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Helicobacter pylori Proteins Response to Nitric Oxide Stress

Wei Qu[1†], Yabin Zhou[1†], Chunhong Shao[1,3], Yundong Sun[1], Qunye Zhang[1], Chunyan Chen[2*], and Jihui Jia[1*]

[1]*Department of Microbiology and Key Lab for Experimental Teratology of Chinese Ministry of Education, School of Medicine, Shandong University, Jinan 250012, P. R. China*
[2]*Department of Hematology, Qilu Hospital, Shandong University, Jinan 250012, P. R. China*
[3]*Clinical Laboratory, Provincial Hospital Affiliated to Shandong University, Jinan 250021, P. R. China*

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Helicobacter pylori **is a highly pathogenic microorganism with various strategies to evade human immune responses. Nitric oxide (NO) and reactive nitrogen species (RNS) generated via nitric oxide synthase pathway are important effectors during the innate immune response. However, the mechanisms of *H. pylori* to survive the nitrosative stress are not clear. Here the proteomic approach has been used to define the adaptive response of *H. pylori* to nitrosative stress. Proteomic analysis showed that 38 protein spots were regulated by NO donor, sodium nitroprusside (SNP). These proteins were involved in protein processing, antioxidation, general stress response, and virulence, as well as some unknown functions. Particularly, some of them were participated in iron metabolism, potentially under the control of ferric uptake regulator (Fur). Real time PCR revealed that *fur* was induced under nitrosative stress, consistent with our deduction. One stress-related protein up-regulated under nitrosative conditions was thioredoxin reductase (TrxR). Inactivation of *fur* or *trxR* can lead to increased susceptivity to nitrosative stress respectively. These studies described the adaptive response of *H. pylori* to nitric oxide stress, and analyzed the relevant role of Fur regulon and TrxR in nitrosative stress management.**

Keywords**:** *Helicobacter pylori*, nitrosative stress, proteomics

Helicobacter pylori is a prevalent Gram-negative microaerophilic bacterium that colonizes the mucus layer in the stomach and causes gastritis, peptic ulcer, gastric cancer, and so on (McGee and Mobley, 2000). The human innate immune response is activated while *H. pylori* invades the human gastric mucosa. NO is an important effector possessing antimicrobial activity and immunomodulatory effect (Zaki *et al.*, 2005). NO and reactive nitrogen intermediates (RNI) play protective roles in the acute and persistent phases of *H. pylori* infection.

When NO diffuses into bacterial cytoplasm, peroxynitrite is formed via interaction with O_2^- (Raupach and Kaufmann, 2001). Subsequently, peroxynitrite can oxidate, S-nitrosate microbial proteins, nucleic acids and lipids, exerting toxic effect (Zaki *et al*., 2005). On the other hand, NO and RNI can react with thiols to form S-nitroso compounds (RSNO or RSOH), therefore cell respiration and metabolism of *H. pylori* can be inhibited. NO also nitrosylates free or enzyme-bound Fe(II), such as cytochrome P450. Deoxyribonucleic acid (DNA) damage induced by NO and RNI is another toxic effect to *H. pylori* and host tissues (Siomek *et al.*, 2006). Recently, it is determined that the death rate of inducible nitric oxide synthase (iNOS-/-) knockout mice is higher than wild-type mice after *H. pylori* infection (Nam *et al*., 2004). Thus, tolerance to nitrosative stress is crucial for *H. pylori* survival and infection in the stomach.

So far, there are some studies focused on NO resistance in *H. pylori*, and several proteins contributed to NO resistance have been discovered, such as arginase, urease, and peroxidoxins (Bryk *et al.*, 2000; Kuwahara *et al*., 2000; Chaturvedi *et al.*, 2007). The alkylhydroperoxide reductase (AhpC) catalyzes the conversion of $OONO^-$ to NO_2 (Chen *et al.*, 1998; Bryk *et al.*, 2000). However, some homologues of the NO detoxifying enzymes present in other bacteria, including flavorubredoxin NorV, flavohemoglobin HmpA and nitrite reductase NrfA have not been found in *H. pylori* genome. Proteomes can provide snapshots of protein expression profiles of particular cells under defined conditions. They have been widely used in studies designed to characterize the differential protein compositions on various biological systems. However, proteomic analysis of the nitrosative stress response has not been studied in *H. pylori* to date.

Here we simulated the antimicrobiol process of NO in *H. pylori in vitro*, with sodium nitroprusside (SNP) as NO donor. The holistic protein expression of *H. pylori* treated with SNP was investigated using the proteomic technologies. Then real time PCR revealed the transcriptional change of *fur* and *trxR* under nitrosative stress in *H. pylori*. Deletion mutants were constructed, and their NO sensitivity was compared with wt-*H. pylori* for further understanding the functional roles.

