bryk *et al.*, 2000).

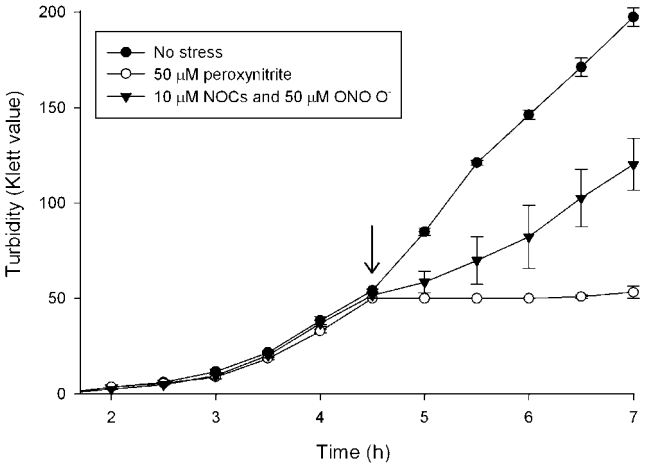

Fig. 4. NO alleviates the susceptibility of an *nsrR* knockout mutant to peroxynitrite. Cells were grown in defined minimal medium. At a turbidity of 45 Klett units, 10 μM NOC-5 and NOC-7 was added to the appropriate cultures and, at a turbidity of 50 Klett units, 50 μM peroxynitrite was added (arrow). Turbidity was measured every 30 min using a Klett meter to assess the effect on growth. $n \geqslant 3$; values shown are mean ± SEM.

The role of KatG in response to peroxynitrite

Due to the upregulation of *katG* seen in the RT-PCR we investigated the mechanism by which this enzyme may protect the cell in the presence of peroxynitrite. Firstly, we analysed the consequences for growth of a *katG* deletion mutant (XF1000) upon injection of 50 μM peroxynitrite. This strain showed a slightly greater lag in growth after injection with the stress (Fig. 5b) compared with wild-type (Fig. 1a). This lag was particularly evident within the first hour after exposure.

Pure KatG enzyme was assayed next for its ability to enhance the breakdown of peroxynitrite. Increasing concentrations of KatG (0–25 μM) were able to enhance the breakdown of 10 μM peroxynitrite in a concentration-dependent manner (Fig. 5c). This is in agreement with previous studies of the peroxynitritase activity of KatG (McLean *et al.*, 2010a; Wengenack *et al.*, 1999). Further work was undertaken to ascertain the possible effect of KatG on nitration of free tyrosine by peroxynitrite *in vitro*. Tyrosine (5 mM) was incubated with 1 mM peroxynitrite and varying concentrations of KatG (0–40 μM). 3-Nitrotyrosine formation was determined by measurement of the absorbance at 430 nm. KatG was able to partially intercept peroxynitrite and reduce the level of nitration (Fig. 5d). At 40 μM KatG, nitration was reduced by approximately 50 % compared with nitration in the absence of enzyme.

