Flavohemoglobin protects uropathogenic *Escherichia coli* against nitrosative stress; implication for urovirulence

Lovisa Svensson[1], Mirjana Poljakovic[2], Susanne Säve[1], Nicola Gilberthorpe[3], Thomas Schön[4], Sigge Strid[5], Robert K. Poole[3] and Katarina Persson[1]

[1] *School of Pure and Applied Natural Sciences, University of Kalmar, Kalmar, Sweden*
[2] *Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden*
[3] *Department of Molecular Biology and Biotechnology, University of Sheffield, UK*
[4] *Department of Clinical Microbiology, Kalmar County Hospital, Kalmar, Sweden,*
[5] *Stensö Health Center, Kalmar County Council, Kalmar, Sweden*

During the course of urinary tract infection (UTI) nitric oxide (NO) is generated as part of host response. The aim of this study was to investigate the significance of the NO–detoxifying enzymes flavohemoglobin (Hmp) and flavorubredoxin (Norv) in protection of uropathogenic *Escherichia coli (*UPEC) against nitrosative stress. *Hmp* (J96Δhmp) and *norV* (J96ΔnorV) knockout mutants of UPEC strain J96 were constructed using a single-gene deletion strategy. Bacterial tolerance and expression of *hmp* and *norV* in response to the NO-donor DETA/NO was evaluated in Luria broth and urine from healthy volunteers. Bacterial NO consumption and respiratory inhibition were assessed when exposed to NO. Expression of *hmp* and *norV* from *E. coli* originating from patients with UTI was evaluated using real-time PCR. The colonizing ability of J96 wild-type (wt) compared to an *hmp*-deficient mutant was assessed using a competition-based mouse UTI model. The viability of J96Δhmp and J96ΔnorV was significantly reduced compared to the wild-type strain after exposure to DETA/NO. The *hmp* expression in DETA/NO-exposed cultures was similar in J96wt and J96ΔnorV, while J96Δhmp showed an increased *norV* expression compared to J96wt. The NO consumption in J96Δhmp, but not in J96ΔnorV, was significantly impaired compared to J96wt. An up-regulation of *hmp* expression was found in *E. coli* isolated from all UTI-patients while *norV* expression increased in 50% of the patients. In the mouse UTI model, the *hmp*-mutant strain was significantly out-competed by the wild-type strain in the bladder and kidney. Hmp and NorV contribute to the protection of UPEC against NO-mediated toxicity *in vitro*. Screening UPEC isolates from UTI patients revealed an increased *hmp* expression in all patients which confirms that *hmp* expression occurs *in vivo* in the infected human urinary tract. The ability to colonize the mouse urinary tract was impaired in the *hmp*-deficient mutant compared to the wild-type strain. NO-detoxification by Hmp is suggested to be an important characteristic for UPEC in protection against nitrosative stress and may be a virulence-facilitating factor.

Key words: flavohemoglobin, nitric oxide, uropathogenic *E. coli,* nitrosative stress

Nitric oxide (NO) is a highly reactive and diffusible radical that plays an important role in a wide range of physiological processes. NO is involved in the early innate host response and takes part as a key component in defense against a variety of pathogenic micro-organisms [1]. In activated phagocytic cells, high levels of NO are produced by the inducible NO synthase (iNOS) and in combination with various macro-molecules or superoxide generated by the phagocyte NADPH oxidase, reaction of NO leads to further reactive nitrogen intermediates (RNIs) such as peroxy-nitrite ($ONOO^-$) [2]. Microbial targets of RNI include thiols, metal centers, lipids and DNA. NO itself is considered to be bacteriostatic and NO-related toxicity includes inhibition of respiration [3] and reduced cell division by disrupting DNA replication [4]. In approxi-mately 80 % of the community-acquired urinary tract infections (UTIs) the cause of the infection is a uro-pathogenic *Escherichia coli* (UPEC) strain. Flavo-hemoglobin (Hmp) and flavorubredoxin (NorV*)* are the two main NO-detoxifying systems in *E. coli* and are up-regulated in response to NO and nitrosating agents such as S-nitrosoglutathione [5, 6, 7, 8]. The main function of Hmp is to catalyse the conversion of NO to nitrate [9, 10], but Hmp can also operate in anaerobic conditions reducing NO to nitrous oxide (N_2O) [11]. The regulation of *hmp* transcription is complex involving several regulators including Fnr [12] and MetR [13]. Recently, it was discovered that the regulator NsrR represses *hmp* transcription in the absence of NO or nitrite [14, 15]. NsrR is proposed to contain a NO-labile [Fe-S] cluster responsible for the NO-sensing capacity [14]. The importance of Hmp as a mechanism of protection from endogenous host-derived NO has so far been shown in human macrophages challenged with *Salmonella enterica* and non-pathogenic *E. coli* [16, 17, 18]. NorV (flavo-rubredoxin) and its associated oxidoreductase NorW are regulated by the product of the divergently transcribed regulatory gene *norR*. The NorV protein is considered to govern NO-detoxification during an-

Author for correspondence: Katarina Persson PhD, School of Pure and Applied Natural Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden. Email: katarina.persson@hik.se

aerobic or microaerobic conditions by reducing NO to N_2O at the expense of NADH [19, 20].

NO seems to have a particularly important role in the control of intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Salmonella* [21]. In addition, detrimental effects of NO and RNI on non-pathogenic *E. coli* K-12 strains are also well established [3, 7, 10, 22, 23]. Studies in humans and mice have shown that urinary neutrophils and urinary tract epithelial cells are able to express iNOS in response to UPEC infection [24, 25, 26] and NO levels have been documented to increase in the urinary bladder in patients suffering from UTI [27]. Furthermore, infected urine contains nitrite as a result of bacterial nitrate reductase and acidified urine releases NO from nitrite [28]. The growth of the common urinary pathogens *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus saprophyticus* was reduced by the addition of nitrite to acidified urine [29]. During anaerobic respiration of *E. coli*, nitrate and nitrite are dominant electron acceptors [30]. NO is detectable in *E. coli* cell suspensions during nitrate respiration, and its production is dependent on nitrite reductase activity [31].

NO-detoxification may be an important characteristic for UPEC to resist NO generated as part of host response during UTI. This is supported by reports that UPEC are more able to resist nitrosative stress than non-pathogenic *E. coli* K-12 strains [22, 23]. Studies regarding the functional role of NO-detoxifying systems in UPEC are still lacking. In order to investigate the importance of Hmp in NO-tolerance and urovirulence we used a single-gene deletion strategy to construct a UPEC strain lacking *hmp* or *norV*. The expression of *hmp* and *norV* in UPEC collected from women with UTI were analysed using real time-PCR. The importance of Hmp as a factor for UPEC virulence was investigated in a competition model where mice were infected with a mix of wild-type and *hmp*-deficient UPEC.

MATERIALS AND METHODS

Strains, media and growth conditions

The UPEC strain J96 was originally isolated from a patient with acute pyelonephritis. The *hmp* and *norV* knockout mutants were constructed from wild-type (wt) J96 using a single-gene deletion strategy [32]. Plasmids were received from *E. coli* genetic stock centre (CGSC) (Yale University, New Haven, USA). The open reading frame was replaced with a kanamycin cassette flanked by FLP recognition target sites with primers (Table 1) designed to create in-frame deletions after the excision of the resistance cassette [33]. The J96 wild-type is denoted J96wt and the *hmp* and *norV*- deficient mutants J96Δhmp and J96ΔnorV, respectively. For the mouse UTI experiments, the kanamycin resistance cassette was not removed in order to be able to separate the wild-type from *hmp*-deficient mutant (J96Δhmp:km).