† These authors contributed equally to this work.
∗ To whom correspondence should be addressed.
(Tel) 86-531-8838-2672; (Fax) 86-531-8838-2502
(E-mail) Chunyan Chen: chency@sdu.edu.cn
Jihui Jia: jiajihui@sdu.edu.cn

Materials and Methods

Bacterial strain and culture conditions

H. pylori 26695 was provided by Dr. Zhang Jianzhong from Chinese Disease Control and Prevention Center. Bacteria cells in Brucella broth medium containing 10% fetal bovine serum were grown microaerobically to an OD_{600} value of 0.6 to examine the inhibitory effect of SNP on *H. pylori*. Aliquots (20 ml) of the cell cultures were added different concentration of SNP (0, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM) in flasks, under microaerobic condition at 37°C. At 3 h intervals, OD_{600} value was measured, 10 μl suspension was applied to Skirrow agar plate with 5% (v/v) sheep blood under microaerobic condition (5% O_2, 10% CO_2, 85% N_2) at 37°C and incubated for 3~4 days prior to viability assessment.

Protein lysate preparation and two dimensional gel electrophoresis

H. pylori was incubated to logarithmic phase, and the duplicate cultures were treated with 0.5 mM SNP for 6 h. The bacteria harvested by centrifugation were washed three times in sterilized ice-cold PBS (0.01 M, pH 7.4) and resuspended in lysis buffer. The lysis buffer contains 8 M urea, 4% CHAPS, 1% DTT, 4 mM Tris, 1% pharmalyte (pH 3~10), 10 μl/ml protease inhibitor cocktail (Amersham Biosciences, Sweden), 10 μg/ml RNase and 10 μg/ml DNase. After sonication, about 350 μg protein was loaded onto 18 cm IPG strips (pH 3~10, Amersham Biosciences). The IPGphor IEF system (Amersham Biosciences) was used for IEF.

After IEF, the IPG strips were equilibrated for 15 min each in buffer (50 mM Tris-HCl; pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue) with 0.5% (w/v) DTT and 2% (w/v) iodoacetamide respectively. SDS-PAGE was performed with 12% gels using the PROTEANII xi 2-D cell (Bio-Rad, USA). The electrophoresis was conducted at 15 mA per gel for 30 min, and then 30 mA per gel until bromophenol blue front reached bottom of the gel. The gels were silver stained and scanned by ImageScanner II (Amersham Biosciences) at 256 grayscale and 300 dpi degree levels. Image analysis was accomplished using ImageMaster 2D Elite 5.0 (Amersham Biosciences), with the protein map of wild type *H. pylori* acting as the reference. All experiments were performed in triplicate. 38 gel spots whose %vol in the gels between *H. pylori* cultured with and without SNP showed 1.5-fold or greater difference ($P<0.05$) were chosen for in gel digestion.

In gel-digestion and MALDI-TOF/TOF

Based on the 2D gel analysis, 38 gel spots were excised and digested with trypsin (5 mg/ml) overnight at 30°C. Digested peptides were lyophilized and desalted by passing through a C18 ZipTip (Millipore, USA). The resulting peptides were eluted with matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 50% acetonitrile), and spotted onto MALDI target plates, mass spectra were then collected for peptide mass fingerprinting (PMF). PMF was acquired on a MALDI-TOF/TOF-tandom mass spectrometer (Applied Biosystems, USA), mass spectra (MS) and MS/MS were then analyzed with a 50 ppm mass tolerance by GPS Explorer V2.0.1 and Mascot V1.9 based on NCBI SWISSPROT and local *H. pylori* databases (April, 2006 updated). Search parameters in the program allowed for 100 ppm (0.01%) error in the molecular mass.

Real time PCR

H. pylori was incubated to logarithmic phase, and the duplicate cultures were treated with SNP (0.5 mM) for 6 h. The total RNA was isolated using the TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized from MMLV reverse transcriptase and random hexamer primer (MBI). The primers for PCR are listed in Table 1. Each 20 μl PCR reaction mixture contained SYBR Premic *Ex* Taq™ (TaKaRa, Japan), ROX Reference Dye (TaKaRa), 100 ng cDNA, 500 nM forward and 500 nM reverse primer. Prism 7000 (ABI) was used to detect fluorescence with the following protocol for the PCR: one cycle at 95°C for 10 sec and 40 cycles at 95°C for 5 sec and 60°C for 31 sec. The data was normalized to 16S RNA cDNA expression in each sample and three biological replicates were performed.