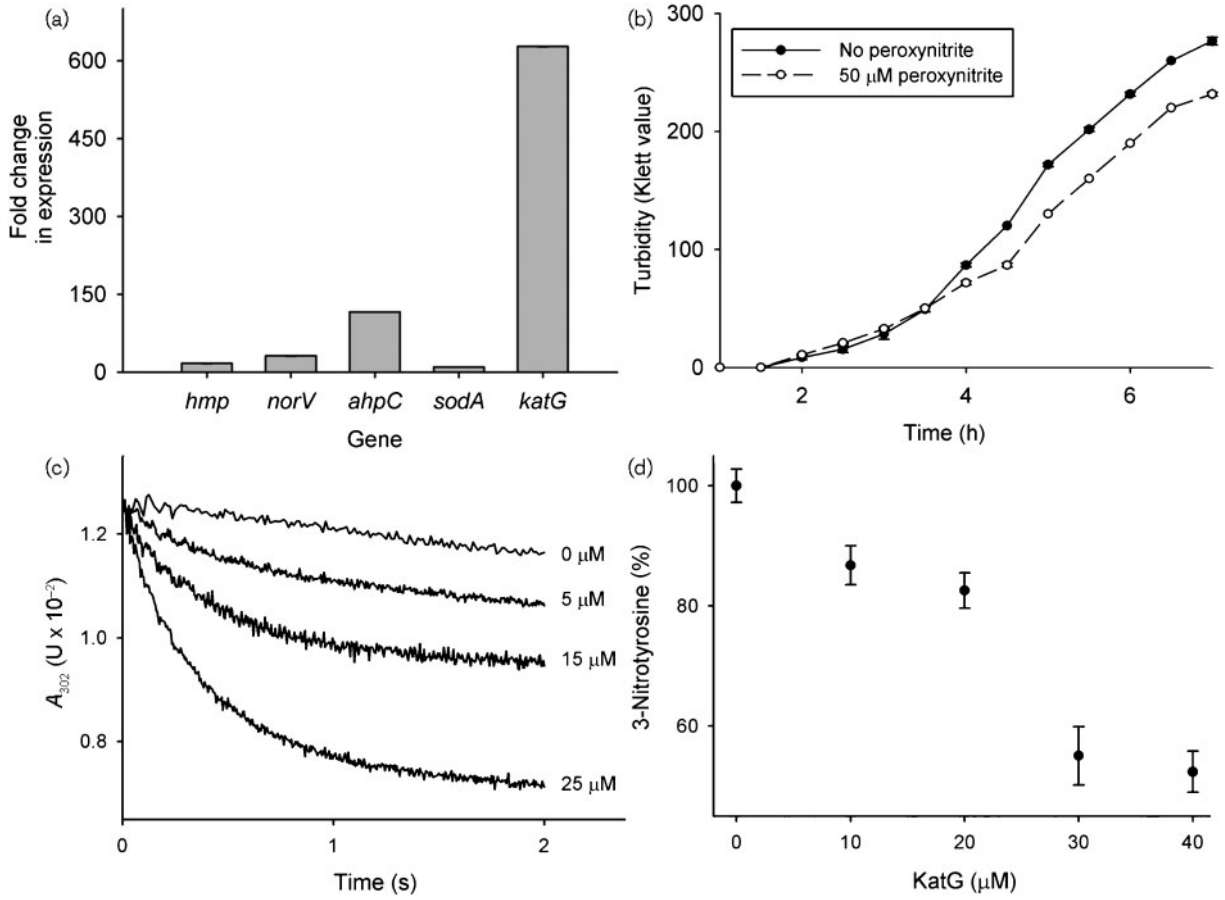

Fig. 5. Real-time PCR of oxidative and nitrosative stress response genes upon exposure to peroxynitrite. (a) Cells grown in batch culture were exposed to 50 μM peroxynitrite for 5 min before aliquots of culture were removed to RNAprotect and processed for RT-PCR analysis. The mean fold change in individual gene expression compared with unstressed cells was calculated. Values are representative of a single experiment ± SD of six technical repeats. Four biological repeats were carried out in total giving similar trends in gene expression levels. (b) The *katG* mutant was grown in batch culture to 50 Klett units before addition of 50 μM peroxynitrite. Cell growth was monitored over a period of 8 h. $n=3$; values shown are mean ± SEM. (c) The decay of 10 μM peroxynitrite was monitored at 302 nm on a stopped-flow spectrophotometer in the presence of varying concentrations of KatG enzyme (0–25 μM). Traces represent the mean of >10 assays. (d) The nitration of 5 mM tyrosine by 1 mM peroxynitrite was monitored spectrophotometrically at 430 nm in the presence of varying concentrations of KatG (0–40 μM). Assays were performed in 100 mM potassium phosphate buffer pH 7.5 and 100 μM diethylenetriaminepentaacetic acid. $n=3$; values shown are mean ± SEM.

DISCUSSION

In order for *Salmonella* to survive and proliferate within a host it must be able to sense and respond to the multitude of defences elicited. To do this, *Salmonella* upregulates the formation of a number of detoxification enzymes, including those responsible for the removal of reactive nitrogen and oxygen species. However, our data highlight the fact that these responses must be tightly regulated and able to change rapidly in response to the environment, as upregulation of the NO detoxification flavohaemoglobin Hmp in the presence of NO aids survival but cells would be highly vulnerable to oxidative stresses such as peroxynitrite should NO levels drop. A recent study highlighted the different redox chemistries apparent under differing and unequal fluxes of NO and $O_2^{-\cdot}$ (Jourd'heuil *et al.*, 2001), conditions that are likely to occur *in vivo* in response to infection where, for example, in the macrophage, there is an early burst of $O_2^{-\cdot}$ formation followed by a later burst in the formation of NO (Vazquez-Torres & Fang, 2001).