Bacteria were maintained on tryptic soy agar (TSA), supplemented with kanamycin (30 μg/ml) when appropriate, and grown in Luria broth (LB, Difco Laboratories, Detroit, MI) or in human urine. Media was inoculated at 1 % with an overnight culture at 37ºC, shaking at 180 rpm unless stated otherwise. Urine samples were collected and pooled from 3 to 5 healthy female volunteers aged 25-50, who had no history of UTI or antibiotic use in the prior 2 months. The urine was filter-sterilized (0.22 μm pore size) and stored at -20ºC for use within two weeks. Nitrite was not detectable in the pooled urine from healthy volunteers.

Growth curves were carried out with J96wt and mutants in LB or pooled human urine at 37°C. Culture growth was monitored by following the turbidity at 600 nm (A_{600}) during 24 hours.

DETA/NO and bacterial viability

Nitrosative stress was induced by the NO-donor DETA/NONOate, (2, 2'-(hydroxynitrosohydrazono)-bis-ethanimine; Alexis Biochemical, Lausanne, Switzerland), a compound that decomposes spontaneously with a half-life of approximately 20 hours [34]. Bacteria were exposed to 1 mM DETA/NO in the exponential growth phase (OD_{600} of 0.2-0.3). After 5 hours exposure in closed vials in static LB or urine bacterial viability (CFU/ml) was determined by serial dilutions on TSA. Bacteria were exposed in static culture in order to keep the environment less aerobic. The viability is expressed as percentage of untreated controls (set to 100%).

Reverse transcriptase-PCR

Total RNA was extracted from bacteria in the exponential growth phase after exposure to 0.5 mM DETA/NO for 30 min in static cultures. RNA extraction was performed using Qiagen RNeasy minikit and RNAprotect Bacteria Reagent with a 1 hour on-column DNAse treatment (Qiagen, Hilden, Germany). RNA concentrations were determined using a spectrophotometer (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, USA). A cDNA synthesis using 0.5 μg total RNA was performed using the Omniscript Reverse Transcriptase (Qiagen) and Random Hexamer (Qiagen). DNA contamination was evaluated in reactions with no reverse transcriptase as template. The cDNA was amplified using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Buckinghamshire, UK), together with PCR specific primers (Invitrogen Life Technologies, Carlsbad, USA), summarized in Table 1. The PCR products were evaluated by electrophoresis on a 2 % agarose gel and visualized by adding 0.5 μg/ml ethidium bromide.

Real time-PCR

RNA was extracted from UPEC in urine samples from UTI patients (*in vivo* samples), and from the corresponding UPEC grown in pooled filtered human

Table 1: Primer sequences for real time-PCR (1-2), reverse transcriptase-PCR (3-5) and single-gene deletion (6-7).

	Primer	Sequence	Size (bp)	Reference
1	*hmp* L	ATATCTCGGCGTCTGGCTGAAG	79	This work
	hmp R	ATCCGGTTTGCGAGTCAAAGAG		
2	*norV* L	GTCGCTTAGCCTGAAAGCGAAAT	68	This work
	norV R	GACCGTGTTCACGGCATAACTTC		
3	*16S* L	GAGTTTGATCCTGGCTCAG	536	[35]
	16S R	GTATTACCGCGGCTGCTG		
4	*hmp* L	AGGGATCCACGCGGCAATTTAAACCGCGTC	520	This work
	hmp R	CCGCATGGAACCAGTTCACTTGT		
5	*norV* L	GTGATTAACCATGCAGAAGAGGAC	1008	This work
	norV R	CTTTAACTACCGTATTCACCGTGC		
6	*hmp* L	GATGCATTTGAGATACATCAATTAAGATGCAAAAAAAGGAAGACCATATG	1264	[33]
	hmp R	GCCGGATGTTTCCATCCGGCAACATCAAATTACAGCACCTTATGCGGGCC		
7	*norV* L	ATTAGCAAGACATCTTTTTAGAACACGCTGAATAAATTGAGGTTGCTATG	1510	[33]
	norV R	GCCGGATGTTTCCATCCGGCAACATCAAATTACAGCACCTTATGCGGGCC		

urine (*in vitro* samples), as described above. Each reaction was carried out in a total volume of 20 µl on 8 well strip (Applied Biosystems, Foster city, USA). Each well contained 10 µl 2 X Power Sybr® Green PCR mastermix (Applied Biosystems), 3.0 pmol of each primer and 25 ng cDNA. The primers used are summarized in Table 1. PCR amplification was carried out in an ABI 7500 thermocycler (Applied Biosystems) with the following thermal cycling conditions; 50ºC for 2 min and 95ºC for 10 min, 40 cycles of 95ºC for 15 s, 60ºC for 1 min. After every run a melting curve was done; 95ºC for 15 s, 60ºC for 1 min and 95ºC for 15 s. All samples were run in triplicates or duplicates. DNA contamination was evaluated in reactions without reverse transcriptase and no-template controls were included as negative controls. Samples were adjusted for DNA background by subtracting mean cycle threshold (Ct) of the sample without reverse transcriptase from the corresponding sample. Samples that did not differ significantly from the DNA background control were not evaluated. Fold increase in *in vivo* samples, compared to *in vitro* samples, was calculated as $2^{\Delta Ctin\ vivo} / 2^{\Delta Ctin\ vitro}$.

Western blot analysis

Cultures in the exponential growth phase were challenged with 0.5 mM DETA/NO for 1 hour. Bacteria were harvested and resuspended in lysis buffer (50 mM Tris-HCl (pH 8.1) 10 % (w/v) sucrose, 1mM EDTA and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by sonication with cooling intervals. The crude extract was ultracentrifuged at 160 000 x g, 4°C for 1 hour. The supernatant was decanted and the protein concentration was determined using the BCA-kit (Sigma-Aldrich St. Louis, Mo). The samples were boiled for 5 min and 5 µg of total protein was separated on a 12% NuPAGE pre-cast gel (Invitrogen Life Technologies, Carlsbad, CA) and transferred onto a Hybond™ P+ membrane (Amersham Biosciences). The membrane was incubated with a rabbit polyclonal antibody against *E. coli* Hmp (1:10 000) [3]. After incubation with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:10 000; Stressgene Biotechnologies, San Diego, CA) detection was performed using ECL plus Western Blotting detection system (Amersham Biosciences). For detection of NorV the membrane was incubated with a rat polyclonal antibody against *E. coli* NorV (1:20 000, kindly provided by Prof. L. M. Saraiva, Lisbon, Portugal) [36] and a secondary HRP-conjugated goat anti-rat antibody (1:10 000; Chemicon International Inc, Temecula, CA, USA).