Construction of mutant *H. pylori* and functional test

The *trxR* and *fur* mutant strain of *H. pylori* 26695 were constructed according to the published protocols (Loughlin *et al.*, 2003). Professor Agnes Labigne kindly provided plasmids pILL570 and pUC18K2. The *fur* mutant *H. pylori* was constructed as following. The genome of *H. pylori* 26695 was extracted to serve as template. The primers are listed in Table 1. Fragment1 containing the 5' region of *fur* gene flanked by *Cla*I and *Eco*RI restriction sites was amplified by PCR using primer pair fur-1/fur-2. Fragment2 containing the 3' region of *fur* gene flanked by *Bam*HI and *Pst*I restriction sites was amplified by PCR using primer pair fur-3/fur-4. The nonpolar kanamycin cassette was excised from pUC18K2 by *Eco*RI/*Bam*HI digestion. These three fragments were ligated with the *Cla*I /*Pst*I digested vector pILL570, generating plasmid pILL570-Fur. Plasmid pILL570-Fur carries a 210 bp deletion of the *fur* gene which was replaced with the kana-

Table 1. Primer sets used in this study

Primers	Sequence (5'-3')
16S rRNAF	GCTCTTTACGCCCAGTGATTC
16S rRNAR	GCGTGGAGGATGAAGGTTTT
furF	TTG CGG CTA AAG AAC AC
furR	GCT AAT CAG CTT GGC TTG
trxF	AAC CGC CCT ACT GCT TA
trxR	GTA TTC CAC CGC TGA GTT G
trxR-1	*CCATCGAT* TCC GCT GTT ATT TGT CCC A
trxR-2	*GGAATTC* ACC CGC TCA CCA CTT CTT T
trxR-3	*CGGGATCC* TTG TTT GCG GCA GGA GAT A
trxR-4	*AAAACTGCAG* TCA CAT AGC CTT GAG TCC C
fur-1	*CCATCGAT* TAC AAT CCT TAC GGC CTC ATA
fur-2	*GGAATTC* GAT TTC TTC AGG GCT TAG GTG
fur-3	*CGGGATCC* CCG CCA GAA TGA AGT CGT TAA
fur-4	*AAAACTGCAGA* TGC CAA AGA AAG CGA GAA AGG

Italics indicate nucleotides that were added at the 5' end to create a restriction site. Restriction sites for *Cla*I (trxR1-1, fur-1), *Eco*RI (trxR-2, fur-2), *Bam*HI (trxR-3, fur-3), and *Pst*I (trxR-4, fur-4) are underlined.

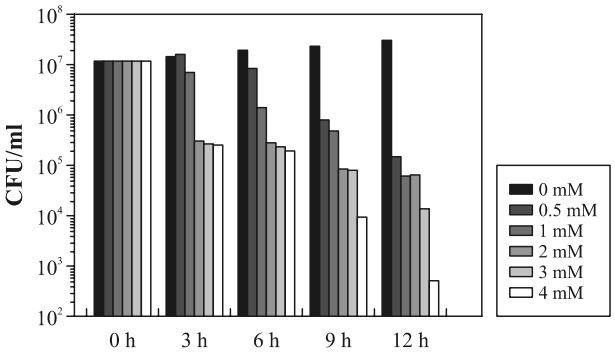

Fig. 1. Survival of *H. pylori* 26695 in the presence of nitrosative stress. *H. pylori* cells were grown to logarithmic phase, and then exposed to various concentrations of SNP for 0, 3 h, 6 h, 9 h, 12 h. Data from a single representative experiment are shown.

mycin cassette. Then, the mutant vector pILL570-Fur was electrotransformed into *H. pylori* 26695 as conducted in the previous study (Kim *et al.*, 1999). Genomic DNA was extracted from putative recombinants, and correct recombination was evaluated by PCR using primer pairs fur-1/fur-4. The *trxR* gene was inactivated as above.

Wt-*H. pylori* and the mutant strains were grown in Brucella broth containing 10% fetal bovine serum with SNP (0.1 mM) or without. OD_{600} was monitored at 12 h intervals. Cell cultures at logarithmic phase were treated with a lethal concentration (1 mM) of SNP or left untreated. Aliquots were plated onto agar plates per 3 h to assess viability.

Results and Discussion

Concentration-dependent SNP (NO) toxicity in *H. pylori*

SNP was used as the NO donor to analyze the effects of NO and RNI on *H. pylori* in this work. We examined the effects of SNP of various concentration (0~4 mM) and exposure time (0~12 h) on the growth and cell viability of *H. pylori* to obtain the appropriate concentration and exposure time of SNP. Figure 1 showed that SNP inhibited growth of logarithmic-phase *H. pylori* in a time-dependent fashion in this short period. Therefore, changes in protein expression were examined with 0.5 mM SNP for 6 h to ensure that the protein changes observed were in response to nitrosative stress and not due to severe nitric oxide-mediated toxicity.

NO can be released by SNP indirectly following nitrosation of a thiolate group and subsequent degradation of S-nitrosothiol. The positive attribute of NO donors is that, compared with authentic NO, they are more convenient and workable. The drawback is that it will introduce some responses to SNP derived species, rather than the nitrosating agent per se. Florio *et al.* (2006) has measured the concentration of SNP derived peroxynitrite in culture supernatants of *BCG* exposed to SNP and found its bactericidal effect versus NO was negligible. Therefore, SNP used as NO donor was feasible, credible, and typical.