Previous work on the reactions and toxicity of peroxynitrite have utilized authentic peroxynitrite synthesized in a variety of ways, as well as generators of peroxynitrite such as SIN-1 and the use of NO donors and enzymic sources of $O_2^{-\cdot}$. Each method has advantages and pitfalls; peroxynitrite can be synthesized for use via the reaction of acidified H_2O_2 and nitrite (Saha *et al.*, 1998) and use of peroxynitrite sourced in this manner has the advantage of utilizing authentic peroxynitrite. However, possible

contaminants include H_2O_2 and nitrite as unused substrates in synthesis and must be controlled. A common criticism of the use of bolus additions of peroxynitrite is that they do not reflect *in vivo* conditions and so generators of peroxynitrite are also employed to study peroxynitrite toxicity as they are thought to better reflect the conditions found *in vivo*. However, these methods are also not without their pitfalls, SIN-1 has been widely utilized in this manner but data in the literature regarding this molecule have been conflicting. Whilst some studies show that SOD has a protective effect against SIN-1 due to reaction with $O_2^{-\cdot}$ and that catalase has no effect (Ishii *et al.*, 1999), other reports have shown that SOD has no protective effect (Lepore *et al.*, 1999) or even exacerbates toxicity (Gergel *et al.*, 1995), and further work has demonstrated that catalase is able to protect against SIN-1 toxicity (Volk *et al.*, 1995). These disparities were recently investigated and Konishi *et al.* (2009) were able to show that cytotoxicity to neuronal PC12 cells is mediated by thiol-sensitive short-lived substances generated through SIN-1 decomposition in media and not just as a result of peroxynitrite toxicity. Furthermore, the type of media in which SIN-1 decomposed had a significant impact upon the toxicity of the secondary species formed (Konishi *et al.*, 2009). This study highlights the importance of appropriate controls and the highly reactive nature of the substrates as well as peroxynitrite in isolation. Further work has been undertaken utilizing the simultaneous addition of NO donors (such as spermineNONOate) and an enzymic source of $O_2^{-\cdot}$, xanthine oxidase, with either hypoxanthine or luminaze as a substrate (Jourd'heuil *et al.*, 2001). This approach allowed the investigation of adding unequal fluxes of NO and $O_2^{-\cdot}$ on the reactivity of peroxynitrite and other reactive species. The authors of this study found that excess of either substrate led to inhibition of the oxidative chemistry associated with the production of peroxynitrite. The story *in vivo* is further complicated by the reaction of peroxynitrite and its substrates with carbonate and a variety of biomolecules. It is evident that the source of peroxynitrite and conditions used will have a major impact upon any study.

In this report, we chose to add authentic peroxynitrite to specifically study the effects of this molecule upon changing conditions within the cell (i.e. increases in $O_2^{-\cdot}$ due to Hmp activity). A bolus addition of peroxynitrite corresponding to a final concentration of 50 μM is thought to correlate to a steady state concentration of 1.4 μM under physiological pH and temperature (Zhu *et al.*, 1992). This concentration is therefore well within the concentration expected *in vivo*, as rat alveolar macrophages have been reported to produce up to 1 mM peroxynitrite min^{-1}, with concentrations inside the phagocyte likely to be higher (Ischiropoulos *et al.*, 1992).

How is peroxynitrite generated by any of these means detoxified in the cell? Bryk and co-workers have shown that AhpCF is able to enhance the breakdown of peroxynitrite in *S.* Typhimurium with a second-order rate constant of $1.5 \times 10^6\ M^{-1}\ s^{-1}$ (Bryk *et al.*, 2000). This may explain the upregulation of *ahpC* seen in our RT-PCR data, as the cell is likely to utilize this enzyme and its corresponding reductase as a mechanism of peroxynitrite detoxification. KatG has also been implicated in the detoxification of peroxynitrite due to its ability to act as a peroxynitritase (McLean *et al.*, 2010a; Wengenack *et al.*, 1999). Here, we confirm that KatG from *S.* Typhimurium enhances the breakdown of peroxynitrite and reduces the nitration of free tyrosine to approximately 50 % of that in its absence (Fig. 5). However, the protection from nitration afforded by KatG requires an enzyme concentration that is an order of magnitude higher than the same protection given by bovine liver catalase (Gebicka & Didik, 2009). We suggest that, while KatG is able to act as a peroxynitritase and reduce nitration, there are other proteins able to catalyse these reactions more efficiently. The major role of *katG* upregulation seen in our RT-PCR analyses may be to protect the cell from general oxidative stresses, responding to secondary species such as hydroperoxides formed by the reactions of peroxynitrite (Claiborne & Fridovich, 1979; Hébrard *et al.*, 2009), rather than to directly break down peroxynitrite per se. Due to the highly reactive nature of peroxynitrite it is sensible to assume that *Salmonella* would need to upregulate genes that are able to detoxify the plethora of secondary reactive species formed upon exposure.

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