NO consumption and respiration rate in J96wt, J96Δhmp and J96ΔnorV

The method was adapted but slightly modified from previous studies [3, 18]. Cultures in LB were grown aerobically until reaching an OD_{600} of 0.7-1.0, harvested by centrifugation and resuspended in 1 ml PBS. The buffer was saturated with air in a Clark-type polarographic oxygen electrode system (Rank Brothers Ltd, Cambridge, UK), comprising a water-jacketed (37 °C) Perspex chamber stirred magnetically. Bacterial suspension (300 µl) was added, a close-fitting lid applied to the chamber, and an ISO-NOP NO sensor (2 mm diameter, World Precision Instruments, Sarasota, USA) was inserted through a custom-made capillary hole in the lid. Oxygen levels in the chamber were allowed to fall by ~60 % through respiration before 100 µl of anoxic, NO-saturated solution was injected into the chamber using a Hamilton microsyringe. Oxygen consumption and NO levels were measured until the chamber contents became anaerobic. A NO-saturated solution was made by acidified nitrite as previously described [12]. Respiratory inhibition (in minutes) was calculated from the time point when NO was added to the time point when the respiration resumed. NO

consumption rate was calculated from normalized NO traces and expressed as the half-time (in seconds) when 50 % of added NO had disappeared.

Mouse UTI model

The experimental protocol has been approved by The Animal Ethics Committee of the Linköping University, Sweden. Female C3H/HeN mice (10-12 weeks of age) were purchased from Taconic (Ejby, Lille Skensved, Denmark). One week prior to the experiment the mice were put on a diet of nitrite/nitrate free pellet (Altromin 1324N, Altromin Special Animal Feed GmbH & Co., Lage, Germany) and tap-water *ad libitum*. Three days prior to infection, urine was collected and the neutrophil content was determined using a Bürker chamber. Mice with >50 x 10^4 leukocytes/ml were excluded from the study. A competition model of ascending UTI was used to asses the urinary colonizing ability of J96wt compared to the J96Δhmp:km. Bacteria were prepared and grown overnight on TSA plates and harvested in sterile PBS. The two strains were combined and washed twice in PBS by centrifugation at 5000 x g for 10 min. The pellet was diluted in sterile PBS to a final concentration of 2 x 10^9 CFU/ml.

The bladder was emptied by gentle compression of the abdomen and urine was saved for pre-infection measurements of nitrite and neutrophil content. Under isoflurane anaesthesia (Baxter Medical AB, Kista, Sweden), 100 µl of the bacterial mixture was instilled into the bladder using a soft polyethylene catheter (outer diameter 0.61 mm, KEBOLab, Malmö, Sweden) dipped in a lubricating gel (ACO, Stockholm, Sweden). The catheter was immediately withdrawn after inoculation and no further manipulations were carried out. The animals were placed in the cages after instillation and allowed food and water *ad libitum.*

Determination of bacterial titer in tissue homogenate

Mice were sacrificed by cervical dislocation 6, 12, 24 and 48 hours post-infection. Prior to sacrifice urine samples were collected for determination of nitrite levels and neutrophil content. Urine samples were centrifuged at 5000 x g for 15 min and stored at -20ºC before nitrite analysis. The bladder and one kidney were aseptically harvested and homogenized in 0.5 ml sterile PBS using 1.5 ml homogenisation tubes (Kontes Glass Company, Vineland, NJ). The homogenate was serially diluted and plated onto TSA plates with or without kanamycin. CFU was enumerated after growth overnight at 37ºC. The relative bacterial loads for wild-type and mutant bacteria were determined by replica plating onto TSA plates and TSA-kanamycin (30 µg/ml) plates. The ratio of the two test strains (J96Δhmp:km/J96wt) in post-mortem tissue-homogenates (out-put ratio) was adjusted for the test strains ratio in the inoculum suspension (input ratio) to derive the competitive index (CI) for each bladder or kidney. Strain identity in post-mortem colonies was verified using colony PCR with *hmp*-specific primers (Table 1).

Urinary nitrite analysis

NO is rapidly converted to more stable end products. In water and buffers the main end product is nitrite [37]. Urinary nitrite concentration was determined by Griess assay [38]. Briefly, 10 µl centrifuged mouse urine or 100 µl human urine were mixed with 100 µl of Griess reagent (one part 0.1 % N-(1-naphtyl) ethylene-diamine dihydrochloride and one part 1 % sulphanilamide) in a 96-well plate and incubated for 10 min in room temperature before measuring absorbance at 540 nm (Spectracount™ Packard, Canberra, Australia). The readings were compared to a standard curve of sodium nitrite with a lower detection limit of 1 µM nitrite.

Clinical study

In a prospective clinical study of symptomatic UTI, female patients with dysuria were included after informed consent. Urine samples were collected from 39 patients (mean age: 35.4 years) and tests of leukocyte esterase, nitrite and bacterial cultures were performed. Urine (10 ml) was transferred to a 50 ml tube containing 20 ml RNAprotect Bacteria Reagent, mixed and kept at 4ºC. The samples were centrifuged (5000 x g, 4ºC, 10 min) and the bacterial pellet was frozen at - 20ºC until RNA extraction. The clinical study was approved by the Medical Ethics Committee of the Linköping University, Sweden.

Statistical analysis

Data are given as mean ± standard error of the mean (SEM). Student's unpaired t-test or ANOVA followed by Bonferroni-Dunn test was used for statistical analysis where appropriate. CI was tested using the Wilcoxon rank sum test where one (1) was considered to be the theoretical mean, giving a ration of 1:1. Statistical significance was considered at $p<0.05$.

RESULTS

Expression of flavohemoglobin and flavorubredoxin after DETA/NO exposure in LB media or urine

Transcription of *hmp* and *norV* in J96wt was increased in response to DETA/NO in LB media (Figure 1A). Western blot analysis confirmed a marked induction of Hmp and NorV protein in response to DETA/NO (Figure 1B). The expression of *hmp* and *norV* when exposed to DETA/NO in urine from healthy volunteers was assessed in J96wt, J96Δhmp and J96ΔnorV using reverse transcriptase-PCR. In urine, the transcription pattern of *hmp* and *norV* in response to DETA/NO was similar to in LB media (Figure 1C). When exposed to DETA/NO, J96Δhmp and J96ΔnorV did not up-regulate *hmp* or *norV*, confirming that the mutations had been successfully accomplished (Figure 1C). The

hmp expression in DETA/NO-exposed cultures was similar in J96wt and J96ΔnorV, while J96Δhmp showed an increased *norV* expression compared to J96wt (Figure 1C).

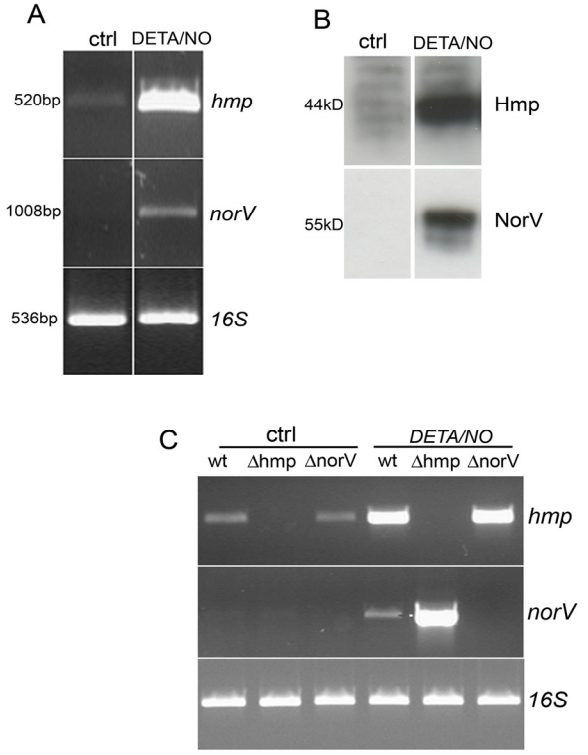

Figure 1. (**A**) Transcriptional expression of *hmp*, *norV* and *16S* in unstimulated J96 (ctrl) and in J96 exposed to 0.5 mM DETA/NO in LB media for 30 min. (**B**) Western blot analysis showing protein expression of flavohemoglobin and flavorubredoxin in unstimulated J96 (ctrl) and in J96 exposed to 0.5 mM DETA/NO in LB media for 1 h. (**C**) Transcription of *hmp*, *norV* and *16S* in unstimulated bacteria (ctrl; lanes 1-3) and in bacteria exposed to 0.5 mM DETA/NO (lanes 4-6) for 30 min. These experiments were performed in urine from healthy volunteers in J96 wildtype (wt) and *hmp* (Δhmp) and *norV* (ΔnorV) mutants.