Translational responses to nitric oxide stress in *H. pylori*

For a deeper understanding of the proteomic response to RNI, *H. pylori* at logarithmic phase was treated with 0.5 mM SNP for 6 h. The resulting protein lysates were compared to lysates of untreated cells. 2-DE analysis was repeated in three times using independent grown cultures. Figure 2 showed 38 protein spots displayed differential expression on the 2D maps of *H. pylori* treated with SNP or not. All these protein spots were identified using peptide mass fingerprinting (PMF) and classified according to their known or postulated functions in Table 2. Figure 3 depicts the response of *H. pylori* to nitrosative stress comprised of complex physiological mechanisms. They are discussed as follows.

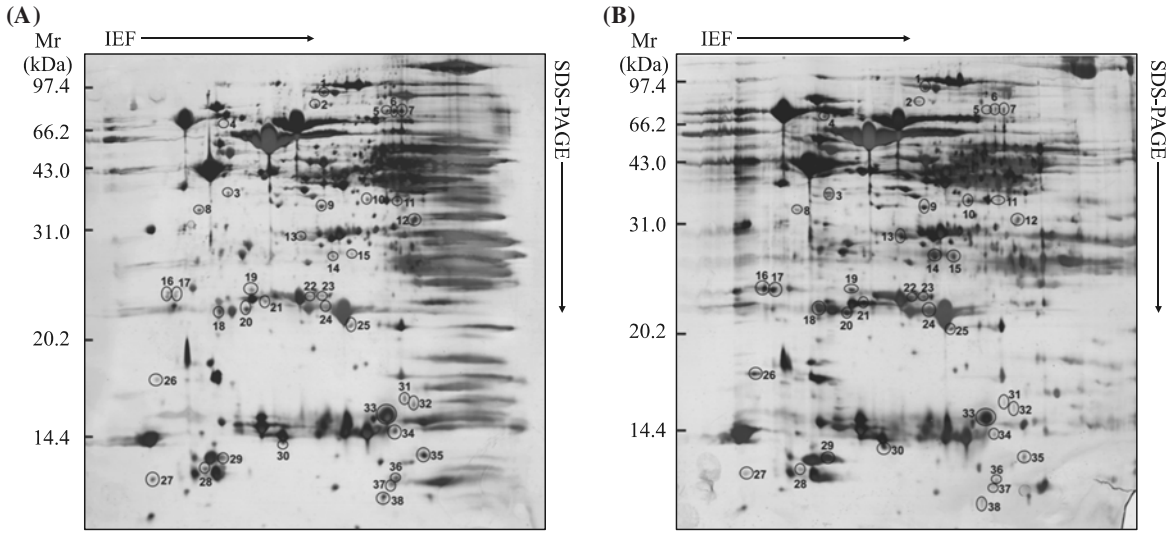

Fig. 2. Comparison of proteomic profiles of *H. pylori* 26695 with and without SNP. (A) 2-DE map of *H. pylori* 26695 without SNP; (B) 2-DE map of *H. pylori* 26695 exposed to 0.5 mM SNP for 6 h.

Table 2. Summary of protein spots showing altered expression in response to nitric oxide

Function class	Spot no.[a]	Proteins (genes)	TIGR ORF no.[b]	Gi	Mr[1] (kD)	Mr[2] (kD)	Top score	Sequence coverage	%vol ratio[c]
Protein processing	1	ATP-dependent protease binding subunit (*clpB*)	Hp0264	2313356	96.6	97.4	133	25%	1.91
	4	ribosomal protein S1 (*rps1*)	Hp0399	2313498	62.8	71.4	165	37%	+[d]
	8	aminopeptidase a/i (*pepA*)	Hp0570	2313690	54.4	35.0	391	32%	0.62
	11	branched-chain-amino-acid amino-transferase (*ilvE*)	Hp1468	2314646	37.6	36.0	148	22%	0.15
	16	aminopeptidase a/i (*pepA*)	Hp0570	2313690	54.4	23.8	391	32%	6.53
	17	aminopeptidase a/i (*pepA*)	Hp0570	2313690	54.4	23.8	413	36%	9.09
	21	translation elongation factor EF-P (*efp*)	Hp0177	2313266	20.8	22.8	130	49%	6.63
Virulence factor	2	cag pathogenicity island protein (*cag26*)	Hp0547	2313664	132.3	81.8	218	22%	0.33
	27	paralysed flagella protein (*pflA*)	Hp1274	2314439	92.5	8.0	66	21%	0.30
General stress protein	3	chaperone and heat shock protein (*groEL*)	Hp0010	2313084	58.2	37.5	245	31%	4.18
	10	thioredoxin reductase (*trxR*)	Hp0825	2314321	36.0	36.1	90	58%	3.67
	13	thioredoxin reductase (*trxR*)	Hp0825	2314321	36.0	30.1	234	68%	1.82
	14	co-chaperone (*groES*)	Hp0011	2313085	13.0	28.6	128	46%	4.47
	15	co-chaperone (*groES*)	Hp0011	2313085	13.0	29.0	140	55%	4.70
	25	thiol:disulfide interchange protein (*dsbC*)	Hp0377	2313480	25.3	21.0	135	27%	1.82
	28	hydrogenase expression/formation protein (*hypA*)	Hp0869	2313996	13.2	9.3	254	87%	0.43
	26	flavodoxin (*fldA*)	Hp1161	2314319	17.5	18.0	150	46%	12.21
Cell division	5	glucose inhibited division protein (*gidA*)	Hp0213	2313303	69.6	76.6	225	38%	0.28
	6	glucose inhibited division protein (*gidA*)	Hp0213	2313303	69.6	76.6	254	32%	0.20
	7	glucose inhibited division protein (*gidA*)	Hp0213	2313303	69.6	76.6	283	29%	0.49
Antioxidant proteins	22	superoxide dismutase (*sodB*)	Hp0389	2313490	24.6	23.2	251	61%	2.53
	23	superoxide dismutase (*sodB*)	Hp0839	2313490	24.6	23.2	298	66%	2.44
	24	alkyl hydroperoxide reductase (*tsaA*)	Hp1563	2314747	22.2	22.0	442	82%	2.81
	30	neutrophil activating protein (*napA*)	Hp0243	2313332	16.9	13.1	364	73%	9.71
	33	adhesin-thiol peroxidase (*tagD*)	Hp0390	2313491	18.3	16.0	64	48%	2.05
Metabolism	9	Holliday junction DNA helicase (*ruvB*)	Hp1059	2314203	37.3	34.7	264	42%	1.70
	12	aldo-keto reductase, putative	Hp1193	2314358	37.1	32.5	362	65%	0.48
	18	adenylate kinase (*adk*)	Hp0618	2313740	21.2	22.3	463	76%	3.79
	31	(3R)-hydroxymyristoyl-(acyl carrier protein) dehydratase (*fabZ*)	Hp1376	2314546	18.2	17.5	165	49%	0.29
	32	deoxyuridine 5'-triphosphate nucleotidehydrolase (*dut*)	Hp0865	2313993	15.8	16.8	327	73%	0.24
	38	phosphomannomutase (*algC*)	Hp1275	2314440	51.7	5.6	240	48%	0.27
Unknown function	19	conserved hypothetical protein	Hp0813	2313945	23.4	24	209	45%	4.05
	20	*H. pylori* predicted coding region HP1029	Hp1029	2314185	20.3	22.0	191	57%	1.77
	29	*H. pylori* predicted coding region HP1482	Hp1482	2314669	10.0	11.5	107	62%	4.02
	34	*H. pylori* predicted coding region HP1542	Hp1542	2314731	14.6	14.4	259	58%	0.19
	35	*H. pylori* predicted coding region HP0614	Hp0614	2313745	13.0	12.3	156	59%	0.16
	36	*H. pylori* predicted coding region HP0902	Hp0902	2314040	11.0	8.5	137	52%	0.36
	37	*H. pylori* predicted coding region HP0902	Hp0902	2314040	11.0	7.5	276	63%	0.44

[a] Spot numbers refer to the proteins labeled in Fig. 2
[b] TIGR ORF no. follows the nomenclature of *H. pylori* strain 26695
[c] %vol ratio for each protein derived from *H. pylori* cultured at medium with SNP with respect to the protein derived from wt *H. pylori*
[d] "+" shows a spot that was absent in the untreated control; Mr[1] molecular weight of protein spot from NCBI; Mr[2] molecular weight of protein spot observed from the gel.

Nitric oxide regulated the expression of proteins involved in protein processing

The proteins highly induced during nitric oxide stress included ribosomal protein S1 (Rps1), aminopeptidase a/i (PepA), and translation elongation factor EF-P (Efp), as well as ATP-dependent protease binding subunit (ClpB). They

represented a major class of nitric oxide responsive proteins that were up-regulated. EF-P is involved in the delivery of aminoacyl-tRNA to the ribosome and ClpB has been reported to be involved in the acid stress adaptation in *P. freudenreichii* (Leverrier *et al.*, 2004). Branched chain amino acid aminotransferase (IlvE) catalyzes the last step of the synthesis of the hydrophobic amino acids valine, leucine, and isoleucine. Unexpectedly, IlvE was down-regulated. Several amino acid synthetic proteins have been previously detected after nitric oxide stress in *C. neoformans*, and after bile salt stress in *Listeria monocytogenes* (Duché *et al.*, 2002; Missall *et al.*, 2006). Given the importance of these proteins for cell nutrition and maintenance in *H. pylori*, they would have some indirect or secondary effect on protection from nitrosative stress.