Growth of J96wt, J96Δhmp and J96ΔnorV in LB media or urine

No difference in growth ability between the mutants and J96wt grown *in vitro* in LB media was observed (Figure 2A). When monitored in urine from healthy volunteers, the overall growth rate was lower than in LB, but mutants showed no consistent difference in growth ability compared with the wild-type (Figure 2B).

Effect of DETA/NO on the viability of J96wt, J96Δhmp and J96ΔnorV

The viability of J96wt was unaffected (121±14% of control) when exposed to DETA/NO in LB media. However, the viability was significantly reduced in J96Δhmp (51±19%, $p<0.05$) and J96ΔnorV (46±8.6%, $p<0.01$) (Figure 3A). In urine from healthy volunteers, the viability of J96wt was reduced to 61±7.8% in response to DETA/NO. Mutations of *hmp* or *norV* further reduced the viability in response to DETA/NO to 42±4.1% ($p<0.05$) and 42±6.2% ($p<0.05$) for J96Δhmp and J96ΔnorV, respectively (Figure 3B).

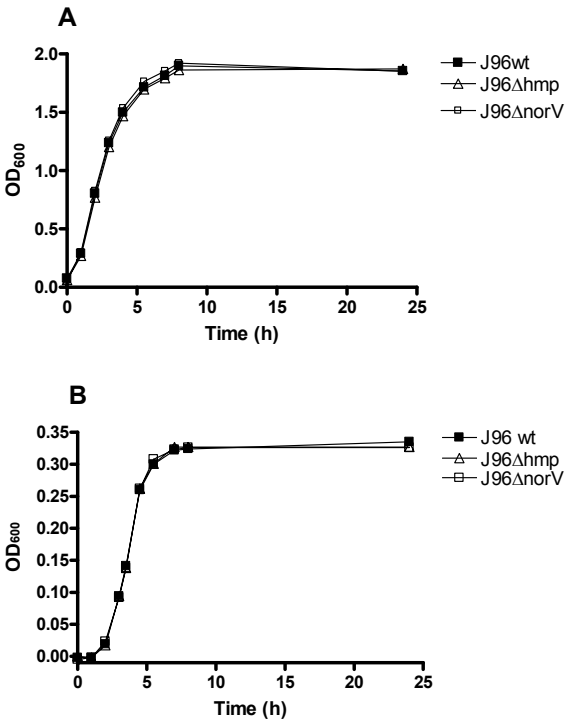

Figure 2. Representative growth curves in (**A**) LB media and (**B**) urine from healthy volunteers for J96wt, J96Δhmp and J96ΔnorV by measuring OD_{600} during 24 h. Similar curves were obtained twice in LB and four times in urine.

Inhibition of respiration and NO consumption

Injection of a NO-solution to bacterial suspensions immediately inhibited oxygen uptake (Figure 4). In J96wt the time of inhibition was 2.0±0.18 min and it stringently correlated with the rapid upward excursion of the NO electrode output. When NO levels had reached negligible amounts, oxygen uptake resumed and progressed at a similar rate as before NO addition (Figure 4A). Oxygen uptake in J96Δhmp was severely affected by NO addition and time of inhibition was prolonged compared with J96wt (4.6±0.25 min, $p<0.001$) (Figure 4B, Table 2). The initial respiratory rate was not restored during the time of measure. The half-time for disappearance of NO (135±15s) in J96Δhmp was significantly prolonged compared to J96wt (56±9.5s, $p<0.001$). There was no significant difference in NO consumption or time of respiratory inhibition between J96wt and J96ΔnorV under the experimental conditions used (Figure 4C, Table 2). In the absence of NO, respiration rates did not differ between the strains.

Table 2. The effect of a NO-saturated solution on respiration and NO consumption in J96wt, J96Δhmp and J96ΔnorV. NO-induced inhibition of respiration is expressed in minutes and NO consumption is calculated as half-time for NO disappearance. Data are expressed as mean ± SEM (n = 6-8), *** p<0.001 vs J96wt

	Inhibition of respiration (min)	NO consumption (s)
J96wt	2.0±0.18	56±9.5
J96Δhmp	4.6±0.25***	135±15***
J96ΔnorV	1.3±0.18	42±6.9

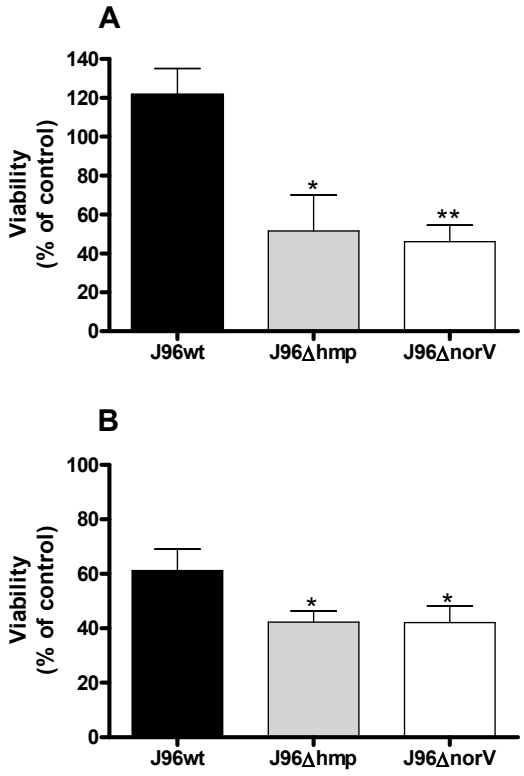

Figure 3. The effect of DETA/NO on bacterial viability (% of control) in (**A**) LB media and (**B**) urine from healthy volunteers. J96wt, J96Δhmp and J96ΔnorV were challenged with 1.0 mM of DETA/NO for 5 h. Untreated controls are set to 100%. Data are expressed as mean ± SEM (n = 4-5), * p<0.05, ** p<0.01 vs J96wt.

Expression of flavohemoglobin and flavorubredoxin in UPEC isolated from UTI patients

Urine samples were collected from a total of 39 patients and 19 samples were verified as *E. coli* monocultures. Urinary nitrite concentrations were elevated (≥1 μM) in 12 of 19 patients with values ranging from 1.0-313 μM (Table 3). Pyuria was found in all patients as verified by a positive leukocyte esterase test. RNA was extracted from all samples and 14 of 19 samples yielded enough RNA for the real time-PCR assay. Control PCR experiments, using human neutrophil cDNA as template, confirmed that the *hmp* and *norV* primers were bacteria-specific.