Proteins involved in oxidative stress response were induced in response to nitric oxide

In this experiment, four proteins involved in oxidative stress were induced during nitric oxide stress. AhpC not only acted as a peroxide reductase in reducing organic hydroperoxides but also prevented the protein misfolding under oxidative stress as a molecular chaperone (Chen *et al.*, 1998; Chuang *et al.*, 2006). Its induction was consistent with previous reports suggesting that AhpC of *S. typhimurium* can protect bacterial and human cells against RNI, and deletion of *ahpC* made it hypersusceptible to RNI (Chen *et al.*, 1998). NapA induces the production of oxygen radicals from host cells and plays an identifiable role in protecting *H. pylori* from iron-mediated oxidative DNA damage (Wang *et al.*, 2006). It was also detected after human bile stress in *H. pylori*, as well as after iron and nickel change (Shao *et al.*, 2008a; Sun *et al.*, 2008). SodB is a classic antioxidant protein contributed to oxidative stress resistance. In 2005, the protein change mode of *H. pylori* under oxidative stress was analyzed (Chuang *et al.*, 2005). 11 differentially expressed protein spots under oxidative stress were identified, among which six proteins (TagD, AhpC, Adk, NapA, Hsp60, GroEL) were also found in our result. This reflected the high relevance between oxidative stress response and nitrosative stress response in *H. pylori*, showing that they may use some detoxifying proteins and regulators in common.

Various general stress response proteins altered by nitric oxide stress

General stress response proteins are generally involved in maturation of newly synthesized proteins, protein targeting to membranes, refolding and degradation of denatured proteins, as well as DNA repair (Segatori *et al.*, 2006). In the 2-DE map of *H. pylori* with SNP, chaperone, co-chaperone (GroES), flavodoxin (FldA), and thioredoxin reductase (TrxR), as well as thiol:disulfide interchange protein (DsbC) were induced. Interestingly heat shock protein (GroEL) appeared to be the products of degradation because their observed Mr value was smaller than the theoretical one, and the nitrosative stress enhanced the degradation of GroEL. DsbC can catalyze the rearrangement of mispaired cysteine residues, in charge of the folding of proteins containing multiple disulfide bonds. FldA acts as electron carriers, involved in energy metabolism. Some of them have been reported to be induced in various stress responses in *H. pylori* and other bacteria, such as after bile, acid or nickel stress (Duché *et al.*, 2002; Shao *et al.*, 2008b; Sun *et al.*, 2008). Particularly, TrxR was chosen for further analysis. Its deletion mutant was constructed and analyzed.

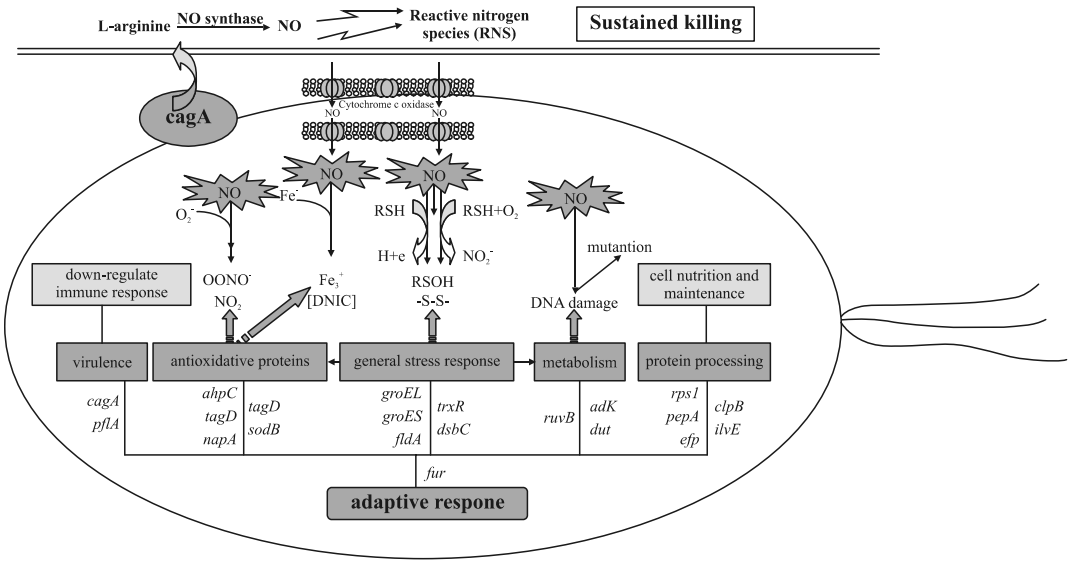

Fig. 3. A simple model of the nitrosative stress response in *H. pylori*. The human innate immune response is activated while *H. pylori* invades the human gastric mucosa. NO can diffuse across cell membranes and through the cytoplasm, then reacting rapidly with iron centres, thiols and superoxide. Meanwhile, *H. pylori* use some mechanisms for coping with the toxic effects exerted by nitrosative stress. *H. pylori* can also down regulate the expression of virulence (CagA, PflA) to alleviate the immune response. Eventually, *H. pylori* can escape the unfavourable environment.