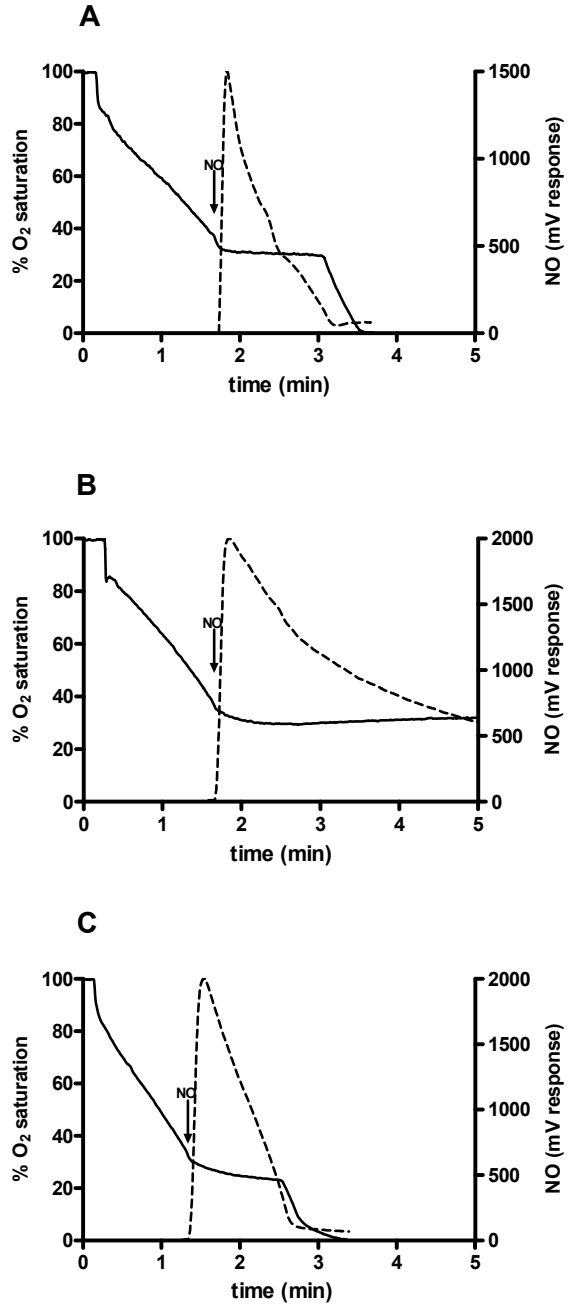

Figure 4. Representative traces of NO consumption and respiration for (**A**) J96wt, (**B**) J96Δhmp and (**C**) J96ΔnorV. The filled line tracing shows the decrease in oxygen level due to bacterial respiration. When approximately 40% of the dissolved oxygen remains a NO-saturated solution is added to the chamber (arrow). The dotted line tracing represents NO levels.

DNA-background was detected in the majority of the samples and therefore all Ct values were corrected for DNA background. An up-regulation of *hmp* expression was found in *E. coli* from all 14 patients and also if the same isolates were exposed to urine from healthy volunteers *in vitro* (Table 3). The *hmp* expression was consistently higher in *E. coli* from patients compared

Table 3. Results from the real time-PCR analysis showing *hmp* and *norV* expression expressed as ΔCt (Ct^{sample} -$Ct^{DNA\text{-}background}$) in *E. coli* from patients (*in vivo)* and when the same isolate was exposed to urine from healthy volunteers (*in vitro).* The fold increase in *hmp* expression *in vivo* compared to *in vitro* is calculated as $2^{\Delta Ct\text{-in vivo}}$ / $2^{\Delta Ct\text{-in vitro}}$. Nitrite concentrations (μM) in urine collected from UTI patients are also given. nd; not detectable, -; not analysed, ns; not significantly different from the DNA-background.

Patient	Nitrite (μM)	ΔCt ***hmp in vivo***	ΔCt ***Hmp in vitro***	Fold increase ***hmp***	ΔCt ***norV in vivo***	ΔCt ***norV in vitro***
3	nd	-	-	-	-	-
4	nd	9.80	2.63	144	7.27	1.22
6	nd	-	-	-	-	-
7	nd	6.26	3.13	8.58	ns	ns
8	nd	7.11	5.48	24.8	3.68	ns
10	3.9	9.95	3.05	120	ns	0.81
11	nd	13.8	2.67	2242	9.12	ns
12	1.0	8.37	2.29	67.7	ns	1.63
13	41	7.42	2.36	33.4	ns	1.78
17	113	16.4	4.13	6295	13.2	ns
18	nd	7.33	3.61	13.2	ns	ns
19	1.6	8.91	5.04	14.6	2.31	0.43
20	313	6.47	2.78	12.9	ns	ns
21	313	-	-	-	-	-
29	2.2	-	-	-	-	-
30	3.9	11.7	2.80	465	8.3	ns
34	6.5	7.16	2.24	79.1	ns	ns
35	249	8.88	3.68	36.8	9.66	ns
36	1.0	-	-	-	-	-

with the expression *in vitro*. The *norV* transcription was elevated in *E. coli* from seven patients (Table 3).

Urovirulence of J96wt compared to J96Δhmp in a mouse UTI model

Neutrophils are the crucial effector cells in UTI and urine neutrophils were monitored in order to assess the host response at each time point (Figure 5A). In pre-infected urine the mean neutrophil content was 6.9 x 10^4 PMN/ml. The Infection triggered a neutrophil response that peaked 12 h post-infection (mean 6.0 x 10^6 PMN/ml, $p<0.01$). The neutrophil numbers in urine remained significantly ($p<0.05$) elevated 24 h post-infection but returned close to pre-infected levels after 48 h (Figure 5A). The nitrite concentration was 22±7.1μM in pre-infected urine with a non-significant peak after 12 h (50 ± 26 μM, Figure 5B). The colonizing ability of a mixture consisting of J96wt and the isogenic mutant J96Δhmp:km was evaluated in a competition UTI model (Figure 6). The *hmp* gene is a monocistonic transcript making polar effects and unrelated phenotypes unlikely and therefore complementation of the *hmp* mutant was not attempted.

No difference in P or type 1 fimbrial expression was found between J96wt and the mutant as determined by agglutination of human A_1P_1 erythrocytes and guinea pig erythrocytes, respectively (data not shown), suggesting that adhesion to host cells was unaffected by the mutation. The *hmp* mutant strain was significantly out-competed by the wild-type strain

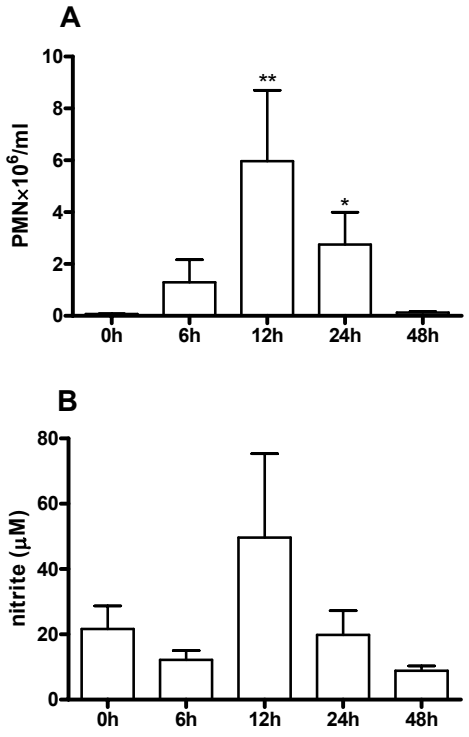

Figure 5. (**A**) Total PMN/ml in mouse urine samples 6, 12, 24 and 48 hours post-infection. The number of PMN/ml in pre-infected urine is shown at time 0 h. (**B**) Nitrite concentrations in mouse urine samples 6, 12, 24 and 48 hours after infection. The pre-infected nitrite levels are shown at time 0 h (n = 31). Data are given as mean ± SEM (n = 4-10); * $p<0.05$, ** $p<0.01$ vs 0h.

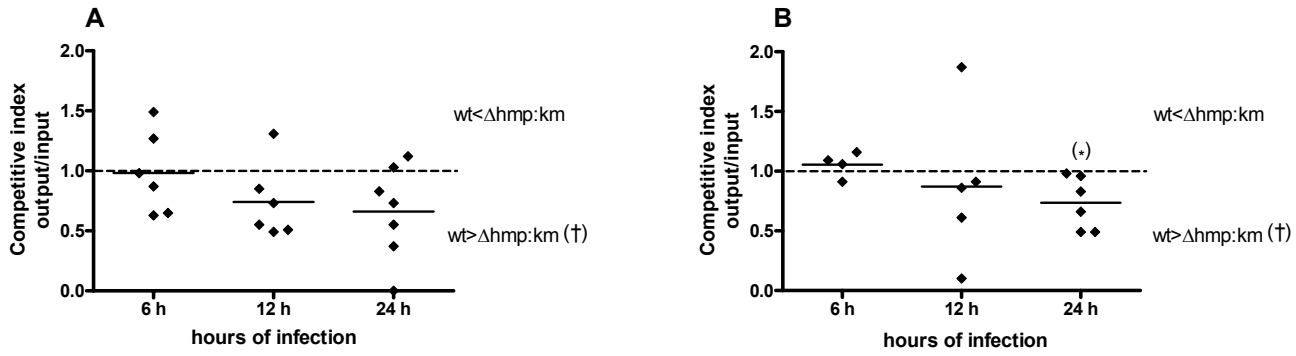

Figure 6. Comparative mouse urinary tract colonization ability of a UPEC strain and an *hmp*-deficient mutant. C3H/HeN mice were transurethrally inoculated with a 1:1 ratio of J96wt and J96Δhmp:km. Outcomes after 6, 12 and 24 hours cultures in (**A**) bladder and (**B**) kidney are shown as competitive index (CI), which is the relative prevalence of the two strains in the post-mortem cultures (output ratio), corrected for the inoculated suspension (input ratio). Values <1, below the dotted line, indicate a competitive advantage for the wild-type over the *hmp*-deficient mutant. Values >1 indicate the reverse. * $p<0.05$ vs hypothetical value (1.0) 24 hours post-infection, † $p<0.05$ vs hypothetical value (1.0) overall post-infection.

in the bladder ($p<0.05$) and in the kidney ($p<0.05$) when evaluating all time-points together (Figure 6). After 6 h of infection the CI values in the bladder and kidney were 0.98±0.14 and 1.05±0.05, respectively, indicating equal colonization of the J96wt and J96Δhmp:km. When the infection had proceeded for 12 h, the CI in the bladder was 0.74±0.13 and in the kidney 0.87±0.29. The *hmp*-deficient mutant was significantly out-competed by the wild-type strain in the kidney CI = 0.74±0.09 ($p<0.05$, Figure 6B) after 24 h. The CI in the bladder 24 h post-infection was 0.66±0.15 ($p = 0.078$). After 48 h the infection was generally cleared (data not shown).

DISCUSSION

The significance of NO as part of host response against bacterial infections is widely recognised [2]. Thus, pathogenic bacteria such as UPEC need to posses mechanisms to antagonize the antimicrobial actions of NO in order to establish themselves in the urinary tract. In this work we demonstrate 1) that *norV* and *hmp* play a role in protecting UPEC against nitrosative stress during growth in rich LB media and in human urine 2) that UPEC lacking *hmp* exhibit overall lower colonization ability in the urinary tract compared to wild-type bacteria and 3) that *hmp* transcription is increased in UPEC isolated from UTI patients.

An *hmp* and a *norV* deficient mutant of UPEC strain J96 were constructed in order to assess the functional roles of these enzymes for protection against nitrosative stress. There was no difference in growth ability between the strains and although some variations were noted in urine cultures from healthy volunteers the data did not indicate that Hmp or NorV is crucial for UPEC growth. In line with previous findings [23], J96 were able to endure high concentrations of NO in rich media. In urine from healthy volunteers there was a more prominent effect of NO-exposure and the survival of J96wt was decreased by approximately 40 %. The different sensitivity in rich LB media compared to urine may be due to the fact that the bacteria have less ability to respond to nitrosative stress in the nutrient-deficient urine. NO release from DETA/NO is pH-dependent [34] and it is a possibility that the lower and more unstable pH in urine, compared with the buffered LB medium, results in higher concentrations of NO in urine. In addition, NO released from DETA/NO may react with amino acids in the rich LB media thus resulting in a higher free NO concentration in urine.

In both LB media and urine from healthy volunteers, the survival of J96Δhmp and J96ΔnorV was significantly reduced when exposed to NO as compared to the wild-type. Previous studies have shown that *hmp*-deficient non-pathogenic *E. coli* are less capable of surviving NO and related species [39, 40, 41]. In the *hmp*-mutant, there was a compensatory up-regulation of *norV* transcript in response to DETA/NO. One possible explanation for the increased *norV* expression is that J96Δhmp may be exposed to higher concentrations of NO due to impaired NO detoxification. This explanation is supported by the NO consumption data that demonstrated prolonged NO exposure in J96Δhmp. However, the NO-induced increase in *norV* expression in J96Δhmp was not able to compensate for the lack of *hmp* suggesting that Hmp is important for NO detoxification. The reduced survival of the J96ΔnorV is more surprising since the NO-reducing activity of NorV has been shown to be oxygen sensitive [10]. On the other hand, NorV has been suggested to play a role in protecting *E. coli* from NO during microaerobic conditions [19] and *norV* transcription was also activated in UPEC by DETA/NO in our experimental conditions. The decreased viability of the J96ΔnorV mutant may indicate that the oxygen level in the static culture in our experiments is

microaerobic rather than aerobic. In hypoxic conditions the dioxygenase activity of Hmp have been shown to be reduced [42], which may explain why Hmp was unable to compensate for the *norV*-deficiency. Taken together, our results suggest that, under the experimental conditions used, both Hmp and NorV seem to be important for NO protection in UPEC.