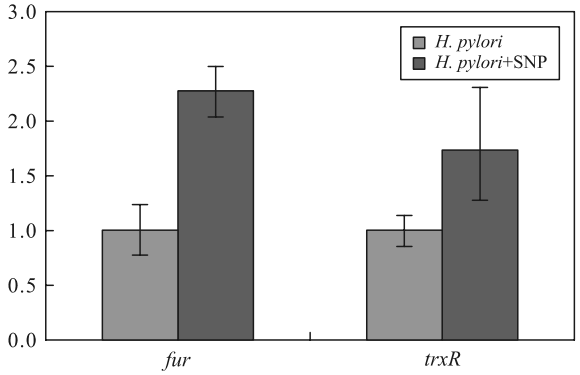

Fig. 4. Real time PCR results of RNA from *H. pylori* SNP exposed versus without for *fur* and *trxR*. Signals were normalized to 16S rRNA levels.

Nitric oxide stress affected expression of virulence genes
In our result, a major virulence protein of *H. pylori*, cytotoxin associated gene A (CagA) was down-regulated surprisingly under the exposure of nitrosative stress. Infection with the *cagA*-positive *H. pylori* was associated with higher grades of gastric mucosal inflammation, atrophic gastritis as well as gastric carcinoma (Hatakeyama and Higashi, 2005). Therefore, it was likely to be favorable to alleviate the immune response. In enterohemorrhagic *Escherichia coli*, NO can inhibit Shiga-toxin synthesis, then acting as a potential protective factor limiting the development of hemolytic syndromes (Vareille *et al.*, 2007). Down regulation of paralysed flagella protein (PflA) resulted in reduced mobility. Nonmotile mutant *H. pylori* cannot colonize the stomach suggesting that motility is indispensable to gastric colonization and infection (Dhar *et al.*, 2003). NO might thus down regulate the inflammatory response in favor of the survival of *H. pylori* under the adverse circumstance.

Several proteins involved in substance metabolism and unknown functions were regulated by nitric oxide stress
Two enzymes (AlgC and FabZ) related to metabolisms of sugar and lipid were down-regulated after nitric oxide exposure. This finding may suggest that *H. pylori* has a lower level of metabolism under the environmental stress condition. The sensitivity of metabolic enzymes to nitrosative stress may partly account for the nitric oxide toxicity in *H. pylori*. Holliday junction DNA helicase (RuvB) was slightly induced in our result. It promotes strand exchange during homologous recombination and scans the DNA during branch migration (Donaldson *et al*., 2006). Adenylate kinase (Adk) is essential in intracellular nucleotide metabolism, RNA and DNA biosynthesis. Deoxyuridine 5'-triphosphate nucleotidehydrolase (Dut) is also involved in nucleotide metabolism; it hydrolyzes dUTP to dUMP and pyrophosphate. Therefore, they were likely to relate to DNA repair in the adverse circumstances. Besides, there were several unknown proteins altered by nitric oxide stress.

Effect of SNP on *trxR* and *fur* mutant *H. pylori*
Several proteins (Dut, AhpC, NapA, TagD, HypA, and

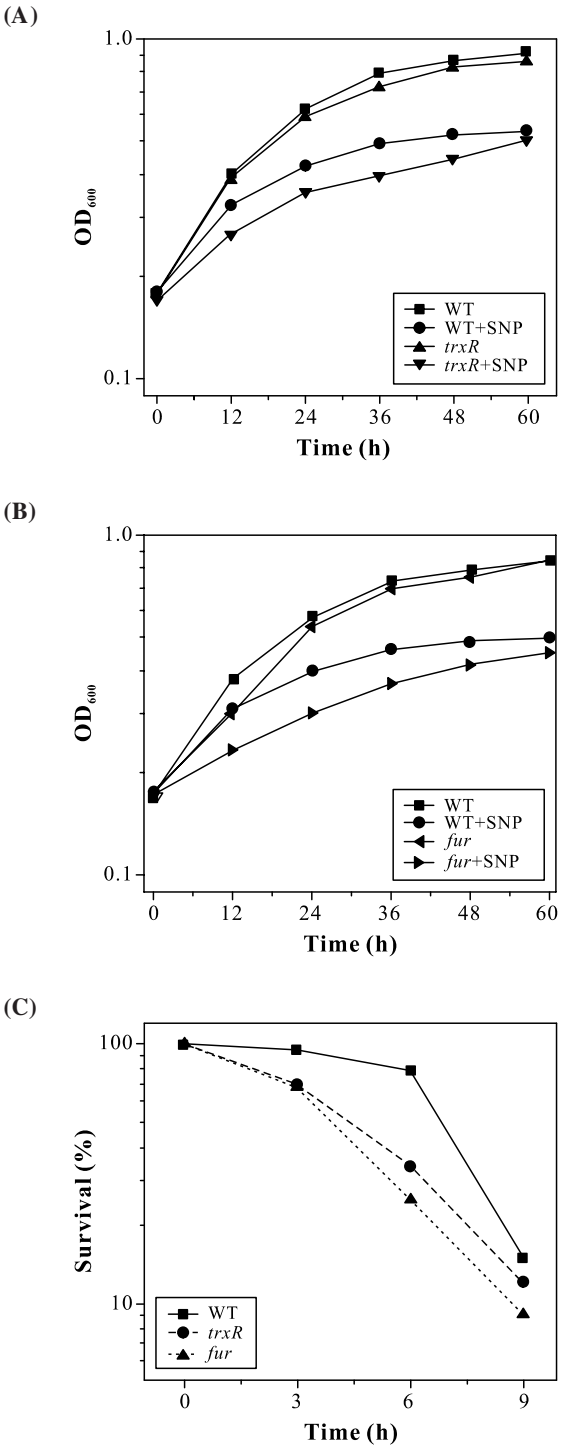

Fig. 5. *H. pylori trxR* and *fur* mutants were sensitive to nitrosative stress. *H. pylori* 26695 and the mutant strains were inoculated to a starting OD_{600} value of 0.17, and the duplicate culture was treated with SNP (0.1 mM); growth rate was monitored spectrophotometrically (Fig. 5A and B). Cell suspensions at logarithmic phase were incubated with 1 mM of SNP or left untreated for 9 h. Every three hours, aliquots were removed, serially diluted and plated onto agar plates to determine viability as CFU. Survival% was calculated as the CFU after treated with SNP divided by the CFU in the same culture without SNP (Fig. 5C). Data from a single representative experiment are shown.

SodB) found in our result also belonged to Fur regulon identified by microarray and other proteomic analysis (Lee *et al.*, 2004; Ernst *et al.*, 2005). Fur is a bacterial global regulator that involves iron distribution and uses iron as a cofactor to bind to specific DNA sequences to lead to transcriptional repression of iron-regulated genes (D'Autreaux *et al.*, 2002). When the iron level reduces to a low level, the active Fur repressor releases Fe^{2+} and is no longer able to bind to specific DNA sequences. Nitrosylation of the Fur bound ferrous iron by RNS can lead to inactivation of its repressor activity and thus derepressing Fur repressed genes. Derepressed expression of Fur regulated genes has also been proved to contribute to SNP resistance in *Bacillus subtilis*, *E. coli*, and *Staphylococcus aureus* (D'Autreaux *et al.*, 2002; Moore *et al.*, 2004; Richardson *et al.*, 2006). To analyze the role of Fur regulon in *H. pylori* resistance to nitrosative stress, we examined the expression of *fur* in *H. pylori* using real time PCR. The induction of *fur* by NO was depicted in Fig. 4. Besides, a *fur* mutant was constructed and analyzed. Fig. 5B showed the growth curve of *fur* mutant *H. pylori* under nitrosative stress. The growth of *fur* mutant was impaired, perhaps due to elevated intracellular iron, leading to Fe-nitrosyl complexes toxic to the bacteria. It also strongly implicated that derepression of Fur-regulated genes help alleviate the nitrosative stress. As to the failure to detect Fur protein in proteomic analysis, it can be attributed partly to the drawback of gel-electrophoretic proteomic analysis to visualize the proteins of low abundance.

Real time PCR analysis showed that NO stress also stimulated the *trxR* transcription (Fig. 4). Figure 5A depicted the growth curve of *trxR* mutant *H. pylori* with and without SNP. We can find from Fig. 5A that the inactivation of *trxR* significantly enhanced *H. pylori* susceptibility to NO, indicating its effect on NO resistance. It is known that thioredoxin system may transfer electron from NADPH/NADH to AhpC, Bcp, and Msr (Alamuri and Maier, 2004; Wang *et al.*, 2005). It is in accordance with the fact that TrxR of *C. neoformans* is necessary to the survival in the oxidative environment of macrophages and important to virulence of this fungal pathogen (Missall and Lodge, 2005). To further validate the result, Fig. 5C depicted the effect of nitrosative stress on the viability of wild-type *H. pylori*, *trxR*, and *fur* mutants. A defect of *trxR* or *fur* significantly reduced the viability of the bacterium. The present data strongly suggested that *trxR* and *fur* were involved in the adaptive response to NO stress in *H. pylori*.

Conclusion

The present work established the first proteomic profile for *H. pylori* under nitrosative stress condition. 38 proteins showing a 1.5-fold or higher difference were mainly involved in protein processing, antioxidation, general stress response and respiration, as well as virulence of the bacteria. This study also revealed that *trxR* and *fur* were connected to the NO stress resistance. In conclusion, this proteomic analysis-based approach has found a series of targets for future physiological studies and has certainly shed new light on designing and constructing drugs to suppress the bacteria infection.

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