We next examined if the J96hmp and J96norV mutants were defective in their ability to detoxify NO. Respiratory inhibition in response to NO is well established in non-pathogenic *E. col*i [3, 6, 43]. Respiration of UPEC was also significantly inhibited by NO and the inhibition was initiated momentarily upon NO addition and resumed after the NO levels decreased. A significant reduction in time of NO consumption was found in J96Δhmp, suggesting that Hmp in UPEC, like in non-pathogenic *E. coli* [39], is involved in detoxification of NO. The respiratory rate in J96Δhmp never recovered to the pre-stimulated rate suggesting that there are irreversible effects of the prolonged NO exposure. Similar effects were also seen in an *hmp*-deficient mutant of *Salmonella enterica* [18]. The effect of NO on respiration in J96ΔnorV was similar to the effects observed in J96wt suggesting that NorV activity is not important for NO detoxification in UPEC during the present conditions. In line with our findings, deletion of *norV* showed little effect on NO consumption in a non-pathogenic *E. coli* in aerobic conditions [6, 19]

Previously, a DNA-based microarray has shown that *hmp* was among the top 50 genes up-regulated in UPEC strain CFT073 in a mouse UTI-model [44]. Moreover, increased transcription of *hmp* and *norV* was reported in an asymptomatic bacteriuria *E. coli* strain from three patients based on microarray analysis [45]. In this study we investigated *hmp* and *norV* expression in UPEC isolated from women with symptomatic UTI using real time-PCR. Urine samples from 14 of 19 patients yielded sufficient bacterial RNA for the analysis and no pooling of samples were needed. Besides bacteria, the urine samples also contain leukocytes and most likely some exfoliated epithelial cells. However, the primers used were verified to be bacteria-specific. A considerable increase in *hmp* expression was detected in UPEC isolated directly from patients compared to when the same strain was grown in uninfected urine from healthy volunteers *in vitro*. This suggests that UPEC encounter a greater nitrosative stress during UTI than when grown in uninfected urine. There may be several sources of nitrosative stress in the urinary tract during UTI. Host-derived production of NO from iNOS by uroepithelial cells and neutrophils is most likely a major source [25] [24, 29]. Indeed, the fact that all urine samples were positive for leukocyte esterase indicates that the host response was activated in the UTI patients. Small amounts of NO may also be generated non-enzymatically from urinary nitrite [28, 29] and by bacterial nitrite reductase [31] or nitrate reductase [46], in particular if the urine becomes acidified and the bacteria experience low oxygen levels during the infection. The *hmp* gene is also inducible by nitrite [12] and in our study urinary nitrite levels were elevated in 9 out of 14 patients. However, no correlation was found between *hmp* expression and urinary nitrite levels.

Expression of *hmp* was also detected in UPEC grown in nitrite-negative urine from healthy volunteers. It is possible that trace amounts of NO, formed in uninfected urine [28], may cause this basal *hmp* transcription. An increased *norV* expression was detected in seven patients (50 %). UTI patients demonstrate a decrease in urinary oxygen levels compared with healthy subjects [47] which may explain why the oxygen-sensitive *norV* expression was more prominent *in vivo*. The finding that both *hmp* and *norV* expression were detected in patients suggest that UPEC may be exposed to various oxygen levels during the course of the infection, and therefore need NO-detoxifying systems which function at different oxygen concentrations. Thus, the fact that gene expression of NO-detoxifying enzymes was increased in UPEC-isolates from UTI patients strongly implies that UPEC faces nitrosative stress in the infected human urinary tract.

To investigate whether the NO-detoxifying effect of Hmp contributes to UPEC virulence during UTI we used a well-established mouse model of ascending UTI. A competition model was chosen since it eliminates the variation in host response between animals and gives a more sensitive measurement of the ability of a mutant to survive *in vivo* compared to a wild-type strain. Hmp has been identified as important for protection of non-pathogenic *E. coli* against NO-mediated killing by human macrophages [17]. Furthermore Hmp was required for *Salmonella* virulence in mice [48]. However, so far no studies have addressed the significance of Hmp in urovirulence. An activated host response was verified by the influx of PMN cells, which peaked 12 h after bacterial infection and by the urinary nitrite concentrations that were slightly elevated 12 h post-infection. The mice were on a nitrite/nitrate deficient diet and the increase in urinary nitrite is therefore believed to mainly originate from iNOS-derived NO rather than from bacterial conversion of nitrate. The time-course of elevated urinary nitrite correlated well with the PMN influx suggesting that PMN cells are the main source of NO. Supporting this, a massive influx of iNOS-positive PMN cells were detected in C3H/HeN mice at 6 and 12 h after UPEC-induced UTI [24]. The *hmp*-mutant exhibited a significant colonization deficiency in both the bladder and kidneys, relative to the wild-type strain, when evaluating the entire infection experiment. These data suggest that Hmp confer a fitness advantage in colonization and persistence in the urinary tract. In the time-course study, there was no significant impairment in colonizing ability of J96Δhmp at 6 and 12 h post-infection but after 24 h the J96wt significantly out-competed the *hmp*-deficient mutant in

the kidney. NO does not appear to be part of the first line of host defense in UTI [26] and uroepithelial iNOS expression in infected C3H/HeN mice was detectable first after 24 h of infection [24]. The fact that the colonizing ability of J96Δhmp is preferentially attenuated 24 h post-infection may suggest that a combined NO release from neutrophils and urothelium results in a stronger nitrosative stress response over time that the *hmp*-deficient mutant is less able to withstand. Recently, studies in an *hmp*-deficient *E. coli* K-12 strain revealed an unexpected phenotype characterized by loss of flagella and a motility defect [17]. It is not known if the *hmp*-deficient UPEC strain also exhibits an aflagellated phenotype. The role for flagella in UPEC colonization is not fully elucidated but non-motile strains are not less uropathogenic in mice than motile strains [49]. Experiments performed in a similar ascending mouse model of UTI as in our study found that genes involved in motility were down-regulated in UPEC during infection [44].

In summary, we show that Hmp contributes in protecting UPEC against NO-mediated toxicity *in vitro*. A competitive mouse infection model demonstrated that colonization of the UPEC *hmp*-deficient mutant was reduced compared to the wild-type strain, suggesting that Hmp may be a virulence-facilitating factor in UPEC. Screening UPEC isolates from UTI patients for *hmp* expression revealed an increased expression in all patients which confirms that *hmp* expression occurs in the infected human urinary tract.

ACKNOWLEDGEMENT

This project was supported by the Swedish Medical Research Council (12601), the Medical Research Council of Southeast Sweden, a donation from Mrs Kerstin Heyman and the Faculty of Natural Science at the University of Kalmar. We thank the staff at Stensö Health Center for valuable assistance with the clinical study.

REFERENCES

1. De Groote, M.A. and F.C. Fang, NO inhibitions: antimicrobial properties of nitric oxide. Clin Infect Dis, 1995. **21 Suppl 2**. S162-5.
2. Fang, F.C., Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol, 2004. **2**(10). 820-32.
3. Stevanin, T.M., N. Ioannidis, C.E. Mills, et al., Flavohemoglobin Hmp affords inducible protection for Escherichia coli respiration, catalyzed by cytochromes bo' or bd, from nitric oxide. J Biol Chem, 2000. **275**(46). 35868-75.
4. Schapiro, J.M., S.J. Libby, and F.C. Fang, Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. Proc Natl Acad Sci U S A, 2003. **100**(14). 8496-501.
5. Mukhopadhyay, P., M. Zheng, L.A. Bedzyk, et al., Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species. Proc Natl Acad Sci U S A, 2004. **101**(3). 745-50.
6. Flatley, J., J. Barrett, S.T. Pullan, et al., Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. J Biol Chem, 2005. **280**(11). 10065-72.
7. Justino, M.C., J.B. Vicente, M. Teixeira, et al., New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. J Biol Chem, 2005. **280**(4). 2636-43.
8. Pullan, S.T., M.D. Gidley, R.A. Jones, et al., Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. J Bacteriol, 2007. **189**(5). 1845-55.
9. Poole, R.K. and M.N. Hughes, New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol Microbiol, 2000. **36**(4). 775-83.
10. Gardner, A.M. and P.R. Gardner, Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, Escherichia coli. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. J Biol Chem, 2002. **277**(10). 8166-71.
11. Kim, S.O., Y. Orii, D. Lloyd, et al., Anoxic function for the Escherichia coli flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett, 1999. **445**(2-3). 389-94.
12. Poole, R.K., M.F. Anjum, J. Membrillo-Hernandez, et al., Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12. J Bacteriol, 1996. **178**(18). 5487-92.
13. Membrillo-Hernandez, J., M.D. Coopamah, A. Channa, et al., A novel mechanism for upregulation of the Escherichia coli K-12 hmp (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region. Mol Microbiol, 1998. **29**(4). 1101-12.

14. Bodenmiller, D.M. and S. Spiro, The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. J Bacteriol, 2006. **188**(3). 874-81.
15. Nakano, M.M., H. Geng, S. Nakano, et al., The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. J Bacteriol, 2006. **188**(16). 5878-87.
16. Stevanin, T.M., R.K. Poole, E.A. Demoncheaux, et al., Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages. Infect Immun, 2002. **70**(8). 4399-405.
17. Stevanin, T.M., R.C. Read, and R.K. Poole, The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: Links with swarming motility. Gene, 2007.
18. Gilberthorpe, N.J., M.E. Lee, T.M. Stevanin, et al., NsrR: a key regulator circumventing Salmonella enterica serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages. Microbiology, 2007. **153**(Pt 6). 1756-71.
19. Gardner, A.M., R.A. Helmick, and P.R. Gardner, Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli. J Biol Chem, 2002. **277**(10). 8172-7.
20. Gomes, C.M., A. Giuffre, E. Forte, et al., A novel type of nitric-oxide reductase. Escherichia coli flavorubredoxin. J Biol Chem, 2002. **277**(28). 25273-6.
21. Bogdan, C., M. Rollinghoff, and A. Diefenbach, Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol, 2000. **12**(1). 64-76.
22. Bower, J.M. and M.A. Mulvey, Polyamine-mediated resistance of uropathogenic Escherichia coli to nitrosative stress. J Bacteriol, 2006. **188**(3). 928-33.
23. Svensson, L., B.I. Marklund, M. Poljakovic, et al., Uropathogenic Escherichia coli and tolerance to nitric oxide: the role of flavohemoglobin. J Urol, 2006. **175**(2). 749-53.
24. Poljakovic, M., M.L. Svensson, C. Svanborg, et al., Escherichia coli-induced inducible nitric oxide synthase and cyclooxygenase expression in the mouse bladder and kidney. Kidney Int, 2001. **59**(3). 893-904.
25. Wheeler, M.A., S.D. Smith, G. Garcia-Cardena, et al., Bacterial infection induces nitric oxide synthase in human neutrophils. J Clin Invest, 1997. **99**(1). 110-6.
26. Mysorekar, I.U., M.A. Mulvey, S.J. Hultgren, et al., Molecular regulation of urothelial renewal and host defenses during infection with uropathogenic Escherichia coli. J Biol Chem, 2002. **277**(9). 7412-9.
27. Lundberg, J.O., I. Ehren, O. Jansson, et al., Elevated nitric oxide in the urinary bladder in infectious and noninfectious cystitis. Urology, 1996. **48**(5). 700-2.
28. Lundberg, J.O., S. Carlsson, L. Engstrand, et al., Urinary nitrite: more than a marker of infection. Urology, 1997. **50**(2). 189-91.
29. Carlsson, S., N.P. Wiklund, L. Engstrand, et al., Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. Nitric Oxide, 2001. **5**(6). 580-6.
30. Cole, J., Nitrate reduction to ammonia by enteric bacteria: redundancy, or a strategy for survival during oxygen starvation? FEMS Microbiol Lett, 1996. **136**(1). 1-11.
31. Corker, H. and R.K. Poole, Nitric oxide formation by Escherichia coli. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. J Biol Chem, 2003. **278**(34). 31584-92.
32. Datsenko, K.A. and B.L. Wanner, One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A, 2000. **97**(12). 6640-5.
33. Baba, T., T. Ara, M. Hasegawa, et al., Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol, 2006. **2**. 2006 0008.
34. Keefer, L.K., R.W. Nims, K.M. Davies, et al., "NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. Methods Enzymol, 1996. **268**. 281-93.
35. Fujii, T., Y. Aritoku, H. Agematu, et al., Increase in the rate of L-pipecolic acid production using lat-expressing Escherichia coli by lysP and yeiE amplification. Biosci Biotechnol Biochem, 2002. **66**(9). 1981-4.
36. da Costa, P.N., M. Teixeira, and L.M. Saraiva, Regulation of the flavorubredoxin nitric oxide reductase gene in Escherichia coli: nitrate repression, nitrite induction, and possible post-transcription control. FEMS Microbiol Lett, 2003. **218**(2). 385-93.
37. Ignarro, L.J., J.M. Fukuto, J.M. Griscavage, et al., Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proc Natl Acad Sci U S A, 1993. **90**(17). 8103-7.
38. Green, L.C., D.A. Wagner, J. Glogowski, et al., Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem, 1982. **126**(1). 131-8.

39. Gardner, P.R., G. Costantino, and A.L. Salzman, Constitutive and adaptive detoxification of nitric oxide in Escherichia coli. Role of nitric-oxide dioxygenase in the protection of aconitase. J Biol Chem, 1998. **273**(41). 26528-33.
40. Hausladen, A., A.J. Gow, and J.S. Stamler, Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc Natl Acad Sci U S A, 1998. **95**(24). 14100-5.
41. Membrillo-Hernandez, J., M.D. Coopamah, M.F. Anjum, et al., The flavohemoglobin of Escherichia coli confers resistance to a nitrosating agent, a "Nitric oxide Releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J Biol Chem, 1999. **274**(2). 748-54.
42. Gardner, A.M., L.A. Martin, P.R. Gardner, et al., Steady-state and transient kinetics of Escherichia coli nitric-oxide dioxygenase (flavohemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. J Biol Chem, 2000. **275**(17). 12581-9.
43. Hernandez-Urzua, E., C.E. Mills, G.P. White, et al., Flavohemoglobin Hmp, but not its individual domains, confers protection from respiratory inhibition by nitric oxide in Escherichia coli. J Biol Chem, 2003. **278**(37). 34975-82.
44. Snyder, J.A., B.J. Haugen, E.L. Buckles, et al., Transcriptome of uropathogenic Escherichia coli during urinary tract infection. Infect Immun, 2004. **72**(11). 6373-81.
45. Roos, V. and P. Klemm, Global gene expression profiling of the asymptomatic bacteriuria Escherichia coli strain 83972 in the human urinary tract. Infect Immun, 2006. **74**(6). 3565-75.
46. Gilberthorpe, N.J. and R.K. Poole, Nitric oxide homeostasis in salmonella typhimurium: Roles of respiratory nitrate reductase and flavohemoglobin. J Biol Chem, 2008.
47. Giannakopoulos, X., A. Evangelou, V. Kalfakakou, et al., Human bladder urine oxygen content: implications for urinary tract diseases. Int Urol Nephrol, 1997. **29**(4). 393-401.
48. Bang, I.S., L. Liu, A. Vazquez-Torres, et al., Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp. J Biol Chem, 2006. **281**(38). 28039-47.
49. Johnson, J.R., Virulence factors in Escherichia coli urinary tract infection. Clin Microbiol Rev, 1991. **4**(1). 80-128.